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REVIEW

THE RACE TO CURE DIABETES: HOW FAR ARE WE FROM A BREAKTHROUGH?

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ABSTRACT

Currently recognized as a major public health concern of both highly industrialized and developing countries, diabetes, a metabolic disorder affecting more than 220 million people worldwide, warrants a definitive cure. Exogenous insulin supply, the current treatment, is not fully capable of achieving tight control of glucose regulation, while pancreatic islet transplantation is not a viable solution due to the shortage of donors. This mini-review discusses progressive therapeutic initiatives launched in the quest for the cure of diabetes, from gene therapy to cellular replacement using embryonic or mesenchymal stem cells converted to β-pancreatic cells, immunotherapy and combinations of the above. Although the article addresses mainly autoimmune-derived diabetes, type 2 diabetes mellitus will equally benefit from β-cell replacement and regenerative therapies.

Key words: diabetes, embryonic stem cells, mesenchymal stem cells, gene therapy, autoimmune, immunotherapy, transplantation

BACKGROUND

Frederick Banting, one of the co-discoverers of insulin, recognized in his Nobel Prize lecture in 1923 that 'Insulin is not a cure for diabetes; it is a treatment'. After almost a century since the revolutionary discovery, exogenous insulin supply still represents the main therapeutic option for Type 1 diabetes (T1D) patients and is beneficial for some severe Type 2 diabetes (T2D) cases, but it is far from being perfect and it may seem primitive from a current medical perspective. Insulin therapy is not fully capable of achieving a tight control of glucose metabolism, being often associated with an increased risk of hypo- or hyperglycemic episodes ("the yo-yo effect"). Even the latest insulin analogues fail to prevent long-term complications of high blood glucose levels that include severe damage to the microvasculature and nerves (1) and ensuing diseases, like diabetic nephropathy or diabetic retinopathy.

Transplantation of pancreatic islets under the Edmonton protocol (2) offers a better approach than simply administering insulin. However, it is obvious that only a small number of patients worldwide will be able to benefit from this therapeutic approach that would still require long-term immunosupression regimens and patient monitoring (3). Islet allotransplantation can provide insulin independence for a limited period of time (4), but it represents proof-of-concept that cell-based treatments for T1D can be effective.

In Type 1 diabetes mellitus, the insulin-secreting β-cells in the pancreatic islets of Langerhans are selectively and irreversibly destroyed by an autoimmune attack. As diabetes is caused by the destruction of only one type of highly specialized cell, it follows logically that therapeutic approaches should focus on replacing this particular cell type. Overall, two distinctive pathways can be considered as means of obtaining functional β-cells: gene-therapy or factor-based differentiation of stem or progenitor cells. Gene-therapy refers to the in vivo or in vitro transfer of a foreign gene into any type of cell, allowing it to produce insulin. The foreign gene can be insulin or another gene (e.g. transcription factors) that can in turn activate or repress on demand the insulin gene. The factor-based approach involves exposing stem cells to a cocktail of growth factors and cytokines over an extended period of in vitro culture, followed by transplantation of the differentiated insulin-producing cells into the receiving diabetic patient. In the present review, we wish to highlight the successes attained in each of these fields and also the hurdles still needed to be overcome before a safe, effective, and widespread therapeutic application is possible. Although the long road from treatment to cure is still unwinding, the new developments in gene and cell-based therapy could remove in the near future diabetes from the list of incurable, chronic diseases. In the same time, personalized genomics that would allow identification of certain risk genes predisposing to diabetes mellitus could dramatically improve disease prevention.

NEW CONCEPTS, OLD PROBLEMS

Embryonic and adult stem cells for diabetes

The minimum properties sought for in a surrogate β-cells have been considered before (5, 6). One requirement is that a significant number of replacement cells will be needed as current transplantation protocols replace approximately 2-4x 10^9 β -cells per recipient and a significant fraction is lost immediately post-transplant due to procedure or host factors. Mature β-cells, although long-lived, have very limited proliferative capacity, unlike progenitor or stem cells, which represent a more suitable candidate for expansion and directed in vitro differentiation due to their intrinsic plasticity. However, an essential prerequisite of any potential stem cell therapy and its translation into clinics is to ensure that differentiation was efficient and no residual stem cells are implanted into patients. These uncommitted stem cells might prove detrimental on account of spontaneous differentiation to divergent lines or unchecked proliferation resulting in the formation of tumors.

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The most important question is which stem cells would represent an appropriate starting material: embryonic or adult stem cells? The use of allogeneic or xenogeneic embryonic stem cells will most likely generate immune rejection that has to be controlled in addition to underlying autoimmunity, while the patient's own tissue stem cells will be less likely to pose this problem (7) although the issue of autoimmune response may still pose difficulties.

In recent years, many research groups have reported the creation of insulin-producing cells from tissue progenitor or stem cells by means of transdifferentiation (i.e. the switching of a committed cell's fate towards another distinct phenotype); however, earlier, promising studies have so far failed to translate into reliable protocols for generating functional β-cells (8, 9). Bone-marrow derived mesenchymal stem cells (MSCs) have been a favorite candidate for differentiation studies due to their availability, ease of isolation, apparent plasticity and immunosuppressive properties (10, 11). However, recent studies demonstrated that MSCs enhance the regeneration of endogenous β-cells in experimental diabetes mouse models rather than repopulate the pancreas by transdifferentiation (12). Microenvironment manipulation differentiation studies on other types of adult stem cells, including neural progenitor cells (13), umbilical cord-blood stem cells (14), multipotent acinar pancreatic stem cells (15), liver progenitor cells (16), in the absence of any genetic manipulation, resulted in only partial successes.

The first notable progress in the field of factor-based differentiation towards β-cells came from studies that applied developmental principles to stem cell biology, recapitulating embryonic differentiation in vitro (17, 18). The protocol required first a definitive endoderm specification of the embryonic stem cells, then the step-wise generation of pancreatic progenitors, endocrine progenitors and mature β-cells. Human embryonic stem cells were exposed initially to activin A, a member of the TGFβ family, in the absence of serum, resulting in the efficient formation of definitive endoderm. Later, TGFβ signaling was eliminated and the cell cultures were exposed to retinoic acid, which initiated the endocrine lineage commitment. The Baetge group (19) arrived at this model by quantitatively monitoring gene expression in the differentiating human embryonic stem cells after manipulation of culture environment using a combination of growth factors and signaling molecules. The same group reported more recently the obtaining of glucose-responsive mature endocrine cells in mice after a three months period of in vivo incubation (20).

It is not surprising that the embryonic stem cells were the first to be differentiated to β-cells, given their well-documented plasticity. Additionally, these cells can proliferate indefinitely in vitro, in an undifferentiated state, thus being capable of supplying the high number of cells required for transplantation. However, long-term immunosuppression would be a prerequisite in this case, the embryonic stem cells and their insulin-producing derivatives behaving essentially as an allograft upon transplantation.

An alternative that could circumvent the ethical considerations and immune rejection issues associated with embryonic stem cells seem to be the induced pluripotent stem cells (iPS), a sort of surrogate ESC pioneered by Yamanaka's laboratory in 2007 (21, 22). The discovery that human somatic cells, including those from patients with T1D (23), can be reprogrammed to a state of pluripotency highly similar to that of embryonic stem cells, marked a turning point in regenerative medicine. These autologous iPS cells, can be differentiated into insulin producing cells by a protocol similar to that employed for hESC (24) and used as an autograft that would obviously not require immunosupression. Original methods for obtaining iPS cells involved the viral transduction of four transcription factors associated with pluripotency (Oct4, Sox2, Klf4 and c-Myc) in the target cells, but subsequent studies reported the generation of iPS cells using nonintegrative transfer vectors better suited for future clinical applications, and even membrane-soluble recombinant proteins (25). Although a promising alternative to ESC, the iPS cells still need to be more thoroughly characterized and their features and potential limitations understood correctly before any clinical studies are to be performed.

The functional architecture of the β-cell

The pancreatic β-cell is a highly differentiated and complex cell that harbors several mechanisms critical for the regulated transcription and translation of proinsulin, a controlled secretory pathway and a stimulus-secretion coupling, which may not be individually unique to β-cells, but together they sustain the homeostatic purpose of β-cells. The β Langerhans cell is able to monitor circulating glucose levels because it expresses the high capacity glucose transporter GLUT2 and glucokinase. These elements would be necessary characteristics of any candidate cell, however they are not sufficient to reconstruct the glucose-responsive system of the actual β-cells, as it was demonstrated by the attempt to express GLUT2 and GK in intermediate pituitary cells, which resulted in a defective glucose metabolism and induction of apoptosis (26). In vivo, insulin secretion is also influenced by other fuel secretagogues (27), such as leucine, and can be regulated by incretin hormones (GIP, GLP-1) (28) or as a result of neuronal input. The response of engineered cells to all of the stimuli above, in a tightly coordinated fashion, does not seem very likely to be achieved with current gene therapy tools.

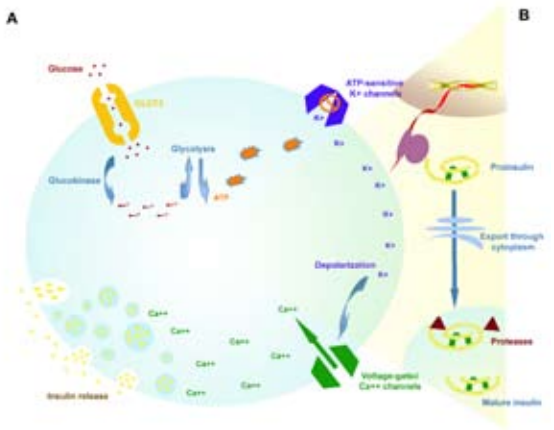

Fig. 1 A Signal transduction in β-cells. Glucose is transported into the β-cells on the high-capacity GLUT2 transporter and metabolized within the cell, generating ATP, which binds to ATP-sensitive K^+-channels, leading to the closure of those channels. The inhibition of K^+ efflux leads to plasma membrane depolarization and opening of voltage-dependent Ca^{2+}-channels. Extracellular Ca^{2+} enters β-cells resulting in elevated concentrations of intracellular Ca^{2+} and initiation of insulin secretion. **B** Insulin translation and post-translational processing. After insulin is released from the β-cell by exocytosis, specific transcription factors bind to the insulin gene promoter and upregulate insulin transcription into preproinsulin mRNA, leading to proinsulin biosynthesis in the ER. Proinsulin is exported to the Golgi vesicles where it will be packed into secretory granules. Only here will proinsulin be cleaved by PC2 and PC3 endoproteases and carboxypeptidase H to yield mature insulin and C-peptide.

Insulin secretion can be stimulated within minutes after exposing the β-cell to a high glucose concentration. Glucose is transported and metabolized within β-cells, generating ATP, which binds ATP-sensitive K^+-channels, leading to the closure of channels. The inhibition of K^+ efflux leads to β-cell plasma membrane depolarization and opening of voltage-operated Ca^{2+}-channels. Extracellular Ca^{2+} enters β-cells resulting in elevated intracellular Ca^{2+}, activation of Ca^{2+}-sensitive downstream signaling and rapid discharge of insulin stored in secretory granules by exocytosis (29) (Figure 1). This first phase of insulin secretion is followed by a gradually increasing second phase that lasts as long as glucose stimulation is applied (30). Normal glucose homeostasis absolutely depends on rapid and biphasic kinetics of insulin release.

In order for insulin to be released from mature insulin secretory granules as a biologically active protein, it must be first cleaved from proinsulin by β-cells-specific endoproteases PC2 and PC3 and carboxypeptidase-H. In turn, proinsulin production in the β-cell is predominantly regulated at the level of translation (31, 32) by heteronuclear mRNA processing and control of preproinsulin stability. In the long-term, translational control is supplemented by transcriptional regulation involving a variety of transcription factors, and among these Pdx-1, NeuroD1 and MafA play a crucial role.

Should we make a β-cell?

If we consider all the fine-tuning mechanisms we need to engineer and put into a candidate β-cell, the answer is a definite 'No'. It is also negative from the point of view of Type 1 diabetes as an autoimmune chronic condition associated with the perpetual generation and activation of autoreactive T cells recognizing pancreatic β-cell autoantigens. Presently, there is still no complete list of these β-cell autoantigens, but the closer we will come to β-cell identity, the more likely the newly engineered transplanted cell will be subject to autoimmune destruction. A very important question is how close do we have to come to imitating a β-cell in order to achieve a normal metabolic control of glycemia and still somehow avoid (self) destruction by the diabetic patient's immune system.

Gene-therapy for diabetes

The possibility of ectopic insulin expression by gene transfer in a non-β-cell has been explored since 1983 and further experiments have shown that simple replacement of insulin expression is not likely to be useful as a therapy for T1D unless it contains a system to regulate insulin levels in response to minute variations of blood glucose. Considering the complex machinery of stimulus-secretion coupling in β-cells, a successful gene therapy for diabetes should deliver in the target cells, separately or in combination, at least two key elements: a regulatory system responsive to glucose concentrations, and appropriate processing of proinsulin into mature, biologically active insulin.

One method to achieve glucose-regulated insulin release involves glucose-responsive promoters linked to the insulin gene that ensure activation of transcription of the insulin gene under hyperglycemic conditions and inhibition under hypoglycemic conditions. Several research groups have experimented with liver-specific LPK (L-type pyruvate kinase) and glucose-6-phosphatase promoters in liver cells (33). Still, transcriptional stimulation of gene expression takes no less than 2 hours, while the 'off' response depends on the half-life of preproinsulin mRNA, which is at least of 6 hours (34), not even comparable to the few minutes response time necessary in vivo.

Another hurdle of insulin gene therapy - correct proinsulin processing in non-β cells - has been overcome by engineering a mutated version of the proinsulin gene, in which the aminoacid cleavage sites recognized by β-cell-specific proteases have been replaced with the sites recognized by the more ubiquitary protease furin (35). A different approach focused on expressing a modified single-chain insulin (36), lacking C-peptide, which has a higher biological activity than proinsulin, but still only about 30% of that of the normally-occurring insulin, and does not require any enzymatic cleaving.

Obviously, such somatic insulin gene therapy that attempts to replace every element involved in the physiological function of β-cells, has very small chances of being successful. Non-β-cells possessing some of the characteristics of our target are better suited in the experimental design. For instance, hepatocytes have a glucose-sensing system, involving GLUT2 and glucokinase, but do not contain proinsulin processing enzymes and secretory granules, while neuroendocrine cells on the other hand contain components of the regulated secretory pathway, including PC2 and PC3 and secretory granules. Another type of cells has received some attention – the K cells (37), an endocrine cell type located in the gut that can secrete the incretin hormone GIP. These cells seem to be a very good target for insulin gene therapy as they show glucose responsiveness and exocytosis and contain the necessary endoproteases.

Taking their cue from recent work on iPS cell formation (21, 22, 38), the group of Douglas Melton pointed to the possibility of converting one type of cell into another directly, without reverting to an undifferentiated pluripotent state first and exemplified this concept by reprogramming, in vivo, adult pancreatic exocrine cells to β-cells (39). The key point of this strategy, similar to that used for iPS cells, is the delivery of a combination of β-cell specific transcription factors singled out by mutational studies on pancreatic embryogenesis (40). Three transcription factors (Ngn3, Pdx1 and MafA) were transduced into the murine pancreas by means of adenoviral vectors, resulting in extra-insular secretion of insulin from cells that closely resemble β-cells in size, shape and gene expression. It is still unclear whether the same method can be successful in vitro, in the absence of signals from the pancreatic microenvironment, however, it might prove to be a more logical alternative to the laborious genetic reconstruction of β-cell mechanisms of metabolic control.

Combined therapy for type 1 diabetes

Cell replacement therapeutic options, as presented above, are purposefully designed to restore the diminishing β-cell mass in T1D subjects; unfortunately, the newly transplanted β-cells will require protection from the recurrence of islet-specific autoimmune attack. Ideally, we should address both the cause and the consequence of type 1 diabetes (41) in a combined therapy pairing β-cell replacement or regeneration with immunomodulation. Self-reactive effector T lymphocytes responsible for the selective destruction of insulin-producing cells could be tempered by enhancing local suppressor lymphocyte subpopulations, explicitly CD4+CD25+FoxP3+ regulatory T cells (Tregs) (42), as means of immunotherapy. The loss of frequency or function of Tregs has already been proposed as one mechanism that contributes to the development of type 1 diabetes (43), therefore a method of controlled induction of functional Tregs without causing generalized immune suppression would likely prove beneficial, if not necessary, as an adjuvant therapy for diabetes. Nevertheless, other pathways of immune intervention have been explored besides the expansion of Tregs: encapsulated islets as synthetic immune barriers, induction of apoptosis in the effector T cells, overexpression of anti-apoptotic genes in the pre-diabetic islets (44), co-infusion of mesenchymal stem cells with transplanted β-cells (11), use of anti-inflammatory agents, such as IL-1R antagonists, for improving β-cell survival (45).

Mesenchymal stem cells (MSCs), which generate minimal immune reactivity and have anti-inflammatory (46) and immunomodulatory (47-49) effects, have become favourites in recently initiated or on-going clinical trials involving the use of stem cells for the treatment of diabetes mellitus, matching the more comprehensive interest in the potential of MSCs to alleviate abnormal immune responses in graft-versus-host-disease (50), solid organ transplantation (51) and Crohn's disease, among others. The precise therapeutic mechanism through which MSCs would be able to treat T1D, either by release of regulatory cytokines such as TGF-β or IL-10 or by cell-cycle inhibition of lymphocyte proliferation (52), has yet to be conclusively determined. Furthermore, should we be able to unlock the transdifferention capacities of MSCs in the direction of β-cell replacement, this cell type might become one of the most useful therapeutic tools, linking immunomodulation with pancreatic repopulation in type 1 diabetes.

Figure 2 is depicting the most current therapeutic tools that could be employed in the treatment of type 1 diabetes in the near future.

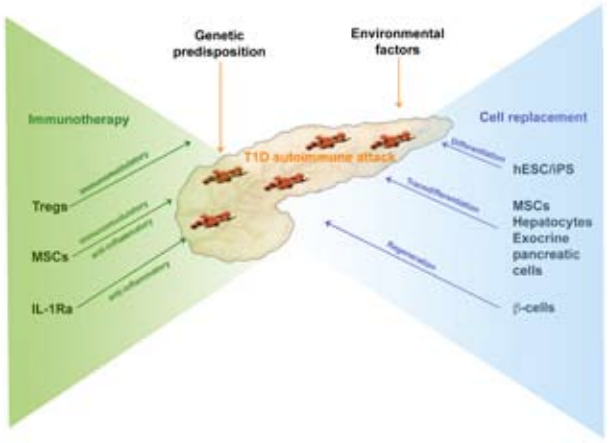

Fig. 2. Therapeutic strategies for type 1 diabetes. Insulin producing cells can be generated from various sources, such as embryonic stem cells (hESC) or autologous adult stem cells and reprogrammed somatic cells, or regenerated from existing β-cells. For a successful 'cure' of diabetes, cell-replacement approaches should be combined with immune intervention that would address the underlying cause of diabetes by blocking the islet-directed autoimmune attack.

