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# Journal of Experimental and Integrative Medicine

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# Journal of Experimental and Integrative Medicine

**Volume 3 – Issue 4 – October 2013**


## TABLE OF CONTENTS


**REVIEW ARTICLE**

**Adaptation to cold of homeothermic organism: changes in afferent and efferent links of the thermoregulatory system** 255-265
Tamara Vladimirovna Kozyreva

**ORIGINAL ARTICLES**

**Differentiation of norm and disorders of schizophrenic spectrum by analysis of EEG correlation synchrony** 267-278
Alexey Pavlovich Kulaichev, Natalia Leonidovna Gorbachevskaya

**Sweating and thirst perception in premenopausal, perimenopausal and postmenopausal women during moderate exercise** 279-284
Emmanuel Amabebe, Sonia I. Omorodion, Janet O. Ozoene, Andrew C. Ugwu, Leonard F. Obika

**Neuropathies of spinal cord development in rat pups maternally fed with fried potato chips** 285-292
Abdelalim A. Gad-Allah, Hassan I. El-Sayyad, Effat M. El-Shershaby, Ibrahim M. Abdelatif

**Purine metabolism and oxidative stress in children with autistic spectrum disorders** 293-297
Faisal Gh. Al-Rubaye, Taha Shawi Morad

**PARP-1 expression against Epstein-Barr virus LMP-1 and BZLF-1 in undifferentiated nasopharyngeal carcinoma** 299-304
Anggun I. Budiningrum, Achmad Rofi'i, Suharjono Suharjono, Fatchiyah Fatchiyah

**Effect of yogic practices on age related changes in oxygen metabolism and antioxidant-redox status** 305-312
Rameswar Pal, Som Nath Singh, Koushik Halder, Omveer Singh Tomer, Awadh Bihari Mishra, Mantu Saha

**Ethyl acetate extract of *Squilla oratoria* suppresses growth of HepG2 cells by inducing S phase arrest** 313-322
Xiangwei Qi, Xia Kong, Xiangning Zhang, Peichun Huang

**Protection against carbofuran-induced toxicity in rat tissues and plasma by *Ipomoea aquatica* Forsk crude extract** 323-329
Sanjukta Datta, Santinath Ghosh, Pubali Dhar

**Effect of methanolic extract of *Physalis minima* on gastric inflammation and gastric ulcers formation** 331-335
Umi Kalsum, Mulyohadi Ali, M. Aris Widodo, Handono Kalim

**Antioxidant activity and phytochemical composition of *Cynometra cauliflora*** 337-341
Azalina Farina Abd Aziz, Mohammad Iqbal

**HYPOTHESIS**

**Cosmological dark matter and ensoulment** 343-346
M. Kemal Irmak

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**Invited Review**

# Adaptation to cold of homeothermic organism: changes in afferent and efferent links of the thermoregulatory system

**Tamara Vladimirovna Kozyreva**

*Institute of Physiology and Fundamental Medicine, Russian Academy of Medical Sciences, **Novosibirsk, Russia***

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**Corresponding Author**
Tamara Vladimirovna Kozyreva
Department of Thermophysiology, Institute of Physiology and Fundamental Medicine, Russian Academy of Medical Sciences, Timakov str. 4, Novosibirsk, 630117, Russia.
Kozyreva@physiol.ru

**Key Words**
Cold adaptation; Gene expression; Ion channel; Muscle activity; Respiration; Thermoregulation; Thermoreceptors

**Abstract**

This review focuses on mechanisms of cold adaptation and with the interaction of the afferent and efferent links of the system of thermal homeostasis found through major research advances in our department.

Certain mechanisms of adaptive changes in metabolic and heat loss processes were disclosed mostly concentrated on muscle and respiratory functions. It was shown that, as a result of cold adaptation, there occur changes in the functional characteristics of the central and peripheral thermoreceptors, which form the input signal and determine the regulatory parameters of the system of thermal homeostasis. The adaptive changes in the afferent link are consistent also with the re-arrangement in the work of the respiratory system. The accumulated facts give grounds for believing that the important role of thermoreceptors in maintenance of adaptive re-arrangement is due to the direct and feedback relation to the neurohumoral systems of the organism.

The direct relation makes possible the implementation of a wide range of effector responses to thermal stimulus; while the feedback relation makes possible various modulations of the thermoreceptors, which are the initial link of the thermoregulatory system.



Problems of ecological physiology are fundamental to the phenomena of life. Indeed, the living organism emerges, develops and lives constantly interacting with the environment. The possibility to adapt to different environmental conditions allows the living organism to retain some freedom and independence from the environment and, ultimately, provides survival.

Temperature is one of the most significant natural ecological factors, which, in contrast to the human made ones, cannot be abolished. Changes in the seasons of the year, in climatic zone, alternation of day and night, shift in professional occupation are all related to alterations of temperature conditions. Conquest of Siberia, the northern territories, the circumpolar areas where low temperatures prevail draw special attention to questions whether humans and animals can adapt to cold, to what extent and through what mechanisms.

The mechanisms underlying the adaptation to cold are complex. The system of thermal homeostasis, which ensures the thermal regime of chemical reactions in the living organism, like all homeostatic systems, is subdivided into afferent and efferent links. Our many years of research allowed us to consider the questions of the possible changes and interactions of these links under long-term adaptation of the homoeothermic organism to cold. These considerations are of great interest in basic and applied sciences. In this review the main attention will be concentrated on the data concerning the adaptive changes in afferent link and such components of efferent link as muscles and respiratory system.

## THE EFFERENT LINK

Study of the thermoregulatory mechanisms and of the thermal adaptation processes was focused for a long time on only the functions of the effector structures. Study of the organism surviving in the cold allowed us to disclose a set of adaptive changes in the functioning of the effector organs and tissues directed towards conservation and increase in heat production.

Heat conservation in the organism during cold adaptation promotes first of all a reduction in heat loss from body surface. This can be achieved by different ways: an increase in subcutaneous fat, hair coat, and piloerection in animals, a decrease in the mean weighted skin temperature in humans, and also through behavioral responses [1-4].

**Thermogenesis**, the production of heat energy, is an essential component of the homeostatic repertoire to maintain body temperature in mammals and birds during the challenge of low environmental temperature. The primary sources of regulated metabolic heat production are mitochondrial oxidation in brown adipose tissue, increases in heart rate and shivering in skeletal muscle. Thermogenesis is regulated in each of these tissues by parallel networks in the central nervous system, which respond to peripheral afferent signals from cutaneous and core body thermoreceptors as well as to signals from brain thermosensitive neurons to activate the appropriate sympathetic and somatic efferents.

In many works, an increase in heat production after cold adaptation has been related to an increase in nonshivering thermogenesis, especially in brown adipose tissue [5-8]. However, a decrease in shivering occurs after cold adaptation suggested changes in shivering thermogenesis, too. In fact, as the studies have shown [9, 10], long-term cold exposure produces adaptive changes in heat production in the skeletal muscles. This is associated with an increase in the number of tonic slowly contracting muscle fibers with an increased heat producing capacity [11] more heat is produced during muscle contraction, thereby heat production is increased in the cold at a decreased level of shivering and thermoregulatory tone. These data were obtained first in muscle *in situ* using methods of precision thermometry, measuring oxygen consumption and electrical muscle activity [9, 10]; they were later supported by calorimetric measurement data obtained *in vitro* in diaphragmatic and heart muscles of cold adapted animals [12-14].

Changes in heat production by the muscles during cold adaptation were observed not only for thermoregulatory responses, but also for exercise [15]. Enhancement of the heat production processes as a result of cold adaptation produces a decrease in the efficiency of muscular work. An increase in the energy cost takes place in the period of recovery [16]. A decrease in the strength of isometric contraction is observed in cold adapted animals, too. However, a fall in the strength of contraction during a 10 sec of muscle tetanus becomes less expressed after adaptation to cold; this may be regarded as a decrease in muscular fatigue [17] that can be due to $Ca^{2+}$ increase in muscle mitochondria [18, 19] and is consistent with an increase in the number of tonic muscular fibers during adaptation to cold [11, 20]. Furthermore, adaptation to cold produces a shift of the optimum regimen of muscular contraction (corresponding to a load with maximum efficiency) toward smaller loads and deceases maximum efficiency itself [21]. A decrease in the efficiency of the cardiac muscle work can also contribute to the energy cost of physical exercise after cold adaptation of the whole organism [12]. Obviously, the described changes in muscle energetics, by promoting heat production, lead to deterioration of work capacity, which is the "cost" of adaptation.

**The sympathetic nervous system** plays an important role in the thermogenic mechanisms during adaptation to cold. It was believed that its influence is implemented through an activation of non-shivering thermogenesis [5-8]. However, the studies of our laboratory demonstrated that noradrenaline administration at doses producing a clear-cut calorigenic effect in the whole organism is accompanied by an increase of heat release during contraction occurring mainly in the secondary phase of heat production [22]. In such a case, the heat effect of contraction caused by noradrenalin injection in cold adapted animals proved to be 2.3 times stronger than in the controls. This is also another evidence for the enhanced sensitivity of muscular adrenoceptors to noradrenalin. Studies on the muscle oxygen consumption before and after noradrenaline administration demonstrated that noradrenaline does not affect oxygen consumption by the muscles at rest, yet increases the caloric expenditure of muscle work, on average by 90%. An increase in heat production under the effect of noradrenaline was observed also in the rat isolated diaphragm during its contraction [14]. Thus, it may be believed that noradrenaline acts as a physiological regulator of the extent to which muscular contraction is efficient.

The nature of the sympathetic control of thermogenesis is predominantly β-adrenergic, and increased sensitivity to catecholamine in cold adaptation is mainly developed by reorganization of the mechanisms of β-adrenergic coupling [6, 7]. Treatment of animals with the β-adrenoceptor antagonist propranolol on the background of noradrenaline effect was associated with decrease in body temperature and an decrease in the heat production of muscular contraction to values close to baseline [23]. These facts are evidence of the β-adrenoceptor mediated role of noradrenaline in the adaptive reorganization of muscular heat production during prolonged cold exposure.

Change in oxidation-phosphorylation coupling in the respiratory chain during ATP resynthesis is one way of decreasing the efficiency of muscular contraction in the coursed organism adaptation to cold [24]. As indicated,

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the heat effect of muscular contraction after noradrenaline administration increases due to an increase in the secondary phase of heat production in muscles, which, as a rule, is explained by ATP re-synthesis after contraction. It is also known that noradrenaline, an activator of lipolysis raises the concentration of free fatty acids in blood and muscles [18, 25]. Free fatty acids through their uncoupling action can alter the process of oxidative phosphorylation [26]. Change in the heat effect of muscular contraction resulting from cold adaptation may also be a consequence of changes in the regulation of the motor act itself, including the mechanism for excitation in the neuromuscular junction, the muscular membrane, and the sarcoplasmic reticulum.

**The respiratory system** is a significant component of the efferent link of the thermoregulatory system. The problem of temperature effect on the implementation of chemoreceptor regulation was widely discussed in the literature [27-30]. It is known that carbon dioxide acts both on the central, peripheral chemoreceptors, and on thermoreceptors of the brain and upper airways [31-33]. An increase in the concentration of carbon dioxide in the inspired air produces a decrease in the tidal volume and respiratory rate with a prolongation of expiration [34]. Long repeated cold exposures result in an increase in carbon dioxide concentration in the last portion of expired air in human, this is associated with a close correlation between this parameter and the oxygen utilization (oxygen percent by volume, which is used from 100 ml of inspired air) [35]. In humans adapted to cold, the ventilator response to a hypercapnic stimulus is decreased during recurrent respiration [35]. This may be evidence of decreased sensitivity of the respiratory center to carbon dioxide. As a result of cold adaptation in human respiratory rate and pulmonary ventilation volume decrease, however, oxygen supply of the organism is then unimpaired, because its utilization from inspired air increases [36].

An increase in oxygen utilization at low environmental temperature allowing to maintain pulmonary ventilation at a low level can be understood as an adaptation to diminish the metabolic cost of thermoregulation through a reduction in respiratory heat loss [36, 37]. The ability for increased utilization of oxygen from the inspired air by living organisms inhabiting in conditions of cold is provided by structural reorganization in the oxygen transport system [38, 39]. Similar changes were observed in human. In fact, the mucosal layer of the trachea and large bronchi of the "northern" lung is distinguished by vascularization (copious blood supply); the lumens of the small bronchi and bronchioles are enlarged. As a result of alveolar hypertrophy, widening of pulmonary capillaries and the formation of new capillaries, alveolar surface increases by about 20%. The aerohematic barrier becomes thinner and the areas of working zones larger [40]. The abundance of blood capillaries promotes a greater inflow of blood to the aerohematic barrier; shortening of diffusion distance provides a more effective gas exchange in the lungs of the adapted inhabitants of the north. An increase in the amount of surfactant on alveolar surface promotes also improved oxygen diffusion [41, 42].

Simultaneous continuous registration of temperature and humidity of expired air in standard conditions of thermal comfort and total moderate cooling demonstrated that in cold adapted men the temperature of expired air is lower by 1.2°C on average, and with each 1 liter of expired air they have less losses of moisture by 4.3 ml than in the control [43, 44]. This promotes less respiratory heat loss due to convection and evaporation. Calculation showed that at air 26°C temperature and 5 mg/l humidity respiratory heat loss in human of control group was 155 cal/min, while in the cold adapted individuals it was 116 cal/min, *i.e.*, by 25% smaller than in control. These differences become more marked with the air temperature decrease and its humidity increase: at 13°C and 10 mg/l humidity respiratory heat loss in cold adapted man was by 40% smaller than in controls (191 and 114 cal/min, respectively).

Changes in the dynamics of the respiratory cycle may be a mechanism of a decrease in the temperature and humidity of the expired air. Longer expiration time in cold adapted humans not only produces lower respiratory rate when they are in warm conditions and especially in the cold, it provides also longer contact of warm moist alveolar air with mucosa of the upper respiratory tract cooled during inspiration. This promotes more effective return of heat and moisture expended for conditioning inspired air. It may be reasoned that the observed functional and morphological rearrangements of the respiratory system during cold adaptation are targeted toward optimal resolution of two tasks: ***(1)*** defense of respiratory pathways from cold damage; and ***(2)*** maintenance of energy and thermal homeostasis. Thus, as a result of cold adaptation, considerable changes in the functioning of effector organs take place. The scheme of these changes is presented in Fig.1.

Cold adaptation, also repeated cold exposures in both humans and animals, cause shifts of the thresholds of the thermoregulatory responses to a range of lower temperatures. The thermal thresholds for shivering and metabolic response elevated, *i.e.* the organism can be stronger cooled without starting cold-defense responses [45-47]. This is unquestionable evidence for changes in the regulatory characteristics of the system of thermal homeostasis. The presence of regulatory shifts suggests changes in the nature of the functioning of the central

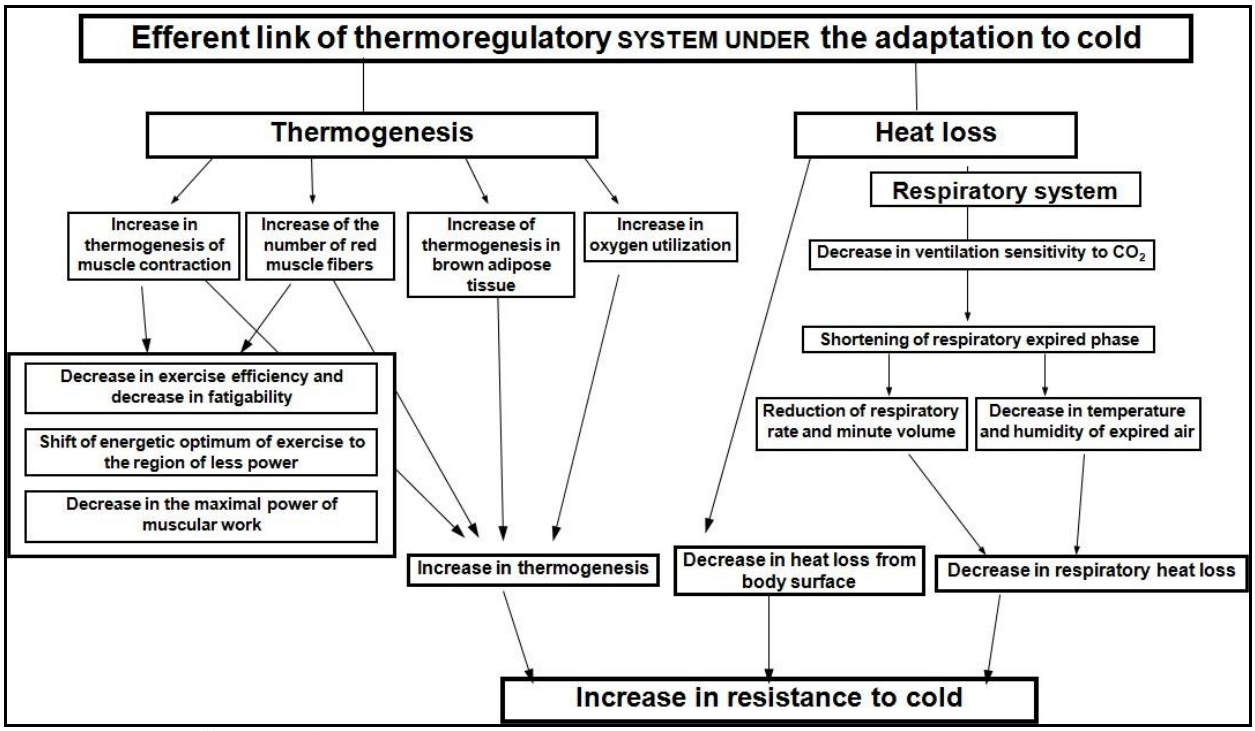


**Figure 1.** Changes in efferent link of thermoregulatory system during adaptation to cold environment.

and peripheral thermoreceptors. These receptors forming the afferent signal actually determine the organism's response to changes in thermal conditions. However, the significance of the receptors, in terms of sensory structures that play an important role in the organism's adaptation, as well as the mechanisms implementing the specific role of the receptors have been very scantily studied. Investigation of the thermoregulatory mechanisms and of thermal adaptation was restricted to the function of the effector structures only. Explanations for the adaptive mechanisms were offered from this vantage point.

## AFFERENT LINK

### Central thermoreceptors

In the central mechanism for body temperature regulation, the hypothalamic preoptic area (POA) plays pivotal roles by receiving and integrating temperature information from the skin and their own thermoreceptors and then by sending command signals to peripheral thermoregulatory effectors [48-52]. Thermo-sensitive hypothalamic neurons mostly orient their dendrites medially to have the local temperature information while thermo-insensitive neurons have a different dendrite orientation [53, 54].

Let us consider how the characteristics of impulse activity of the hypothalamic neurons and skin thermoreceptors, temperature sensation, and by thermoreceptors regulated temperature thresholds for cold defense response change after the long-term adaptation to cold.

Changes in the activity patterns and in the thermal sensitivity of the central and peripheral thermoreceptors were conducted in rats using Hart's [55] method of long-term cold adaptation: the rats were exposed to an environmental temperature of +5ºC for 6 weeks in solitary chambers. Food and water were provided *ad libitum*. The controls were maintained under similar conditions for the same time, with the difference that the temperature was +20ºC. At the end of cold adaptation the rats in the cold have the same skin and deep body temperatures as control rats in the warm environment [56]. This supports the idea that there should be another regulatory pattern of the temperature homeostasis maintenance.

While studying the central thermoreceptors in rat brain slices [57, 58] thermosensitive neurons of three types are distinguished in the preoptic area of the hypothalamus in both control and cold adapted rats: ***(1)*** neurons sensitive to changes in the low temperature range of 35-38°C; ***(2)*** neurons sensitive to changes in the high temperature range of 38-41°C; and ***(3)*** neurons sensitive to temperature changes in the whole range of studied temperatures 35-41°C.

After the adaptation to cold, the portion of neurons sensitive in the low temperature range decreases. On the contrary, the portion of neurons sensitive in the high temperature range increases and their portion is markedly prevalent. This may evidence for a decrease in the central hypothalamic sensitivity in the low temperature range and for its increase in the high temperature range. It is of interest that a nocturnal

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increase in the number of warm sensitive neurons in the hypothalamic POA is observed when body temperature at this time is maintained at a lower level compared to its diurnal [59].

The question on the ***basis of thermosensitivity***, *i.e.* on the cellular mechanisms due to which the impulse activity of neuron can be changed, remained unclear for a long time. The discovery of the cell membrane thermosensitive proteins, in particular, thermosensitive transient receptor potential (TRP) ion channels, allowed approaching this issue. Thermosensitive TRP ion channels are proteins which have intracellular amino- and carboxyl-terminals, six transmembrane domains, and a loop between segments 5 and 6; this loop participates in the formation of cation-permeable channel pore [60]. Thermosensitive TRP ion channels, under the effect of temperature, can alter penetration of ions into the cell, which in turn can lead to changes in membrane potential with high $Q_{10}$ (10-degree temperature coefficient), varying from 10 to 20 [60, 61]. Thermosensitive TRP ion channels, according to the opinion of many authors, are the primary detectors of temperature changes in homeotherms [62-65].

Currently, it is considered that the temperature range perceived by most mammals may be covered by temperature-activated TRP ion channels [65-67]. The temperature below 17°C is perceived by TRPA1, below 28°C by TRPM8, TRPV4 ion channel is activated in the temperature range of 25-42°C, TRPV3 in the range of 31-39°C, TRPV1 at 42°C and higher temperatures, and TRPV2 at 52°C and higher temperatures [63]. It is possible, that such property of neurons as temperature sensitivity or, alternatively, temperature insensitivity as well as modifications in neuron response to temperature stimulus may be due to different composition of thermosensitive and non-thermosensitive ion channels.

We have studied the ***gene expression*** of the six best known thermosensitive TRP ion channels, namely TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8, by the method of quantitative reverse transcription polymerase chain reaction (RT-PCR) in different brain regions in rats, adapted and not adapted to cold [68, 69]. Our results provide evidence indicating that the expression of the investigated genes was identified in all the examined brain regions (hypothalamus, frontal cortex, hippocampus and midbrain). It was found rather high expression of the genes of warm sensitive ion channels activated at temperatures above 30ºC; in contrast, the gene expression level of cold sensitive TRPM8 and TRPA1 proved to be much lower. The expression of the genes of thermosensitive TRP ion channels was, mostly, greater in the hypothalamus than in the other brain regions.

***What was the effect of cold adaptation on expression level of the genes for the thermosensitive TRP ion channels?*** It was established the decrease in the expression of the TRPV3 gene in the cold adapted animals, that suggested the participation of this ion channel in providing the thermal sensitivity of the hypothalamic neurons and its adaptive changes. It is known that TRPV3 ion channel is active at 31-39°C [63], *i.e.* in the physiological temperature range. The thermal ranges of activation of TRPV3 ion channel and those neurons (see above) whose number decreased as a result of adaptation overlap. From comparisons of these facts it was inferred that precisely TRPV3 ion channel is responsible for ensuring; ***(1)*** thermal sensitivity of that portion of hypothalamic neurons that is sensitive in the temperature range 35-38°C, and ***(2)*** their changes (decrease) arising from cold adaptation.

It may be concluded that thermal adaptation can affect processes enfolding at the level of mRNA expression, while change in TRP ion channel genes as such are one of the molecular mechanisms of change in thermosensitivity of hypothalamic neurons under long-term thermal exposures. It should be noted that the observed changes are hypothalamus-specific and there are no changes in the gene expression of investigated thermosensitive TRP ion channels in other investigated brain structures (prefrontal cortex, hippocampus and midbrain) after long-term adaptation to cold [69].

In another work of our laboratory the increase in serotonin 5-$HT_{2A}$ receptors mRNA in the hypothalamus in cold adapted animals was also shown [70]. It is well known that when the 5-$HT_{2A}$ receptors are activated, responses are targeted at both increase in heat production and decrease in heat loss [71, 72]. Taken together, these processes can improve the defense of the homeotherms from the effect of decreased environmental temperatures. The adaptation to cold does not increase the expression of the 5-$HT_{2A}$ receptor mRNA in the other brain structures, moreover, in the frontal cortex the changes reverse to those observed in the hypothalamus: 5-$HT_{2A}$ receptor mRNA amounts falls significantly. The observed changes in the expression of 5-$HT_{2A}$ receptors are also hypothalamus-specific as for the changes in TRPV3 ion channel expression.

Thus, long-term cold adaptation causes changes in the hypothalamic thermosensors at the mRNA expression level. It can be suggested that a mechanism for changes in thermosensitivity of hypothalamic neurons under the adaptation to cold may be connected with two mechanisms related to change in the proportions of TRP ion channels and to change in the proportions of receptors of different mediators at the membrane of hypothalamic neurons. Obviously, these mechanisms can supplement each other.

**Peripheral skin thermoreceptors**

As for the peripheral thermal receptors, the view was long held that they do not undergo adaptive changes. At variance with this view, our studies in rats [73, 74] as well as studies in cats [75] demonstrated that the pattern for the function of the skin cold receptors substantially changed during the organism's adaptation to cold.

According to our results [56, 73, 74, 76] two groups of cold receptors are clearly distinguished in the area of *n. sapheni* innervations in the control animals. These groups differ by their frequency characteristics and temperature range of the maximal activity; static (discharge rate at a constant temperature), and dynamic (transient increase in discharge rate during rapid change in temperature). One group, the low frequency receptors, whose static activity was less than 1 imp/sec at skin temperature of 34-36ºC, showed maximum thermal sensitivity in the range of lower temperatures of 24-25ºC. Second group, most skin cold receptors, had a static activity from 1-4 imp/sec, and their maximum static and dynamic activity was in the 28-30ºC temperature range.

After the organism's adaptation to cold, the portion of the low frequency cold receptors, the receptors most sensitive in the low temperature range, decreased considerably (they virtually disappeared). Cold receptors with a maximum at 28-30ºC showed a decrease in their static activity and their dynamic response to a rapid fall in temperature decreased 2-fold after adaptation to cold. However, a group of high frequency cold receptors with maximum activity shifted to the high temperature range (34-35ºC) appeared.

Thus, under long-term adaptation to cold the sensitivity of both the central and peripheral thermoreceptors decreases in the low temperature range and increases in the range of high temperature. This is consistent with the data indicating that, after adaptation to cold, the organism admits a greater reduction in body temperature, without triggering cold defense responses and also with the observation that it is easier to produce overheating and switch on heat loss responses in cold adapted animals [45-47, 77, 78].

**Temperature sensation**

The inflow of afferent thermal information depends on the amounts of impulse activity and the number of functioning receptors. We made an attempt to estimate how the number of functioning (sensitive) skin cold receptors may change in humans long exposed to cold, because in animals this is impossible. It is known that every cold/warm spot 1 mm in diameter is innervated by at least one cold/warm receptor [79, 80]. Thus, the number of functioning cold/warm receptors may be estimated from that of the sensitive cold/warm spots. The number of cold and warm spots was tested with the temperature of thermode as +3-4ºC for cold spots and as +41ºC for warm spots. It proved that in builders that work not less than 1-3 years out of doors in winter time in the conditions of Siberia and the Far North the number of cold spots is decreased [81, 82]; *e.g.*, it decreased 2-fold in the arm. The number of warm spots was unaltered. Studies we performed in climatic chamber showed that the ambient temperature is sensed as "cold" by an unclothed subject and is directly related to the number of cold spots in the arm [82, 83]. This allows concluding that the organism's adaptation to cold results also in a decrease in the number of functioning cold receptors and in a reduction of cold sensation (perception), and the elevation in the cold sensation threshold.

**Thermoreceptor activity and the formation of the effector response**

The rate of external cooling is of particular importance for the skin thermoreceptor discharge rate, *i.e.* for the formation of the afferent thermal signal. Studies concerned with the registration of the skin receptor impulse activity have demonstrated that the dynamic response of the cold receptors increases [84] and the cold sensation threshold decreases with increase in cooling rate [85]. Our studies established a dependence of the thermal thresholds for the cold defense responses on cooling rate in control and cold adapted animals [47, 86-88]. At low rates of external cooling, when skin temperature changes slower than 0.01-0.02ºC/sec and the dynamic activity of the cold receptors is low, if at all present, the contribution of both the deep body and skin temperatures are required to trigger the effector responses (for example, at slow cooling the metabolic response is initiated when both the skin and rectal temperature are lowered), that is the involvement of both the central and peripheral thermoreceptors. At high cooling rates (> 0.03ºC/sec), in the presence of the dynamic activity of the skin cold receptors, the initial phase of metabolic response can be observed even when the core temperature is unaltered, *i.e.* the afferent signal from skin thermoreceptors is enough to initiate the response. It should be noted that with increasing in value of dynamic activity of the skin cold receptors (observed at an increase in cooling rate) the threshold of the metabolic response lowers. As indicated above, after the organism has adapted to low temperature, the dynamic response of the cold receptors decreases considerably and the patterns of slow cooling spread over to a wider range of cooling rates (up to 0.33ºC/sec compared with 0.13ºC/sec in the control) and the threshold for metabolic response becomes less dependent on the cooling rate. Thus, the adaptive changes in the peripheral thermoreceptors activity manifest in the effector response character.

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The possible mechanism of these adaptive changes in thermosensitivity can be the influence of *noradrenaline* on thermoreceptors. Our studies demonstrated the following: noradrenaline affects both the static and dynamic activities of the skin cold receptors [89]. The effect may be different, depending on the functional characteristics of the skin cold receptors. The low frequency cold receptors, as it was mentioned above, showing maximum sensitivity in the low temperature range (24-25ºC), at elevated noradrenaline concentration, increased their activity and sensitivity to cooling. It will be recalled that precisely the activity of these receptors decreases during adaptation to cold. In contrast, the high frequency cold receptors showing maximum activity and sensitivity in the higher temperature range (28-30ºC) decreased their activity and sensitivity to cooling under the effect of noradrenaline. The latter is coincident with the observations made for the organism's long term adaptation to cold.

Judging by our results in humans [56, 83], iontophoretic noradrenaline application to the skin produced a decrease in the number of cold spots, *i.e.* the number of functioning cold receptors. The number of warm spots (functioning warm receptors) did not change. Hence, an increase in noradrenaline concentration in the skin or increased sensitivity to noradrenaline in cold receptors after adaptation to cold can produce a decrease in the number of functioning cold receptors which leads to an elevation in the cold sensation threshold. The described mechanism is one of possible epigenetic mechanisms of adaptive changes in afferent link of thermoregulatory system. Recently, there are also some data indicating that modulation of TRPM8 activity by different chemical agents unveils an important flexibility in the temperature-response curve of TRPM8 channels and cold thermoreceptors. These results indicate that post-translational modification of TRP ion channels can be an important mechanism in modulating cold thermoreceptor function [90].

It is interesting to note that there is also a ***genetic mechanism of different themosensitivity*** in human. We have obtained the data that different number of functioning skin cold receptors can be also due to single nucleotide polymorphism of gene of cold sensitive ion channel TRPM8 [91]. In Russian ethnic group it was shown the presence of 20.3% of subjects with the heterozygous genotype containing the C allele of the single nucleotide polymorphism rs11562975 (GC) located in exon 6 of the gene encoding the cold sensitive ion channel TRPM8. The subjects with the heterozygous genotype GC containing the C allele are characterized by increased sensitivity to cold and reduced sensitivity to menthol, an agonist of the TRPM8 ion channel, compared to the subjects with the homozygous genotype GG. It is possible that variable single nucleotide polymorphisms may change thermosensitivity in different way. Taking into account that according to our recent results, activation of TRPM8 ion channel by its agonist menthol significantly affects not only thermoregulatory but also immune response [92, 93] the polymorphism of this channel may be connected with the resistance of organism to different infections especially in the cold environment. Some changes in immune response after long-term adaptation to cold was also observed, *i.e.* the decrease in antigen binding but increase in antibody forming functions [94]. Additional investigations are necessary to answer the question about the thermosensitivity dependence on epigenetic and genetic factors as well as about their interrelation and participation in adaptive mechanisms in thermoregulatory and immune systems.

Thus, based on the above observations, the changes in afferent link brought about by the organism's adaptation to cold are as follows (Fig.2): ***(1)*** a decrease in the sensitivity of the hypothalamic neurons in the low body temperature range, most likely due to decrease in TRPV3 gene expression; ***(2)*** an increase in the sensitivity of the hypothalamic neurons in high temperature range; ***(3)*** a decrease in the dynamic and static activities of the high frequency skin cold receptors; ***(4)*** a considerable decrease in the portion of the active low frequency skin cold receptors with maximal sensitivity in the range of low skin temperature in animals; ***(5)*** a decrease in the number of functioning skin cold receptors providing sensation of low temperatures in human; and ***(6)*** an elevation of the threshold for cold sensation and cold defense effector responses.

The respiratory characteristics were compared with the cold spot number of the forearm and foot skin areas. It was revealed that in subjects with a greater cold spot number in tested areas the pulmonary ventilation and respiratory rate were greater, while the oxygen utilization was reduced [83, 95]. Regression analysis of the results allowed us to establish a direct correlation between the number of functioning (sensitive) cold receptors in the forearm area and respiratory parameters in human such as respiratory minute volume and respiratory rate, whereas the correlation with oxygen utilization was inverse. The inverse dependence of the respiratory minute volume and oxygen utilization on the number of functioning cold receptors presumably clarifies why there is no relation between oxygen consumption and the number of functioning skin cold receptors. That means that the same level of oxygen consumption can be reached by different strategies: either increased respiratory minute volume or increased level of the oxygen utilization.

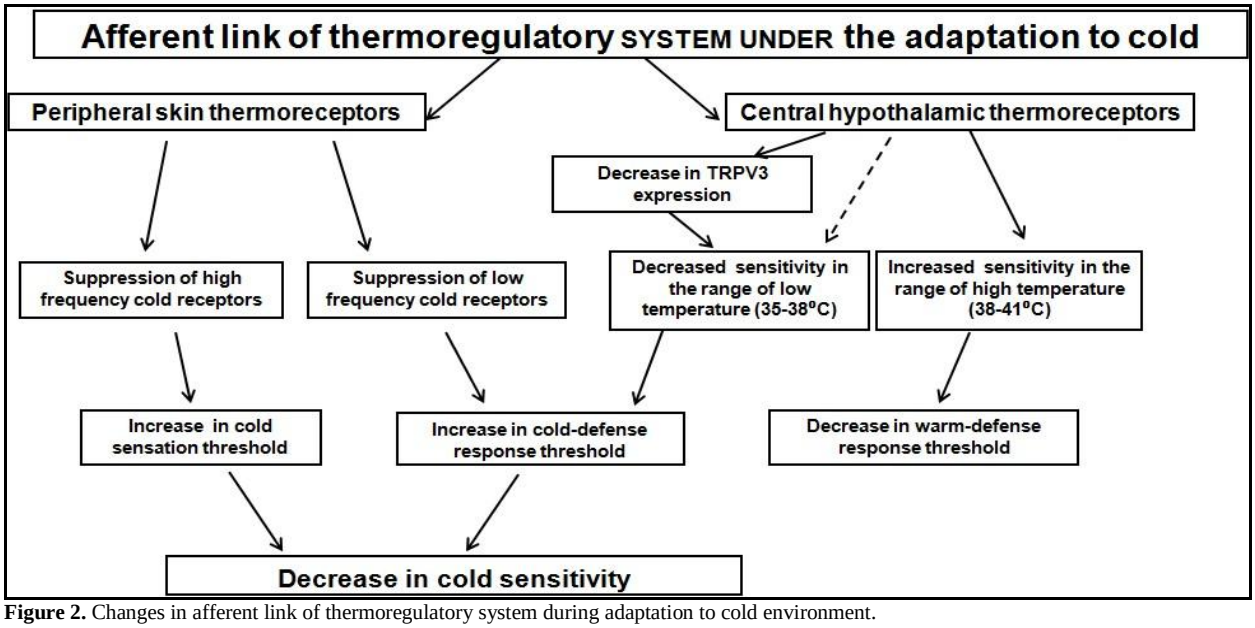


**Figure 2.** Changes in afferent link of thermoregulatory system during adaptation to cold environment.

These facts justified the assumption that changes in the peripheral temperature input are involved in the reorganization of the system of external respiration during cold adaptation of human to low temperatures. In fact, a decreased number of sensitive cold receptors in persons regular subjected with low temperatures (working outdoors in winter in conditions of Siberia) is accompanied by an increased oxygen utilization when compared with those not adapted to low temperatures and having greater number of cold spots in this area [83]. A decrease in pulmonary ventilation due to reduced ventilator sensitivity to carbon dioxide provides smaller respiratory heat loss [35], whereas the increase in oxygen utilization provides increased heat production [36].

It was mentioned that the effect of biologically active substances may underlie adaptive changes in the functioning of thermal receptors during cold adaptation. We have previously considered some facts that evidence for the participation of noradrenaline in re-organization of the effector link of the thermoregulatory system during cold adaptation, especially in an increase in the β-adrenergic sensitivity of muscle and brown adipose tissue, which contribute significantly to increased heat production. As for the afferent link, we obtained experimental proof that noradrenaline has, in principle, a modulating influence on the impulse activity of thermosensitive neurons of the hypothalamus and the skin cold receptors in the rat and on the number of cold sensitive receptors (cold spots) in human [57, 74, 83, 89, 96, 97]. It was shown also that the sensitivity of central and peripheral thermoreceptors to noradrenaline changes after cold adaptation [57, 87, 96]. The sensitivity of the skin cold receptors to noradrenaline increases after long-term exposure of the organism to cold that of the central receptors on the contrary decreases. According to the available data, change in calcium ion concentration may be a factor whose effect alters adrenergic receptor sensitivity [98].

The performing experiments demonstrated that long-term adaptation to cold results in a significantly reduction in the concentration of blood calcium ions both in animals and humans [99]. This reduction may be related to a greater accumulation of these ions by muscle cell mitochondria [18]. Comparison of the number of cold spots with the concentration of blood calcium demonstrated a direct correlative relation between these two parameters. Decreased level of the concentration of blood calcium ions is accompanied by a smaller number of cold spots, *i.e.* by a reduced cold sensitivity [99]. Artificial increase in the concentration of blood calcium ions produces an increase in the number of cold spots [100]. The reduction in the concentration of calcium ions in cold adapted individuals is fully consistent with the decrease in the number of cold spots observed in them. It is also in agreement with the data that cold sensitive ion channels (TRPM8 and TRPA1) localized in sensory fibers are $Ca^{2+}$ dependent [65, 101].

The increase in β-adrenergic sensitivity of muscles and brown adipose tissue after cold adaptation can be related with the lowering of blood calcium ions. This relation appears possible because of the well known inhibitory role of calcium in the regulation of the secondary mediator of β-adrenoceptors, namely cyclic adenosinemonophosphate (cAMP) [98]. The nature of adrenergic receptors causing sensitivity of thermosensors to noradrenaline is unknown. It requires further study. It is known, however, that calcium ions

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suppress β-adrenoceptors, but increase α-adrenoceptor sensitivity, being their secondary mediator. The existence of α-adrenoceptors in the central thermosensors, which decrease the sensitivity to noradrenaline after cold adaptation, may be suggested. As for the increase in sensitivity to noradrenaline of the peripheral skin thermoreceptors after cold adaptation on the background of a decreased concentration of calcium, the nature of changes in their adrenosensitivity is unknown.

During the process of adaptation to low environmental temperatures under the effect of the initial action of cold on thermoreceptors, the concentration of blood noradrenaline rises, this in turn can result in a lowering of the level of blood calcium ions (a series of experiments established that injection of exogenous noradrenaline produces a decrease in the concentration of calcium ions in blood) [97, 102]. This results in change in noradrenaline sensitivity of a number of peripheral tissues, including the effector organs and receptor structures. The accumulated facts justify the belief that both the central and peripheral thermoreceptors have an important role in the establishment and maintenance of adaptive re-arrangement. This role of thermoreceptors is presumably due to their direct and inverse relation to neuro-hormonal system in the organism. The direct relation makes possible to realize a wide range of effector responses to thermal stimulus, while the inverse relation makes possible various modulations of the thermoreceptor activity, which is input of the thermoregulatory system.

Based on the above results, cold adaptive rearrangements in afferent link in the homeotherms may be represented by an overall scheme (Fig.2). The consequences of cold adaptation are changes in the functional characteristics of thermoreceptors: the sensitivity of the neurons of the hypothalamus in the low temperature range diminishes due to decrease in TRPV3 gene expression, the dynamic and static activity of the skin cold receptors considerably reduces, and this can lead to a decrease in the number of cold spots (sensitive cold receptors). There follows as a consequence a rise of the threshold of cold sensations and cold defense responses of the organism. It seems that high frequency skin cold receptors are mostly important for temperature sensation and low frequency skin cold receptors for initiation of cold-defense response.

Thus, the observed adaptive re-organization in the organism exposed to long-term cold promotes a decrease in information flow and a reduction in energy expenditures for maintenance of thermal homeostasis at low environmental temperature. It should be noted that the adaptive re-organization cannot be completely explained yet and requires following studies.

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**Original Article**

# Differentiation of norm and disorders of schizophrenic spectrum by analysis of EEG correlation synchrony

**Alexey Pavlovich Kulaichev[1], Natalia Leonidovna Gorbachevskaya[2]**

*[1]Moscow State University; [2]Mental Health Research Center, Russian Academy of Medical Sciences;*
***Moscow, Russia***

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**Corresponding Author**
Alexey Pavlovich Kulaichev
Faculty of Biology,
Moscow State University,
119234, Lenin Hills, 1/12,
Moscow, Russia.
akula-@mail.ru

**Key Words**
Discriminant classification;
Electroencephalography;
Envelope correlation;
Schizophrenia;
Schizotypal disorder

**Abstract**

***Objectives:*** Experimental work was designed to find the integrated differences in electroencephalography (EEG) synchrony between normal people and patients with disorders of schizophrenic spectrum.

***Methods:*** In this study EEG recording have been performed in a state of quiet wakefulness with eyes closed for three groups of 8-15 years old adolescents: normal group and two groups of mental disorders in nosological categories F20 and F21 according to International Classification of Diseases (ICD)-10. We have used the alternative method for EEG synchrony estimating based on correlation between envelopes of EEG signals. This method was previously proven as a highly sensitive tool of differentiation of psychopathological and functional states.

***Results:*** As a result of research, the complex picture of significant topographical, inter-hemispheric, regional and age distinctions was revealed, in which many of fragmentary results previously received by other researchers found their confirmation. One of the basic features of the received integrated picture of pathology is existence of extended zones of sharply lowered EEG synchrony dividing local and isolated areas in frontal and occipital regions mainly of normal or sometimes increased EEG synchrony. The received results completely fit into the framework of the theory of disintegration of cortical electric activity in cases of disorders of schizophrenic spectrum.

