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CLINICAL SIGNIFICANCE OF CIRCULATING INTERLEUKIN-8 CHANGES IN UNTREATED PATIENTS WITH ACUTE PSORIASIS

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ABSTRACT

Psoriasis in its various forms is one of the most common chronic inflammatory skin diseases worldwide. Interleukin-8 (IL-8) is a pro-inflammatory cytokine. IL-8 is a potent chemoattractant for neutrophils, T cells and basophils.

The aim of this study was to compare the levels of IL-8 in adult patients with acute psoriasis prior to therapy and those of healthy subjects and to investigate the relation of IL-8 levels to the severity of the disease. Serum levels of IL-8 were investigated in 39 untreated patients (28 women and 11 men, aged 33.76±4.2 years, age range 25-50) with acute psoriasis and 12 healthy subjects (8 women and 4 men, aged 34.9±7.7 years, aged range 27-54). The production of IL-8 was measured by ELISA technique. Cytokine concentrations (pg/mL) were expressed as the mean of duplicate samples. Results are given as the mean±SD. The mean value of IL-8 of the patients group was 934±312 pg/mL (range from 629 pg/mL to 1246 pg/mL) and that of controls was 417±126 pg/mL (range 296 pg/mL to 539 pg/mL). Results were analyzed using a paired Student's t-test. Wilcoxon's rank-sum test was used as a two-tailed test for statistical analysis of group differences. A p-value of less than 0.05 was considered significant. These analyses demonstrated a significant increase in IL-8 production in acute psoriasis ($p<0.01$). No correlation was found between serum IL-8 levels and disease severity. Our data suggest that IL-8 may be of pathophysiological importance.

Key words: psoriasis, inflammation, serum IL-8

INTRODUCTION

Psoriasis in its various forms is one of the most common chronic inflammatory skin diseases worldwide. It ranges from mild to severe, depending on how much of the skin is involved, but in more severe cases almost the entire skin can be affected. Psoriasis affects both males and females equally and is independent of race. There are two ages of onset. Early onset disease (Type 1), which peaks at between 15 and 35 years of age, is the most common and is often associated with a positive family history. Late onset disease (Type 2) occurs at a peak age of between 55 and 60 years. Although the precise aetiology of psoriasis is not known, its pattern (at least in the case of Type 1 disease), fits that of an autoimmune disease. The onset of type 1 disease occurs around the time of adolescence and its appearance is gradual rather than sudden (1-4).

Interleukin-8 (IL-8) is a pro-inflammatory cytokine with chemoattractive and major activator properties on neutrophils. The stimulation of T-cells involves activation signals from antigen presenting cells (APCs). IL-8 is a cytokine with high specificity for neutrophils, on which it has chemoattractant and major activator properties. It may be produced by a large number of cells: circulating monocytes, macrophages, endothelial cells and even activated neutrophils. The few studies in literature on the IL-8 behaviour refer to psoriasis (5-8).

This study proposed investigation of IL-8 behaviour in acute psoriasis prior to therapy and to demonstrate the hypothesis that IL-8 may have a pivotal role in determining the outcome of disease.

MATERIAL AND METHODS

In order to provide a comparison with previously published results in this study serum levels of IL-8 were investigated in 39 untreated patients (28 women and 11 men, aged 33.76±4.2 years, age range 25-50) with acute psoriasis and 12 healthy subjects (8 women and 4 men, aged 34.9±7.7 years, aged range 27-54). Blood was harvested by venous puncture, in aseptic conditions and with minimal venous compression at the time of admission in hospital. The production of IL-8 was measured by ELISA technique. Cytokine concentrations (pg/mL) were expressed as the mean of duplicate samples. Results are given as the mean±SD. The IL-8 concentration was calculated based on a standard curve for each determination. Results were analyzed using a paired Student's t-test. Wilcoxon's rank-sum test was used as a two-tailed test for statistical analysis of group differences. A p-value of less than 0.05 was considered significant.

The project was approved by the Ethics Committee

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RESULTS

Analyzing the behaviour of IL-8 in relation with acute psoriasis it is to be noted that increased serum IL-8 levels in 39 of the patients with psoriasis prior to therapy as compared with those of healthy controls. The mean value of IL-8 of the patients group was 934±312 pg/mL (range, 629 pg/mL to 1246 pg/mL) and that of controls was 417±126 pg/mL (range 296 pg/mL to 539 pg/mL). These analyses demonstrated a significant increase in IL-8 production in acute psoriasis ($p<0.01$). No correlation was found between serum IL-8 levels and disease severity. Our findings that IL-8 is increased in acute psoriasis could have pathogenetic implications.

DISCUSSION

Our investigations demonstrate the major importance of IL-8 in psoriasis. The stimulation of T-cells involves activation signals from antigen-presenting cells (APCs). These high IL-8 levels in acute psoriasis point to inflammatory process in which IL-8, a pro-infammatory cytokine may be involved. Our results correspond well with finding regarding high cutaneous IL-8 protein expression, as determined by immunohistochemistry (7).

A publication has implied that production of IL-8 may be under genetic control via a polymorphism at a functionaly active site on the IL-8 promoter gene (9).

Among cytokines, TNF-α and IL-1 can induce IL-8 production. IL-8 sends signals through the neutrophil receptors bound to protein G. The signals received by the chemotactic receptors of the neutrophils are essential for diapedesis and exocytosis of the neutrophil granulations, rich in gelatinase, whose preferred substrate is type IV collagen, a major component of the basal membrane. T helper 2 (Th2) type responses are also characterized by production of IL-8. The local and systemic production of cytokines is responsible for the majority of pathological and clinical consequences. IL-8 detection in the systemic circulation of the patients with acute psoriasis is essential for the investigation of the neutrophil involvement in the pathophysiology of psoriasis. Some evidence accumulated that epidermal cells are able to synthesize and release IL-8. These observations suggest that the IL-8 may be crucial in determining the degree of T-cell proliferation. In addition to this local IL-8 overexpression, endotoxin-stimulated whole blood from psoriatic patients showed a tendency for higher IL-8 formation capacity. IL-8 overexpression may be a central phenomenon for the pathogenesis of psoriasis and that its normalization would be beneficial (6,7,10).

Neutrophils have been much investigated, both because this type of cell is a key factor in the inflammatory response and because the cytokines secretion may significantly influence the direction and course of the disease. The few existing reports on IL-8 in psoriasis are controversial. Chemotaxis, adhesion or migrations of neutrophils in the vascular walls are essential inflammatory phenomena that may be partially mediated by IL-8. An interaction between T-cells and APCs is initiated when a specific antigen, presented in association with the MHC on the APC, binds to T-cell antigen receptors. It is important to caution that this is just one step in a very long process, although it may be an important one. New treatments based on T-cell pathophysiology have already shown considerable promise (8,11-13).

Altogether, our study enforces earlier data that IL-8 is produced at local level to focus and amplify inflammatory responses. Evidently, however, these local processes are not reflected by the systemic IL-8 level even in cases of severely involved psoriatic skin. Consequently, serum IL-8 levels do not provide diagnostic or prognostic criteria in psoriasis.

The neutrophil behaviour in patients with psoriasis constitutes the subject of current studies. Further investigations are necessary to assess safety aspects as well as effects on disease activity and related immunological parameters.

CONCLUSION

IL-8 overexpression may be a central phenomenon in psoriasis. The present study provides further evidence that IL-8 deregulation may play a key role in psoriasis, may contribute to exacerbate psoriasis. The precise nature of IL-8 over-expression is still unclear.

The IL-8 increase in acute psoriasis proves the involvement of neutrophils in the pathogenesis of the disease, IL-8 being one of the most powerful chemotactic factors for neutrophils.

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SEMNIFICATIA CLINICA A MODIFICARILOR INTERLEUKINEI-8 CIRCULANTE LA PACIENTII CU PSORIASIS ACUT NETRATAT

REZUMAT

Psoriazisul sub diverse forme este una dintre cele mai frecvente boli inflamatorii cronice ale pielii întâlnită pretutindeni. Interleukina-8 (IL-8) este o citokină proinflamatorie. IL-8 este un chemoatractant puternic pentru neutrofile, celulele T şi bazofile.

Scopul acestui studiu a fost să comparăm producerea de IL-8 la pacienţii cu psoriazis acut cu cea de la subiecţii aparent sănătoşi şi să investigăm relaţia dintre concentraţia IL-8 şi severitatea bolii. Concentraţia de IL-8 în ser a fost investigată la 39 pacienţi netrataţi (28 femei şi 11 bărbaţi cu vârsta medie 33,76±4,2 ani, cu limite între 25-50 ani) cu psoriasis acut şi 12 subiecţi aparent sănătoşi (8 femei şi 4 bărbaţi cu vârsta medie 34,9±7,7 ani, cu limite între 27-54 ani). Concentraţia de IL-8 a fost măsurată folosind tehnica ELISA. Concentraţiile citokinei (pg/ml) au fost exprimate ca media probelor lucrate în duplicat. Rezultatele sunt exprimate ca media±DS. Concentraţia de IL-8 la lotul de pacienţi a fost 934±312 pg/ml (cu limite între 629-1246 pg/ml), la lotul martor a fost 417±126 pg/ml (cu limite între 296-539 pg/ml). Rezultatele au fost analizate folosind testul Student pe perechi. Testul Wilcoxon a fost folosit pentru analiza statistică a diferenţelor între grupuri. Valoarea $p<0,05$ a fost considerată semnificativă. Există creştere semnificativă de IL-8 în psoriazisul acut ($p<0,01$). Nu s-a găsit corelaţie între concentraţiile de IL-8 în ser şi severitatea bolii. Rezultatele sugerează că IL-8 prezintă o importanţă fiziopatologică.

Cuvinte cheie: psoriazis, inflamaţie, IL-8 în ser

HYPERALGESIA – AN OVERVIEW

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ABSTRACT

The current article is designed as an overview of the most recent data on hyperalgesia, emphasizing the following aspects: hyperalgesia – symptom and/or disease, molecular bases of hyperalgesia, types, pathways, cells, mediators and receptors involved, opioid-induced hyperalgesia, the role played by inflammation, the involvement of hippocampus in the exaggerate persistent painful sensation and the experience of hyperalgesia according to individual factors such as age, gender, anxiety.

Key words: hyperalgesia, nociception, sensitization, opioid, nocebo, inflammation.

INTRODUCTION

Pain is certainly the most common symptom of trauma or diseases, which determines patients to seek medical assistance. Although it seems paradoxical given its unpleasant nature, pain is more and more considered a precious advantage that contributes to the integrity of the human body.

Essential to the pain sensation are the nociceptors – nerve cell endings/receptors – that send information to the central nervous system regarding the place and the nature of the injury, as well as input about the intensity of the pain response that is to follow. By means of the nervous system, a stimulus of nociceptive intensity will cause a series of events that will loop to the site of the injury to facilitate the immune response which will influence the severity and duration of pain.

Due to excessive mechanical, chemical or thermal stimulation, the nerve endings of myelinated and unmyelinated fibers become activated. This aspect is of ultimate importance for survival, since acute nociception is one of the most intriguing defense mechanisms of the human body. Like all the senses, acute nociception sends information about the internal or external environment, acting as an alarm system meant to provide vital input for survival. A metaphor for the lack of pain is the case of congenital insensitivity to pain that leads to a significantly lower life expectancy due to injuries/trauma of which there is no awareness.

At the other end, when nociception persists for a long time, it ceases to be a symptom and it becomes a pathology in itself. As long as pain sends a message about a threat/injury, it is a valuable protective tool. Still, when it ceases to serve this purpose and it continues long after the threat stopped, it not only becomes useless, but becomes pathological.

Chronic pain occurs due to the interaction of environmental and neurogenic factors. What starts as local injury, ischemia or inflammation determines chronic alterations of the environment, impacting the function of the sensory nerve fibers. One of these alterations that occur during chronic pain is a peripheral and central sensitization that leads to hyperalgesia and allodynia.

Definition

Hyperalgesia is increased pain sensitivity due to a decrease in threshold, an increase in suprathreshold response and spontaneous activity. It needs to be distinguished from *allodynia*, which is a nociceptive reaction triggered by nonnociceptive stimuli.

Hyperalgesia is characterised by a leftward shift in the stimulus-response function. The electrophysiological correlate for hyperalgesia is a sensitization of nociceptive neurons that can be peripheral, central or mixed.

Hyperalgesia – symptom or disease?

The function of the nociceptive system implies protective responses to acute painful stimuli. This system adapts to the increased vulnerability of the damaged tissue by lowering the nociceptive thresholds that usually happens in case of hyperalgesia. Thus, hyperalgesia per se is not negative, but a normal reaction of the nociceptive system meant to prevent further tissue damage.

Still, sometimes, the intensity, duration or location of pain may not accurately reflect the threat, which is the case with hyperalgesia prolonged longer than the action of the stimulus/damage. Also, hyperalgesia may reflect an alteration of the normal function of the peripheral or central nervous system. Thus, when hyperalgesia ceases to be protective and starts being maladaptive and the pain ends serving a purpose, the pain itself becomes a disease that needs to be addressed (16).

Molecular basis

In a matter of minutes or hours, at the site of the injury or inflammation a number of inflammatory mediators and products that result from tissue breakdown are released: proteases, histamine, serotonin, bradykinin, nitric oxide, prostanoids, cytokines, ATP. This "inflammatory soup" irritates or sensitizes the nerve endings that determines nociceptive signaling and neurogenic

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inflammation – which leads to the release of neuropeptides (14). Through the sympathetic efferents, catecholamines are released. This mixed inflammation, immunogenic and neurogenic, results in chronic pain (12).

Bradykinin induces pain and hyperalgesia that can be blocked by inhibitors of prostaglandin synthesis or by sympathectomy.

Prostaglandins play an important role in determining hyperalgesia. Still, PGE1, which plays the leading role in inducing hyperalgesia is not found in inflammatory exudates, while PGE2, which is found in exudates, has a significantly lower hyperalgic effect (11).

Recent studies suggest that in inducing hyperalgesia there are other cells that become involved, such as:

- *Polymorphonuclear leukocytes* (PMNLs). Leukotriene B4 is also involved in hyperalgesia, dependent to PMNLs. The incubation of LTB4 with PMNLs in vitro releases a hyperalgesic factor in the supernatant which is a product of the 15-lipooxygenase pathway of arachidonic acid metabolism (9, 10, 13).
- *The sympathetic postganglionic neurons.* A number of studies suggest that sympathetic post-ganglionic neuronal activity exacerbates hyperalgesic states in some patients with nerve injury, that suffer from causalgia or reflex sympathetic dystrophy (11).

The mechanisms and pathways that are involved in mediation of nociception are transcriptional regulated. A major role in the regulation of the inflammatory milieu is played by the *nuclear factor (NF)-κB* through the control of gene expression/suppression. An association has been established between (NF)-κB and pain/nociception, probably by regulation of the inflammatory loop and the biosynthesis of pro-inflammatory mediators. There is an indication that the effective inhibition of this factor, the associated upstream kinases and the pathways that regulate its nuclear translocation could be areas of interest for a new strategy meant to alleviate inflammation and inflammatory-related pain (6).

Types of hyperalgesia

There are three types of hyperalgesia:

1. Primary hyperalgesia is the hyperalgesia that occurs at the site of the injury due to sensitization of nociceptive nerve endings. Nowadays, there are ongoing studies that suggest that an equally important part is played by an altered processing in the central nervous system.

2. Secondary hyperalgesia occurs in an adjacent or remote area of the injury site. It is due only to the changes in the processing of sensory information in the CNS.

3. Reffered hyperalgesia can occur in the skin, remote from the site of injury.

PRIMARY HYPERALGESIA. There are a number of stimuli that can determine this type of hyperalgesia, such as heat, mechanical stimuli and inflammation.

Hyperalgesia to heat occurs after a skin lesion and after inflammation. Sensitization of primary afferent nociceptors to heat account for this type of hyperalgesia. An interesting fact is that a burn injury to the glabrous skin of the hand determines hyperalgesia to heat, but does not lead to sensitization of CMHs, while a burn injury to the hairy skin determines such a sensitization. This suggests that, according to the skin type, the response may differ. Hyperalgesia to heat can develop after inflammation. When inflammatory mediators are injected into a receptive field of nociceptive afferents, a marked sensitization to heat stimuli occurs.

Hyperalgesia to mechanical stimuli can also occur both after skin injuries and after inflammation by a number of mechanisms:

Nociceptor sensitization. After a burn injury, nociceptors are sensitized to heat stimuli, but the mechanical threshold of nociceptors is not lowered. Still, nciceptors are sensitized to mechanical stimuli in inflammation.

Spatial summation. If heat is applied at an edge of the receptive field of a nociceptor, the receptive field expands into the area of injury. Thus, a mechanical stimulus in the area of injury evokes a response in a greater number of nociceptive afferents, which will lead to spatial summation in the CNS that will determine an increased pain.

Central sensitization plays a major role in secondary hyperalgesia, but it seems to be involved to a certain level in primary hyperalgesia to mechanical stimuli.

Inflammatory mediators

The thermal hyperalgesia associated with induced inflammation or to specific inflammatory mediators (bradykinin, ATP, prostaglandin E2, proteases) depends strongly on the presence of TRPV1 ion channel. The activity of TRPV1 can be sensitized by all of these mediators via a number of mechanisms.

The most direct mechanism by which mediators sensitize TRPV1 is by generating co-agonists such as protons or arachidonic acid metabolites. Other mechanisms involve direct phosphorylation by serine/threonine kinases and the alteration of the phospholipids content of the membrane. Regardless the mechanism, one thing is certain: the nociceptors have developed many ways to respond to inflammation by becoming hypersensitive (2).

SECONDARY HYPERALGESIA occurs as a response to mechanical stimuli, but not to heat stimuli. There are two types of mechanical hyperalgesia: punctate (static) and stroking (dynamic).

Punctate hyperalgesia is hyperalgesia in response to sharp stimuli that normally generate pain (pin prick). Stroking hyperalgesia refers to pain to a light moving touch, but not blunt pressure.

An explanation for secondary hyperalgesia is that the sensitization that starts at the site of the injury then spreads to healthy tissue. Still, there is data suggesting that this spreading does not necessarily take place. For instance, burn injury to a half of the receptive field is not accompanied by sensitization in the

uninjured receptive field. Another interesting thing is that the area of flare is generally smaller than the area of hyperalgesia.

While both punctate and stroking hyperalgesia present central sensitization, there are some differences between them:

1. *Punctate hyperalgesia* occurs under the action of a punctate stimuli and, compared to the stroking hyperalgesia, the area involved is larger and the pain lasts longer.
2. *Stroking hyperalgesia (allodynia)* is triggered by a light stroking, lasts a shorter period of time and it involves a smaller area. (2)

Opioid-induced hyperalgesia

Opiates have been used for a long time in the treatment of chronic pain, such as the pain associated with cancer. Paradoxically, studies have shown that opiates used to treat pain can induce hyperalgesia.

Opioid-induced hyperalgesia is a condition that is translated clinically as hyperesthesia and allodynia. It occurs in some of the patients that receive chronic opioid therapy. Clinical reports that date back to the late 19th century documented that hyperalgesia goes hand in hand with opioid addiction. Later studies suggested that pain sensitivity is different in subjects with opioid addictions compared to those who are not addicted (1). Research also indicates that hyperalgesia occurs in the context of short-term and continuous therapy in which physical dependence and withdrawal did not play a role.

There are a number of mechanisms associated with this type of hyperalgesia. A possible mechanism concerns the NMDA-receptor. Changes mediated via this receptor in the dorsal horn occur after a long exposure to opioid drugs resulting in hyperalgesia. Glutamate-associated activation of NMDA-receptors generates spinal neuron sensitization. NMDA-receptor-cellular mechanisms mediate irreversible neurotoxic changes, including apoptosis.

Other studies have shown that hyperalgesia is closely linked to the increased excitatory peptide neurotransmitters such as CCK which activate spinal pathways that up-regulate spinal dynorphin. These substances have a pro-nociceptive action, causing central sensitization – hypersensitivity of the spinal cord to nociceptive inputs from the periphery (4).

Tolerance and opioid-induced hyperalgesia may be indistinguishable clinically. Pharmacological tolerance, opioid induced pain and the hyperalgesia of neuropath pain are closely linked. It is not clear which of the phenomena produces the clinical syndrome of opioid tolerance. Studies suggest that this abnormal sensitivity is closely related to the mechanism by which neuropath pain occurs. The pro-nociceptive actions of long-term opiate administration need a certain interaction with opiate receptors and probably are not due to the accumulation of excitatory metabolic products. The exact mechanism by which this event takes place remains unknown, but some recent studies suggest that plasticity initiated by opiate receptor interaction is involved (5). Other studies also emphasize the role played by the pain vulnerability that occurs as a result of events that took place earlier in life (15).

Contribution of individual factors

1. Gender. In many studies it has been suggested that women are more sensitive to pain than men are due to enhanced central pain processing and psychological factors. Still, a study performed by M.T. Jensen and K.L. Petersen shows that there are only small differences between men and women regarding nociception and neuronal sensitization (7).

2. Age. Because of ethical concerns, studies of pain on children are scarce. Still, the existing data suggests that, due to the immaturity of sensory processing in the spinal cord, children have lower thresholds for sensitization and excitation. In the neonate, hyperalgesia is explained partially by the substantial upregulation of neurotrophins as a response to the damaged tissue. This phenomenon is a few times amplified in neonates compared to adults. Also, because of the higher plasticity in the peripheral and central connections in neonates, early damage during infancy can lead to structural and functional alterations in pain pathways that persist in adulthood. Another age-related category that is at risk for hyperalgesia is the elderly. Since chronic pain is found more often in the elderly population – two times compared to pain in younger individuals – and its management is often poor, it is no surprise that hyperalgesia is found more frequent in the elderly, compared with younger adults.

3. Emotions. J. Kong et colab. performed a study on the neural mechanisms of hyperalgesic nocebo effect (also called "negative placebo effect"), since it is sometimes incriminated as a factor for a number of symptoms and adverse reactions in medical care. They showed, by means of functional MRI, that there is a link between the left frontal operculum and hippocampus and the pain network – insula, operculum, ACC and left S1/M1 (8). Thus, nocebo hyperalgesia may take place through an affective-cognitive pain pathway, with the hippocampus playing a major part.

4. Anxiety. It has been demonstrated that the negative expectations of a subject of pain increase when an inert substance is administered will facilitate pain transmission and induce hyperalgesia by means of anticipatory anxiety. Through an experimental study on ischemic pain induced to healthy volunteers, F. Benedetti et colab showed that verbally induced nocebo hyperalgesia is associated to a hyperactivity in the hypothalamic-pituitary-adrenal axis. Their findings emphasise that there is a close link between anxiety and the nocebo hyperalgesia, in which colecystokinergic systems play a major part (3). Studies have demonstrated that the analgesic placebo is mediated by endogenous opioids, while the hyperalgesic nocebo effect is due to the opposite activation of endogenous opioidergic and CCKergic systems.

CONCLUSION

Hyperalgesia, as a symptom, is part of the intricate defense mechanism of the human body. Still, a prolonged intense painful sensation that serves no purpose in the alarm system of the body becomes an entity that needs to be addressed and taken

seriously. From the three types of hyperalgesia, two are more important: primary hyperalgesia that occurs at the site of the injury due to the sensitization of nerve endings and secondary hyperalgesia that takes place due to alterations in the processing of sensory information in the central nervous system. In hyperalgesia, at a molecular basis, a major role is played by the inflammatory mediators that constitute the "inflammatory soup" that sensitize the nerve endings. Recently, it has been demonstrated that polymorphonuclear leukocytes are involved in the hyperalgesia through leukotriene B4, as well as the sympathetic postganglionic neurons. Opiod-induced hyperalgesia is a type of hyperalgesia that occur after administration of opioid medication or drugs. Changes mediated via the NMDA-receptor in the dorsal horn occur after a long exposure to opioid drugs resulting in hyperalgesia.

There are a number of individual factors that alter the experience of pain in certain groups of individuals: children (neonates included) and elderly are populations at risk for hyperalgesia. There is little difference between hyperalgesia in men and in women, and the few differences present are due to socio-psychological factors and to enhanced central pain processing.

The emotional life of the individual alters one's experience of pain. It has been shown that the left frontal operculum and the hipppocampus are linked to the pain pathways. Also, by means of anticipation and nocebo effect, subjects that have a higher level of anxiety will experience pain more dramatically.

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HIPERALGEZIA – PRIVIRE DE ANSAMBLU

REZUMAT

Articolul de fata este menit a fi o trecere in revista a ultimelor date din literatura referitoare la hiperalgezie, subliniind urmatoarele aspecte: hiperalgezia – simptom si/sau boala, bazele ei moleculare, tipuri, mecanisme, celule, mediatori si receptori implicati, hiperalgezia indusa de opioide, rolul jucat de inflamatie, implicarea hipocampului in senzatia dureroasa exagerata persistenta si experienta particulara a hiperalgeziei in functie de factori individuali precum varsta, sex, nivel de anxietate.

Ca simptom, hiperalgezia este parte integranta a mecanismului complex de aparare al organismului uman. Totusi, persistenta unei senzatii dureroase intense care nu slujeste niciunui scop in sistemul de alarma al organismului devine o entitate care trebuie solutionata si luata in serios. Dintre cele trei tipuri de hiperalgezie, doua sunt mai importante: hiperalgezia primara, care se produce la locul leziunii datorita sensibilizarii terminatiilor nervoase si hiperalgezia secundara, care se produce datorita procesarii modificate a informatiei senzoriale la nivelul sistemului nervos central. In hiperalgezie, la nivel molecular, un rol important il joaca mediatorii inflamatiei, care alcatuiesc „supa inflamatorie" ce sensibilizeaza exagerat terminatiile nervoase. Recent, s-a demonstrat ca leucocitele polimorfonucleare sunt implicate in hiperalgezie prin leucotriena B4. Hiperalgezia indusa de opioide este un tip particular de hiperalgezie care se produce dupa administrarea medicatiei opioide. Dupa expunerea prelungita la acest tip de medicatie sau dupa consumul de droguri opioide apar modificari la nivelul cornului posterior al maduvei ce implica receptorul NMDA, rezultatul fiind hiperalgezia.

Experienta hiperalgezica este resimtita diferit in functie de varsta si starea emotionala a subiectului, un rol important in amplificarea durerii revenind anxietatii.

Cuvinte cheie: hiperalgezie, nociceptie, sensibilizare, opioid, nocebo, inflamatie.

REVIEW

PARTICULARITIES OF ASSOCIATION BETWEEN DIABETES MELLITUS AND THYROID DISEASES

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ABSTRACT

Diabetes mellitus and thyroid diseases represent two endocrinopathies frequently in general population. Because insulin and thyroid hormones are implicated in cellular metabolism, excess or deficiency of one of these determinate functional disorders of another.
Autoimmune pathology appears by almost all endocrine glands and affects them in a variable incidence. Type 1 diabetes associated with certain endocrine disorders, pictures the autoimmune polyglandular syndromes.
The purpose of this review is to show epidemiological, ethiopatogenic, diagnostic and therapeutic particularities of association between diabetes mellitus and thyroid diseases.

1. EPIDEMIOLOGY

The thyroid disorders are common in the general population; their prevalence increases with age. The screening for thyroid disorders is indicated in certain high-risk groups as new born and elderly people (31). The hypothyroidism is the most common thyroid disorder in adults and older women. Its origin is usually autoimmune, presenting as atrophic primary hypothyroidism or Hashimoto thyroiditis. It may be also secondary to radioactive iodine treatment or thyroid surgery. In rare cases, occurs secondary hypothyroidism to hypothalamic or pituitary disease (31). By contrast, the hyperthyroidism is less common, with a ratio female/male 9/1. The Graves's disease is most common and usually affects young adults. The toxic multinodular goiter usually occurs in older people (31).