CONCLUSIONS

The standards we set for a 'cure' in type 1 diabetes are proportionally high with respect to the complex immunologic and metabolic disruption elicited by the disease. While we do expect to obey the first principle of medicine, that is the treatment should not aggravate the disease, a life-long regimen of immunosuppression following transplantation is barely acceptable. Strategies focused on replacing β-cells either by a milieu-based differentiation or genetic engineering of adult or embryonic stem cells stem cells portend a new therapeutic era for type 1 diabetes management should we overcome the two most stringent requirements for a successful clinical application: that the surrogate β-cells are able to secrete insulin in response to metabolic stimuli and not constitutively, and that they circumvent acute rejection, chronic rejection and the reactivation of pre-existent autoimmune diabetes following transfer into the receiving diabetic patient.

Considering a pre-emptive approach to arrest the development of full-onset diabetes by determining people at risk of manifesting the disease in a preclinical phase and treating them to prevent islet damage, we are still far from reconstructing the etiology of type 1 diabetes. Genome-wide association studies (GWAS) aimed at identifying single nucleotide polymorphisms responsible for susceptibility to certain autoimmune conditions uncovered 41 distinct loci that increase the risk for type 1 diabetes. Environmental factors, such as infectious agents, chemicals and stress are also known to play an important part leading to the accelerated destruction of Langerhans islets, while the exact molecular mechanisms and causative autoantigens are yet to be detailed.

As for future prospects, we expect a steep improvement in the current knowledge of the predisposing factors and progression of type 1 diabetes, before any breakthrough definitive treatment is possible. Nevertheless, we are confident that the solution is not beyond our current or near-future scientific endeavors.

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INOVATII BIOMEDICALE PENTRU DIABETUL ZAHARAT: VINDECARE SAU TRATAMENT?

REZUMAT

Considerat in prezent o problema majora de sanatate publica atat in statele industrializate cat si in tarile in curs de dezvoltare, diabetul zaharat, defect metabolic ce afecteaza global mai mult de 220 de milioane de persoane, necesita urgent o solutie terapeutica. Tratamentul curent, ce presupune administrarea exogena de insulina, nu asigura un control eficient al glicemiei, in timp ce transplantul de insule pancreatice este limitat de numarul redus de donatori. Scopul prezentului articol este de a compara initiative terapeutice inovatoare explorate ca posibile solutii pentru diabet: terapie genica si terapie cu celule stem embrionare sau celule stem mezenchimale diferentiate catre celule β-pancreatice, imunoterapie si combinatii ale acestora. Desi articolul discuta in principal diabetul de tip autoimun, optiunile de regenerare a masei de celule β-pancreatice secretoare de insulina pot fi aplicate cu succes comparabil in tratamentul diabetului de tip 2.

Cuvinte cheie: diabet, celule stem embrionare, celule stem mezenchimale, terapie genica, boala autoimuna, imunoterapie, transplant.

REVIEW

INTERLEUKIN-24

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ABSTRACT

Gene therapy could prove to be an alternative strategy for use in cancers resistant to conventional therapies. One of the choices is to use a therapeutic gene which codes for a protein with antitumor properties. Such a gene has been shown to be the interleukin-24 (IL-24) gene. In this review we discuss the genomic profile, localization, expression and functions of interleukin-24.

Key words: interleukin-24, mda7, il-10 cytokine family, cancer, apoptosis

INTRODUCTION

IL-24 has been identified in 1993 by substration hybridization techniques, using human melanoma cells, and after inhibiting their growth and inducing differentiation with IFN-β and mezerein (MEZ)(1). Immunohistochemistry assays showed protein expression in the melanoma cells, but the expression was significantly decreased in the later, invasive phases of the melanoma (2). It was therefore suggested that the loss of MDA-7 expression is critical in the progression of the melanoma and that this protein is a viable candidate for melanoma gene therapy (3).

The protein was originally named mda-7 (melanoma differentiation associated gene), but following the discovery of its gene's chromosomal localization and its homology with IL-10, mda-7 was classified as a citokine and renamed IL-24.

THE IL-10 FAMILY OF CYTOKINES

Nowadays IL-24 is considered to be part of the IL-10-like family, together with IL-10, IL-19, IL-20, IL-22, IL-26, IL-28. IL-10 is known to have immunomodulatory effects on many different cell types; it acts as inhibitor to a number of cytokines, such as IFN-γ, TNF, IL-2, GM-CSF (4). The other related interleukins have various functions: IL-19 production by B lymphocytes and monocytes is up-regulated by their stimulation with GM-CSF and LPS (5), IL-20 regulates the proliferation and differentiation of keratinocytes (6), while IL-22 is responsible for the production of acute-phase proteins in inflammation (7). IL-20 over expression in transgenic mice is lethal during the neonatal period and it produces abnormalities of the epidermal differentiation[6]. IL-28 is involved in the acquired immune response.

The amino acid sequence for IL-24 across species is highly homologous, unlike the lower homology (20-30%) which exists between the different members of the IL-10 family (8).

THE STRUCTURE AND LOCATION OF THE IL-24 GENE

The human IL-24 gene is located on chromosome 1q32-33 (Fig 1), in a region spanning 195 kb and it is part of a cluster of genes that also contains genes for other members of the IL-10 family: IL-10, IL-19, IL-20 (9). The ARNm for IL-24 is approximately 2 kb, and the protein has a molecular weight of 23.8 kDa (10). The gene consists of 7 exons and 6 introns (9). The reading frame lies between untranslated regions of 274, and 823 bp. The untranslated region from the antisense strand contains 3 polyadenylation signals which are essential for the posttranscriptional stability of the ARNm. By using sequencing techniques, a signal peptide of 49 amino acids was identified, which allows the splicing and the secretion of the protein. Sequencing also revealed 3 positions where glycosilation is possible – amino acids 95, 109, and 126. The different glycosilation possibilities allow for different molecular weights for IL-24 (11).

Fig. 1. Chromosome 1

HOMOLOGOUS MOLECULES

IL-24 is highly conserved across species, as proven by the fact that murine and rat equivalents for IL-24 share a structural homology of 69 (12) and 82% (13), respectively. Both rat and murine IL-24 have a molecular weight of 23 KDa11, while human glycosilated IL-24 has a molecular weight of 33 KDa. This glycosilation has no effect on the binding properties, nor on the activity of the protein, as shown by the fact the murine IL-24 can bind to and activate human IL-24 receptors (14). The gene's localization in the IL-10 gene cluster is identical for the human, rat and mouse counterparts (9). The human IL-24 gene is located on chromosome 1, among a gene sequence that codes for IL-10, IL-19, IL-20, and IL-24, while murine IL-24 lies on chromosome 13q13, in the same gene cluster. The organisation and the size of the exons are similar for humans and mice, with the exception of the human exon 2, which is the signal peptide. Therefore, murine exons 3-5 and the coding region form exon 6 are identical with human exons 4-6 and the coding region from exon 7, coding the corresponding amino acids (14). The rat homologous gene for IL-24 has been identified in fibroblasts during tissue repair processes and named c49a (13). The murine equivalent for IL-24 is expressed by Th2 lymphocytes stimulated by IL-4 and it was named FISP (12) (IL-4-induced secreted protein).

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c49a/mob-5

There seem to be some differences between the human and rat orthologous. Different sets of up or downregulated genes have been found in rat fibroblasts during tissue repair processes (13). By comparing amino acid sequences from C49A and IL-24, a 58,7% homology has been found, which seems to suggest that these molecules are related, and not true homologues (11). C49A is expressed in injured dermal cells, which led to the conclusion that it is involved it proliferation processes. Another IL-24-like protein, MOB-5, has been isolated from embryonic rat fibroblasts (15). This is identical with C49A, with the exception of two amino acids. The mob-5 expression is induced by the H-ras and K-ras oncogenes, and it seems to also have a role in proliferation (16). In conclusion, even though C49A and MOB-5 are similar to IL-24, they stimulate proliferation, and don't inhibit it.

FISP

FISP is expressed in lymphocytes which have been induced to differentiate towards Th2 cells, and the expression is regulated by T cell receptors and IL-4. The protein is 93% homologous with the rat c49a/mob-5 and 69% homologous with the human IL-24. Even though FISP functions are not fully known, there is some evidence that they are similar to those of IL-24. For example, inhibition of the growth of liver tumor cells has been induced by intramuscular introduction of FISP in mice. This seems to suggest that IL-24 functions are more similar to those of FISP, than to those of c49a/mob-5 (17).

IL-24 EXPRESSION

Human IL-24 has been detected in various cell types, such as cultivated melanocytes (13), and cells which are part of the immune system – periferal blood mononuclear cells (PBMC) activated with either concanavalin A (15) and phyto-hemagglutinin (18), or IL-4 and LPS (19), splenocytes (13), thymocytes (10), but also in tumor cells. Like other members of the IL-10 family, IL-24 can be expressed by Th2 cells stimulated with protein kinase C and IL-4 (20). IL-24 expression can be detected by using RT-PCR (Reverse Transcription Polymerase Chain Reaction) in activated T cells and monocytes stimulated with LPS (21). Also, an elevation of IL-24 (8) expression has been detected in vivo, in skin injuries, and the high level was maintained for several days, returning to base level after approximately 14 days (13). Considering the fact that IL-24 expression was also elevated before and during the proliferation phase of the tissue repair process, it is likely that IL-24 is involved in cell proliferation.

IL-24 RECEPTORS

The IL-24 receptors belong to the class II cytokine receptors family, along with other receptors for IL-10-like interleukins (22). They consist of two chains – R1 and R2 – of different intracytoplasmic lengths (23). There are 3 subtypes of R1 chains (IL-10R1, IL-20R1, and IL-22R1), and 2 subtypes of R2 chains (IL-10R2 and IL-20R2) (24). The R1 chain contains a long cytoplasmic domain and represents the main signalling component, while the R2 chain has only a short cytoplasmic domain (23). The IL-20 specific receptor consists of two subunits – IL-20R1 and IL-20R2, which connect on the surface of the keratinocytes, in order to form the functional receptor for IL-20. It has recently been observed that IL-20 can also bind to a receptor which contains IL-22R1. The IL-22 receptor is made up from the IL-22R1 and IL-10R2 (25). IL-24 can bind to either IL-20R1/IL-20R2, or IL-22R1/IL-20R2. The most common type of receptor is IL-20R1/IL-20R2, which is common in most tissues throughout the body, while IL-22R1/IL-20R2 can only be found in the liver, colon, small intestine, and pancreas. After binding with either of the two receptors, IL-24 activates the STAT3 pathway (26). However, it has been shown that the activation of the JAK/STAT pathway is not indispensible for inducing apoptosis, and cell death is induced by intracellular IL-24 through a mechanism independent from receptors. Intracellular IL-24 cand be found at the endoplasmic reticulum, where it induces a stress response, which leads to the apoptosis of the tumor cells through different pathways, which depend or not on mitochondria (26).

Unlike the IL-10 receptor (IL-10R1/IL-10R2), which is expressed by most hematopoietic cells, the expression of IL-24 receptors seems to be restricted by the presence of the IL-20R2 in some non-hematopoietic tissues, such as skin, lungs, testicles, and ovaries, which suggests a pleiotropic role for IL-24. The over expression of IL-24 can be found in psoriatic skin, which suggests a connection between overactivation of IL-24 and IL-24 (which share the same receptors) and disease (27).

THE BIOLOGICAL FUNCTIONS OF IL-24

IL-24 can perform its functions in two different ways: like a normal cytokine, through the specific receptors, or independent of its receptors. The latter manner only applies to certain types of cancer cells.

Receptor Dependent Functions

One of the most important physiological functions of IL-24 is to be involved in tissue repair processes. The target cells for this function are keratinocytes. In certain skin pathologies, such as psoriazis, monocytes migrate directly beyond the dermic layer of the skin and secrete IL-24 which leads to persistent proliferation of keratinocytes (28).

In vitro, IL-3-dependent murine proB cells proliferate in the presence of IL-24. IL-24 can also function as growth factor for B lymphocytes (14) and stimulate the expression of other cytokines, such as TNF-α, IL-6, and IFN-c by PMBC (15), which suggests an involvement in inflammation. Ras oncogenes can induce not only IL-24 expression, but also the expression of their receptors, which suggests a paracrine or autocrine function for IL-24, which contributes to the survival and proliferation of tumor cells (29).

It has also been shown that it has antiangiogenic properties through a receptor-mediated mechanism (30), although no specific receptors have been found on the endothelial cells.

Receptor Independent Functions

Several studies have shown an antitumor effect of IL-24 which is not mediated by receptors. Most of these studies have produced IL-24 overexpression with the aid of an adenoviral vector, this construct being named Ad.mda-7.

Immunohistochemistry and RNA studies on melanocytes have shown the messenger RNA expression for IL-24, as well as the protein levels. IL-24 expression decreases with the progression of the melanoma, from the radial growth phase, to the vertical phase (2). This suggests the fact that IL-24 has a tumor suppression role, and the loss of expression is a critical step in melanoma progression from noninvasive tumor to invasive malignity with metastatic potential (31).

It has been shown that IL-24 overexpression induces apoptosis in various types of tumor cells – melanomas (3), malignant gliomas (4), prostatic (32), ovarian (33), and lung carcinomas (34), and on the other hand it does not inhibit the growth of normal cells – for example, fibroblasts (32), breast (35) and prostatic epithelium (32), astrocytes (31). The antitumor activity does not seem correlated

with the status of tumor suppresor genes (p53, p21) (11).

IL-24 performs its antitumor effects by different mechanisms. One such mechanism might be modifying the ratio between pro- and antiapoptotic intracellular proteins – IL-24 tips the balance towards apoptosis mediated by BAX and other related proteins. Inside the cell there is a delicate balance between the molecules which promote or inhibit apoptosis (36). Proteins such as BCL-2, BCL-XL, MCL-1, BCL-W, and Ad-E1B protect cells from apoptosis, while proteins such as BAX, BAD, BAK, and BCL-XS stimulate it (37). This effect has been proven on breast cancer cells with different p53 genotypes, which suggests the fact that it is a p53-independent mechanism. IL-24 modified the balance between these proteins, upregulating the BAX protein, raising the BAX/BCL-2 ratio, and tipping the balance towards apoptosis (35). Although Bax is considered to be p53-dependent, the abillity of IL-24 to upregulate the protein levels independently from p53 seems to suggest alternative pathways for tumor cell apoptosis. Also, ectopic BCL-2 (or its viral equivalent Ad-E1B) can prevent the inhibition of tumor cell growth, which suggests a possible role of mitochondria in apoptosis (33).

IL-24 can also activate tumor cell apoptosis through the MAPK (mitogen activated protein kinase) pathway, by activating p38. This activation inhibits cell growth in melanomas and malignant gliomas and it expresses the GADD gene family (DNA damage inducible) (38). It has also been observed that blocking p38 or GADD expression saves the melanoma cells from apoptosis. In the case of tumor lung cells, the Ad.mda-7-induced apoptosis correlates with the upregulation of RNA-dependent protein kinase (PKR), which is a mediator for antiviral and antitumoral responses in target cells (39).

It can also inhibit tumor angiogenesis. This has been shown in vitro, by using human umbilical vein endothelial cells (HUVEC) (18). Other cytokines from the IL-10 family have antiangiogenic effects, as well. IL-10, besides its immunosuppressive actions, also inhibits tumor cells growth when it is over expressed in tumor cells, by suppressing angiogenesis.

Other studies have shown a possible bystander antitumor effect of IL-24, in the case of pancreatic cancer (40).

CONCLUSIONS

So far, other candidate molecules for gene therapy have not met the high expectations. It seems that IL-24's specificity does not allow for toxic effects on normal, healthy cells. Also, its growth suppression and apoptosis actions maninfest themselves in a large spectre of cancers: melanoma, osteosarcoma, glyoma, mesothelioma, breast, cervical, colorectal, kidney, liver, ovarian, pancreatic carcinomas.

These present findings suggest that IL-24 is worthy of interest as therapeutic gene for cancer and it is to be hoped that future studies will provide additional data and enhance the benefits for patients.

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INTERLEUKINA 24

REZUMAT

Terapia genică se poate dovedi a fi o strategie alternativă utilă în cazul afecțiunilor maligne rezistente la terapii convenționale. Una dintre posibilitățile de tratament o reprezintă utilizarea unei gene terapeutice care codează o proteină cu proprietăți antitumorale. O astfel de genă este cea pentru interleukina-24 (IL-24). În acest raport trecem în revistă profilul genic, localizarea cromozomială, expresia şi funcțiile interleukinei-24.

Cuvinte cheie: interleukina-24, mda7, familia citokinica IL-10, cancer, apoptoza

HEALTH, LONGEVITY AND ECOLOGY - AN INTEGRATED PARADIGM

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ABSTRACT

Present day stage of human civilization requires new concepts for better understanding evolution mechanisms and tendencies, as well as rapid changes and reevaluations. For this reason our paper substantiates a novel and neccesary integrated conception: global (human & Earth) health, longevity and ecology. The paradigm reunites into a new vision three Gaia precepts: globalization, ecological strategy, and global (human & Terra) soundness. As a matter of fact, all world institutions support and supervise these international programmes, i.e. Amsterdam Declaration on Global Change, July 13, 2001 and Amsterdam Declaration of the Club of Rome on Climate, Energy and Economic Recovery, October 27, 2009. For mankind this new concept represents the reconsideration and improvement of holistic conception from Indo-Chinese and Greek-Roman antiquity, the 2nd Renaissance of human spirit and a new beginning for humanity future.

Key words: original holistic paradigm; globalization; ecological strategy; (human & Terra) global vitality; ecological organizations, programmes and actions

A planet is passing by the Earth and asks it:
- How are you, sister? I haven't seen you for millions of years!
- Not so well… you know… I've got Homo Sapiens…
- Don't worry, it'll pass! I had it myself!

A joke, but also a sad reality

INTRODUCTION

Nowadays mankind needs novel prospects for rapid and better understanding evolution mechanisms and tendencies, as well as fast transformations and reevaluations (13).

For this reason, the authors have introduced a new and essential paradigm: global (human & Earth) health, longevity and ecology.

EARTH COMMANDMENTS

At present there are three Gaia concepts-precepts, which have to be developed, implemented and supervised: globalization, ecological strategies, and global (human & Earth) soundness. In addition, they can form an integrated paradigm with immediate positive consequences, which is more easily followed and controled.

Globalization is and will be a new stage of civilization and signifies the local, national, regional and global integration and cooperation of all countries (1, 5). Ecological strategies mean a coherent ensemble of programmes, resolutions, actions, results and effects (12, 17). Global (human & Terra) soundness represents the unification of health (22), longevity (21) and ecology (13) concepts, concerning both human being and Earth.

GAIA CONCEPT I - GLOBALIZATION

Globalization precept can be assimilated and represented by Atlas from Greek mythology. Iapetus's son, one of the Twelve Titans, Atlas (8, 10) was punished by Zeus to sustain the vault of Heaven on his shoulders (the Earth or the Atlas mountains, in other sources). On Terra, Atlas is present everywhere: in Naples, Italy as a 2nd century Roman copy of a Hellenistic work, in New York, NY, USA as a sculpture in front of Rockefeller Center, in Melbourne, Australia on a building etc.