***Conclusion:*** The used method provides close to 100% reliability of tripartite classification of norm and two pathology groups separately, it allows revelation of many authentic correlations between EEG synchrony estimations and psychometric indices, its results are consistently reproducible for different groups of patients and examinees, which opens up opportunities and prospects for its use as an auxiliary quantitative differential indicator.



## INTRODUCTION

Among numerous papers devoted to electroencephalography (EEG) differences between norm and schizophrenia, relatively few studies relate to differences in EEG synchrony in a state of quiet wakefulness as it follows from the special review [1]. However, classificatory sensitivity of EEG synchrony estimations is significantly higher compared with amplitude spectrum [2-4], power spectrum [1] and some other measures [4]. To a large extent this is determined by the fact that estimates of EEG synchrony have a significantly lower intra-individual variability, which according to our data obtained at different experimental material and estimated by variation coefficient is 8-12 against 23-41% for average amplitude spectrum and against 86-95% for power spectrum. So by EEG synchrony estimations it is possible to receive reliability of comparable distinctions at smaller sample volumes and reliability of smaller distinctions under comparable sample volumes.

Results obtained by different researchers are rather fragmentary and contradictory, that was noted in the discussion [5]. Some researchers have found that compared with the norm at schizophrenia a coherence is lower, namely: ***(a)*** intra- and inter-hemispheric coherence in all domains [6]; ***(b)*** violated left

hemispheric *F-T* connections [7]; ***(c)*** a coherence in delta (δ) and theta (θ) domains at *Fp1-F7* derivations and in alpha (α) domain at *F7-F8* [8]; ***(d)*** a coherence in δ domain in temporal lobe [9]. Other studies on the contrary have shown that for schizophrenia compared with the norm a coherence is higher, namely: ***(a)*** intra- and inter-hemispheric one in θ domain and intra-hemispheric one in α domain [10]; ***(b)*** inter-hemispheric one in δ and beta (β) domains at *O1-O2* and in δ domain at *T5-T6* [11]; ***(c)*** intra-hemispheric one in general [12] or only in δ domain [13]. It is significant that most of the cited works were published about ten and more years ago. Probably, such a situation is caused by the fact that coherence function is unstable indicator of EEG synchrony [14-17]. The observed inconsistency of results makes it actual to use alternative approaches for the evaluation of EEG synchrony in this field.

## MATERIALS AND METHODS

EEG recording was carried out in a state of quiet wakefulness with eyes closed. The electrodes were placed according to 10-20% system in 16 cortex areas (*O1, O2, P3, P4, C3, C4, F3, F4, T5, T6, T3, T4, F7 and F8*); united ears electrodes were used as referents (*A1+A2*); the bandwidth was 0.5-35 Hz; sampling rate was 200 Hz. For the analysis we selected the fragments free of artifacts with a duration of 41 seconds (8196 discrete time slots). The analysis was carried out in five standard frequency domains: δ 0.5-4 Hz, θ 4-8 Hz, α 8-13 Hz, β-1 13-20 Hz, β-2 20-32 Hz.

The group of patients with disorders of schizophrenic spectrum was diagnosed according to International Classification of Diseases (ICD)-10 in Mental Health Research Center, Moscow and it consisted of 125 boys (8-15 years old). For 45 of them (age 11.5 ± 2.2 years), the diagnosis made was schizophrenia, childish type (F20), and for 80 adolescents (age 11.9 ± 2.5 years) schizotypal disorder (F21). Control group (N, norm) included 36 pupils from Moscow's schools without documented mental deviations (age 12.2 ± 2 years). Parents of all examinees gave the written permission for carrying out researches and publication of their results.

In this study we used the alternative approach to similarity estimation between bioelectric activity of different cerebral areas: the analysis of EEG correlation synchrony (ACS) was proposed and detailed previously [4]. It estimates the degree of EEG synchrony by correlation coefficient between envelopes of EEG records preliminary filtered in a given frequency range. Here it is appropriate to emphasize that as an envelope representing a change of EEG amplitude modulation, the synchrony estimation constructed on its basis has the direct and important physiological sense (unlike coherence). Indeed, the EEG amplitude increases with increase of synchrony of postsynaptic potentials, so the correlation of EEG envelopes estimates the degree of synchrony in change of such intra-neuronal synchronism.

An ordered sequence of such correlations between nearby derivations (in our case, between 36 EEG derivation pairs) have been named 'profile of synchrony' (PS) and such profiles as topographic patterns of EEG synchrony (for group of subjects we have an array or a matrix of profiles) are the source material for the further analysis. This method has already demonstrated its high efficiency for a similar problem [4] as well as for differentiation of night sleep stages, *i.e.* functional states [3].

Below for evaluation of pairwise sample differences we use the nonparametric Wilcoxon test since a large part of sample distributions differs from normal law. For evaluation of group differences we also apply the two-way analysis of variance (ANOVA) with repeated measures design (number of repeated measures is equal to number of subjects in compared groups). We also use the designations of groups: F20, F21, N and the designation of frequency domains: δ, θ, α, β-1, β-2.

## RESULTS

### Analysis of records on consistency

In any statistical sample due to influence of casual, uncontrolled in experiment factors there are outliers, and also among measurements there are more consistent and less consistent ones. For reliable separation of prevailing parities it is desirable preliminary to clear samples from outliers as well as from less consistent measurements. In our case, a role of random factors can be acted by: ***(1)*** instrumental factors such as differences in position of electrodes concerning anatomic cortex structures, changes in inter-electrode resistance, *etc*; ***(2)*** personal factors such as differences in individual EEG characteristics, differences in current physiological and psychological state, *etc*; ***(3)*** classifying factors such as patients belonging to nosology not differentiated or not clearly differentiated in ICD-10 [18], subjective judgments of psychiatrists, *etc*. Therefore, in each of two groups of patients it is desirable to get rid of influence of such extraneous casual factors by extracting among each of groups a central compact "kernel" of highly consistent measurements. In connection with the representative statistical volume of available samples, such selection of compact "kernel" is considered to be possible to perform.

For this purpose we used the method, which was proposed previously [4] and showed its effectiveness for a similar task as well as for differentiation of

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functional states [3]. Its essence is calculation of the average correlation of PS of each subject with profiles of synchrony of all other subjects. This average correlation estimates the average personal consistency of topographic distribution of EEG synchrony on scalp. As a result, a growing sequence of such estimates (rank-ordered sample) is formed. Using this chart we select subjects, averaged correlations of which exceed 0.4-0.5 and number of which is not less than 50% of original sample.

Since our analysis is carried out in 5 frequency domains, in order to perform the abovementioned selection, the estimates should be used that averaged over 5 domains. In the variational series for F20 and F21 groups (Fig.1a) we can see the presence of outliers and of several subgroups of different degree of consistency. Fig.1b presents variational series of highly consistency subgroups of F20, F21 and N subjects. The fact draws the attention that N subgroup is characterized by less averaged consistency (0.5) compared with F20 and F21 subgroups (0.52 and 0.55, respeptively). This confirms the conclusion [15] that a sample from a less representative general population related to a particular type of pathology turns out to be more consistent than a sample from a much larger population related to psychological norm, or in other words according to winged expression: every "healthy" man is healthy in its own way but every "sick" one is sick alike.

It is necessary to emphasize, that in this study not only the usual problem of differentiation of norm and pathology was considered, but at the same time also the non-depicted earlier in literature more complex task of detection of subtle differences between the two close nosology. Such a formulation of the task proves advisability and necessity for the following analysis of use of the highly consistent EEG records (Fig.1b): ***(1)*** F20 subgroup included 23 patients in age of $11.2 \pm 2.1$; ***(2)*** F21 subgroup included 41 patients, in age of $12.2 \pm 2$. As anyone can see, the selected subgroups reproduce the age ratio of initial groups in a well-balanced way, and on this basis they are also quite suitable for the further analysis.

In a case of larger volume of experimental data the second stage could be completed of the source material purification, which consists in removal of records, synchrony profiles of which contain two or more values exceeding three standard deviations. A simple statistical calculation shows that probability of occurrence of such a "complex" outlier among 36 variables of synchrony profile is 0.054.

**Discriminant classification**

The results of some our researches, in particular [3, 4], have shown that linear discriminant classification of groups of subjects corresponding to different nosology, therapeutic treatment, functional states, social, age and sexual categories is the effective primary indicator of prospectivity of a further research. If such a classification of originally specified groups gives a significant number of errors (over 20-30%), then such groups are slightly differing by their EEG indicators or are strongly internally heterogeneous, and if so further detailed analysis of their differences is as a rule unproductive.

The results of the classification are given in Table 1. Let us note the following: ***(1***) θ domain provides the lowest (on average) percentage of classification errors, which confirms the previous results [4]; ***(2)*** β-2 domain is the next one by its discriminant sensitivity; ***(3)*** association of indicators of these two frequency domains gives the exact classification of three groups; ***(4)*** presence of small errors of classification shows that: ***(a)*** the performed selection of subjects assured sufficient consistency of each pathology group; ***(b)*** a detailed analysis of intergroup differences promises fruitful results.

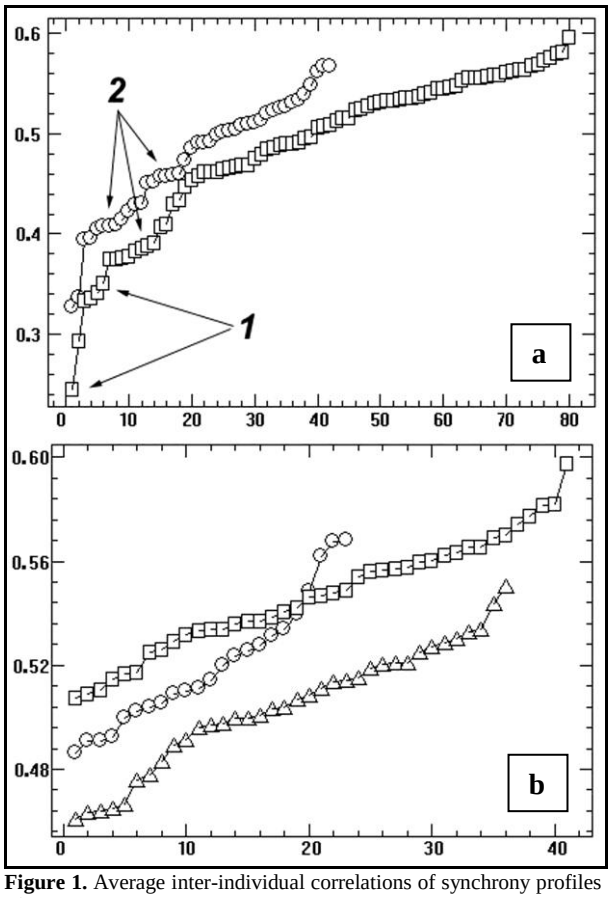


**Figure 1.** Average inter-individual correlations of synchrony profiles of EEG records **(vertical)** in its ascending order **(horizontal)**: **(a)** all records of F20 and F21 groups; circles, F20 group; squares, F21 group; **(b)** highly consistent records of F20 and F21 groups, and all records; triangles, N group; ***(1)*** outliers, ***(2)*** less harmonized subgroups.

**Table 1.** Errors of discriminant classification (in percentage) between the norm and the pathology (F20 + F21 ↔ N) and between two pathology categories (F20 ↔ F21) depending on a frequency domain

| Frequency domain | $F20_m + F21_m \leftrightarrow N_m$ | $F20_m \leftrightarrow F21_m$ | Mean value |
|---|---|---|---|
| δ | 4 | 7 | 5.5 |
| θ | 4 | 3 | 3.5 |
| α | 2 | 10 | 6 |
| β-1 | 3 | 10 | 6.5 |
| β-2 | 1 | 9 | 5 |
| θ + β-2 | 0 | 0 | 0 |
| Mean value | 2.3 | 6.5 | |

The obtained results favorably differ from a number of alternative approaches using other indicators and more sophisticated methods for classification by normal and schizophrenic patterns of EEG, where the number of errors makes: 23% [19], 12.5% [20], 5.5-13.5% [21], 25-28.2% [22], 18.6% [23]. Only in the study by Kaplan *et al* [24] the accuracy of classification has been achieved close to 100%, however, the revealed there set of rules was able to achieve a unidirectional separation of schizophrenia from the norm, but not vice versa.

It is also interesting to compare these results with discrimination by usage of spectral estimations. Let's restrict ourselves to θ domain which is the best one for minimizing errors. The usage of spectrum amplitude averaged in frequency domain [mcV] gives **'(9 + 25) / 2 = 17%'** classification errors in average (9%, 25% and 17% correspond to three columns of Table 1); a usage of averaged power estimates [$mcV^2$] gives **'(15 + 29) / 2 = 22%'** errors; the logarithm of power [$2\log(mcV^2)$] often used in studies gives **'(10 + 22) / 2 = 16%'** errors. This once again confirms the above given conclusion on the higher discriminating sensitivity of EEG synchrony estimates.

### Local relations of synchrony

In order to determine directions and prospects for further analysis it is necessary, first of all, to examine the overall detailed picture of relations of EEG synchrony between normal and pathological groups. For each of three groups we compute the average values of synchrony in each derivation pair and scrutinize intergroup ratios of greater/lesser synchrony (Figs.2&3)

At the topograms in Figs.2&3, first of all, our attention is drawn to the crosswise area of sharp decrease in synchrony of pathology groups ("downfall") in comparison with the norm, including sagittal inter-hemispheric and axial-central segments. It's possible that this indicates significant violations of inter-

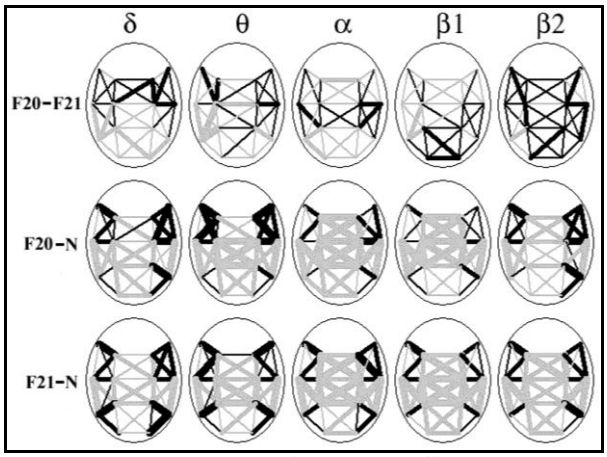


**Figure 2.** Topographic maps of intergroup differences (compared groups are designated at the left) in averaged synchrony for all derivation pairs in 5 frequency domains (specified at top). **Black lines** specify the more high synchrony in the first of two compared groups; **gray lines**, the smaller synchrony; **three gradation of lines thickness** specify the absolute difference in averaged synchrony (ΔS) between two compared groups as it increases: ΔS < 0.05; ΔS < 0.1; ΔS > 0.1.

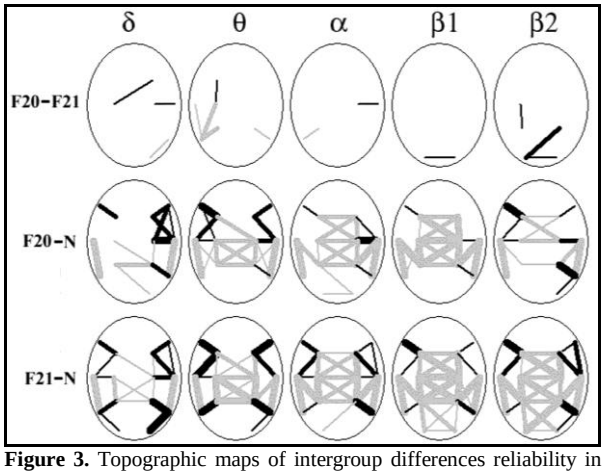


**Figure 3.** Topographic maps of intergroup differences reliability in averaged synchrony for derivation pairs in 5 frequency domains. **Three gradation of lines thickness** specify the significant level of null hypothesis: 0.01 < P < 0.05; P < 0.01; P < 0.001. Other notations are similar to Fig.2.

hemispheric and frontal-occipital relationships at disorders of schizophrenic spectrum. At comparison of two pathology groups (F20-F21), in many frequency domains, we also observe distinctive regional and inter-hemispheric areas of increase/decrease of synchrony.

Due to observed regional structure of intergroup synchrony relations with a purpose of identification of statistically significant patterns it is more appropriate now to consider separately inter-hemispheric and averaged regional intra-hemispheric ratios.

### Interhemispheric synchrony

For each group and each frequency domain there were calculated average values of synchrony between

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derivations *F3-F4*, *C3-C4*, *P3-P4* and *O1-O2*. The results are presented in Fig.4. From comparison of the charts and the statistical distinctions, first of all, it should be noted that;

***(1)*** In most cases, there can be observed a reduction of synchrony in **'center → vertex → occiput'** direction. Jonckhreere test, which takes an orientation of factor effect into account, reveals the existence of such trends at $P = 0.03\text{-}10^{-7}$ for all groups and domains (except for F20 group in β-2 domain). The reduction of synchrony in **'front → center'** direction is observed for all groups in α domain ($P = 0.0002\text{-}10^{-7}$) and for pathology groups also in θ domain (P = 0.016-0.0012). This conclusion coincides with the results of Borisov *et al* [5].

***(2)*** In most cases (68% from 40 comparisons, P = 0.04-0.0004) there is observed the higher synchrony in N group in relation to F20 and F21 groups, and in 23% cases this ratio is manifested in a form of trend of mean values. This conclusion coincides with the results of Borisov *et al* [5] and Strelets *et al* [6] being opposite to some fragmentary conclusions [10, 11]; the latter ones however are distinguished by statistically small volumes of samples included 8 and 11 patients.

***(3)*** Local differences between F20 and F21groups are observed only in *O1-O2* occipital pair in β-1 (ð = 0.04) and β-2 (ð = 0.03) domains, and in both cases, the synchrony values for F20 group do not differ from the norm (ð = 0.46), but for F21 group these values are significantly lower (P = 0.043).

***(4)*** However, in Fig.4 for F20 and F21 groups in sagittal neighboring derivation pairs we see systematic differences between them that the analysis of variance allows to reveal when the second factor is regional one (2 factor gradations): ***(a)*** increase of synchrony in F21 group (with the convergence to the norm) in *F-C* region in θ domain (P = 0.00005); ***(b)*** increase of synchrony in F20 group (with the convergence to the norm) in *F-C* region in β-1 (P = 0.00001) and β-2 (P = 0.004) domains.

***(5)*** For differences between front-occiput regional synchrony (*F-O*) there is only one distinction between F20 and F21 groups in β-1 domain (P = 0.01)

**Regional intra-hemispheric differences**

For each group and for each frequency domain there were calculated average values of synchrony for six regions: for the left and right frontal regions ($F_L$ and $F_R$, respectively) comprising the values of synchrony between *F7*, *F3*, *T3*, *C3* and *F8*, *F4*, *C4*, *T4* derivations; for the left and right central ones ($C_L$ and $C_R$) including synchrony between *T3*, *C3*, *T5*, *P3* and *C4*, *T4*, *P4*, *T6* derivations; for left and right occipital ones ($O_L$ and $O_R$) including synchrony between *T5*, *P3*, *O1* and *P4*, *T6*, *O2* derivations. The results are presented in Fig.5.

From comparison of the charts and shown statistical differences, first of all, it should be noted that;

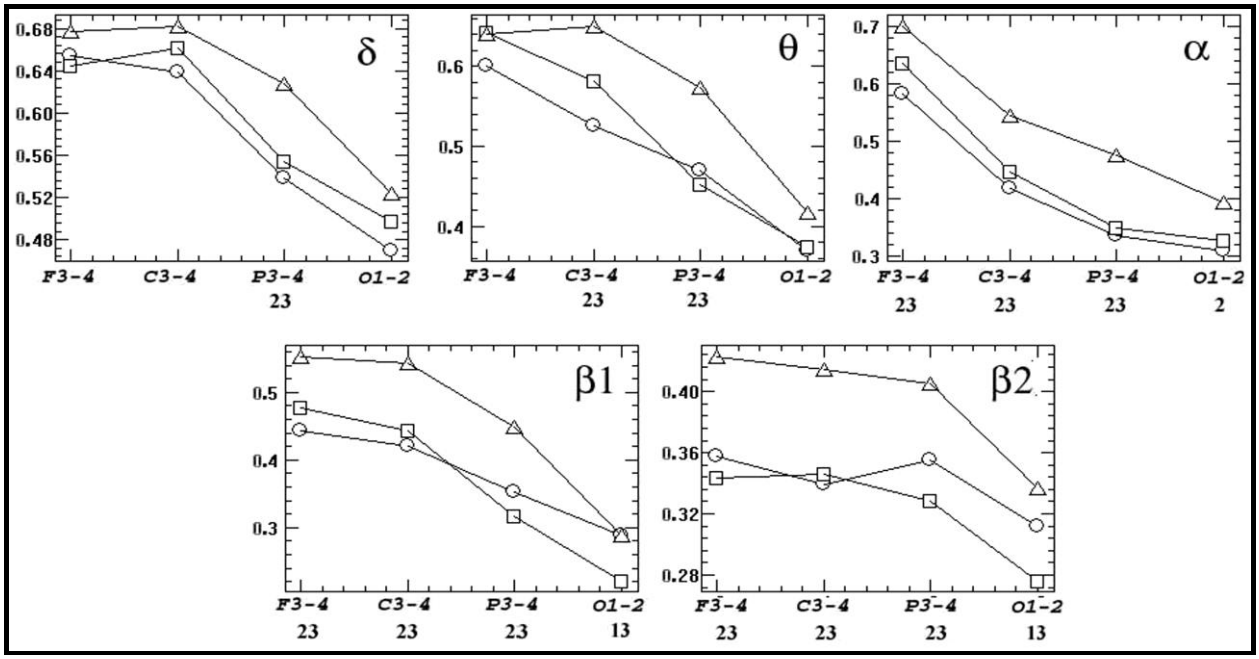


**Figure 4.** Differences in inter-hemispheric synchrony for 5 frequency domains (P = 0.04-0.0004). The values averaged for each group synchrony (vertical axes) are shown for derivation pairs: *F3-F4*, *C3-C4*, *P3-P4* and *O1-O2* (horizontal axes). **Group markers**: **circles**, F20; **squares**, F21; **triangles**, N. **Below graphics**, the designation of reliable intergroup differences is shown in number notation: **1**, F20-F21; **2**, F20-N; **3**, F21-N.

***(1)*** In N group there is observed: ***(a)*** approximate equality of synchrony in frontal-central $F_L$, $F_R$, $C_L$, $C_R$ regions (except its decrease in α domain, P = 0.02-0.0007); ***(b)*** reduction of synchrony in the occipital $O_L$, $O_R$ area (P = 0.048-$10^{-5}$, except β-2 domain).

***(2)*** In F20 and F21 groups it is observed a sharp decrease of synchrony in central region compared with frontal and occipital ones. In most cases the differences between $F_L$-$C_L$, $F_R$-$C_R$, $C_L$-$O_L$, $C_R$-$O_R$ manifest itself with high confidence (76% reliable differences from 50 comparisons, P = 0.033-$10^{-8}$).

***(3)*** Synchrony in N group compared with F20 and F21 groups is as follows: ***(a)*** it is significantly higher in central region (95% reliable differences from 20 comparisons, P = 0.01-$10^{-7}$), which coincides with the results of Borisov *et al* [5], Strelets *et al* [6] and Winterer *et al* [9]; ***(b)*** in some cases it is lower in frontal and occipital regions (30% reliable differences from 40 comparisons, P = 0.049-0.001), which partially coincides with the results of Mann *et al* [12], Merrin *et al* [10], Strelets *et al* [6] and Wada *et al* [13].

***(4)*** Local intraregional differences between F20 and F21 groups are detected in $C_L$ and $O_R$ regions in θ domain (P = 0.04) and in $O_L$ region in α domain (P = 0.047). Additionally, in Fig.5, the macro regional intergroup differences (for both hemispheres) are also observed, and analysis of variance allows to reveal those differences in case that as a second factor we use left and right regions: ***(a)*** reduction of synchrony in F20 group in occipital $O_L$-$O_R$ area in δ (P = 0.007), θ (P = $10^{-6}$) and α (P = 0.0002) domains with its convergence to the norm and increase of synchrony in central $C_L$-$C_R$ area in β-2 domain (P = 0.008); ***(b)*** reduction of synchrony in F21 group in frontal $F_L$-$F_R$ area in β-2 domain (P = 0.004) with its convergence to the norm.

***(5)*** Comparing of the difference between frontal synchrony and occipital one reveals differences between F20 and F21 groups in δ domain for $F_R$-$O_R$ remainder (P = 0.03) and for remainders between $F_L$-$O_L$ (P = 0.02) and $F_R$-$O_R$ (P = 0.03) regions in θ domain.

**Regional asymmetry**

Visually, in Fig.5 we can note some signs of right-sided asymmetry; most distinctly they appeared in F20 and F21 groups. Statistical comparison of mean values for left and right regions reveals the presence of right-sided asymmetry (P = 0.048-0.007) in occipital $O_L$-$O_R$ area for F20 group in δ, α and β-2 domains and for F21 group in α, β-1 and β-2 domains, and also in central $C_L$-$C_R$ area for F20 and N groups in β-1 domain. Differences in asymmetry coefficient calculated by the formula **'(L x R) / (L + R)'** are detected in central $C_L$-$C_R$ area in θ domain (P = 0.035-0.018) between F20 and N groups and between F20 and F21 groups.

On the one hand, these asymmetries are not that numerous so to indicate a general pattern; on the other hand, no case of asymmetry is revealed in N group.

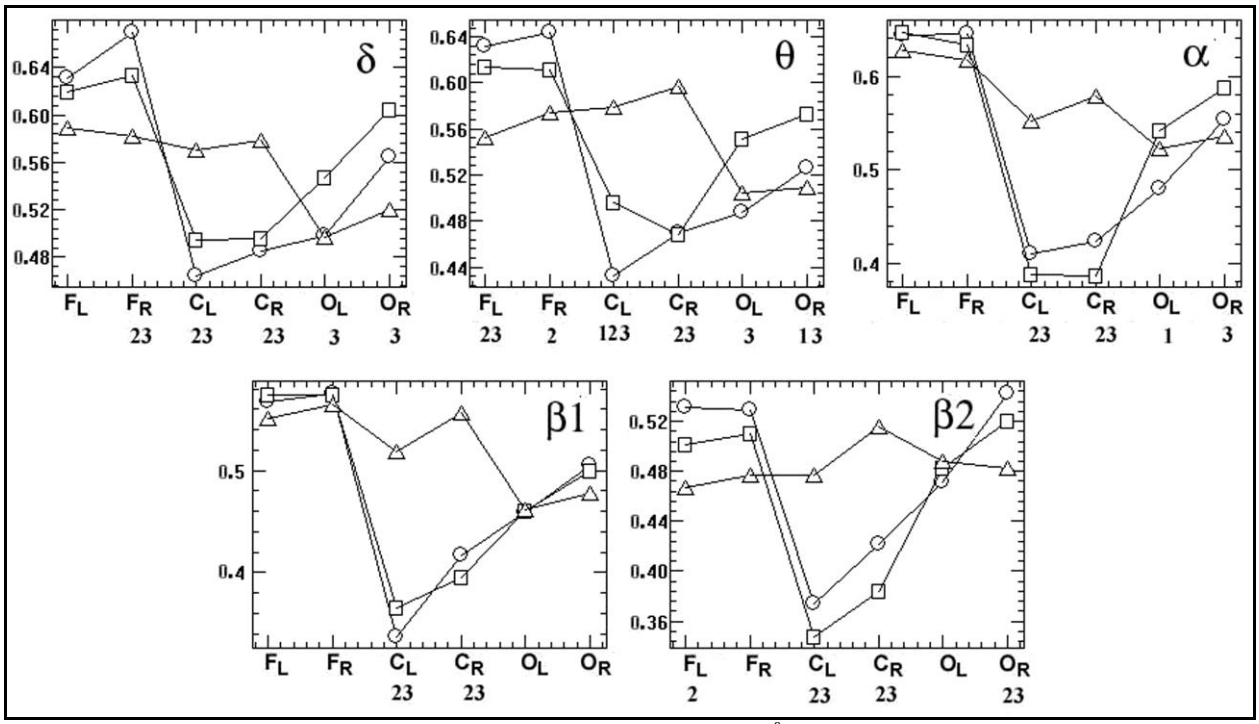


**Figure 5.** Regional intra-hemispheric differences in frequency domains (P = 0.033-$10^{-8}$). The averaged values of synchrony for each group (vertical) in order of regions (horizontal): $F_L$, $F_R$ (frontal left and right), $C_L$, $C_R$ (central left and right), $C_L$, $C_R$ (occipital left, right); other notations are similar to Fig.4.

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**Age and sex differences**

In order to identify age-related differences we divide each group into two subgroups in age ranges of 8-11 and 12-15 years (the respective number of subgroups is 16 and 7 boys for F20 category, 16 and 20 boys for F21, category and 22 and 19 boys for N category). Now let's make a comparison of these subgroups. The results are presented in Table 2, from a consideration of which we can make the following conclusions:

***(1)*** In all detected cases, the differences are associated with an increase in synchrony with age, and this indicates a presence of systematic tendency;

***(2)*** Intraregional changes of synchrony are most representative in N group and intra-hemispheric ones in F20 group;

***(3)*** In a case of the pair comparison of three N, F20, F21 groups, the most of changes in synchrony topographically do not coincide, except for following cases: in α domain in $F_R$ region for N, F21 groups, in β-1 and β-2 domains in $C_L$ region for N, F20 groups, in α domains for *C3-C4* derivation pair for F20, F21 groups and for *P3-P4* derivation pair for N, F20 groups;

***(4)*** If we compare the results of Table 2 with the charts at Figs.4&5, then the convergence of EEG synchrony with the age to the norm is observed in pathology groups in inter-hemispheric connections predominantly in α domain, whereas as for relative intra-hemispheric relations, the situation is reversed: in $C_R$ region differences increase and in $F_L$, $F_R$, $O_R$ regions the higher synchrony observations are leveled in pathology groups in relation to norm.

Revealed age differences may indicate an identification feasibility of the differences between norm and pathology within specific age categories in a case of presence of much more voluminous experimental material.

The scope of this article do not allow to consider our available results of analysis of female adolescents, topography of distribution of EEG synchrony of which in control and pathology groups has a number of significant local differences and yet maintains the marked phenomenon of cross−shaped "downfall" in EEG synchrony at pathology. However, it certainly indicates that such studies should be performed with taking the gender into account.

**Comparison with psychometric measures**

For assessment of cognitive functions of patients, violation of which is one of the main consequences of schizophrenia, the following four psychometric indices were used:

***Volume of direct reproduction (VDR);*** defined by the technique of memorization of 10 words under verbal presentation (developed by A.R. Luria in 1962). This technique is intended to assess the status of voluntary verbal memory, fatigue, activity of attention, storing, preservation, reproduction, voluntary attention, *etc*.

***Volume of simple and difficult paired associates (VSA, VDA)/paired-associates learning (PAL);*** this technique is intended to study the memory and memory processes;

***Runtime of Schulte tables execution (TS);*** this technique is applied to research a rate of sensorimotor reactions and characteristics of attention, level of intellectual working capacity.

**Table 2.** Authentic age changes in intra-hemispheric and inter-hemispheric EEG synchrony in frequency domains δ, θ, α, β-1 and β-2. Remainders are represented between average values of synchrony in subgroups of 8-11 and 12-15 years old; the significance values are shown in brackets.

| Group | Localization | δ | θ | α | β-1 | β-2 |
|---|---|---|---|---|---|---|
| **F20** | $F_R$ | | | | 0.13 (0.01) | 0.14 (0.01) |
| **F21** | $F_L$ | | | 0.1 (0.005) | | |
| **F21** | $C_R$ | | | 0.07 (0.01) | | |
| **N** | $F_L$ | 0.09 (0.02) | 0.11 (0.001) | 0.09 (0.02) | | |
| **N** | $F_R$ | 0.1 (0.01) | 0.1 (0.004) | 0.12 (0.001) | 0.05 (0.04) | 0.07 (0.01) |
| **N** | $C_R$ | 0.1 (0.01) | 0.07 (0.02) | 0.09 (0.03) | 0.06 (0.02) | 0.08 (0.01) |
| **N** | $O_R$ | 0.1 (0.006) | | | 0.09 (0.002) | 0.08 (0.01) |
| **F20** | *F3-F4* | | | | 0.13 (0.03) | 0.1 (0.01) |
| **F20** | *C3-C4* | | | 0.15 (0.047) | | 0.08 (0.04) |
| **F20** | *P3-P4* | | 0.1 (0.04) | 0.17 (0.02) | | |
| **F20** | *O1-O2* | 0.16 (0.02) | | | | |
| **F21** | *F3-F4* | | | 0.1 (0.01) | | |
| **F21** | *C3-C4* | | | 0.11 (0.04) | | |
| **F21** | *O1-O2* | | | 0.12 (0.006) | | |
| **N** | *P3-P4* | | | 0.1 (0.01) | | |

Between these indices for both groups of patients there were found no significant correlations (except VDA and TS, P = 0.49), which indicates that there is no strong functional dependencies between those indices for analyzed samples of patients.

The proximity of estimates of EEG synchrony to psychometric indices was assessed by Pearson correlation coefficient *r* critical value of which for those samples is $r_{cr} < 0.31$ at P = 0.05. Fig.6 shows the identified significant correlations with local estimates of EEG synchrony between derivation pairs in the range of average and above average correlation values ($r = 0.45$-$0.75$, P = 0.03-0.008). In addition, it is interesting to calculate correlations with the average estimates of regional intra-hemispheric synchronities as well as of differences between them that characterize the magnitude of decrease of EEG synchrony in $C_L$, $C_R$ regions in relation to neighboring $F_L$, $F_R$, $O_R$, $O_L$ regions. These correlations are presented in Table 3. The received results allow making the following conclusions:

***(1)*** The greatest number of significant correlations with the psychometric indices is revealed for F20 group (25 *vs* 9 for F21 group); it is quite consistent with the fact that for schizophrenia category (F20) the violations of cognitive processes estimated by these psychometric indices are more expressed.

***(2)*** The greatest number of significant correlations belongs to "downfall" of synchrony for pathology groups in central axial area and to its remainders with with neighboring regions: 19 significant correlations against 11 for other areas and derivation pairs.

***(3)*** In rank-order of total numbers of significant correlations, the frequency domains are ranked as follows: β-2 = 11, θ = 9, α = 9, δ = 4 and β-1 = 4 correlations. With respect to local correlations (Fig.6) β-2 and θ domains have the obvious advantage as well as in a case of discriminant classification; the leading place of β-2 domain can be determined by its greater relationship with cognitive activity.

***(4)*** In rank-order of significant correlations, the psychometric indicators are ranged as follows: VDA = 13, VSA = 11, TS = 10 and VDR = 8 correlations. According to average value of correlations, the TS index has a considerable advantage ($r = 0.7$) in comparison with VDR (0.49), VSA (0.48) and VDA (0.5). The last would seem to indicate that in F20 group (which shows the most number of correlation), the features of attention and mental performance are more vulnerable compared with the capabilities of memorizing and reminiscence

***(5)*** Signs of correlations for VDA, VSA are opposite to ones for TS, which corresponds to their psychometric ratio.

***(6)*** In high-frequency domains (β-1, β-2) compared with mid-frequency domains (θ, α), in most cases there are inversion of signs of correlations, which can be a result of opposite relationship between the activity of these domains and the cognitive abilities.

Let us note that in recent years we can see an increasing interest of researchers to comparison of different estimates of EEG synchrony on one hand and psychometric and syndromic indicators of schizophrenic spectrum disorders on the other hand. These studies reveal the following significant correlations: 0.36-0.52 [25], 0.27-0.39 [26], 0.37-0.82 [27] for a small group of 14 patients, 0.37-0.55 [28], 0.38-0.49 [29]. In this comparison, the numerous received by us significant correlations between EEG synchrony estimates and psychometric indices in a range of 0.45-0.75 look rather perspective.

**Reproducibility of results**

In order to test the stability of our results obtained on the basis of the here stated methodology, we analyzed another EEG data which has been recorded in 2001-2004 and discussed earlier [5]. Two groups of male adolescents 10-12 years old include: F20 group of 18 patients (in age range of 12.1 ± 0.93) and control group of 25 pupils (in age range of 12.1 ± 0.53). The results turned out to be similar to Figs.4&5; they are shown in

**Table 3.** Correlations between psychometric measures with intra-hemispheric regional synchrony and with remainders between regional synchrony for frequency domains δ, θ, α, β-1 and β-2.

| Indice | δ | θ | α | β-1 | β-2 |
|---|---|---|---|---|---|
| **VDR** | | -0.41 $F_R$-$C_R$ | -0.53 $F_R$<br>-0.50 $F_R$-$C_R$ | | |
| **VSA** | | | -0.50 $F_R$-$C_R$ | | |
| **VDA** | -0.47 $O_L$-$C_L$ | -0.43 $F_R$-$C_R$ | | -0.54 $O_L$-$C_L$ | -0.47 $O_R$-$C_R$ |
| **TS** | -0.56 $C_L$<br>-0.47 $C_R$ | | -0.52 $F_R$<br>-0.57 $C_L$<br>-0.49 $C_R$<br>+0.48 $O_R$-$C_R$ | | |

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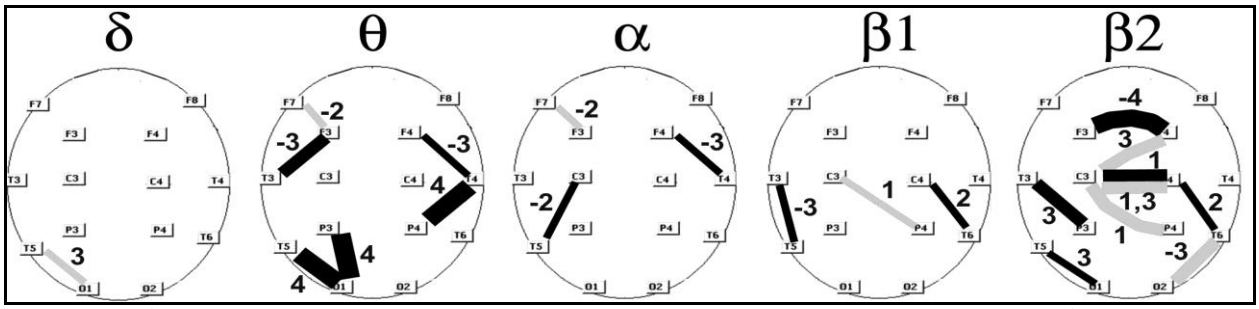


**Figure 6.** Significant correlations between synchrony estimates and psychometric measure (P = 0.03-0.008) with the following numbering notation: **(1)** volume of direct reproduction by technique of memorization of 10 words under verbal presentation; **(2)** volume of simple binary associations; **(3)** volume of complex binary associations; **(4)** runtime of Schulte tables execution. Color of lines indicates the group of patients: **black** F20, **gray** F21; **three grades of lines thickness** indicate the absolute value of correlations: 0.45-0.49, 0.5-0.59, 0.6-0.75. The figures at lines indicate the numbering notation of psychometric measure; **minus** indicates a negative correlation.

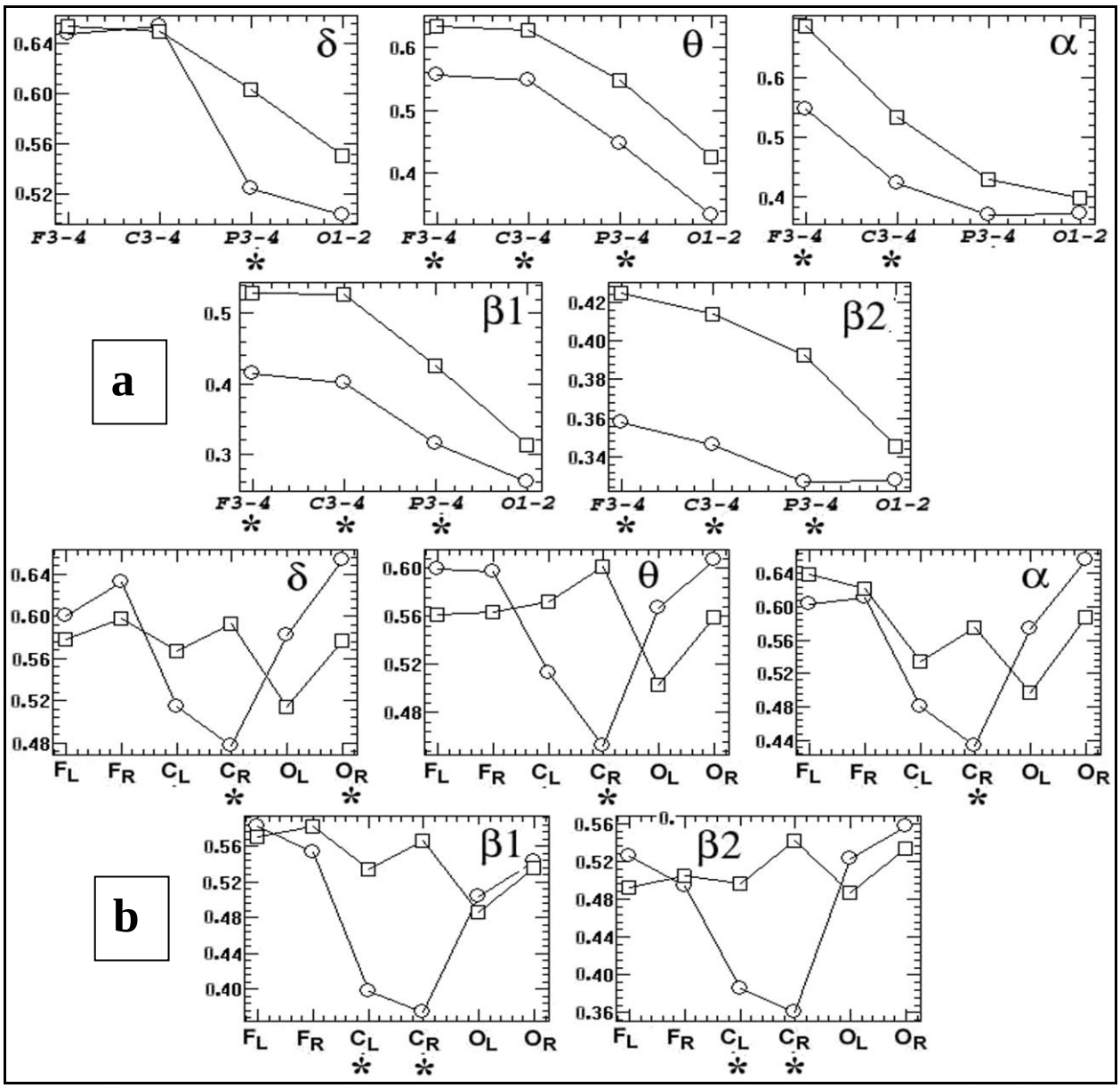


**Figure 7.** Differences between synchrony for N and F20 group records as discussed earlier [5]; asterisks denote cases of significant group differences (P = 0.47-$10^{-5}$). **(a)** Inter-hemispheric synchrony, **(b)** regional intrahemispheric synchrony. Remaining notation is similar to Figs.4&5.