The patients with diabetes mellitus (DM) have an increased prevalence of thyroid disease compared with non-diabetic population (31). As patients with a specific organ autoimmune disease are at risk of developing other autoimmune diseases, and the thyroid disorders are more common in women, it is not surprising that 30% of the women with type 1 diabetes have thyroid disease. The rate of postpartum thyroiditis in diabetic patients is three times higher than in healthy women (31).

A number of studies also indicate a high prevalence of thyroid disorders in patients with type 2 diabetes, the hypothyroidism is the most common disorder encountered (6). The thyroid disorders into the general population have a prevalence of approximately 6.6%. In patients with diabetes, the thyroid disease prevalence is between 10.8% and 13.4%, consisting of: clinical hypothyroidism (36%), sub-clinical hypothyroidism (41%), hyperthyroidism (12%) and postpartum thyroiditis (11%) (31).

1.1. Epidemiology of type 1 diabetes associated with thyroid diseases

Type 1 diabetes is commonly associated with endocrine and systemic diseases with autoimmune etiology of type: Graves-Basedow disease, Hashimoto's thyroiditis, Addison's disease, celiac disease, pernicious anemia, myasthenia gravis, vitiligo, etc. (6). For people with type 1 diabetes, ≈1-100 patients will develop Graves' disease (3) and ≈ 1-20 patients are generally affected by hypothyroidism (8). The frequency of association between type 1 diabetes with hypothyroidism and hyperthyroidism varies from 3.2% to 4.6% and from 0.7% to 4% (22). Addison's disease is extremely rare in the general population, affecting ≈ 1-200 patients of population with type 1 diabetes (8). Celiac disease occurs in 1 from 20 patients with type 1 diabetes, pernicious anemia in 1 from 50 patients with type 1 diabetes (8). A particular association of type 1 diabetes with hypo- or hyperthyroidism is characteristic to polyglandular autoimmune syndrome. There are three polyglandular autoimmune syndromes.

Polyglandular autoimmune syndrome type I (PAS - I) is an autosomal recessive transmitted disease caused by a mutation in the short arm of chromosome 21, characterized by the triad: cutaneous-mucosal candidacies, hypoparathyroidism and Addison's disease.

The suffering begins in childhood; coetaneous-mucosal candidacies are the first manifestation, usually followed by hypoparathyroidism and Addison's disease (2, 20). Type 1 diabetes occurs in less than 4% of affected children, but increased to 12% in adults.

- *Frequency:* international, PAS-I is a very rare disorder. The largest number of patients was reported in Finland, where the prevalence was estimated at 1 to 25.000 subjects.
- *Mortality/Morbidity:* appear to be equivalent to individual components of the syndrome.
- *Predominance of sex*: the ratio F/M is 0.8 to 1.5/1; the numbers from 1998 indicate a ratio of 2.4/1
- *Age:* PAS - I occur in children aged 3-5 years or in early adolescence, but always appear until the third decade of life.

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Polyglandular autoimmune syndrome type II (PAS - II) (24, 4) is the most common immuno-endocrinopathies. It occurs in adulthood, especially affecting women. It is characterized by the appearance into the same person of two or more of the following diseases: Addison disease, Graves-Basedow disease, thyroiditis of autoimmune etiology, type I diabetes mellitus, primary hypogonadism, myasthenia gravis, and celiac disease. Most diseases are associated with the following HLA haplotypes: A1, B8, DR3 (DQA1 * 0501, DQB1 * 0201) and DR4 (DQA1 * 0301, DQB1 * 0302). Since most syndrome components have a long prodromal phase in which auto antibodies are present before the development of the disease, the current efforts are focused in order to determine if immunosuppressive or immunomodulatory medications can prevent or stop the destructive processes.

• *Frequency:* in U.S. about 14 to 20 per 1 million people are affected by this syndrome.

• *Mortality/Morbidity:* so far, the mortality and morbidity rate of PAS - II has not been estimated from the clinical point of view, but it is consider that they should be equal with the mortality and morbidity of individual components.

• *Predominance of sex*: the ratio F/M of PAS - II is 3 - 4/1.

• *Age:* PAS - II appears in the third and fourth decade of life.

Polyglandular autoimmune syndrome type III (PAS - III) (3) is a PAS II syndrome but without adrenocortical involvement. It includes a group of autoimmune disorders generally characterized by severe glandular deficiency. A quarter of the patients with a gland hypofunction also present other endocrine diseases. This syndrome is associated with the following diseases: celiac disease, hypogonadism, myasthenia gravis, sarcoidosis, Sjogren syndrome, rheumatoid arthritis, gastric cancer, malabsorption due by pancreatic exocrine deficiency, and can be classified into three subcategories:

- PAS III A - autoimmune thyroiditis with DM type1
- PAS III B - autoimmune thyroiditis with pernicious anemia
- PAS III C - autoimmune thyroiditis with vitiligo and/or alopecia and/or other autoimmune diseases

• *Frequency:* exact prevalence of PAS III is unknown.

• *Mortality/Morbidity:* are determined by individual components of the syndrome.

• *Predominance of sex*: PAS III is more common in women than in men.

• *Age:* PAS III is typically seen in middle-aged women, but can occur in people of any age.

DM is associated with endocrine disorders also in other polyglandular autoimmune syndromes (7), found with a much lower frequency.

Type B insulin resistance syndrome is caused by the presence of insulin antireceiver antibodies. A third of patients have associated autoimmune suffering as systemic lupus erythematosus, autoimmune thyroid disease. Although blood glucose levels are elevated secondary extreme insulin resistance, ketoacidosis is not characteristic. The patients may present spontaneous remissions and severe hypoglycemia (secondary insulin-like effects of insulin antireceiver antibodies, effects demonstrated in vitro).

POEMS syndrome includes DM, primary gonad failure, sensory and motor neuropathy, and bone lesions, hyperpigmentation.

DIDMOAD syndrome is an autosomal recessive disorder that includes diabetes insipidus, DM, optic atrophy, deafness. DM is usually the first manifestation in children.

Trisomy 21 (Down syndrome) is associated relatively frequent with the presence of type 1 diabetes and thyroiditis. This suggests that the chromosomal abnormalities influence autoimmune processes or that the susceptibility to develop autoimmune diseases can be associated with chromosomal disorders.

1.2. Epidemiology of type 2 diabetes associated with thyroid disease

In the case of association of IGT and IFG with thyroid disorders, they usually occur as a result of excess thyroid hormones. It was found that these associations are more common in women. In a U.S. study shows that at patients with thyroid disease, glucose intolerance was present in 38% of cases, and incidence of clinical diabetes was ≈ 2-3% (29,30). Other authors have obtained a prevalence of thyroid disorders in patients with type 2 diabetes of 2.5%, the most common endocrine disorder were subclinical hypothyroidism (4.1%) (23).

DM type 2 is frequently associated with hyperthyroidism (Graves-Basedow disease and toxic multinodular goiter). DM type 2 is present in 11% of patients with Graves-Basedow disease and in 5% of those with toxic multinodular goiter. The glucose intolerance is also frequently associated with hyperthyroidism; its prevalence is much higher compared to type 2 diabetes (72.3%). In the case of the toxic multinodular goiter incidence of impaired glucose tolerance was much higher, namely 85%, and in the case of the Graves-Basedow disease around 54% (21).

Also, in the case of euthyroid diffuse goiter, its prevalence in the people with DM varies between 3.4% and 17%; being discussed the treatment with sulfonylurea involvement in its appearance (11).

2. ETIOPATHOGENESIS

2.1. Pathogenic mechanisms involved into the association between type 1 diabetes with thyroid endocrine disorders

Autoimmune diseases are divided into "organ *specific*" (autoimmune disease of the pancreatic islet, brain, thyroid, parathyroid, anterior pituitary gland, adrenal cortex, skin, ovaries, and gastrointestinal tract) and "*organ unspecific"* (systemic lupus erythematosus and pernicious anemia).

Autoimmune response may be then developed, either from an organ-specific antigen, or to an antigen with ubiquitous distribution, with a wide distribution in the body.

Both at the humans and animals, there is the possibility of associating of many organ specific diseases. For example, DM type 1 may be associated with the chronic gastritis/pernicious anemia, vitiligo and thyroid disorders in PAS III, which indicates the likelihood of common immunological factors, genetic and environmental issues that arise in the pathogenesis of affected organs.

2.2. Genetic susceptibility

The genetic factors play an important role into individual susceptibility at autoimmune diseases, as demonstrated by numerous epidemiological studies. In some families there is an association of several autoimmune endocrine disorders, as between autoimmune endocrine disorders and other systemic immunopathies, which are more common in monozygotic twins than in dizygotic (16).

The most autoimmune diseases have a polygenic transmission, with certain genes susceptible to their occurrence. Appearance risk of an autoimmune disease differs from one gene to another (10). It seems likely that the location of several susceptible genes on the same *loci* predisposing the person to the concomitant development of many autoimmune diseases (12,26).

An important role in autoimmune disease susceptibility have HLA class I and II, some having protective role and other enabling role in appearance of autoimmune diseases (25).

In last time was incriminated the gene which encoding cytotoxic T lymphocyte associated with molecule 4 (CTLA-4). The polymorphism of this gene leads to decrease of the inhibitory signal mediated by CTLA-4, causing autoimmune diseases (1, 15, and 27).

Studies in Japan have shown the role of CTLA-4 polymorphism in susceptibility of children to present type 1 diabetes and autoimmune thyroid disease associated. Thus, the G allele frequency was 63.9% in patients with type 1 diabetes; at those with GAD + it was 72.9%. In patients with Graves-Basedow disease their frequency was around 78.6 % (19,14).

Association of this gene polymorphism with type 1 diabetes and autoimmune thyroid disease has been confirmed by other studies in Caucasian patients and in other ethnic groups (19,14).

The predisposition for developing autoimmune disease is likely caused interaction susceptible gene- protective gene, as of antigen ability to target organ damage (28,13).

It is widely recognized that at the same person may appear more organ-specific endocrinopathies. The main diseases characterized by the combination of several immune diseases such are called polyglandular autoimmune syndromes. There are three polyglandular autoimmune syndromes, PAS I and PAS II such as independent entities, while PAS III is considered a subset of PAS II. The main characteristics of these syndromes are described in **Table I.**

Table I. Polyglandular autoimmune syndromes - clinical and etiopathogenic characteristics (17)

	PAS I	PAS IIA (PAS II)	PAS IIB (PAS III)
Onset age	Children (peak < 10 years)	Adult (peak 30 years)	Adult (peak 30 years)
Genetic	Gene AIRE and the disease components influenced by HLA-DR/DQ genotype	HLA-DR3, -DR4	HLA - DR3, -DR4
Clinical symptoms			
Addison disease	++	++	-
Hypoparathyroidism	++	-	
Chronic coetaneous-mucosal candidacies	++	-	-
Graves disease	-	+	+
Hashimoto thyroiditis	+/-	++	++
Pernicious anemia	+ (early onset)	+	++ (late onset)
DM type 1	+/-	++	+
Gonad deficiency	++ (Female)	+/-	+/-
Vitiligo	+	+	+
Active chronic hepatitis	+	-	-
Alopecia	+ (universal)	-	-
Malabsorption	+	-	-
Celiac disease	+	+	+
Hypopituitarism	+	+/-	-
Myasthenia gravis	-	+/-	+/-

The triggers of autoimmune process

The triggers of autoimmune process are represented by exogenous environmental factors, which acting on genetically prone land. Their role is demonstrated by the study of populations genetically similar but which are living in different living conditions (18).

Infectious agents - are represented by involvement of some viruses' type: mumps virus, varicella-zoster virus, cytomegalovirus, reovirus type 3, and measles virus for type 1 diabetes and as rubella virus, hepatitis C virus in the case of the chronic autoimmune thyroiditis (17).

The non-infectious agents - are represented by iodine, sex, estrogen, pregnancy, certain medications and stress. The role of iodine in the development of immune thyroid disease is controversial. Some authors report an increased incidence of chronic Hashimoto's thyroiditis and of Graves- Basedow disease with increasing of iodine intake. Other authors found no significant correlation between iodine intake and occurrence of autoimmune thyroid diseases (17).

The mechanism by which iodine interacts with immune system is not known. It seems that iodine would have a modulator effect on thyroid growth factors (17). Regarding the gender of the subjects, at all autoimmune thyroid diseases has been a net predominance of females, determined primarily by genetic factors, and then by the hormone (17). Another factor involved could be hormonal. Thus it appears that estrogen affects immune system by effect exerted on B and T cells (5). Pregnancy can

induce immunosuppression, followed subsequently by immune system hyperactivity. This is demonstrated by the relatively frequent occurrence of Graves-Basedow disease or postpartum thyroiditis at 3-6 months after birth (17).

The drugs can impair immunity link. The main drugs involved in altering immune process are cytokines and interferon α. The cytokines may exacerbate a preexisting autoimmune disease. The interferon α determined in predisposed patients to autoimmune thyroid disease, occurrence of anti-thyroid antibodies, but does not induce the formation of new autoantibodies (17).

Acute stress, as chronic one determined immune suppresses through antigen-nonspecific mechanisms, possible, secondary of the cortisol and CRH's effects on immune system. Induced immune suppression may be followed by a hyperactivity of immune system (17).

The performance of autoimmune process

Autoimmune nature of autoimmune glandular syndrome component is supported by the following factors: the presence of chronic inflammatory infiltrate composed mostly of lymphocytes, association of components with expression of HLA class II genes (HLA-DR/DQ), as the presence of auto antibodies that response to specific antigens.

There are three main classes of antigens that cause specific auto antibodies (17):

- molecular surface receptors (e.g. TSH receptor involved in Graves disease and in atrophic thyroid disorders, insulin receptors in autoimmune insulin resistance syndrome)
- intracellular enzyme (thyroid peroxidase in Hashimoto thyroiditis and 21-hydroxylase in Addison's disease)
- proteins secreted as hormones produced by affected organs (thyroglobulin in Hashimoto's thyroiditis, insulin and proinsulin in type 1 diabetes)

Autoimmune thyroid diseases symptoms are caused by: action of thyroid auto antibodies, thyroid infiltration with lymphocytes (cell aggregation in tissues causing inflammation and cell destruction), action of cytokines released by T cells during autoimmune process, as of suppressor T lymphocytes (CD8 +) and natural killer (NK) decrease.

The thyroid autoantibodies exert its effects either directly on thyroid cells, or on the TSH receptor, blocking or stimulating the production of thyroid hormones. Autoimmune thyroid diseases may occur in the same patient at different intervals during life. The patients with Hashimoto thyroiditis may develop later Graves disease and the most patients with Graves' disease may present hypothyroidism symptoms before diagnosis. The predominant symptoms depend on existing blood auto antibodies (auto antibodies that stimulate TSH receptor causes symptoms of hyperthyroidism, and those who block it symptoms of hypothyroidism).

2.3. Pathogenic mechanisms involved in association between type 2 diabetes with thyroid endocrine disorders

The hypothyroidism reduces glucose absorption from the gastrointestinal tract. Peripheral glucose uptake is also delayed. At the same time, glycerol release from adipose tissue is slowed, and availability of amino acids and glycerol for gluconeogenesis is low.

Despite easily demonstrate of the carbohydrate metabolism abnormalities in hypothyroidism, the clinical manifestations of these abnormalities are rarely obvious. The patients with hypothyroidism may have a flattened glucose tolerance curve despite the concomitant reduction in peripheral glucose uptake. Although hypoglycemia is sometimes stated as a manifestation of hypothyroidism, is rarely an isolated sign of thyroid hormone deficiency, and the presence of hypoglycemia in a patient with hypothyroidism should suggest the presence of hypopituitarism. Occurrence of hypothyroidism in a patient with insulin dependent diabetes can lead to decrease exogenous insulin requirements (by decreasing insulin degradation rate and decreased appetite). These patients, however, may have ketoacidosis. Inverse, the correction of hypothyroidism in a patient insulin dependent usually requires insulin dose increase (9).

The hyperthyroidism is described as an increase in thyroid hormones, whereas the thyrotoxicosis refers to events resulting from increases in serum thyroid hormones. Graves' disease (known as Basedow's disease in Europe) is the most common cause of thyrotoxicosis, other causes including toxic adenoma, toxic nodular goiter and thyroiditis. The features of Graves' disease include: diffuse goiter, ophthalmopathy and symptoms of thyrotoxicosis, especially nervousness, sweating, weight loss and palpitations.

The Graves-Basedow disease may be associated with type 1 diabetes in polyglandular autoimmune syndrome type II, but thyrotoxicosis itself is diabetogenic. The glucose intolerance occurs in 50% of cases, but frank diabetes only in 2-3% of cases. When the hyperthyroidism occurs at insulin dependent patients, the diabetes control deteriorates and increase insulin requirements to about half of patients. These changes are reversible with treatment of hyperthyroidism.

Several factors are involved in glucose intolerance from thyrotoxicosis. Thus, intestinal absorption of glucose can be enhanced through accelerating gastric emptying and increase portal venous flow. The hepatic glucose production can be increased due to increased gluconeogenesis, glycogenolysis, and increased expression of glucose transporter protein GLUT-2 (the main transporter of glucose efflux at the plasma membrane of hepatocytes).

Increase of basal hepatic glucose production and its suppression impossibility by insulin is the main cause of hyperglycemia in subjects at who were given experimental thyroid hormones. It has also been described in liver and peripheral insulin resistance, which probably underlies exaggerated plasma insulin response to glucose administration, observed in some non-diabetic subjects with thyrotoxicosis. It was also revealed existence of β-cell dysfunction in some cases of thyrotoxicosis, by increasing concentrations of fasting and postprandial proinsulin, which may induce diabetes through a lower insulin response to

increased blood glucose (9).

In thyrotoxicosis, the patients may have impaired glucose tolerance but a DM induced only through thyroid hormones excess is unlikely. There is much more discussion regarding involvement of triiodothyronine (T_3) deficiency in etiology of type 2 diabetes. Numerous studies suggest that glucose homeostasis is the result of synergistic regulation by T_3 and insulin of a gene transcription involved in glucose and lipid metabolism. The triiodothyronine chronic deficiency should be responsible for the metabolic alteration from diabetes, hypertriglyceridemia, central obesity (7).

3. DIAGNOSIS

The diagnosis of thyroid dysfunction in patients with diabetes, based solely on clinical manifestations, can be difficult. An unsatisfactory glycemic control can cause hyperthyroidism similar symptoms, such as weight loss despite increased appetite, and fatigue. On other hand, the severe diabetic nephropathy can be omitted in patients with hypothyroidism because these patients may present edemas, fatigue, and pallor and weight gain (31).

To further complicate the diagnostic process, unbalance DM, with or without complications, can cause alterations of thyroid function tests, which can occur in patients without associated thyroid disease. The typical changes are represented by the decrease of T_3, determined by the decrease of T_4 conversion in T_3, decreased serum T_4, due to lower fixing on protein and lower serum TSH (31).

The most sensitive test to detect the thyroid dysfunction is the determination of TSH, which allows the diagnosis with certainty of the hypo-and hyperthyroidism. It also allows the diagnosis of subclinical thyroid dysfunction; characterized only through adjusting the level of TSH, with T_3 or T_4 in the normal range, the patients with this type of dysfunction is usually asymptomatic (31).

The thyroid dysfunction can produce significant biochemical and clinical changes. Thus, the subclinical hypothyroidism may increase LDLc and aggravating pre-existing dyslipidemia, increasing the risk of arteriosclerosis. On other hand, the subclinical hyperthyroidism may increase the risk of cardiac arrhythmias and can exacerbate a pre-existing coronary heart disease. Because the patients with diabetes are an increased risk for cardiovascular disease, the diagnosis and treatment of associated thyroid disease is important (31).

The presence on antiperoxidase thyroid antibodies (TPO) has a role in predicting occurrence of autoimmune thyroid disease, particularly of the hypothyroidism. Autoantibodies may occur long before obvious clinical manifestations of autoimmune thyroid disease and predict the appearance of clinical disease. The patients with polyglandular autoimmune syndrome may present autoantibodies against the same antigen. At the patients with present AB antiTPO must make their annual screening for early detection of thyroid dysfunction and establishment of an appropriate treatment (17).

Also, at all the patients with diabetes should be performed a screen for adrenal autoantibodies, celiac disease, steroid, gastric parietal and thyroid even at diagnosis. Also, at the patients who have one of the diseases listed above must be confirmed existence of diabetes by determining the ICA, GAD, IA_2, in absence of characteristic clinical manifestations (17).

At the family members of patients with diabetes must also determine autoantibodies, especially if the diabetic patient has thyroid autoantibodies. Identifying of the autoantibodies presence should be followed by hormonal assessment and the treatment of thyroid disease (17).

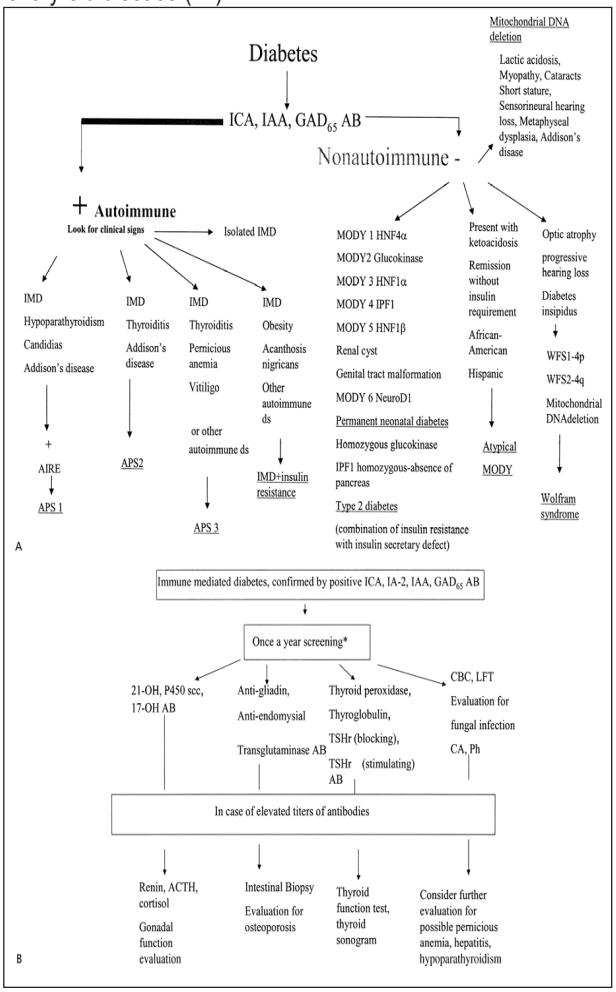

Fig.1. Diagnostic workup for the association between immune-mediated (type 1) diabetes mellitus (IMD) and other autoimmune diseases (A. Kukreja and N. MacLaren - 2005) (17)

4. THERAPEUTIC PARTICULARITIES

The clinical hypothyroidism should be treated through hormone replacement therapy. The most used replacement therapy is with L-thyroxin. Usual dose is 1.6 μg L-thyroxin/kg. Often, in the cases of mild thyroid failure, initial dose may be lower and it is adjusted according to TSH value at every 23 months. With the normalization of TSH levels and titrating L-

thyroxin, the TSH should be monitored annually. In the case of aggravation thyroid failure is necessary to increase the dose of thyroxin. The L-thyroxin therapy in subjects with diabetes may exacerbate a pre-existing coronary artery disease, by increasing myocardial contractility and heart rate. Therefore, it is better to start with a low dose, around 25 μg/day, followed by a slow growth, monthly with 25 μg /day, monitoring of clinical status and TSH level (31).

T**he subclinical hypothyroidism** should be treated in patients who present increased LDLc, which is worsened by hypothyroidism, which present the AB antiTPO detectable in serum (which means a possible transition to clinical hypothyroidism) and in patients with clinical manifestations (31).

The hyperthyroidism can cause adverse effects such as worsening of glycemic control and coronary artery disease. For this reason, it is indicated definitive treatment with iodine radiotherapy, whenever is possible. There are no contraindications for antithyroid medication at the diabetic patients, but the Graves-Basedow disease long-term remission is less than 40%. The patients with toxic multinodular goiter or with autonomous thyroid nodules should be treated definitive with iodine radiotherapy or surgery (31).

The key of the successful management of the patients with autoimmune endocrinopathies is to identify and treat autoimmunity before it cause significant morbidity and mortality. The treatment of organ insufficiencies is identical whether they occur isolate or in the polyglandular autoimmune syndrome. The replacement therapy remains the cornerstone of the clinical management (17).

Educating patients regarding the nature of the disease is often critical for early recognition of associated autoimmune disorders, and as in any chronic disease, the psychosocial support should be evaluated individually (17). The genetic counseling is also justified at affected family members who required carrying out specific tests (28).

Emergency identification should be performed in all patients with polyglandular autoimmune syndrome because use of high doses of corticosteroids in acute stress usually prevent adrenal crisis in the patients with Addison's disease, in those with adrenal auto antibodies and increased risk of adrenal insufficiency. Some authors believe that exogenous supplement with glucocorticoids administered during acute stress is indicated also at asymptomatic individuals with biochemical changes of asymptomatic adrenocortical disease (17). From all endocrine components of the polyglandular autoimmune syndrome, only diabetes has not a favorable prognosis after hormonal replacement therapy and appropriate monitoring (17).

Due to long-term vascular complications, the diabetes is a candidate for aggressive therapeutic interventions. For example, the studies results which have used cyclosporine A and azathioprine for the newly diagnosed diabetes treatment have demonstrated some metabolic benefits, but not for long term, even with continued immunotherapy. As a result, any immunomodulating therapy should be considered experimental and should be prescribed only in the controlled trials. Consequence of identification of new auto antigens and better knowledge of the pathogenesis of the disease, selective therapies may be introduced that does not cause generalized immunosuppression (17).

Also, in the near future is predicting curative organ transplantation. The transplantation of pancreas or pancreatic islands has been used in patients with diabetes and chronic kidney disease. Adrenal gland transplantation was successfully performed in experimental rodents and humans (17).

In conclusion, thyroid damage is common in the patients with diabetes and may cause significant metabolic changes. Therefore, thyroid function regular screening is indicated in all the patients with diabetes for establishment of early treatment of subclinical thyroid dysfunction (17).

The best screening test is the TSH determination. At the patients with type 1 diabetes is useful to determine the presence of anti-TPO antibodies. If they are present, it is indicate annual determination of TSH. In their absence, the TSH determination should be performed at every 2-3 years (17).

At the patients with type 2 diabetes, the TSH determination is performed at diabetes diagnosis and then at a minimum 5 years interval (17).

In the case of the patients with thyroid disease is obligatory annual determination of glycemia. In the case of elevated blood glucose levels, according to the values obtained, it is necessary employment in one of the category: type 1 diabetes, type 2 diabetes or IGT (17).

If the thyroid disease is on autoimmune nature it is better to determine the diabetes specific antibodies for identify the presence of the type 1 diabetes (17).

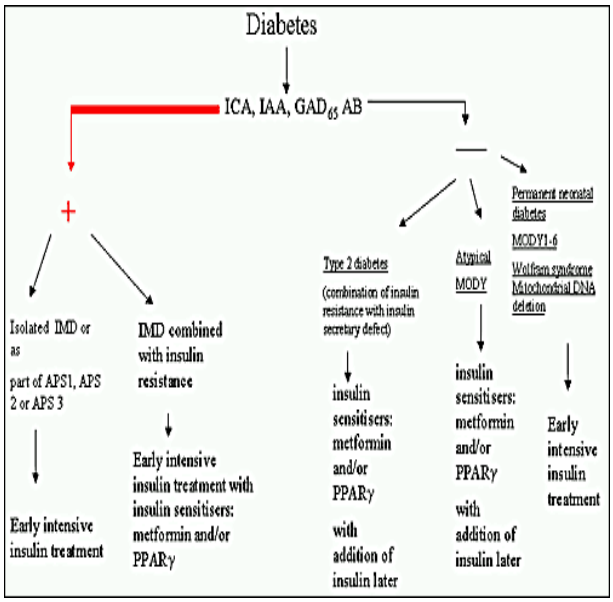

Fig.2. Algorithm of treatment of different forms of diabetes (by a. Kukreja and n. Maclaren - 2005) (28)

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PARTICULARITATI ALE ASOCIERII DINTRE DIABETUL ZAHARAT SI AFECTIUNILE TIROIDIENE

REZUMAT

Diabetul zaharat şi afecţiunile tiroidiene reprezintă două endocrinopatii frecvent întâlnite în populaţia generală. Deoarece insulina şi hormonii tiroidieni sunt implicaţi în metabolismul celular, excesul sau deficitul unuia dintre aceştia determină tulburări funcţionale ale celuilalt.