Gaia global concept was introduced by Lovelock (14), former NASA scientist, as Gaia hypothesis and theory. This global ecological concept demonstrates that the bio-sphere and geo-sphere - the physical components of the Earth (atmo-, cryo-, hydro- and litho-spheres) are closely integrated to form a complex interacting system. His role is to maintain the climatic and bio-geo-chemical conditions on Terra in a preferred homeostasis. In this way, the Earth can be considered as a unique global organism.

Globalization, considering its general features, can be defined (2, 3, 4):
- central force that presides over the restructuring of today's world;
- most extended process of socio-historical and politico-economic transformations from humankind history;
- most challenge of XXI century, involving in the same direction whole human sociery and whole geo-sphere (geo-space, geo-system);
- new philosophico-political and historico-social paradigm;
- power of the most important historical-cultural, scientific-military-economic, socio-political and IT-communication factors, trends and conceptions that are reshaping and restructuring today's world.

Globalization represents and has new values and objectives (political, economic, social, juridical, ethical, ideological), as well as restrictions and penalties, but no negative forces against biosphere, geosphere and civilization (6, 7).

GAIA CONCEPT II - ECOLOGICAL STRATEGIES

Construction of future global ecological society, planned to be carried out

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and consolidated in this century, will necessitate the establishment of equilibrated relations between natural, ecological, anthropological, demographic, social and economic factors. Principles of sensible mankind eco-development must be scientifically proved and afterwards placed firmly at the foundation of future ecological civilization. There are a lot of global and regional institutions, organizations, agencies and programmes:

- UNEP - United Nations Environment Programme, Stockholm, 1972;
- UNCED - United Nations Conference on Environment and Development, 1992;
- NAAEC - North American Agreement on Environmental Cooperation, 1994;
- EEA - European Environment Agency, established by EEC Regulation 1210/1990;
- IPCC - Intergovernmental Panel on Climate Change;
- IGBP - International Geosphere-Biosphere Programme;
- IHDP - International Human Dimensions Programme on Global Environmental Change;
- WCRP - World Climate Research Programme;
- DIVERSITAS - International Biodiversity Programme.

The last four International Global Change Research Programmes presented and introduced their conclusions in the Amsterdam Declaration on Global Change, Amsterdam, NL, July 13, 2001 (18):

- The Earth System behaves as a single, self-regulating system comprised of physical, chemical, biological and human components;
- Human activities are significantly influencing Earth's environment in many ways;
- Global change cannot be understood in term of a simple cause-efect paradigm. Human-driven changes cause multiple effects that cascade through the Earth system in complex ways;
- Earth system dynamics are characterised by critical threshold and abrupt changes. Human activities could inadvertently trigger such changes with severe consequences for Earth's environment and inhabitants.
- In term of some key environmental parameters, the Earth System has moved well outside the range of the natural variability exhibited over the last half million years at list. The Earth is currently operating in a no-analogue state.

Recently, the Amsterdam Declaration of the Global Assembly - the Club of Rome

"Climate, Energy and Economic Recovery" Amsterdam, NL, October 27, 2009 reaffirms the actuality and acuteness of this problem (19):

The Club of Rome calls for urgent action to avert the growing risk of catastrophic climate change. The most recent scientific data presented to the Assembly by the world's top climate scientists demonstrate the accelerating impacts of climate change on the natural systems of the planet... Governments have directed trillions of dollars to stabilize the financial system: we call for the required levels of finance to salvage the future of the planet...

The Club of Rome urges governments to adopt a strong climate treaty that will be fair, that will contribute to energy and economic security, that will respond to the growing urgency of the risks of catastrophic climate change...

We emphasize that the transition to an equitable, sustainable low-carbon society must also engage the business and investment community, civil society and communities at large. There is enormous potential for business in rapidly emerging markets for new sustainable low carbon products. Business leaders must commit to the re-design of business models, to innovative solutions and to new energy and resource-efficient products. They have a responsibility to regain pub-lic trust in the ethical and sustainable basis of banking and business activities.

The goals of concerted international climate action must be (19):

• To adopt, at the UN Climate Conference in Copenhagen, legally binding agreements that will initiate immediate action to achieve a stable climate with atmospheric concentrations of CO_2 not exceeding 350 ppm.

• To establish financial mechanisms, including a carbon market, that will enable countries, companies and communities to reduce their net carbon emissions to the levels required.

• To accelerate support for adaptation and humanitarian assistance in developing countries as an integral component of national development so as to reduce the impacts of climate change (16).

• To promote the development of new models and strategies for growth, development and globalization which place a real value on natural capital and ecosystems services - including the removal of CO_2 from the atmosphere.

• To agree upon an international study, to be concluded within one year, which will propose how the framework of international policies and institutions must be adapted to meet the connected, systemic challenges of the 21st Century.

GAIA CONCEPT III - HEALTH, LONGEVITY, ECOLOGY

Construction of the Gaia IIIrd concept - Health, Longevity, Ecology - can be exemplified by three representative goddesses in these fields (8, 9, 10):

- Hygieia, goddess of health; her name is the the source of word hygiene.
- Hebe, goddess of eternal youth - longevity, the cupbearer for the gods and goddesses of Mount

Olympus, and

- Gaia, the primal Greek goddess personifying the Earth, as Gaia ecological concept.

Hygieia, goddess of Health, daughter of the god of medicine, Asclepius
1st century Roman statue

Hebe, goddess of Eternal Youth - Longevity, daughter of Zeus and Hera, Roman equivalent - Juventas
Antonio Canova's (1757-1822) marble sculpture

Gaea - Gea - Gaia, considered a Mother Goddess or Great Goddess
Pre-Olympian Greek Earth goddess, sprang from primordial Chaos,
Terra Matter or Tellus - her equivalent in the Roman pantheon

Indeed, these three notions can and must be unified, both for human being and Earth as integrated conceptions, as well as between them. The result is and will be the global (human and Terra) soundness, or global (human & Earth) health, longevity and ecology.

Surroundings (bio- and geo-spheres) are in continuous transformations (alterations or good variations), and also humans are still in evolution (bad or good modifications). In our days the environment configuration and human evolution depend in the greatest measure of human thinking, decisions, behaviours and actions (15). So, the human interventions on the outside (environment), and on the inside (ourselves) are and will be decisively for global (human & Earth) health, longevity and ecology in the near future.

These can determine:

• the normality of surroundings and humans with harmonious interconnections, or

• unfortunately the abnormality and destruction, bringing about the aggressive environment and fragile human beings with total disagreement and antagonism between them.

By his irrational actions, man becomes an enemy of himself (dangerous and wrong life styles) and also of the natural environment (qualitative and quantitative large demolitions). With good reason, George Crockcroft (b. 1932, pseudonym Luke Rhinehart) said Social consequences of a nation formed by normal people are evident: poverty, conflicts, violence, war and a generalized gloom.

Therefore, humanity must urgently change and may act as holistic strategy in three interconnected important directions:

• remplacement of diseases of life style (pro-impairment, pro-aging and pro-pathologies), by health promotion (24), stress-related diseases prevention (20) and pro-longevity concepts (23);

• substitution of surroundings destructive actions for an upright and stimulative partnership with nature;

• understanding the necessity for fast implementation and combination of global and regional strategies with national and local measures, regarding global (human & Earth) health, longevity and ecology.

The change of human behaviour is one of decisive solution. In this respect, the famous words said in 1949 by Contantin Brâncuşi are very suitable in our days: If man manages to eliminate his Ego, he will be able to hear the heartbeat of nature and her whispers (11). Humans can be able to apply the Gaia concept, mission and philosophy, namely living organisms must improve themselves and their environment too.

In few but cardinal words, humanity must define the question-answer, choice, pathway-solution and also the action-result To be or not to be, that is the question - a very great soliloquy on the very great topic of human being (W. Shakespeare, Hamlet, Act 3, Scene 1).

CONCLUSIONS AND FUTURE ADVANCES

Photograph of the Earth,
Apollo 17,
taken from Appolo 17, in 1972
Mission insignia

The three Gaia philosophy and concepts (• globalization • ecological strategies • human & Earth soundness) will determine the future of minkind and of our planet too.

For human civilization they represent:

- the reconsideration and improvement of holistic conception from Indo-Chinese and Hellenic-Roman antiquity;

- the 2nd Renaissance of human spirit; and

- a new beginning for human future started with Gaia ESA (European Space Agency) astrometry space mission (spring 2012-2017), with the obiectives to create the largest and most precise three dimensional chart of our Galaxy (about one billion stars in our Galaxy and troughout the Local Group).

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SANATATE, LONGEVITATE SI ECOLOGIE - O PARADIGMA INTEGRATA

REZUMAT

In zilele noastre, civilizatia umana are nevoie de noi concepte pentru o mai buna intelegere a mecanismelor si tendintelor evolutive, precum si a schimbarilor si reevaluarilor produse cu rapiditate. Din aceasta cauza, lucrarea noastra prezinta si demonstreaza o noua si necesara conceptie integrata: sanatatea, longevitatea si ecologia globala (umana si a Pamantului). Paradigma reuneste intr-o noua viziune trei precepte Gaia: globalizare, strategie ecologica si sanatate globala (umana si a Pamantului). De altfel toate institutiile lumii sprijina si supravegheaza aceste programe internationale, ca de ex. prin Declaratia de la Amsterdam asupra Schimbarii Globale, 13 iulie 2001 si Declaratia de la Amsterdam a Clubului de la Roma asupra Restabilirii Economice, Energetice si Climatice, 27 octombrie 2009. Acest nou concept reprezinta pentru omenire reconsiderarea si imbunatatirea conceptiei holistice din antichitatea Indo-Chineza si Greco-Romana, a doua Renastere a spiritului uman si un nou inceput pentru viitorul umanitatii.
Cuvinte cheie: paradigma holistica originala; globalizare; strategie ecologica; vitalitate globala (umana & a Terrei); organizatii, programe si actiuni ecologice

INSULIN GROWTH FACTOR 1 INVOLVEMENT IN THE ETIOLOGY OF PECTUS EXCAVATUM IN CHILDREN

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ABSTRACT

Pectus excavatum (PE) is the most frequent anterior chest deformity occurring in approximately 1 in 1000 live births. Despite the excellent achievements in the surgical treatment of the disease the etiology of PE is yet to be clarified. Nowadays the most accepted etiopathogenic theory is that the posterior displacement of the sternum is caused by the overgrowth of the disturbed costal cartilages. Insulin Growth Factor 1 (IGF1) is the main anabolic growth factor for human hyaline cartilages and plays a key role in cartilage homeostasis, promoting proteoglycans synthesis and cell division. In this article we present the results of our study regarding the involvement of IGF1 in the etiology and pathogenesis of PE in children. Samples of the deformed costal cartilages from 25 children with PE were obtained during the surgical intervention for the correction of the disease. Cartilage samples were obtained during autopsy from 17 children in whom the cause of death was unlikely to affect the cartilage. The samples were immunohistochemically stained with fluorescent antibody for phosphorylated IGF1 receptor (IGF1R). The majority of the samples were negative for phosphorylated IGF1R and there were no significant differences between the two groups ($p > 0.05$). In both PE patients and in general populations, the majority of the costal chondrocytes doesn't reveal activated IGF1R. PE in children is not produced by a defect of the IGF1 axis.

Key words: pectus excavatum, IGF1, etiology, growth factor

INTRODUCTION

Pectus excavatum (PE) is the most frequent anterior chest deformity occurring in approximately 1 in 1000 live births (1). The deformity consists in the posterior depression of the sternum and the lower costal cartilages (2). Despite the excellent achievements in the surgical treatment of the disease the etiology of PE is yet to be clarified. Several etiopathogenic theories were proposed over time and the most accepted today is that the posterior displacement of the sternum is caused by the overgrowth of the disturbed costal cartilages (3). Previous histological studies found no significant differences regarding the number, shape, area of the cell and nucleus between cartilages from PE patients and normal (4, 5). This indicates that the overgrowth of the cartilages has a global pattern with equal involvement of the cells and the matrix.

Insulin Growth Factor 1 (IGF1) is the main anabolic growth factor for human hyaline cartilages (6). IGF1 is it plays a key role in cartilage homeostasis, promoting proteoglycans synthesis and cell division (7). Compared with other growth factors, IGF1 has the most powerful anabolic effect over chondrocytes in culture (8). Approximately 80% of the circulating of IGF1 is produced in the liver under the direct stimulation of the pituitary growth hormone (GH) (9). Despite of that the complete ablation of the liver production of IGF1 has little effect on the somatic growth indicating that alternative autocrine and paracrine mechanism are involved (10). In the serum it circulates bind to one of the 6 specific binding proteins (IGF1BP) (11). IGF1BP prolong its life time and, because their affinity for IGF1 is greater than IGF1 receptor (IGF1R) they are the major factor influencing the biodisponibility of the IGF1 (11). IGF1 has a specific receptor displayed by the majority of the tissues in the human body (12).

In this article we present the results of our study regarding the involvement of IGF1 in the etiology and pathogenesis of PE in children.

MATERIAL AND METHODS

Samples of the deformed costal cartilages from 25 children with PE, age 5 to 18 years, mean 12 years, were obtained during the surgical intervention for the correction of the disease. Costal cartilage excision is a regular part of the Ravitch surgical procedure for the correction of PE (13). Costal cartilage samples were obtained during autopsy from 17 children in whom the cause of death was unlikely to affect the cartilage, age 1 to 19 years, mean 9.5 years. The samples were cut perpendicular to the long axis of the cartilage and apart from the costo-chondral and the chondro-sternal junction. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Three sections were cut at 3 µm thickness from each cartilage sample. Prior to staining, each slide was dewaxed and rehydrated by standard protocol.

The samples were immunohistochemically stained with fluorescent antibody for phosphorylated IGF1R (phospho Y1158) (Alexa Fluor® 488) antibody (ab20493), Abcam®, UK using standard over night incubation technique. With this antibody the chondrocytes membrane expressing phosphorylated IGF1R is stained in green. The slides were examined at Nikon Eclipse E800 microscope with fluorescence filters. Triband and green filters were used and at least one photographic image was obtained for each sample.

Positive control was obtained using cultured fibroblast stimulated with IGF1

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and MDA-MB-231 tumoral cells (Fig. 1).

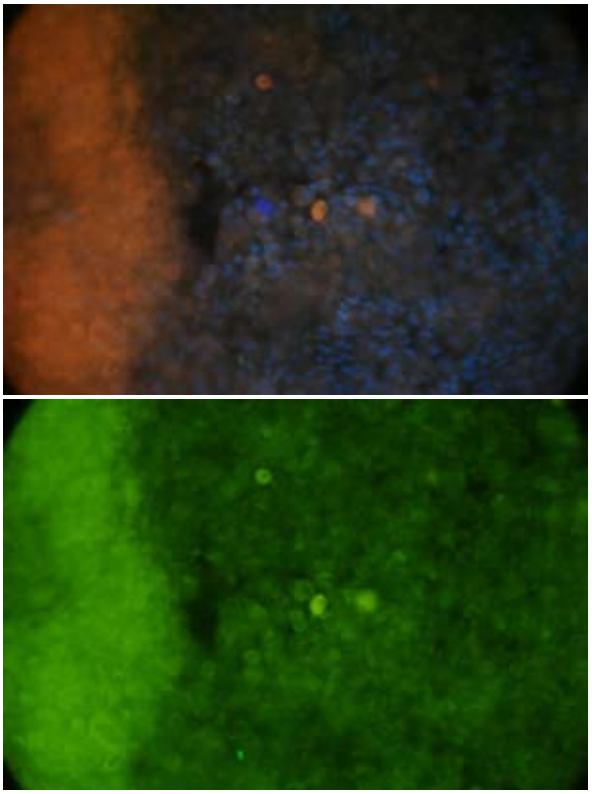

Fig. 1. Immunofluorescence for IGF1R. Cultured fibroblasts stimulated with IGF1, triband filter left, green filter right (400X)

The chondrocytes were considered positive if the cell membrane was completely stained in green. The samples were considered positive if they had large areas of positive cells outside the subperichondral zones and in the nearby of the vascular channels.

Statistical analyzing was performed with the help of SPSS v1.7 software Differences between groups we used chi-square test for non-numeric variables; level of significance for p was set at 0.05.

RESULTS

We found a total of 14 specimens positive for phosphorylated IGF1R, 7 for the experimental and 7 for the control group (Fig. 2). There were no significant differences between the two groups ($p > 0.05$) (Table 1). The majority of the samples were negative for phosphorylated IGF1R 72% for the experimental group and 55% for the control group (Fig. 3). Positive cells were found nearby vascular channels only in 2 samples from the experimental and 3 from the control group (Fig. 4).