Fig.7 (identified significant differences showed $P = 0.047\text{-}10^{-5}$). As you can see, these charts are in good agreement with Figs.4&5 with the exact reproduction of the phenomenon of cross-shaped "downfall" in EEG synchrony for F20 group. The separate and not numerous distinctions can be a consequence of narrower age range of the used groups. The discriminant classification gives an unmistakable separation of normal and pathological groups in all frequency domains. Thereby, the ACS-method possesses sufficient accuracy and stability, yielding almost identical results on various groups of examinees and patients.

## DISCUSSION

The results of our complex analysis reveal the complicated picture of regional, inter-hemispheric and age differences in EEG synchrony between two disorders of schizophrenic spectrum and the norm, including interchanging cortical areas with oppositely directed ratios of higher, lesser or equal synchrony. Apparently, this is caused the apparent inconsistency of fragmentary results obtained by other researchers as noted in the introduction. Disclose of complete picture of EEG synchrony relations in these studies could be prevented by: ***(a)*** uncertainties of coherent analysis [15]; ***(b)*** small volume of experimental data [10, 11, 27]; ***(c)*** absence of selection of EEG records on consistency; ***(d)*** absence of separation of groups according to nosological type, age and sex. However, many of particular conclusions of other researchers find their counterparts in the considered complex picture: local cases of increase of intra-hemispheric coherence in schizophrenics [6, 10, 12, 13], its decline in central region [5, 9], reduced inter-hemispheric synchrony [5, 6], a violation of frontal-temporal relationships [7].

One of distinctive and stable components of above considered picture of mental disorders in comparison with the norm is the presence of the vast areas of low synchrony separating isolated intra-hemispheric (frontal and occipital) areas with synchrony near to normal level. The presence of such a reduction and detection of right-sided asymmetry can indicate a substantial violations of inter-hemispheric and frontal-occipital relationships for schizophrenic and schizotypal disorder, which fits into framework of the well-known theory of disintegration of cortical electrical activity [30, 31] ascending to Bleuler's studies (1911, 1913). Apparently, in schizophrenic process, a tendency to disintegration comprises cortical neuronal substrate at different levels, *i.e.* from local neuronal ensembles to spatially separated neural networks, which causes serious disturbances in their interaction [5]. It is considered that one of direct consequences of this disintegration is represented by observed violations of cognitive and behavioral functions at patients with schizophrenic disorders.

Our additional task of differentiation of two closely related F20 and F21 categories among the block of disorders of schizophrenic spectrum is especially complicated because among experts there is still no consensus on a safe separation criteria for schizophrenia and schizotypal disorder [18]. The significant differences between F20 and F21 groups appear mainly in frontal and occipital areas in certain frequency domains. With this in occiput an inter- and intra-hemispheric synchrony for schizophrenia (F20) in some cases was closer to normal, whereas for schizotypal disorder (F21) intra-hemispheric synchrony is higher than normal, but inter-hemispheric synchrony is below than normal. Certain relationships of this kind are also observed in parietal, temporal and central areas. Apparently, this is due to the fact that criteria of schizotypal disorder includes, in particular, the presence of unusual phenomena of perception including somatosensory, auditory and visual illusions or hallucinations, and as a result there can be more drastic deviations of EEG synchrony from the norm in areas of primary projection of corresponding analyzers.

On the other hand, in frontal and some central cortex areas in F20 group there are observed greater deviations of inter-hemispheric and intra-hemispheric synchrony estimates from the norm than in the case of schizotypal disorder. This is consistent with concept of greater safety of frontal cortex at patients of F21 categories [18]. It is significant that most such deviations in intra-hemispheric synchrony manifest themselves in β-2 domain, whose activity is directly related to cognitive activity, and namely violations of cognitive processes are most typical just for schizophrenia pathology [18].

We note also that most of patterns on charts like Figs.2-5 also appear when we analyze full amount of data (125 patients), but the performed selection of highly consistency subgroups (64 patients) improved considerably the reliability of conclusions about observed differences. Moreover, re-calculating of previous EEG records [5] by the here used methodology confirms all the above mentioned inter-hemispheric and regional relationships with high numerical accuracy. That proves the stable reproducibility of results in different groups of patients by the use of ACS-method.

These results demonstrate the high efficiency of ACS-method in differentiation of normal examinees from patients of different mental disorders by EEC and according to its classifying efficiency θ and β-2 frequency domains have noticeable advantage. It should also be emphasized that the efficiency for classification of θ domain was found in our previous

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paper [4]; in the same paper there was shown an advantage of ACS-method in comparison with other methods of classification and with other EEG indices.

The present study also showed that for the reliable differentiation on EEG of various subcategories within such the complex and multidimensional nosology as psychiatric disorders of schizophrenic spectrum, it is necessary to use: ***(1)*** a bigger volume of experimental data than it takes place in most cited studies: ***(2)*** separate study of different nosology, age categories and sexual groups; ***(3)*** preliminary extraction of highly consistent EEG records for elimination of extraneous factors influence.

Apparently the real progress towards development and implementation of efficient numerical methods for differentiation of norm and various forms of mental pathology by EEG is possible upon condition of international cooperation and coordination of researches. It also requires a formation of an integrated bank of EEG records from the data of various research and clinical centers differentiated by separate nosology, functional states, sex, age and other characteristics. One of possible mechanisms for this integration may be obligation to upload EEG records in standard European Data Format (EDF) in such a bank and do it for all articles published in leading scientific journals. In addition, such publicly-accessible bank will make the results and theoretical conclusions of EEG studies to be the falsifiable in sense of Karl Popper. For the purification of such a bank from influence of extraneous random factors a technique can be used similar to above discussed extraction of highly consistent EEG records.

Considered multidimensional results on distinctions of the norm and two groups of deviations of schizophrenic spectrum confirms in particular; ***(a)*** the revealed numerous significant correlations of EEG synchrony estimates with psychometric indices, ***(b)*** the high classifying sensibility of the used ACS-method, near 100% reliability, and ***(c)*** the reproducibility of results for different groups of patients and examinees. All this shows that EEG correlation synchrony measures can be perspective for the use as auxiliary quantitative estimates (in addition to ranking expert estimates) at diagnostics of mental deviations of schizophrenic spectrum.

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**Original Article**

# Sweating and thirst perception in premenopausal, perimenopausal and postmenopausal women during moderate exercise

**Emmanuel Amabebe[1,2], Sonia I. Omorodion[2], Janet O. Ozoene[2], Andrew C. Ugwu[2], Leonard F. Obika[2]**

*[1]Physiology Department, Niger Delta University, Wilberforce Island, Bayelsa State;*
*[2]Physiology Department, University of Benin, Benin City, Edo State;* ***Nigeria***

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**Corresponding Author**
Emmanuel Amabebe
Department of Physiology,
Niger Delta University,
Wilberforce Island
Bayelsa State, Nigeria
emmanuelamabebe@yahoo.com

**Key Words**
Exercise, Menopause,
Sweating, Thirst perception

**Abstract**

***Objective:*** We examined the sweat rate (SR), sweat volume (SV), sweat sodium concentration ($S[Na^+]$) and changes in thirst perception (TP), in premenopausal (preM), perimenopausal (periM) and postmenopausal (postM) women after moderate exercise.

***Methods:*** Thirty healthy women comprising preM (22.5 ± 0.8 yrs, n = 10), periM (46.5 ± 1.1 yrs, n = 10) and postM (52.2 ± 0.9 yrs, n = 10) participated in the study. All participants gave informed consent. TP was rated using the visual analogue scale. Sweat was obtained with sweat suction apparatus from a 120 $cm^2$ circular area marked on the skin of the face and neck, after moderate exercise, *i.e.* a 15 min walk on the treadmill at a speed of 4.2 km/h at 27°C. The start time was noted, SR, SV and $S[Na^+]$ were determined.

***Results:*** Exercise induced a significant change in TP in all groups, of which the postM women exhibited the lowest thirst ratings. Although there was no significant difference in SV and $S[Na^+]$ between the groups, the periM women showed a significantly higher SR compared to both preM and postM groups. A significant and positive correlation existed between TP, SR, SV and $S[Na^+]$ values.

***Conclusion:*** These data indicate that an increase in sweat rate and volume produces a concomitant increase in thirst perception in women during moderate exercise, but sodium loss is reduced.



## INTRODUCTION

Menopause is defined as the cessation of menstruation resulting from loss of ovarian follicular activity and a decline in estrogen secretion by the ovaries. It occurs at an average age of 51.4 years, though may vary between 40-58 years of age. The menopausal transition (perimenopause) with a median age of 47 years is a period of increased menstrual cycle irregularities or skipped menses and concludes with the final menstrual period. Postmenopause begins at that time, although it is not recognized until after 12 months amenorrhea [1-4].

Sweating and increased thirst are common symptoms of menopause and are also important factors during exercise [5-9]. Excessive sweating, including night sweats and hot flushes are the most common menopausal vasomotor symptoms occurring in women of all ethnic groups [10]. They are possibly due to changes in the central nervous system related to estrogen withdrawal [11-14], and the consequent burst of gonadotropin-releasing hormone (GnRH) which acts on GnRH receptors in the septal area of the hypothalamus eliciting thermoregulatory skin vasomotion [15]. Elevated brain norepinephrine acting on central $\alpha_2$-adrenoceptors has also been shown to be involved in narrowing the thermoneutral zone and initiating hot flushes in the absence of the modulatory effect of estrogen on brain adrenergic receptors [16]. These vasomotor symptoms occur in about 74% of

menopausal women [17] and more frequently in perimenopausal women (88%) [18-20].

Loss of water and electrolytes, such as sodium in sweat, is an important factor during exercise, especially in hot weather [21]. Sodium is the most important sweat electrolyte with an average concentration of 0.9 g/l [22]. This is higher in premenopausal women than in perimenopausal and postmenopausal women due to the increase in salt and water retention and renin-angiotensin-aldosterone stimulation during the menstrual cycle [23-25]. These studies contrast with an earlier report that perimenopausal and postmenopausal women have higher sodium concentration than premenopausal women [26]. Several factors including sweat rate and heat acclimation of the individual influence the solute content of sweat [27], *e.g.* human sweat sodium concentration decreases with heat acclimation [28, 29].

Sweat rates vary between 0.7-1.4 l/h (women) and 1.2-2.5 l/h (men) in hot humid conditions. Under normal and hot environmental conditions, females sweat less than males [30, 31]. Sweat rate is higher in perimenopausal women than in premenopausal and postmenopausal women [1, 32, 33].

Dry mouth and increased thirst are frequently observed among menopausal women and are typically associated with hormonal changes and neurological complications. Estrogen reduces the osmotic threshold for the synthesis and release of arginine vasopressin (AVP) by direct action on hypothalamic receptors or via catecholaminergic or angiotensinogernic neuronal projections to these hypothalamic nuclei [24, 25]. The excessive loss of body fluid that accompanies sweating due to estrogen withdrawal can result in a rise in plasma sodium concentration and osmolality (hyperosmolality), stimulating increased thirst sensation [34-36]. Although menopause is associated with increased thirst, thirst sensation decreases with aging and is significantly impaired in healthy older adults [37-41].

In this study, we examined the association between sweat rate, volume, sodium concentration and thirst perception (TP) in premenopausal (preM), perimeno-pausal (periM) and postmenopausal (postM) women during moderate exercise.

## MATERIALS AND METHODS

### Subjects

Thirty healthy women comprising of preM, periM and postM (n = 10 for each) women, defined according to their ages and menstrual cycle history, participated in the study. All subjects were active but none of them were athletically trained as defined by the absence of a regular exercise program during the last six months before the experiment [42]. Exclusion criteria for this study included any history of smoking, diabetes, musculoskeletal or cardiopulmonary diseases and pregnancy.

### Procedure

Subjects reported to the laboratory on the day of the experiment. Participants were adequately informed of the experimental procedures and consent was obtained. Permission for the use of human subjects for the study was obtained from the Ethical Committee of the University of Benin, Nigeria. A period of 30 min was allowed for acclimatization and questionnaires were issued to obtain medical and menstrual histories. Measurements of some anthropometric parameters were obtained. *e.g.* the height (HT, m) and weight (WT, kg) were measured using a meter rule and digital weighing scale, respectively, and the body mass index (BMI) was calculated using the following formula:

$$\mathbf{BM = WT\ (kg) / HT\ (m^2)}$$

Baseline (resting) blood pressure (BP) was measured in the seated position from the right arm supported at heart level. Measurements were made with the aid of the stethoscope and sphygmomanometer. The average of three consecutive readings was recorded as the normal blood pressure [43].

The pulse rate was obtained from the right radial artery during exercise. The skin and room temperatures were measured with the aid of skin temperature and room temperature thermometers, respectively.

### Thirst rating

Thirst perception (TP, cm) was rated before and after moderate exercise using the visual analogue scale (VAS) [41, 43, 44]. Subjects rated their TP by making a mark across the 10 cm scale, the ends of which were labeled “very thirsty” and “not thirsty”. This was done after adequate instructions on completing the VAS. Thirst responses defined by this method are highly reproducible within an individual [25, 45] and correlate well with the volume of water drunk subsequently [44, 46].

### Sweat sample collection and handling

Before sweat collection, the treadmill was calibrated using the Bruce Treadmill Protocol [47]. Sweat sample was obtained with sweat suction apparatus from a 120 $cm^2$ circular area marked on the skin of the face and neck [48], after a moderate exercise, *i.e.* a 15 min walk on the treadmill at a speed of 4.2 km/h at 27ºC.

Each sweat sample was collected into a sweat collection bottle and preserved at -4°C to prevent evaporation prior to laboratory analysis. Sweat rate (SR) was determined using the formula below [32, 49]:

$$\mathbf{SR = volume\ of\ sweet\ collected\ /\ exercise\ time}$$

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### Statistical analysis

Data are presented as mean ± SEM. Differences in TP changes, SR, sweat volume (SV) and sweat sodium ion concentration ($S[Na^+]$) within and between the groups were analyzed using the Student t-test and one way Analysis of Variance (ANOVA). A linear regression plot and correlation were performed for possible association between TP, SR, SV and $S[Na^+]$. Changes were considered statistically significant when $P < 0.05$.

## RESULTS

Table 1 shows the anthropometric data of all participants. Table 2 presents the TP ratings before and after moderate exercise in the different groups; it was recorded that exercise induced a significant change in TP ($P < 0.05$) especially within the periM subjects, but the postM subjects exhibited the least thirst ratings with no significant change in TP after moderate exercise.

In addition to the change in TP, the periM women had a significantly higher SR compared to the other groups ($P < 0.05$). However, the SV and $S[Na^+]$ did not differ significantly (Table 3).

The relationship between TP and SR, SV and $S[Na^+]$ is shown in Fig.1. A significant ($P < 0.001$) and positive correlation existed between TP *vs* SR, SV and $S[Na^+]$. However, a linear regression plot indicated an inverse relationship (negative correlation) between $S[Na^+]$ *vs* SR and SV as shown in Fig.2.

## DISCUSSION

The mean ages of the subjects in this study are similar to the values of previous reports [1-4, 32], which is critical for comparison between these groups. Our findings indicate that, in women of various ages, moderate exercise induced a significant change in thirst perception especially within the periM subjects. The postM women exhibited the lowest thirst ratings and recorded no significant change in TP after exercise. This supports the report that older adults exhibit diminished thirst sensation [37-41]. Sweat rate was significantly higher in the periM women compared to the other groups. This observation was also reported by Minkin and Wright [32] and Rodstrom *et al* [33], and is due to hot flushes and sweating characteristic of this menopause transition stage caused by the dramatic drop in ovarian estrogen levels and thermoregulatory vasodilatory effect of GnRH [50, 51]. Contributions from elevated sympathetic stimulation via central $\alpha_2$-adrenergic receptors have also been documented [52, 53].

**Table 1.** Anthropometric data

| | Premenopausal women (n = 10) | Perimenopausal women (n = 10) | Postmenopausal women (n = 10) |
|---|---|---|---|
| **Age, years** | 22.5 ± 0.8 | 46.5 ± 1.1 | 52.2 ± 0.9***** |
| **Wight, kg** | 61.8 ± 4.2 | 68.1 ± 3.0 | 72.1 ± 1.9***** |
| **Height, m** | 1.6 ± 0.02 | 1.6 ± 0.01 | 1.6 ± 0.02 |
| **BMI, kg/m$^2$** | 23.7 ± 1.3 | 28 ± 1.2 | 28.4 ± 0.7***** |
| **SBP, mmHg** | 113.4 ± 2.7 | 123 ± 2.1 | 120.2 ± 2.8***** |
| **DBP, mmHg** | 73.7 ± 2.3 | 80 ± 2.6 | 82 ± 2.9 |
| **Pulse rate/min (during exercise)** | 110.8 ± 4.7 | 113.1 ± 1.9 | 114.6 ± 2.2 |
| **Skin temperature, °C** | 36.9 ± 0.2 | 36.2 ± 0.1 | 36.2 ± 0.2 |

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; values are mean ± SEM; *****$P < 0.05$.

**Table 2**. Thirst perception ratings (cm) before and after moderate exercise (mean ± SEM)

| | Premenopausal women (n = 10) | Perimenopausal women (n = 10) | Postmenopausal women (n = 10) |
|---|---|---|---|
| **Before** | 4.49 ± 0.8 | 3.72 ± 0.4 | 2.12 ± 0.3 |
| **After** | 6.55 ± 0.8 | 6.22 ± 0.4***** | 3.11 ± 0.5 |

*****$P < 0.05$

**Table 3**. Thirst perception change, sweat rate, sweat volume and sweat sodium concentration expressed as mean ± SEM

| | Premenopausal women (n = 10) | Perimenopausal women (n = 10) | Postmenopausal women (n = 10) |
|---|---|---|---|
| **TP change, cm** | 2.1 ± 0.5 | 2.2 ± 0.2 | 0.99 ± 0.2***** |
| **Sweat rate, ml/min** | 0.07 ± 0.02 | 0.12 ± 0.01 | 0.06 ± 0.01***** |
| **Sweat volume, ml** | 1.3 ± 0.4 | 1.7 ± 0.2 | 0.9 ± 0.1 |
| **Sweat $Na^+$ concentration, mmol/l** | 125.4 ± 18.7 | 77.2 ± 8.8 | 101.3 ± 10.6 |

*****$P < 0.05$

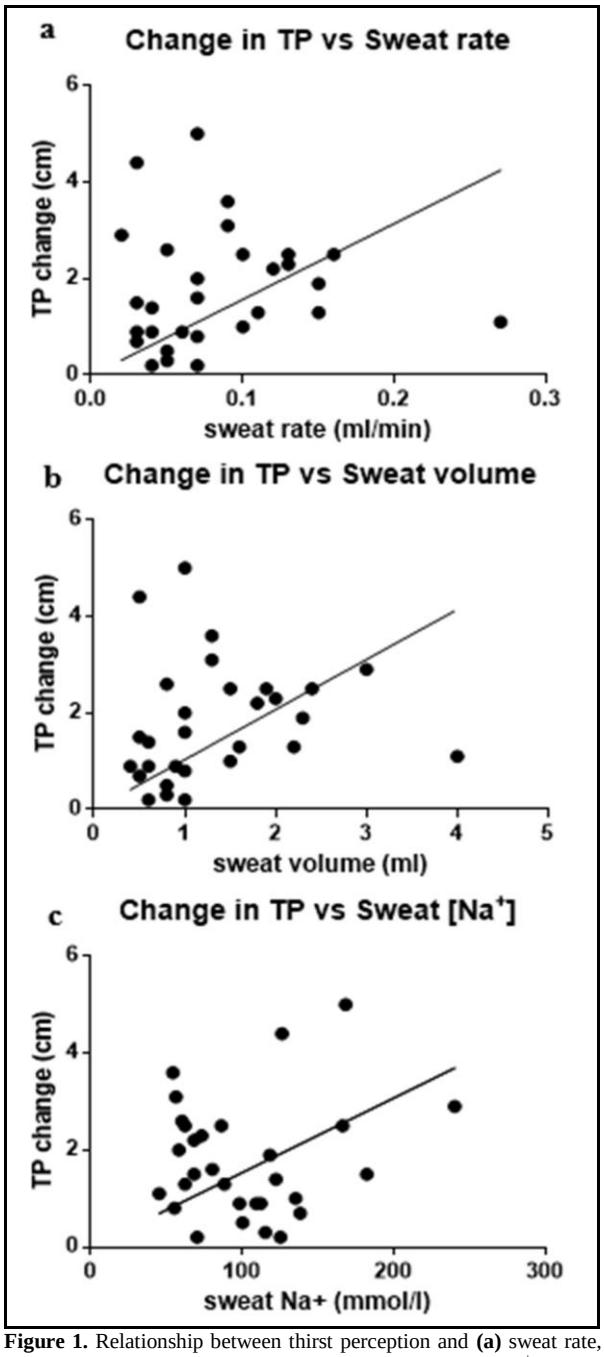


**Figure 1.** Relationship between thirst perception and **(a)** sweat rate, **(b)** sweat volume, and **(c)** sweat sodium concentration (S[$Na^+$]).

The periM women also recorded the highest sweat volume. Although this was not statistically significant, it could be attributed to the increased sweat rate owing to the interdependence of both factors. There was no significant difference in the sweat sodium ion concentration between the groups with the periM women excreting the lowest amount despite having the highest sweat rate and volume. S[$Na^+$] was higher in preM women compared to the other groups. Liberman [23] gave a similar report and attributed it to the increased salt and water retention during the menstrual cycle as a result of high estrogen levels around this period [24, 25]. This counteracts the observation of Drinkwater *et al* [26] who stated that sweat sodium is highest during periM.

Sweat sodium loss has been shown to increase with exercise in females [54], but we observed that as sweat rate and volume increased, the sweat sodium loss decreased. This could be due to aldosterone secretion from the adrenal cortex during exercise which enhances the reabsorption of sodium from the sweat glands, thereby reducing sweat osmolarity and preventing electrolyte depletion [29, 55, 56]. However, the rate of secretion and composition of sweat vary with duration of sweating, period of sweat collection, climate and muscular activity. Gender, age, genetics, level of training (fitness), and heat acclimation also affect sweat rate and S[$Na^+$] [30, 31, 57-59].

A significant and positive relationship existed between TP and SR, SV and S[$Na^+$]. Increase in SR and volume produced a concomitant increase in TP during moderate exercise because sweating, which results in water and electrolytes deficit, causes progressive depletion of circulating blood volume (hypovolemia), plasma hyperosmolality and dehydration. Decreased blood volume and dehydration decreases sweating and stimulate thirst [46, 60-65].

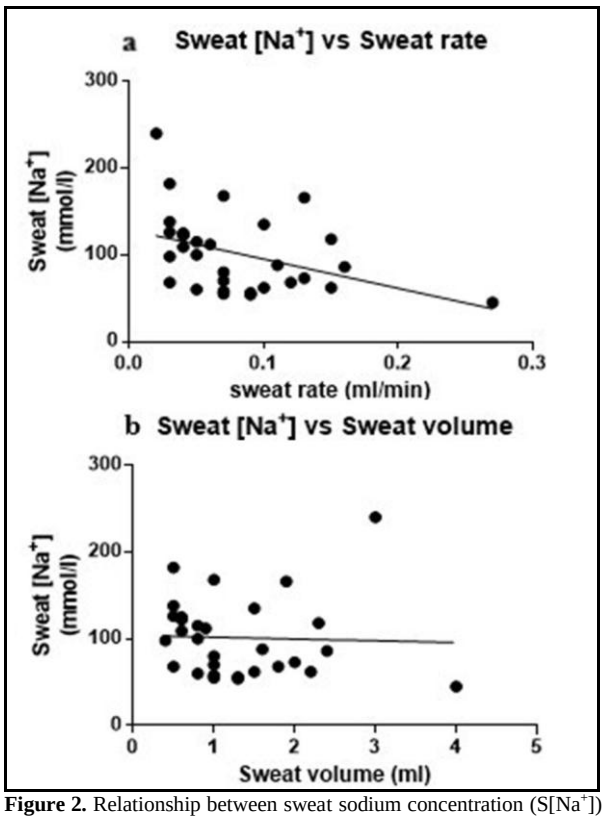


**Figure 2.** Relationship between sweat sodium concentration (S[$Na^+$]) and **(a)** Sweat rate or **(b)** Sweat volume.

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Additional large-scale studies involving the measurement of plasma osmolality, urine output and volume of water drunk after exercise are required to further elucidate thirst mechanisms and water homeostasis during menopause. These parameters can be correlated with serum hormonal levels (*e.g.*, estrogens and androgens).

In summary, during moderate exercise in women, the sweat rate, sweat volume and change in thirst perception are highest around the perimenopausal stage, but the amount of sodium lost in sweat is decreased. Thirst sensations decrease with ageing. There is a linear relationship between sweating and thirst perception, and as sweating increases, sodium loss is reduced.

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**COMPETING INTERESTS**

The authors declare that they have no conflicts of interest.

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**Original Article**

# Neuropathies of spinal cord development in rat pups maternally fed with fried potato chips

**Abdelalim A. Gad-Allah[1], Hassan I. El-Sayyad[2], Effat M.El-Shershaby[2], Ibrahim M. Abdelatif[2]**

*[1]Deanship, Jazan University, Kingdom of Saudi Arabia;*
*[2]Faculty of Science, Mansoura University, **Mansoura, Egypt***

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**Corresponding Author**
Hassan I. El-Sayyad
Department of Zoology,
Faculty of Science,
Mansoura University,
Mansoura, Egypt.
elsayyad@mans.edu.eg

**Key Words**
Fried potato chips;
Neuropathy;
Rat pups;
Spinal cord

**Abstract**

***Objective:*** Acrylamide is a neurotoxic material and recently elevated levels of acrylamide in varieties of foodstuffs were reported. The present study aimed to illustrate the demyelination of spinal cord of pups maternally fed a diet containing fried potato chips.

***Methods:*** Eighty fertile virgin female Wistar rats were made pregnant after mating with healthy male. Zero dates of gestation were determined and dams were arranged into three groups as control, acrylamide-treated (15 mg/kg body weight, p.o.) and 50% fried potato chips containing diet group. Treatments were carried every other day from 6$^{th}$ day of gestation until 3-week post-partum. The cervical spinal cord was separated and subjected for SDS-PAGE analysis and light and transmission electron microscopy.

***Results:*** Comparing with acrylamide-treatment, protein expression in spinal cord of pups maternally fed with fried potatoes was altered. Necrosis of motor neuronal cells within grey matter, hyperplasia of ependymal lining cells and fragility of white matter was detected. At ultrastructural level, the sensory and motor neuronal cells showed convoluted nuclear envelope and either chromatolysis or compacted chromatin material. Fragmentation of rough endoplasmic reticulum and damage of mitochondria become well evident in pups maternally fed with potato chips. The neuronal axons possessed vacuolation and demyelination associated with apparent damage of mitochondria.

***Conclusion:*** Supplementation of fried potato chips exerted neurotoxicity either directly through their content of acrylamide or via its metabolite glycidamide. Both components were reported to find their way across the placenta during gestation and breast milk during the lactation period, interfering with spinal cord differentiation and adversely affected demyelination.



## INTRODUCTION

French fries and potato chips are common parts of children's menus in fast-food restaurants, over the past 30 years; these familiar foods contain high levels of toxic and carcinogenic by-products, mainly acrylamide [1, 2]. Recently, high levels of acrylamide were unexpectedly detected in widely consumed food items, notably french fries, potato chips and bread [3]. Hirvonen *et al* [4] analysed the food consumption data over 2038 adults (25-74 years old) and 1514 children of 1, 3 and 6 years of age, with the data on foods' acrylamide content and found that acrylamide exposure was highest among the 3-year-old children and lowest among 65-74-year-old women. Among adults, the most important source of acrylamide exposure was coffee, followed by casseroles rich in starch, then rye bread. Among children, the most important sources were casseroles rich in starch and then biscuits and, finally, chips and other fried potatoes.

Acrylamide is a major environmental chemical found in tobacco smoke reaching 1 to 2 µg of acrylamide per cigarette [5] as well as widely used for industrial application such as soil conditioning, wastewater treatment and cosmetic, paper and textile industries [6]. Moreover, long-term acrylamide-treatment was found to induce neurotoxicity [7-10], especially axonal damage [11].

Recently, experimental studies revealed that ingestion of diet containing deep-fried potato chips led to fetal growth defects and development of histopathological lesions in hepatic, renal and myocardial tissues [12]. We previously reported that rat pups maternally fed on fried potato chips possessed markedly retarded cerebellar cortex which pointed out the close relation of acrylamide in fried potato chips [13]. The present work aimed to illustrate the developmental defects of neuronal cells and myelination of their axons in rat pups maternally fed on fried potatoes during suckling period.

## MATERIALS AND METHODS

### Acrylamide treatment

Acrylamide of highest purity 99.9% supplied from Sigma (St. Louis, MO, USA) was used in the present work. The applied dose of 15 mg/kg body weight was dissolved in 0.2 ml saline solution and orally dosed by a stomach tube to pregnant rats from 6$^{th}$ day of gestation until parturition and continued weekly until the pups became 3-week-old.

### Fried-potato chips supplementation

Fried potato chips were supplied from the market and mixed with standard diet at a concentration of 50% and used for feeding the pregnants from the 6$^{th}$ day of gestation until parturition as well as 1, 2 and 3 weeks post-partum.

### Experimental work

Eighty fertile virgin females and males of Wistar rats weighing 150-180 g were obtained from Hellwan Animal Breeding Farm (Ministry of Health, Cairo, Egypt) and used for experimentation. Rats were housed in individual cages and maintained at 23°C with a 12 h light-dark cycle. Free access of standard diet composed of 30% protein, 20% grinding yellow maize, 15% carbohydrates, 2.5% minerals, butterfat 4%, moisture 7%, fiber 13% and vitamins was supplied. Free excess of water was allowed *ad libitum*. The rats were kept under good ventilation.

Females were made pregnant by keeping them with fertile males overnight and examining in the next morning for the presence of vaginal plugs and sperms in their vaginal smears to give a precise determination of the onset of gestation. The pregnants were arranged into three groups (n = 20) including control, acrylamide-treatment and fried potato chips.

### Sodium dodecyl sulfate-polyacrylamides gel electrophoresis (SDS-PAGE)

A set of five pups per mothers of five colonies of both control and experimental groups were sacrificed at the end of treatment, and biopsies of cervical spinal cord were incised and kept frozen at -20ºC for SDS-PAGE according to Laemmli [14]. The gel used for SDS-PAGE is made out of acrylamide and composed of two layers; the top one is the stacking gel and the lower layer called separating gel. SDS-PAGE of the plasma membranes-enriched preparation (PMEP) proteins was performed on 4.8% stacking and 11.5% separating gels. Prior to electrophoresis, PMEMs containing 100 µg of proteins were re-suspended in 0.2 ml sample loading buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 0.1 M DTT, 20% glycerol and a trace of bromophenol blue). After centrifugation at 12,000g for 12 min, the supernatant was loaded into gel wells. The SDS-PAGE was run at 20 mA on the polyacrylamide stacking gel and at 40 mA on the separating gel. After completion of electrophoresis, the separated protein bands were visualized using Coomassie brilliant blue G-250 [15]. A low molecular weight calibration kit (Bio-Rad) was used as the standard molecular weight marker.

### Light microscopic investigations

The cervical spinal cord of pups at parturition as well as 1, 2 and 3 weeks of age of both control and study groups were separated and immediately fixed in 10% phosphate buffered formalin. The specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten Paraplast at 58-62°C. Five micrometer histological sections were carried out and stained with Harris hematoxylin & eosin.

### Transmission electron microscopic (TEM) investigation

The cervical spinal cord at 2 and 3 weeks of pups were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, (pH 7.4) overnight at 4ºC. The tissue was washed with cacodylate buffer and postfixed with 1% osmium tetroxide for 2 h. Then re-washed again with 0.1 M cacodylate buffer, serially dehydrated in ethanol and propylene oxide and embedded in Eponate-resin (Ted Pella, Redding, CA, USA). Eighty nanometer thin sections were cut with a diamond knife on a ultratome (LKB Instruments, Bromma, Sweden), mounted onto 300 mesh copper grids and stained with saturated uranyl acetate in 50% methanol and then with lead citrate. The grids were viewed in a transmission electron microscope (Jeol Ltd., Tokyo, Japan).

## RESULTS

### Proteomic (SDS-PAGE) analysis

After fractionation by polyacrylamide gel electrophoresis, there were marked alterations in experimental groups. In 2-week-old pups, experimental groups expressed extra formation of stressed protein band, however both treatments lacked expression of one protein band: at 43kDa, acrylamide treatment revealed missing of two expressed protein bands. In 3-week-old

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pups, fried potato group was less susceptible compared to acrylamide treatment at 200 kDa; a defect was detected only in one expressed protein compared with missing of two proteins in acrylamide group. However, at 43 kDa new expression of a protein band was detected in both experimental groups. Expressions of protein bands were illustrated in Fig.1.

**Light microscopic observations**

In control 2-week-old pups, the multipolar neurons attained more growth and possessed prominent centrally nuclei and thin basophilic cytoplasm. In the ventral column, the neuronal cells are much larger and differentiated from those of the dorsal column (Fig.2A1-A2). However, in those maternally treated with acrylamide or fed on diet containing fried potato chips, there was a considerable atrophy of ependymal canal associated with widespread pyknotic nuclei in their lining cells. The apical and ventral margin of the ependymal canal showed massive necrosis. The grey matter possessed hyalinization and massive necrosis of their neuronal cells. Many of the multipolar neuronal cells showed eosinophilic appearance. The white matter showed marked fragility and spongy appearance (Fig.2B1-C2).

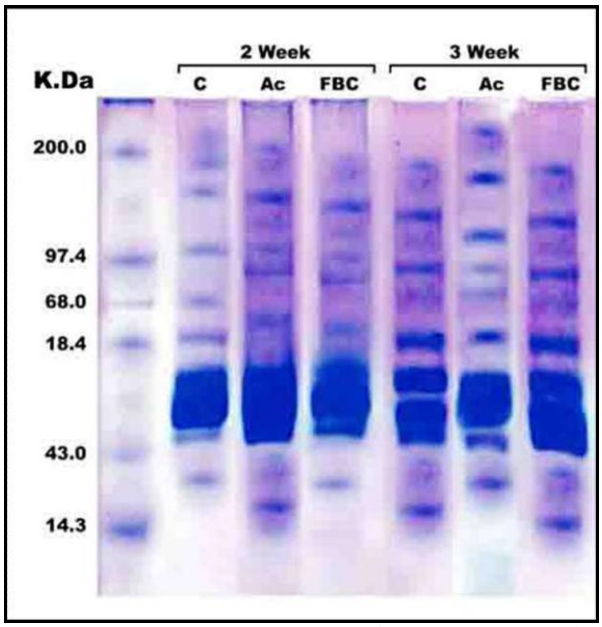


**Figure 1**. SDS-PAGE protein expression of cervical spinal cord of 2- and 3-week-old control pups and those maternally fed with diet containing fried potatoes or acrylamide. C, control; Ac, acrylamide; FBC, fried potato chips.

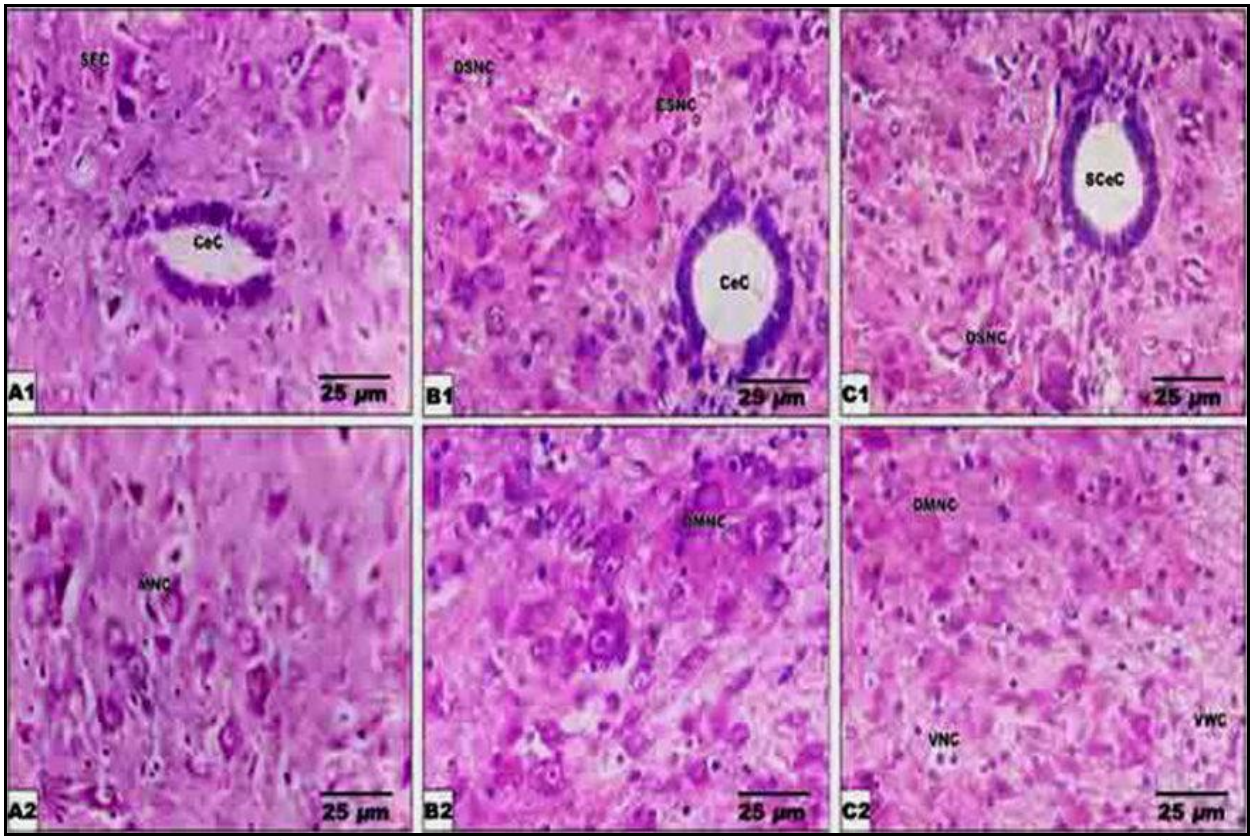


**Figure 2.** Photomicrographs of transverse histological sections of spinal cord of 2-weeks-old pups. **A1-A2:** controls showing differentiated ependymal canal and multipolar neuronal cells; **B1-B2:** maternally treated with acrylamide showing bilateral apical degeneration of ependymal lining cells; **C1-C2:** maternally fed with fried potatoes showing degeneration of neuronal cells similar to acrylamide treatment (H&E). CeC, central canal; DSNS, degenerated sensory neuron cell; DMNC, degenerated motor neuron cell; MNC, motor neuron cell; SNS, sensory neuron cell.

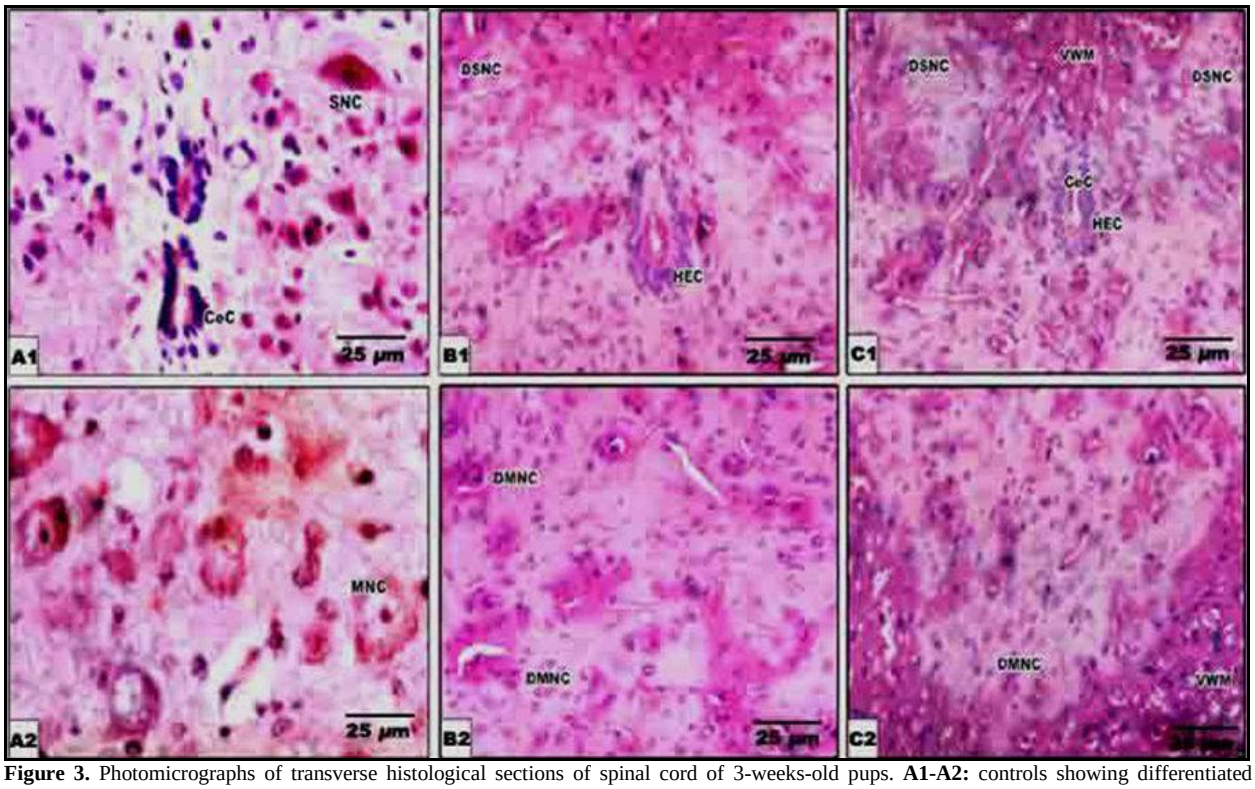


**Figure 3.** Photomicrographs of transverse histological sections of spinal cord of 3-weeks-old pups. **A1-A2:** controls showing differentiated ependymal canal and multipolar neuronal cells; **B1-B2:** maternally treated with acrylamide showing hyperplastic ependymal lining cells and marked neuronal cell death; **C1-C2**: maternally fed with potato chips showing similar neuronal dysfunction to acrylamide treatment (H&E). CeC, central canal; DSNS, degenerated sensory neuron cell; DMNC, degenerated motor neuron cells HEC, hyperplastic ependymal cell; MNC, motor neuron cell; SNS, sensory neuron cell; VWC, vacuolated white matter.