Procesele autoimune interesează aproape toate glandele endocrine, care sunt afectate într-o incidenţă variabilă. Diabetul zaharat tip 1 asociat cu anumite endocrinopatii realizează tabloul poliendocrinopatiilor autoimune.

Scopul acestei treceri în revistă este de a arăta particularităţile epidemiologice, etiopatogenice, diagnostice şi terapeutice ale asocierii dintre diabetul zaharat şi afecţiunile tiroidiene.

THE EFFECTS OF HYPOXIA ON THE PROLIFERATION AND APOPTOSIS OF HUVEC CULTURES IN VITRO

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ABSTRACT

Objective: Endothelial cells that line blood vessels are anatomically positioned at the interface of the blood and tissue exchange, and thus, endothelial cells are especially influenced by hypoxemia. The impact of hypoxia on endothelial cells is complex and includes changes in metabolism, gene expression, and induction of specific cell surface proteins. With this aim, we investigated the effects of anoxia on the proliferation rates and apoptosis of HUVEC cell cultures.

Methods: *Cell source*: Commercial human umbilical vein endothelial cells (HUVEC) were bought from European Collection of Cell Cultures (ECACC,Porton Down,Salisbury, UK) and multiplied in standard medium: RPMI, supplemented with 10% fetal calf serum (FCS), gentamicin 50µg/ml, amphotericin 100µg/ml (Biochrom AG, Germany). Cell cultures in the 23rd to 28th passages were used. *Hypoxia exposure* of the HUVEC cells. Growth medium was replaced with fresh medium equilibrated with hypoxic gas mixture, and cells were placed in the hypoxic chamber () to a gas mixture of 5%CO_2, 95% N_2, humidified with water vapors, at 37°C for 24h, respective 72h. *Cytotoxicity assay* was done using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, USA). *Apoptosis induction assessment* was done using Annexin V-FITC staining (BD Pharmingen, USA).

Results: Hypoxia exposure diminished the proliferation rate, in a time dependent manner (p hypoxia 24h versus hypoxia 72h, p= 5,544744E-2). The inhibition of proliferation was significant when compared to the controls (p hypoxia 24h versus control 24h, p= 9,3E-4; respective p hypoxia 72h versus control 72h, p= 3.08209E-12). The effect of hypoxia on cell proliferation at 24h was not cytotoxic, with a diminished proliferation rate with only 5.7%, however 72h of hypoxia exposure greatly diminished the proliferation, having a citotoxic effect, with a 27% of proliferation rate reduction. 72h of hypoxia exposure greatly diminished the cell viability, thus the cells lost the surface adherence and were washed away during the annexin V staining procedure. This explaines why the % of apoptotic cells is diminished at 72h of exposure when compared to 24h of hypoxia.

Conclusions: Hypoxia and reoxygenation of the HUVEC cultures were stressful events that significantly decreased the cells proliferation rate, viability, induced morphological changes in the exposed cultures, and increased apoptosis.

Key words: hypoxia, HUVEC cell culture, apoptosis, proliferation.

INTRODUCTION

Tissue hypoxia frequently accompanies a variety of vascular diseases, including thrombosis, atherosclerosis and ischemia/reperfusion injury. Endothelial cells that line blood vessels are anatomically positioned at the interface of the blood and tissue exchange, and thus, endothelial cells are especially influenced by hypoxemia. Because of this critical location, endothelia serve as a buffering monolayer during episodes of decreased oxygen tension. Thus, the ability of these cells to tolerate and respond to hypoxia is critical to their survival. The impact of hypoxia on endothelial cells is complex and includes changes in metabolism, gene expression, and induction of specific cell surface proteins (1). Moreover, hypoxia often occurs in conjunction with, or as a direct result of, other inflammatory processes. Under such conditions, the endothelium is bathed in a myriad of soluble factors, including cytokines, bioactive lipids and bacterial endotoxines (2).

Experimental and clinical studies indicate that hypoxia plays a fundamental role in the pathogenesis of a variety of diseases, including cardiovascular, hematological and pulmonary disorders and cancer (3, 4, 5)

MATERIAL AND METHODS

Cell source: commercial human umbilical vein endothelial cells (HUVEC) were bought from European Collection of Cell Cultures (ECACC, Porton Down, and Salisbury, UK) and multiplied in standard medium: RPMI, supplemented with 10% fetal calf serum (FCS), gentamicin 50µg/ml, amphotericin 100µg/ml (Biochrom AG, Germany). Cell cultures in the 23rd to 28th passages were used.

Hypoxia exposure of the HUVEC cells. Growth medium was replaced with fresh medium equilibrated with hypoxic gas mixture, and cells were placed in the hypoxic chamber to a

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gas mixture of 5%CO_2, 95% N_2, humidified with water vapors, at 37°C for 24h, respective 72h. The gases composition was measured using an gas cromatograph (Shimazu, Japan) and the gas flow was continuously monitored using a flowmeter. Cells were then allowed to reoxygenate for 2h in standard cell culture conditions.

Cytotoxicity assay was done using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, USA). All the experiments were conducted in triplicate. The cells seeded at a density of 10^4/well in ELISA 96 wells micro titration flat bottom plaques (TPP, Switzerland), were settled for 24h at 37ºC and 5% CO_2 in a humidified atmosphere, then incubated in hypoxic conditions for 24, respective 72h. Duplicate cultures, maintained in standard growth conditions were used as controls. HUVEC cultures were then exposed to 20µl of MTS/PMS mixture in 100µl fresh medium/well, for 2h, then absorbance at 490nm was measured using an ELISA plate reader. (Tecan, Austria).

Apoptosis induction assessment was done using Annexin V-FITC staining (BD Pharmingen, USA). Cells seeded at a density of 10^4/cm^2 were settled for 24h at 37ºC and 5% CO_2 in a humidified atmosphere, then incubated in hypoxic conditions for 24h, respective 72h. Duplicate cultures, maintained in standard growth conditions were used as controls. Cells were then washed and stained with Annexin V-FITC, according to the manufacturer's instructions, then observed under fluorescent microscope (Olympus BX41 equipped with an Olympus E330 camera driven by software Olympus Master version 1.41EX) (6,7). Apoptotic cells were scored by eye, at a magnification of 40× and at least 200 nuclei per condition were examined. Pictures were taken (8,9).

RESULTS

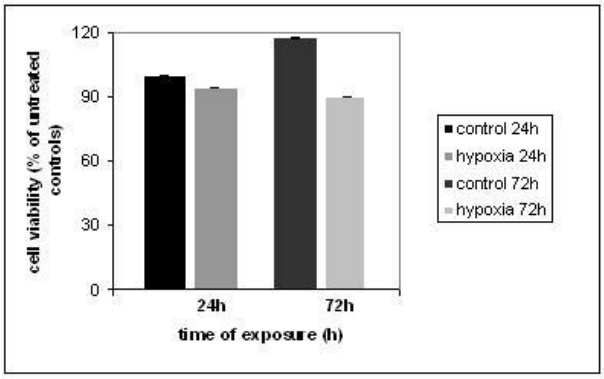

Fig.1. HUVEC proliferation after exposure of the cultures to hypoxia for different periods of time

As seen in Figure 1. The cultures significantly proliferated with time elapsed (p control 24h versus control 72h, p= 3.*09398E-12*). Hypoxia exposure diminished the proliferation rate, in a time dependent manner (p hypoxia 24h versus hypoxia 72h, p= 5.544744E-2). The inhibition of proliferation was significant when compared to the controls (p hypoxia 24h versus control 24h, p= 9.3E-4; respective p hypoxia 72h versus control 72h, p= 3.08209E-12). The effect of hypoxia on cell proliferation at 24h was not cytotoxic, with a diminished proliferation rate with only 5.7%, however 72h of hypoxia exposure greatly diminished the proliferation, having a cytotoxic effect; with a 27% of proliferation rate reduction.

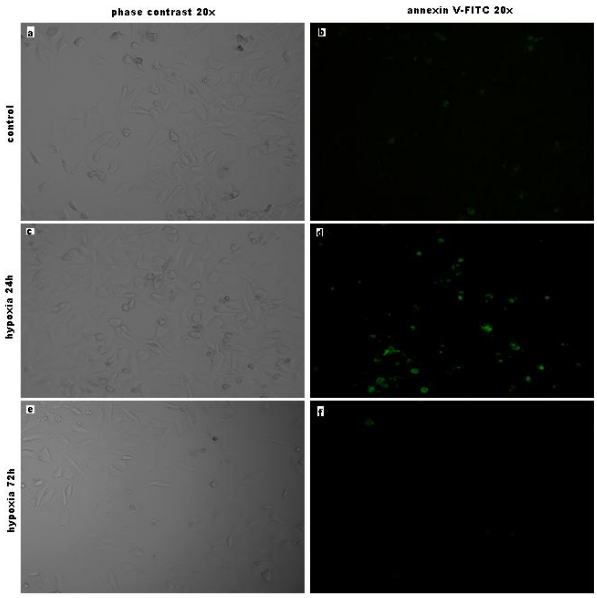

Fig.2. Apoptosis induction in the HUVEC cultures following exposure to hypoxia for 24h respective 72h

Comparative images of HUVEC cultures between the phase contrast and green fluorescence microscopy after exposure to hypoxia; a, b-controls,; c,d- hypoxia exposure for 24h; e,f- hypoxia exposure for 72h; apoptotic cells are shown in fluorescent green; the cells undergoing apoptosis in the controls (b)are in a limited number, compared to the images from 24h hypoxia(d), while the total number of cells is comparative (a, c); after 72h of hypoxia exposure, the total cell number is deeply decreased (e), with a diminished number of apoptotic cells(f); apoptotic bodies are shown by arrows, original magnification 20x, photos taken through an fluorescent Olympus BX41 microscope.

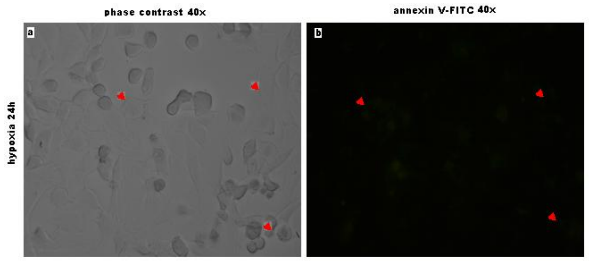

Fig. 3. Detailed images of the HUVEC cultures exposed to hypoxia for 24h, which underwent apoptosis

Comparative images of HUVEC cultures between the phase contrast and green fluorescence microscopy after exposure to

hypoxia; a-phase contrast, b-green fluorescence; arrows indicate the presence of apoptotic bodies; the culture exposed to hypoxia showed pleiomorphism with normal shaped cells; round cells that exhibited retraction of cytoplasmic elongations and loss of surface adherence, as well as flattened, bigger cells which showed apoptotic bodies.

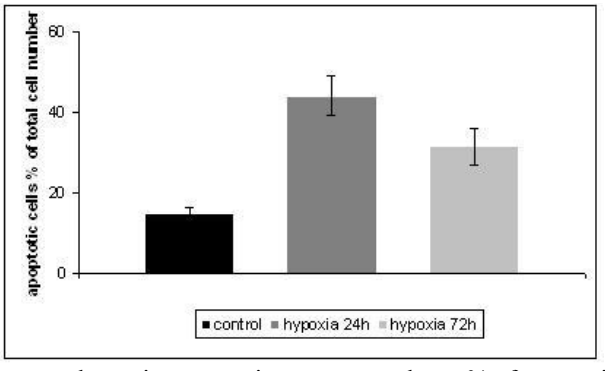

Fig. 4. Apoptotic cells after exposure to hypoxia, apoptosos was assessed as a % of apoptotoc cells compared to the total cell number

72h of hypoxia exposure greatly diminished the cell viability, thus the cells lost the surface adherence and were washed away during the annexin V staining procedure. This explaines why the % of apoptotic cells is diminished at 72h of exposure when compared to 24h of hypoxia.

DISCUSSIONS

Our cultures were exposed to anoxia conditions for 24h, respective 72 h, and then allowed to re oxygenate for 2 h in normal cell growth conditions. These experimental conditions are different compared to the studies in literature (10, 11, 12, 13), since other authors used a very low amount of oxygen atmosphere surrounding the cells. By these conditions we tried to create a very stressful environment for the cell cultures, comparable to that encountered in areas that suffer a dramatic decrease of blood supply, such as infarctation or thrombosis. Moreover, we were interested in finding the effects of re oxygenation on the survival and proliferation capabilities of our cultures since they are directly involved in recuperation of these events.

As expected, hypoxia had an inhibitory, time dependent effect on HUVEC cells proliferation. This effect was due partly to the re oxygenation, because we allowed the cells to recover from hypoxia for 2h in the humidified incubator at 37C, after which, the MTS test was performed. As expected the cell proliferation in the controls was maintained in cell growth conditions. This is a positive indicator for the cultures ability to multiply in normal conditions and underlines the importance of lack of oxygen in the cell proliferation inhibition.

This is supported by other studies which showed that HUVEC's exposed to hypoxia experienced decreased proliferation, reduced S-phase ratio, and increases in apoptosis under the condition of hypoxia (10).

In a study done by Bednarek et al., early passage HUVEC's exposed to hypoxia for 12, 24 and 36 h, showed decreased proliferation rates assessed through Brdu method when compared to normoxia treated cells. Significant differences in proliferation rates were found only between 24 hours hypoxic group and the control group (11).

Exposure to hypoxia for 24 h, respective for 72 h of the HUVEC's allowed viability of 90% of the culture compared to the initial number of cells. This shows that the culture has the ability to recover from stress, after relatively long periods of hypoxia exposure.

The HUVEC cultures showed a peak of apoptosis induction after 24h of hypoxia induction (figure 2 c,d), with a decreased Annexin V-FITC staining in the cultures exposed to hypoxia for 72h (figure2 e,f). Annexin V staining reveals the early apoptosis, thus if the cells are already experiencing the late stages of the activation of cell death, the staining will show false negative results. Moreover, this can be partially explained by increased number of cells which lost their adherence to the plaque surface and were discarded with the medium after 72h of hypoxia exposure (figure 2. e). This is showed by the diminished number of cells in the contrast phase image (figure 2. e).

HUVEC cultured in 1% oxygen followed by reoxygenation activated the classical complement pathway resulting in C3 deposition. There was an increase in apoptotic cells in these cultures that was demonstrated by binding of fluorescein isothiocyanate-Annexin V and staining for hypodiploid nuclei (12).

This is consistent with the data reported by others. HUVEC plated onto fibrin gels in low-serum culture medium underwent rapid and profound morphological changes within 12 to 48 hours of hypoxia; their characteristic cobblestone organisation was transformed into a network of cord-like or tube-like structures. It is likely that hypoxia induces modification in the factors that integrate matrix information and cytoskeletal organisation in order to contract fibrin (13).

CONCLUSIONS

Hypoxia and reoxygenation of the HUVEC cultures were stressful events that significantly decreased the cells proliferation rate, viability, induced morphological changes in the exposed cultures, and increased apoptosis.

Even if the viability of the cells was altered, there remained a relatively high amount of viable cells that could be able to support proliferation of the culture, thus recovering, when normal conditions are reestablished.

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EFECTELE HIPOXIEI ASUPRA PROLIFERARII SI APOPTOZEI CULTURILOR HUVEC IN VITRO

REZUMAT

Obiectiv: Celulele endoteliale sunt pozitionate la interfata dintre sange si tesuturi, astfel, fiind influentate in mod special de hipoxemie. Impactul hipoxiei asupra celulelor endoteliale e complex si include modificari in metabolismul celulelor, expresia genica si a proteinelor de suprafata. Cu acest scop am investigat efectele anoxiei asupra proliferarii si apoptozei culturilor de celule HUVEC.
Metoda: am utilizat celule endoteliale umane-linie comerciala (HUVEC – human ombilical endothelial cells) aflate in pasaje 22-28. Anoxia a fost realizata prin incubarea celulelor cu un amestec gazos care contine 5%CO_2, 95% N_2, in atmosfera umeda, la 37°C. Evaluarea proliferării celulelor s-a făcut cu Cell Titer Aqueous 96R (Promega, Madison, SUA). Evaluarea apoptozei celulare s-a facut prin coloratie cu Annexina V (BD Pharmingen).
Rezultate:expunerea la hipoxie diminueaza rata de proliferare, intr-o maniera dependenta de timp (p hipoxie 24h versus hipoxie 72h, p= 5,544744E-2). Inhibitia proliferarii a fost semnificativa statistic la celulele mentinute in mediu anoxic comparativ cu celulele expuse la normoxie (p hipoxie 24h versus control 24h, p= 9,3E-4; respectiv p hipoxie 72h versus control 72h, p= 3.08209E-12). Efectul hipoxiei asupra ratei de proliferare la 24h nu a fost citotoxic, cu o scadere a ratei de proliferare de doar 5,7%, totusi expunerea timp de 72h la hipoxie a avut efect citotoxic, cu o scadere a ratei de proliferare de 27%.
Expunerea timp de 72h la anoxie a redus dramatic viabilitatea celulara, astfel incat celulele si-au pierdut aderenta si au fost spalate in cursul procedurii de fixare a anexinei. Aceasta explica scaderea procentului de celule apoptotice la 72h comparative cu 24h.
Concluzii: hipoxia si reoxigenarea culturilor de celule HUVEC sunt evenimente stresante care reduc semnificativ rata de proliferare celulara, viabilitatea, induc schimbari morfologice si amplifica apoptoza.
Cuvinte cheie: hipoxie, culturi de celule HUVEC, apoptoza, proliferare.

EXPRESSION AND SIGNIFICANCE OF KI-67 ANTIGEN IN PULMONARY CANCER. RELATIONSHIP BETWEEN KI-67 AND OTHER PROGNOSTIC FACTORS

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ABSTRACT

We assessed the proliferative activity in 62 primary pulmonary carcinomas by: determining the percent of Ki-67 immunoreactive cells (stained with MIB-1 antibody), comparing Ki-67 score with the fraction of PCNA positive cells (marked with PC10 antibody) and by evaluating nucleolar organizer regions (NORs) using the histochemical method of staining with coloidal silver and comparing Ki-67 score and the fraction of PCNA positive cells with the mean number of AgNOR per nucleus.

We followed the relationship between Ki-67 growth rate, p53 immunoreactivity and the level of epidermal growth factor receptor (EGFR), by staining sections with anti-p53 (DO-7) and anti-EGFR (31G7); tissue sections fixed in formalin and embedded in paraffin were immunohistochemically stained using the Avidin Biotin Complex (ABC) method.

The results obtained show: (1) a Ki-67 positivity rate significantly correlated with histological grade (G3) of the tumor ($p<0.001$; ES) and high cellularity (C3); (2) a statistically insignificant relationship between Ki-67 expression and lymph node involvement ($p=0.924448$; NS), invasion of pleura and/or thoracic wall ($p=0.825267$; NS), local relapses ($p=0.581481$; NS) and distant metastases ($p=0.646270$; NS); (3) prevalence of p53 positive expression in poorly differentiated pulmonary tumors, with high proliferation rate ($p<0.001$; ES); (4) an increased level of EGFR expression in highly proliferative tumors, diagnosed in an advanced stage of the disease, which reflects the invasive character and high potential for metastases of Ki-67+ EGFR+ pulmonary carcinomas; (5) a weak correlation between mean of AgNOR per nucleus and IHC markers of proliferative activity.

The Ki-67 and PCNA positivity rate, correlated with cellularity of the tumor, together with clinical stage and histological differentiation, offer useful information in anticipating the evolution and prognosis of pulmonary cancer.

Key words: lung carcinoma**,** Ki-67, PCNA, immunohistochemistry.

INTRODUCTION

Current methods for estimating tumor malignancy and prognosis of patients with pulmonary cancer also refer to the proliferation rate of tumor cells. Proliferative activity of a tumor can be investigated through various methods, among which the immunohistochemical (IHC) ones proved to be a simple and credible tool in assessing cell proliferation (Veronese SM, 1993; Yoshida YA, 1994).

Ki-67 antigen is a marker of cell cycle and proliferation, commonly used to estimate proliferation coefficient of a cell population (Seigneurin DG, 1991). It indicates growth fraction and the number of cells that can be found in the active division (Yoshida YA, 1994). Ki-67 is a protein doublet with molecular weight of 345 and 395kDa (Seigneurin DG, 1991; Rowlands D, 1994), respectively, being part of the chromosome coating layer.

In most malignant tumors, the percentage of Ki-67 positive cells can be correlated with aggressiveness or tumor progression parameters; this gives its practical interest in tumor histopathology, although its biochemical nature and function are still little known (Seigneurin DG, 1991).

Ki-67 is a nuclear antigen expressed in all phases of cell cycle, except G0 (Jasani B, 1993); it is detected during phases G1, S, G2 and M of the cell cycle and is not evident in transit cells from pausing state to G0 (Seigneurin DG, 1991; Martin B, 2004).

Because in pulmonary cancer data referring to the prognostic value of Ki-67 proliferation index are little and the relationship between Ki-67 positive rate and prognosis is still unclear, this paper wants to analyze by means of IHC the proliferative activity on a group of 62 pulmonary carcinomas; we present aspects of correlating the Ki-67 score with histological subtype of the tumor, cellularity and differentiation degree, as well as with other IHC markers that assess proliferation activity.

MATERIAL AND METHODS

The studied material represented by pulmonary biopsies obtained by bronchoscopy and surgical resection pieces, was made up of 62 cases from patients with lesions that were clinically diagnosed as pulmonary displasias or cancer. The entire histological material was fixed in 10% formalin and included in paraffin.

The common morphologic investigation was made on sections stained with hematoxylin-eosin and tricrom Gomori, which helped in classifying and establishing the differentiation degree of the lesions.

We assessed the proliferative potential of the studied lesions by identifying the nuclear antigen Ki-67, applying the

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IHC staining method of the Avidin-Biotin Complex (ABC). After pretreatment by boiling for 60 minutes at 80-90°C in citrate buffer pH6, sections were incubated over night at 4°C with MIB-1 primary antibody (MIB-1 – Dianova, Hamburg, Germany) diluted 1/800. Visualization with AEC (3-amino, 9-etilcarbazole), followed by a light counterstain of the nuclei with hematoxylin, allowed highlighting of Ki-67 in the form of a final reaction product, red and with nuclear localization.

In what concerns the proliferative activity, on the same number of cases we tested a variety of antibodies with specificity for p53 protein (DO-7, DAKO, Glostrup, Denmark – diluted 1/500) and for proliferation cell nuclear antigen (PCNA) (PC10 – DAKO, Glostrup, Denmark – diluted 1/800) and we identified the nucleolar organizer regions (NORs) applying the histochemical method of staining with silver.

In 15 pulmonary carcinomas – other than those with small cells (NSCLC) we also examined the expression of epidermal growth factor receptor (EGFR).

Quantification of Ki-67 immunoreaction

The IHC marking of the sections with MIB-1 antibody identified a staining pattern limited to the nucleus. We assessed Ki-67 immunoreactivity by evaluating tumor cells from evenly colored areas, avoiding necrosis and hemorrhagic foci, as well as areas of inflammation. We considered as positive any detectable nuclear staining (dotted or diffuse).

For the quantification of Ki-67 immunoreaction we determined the percent of reactive cells. Given the heterogeneity in staining distribution and intensity, to avoid subjectivity and difficulties in assessing the results, we used a semi-quantitative score (corresponding to staining intensity and the percent of reactive nuclei), that allowed the classification of Ki-67 immunoreactivity in three score groups:

- Low score (< 25% of tumor cells stained),
- Intermediate score (26-75% of tumor cells stained),
- High score (76-100% of tumor cells stained).

The marking of sections using PC10 antibody identified a staining pattern confined to the nucleus.

Silver staining of sections allowed visualization AgNOR like some black or dark brown "drops", distinct or grouped, with nucleolar or/and nuclear localization. For the numerical assessment of NOR we counted 100 consecutive nuclei, calculating the mean AgNOR per nucleus for each case, and for the measurement of maximum diameter of NOR we used the ocular micrometer.

We interpreted as p53 positive reaction the distinct, homogenous or granular nuclear staining.

Positive EGFR immunoreaction was indicated by the dark brown staining of cell membrane and cytoplasm.

Statistical analysis was performed using the EpiInfo 6.04, EpiInfo 3.2.2 and OpenEpi 2.3.1 programs and consisted in computing the frequency counts and percentages for the qualitative variables. The comparision of the percentages and the means was performed using the chi-square teste and unpaired t -test.

RESULTS

In the majority of cases examined we observed heterogeneity of immunoreactions (concerning intensity of staining and nuclear marking) (Fig. 1) and a variety of marking types: isolated nucleolar marking (delicate, dotted), nucleolar and nucleocytoplasmatic marking, intense and diffuse marking of the entire nucleus and intense marking of the nucleus and cytoplasm.

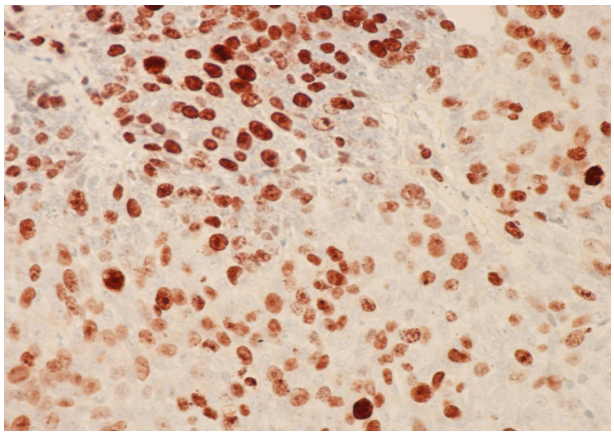

Fig. 1. Ki-67 immunoreaction with intense and granular nuclear pattern of staining. ABC method, viz. AEC x 400.

We remarked the absence of Ki-67 immunoreaction on the negative control section and in normal pulmonary tissue. In normal surface pulmonary epithelium and in hyperplastic basal cells we noted the presence of rare, dispersed positive nuclei.

While in the squamous metaplastic pulmonary epithelium without atypia, MIB-1 immunostaining marked isolated reactive nuclei, in atypical squamous or dysplastic metaplasia and in intraepithelial carcinomas associated with invasive squamous carcinomas, we remarked the presence of a large number of Ki-67 positive nuclei, with anarchic distribution throughout the epithelium (Fig. 2). We observed positive reaction for Ki-67 (with rare reactive nuclei) in pulmonary glands and in the nuclei of stoma lymphocytes, and in a small number of cases we noted a clear and consistent staining of elastic fibers from alveolar septa and vascular walls.

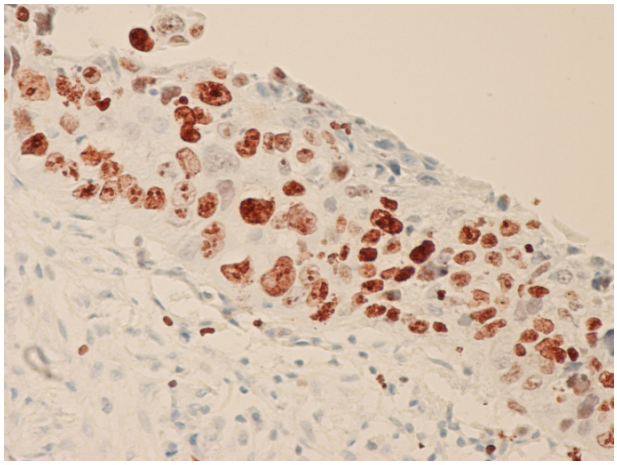

Fig.2. Intraepithelial carcinoma with Ki-67 positive nuclei dispersed throughout the entire thickness of the epithelium. ABC method, viz. AEC x 400.