Table I. The results of the immunohistochemistry staining for phosphorylated IGF1R

Group	Samples	Positive
Experimental	25	7 (24%)
Control	17	7 (41%)
		p= 0.374

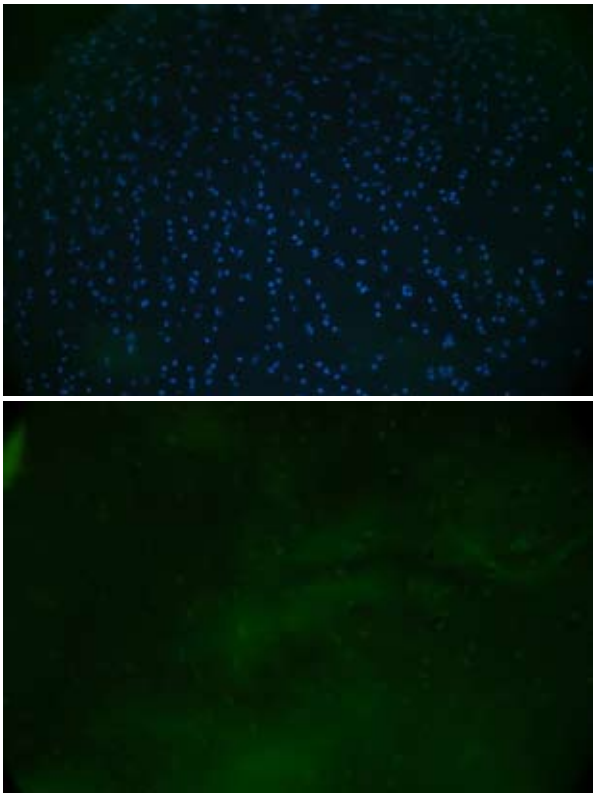

Fig. 2a. Positive cells for phosphorylated IGF1R, 10 years old patient, experimental group, triband filter left, green filter right (400X)

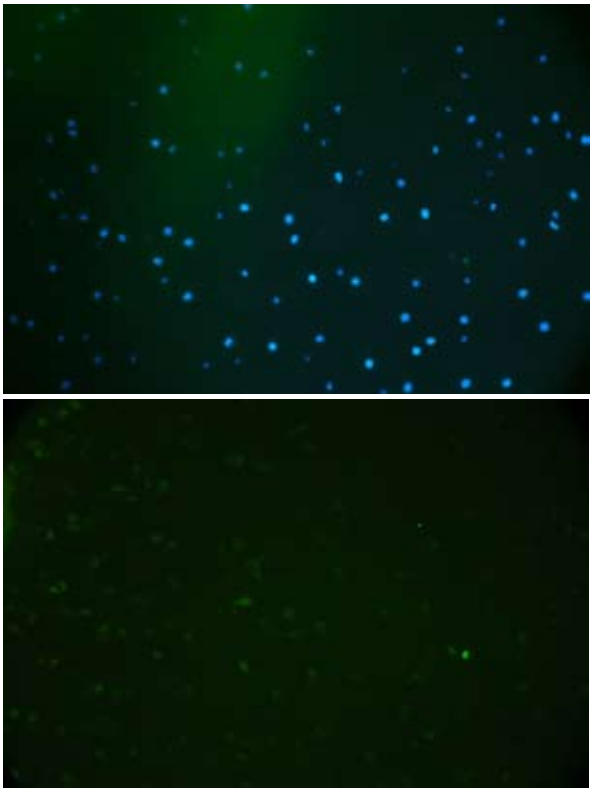

Fig. 2b. Positive cells for phosphorylated IGF1R, 1 year old patient, control group, triband filter left, green filter right (400X)

Fig. 3a. Negative cells for phosphorylated IGF1R, 10 years old patient, experimental group, triband filter left, green filter right (400X)

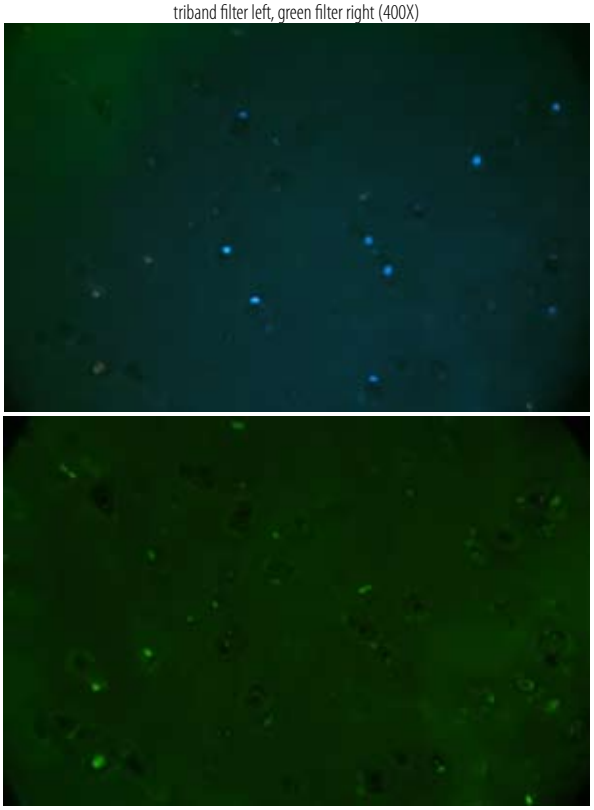

Fig. 3b. Negative cells for phosphorylated IGF1R, 18 years old patient, control group, triband filter left, green filter right (400X)

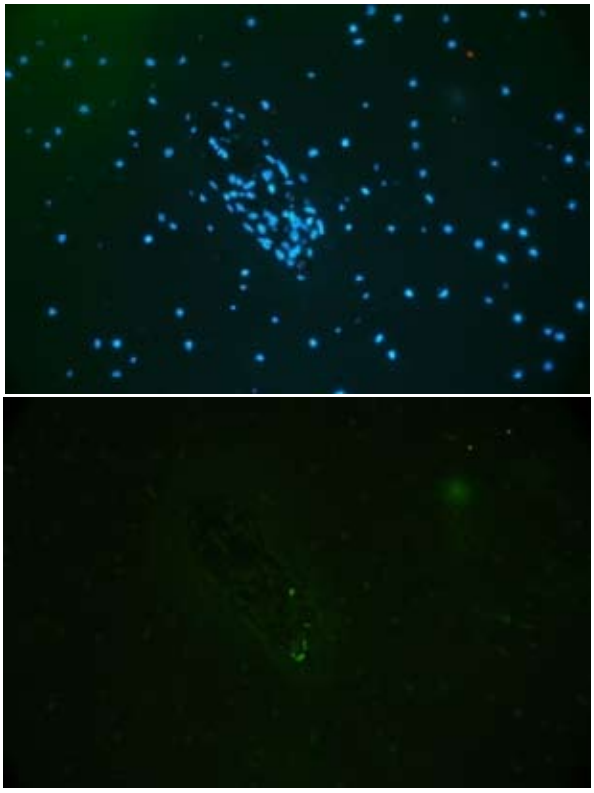

Fig. 4. Positive cells for phosphorylated IGF1R in the nearby of a vascular channel, 6 years old patient, experimental group, triband filter left, green filter right (400X)

DISCUSSIONS

PE is caused by the overgrowth of the costal cartilages (3). There are some elements that suggest that the IGF1 axis is involved in the etiology of the disease. First of all, IGF1 is the main anabolic factor for human hyaline cartilage (7). Under IGF1 stimulation the number of chondrocytes, the quantity of proteoglycans and collagen II increase in superior manner to any other growth factor (8). More than that, IGF1 is a factor of differentiation of the mesenchymal cells towards sulfated proteoglycans producing chondrocytes (7). Another element suggests the possibility of the IGF1 involvement is the fact that the curve of plasmatic level of the IGF1 during childhood and puberty is following the curve of the progression of the chest wall deformity (2, 14). Serum levels of IGF1 increase progressively from birth reaching the maximum trough puberty and decreases after that (14). Parallel to that the deformity of the chest wall in most of the patients with PE is minor or absent at birth, increases slowly or remain stable during childhood and aggravate rapidly during puberty (2). After puberty the deformity usually remains stable (2).

In the same time there is no other evidence that PE patients suffer of a disturbance of the GH - IGF1 axis. The tallness the majority of the PE patient is between the limits of the general population (34). But this doesn't eliminate the possibility of involvement of the autocrin mechanism of the IGF1 axis. This was the reason why we decided to choose to determine the phosphorylated IGF1R in the costal cartilages of children with PE and not go for an assessment of the plasmatic level of the IGF1.

The IGF1 and his receptor are major factors influencing the proliferation, survival and treatment resistance of tumoral cells (15). IGF1R is present in almost all solid and blood tumors and is one of the main targets in the antitumoral treatment (16). The IGF1R blockade using monoclonal antibodies is a promising treatment option in the treatment f cancer (17, 18). By analogy, IGF1R blockade may be used

as a mean for stopping the overgrowth of the costal cartilages in PE and may be used as an alternative to the surgical treatment. In order to be efficient and to avoid systemic side effects the blockade should be made strictly local or by antibodies specific for costal chondrocytes. These are now only speculations and more research is to be done in this direction. We, by conducting this research have made the first steps to finding an alternative nonsurgical treatment for PE.

The results of our study in PE patients the number of chondrocytes revealing phosphorylated IGF1R is similar to general population. The presence of IGF1R in human chondrocytes was already demonstrated (19).The antibody used by us targeted the phosphorylated IGF1R. This doesn't imply the absence of the IGF1R in the chondrocytes but only the ones activated by IGF1. This indicates than when the experiments were done the level of IGF1 in the cartilage was low. In the same time, the fact that nearby the vascular channels the cells was positive for phosphorylated IGF1R and in the rest of the cartilage was not, indicates that is happening duet o circulating IGF1 stimulation. This means that the autocrine/ paracrine mechanism have poor involvement in the IGF1 mediated growth of the cartilage. For PE the absence of activated IGF1R is an indication that the overgrowth of the costal cartilages does not cur under a hyperstimulation with IGF1 by non autocrin, paracrin or endocrine mechanism.

CONCLUSIONS

1. In both PE patients and in general populations, the majority of the costal chondrocytes doesn't reveal activated IGF1R. IGF1R is expressed by the costal chondrocytes mainly in the nearby of the vascular channels and in the subperichondral space. There are no differences between PE patients and general population regarding the number and the distribution of the chondrocytes reveling phosphorylated IGF1R.

2. PE in children is not produced by a defect of the IGF1 axis. In the same time, this doesn't imply that this axis can't be used as a therapeutic way in blocking the overgrowth of the costal cartilages in PE patients.

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IMPLICAREA FACTORULUI DE CRESTERE INSULINIC 1 ÎN ETIOLOGIA PECTUS EXCAVATUM LA COPII

REZUMAT

Pectus Excavatum (PE) este cea mai frecventă malformatie a peretelui anterior al toracelui şi are o incidentă de aproximativ 1 la 1000 de nou-născuți. În ciuda realizărilor semnificative în tratamentul chirurgical al bolii, cauza maladiei nu a fost încă stabilită. Mai multe teorii etiopatogenice au fost elaborate de-a lungul timpului, dar cea mai acceptată astăzi este cea conform căreia înfundarea sternului se produce datorită creşterii în exces a cartilajelor costale. IGF1 este principalul factor de creştere anabolizant pentru cartilajul hialin Si are un rol esențial în homeostazia acestuia, stimulând secreția de proteoglicani Si diviziunea celulară. În acest articol prezentăm rezultatele studiului nostru privind implicare IGF1 în etiologia Si patogenia PE. Specimene de cartilaj costal au fost obținute în timpul operației pentru corecția PE de la 25 de pacienți. Un număr de 17 specimene de cartilaj costal au fost obținute de la cadavre a căror cauză de deces a fost foarte puțin probabil să afecteze cartilajul costal. Specimenele au fost colorate prin tehnica imunohistochimică directă cu anticorp fluorescent pentru receptor IGF1fosforilat (IGF1R). Majoritatea specimenelor au fost negative pentru IGF1R fosforilat iar între cele două loturi nu există diferențe semnificative ($p > 0,05$). Atât la pacienții cu PE cât şi la populația normală, majoritatea condrocitelor costale nu exprimă IGF1R activat. La originea PE la copil nu se află un defect al axei IGF1.

Cuvinte cheie: pectus excavatum, IGF1, etiologie, factor de creştere

COMPARISON BETWEEN DIFFERENTIATION POTENTIAL OF DOG PERIOSTEUM – DERIVED CELLS AND BONE MARROW-DERIVED CELLS TO OSTEOGENIC, CHONDROGENIC AND ADIPOGENIC LINEAGES

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ABSTRACT

The aim of the present study is to investigate the probability of dog periosteum-derived cells and bone marrow-derived cells to differentiate along the osteogenic, chondrogenic and adipogenic lineages. Results of this experiment aim to highlight the similarities and differences between the cells obtained as a result of periosteum-derived culture and bone marrow-derived cells cultivated in three differentiation media. While the dog bone marrow derived-cells differentiate towards all three lineages, with variable intensity, thus confirming the results of other research studies in this field referring to other species, canine periosteum-derived cells obtained using culture of periosteal explants, differentiate mostly towards the osteoblastic and chondrogenic lineage, and to lesser extent towards the adipogenic lineage; the cells obtained using the digestion method of periosteal fragments positively differentiate towards all the three lineages: osteoblastic, chondrogenic and adipogenic. In addition, it was demonstrated that cryopreserved studied cells have osteogenic, chondrogenic and adipogenic differentiation capacity.

Key words: periosteum-derived cells (explant and enzymatic isolation methods), bone marrow-derived cells, differentiation potential

INTRODUCTION

There is a difference between bone formation during embryonic life and postnatal bone growth, when osteoprogenitor cells directly differentiate into bone forming osteoblasts. During the fracture healing process 2 distinct phenomena are developing: osteogenesis as well as chondrogenesis. It is known that local micro-environmental conditions may be important in directing the differentiation of the periosteum-derived cells towards either osteogenesis or chondrogenesis. As such, periosteal cells could be used as an in vitro culture model to study the osteogenic capacity of biomaterials developed for bone regeneration therapy. Some studies to evaluate the osteogenic potential of biomaterials were performed which used periosteum-derived cells from human, rabbit, pig and bovine origin (1,2,3). However, dog periosteum-derived cells were not yet analyzed with regard to their suitability to study cell/biomaterial interactions.

The strategy for obtaining cell populations with predictable tissue formation capacity would be dependent, at least in part, on the presence of either multiple functionally distinct cell subsets or more primitive stem cells capable of multi-lineage differentiation. It is important to identification and separation of the cell subset with the desired tissue-forming capacity from cell subpopulations with unwanted differentiation potential. In the later case, cell product manufacturing, including culture techniques, scaffold technologies, or growth factor treatments, could be focused in the commitment to the desired tissues and in the prevention of unwanted/heterotopic tissue formation.

MATERIALS AND METHODS

Isolation and cultivation of dog periosteum-derived cells and bone marrow derived-cells

Biologic material was harvested from the five common-breed dogs. For harvesting the periosteal flaps the skin and underlying muscular-fibrous connective tissue were removed to expose the periosteum. The periosteal flaps were detached using a periosteal elevator and processed by 2 methods: explant method and enzymatic digestion (4). The periosteal explants were placed with their osteogenic (cambium) side in direct contact with the culture plate in order to provide the adherence for explant culture, while the other periosteal flaps were exposure to collagenase for digestion and cellular suspensions were plated into culture flasks. Bone marrow was extracted by aspiration from the dog humerus and used in order to isolate the mesenchymal stem cells. The Ficoll-Paque technique for the isolation of mononuclear cells, followed by the separation of mesenchymal stem cells by adherence to plastic, has been chosen.

The nutritive medium used for periosteal cells was culture medium HAM/F 12: DMEM (Dulbecco's Modified Eagle's Medium) (1:1) with L-glutamine + 10% fetal bovine serum + 1% Penicillin/Streptomycin + 3ng/ml bFGF (basic Fibroblast Growth Factor) and for bone marrow-derived cells was MEM Alpha Medium (low glucose) + 10% fetal bovine serum + 1% Penicillin / Streptomycin + 3ng/ml bFGF. Culture medium was replaced every 3 days. The morphology was evaluated by phase contrast and light microscopy.

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Expression of Vimentin

Monoclonal Antibody to Vimentin (Clone V9) is use to identify qualitatively by light microscopy tissues of mesenchymal origin using immunohistochemical staining methods.

The cells from Nalgene Nunc Permanox chamber slides were fixed with 100% methanol (-20°C, 10 minutes). The fixed cultures were washed twice with phosphate buffer saline and incubated for 30 minutes with anti-vimentin primary antibody diluted in phosphate buffer saline, at room temperature, on shaker. After washing with phosphate buffer saline, biotinylated secondary antibody was added and the cells were incubated for another 30 minutes, at room temperature, on shaker. After washing with phosphate buffer saline, the cells were incubated for 20 minutes with peroxidase-conjugated streptavidin followed by a 10 minutes incubation period with AEC (3-amino-9-ethylcarbazole) peroxidase substrate. After washing with tap water and counterstaining with hematoxylin (30 seconds), the Permanox slides were mounted in an aqueous mounting medium and the cover slip was applied.

Culture condition for differentiation to osteogenic, chondrogenic and adipogenic lineages

At passage 2 (P2), cells obtained from the two cellular sources were re-seeded in culture flasks characteristic for evaluation each type of differentiation towards the three lineages: osteoblastic, chondrogenic and adipogenic. Cell seeding required different cellular concentrations: $15x10^3$cells/cm^2 for osteoblastic differentiation, $4x10^4$ cells/cm^2 for adipogenic differentiation, and $3x10^4$ cells/cm^2 for monolayer chondrogenic differentiation. Ready-to-use Miltenyi Biotec differentiation media were used: NH OsteoDiff Medium, NH ChondroDiff Medium and NH AdipoDiff Medium, supplemented with 1% antibiotic solution.

Analysis of the differentiation potential into the osteogenic lineage

After 10 days culture in osteogenic medium, the alkaline phosphatase presence in the cultures, the potential to form a mineralized matrix and the collagen I immunolocalization were determined histochemically as well as imunohistochemically.

a. Alkaline phosphatase histochemical staining

A ready-to-use precipitating substrate system for alkaline phosphatase is 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/ NBT) liquid substrate system. This substrate system produces an insoluble NBT diformazan end product that is blue to purple in color and can be observed visually.

After rinsing the cells on the Nunc Permanox chamber slides with cold (4°C) phosphate buffer saline, the cells were fixed with acetone (-20°C) for 5 minutes. The fixed cultures were washed with cold distilled water and allowed to dry for 30 minutes. The cultures were incubated for 10 minutes with BCIP/ NBT liquid substrate system at room temperature. The reaction was stopped by removing the substrate solution and washing with distilled water. The Nunc Permanox slides were mounted in an aqueous mountant.

b. The potential to form a mineralized matrix

The mineralization of the bone matrix was analyzed histochemically by Von Kossa staining. Reveals calcium salts (phosphate, carbonate, sulfate, oxalate). Calcium phosphate deposits can be detected by the Von Kossa technique in which phosphate deposits are stained black.

The cultures were rinsed with cold (4°C) phosphate buffer saline and fixed with 4% formaldehyde (4°C, 10 minutes). After washing with cold distilled water, the fixed cultures were covered with a 5% silver nitrate solution and kept for 30 min in a dark room. Afterwards, they were rinsed and covered with distilled water and exposed to ultraviolet light for 1 hour. The cultures were rinsed in distilled water and treated for 2 minutes with a 5% sodium thiosulphate solution. After washing with tap water, counterstaining with hematoxylin solution for 30 seconds, followed by washing with tap water, the Nunc Permanox slides were mounted with an aqueous mounting medium.

c. Collagen I immunolocalization

After rinsing the cultures on the Nunc Permanox chamber slides with cold (4°C) phosphate buffer saline, the cells were fixed with 4% formaldehyde (4°C, 8 minutes). The fixed cultures were washed with cold distilled water and let to dry. The cultures were incubated for 30 minutes with anti-collagen primary diluted antibody, on shaker. After washing with phosphate buffer saline, biotinylated secondary antibody was added and the cells were incubated for another 30 minutes, on shaker. After washing with phosphate buffer saline, the cells were incubated for 30 minutes with peroxidase-conjugated streptavidin followed by a 10 minutes incubation period with AEC (3-amino-9-ethylcarbazole) peroxidase substrate. After washing with tap water and counterstaining with hematoxylin, the Permanox slides were mounted in an aqueous mountant.

Visualization is based on enzymatic conversion of a chromogenic substrate AEC into a colored red precipitate by horseradish peroxidase (HRP) at the sites of antigen localization, which can then be viewed using bright-field microscopy.

Analysis of the differentiation potential into the chondrogenic lineage

After 24 days under a chondrogenic environment, the dog periosteum-derived cells and bone marrow-derived cells from monolayer cultures were processed for histochemical staining. Safranin O and Alcian Blue were used to stain proteoglycans in the extracellular matrix, a positive indicator of cartilaginous tissue.