In control 3-week-old pups, numerous multipolar neuronal cells of varying sizes are clearly identified. The cell structural pattern attained much more maturity. The white matter composed of dense nerve endings (Fig.3A1-A2). In those maternally treated with acrylamide or fed with diet containing fried potatoes, there was a considerable damage of the spinal cord. Most of the histopathological lesions were detected around the central canal and consisted of severely necrotic areas. The gray matter showed apparent chromatolysis of the motor neurons. Vacuolation of the white matter becomes more apparent. There was a close similarity between the pathology of acrylamide and feeding on diet containing fried potatoes (Fig. 3A1-C2).

**Transmission electron microscopic observations**

In control 2-week-old pups, the motor neuronal cells possess large, round centrally located nuclei and prominent nucleoli. The nuclear envelope is membranous, with peripheral chromatin arrangement. The cytoplasm is rich in rough endoplasmic reticulum, spherical and elongated mitochondria and Golgi complex .The white matter exhibits the presence of myelinated axons. Each axon is formed of several lamellate sheets with markedly enlarged extracellular spaces separating the axon from its myelin sheath (Fig.4A1-A2). In those maternally treated with acrylamide or supplemented fried potato chips, the neuronal cells possessed chromatolysis of their nuclei. The cytoplasmic organelles exhibited vesicuolated rough endoplasmic reticulum and swollen of mitochondria with missing of their internal compartments. The Golgi apparatus was abnormal degenerated. The white matter revealed marked variations in the size of demyelinated axons. Demyelination becomes prominent in many of the axons. Others were invested with varying amounts of myelin in different stages of breakdown. Vacuolation of myelinated axons was detected in many of them. In 2-week-old pups maternally fed with diet containing fried potatoes, the spinal cord exhibited closely similar alteration of both neuronal cells and nerve axons (Fig.4B1-C2).

In control 3-week-old pups, the gray matter reveals prominent distribution of neurons with centrally located nuclei having peripheral marginated heterochromatin on nuclear envelope and abundant euchromatin. The cytoplasm possesses dense arrangement of rough endoplasmic reticulum and free polysomes in between the cristae. A moderate distribution of mitochondria and Golgi apparatus is detected. The white matter possesses abundant distribution of axons closely

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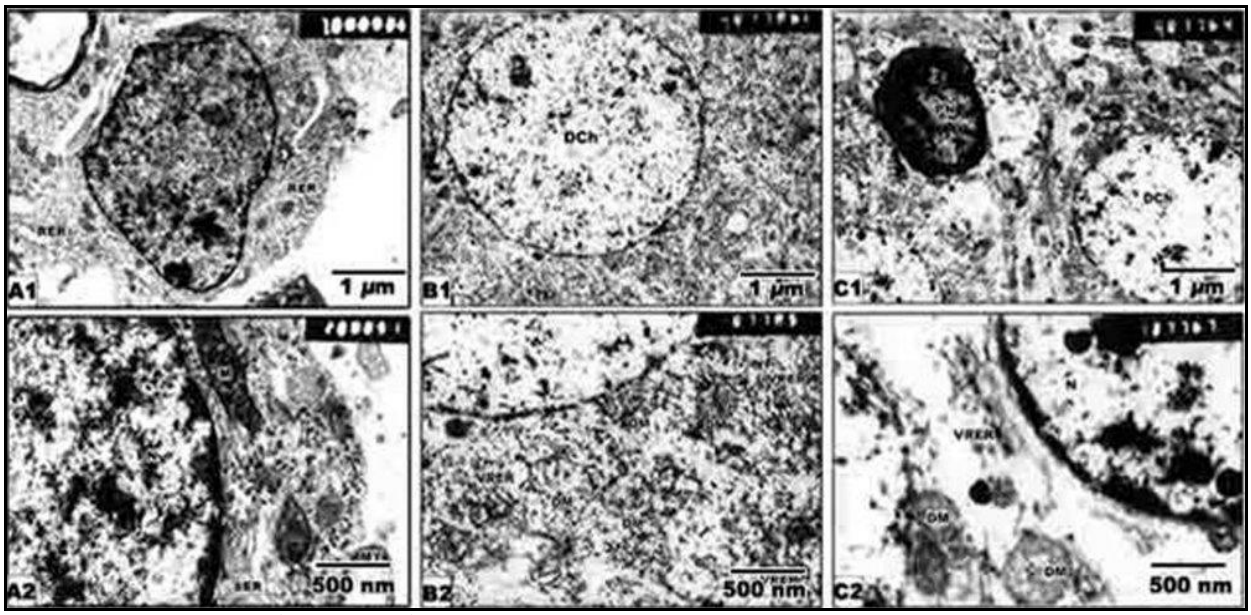


**Figure 4.** TEM micrographs of spinal cord of 2-weeks-old pups. **A1-A2:** controls showing multipolar neuronal cells with normal nuclei and cytoplasm rich in rough endoplasmic reticulum, mitochondria and ribosomes; **B1-B2:** maternally treated with acrylamide showing karyolysed nuclei and degenerated cytoplasmic organelles; **C1-C2:** maternally fed with fried potatoes showing either karyolysis or pyknosis of nuclei and degeneration of cytoplasmic organelles (lead citrate & uranyl acetate). RER, rough endoplasmic reticulum; VRER, vesiculated rough endoplasmic reticulum; N, nucleus; M, mitochondria; DM, degenerated mitochondria; DCh, degenerated chromatin; PN, pyknotic nuclei.

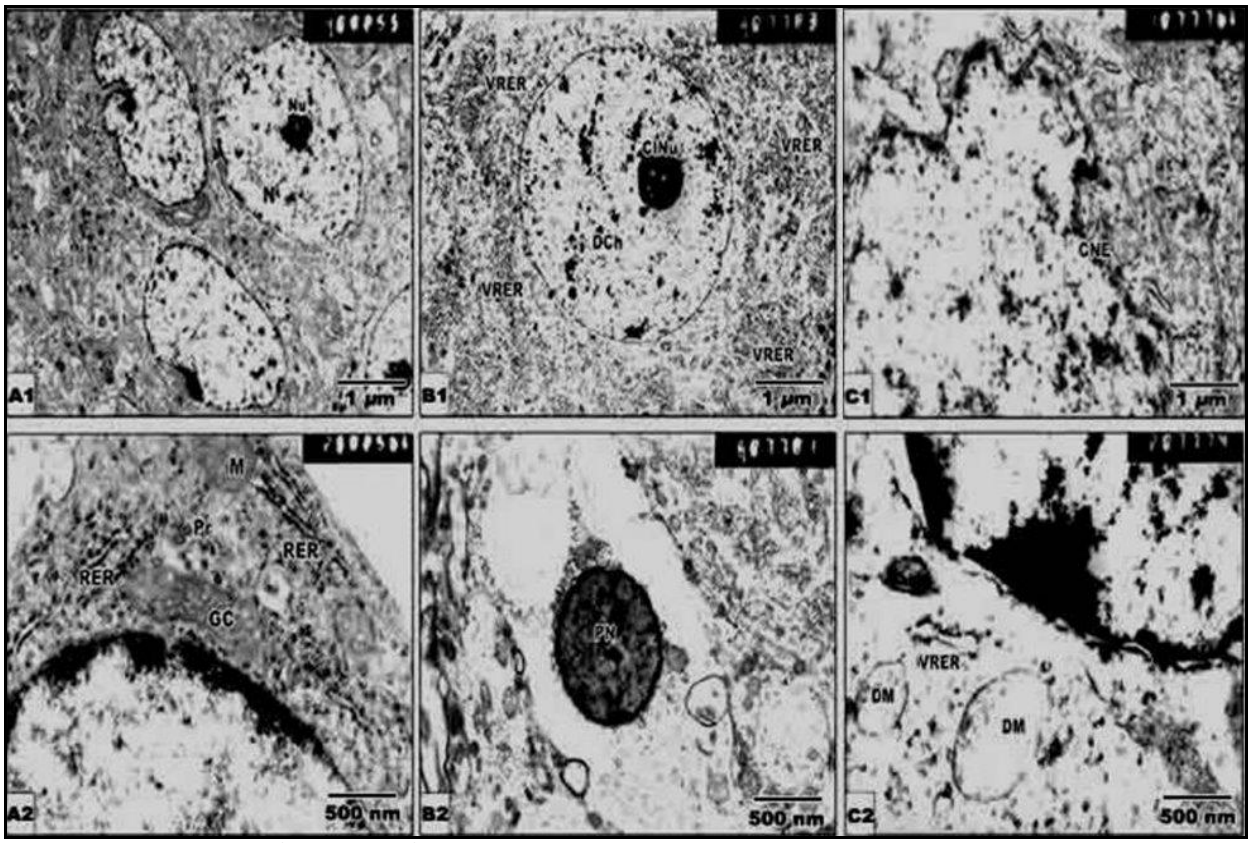


**Figure 5.** TEM micrographs of spinal cord of 2-weeks-old pups. **A1-A2:** controls showing multipolar neuronal cell with normal nuclei and cytoplasm rich in rough endoplasmic reticulum, mitochondria and ribosomes; **B1-B2**: maternally treated with acrylamide showing either karyolysed or pyknotic nuclei and degenerated cytoplasmic organelles; **C1-C2**: maternally fed with fried potatoes showing karyolysis or pyknosis of nuclei and degeneration of cytoplasmic organelles (lead citrate & uranyl acetate). PN, pyknotic nuclei; SM, swollen mitochondria; VRER; vesiculated rough endoplasmic reticulum.

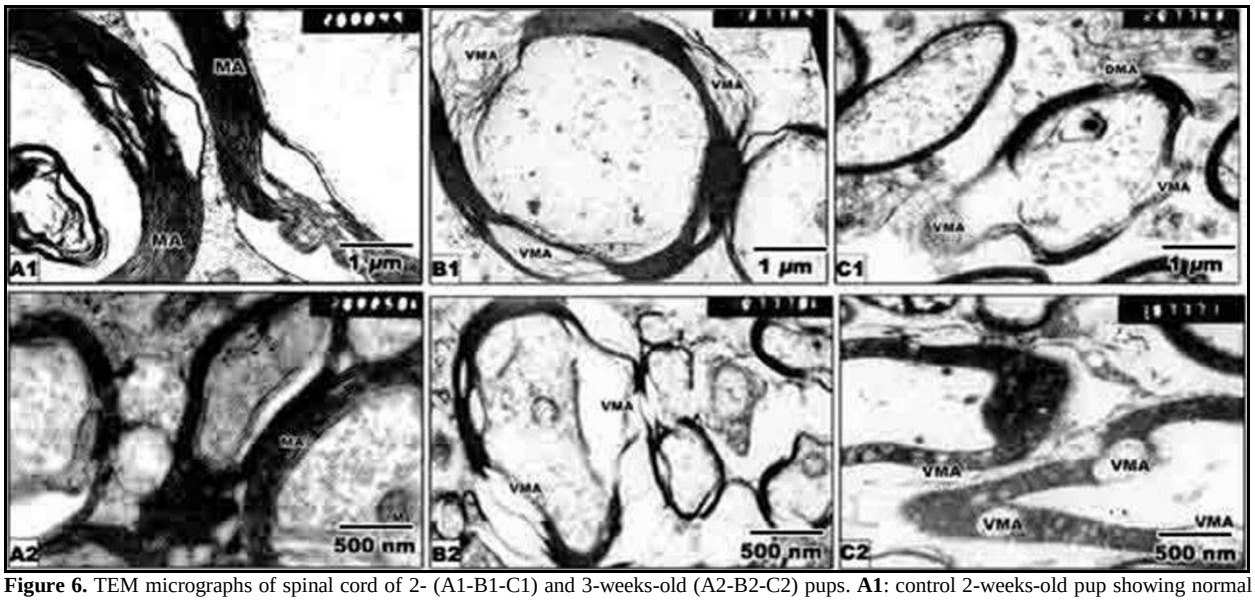


**Figure 6.** TEM micrographs of spinal cord of 2- (A1-B1-C1) and 3-weeks-old (A2-B2-C2) pups. **A1**: control 2-weeks-old pup showing normal myelinated axons; **A2:** control 3-weeks-old pup showing normal myelinated axons; **B1-B2:** 2- and 3-week-old pup maternally treated with acrylamide showing vacuolar degeneration of myelinated axons; **C1-C2:** 2- and 3-week-old pup maternally fed with fried potato chips showing similar demyelination (lead citrate & uranyl acetate). DMA, degenerated myelinated axon; MA, myelinated axon; VMA, vacuolized myelinated axon.

aligned with each other. Each axon is ensheathed by several layers of electron-dense myelin membranes regularly arranged in a circular manner. The axons, containing characteristic neurofibrils and mitochondria, are surrounded by myelin sheaths of relatively uniform thickness. Few numbers of non-myelinated axons are detected. The cytoplasm of the axons possessed abundant microtubules, mitochondria and vesiculated rough endoplasmic reticulum (Figs.5&6A1-A2).

In those pups maternally treated with acrylamide or fed on diet containing fried potatoes, the neuronal cells possessed nuclei with densely aggregated chromatin material. The cytoplasm exhibited vesicuolated rough endoplasmic reticulum, swollen mitochondria and abnormally degenerated Golgi apparatus. The white matter possessed massive destruction of nerve axons with either totally or partially degenerated myelin. The myelin lamellae showed massive detachment, separation, dissolution and vacuolation. Breakdown may start within or at the inner or outer surface of the sheath and yield in some axons a cleft of the demyelinated axons. The inner cytoplasm compartment becomes degenerated. The axolemma lacked normal regularity (Figs.5&6A1-C2).

## DISCUSSION

From the present findings, the spinal cord of pups maternally fed diet containing 50% fried potato chips developed neuropathological alterations in both gray and white matter at 1, 7, 14 and 21 days. There was a marked damage of neuronal cells of gray matter. The number of multipolar sensory and motor neuronal cells was comparatively reduced. The axons showed massive spongiform vacuolation prominent in the white matter. At the ultrastructural level, the neuronal cells showed apparent vesiculation of rough endoplasmic reticulum with derangement of ribosomes, swollen of mitochondria with missing of cristae and degeneration of Golgi complex. The detected findings clarified close similarities of pathological alterations between acrylamide-treated and potato chips fed pups.

Similar findings of acrylamide neurotoxicity were reported in cerebellar purkinje cells of mature rats [16] or in pups maternally received either fried potatoes or acrylamide [13]. The similarities of neuropathological alterations may be attributed to the generation of acrylamide in fried potatoes and which is absorbed during feeding and consequently exerted its toxicological aspects. Tareke *et al* [17] showed that acrylamide was formed by heating certain starch-based foods, such as potatoes, bread and processed cereals, above 120°C. French fries and potato chips exhibit relative high values of acrylamide. The acrylamide level of potato chips was shown to be increased depending on the elevated temperature degree of cooking [18]. The higher susceptibilities of pups may be attributed to the primitive growth and less differentiated neuronal tissues.

Acrylamide can be generated during the heating of specific foodstuffs as a result of a Millard reaction between amino acids and sugars [19, 20]. Potatoes and cereals which had the highest measured levels of acrylamide were found to be rich in asparagine [21].

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Our findings reported vacuolation and demyelination of spinal axons in 2- and 3 weeks-old neonates maternally fed with diet containing fried potato chips compared to acrylamide treatment. A considerable thinning of lamellated myelin was detected. According to Confavreus *et al* [22], axonal degeneration is a key of the progressive disability detected in multiple sclerosis. Acrylamide exposure was found to cause nerve terminal damage in both the central and peripheral nervous system [10, 23].

Proteomic analysis of spinal cord altered protein expression clarified disruption of protein synthesis as a result of acrylamide cytotoxicity. The present findings were confirmed by the work of Lakshmi *et al* [24] who reported increased levels of lipid peroxidation, protein carbonyl content, hydroxyl radical and hydroperoxide in cerebellar cortex of rats received acrylamide-treatment.

Zhang *et al* [25] mentioned that acrylamide form adducts with the nucleophilic sulfhydryl groups on cysteine residues leading to dissociation of the transcription factor, nuclear factor erythroid 2-related factor (Nrf2) which up-regulated gene expression of phase II detoxification enzymes and consequently, impair the protection of neuronal regions.

Axonal damage may be attributed to hypoxia caused by the acrylamide-formed hemoglobin adduct decreasing oxygen transport [26] and consequently leading to disruption of microcirculation, production of toxic metabolites and diminishing mitochondrial energy metabolism [27]. Mitochondria are the major source of free radicals within the cell and are thought to play a key role in many neurodegenerative diseases [28]. Decreased number of mitochondria per axon has been described in animal models of demyelination [28, 29].

In conclusion, maternal supplementation of fried potato chips during gestation and suckling period led to consume the large amount of acrylamide generated during cooking as well as its metabolite glycidamide. Both components pass across the placenta during gestation and breast milk during lactation period interfering with spinal cord differentiation. These led to demyelination and cell death of sensory and motor neuron cells, possibly as a result of liberation of free oxygen radicals from the damaged mitochondria.

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**Original Article**

# Purine metabolism and oxidative stress in children with autistic spectrum disorders

**Faisal Gh. Al-Rubaye[1], Taha Shawi Morad[2]**

*[1]Department of Chemistry & Biochemistry, [2]Department of Medical Biology, College of Medicine, Al-Nahrain University, **Baghdad, Iraq***

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**Corresponding Author**
Faisal Gh. Al-Rubaye
Department of Chemistry & Biochemistry,
College of Medicine,
Al-Nahrain University, Baghdad, Iraq.
faisal3ghazi@yahoo.com

**Key Words**
Adenosine diaminase;
Autism,
Malondialdehyde;
Superoxide dismutase

**Abstract**

***Objectives:*** Autism and related autism spectrum disorders (ASD) are heterogeneous neurodevelopmental disorders behaviorally defined by significant deficits in social interaction and communication and by the presence of restricted interests and repetitive behaviors. It has been suggested that oxidative stress and abnormal purine metabolism may play a role in the pathogenesis of ASD, but the literature reports somewhat contradictory results. The aim of this study is to assess the status of purine metabolism - expressed as serum adenosine diaminase (ADA) - and oxidative stress - expressed as serum malondialdehyde (MDA) and serum superoxide dismutase (SOD) - in male children with autism.

***Methods:*** The present study is a cross-sectional study performed at Al-Kadhimiya Teaching Hospital, Baghdad, Iraq including measurement of serum ADA in boys with ASD. A total of 35 patients (age range 14-19 years) with autism were involved in this study together with a matching group of 40 apparently healthy boys (age range 14-16 years) who were included as controls.

***Results:*** Serum ADA and SOD were significantly lower in boys with autism accompanied by significant higher serum MDA levels when compared with controls.

***Conclusion:*** Patients with ASD have impaired purine metabolism and increased oxidative stress which was supported by low levels of ADA and SOD, and high level of MDA. Further biochemical or genetic studies are required to explore the nature of autism.



## INTRODUCTION

Autism and related autism spectrum disorders (ASDs) are heterogeneous neurodevelopmental disorders behaviorally defined by significant deficits in social interaction and communication and by the presence of restricted interests and repetitive behaviors. Despite intense research, the etiology of autism remains largely unknown but is likely multifactorial, including biologic, genetic, and environmental factors [1].

It has been suggested that oxidative stress may play a role in the etiopathogenesis of ASD [2]. Oxidative stress is defined as the disruption of the normal intracellular balance between reactive oxygen species (ROS), produced either during aerobic metabolism or as a consequence of pathologic processes, and antioxidant defence mechanisms [3]. Oxidative stress, in turn, induces the secretion of numerous vasoactive and pro-inflammatory molecules [4] leading to neuro-inflammation [5]. Oxidative stress has been suggested to underlie several other mental disorders, including schizophrenia and bipolar disorder [6], and neurodegenerative pathologies such as Alzheimer disease [7]. Oxidative stress is the result of increased production of pro-oxidant species or decreased antioxidant defences; glutathione redox status has indeed been found to be decreased in autistic patients, also in the post-mortem analysis of Autistic brain tissues [8].

Oxidative stress can be detected by studying a panel of different markers [9], some of which such as DNA, proteins and polyunsaturated fatty acids (PUFAs) residues are pathognomonic for oxidative damage of biomolecules. It is worth mentioning that lipid peroxidation was found to be elevated in autism and that PUFA are important for neurodevelopment [10]. Several markers of oxidative stress are available such

as malondialdehyde (MDA), a marker of lipid peroxidation [11].

Inborn errors of purine metabolism have been implicated as a cause for some cases of autism [12]. Adenosine deaminase (ADA) is an enzyme involved in purine metabolism. It is needed for the breakdown of adenosine from food and for the turnover of nucleic acids in tissues. It has also been proposed that ADA, in addition to adenosine breakdown, stimulates release of excitatory amino acids and is necessary to the coupling of adenosine receptors and heterotrimeric G proteins, which may be altered in some autistic patients [13]. However, the full physiological role of ADA is not yet completely understood [12].

Given together, this study was conducted to assess the status of some biological markers related to purine metabolism and oxidative stress in male children with ASD.

## MATERIALS AND METHODS

### Subjects

The study was a cross-sectional study carried out in the Psychiatry Department at Al-Kadhimiya Teaching Hospital, Baghdad, Iraq. The diagnosis of autism was confirmed in all subjects by a consultant psychiatrist using the Autism Diagnostic Interview-Revised (ADI-R) [14], the Autism Diagnostic Observation Schedule-Generic (ADOS-G) [15], and the Developmental, Dimensional and Diagnostic Interview (3di) [16] criteria. All patients had simplex autism, and all were negative for Fragile X. None of the patients were on special diets or alternative treatments.

Exclusion criteria included presence of organic aciduria, dysmorphic features, diagnosis of Fragile X or other serious neurological (*e.g.* seizures) or psychiatric (*e.g.* bipolar disorder) conditions, known medical conditions including endocrine, cardiovascular, pulmonary, liver, kidney or other diseases. The protocol for the study was approved by the Ethical Committee of Al-Nahrain Medical College, and informed consent was signed by each subject.

### Blood samples

Five milliliters of random venous blood were withdrawn from each patient, in supine position, without application of tourniquet. Samples were transferred into clean new plane tube, left at room temperature for 15 min for clotting, centrifuged at 1,800g for 10 min at 4°C, and the separated serum was transferred into eppendorf tubes that were stored at -20°C until analysis.

### Biochemical analyses

***Serum adenosine deaminase*** was measured by an ELISA kit (USCN Life Sciences; Wuhan, PR China) which relays on a sandwich enzyme immunoassay for the in vitro quantitative measurement of ADA in human serum [17]. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to ADA. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for ADA. Next, avidin conjugated to horseradish peroxidase (HRP) is added to each microplate well and incubated. Then a tetramethylbenzidine (TMB) substrate solution is added to each well. Only those wells that contain ADA, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 ± 10 nm. The concentration of ADA in the samples is then determined by comparing the optical density (OD) of the samples to the standard curve. The detection range was 0.312-20 ng/ml; the standard curve concentrations used for the ELISA's were 20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, and 0.312 ng/ml.

***Serum superoxide dismutase*** (SOD) was measured using the inhibition rate of 2-(4-indophenyl)-(4-nitrophenol)-5-phenyltetrazolium chloride reduction method (modified method of Sun *et al* [18]) via a commercial kit (Randox; Antrim, Northern Ireland, UK). One unit of SOD activity was defined as the amount of protein that inhibits 2-(4-indophenyl)-(4-nitrophenol)-5-phenyltetrazolium chloride reduction rate by 50%.

***Serum malondialdehyde*** was determined by the method of Draper and Hadley [19] based on the reaction of MDA with thiobarbituric acid (TBA) at 95°C. In the TBA test reaction, MDA and TBA react to form a pink pigment with an absorption maximum at 532 nm. The MDA concentrations were calculated using the molar extinction coefficient of $1.5 \times 10^5$. The results were expressed as nmol/ml serum.

### Statistical analysis

Statistical analysis was done using descriptive (mean and standard deviation) and inferential statistics (t-test) to test the significancy of mean difference. When P value was less than 0.05, the difference was considered statistically significant.

## RESULTS

### Subjects

A total of 35 male patients with ASD were enrolled in this study: age range 14-19 years (mean age 15.3 ± 2.8 years). Another 40 apparently healthy male subjects were used as healthy controls: age range 14-16 years (mean age 14.23 ± 2.2 years) (Table 1).

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All participants were screened via parental interview for current and past physical illness.

**Biochemical outcome**

***Serum Adenosine deaminase*** was found to be highly significant lower in ASD group compared to healthy controls ($P < 0.001$, Table 2).

***Serum superoxide dismutase*** was significantly less in ASD group compared to healthy controls ($P < 0.05$, Table 2).

***Serum malondialdehyde*** was significantly higher in ASD group than healthy controls ($P < 0.05$, Table 2).

**Table 1.** Demographic criteria of patients and control subjects (range and mean ± SD)

| Group | ASD group | Healthy controls |
|---|---|---|
| **Number (subjects)** | 35 | 40 |
| **Mean age (years)** | 15.3 ± 2.8 | 14.2 ± 2.2 |
| **Age range (years)** | 14-19 | 14-16 |

**Table 2.** Serum adenosine deaminase (ADA), superoxide dismutase (SOD) and malondialdehyde (MDA) levels (mean ± SD)

| Variable | ASD | Control | P |
|---|---|---|---|
| **ADA (ng/ml)** | 9.2 ± 1.9 | 16.5 ± 3.4 | < 0.001 |
| **SOD (U/ml)** | 0.61±0.21 | 0.66±0.41 | < 0.05 |
| **MDA (nmol/ml)** | 0.75±0.17 | 0.69±0.12 | < 0.05 |

## DISCUSSION

Autism is a complex neurodevelopmental disorder that is thought to involve an interaction between multiple, variable susceptibility genes [20], epigenetic effects [21], and environmental factors [22]. The apparent increase in the diagnosis of ASD from 4-5 per 10,000 children in the 1980s to 30-60 per 10,000 children in the 1990s has raised great concern [23]. This increased prevalence of autism has enormous future public health implications and has stimulated intense research into potential etiologic factors and candidate genes.

Because abnormal purine metabolism and low activity of adenosine deaminase have been reported in other neurologic disorders, including Alzheimer disease, Parkinson disease, schizophrenia, and Down syndrome [24], the activity of ADA was measured in a group of autistic children with respect to the control children [25]; in the present study the results were within the range of values previously found in several other studies.

The observed imbalance in ADA activity in the autistic children is complex and not easily explained by perturbation of a single pathway or isolated genetic or nutritional deficiency. Moreover, a possible relationship between the losses of adenosine homeostasis due to the currently reported reduced activity of ADA, and the impairment of neurotransmitter profile is suggested in patients with autism. This is also supported by the findings of a recent work by El-Ansary [26] who showed a low plasma and high brain serotonin and dopamine concentration in patients with autism compared with control subjects. Based on these results, it seems likely that tight control of adenosine levels could play an important role in brain development and neural plasticity [27], and that any dysfunction in homeostatic control of adenosine, an important modulator of the brain immune system, could upset the balance between pro-inflammatory and anti-inflammatory cytokines, which is crucial for normal brain development [28].

It is possibly relevant that, in autistic children, decreased activity of ADA has been shown to be associated with increased frequency of ADA polymorphisms [29]. The observed increase in adenosine could be due to either an inhibition of adenosine kinase or an increase in 5-nucleotidase, both of which have been shown to occur with oxidative stress [30]. Elevated intracellular adenosine has been shown to increase oxidative stress [30].

In the present work, among the oxidative stress parameters that were evaluated in autistic group compared to healthy controls, a significant increase in MDA, a marker of lipid peroxidation, accompanied by significant reduction in SOD, an enzyme reflecting free radical scavenging activity, was observed. Similar findings were reported by other groups [31, 32]. This finding suggests that oxidative stress was involved in the pathogenesis of conditions related to the neuronal tissues. The fatty acid composition of the brain and neural tissues is characterized by high PUFA concentrations which play a very important role in signal transduction [33], neuro-inflammation [34] and cellular repair and survival [35]; this tissue appears to be involved by peroxidation process.

Alternatively, a genetic predisposition to environmental agents or conditions that promote oxidative stress could contribute to the abnormal metabolic profile observed in the autistic children. The contribution of MDA-related oxidative injury to autism was explored by autoimmunity as an etiological factor of autism [36]. Autoimmunity is an abnormal immune reaction in which the immune system becomes primed to react against body organs. It can result from an immune response against altered self proteins, *e.g.* modified by adduction of lipid-derived electrophiles generated by oxidative injury. Immunoglobulin (Ig)G, IgA, IgM and myelin basic protein anti-brain auto antibodies were reported to be present in high percentage of sera from children with autism compared to healthy children [37].

In conclusion, the present study indicates that abnormal purine metabolism and oxidative stress might contribute to the pathogenesis of ASD. This suggestion is supported by the finding of low serum ADA and SOD activities and high levels of serum MDA in autistic boys with respect to healthy boys. Further studies on other markers of purine metabolism and oxidative stress, in addition to genetic study in a larger number of children (boys and girls) with ASD are required to explore the nature of ASD.

**COMPETING INTERESTS**

The authors declare that they have no conflict of interest.

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**Original Article**

# PARP-1 expression against Epstein-Barr virus LMP-1 and BZLF-1 in undifferentiated nasopharyngeal carcinoma

**Anggun I. Budiningrum[1], Achmad Rofi'i[2], Suharjono Suharjono[1], Fatchiyah Fatchiyah[1]**

*[1]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, East Java; [2]Departments of Otorhinolaryngology, Head and Neck, Ulin General Hospital, Faculty of Medicine, University of Lambung Mangkurat, Banjarmasin, South Kalimantan;* ***Indonesia***

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**Corresponding Author**
Fatchiyah Fatchiyah
Faculty of Mathematic and Sciences, University of Brawijaya, Jl. Veteran, Malang 65145, Indonesia.
fatchiya@ub.ac.id; fatchiya@gmail.com

**Key Words**
BZLF-1;
Epstein-Barr virus,
LMP-1;
Nasopharyngeal carcinoma;
PARP-1

**Abstract**

***Objective:*** This study aims to determine the poly(ADP-ribose) polymerase (PARP)-1 expression levels against the Epstein-Barr virus (EBV) latent membrane protein (LMP-1) and BamHI-Z leftward reading frame (BZLF)-1 of nasopharynx tissues and serum of nasopharyngeal carcinoma (NPC) patients and characterize the histopathology of necrosis cells of NPC tissues.

***Methods:*** 32 tissues sections consisting 24 NPC and 12 polyp tissues, and also blood serum of patients were taken from Ulin General Hospital, Banjarmasin. Tissue samples were analyzed by using immunofluorescence staining in order to examine LMP-1 and PARP-1 expression. Analyses were done by using immunoblotting with anti-LMP-1, anti-BZLF-1, anti-PARP-1, and anti-caspase-3 antibodies. Histopathological profiles of necrosis cells were analyzed by using hematoxylin-eosin staining and necrotic cells were counted by using the standard software of the microscope.

***Results:*** LMP-1, BZLF-1 and PARP-1 proteins have high levels of expression in patients with NPC. LMP-1 expression of NPC patients and control sera are equal. Interestingly we found that 35 kDa of BZLF-1 and 55 kDa PARP-1 only expressed in NPC patients, meanwhile caspase-3 expression was negative. Histopathologically, NPC tissues were more dominated by karyorrhexis than pyknosis and karyolysis.

***Conclusions:*** This study showed that the expression level of PARP-1 in NPC tissues was increased against LMP-1 and BZLF-1 EBV to protect DNA damage of the recipient cells. This mechanism probably stimulated cell metamorphosis and consequently provoked necrosis.



## INTRODUCTION

Nasopharyngeal carcinoma (NPC) is the most malignant type of carcinoma in the head and neck [1]. Early diagnosis of NPC still remains a problem, due to nonspecific early symptoms. The Epstein-Barr virus (EBV) infection is one of etiologic factors of NPC. EBV will interact with nasopharyngeal epithelial cells through binding between viral envelope gp350/220 and CD21. This interaction induce the expression of latent membrane protein (LMP)-1 in the membrane of EBV [2]. LMP-1 is an early oncoprotein which affect downstream signal for cell proliferation, anti-apoptosis and metastasis [3, 4].

On early lytic phase, LMP-1 will activate BamHI-Z leftward reading frame (BZLF)-1 [5]. BZLF-1 plays role for switching from latent into lytic phase and replication of EBV [5]. The integration between EBV and host DNA will activate poly(ADP-ribose) polymerase (PARP)-1 to prevent DNA damage. Over-activation of PARP-1 depletes $NAD^+$ and ATP, and is associated with necrosis [6]. The morphology of cell necrosis is divided into nuclear shrinkage (pyknosis), nuclear fragmentation (karyorrhexis), and nuclear fading (karyolysis) [6].

Previous study shows that the expression of BZLF-1 induces PARP-1 of undifferentiated NPC, even though the expression intensity of BZLF-1 and PARP-1 are not correlated with percentage of necrotic cell [7]. This study aims to investigate the expression of LMP-1, BZLF-1 and PARP-1, and the characteristic of necrosis cell in undifferentiated NPC.

## MATERIALS AND METHODS

### Ethical considerations

The study was approved by the Ethical Review Committee of Faculty of Medicine, University of Lambung Mangkurat, Banjarmasin, and South Kalimantan, Indonesia. For appropriate management, all individuals participated in the study, after having information and consent, and abnormal results were firstly communicated with their physician.

### Subjects

This study was mainly conducted on Central Laboratory of Life Sciences, University of Brawijaya, Malang, and East Java, Indonesia. Twenty-four biopsy specimens from untreated NPC patients were recruited from Department of Otorhinolaryngology, Ulin General Hospital, Faculty of Medicine, University of Lambung Mangkurat, Banjarmasin, and South Kalimantan, Indonesia. As control, we obtained twelve nasal polyp patients. These patients were not taking any drugs before samples were taken. Informed consent was obtained from all patients and controls. In addition, we also compared the level LMP-1, PARP-1, and BZLF-1 expression in blood serum of nasopharyngeal carcinoma patients (histological confirmed to be undifferentiated WHO type II) compared with normal nasopharyngeal tissue.

### Double-labeling immunofluorescence staining of LMP-1 and PARP-1

Immunofluorescence staining was conducted to determine the LMP-1 and PARP-1 protein expression on NPC and nasal polyp tissue. Slides were incubated at 37ºC (overnight), then deparaffinized with xylol, absolute ethanol, ethanol 90% and 70% (5 min). Washing solution was phosphate buffered saline (PBS, pH 7.4). Slides washes were heated in citrate buffer (pH 6) in a high temperature microwave (10 min). Non specific protein-binding sites were blocked by incubation of 2% skim milk in PBS. Slides were then washed and incubated with primary anti-LMP-1 rabbit (1:1500 dilution; DB Biotech, Kosice, Slovakia) and anti-PARP-1 mouse monoclonal (1:1500 dilution; Novus Biologicals, Littleton, CO, USA) antibodies for 1 h in dark conditions. Slides were then washed and incubated with secondary mouse anti-rabbit IgG-FITC (1:1500; Santa Cruz; USA) and goat-anti mouse IgG-R (1:1500; Santa Cruz; USA) antibodies for 1 h in dark conditions. Slides were then washed and the LMP-1 and PARP-1 expressions were analyzed using scanning laser confocal microscopy (Olympus) [8]. Slides were captured on three fields. LMP-1 and PARP-1 expression was quantified with the Olympus FluoView software (ver 1.7a) and the average expression of LMP-1 and PARP-1 were calculated on NPC and polyp tissue.

### Histological analysis of cell necrosis

In order to obtain the distribution of necrotic cells in NPC and polyp tissue, we count the cells with morphology of pyknosis, karyorrhexis and karyolysis using CellSens software Standard on microscope (Olympus BX53). The necrotic cells were counted on three fields and the average was calculated. Necrotic cells were measured by nuclear morphology involving nuclear shrinkage (pyknosis), nuclear fragmentation (karyorrhexis) and nuclear fading (karyolysis) [6].

### Immunoblotting of LMP-1, BZLF-1, PARP-1 and caspase-3

Proteins' serum (1 mg/ml) were separated on 12.5% SDS-polyacrilamide gels electrophoresis and blotted onto nitrocellulose membrane. Non specific protein-binding sites were blocked by overnight incubation of 5% skim milk in PBS. The membrane was subsequently incubated overnight at 4°C with mouse anti-EBV monoclonal antibody (Lifespan Biosciences, Seattle, WA, USA) for BZLF-1, anti-caspase-3 rabbit polyclonal antibody (Thermo Scientific, USA), anti-LMP-1 rabbit, and anti-PARP-1 mouse monoclonal antibodies. The membranes were washed and incubated for 1 to 2 h at room temperature with peroxides-labeled polyclonal anti-rabbit IgG and anti-mouse IgG (KPL, Gaithersburg, MD, USA). The antigen-antibody complexes were visualized with Western Blue® Stabilized Substrate (KPL). The reaction was stopped by the addition of sterile aquadest. Membrane-labeled was visualized by ChemiDoc Imaging (Bio-Rad) and counted the intensity of specific protein bands by Quantity One software.

### Statistical analysis

Intensity of LMP-1 and PARP-1 expression in NPC and polyp tissue were analyzed by independent samples t-test. Histological profiles were analyzed by one-way analysis of variance (ANOVA). Correlation between LMP-1 and PARP-1 expression with cell morphology of necrosis was also analyzed. Density data of LMP-1, BZLF-1, PARP-1 and caspase-3 was analyzed by descriptive tests. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS v.16.0.

## RESULTS

LMP-1 and PARP-1 proteins are expressed in both of NPC and polyp tissues as shown in Fig.1. LMP-1 and PARP-1 are expressed in tubular epithelium and the stroma in both NPC and polyp tissues; the level of both protein expressions in polyp tissue were lower than in NPC tissue. The average value of LMP-1 and PARP-1 intensity in NPC tissues was insignificantly higher compared with polyp tissue; $1068.11 \pm 302.04$ and $1535.74 \pm 384.93$ *vs* $884.97 \pm 123.67$ and $1303.07 \pm 236.88$ intensity/μm, respectively.

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Cell morphology analysis in NPC and polyp tissue showed pyknosis, karyorrhexis and karyolysis as to see in Fig.2. Cells undergoing pyknosis are counted as $20 \pm 10$ and $20 \pm 9$, karyorrhexis as $438 \pm 158$ and $297 \pm 95$, and karyolysis as $28 \pm 12$ and $18 \pm 9$ in NPC and polyp tissues, respectively. The correlation analyses between cell morphology and the intensity of LMP-1 and PARP-1 expressions showed positive correlation with karyorrhexis, however negative correlation with pyknosis and karyolysis ($P < 0.05$). After calculating the average number of cells in NPC and polyp tissue, we then calculated the intensity of LMP-1/cells and PARP-1/cells to determine LMP-1 and PARP-1 expression in each cell. From these results, it was recorded that LMP-1 and PARP-1 expression is higher in NPC tissue than polyp; 4.19 and 6.02 *vs* 2.76 and 4.07 intensity/μm, respectively.

Immunoblotting analysis was performed to investigate the expression of LMP-1, BZLF-1, PARP-1 and caspase-3 in sera of NPC patient compared to healthy controls. The LMP-1 protein (60 kDa) was expressed equally in both of NPC patients and healthy control as seen in Fig.3. We found the 60 kDa of BZLF-1 in all samples but 35 kDa of BZLF-1 only in NPC patient's serum. We predict that the 35 Da of BZLF-1 is one of the protein markers of EBV lytic cycle in NPC cells. The PARP-1 protein (55 kDa) only expressed in NPC group; conversely, caspase-3 (32 kDa) only expressed in healthy control.