We identified Ki-67 immunoreactivity in 53 of the 62 primary pulmonary carcinomas analyzed (85.4%), the positive reaction being characterized by a nuclear immunostaining with variable intensity from case to case. The percent of reactive nuclei varied widely, differing with the histological grade. While typical carcinoids and bronchioalveolar carcinomas evaluated presented low values of the proliferative activity, with rare Ki-67 immunoreactive cells (LI Ki-67 < 5%), small cell carcinomas presented the highest growing rate, the majority of cases (10 of 12 cases; 83.3%) presenting intermediate of high immunoreactivity, with over 25% Ki-67 reactive nuclei (Table I) and intense marking of the whole nucleus (Fig. 3).

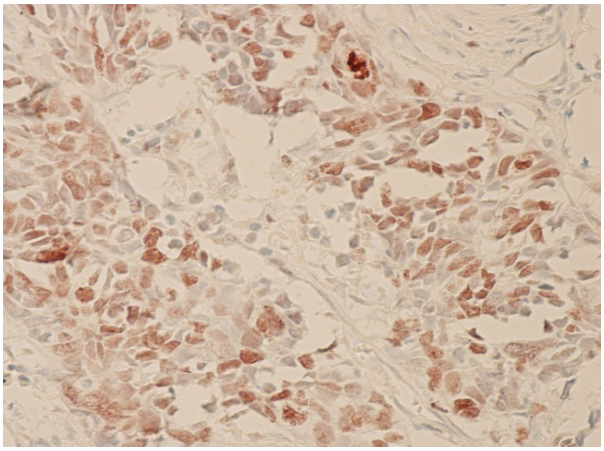

Fig.3. Small cell pulmonary carcinoma with high Ki-67 score (>75% reactive tumor cells). ABC method, viz. AEC x 400.

The 7 undifferentiated big cell carcinomas, positive for Ki-67, were characterized by an evident heterogeneity of Ki-67 expression, with variations of staining intensity, but especially of the types of marking (Fig. 4).

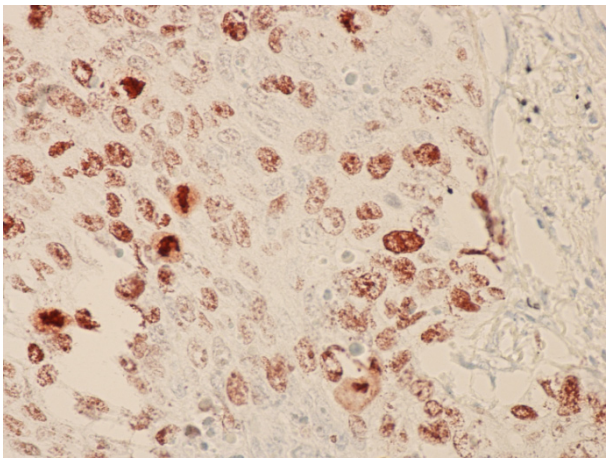

Fig.4. Undifferentiated big cell carcinoma. Heterogeneous Ki-67 expression with variations of marking types. ABC method, viz. AEC x 400.

We found an intense proliferative activity and a similar heterogeneity of marking in adenocarcinomas (Fig. 5) and in squamous cell carcinomas (Fig. 6).

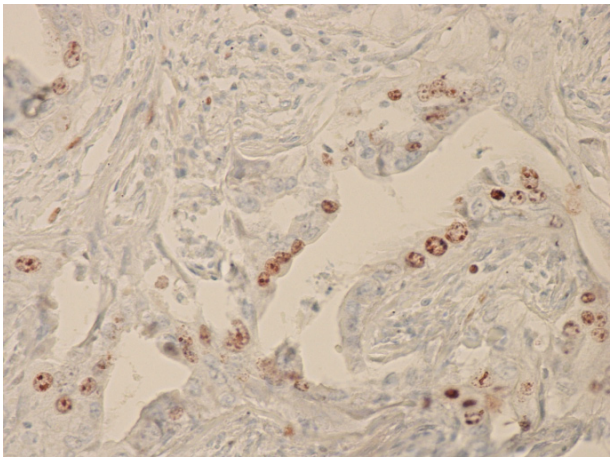

Fig.5. Acinar pulmonary carcinoma. Positive Ki-67 immunoreaction with isolated Ki-67 reactive nuclei. ABC method, viz. AEC x 400.

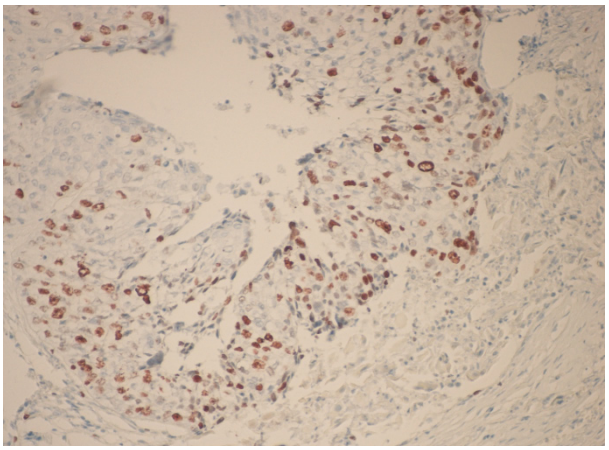

Fig.6. Squamous cell carcinoma. Positive Ki-67 immunoreaction in the nuclei of tumor cells. ABC method, viz. AEC x 200.

Ki-67 was expressed by primary pulmonary tumors, but also by corresponding metastases, hepatic metastatic lesions, metastases from lymphatic nodules and submandibular salivary glands, being characterized by an intermediate or high proliferative activity.

We grouped pulmonary carcinomas with positive Ki-67 immunoreaction based on histological type and tumor differentiation degree. Using cellularity as criterion, we divided the studied tumors in three groups; the cellularity of tumors was assessed on a scale from 1 to 3, the third degree having the highest degree of cellularity.

Analyzing the relationship between the reminded morphological parameters and tumor proliferative activity, we observed a significant relationship between cellularity (C), tumor histological degree (G) and growth rate appreciated through Ki-67 score (Table I).

Table I. Ki-67 positivity rate correlated with cellularity and histological grade.

Variable (n)	Ki-67 negative n (%)	Ki-67 positive n (%)	Ki-67 score groups Low (<25%)	Ki-67 score groups Intermediate and high (25-100%)
Histological type SCLC (12) NSCLC (47) Carcinoid (3)	0 8 (17%) 1 (33%)	12 (100%) 39 (83%) 2 (66%)	2 (16.6%) 9 (48.7%) 1 (50%)	10 (83.3%) 20 (51%) 1 (50%)
Cellularity (C) C1 (15) C2 (25) C3 (22)	4 (26.6%) 3 (12%) 2 (9%)	11 (73.3%) 22 (88%) 20 (90.9%)	9 (81.8%) 7 (31.8%) 6 (30%)	2 (18.1%) 15 (68.1%) 14 (70%)
Histological grade (G) G2 (17) G3 (45)	7 (41.1%) 2 (4.1%)	10 (58.8%) 43 (95.5%)	5 (50%) 17 (39.5%)	5 (50%) 26 (60.4%)
Number of cases	9 (14.5)	53 (85.4%)	22 (41.5%)	31 (58.4%)

n = number of cases; SCLC = small cell lung carcinomas; NSCLC = non-small cell lung carcinomas

The percent of Ki-67 positive cells correlated with the lowest differentiation degree (G3) (p<0.001; ES) and the third degree of cellularity (C3) was seen more frequently in small cell carcinomas and in dedifferentiated areas of pulmonary carcinomas, other than those with small cells.

Among the patients with G2 histological grade of the tumor, 7(41.1%) were Ki-67 negative and 10 (58.8%) Ki-67 positive (p=0.672875; NS), while 2 (4.1%) G3 patients were Ki-67 negative and 43 (95.5%) were positive for this antigen (p<0.001; ES).

We analyzed the possible prognostic significance of Ki-67 expression on subgroups of patients that were classified based on lymphatic nodules and metastasis potential (Table II).

Table II. The relationship between Ki-67 expression and the evolutive character of tumors

Ki-67 immunoreaction (n)	Positive lymph nodes	Invasion of pleura ± thoracic wall	Local relapses	Distant metastases
Ki-67 negative (9)	4 (44.4%)	1 (11.1%)	1 (11.1%)	1 (11.1%)
Ki-67 positive (53)	21 (39.6%)	11 (20.7%)	6 (11.3%)	13 (24.5%)
p	p=0.924448 (NS)	p=0.825267 (NS)	p=0.581481 (NS)	p=0.646270 (NS)

NS = not significant

In the group of the 53 carcinomas expressing Ki-67 nuclear antigen we found implication of lymph nodes in 21 (39.6%) cases, invasion of pleura ± extension into the thoracic wall in 11 (20.7%) cases, local relapses in 6 (11.3%) cases and distant metastasis in 13 (24.5%) cases; all these tumors were characterized by an unfavorable evolution, resulting in 14 deaths within 21 months from diagnosis.

We did not observe a statistically significant relationship between Ki-67 expression and lymph node involvement (p=0.924468; NS), invasion of pleura and/or thoracic wall (p=0.825267; NS), local relapses (p=0.581481; NS) and distant metastases (p=0.646270; NS).

We analyzed tumor proliferative rate (appreciated by Ki-67 score) in relation to p53 immunoreactivity (Table III).

Table III. Ki-67 immunoreaction correlated with p53 expression in pulmonary carcinomas

Ki-67 score	p53 immunoreaction p53 negative (n=30)	p	p53 positive (n=32)	p
Ki-67 negative (n=9) ***Ki-67 positive (n=53)***	7 (23.35%) 23 (76.7%)	p=0.003431 (VS)	3 (9.3%) 29 (90.6%)	p<0.001 (ES)
Low score (<25% reactive nuclei) ***Intermediate and high score (>25% reactive nuclei)***	14 (60.8%) 9 (39.2%)	p=0.121395 (NS)	8 (27.5%) 21 (72.4%)	p=0.000465 (ES)

VS = very significant; NS = not significant; ES = extremely significant

29 (90.6%) of the 32 carcinomas p53 positive presented cells expressing Ki-67 nuclear antigen (p=0.001; ES), 21 cases (72.4%) having an intermediate and high Ki-67 score (Table III), with more than 25% reactive nuclei) (p=0.000465; ES)

In the subgroup of p53 negative tumors we noted the presence of a large number of cases (23 cases; 76.6%) that expressed positive immunoreaction for Ki-67 (p=0.003431; VS), significantly higher than the number of patients p53-, Ki67-; in 14 (60.8%) of these cases the antibody used (MIB-1) stained < 25% of tumor cells.

In the p53 positive group, the number of patients with intermediate and high Ki-67 score is significantly higher than the number of patients with low score (p=0.000465; ES), while in the p53 negative patients, the difference between intermediate/high and low Ki-67 score was not significant (p=0.121; NS).

Among p53 negative patients, the number of Ki-67 positive tumors is significantly higher than Ki-67 negative tumors (p=0.0034; ES).

To appreciate the significance of Ki-67-EGFR co-expression, we followed a possible relation between immunohistochemically determined growth fraction and EGFR expression. We identified positive Ki-67 immunoreaction especially in stage III and IV tumors (9 cases); similarly, EGFR expression was proved to be more frequently positive in advanced tumor stage than in early stage, thus demonstrating a relationship between these markers and invasive characteristics of the tumor.

The majority of T3 and T4 tumors (7 of 9 cases; 77.7%)

presented high values of proliferative activity (Ki-67 score >25%) and positive EGFR immunoreactions (with a mean of EGFR absolute staining score of 3), as compared with tumors presenting less invasive features. Also, involvement of lymph nodes and distant metastasis were more frequently observed in Ki-67+ EGFR+ cases (5 cases).

For a better definition of the prognostic roleof Ki-67 – EGFR co-expression, we analyzed these two parameters after classifying tumors into 4 groups (based on immunoreaction positivity) (Table IV). group 1 included Ki-67- EGFR- tumors; group 2 comprised Ki-67- EGFR+; in the 3rd group we included tumors with Ki-67 positive (low score; Ki-67 <25%) and EGFR positive immunoreactions; in the 4th group, EGFR positive tumors with intermediated and high Ki-67 score (Ki-67 >25%).

Table IV. Prognostic significance of Ki-67 – EGFR co-expression

Tumor group	Ki-67- EGFR-	Ki-67- EGFR+	Ki-67<25% EGFR+	Ki-67>25% EGFR+
Number of patients	1	3	3	8
Survival rate (months)	40	11	9	7

The results obtained show the highest survival rate (of 40 months), but one that was not attained at the last visit, in the case of a stage II patient with a Ki-67- EGFR- tumor; relative to this aspect, the analysis of survival rate in relation with Ki-67 and EGFR expression suggests that positive Ki-67 immunoreaction (> 25%) and simultaneous over-expression of EGFR anticipates a group of pulmonary tumors with poor prognosis and the tendency towards a shorter survival.

Analyzing the relationship between Ki-67 and PCNA positivity rate (Table V), we observed a higher percentage of tumors with intermediate and high PCNA score (39 cases; 73.5%), as compared with tumors positive for Ki-67 nuclear antigen from the same score groups (31 cases; 58.4%), but statistically insignificant (p=0.074989).

Table V. Ki-67 and PCNA positivity rate

Ki-67 and PCNA immunoreaction	Nr. of cases	Score groups Low score (<25%)	p	Intermediate and high score (25-100%)	p
Ki-67 positive	53	22 (41.5%)	p=0.215815 (NS)	31 (58.4%)	p=0.074989 (NS)
PCNA positive	53	14 (26.4%)		39 (73.5%)	

NS = not significant

In the attempt of defining the relationship between the number AgNOR and immunohistochemical markers of proliferative activity, we compared the percentage of Ki-67 positive and PCNA positive cells with mean AgNOR. In this sense we grouped pulmonary tumors according to Ki-67 and PCNA scores (Table VI), calculating the mean value of AgNOR/nucleus for each score group.

Table VI. The relationship between mean number of AgNOR/nucleus and IHC markers of proliferative activity (Ki-67 and PCNA)

Ki-67 (MIB-1)	Score groups Low (<25%)	Intermediate (26-75%)	High (>75%)
Mean AgNOR/ nucleus	3.19	3.5	3.57
PCNA (PC10)	**(<25%)**	**(26-75%)**	**(>75%)**
Mean AgNOR/ nucleus	3.04	3.24	3.41

Although the differences are not significant, the results we obtained show a tendency towards a higher mean number of NORs, directly related to an immunoreactivity increase for Ki-67 antigen and PCNA, suggesting a weak correlation between mean AgNOR and tumor growth rate appreciated by immunostaining with MIB-1 and PC10.

DISCUSSION

In the intent of appreciating the prognosis of patients with pulmonary cancer, we investigated several parameters related to aggressiveness of malignant growth, cellularity, positivity rate for Ki-67 and PCNA. For the evaluation of proliferative activity we used, in 62 primary pulmonary tumors, the following methods: (1) determining the percent of immunoreactive cells for Ki-67 antigen and PCNA and classifying Ki-67 and PCNA immunoreactivity in 3 score groups; so, we evaluated the proliferative activity shown through MIB-1 antibody and PCNA staining; (2) assessing of NORs using the histochemical method of staining with colloidal Ag and (3) comparing Ki-67 score and the fraction of PCNA positive cells with mean number of AgNOR per nucleus.

MIB-1 recognizes a nuclear antigen that is associated with all phases of cell cycle, except G0. Its presence in cells from the cell cycle and its absence from G0 cells allows the determination of proliferation coefficient, the result being expressed in percent of stained cells – so, the percent of Ki-67 positive cells could be considered as measurement of growth fraction of the studied cell population (Seigneurin DG, 1991).

Studying the expression of Ki-67 antigen in normal and squamous metaplastic bronchial epithelium without atypia, we identified rare dispersed reactive nuclei, the majority being situated in the basal layer. But, we observed a large number of reactive nuclei throughout all the thickness of the epithelium in atypical squamous metaplasia and intraepithelial carcinomas associated with invasive squamous tumors, in accord with Pendleton's observations (1993) about Ki-67 expression in bronchial squamous metaplasia.

We observed positive immunoreactions and heterogeneity of Ki-67 expression in 53 (85.4%) of the 62 primary pulmonary tumors immunostained with MIB-1. IHC detection of Ki-67 antigen expression combined with other clinical-morphological and biological parameters proved to have a real value in appreciating the prognosis of patients with pulmonary cancer.

A significant relationship can be noted between Ki-67 staining and other prognostic traces; so, the percent of Ki-67 positive cells (Ki-67 score) appears different through the various histological subtypes of pulmonary cancer, according to the stage and degree of tumor differentiation, and thus confirming its prognostic validity. Correlating Ki-67 score groups with histological types of pulmonary cancer, we observed that most small cell carcinomas (83.3%) show >25% Ki-67 reactive nuclei, with an intermediate or high Ki-67 score, while only 51.2% of non-small cell carcinomas are in the same score groups.

Typical carcinoids and bronchioloalveolar carcinomas presented a Ki-67 staining index <5% (low Ki-67 score), values that are much lower than in small cell carcinomas and conventional pulmonary adenocarcinomas; these differences, similar to those observed by Kitamura (1995) and Przygodzki (1996), seem to be related to the degree of atypia of the lesions, indicating the proliferation of a limited number of cells.

The data from literature referring to the prognostic value of Ki-67 staining index are few, and the relationship between Ki-67 positivity rate and prognosis is still unclear (Hashimoto K, 2004). Bouzubar et al. (1989) sustain that tumors that stain over 20% with Ki-67 have a high risk of relapse; Wintzer et al. (1991) reported a more severe prognosis in patients with Ki-67>16% values, while Sabin (1991) noted a lower probability of survival at 5 years of patients with Ki-67>13% values (Veronesse SM, 1993).

In the study of Thaler (1987), detection of Ki-67 antigen proved to be superior to the determination method of differentiation histological grade or of mitotic index, and he considers that no matter the histological type, all tumors with over 5% Ki-67 positive cells have an adverse clinical course (Veronesse SM, 1993).

The results of Nakano and Oka (1993) sustain that high proliferative activity is correlated with high tumor malignity, with relapses and high metastasis rate. The higher the proliferative activity of a tumor, the higher its malignant potential, and survival (from the moment of diagnosis) is reduced (Pugsley JM, 2002; Skov BG, 2010).

Our results point out a suggestive relationship between Ki-67 immunostaining, advanced disease stage (III and IV) and high cellularity (C3), histological type of small cell carcinoma and poor tumor differentiation (in accord with similar observations reported by Kawai T, 1994); we found these parameters to be closely correlated between them, as well as with prognosis, which suggests the possibility of their use as factors in appreciating clinical behavior. Involvement of lymph nodes and high potential of metastasis correlated well with tumors expressing Ki-67 nuclear antigen, characterizing a group of neoplasms with rapid evolution and death in the first two years from diagnosis.

Given the allegation of the relation between gene increase or overexpression and tumor proliferative activity (Ştefănescu DT, 1996), we analyzed the relationship between growth rate and p53 immunoreactivity, and on the other hand we followed the relation between proliferative activity and EGFR positivity rate (estimated by calculating Ki-67 score and EGFR absolute staining score).

We found a p53 overexpression in 29 (90.6%) of the 32 Ki-67 positive pulmonary tumors and in 21 (72.4%) of tumors with intermediate or high Ki-67 score (with >25% reactive nuclei), these results suggesting the prevalence of p53 positive immunoreactions in poorly differentiated pulmonary tumors with high proliferation rate.

The observations concerning the relationship between p53 and Ki-67 antigen expression in pulmonary cancer are controversial (Maddau C, 2006). In this respect, evaluating the prognosis significance of Ki-67 and p53 expression in resection non-small cell pulmonary carcinomas, Scagliotti (1994) does not remark any significant correlation; but, the author observes a relatively high risk of recurrence and a lower rate of survival of patients with >25% Ki-67 positive tumor cells, our results confirming these last observations.

In the group of tumors with positive Ki-67 immunoreaction we noted a high number of p53 negative cases; these results probably suggest that p53 gene mutations are not the only mechanisms involved in acquiring the proliferative potential.

Little is known about a possible relationship between growth fraction immunohistochemically determined and EGFR expression in pulmonary carcinoma. In 15 of the 62 carcinomas immunohistochemically studied we analyzed EGFR expression and its relationship with proliferative activity estimated through Ki-67 score. We remarked that Ki-67 positivity is in close relation with EGFR positive immunoreactions; all Ki-67 positive tumors expressed EGFR, while only 3 Ki-67 negative tumors proved positive EGFR reaction. So, it seems that high expression of Ki-67 and EGFR could be related with tumor evolution.

EGFR expression in high proliferative tumors (Ki-67 score of 75-100%) diagnosed in an advanced stage of the disease, with positive lymph nodes, suggest that simultaneously positive expression of Ki-67 antigen and EGFR (Ki-67+ EGFR+) reflects the invasive character of pulmonary cancer, being considered indicators of malignancy. These results clearly indicate a tight relationship between growth rate (Ki-67>25%), high EGFR expression and clinical-pathological features of an invasive tumor behavior, and Ki-67+ EGFR+ co-expression is an indicator or poor prognosis, high invasion and metastasis potential in patients with pulmonary cancer.

The observations from literature regarding the relationship between PCNA and Ki-67 antigen expression in pulmonary cancer are rather controversial (Koohdani F, 2009). While Rowlands (1994) notes lack of a relationship between Ki-67 and PCNA score, in our study PCNA immunoreaction corresponded rather well to Ki-67 growth fraction; but, we tracked a higher number of tumors with intermediate and high PCNA score, as compared to Ki-67 positive tumors from the same score groups.

The relationship between positive PCNA immunoreactions, Ki-67 positivity rate and metastasis potential (involvement of lymph nodes and distant metastasis) suggest the predictive value of these factors linked to proliferation, in pulmonary cancer. We can say that tumor cells with high proliferative activity anticipate a higher malignancy of the disease, consisting in a

higher frequency of relapses and metastases, confirming the observations of Kawai and Suzuki (1994).

If, as suggested, AgNOR plays a role as cell proliferation markers (Seigneurin DG, 1991; Crocker J, 1987), then correlations with mitotic activity and Ki-67 and PCNA immunoreactivity are to be expected. In this respect, to determine how much the number of AgNOR is related to proliferative activity, we studied the relationship between the mean number of AgNOR per nucleus and tumor growth rate estimated through assessment of Ki-67 and PCNA score.

We observed a tendency of increasing mean AgNOR value in parallel with growing of tumor cell proliferative activity (appreciated through Ki-67 and PCNA immunostaining). The weak correlation between the number of AgNOR and immunohistochemical markers for proliferative activity, also found in the study of Rowlands, pleads for the widely accepted idea that AgNOR mean number can be a valuable parameter, useful in predicting tumor malignant potential (Fushiki S, 1996; Cornianu M, 1999).

The results obtained, demonstrating the increase of AgNOR mean number parallel with the increase of Ki-67 antigen and PCNA immunoreactivity, are in accord with the observations of other authors, pointing out that these parameters can reflect the state of cell proliferation (Aleen JP, 1992); on the other hand, AgNOR mean number seems to reflect closely the rate of tumor growth and can be used as index of proliferative activity (Ogura S, 1989; Cornianu M, 1999).

CONCLUSIONS

- We identified cells that express Ki-67 antigen (MIB-1 positive cells) in 53 (85.4%) from the group of 62 pulmonary carcinomas studied immunohistochemically. After correlating the groups of Ki-67 score with other prognostic variables, we noted a relationship between Ki-67 immunostaining, advanced stage of the disease (III and IV) and high cellularity (C3), histological type of small cell carcinoma and poor tumor differentiation. These parameters are intimately correlated between them, as well as with prognosis, suggesting the possibility of their use as factors in appreciating proliferative status and clinical behavior.
- Lymph node involvement and high metastasis potential in patients with Ki-67 positive tumors characterized a group of carcinomas with short evolution and death because of the disease in the first two years from diagnosis.
- 29 (90.6%) of the 32 p53 positive carcinomas presented cells expressing Ki-67 antigen, 21 cases (72.4%) having an intermediate and high Ki-67 score (with >25% reactive nuclei); the results obtained show the prevalence of positive p53 immunoreaction in poorly differentiated pulmonary tumors with a high proliferation rate.
- All Ki-67 positive tumors expressed EGFR. EGFR overexpression in highly proliferative tumors (with >75% Ki-67 reactive nuclei) diagnosed in the advanced stage of the disease, with positive lymph nodes, suggest that simultaneous expression Ki-67+ EGFR+ is an indicator of poor prognosis, invasion and increased metastasis potential in patients with pulmonary cancer.
- PCNA immunoreaction corresponded rather well to Ki-67 growth fraction; the relationship between PCNA immunostaining, Ki-67 positivity rate and metastasis potential shows that high proliferative activity anticipates a higher malignancy of the disease, consisting in a higher frequency of relapses and metastases.

The weak correlation between mean AgNOR per nucleus and IHC markers for proliferative activity, suggested by the tendency of increase of the mean AgNOR number, proportional with the increase of immunoreactivity for Ki-67 antigen and PCNA, sustains that these parameters can reflect the state of cell proliferation.

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EXPRESIA SI SEMNIFICATIA ANTIGENULUI KI-67 IN CANCERELE PULMONARE. RELATIA DINTRE KI-67 SI ALTI FACTORI DE PROGNOSTIC

REZUMAT

Am apreciat activitatea proliferativă în 62 de carcinoame pulmonare primitive, prin: determinarea procentului de celule Ki-67 imunoreactive (marcare cu anticorpul MIB-1), prin compararea scorului Ki-67 cu fracţia de celule PCNA pozitive (evidenţiate cu anticorpul PC10), şi prin evaluarea regiunilor de organizare nucleolară (NORs) prin metoda histochimică de colorare cu argint coloidal şi compararea scorului Ki-67 şi a fracţiei de celule PCNA pozitive cu numărul mediu al AgNOR per nucleu.

Am urmărit relaţia dintre rata de creştere Ki-67, imunoreactivitatea p53 şi nivelul receptorului pentru factorul epidermal de creştere (EGFR), prin colorarea secţiunilor cu anti-p53 (DO-7) şi anti-EGFR (31G7); sectiunile tisulare fixate in formol si incluse la parafina au fost colorate imunohistochimic prin metoda Avidin Biotin Complex (ABC).

Rezultatele relevă: (1) o rată de pozitivare Ki-67 corelată semnificativ statistic cu gradul histologic (G3) al tumorii ($p<0,001$; ES) şi celularitatea crescută (C3); (2) o relaţie nesemnificativă statistic între expresia Ki-67 şi implicarea limfonodulilor ($p=0.924448$; NS), invazia pleurei şi/sau a peretelui toracic ($p=0.825267$; NS), recidivele locale ($p=0.581481$; NS) şi metastazele la distanţă ($p=0.646270$; NS); (3) prevalenţa expresiei p53 pozitive in tumorile pulmonare slab diferenţiate, cu rată crescută de proliferare ($p<0.001$;ES); (4) nivelul crescut al expresiei EGFR în tumorile înalt proliferative, diagnosticate în stadiul avansat de boală, ce reflectă caracterul invaziv şi potenţialul crescut de metastazare al carcinoamelor pulmonare Ki-67+ EGFR+; (5) o slabă corelaţie între media AgNOR per nucleu şi markerii IHC ai activităţii proliferative.

Rata de pozitivare Ki-67 şi PCNA corelată cu celularitatea tumorii, aduc alături de stadiul clinic şi diferenţierea histologică un plus de informaţie utilă în anticiparea evoluţiei şi în prognozarea cancerului pulmonar.