Safranin O stains proteoglycans and glycosaminoglycans. The cultures on the Nunc Permanox chamber slides were rinsed with phosphate buffer saline and fixed with formaldehyde and then ethanol 50% for 10 minutes. After washing with distilled water, the fixed cultures were covered with a 0.1% Light Green solution for 2 minutes. The cultures were rinsed in distilled water and treated for 30 seconds with 1% acetic acid and then with 0.1% Safranin O for 5 minutes, followed by washing with distilled water. The cultures were treated with 50% ethanol for 10 seconds. The Permanox slides were washed with distilled water and were mounted in an aqueous mountant.

For Alcian Blue stain, the cultures were fixed in the same way and treated with a hematoxylin solution for 3 minutes. After washing with tap water, the fixed cultures were covered with 1% Alcian Blue solution in 3% acetic acid for 20 minutes, followed by washing with tap water. The cultures were treated with 50% ethanol for 10 seconds and mounted.

Analysis of the differentiation potential into the adipogenic lineage

After 21 days of adipogenic stimulation, the dog periosteum-derived cells and bone marrow-derived cells from monolayer cultures were rinsed with phosphate buffer saline and fixed in 10% formalin for 30 minutes at room temperature. After washing with sterile water, the fixed cultures were treated with 60% isopropanol for 2 minutes and then incubated with freshly prepared Oil Red O working solution for 5-10 minutes to stain lipid vacuoles. After washing with tap water, the Permanox slides were mounted in an aqueous mountant.

RESULTS

Bone marrow and periosteum surrounding the femoral bone of five adult dogs were used. Cells from the osteogenic layers of the periost were obtained by explant cultures of periosteal fragments. Cells migrated from the tissue within 3-5 days after harvest. In the same time interval of 3-5 days, periosteum-derived cells processed by digestion method and bone marrow-derived cells induced the occurrence of fibroblast-like adherent cells within the culture flasks. Cellular confluence for studied cellular types was reached within 10-14 days. All samples gave rise to primary cell cultures with a fibroblast-like morphology, spindle-shape, and increased confluence and next passage cultures showed adherent cells uniformly distributed over the tissue

culture dish with cells merely having a fibroblast-like morphology (4).

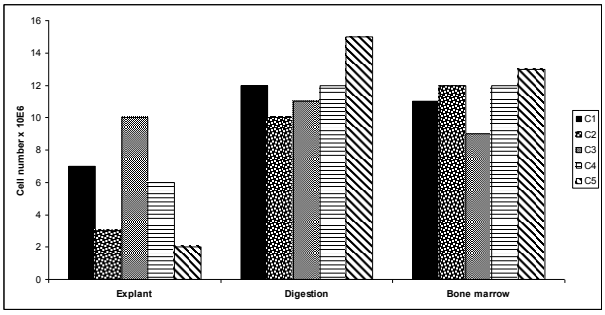

Fig. 1. Comparison between total cell count at P2 of periosteum-derived cells (explant and enzymatic digestion isolation methods) and bone marrow-derived cells

In order to characterize the cellular types that would be further used in differentiation process towards osteogenic, chondrogenic and adipogenic lineages we analyzed the degree of Vimentin expression. Vimentin is a protein present in the cells of mesenchymal origin. It belongs to the class of intermediate filaments of the cell associated with both the nuclear and plasma membranes. Dog periosteum-derived cells and bone marrow-derived cells are characterized immunohistochemical by their positive expression of Vimentin (Figure 2).

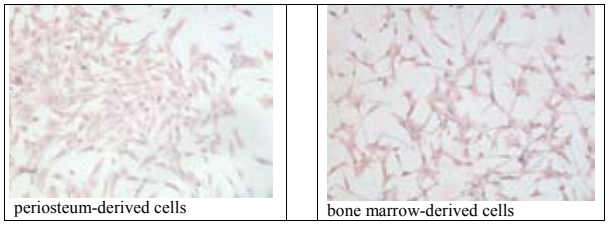

Fig.2. Expression of vimentin (x100)

Osteogenic differentiation was evaluated based on alkaline phosphatase presence in the cultures (an early marker), the potential to form a mineralized matrix and the collagen I immunolocalization. The mineralization of a collagen contained by extracellular matrix represents late markers. Osteoblasts can be identified morphologically by their cuboidal appearance and by their association with newly synthesized bone matrix.

Committed osteogenic cells are histologically characterized by their positive expression of alkaline phosphatase, an enzyme that is involved in the bone matrix mineralization. BCIP/NBT liquid substrate system produces an insoluble nitro blue tetrazolium diformazan end product that is blue to purple in color (Figure 3). It can be shown that within the same time interval, the osteogenesis process is more advanced in periosteum-derived cells than in bone marrow-derived cells.

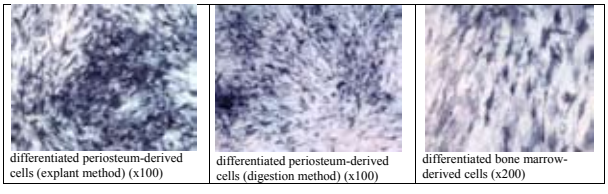

Fig 3. Osteogenic differentiation. a) Alkaline phosphatase activity

The potential to form a mineralized matrix is highlighted by the osteoblasts specialized functions to secrete mineralization factors like calcium salts. Calcium phosphate deposits can be detected by the Von Kossa technique in which phosphate deposits are stained black with silver (Figure 4).

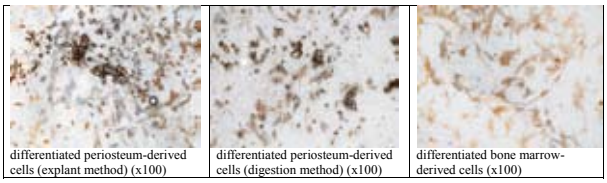

Fig.4. Osteogenic differentiation. b) Mineral deposits stained by the von Kossa technique

In the differentiated cultures from periosteum-derived cells, the mineral deposits showed much intense than the same marker in differentiated cultures from bone marrow-derived cells, which suggest that within the same time interval, the osseous mineralization process is faster in the first case, with the specification that cells obtained by explant method seemed to induce the most intense mineralization. In the 10th day of differentiation towards the osteoblastic lineage, collagen I synthesis can be observed within the cells (Figure 5).

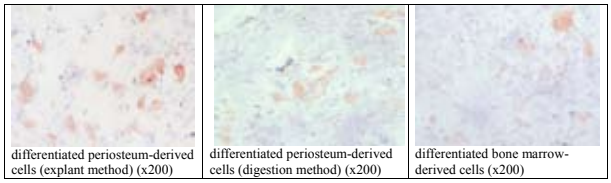

Fig.5. Osteogenic differentiation. c) Collagen I immunolocalization

After three weeks (24 days), the monolayer culture of dog periosteum-derived cells and bone marrow-derived cells in chondrogenic medium showed that the cells were differentiated into chondrocyte-like cells. The histochemical results of Alcian Blue and Safranin-O staining showed that the differentiated chondrocyte-like cells produced chondrocyte-specific extracellular matrix (Figures 6 and 7).

In monolayer culture, periosteal cells begin to form chondrocyte colonies and continued to expand forming a tightly organized mass of cells. Tendency to form chondrocyte colonies is more evident in the cultures differentiated from periosteum-derived cells than those differentiated from bone marrow-derived cells.

Adipogenic differentiation was evaluated based on production and accumulation of neutral lipids in differentiated cells. Adipocytes are rounded and filled with lipid droplets, which might fuse to form vacuoles that can be stained by Oil Red O, a lipophilic red dye (Figure 8).

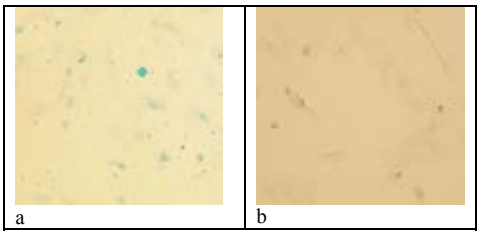

Fig.6. Chondrogenic differentiation of periosteum-derived cells (explant method). Staining for Alcian Blue (a) and Safranin-O (b), (x200)

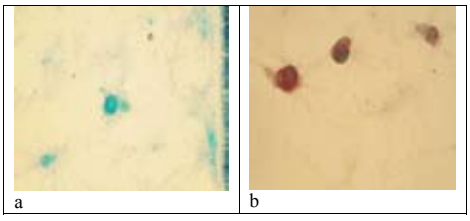

Fig.7. Chondrogenic differentiation of bone marrow-derived cells. Staining for Alcian Blue (a) and Safranin-O (b), (x200)

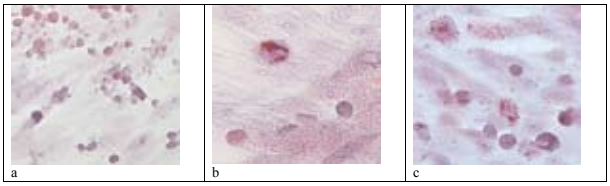

Fig.8. Adipogenic differentiation of periosteum-derived cells (a. explant method – x200, b. digestion method – x400) and bone marrow-derived cells (c) (x400). Staining for Oil Red O

Our findings indicate that there are special characteristic features in differentiation of studied cells to adipogenic lineage according to isolation methods of dog periosteum-derived cells. The adipogenesis ability of bone marrow-derived cells is greater than that of periosteum-derived cells, especially than periosteum-derived cells processed by explant method.

DISCUSSION

In bone fracture healing, it is important that restoration of the damaged area results in the formation of new bone, having the same integrity as the surrounding bone tissue. To determine a suitable cell source, we isolated dog mesenchymal stem cells from periosteum and bone marrow from the same subjects and compared their properties for expansion, multipotentiality and morphology.

The "conventional" mesenchymal stem cells are those obtained from bone marrow (5,6). However, there are increasing reports that mesenchymal stem cells can be isolated from various other tissues as well (7,8,9). From periosteum were isolated and characterized periosteum-derived progenitor cells by various surface markers of mesenchymal stem cells (10). Mesenchymal stem cells have been assumed to be similar irrespective of their original tissue source since they all have self-renewal and multidifferentiation potential with common surface epitopes (11,12). However, the properties of mesenchymal stem cells can be affected by their preparations (13,14) which have not been properly controlled for in some studies.

Present study aims to reveal the similarities and differences between the cells obtained as a result of canine periosteal-derived cells cultivated using explant/digestion method and canine bone marrow-derived cells, in three different differentiation media. The expansion potential of dog periosteum-derived cells (digestion method) is no lower than that of bone marrow-derived cells cultivated in growth medium, with the specification that explant method provides a relatively reduced amount of periosteal cells than the enzymatic method, as demonstrated in our previous study (4). Cell types used in present study have a remarkable viability and proliferative capacity. No obvious differences in morphology were noted between the studied cells. However, cells committed to differentiation have been found to have lower proliferative potential than cells cultivated in growth medium, thus confirming the results revealed in other similar studies (15). From the cells induced toward differentiation, we concluded that explant method-obtained periosteal cells had the highest proliferation rate, with an increased tendency to form cellular aggregates and colonies. This tendency appears in osteogenic and chondrogenic differentiation (Figures 3,6). Thus, the use of periosteal cells may shorten the cell culture period, thereby reducing both cost and the risk of contamination.

Periosteum-derived cell preparations can form cartilage and bone in vitro and in vivo (16,17) as well as adipocytes in vitro (18) (digestion method). The present study focuses on characteristic features regarding the differentiation potential of canine periosteum-derived cells, depending on the method chosen for processing of the harvested periosteal fragments. While the dog bone marrow derived-cells differentiate towards all three lineages, thus confirming the results of other research studies in this field referring to other species, canine periosteum-derived cells obtained using culture of periosteal explants, differentiate mostly towards the osteoblastic and chondrogenic lineage, and to lesser extent towards the adipogenic lineage; the cells obtained using the digestion method of periosteal fragments positively differentiate towards all the three lineages: osteoblastic, chondrogenic and adipogenic. A possible explanation could be that the cells obtained from the osteogenic part of the periosteum (cambium) using the explant method are mainly precursors of the osteoblatic/chondrogenic lineage, while the periosteal cells obtained using the digestion method could contain cambium cells contaminated with other cellular types derived from the fibrous upper layer. Periosteum is at the boundary between the bone and the surrounding soft tissues and contains multiple cell types (cells of the cambium layer, fibrous layer, and vascular pericytes) that could potentially function as progenitor cells. This issue has important implications in the preparation of cellular products for clinical applications.

The round cuboidal cells are considered to be the osteo/chondroprogenitor cells (19, 20). According to Izumi et al. (19), alkaline phosphatase expression in the periosteum is not from fibroblasts or chondrocyte precursors, but from osteoblasts and osteoblast precursors. This suggests that the periosteal cells culture is enriched in alkaline phosphatase-positive progenitor cells or assumes that almost all the cells are able to differentiate into osteoblasts. By Hanada et al. (20), exist 2 types of cells, alkaline phosphatase-positive and alkaline phosphatase-negative cells present at early culture periods. These alkaline phosphatase-negative cells are not only contaminating fibroblasts from the outer layer of periosteum, but also undifferentiated mesenchymal progenitor cells capable of differentiating into the osteogenic lineage.

For the purpose of treating defective articular cartilage, mesenchymal stem cells have been isolated from bone marrow, synovial membranes, periosteal tissue, and umbilical cord blood (10,21,22,23). Periosteum has the potential to differentiate into neocartilage. It contains a lot of progenitor cells that directly differentiated into chondrocytes and have a chondrogenic potential regardless of passage number and donor age (15,24), therefore periosteum-derived cells can be used for hyaline cartilage treatment. In vitro chondrogenesis as a monolayer culture system was performed to evaluate the chondrogenesis potential of cells derived from periosteum and bone marrow. Histologically, all samples exhibited a cartilage matrix that stained with Alcian Blue and Safranin O (Figures 6,7).

The cryopreserved studied cells have been tested for their differentiation capacity. In the present study, we demonstrated that dog periosteum-derived cells can be differentiated into mesodermal cell types, such as osteoblasts, chondrocytes and adipocytes, just like dog bone marrow-derived cells and could be used in place of bone marrow-derived cells which are widely used, as a useful source of bone regeneration. According to other studies, the periosteum-derived cells osteogenic potential persists even after prolonged cultivation in osteogenic medium. Longer culture periods (3–4 months) can be performed before final differentiation is obtained, making these cultures suitable for long-term in vitro evaluation of biomaterials (25,26). Moreover, sometimes harvesting periosteum-derived cells is easier and less invasive than yielding bone marrow-derived cells such in bone reconstructive surgery, general dental medicine -regeneration of alveolar bone in periodontitis and implant dentistry. Therefore, periosteum-derived cells could be candidates for tissue engineering and cell-based therapeutic strategies.

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ANALIZA COMPARATIVA A POTENTIALULUI DE DIFERENTIERE A CELULELOR DERIVATE DIN PERIOSTUL SI MADUVA OSOASA CANINA SPRE LINIILE OSTEOGENICA, CONDROGENICA SI ADIPOGENICA

REZUMAT

Scopul acestui studiu a fost investigarea posibilitatii celulelor periostale si a celor derivate din maduva osoasa canina de a se diferentia spre liniile osteogenica, condrogenica si adipogenica. Rezultatul acestui experiment doreste sa evidentieze asemanarile cat si deosebirile intre celulele obtinute in urma cultivarii celulelor periostale si a celulelor derivate din maduva osoasa in trei medii variate de diferentiere. In timp ce celulele derivate din maduva osoasa canina se diferentiaza spre cele trei linii celulare, confirmand rezultatele altor cercetari in domeniu cu aplicatii pe alte specii, celulele periostale canine se diferentiaza diferit, in functie de metoda aleasa pentru prelucrarea fragmentelor de periost recoltat. Astfel, celulele periostale obtinute prin cultivarea explantelor de periost se diferentiaza cu precadere spre linia osteoblastica si condrogenica si mai slab spre linia adipogenica, in timp ce celulele obtinute prin metoda digestiei enzimatice a fragmentelor de periost se diferentiaza pozitiv spre toate cele trei linii celulare: osteo-, condro- si adipogenica. In plus, s-a putut demonstra ca celulele crioprezervate prezinta capacitate de diferentiere osteogenica, condrogenica si adipogenica.

Cuvinte cheie: celule derivate din periost, celule derivate din maduva osoasa, metode de izolare, potential de diferentiere

ARISTOLOCHIA CLEMATITIS IN MEDICINE: THE GOOD AND THE BAD

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ABSTRACT

Aristolochia clematitis, member of the *Aristolochiaceae* family is a medicinal plant used from time immemorial as a natural remedy, in obstetrics, in the treatment of snake bites, therapy of arthritis, gout, rheumatism, and festering wounds. Only recently *Aristolochia* plant use as a natural or pharmacologic remedy was banned by the Food and Drug Administration (FDA), because it contains aristolochic acids, carcinogenic and nephrotoxic compounds, considered to be responsible for the development of Aristolochic Acid Nephropathy (AAN) (formerly known as the Chinese Herbs Nephropathy (CHN)) and induction of upper urinary tract tumors. An interest correlation has also been made between *Aristolochia clematitis* and the etiology of Balkan Endemic Nephropathy (BEN).

The purpose of this review is to provide more insight into the role of *Aristolochia clematitis* as factor or cofactor in the above-mentioned diseases and to explain why the plant has been blacklisted by the current scientific community after centuries of being considered a powerful remedy in the natural pharmacopoeia.

Key words: *Aristolochia clematitis*, Balkan Endemic Nephropathy, carcinogenesis, nephrotoxicity

INTRODUCTION

Aristolochia clematitis (Figure 1a, b), member of the *Aristolochiaceae* family, popularly named in Romania *„Mărul Lupului"*, *„Curcubetica"*, *„Buruiana de remf"*, is a medicinal plant that has a very long history of medicinal use, though it is rarely used by herbalists nowadays. It is an aromatic tonic herb that stimulates the uterus, reduces inflammation, controls bacterial infections, and promotes wound healing (1).

Fig. 1a. *Aristolochia clematitis* leaves and flowers

Fig. 1b. *Aristolochia clematitis* fruit

Herbal drugs derived from *Aristolochia* species have been known since antiquity and were used in the treatment of skin infections, and in the treatment of snake bites in ancient Egypt and India. Contemporary traditional medicine has used Aristolochia plant extracts for the therapy of arthritis, gout, and rheumatism (2). Externally, various parts of the plant are used in the treatment of slow-healing cuts, eczema, infected toe and finger nails, but internal consumption can cause uterine bleeding and especially damage to the kidneys (1).

The plant contains a complex of acids so called "aristolochic acids", which stimulate white blood cell activity and speeds the healing of wounds if it is used in the right concentration (1). Aristolochic acids (AA) are a mixture of structurally related nitrophenanthrene carboxylic acid derivatives (Figure 2) found primarily in the genus *Aristolochia* and *Asarum* (3).