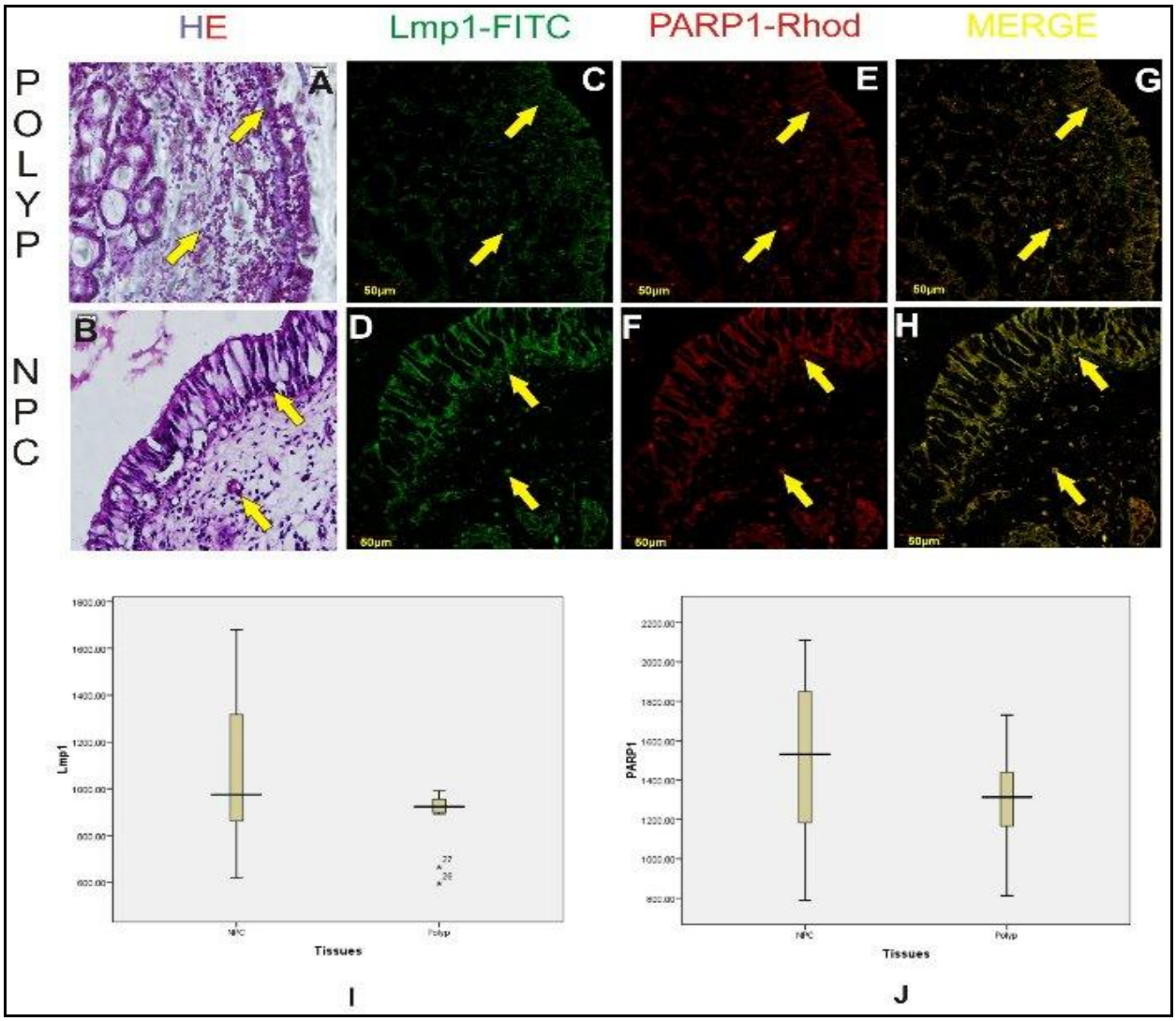


**Figure 1**. LMP-1 and PARP-1 expression in NPC and polyp tissues. **(A-B)** Hematoxylin-eosin staining. **(C-D)** Immunofluorescence of LMP-1 was labeled with FITC, so LMP-1 expression is to see by green color, **(E-F)** and PARP-1 expression was labeled with rhodamin, so PARP-1 is shown by the red ones; **(G-H)** LMP-1 and PARP-1 expression **(MERGE) is shown** by yellow color. **(I-J)** Expression levels of LMP-1 and PARP-1 in NPC and polyp tissues. Expression levels of LMP-1 (green) and PARP-1 (red) were higher in NPC than polyp tissues. Expressions of LMP-1 in polyps show that this tissue has EBV infections as well as NPC tissues.

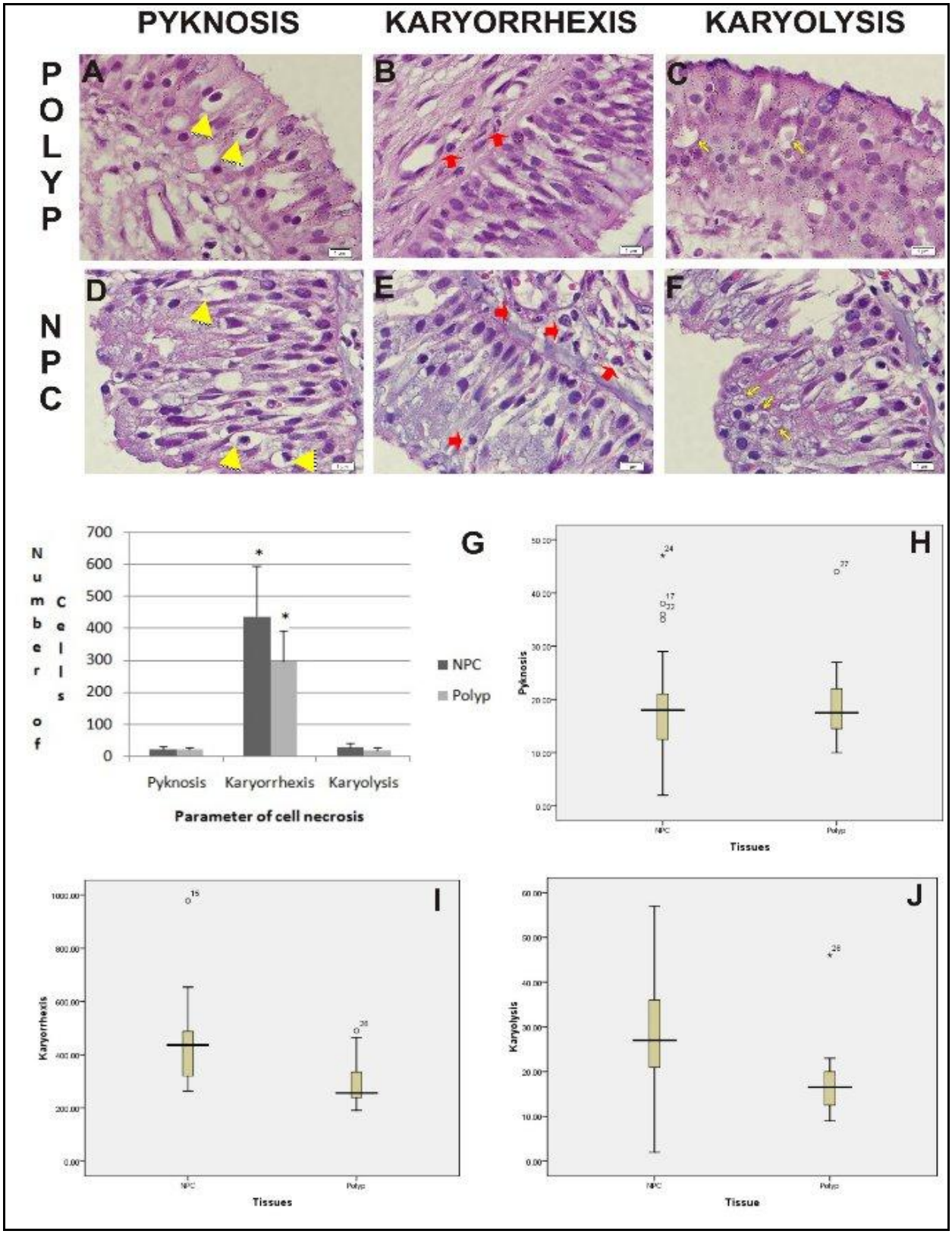


**Figure 2**.Histopathological profiles of cell morphology in both NPC and polyp tissues (HE, x1000). **(A-D)** Pyknosis is shown by yellow arrow; **(B-E)** karyorrhexis by red; and **(C-F)** karyolysis by yellow arrows. **(G)** The comparisons of necrosis parameters. Average values of **(C)** pyknosis, **(D)** karyorrhexis, and **(E)** karyolysis in NPC and polyp tissues. Karyorrhexis was determined to be the dominant morphologic change in NPC and polyp tissues.

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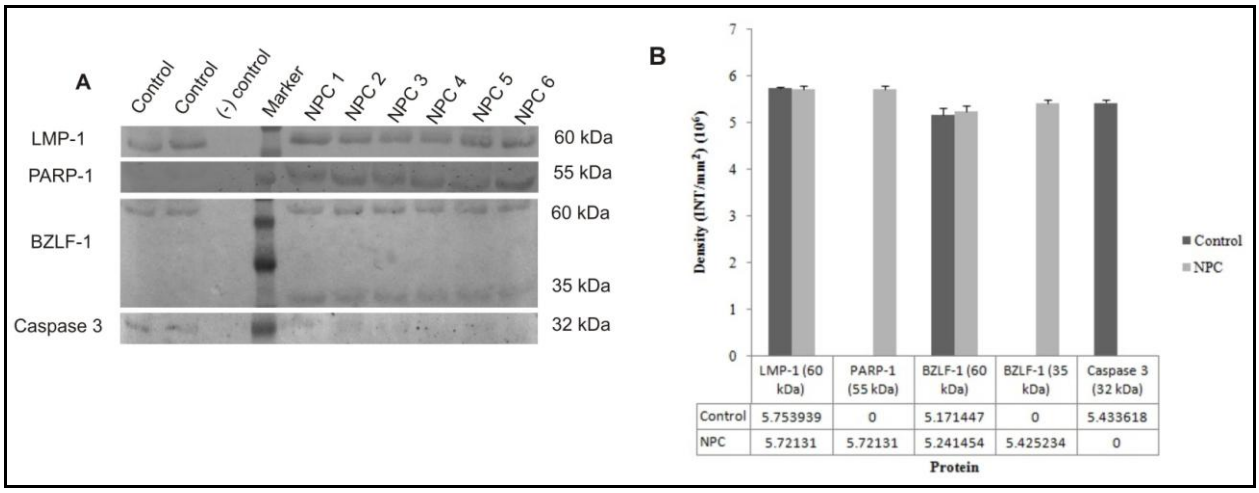


**Figure 3**. **(A)** Immunoblotting and **(B)** density level of LMP-1, PARP-1, BZLF-1 and caspase-3 proteins in NPC patient's serum. LMP-1 as marker of EBV latent infection has 60 kDa and was detected in both NPC and control sera. PARP-1 as DNA protective enzyme was found in 55 kDa and represented high expression in NPC serum. BZLF-1 was found at two molecular weights (60 and 35 kDa) due to a proteolytic process; BZLF-1 35 kDa as marker of EBV lytic cycle infection was detected only in samples derived from NPC patients. On the other hand, caspase-3 (32 kDa) only expressed in healthy control's serum; this indicates that the apoptotic process was inhibited in NPC patients, so cells were differentiated to necrosis.

## DISCUSSION

In this research, we found higher expression of LMP-1 in NPC and polyp tissues however it does not reach statistical difference. According to the immunoblotting analysis, similar LMP-1 expression in both healthy control and NPC patients is consistent with previous studies. EBV infection is considered to play a causative role in the pathogenesis of nasal polyps [9, 10]. EBV infects more than 90% of the adult people, and upon infection, the individual remains as a long life carrier of the virus [11].

The lytic form of EBV requires transmission of the virus from cell to cell and is signed by BZLF-1 expression. As shown in Fig.3 that the 35 kDa protein of BZLF-1 was only found in NPC, suggests that this protein is degraded by a protease due to proteolytic process, meanwhile the 60kD protein is present in latent phase. Another study reported differently that BZLF-1 is only expressed in the lytic but not latent form and suggested that BZLF-1 activates all of the EBV genes transcription required for the lytic replication, consequently leading to undifferentiated NPC progression [12].

PARP-1, as DNA protective enzyme of host cells, showed high levels in NPC patients. Our previous study reported that p53 expressed also highly in NPC tissues [13]. We believe that when proteolysis proceeds BZLF-1 interacts with p53 and prevents transcription genes target such as interferon (INF)-γ and major histocompatibility complex (MHC) proteins [14].

The apoptotic pathway of PARP-1 regulation is inhibited by LMP-1, so cells become necrotic and the protective mechanism of host cells stimulates severe inflammation [15, 16]. In this study, we found that the expression of LMP-1 and PARP-1 in both NPC and polyp tissues has localized in epithelium and stroma (see Fig.1). In order to detect the mechanisms of cell death in NPC patients, we detected that caspase-3 was negatively expressed in NPC; this result indicated a cell death mechanism through necrosis. This finding is in line with an earlier study performed in Jurkat T cells which reported that the necrotic cleavage of PARP-1 is caused in part or in totality by lysosomal proteases released during necrosis [17].

Necrosis was characterized with nuclear morphological changes in the form of pyknosis, karyorrhexis, and karyolysis. Pyknosis was characterized by condensation of the nucleus and clumping of chromatin; karyorrhexis was characterized by fragmentation of the nucleus; and karyolysis was characterized by lysis of nucleus, due to action of DNAse and RNAse [6]. Karyorrhexis was found to be higher than other necrosis types in NPC tissue (Fig.2G). This result is also consistent with our previous study [7].

In summary, we found that a BZLF-1 protein at a specific molecular weight of 35 kDa may be a potential marker of undifferentiated NPC. We also found that cell death mechanism in undifferentiated NPC is in preference to necrosis. This finding will open a new dimension in diagnostic and management approaches of undifferentiated NPC.

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**COMPETING INTERESTS**

The authors declare that there are no conflicts of interest.

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GESDAV

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**Original Article**

# Effect of yogic practices on age related changes in oxygen metabolism and antioxidant-redox status

**Rameswar Pal[1], Som Nath Singh[2], Kaushik Halder[3], Omveer Singh Tomer[3], Awadh Bihari Mishra[1], Mantu Saha[3]**

*[1]Centre for Advanced Research and Training in Yoga (CARTY), [2]Nutrition Division, and [3]Work Physiology & Yoga; Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur,* ***Delhi, India***

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**Corresponding Author**
Mantu Saha
Work Physiology & Yoga,
Defence Institute of Physiology and Allied Sciences,
Lucknow Road, Timarpur,
Delhi – 110054, India.
wpy.dipas@gmail.com

**Key Words**
Aging; Antioxidant; Oxidative stress; Oxygen metabolism; Yoga

**Abstract**

***Objective:*** The aim of the study was to evaluate the effect of yogic practice on age related changes in antioxidants and redox status, resting metabolism and energy expenditure.

***Methods:*** The study was conducted on 60 healthy male volunteers of three age groups *viz* 20-29 years, 30-39 years and 40-50 years. In addition to their routine activities, volunteers practiced yogasana, pranayama and meditation for a period of 3 months. Blood samples were collected in fasting condition before and after 3 months of yogic practice for the estimation of biochemical parameters.

***Results:*** Oxygen consumption and energy expenditure were decreased with the advancement of age and after yogic practice. Respiratory quotient was increased with the age and decreased after yogic practice. Advancement of age showed progressive shifting of redox status towards the oxidized state, which restored by yogic practice. Lowered levels of reduced glutathione, the ratio of reduced to oxidized glutathione, total antioxidant status, vitamin C and vitamin E as well as the activity of enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione S-transferase were associated with aging. The regular yogic practice helps to improve in these above mentioned parameters. Hydroperoxides, protein carbonyl, malondialdehyde and glutathione peroxidase levels were found to be higher due to progression of age. These have been decreased after yogic practice.

***Conclusion:*** Regular yogic practices have the ability to revert back with the changes in antioxidant and redox status due to advancement of age.



## INTRODUCTION

Aging is characterized as a progressive decline in biological functions. Progressive decline in physiological, metabolic, cognitive, neuro-endocrine functions and biochemical parameters take place with advancement of age. Studies have been reported alteration of autonomic functions due to advancement of age [1]. Age related decline in resting energy expenditure (REE) were reported [2]. The positive correlation between reactive oxygen species (ROS) and age has been well documented. The oxidative stress theory or free radical theory of aging states that damage to cellular macromolecules via free radical production in aerobic organisms is a major determinant of life span [3]. Antioxidant capacity and oxidative damage during aging in several tissues of many species has been reported [4]. Some contradictory data is also available on changes of oxidative stress markers in plasma and erythrocytes of the healthy population during aging. Studies have been reported age dependent changes in superoxide dismutase (SOD), catalase (CAT), nitric oxide (NO), malondialdehyde (MDA), reduced glutathione (GSH) and membrane sulfhydryl (SH) groups [5, 6]. Age related decrease in glutathione peroxidase (GPx) activity is positively correlated with the age dependent decline of total antioxidant capacity in plasma [7]. There is age dependent alteration of glutathione S-transferase in human lymphocytes [8].

Yoga is believed to be more than five thousand years old and originated from one of six orthodox

philosophies evolving out of the transcendentalism of ancient India. The word 'yoga' derived from Sanskrit root 'yuj' meaning 'union'. Yoga is commonly translated as "union" and the combination of heart, mind, and body. Practice of asanas, pranayamas and meditation result in reduced mental stress. It provides the practitioner with psychological-physiological fitness and holistic health. Regular yogic practices help in the constructive development of body and mind. Several studies have reported improvement in physiological, physical and psychological functions [9]. It has been reported that yoga improves also physical performance (aerobic capacity, anaerobic power and anaerobic threshold), body composition by decreasing fat and increasing lean body mass [10-12]. Yoga has a positive effect on autonomic functions to maintain the homeostasis [13]. Studies reported that yoga has a profound effect on stress and inflammation [14]. Studies showed that yoga can decrease oxidative stress and improve antioxidant and redox status [15-17]. Improvement of GSH and total antioxidant status was reported [15]. Other studies showed that short term and long term yogic practice have a beneficial role in the management of oxidative stress [16, 17]. Yoga is well known to all of its therapeutic implications [18].

Studies showed that endurance exercise has a beneficial effect on oxidative stress. Endurance exercises reduce oxidative stress in rats [19]. Positive role of exercise over the activity of antioxidant enzymes such as CAT, GPx as well as SOD has been well documented [20]. Studies showed that exercise (swimming, treadmill running and other form of physical training) has a beneficial role to reduce age associated ROS production in rat brain [21]. It needs more space, and good health to perform exercise. It is also very difficult to perform the exercise in adverse condition such as in extreme cold, extreme heat and in rainy seasons but yoga may be practiced in limited space at any environmental conditions and may be practiced by ill or healthy persons.

Keeping in mind the benefits of yoga, the present study was designed to evaluate the effect of yogic practice on age related changes on impairment of age associated decline in antioxidant and redox status including vitamin C, vitamin E, as well as hydroperoxides, lipid peroxidation products and antioxidant defense enzymes such as CAT, glutathione S-transferases (GST) and GPx with resting oxygen consumption as well as resting energy expenditure.

## MATERIALS AND METHODS

### Study volunteers

A total of 60 healthy male volunteers was randomly chosen from Indian Air Force for this study and were divided into three age groups *viz* 20-29, 30-39 and 40-50 years designated as groups A, B and C, respectively. Participants were briefed about study protocol approved by the Institutional Ethics Committee and written consent was obtained from each volunteer; data has maintained properly under report No DRDO-DIPAS-DLS-TR-09-2013. Volunteers had similar patterns of daily activity and diet. In addition to their routine activities, volunteers practiced yogasana, pranayama and meditation for 3 months. None of the volunteers had any previous exposure to yogic exercises/practices.

The anthropometric and physiological parameters were recorded. Blood samples were also collected before and after 3 months yoga training period for biochemical estimation.

### Yoga protocol

The participants were trained in selected yogic practices for 6 days in a week. Yoga training was imparted by a qualified yoga instructor. The training consisted of one practical session (60 min). The yogic module is depicted in Table 1.

### Anthropometric measurements

Anthropometric parameters of the volunteers were measured with minimal clothing (only inner wears). Body weight (BW) in kilograms was recorded using an electronic weighing machine (Delmar, India) least counting 0.1 kg. The standing height from the sole of the feet to the vertex in erect body position was measured in centimeters using anthropometric rod. Body mass index (BMI) was calculated as the ratio of weight to height in squared meter ($kg/m^2$).

### Resting physiological parameters; *Oxygen consumption ($VO_2$), carbon dioxide elimination ($VCO_2$) and energy expenditure (REE), respiratory quotient (RQ)*

Portable computerized equipment (Cosmed $K4b^2$, Rome, Italy) was used to measure $VO_2$ and $VCO_2$. Prior to each test, the equipment was calibrated as per standard method. The standard gas mixture was used to calibrate the oxygen and carbon dioxide. The $O_2$ and $CO_2$ sensors were calibrated with the same gas mixture (15.06% $O_2$, 5.97% $CO_2$) used to calibrate the reference system. RQ and REE was calculated by the software provided with the system.

### Biochemical estimation

#### *Collection of samples*

Fasting venous blood samples of the volunteers were collected from an antecubital vein in EDTA treated vial in the morning in resting condition. The aliquot of 0.5 ml of whole blood was treated with equal volume of 10% metaphosphoric acid (MPA) and 1 ml 10% trichloroacetic acid (TCA) was mixed and kept in a

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**Table 1.** Details of yogic module practiced by volunteers

| |
|---|
| **Cleansing processes (2 min)** |
| 1. Kapalbhati (rapid shallow breathing) |
| **Yogasanas (yogic postures) (40 min)** |
| 1. Suryanamaskar (sun salutation in 12 different postures, 1 round) |
| 2. Padmasana |
| 3. Yogamudra |
| 4. Matsayasana |
| 5. Suptapavanmuktasana |
| 6. Pavanmuktasana |
| 7. Paschimottanasana |
| 8. Vajrasana |
| 9. Suptavajrasana |
| 10. Gomukhasana |
| 11. Sarvangasana |
| 12. Halasana |
| 13. Karnapedasana |
| 14. Bhujangasana |
| 15. Shavasana (relaxed supine posture) |
| **Pranayama (breathing exercises) (10 min)** |
| 1. Bhastrika (forceful expulsion of breathing) |
| 2. Anulom-vilom (alternative nostril breathing) |
| 3. Bhramri (producing buzzing sound of bee with closed ear and lips) |
| **Meditation (8 min)** |
| 1. Omkar meditation (Om Chant) |

separate vial. The remaining blood sample was centrifuged at 1,000g for 15 min to collect plasma and RBC pellet. Samples were maintained at –20°C at a field location brought to laboratory and stored at –80°C until assayed.

***Reduced (GSH) and oxidized (GSSG) glutathione***

Metaphosphoric acid and TCA treated whole blood was centrifuged at 10,000g for 15 min and the clear supernatant obtained. Supernatant was used to estimate the GSH and GSSG by using fluorimetric method [22].

***Total antioxidant status (TAS)***

It was measured in plasma as an 2,2'-Azino-di-(3-ethylbenzthiazoline sulphonate (ABTS) radical cation decolorizing assay using a commercial kit (Randox Laboratories, Antrim, UK)

***Vitamin C***

Supernatant obtained from MPA and TCA treated whole was used to estimate vitamin C [23]. The color attained due to coupling reaction of 2,4-dinitrophenyl hydrazine (DNPH) and vitamin C; the density of color was measured at 540 nm using a spectrophotometer.

***Vitamin E***

The estimation of vitamin E present was carried out using plasma sample [24]. Ferric ion was reduced to ferrous ion by vitamin E present in the lipid residue and forms a pink colored complex with bathophenanthroline orthophosphoric acid. Absorption due to the pink complex was measured at 536 nm using spectrophotometer.

***Protein carbonyl (PC)***

Protein carbonyl content was estimated using plasma. DNPH reacts with PC forming a Schiff base to produce the corresponding hydrazone which can be analyzed spectrophotometrically [25].

***Malondialdehyde (MDA)***

Malondialdehyde content was estimated using supernatant obtained from MDA- and TCA-treated whole blood [26]. Supernatant was mixed with equal volume of thiobarbituric acid solution and kept it in 90°C for 30 minutes; the color density was measured spectrophotometrically and the value was calculated by using extinction coefficient.

***Glutathione reductase (GR)***

Red blood cell (RBC) lysate was used to measure the GR activity [27]. The final reaction mixture contains GSSG and nicotinamide adenine dinucleotide phosphate (NADPH); these are incubated at pH 7.5 and change in optical density (OD) was measured at 340 nm for 3 min at an interval of 30 sec.

***Glutathione peroxidase (GPx)***

It was measured in RBC lysate by using a commercial kit (Cayman, Ann Arbor, MI, USA).

***Superoxide dismutase (SOD)***

Superoxide dismutase was measured also in RBC lysate by using the Cayman kit.

***Catalase (CAT)***

Catalase was measured spectrophotometrically [28]. Briefly the RBC lysate was treated with $H_2O_2$ and absorbance was measured at 240 nm wavelength for 1 min at an interval of 30 sec.

***Glutathione S-transferase (GST)***

The estimation of GST using 1-chloro-2,4-dinitrobenzene was carried out using RBC lysate [29]. In brief RBC lysate was incubated with GSH, 1-chloro-2-4-dinitrobenzenes (CDNB) and phosphate buffer in the pH 6.5 and change in OD was measured at 340 nm for 3 min at an interval of 30 sec.

***Glucose 6 phosphate dehydrogenase (G6PDH)***

The estimation of G6PDH was carried out using RBC lysate [30]. RBC lysate was incubated with magnesium chloride, glucose 6 phosphates, nicotinamide adenine dinucleotide phosphate ($NADP^+$) and buffer at pH 7.4 and change in OD was measured at 340 nm for 3 min at an interval of 30 sec.

**Statistical analysis**

Comparison of before and after yogic practice in each group was made using paired t-test; P values of less than 0.05 were considered significant. All intergroup comparisons were done by one-way ANOVA. When significant differences were found, Tukey-Kramer multiple comparisons tests were done as a post hoc analysis. All statistics were made using SPSS (v. 17). Data shown are mean ± standard error of mean (SEM).

## RESULTS

**Anthropometric data**

Anthropometric data are summarized in Table 2. BW and BMI were found significantly higher due to advancement of age in group B ($P < 0.01$) and C ($P < 0.001$) in respect of group A. BW did not show any significant change following yogic practices in group A and group C, though there was a trend of increment of BW (1.86%) following yogic practice in group A. A significant ($P < 0.01$) reduction of BW was noted in group B, while BW was slightly (1.09%) decreased in group C following yogic practice.

BMI was significantly higher in group B ($P < 0.01$) and C ($P < 0.001$) in respect of group A. BMI did not show any significant change following yogic practices in group A and group C, though there was a trend of increment of BMI (1.81%) following yogic practice in group A. A significant ($P < 0.01$) reduction BMI was noted in group B, and BMI was slightly (0.79%) decreased in group C following yogic practice.

**Resting physiological variables**

Resting physiological variables are illustrated in Table 3. $VO_2$ and $VCO_2$ were lowered due to progression of age. $VO_2$ was significantly lower in group B ($P < 0.01$) in and group C ($P < 0.001$) in respect of group A. A further significant ($P < 0.05$) reduction in $VO_2$ was noted following yogic practice in all the age groups. $VCO_2$ was also decreased due advancement of age. $VCO_2$ was further decreased significantly ($P < 0.05$) in group B and group C following yogic practice. A marginal reduction (7.02%) of $VCO_2$ was noted in the group A following yogic practice.

RQ was observed higher due to advancement of age. RQ was significantly ($P < 0.05$) higher in group C in respect of group A. RQ was significantly ($P < 0.001$) reduced in groups B and C following 3 months of yogic practice. A marginal reduction (8.75%) of RQ was noted in group A following yogic practice. REE was decreased significantly due to advancement of age in groups B ($P < 0.05$) and C ($P < 0.01$) in respect of group A. REE tended to reduce further due yogic practice; a significant ($P < 0.05$) reduction was noted in group A, a marginal reduction of 2.04% in group B, but no change in group C was noted.

**Non-enzymatic antioxidant**

Non-enzymatic antioxidant variables are depicted in Table 4. Advancement of age lowered the GSH level, which was significantly improved after yogic practice. Improvement of the GSH level was significant ($P < 0.001$) in the entire groups. GSSG was observed higher due to advancement of age and a reduction was noted after yogic practice; it decreased significantly in groups A ($P < 0.05$), B ($P < 0.05$) and C ($P < 0.001$). GSH/GSSG ratio was noted the same trend of reduction as GSH due to advancement of age but the increment of this ratio was significant ($P < 0.001$) in groups A, B and C following yogic practice.

Advancement of age lowered the TAS level. Improvement of levels of TAS was noted in the entire groups whereas it was found significant ($P < 0.01$) in groups B and C. A marginal increment (7.93%) of TAS was noted in group A following yogic practice.

Levels of vitamin C were found decreased due to progression of age and which improved following 3 months of yogic practice. Yogic practice caused significant improvement of vitamin C levels in groups A ($P < 0.001$), B ($P < 0.001$) and C ($P < 0.01$). Levels of vitamin E showed similar pattern of decrement as vitamin C associated with age. The vitamin E level was found to be decreased significantly ($P < 0.05$) in group C in respect of group A. Yogic practice showed a significant improvement of vitamin E in groups A ($P < 0.01$), B ($P < 0.01$) and C ($P < 0.001$).

Age associated increase of hydroperoxide level was observed. Hydroperoxides were decreased by yogic practice in groups A (16.18%), B (16.20%) and C (14.63%) following yogic practice. Lipid peroxidation and protein oxidation products MDA and PC, respectively, followed the same pattern of increment due to advancement of age and were found to be decreased after 3 months of yogic practices. Levels of MDA decreased significantly ($P < 0.01$) in group A, however only marginal levels of 12.84% and 14.63% reduction was observed in groups B and C, respectively, following yogic practice. Furthermore, marginal reduction of 15.91%, 13.96% and 6.04% for the levels of PC were detected in groups A, B and C, respectively, following yogic practice.

**Activity of antioxidant enzymes**

Activities of antioxidant enzymes are illustrated in Table 5. Activities of SOD were observed lower due to advancement of age. SOD activity increased significantly following 3 months of yogic practices in group A ($P < 0.01$), group B ($P < 0.05$) and group C ($P < 0.05$). The activity of CAT was also declined due to advancement of age. A total 3 months of yogic practices enhanced the activity of CAT marginally in groups A (28.37%), B (15.27%) and C (4.31%).

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**Table 2.** Physical characteristics of volunteers (mean ± SEM)

| | 20-29 yrs (A) | | 30-39 yrs (B) | | 40-50 yrs (C) | |
|---|---|---|---|---|---|---|
| **Parameters** | **Before** | **After** | **Before** | **After** | **Before** | **After** |
| **Height (cm)** | 171.5 ± 1.6 | - | 169.8 ± 1 | - | 170.1 ± 1.4 | - |
| **Weight (kg)** | 64.4 ± 1.4 | 65.6 ± 1.3 | 71.9 ± 1.5** | 71.1 ± 1.6## | 73.3 ± 1.6*** | 72.5 ± 1.6 |
| **BMI** | 22 ± 0.6 | 22.4 ± 0.6 | 24.9 ± 0.4* | 24.6 ± 0.4## | 25.3 ± 0.5*** | 25.1 ± 0.5 |

(*) in comparison to group A; (#) in comparison to the respective value before yogic exercise. *P < 0.05; ##,**P < 0.01; ***P < 0.001.

**Table3.** Effect of yogic practice on resting oxygen metabolism of different age groups (mean ± SEM)

| | 20-29 yrs (A) | | 30-39 yrs (B) | | 40-50 yrs (C) | |
|---|---|---|---|---|---|---|
| **Parameters** | **Before** | **After** | **Before** | **Parameters** | **Before** | **After** |
| **$VO_2$ (l/min)** | 326.2 ± 17.6 | 280.4 ± 17.3* | 254.6 ± 14.0** | 225.9 ± 9.48# | 249.4 ± 15.3*** | 210.7 ± 56.4# |
| **$VCO_2$ (l/min)** | 220.7 ± 13.7 | 205.2 ± 13.5 | 218.1 ± 16.3 | 184.4 ± 9.3# | 213.9 ± 12.2 | 167.5 ± 10.5# |
| **RQ** | 0.8 ± 0.05 | 0.73 ± 0.02 | 0.89 ± 0.02 | 0.78 ± 0.03### | 0.94 ± 0.02* | 0.8 ± 0.02### |
| **REE (kj/min)** | 6.3 ± 0.34 | 5.5 ± 0.34* | 4.9 ± 0.35* | 4.8 ± 0.25 | 4.2 ± 0.14** | 4.2 ± 0.24 |

(*) in comparison to group A (before yogic exercise); (#) in comparison to the respective value before yogic exercise. *,#P < 0.05; **P < 0.01; ***,###P < 0.001. $VO_2$: oxygen consumption; $VCO_2$: carbon dioxide elimination; RQ: respiratory quotient; REE: resting energy expenditure.

**Table 4:** Effect of yogic practices on antioxidant and redox status of different age groups (mean ± SEM)

| | 20-29 yrs (A) | | 30-39 yrs (B) | | 40-50 yrs (C) | |
|---|---|---|---|---|---|---|
| **Parameters** | **Before** | **After** | **Before** | **Parameters** | **Before** | **After** |
| **GSH (nmol/ml)** | 293.5±25.9 | 450.1±20.6*** | 289.9±17.9 | 388.1±18.4### | 259.5±19.2 | 360.8±15.9### |
| **GSSG (nmol/ml)** | 411.8±14.9 | 355.8±19.3* | 422.2±15.9 | 361±10.8# | 451.2±16.8 | 384.4±13.9### |
| **GSH / GSSG** | 0.72±0.06 | 1.34±0.09*** | 0.69±0.04 | 1.1±0.1### | 0.58±0.04 | 0.96±0.05### |
| **TAS (mmol/ml)** | 2.9±0.08 | 3.13±0.12 | 2.81±0.03 | 3.08±0.07## | 2.61±0.09 | 3.01±0.08## |
| **Vitamin C (mg/dl)** | 1.07±0.14 | 2.45±0.31*** | 1.26±0.14 | 2.76±0.3### | 0.98±0.13 | 1.70±0.25## |
| **Vitamin E (µg/ml)** | 15.3±1.16 | 19.9±2.79** | 14.6±5.44 | 18.5±0.52## | 11.5±0.98* | 16.7±0.46### |
| **Hydroperoxide (µmol/ml)** | 380.8±28.8 | 319.2±41.9 | 402.5±37.6 | 337.3±33.4 | 423.7±40.7 | 357.5±20.1 |
| **MDA (nmol/ml)** | 6.61±0.23 | 5.69±0.12** | 6.93±0.38 | 6.04±0.28 | 7.79±0.37 | 6.65±0.39 |
| **PC (nmol/ml)** | 98±10.51 | 82.4±7.6 | 110.3±11.49 | 94.9±7.79 | 114.1±11.05 | 107.2±11.95 |

(*) in comparison to group A (before yogic exercise); (#) in comparison to the respective value before yogic exercise. *,#P < 0.05; **,##P < 0.01; ***,###P < 0.001. GSH: reduced glutathione; GSSG: oxidized glutathione; GSH/GSSG: ratio of reduced and oxidized glutathione; TAS: total antioxidant status; MDA: malondialdehyde; PC: protein carbonyl.

**Table 5.** Effect of yogic practice on antioxidant defence enzymes of different age groups (mean ± SEM)

| | 20-29 yrs (A) | | 30-39 yrs (B) | | 40-50 yrs (C) | |
|---|---|---|---|---|---|---|
| **Parameters** | **Before** | **After** | **Before** | **Parameters** | **Before** | **After** |
| **SOD (u/ml)** | 237.9 ± 6.9 | 309.9 ± 17.1## | 224.3 ± 14.2 | 298.9 ±19.7# | 209.4 ± 7.5 | 288.4 ± 19.4## |
| **CAT (µmol/ml/min)** | 194.9 ± 16.6 | 250.2 ±21.3 | 139.2 ± 22.4 | 160.5 ± 18.8 | 136.6 ± 13.5 | 142.5 ± 16 |
| **GPx (µmol/ml/min)** | 7.58 ± 0.2 | 5.79 ± 0.29### | 8.26 ± 0.15 | 6.11 ± 0.3### | 8.27 ± 0.37 | 6.22 ± 0.25### |
| **GR (µmol/ml/min)** | 0.29 ± 0.04 | 0.39 ± 0.07 | 0.27 ± 0.03 | 0.31 ± 0.02 | 0.22 ± 0.02 | 0.3 ± 0.02# |
| **GST (mmol/ml/min)** | 16.8 ± 1.37 | 25.4 ± 1.87## | 14.8 ± 0.93 | 22.7 ± 2.22## | 13.6 ± 1.53 | 19.6 ± 1.21## |
| **G6PDH (nmol/ml/min)** | 177.9 ±16.9 | 186.3 ± 10.6 | 161.3 ± 9.1 | 176.2 ± 9.7 | 134.5 ± 9.7 | 153.6 ± 9.2 |

(#) in comparison to the respective value before yogic exercise. #P < 0.05; ##P < 0.01; ###P < 0.001. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione S-transferase; G6PDH: glucose 6 phosphate dehydrogenase.

GPx activity also increased due to advancement of age. Yogic practice caused decrease in activity of GPx significantly ($P < 0.001$) in all the age groups. The activity of GR was lower with advancement of age and improvement was recorded after 3 months of yogic practices. Activities of GR was improved significantly ($P < 0.05$) in group C. A marginal improvement of 37.48% and 14.81% in the GR activity was noted in group A and group B, respectively, following yogic practices. GST activity was lowered due to advancement of age and was significantly ($P < 0.01$) improved after 3 months of yogic practices in all entire groups. Advancement of age reduced the activity of G6PDH. A marginal enhancement of 4.72%, 9.24% and 14.20 % was noted in groups A, B and C, respectively, following yogic practices.

## DISCUSSION

Progression of age increases the body surface area and also fat deposition in the body, which causes an increment of BW and BMI. There are conflicting reports in body weight changes after yogic exercises in literature as in some reports it was found to increase while other noted decrease [31, 12]. However in the present study decrease in body weight in higher age with yogic practice was observed.

It is well established that advancement of age influence fat deposition, which may decrease REE as well as $VO_2$ and $VCO_2$ [2]. Present study also supports this phenomenon. In this study the decrement of REE as well as $VO_2$ and $VCO_2$, and increment of RQ due to advancement of age were noted. REE as well as $VO_2$, $VCO_2$, and RQ were decreased after yogic practice. Studies explained decrement of $VO_2$, $VCO_2$, and respiration rate after yogic practices due to decreased stress and relaxed body and mind [32].

This study shows that SOD activity increases significantly and there is a trend to increase in CAT activity after yogic practice. Earlier studies have also reported that the yogic practice help in the increase of SOD activity [18]. SOD spontaneously catalyses superoxide ($O_2^{\bullet -}$) and generate hydrogen peroxide ($H_2O_2$) through a rapid dismutation reaction. This means that wherever $O_2^{\bullet -}$ is generated it will increase the formation of $H_2O_2$. SOD and CAT are metalloproteins that catalyse $O_2^{\bullet -}$ and $H_2O_2$ respectively. SOD catalyses the formation of $H_2O_2$ from two $O_2^{\bullet -}$, whereas CAT catalyses the formation of oxygen ($O_2$) and water from $H_2O_2$ molecule. Decrement of SOD and CAT activity due to advancement of age may increase the accumulation of hydroperoxides in the body whereas increment of SOD and CAT activity after yogic practice will lead to catalyze more $H_2O_2$ and produce more $O_2$ and water. Hence hydroperoxide level was decreased after yogic practice. Studies reported the alteration of SOD and CAT due to advancement of age [5].

Advancement of age increases the activity of GPx and decreases the activity of GR. Yogic practices decreased the activity of GPx and increased activity of GR. GSH level as well as the ratio of reduced to oxidized glutathione (GSH/GSSG), another important and sensitive marker of the antioxidant system, increased significantly after yogic practice. These may be due to the improvement of the antioxidant defense mechanism by yoga. Age related shifting towards oxidized state was reported whereas studies stated the shifting of individual towards a reduced state due to yogic practice by improving GSH and GSH/GSSH ratio through yogic practice [4, 15]. GSH protects the cell from oxidative damage by catalyzing $H_2O_2$ in the presence of GPx. GSSG, produced upon reduction of hydroperoxide by GPx is recycled to its reduced state by GR using NADPH as hydrogen donor. Here the production of NADPH is coupled with G6PDH. Advancement of age alters the activity of GPx, GR and G6PDH which decrease the level of GSH and GSH/GSSG, and increase GSSG. This study showed that yoga increased GSH and decreased GSSG by altering the activities of GPx and GR.

Glutathione S-transferases are multifunctional enzymes which play a key role in cellular detoxification. The enzymes protect cells against toxicants by conjugating them to GSH, thereby neutralizing their electrophilic sites, and rendering the products more water-soluble [33]. The GSH conjugates are metabolized further to mercapturic acid and then excreted [34]. Advancement of age decreased GST activity, which was increased significantly after yogic practices; this also acts as a defense mechanism of the body against ROS. GST also catalyze the thiol groups of GSH and possible alkylating agents, allowing GSH to carry out its detoxifying function.

Levels of vitamin C and E decreased due to advancement of age and increased after yogic practice. Vitamin C and E have a profound effect on the body; during lipid peroxidation vitamin E acts as a potent chain-breaking antioxidant, intercepting lipid peroxyl radicals and forming the vitamin E radical as a product. By accepting the electron from the vitamin E radical, vitamin C regenerates vitamin E, and the vitamin C radical is being formed; this radical either can be excreted from the body via urine or regenerated to vitamin C via electron donation from GSH. It was also reported that both vitamin E and vitamin C have the positive role to scavenge superoxide and hydroxyl radicals in lipid and aqueous phase, respectively, *i.e.* inhibiting lipid peroxidation and oxidative damage to other macromolecules.

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Shifting of the individual towards oxidized state was observed due to advancement of age may cause a decrement of TAS. This is also supported by earlier studies [4]. TAS has been seen to improve significantly after yogic practice denoting a marked improvement in the overall cellular antioxidant level. Studies also reported that the yogic practice helped in the improvement of TAS [15].

Oxidative stress generally damages the membrane polyunsaturated fatty acids leading to the generation of MDA. Earlier studies support this fact [5]. This biochemical study implies that the products of lipid peroxidation were decreased with yogic practice. This is also supported by previous reports [17]. Studies also showed that the yogic practice decreased MDA level in diabetic patients [18]. Oxidation of proteins accumulates PC due to advancement of age which was decreased due to yogic practice. Above mentioned literatures and this study showed the shift of antioxidant and redox status towards oxidized state with regard to advancement of age; this may due to the production of more oxidized products such as PC via oxidation of proteins and MDA via lipid peroxidation. The yogic practice shifted the individuals towards a reduced state; a possible reason may be less accumulation of PC in the body.

In conlusion, this study showed that advancement of age shifts the individual towards the oxidized state of antioxidant and redox status. The selective yogic training helped to improve GSH GSSH/GSSG and activity of antioxidant defense enzymes which may shift the individual towards a more stable reduced state. Yoga helped to relax the body by decreasing resting $O_2$ consumption and REE. Yoga may help to reduce aging processes by increasing antioxidants of the practitioner. Yoga may also improve the antioxidant defense mechanism and the practitioner goes towards a redox homeostasis.