Cuvinte cheie: carcinom pulmonar, Ki-67, PCNA, imunohistochimie

DETECTION OF ANTI-p80 AUTOANTIBODIES IN PATIENTS WITH ALZHEIMER'S DISEASE

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ABSTRACT

The protein p80 coilin is a nuclear autoantigen that strongly accumulates in Cajal bodies and is considered a marker for Cajal bodies which are ubiquitous nuclear structures frequently observed in close proximity to nucleoli. The protein p80 coilin is a target for autoantibodies identified in the sera of patients with diverse autoimmune features. Several clinical studies have investigated the distribution of anti-p80 coilin antibodies in various diseases. However, the clinical significance of anti-p80 coilin antibodies is not fully understood.

The objective of this study was to evaluate the anti-p80 coilin autoantibodies in serum of patients with Alzheimer's disease and to analyze the link between p80 coilin autoantibodies outcome of the disease.

Serum samples from 47 patients with Alzheimer's disease 27 women and 20 men (mean age 70.43±10.82 years, range 40-89 years) as compared to 47 control subjects, 25 women and 22 men (mean age 70.17±10.64 years, range 40-89 years). Patients with evidence of infectious or inflammatory disease were excluded. For screening of anti-p80 coilin antibodies, indirect immunofluorescence was performed with HEp-2 cell slides.

Anti-p80-coilin antibodies produce a unique pattern of immunofluorescence staining called nuclear dots. Five patients with Alzheimer's disease showed the nuclear dot pattern by immunofluorescence. Our data indicate that 10% were positive for anti-p80-coilin antibody.

In conclusion, immunological disturbances seem to be a common feature of patients with Alzheimer's disease in the form of local brain tissue inflammatory reactions and autoagressive processes directed against the brain cells. Increased plasma levels of the inflammatory marker IL-6 are associated with worse of disease. Interleukin-6 assists in risk stratification of patients with Alzheimer's disease. Autoantibody to p-80 coilin may be useful for the elucidation of the structure and function of the coiled body.

Key words: Alzheimer's disease (AD), anti-p80 coilin autoantibodies, chronic neuroinflammation

INTRODUCTION

The cause of Alzheimer's disease remains unknown. There is a growing of body evidence which supports the hypothesis of faulty immune regulation and autoimmunity or inflammatory processes as viable mechanisms of the pathogenesis of the disease. There are not publications reporting anti-p80 autoantibodies in the serum of patients with Alzheimer's disease. Several clinical studies have investigated the distribution of anti-p80 coilin antibodies in various diseases. P80-coilin, a nuclear autoantigen with a molecular weight of 80 kda, is one of several proteins localized in structures called "coiled bodies". Historically, these nuclear structures were first described by the cell biologist Ramon y Cajal and subsequently identified as "Cajal bodies". p80 coilin is a nuclear autoantigen that strongly accumulates in Cajal bodies (CB) and is considered a marker for CBs. No clear clinical features have been associated with anti-p80 autoantibodies, and the epitopes recognized by these antibodies are entirely unknown. Anti-p80 coilin antibodies are present in some patients with clinical disease but with no discernible pattern (1-3)

MATERIALS AND METHODS

47 serum samples obtained from AD patients were analyzed for anti-p80 coilin antibodies by indirect immunofluorescence. All AD patients fulfilled the following criteria: 1) slow progressive decline of intellectual function; 2) a score on the clinical dementia rating scale more than 0.5; 3) a score on the short portable mental status questionnaire (SPMSQ) of less than 20; 4) a score of 7 or less on the Hachinski-scale; and 5) no evidence for abnormalities on CT-scan other than cerebral atrophy, and no evidence for focal dysfunction in the EEG. Exclusion criteria were: infections, neoplasias, surgical interventions, autoimmune disorders, concurrent major renal or hepatic disorders, major trauma in previous month. Informed consent was obtained from all patients and controls, and approval was also obtained from the Ethics Committee. Sera were diluted at 1:40 in PBS. For screening of anti-p80 coilin antibodies, indirect immunofluorescence (IIF) was performed with HEp-2 cell slides.

We used Fisher's exact test for the analysis of autoantibody frequency, and unpaired t-test. P values of less than 0.05 were considered significant.

RESULTS

Anti-p80 coilin antibody produces a unique pattern of immunofluorescence staining called nuclear dots characterized by the presence of up to six discrete nuclear bodies in interphase cell nuclei. Our data indicate that 10% were positive for anti-p80 coilin antibody. Autoantibody to p-80 coilin may be useful for the elucidation of the structure and function of the coiled body. Five patients with Alzheimer's disease showed the nuclear dot pattern by immunofluorescence, and reacted by immunoblotting with 80-kDa protein in a nuclear extract from HeLa cells. Anti-p80 coilin should be considered natural autoantibodies present in the sera of healthy individuals. The decreased prevalence of anti-p80 coilin autoantibodies in the serum of older individuals suggests that titers may decrease with age. Longitudinal studies are required to clarify this. Anti-p80 coilin in patients with Alzheimer's disease

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may reflect the specific impairment of helper T cell activity for B cells that produce anti-p80 coilin.

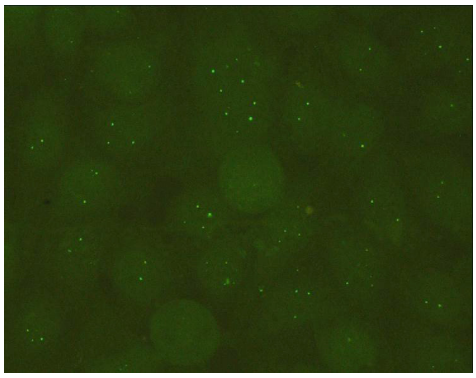

Fig.1. The coiled body pattern by indirect immunofluorescence on HEp-2 cell substrate. Discrete round bodies (zero to 6 per cell) randomly distributed in the nucleoplasm of interphase cells (often located in the vicinity of the nucleolus)

DISCUSSION

The coiled bodies appear as a tangle of coiled, electron-dense threads roughly 0.5 mm in diameter. The only epitope reported to date that can be used as an unambiguous marker for coiled bodies is a protein called p80 coilin. Although there is certainly a pool of this protein localized diffusely throughout the nucleoplasm, antibodies against coilin typically stain a few discrete foci per nucleus. Coiled bodies are highly enriched in several classes of snRNPs, nucleolar 9 e.g., fibrillarin, topoisomerase I) and cell-cycle conrol proteins (e.g., cyclin E, cyclin H), as well as several basal transcription factors. Of note, coiled bodies play a major role in directing intranuclear snRNA sorting and might even be an obligate waystation in the biogenesis pathways of snRNPs and snoRNPs. The protein p80 coilin is a target for autoantibodies identified in the sera of patients with diverse autoimmune features (4-6).

Coilin autoantibodies are rarely detected. This autoantibody is sometimes found in patients with some allergic diseases but rarely in patients with connective tissue disease (CDT). The clinical significance of many other antinuclear antibodies (ANAs) is not understood. One such ANA is directed against p80 coilin is a specific marker of Cajal bodies (CBs) (7,8).

We find these results very promising for the consideration of future treatment of AD patients**.** The preliminary results need be confirmed by controlled studies.

CONCLUSION

Anti-p80 colin autoantibodies are found during ANA screening with HEp-2 cells. We conclude that the presence of anti-p80-coilin autoantibodies is not associated with any particular clinical syndromes nor is it diagnostic of any conditions.

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DETECTIA AUTOANTICORPILOR ANTI-P80 LA PACIENTII CU ALZHEIMER

REZUMAT

Proteina p80 coilin este un autoantigen nuclear, care se acumuleaza la nivelul corpilor Cajal, fiind considerata un marker al acestora, care sunt structuri nucleare ubicuitare, frecvent observate in apropierea nucleolilor. Proteina p80 coilin reprezinta tinta autoanticorpilor identificati in serul pacientilor cu diferite caracteristici autoimmune. Multiple studii clinice au investigat distributia anticorpilor anti-p80 coilin in diferite afectiuni. Cu toate acestea, semnificatia clinica a anticorpilor anti-p80 coilin nu este pe deplin inteleasa.

Obiectivul acestui studiu a fost evaluarea autoanticorpilor anti p-80 coilin in serul pacientilor cu Alzheimer si analizarea legaturii dintre acestia si evolutia bolii.

Au fost utilizate probe de ser de la 47 de pacienti cu Alzheimer, 27 de femei si 20 de barbati (varsta medie 70.43±10.82 ani, cu varste cuprinse intre 40-89 ani) si comparate cu 47 subiecti de control, 25 femei si 22 barbati (varsta medie 70.17±10.64 ani, cu varste cuprinse intre 40-89 ani). Au fost exclusi pacientii care au prezentat semne ale unei afectiuni inflamatorii sau infectionase. Pentru screening-ul anticorpilor anti-p80 coilin, a fost utilizata metoda de imunofluorescenta indirecta, folosind lame Hep-2.

Anticorpii anti-p80-coilin produc un tip unic de imunofluorescenta, numit puncte nucleare. Cinci dintre pacientii cu Alzheimer au prezentat aceasta imunofluorescenta nucleara. Datele noastre indica faptul ca 10% dintre probele selectate au fost pozitive pentru anticorpii anti-p80 colilin.

In concluzie, perturbarile imunologice par sa fie o caracteristica comuna la pacientii cu Alzheimer, sub forma reactiilor inflamatorii locale cerebrale si procese auto-agresive orientate impotriva celulelor cerebrale. Cresterea plasmatica a nivelului interleukinei IL-6, marker al procesului inflamator, este asociata cu un prognostic negativ in evoulutia bolii. IL-6 este implicata in stratificarea riscului la pacientii cu Alzheimer. Autoanticorpii pentru p-80 coilin ar putea fi utili pentru elucidarea structurii si functiei “coiled body”.

Cuvinte cheie: Alzheimer (AD), autoanticorpi anti-p80 coilin, neuroinflamatie cronica

PREVALENCE OF HIGH RISK GENOTYPES OF HUMAN PAPILLOMA VIRUS AMONG FERTILE WOMEN IN THE WESTERN PART OF ROMANIA

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ABSTRACT

Human Papilloma viruses (HPV) are widely distributed, producing epithelial and mucous tumors and are involved in malignant genital pathogenesis. There are more than 100 known genotypes of HPV, of which approximately 40% infect genital mucosa.
During 01.03.2007 – 19.09.2009, Bioclinica Laboratories, tested 9245 women for detecting HPV infection. 38.27% were positive for at least one genotype, from which, 66.31% were high risk genotypes.
DNA extraction was automated performed (Magna Pure Roche) and genotyping by LINEAR ARRAY HPV Genotyping Test (Roche).
From the high-risk one's, type 16 was detected the most (20.68%), followed by genotypes 31 (13.89%), 51 (12.39%), 33 (8.75%), 66 (7.96%), 18 (6.78%), 52 (5.87%), 58 (5.37%), 68 (3.70%), 45 (3.55%), 39 (3.08%), 56 (3.05%), 59 (2.85%) and 35 (2.06%).
Keywords: HPV, genotype, prevalence, high risk

INTRODUCTION

Human Papilloma viruses (HPV) are widely distributed, producing epithelial and mucous tumors and are involved in malignant genital pathogenesis (14). In Romania, cervical cancer is up to 15% of total number of malignancies, being first as a genital cancer (aprox. 67% of genital cancers) and second place as cancer death among women (1).

Cervical cancer has an overall good prognosis; survival at 5 years is 100% in phase 0, 91% in phase I, 83% in IIA phase, 66% in IIB, 45% in IIIA, 36% in IIIB, and 10-14% in phase IV. Due to this, the main aim of cervical cancer treatment is a curative (local control), except phase IV, where the treatment is palliative (12).

Epidemiological studies revealed that, the incidence of cervical cancer is considerably increased among women with a low life status, early start of sexual life, sexual promiscuity, multiple pregnancies and births, and among smokers.

Main etiology of cervical cancer and pre-neoplasic lesions is due to human papilloma virus infection (8). The viruses are small (45-55 nm), spherical, non-enveloped. HPV genome is formed from approximate 7600 base pairs. All supposed codifying sequences (open reading frames - ORF) are arranged on one DNA strand and all papillomaviruses have the same organization. Various protein products are derived from these ORF's.

Anogenital HPV subtypes are classified in "low risk", for example HPV6 and HPV11, which are associated with squamous lesions, rarely causing cancer, and "high risk", HPV16, HPV18, associated with cervical intraepithelial neoplasia or squamous lesions which can progress to cancer (2, 3, 9, 13). Experimental studies showed that most cervical neoplasia, intraepithelial or invasive one, are caused by an HPV infection. High risk DNA-HPV can be found in 90% of human cervical cancers. Most of the infections cannot be detected even with the slightest method of DNA detection. Papanicolau test can detect some abnormalities in the cervix. Both partners require examination and treatment, to avoid reinfection; for relapses prevention, a permanent and prolonged medical surveillance is recommended.

Papillomaviruses do not grow in cell cultures. Thus, the laboratory diagnose implies:

A. Microscopically examination of lesions
B. Electronic microscopy
C. Serology
D. Molecular biology techniques.

Papillomaviruses have tropism for squamous epithelial tissue of skin and mucous membranes. At this level, the virus is replicating and induces tissue proliferation, forming tumors. Infection is by close contact (including sexual) through some lesions, even very small. Viral replication and gene expression depends on differentiation degree of the infected epithelial cell, causing persistent infections at the basal layer and active infections in keratinocytes. Usually, the infection is localized and spontaneously regressing. In some papillomaviruses, viral genome can persist intracellular (viral DNA integration in host cell), causing recurrences.

Clinical syndromes of HPV infection are:

- Skin verruca
- Benign tumors of head and neck
- Genital condylomatosis

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- Cervical dysplasia and neoplasia
- Cervical intraepithelial neoplasia
- Bowen disease
- Invasive cervical carcinoma (4, 5, 6, 7)

Motivation for this study was represented by increasing frequency of cervical cancer and the ability of ambulatory testing of circulating Human Pappiloma virus genotypes (responsible for this type of cancer), combined with increased addressability of the female patients. Thus, the study reveals the frequency of circulating genotypes of HPV among females, as well as persistency in time.

MATERIAL AND METHODS

The samples for this study were obtained from female patients who addressed themselves at Bioclinica Laboratory for HPV-DNA detection, during 01.03.2007 – 19.09.2009. 9245 cervical samples were analyzed, from 8858 patients, age varying from 12 to 73 years old. From 8858 patients, 413 were present again for re-testing, a good opportunity for surveillance of virus persistency. Cervical samples were collected in specialist's office, with the kit provided by the laboratory. Exfoliated cytology was used to obtain the samples, this being a non-invasive collection method for screening in a normal gynecology check, collection is not based on the presence of a lesion. The sample's quality depends on collector's type and the anatomical site, for women, cervical samples being the most reliable.

Age distribution among patients show an increasing trend until 29 years of old (570 patients), after that, the addressability decreases progressively (Figure 1).

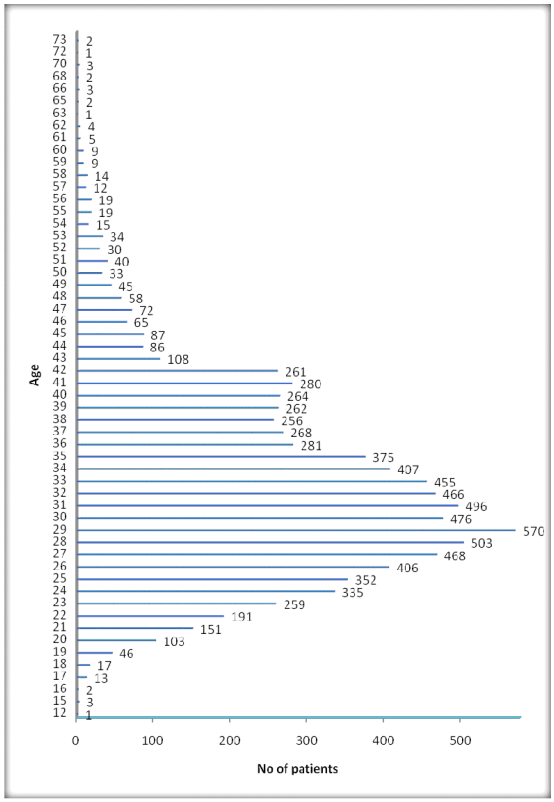

Fig.1. Age distribution

DNA extraction was performed automatically (MagnaPure Roche) and genotyping by LINEAR ARRAY HPV Genotyping Test (Roche). LINEAR ARRAY HPV Genotyping Test is a qualitative in vitro assay for detection of HPV in clinical samples. The test uses polymerase chain reaction for amplifying the target DNA, followed by hybridization, detecting 37 anogenital genotypes of HPV (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108).

RESULTS AND DISCUSSIONS

From 9245 tested samples, 3538 were positive for at least one genotype (38.27%) and 5107 were negative (61.73%) (Figure 2).

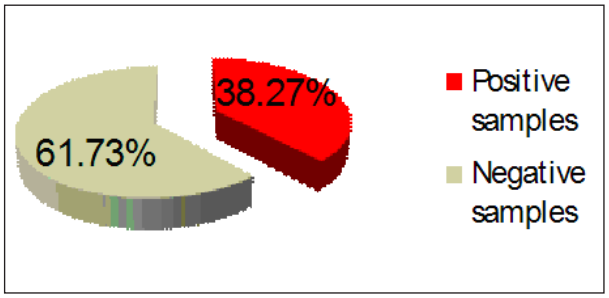

Fig.2. Distribution of positive and negative samples

Distribution of positive results versus negative results, revealed that at 21-25 age group, the positive samples were more than negative's, unlike other age groups, where the negative samples were predominant (Table I).

Table I. Age distribution of positive and negative samples

Age (years)	Negative samples	Positive samples	Age (years)	Positive samples	Negative samples	Age (years)	Positive samples	Negative samples
12	1	0	33	310	145	52	23	7
15	2	1	34	279	128	53	25	9
16	0	2	35	260	115	54	9	6
17	6	7	36	198	83	55	11	8
18	8	9	37	186	82	56	16	3
19	18	28	38	173	83	57	12	0
20	57	46	39	170	92	58	9	5
21	61	90	40	188	76	59	7	2
22	78	113	41	199	81	60	7	2
23	117	142	42	185	76	61	5	0
24	151	184	43	75	33	62	4	0
25	174	178	44	61	25	63	0	1
26	225	181	45	60	27	65	2	0
27	253	215	46	52	13	66	2	1
28	282	221	47	59	16	68	2	0
29	352	218	48	41	17	70	2	1
30	294	182	49	35	10	72	1	0
31	320	176	50	27	6	73	2	0
32	290	176	51	30	10			

Identified DNA-HPV genotypes were 66.31% high-risk ones, 10.44% low-risk, and the rest, 23.25% - other HPV genotypes (Figure 3).

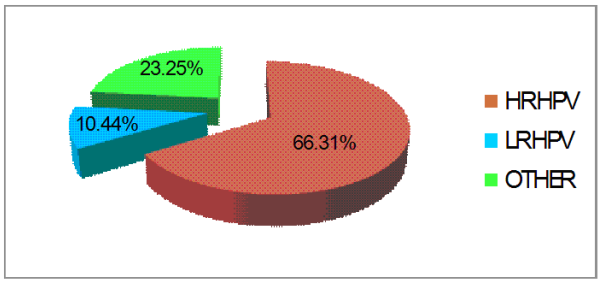

Fig.3. Genotype distribution among positive samples

High risk genotypes of HPV, detected in this study, are represented by: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. From the high-risk one's, type 16 was detected the most (20.68%), followed by genotypes 31 (13.89%), 51 (12.39%), 33 (8.75%), 66 (7.96%), 18 (6.78%), 52 (5.87%), 58 (5.37%), 68 (3.70%), 45 (3.55%), 39 (3.08%), 56 (3.05%), 59 (2.85%) and 35 (2.06%) (Table II).

Table II. High risk genotypes distribution

HR HPV genotype	No of detections	Percent
16	704	20,68%
31	473	13,89%
51	422	12,39%
33	298	8,75%
66	271	7,96%
18	231	6,78%
52	200	5,87%
58	183	5,37%
68	126	3,70%
45	121	3,55%
39	105	3,08%
56	104	3,05%
59	97	2,85%
35	70	2,06%
Total	**3405**	**100%**

Papilloma virus persistency at genital level is considered to be a risk factor for developing a cervical neoplasia. From 8858 female patients included in the study, during 30 months, only one patient was tested 5 times, 3 patients – 4 times, 25 – 3 times and 384 – 2 times. The patient tested 5 times (23 years old) was positive every time. In all 5 tests, genotype 58 persisted in time. The 3 patients tested 4 times, one was positive for every test, one negative, and one was intermittent positive/negative each time. 25 patients were tested 3 times. They had various results regarding the presence or the absence of infection. Only 2 of them had negative results each time, and 8 were constant positive. 384 patients were tested twice. 125 were negative in both testing, 117- positive, 120 – eliminated the virus, and 22, although a first test was negative, the second one showed an infection. Distribution of persistent genotypes over time, revealed that genotype 16 persisted for 4, 8, 12 and 16 months, followed by genotype 66 – at 8, 12, 16 months.

All existent prophylaxis guides for cervical cancer, have practical recommendations for splitting screening groups, the time between testing and strategies to be followed for special groups of patients.

Optimal age for beginning of screening is different due to factors considered by various authors:

• From 25 years of old ;

• From 21 years of old, or after 3 years from the sexual life debut;

• From 18 years of old because of the high frequency of sexual activity at this age;

• From 30 years of old for new programs based on HPV-DNA testing; from 25 years for existent programs (classical cytology)

Now, there are no scientific arguments to establish the optimal age for screening start.

The age from which the screening is no longer justified:

• From 60 years;

• From 65 if by this age a proper screening was performed, the results were negative and the woman did not belong to any risk groups;

• From 70 years old if there are three successive negative cytology results and no positive results in the last 10 years. At the same time, testing is recommended for older women if before they were not tested or there are no results from previous testing;

• Patients more than 70 years old, DNA-HPV positive, must continue testing in the recommended screening program;

• From 65 – if the last two smears were negative

• there are no limits in age for screening

Screening should be performed every 3-5 years, but no less than once every 3 years, if the procedure is based on classical smears, or every 2 years if the liquid based cytology is used. The period can be prolonged to 2-3 years for patients above 30 years old, considering the associated risk factors and the result from previous cytology exam. This recommendation is based on sensibility of cytology exam.

A female patient, initially HPV negative, can be infected, becoming HPV positive, but the way to develop cervical cancer is depending on the involved genotype, persistency in time, associated factors: age, immunity, co-infection with other genotypes, life style, multiple births, smoking, use of contraceptive devices etc. (10, 11, 15, 16)

CONCLUSIONS

Considering the complications which can occur from this infection, with severe repercussions, a management revision of infections on fertile age is required, by approaching this problem from different pathways, as a diagnosis and treatment, and also as evaluating the knowledges, atitudes, behaviour for best corrective or preventive measures. It implies immediat measures for primary and secondary prophylaxis.

Some of those, more important are: education, mass screening, correct management of cases and partners, increasing the quality and accesability of medical services.

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PREVALENTA GENOTIPURILOR CU RISC CRESCUT A VIRUSULUI PAPILLOMA UMAN LA FEMEILE DE VARSTA FERTILA, IN PARTEA DE VEST A ROMANIEI

REZUMAT

Papillomavirusurile umane (HPV) sunt larg răspândite în populaţie, producând tumori epiteliale şi ale mucoaselor, şi sunt implicate în patogenia afecţiunilor maligne genitale. Se cunosc mai mult de 100 de genotipuri HPV, din care aproximativ 40% infectează mucoasa genitală.

În perioada 01.03.2007 – 19.09.2009, în cadrul laboratorului Bioclinica au fost testate 9245 de femei pentru detectarea infecţiei HPV. 38,27% dintre acestea au fost pozitive pentru cel puţin un genotip, dintre care 66,31% au fost genotipuri cu risc crescut. Extracţia ADN-ului s-a efectuat în sistem automat (Magna Pure Roche), iar genotiparea cu LINEAR ARRAY HPV Genotyping Test (Roche).

Dintre genotipurile cu risc crescut, tipul 16 a fost detectat în cel mai mare procent (20,68%), urmat de genotipurile 31 (13,89%), 51 (12,39%), 33 (8,75%), 66 (7,96%), 18 (6,78%), 52 (5,87%), 58 (5,37%), 68 (3,70%), 45 (3,55%), 39 (3,08%), 56 (3,05%), 59 (2,85%) şi 35 (2,06%).

Cuvinte cheie: HPV, genotip, prevalenta, risc crescut

PSYCHOLOGICAL CHARACTERISTICS AND PERSONALITY TRAITS IN PRODROMAL STAGES OF BODY DYSMORPHIC DISORDER

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ABSTRACT

Objective: In a preliminary study on Romanian population, the authors examined the prevalence and correlates of certain psychological characteristics and personality traits regarding the Adonis Complex, a debilitating and chronic condition characterized by one's preoccupation with thoughts concerning muscle size and shape, as well as the specific behaviour that derives from this. **Method:** Levels of anxiety and depression were investigated, as well as the prevalence of certain personality traits, similar to personality disorders as described in the DSM-IV-TR and ICD-10, and several characteristics concerning the Adonis Complex. This study used the Hamilton Anxiety Scale, Beck Depression Inventory, the Altered Personality Questionnaire, and the Adonis Complex Questionnaire to characterize the diagnostic status of a population-based sample of 30 males who work out in fitness gyms at least 3 times a week. **Results:** The presence of possible symptoms for muscle dysmorphia was significantly associated with the presence of mild depression and moderate or severe anxiety symptoms. Significant correlations were found between depression and anxiety symptoms and certain characteristics related to muscle dysmorphia. Research subjects also exhibited certain personality features, similar to the manic, emotionally unstable, paranoid and elated personality disorders. **Conclusions:** The authors found that the presence of muscle dysmorphia characteristics was linked to the presence of depression and anxiety symptoms, which is similar to findings in other clinical studies. Their estimate of the personality trait prevalence is consistent with data from other studies and suggests a prevalence similar to that of other serious psychiatric disorders (e.g., muscle dysmorphia comorbidity with personality disorders). These prevalence data encourage further research in order to better understand and identify several characteristics of the Adonis Complex.

Key words: Adonis Complex, muscle dysmorphia, anxiety, depression, altered personality traits, avoidance behavior

"While the general population is concerned with appearance, we are obsessed with it... I plan my meals. I time them. I don't eat for the pleasure of it. I eat according to my current goal" (1)

INTRODUCTION

The Adonis Complex, also known as *reverse anorexia nervosa*, bigorexia or muscle dysmorphia, can be defined as one's preoccupation with thoughts concerning appearance, especially musculature, as well as gaining weight without actually getting fatter (2). According to Olivardia (3), diagnostic criteria for muscle dysmorphia have three main components:

A. Excessive pathological preoccupation with thoughts concerning muscularity, especially the idea that the body is not muscular or lean enough;

B. A clinically significant impairment in life activities (e.g. decreased social functioning) as a result of the preoccupation with insufficient musculature, namely:

1. Giving up on social/professional/enjoyable activities in favour of the compulsive need for strict weight-lifting schedule, and a particular diet;

2. Avoidance of social situations that imply body exposure, and if such a situation is unavoidable, the individual experiences intense subjective distress;

3. This preoccupation affects the individual's social/professional/interpersonal functioning;

4. Continued harmful behaviours (e.g. excessive physical training or strict diet) even if the individual is knowledgeable about potential dangers, even if adverse reactions occur or he/she suffers physical damage.

A positive diagnosis of muscle dysmorphia requires the presence of at least two out of four criteria.

5. Muscle dysmorphia and its associated behaviours are based on the individual's preoccupation with thoughts of not being muscular enough, and thus it can be differentiated from body dysmorphic disorder or other eating disorders, if the associated behaviours are manifestations of a significant preoccupation with body build or musculature as opposed to a preoccupation with food intake.