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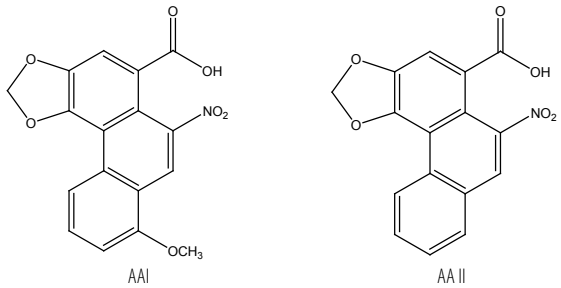

Fig. 2. AA I and AAII chemical structure

Consumption of products containing aristolochic acid has been associated with permanent kidney damage, sometimes resulting in irreversible kidney failure that has required kidney dialysis or kidney transplantation. In addition, some patients have developed certain types of cancers, most often occurring in the urinary tract.

AAN, a rapidly progressive interstitial nephropathy leading to end-stage renal disease and urothelial malignancy, was initially reported in a Belgian group in 1992 of more than 100 patients after the intake of slimming pills containing a Chinese herb, *Aristolochia fangchi* (4). This was an accidental exposure due to a mistake that occured during the preparation of the pills, when *Aristolochia* roots and leaves were inadvertenly used to replace a rather inocuous plant (*Stephania tetrandra*) that was supposed to be included in the pills. Subsequently, the disease has been described in other European countries as well, also in patients that have taken similar pills.

BEN is a chronic tubulointerstitial disease occuring almost exclusively in farming villages situated in valleys of tributaries of the Danube River, in several countries of the Balkan Peninsula (Bosnia, Bulgaria, Croatia, Romania and Serbia). This geographic distribution has been constant since the disease was first described in the late 1950s. The most significant epidemiologic features of BEN are its focal occurrence in certain villages, a familial but not inherited pattern of disease, initial manifestation after residence in an endemic village for 15 years or more, and strong association with urothelial cancers, similar to those encountered in AAN (5).

Aristolochic Acid Nephropathy (AAN)

Initially called Chinese-herbs nephropathy, AAN appears to be the dramatic consequence of the substitution in the slimming pills originating in China of *Stephania tetrandra* with *Aristolochia fangchi*, the latter rich in nephrotoxic aristolochic acids; the substitution occurred because both herbs have very similar common names in *Pin Yin (Han Fang Ji* and *Guang Fang Ji*) (4). This two plants belong to the same therapeutic *"Fang Ji"* family in traditional Chinese medicine and generally their common *Pin Yin* name is being used (4).

Nephrotoxicity manifested by progressive atrophy of renal proximal tubules and development of a characteristic form of interstitial fibrosis involving the outer renal cortex and progressing toward the medulla. The process tends to spare glomeruli and is associated with less inflammation than most other types of interstitial nephritides. Importantly, the syndrome was associated with a high prevalence of urothelial cell carcinomas, which often occurred years after the onset of chronic renal disease. Moreover, in contrast with most other urothelial cell tumors, cancers associated with AAN tend to develop in the upper urinary tract (6).

Because aristolochic acids were identified in the slimming pills taken by the patients with AAN, their were thought to be the main culprit of both the renal failure and the cancers. In order to demonstrate their toxicity several *in vitro* and *in vivo* experiments ensued. Some *in vitro* experiments assesed the cytotoxicity of *Aristolochia* plants' extracts on different cell types, like human renal epithelial HK-2 cell line, mesenchymal stem cells, with different concentrations of aristolochic acids and also extracts from different parts of the plant (7). The AA toxicity was evaluated also in vivo, on rabbits, rats and mice, reproducing to a large extent the kidney pathology and tumorigenesis encountered in patients with AAN. These studies have demontrated beyond any doubt the central role of AAs in the pathogenesis of AAN/CHN (8).

Balkan endemic nephropathy (BEN)

BEN, a chronic tubulointerstitial fibrosis with slow progression to end-stage renal disease and urothelial malignancy (4), is so named because it is presently known to affect only rural inhabitants of Romania, Bulgaria, Bosnia, Serbia, Croatia, in villages located along rivers of the Danube River basin (Figure 3) (5,8,9).

Fig. 3. Map showing the geographical distribution of the known Balkan nephropathy areas (9)

About 25,000 people suffer or are suspected of having BEN, whereas the total number of people at risk in this countries may exceed 100,000 (4). The adults in their fourth or fifth decade of life are usually affected by the disease, with end-stage renal failure developing by their sixth decade (8). The most important epidemiologic features of BEN include its focal occurrence in certain farming villages, a familial but not inherited pattern of disease, and strong association with upper urinary tract urothelial malignancies (5).

This disease is relatively asymptomatic and the patient becomes symptomatic only when the advanced renal failure becomes manifest. The clinical and paraclinical features are those of chronic renal failure, including severe anemia, polyuria, polydipsia, nicturia, abnormalities on urinalysis, small and shrunken kidneys, and rarely hypertension (8). Upper urinary tract cancers are also frequently diagnosed in BEN.

The etiology of BEN remains obscure despite all the research and studies conducted over the past 50 years (5). However, based on the evidence accumulated during the last several decades, it can be stated that BEN is an environmentally induced disease. There are three current theories that attempt to explain the environmental cause of BEN: the aristolochic acid hypothesis, which assumes that the disease is produced by chronic intoxication with *Aristolochia clematitis* seeds; the mycotoxin hypothesis, which claims ochratoxin A to be the main culprit of BEN; the Pliocene lignite hypothesis, which proposes that the disease is caused by long-term exposure to polycyclic aromatic hydrocarbons and other toxic organic compounds leaching into the well and spring drinking water from low-rank coals found in the vicinity of the endemic settlements. As not all the people living in the endemic villages develop BEN it is logically assumed that a genetic susceptibility is also involved in disease emergence (8). The gene-environment interaction is translated into peculiar xenobiotic substance metabolism (controlled by cytochrome P450 and other enzymes) that increases the risk to develop BEN only in those people that bear certain gene variants coding for those detoxification enzymes.

The „AA hypothesis"in BEN was formulated for the first time in 1969 by Ivic (14), based on the observation that *Aristolochia clematitis* plant occurs with higher frequency in BEN endemic compared to nonendemic regions, and it often grows

as a weed in fields cultivated with wheat. Ivic formulated the idea that during the annual harvest the wheat grain is mixed with *Aristolochia* seeds (which contain a tenfold amount of Aas compared to the leaves) and as a consequence the bread made from the contaminated flour is tainted with small amounts of AAs. Thus, residents of the endemic region who ingest this kind of bread may be exposed, over time, to cummulative toxic amounts of AAs (5).

Although the flour contamination hypothesis could be standing for BEN countries like Croatia or Serbia, it is contradicted by the hilly topography of the endemic, as well as nonendemic, villages in Romania, where wheat cultivation has been very limited. Other crop plants, like staple corn, are much more frequently cultivated here but to what extent AA can contaminate the corn seeds and enter the human food chain are open questions. Another topic of discontent is the medicinal use of *A. clematitis*, which has been consistently reported only for Romania although without any significant difference between endemic and nonendemic households, but not for the other BEN afflicted countries.

Analyzing the description of the clinical, morphological and biological features of AAN and BEN striking similarities can be observed. Another argument for the role of AAs as etiologic factors/cofactors in both types of nephropaties is the recent detection of AA-DNA adducts in kidney and urinary tract tissues from patients with AAN, and also in the renal tissues of some patients with BEN; such adducts were claimed to be absent in patients with chronic renal diseases or with upper urinary tract malignancies from non-endemic areas (7-12)

Based on this kind of information, in 2001, the Food and Drug Administration (FDA) issued warnings and an import alert that herbal products are unsafe if they contain or are suspected to contain aristolochic acids. Despite the action of the FDA and the effort of many countries, 19 products containing AAs and 95 products suspected to contain AAs were for sale on the Web in 2003 (13) and are currently sold in many open markets in Romania.

The International Agency for Research on Cancer also classifies products containing *Aristolochia* species as group 1 human carcinogens.

CONCLUSIONS

Considered an "aristocratic" plant in ancient times (hence the name), *Aristolochia* was used in folkloric medicine to succesfully treat various ailments, from snake bites to cancer. It is still used as a medicinal plant in several rural places in Eastern Europe and elsewhere. For instance, in the Balkan Peninsula, dried and powdered leaves are mixed with pig fat and used by some people for topical application for arthritic pain or bruises; leaf decoction (usually one or two leaves boiled in one liter of water) is also used by women for vaginal bath, however, such uses would imply very little or insignificant exposure to AA. Topical absorption of traces of AA might occur though, sufficient to accumulate for detection in tumours, but extensive GC-MS urinalysis of samples from Romanian nephropathy villages by one of us (CT) has failed to find any trace of AA. Infrequent use of such *A. clematitis* preparations might in part be responsible for the negative results.

In spite of being declared "bad" by the local health authorities in many countries, in the folkloric wisdom *Aristolochia* has always been considered to be a "good" plant, provided several precautions were taken (i.e, only leaves and roots collected during the flowering season and in very small amounts should be used). Any rare intoxications with long term consequences like kidney damage or cancer may have emerged due to improper administration and/or dosage, but this is the case with most pharmaceutical drugs currently found in the drugstores.

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ARISTOLOCHIA CLEMATITIS IN MEDICINA: BENEFICII SI EFECTE ADVERSE

REZUMAT

Aristolochia clematitis, membra a familiei Aristolochiaceae este o planta medicinala folosita din timpuri imemoriale ca remediu natural in afectiunile obstetrice, in tratamentul muscaturilor de sarpe, terapia artritei, gutei, reumatismului si vindecarea ranilor. Folosirea plantei Aristolochia ca remediu natural sau farmacologic a fost doar recent interzisa de Food and Drug Administration (FDA), deoarece contine acizi aristolohici, compusi carcinogenici si nefrotoxici, care sunt considerati a fi raspunzatori de dezvoltarea nefropatiei aristolohice (AAN) (cunoscuta anterior ca nefropatia plantelor chinizesti (CHN)) si de inducerea tumorilor de tract urinar superior. De asemenea, a fost observata o corelatie interesanta intre Aristolochia clematitis si etiologia Nefropatiei Endemice Balcanice (NEB).

Scopul acestui studiu este de a furniza mai multe date despre rolul Aristolochia clematitis ca factor si cofactor in afectiunile amintite anterior si de a explica de ce aceasta planta a fost recent introdusa pe „lista neagra" de catre comunitatea stiintifica, dupa secole in care a fost considerata un remediu important al farmacopeei naturale.

Cuvinte cheie: Nefropatie Endemica Balcanica, carcinogeneza, nefrotoxicitate

BETA-ADRENERGIC STIMULATION AND PULMONARY FUNCTION MODULATION IN ASTHMA AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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ABSTRACT

Adrenergic receptors have a wide distribution in the human body. There has been a progression in the discovery of their various subtypes, at least nine such receptors having been described so far. The message stimulation and translation of these receptors is extremely complex, going beyond the classical description of the second-messenger activation (cyclic adenylmonophosphate – cAMP); there is a complex chain of events which involves the participation of multiple protein isoforms/subunits (G protein, adenyl cyclase, phosphokinase, etc.) as well as their blockers. If the possible genotypic differences of β2 receptors are also taken into consideration, then it would be possible to predict the different clinical responses to therapy[1]. On the other hand, the condition of the patient, whether aggravated or stable, may also influence the response to treatment. Bronchial asthma and chronic obstructive pulmonary disease (COPD) offer a wide possibility of using short- or long-term-acting β-antagonists. Their administration in association with inhaled corticosteroids (ICS) enhances both their effectiveness and their safety.

Key words: β2 receptors, stimulation, asthma, COPD

ELEMENTS OF PHARMACOLOGY OF B-RECEPTORS

Pharmacological involvement in the airways is possible due to the receptors existing in the smooth muscles of the airways, in the secretory cells, in the bronchial epithelium and in the bronchial and pulmonary vessels. The lungs contain both sympathetic (adrenergic) and parasympathetic (cholinergic) receptors; their names come from their respective neurotransmitter: norepinephrine (also known as adrenalin), and acetylcholine. As cholinergic receptors can be detected both in the neuromuscular junction and in the parasympathetic lymph nodes, the term muscarinic agent refers only to the cholinergic receptors acting in the neuromuscular junction. Each type of receptor may have stimulating agent, the so-called agonists, or, on the contrary, inhibiting agents, called blockers. In their turn, adrenergic receptors have been divided into two main groups, α and β, although at least nine distinct subtypes have been decoded based on their respective genes: α_{1A}, α_{1B}, α_{1D}, $\alpha_{2A/D}$, α_{2B}, α_{2C}, β_1, β_2, β_3.

Thus, α-adrenergic stimulation generates vasoconstriction and subsequently increases blood pressure, β-adrenergic stimulation increases heart rate and inotropism, causes bronchodilatation, stimulates mucociliary activity and induces a mild inhibition of inflammatory mediator release. Bronchodilatation, due to β-adrenergic stimulation, is the desired effect in the therapy of obstructive bronchial syndromes consequently the present study focuses only on the modulation of β_2-receptor function.

Both α and β receptors have to be linked to/with G proteins. There are different forms of α, β and γ subunits, generating a great heterogeneity of G proteins involved in the signal-translation mechanism. Gs and Gi proteins stimulate and, respectively, inhibit adenylate cyclase, modulating cAMP concentration. Subsequently, a rise in the cAMP induces the activation of cAMP-dependent proteinkinase cascade. The finding that Gs can also activate membrane C^{++} channels showed that G protein can control several effectors. Other G proteins, such as Gq, Go, Gpic, associate receptors with C phospholipase activation or Gk, which is connected with the K^+ channel. The activity of another enzyme, MLCK (Myosin-Light-Chain-Kinase), is essential for the activation of actomyosin interaction. MLCK activity is mostly controlled by calmodulin intracellular Ca^{++}. Airway relaxation modulators may alter MLCK phosphorilation by regulating certain phosphoproteinphosphatase (1) (Fig. 1) (3,4).

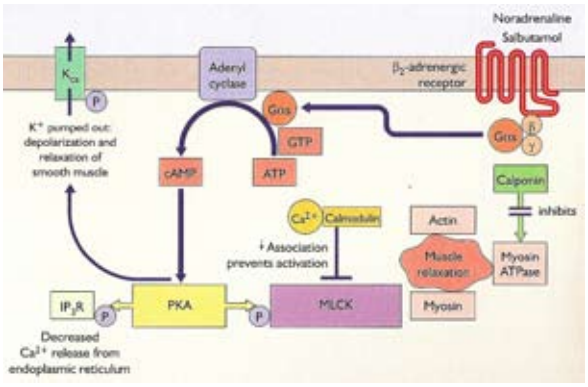

Fig. 1. The relaxation of the smooth muscle is determined by the association with an agonist such as adrenalin or salbutamol to the β receptor. The association takes place in the receptor's Gs component which will stimulate adenyl cyclase, inducing the transformation of adenosine triphosphate (ATP) into adenosine monophosphate (cAMP). An increase in the cAMP concentration will activate A protein kinase (APK) which will phosphorilate MLCK. The latter will decrease its association with calcium/calmodulin. Besides, inositol triphosphate receptors (IP3R) from the endoplasmic reticulum (ER) are phosphorilated by the APK. This will, in turn, lead to a decrease in calcium release from ER in the cytoplasm. These two pathways will induce the depolarization and relaxation of the smooth muscle cell.

Thus, up to the moment of bronchial relaxation, there is a cascade of events involving multiple genotypic adenyl cyclase (AC), protein kinase (PK) and phosphodiesterase (PD) variants, as well as various blockers (scaffolds) (Fig. 2) (1). If all these are also associated with possible genotypic differences (substitution of Arg with Gly in the position B-16) of β2 receptors, this could predict different clinical responses

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to treatment. On the other hand, response to treatment can also be influenced by the condition of the patient, whether aggravated or stable.

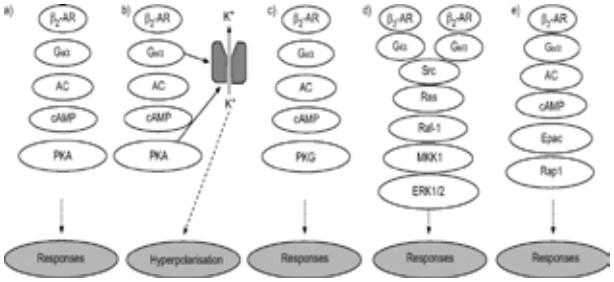

Fig. 2. The translation of β2 adrenergic receptor stimulation is mediated by a complex cascade of events which involves the participation of multiple G, AC, PK and PDE protein isoforms/ subunits, as well as of their scaffolds. Thus, (a) the interaction of the agonist with the β2 receptor (β2-AR) in the bronchial muscle cell membrane triggers the release of the α-subunit-stimulating G protein (Gs) from an αβγ heterotrimetric complex. Subsequently, the released Gsα will boost the activity of one or more AC isoforms, resulting in the intensification of cAMP formation from ATP. cAMP will be associated with the PK (A) regulating subunit which, by target protein phosphorilation, will induce cell bronchodilatation response. (b) K^+ channels may also be targets for the PKA, opening after phosphorilation and producing a flow of K^+, which will induce a diminishing of the excitability and the subsequent bronchial relaxation. The opening of the K^+ channels may also take place through the direct interaction of Gs with the respective channels, independent from cAMP and PKA. (c) The β2-AR agonist couple may induce an increase in the cAMP concentration triggering bronchial relaxation and PKG activation. (d, e) Still, the cascade initiated by β2-AR linking may follow the pathway of tyrosine kinase activation (Src), either through the $G_{i\alpha}$ or the $G_{s\alpha}$ subunit, leading to the formation of Ras, Raf-1 or MKK-1 (mitogen-activated protein kinase linase) and ERK (extracellular signal-regulated kinase) activation; or, independent from PKA, cAMP activation can, through Epac (exchange proteins directly activated) lead to the same result (bronchodilatation) via Rap-1.

There is an extremely varied range of β-adrenergic agonist agents which differ through their affinity to a certain subtype of receptor (α, β1, β2, etc), and through their pharmacodynamic properties (onset of action, peak intensity, length of action, etc). A general classification is also the one that delimits β-agonists to ultrashort-acting agents (epinephrine, isoproterenol, isoetharine), short-acting agents (metaproterenol, terbutaline, salbutamol, pirbuterol), and long-acting agents (salmeterol, fenoterol).

The former, being all unspecific β2 catecholamines, had a high cardiac adverse potential (tachiarrhythmias, increased blood pressure). Due to the strong effect of α1 and of their vassoconstrictive effect, they are only used as ingredients in nasal decongestants or to limit severe episodes (anaphylaxis, croup).

Short-acting β-agonists (4-6 hours) also lack 2 specificity (with the exception of levalbuterol, the R-isometric form of albuterol, which has β2 affinity), have been replaced with long-acting β-agonists (~ 12 hours), which are also β2 specific. The latter refer to salmeterol, which was introduced in 1994 and to formoterol, introduced in 2001. Both drugs have a duration of action up to 12 hours, only differing through the onset of their action (~ 20 minutes for salmeterol, and only 5 – 15 minutes for formoterol), and through the rapidity with which they reach their peak effect (faster for formoterol, similar to that of salbutamol). On the other hand, it should be taken into account the fact that salmeterol is a partial agonist, while formoterol is a full-agonist, with greater relaxing effect, but from the point of view of β2/ β1 selectivity = 85,000/400 = samleterol/formoterol (5).