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**Original Article**

# Ethyl acetate extract of *Squilla oratoria* suppresses growth of HepG2 cells by inducing S phase arrest

**Xiangwei Qi[1], Xia Kong[1], Xiangning Zhang[1,2], Peichun Huang[1,2]**

*[1]Department of Pathophysiology, [2]Key Laboratory for Medical Molecular Diagnostics of Guangdong Province, Sino-American Cancer Research Institute, Guangdong Medical College,* ***Guangdong, China***

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**Corresponding Author**
Xiangning Zhang[1] & Peichun Huang[2]
Department of Pathophysiology,
Guangdong Medical College,
1 Xincheng Road,
Song-Shan Lake (SSL) Science
Technology and Industrial Park,
Dongguan, Guangdong 523808, China.
[1]zhangxn_2006@126.com
[2]huangpc@gdmc.edu.cn

**Key Words**
Antitumor agent; Cell cycle; Cyclin; Marine drug; Proliferating cell nuclear antigen; Xenograft.

**Abstract**
***Objective:*** The oceans and seas are a rich source of organisms from which anti-cancer drugs can be isolated and developed. Marine organisms have been screened in our laboratory, and organic solvent extracts of *Squilla oratoria* (ESO) have been shown to possess cytostatic effects on cancer cell lines of diverse origins. To explore the underlying mechanisms, the growth inhibition by ESO was investigated in the present study.
***Methods:*** Human hepatocellular carcinoma (HCC) derived cells (HepG2) were used. The cells were challenged with ESO, cell cycle profile was assayed, and level of proliferating cell nuclear antigen (PCNA) expression and that of cyclin D1 and cyclin A were evaluated with flow cytomtery. The *in vivo* antitumor effect of ESO was tested in nude mouse xenografts. PCNA expression was evaluated immunohistochemically in nude mouse xenograft tissues.
***Results:*** With the increase in dose of injected ESO, expression of PCNA by human HCC xenografts increased. ESO inhibited the growth of human HCC HepG2 cells both *in vitro* and *in vivo*. The effect was correlated with arrest of the cell cycle in S phase. Expression of PCNA, which is a cell-cycle regulator that promotes S phase entry, was elevated in both cell lines and xenografts whereas that of cyclins that promote M phase entry was down-regulated by exposure to ESO.
***Conclusion:*** Growth inhibition was explained by arrest of the cell cycle in S phase and down-regulation of molecules that promote cells to enter S phase.



## INTRODUCTION

It is generally agreed that the cancer is a disorder of heredity defects in which multiple genetic lesions have been identified. The loss-of-functions of tumor suppressor genes, and gain of transforming ability of oncogenes lead to the aberrant regulation of multiple biological activities in malignant cells including cell cycle progression, response to apoptotic stimuli, cell adhesion and motility, and angiogenesis [1]. The efficacy of anticancer therapy demands precise targeting to the defects, so as to accurately eradicate malignant clones arisen within the body of the affected individuals.

Today, hepatocellular carcinoma (HCC) remains a major health problem due to its high incidence in the population and as an important cause of cancer-related death. In 2008, half of the new liver cancer cases and deaths were estimated to occur in China [2]. HCC represents the major histological subtype, accounting for 70% to 85% of the total liver cancer burden worldwide [3]. Multiple cytogenetic as well as molecular genetic lesions have been identified in tumor and cell lines of HCC, and proposed to be responsible for its occurrence, and the aberrations provide clues for design of anti-HCC therapy.

Among the aberrations implicated in the genesis of HCC, gain of chromosome 1q copy, loss of chromosome 4q, 8p [4, 5] and 16q [ 6, 7] are frequent. The loss of 16q24.1-q24.2 in prostate cancer has been associated with aggressive behavior of the disease, recurrent growth, poor differentiation of the tumor, and a poor prognosis for the patient [9]. The best-studied tumor suppressor gene is E-cadherin (*CDH1*) at 16q22.1. Interestingly, expression of the E-cadherin gene is

reduced by a hypermethylation mechanism, although no somatic mutations of the E-cadherin gene in HCC have yet been described [10, 11].

Resection and transplantation are the only curative treatments available but are greatly hampered by high recurrence rates [3]; thus, more effective therapeutic approaches are needed for the treatment of HCC. One therapeutic approach includes using highly effective and minimally cytotoxic drugs derived from natural products in addition to the routinely used chemotherapeutical agents. Similar to other chemotherapeutic agents routinely employed in anticancer treatments, compounds derived from natural products exert cytostatic or growth-inhibitory effects on target cells by induction of apoptosis or inhibition of proliferation by arresting the cell cycle, inducing differentiation of malignant cells, modifying host immunity, or inhibiting angiogenesis [12].

The oceans and seas comprise approximately three-quarters of the global surface and are rich in such natural resources such as medicinal materials. To date, several bioactive antitumor compounds isolated from marine organisms have shown promising clinical application. Dolastatin 10 and auristatins were originally discovered as constituents of the sea hare *Dolabella auricularia* and have entered human cancer clinical trials [13].

The agents in combination are more effective in inhibiting cancer cell proliferation than either agent alone [14]. A new antimitotic natural product, cyanobacterial style with features of both dolastatins 10 and 15 was isolated from the same Floridian *Symploca* sp sample that produced the histone deacetylase inhibitor largazole. Both agents in combination are more effective in inhibiting cancer cell proliferation than either agent alone. Pico, a synthetic bryostatin-1-like compound, caused increased expression of the pro-apoptotic protein Bak in human mantle lymphoma cell lines, showing a potent apoptotic effect [15].

Mycoepoxydiene (MED) is a polyketide isolated from a marine fungus associated with mangrove forests. It induces cell cycle arrest at G2/M phase in HeLa cells. MED-associated apoptosis was characterized by the formation of fragmented nuclei, poly(ADP-ribose) polymerase (PARP) cleavage, cytochrome c release, caspase-3 activation, and an increased proportion of sub-G1 cells. These results provide evidence that MED is a novel antitumor agent with low toxicity to host cells [16]. Therapeutic effects have also been demonstrated for other, less chemically defined extracts from marine organisms. Marine sponges harbor novel and undefined compounds with anti-neoplastic activity. Among the sponges that have been studied *Crambe crambe* marine sponge extract (CR) strongly reduced viability of tumor cells at a dilution of 1:1000 but was less toxic to normal fibroblasts and endothelial cells [17]. The cytotoxicity of the extracts of three soft corals, *Cladiella australis*, *Clavularia viridis* and *Klyxum simplex*, on human oral squamous cell carcinoma cells (SCC25) has also been demonstrated. They sensitized SCC25 cells in the G0/G1 and S-G2/M phases with a concomitant, significantly increased sub-G1 fraction, indicating cell death by apoptosis [18]. It has previously been demonstrated that extracts of soft coral of the genus *Sinularia* exhibit cytotoxic effects towards SCC25 and HaCaT cells (immortalised human keratinocyte cell line) by reducing cell attachment [19].

*Squilla oratoria* (or *Oratosquilla oratoria*) is a species of mantis shrimp *Stomatopoda* that was first identified by De Haan in 1844 and is found in the Western Pacific [20]. Since 1988, several marine organisms obtained from the local marketplace have been screened for antitumor activities in our laboratory. The organic solvent extract of *Squilla oratoria* (ESO) was shown to inhibit the proliferation of CNE-2Z cells (human nasopharyngeal carcinoma cell line) in a dose-dependent manner, accompanied by decreased telomerase activity [21]; it also remarkably inhibited the tumor uptake of nasopharyngeal carcinoma (NPC) xenograft in nude mice, with an activity comparable to that of 5-fluorouracil. However, the mechanisms of *in vitro* and *in vivo* tumor growth inhibition remain to be elucidated.

As an anticancer agent under development, ESO inhibits proliferation of cancer cells and suppresses tumor formation. The effect may be due to regulation of cell cycle entry. In the present study, the effect of ethyl acetate ESO on regulation of the cell cycle in HCC-derived HepG2 cells and the possible mechanisms involved were explored.

## MATERIALS AND METHODS

### Cells and reagents

HepG2 cells derived from HCC were purchased from Cell Bank of Institute of Life Science, Chinese Academy of Science, Shanghai, China and were thawed from a liquid nitrogen stock. The anticancer, therapeutic drug cisplatin (CDDP) was purchased from Haosen Pharmaceutic Inc. (Hangzhou, China). Cells were maintained in RPMI 1640 (Gibco Life Technology, Invitrogen; Guangzhou, China) supplemented with fetal calf serum (Evergreen Inc.; Hangzhou, China). Dimethyl sulfoxide (DMSO), methylthiazolyl diphenyl tetrazolium bromide (MTT), trypan blue, Giemsa's dye, and propidium iodide (PI) were purchased from Sigma through Sino-American Biotechnology, Inc (Luoyang, China). Fluorescein isothiocyanate (FITC)-conjugated anti-PCNA (proliferating cell nuclear antigen), anti-cyclin A, and

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anti-cyclin D1 and FITC-labeled conjugated anti-CD3 were purchased from Becton Dickinson (San Jose, CA, USA).

**Isolation and purification of the ESO**

Fresh *Stomatopoda, Squilla oratoria* was purchased from the local marketplace. The anticancer components were isolated by extraction with 95% ethanol, immersing the coarse isolate in ethanol 3-4 times. Primary extracts were obtained and further extracted with petroleum ether, ethyl acetate, and n-butanol saturated with distilled water. Three jelly-like extracts were obtained, *i.e.* termed extracts A, B and C. Their weight and extraction rates after evaporation of the solvent are shown in Table 1.

**Cell culture and MTT assay**

The effects of ESO on growth of the human HCC line HepG2 at different time courses were evaluated by the MTT assay. The inhibitory rates at each time course were calculated and $IC_{50}$ values were calculated by the Karber equation [22]. The extract with the strongest effect was chosen for the *in vitro* experiments. HepG2 cells grown to log phase were used. Cell viability was determined by trypan blue expulsion and aliquots with > 95% viable cells were adjusted to the desired cell density and added to 96-well culture plates. Cells were incubated at 37°C, 5% $CO_2$ overnight and fresh medium with ESO at concentrations of 0, 100, 200 or 400 μg/ml was added. The positive control contained an equal volume of CDDP at a final concentration of 10 μg/ml. Each concentration of ESO and the CDDP were added in quadruplicate and further incubated for 24, 48 and 72 h. MTT was diluted with serum-free RPMI 1640 medium and 50 μl of the solution was added to each well of the culture plate and incubated for 4 h. The medium was removed and replaced by 100 μl of DMSO (150 μg/ml), and the plates were rocked before the samples were visualized with an ELISA reader (BioTek; Beijing, China), with absorbance at 570 nm (OD). Mean values for each group were calculated, and the data were analyzed with SPSS 15.0 software. The Karber formula was used to calculate the $IC_{50}$ of the drug:

$$\mathbf{LogIC_{50} = XK - I\ (\Sigma P - 0.5)}$$

-XK; the log of maximal dose

-ΣP, the sum of the inhibitory rates in each group

$$\mathbf{GI = \frac{1 - OD\ of\ experimental\ groups}{OD\ of\ control\ group} \times 100\%}$$

-GI; growth inhibitory rate: an inhibitory rate > 50% is defined as highly sensitive, 30-49% as moderately sensitive, and < 30% as insensitive.

**Flow cytometry**

For analysis of DNA content, cells in log phase were seeded onto Falcon flasks and incubated overnight in 5% $CO_2$ before switching to fresh complete medium mixed with ESO at final concentrations of 100, 200 or 400 μg/ml; equal amount of RPMI medium was added as a blank control. After culturing for 24 and 48 h, the cells were harvested, washed twice with sterile PBS, and fixed with pre-cooled 70% ethanol overnight at 4°C. After washed with cold PBS and resuspended in PBS supplemented with PI at a final concentration of 10 μM, DNA contents of cells in each group were assayed with fluorescence-activated cell sorting (FACS) (EPICS XL/XL-MCL; Beckman Coulter; Guangzhou, China). The cell cycle profile was analyzed with the MultiCycle AV DNA analysis software.

For detection of PCNA, cyclin A and cyclin D1 expression in cells treated with ESO, the cells were manipulated as described above, fixed with 4% PFA (poly formaldehyde) and permeabilized. They were either co-incubated with conjugated primary antibodies or conjugated with secondary antibodies after co-incubation with primary antibodies for an appropriate time as recommended by the manufacturer. Isotype Ig was used as a negative control. The expression of PCNA, cyclin A and cyclin D1 was evaluated.

**Table 1.** Extracts isolated from fresh *Squilla oratoria* using different organic solvents

| Extract | Weight | Extraction rate |
|---|---|---|
| **A** | 0.45 kg | 0.5% |
| **B** | 0.21 kg | 0.2% |
| **C** | 0.15 kg | 0.18% |

A: petroleum ether; B: etheyl acetate; C: n-butanol, from 80 kg *Squilla oratoria*.

**Establishment of human HCC xenograft in nude mice**

HepG2 cells in log phase were harvested and evenly dispersed in complete medium. Viability of the cells was detected by trypan blue exclusion. Suspensions with at least 95% viable cells were collected. And the cells were washed with serum-free RPMI medium, then suspended with serum-free medium, adjusting the cell density to $10^7$ cells/ml. Cells were inoculated in nude mice. Both sexes of Balb/c nude mice, aged 4-6 weeks, and weighing 18-22 g, were maintained in a specific pathogen free (SPF) environment until use. The experiments were manipulated in accordance with the guidelines established by the college ethics committee. They were numbered before inoculation with the cancer cells. HepG2 cells were injected subcutaneously at the dorsal skin.

**Administration of ESO to the xenograft**

The mice were randomly divided into 6 groups according to the size of the HCC xenograft when the tumors with HepG2 cells grew to a size of 100 $mm^3$. The groups were defined as high concentration

(1300 mg/kg), medium concentration (650 mg/kg) and low concentration (325 mg/kg) of ESO, normal saline (NS), solvent and CDDP (5 mg/kg) groups. The route of drug administration for each group was intraperitoneal. The drugs were injected every other day for two continuous weeks. Prior to each administration, the volume of the xenografted tumors was calculated after measurement of the length in different directions with a caliper using the formula:

$$v = ab^2 / 2$$

-'a' is the length ($mm^3$) and 'b' is the short radius (in $mm^3$)

At the end of the observation period, the mice were sacrificed by cervical dislocation. The xenografted tumors were excised and weighed. The tumor inhibitory rate was calculated by the formula:

$$(C - T) / C \times 100\%$$

-C; the mean weight of the control group

-T; the mean weight of the drug-treated groups.

Excised tumors were fixed with neutral buffered formaldehyde for 24 h. After dehydration, they were embedded with paraffin and continuous sections were prepared. Hematoxylin-eosin (HE) staining was performed after the sections were mounted onto clean slides. Morphology of the tumors was viewed microscopically.

**Immunohistochemical detection of PCNA expression by nude mice xenografts**

The paraffin sections were deparaffinated with benzene, and hydrated with gradient concentrations of alcohol, and then the endogenous peroxidase activity was blocked with $H_2O_2$. Antigenic determinants were retrieved with a microwave oven. After blocking with sheep serum for 1 h at room temperature, the sections were co-incubated with anti-PCNA primary antibody overnight at 4°C. They were then washed with PBS and conjugated with secondary antibodies for 30 min at room temperature, developed with diaminobenzene for 13 min and counterstained with HE. The slides were dried and sealed, then were viewed under a light microscope, and photographed. At least five fields were chosen randomly and 500 cells were counted. The reactivity was graded as brown (positive) and no staining (negative).

**Statistical analysis**

All quantitative data were obtained from three independent tests and presented as mean ± SD. Student's t-tests and Chi-square tests were used. In all cases, $P < 0.05$ was considered statistically significant.

## RESULTS

**Extraction and isolation of different components from the *Stomatopoda, Squilla oratoria***

To study the biological effect of the organic solvent extract of *Squilla oratoria*, 80 kg of the *Stomatopoda* was purchased from the marketplace. The isolate was immersed in 95% ethanol after mashing. The lysate appeared as a brown jelly. The ethanol extract was further extracted with petroleum ether, ethyl acetate and n-butanol at 56°C, components A, B and C were obtained and their weights and rates of extraction were determined (Table 1).

**Growth inhibition on HepG2 cells by ESO isolated with different organic solvents**

Cells grown for different time courses were used to study the effects exerted by *Squilla oratoria*. The inhibitory effect of extracts A, B and C on the growth of HepG2 cells, assayed with the MTT method, is shown in Table 2. At each time course, the extracts inhibited growth of the HepG2 cells in a dose-dependent manner. The $IC_{50}$ values on three time windows were calculated with a modified Karber formula. The result suggested the highest value for extract A, followed by C, and then B.

**Effect of ethyl acetate ESO on the cell cycle status of HepG2 cells**

To explore the mechanisms underlying the growth inhibitory effect of ethyl acetate ESO on hepatoma cells, we performed a FACS-based DNA content assay to determine how cell cycle entry is regulated by the extract from the marine organism. As shown in Figs.1A-D & 1F-I, the populations of G1 and G2/M were reduced while the population of S phase was increased with increasing extract concentration at 24 h and 48 h after challenge with ESO.

**The effect of ethyl acetate ESO on the level of cell cycle regulators**

The entry to individual phases of the cell cycle is regulated by various factors including cyclins. Increases in levels of the PCNA are responsible for the progression to S phase from G1. The levels of PCNA were tested, therefore, in HepG2 cells exposed to ethyl acetate and in a control group. As shown in Figs.2A-D, the PCNA level increased with increasing concentration of extract, and was significantly higher than the control. The levels of a cyclin regulating M phase entry, cyclin A, and a cyclin regulating G1-S phase progression, cyclin D1, however, were reduced with increasing concentrations of the ethyl acetate extract when challenging HepG2 cells ($P < 0.05$), as suggested in Figs.2F-I & 2K-N. The data suggest that changes in the cyclin regulating G2/M entry presented a different pattern than those in cyclin D1 and cyclin A ($P < 0.01$).

**The effect of ethyl acetate ESO on the growth of HepG2 xenografts in nude mice**

To further confirm the *in vivo* growth inhibitory effect of ESO on cancers cells, a human HCC xenograft model in nude mice was established. The mice bearing

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tumors were divided into drug-challenged and control groups.

Mice were randomly divided into six groups to be injected with saline, solvent, high, medium, or low doses of EOS or CDDP. The gross morphology of the xenografted tumor suggested that in comparison with the saline and solvent control groups, the tumor in mice of drug-administered groups appeared to be reduced in size (Figs.3A-F). As shown in Fig.3G, from day 5 of administration onward, the volume of the tumor on administration with ethyl acetate with high, medium, and low doses was reduced in a dose-dependent manner.

**Table 2.** Growth inhibition on cells exerted by *Squilla oratoria* extracts isolated with different solvents

| Group | Dose (μg/ml) | Inhibitory rate (%) | | |
|---|---|---|---|---|
| | | 24 h | 48 h | 72 h |
| **A** | 200 | 4.06 ± 0.09 | 6.05 ± 0.07 | 10.15 ± 0.05 |
| | 400 | 9.37 ± 0.01 | 14.83 ± 0.10 | 21.58 ± 0.05 |
| | 800 | 23.9 ± 0.05 | 30.02 ± 0.04 | 33.5 ± 0.15 |
| **B** | 200 | 19.63 ± 0.12 | 46.79 ± 0.07 | 61.87 ± 0.03 |
| | 400 | 46.4 ± 0.06 | 57.97 ± 0.08 | 71.34 ± 0.06 |
| | 800 | 66.96 ± 0.05 | 84.85 ± 0.06 | 87.77 ± 0.03 |
| **C** | 200 | 6.85 ± 0.01 | 17.53 ± 0.08 | 32.71 ± 0.15 |
| | 400 | 21.08 ± 0.05 | 38.06 ± 0.05 | 57.35 ± 0.08 |
| | 800 | 52.61 ± 0.03 | 57.45 ± 0.1 | 63.08 ± 0.06 |
| **DDP** | 10 | 70.22 ± 0.08 | 88.12 ± 0.09 | 90.34 ± 0.12 |

The cells were exposed to extract with **(A)** petroleum ether, **(B)** ethyl acetate and **(C)** n-butanol; the data were mean ± SD derived from 6 independent tests.

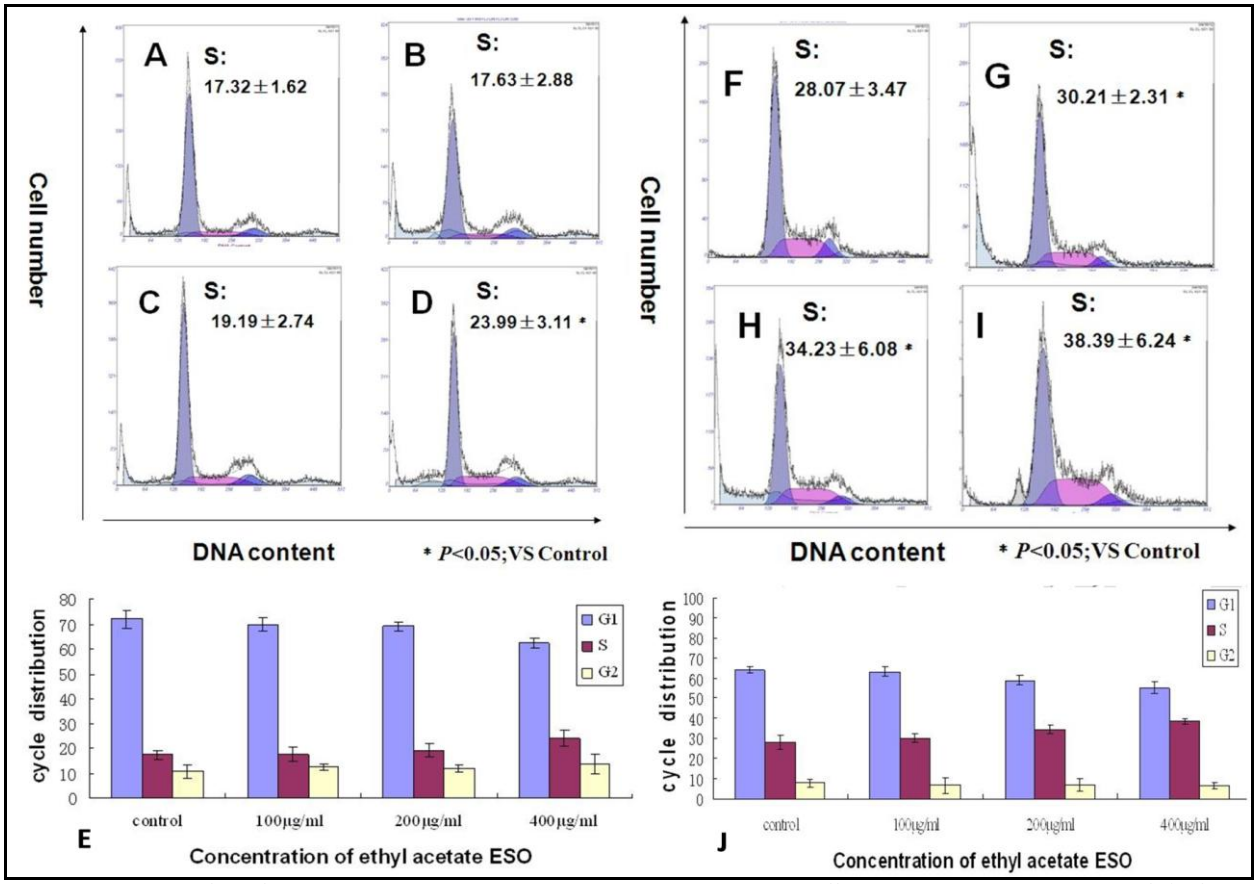


**Figure 1** Cell cycle profiles of HepG2 cells treated with ESO. HepG2 cells were treated with different doses (0, 100, 200 and 400 μg/ml) of ethyl acetate ESO for 24 h (**A, B, C, D**) or 48 h (**F, G, H, I**). Cell cycle profile was then analyzed by FACS-based DNA contents assay. Mean fluorescence intensity of S phase was indicated. **E, J**: change of cell cycle in HepG2 cells when incubated with 0, 100, 200, and 400 μg/ml ESO; height of the histograms represents means ± SD of the mean fluorescence of each cell cycle phase registered from at least three independent tests.

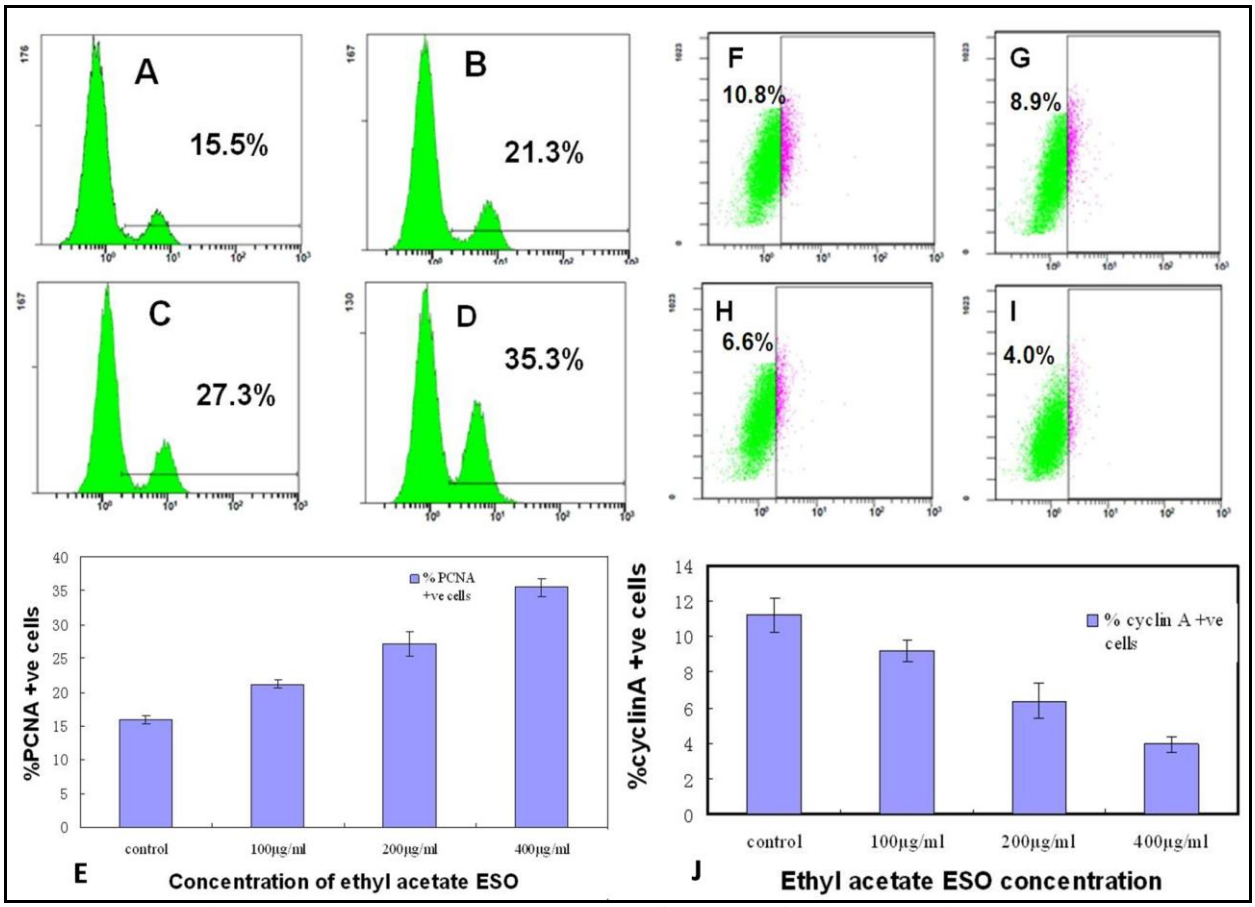


**Figure 2.** Expression of cell cycle regulators by HepG2 cells exposed to different doses of ESO detected by flow cytometry. **A-D**: expression of PCNA in cells treated with 0, 100, 200 and 400 μg/ml ethyl acetate ESO. **E**: the effect of ethyl acetate ESO on the expression of PCNA by HepG2 cells; height of the histogram represents mean ± SD of the mean fluorescence of the positive cell population registered from at least three independent tests. **F-I**: expression of cyclin A in cells treated with 0, 100, 200 and 400 μg/ml ethyl acetate ESO; the gated dots at the left side represent populations of cyclin A positive cells. **J**: the effect of ethyl acetate ESO on the expression of cyclin D by HepG2 cells; height of the histogram represents means ± SD of the mean fluorescence of the positive cell population registered from at least three independent tests.

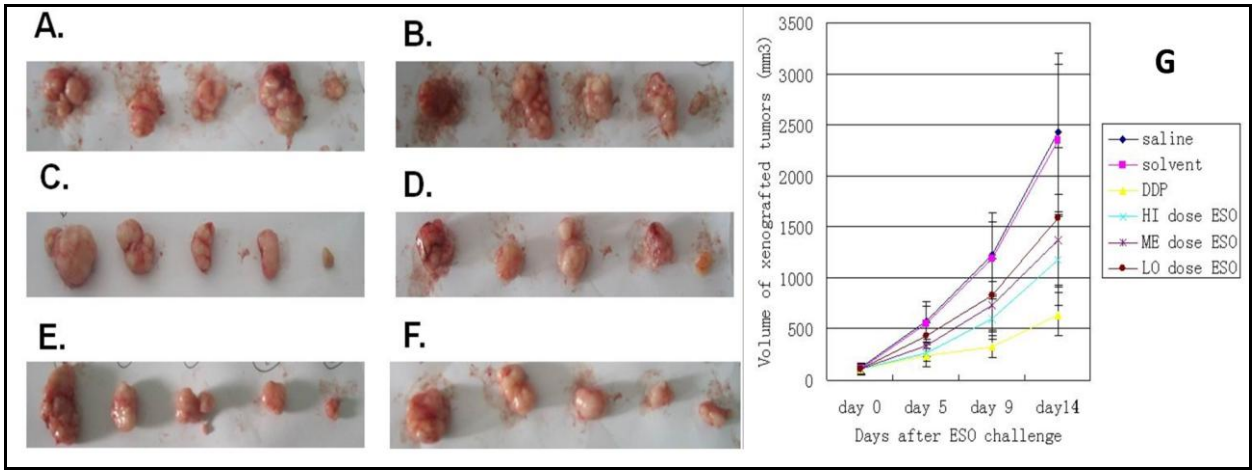


**Figure 3.** Xenografted tumors from nude mice challenged with different doses of ESO. The sizes of subcutaneous tumors excised from ESO-treated groups of mice (high, medium and low doses) and the CDDP-treated were compared with those of saline and solvent treated groups. **A:** saline control group; **B:** solvent control group; **C:** CDDP control group; **D:** high ESO dose group; **E:** medium ESO treated group; **F:** low ESO dose group; **G:** Change of tumor weight of the xenografted HCC on challenge with different doses of ESO. The volume of the tumors was calculated as described in materials and methods. The values represent mean ± SD derived from at least three independent tests. HI: High; ME: medium; LO: Low.

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**Table 3.** Profile of DNA contents of HepG2 cells on exposure to ethyl acetate ESO

| Concentration | | G1 | S | G2/M |
|---|---|---|---|---|
| **Control** | **24 h** | 71.99 ± 3.70 | 17.32 ± 1.62 | 10.67 ± 2.73 |
| | **48 h** | 64.01 ± 1.61 | 28.07 ± 3.47 | 7.91 ± 1.87 |
| **100 μg/ml** | **24 h** | 69.85 ± 2.73***** | 17.63 ± 2.88 | 12.53 ± 1.19 |
| | **48 h** | 63.09 ± 2.49 | 30.21 ± 2.31***** | 6.68 ± 3.94 |
| **200 μg/ml** | **24 h** | 69.07 ± 1.85***** | 19.19 ± 2.74 | 11.73 ± 1.44 |
| | **48 h** | 58.85 ± 2.41 | 34.23 ± 6.08***** | 6.91 ± 8.37 |
| **400 μg/ml** | **24 h** | 62.42 ± 1.77***** | 23.99 ± 3.11***** | 13.58 ± 3.96***** |
| | **48 h** | 55.23 ± 6.78***** | 38.39 ± 6.24***** | 6.37 ± 0.96***** |

*****P < 0.05 *vs* control; the data were presented as % of total population assayed, with mean ± SD calculated from results of three independent tests.

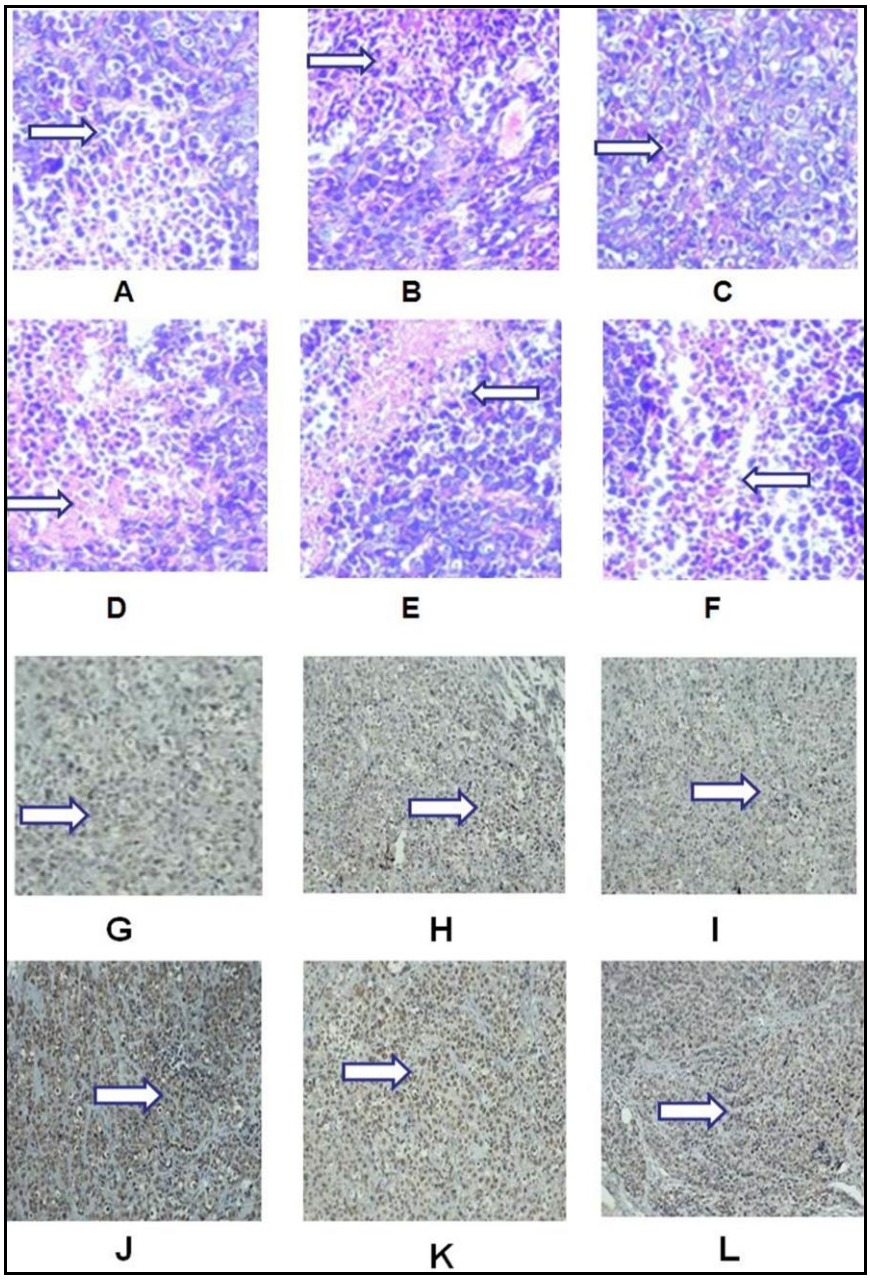


**Figure 4.**
Expression of PCNA in xenografted tumors detected by immunohistochemistry.
**A-F:** microscopic view of the nude mouse HepG2 xenograft (HE staining, magnification 20×10); cells intensely stained with eosin staining as pointed by arrows, suggested necrosis, and the number of such cells increased with the doses of ESO challenged.
**G-L:** expression of PCNA by HepG2 xenograft detected by immunohistochemistry; the brownish staining suggested positive signals. Typically positive cells were pointed by arrows, and the intensity of PCNA signals tended to increase with dose of ESO challenged.
**A, G:** saline control;
**B, H:** solvent control.
**C, I:** CDDP control;
**D, J:** high-dose ESO;
**E, K:** medium-dose ESO;
**F, L:** low-dose ESO.
Semi-quantitative data are shown in Table 5.

**Table 4.** Effect of ethyl acetate ESO on level of cell cycle regulators in HepG2 cells

| Group | Control | 100 μg/ml | 200 μg/ml | 400 μg/ml |
|---|---|---|---|---|
| **PCNA (%)** | 15.9 ± 0.61 | 21.23 ± 0.65***** | 27.17 ± 1.8***** | 35.55 ± 1.3***** |
| **Cyclin D1(%)** | 16.54 ± 2.91 | 9.1 ± 3.30***** | 4.68 ± 0.69***** | 2.62 ± 0.58***** |
| **Cyclin A (%)** | 11.22 ± 0.96 | 9.17 ± 0.68***** | 6.41 ± 1.01***** | 3.97 ± 0.45***** |

*****P < 0.01 *vs* control; the figures were presented as mean ± SD with data derived from at least three independent tests.

**Table 5.** Effect of ethyl acetate ESO on PCNA levels in nude mouse HCC xenografts

| Group | Negative (-) | Positive (+ ~ +++) |
|---|---|---|
| **Normal saline** | 57% (285/500) | 43% (215/500) |
| **Solvent control** | 55.5% (273/500) | 45.5% (227/500) |
| **DDP control** | 43% (215/500) | 57% (285/500) |
| **ESO high dose** | 1.4% (7/500) | 98.6% (493/500)***** |
| **ESO medium dose** | 9.4% (47/500) | 90.6% (453/500)***** |
| **ESO low dose** | 18.8% (94/500) | 81.2% (406/500)***** |

*****P < 0.01 *vs* control

Light microscopic analysis of HE-stained sections showed that the tumor cells appeared large in size, round or oval in shape, with diverse nuclear morphology, and were clustered in a nest. The tumors were found to be rich in blood vessels where tumor embolism was observed, and there was massive necrosis in the tumor-bearing tissues as manifested by intense eosin staining and the degree of necrosis increased with dose of ESO challenged (Figs.4A-F). On day after administration of drugs, the xenografted tumors were harvested and expression of PCNA was detected with immunohistochemistry. The most intense brown signal was in the high-dose ESO group (Figs.4G-L, Table 5).

## DISCUSSION

HCC is the sixth most common neoplasm and the third most frequent cause of cancer death worldwide [2, 23]. Multiple cytogenetic lesion as well molecular genetic aberrations are frequently identified in HCC, and the genes involved are responsible to the malignancy, and such phenotypic changes, like evasion of apoptosis, abnormal cell cycle regulation, active angiogenesis greatly contribute to genesis of tumors, and also serve as appropriate target s for antitumor therapies[2].

In spite of progresses in diagnosis and modalities, incidence of HCC remains increase worldwide. It is exceptionally high in Asia and Africa, the number of new cases in America and Europe is rapidly increasing, making HCC a worldwide health problem. Even with aggressive treatment, HCC usually has a poor prognosis, with a 5-year survival rate as low as 25-39% after common treatments, such as surgery, chemotherapy, and radiotherapy [24-27]. Hepatic resection is the treatment of choice for HCC patients without cirrhosis, as these patients tolerate major resections with low rates of life-threatening complications [28]. Recurrence with high frequency reduces efficacy and survival of patients who receive surgical treatment. Radio- and chemotherapy or transplantation has achieved some success. The fact suggests that effective therapy is a necessity for general curative aim. High effect with low side effect drugs isolated from natural products, targeting multiple targets would well meet the demand for successful treatment of HCC. It has been reported that drugs of such origin presented promising application in clinical therapy of HCC [29].

*Squilla oratoria* is a *Stomatopoda* widely inhabiting the Pacific Ocean and the seas mostly in the western Pacific region. Our previous study on organic solvent extracts of *Squilla oratoria* (ESO) demonstrated its anti-proliferative effects on multiple cell lines of different origins. The most potent effect was seen with the ethyl acetate extract, which was speculated to be composed of either single or mixed bio-alkaloid compounds. The underlying mechanisms of the inhibitory effects remain to be elucidated. The present study suggests that growth inhibition could be attributed to its potential to regulate the cell cycle.

Cell cycle checkpoints are mainly regulated by several kinds of cyclin-cyclin dependent kinase (Cdk) complexes. For years, natural products have been the cornerstone of the pharmacology of anticancer chemotherapy [30]. Thus, the interest in marine natural products lies in their biodiversity, and in turn, the multitude of interactions between these organisms and their environment [31].

Compounds derived from natural products impair cell cycle progression by dysregulation of cycle entry or microtubule formation [32]. Pectenotoxins, which are a

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group of natural toxins present in marine sponges and some shellfish, are toxic to humans. The most toxic compound in the group is pectenotoxin-2 (PTX-2) significantly suppressed entry into the G2/M phase of the cell cycle through down-regulation of cyclin B1 and cdc25C expression [33].

The present study revealed that exposure of HepG2 cells to ESO leads to arrest at S phase. In agreement with previous reports, we observed a decrease of cyclin A level correlated with the alteration of cell cycle profile, suggesting that ESO may disrupt cell cycle entry through modulation of the expression of some cyclins. The decreased level of cyclin A may prevent the cells from progressing further to the G2/M phase, causing accumulation of cells in the S phase.

Another major finding in the present study regarding the cell cycle regulator is the elevated level of PCNA in cells when challenged with ESO. PCNA is a cyclin controlling S phase entry; its level is normally altered in cells undergoing S phase arrest. It is the molecular coordinator in the core DNA synthesis machinery [34], forming a molecular platform to recruit proteins involved in DNA synthesis, cell-cycle control, and DNA-damage response and repair [35]. It is generally believed that PCNA is a molecule that supports proliferation [36], but in our experiment it was speculated that it mainly plays a role in promoting S phase entry of the cells without further supporting cell cycle progression.

The molecular basis of ESO-induced S-phase arrest is not completely clear. Future efforts to elucidate the possible interactions between cyclins, Cdk and other molecular partners are required. Our study provided some basic evidence for possible therapeutic targets of the marine anti-cancer drug. In conclusion, ethyl acetate extract of *Squilla oratoria* (ESO), from a marine *Stomatopoda* exerts *in vitro* and *in vivo* cytostastic effects on the HCC derived line HepG2, correlating with its modulation of level of cell cycle regulators. The present data suggested ESO as an anticancer agent to be developed.