According to Olivardia (3), muscle dysmorphia was primarily diagnosed in 1997, in a historical context that witnessed a particular growth in the amount of men concerned with their physical appearance, especially with their body size and form, particularly during the last three decades of the 20th century. Thus, according to general statistics (4), during the last 30 years people's dissatisfaction with physical appearance has become three times more important (from 15% in 1972 to 34% in 1985, and to 43% in 1997).

Regarding the causes of this disorder, it is generally considered that there are a few possible risk factors which contribute

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to this disorder:

1. The genetic (biological) factor implies a certain serotonin irregularity, similar to that found responsible for obsessive-compulsive disorder (3,4);

2. Certain personal experiences in early childhood, mostly puberty and adolescence (bullying, teasing); lack of love as a disharmony element responsible for the disorder, which may lead to rejection and low self esteem;

3. The socio-cultural factor, especially certain influences through mass-media, promoting the so-called "real man", who is physically attractive, strong, with an extremely muscular body; at a very young age children come to interact with this over-sized masculine figure, through several toys which represent certain film/cartoon/story/comic book characters. In the 2000 book, *The Adonis Complex,* Olivardia and Pope argue that muscle dysmorphia is fuelled by the portrayal of overly fit characters of unattainable musculature in children's cartoons, by observing the changes their appearance has undergone since the '70s, from a so-called normal musculature to a hypertrophied body. Another feature taken into consideration by these authors refers to the fact that, during the past 30 years, women have gradually become more independent, witnessing a change in their social status. Nowadays, not only men get to play the head of the family part, by providing financial safety and social stability, as most of the professional activities can be carried out by both men and women. Thus, as the authors note, in search for their manhood, men have developed a compensation mechanism, by becoming more and more concerned with their bodies, especially with strength and bodily size; these features remain a male characteristic, due to the fact that "musculature is regarded as a symbol of manhood/masculinity" (4).

Several hypotheses regarding the origins of muscle dysmorphia

The **cognitive explanation** is one of the most common theories. It searches for possible causes of the disorder in a cognitive vicious circle of dysfunctional thoughts. When a person has a negative appraisal of internal body image, it influences the external representation of appearance. That triggers processing self as an aesthetic object which results in negative internal body image. So, people are not only sensitive to bodily cues, but also threatened by them. In people vulnerable to somatoform disorders it can imply an over interpretation of bodily imperfections.

The **psychodynamic explanation** states that these are unresolved conflicts from childhood and extremely difficult feelings that are responsible for the disorder. The disorder provides a means for people to express their emotions that otherwise would be too difficult to express. In this case emotions are converted into more tolerable physical symptoms. The purpose of such conversion is to communicate extreme feelings in "physical language". Therefore a preoccupation with musculature could be treated as an individual's unconscious displacement of sexual or emotional conflict (or feelings of guilt, or even poor self-image).

The **cognitive-behavioural explanation** states that the responsibility for muscle dysmorphia is shared by: cultural factors, biological predispositions, psychological vulnerabilities and early childhood experiences. Cultural factors manifest themselves in an exaggerated emphasis on appearance, physical strength and attractiveness. People compare themselves with others and even little deviations from the pattern perceived as ideal lead to extremely negative appraisals. Next, some people are biologically predisposed to a constant drive for perfection. Psychological vulnerability is mostly based on low self-esteem. Childhood experiences connected with the disorder are: disharmonious family background, bullying, teasing. This may produce feelings of being unloved, insecure and rejected.

According to the **biological explanation** a serotonin irregularity is mostly responsible for the disorder.

The **evolutionary perspective** links the causes of this disorder to the progressive alignment of men and women in most aspects of every day life. Due to women's social emancipation, men have gradually lost their traditional identity as food and income providers, family protectors and potential fighters. While women gained in independence, men began to suffer a lack of identity, and secondarily physical appearance became more and more important to them. The need for redefining masculinity can be seen as the need for increasing muscularity, as the latter is considered to be a symbol of masculinity.

Regarding the **prevalence** of this disorder, muscle dysmorphia is most common in males and often starts in the late teens, although sometimes, they can be older. Olivardia and others found in a 2000 study on American populations that the average onset age was 19.4 years (3). The preoccupation with muscularity is considered more frequent in young males, in comparison with men over 45 years of age who show a lower degree of concern. However, the desire to work out the upper body seems to appear in younger boys as well (between 5 or 6 years of age) (4).

From a **psychopathological** point of view, muscle dysmorphia involves several changes on the following levels of psychological functioning:

A. THE COGNITIVE LEVEL – psychopathology changes regarding the following psychological processes:

1. Perception – pathological delusions regarding one's body (the individuals perceive themselves as too skinny and underweight);

2. Attention – selective attention, prevailing long-term focus on one's body (individuals selectively focus their attention on perceived physical "defects", they are hypervigilant to even small deviations from perceived ideal and they ignore information that their body image is not consistent with reality);

3. Cognition – pathological changes such as:

a. Delusional thoughts and beliefs (negative, distorted and repeated convictions regarding one's physical appearance, with a tendency towards certainty and incorrigibility);

b. Recurrent thoughts of worthlessness, helplessness, hopelessness, and dissatisfaction;

c. Self criticism;

d. Obsessive thoughts concerning muscular growth, the

need for excessive exercising, and a special high-protein diet;

e. Low self-esteem;

f. Frequent comparisons with others or with certain so-called "ideal" figures.

B. THE EMOTIONAL LEVEL – individuals frequently exhibit a low mood, irritability, and melancholy. Also, they often experience intense feelings of dissatisfaction related to their perceived appearance, self-hatred, as well as strong feelings of shame. Other common symptoms include anxiety and subjective distress, as well as chronic feelings of emptiness. In severe cases, individuals may exhibit symptoms of phobia, characterized by intense fear of not living up to other people's expectations.

C. THE BEHAVIORAL LEVEL – refers to regular and excessive physical training, as well as keeping a strict diet.

Nowadays it is generally considered that muscle dysmorphia is based on a **negative body image** which derives from:

- the excessive, time consuming preoccupation with bodily size and form, causing significant social and professional impairment;
- dissatisfaction with one's perceived body image, causing low self esteem, depression, and social anxiety.

In this regard, several studies assert the fact that one's biased perception of the actual size and shape of their own body may be based on certain cortical deficits associated with certain emotional processes that enhance one's tendency to focus exclusively on the size and shape of their bodies. Thus, specific perceptive disturbances related to one's body image should ultimately be seen as coming from certain cortical deficits which in turn cause visual and spatial disturbances; all in all this perspective might provide an answer to why certain people tend to either underestimate or overestimate their bodily features (5).

In term of clinical presentation, symptoms vary in severity on a continuum. Common symptoms are:

- Frequent and repeated mirror and other reflective surfaces checking;
- Periodic measurements of the shape and muscle mass;
- Preference for clothing designed to hide the physical form (large clothes);
- Avoiding situations requiring body exposure;
- Concern to engage regularly and rigorously intense exercise/ excessive (daily, several times a day);
- Physical training in spite of physical injury (muscle tear, stretching of ligaments, tendons or joints);
- Ignoring/ avoiding social or family events and job responsibilities in order to exercise;
- Main concern for a strict diet rich in protein and fat-free and sticking to this;
- Constant dissatisfaction related to the shape and size of muscle mass;
- Consumption of anabolic steroids (amphetamines, HCG, L-DOPA, estrogen blockers, steroids).

The benefits of this behaviour (efficiency in building muscle mass, muscle sustainability) are accompanied by a series of side effects, among which we mention:

- Non-psychiatric effects: increased risk for cardiovascular disease, stroke, prostate cancer;
- Psychiatric effects: irritability, emotional instability, tendency to aggressive manifestation, verbal and physical aggression, impaired reasoning, megalomaniacal ideation, severe depressive episodes, physical and psychological dependence.

In severe cases, the concern for physical exercises (training) becomes an obsessive idea and the urge to perform theme turns in to compulsion. Missing a training session causes intra-psychic tension and anxiety, accompanied by feelings of vulnerability, ideas of incapacity and guilt.

What comorbidity is concerned, muscle dysmorphia is most frequently associated with mood disorders (depression or bipolar disorder), anxiety disorders, eating disorders, obsessive-compulsive disorder, schizophrenia, Cluster B personality disorders (particularly Narcissistic personality disorder type) (6). Approximately 58% of patients with muscle dysmorphia have been found to suffer from mood disorder like unipolar depressive disorder or bipolar disorder (hypomanic episodes while using steroids) (3).

There is still a debate about comorbidity with anxiety disorder or classification in anxiety disorder group. Thus, patient suffering from bigorexia show anxiety symptoms, especially when they are unable to meet rigorous physical training program and diet. According to the literature, anxiety disorder are present in 29% of those with muscular dysmorphia (3), while statistics show that the same percentage of those diagnosed with Adonis Complex describe a history of anorexia or bulimia nervosa (3). Despite these data, the two disorders differ in that the characteristic manifestation of generalized anxiety disorder (muscle tension, irritability, psychomotor restlessness, sleep disturbances, etc.) are not necessarily manifest in those with muscle dysmorphia.

There are also certain similarities between muscle dysmorphia and obsessive-compulsive disorder, regarding certain clinical features, namely: obsessive thoughts (related to muscular growth or not being muscular enough), compulsive behaviour (such as excessive physical training, adherence to a strict diet), feelings of discomfort, anxiety, distress, low mood particularly when individuals find themselves incapable of following their strict training or dieting schedule.

Some studies have found certain similarities between muscle dysmorphia and social phobia, regarding individuals' reaction in certain social situations that require dealing with other people's attention (7).

In addition to this, some authors have found several similarities as well as differences between muscle dysmorphia and cluster B personality disorders (particularly narcissistic personality disorder) (7). On the one hand, both categories of disorders refer to identity related concerns as well as unstable interpersonal relations; on the other hand, while narcissistic individuals have a need for being the centre of attention and for being socially successful, individuals with muscle dysmorphia avoid similar situations consciously and knowingly, for fear of rejection, thinking that others might notice and mock their lack of muscularity. Also, individuals with muscle

dysmorphia often report working out in order to gain acceptance from others rather than exhibiting ideas about their influence on others, as in the case of the narcissistic psychopath. Finally, individuals with muscle dysmorphia don't usually exhibit the impulsive behaviours common to cluster B personality disorders.

There are also several features which link muscle dysmorphia to eating disorders, such as: disturbed perception of bodily size and shape (8); feelings of lack of control related both to binge eating periods (in bulimia nervosa) and to the incoercible need for training and dieting (in muscle dysmorphia); similar aetiology which includes: dissatisfaction with one's body image, low self esteem, emphasis on social and cultural influence (mainly through mass-media). However, on a behavioural level, these disorder types involve distinctive compensatory behaviours, namely: fasting (abstinence from food) in anorexia nervosa, eating according to a special high-protein and low-fat diet in muscle dysmorphia, and the use of laxatives, enemas or diuretics to avoid gaining weight in bulimia nervosa.

Last but not least, individuals with muscle dysmorphia frequently end up neglecting work, school, family, and other social aspects in life because of excessive exercising in order to continuously gain muscle size/strength; this type of secondary behaviour turns out to be repetitive (1 to 5 training sessions each day), as well as time consuming (more than 200 minutes each day) (4).

Due to the fact that muscle dysmorphia was only recently described and diagnosed as a mental disorder, there has been considerable controversy over the effectiveness of certain treatment methods. Nowadays, the complete treatment regimen for muscle dysmorphia requires a multidisciplinary approach, involving a team of mental health professionals, including psychiatrists, nutritionists, and psychologists (9). Patients are usually required to fill out several questionnaires in order to assess both individual characteristics and different levels of severity before a specific treatment is instituted.

MATERIALS AND METHODS

The sample involved in the present study consisted of 60 male subjects between 18 and 40 years old, who were divided into two samples, as follows: the research sample consisted of 30 subjects who attend body-building gyms at least 3 times a week, for at least 1 year, and who spend at least 1 hour a day performing physical training in order to improve their physical appearance (not for taking part in competitions). The objective of the present study was investigating whether there would be any cases of muscle dysmorphia (mostly in early stages of development) among these men. The second sample, the so-called control sample, consisted also of 30 individuals who were selected following certain sample criteria, such as: gender, age, marital, and professional status.

Both samples had to fill out the following standard questionnaires: the Hamilton Anxiety Rating Scale, the Beck Depression Inventory, a screening questionnaire regarding possible issues related to physical appearance, the Adonis Complex Questionnaire, and the Altered Personality Questionnaire (H. Schmiescheck). Regardless what sample they belonged to, all subjects had to fill out each of the questionnaires.

RESULTS

1. Comparative sample analysis. Socio-demographic features

The mean age of the research sample was 22.9 years (with a standard deviation of 3.67), while for the control sample the mean age was 23.96 years (with a standard deviation of 13.64). Comparative sample distribution is shown below:

Age	Research sample		Control sample	
	Subjects. no	%	Subjects. no	%
<21 years old	3	10.00	3	10.00
21-30 years old	26	86.67	26	86.67
31- 40 years old	1	3.33	1	3.33

According to stratified sampling, the percent distribution of age categories is the same in both the control and the research sample.

Due to the fact that both the population mean and the standard deviations are unknown variables, associated with the clinical nature of the research sample, and the construction of the control sample using quote sampling, the Liliefors test had to be used in order to estimate data variance. As a result the normal distribution hypothesis proves false, thus requesting the use of non-parametric tests for data analysis.

Clinical parameters analysis

Levels of anxiety, as measured with Hamilton Anxiety Scale

The Hamilton Anxiety Scale consists of 14 items that quantify several anxiety related features, such as: anxious mood, fear, insomnia, cognitive symptoms, depression, various physical behaviours and symptoms, and muscular strain. Each item is scored on a scale of 0-5 (0 = lack of symptoms, 5 – severe and impairing symptoms). The scores range from 0 to 56, any score higher than 14 indicates clinically significant anxiety, dividing the remaining categories as follows: 14-17 for mild anxiety levels, 18-24 for moderate anxiety levels, and 25-30 for severe anxiety levels.

a) Case distribution in the research sample depending on anxiety levels is shown below:

Anxiety level	Research sample			
	Subject no.	Cumulative frequency	Percent	Cumulative percent
Minimal or no anxiety (score < 14)	25	25	83.3	83.3
Mild anxiety level (scores 14-17)	1	26	3.3	86.6
Moderate anxiety level (scores 18-24)	3	29	10.0	96.6
Severe anxiety levels (scores 25-30)	1	30	3.4	100.0

Up to this point research data show that 13.4 % of the subjects included in the research sample exhibit moderate and severe levels of anxiety.

b) Case distribution in the control sample depending on anxiety levels is shown below

Anxiety level	Control sample			
	Subjects no.	Cumulative frequency	Subjects no.	Cumulative percent
Minimal or no anxiety (score < 14)	29	29	96.7	96.7
Mild anxiety level (scores 14-17)	1	30	3.3	100.0
Moderate anxiety level (scores 18-24)	0	30	0.0	100.0
Severe anxiety levels (scores 25-30)	0	30	0.0	100.0

None of the subjects in the control group exhibits severe or even moderate anxiety, while only one subject was found to report mild anxiety; all in all, 96.7% of subjects in the control sample exhibit no clinically significant anxiety. The difference between the two percentage values (13.4% vs. 0.0%) is statistically significant (p = 0.042), thus results indicate that research subjects exhibit moderate and severe anxiety in a significantly greater respect than control subjects.

By comparing the mean values between the two samples, we get the following results which are shown below:

Sample	Median Value	Minimal value	Maximum value	Standard deviation
Control sample	3.0	0.0	14.0	4.36
Research sample	2.0	0.0	28.0	8.10

Thus, in spite of the small number of subjects in both samples, subjects in the research sample tend to display moderate and severe anxiety in a greater proportion than the control sample, keeping in mind that this is not the case of a normal distribution of variables. We believe that by including more cases in the analysis we could get more significant results, concerning differences between the two samples.

Results of the non-parametric Mann-Whitney U test are as follows:

Sum of ranks in the control sample	Sum of ranks in the research sample	U	Z	p-level
900.5	929.5	435.5	-0.214	p=0.826>0.05 not statistically significant

Sample differences are not statistically significant, showing that all in all there are no notable differences between the two samples concerning anxiety levels. Yet we believe that this result can be determined by the relatively small number of subjects included in the present study.

Levels of depression, as measured with Beck Depression Inventory

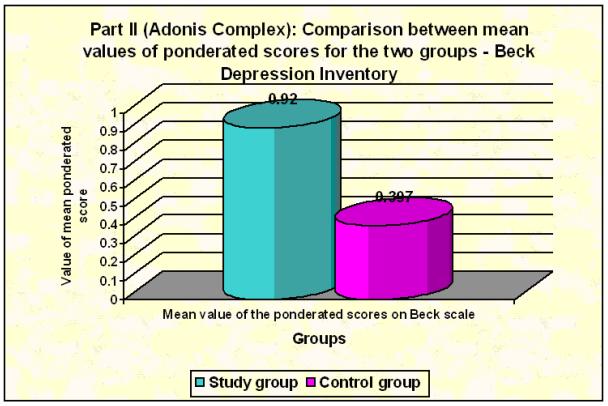
The inventory contains 21 questions, each answer being scored on a scale value of 0 to 3. The cut-offs used differ from the original: 0–13: minimal depression; 14–19: mild depression; 20–28: moderate depression; and 29–63: severe depression. Higher total scores indicate more severe depressive symptoms. Level categories are as follows:

0.5 – 1.2 – Mild depression
1.2 – 2 – Moderate depression
2 – 2.5 – Severe depression
2.5 – 3 – Severe depression, risk of suicide
The sample situation is as follows:

Sample	Median value	Minimum	Maximum	Standard deviation
Control sample	0.28	0.0	1.67	0.39
Research sample	1.0	0.0	2.19	0.65

Results show the fact that the mean value in the research sample as well as the control sample indicates the absence of depression (<0.5).

A chart showing these differences is given below:



To determine whether the above mentioned differences are of statistic significance, we used the non-parametric Mann-Whitney U test. Results are shown below:

Sum of ranks in the control sample	Sum of ranks in the research sample	U	Z	p-level
1118.0	712.0	247.0	3.00	p=0.002<0.05 Statistically significant

There is a statistically significant difference concerning depression between the two samples, namely general depression level is higher in the research sample than in the control sample, corresponding to a mild depression level (0.92).

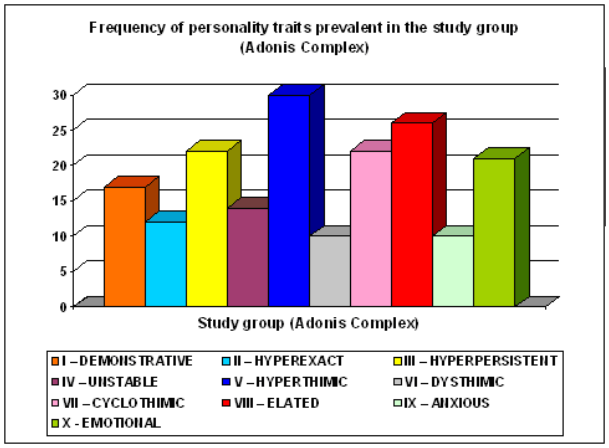
Comparing frequencies of altered personality traits in the two samples

Considering the particular way of scoring and interpreting the Altered Personality Questionnaire, we compared frequencies in the two samples, concerning the prevalence of all 10 personality types and their corresponding features.

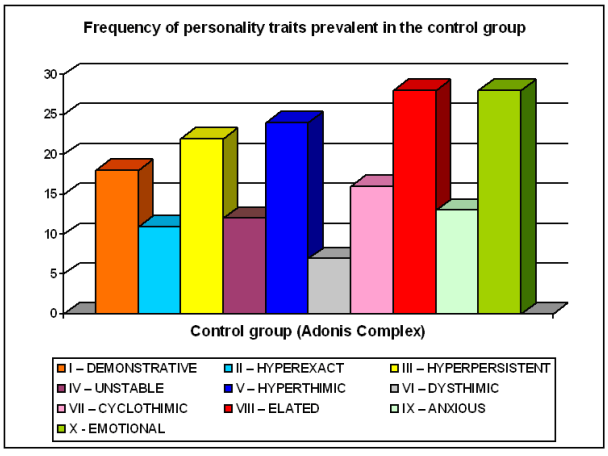
Generally a particular personality feature is taken into consideration if subjects report at least 50% of its characteristics (o score of 50% or more). Results are shown below:

Personality feature	Research sample		Control sample	
	Subjects no.	%	Subjects no.	%
I – Histrionic	17	56.7	18	60.0
II – Obsessive-compulsive	12	40.0	11	36.7
III – Paranoid	22	73.3	22	73.3
IV – Borderline	14	46.7	12	40.0
V – Manic	30	100.0	24	80.0
VI – Low mood	10	33.3	7	23.3
VII – Emotionally unstable	22	73.3	16	53.3
VIII – Elated	26	86.7	28	93.3
IX – Anxious	10	33.3	13	43.3
X - Emotional	21	70.0	28	93.3

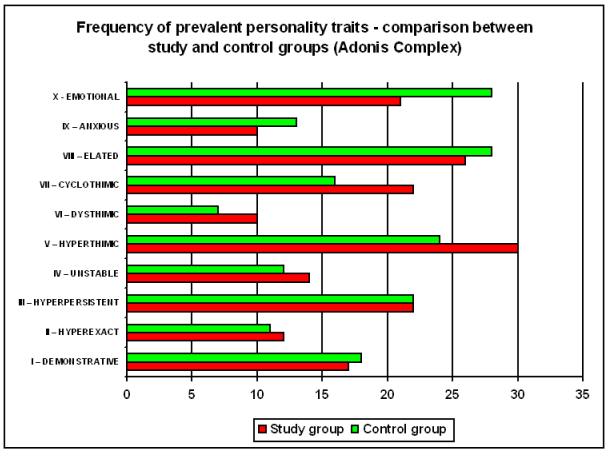
A graph showing personality profiles for both samples is shown below:



Thus, in the research sample there is a prevalence of personality traits corresponding to following personality types: manic (100%), elated (86.7%), paranoid (73.3%) and emotionally unstable (73.3%). These are common features in 60% of the research subjects.

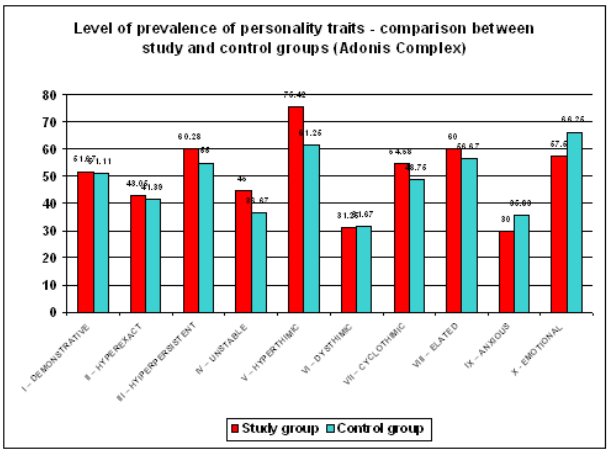


Several personality features, corresponding to the manic, elated, and paranoid personality type are also present in the control sample (60% of the subjects); however, taking the following graph into account, all altered personality traits (except those corresponding to the elated type) are less frequent in the control sample than in the research sample. The χ^2 test does not show significant differences between frequencies.



Thus, results indicate that personality features related to the manic, emotionally unstable, low mood, obsessive-compulsive, and borderline personality types prove to be more frequent in the research sample, while in the control sample there is a higher frequency regarding other personality types, such as the emotional, anxious, elated, and histrionic type.

All in all, most important frequency differences were found between the two samples, namely concerning the manic personality type (100% in the research sample vs. 80% in the control sample), as well as the emotionally unstable type (73.3% in the research sample vs. 53.3% in the control sample). According to the classical interpretation of this questionnaire, we have identified several altered personality traits in the research sample, corresponding to the manic, paranoid, elated, emotional, emotionally unstable, and histrionic types, while results regarding the control sample indicate other features, related to the emotional, manic, paranoid, and emotionally unstable personality types.



As far as the research sample is concerned, one can notice the high intensity of certain personality features, like those corresponding to the manic, paranoid, and emotionally

unstable types; the comparison test Mann-Whitney indicates statistically significant differences between the two samples only what the manic personality type is concerned. Results are shown below:

Altered personality features	Sum of ranks in the research sample	Sum of ranks in the control sample	U	Z	p
I – Histrionic	920.0	910.0	445.0	0.074	0.941
II – Obsessive-compulsive	933.0	897.0	432.0	0.266	0.790
III – Paranoid	993.0	837.0	372.0	1.153	0.249
IV – Borderline	1001.0	829.0	364.0	1.276	0.203
V – Manic	**1121.0**	**709.0**	**244.0**	**3.047**	**0.002 Statistically significant**
VI – Low mood	905.5	924.5	440.5	-0.140	0.883
VII – Emotionally unstable	982.5	847.5	382.5	0.998	0.318
VIII – Elated	969.5	860.5	395.5	0.806	0.424
IX – Anxious	858.5	971.5	393.5	-0.835	0.403
X - Emotional	803.5	106.5	338.5	-1.648	0.099

All in all, we can conclude that subjects in the research sample (Adonis complex) exhibit certain personality traits corresponding to the manic type more frequently and at a higher intensity level than subjects in the control sample.

The Adonis Complex Questionnaire

The questionnaire contains 13 items, each with 3 possible answers, scoring from 1 to 3 (answer a = 1, answer b = 2, answer = 3), corresponding to the frequency/intensity of each measured factor.

The measured features are as follows:

• subject's preoccupation regarding physical appearance (amount of time spent thinking about this issue), as well as the feelings involved;

• active avoidance behaviour due to this preoccupation;

• active behaviour in order to correct the perceived defect (amount of time spent exercising, dieting, consumption of diet supplements);

• social impairment due to subject's behaviour and beliefs related to muscle size and shape (amount of money spent, avoidance of interpersonal intimacy, social impairment);

• risk behaviour: health issues related to the above mentioned behaviour (substance abuse, extreme training methods).

The total score is obtained by adding the scores obtained for each of the 13 items.

What our sample is concerned, results show that there are more subjects in the research sample than in the control sample who: spend more time worrying about their physical appearance, more frequently feel unhappy due to this preoccupation; spend more time performing grooming activities and physical training, and more frequently tend to abuse dietary supplements in order to improve muscle size and shape.

These preoccupations related to physical appearance greatly influence subjects' daily life: most of the subjects in the research sample (13.3% vs. 0% in the control sample) report that the amount of money spent in order to improve their appearance have frequently caused financial issues. In addition to this, most subjects in the research sample have report that this preoccupation has „sometimes" or even „frequently" affected their social interactions, the level of intimacy, and work performance, as well as several other social activities.

What risk substance consumption is concerned, both samples reported „never/rarely" or „sometimes" exhibiting this behaviour, with comparable scores in both cases.

All in all, results indicate a distinct profile for subjects in the research sample, compared to those in the control sample, featuring: excessive preoccupation for muscle size and shape, social impairment, active behaviour used for mending this issue, namely a special diet, as well as intense and excessive physical training.

In order to check if there is a significant difference between the two samples, we used the Mann-Whitney comparison test. Results indicate significant differences regarding the intensity of preoccupations related to physical appearance, prolonged physical training, as well as the total score.

Correlations between general test scores obtained by the research sample (subjects with muscle dysmorphia)

We used the correlation test Spearman R in order to check whether there are any significant correlations between subjects' scores regarding the evaluation methods we used. Results indicate the following significant correlations:

• between the general BDI score and the total Hamilton score ($R = 0.599$, $p < .05$), namely subjects who exhibit higher levels of depression also tend to report higher levels of anxiety;

• the more severe a subject's level of depression, the weaker the belief that his physical appearance is normal ($R = -0.644$, $p < .05$);

• the more severe a subject's anxiety level, the more he will try to avoid social contact ($R = 0.414$, $p < 0.05$), make use of more methods in order to improve muscle size and shape ($R = 0.375$, $p < .0.05$), feel more unhappy because of excessive preoccupation related to physical appearance ($R = 0.421$, $p < .05$), and avoid being seen by others ($R = 0.431$, $p < .0. 05$).