BENEFICIAL EFFECTS OF B-AGONISTS

The competition for the validation of the best therapeutic conduct started between short-acting β-agonists (i.e. salbutamol) and long-acting β-agonists (i.e. salmeterol); later on, the competition continued between long-acting β-agonists and combination drugs [inhaled corticosteroids (ICS) + long-acting β-agonists].

The results showed that salmeterol inhaled twice a day is more effective than salbutamol inhaled four times a day in lessening the symptoms of moderate bronchial asthma patients requiring maintenance therapy. There was no worsening of asthma control after using salmeterol for three months. At the same time, the pulmonary function (AUC greater for FEV1) was improved as compared to placebo or salbutamol (6).

Both salmeterol (study conducted on 411 patients with COPD) (5) and formoterol (study conducted on 780 patients with COPD) (7) are better than ipratropium in producing constant long-term bronchodilatation (12 hours). In a 21-week study, the association of salmeterol with ICS resulted in a greater improvement of the pulmonary function than doubling the ICS dosage (p=0.001).

The association of a long-acting β-agonist is recommended when an average dose of ICS is not enough to control symptoms.

The bronchodilating effect of salmeterol remains similar in time. There is no indication of tachyphylaxis ($p<0.001$ vs. placebo) (9). There was a direct correlation between the deterioration of the pulmonary function (assessed by FEV1 or the degree of dyspnea, BDI) and the quality of life (determined by the SGRQ questionnaire). Bronchodilators, obviously influencing FEV1 in asthma and less in COPD, will improve the patient's health (10). Seretide offers instant relief from the very beginning and a very good long-term effect (11). The quality of life will also be improved indirectly if we take into consideration a definite decrease of exacerbations (12,13).

Several studies have shown that combined therapy is more effective than each drug taken separately, having the advantage of easier administration and greater compliance (14,15).

Still, as formoterol has a faster onset than salmeterol, is preferred both in rescue therapy and in controller therapy when used in combined therapy (budesonide + formoterol). Both components, administered "in need", ensure a higher protection against severe exacerbations in patients receiving combined maintenance therapy, thus improving low-medication asthma control (16,17).

Other beneficial effects have been noticed besides bronchodilation:

1. hyperinflation decreases as a direct consequence of bronchodilation (18);
2. mucociliary clearance improves (19,20);
3. various β-agonists increase muscle mass and strength, lessen fatigue, showing potential for improved skeletal (and respiratory) muscle fatigue (21);
4. possible antiinflammatory action by mastocyte stabilization;
5. decreased plasma exudation in the airways by closing the inter-endothelial-cell spaces in the post-capillary venules (22);
6. bacterial adherence decreases in bronchial epithelial cells (23).

ADVERSE EFFECTS

β-agonists may induce various adverse effects: cardiac (tachiarrhythmias, palpitations, lengthening of QT), muscular (tremors, cramps), central nervous (distress, dizziness, anxiety, nausea), metabolic (hypokalemia, hyperglycemia), respiratory (tachyphylaxis, loss of bronchoprotection, bronchospasm, hypoxemia). Association of teophylines increases cardiovascular risk. There are two strategies aimed at diminishing adverse effects and increasing pulmonary effectiveness: using inhaled drugs, and including the drugs in the so-called combined medication.

The reason for using combined medication (ICS + inhaled β-agonists) is supported by an existing mutual amplification relation (synergy) of using corticosteroids and β2-agonists:

- β2-agonists: activate steroid receptors, increase the number of steroid receptors, prevent the down regulation of these receptors, their overall effect being

an amplification of the steroid effect;

- steroids: increase β2-receptor gene transcription, facilitate the association of β2 receptors with adenyl cyclase, their overall effect being an increased desensitization of β2 receptors.

There has been a certain reserve towards these products due to the fact that these agents are systematically absorbed in the lungs and reach the heart without being metabolized in the liver ("first pass"). Even "selective" β2-agonists may increase inotropic and chronotropic response, facilitating atrial and ventricular ectopy (24,25). As shown above, the administration of inhaled substances significantly diminished this risk, and the fact that long-acting drugs are also β2-selective seems to have alleviated all fears.

However, the SMART study (Salmeterol Multicenter Asthma Research Trial) has reactivated suspicions. The trial was aimed at assessing salmeterol safety in bronchial asthma patients; it was a double-blind randomized study lasting 28 weeks that included 26,355 patients (71% Caucasians, 18 % African-Americans and 8% Hispanics) who received either salmeterol or placebo besides their usual medication (26).

The death rate was greater (13 out of 13,176) in the group that received salmeterol vs. the placebo group (3 out of 13,179), i.e., 0.10% vs. 0.02%, which led to the study being prematurely discontinued. However, statistically, the incidence of primary events was significantly greater in the African-Americans who received salmeterol vs. the placebo group (RR=4.1)

Patients who reported using ICS at the beginning of the trial had an occurrence rate of key primary and secondary events similar for both salmeterol and placebo groups (RR=1.2). Patients who did not report using ICS at the beginning of the study, the asthma-related death rate or the development of life-threatening events was significantly higher in the salmeterol group vs. the placebo group (RR=1.6). Whether considering the asthma-related death rate or the development of life-threatening events, differences were even greater in the African-American subgroup who did not use ICS (RR=5.61).

A careful analysis of the SMART data did not yield any clear explanation of these results. Still, possible explanations point rather to a genetic predisposition, to ICS neutralization, or to patient-related factors such as late diagnosis or inconsistent medication, than to a pharmacological effect. Consequently, the GINA guidelines maintain that "long-acting β2-agonists are not a substitute for inhaled or oral glucocorticosteroids, and should only be used in combination with an appropriate dose of inhaled glucocorticosteroid as determined by a physician (27)."

The SMART results are similar to those of another trial, Salmeterol Nationwide Surveillance (SNS) that was conducted for 16 weeks in 1990, and included 25,180 asthma patients from England. SNA showed that the incidence of asthma- and respiratory-related deaths, although statistically insignificant, was greater in salmeterol patients (12/16787) vs. salbutamol patients (2/8393) combined with their usual asthma treatment (28).

In 2004, Salpeter (29) published a meta-analysis conducted on 19 studies. His conclusion was that in long-term studies, the use of β2-agonists was associated with the RR increase of cardiovascular adverse reactions vs. placebo (99=2.45, 95% CI, 1.59, 4.05, p=0.00001). The most common adverse reaction was sinusal tachycardia, which was significantly increased by β2-agonists vs. placebo (RR=3.06, 95% CI, 1.7, 5.5), while the combined incidence of other adverse effects (ventricular tachycardia, atrial fibrilation, syncope, cardiac failure, myocardial infarction, heart arrest, sudden death)was not statistically significant (RR=1.61, 95% CI, 0.76, 3.42).

But the problem with this meta-analysis is both that it lacks homogeneity (it included both asthma and COPD) and that it lacks clarity: we do not know the number of persons that also received ICS, nor the number of patients that received short-acting β-agonists, long-acting β-agonists or combined therapy (29).

Researching MEDLINE, EMBASE, CINAHL and Cochrane, Gustavo Rodrigo identified 27 studies including 8,400 patients, dealing with the safe use of long-acting β-agonists in stable COPD. The analysis of the studies that reported deaths did not show any significant differences between the patients who used long-acting β-agonists and those who received placebo (RR=0.90; 95% CI, 0.76 to 1.06; p=0.20).

The study represents a turning point in the traditional view according to which the use of long-acting β-agonists would represent a risk for patients with COPD associated with cardiovascular disease (30).

Still, precaution should be taken in using short-acting β-inhalers (as MDI) in smoker cardiovascular COPD patients, where the risk of an acute cardiac ischemic event (infarction or angina) is dose-dependent: OR=1.38 for those who used 1-2 canisters/month, OR=1.58 for those who used 3-4 canisters/month, and OR=1.93 for those using 6 or more canisters/month. This situation is the result of the fact that dyspnea or thoracic discomfort, often induced by myocardial ischemia, is misinterpreted as being of pulmonary nature and, consequently, β-agonists that will keep being used leading to a heart attack as a result of overdose. Administration of β-blockers significantly reduces and even eliminates cardiac risk (31).

CONCLUSIONS

Between 1994 – 1998, salmeterol was approved for the treatment of bronchial asthma and of COPD, currently being used in over 100 countries, either as a unique drug or in combination with fluticasone (Seretide, Viane, Advair).

In 2006, the FDA reported a worldwide exposure to salmeterol, estimating 45.2 million patients/year, as follows (32):

- 24.3 million patients/year for salmeterol;

- 20.9 million patients/year for salmeterol associated with fluticasone propionate in a single device.

Following the publication of the results of the most representative meta-analyses regarding the widespread use of this medication, we can conclude that the use of long-acting β2-agonists both in asthma and in COPD is safe, and they should be associated with ICS which enhance both their effectiveness and their safety. Special precautions are needed in patients with severe cardiac disorders or thyroid disorders (33)

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STIMULAREA BETA-ADRENERGICA SI MODULAREA FUNCTIEI PULMONARE IN ASTM SI IN BRONHOPNEUMOPATIA CRONICA OBSTRUCTIVA

REZUMAT

Receptorii adrenergici au o largă distribuție în corpul uman, stimularea lor însoțindu-se de o pleiadă variată de efecte. Decodificarea genelor de transcripție a demonstrat existența a nu mai puțin de 9 subtipuri. Distribuția receptorilor Beta (β1, β2, β3) arată o predominanță β1 la nivelul sistemului cardiovascular și β2 la nivelul aparatului respirator. Stimularea receptorilor β2 s-a dovedit că pe lângă efectul primordial de brohodilatație este susceptibilă, într-o măsură mult mai redusă, de îmbunătățirea clearanceului mucociliar, de o activitate antiinflamatorie, de reducerea exudării plasmatice în căile aeriene, de reducerea aderenței bacteriene la celulele epiteliale bronșice, și chiar are potențialul de a crește masa musculară scheletică, puterea acesteia și ameliora fatigabilitatea, etc.

Mecanismul prin care este posibilă inducerea acestor efecte depășește clasicul releu al "celui de-al doilea mesager", - rectae, creșterea adenosinmonofosfatului ciclic (AMPc) pe calea activării adenyl ciclazei, - antrenând multiple izoforme/subunități de proteine (proteina G, adenilciclaze, phosphokinase, etc) care interacționează în cascadă. Fenotipurile enzimatice codificate genetic, sub influiența unui mediu inflamator local aflat în stare de activitate sau nu, a interferențelor cu medicamentele concomitent administrate, a statusului de fumător sau nu, a vârstei etc, vor modula suplimentar răspunsul la stimularea beta terapeutică. Astmul bronșic, cât și bronhopneumopatia cronică obstructivă (BPOC), oferă un câmp larg de aplicare și testare a diferitelor tipuri de agenți β2 agoniști (de scurtă, lungă și ultralungă durată de acțiune), cât și a combinațiilor care le sporesc eficiența și siguranța (asocierea în același preparat cu un corticosteroid).

Cuvinte cheie: receptori β2, stimulare, astm, BPOC

MATHEMATICAL MODELS OF BREAST CANCER RISK CALCULATION AND ITS APPLICATIONS

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ABSTRACT

A complete assessment of a woman's risk for breast cancer is important. The five year breast cancer risk can vary from 0.4% for a woman age 40 years with no risk to 6% for a woman age 49 years with several risk factors. The 2006 American Cancer Society guidelines recommend early mammograms starting at the age of 40 and continuing for as long as a woman is in good health. The large scale introduction of mammography screening has had as consequences the increasing of breast cancer rate, but also the mortality reduction through the diagnostic in early stages. Current evidence shows variation among women in terms of benefits and harms associated with screening mammography. A new method of calculating breast cancer risk can help to predict a woman's risk of developing an invasive form of the cancer. The technique can provide useful information to women who may be contemplating the risks and benefits of breast cancer preventative strategies. The Gail Model is one of the models that have been developed to estimate a woman's risk of developing breast cancer. The model incorporates a series of questions related to breast cancer risk factors and answers are calculated into a Gail risk score. The model uses a woman's own personal medical history (number of previous breast biopsies and the presence of atypical hyperplasia in any previous breast biopsy specimen), her own reproductive history (age at the start of menstruation and age at the first live birth of a child) and the history of breast cancer among her first-degree relatives (mother, sisters, daughters) to estimate her risk of developing invasive breast cancer over specific periods of time. The Breast Cancer Risk Assessment Tool is an interactive tool designed by scientists at the National Cancer Institute (NCI) and the National Surgical Adjuvant Breast and Bowel Project (NSABP) to estimate a woman's risk of developing invasive breast cancer (NSABP model 2).Because of the complexity of interpretation of some of the input information, the information should be entered by a health professional with some experience in oncology. The combination of these mathematical models with other early diagnostic methods such as mammography or preventive chemotherapy or other can lead to survival rates improving in breast cancer.

Keywords: breast cancer risk, assessment tool, survival rates

INTRODUCTION

Invasive breast cancer is the most common carcinoma in women; it accounts for 22 % of all female cancers. The areas of high risk are the populations of North America, Europe and Australia; the risk is low in the less developed regions of sub-Saharian Africa and Southern and Eastern Asia, including Japan. The prognosis of the disease is very good if detected at an early stage. Breast cancer incidence, as with most epithelial tumours, increases rapidly with age.

The aetiology of breast cancer is multifactorial and involves diet, reproductive factors (young age at menarche, older age at first full-term birth, nulliparity, older age at menopause, oral contraceptives) and related hormonal imbalances.

For almost half a century, the events of reproductive life have been considered to be risk factors for breast cancer in women. Breast cancer occurs more frequently among women who have an early menarche, remain nulliparous or, if parous, have few children with a late age at first delivery. Infertility appears to be a risk factor as may be lack of breast- feeding. Also, late age at menopause increases the risk. Recent data indicates that the age at any delivery, not just the first is associated with breast cancer risk, with deliveries occurring before the age of 30 having a protective effect.

Another important risk factor is exogenous hormones. Two major types of hormonal compounds have been evaluated in relation to breast cancer: oral contraceptives and menopausal replacement therapy. The evidence suggests a small increase in the relative risk associated with the use of combined oral contraceptives, especially among current and recent users, which is not related to duration of the use and type or dose of preparation and may be partly linked to detection bias. Data on injectable pure progestogen contraceptives shows relative risks from 1 to 3 wich are not statistically significant.

Epidemiological studies on postmenopausal estrogen therapy show a small increase in risk with longer duration of use in current and recent users. Information on the effect of postmenopausal estrogen- progesterone therapy was provided in only a minority of studies, but indicates that the increased relative risk in long- term users is not significantly different from that for long-term use of estrogens alone. Yet it should be noted that, among hormone replacement therapy users, there is an over representation of tumours that, with regard to tumour stage, type and grade are associated with a more favorable prognosis.

There is overwhelming evidence from epidemiological studies that sex steroids (androgens, estrogens, progestogens) have an important role in the developement of breast tumours. Breast cancer incidence rates rise more steeply with age before menopause than after, when ovarian synthesis of estrogens and progesterone ceases and ovarian androgen production gradually diminishes.

The estrogen excess hypothesis is central, stipulating that breast cancer risk depends directly on breast tissue exposure to estrogens. In vitro studies show increases breast cell proliferation and inhibition of apoptosis. Animal studies show increased rates of tumour development when estrogens are administered. The risk is higher among postmenopausal women who have elevated plasma levels of testosterone and androstenedione, reduced levels of sex hormone-binding globulin (SHBG), and increased levels of oestrone, oestradiol and bioavailable oestradiol not bound to SHBG.

A second major theory , the estrogen plus progesterone hypothesis, postulates that , compared to exposure to estrogens alone (as in postmenopausal women not using exogenous hormones) , risk of breast cancer is futher increased in women who have elevated plasma and tissue levels of estrogens in combination with progestogens. This theory is supported by observations that proliferation of mammary epithelial cells is increasing during the luteal phase of the menstrual cycle,

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compared to the follicular phase. Among pre-menopausal women, several studies have not shown any clear association between breast cancer risk and circulating levels of androgens, estrogens or progesterone.

In addition their research showed a higher risk among long term oral contraceptives users; especially those had started to take the pill before age 18. Women who have a first- degree family of breast cancer and oral contraceptives exposure may want to be particularly vigilant regarding appropriate breast cancer screening practices.

A metabolic consequence of excess body weight and lack of physical activity is development of insulin resistance. Elevated insulin levels may lead to increased ovarian and / or adrenal synthesis of sex steroids, particularly of androgens, and decrease the hepatic synthesis and circulating levels of SHBG. Especially in postmenopausal women, elevated plasma androgens lead to increased estrogen formation in adipose tissue and hence to increased levels of oestrone and oestradiol. The hypothesis that chronic hyper-insulinemia might explain the observed associations of breast cancer risk with low plasma SHBG and elevated androgens and estrogens, among postmenopausal women has, however, received only limited support . Insulin-growth factor I (IGF-I) and IGF- binding proteins (IGFBP) appear to be significant risk predictors.

Recent studies have shown a fairly consistent though small effect of alcohol consumption on breast cancer risk. In a summary analysis of epidemiologic studies, breast cancer risk increased between 40 and 70 percent with about two drinks daily (9).

A part of these risk factors have been considered for the conception of some tools or mathematical models for the breast cancer risk evaluation. This paper aims for the presentation of two tools such as the ones above and their applicability. The Gail model is named after Mitchell H. Gail who published several famous papers describing the scientific basis for the risk calculation. National Surgical Adjuvant Breast and Bowel Project is an important organization that has conducted many important research studies. The NSABP used the original Gail model and modified it for a research study. That is why it is called NSABP model 2 or Gail model 2, but that would be even more confusing.

The Gail model

The Gail Model incorporates a series of questions 1 through 6 related to breast cancer risk factors and answers are calculated into a Gail risk score, a summary relative risk (3). The model uses a woman's own personal medical history (number of previous breast biopsies and the presence of atypical hyperplasia in any previous breast biopsy specimen), her own reproductive history (age at the start of menstruation and age at the first live birth of a child), and the history of breast cancer among her first-degree relatives (mother, sisters, daughters) to estimate her risk of developing invasive breast cancer over specific periods of time. A medical history of ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) increases the risk of developing invasive breast cancer.

The Gail model has been tested in large populations of white women and has been shown to provide accurate estimates of breast cancer risk (2, 13, 11). In other words, the model has been "validated" for white women. It has also been tested in data from the Women's Health Initiative for African American women and the model performs well, but may underestimate risk in African American women with previous biopsies. The model still needs to be validated for Hispanic women, Asian women, and other subgroups, and results should be interpreted by a health care provider for women with special risk factors, such as women treated for Hodgkin's disease with radiation to the chest and carriers of gene mutations that increase breast cancer risk. Researchers are conducting additional studies, including studies with minority populations, to gather more data and to test and improve the model. While race/ethnicity is included in the calculation, it does not influence breast cancer risk as much as other factors. The model for African American women was derived from the Women's Contraceptive and Reproductive Experiences (CARE) Study and NCI's SEER Program. For Hispanic women, part of the model is derived from white women who participated in the Breast Cancer Detection Demonstration Project and from SEER data. The risk estimates for Hispanic women are therefore subject to greater uncertainty than those for white women. Calculations for American Indian, Alaskan Native, Asian, and Pacific Islander women are based entirely on data for white women and may not be accurate. Researchers are conducting additional studies, including studies with minority populations, to gather more data and to increase the accuracy of the tool for women in these populations.