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**COMPETING INTERESTS**

None to declare

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GESDAV

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**Original Article**

# Protection against carbofuran-induced toxicity in rat tissues and plasma by *Ipomoea aquatica* Forsk crude extract

**Sanjukta Datta[1], Santinath Ghosh[1], Pubali Dhar[2]**

*[1]Department of Chemical Technology, University College of Science and Technology;*
*[2]Food and Nutrition Division; University of Calcutta;* ***Kolkata, India***

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**Corresponding Author**
Sanjukta Datta
Department of Chemical Technology,
92, A.P.C. Road, Kolkata-700 009
West Bengal, India
sanj_9231@yahoo.co.in

**Key Words**
Antioxidant enzymes; Carbofuran; DNA fragmentation; Oxidative stress; Polyphenols

**Abstract**

***Objective:*** Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), a commonly used carbamate, induces oxidative stress through free radical generation. Role of green leafy vegetables against such toxic compounds have been well established. Hence, this study aimed to evaluate the deleterious effects of carbofuran on brain and plasma in albino male rats of Charles Foster strain and whether *Ipomoea aquatica* crude extract (IAE) can protect body cells and tissues against oxidative insult.

***Methods:*** The rats were divided into 4 groups: one was treated with an oral dose of 0.1 mg/kg b.wt of carbofuran alone; 20 mg of polyphenolic compound expressed as gallic acid equivalents per kg b.wt was fed to another group; a third group was gavaged both the doses; the final group served as control and provided normal diet.

***Results:*** Evaluations based on altered activities of antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic glutathione in carbofuran treated rats showed the protective side of IAE. Also, increase in the total cholesterol levels in brain and plasma and DNA fragmentation in bone marrow cells were attenuated positively in the presence of IAE.

***Conclusion:*** The present study gives an insight into the protective role of plant polyphenols in minimizing the ill-effects of carbofuran.



## INTRODUCTION

Pesticides are occasionally used indiscriminately in large amounts causing environmental pollution and therefore, are a great cause of concern. Large numbers of xenobiotics have been identified to have potential to generate free radicals in biological systems [1, 2]. Free radicals have become an attractive means to explain the toxicity of numerous xenobiotics that interact with various tissue components resulting in dysfunction. Pesticides comprise an important source of reactive oxygen species (ROS) which may occur through processes like mitochondrial electron transport chain and subsequent accumulation of reduced intermediates [3], inactivation of antioxidant enzymes and depletion of radical scavengers [4-6].

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), is a commonly used broad spectrum carbamate. The carbamate group of pesticides is known to persist in nature for a relatively small duration of time and to act quickly on the target pests, and hence acting as substitutes for organophosphates and organochlorates. However, they exhibit the potential to generate serious neurotoxic, neurobehavioral and neuropsychological consequences in non-target organisms, and therefore, their indiscriminate use are of great concern for human health [7]. The environmental exposure to carbamates in the general population is mainly due to the ingestion of contaminated food and water [8].

Carbofuran exposure has been shown to alter neurotransmitter concentration, the isoenzyme pattern of creatinine kinase and total adenine nucleotides [9]. Oral administration of carbamates like carbofuran has been shown to produce neuronal injury by excessive generation of ROS and nitrogen species leading to lipid peroxidation (LPO) [10-12], mitochondrial dyshomoeostasis or damage, reduction of neuronal

energy level and increased cytochrome c oxidase (CCOX) activity. The lipophilic nature of carbofuran has been reported to cause oxidative injury resulting in perturbations in the membrane structure and functions [13]. However, to counteract the ill-effects of such toxic insults in the body tissues, antioxidant defenses like superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR) play a vital role. Most of them act as the first line of defense and hence their crucial role in protecting the body against ROS generation is of much consideration.

Antioxidants in the form of various food stuffs like fruits and vegetables have already gained attention that have the capacity to protect living systems against tissue injuries and damages. Green leafy vegetables are rich sources of many nutrients and their beneficial role has partly been attributed to the antioxidant components present in them of which the major portion is formed by the flavonoids, isoflavones, lignans, catechins and isocatechins [14, 15]. Sulfur containing phytochemicals glucosinolates and S-methylcystein sulfoxide in cruciferous vegetables are effective against carcinogens [16]. Reports suggest that orally administered butanolic extract of *Paronychia argentea* protects against chlorpyrifos ethyl induced toxicity possibly through the inhibition of increased LPO in addition to inhibition of triglyceride accumulation, plasma membrane destruction and neutrophil infiltration in the liver tissue [17]. In the author's laboratory, it has been observed that the leafy vegetable extracts of *Enydra fluctuans* prevents LPO by inhibiting the production of free radicals and also by protecting the rats from the deleterious effects of acephate by altering the antioxidant levels in their body to a great extent [18]. High antioxidant intake has been shown to reduce cancer risk and may also mitigate the effects of oxidative DNA damage, which is hypothesized to be causally linked to carcinogenesis. Studies of damaged cells by methods like comet assay, chromosomal aberrations, micronuclei tests have provided much understanding in the phenomenal changes that take place in the body cells due to the deleterious effects of the commonly used carbamates with regard to their relatively lower toxicity levels [19, 20].

Thus, in the present study, we have selected one such green leafy vegetable, *Ipomoea aquatica*, which is locally available almost throughout the year, and tried to evaluate its efficacy in protecting against carbofuran toxicity, based on the antioxidant enzyme concentrations and total cholesterol levels in rat brain and blood components, and also the DNA damage done to bone marrow cells.

## MATERIALS AND METHODS

### Chemicals

Methanol, hematoxylin and $H_2O_2$ were purchased from Sisco Research Laboratory (SRL, Mumbai, India). GSH, GR, nicotinamide adenine dinucleotide phosphate (NADPH), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or also known as Ellman's reagent) were purchased from Sigma Chemicals (St. Louis, MO, USA), and bovine serum albumin (BSA) standard from E. Merck (Mumbai, India). Carbofuran pesticide was supplied by Anu Products Limited (Haryana, India).

### Preparation of leafy vegetable extract

The leafy vegetable, *Ipomoea aquatica*, was collected from the local market and was authenticated by the Central National Herbarium, Shibpur (vide letter no CNH/120/2011/Tech. II/607). The polyphenolic compounds were extracted by following the methods developed in our laboratory [21]. Briefly, the whole plants (stem and leaves) of *I.aquatica* were washed thoroughly, oven dried and ground to powder. It was then extracted using 80:20 methanol:water and concentrated in a rotary evaporator. The concentrates were pooled and the final concentrate was lyophilized to obtain the dry matter. The required amount for the dose (20 mg polyphenolic compounds expressed as gallic acid equivalents/kg b.wt) was dissolved in water to obtain the water extract. It was then stored at –40°C for further use. The total polyphenol content was obtained following the methods of Matthaus *et al* [22] and chromatographic analysis was done following the methods of Siddhuraju and Becker [23].

### Animal diet and treatment

Male albino rats of Charles Foster strain, weighing 100-130 g, were caged singly and provided with balanced diet and water *ad libitum*. The diets composed of fat free casein, 18%; fat, 20% (sunflower oil); starch, 55%; salt-mixture, 4% [composition of salt mixture No.12 (in g): NaCl 292.5, $KH_2PO_4$ 816.6, $MgSO_4$ 120.3, $CaCO_3$ 800.8, $FeSO_4.7H_2O$ 56.6, KI 1.66, $MnSO_4.2H_2O$ 9.35, $ZnCl_2$ 0.5452, $CuSO_4.5H_2O$ 0.9988, $CoCl_2.6H_2O$ 0.0476]; cellulose, 3%; one multivitamin capsule (vitamin A IP 10,000 U, thiamine mononitrate IP 5 mg, vitamin B IP 5 mg, calcium pantothenate USP 5 mg, niacinamide IP 50 mg, ascorbic acid IP 400 U, cholecalciferol USP 15 U, menadione IP 9.1 mg, folic acid IP 1 mg, vitamin E USP 0.1 mg) per kg of diet. The diets were adequate in all nutrients. They were maintained at 12 h light/dark conditions. The animal experiment was carried under the supervision of the Animal Ethical Committee of the Department of Chemical Technology, University of Calcutta.

The animals were divided into 4 groups comprising 8 rats in each group. Group I served as the control and was provided with normal diet. The animals in group II

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were gavaged with the pesticide carbofuran only at a dose of 0.1 mg/kg b.wt. Group III animals were administered by gavaging the leafy vegetable extract at a dose of 20 mg/kg b.wt. Group IV animals were given the pesticide along with the leafy vegetable extract at the doses earlier mentioned. The dose used for the pesticide was calculated as $1/100^{th}$ of the $LD_{50}$, so that it showed no adverse effects or mortality in the animals.

All the treated rats were gavaged for 14 days and were sacrificed under mild anesthesia, blood was collected and the brain tissues were immediately excised, blotted, and stored frozen (–40°C) for further analysis.

**Enzymatic and non-enzymatic antioxidant activities**

The tissues were first homogenized in phosphate buffer (50 mM potassium phosphate buffer at pH 7 for CAT and 1 M potassium phosphate buffer at pH 7 for SOD, GSH and GPx) and centrifuged. The supernatant was then used for measuring the following antioxidant enzymes. CAT was measured according to the method of Aebi [24]. CAT activity was measured spectrophotometrically and expressed as U/mg protein by the rate of decrease of hydrogen peroxide at 240 nm. SOD activity was assayed by measuring the auto-oxidation of hematoxylin as described by Martin *et al* [25]. GSH was determined by the method of Ellman [26]. Total activity of GPx (EC 1.11.1.9) was determined in the tissue homogenates and plasma according to Paglia and Valentine [27].

**Protein quantification**

The total protein was determined by the method of Lowry *et al* [28].

**Extraction of tissue lipids**

Tissue lipid was extracted by the method of Folch *et al* [29]. One gram of tissue (brain) and plasma were homogenized with 1 ml of 0.74% potassium chloride and 2 ml of different proportions of chloroform and methanol for 2 min and then centrifuged. The mixture was left overnight and the chloroform layer was filtered through a Whatman filter paper (No.1). The chloroform layer was dried and the tissue lipid contents were measured.

**Total cholesterol**

The total cholesterol from the extracted lipid was determined by the standard kit method.

**DNA fragmentation assay**

DNA fragmentation of bone marrow cells was determined by the diphenylamine assay of Taylor [30]. One milliliter of cell suspension (not less than $5 \times 10^5$ and no more than $5 \times 10^6$, in order to obtain an OD 600 for DNA > 0.04 and < 1.200) was delivered in tubes labeled B (bottom) and centrifuged at 200g at 4°C for 10 min. Supernatants were carefully transferred in new tubes labeled S (supernatant). To the pellet in tubes B, 1 ml Tris-EDTA buffer (TTE; pH 7.4 with 0.2% Triton X-100) solution was added, vortexed vigorously and then centrifuged at 20,000g for 10 min at 4°C. Supernatants were carefully transferred in new tubes labeled T. One milliliter TTE solution was added to tubes B and 1 ml of 25% trichloroacetic acid (TCA) were added to tubes T, B and S and vortexed vigorously. They were then allowed to precipitate overnight at 4°C. After incubation, precipitated DNA was recovered by pelleting for 10 min at 20,000g at 4°C. Supernatants were discarded by aspiration. The DNA was hydrolyzed by adding 160 ml of 5% TCA to each pellet and heating them for 15 min at 90°C in a heating block. To each tube was added 320 ml of freshly prepared DPA solution, then vortexed and color development was allowed for about 4 h at 37°C or overnight at room temperature. Two-hundred milliliter aliquots of colored solution (ignoring dark particles) were transferred from each tube to a well of a 96-well microtiter plate and measured at 600 nm in an ELISA reader. The percentage of fragmented DNA was calculated using following formula:

$$\% \text{ fragmented DNA} = (S + T) / (S + T + B) \times 100$$

-S, T and B are the OD 600 of fragmented DNA in the S, T and B fractions, respectively

**Statistical analysis**

The data was expressed as mean ± SD. Differences among the experimental groups were analyzed using one-way ANOVA and the comparisons between the means were carried out using the Tukey test; $P < 0.05$ was considered as statistically significant in all the experiments.

## RESULTS

**Total polyphenol content and component identification of the extract**

The total polyphenol content of *I.aquatica* extract was noted as 55.95 ± 0.002 mg/g gallic acid equivalents. The active components present were analyzed by high performance liquid chromatography (HPLC; see Fig.1). It was observed that the polyphenolic extract mainly contained flavonoids like rutin, apigenin and quercetin as major components along with some unidentified components.

**Enzymatic and non-enzymatic antioxidant activities**

A significant increase ($P < 0.05$) in the GSH level in brain and a decrease in plasma was observed in the carbofuran treated group (group II). However, a significant increase in the GSH level had been observed in plasma in the *I.aquatica* treated group (group III). The GSH level had reached a near normal status in case

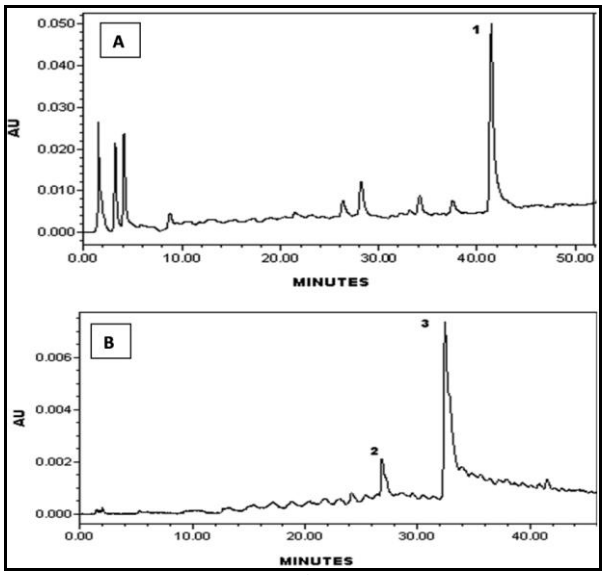


**Figure 1.** HPLC chromatogram of *Ipomoea aquatica* extract at **(A)** 280 nm, and **(B)** 370 nm. The peaks identified are; **(1)** apigenin, **(2)** rutin, and **(3)** quercetin

of plasma in the group treated with the *I.aquatica* extract (IAE) along with carbofuran (group IV) (Table 1).

The GPx activity in brain in the carbofuran treated group had shown a significant ($P < 0.05$) increase, whereas, a decrease had been noticed in case of plasma. However, restoration of the enzyme level to the normal status had been observed in brain tissue of the group treated with the IAE along with carbofuran (Table 2).

A significant increase in the activity of CAT enzyme had been observed in plasma and brain. However, the level of the enzyme seemed to have been restored significantly to a normal state in the brain tissue of the group treated with IAE along with the pesticide (Table 3).

The level of SOD activity showed a significant decrease ($P < 0.05$) in the carbofuran treated group in the brain tissue which seemed to have gained a significant increase in the group treated with IAE along with carbofuran (Table 4).

### Total cholesterol

The total cholesterol levels in plasma and brain had significantly increased in the carbofuran treated rats. However, treatment with IAE did have a protective effect by minimizing the level of cholesterol in plasma and brain (Table 5).

### DNA fragmentation in bone marrow cells

Fragmentation of bone marrow cell DNA was observed in rats treated with carbofuran (group IV). IAE seemed to have prevented it to a certain extent in case of the group treated with both carbofuran and IAE (Table 6).

**Table 1.** Effect of IAE on glutathione content in plasma and brain tissue of carbofuran treated rats (mean ± SD, n = 8)

| Groups | GSH content in different tissues of different groups (μmol/mg protein) | |
|---|---|---|
| | Plasma | Brain |
| **Group I** | 15.83 ± 0.092 | 239.1 ± 0.371 |
| **Group II** | 10.97 ± 0.181[a] | 262.72 ± 1.565[a] |
| **Group III** | 23.95 ± 1.321[b] | 243.82 ± 1.679 |
| **Group IV** | 12.83 ± 0.126 | 255.29 ± 1.786 |

Group I, control; group II, treated with carbofuran; group III, treated with IAE alone; group IV, treated with both IAE and carbofuran. Significantly different from [a]groups I, III and IV, and [b]groups I, II and IV ($P < 0.05$).

**Table 2.** Effect of IAE on GPx activity in plasma and brain tissues of carbofuran treated rats. (mean ± SD, n = 8)

| Groups | GPx content in different tissues of different groups ( U/mg protein) | |
|---|---|---|
| | Plasma | Brain |
| **Group I** | 0.0175 ± 0.014 | 0.127 ± 0.079 |
| **Group II** | 0.016 ± 0.003[a] | 0.531 ± 0.010[a] |
| **Group III** | 0.023 ± 0.001 | 0.245 ± 0.009 |
| **Group IV** | 0.016 ± 0.002 | 0.151 ± 0.014 |

Group I, control; group II, treated with carbofuran; group III, treated with IAE alone; group IV, treated with both IAE and carbofuran. [a]Significantly different from groups I, III and IV in brain and only groups I and III in plasma ($P < 0.05$).

**Table 3.** Effect of IAE on CAT activity in plasma and brain tissues of carbofuran treated rats (mean ± SD, n = 8)

| Groups | CAT activity in different tissues of different groups (U/mg protein) | |
|---|---|---|
| | Plasma | Brain |
| **Group I** | 0.115 ± 0.002 | 0.73 ± 0.032 |
| **Group II** | 0.276 ± 0.011[a] | 1.145 ± 0.197[a] |
| **Group III** | 0.132 ± 0.013 | 0.653 ± 0.021 |
| **Group IV** | 0.274 ± 0.004 | 0.654 ± 0.005 |

Group I, control; group II, treated with carbofuran; group III, treated with IAE alone; group IV, treated with both IAE and carbofuran. [a]Significantly different from groups I, III and IV in brain and only groups I and III in plasma ($P < 0.05$).

**Table 4.** Effect of IAE on SOD activity in plasma and brain tissues of carbofuran treated rats (mean ± SD, n = 8)

| Groups | SOD activity in different tissues of different groups (U/mg protein) | |
|---|---|---|
| | Plasma | Brain |
| **Group I** | 1.065 ± 0.029 | 9.951 ± 0.262 |
| **Group II** | 0.482 ± 0.025[a] | 3.607 ± 1.012[a] |
| **Group III** | 1.295 ± 0.105 | 5.409 ± 0.81 |
| **Group IV** | 1.423 ± 0.067 | 8.709 ± 0.554 |

Group I, control; group II, treated with carbofuran; group III, treated with IAE alone; group IV, treated with both IAE and carbofuran. [a]Significantly different from groups I, III and IV ($P < 0.05$).


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**Table 5.** Effect of IAE on the total cholesterol level in plasma and brain tissues in the rats treated with carbofuran

| Groups | Total cholesterol in plasma (mg/ml) and brain tissues of different groups (mg/g) | |
|---|---|---|
| | Plasma | Brain |
| **Group I** | 0.46 ± 0.075 | 3.87 ± 0.447 |
| **Group II** | 1.06 ± 0.075[a] | 10.91 ±1.094[a] |
| **Group III** | 0.33 ± 0.023 | 1.43 ± 0.292 |
| **Group IV** | 0.36 ± 0.004[b] | 3.64 ± 0.348[b] |

The values are expressed as mean ± SD; n= 8. Group I, control; group II, treated with carbofuran; group III, treated with IAE alone; group IV, treated with both IAE and carbofuran. Significantly different from [a]groups I, III and IV, and [b]groups I and II in plasma and groups II and IV in brain ($P < 0.05$).

**Table 6.** DNA fragmentation in bone marrow cells of rats treated with carbofuran (mean ± SD, n = 8)

| Groups | Fragmented DNA (%) |
|---|---|
| **Group I** | 0.73 ± 0.071 |
| **Group II** | 6.98 ± 0.11[a] |
| **Group III** | 1.22 ± 0.002 |
| **Group IV** | 3.73 ± 0.012 |

Group I, control; group II, treated with carbofuran; group III, treated with IAE alone; group IV, treated with both IAE and carbofuran. [a]Significantly different from groups I, III and IV ($P < 0.05$).

## DISCUSSION

Phytochemicals in the form of phenolic compounds are a crucial part of nutrition provided by vegetables and fruits, including the green leafy ones [31]. Among the various polyphenolic compounds, flavonoids form the major class including the flavones, isoflavones, flavonols, flavanols, *etc* and leafy vegetables are a good store house for these components [32]. Flavonoids in plants are generally present in their glycosylated and sulphated derivative forms, which are readily absorbed by the body cells [33]. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a commonly and widely distributed flavonoid group present in plants, mainly in the glycosidic forms such as rutin (5,7,3',4'-OH, 3-rutinose). In the present study, these common flavonoids were identified in *I.aquatica* extract. Besides quercetin, several of its derivatives and other flavonoids like myricetin, luteolin and apigenin have been identified and reported in *I.aquatica* which correlates with our finding to quite a good extent [34, 35]. They have been best described as free radical scavengers and mediators of peroxidation reactions in the body. Hence, their role as protectors of body cells and tissues against stress conditions well explains the present situation.

Glutathione mediated detoxification in the body, forms the most important antioxidant defense lines in cells. Formation of less toxic intermediates, by conjugation reactions, protects the body cells by reducing the injury levels [36, 37]. The importance of such detoxification in the body cells becomes evident, when the GSH level, together with its coupled enzyme system gets disrupted due to excessive consumption [38, 39]. Depletion in glutathione level due to carbamates like aldicarb and propoxur has been reported earlier [40-42]. This was observed in case of plasma GSH in the present study, where the levels declined in the presence of the pesticide. A simultaneous increase in both SOD and CAT was also observed, probably due to the depletion of GSH levels in the body. However, an increase in the GSH and GPx levels was noticed in rat brains in presence of the carbamate, which might be an outcome of the reduced activity of SOD that might have enhanced the activities of both GSH and GPx. The decrease in the activity of SOD in carbofuran-intoxicated animals may be due to the consumption of this enzyme in converting the $O_2\bullet^-$ to $H_2O$. The dismutation of $O_2\bullet^-$ to $H_2O$ is catalyzed by SOD which contains both copper and zinc. Also, the high levels of hydroperoxides in the tissues of the carbofuran treated animals might have triggered this action. To reduce these peroxides to stable non-radical lipid alcohols, GPx utilizes GSH thus oxidizing them to GSSG which is regenerated by GR [43], thus, bringing GSH to the normal levels. Supplementation of IAE has shown an alteration in their levels, so as to help the body regain their normal or near normal activity status.

The disruption of formation of lipoprotein has been reported by Hassan *et al* [44] as one of the factors leading to accumulation of cholesterol in carbofuran treated mice. Data presented in this investigation showed that the carbamate compound caused a general hypercholesterolemia. Cholesterol is usually obtained in the diet but, if necessary, sufficient for normal requirement can be synthesized in the liver, intestine and other tissues. Virtually, all nucleated cells have the capacity to synthesize this compound, but the quantitatively important tissue is the liver [45]. It is reasonable to suggest that the carbamate compound had increased tissue lipogenesis. Probably, this had been achieved through acceleration of acetyl CoA which was supposed by Newsholme and Leech [45] to be the precursor of cholesterol biosynthesis. On the other hand, no significant changes were noticed with groups treated with the antioxidants. In fact, the ameliorative effect of the leafy vegetable extract can be well understood from the presented data.

It had been reported that serum cholesterol level had increased in the rats exposed to benzene hexachloride cyclohexane (BHC). Plasma cholesterol level is considered valuable indicator of drug induced disruption of lipid metabolism and development of fatty liver and altered cholesterol levels are implicated in impaired biliary excretion. The increased cholesterol

levels are also suggestive of the inhibition of steroidogenesis in the testis and adrenal. Marked dose-dependent increase of serum cholesterol in BHC fed rats suggests increased synthesis and accumulation of cholesterol in the liver, kidney and testis and/or impaired biliary function [46]. In the present study, cholesterol increase in the tissues might be due to the inhibition in the activity of enzymes involved in cholesterol break up that resulted in deposition of cholesterol into the cell. Similar results were also reported in rats treated with dimethoate [47].

DNA fragmentation is one of the earliest events in apoptosis. In the late apoptotic process, the cells break into a number of membrane-bound apoptotic bodies containing one or more fragments of nucleus. Case studies on human subjects working as floriculturists have been reported recently where pesticides have caused DNA fragmentations [48]. Several studies on Indian farmers have confirmed the fact the pesticides have a huge impact on the DNA constitution of the body cells and tissues [49, 50]. Chromosomal aberrations, sister chromatid exchanges and micronuclei formations in cells are common aspects of pesticide toxicity. However, it is noteworthy to mention that data confirming DNA fragmentations in the bone marrow cells by the method presently used is almost lacking. Hence, from the present study, it can also be concluded that this assay is a simple method of assessing DNA damage in bone marrow cells.

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**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

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**Original Article**

# Effect of methanolic extract of *Physalis minima* on gastric inflammation and gastric ulcers formation

**Umi Kalsum[1], Mulyohadi Ali[1], M. Aris Widodo[1], Handono Kalim[2]**

*[1]Department of Pharmacology, Faculty of Medicine;*
*[2]Department of Internal Medicine, Saiful Anwar General Hospital, Faculty of Medicine;*
*University of Brawijaya,* ***Malang, East Java, Indonesia***

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**Corresponding Author**
Umi Kalsum
Department of Pharmacology,
Faculty of Medicine,
University of Brawijaya,
Malang, East Java, Indonesia.
dr.umikalsum@gmail.com

**Key Words**
Aspirin; Gastric ulcer; Indomethacin; Inflammation; Steroids

**Abstract**

***Objective:*** This study aimed to investigate the effect of methanolic extract of *Physalis minima* L (MEPM) in gastric inflammation and gastric ulcer formation.

***Methods:*** For anti-inflammatory activity, we divided 30 male rats into five groups (n = 6), including the control group, gastric inflammation group, and gastric inflammation + MEPM groups (600, 1200 and 1800 mg/kg BW). Gastric inflammation was performed by oral administration of indomethacin at dose 30 mg/kg BW six hour prior to administration of MEPM. Phospholipase A2 was analysed by Western blotting. For gastric ulcer study, we compared the formation of ulcers between nonsteroidal anti-inflammatory drugs (aspirin) and MEPM. We divided 42 rats into seven groups (n = 6), including the control, aspirin (250, 500 and 750 mg/kg BW) and MEPM (600, 1200 and 1800 mg/kg BW) groups. Twenty four hours later the rats' gastric and duodenal tissues were removed and ulcers formation were analysed histologically.

***Results:*** MEMP inhibit phospholipase A2 at the dose of 1200 mg/kg. The gradation of gastric ulcers was higher in aspirin compared to MEMP. At the dose of 600 mg/kg, MEPM does not induce gastric ulcers, but aspirin induces ulcers just at 250 mg/kg.

***Conclusion:*** Methanolic extract of *Physalis minima* L exerts anti-inflammatory action due to its effect to inhibit phospholipase A2 expression. In addition, the gastric ulcers formation side effect of MEMP was lower compared to aspirin.



## INTRODUCTION

Inflammation is a pathophysiologic process mediated by various signaling molecules produced mainly by leukocytes, macrophages and plasma cells [1]. Steroids are known to be the best medicine used in treating acute inflammation, but have side effects when used for long periods of time, such as lowering the resistance of infection. Non-steroidal anti-inflammatory drugs (NSAIDs) are also used to treat inflammation, but they bring about side effects such as gastrointestinal bleeding [2].

Over-activation of the inflammatory mediators may contribute to gastric and intestinal mucosal damage [3]. Inflammation of the gastrointestinal (GI) tract can affect the functioning of the mucosal barrier, thereby influencing its protective activity. Drug-induced damage to the GI tract has become a global problem due to widespread as well as the indiscriminate use of NSAIDs. Therefore, effective management of GI ulceration would primarily depend on the reduction of the aggressive factors, improved generation of protective factors or a combination of both. Advances in natural product chemistry have led to the purification and characterization of a number of chemical compounds with potent anti-ulcer activity [4].

*Physalis minima* L (Indonesian name as ciplukan) is wild vegetation found in the garden or wetland. This herb is commonly known as the bladder cherry and belongs to the Solanaceae family [5]. *P.minima* is an annual herb found throughout India, Baluchistan and Afghanistan, Tropical Africa and Australia, and is reported as one of the important medicinal plants in an

Indian traditional system of medicine. The plant majorly contains phenols, alkaloids, steroids and flavonoids [6]. Steroids of *P.minima* are physalin, resin, alkaloids, saponin and sterol. It contains also fatty acid. Several steroid compounds were found in the leaves of this plant, such as 13,14-seco-16,24-cyclosteroid consisted of physalin B, 5, 6β-epoxyphysalin B, withaphysalin A, withaphysalin B, physalin D and physalin L [7].

Phospholipase A2 (PLA2) catalyzes hydrolysis of the sn-2 fatty acyl ester bond of phosphoglycerides, releasing free fatty acids and lysophospholipids. One of the fatty acids that can be released from membrane stores by the activity of PLA2 is arachidonic acid, the critical precursor for biosynthesis of diverse eicosanoids, including prostaglandins, thromboxanes, and leukotrienes. At least 15 human genes encode different PLA2 isoenzymes, including both secreted and cytosolic forms [8]. PLA2 is now generally considered playing a major role in various inflammatory diseases including *Helicobacter pylori*-associated gastritis or duodenitis [9]. Damage to the gastrointestinal surface protection system and the breakdown of complex membrane lipids activate PLA2, a key enzyme in the production of inflammatory lipid mediator. High concentrations of PLA2 have been reported in gastric mucosa [10].

None of the previous studies mentioned above revealed the potential effects of *Physalis minima* L as an gastric anti-inflammatory agent or its ulcer formation potential. Therefore, this study will attempt to analyze the potential effects of *P.minima* to reduce PLA2 activity as a marker of gastric inflammation. Besides, this study also compared the potential side effect (ulcer formation) of *P.minima* with aspirin as a standard NSAID. The hypothesis of this study is that the methanolic extract of *Physalis minima* L (MEPM) can reduce inflammation in the gastric mucosa and have minimal side effects compared to NSAIDs.

## MATERIALS AND METHODS

### Extraction

Methanolic extract was obtained in three steps, including drying, extraction, and evaporation. The drying process was done by cutting clean leaves and stem of *Physalis minima* L into small pieces, and then heated at 60-70ºC. The drying samples were then blended and filtered using a mesh at size 60. A hundred grams of powder were added to 900 ml methanol in a 1 l Erlenmeyer jar. To obtain maximal evaporation, the mixture was incubated overnight. Then the upper layer was collected and connected to an evaporation apparatus. After separation, gas chromatography analysis was conducted to the MEPM.

### Gas chromatography

Gas chromatography was performed using Rtx®-5MS columns (Restek Corporation, Bellefonte, PA, USA); length: 30 m x 0.25 mm; gas: helium; rate of column: 37.8 ml/min; injector temperature: 320ºC; column temperature: 150ºC; interface temperature: 320ºC.

### Animals

Seventy-two female Wistar rats, weighing 130-160 g, purchased from Central Animal House of Bandung were housed in an air-conditioned room at 24 ± 2ºC and 65-70% relative humidity with a 12 h light-dark cycle. The protocol used in this study was approved by the Ethic Committee for Animal Experimentation of the University of Brawijaya. Diets were prepared following American Institute of Nutrition (AIN) recommendations. The animals were given water *ad libitum* during the experimental period. The composition of diet is 66% comfeed PAR-s, 33% wheat powder and water.

### Gastric inflammation

Gastric inflammation was performed according to Whiteley and Dalrymple [11]. We divided 30 rats into five groups (n = 6 each), including the control group, gastric inflammation group, and gastric inflammation + MEPM groups at doses of 600, 1200, and 1800 mg/kg body weight. Gastric inflammation was induced by indomethacin (30 mg/kg BW per oral) six hours prior to administration of MEPM.

### Gastric ulcers

To examine the formation of gastric ulcers, we compared MEPM with aspirin, the standard non-steroidal anti-inflammatory drug. For this reason, 42 rats were divided into seven groups (n = 6 each), including the control, aspirin (250, 500 and 750 mg/kg BW) and MEPM (600, 1200 and 1800 mg/kg BW) groups. Twenty-hours after drugs administration the rats were killed by ether inhalation.

### Phospholypase A2 analysis

The gastric tissue was digested and centrifuged at 6,000 rpm for 15 min at 4ºC. The supernatant was then collected and added cold ethanol absolute at a ratio of 1:1. The samples were kept at 4ºC overnight, then again centrifuged at 10,000 rpm for 15 min at 4ºC. Pelet was dried until no smell of ethanol remained, then added to 20 mM Tris buffer and was ready for Western blotting procedure. SDS-PAGE electrophoresis was performed to separate protein with different molecular mass. Subsequently, this protein was transferred from gel polyacrilamide into nitrocellulose membranes. Labeling was performed by rabbit polyclonal PLA2 (ab58375; Abcam, Cambridge, UK). The binding between antigenic protein and antibody was then stained by Commasie blue.

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### Ulcer formation and histological analysis

The stomach was opened along the greater curvature, the lumen was rinsed with ice-cold saline, and the mucosa was examined macroscopically. The gastric and duodenal tissues were evaluated by gross examination and scored as follows (Lanza score): **0,** no evidence of ulceration; **1,** hyperemia and redness in mucosa; **2,** one until two ulcerations or hemorrhaging ulcers; **3,** 3-10 ulcerations or hemorrhaging ulcers; **4,** > 10 ulcerations or hemorrhaging ulcers. Erosions are defined as flat, white-based mucosal breaks of any size. Ulcers are defined as mucosal breaks of at least 3 mm or more [12]. Subsequently, the gastric and duodenal tissues were formalin-fixed, and paraffin-embedded tissue blocks were prepared for histopathological examination. The sections of these blocks were stained by hematoxylin and eosin (H&E) [13].

### Statistical analysis

Lanza scores are presented as mean ± SD and differences between groups were analyzed using one-way analysis of variance (ANOVA) with SPSS 17.0 software. Post hoc test was used if the ANOVA was significant; $P < 0.05$ was considered statistically significant.

## RESULTS

### Steroid compounds in *Physalis minima*

We identified several steroid compounds in *Physalis minima* including fucosterol (6.52%), campesterol (4.29%), stigmasterol (2.48%) and γ-sitosterol (1.8%).

### Effect of MEPM on gastric inflammation

The level of PLA2 was analyzed by Western blotting as seen in Fig.1. MEPM inhibited PLA2 expression at the dose of 1200 mg/kg BW. There is no inhibition of PLA2 expression at doses of 600 and 1800 mg/kg BW.

### Effect of MEPM on gastric ulcers formation

Histology of gastric tissue is given in Fig.2. Epithelial disruption identified as marker of ulcer formation was apparent. Table 1 shows the Lanza scores of study groups. The grade of gastric ulcers was significantly higher in aspirin-received groups than those of control animals ($P < 0.05$). At the dose of 600 mg/kg BW, MEMP did not induce gastric ulcers, but aspirin induced ulcers at just 250 mg/kg BW.

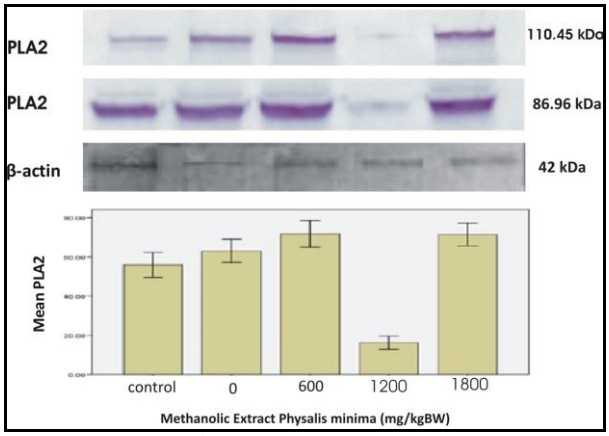


**Figure 1.** The level of phospholipase A2 was analyzed by Western blotting. MEMP inhibit the expression of phospholipase A2 at 1200 mg/kg BW. Interestingly, at lowest (600 mg/kg BW) or highest dose (1800 mg/kg BW), MEMP could not inhibit the expression of phospholipase A2.

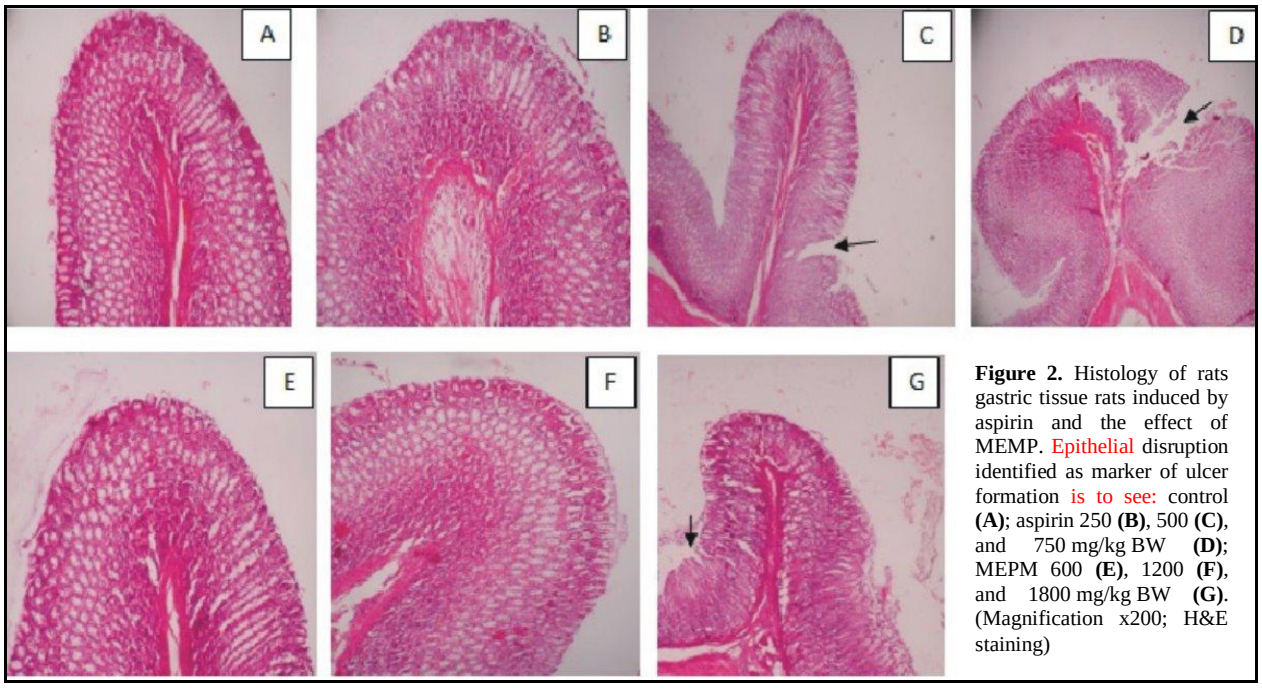


**Figure 2.** Histology of rats gastric tissue rats induced by aspirin and the effect of MEMP. Epithelial disruption identified as marker of ulcer formation is to see: control **(A)**; aspirin 250 **(B)**, 500 **(C)**, and 750 mg/kg BW **(D)**; MEMP 600 **(E)**, 1200 **(F)**, and 1800 mg/kg BW **(G)**. (Magnification x200; H&E staining)

**Table 1.** Lanza scores of different groups

| | Control | Aspirin (mg/kg BW) | | | MEPM (mg/kg BW) | | |
|---|---|---|---|---|---|---|---|
| | | 250 | 500 | 750 | 600 | 1200 | 1800 |
| **Lanza score** | 0 | $1.6 \pm 0.54^{a}$ | $5 \pm 0.7^{ab}$ | $10.2 \pm 0.83^{abc}$ | $0^{bcd}$ | $1.4 \pm 0.54^{acde}$ | $3.2 \pm 0.83^{abcdef}$ |

Values are presented as means ± SD; $P < 0.05$ in comparison with [a]control, [b]aspirin 250, [c]aspirin 500, [d]aspirin 750, [e]MEPM 600, and [f]MEPM 1200 groups.

## DISCUSSION

The gastric mucosa is continuously exposed to noxious substances and has specific defense mechanisms for maintaining its structural integrity. The epithelial surface secretes a barrier consisting of water, mucin bicarbonate and prostaglandins [14]. Phospholipids also play an important role in the preservation of gastrointestinal homeostasis. The enzyme PLA2 is capable of hydrolyzing membrane phospholipids, which in the presence of high gastric acidity lead to mucosal damage. PLA2 mediated hydrolysis of membrane lipids results in membrane perturbation, cell degranulation and stripping of cell surface receptors resulting in gastric ulcer. High concentrations of PLA2 have been reported in the gastric mucosa and PLA2 inhibitors are known to modulate proton conductance across cell membranes and thus can offer gastric mucus protection from enzymatic breakdown [15]. The expression of cytosolic PLA2 in cells of the intestinal tract has been thoroughly investigated and has also been correlated with the development of several inflammatory diseases [16].

In the present rat model of gastric inflammation, MEMP inhibits the expression of PLA2 at a dose of 1200 mg/kg BW. This finding might be due to active steroid compounds acting as anti-inflammatory agents. Based on chromatographic and spectroscopic analyses, previous studies showed that the extract of *Physalis minima* contains physalins B, F and K [6]. In the present study, steroid compounds analyzed by was chromatography on MEPM were found to be fucosterol, campesterol, stigmasterol, and γ-sitosterol. Fucosterol attenuates inflammatory cytokine expression by deactivating mitogen-activated protein kinases [17] and inhibiting the nuclear factor kappa-B (NF-κB) [18]. Stigmasterol inhibits inflammation mediators via the blockade of NF-κB distribution into the nucleus. Besides, campesterol also presented anti-inflammatory effects in a murine model of inflammation [19, 20]. Interestingly, at lowest or highest dose, MEMP could not inhibit the expression of PLA2; this may be due to the pro-inflammatory action of active compounds in MEPM as to see in Fig.1.

The gastric mucosa is one of the most important tissues in an organism, because of its function, structure, and the pathological processes that can take place in this tissue [21]. Gastric ulceration is related to more than one factor, including *Helicobacter pylori* infection, stress, mucosal mucus secretion, gastric irritants and gastric acidity [22]. To compare side effects between aspirin and MEMP, we analyzed gastric ulcers formation in rats. MEMP did not induce gastric ulcers at 600 mg/kg BW, but aspirin induced ulcers at just 250 mg/kg BW. Aspirin induces gastric ulcer formation through the blockade of the cyclooxygenase pathway; thus, shifting the arachidonic acid metabolism to the 5-lipoxygenase pathway, which in turn led to enhanced production of leukotrienes, consequently leading to glandular disruption, decline in mucus production, excessive ulceration, and subsequent hemorrhagic ulcers [4, 7, 23]. Totally, the grade of gastric ulcers was significantly higher in aspirin-received groups compared to MEMP-administered groups.