DISCUSSION

Following data analysis up to now, several conclusions can be drawn, regarding the existence and possibility of identifying certain characteristics, as well as personality traits concerning individuals who might suffer from the Adonis Complex.

Regarding the two samples involved in this study, results indicated the following:

1. Subjects in the research sample exhibit higher levels of moderate and severe anxiety than subjects in the control sample.

2. General depression level in the research sample proves higher than in the control sample (mild depression vs. absence of depression).

3. 60% of the subjects in the research sample exhibit certain altered personality features, mostly corresponding to the manic, elated, paranoid and emotionally unstable types; these features, excluding those related to the elated type, prove to be less frequent in the control sample. Significant differences were found between samples only related to the manic personality type, with higher scores in the research sample.

4. In comparison to the control sample, subjects in the research sample also tend to: spend more time worrying about their appearance, feel unhappier because of their excessive preoccupations with muscle size and shape, spend more time exercising, and are more prone to specific dietary supplement consumption.

5. In addition to this, subjects with muscle dysmorphia report higher levels of anxiety and depression, correlated with a negative self evaluation pattern, as well as a greater level of distress caused by excessive preoccupation related to their physical appearance, sustained by a tendency to avoidance behaviour.

As shown above, results are consistent with other research findings on muscle dysmorphia, even if our samples lack a consistent number of subjects, and our study variables indicate the prevalence of certain aspects only in a small degree. In this case this preliminary study allows us to extend our research and investigation, by adding a larger number of participants, more complex evaluation measures, as well as by sample diversification. Future research will be conducted according to these findings.

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CARACTERISTICI PSIHOLOGICE ŞI TRĂSĂTURI DE PERSONALITATE ÎN COMPLEXUL ADONIS

REZUMAT

Obiective: În acest studiu preliminar pe populaţie românească, autorii au investigat prevalenţa şi corelaţiile între anumite aspecte psihologice şi trăsături de personalitate implicate în circumscrierea diagnostică şi clinică a Complexului Adonis (dismorfiei musculare). **Metodă:** Au fost analizate nivelele de anxietate şi depresie, prevalenţa anumitor trăsături accentuate de personalitate, similare celor descrise la nivelul tulburărilor de personalitate din DSM-IV-TR şi ICD-10, precum şi frecvenţa anumitor caracteristici specifice Complexului Adonis la un lot de 30 de subiecţi de sex masculin. Studiul a utilizat Scala de Anxietate Hamilton, Inventarul de Depresie Beck, Chestionarul pentru Personalităţi accentuate şi Chestionarul pentru evaluarea complexului Adonis, pentru a caracteriza lotul de studiu, alcătuit din 30 de bărbaţi care frecventează sălile de fitness de cel puţin 3 ori pe săptămână. **Rezultate:** Prezenţa posibilelor simptome specifice complexului Adonis a fost asociată cu nivele uşoare respectiv moderate ale anxietăţii, respectiv cu simptome uşoare ale depresiei. La nivelul lotului de studiu s-au înregistrat anumite trăsături accentuate de personalitate, corespondente tulburărilor de personalitate hipertime, paranoide, ciclotime şi borderline. **Concluzii:** Rezultate studiului permit asocierea anumitor caracteristici atribuite complexului Adonis cu indici de depresie şi anxietate la nivel subclinic. Aceste rezultate sunt similare cu alte studii. Prevalenţa trăsăturilor de personalitate se încadrează pe direcţia cercetărilor anterioare, sugerând comorbiditatea tulburării cu anumite tulburări de personalitate. Aceste concluzii permit efectuarea de cercetări viitoare cu scopul de a institui noi metode mai eficiente de a depista şi eventual interveni în cazul persoanelor ce suferă de Complexul Adonis.

Cuvinte cheie: Complexul Adonis, dismorfie musculară, anxietate, depresie, personalitate accentuată, comportament secundar

PARTICULARITIES OF LIPID METABOLISM DISORDERS IN PATIENTS WITH DIABETES MELLITUS AND THYROID DISEASE

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ABSTRACT

Increased serum cholesterol, triglycerides, or both, as well as low HDL cholesterol, characterize dyslipidemia. They may be primary (genetic) or secondary. Their diagnosis is usually biochemical by determining total cholesterol, triglycerides and various lipoprotein fractions. Lipid metabolism disorder treatment consists in lifestyle changes, exercise and administration of lipid-lowering drugs. The main consequence of appropriate untreated dyslipidemia is appearance of cardiovascular disease or worsening of a preexisting one.
Secondary dyslipidemia represent the diseases frequently associated with diabetes mellitus and thyroid disorders. The purpose of this study was to determine the main types of lipid metabolism disorders seen in patients with different changes in glycemic balance and thyroid disorders. General group studied was represented by 733 cases, aged 7-79 years. The study group was subdivided by age criterion in two groups: children and adult group. They used clinical, radiological, biochemical, hormonal, immunological parameters.
Keywords: diabetes mellitus, thyroid diseases, dyslipidemia, hypercholesterolemia, hypertriglyceridemia, cardiovascular disease

INTRODUCTION

Lipid metabolism disorders are frequent diseases in the general population. Increased serum cholesterol (TC), triglycerides (Tg) or both, as well as low HDL-cholesterol (HDLc) characterizes the lipid metabolism disorders. They may be primary (genetic) or secondary. The secondary one is frequently associated with unbalanced diabetes mellitus, obesity, sedentary lifestyle, being more common in industrialized than in developing countries. In all epidemiological and interventional studies, they represent a risk factor for coronary heart disease. Their diagnosis is usually biochemical by determining total cholesterol, triglycerides and various lipoprotein fractions. Lipid metabolism disorder treatment consists in lifestyle changes, exercise and administration of lipid-lowering drugs.

Initial the lipid metabolism disorders were classified by Fredrickson on ultra centrifugation followed by electrophoresis in five types: type I, IIa, IIb, III, IV and V.

Type I may be primary or secondary (very rare). It is due to family deficiency of LPL (lipoprotein lipase), of apoprotein C II or of liver triglycerides lipase (HPL). The disease is rare, being described, in total, about 100 cases to date, and is manifested by episodes of abdominal pain (with an aspect of acute abdomen when the Tg exceed 1500 mg %), recurrent, due to a marked increase in chylomicrons.

Type IIa is primary in the most majority of cases (essential hypercholesterolemia). Familial forms appear through mutations of the responsible gene of LDL receptor synthesis, whose total or partially deficits determine remain of CT outside the cells, resulting in the appearance at the offspring that inherit the gene, of the early and severe arteriosclerosis.

Table I. WHO classification of hyperlipoproteinemias (HLP) (modified) (17)

Type	Cholesterol	Triglycerides	Excess lipoproteins	ELFO	Serum aspect
I	(+)	+++	Chylo	origin	Cream stratum, clear infranatant
IIa	+++	N	LDL	beta	clear
IIb	+++	+	LDL + VLDL	Beta + pre-beta	opalescent
III	++	++	IDL	Broad beta	opalescent
IV	(+)	+++	VLDL	Pre-beta	latescent
V	+	+++	Chylo + VLDL	Chylo at start + pre-beta	Cream stratum, latescent infranatant

Type IIb is present in 0.5% of the population. The primary family form is called familial combined HLP. Genetic defect is not precisely known, it seems that this is an increased of hepatic synthesis of apo B100. The third of those who inherit this tare, the clinical- biologically type is IIa, at the third IIb and at the rest type IV. Even at the same person may be encounter different types at

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different times, depending on influence of environmental factors, diet; characteristic for this type is early arteriosclerosis.

Type III (yellow palms disease, named due by palmary-digital xantomatosis) is rare (1 in 5000) and, in majority, primary. Genetic defect is abnormal inheritance of apoE, which is not recognized by specific hepatic receptors.

Type IV is quite frequently (0.2 to 0.3%) and can be primary (familial endogenous hypertriglyceridemia - unknown genetic defect) or secondary, carbohydrate-induced, were the most frequently associated with diabetes mellitus, but also with obesity, food rich in carbohydrates, alcoholism and estrogen contraceptives.

Type V (endo-and exogenous familial hypertriglyceridemia) is rare ($1/10^6$ subjects). Genetic defect is not precisely known, and the symptoms are similar to type I. Atherosclerotic events may exist, but are rare than in types II, III and IV.

The main causes of lipid metabolism disorders are represented by diabetes mellitus, obesity, hypothyroidism and nephrotic syndrome. The consumption of drugs, alcohol, food rich in carbohydrates, acute pancreatitis and pregnancy may also determine it.

Unbalanced diabetes mellitus (DM), both type 1 and type 2, is one of the most common causes of hypertriglyceridemia, which may be more severe in the presence of ketosis. Usually after restoration of glycemic balance by insulin (in type 1 diabetes) or oral drugs (OAD) (in type 2 diabetes) is restore also the normal blood lipid values.

Hypothyroidism is frequently associated with increased LDLc, mixed dyslipidemia and sometimes with isolated Tg increases.

Nephrotic syndrome is commonly associated with increased LDLc, mixed dyslipidemia and sometimes with isolated Tg increases. Increased levels of proteinuria are correlated with the severity of hyperlipidemia.

Some medications such as high doses of thiazide diuretics or clorthalidon, high doses of beta-adrenergic blockers, except those with intrinsic sympathomimetic activity, estrogen replacement therapy, and oral contraceptives containing high doses of estrogen, tamoxifen and corticosteroids are involved in occurrence of lipid metabolism disorders.

Other causes of lipid metabolism disorders are represented by excessive alcohol consumption, diet rich in carbohydrates (> 60% of calories), acute pancreatitis and pregnancy.

The prevalence of lipid metabolism disorders in U.S. is about 10% in men aged over 30 years and in women aged over 55 years. (5)

The prevalence of severe hypertriglyceridemia, characterized by values of Tg over 2000 mg /dl, is estimated at 1.8/10 000 at adult white race, their prevalence is higher in patients with diabetes mellitus and alcoholism. (5)

The lipid metabolism disorders are associated with an increased risk of cardiovascular disease, especially in case of lower HDL and/or increase LDLc. If these changes are controlled, some studies have shown that elevated Tg values do not correlate with an increased risk of cardiovascular disease. (12). Other studies suggest, however, that elevated Tg is an independent risk factor for cardiovascular disease. (3). Since the metabolism of triglycerides (chylomicrons, VLDL) and HDL metabolism are interdependent and because of Tg level lability, impact of hypertriglyceridemia on cardiovascular risk is difficult to confirm. Randomized clinical trials have used medication that lowers Tg showed decreased coronary events in primary and secondary prevention programs for coronary artery disease. (4, 6, 9, 12)

If the Tg value growth over 1000 mg/dl may appear acute pancreatitis.

Regarding race, it was found that lipid metabolism disorders are more common in Africans than in white American race. (5)

In the prospective Cardiovascular Munster study (PROCAM), medium hypertriglyceridemia was more common in men (18.6%) than in women (4.2%). (1)

The lipid metabolism disorders are more common in males until age 50 years, and then slightly decreases. In women, they continue to increase with age. The Tg values over 150 mg/dl are more common in men from age 30 years and in women from the age of 60 years. (5)

MATERIAL AND METHODS

Investigated population

The study included subjects with diabetes mellitus, which in time present thyroid disease, or subjects with thyroid disease who subsequently present diabetes mellitus.

The study group comprised 733 cases aged 7-79 years. Subjects were divided as follows:

• group of children that included 83 children and adolescents aged 7-17 years (14.57 ± 2.25 years), with a ratio F/M of 5.9/1.

• group of adults that included 650 adults aged 18-79 years (52.03 ± 12.46 years), with a ratio F/M 9.48/1.

Methods of investigation

Methods of investigation were the clinical data - history, present status, and imaging - ultrasound thyroid, biochemistry - carbohydrate metabolism parameters: fasting blood glucose, urine glucose, glycosylated hemoglobin and thyroid hormone investigations and some immunological parameters.

Glucose determination was performed by enzymatic techniques with glucose oxidase. Were considered normal fasting blood glucose between 70-110 mg%, diabetes mellitus - fasting blood glucose values above 126 mg%, impaired glucose tolerance - fasting glucose values between 110-126 mg% and the oral glucose tolerance test (OGTT) at 2 h between 140-200 mg% and fasting impaired glucose tolerance - fasting glucose values between 110-126 mg% and OGTT at 2 h under 140 mg%.

Determination of glycated hemoglobin (HbA1c) was achieved through the DiaStat program for glycated hemoglobin HbA1c who measures the ratio of glycated hemoglobin to total HbA.

Determination of serum levels of TSH, free fraction of serum level of triiodothyronine (FT_3), free fraction of thyroxin (FT_4) were ARCHITECT quantitative method, which is an immunologic determination by chemiluminescence's with small

Chemilumnescent Micro particle Immunoassay (CMIA) The following values were considered normal: TSH = 0.465 to 4.68 mIU/ml, FT_3 = 3.69 -10.4 pmol/l, FT_4 = 10 to 28.2 pmol/l.

Immunological parameters were represented by some markers of thyroid autoimmunity – antiTPO and antiTG antibodies (AB). To determine the serum titers of AB antiTPO Axsym antiTPO kit was used, the method is enzyme immunoassay with micro particles, Meia (Micro particle Enzyme Immunoassay). It was considered normal: AB antiTPO (<35 IU/ml). To determine the serum titers of AB antiTG Axsym antiTG kit was used, the method is enzyme immunoassay with micro particles, Meia (Micro particle Enzyme Immunoassay). It was considered normal: AB. antiTG (<55 IU/ml). Thyroid ultrasound performed in all cases is a non-invasive method of exploration that allows measurement of thyroid volume, thyroid study report with cervical anatomical structures and thyroid parenchyma changes. Appearance of normal thyroid parenchyma is characterized by a high intensity echogenic, homogeneous, easily distinguishable from the neck muscles, which look hypoechogenic. Inflammatory and autoimmune processes are hypoechogenic. The degree of thyroid hypoechogenity was assessed as: discreet +, moderate + + and marked + + +. In autoimmune thyroid disease is found hypoecogenity of thyroid parenchyma. The Graves' disease appears: thyroid volume generally increased, hypoechogenity with different intensities with variable homogeneity. Chronic autoimmune thyroiditis appears: hypoechogenity uneven and normal or increased thyroid volume.

The "fasting" lipid profile in peripheral blood was assessed by determining the total cholesterol (CT), triglycerides (Tg), HDL-cholesterol (HDLc) and LDL-cholesterol (LDLc) fractions, and the ratio TC/HDLc. They used laboratory methods based on the enzyme principle for both CT (Dimension AR, Dade Behring Inc., USA) and Tg and HDLc (REflectron IV, Roche, Switzerland). The LDLc level fraction was calculated according to Friedwald's formula: LDL = CT - (HDLc + Tg/5). Were considered normal: CT <200 mg%, Tg <150 mg% and HDLc> 45 mg% in males and > 50 mg% in females, LDLc <115 mg%, CT/HDLc between 2 to 3.5 (7, 11, and 8).

RESULTS AND DISCUSSION

The group of children and adolescents was represented by 83 subjects, aged 7-17 years (**Tab. II**). All children take in the study had type 1 diabetes.

Table II. Distribution by age and sex of children and adolescents group

Age	Number of cases		Female		Male	
	n	%	n	%	n	%
0- 4 years	-	-	-	-	-	-
5-9 years	2	2.4	2	100	-	-
10–14 years	32	38.56	22	68.75	10	31.25
15–17 years	49	59.04	47	95.92	2	4.08

Dyslipidemia is one of the most common metabolic abnormalities from diabetes mellitus and one of the most important cardiovascular risk factors (in adulthood) present in this disease.

The normal lipid parameters are as follows (**Tab. III):**

Table III. The normal lipid parameters (16)

Lipid parameters	Normal values
Total serum cholesterol (CT)	<190 mg%
Serum triglycerides (Tg)	<180 mg%
HDL-cholesterol (HDLc)	>40 mg%
LDL-cholesterol (LDLc)	<115 mg%

To assess the type of dyslipidemia was used European Atherosclerosis Association classification, which divides them according to severity (**Table IV**):

Table IV. The classification of dyslipidemia according to European Arteriosclerosis Association (16)

Dyslipidemia type	Classification	Lipid fractions (mg %)
Hypercholesterolemia	mild medium severe	CT = 200-239 CT = 240-300 CT > 300
Hypertriglyceridemia	medium severe	Tg = 200-400 Tg > 400
Mixed hyperlipemia	medium severe	CT = 200-300 or Tg = 200 – 400 CT > 300 or Tg > 400

According to this classification, at the group of children take in the study we obtained the results presented in **Table V**.

Table V. Disturbances types of lipid metabolism in children and adolescents group

Dyslipidemia type	Classification	Children and adolescents group (n = 83)	
		n	%
Hypercholesterolemia		6	7.22
	mild	4	4.81
	medium	-	-
	severe	2	2.4
Hypertriglyceridemia		1	1.2
	medium	1	1.2
	severe	-	-
Mixed dyslipidemia		0	0
	medium	-	-
	severe	-	-
Normal		56	67.46
Another changes		20	24.09
- decreased Tg		19	22.89
- decreased CT		1	1.2
- decreased Tg and CT		-	-

In the study group, on the first place were the normal

values (67.46%), followed by other changes in lipid metabolism (24.09%), then by hypercholesterolemia (7.22%) and by hypertriglyceridemia (1.2%).

All the 7 people with lipid metabolism disorders were female. The glycemic balance was unsatisfactory in 4 cases (fasting blood glucose over 150 mg% and HbA1c over 8%). The LDLc was over 160 mg% in 3 cases, and low HDLc was found in two cases.

In type 1 diabetes appear classic" diabetic lipemia" which is characterized by hypertriglyceridemia (Tg = 500-1000 mg %) promptly reversed after insulin therapy and metabolic balance.

The hyperlipemic mechanisms involved are increased of VLDL-Tg production with decreases HDLc, decreased lipoprotein lipase (LPL) activity, increased lipolysis and free fatty acids (FFA). These mechanisms are induced by insulin deficiency or insulin resistance (rare in type 1 diabetes) (16).

Dyslipidemia does not appear in the case of balanced metabolic type 1 diabetes. In the case of type 1 diabetes unbalanced or at onset, are increase Tg and decrease HDLc, eventually grow and LDLc. These changes form the panel of hyperlipoproteinemia type IV (hypertriglyceridemia) and/or type II B hyperlipoproteinemia (mixed hyperlipidemia).

A number of studies in young show that the prevalence of dyslipidemia is rare among them. It is influenced primarily by the level of glycemic control (10). A study in Spain showed that the increase in LDLc was found in 16% of cases, increase in triglycerides in 5% of cases and lower HDLc in 20% of cases. The females' persons were more likely predispose to the presence of dyslipidemia. With metabolic balance occurs also the normalizing of lipid metabolism disorders (15).

The adult group consisted of 650 people, young adults and elderly adults, aged between 17 and 79 years (**Table VI**). The subjects with diabetes mellitus, which in time present thyroid disease, and subjects with thyroid disease who subsequently present glycoregulation disorders or diabetes mellitus represented this.

Table VI. Distribution by age and sex of adult group

Age	Number of cases		Female		Male	
	n	%	n	%	n	%
18 – 19 years	11	1.7	10	90.9	1	9.1
20 – 29 years	29	4.46	27	93.1	2	6.9
30 – 39 years	48	7.38	43	89.58	5	10.42
40 – 49 years	168	25.84	141	83.93	27	16.07
50 – 59 years	219	33.7	209	95.43	10	4.57
60 – 69 years	118	18.15	112	94.91	6	5.09
70 – 79 years	57	8.77	46	80.7	11	19.3

The adult group was subdivided in function of the type of glycemic balance in 4 subgroups (Fig. 1):

- group with type 1 diabetes represented by 60 cases (9.23%)
- group with type 2 diabetes accounted for 290 cases (44.61%)
- group with impaired glucose tolerance (IGT) accounted for 183 cases (28.15%)
- group with fasting impaired glucose tolerance (IFG) accounted 117 cases (18 %)

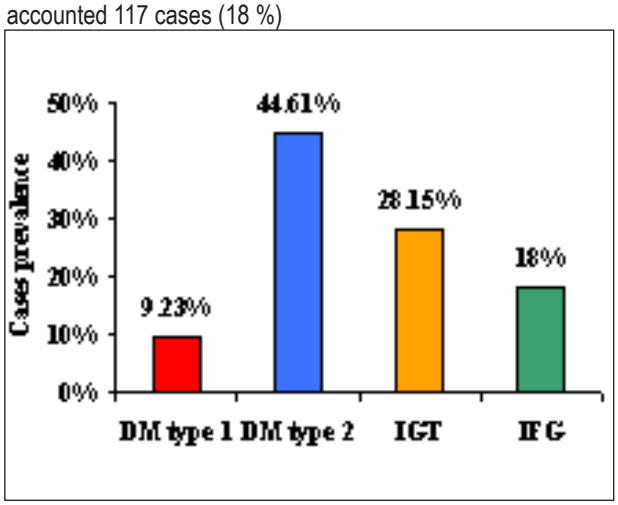

Fig. 1. Distribution of cases by type of changes in glycemic balance

Dyslipidemia does not appear in the case of DM metabolic balance. Dyslipidemia is considered diabetic if it is corrected through optimizing glycemic control (diet, oral hypoglycemic agents or insulin). In general, the characteristics of dyslipidemia in DM are:

- represent 55-65% in unbalanced DM toward to 25% in balanced DM and 20% in non-diabetic population
- the hypertriglyceridemia is predominant toward to hypercholesterolemia (as in the general population)
- it is prevalent in type 2 diabetes (50-70%) toward to type 1 diabetes (20%) due to the combination of dyslipidemia risk factors (14).

It is known that in severe thyroid deficiency appear the following disorders of lipid metabolism:

- the cholesterol is increased (especially LDL-cholesterol)
- the beta-lipoproteins are increased and the ratio beta/alpha-lipoprotein is decreased
- hypertriglyceridemia

Intensity of hyperlipemic syndrome is directly proportional to the degree of thyroid failure. This syndrome can be considered a sign of progression of the disease or of effectiveness of substitution therapy. In the case of subclinical hypothyroidism, it can worsen pre-existing dyslipidemia commonly seen in patients with type 2 diabetes; as a result increases the risk of cardiovascular disease. Adequate replacement therapy with thyroxin will correct the lipid metabolism disorders. In the case of hyperthyroidism the cholesterol is always low. According to this classification, in the group of adults take in the study we obtained results presented in **Table VII.**

Table VII. Types of changes in lipid metabolism in the adult group

Dyslipidemia type/ severity		DM type 1		DM type 2		IGT		IFG	
		n	%	n	%	n	%	n	%
HyperCT		25	41.66	39	13.44	67	36.61	61	52.13
	mild	16	26.66	31	10.68	38	20.76	21	17.94
	medium	9	15	8	2.75	22	12.02	33	28.2
	severe	-	-	-	-	7	3.82	7	5.98
Hyper Tg		2	3.33	21	7.24	12	6.55	3	2.56
	medium	2	3.33	18	6.2	12	6.55	3	2.56
	severe	-	-	3	1.03	-	-	-	-
Mixed HLP		2	3.33	81	27.93	41	22.4	16	13.67
	medium	1	1.66	41	14.13	28	15.3	9	7.69
	severe	1	1.66	40	21.85	13	7.1	7	5.98
Another changes		-	-	5	1.72	4	2.18	5	4.27
Decreased Tg		-	-	1	0,34	3	1.64	2	1.71
Decreased CT		-	-	3	1.03	1	0.54	2	1.71
Decreased Tg and CT		-	-	1	0.34	-	-	1	0.85

In the case of type 1 diabetes group, on the first place was the normal values (51.66%), followed by hypercholesterolemia (41.66%), then by hypertriglyceridemia (3.33%) and by mixed dyslipidemia (3.33%). Between TSH value and lipid fractions was establish a weak, direct correlation ($r = 0.10$, $p<0.001$ for CT and $r = 0.27$, $p<0.001$ for Tg).

In the case of type 2 diabetes group, on the first place was the normal values (31.03%), followed by mixed dyslipidemia (27.93%), then by hypercholesterolemia (13.44%) and hypertriglyceridemia (7.24%). Between TSH and lipid metabolism has established a weak, direct correlation ($r = 0.29$, $p<0.001$ for CT and $r = 0.03$, $p<0.001$ for Tg).

In the case of IGT group, on the first place was hypercholesterolemia (36.61%), followed by normal values (32.24%), then by mixed dyslipidemia (22.4%) and hypertriglyceridemia (6.55%). Between TSH and lipid metabolism has established a weak, direct correlation ($r = 0.42$, $p<0.001$ for CT and $r = 0.27$, $p<0.001$ for Tg).

In the case of IFG group, on the first place was hypercholesterolemia (52.13%), followed by normal values (26.49%), and then by mixed dyslipidemia (13.67%) and other changes (4.27%). Between TSH - lipid metabolism has established a weak, direct correlation ($r = 0.13$, $p<0.001$ for CT and $r=0.05$, $p<0.001$ for Tg).

It is noted that at all types of changes in glycemic control in the adult group predominate hypercholesterolemia and not hypertriglyceridemia, this is likely due to the presence of thyroid disease association.

A study in Kenya shows that in patients with type 2 diabetes hypercholesterolemia were found in 70% of cases, while hypertriglyceridemia only ≈ 30% of cases (13).

CONCLUSIONS

In the case of patients with diabetes mellitus associated with thyroid disease was an increase in the prevalence of lipid metabolism disorders. Among all types of dyslipidemia found the prevalence of hypercholesterolemia and mixed dyslipidemia, which shows a significant influence on thyroid hormone changes on lipid metabolism disorders.

Regarding gender, dyslipidemia was more common in women because the prevalence of thyroid disorders at them.

The thyroid disorders cause significant physiological effects. Subclinical hypothyroidism may increase LDLc and aggravation of preexisting dyslipidemia, increasing thus the risk of atherosclerosis. Subclinical hyperthyroidism may increase the risk of cardiac arrhythmias and may cause worsening of a preexisting ischemic heart disease. Because diabetic patients are an increased risk for cardiovascular disease, early diagnosis and treatment of subclinical thyroid disease is important.

The presence of antithyroid antibodies (antiperoxidase type) allows early detection of autoimmune thyroid disorders, particularly of the hypothyroidism. In those in whom these antibodies are present, thyroid function should be investigated for early detection of any disorder to establish appropriate treatment.

Since dyslipidemia are involved in the occurrence of cardiovascular disease, early detection of patients with diabetes mellitus and associated thyroid disease is necessary.

In the case of patients with type 1 diabetes is necessary to determine antithyroid antibodies antiperoxidase type. If they are present, annual monitoring is required for TSH as early detection of hypothyroidism. If thyroid hypofunction has no major implications for glycemic control, atherosclerotic complications of myxedema adversely affect long-term prognosis of association between diabetes mellitus and hypothyroidism. If they are absent, it is necessary to determine TSH at an interval of 2-3 years.

In the case of patients with type 2 diabetes, TSH determination is necessary at an interval of five years.

Also, to avoid the negative impact of dyslipidemia on the cardiovascular system, it is necessary the correct balance of diabetes, the correct treatment of thyroid disorders associated and the correct treatment of lipid metabolism disorders if they occur.

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PARTICULARITATILE MODIFICARILOR METABOLISMULUI LIPIDIC LA PACIENTII CU DIABET ZAHARAT SI AFECTIUNI TIROIDIENE

REZUMAT

Dislipidemiile se caracterizează prin creşterea colesterolului plasmatic, a trigliceridelor sau a ambelor, precum şi prin scăderea HDL-colesterolului. Ele pot fi primare(genetice) sau secundare. Diagnosticul acestora este de obicei biochimic, prin determinarea colesterolului total, a trigliceridelor şi a diferitelor fracţiuni lipoproteice. Tratamentul tulburărilor metabolismului lipidic constă în modificarea stilului de viaţă, exerciţiu fizic şi administrarea medicamentelor hipolipemiante. Principala consecinţă a dislipidemiilor netratate corespunzător o reprezintă apariţia bolii cardiovasculare sau agravarea uneia preexistente.