In conclusion, methanolic extract of *Physalis minima* L posseses anti-inflammatory action due to an inhibitory effect on phospholipase A2 expression in gastric tissue. In addition, the gastric ulcer formation side effect of *Physalis minima* was lower compared to aspirin as an important member of NSAIDs.

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## COMPETING INTERESTS

The authors declare no conflicts of interest.

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**Original Article**

# Antioxidant activity and phytochemical composition of *Cynometra cauliflora*

**Azalina Farina Abd Aziz, Mohammad Iqbal**

*Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan Kota Kinabalu,* ***Sabah, Malaysia***

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**Corresponding Author**
Mohammad Iqbal
Biotechnology Research Institute,
Universiti Malaysia Sabah, Jalan UMS,
88400 Kota Kinabalu, Sabah, Malaysia.
miqbal2k2008@hotmail.com

**Key Words**
Antioxidant activity;
*Cynometra cauliflora*;
Phytochemical screening;
Total phenolics

**Abstract**

***Objective:*** This study was undertaken to evaluate the antioxidant activity and phytochemical composition of *Cynometra cauliflora*.

***Methods:*** The dry sample of young leaves, matured leaves, stems and barks from *C.cauliflora* were tested for phytochemical screening, total phenolics and flavonoids content. Antioxidant activity of the extract was determined by 1,1-diphenyl-2-picrylhadrazyl (DPPH) assay.

***Results:*** Phytochemical screening showed the presence of tannins, saponins and flavonoids in all parts of *C.cauliflora*. Terpenoids were present in all parts except in bark. The constituent of cardiac glycosides are absent in stem but present in all other parts of the plant. Total phenolic content of young leaves was found to be 1831.47 ± 1.03 mg/g (expressed as milligram gallic acid equivalent per gram of plant extract). The total flavonoid content of young leaves was found to be 33.63 ± 0.25 mg/g (milligram catechin equivalent per gram). The radical (DPPH) scavenging activity was found to be in the following order: young leave > matured leave > stem > bark compared with ascorbic acid using as a standard.

***Conclusion:*** These findings suggested that *C.cauliflora* have antioxidant potential and can be used for the development of natural and safe antioxidant compounds.



## INTRODUCTION

Traditional medicinal plants were believed to be used since time immemorial [1]. The knowledge was passed down from generation to next generation with orally and a lot of medicinal plant remained undocumented [1, 2]. Many plants can be used for producing a variety of bioactive compounds, also known as phytochemicals, and they can provide good source of saponins, tannins, flavonoids and polyphenols [3]. Antioxidant properties of medicinal plants have been widely reported. Medicinal plants are important part of traditional medicine [4]. In recent years, there has been growing interest in establishing the therapeutic potentials of medicinal plants which are seen to be natural, inexpensive and without adverse effects [5]. On the other hand, natural products are highly lucrative for the international marketplace.

Many researchers believed that antioxidants play important role in the body defence system against reactive oxygen species (ROS) or free radicals [6]. Free radicals and ROS are, at least in part, responsible to the aging process, tissue damage and also can cause serious diseases [7]. Many factors can contribute to the production of free radicals damage such as smoking, sun exposure, pollution and exposure to toxic chemicals that can give negative impact to our health. Secondary effects of biotic and abiotic stress are result from the constant production of ROS. Production of ROS is normal in metabolic process, but in case of uncontrolled production it can overwhelm the antioxidant defences [8].

Oxidation is a process which can result in damaging the cell. Antioxidants can prevent or delay the process of oxidation and many scientists believe that antioxidants can reduce the risk for chronic diseases [9]. Many fresh fruits, vegetables and herbal plants have been found to contain a lot of antioxidants [10] and they can be categorized as primary sources of antioxidants. Antioxidant substances can terminate the chain reactions of free radical and ROS before they cause

damage. In addition, antioxidant compounds can scavenge free radicals and protect the cells by delaying the process of lipid peroxidation [11].

*Cynometra cauliflora*, an underutilized fruit, is usually grown in orchards or gardens around houses, and possesses many medicinal values in treating several diseases [12]. *C.cauliflora* belongs to the group of bean family Fabaceae (former name Leguminosae). This genus was established in 1741 [13]. Fabaceae contain over 490 medicinal plants including 20 genera and 31 species used in traditional medicines [14]. *C.cauliflora*, also known as Nam-Nam or Katakpuru (in Kelantan), Hima (in Thailand), Kopi Anjing (in Indonesia), was believed to be native of Malaysia and cultivated in Indonesia (Java Island) and India [15]. The *C.cauliflora* fruit resembles to kidney-shape, and the skin of this fruit is very rough (Fig.1). The immature fruit give sour taste for due to its acid content. The fruit flesh will turn to yellowish-brown during ripen. Systematic investigation of this plant extracts for its medicinal properties could provide an important input to pharmaceutical industry. Therefore, in this study it was aimed to evaluate the phytochemical constituent, total phenolics and flavonoids contents, and antioxidant activity of *C.cauliflora*.

## MATERIALS AND METHODS

### Chemicals

Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhadrazyl (DPPH), gallic acid, aluminum chloride, cathechin, sodium nitrite, sodium carbonate, sodium hydroxide, potassium acetate, hydrochloric acid and sulphuric acid were obtained from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

### Preparation of plant extract

*C.cauliflora* leaves and whole part were collected from Pasir Puteh, Kelantan, Malaysia. The sample was washed under running tap water to remove unwanted dirt and other foreign materials. The sample was air-dried under shade until no moisture left. The dried samples were ground using a heavy duty blender into coarse powder form. The aqueous extract was prepared according to the traditional method [16]. One hundred gram of powdered samples were boiled with distilled water at a ratio of 1:10 for 10 min. Then the extracts were filtered (Whatman Filter No.1) to obtain clear solution. The filtered extracts were then stored at -80°C for 3 days before lyophilisation using a freeze dryer (Labconco). Finally, the extracts were kept at -20°C prior to further analysis.

### Phytochemical screening (qualitative analysis)

Phytochemical screening was carried out using standard methods [17]. The procedures are as follows:

**Figure 1.** *Cynometra cauliflora* fruit and leaves.

***-Tannins;*** a few drops of 0.1% $FeCl_3$ was added to the extract and observed for brownish green or dark blue coloration which indicates the presence of alkaloids.

***-Phylobatannins;*** 1% of HCl acid was boiled with 10 ml of aqueous extract in conical flask. A deposition of a red precipitate occurs if the plant carries phlobatannins.

***-Saponins;*** 10 ml of extract is mixed with 5 ml of distilled water in a test tube and vigorously shaken to obtain stable persistent froth. 3 drops of olive oil are mixed into the froth and observed for formation of emulsion which indicates the presence of saponins.

***-Flavonoids;*** a few drops of 1% $NH_3$ solution was mixed to the aqueous extract of plant sample in a test tube. Yellow coloration is observed if this plant carries flavonoid compounds.

***-Terpenoids;*** 5 ml of aqueous extract of each plant sample was mixed with 2 ml of $CHCl_3$ in a test tube. 3 ml of concentrated $H_2SO_4$ is carefully added to the mixture to form a layer. An interface with a reddish brown coloration is formed that indicates the presence of terpenoids constituent.

***-Cardiac glycosides;*** 1 ml of concentrated $H_2SO_4$ was prepared in a test tube. 5 ml of aqueous extract from each plant sample was mixed with 2 ml of glacial $CH_3CO_2H$ containing 1 drop of $FeCl_3$. The above mixture was carefully added to the 1 ml of concentrated $H_2SO_4$ so that the concentrated $H_2SO_4$ is underneath the mixture. The appeal of brown ring indicates the presence of cardiac glycosides.

### Determination of total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu assay according to a method with a slight modification [18]. Solutions of 1 mg/ml of sample and gallic acid (10, 20, 40, 80, 100, 200 µg/ml) were prepared. Briefly, 200 µl of samples were dispensed into test tubes. Then, 1.5 ml of Folin-Ciocalteu's reagent (Folin-Chocalteu:distilled water 1:9) was added and left in the dark for 5 min at room temperature. Then, 1.5 ml of sodium carbonate solution (60 g/l) was added to each tube mixed and left in the dark at room temperature for 90 min. The total phenolic content was determined by using a spectrophotometer

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(Genesys 20, Thermo Scientific, model 4001/4) at 725 nm. The total phenol content was expressed in milligrams of gallic acid equivalents (GAE) per gram of plant extracts.

### Determination of total flavonoid content

Total flavanoid content was measured by the aluminium chloride colorimetric assay [19]. An aliquot of standard solution of catechin (20, 40, 60, 80 and 100 mg/l) was prepared. 0.5 ml of sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min of incubation, 0.15 ml of 10% $AlCl_3$ solution was added and then allowed to stand for 6 min, followed by adding 2 ml of 4% NaOH solution to the mixture. Immediately after water was added to the sample to bring the final volume to 5 ml, the mixture was thoroughly mixed and allowed to stand for another 15 min. The mixture absorbance was determined at wavelength 510 nm. The total flavanoid content was expressed in milligrams of catechin equivalents (CAE) per gram of plant extracts.

### Determination of free radical scavenging activity

The scavenging activity was determined based on DPPH free radical assay [20]. 5 mg/ml stock solution of plant was prepared into eight different concentrations: 10, 25, 75, 150, 300, 600, 1200 and 2400 µg/ml. 300 µl distilled water was added to each tube also with the control. 2.7 ml of DPPH solution was added, vigorously shaken using vortex machine and left 60 min in the dark. The absorbance was measured by a spectrophotometer at 517 nm. The free radical scavenging activity was calculated as follows:

$$\textbf{DPPH scavenging effect \%} = (A_{control} - A_{test}/A_{control}) \times 100$$

The IC50 value of the sample was determined from the plotted graph from result of radical scavenging activity against the concentration of sample. IC50 value is the the amount of the antioxidant required to decrease the initial DPPH radical concentration to 50%.

## RESULTS

### Qualitative analysis of phytochemical screening

Phytochemical screening using qualitative analysis on different plant parts of *C.cauliflora* aqueous extract showed the presence of following constituents: tannins, saponins and flavonoid are present in all parts of the plant; however, phylobatannins were not detected in any part of the plant; terpenoids are absent in bark, but present in all other plant parts; cardiac glycosides were found in matured leaves, bark and young leaves, but not in stem. The details on phytochemical screening results are shown in Table 1.

### Total phenolic content

The total phenolic content of the *C.cauliflora* aqueous extract is presented in Table 2. Among the four different parts, the highest phenolic compound was found in young leaves (1831.47 ± 1.03 mg GAE/g), followed by matured leaves (1180.47 ± 1.93 mg GAE/g), stem (639.55 ± 1.8 mg GAE/g), and the lowest among in bark (447.2 ± 1.99 mg GAE/g).

### Total flavonoid content

The total flavonoid compound of different parts of *C.cauliflora* aqueous extract ranged from 13.24 to 33.63 mg CAE/g. The details are presented in Table 2.

### Radical scavenging effect of *Cynometra cauliflora*

The DPPH radical scavenging activity of different plant parts of *C.cauliflora* ranged from 66.36 to 258.98% (IC50) in young leaves and bark, respectively. (Fig.2).

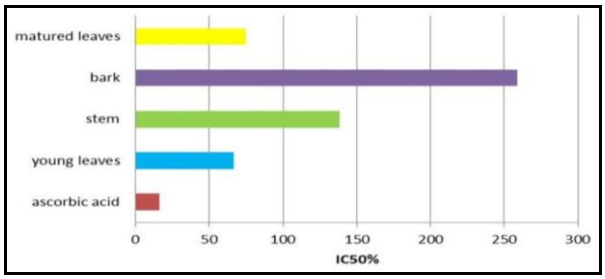


**Figure 2.** Free radical scavenging capacity of *Cynometra cauliflora.*

**Table 1.** Qualitative analysis of phytochemical constituents of *Cynometra cauliflora*

| | Stem | Matured leaves | Bark | Young leaves |
|---|---|---|---|---|
| **Tannins** | + | + | + | + |
| **Phylobatannins** | - | - | - | - |
| **Saponins** | + | + | + | + |
| **Flavonoids** | + | + | + | + |
| **Terpenoids** | + | + | - | + |
| **Cardiac glycosides** | - | + | + | + |

Presence (+) or absence (-) of phytochemical constituents.

## DISCUSSION

Plants contain different phytochemicals that comes with their own biological activity. In recent reports, the presence of bioactive compounds in plants plays a major role to prevent diseases and to improve health [4, 21]. From the present analysis, stem, matured leaves and young leaves of *Cynometra cauliflora* contain terpenoids, which play an important function to healing scar and wound. The presence of tannins also helps in wound healing [17]. Tannins also may prevent microbiological degradation of dietary proteins in the semen [21].

By Malay folk it was believed that *C.cauliflora* can control the diabetes; this plant has been traditionally used for diabetes, which can be explained by the presence of terpenoids that can lower blood glucose level and prevent complications linked to diabetes [21]. Saponins has been found in all parts of the plant; recent reports pointed that saponins have potential for the treatment of hyperglycemia. In medical uses, saponin is a gentle blood cleanser [17, 22, 23]. A previous study reported that, saponins possess hypocholesterolemic and antidiabetic properties [24].

Plant tissues contain variety of bioactive compounds with antioxidant activity and have various therapeutic effects [4, 25]. Recent reports state that the main sources of antioxidants are fruits, plants and vegetables [26]. Phenolic compounds have been reported as antioxidant agents [27]. Flavonoids have been reported as protective antioxidants at various levels [28]. Flavonoids were shown in all parts of *C.cauliflora*. Flavonoids are known to act as antioxidants, which can neutralize unstable and reactive molecules [17]. Previous research reported that, flavonoids could protect membrane lipids from oxidation [27].

In this study, the investigation of the free radical scavenging ability of different parts of *C.cauliflora* was performed by the DPPH scavenging assay. DPPH can measure the ability of antioxidant compounds to scavenge free radical by converting from deep violet colour to discolouration. On the other hand, DPPH assay is a relative stable free radical [25, 26]. This study showed that, $IC_{50}$ value for young leaves is the lowest but bark contains the highest level. Lower $IC_{50}$ value indicates higher antioxidant activity [24]. With all these results, we can conclude that *C.Cauliflora* can be used as a source of safe and natural antioxidant compounds. It could be inferred that *C.cauliflora* has antioxidant activity and possesses the potential to be used to treat or prevent degenerative diseases where oxidative stress is implicated.

**Table 2.** Total phenolic (mg GAE/g extract) and flavonoid (mg CAE/g extract)contents of *Cynometra cauliflora*

| | Total phenolic content | Total flavonoid content |
|---|---|---|
| **Young leave** | 1831.47 ± 1.03 | 21.96±0.3 |
| **Stem** | 639.55 ± 1.8 | 19.65±0.05 |
| **Bark** | 447.2 ± 1.99 | 13.24±0.1 |
| **Matured leave** | 1180.47 ± 1.93 | 33.63±0.25 |

The values are given as the means ± SEM of triplicate tubes.


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## COMPETING INTERESTS

None to declare

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**Hypothesis**

# Cosmological dark matter and ensoulment

**M. Kemal Irmak**

*High Council of Science, Gulhane Military Medical Academy, **Ankara, Turkey***

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**Corresponding Author**
M. Kemal Irmak
Gulhane Askeri Tip Akademisi,
Yuksek Bilim Konseyi
06010 Etlik, Ankara, Turkey.
mkirmak@gata.edu.tr

**Key Words**
Allocortex; Dark matter; Ensoulment; Vomeronasal organ

**Abstract**

Allocortical structures such as hippocampal formation and amygdala are involved in the emotions and memory, and regarded as the seat of personhood. Human body is a composite of a biological organism and an intellective soul. It was suggested that cerebral allocortex is the main region harboring the soul and the beginning of a human person as an individual living organism is at the 13th week of development when an adult type allocortex is already formed.

No experimental data can be sufficient to bring us to the recognition of a soul, but there must be a substance as the basis of personal identity, for without space-occupying substance, there would be no way to account for the soul's ability to interact with the body. It was suggested that the soul substance consists of cosmological dark matter. The dark matter constitutes most of the mass in our universe, but its nature remains unknown. The soul is likely to work into man's physical body directly via that dark matter. We thought that while the soul has a material component as dark matter, there must be an open window to the brain for the entrance of the soul with dark matter.

In this respect, vomeronasal organ which is found in the nasal cavity and which has connections with the brain only between the 12th and 14th weeks of human development - a period including the time of ensoulment at 13th week - seems to be the most appropriate window through which the soul and dark matter can enter the brain.



## ALLOCORTICAL BIRTH AT 13TH WEEK OF DEVELOPMENT

One of the most controversial topics in modern bioethics, science, and philosophy is the beginning of individual human life (personhood) [1-3]. Allocortical birth is a theory which maintains that a fetus becomes a human being when an adult type allocortex is already formed at 13th week [4]. Brain cortex is necessary for the personhood but there exist two different types of cerebral cortex with two different stages of development. The bulk of the brain cortex is a six-layered structure called neocortex (young cortex). The remaining of the cortex is known as allocortex (other cortex) [5]. Allocortex has a relative elementary structure with three basic layers and is composed mainly of the hippocampal formation and amygdala [5]. The most striking feature of the human fetal brain is the early differentiation of allocortical structures [6]. While neocortex does not begin to get organized before 24 weeks [7], an adult type allocortex is already formed at 13th week of development [8-10]. From now on, the macroscopic aspect of the allocortex remains unchanged until birth, and this period is characterized by an increase in its volume [11-19]. Thus, three-layered adult laminar pattern of allocortex appears at 13th weeks [9, 10] and allocortical birth could be located at the occurrence of this first minimal level of structural organization of allocortex at the 13th week of development [5].

Major allocortical structures are hippocampal formation and amygdala. Hippocampal formation plays a key role in memory and emotion [17], and it is essential for the preservation of identity [5]. The human amygdala on the other hand is the area of the brain that is best correlated with emotional reactions [5]. Thus, allocortex can be regarded as the seat of emotion, memory and personhood. At 13th week, fetus has an adult type allocortex by functional and anatomic definition [6, 14, 20, 21] and it is the most reasonable time at which to fix the start of individual human life (personhood) [4].

Allocortical birth theory suggests that an individual human life or personhood cannot begin until the development of the allocortex. At the 13th week, fetus also has distinctive human characteristics and possesses the primordia of all the internal and external organs and parts. Before the allocortex gets organized, what we have is a set of tissues or a series of organ systems. Allocortical birth, the emergence of a mature allocortex is therefore proposed as a reasonable time to demarcate the beginning of individual human life (personhood) [4, 22]. Most religious traditions hold that what makes one a person is the possession of a soul and they treat the human body as a composite of a biological organism and an intellective soul [23, 24]. When the body meets with the soul, it comes to be a human person, with all the attendant rights, especially its basic right to life [25, 26]. If we accept that the soul essentially has the capacity for personhood, it seems that the soul does not begin to exist until there occurs an appropriate seat for the soul in the fetal brain [27]. This position would seem to require that the immortal soul only be infused into a fetus with sufficient cortical development [4, 28]. Therefore, a role was attributed to allocortex to be a seat for the soul to work into man's physical body and the beginning of a human individual's life was suggested as 13th week of development when an adult type allocortex is formed [4]. According to this view, ensoulment occurs when allocortex is formed at 13th week of development and we do not begin to exist until 13 weeks after conception, when there is evidence for the seat of soul or personhood. The soul, it seems, must await the development of the allocortex in order for personhood to become possible.

## SOUL MUST HAVE MATERIAL COMPONENT

Mental functions are powers that the soul has exercised by means of the physical entity called the brain [29]. A question is raised: "How is it possible for an immaterial substance to interact with a physical body?" [29]. No experimental data can be sufficient to bring us to the recognition of a soul, but there must be a substance as the basis of personal identity, for without space-occupying substance, there would be no way to account for the soul's ability to interact with the body [30, 31]. It was suggested that the soul substance consists of cosmological dark matter [32]. The dark matter is a universal connecting medium, filling all space to the furthest limits, penetrating the interstices of the atoms without a break in its continuity. So completely does it fill space that it is sometimes identified with space itself, and universe is built up in this fluid and move through a sea of it [32]. Astrophysical observations indicate that dark matter constitutes most of the mass in our universe, but its nature remains unknown [33]. It is called dark matter since it neither emits nor absorbs light. The existence of dark matter is inferred by its gravitational effects on ordinary matter and radiation [34, 35]. With the conception that the dark matter is the primary form of all substance, that all other forms of matter are merely differentiations of it, then it seems that soul substance which is in this life linked organically with the body can be identical with the dark matter. The soul is likely to work into man's physical body directly via that dark matter [32]. Evidence of the existence of dark matter has been found in large high-energy particle accelerators at the European Organization for Nuclear Research (CERN; (Switzerland) and Fermi National Accelerator Laboratory (Fermilab; IL, USA) [36].

## VOMERONASAL SYSTEM AS A POINT OF ENTRY FOR THE SOUL TOGETHER WITH DARK MATTER

We thought that while the soul has a material component (dark matter), there must be an open window to the brain for the entrance of the soul with dark matter. In this respect, vomeronasal organ (VNO) which is found in the nasal cavity and which has connections with the brain only between the 12th and 14th weeks of human development - a period including the time of ensoulment at the 13th week - seems to be the most appropriate window through which the soul and dark matter can enter the brain [37]. Actually, VNO is said to be the place in the body where the nervous system is closest to the external world [38]. Sensory cells of the vomeronasal system (VNS) are located within the sensory epithelium of the VNO, bipolar in shape with a single dendrite and an axon originating from its soma. The dendrite reaches the surface of the lumen of the VNO to form a dendritic terminal that bears microvilli [39]. The axon leaves the sensory epithelium, forming the vomeronasal nerve with neighboring axons, traveling toward the brain, and terminate on dendrites of second-order neurons in the accessory olfactory bulb [38, 39]. Axons of the second-order neurons of the VNS make close connections with the amygdala and hippocampus [38-40], the seat of emotion, memory and personality; shortly the seat of the soul.

The VNO is a fluid-filled, tubular structure located at the base of the nasal septum that opens into the nasal cavity via a duct at its anterior end [41]. It is a chemoreceptive structure with direct axonal connections to the accessory olfactory bulb in many terrestrial vertebrates [42]. Pheromones presumably bind to the VNO and exert behavioral or physiologic responses, thereby allowing chemical communication between animals of the same species [43]. The effects of pheromones are thought to be mediated by signals from the accessory olfactory bulbs to the amygdala and hypothalamus [41]. The VNS is well developed and

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functional in adult animals [44], while human VNO becomes rudimental before birth [45]. VNO in the human embryo contains bipolar cells similar to the developing vomeronasal sensory neurons of other species, but the structure becomes more simplified later in development [46-52], having no obvious way of communication with the brain. In humans the VNO, including the vomeronasal nerve and associated ganglion cells, is first recognizable at 8$^{th}$ week of development [53]. VNO is well developed during the 12-14$^{th}$ weeks of development [37], but VNO loses receptor cells and becomes a ciliated, pseudostratified epithelium after 14$^{th}$ week of age [54, 55]. Moreover, the vomeronasal nerve connecting the VNO with the accessory olfactory bulb (AOB) degenerates between week 14 and 28 [56, 57] leaving the function of the human VNO unclear. The AOB which is a primary brain center for the VNS [40, 51], is present in human embryos and certain stages of fetuses, but becomes degenerated and it is not identifiable after 7 months [58]. These observations support the view that the VNO functions mainly during the intrauterine period in humans, especially during the period of ensoulment [4, 59]. Thus the development of the vomeronasal structures seems to be limited to a restricted time frame in humans, when they play a role for the ensoulment [48]. We propose here that the human VNO has functions mainly during fetal development when the VNO, along with the vomeronasal nerve, contributes to the transfer of the soul and its dark matter to their proper sites in the brain.

Although no anatomical connection has been demonstrated in adult humans, Monti-Bloch *et al* deduces a physiological connection with the brain because stimulus delivery to the VNO elicited several systemic responses [60-62] such as changes in blood pressure and heart rate, small but significant changes in hormonal levels [62] and some changes in mood [63]. Functional brain imaging studies revealed consistent activation of the hypothalamus, amygdala and cingulate gyrus-related structures during adult human VNO stimulation [60]. Several indirect reports of the presence of pheromone-like substances, influencing human behavior, have also been published [63]. These findings support the view that VNO is strongly related with allocortex harboring the soul even in the absence of anatomical connections. This close relationship may form the basis of the system in humans which detects non-odorant molecules in extreme dilution for homeopathic medicines which appears to trigger a healing response in psychiatric disorders [64].

## CONCLUSION

Allocortex is the primary center harboring the soul and the human life (personhood) begins at the 13$^{th}$ week of development with a delayed ensoulment. Above considerations make it appear likely that the soul may have a component of dark matter; and the soul may enter the brain with dark matter through the window of VNO which is functional and has connections with the brain only during the time of ensoulment.

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## COMPETING INTERESTS

None to declare

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# KEY WORD INDEX


**- A -**

Acceleration **2:** 87
Acetylcholinesterase **1:** 23; **2:** 103
*Achillea millefolium* **2:** 113
Adenosine diaminase **4:** 293
Adenosine triphosphatase **2:** 103
Aging **4:** 305
Alar retraction **1:** 57
Albumin **1:** 3; **3:** 185
Allocortex **4:** 343
Alloxan **3:** 249
Alpha-lipoic acid **2:** 103
Amlodipine **2:** 133
Anemia **3:** 199
Anesthetic modulation **2:** 141
Anthocyanins **1:** 43
Anti-inflammatory **3:** 237
Anticancer **3:** 225
Antihypertensive **2:** 93
Antimicrobial agents **2:** 165
Antimicrobial susceptibility **2:** 165
Antioxidant **1:** 43; **3:** 225; **4:** 305
Antioxidant activity **4:** 337
Antioxidant enzymes **4:** 323
Antitumor agent **4:** 313
Aortic rings **2:** 93
Aphthous ulcer **3:** 171
Apoptosis **3:** 225
Arachnoid granulations **1:** 77
Aspirin **4:** 331
Atherosclerosis **3:** 219
Autism **4:** 293

**- B -**

Bacterial plaque **2:** 165
Biocrystallization **1:** 51
Biomedical journals **1:** 1
Bone minerals **1:** 69
Boron **1:** 9
Brain temperature **1:** 77
Breast cancer **3:** 199
BZLF-1 **4:** 299

**- C -**

*Carapa guianensis* aublet **3:** 231
Carbofuran **4:** 323
Carbon tetrachloride **1:** 43
Caries **3:** 171
Cartilago alaris major **1:** 57
Cartilago nasi lateralis **1:** 57
Cell cycle **4:** 313
Cereals **3:** 205
Cerebellum **2:** 103
Cerebrospinal fluid temperature **1:** 77
Cervical cancer **3:** 225
*Chinnodbhavadi kwath* **3:** 243
Chitosan **2:** 165
Chlorhexidine **2:** 165
Chlorpyrifos **1:** 23; **3:** 213
Cigarette smoke **1:** 69
Coal dust **1:** 69; **3:** 219
Cold adaptation **4:** 255
Comet assay **3:** 231
Cowpea **2:** 159
Cyclin **4:** 313
Cyclophosphamide **2:** 113
*Cymbopogon citratus* **3:** 249
*Cynometra cauliflora* **4:** 337
Cystone® **1:** 51
Cytokine **2:** 137; **3:** 237

**- D -**

Dark matter **4:** 343
Dermal route **1:** 31
Diabetes **2:** 81, **3:** 249
Diabetes mellitus **1:** 3, **3:** 181, 185
Discriminant classification **4:** 267
Disease activity **1:** 9
DNA damage **3:** 231
DNA fragmentation **4:** 323
Dyslipidemia **3:** 205

**- E -**

Electroencephalography **4:** 267
Endothelial damage **3:** 219
Endothelins **3:** 191
Ensoulment **4:** 343
Envelope correlation **4:** 267
Epstein-Barr virus **4:** 299
Erythropoietin **3:** 199
Erythropoietin receptor **3:** 199
Exercise **4:** 279
Exercise-induced hypertension **2:** 133
Experimental research **1:** 1

**- F -**

Female rats **2:** 159
Femur **2:** 153
*Ficus deltoidea* **2:** 93
Fluorescent probe **1:** 3; **3:** 185
Fluoride **3:** 213
Food-grade **2:** 145
Fried potato chips **4:** 285
Functional class **1:** 9

**- G -**

Gastric ulcer **4:** 331
Gene expression **4:** 255
Glucose **3:** 249
Glutarginum **2:** 87

**- H -**

Hela cell line **3:** 225
Hematological parameters **2:** 127; **3:** 213
Hepatoprotective **1:** 43

**- I -**

Indomethacin **2:** 93; **4:** 331
Inducible nitric oxide synthase **2:** 137
Inflammation **1:** 37; **2:** 137; **4:** 331
Inflammatory **3:** 237
Inguinal hernia **1:** 73
Inhalation toxicology **3:** 219
Inhalation **2:** 153
Insuline needles **3:** 181
Integrative medicine **1:** 1
Interdental cleaning aids **1:** 63
Ion channel **4:** 255

**- K -**

Kerosene **1:** 31

**- L -**

L-Arginine **2:** 87
L-Glutamate **2:** 87
L-Name **2:** 93
Lactobacillus **2:** 145
*Lactococcus lactis* **2:** 145
Laparoscopic hernioplasty **1:** 73
Lecithin-cholesterol acyltransferase **3:** 205
Legumes **3:** 205
Lindane **2:** 103
Lipid profile **3:** 219, 249
LMP-1 **4:** 299
Lower lateral cartilage **1:** 57

**- M -**

Macular edema **2:** 81
Male infertility **1:** 17
Malondialdehyde **4:** 293
Mangosteen **2:** 137
Marine drug **4:** 313
Menopause **4:** 279
Methotrexate **2:** 127
Methylgyoxal **3:** 177
Methylphenidate **2:** 121
Migraine **1:** 77
Monoiodoacetate **1:** 37
Mucin activity **3:** 243
Mucositis **3:** 171
Muscle activity **4:** 255

**- N -**

Nasopharyngeal carcinoma **4:** 299
Neuropathy **4:** 285
Nimodipine **1:** 17
Nisin **2:** 145
Non-anemia **3:** 199
Nsr gene **2:** 145

**- O -**

Obesity **3:** 205
Oil extract **3:** 231
*Olea hochstetteri* **2:** 141
Oral hygiene **1:** 63
Oral route **1:** 31
Osteoarthritis **1:** 37
Osteoprotegerin **3:** 177
Oxidative status **3:** 177
Oxidative stress **1:** 31; **4:** 305, 323
Oxygen metabolism **4:** 305
Ozone **3:** 171

**- P -**

Pain **3:** 181, 191
PARP-1 **4:** 299
Particulatte matter 10 **1:** 69
Periodontal treatment **1:** 63
Periodontitis **3:** 171
Peripheral ischemia **2:** 81
Phosphide residue **2:** 159
Phytochemical screening **4:** 337
Phytotherapy **2:** 141
Piperaceae **3:** 225
Polyphenols **4:** 323
Preosteoblast **3:** 177
Proliferating cell nuclear antigen **4:** 313
Proliferation **3:** 199
Pseudotumor cerebri **1:** 77
Pyloric ligation **3:** 243

**- R -**

Radiation-induced effect **1:** 3
Radiation **3:** 185
Ramipril **2:** 133
Rat **2:** 121; **3:** 205
Rat pups **4:** 285
Rats **1:** 17; **2:** 153
Rectus sheath canal **1:** 73
Red radish **1:** 43
Renal calculi **1:** 51
Reproductive hormones **1:** 23
Reproductive toxicity **2:** 113
Resistance marker **2:** 145
Respiration **4:** 255
Resveratrol **2:** 103
Retinopathy **2:** 81
Rheumatoid arthritis **1:** 9; **2:** 127
Rhinoplasty **1:** 57
Rosuvastatin **2:** 127

**- S -**

Schizophrenia **4:** 267
Schizotypal disorder **4:** 267
Scientific publishing **1:** 1
Selective brain cooling **1:** 77
Sensory neurons **3:** 191
Silymarin **1:** 43
Snakehead fish **3:** 237
Sperm **2:** 121
Sperm count **1:** 23
Spermatogenesis **1:** 17; **2:** 121
Spinal cord **4:** 285
Sputum conversion **3:** 237
Steroids **4:** 331
Struvite stones **1:** 51
Superoxide dismutase **4:** 293
Sweating **4:** 279

**- T -**

Temporomandibular joint **3:** 171, 191
Testicular histology **1:** 23
Thermoreceptors **4:** 255
Thermoregulation **4:** 255
Thirst perception **4:** 279
Thymus **2:** 87
Tooth brushing **1:** 63
Total extraperitoneal approach **1:** 73
Total phenolics **4:** 337
Toxicity **2:** 153
Trabecular bone **2:** 153
Trace elements **1:** 9
*Triphala* **3:** 243

**- U -**

Upper lateral cartilage **1:** 57

**- V -**

Vascular reactivity **2:** 93
Visual analogue scale **3:** 181
Vitamin C **1:** 23; **3:** 213
Vitamin E **3:** 213
Vitamins **2:** 159
Vomeronasal organ **4:** 343

**- W -**

Wistar rats **1:** 31

**- X -**

Xenograft **4:** 313

**- Y -**

Yoga **4:** 305

# AUTHOR INDEX


**- A -**

Abdelatif IM **4:** 285
Adamu JY **2:** 141
Adejumo BI **3:** 249
Adiputro DL **2:** 137; **3:** 219
Agadihiremath T **1:** 51
Ahmed J **3:** 171
Akbar IZ **3:** 177
Ali M **4:** 331
Alonso-Laurencio A **3:** 231
Al-Rubaye FG **4:** 293
Amabebe E **4:** 279
Ambali SF **1:** 23
Andreasson S **2:** 81
Anojulu A **3:** 249
Ansari MM **1:** 73
Anturlikar SD **1:** 51
Archana V **2:** 165
Arencibia-Arrebola DF **3:** 231
Arul KS **2:** 165
Asmawi MZ **2:** 93
Avasthi R **2:** 133
Aydogdu A **1:** 63
Ayo JO **1:** 23
Aziz AFA **4:** 337

**- B -**

Baba NA **3:** 213
Bachtiar I **3:** 225
Bakiner SO **3:** 181
Bakir B **1:** 1
Baruah DB **1:** 43
Behera KK **2:** 103
Besbes M **3:** 205
Bhatt DK **2:** 103
Binnal A **3:** 171
Bist R **2:** 103
Bozkirli E **3:** 181
Budiningrum AI **4:** 299

**- C -**

Camara PRS **3:** 191
Chabane FZ **3:** 205
Chaloob R **1:** 17
Chhabra MK **2**: 133
Crespo-Garces Y **3:** 231

**- D -**

Daij NBBA **3:** 205
Darsuni A **3:** 219
Dash RN **1:** 43
Datau EA **3:** 237
Datta S **4:** 323
Dawane JS **1:** 37
Delgado-Roche L **3:** 231
Denny C **3:** 171
Develi S **1:** 57
Dewa A **2:** 93
Dewi FRP **3:** 199
Dhar P **4:** 323
Dimkpa U **3:** 249

**- E -**

El-Sayyad HI **4:** 285
El-Shershaby EM **4:** 285
Ertorer ME **3:** 181
Ewenighi CO **3:** 249
Ezeugwu U **3:** 249

**- F -**

Fatchiyah F **3:** 199; **4:** 299
Fatihu MY **1:** 23

**- G -**

Gabruseva N **1:** 3; **3**: 185
Gad-Allah AA **4:** 285
Gamsizkan M **1:** 57
Ghosh S **4:** 323
Gong F **2:** 145
Gonta S **1:** 3; **3:** 185
Gorbachevskaya NL **4:** 267
Gorbenko G **1:** 3; **3;** 185
Gorial FI **2:** 127
Gupta LK **2:** 133
Gupta R **2:** 133

**- H -**

Habibuddin M **1:** 43
Halder K **4:** 305
Hasanzadeh S **2:** 113, 121
Hassan Z **2:** 93
He S **2:** 145
Hidayat M **3:** 177
Huang P **4:** 313
Hussain SA **1:** 17
Hu X **2:** 145

**- I -**

Iqbal M **4:** 337
Irmak MK **4:** 343
Ismail Z **2:** 93
Iyanda AA **1:** 31; **2:** 159

**- J -**

Jain S **3:** 243
Jalali AS **2:** 113
Jayaramaiah KK **1:** 51

**- K -**

Kalim H **3:** 177; **4:** 331
Kalnina I **1:** 3
Kalnina IE **3:** 185
Kalsum U **4:** 331
Kania N **3:** 219
Karandikar M **1:** 37
Karapinar U **1:** 57
Kareem A **1:** 9
Karthikeyan BV **2:** 165
Katzman P **2:** 81
Kaya A **1:** 77
Khotimah H **2:** 137
Kianifard D **2:** 121
Kianifard L **2:** 121
Kilic C **1:** 57
Kirilova E **1:** 3; **3:** 185
Kirilov G **1:** 3; **3:** 185
Kong X **4:** 313
Kozyreva TV **4:** 255
Kriventsov MA **2:** 87
Kulaichev AP **4:** 267
Kurjane N **1:** 3; **3:** 185
Kurt S **1:** 63

**- L -**

Laksmitawati DR **3:** 225
Lamri-senhadji MY **3:** 205
Lemos TEV **3:** 191
Londahl M **2:** 81
Lutfioglu M **1:** 63

**- M -**

Majeed IA **2:** 127
Malekinejad H **2:** 113
Malla R **3:** 213
Manshor NM **2:** 93
Matheos JC **3:** 237
Mikhael EM **2:** 127
Mishra AB **4:** 305
Morad TS **4:** 293
Moroz GA **2:** 87
Muttaqien F **3:** 219

**- N -**

Nariya M **3:** 243
Noor Z **1:** 69; **2:** 153
Nugrahenny D **3:** 219
Numan IT **1:** 17

**- O -**

Obika LF **4:** 279
Omorodion SI **4:** 279
Onoh GO **3:** 249
Onoh LUM **3:** 249
Onyeanusi JC **3:** 249
Orji E **3:** 249
Oter S **1:** 1
Ozden FO **1:** 63
Ozoene JO **4:** 279
Ozsahin AK **3:** 181

**- P -**

Paliliewu N **3:** 237
Pal R **4:** 305
Pandit V **1:** 37
Patki PS **1:** 51
Permatasari N **3:** 177
Peter ID **2:** 141
Pimane E **3:** 185
Porto RM **3:** 191
Prabhuji MVL **2:** 165
Prenggono MD **3:** 199

**- Q -**

Qi X **4:** 313

**- R -**

Rafiq M **1:** 51
Raina R **3:** 213
Rajan B **3:** 171
Rajopadhye B **1:** 37
Ravishankar B **3:** 243
Razali N **2:** 93
Ribela MT **3:** 191
Rofi'i A **4:** 299
Romdoni R **2:** 137
Rosa OB **3:** 231
Rosario-Fernandez LA **3:** 231

# Author Index


**- S -**

Saha M **4:** 305
Sakallioglu EE **1:** 63
Sandabe UK **2:** 141
Sargowo D **2:** 137
Sariturk C **3:** 181
Sellman A **2:** 81
Setiawan B **1:** 69; **2:** 153; **3:** 219
Sharma KK **2:** 133
Shenoy N **3:** 171
Shittu M **1:** 23
Shukla V **3:** 243
Silova A **1:** 3; **3:** 185
Singh SN **4:** 305
Skesters A **1:** 3; **3**: 185
Soeatmadji DW **3:** 177
Suharjono S **4:** 299
Sulaiman MM **1:** 23
Sultana M **3:** 213
Surachmanto EE **3:** 237

**- T -**

Taleb-senouci D **3:** 205
Tomar R **3:** 243
Tomer OS **4:** 305

**- U -**

Ugwu AC **4:** 279
Uzor S **3:** 249

**- V -**

Verma PK **3:** 213
Vidal-novoa A **3:** 231
Viswanatha GL **1:** 51

**- W -**

Wargasetia TL **3:** 225
Widodo MA **2:** 137; **3:** 219; **4:** 331
Widowati W **3:** 225
Wijaya L **3:** 225

**- Y -**

Yaqub LS **1**: 23
Yazar F **1:** 57
Yelliantty Y **3:** 225

**- Z -**

Zhang D **2:** 145
Zhang L **2:** 145
Zhang X **4:** 313
Zvagule T **1:** 3; **3:** 185

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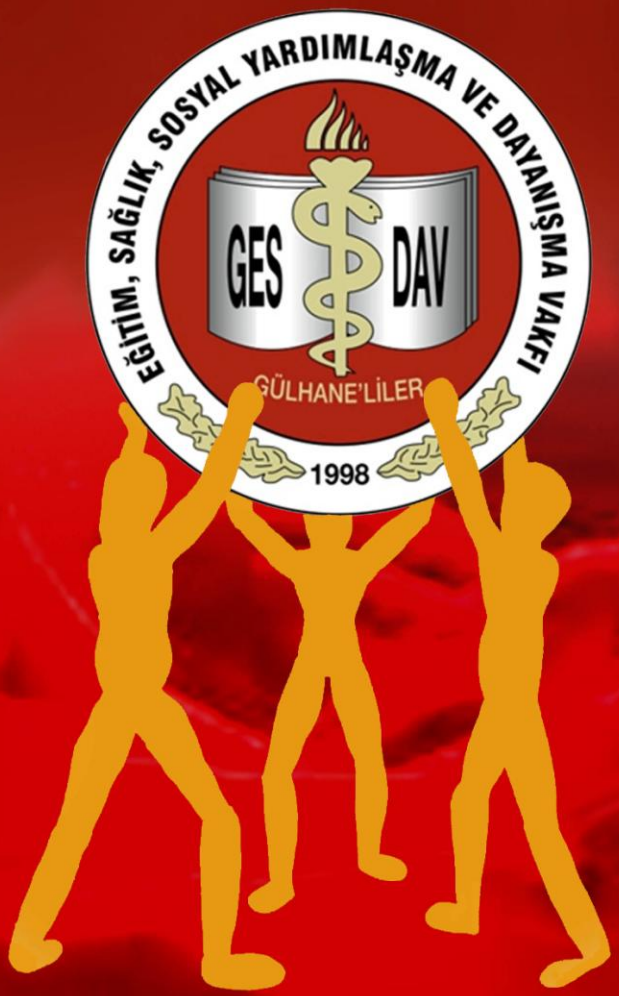


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