Dislipidemiile secundare reprezintă afecţiuni frecvent asociate atât diabetului zaharat, cât şi afecţiunilor tiroidiene. Scopul acestui studiu a fost de a determina principalele tipuri de tulburări ale metabolismului lipidic întâlnite la pacienţii cu diferite modificări ale echilibrului glicemic şi afecţiuni tiroidiene. Lotul general studiat a fost reprezentat de 733 cazuri, cu vârste cuprinse între 7-79 ani.

Lotul studiat a fost subîmpărţit după criteriul vârstei în 2 loturi: lotul de copii şi lotul de adulţi. S-au folosit parametrii clinici, imagistici, biochimici, hormonali, imunologici.

Cuvinte cheie: diabet zaharat, afecţiuni tiroidiene, dislipidemie, hipercolesterolemie, hipertrigliceridemie, boală cardiovasculară

BENEFITS OF THYMECTOMY IN MYASTHENIA GRAVIS

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ABSTRACT

Myasthenia Gravis (MG) is an autoimmune disease, caused by a direct auto-antibodies attack at the level of postsynaptic membrane acetylcholine receptors of the neuromuscular junction, characterized by fluctuating weakness and fatigability of skeletal muscles.

Thymectomy represents an important link in Myasthenia Gravis treatment, because in some patients has determined the remission and improving in others the frequency and the intensity of myasthenic bursts.

Key words: myasthenia, thymectomy, improvement, immune response.

INTRODUCTION

Myasthenia Gravis is a chronic autoimmune disorder of the acetylcholine receptor, which evolve in bursts, being determined by an autoimmune conflict at the level of neuromuscular junction. The cause of Myasthenia Gravis is still unknown. Normal synaptic transmission is interrupted by the auto-antibodies, which blocks the functional $AchR_s$. As a consequence, these auto-antibodies together with other immunological effectors are capable to destroy the receptors, finally resulting in low number of available receptors (1).

Thymic myoid cells may be responsible for the autoimmune response, in our view T cells being sensitized against myoid cells. The significant cytokines activity was reported in several studies and was founded in thymus of MG patients.

Physiopathology and Immunopathology

In brief, specific antibodies for acetylcholine receptors in Myasthenia Gravis are: AchR, MuSK, Titin, Ryanodine, and Rapsyn. Regarding myasthenia, in the 1980's, much immunological research was centered on auto-antibodies. AchR specific antibodies were found to belong of polyclonal IgG, predominantly, but not exclusively to IgG1 and IgG3 (2). They are capable to react with different epitopes on the $AchR_s$ surface (3). A variable and large proportion of the serum antibodies bind to the one main epitope on each on the two-α subunits of the receptor, also called the immunogenic active region (4). AchR rarely recognizes recombinant or synthetic peptide fragments. When activated, the complement system (membrane attack complex), will cause the lysis of muscle cell membrane, and internalization of $AchR_s$ in the membrane, increasing in this manner their degradation rate.

Thymectomy

The right definition of thymectomy with regards on myasthenia gravis is the Keesey point of view, otherwise known as Keesey's algorithm (5).

"Thymectomy is recommended for those relatively healthy patients whose myasthenic symptoms interfere with her personal life, long enough from them to accept a major surgical intervention. Although it is expensive and invasive treatment, thymectomy is currently the only treatment modality that offers any chance of remission without medication. Potential benefit of thymectomy diminishes with the age of patient and the natural involution of the thymus. In addition, surgical risk increases with age. The age at which the risk exceeds the potential benefit, must be individualized for each patient".

Indications for thymectomy

- presence of thymoma, confirmed by CT, indication at any age
- generalized myasthenia without thymoma
- corticotherapy for at least month before surgery
- minimal myasthenic deficit (QMG score < 10 points)
- respiratory capacity > 60

Surgery classes recommended by Myasthenia Gravis Foundation of America for Myasthenia Gravis:

- T1- Trans-cervical thymectomy: standard and extended
- T2- Videoscopic thymectomy: classic (VATS- Video-Assisted Thoracoscopic Surgery) and extended (VATET-Video-Assisted Thoracoscopic Thymectomy)
- T3- Trans-Sternal Thymectomy: standard and extended
- T4-Trans-cervical and Trans-sternal Thymectomy (maximal)

Complications of thymectomy:

Immediate: pneumothorax / hemothorax; atelectasis by bronchus blockage with viscous mucus; hematoma / serohematic

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flooded in anterior mediastinum; pericarditis; cardiac rhythm disorders; paresis of phrenic nerve or recurrent laryngeal nerve; myasthenic crisis.

Late: prolonged angina pectoris; wound or sternum infection; mediastinitis; thymic relapse can occur 5-10 years after thymectomy - a period of improvement or remission of symptoms. This was due to incomplete resection of thymus or persistent autoimmune thymic ectopia of mediastinal fat.

Thymoma

Thymoma is defined as a neoplasm of the epithelial-reticular framework cells of the thymus. In the normal thymus, these cells regularly displayed branching tonofilaments, maculae adherents, elongated processes, and basal lamina. Thymomas are composed of a mixture of neoplastic epithelial cells and non-neoplastic lymphocytes, with proportion among them varying widely from case to case and in different lobules of the same tumor (6). Table I shows the morphological features of thymoma, according with WHO classification, 2004.

Table I. Morphological aspects of thymoma (WHO classification, 2004)

Type A	A tumor composed of neoplastic epithelial cells, spindle or oval in shape, inconspicuous nucleoli, without nuclear atypia, and few or no lymphocytes.
Type AB	A tumor which consists of areas similar to those from A thymoma but mixed with areas lymphocyte-rich, the border between being sharp or less distinct.
Type B1	The tumor resembling the typical thymus appearance, associating areas similar to the thymic cortex with foci with medullary differentiation. The cortical areas are prevalent and in excess compare to the small medullary areas. The neoplastic epithelial cells are scant, small and dispersed in the lymphocytic component.
Type B2	A tumor composed of large plump/polygonal neoplastic epithelial cells, with vesicular nuclei and distinct nucleoli, the tumoral cells are usually outnumbered by the nonneoplastic lymphocytes. The perivascular spaces are common.
Type B3	Tumor composed predominantly of round/polygonal neoplastic epithelial cells; the nucleoli are less prominent, with mild nuclear atypia and with a poor lymphocytic component. The perivascular spaces and squamous metaplasia are common.
Thymic carcinoma	Thymic tumor with loss of organotipical differentiation of the organ and with clear cytological atypia, generally similar with that encountered in other organs. There is a lymphocyte population which is mature.

Myasthenia gravis is associated with thymic hyperplasia in 65% of clinical cases and thymoma in 10 % of them. Thymoma is present in a percentage about 30-45% of myasthenia gravis cases (7).

About the oncogene proteins with a potential antigenic proliferation in thymoma, Bcl-2 oncogene reveals several features that could be taken in discussion. This oncogene Bcl-2 is responsible for the mechanism that desensitizes thymocytes. On the other hand, an interesting remark is the fact that this oncogene protein may trigger the auto reactive thymocyte apoptosis, and its presence in the thymic medulla may be similar with the presence of inflammatory cells, possible mimicking the inflammation of the lymph nodes.

It was already discussed that the development of Myasthenia Gravis in patients with thymoma is probably caused by the sensitization of developing T-cells against autologous muscle-like antigens, including the musacrinc and the acetylcholine receptor, exposed by the neoplastic tissue.

Immunohistological insights

According to Salakou (2001), the presence of apoptotic bodies in hyperplasic thymus and in the thymic carcinoma, may be an important objective that could be investigate with regards on anatomo-pathology of myasthenic thymus (Figure 1 A and B).

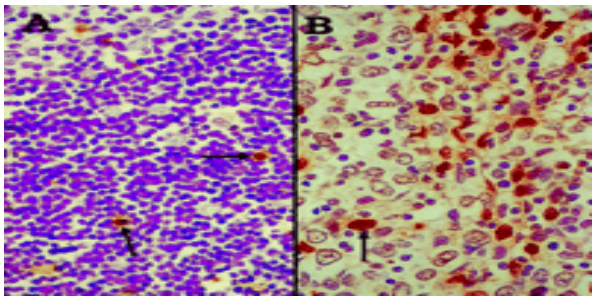

Fig. 1. Apoptotic bodies in hyperplastic thymus (TUNEL-peroxidase 100X) (A) and apoptotic bodies in a thymic carcinoma (TUNEL peroxidase 400X) (B) (After Salakoa, 2001).

The anatomo-pathological and clinical special evidence of patients with Myasthenia Gravis and thymus involvement should outline the following data: gender, age, Osserman classification, duration of the disease prior to surgery (months), medication, and previous plasmapheresis, original histology diagnosis (hyperplasia, atrophy, thymoma, thymic carcinoma, and degenerated thymic lipoma), thymus size (cm^3), thymus weight (gr).

Autoimmune myasthenia gravis can be variable in its clinical characteristics. The clinical data of myasthenia gravis patients subgroups include: Early-onset myasthenia gravis (<40 years, anti-AChR antibodies, thymic hyperplasia), Late-onset myasthenia gravis (>40 years, normal thymus, anti-AChR antibodies, titin antibodies, ryanodine receptors antibodies), thymomatous myasthenia gravis (thymoma, anti AChR antibodies, titin antibodies, ryanodine receptors antibodies, KCNA4 antibodies), anti MuSK myasthenia gravis (anti-MuSK antibodies, normal thymus), anti-AChR and anti-MuSK negative myasthenia gravis (sero-negative generalized myasthenia gravis, thymic hyperplasia, antibodies against clustered AChR-70% of clinical cases) and Ocular myasthenia gravis (anti-AChR antibodies in 50%

clinical cases).

Anyway, individual clinical evidence studies focused on thymectomy, have reported results that shows the validity and benefits of thymectomy *via* surgical techniques. In comparative studies there is not clear evidence yet in order to establish which thymectomy technique is accurate, superior and benefic in the management of myasthenia gravis and patient comfort.

CONCLUSION

Thymectomy represents an important link in the treatment of Myasthenia Gravis because in some patients has determined the disease remission and in others has improved the bursts in frequency and intensity. A correct pre-thymectomy management reduces the post-operator complications rate, the hospitalization period and also the number of the adjacent costs. Thymectomy combined with corticosteroid therapy is more beneficial in treating patients with non-thymomatous Myasthenia Gravis than corticosteroid therapy alone.

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BENEFICIILE TIMECTOMIEI ÎN MIASTENIA GRAVIS

REZUMAT

Miastenia Gravis (MG) este o boală autoimună cauzată de atacul direct al autoanticorpilor asupra receptorilor pentru acetilcolină de la nivelul membranei postsinaptice a joncţiunii neuromusculare, caracterizată prin slabiciune fluctuantă şi fatigabilitate a muşchilor scheletici. Timectomia reprezintă o verigă importantă în tratamentul Miasteniei Gravis, deoarece la unii pacienţi a determinat remisia, iar la alţii ameliorarea puseelor miastenice, ca frecvenţă şi intensitate.

Cuvinte cheie: miastenie, timectomie, ameliorare, răspuns imun

EXPERIMENTAL NEUROPHYSIOLOGICAL ALTERATIONS CAUSED BY COMBINED NANO-MANGANESE EXPOSURE

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ABSTRACT

Manganese (Mn) is a well known heavy metal, causing central nervous system damage after chronic exposure. To model inhalational Mn exposure, we used an animal model system in which male Wistar rats were intratracheally instilled daily with $MnCl_2$ (2.5 or 5.0 mg/kg bw.) dissolved in distilled water; or with nanosuspension of MnO_2 (2.5 or 5.0 mg/kg bw.); or with the combination of 2.5 mg/kg solute $MnCl_2$ and 2.5 mg/kg nanosized MnO_2, administered with a short delay. After the 5 weeks treatment period open field activity of the rats was tested and electrophysiological investigation was performed; spontaneous and stimulus evoked action potentials were recorded from somatosensory, visual and auditory areas of the cortex. Mn could reach the CNS and caused significant alterations in the spectral distribution of the ECoG bands, increased the latency of the evoked potentials and influenced the locomotor activity of the rats. The observed effects showed a kind of additive effect in case of the combined exposure.
Keywords: manganese, nanoparticle, intratracheal instillation, evoked potentials, rat

INTRODUCTION

Manganese (Mn) is an essential micronutrient, cofactor of enzymes (Mn-SOD, GS), but has toxic effects after chronic overexposure. It is often used in industry: in dry cells, coated welding rods, steel alloys, chemical fertilizers or pesticides. Technical applications of manganese (Mn) often results in human exposure, mostly due to inhalation of metal dust and fumes (ATSDR, 2008) causing mitochondrial dysfunction and leading to oxidative stress and excitotoxicity in the neurons (Taylor et al., 2006). Inhibition of voltage-gated Ca-channels and disturbed release of neurotransmitters are also typical of Mn intoxication. Mn evolves its deteriorating effects manly on the central nervous system. Manganism, a chronic human neurological disorder resembling Parkinson's disease (Bowler et al., 2006) often occurs among welders and miners exposed to Mn-containing aerosols (Dobson et al., 2004). These dusts and fumes, arising from soldering and welding contain Mn nanoparticles as well.

Inhalational Mn exposure may result in massive internal doses, depending on the size of the inhaled particles. Microscopic particles cannot pass the blood-brain barrier, but submicroscopic particles (that is, nanoparticles, with dimension 100 nm and below) have high mobility within the organism, so these can have direct access to the CNS (Oberdörster et al., 2000, 2005). According to this, it is worth to pay more attention on the effects of inhaled Mn on the nervous system and its dependence on the different physicochemical forms. In this study we aimed to explore the alterations in the CNS caused by solute and nanosized and their combinations.

MATERIALS AND METHODS

Adult male Wistar rats obtained from the Breeding Centre of the University (300-320 g body weight at start) were housed in an air conditioned room maintained at 22 ºC, with 12-hour light/dark cycle (light on at 06:00) and free access to tap water and standard rodent chow.

Animals were divided into seven groups (with 8 animals each) and were treated intratracheally with $MnCl_2$ dissolved in distilled water (*LD*: 2.5 mg/kg b.w. $MnCl_2$; *HD*: 5 mg/kg b.w. $MnCl_2$) or with MnO_2 nanoparticles (*nLD*: 2.5 mg/kg b.w. MnO_2; *nHD*: 5 mg/kg b.w. MnO_2). In *Comb* group, rats received 2.5 mg/kg b.w. $MnCl_2$ and the same dose of MnO_2 with a short delay. The MnO_2 nanoparticles (NPs) were synthesized by a technique combining ultrasonic and hydrothermal treatment (for details, see Sárközi et al., 2009) at the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics. MnO_2 NPs were suspended in hydroxyethyl cellulose (swollen in phosphate buffered saline) which was used as vehicle and that the rats in vehicle control (*VC*) group were treated with. An untreated control (*Cont*) group was also applied.

Intratracheal instillation (1 ml/kg b. w.) was carried out in brief diethyl ether anaesthesia. The treatment lasted for 5 weeks, performed once a day, 5 days per week.

Before the beginning of the treatment period and on the day following the last instillation, the rats were put into an open field (OF) box to test their spontaneous horizontal and vertical motor activity in 10 min sessions.

Electrophysiological measurement was done after anesthetizing the animals with urethane (1000 mg/kg b. w., i.p.). The left hemisphere was exposed, the animals were put in a stereotaxic device, and silver electrodes were placed on the primary somatosensory (SS), visual (VIS) and auditory (AUD) areas. Spontaneous electrical activity (electrocorticogram, ECoG) was

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recorded from these sites simultaneously for 6 min, and the relative spectral power of the frequency bands was determined (Kandel and Schwartz, 1985).

Stimulus-evoked activity was then recorded via the same electrodes. Somatosensory stimulation was done by electric pulses given trough a pair of needles inserted into the whiskery skin (3-4 V; 1000, 500, 100 ms repetition time). Visual stimulation was performed by flashes (1 Hz) delivered by a high-luminescence white LED directly into the contralateral eye of the rat. For acoustic stimulation, clicks (1 Hz, 40 dB), were applied into the ear of the rat. Fifty stimuli of each modality per rat were applied and the evoked activity recorded. After averaging, latency and duration of the evoked responses was measured.

The body weight of the animals was regularly measured during the 5 weeks of the experiment.

Statistical analysis was done by two-sample t-test.

During the whole procedure, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed.

RESULTS

Mn administration caused significantly slowed body weight gain in all treated groups (vs. *Cont*), from the first week on (Fig. 1).

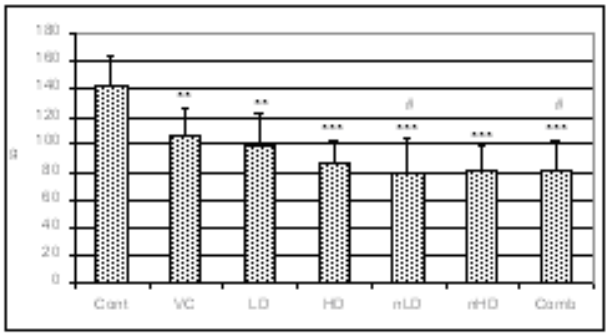
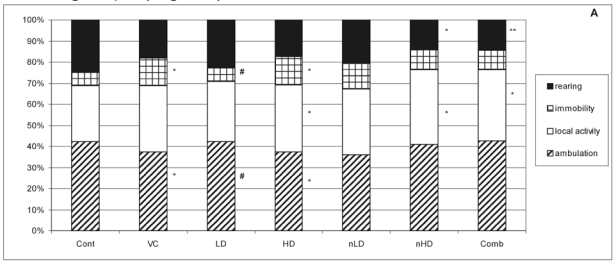


Fig. 1 Body weight gain. *,**,*** p<0.05, 0.01, 0.001 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. *VC*.

The open field test after the 5 weeks treatment period showed decreased motor activity in all the treated rats. Increased immobility and decreased rearing was observed both in the $MnCl_2$ and MnO_2 treated groups. The time spent with local activity increased and showed mild significance in *HD*, *nHD* and *Comb* groups (Fig. 2a). Changes in activity counts were similar to the above mentioned alterations, confirming that the OF activity of the rats shifted to hypomotility, especially in $MnCl_2$ treated and *Comb* groups (Fig. 2b).



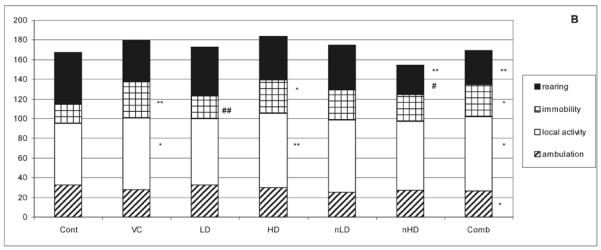


Fig. 2 Effect of $MnCl_2$ and MnO_2 treatment on the OF activity of the rats after the treatment. (A:time B:count) *,**,*** p<0.05, 0.01, 0.001 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. *VC*.

Intratracheal instillation of $MnCl_2$ and MnO_2 significantly altered the spectral distribution of the electrocorticogram. The general trend of the ECoG band spectrum change was different in each modality depending on the treatment type (solute or nano-sized Mn). $MnCl_2$ treatment had a strong effect on theta band and the increase was significant in each cortical area (vs. *VC*). Effect of high dose MnO_2 treatment was mainly seen on the VIS and AUD ECoGs. On the VIS ECoG, the proportion of the fast frequency bands increased and that of the slow bands decreased. On the contrary, spectral distribution of the AUD ECoG showed decrease in fast bands, and increase in slow bands. These changes were significant in case of gamma and delta bands (Fig. 3).

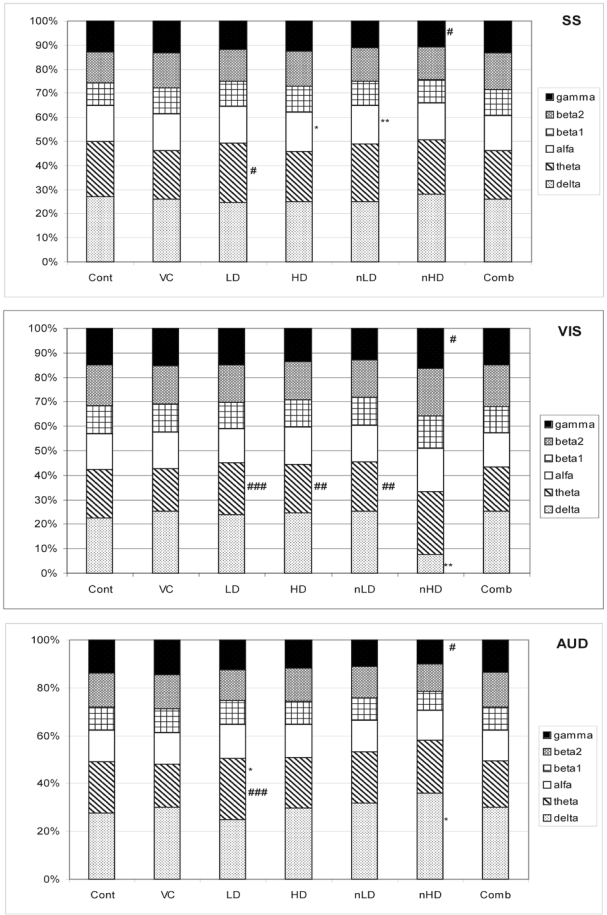


Fig. 3 Frequency spectrum of the spontaneous cortical activity. (SS-somato-sensory, VIS-visual, AUD-auditory area) *,**,*** p<0.05, 0.01, 0.001 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. *VC*.

From the parameters of the cortical evoked potentials, lengthening of the SS latency, caused by $MnCl_2$ treatment, was the most prominent. MnO_2 treatment did not significantly affect the SS latency, but in *Comb* group a combined effect of the two forms of Mn was seen (lower, but significant increase; Fig. 4). SS amplitude was only affected in *nLD* group, where the increase was significant. In case of fast stimulation (10 Hz), the same trend was discovered, but the effect of the combined treatment – strong decrease – appeared to be significant (Fig. 5). Between VIS and AUD latency there was an opposite alteration, similarly to the ECoG findings (Fig. 6).

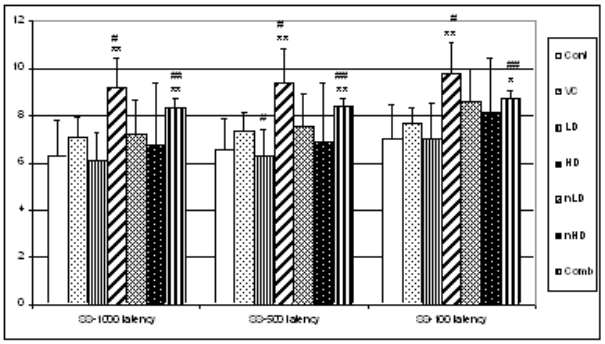


Fig. 4 Comparison of the latency of somatosensory evoked potentials of the treated groups. *,**;*** p<0.05, 0.01, 0.001 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. *VC*.

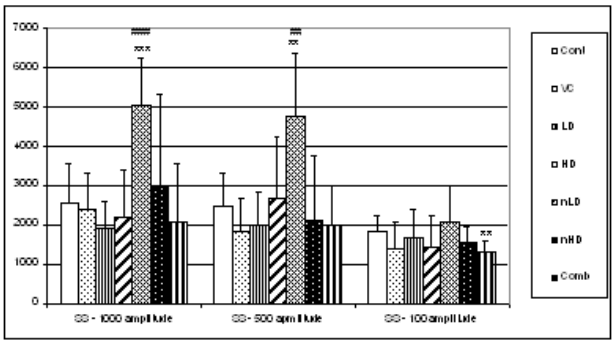


Fig. 5 Comparison of the amplitude of somatosensory evoked potentials of the treated groups. *,**;*** p<0.05, 0.01, 0.001 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. *VC*.

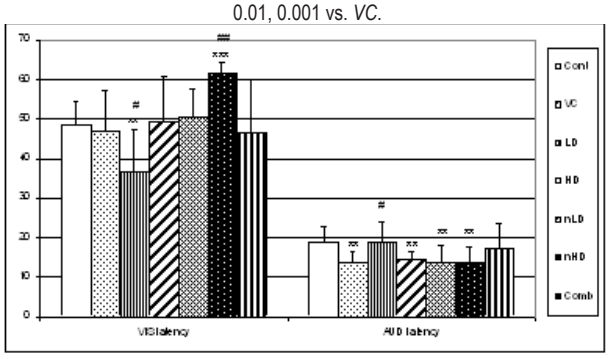


Fig. 6 Comparison of the latency of visual and auditory evoked potentials of the treated groups. *,**;*** p<0.05, 0.01, 0.001 vs. *Cont*; #, ##, ### p<0.05, 0.01, 0.001 vs. *VC*.

DISCUSSION

According to the observed changes in the electrophysiological parameters the applied animal model proved to be suitable for intratracheal Mn intoxication follow up. As it was indicated in previous experiments (9), Mn had access to the CNS and the observed electrophysiological alterations were caused by Mn accumulation in the brain.

In the regulation of OF activity, which is known to be affected by Mn (Normandin et al., 2004), dopaminergic structures play important role (Alexander et al., 1990). Alterations in cortical evoked activity due to Mn can be explained by Mn-dependent inhibition of astrocytic glutamine synthetase (10). Desensitization and slowed action of the thalamocortical afferents (due to enhanced glutamatergic transmission) might cause lengthening of the latency of the cortical evoked potentials. On the other side, nerve conduction can be damaged as well. Mn might also interfere with Ca channels (11) and with mitochondrial energy production (12).

Decrease of slow (delta, theta) and increase of fast (beta, gamma) bands of the ECoG (or the opposite in VIS-AUD ECoG) in the same rats might have resulted from impaired collateral input of the glutamatergic afferents. Altered EEG and event-realated potentials, which was found in this experiment in VIS and AUD recordings, were also described in case of human occupational Mn exposure.

The results further suggested that electrophysiological tests might be more sensitive to the effects of Mn than general toxicological or neurobehavioral ones, which is potentially relevant both in experimental work and in hygienic toxicology.

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ALTERARI NEUROFIZIOLOGICE EXPERIMENTALE DETERMINATE DE EXPUNEREA COMBINATA LA NANOPARTICULE SI MANGAN

REZUMAT

Manganul (Mn) este un metal greu bine cunoscut, care determina injurii la nivelul sistemului nervos dupa expunerea cronica. Pentru a crea un model de expunere inhalatorie la Mn, am folosit un model animal, in care sobolanii masculi Wistar au fost instilati zilnic intra-traheal cu $MnCl_2$ (2,5 sau 5,0 mg/kg corp), dizolvata in apa distilata, sau cu nanosuspensie de MnO_2 (2,5 sau 5,0 mg/kg corp); au fost folosite si combinatii de 2,5 mg/kg solutie $MnCl_2$ si 2,5 mg/kg MnO_2 in suspensie de nanoparticule, care au fost administrate consecutive, intr-un interval de timp scurt. Dupa o perioada de 5 saptamani de tratament, animalele au fost testate din punct de vedere al activitatii si s-au efectuat investigatii electrofiziologice; au fost inregistrate potentiale de actiune evocate si spontane la nivelul ariilor corticale somato-senzoriale, vizuale si auditorii. Mn a ajuns la nivelul SNC si a determinat alterarea semnificativa a spectrului de distributie al benzilor ECoG, a indus latenta potentialelor evocate si a influentat activitatea locomotorie la sobolani. Efectele observate au aratat un efect aditiv in cazul expunerii combinate la ambele solutii.
Cuvinte cheie: mangan, nanoparticlule, instilare intra-traheala, potentiale evocate, sobolan