Although the tool has been used with success in clinics for women with strong family histories of breast cancer, more specific methods of estimating risk are appropriate for women known to have breast cancer-producing mutations in the BRCA1 or BRCA2 genes (10). Other factors may also affect risk and are not accounted for by the tool. These factors include previous radiation therapy to the chest for the treatment of Hodgkin lymphoma or recent migration from a region with low breast cancer rates, such as rural China. A woman who does not have mammograms will have somewhat lower chances of a diagnosis of breast cancer. The risk of developing breast cancer increases with age. The great majority of breast cancer cases occur in women older than age 50. Most cancers develop slowly over time. For this reason, breast cancer is more common among older women. Incidence rates in women before the age of 45 are higher among blacks; after the age of 45, they are higher for whites. Women of higher socioeconomic status, married women, women living in urban versus rural areas and women in northern states have the highest rates.

The questions are:

1.Does the woman has a medical history of any breast cancer or of ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS)?

2. What is the woman's age?

3. What was the woman's age at time of her first menstrual period?

4. What was the woman's age at her first live birth of a child?

Risk depends on many factors, including age at first live birth and family history of breast cancer. The relationship of these two factors is shown in the following table of relative risks. (Table 1) For women with 0 or 1 affected relative, risks increase with age at first live birth. For women with 2 or more first degree relatives, risks decrease with age at first live birth.

5 .How many of the woman's first-degree relatives - mother, sisters, daughters - have had breast cancer?

6. Has the woman ever had a breast biopsy?

6a: How many previous breast biopsies (positive or negative) has the woman had?

6b: Has the woman had at least one breast biopsy with atypical hyperplasia?

7. If known, please indicate the woman's race/ethnicity.

A woman's risk is considered low if her five-year risk of developing breast cancer is less than 1.66 percent; it is considered high if she scores above 1.66 percent. All women who are over 60 have a score of at least 1.66 and are considered high risk, based on the Gail Model. Women younger than 60 have to have the score of a 60-year-old woman [1.66] to be considered high risk. The sensitivity of the Gail model (the number of women who had breast cancer who were actually deemed high risk by the test) was 14, 7% and the specificity (the number of women who did not have breast cancer and who test found to be low risk) was 93, 9%.

NSABP model 2

The Breast Cancer Risk Assessment Tool is an interactive tool designed by

scientists at the National Cancer Institute (NCI) and the National Surgical Adjuvant Breast and Bowel Project (NSABP) to estimate a woman's risk of developing invasive breast cancer. The tool should not be used to calculate breast cancer risk for women who have already had a diagnosis of breast cancer, lobular carcinoma in situ (LCIS) or ductal carcinoma in situ (DCIS). The Breast Cancer Risk Assessment Tool will estimate a woman's risk of developing invasive breast cancer during the next 5-year period and up to age 90 (lifetime risk) based on the woman's age and the risk factor information provided. For comparison, the tool will then calculate 5-year and lifetime risk estimates for a woman of the same age who is at average risk for developing breast cancer. A positive response to any of the questions (except age) equals an elevated risk of breast cancer (2,4).

The internal workings of my NSABP model 2 calculators also start by calculating the summary relative risk, using the basic Gail model method. However, model 2 calculator uses slightly different values for relative risks. Model 2 does not include the risk of developing Ductal Carcinoma In-Situ (DCIS).

The components of this model are:

1. Age at menarche in years
2. Number of Biopsies
3. Number of first degree relatives with breast cancer
4. Biopsy with atypical hyperplasia?
5. Mammographic Density
6. Taking Tamoxifen?
7. Alcohol use
8. LCIS on biopsy?
9. Used Oral Contraceptives?

The answer of to each of the questions above is represented by a score and summary relative risk is calculated multiplying the nine scores between them. After calculating summary relative risk, then the absolute risk is determined using polynomial equations. For the NSABP model 2 calculator, there are separate curves for 5-year and lifetime absolute risk, for ages 20, 30, 40, 50, 60 and 70. The calculated by the tool risk are estimates of absolute breast cancer risk. Absolute breast cancer risk is the chance or probability of developing invasive breast cancer in a defined age interval. One way to evaluate the accuracy of the estimated risk is to determine whether it correctly predicts average risk in a group of women with the same risk factors and age. Although a woman's risk may be accurately estimated, these predictions do not allow one to say precisely which woman will develop breast cancer. In fact, the distribution of risk estimates for women who develop breast cancer overlaps the estimates of risk for women who do not.

Table 1 Relative Risk of Developing Breast Cancer (Gail MH et al., 1999)

Age at first live birth	# of affected relatives		
	0	1	2 or more
20 or younger	1	2.6	6.8
20-24	1.2	2.7	5.8
25-29 or no child	1.5	2.8	4.9
30 or older	1.9	2.8	4.2

CONCLUSIONS

In women after 40 years of age, clinicians should periodically perform individualized assessment of risk for breast cancer to help guide decisions about screening mammography. The most important benefit of screening mammography every 1 or 2 years in women past 40 years of age is a potential decrease in breast cancer mortality, despite the increasing disease incidence. Use of mammography has been associated with increased diagnosis of DCIS. The natural history of DCIS is unknown, as the percentage of these tumors that will progress to more serious disease. Because the evidence shows variation in risk for breast cancer and benefits and harms of screening mammography based on an individual woman risk profile, a personalized screening strategy based on a discussion of the benefits and potential harms o screening and understanding of a woman's preferences will help identify those who will most benefit from screening mammography. Women who score high on the test are encouraged to have increased surveillance and to consider breast cancer risk-reduction options.

Recent guidelines suggest that chemoprevention with tamoxifen may be appropriate for women who have a 5-year risk of breast cancer greater than 1.66% calculated using the Gail model (11).

Given the close balance between the risk and benefits of tamoxifen for most women considering chemoprophylaxis, discovering new strategies to improve the identification of women at very high risk for developing breast cancer is clinically important. Adding information from biological measurements to the risk model may improve prediction of the near-term risk of breast cancer. NAF cytology has the potential to improve prediction models of breast cancer incidence, particularly for high-risk women. Nipple aspiration is a minimally invasive procedure originally developed as a form of Papanicolau test for breast cancer. Prospective cohort studies have shown that cytology information from cells obtained from nipple aspiration predicts breast cancer incidence independent of traditional risk factors (12, 15). Adding NAF cytology results to the predictor variables used to calculate the Gail risk for women modestly improved the discriminatory accuracy of the model (from c-statistic of 0.62 to 0.64). Clinically, the test information may be most useful for women at highest absolute risk by the Gail model because modest differences in relative risk are amplified (6).

Premalignant cell damage in this lining may produce biochemical signals that deliver inflammatory proteins to the site. The presence of C-reactive protein (CRP) in nipple aspirate fluid (NAF) may reflect an inflammatory state indicative of a premalignant breast microenvironment. CRP is differentially present in NAF and varies by Gail model risk factors. CRP in NAF holds promise as a noninvasive biomarker that detects a precarcinogenic breast ductal microenvironment and may contribute to the diagnosis of breast cancer early in the course of the disease when prognosis is most favorable (7).

Researchers are conducting additional studies to gather more data and to determine whether including information on other risk factors can strengthen the mathematical models.

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MODELE MATEMATICE DE EVALUARE A RISCULUI DE CANCER DE SÂN ŞI APLICAȚIILE LOR

REZUMAT

Evaluarea completă a riscului de cancer de sân este importantă în practica medicală. Riscul de cancer de sân la cinci ani poate fi cuprins între 0,4% pentru o femeie de 40 de ani fără factori de risc şi 6% pentru femeia de 49 ani care asociază mai mulți factori de risc. Ghidul Asociației Americane de Cancer din 2006 recomandă mamografii timpurii începând cu vârsta de 40 ani, continuând atât timp cât femeia prezintă o stare bună de sănătate. Introducerea pe scară largă a screening-ului mamografic a avut drept consecințe creşterea ratelor de cancer de sân, dar şi reducerea mortalității prin diagnosticul în stadii incipiente. Date recente arată variații în ceea ce priveşte beneficiile şi prejudiciile asociate screeningului mamografic. O nouă metodă de calcul a riscului de cancer de sân poate aprecia riscul de a dezvolta o formă invazivă de cancer de sân la femei. Tehnica poate furniza informații utile femeilor în ceea ce priveşte riscul şi beneficiile strategiilor preventive ale cancerului de sân. Modelul Gail este unul dintre aceste modele care a fost dezvoltat pentru estimarea riscului de a dezvolta cancer de sân. Modelul cuprinde o serie de întrebări în legătură cu factorii de risc ai cancerului de sân, iar răspunsurile se calculează într-un scor de risc Gail. Modelul utilizează istoricul medical personal al femeii (numărul biopsiilor de sân anterioare şi prezența hiperplaziei atipice în orice biopsie anterioară), istoricul reproductiv (menarha, vârsta primei naşteri) şi istoricul de cancer de sân la rudele de grad I (mama, sora, fiica) pentru a estima riscul său de a dezvolta într-o anumită perioadă de timp o formă invazivă de cancer de sân. Breast Cancer Risk Assessment Tool este un instrument interactive proiectat de specialişti ai National Cancer Institute (NCI) and the National Surgical Adjuvant Breast and Bowel Project (NSABP) pentru a estima la femei riscul de a dezvolta un cancer invaziv (NSABP model 2). Deoarece complexitatea interpretării a unora dintre informațiile introduse se recomandă ca introducerea lor sa fie realizată de către personal cu o oarecare experiență în oncologie. Prin combinarea acestor modele matematice cu alte metode de diagnostic timpuriu cum este mamografia sau chimioterapia preventivă sau altele se poate duce la îmbunătățirea ratelor de supraviețuire în cancerul de sân.

Cuvinte cheie: risc cancer mamar, instrumente de analiza, rata de supravietuire

STROMAL CELLS - TUMOR MICROENVIRONMENT INTERACTIONS – Part I

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ABSTRACT

The complex cellular tumor micro(environment) comprises immunocompetent and inflammatory cells, endothelial cells and fibroblasts. All these cell types may influence the multi-step process of carcinogenesis and malignant phenotype. Fibroblasts are known to take part in immune reaction during tissue damage and injury by modulating local cellular and cytokine milieu, adjusting the kinetics and components of the inflammatory infiltrate, and by modulating the functional status of the immunocompetent cells. Purpose of the study was to investigate stromal cells role in immune suppression, which can profoundly alter tumor cells progression. Mesenchymal stem cells (MSCs) and tumor-associated fibroblasts (TAFs) were used in a comparative study for providing morphological and immunophenotypical characterization of stromal compartment interactions with tumorigenic cells.

Key words: mesenchymal stem cells, tumor-associated fibroblasts, tumor cells, microenvironment

INTRODUCTION

Mesenchymal stem cells (MSCs), most commonly isolated from bone-marrow, are defined as cells that display fibroblastic morphology in cell culture, exhibit a robust self-renewal capacity, and retain the ability to undergo trilineage differentiation into adipocytes, chondrocytes, and osteoblasts. Recent evidence suggests that bone marrow MSC can be mobilized into the periphery to serve as regenerative stem cells at sites of injury and inflammation (1,2). On the other hand, in vivo biology of MSC is poorly understood, several studies demonstrating that MSCs can be selectively recruited into tumors. Following engraftment within tumor stroma, MSC proliferate and acquire an activated phenotype similar tumor-associated fibroblasts (TAF). Tumor-homing properties of MSC have lead to their utility as therapeutic cell-based antitumor protein delivery vehicles (3-6). However, with a greater appreciation for the influential role that the tumor microenvironment can serve during tumor initiation, promotion, and progression, MSC may enhance tumor progression following acquisition of TAF-like characteristics. A more comprehensive delineation of the biological role of MSC within tumor stroma will improve our understanding of tumor-stroma interactions and facilitate future development of MSC-based clinical therapies.

Carcinomas are solid tumors that arise from malignant epithelial cells and represent the most common type of cancer in humans. However, it is becoming increasingly clear that malignant cells are supported by non-epithelial tumor stroma that shape a given tumor microenvironment. Tumor stroma can be generally divided into four main components: 1) tumor vasculature, 2) inflammatory immune cells, 3) extracellular matrix (ECM)/soluble factors, and 4) tumor-associated fibroblasts (TAF). Bidirectional paracrine communication between connective tissue fibroblasts and epithelial cells are vital for normal tissue homeostasis, and a comparable but progressive intimacy continues throughout tumor development.

Tumors are thought to develop their stroma from different sources, so that stromal cells are characterized by Wels et al. as "migratory neighbors and distant invaders" (7). In literature, there are four origins mentioned with this regard: 1) recruitment of resident tissue cells, 2) epithelial to mesenchymal transition, 3) fibroblasts recruitment, and 4) recruitment of bone marrow-derived cells from the circulation (8-11).

Tumors arise from cells that have sustained genetic mutations resulting in deregulation of several of their normal growth-controlling mechanisms. Much of the research concerning the origins of cancer has focused on the genetic mutation within tumor cells, treating tumorigenesis as a cell-autonomous process governed by the genes carried by the tumor cells. However, it is increasingly apparent that the stromal microenvironment in which the tumor cells develop profoundly influences many steps of tumor progression. In various experimental tumor models, the microenvironment affects the efficiency of tumor formation, the rate of tumor growth, the extent of invasiveness, and the ability of tumor cells to metastasize. In carcinomas, the influences of the microenvironment are mediated in large part by paracrine signaling between epithelial tumor cells and neighboring stromal fibroblasts. Paracrine signaling between other cell types within the carcinomas, such as endothelial cells and inflammatory cells may play equally important roles in tumor formation. Although mesenchymal cell fibroblasts can enhance tumor growth and metastasis, there is also evidence that healthy tumor microenvironment can act in a dominant manner to inhibit tumor growth.

Direct contact between bulk tumor cells and bone marrow-derived MSCs or TAFs in coculture models could reveal the true nature of stromal-tumor cell interaction, and could clarify the perspective of using MSCs as a strategy for delivering anti-tumor agents directly into tumors (Figure 1).

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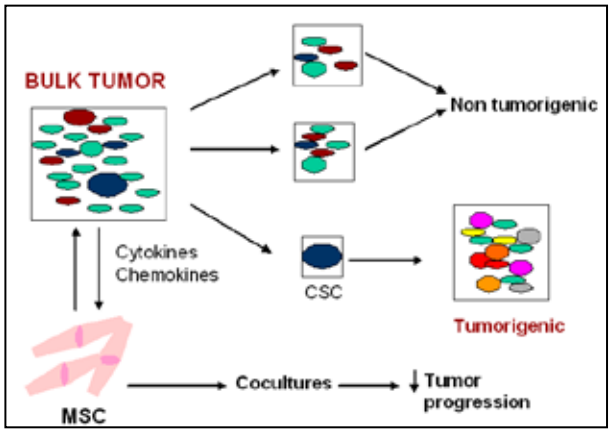

Fig. 1. Concepts regarding formation of tumors and possible role of MSCs/TAFs from tumor stroma in tumor progression and development. Cancer stem cells may generate tumorigenic potential cells, while MSCs/TAFs role is not completely elucidated.

MATERIAL AND METHODS

Cell Isolation and Culture Mesenchymal Stem Cells (MSCs)

Normal human mesenchymal stem cells (MSCs) were obtained from bone marrow of 6 healthy Orthopedics patients undergoing hip replacement surgery. Approximately 10 ml of bone marrow were placed in culture plates, and the fibroblastic-like, plastic adherent fraction, was isolated following multiple passages and used in our experiments. The MSCs were further cultured and expanded in alpha-minimum essential medium (MEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37°C in 5% CO_2 atmosphere. Medium was replaced every three days and when the confluence was 80-90% the cells were passed using 0.25% Trypsin-EDTA solution (Sigma) followed by centrifugation (10 minutes, 300g) and replated in T75 culture flasks at a density of 10,000 cells/cm^2 to ensure optimal proliferation. Starting with passage two, part of the cells were used for further phenotypical analyses and differentiation assays, while MSCs expanded to passages 2-5 were used in the subsequent experiments.

Tumor-associated fibroblasts (TAFs)

Human tumor-associated fibroblasts (TAFs) were isolated using both the explant and collagenase type IV-S from Clostridium histolyticum (Sigma, St. Louis, MO, USA) methods. Breast cancer surgical pieces of approximately 5 cm^2 were obtained form 8 female patients, with the histopathological diagnosis of infiltrative ductal mammary carcinoma. Tissue-isolated cells were washed several times with phosphate buffered saline (PBS, Sigma) solution, and passed through 0.70/0.40 µm strainer filters and were replated as single-cell suspension in adherent plastic culture plates.

Bulk Tumor Cells

Enzymatic digestion: Breast cancer surgical pieces from 20 patients (Ductal infiltrative carcinoma) were processed using Collagenase type IV-S (Sigma) after cutting in small pieces, placed at 37C°, 5% CO_2 for 30 minutes. Cellular suspension was centrifuged 7 minutes at 300g, pellet is washed 3 times using PBS (Sigma) and filtered through 70 µm strainers (BD), then cultured in suspension cell culture flasks. Tumor cells were cultured in 6-well suspension culture flask in presence of Dubelcco's Modified Eagle's Medium (DMEM, Sigma), supplemented with F12, B27, epidermal growth factor (EGF) and fibroblast growth factor (FGF) in variable proportions.

All tissue samples were obtained after signing the informed consent elaborated under an approved protocol, according to the World Medical Association Declaration of Helsinki.

Additional Procedures on Tumor-Derived Cells

After 1 week of suspension cell culture, tumor cells formed spheres, identified using vital staining ethidium bromide/acridine orange (Figure 2). The remaining of the cells were removed by positive microbeads selection using Dead Cell Removal Kit (MACS)-which identifies even early apoptotic cells with an intact cell membrane, followed by Lineage Cell Depletion Kit (MACS)-includes CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, CD235a, so that the morphological aspect of the cells was changed.

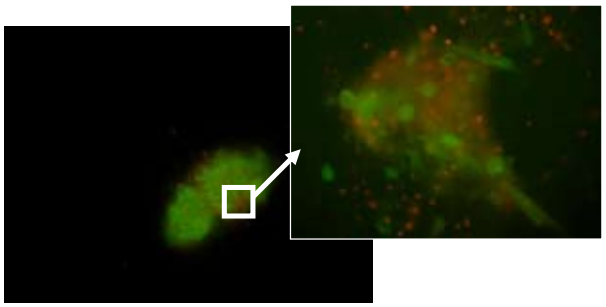

Fig. 2. Vital staining of tumor cells grown in suspension cell cultures. Cellular aggregates stained in green contain viable cells, which were further processed and cultured as single cells suspension.

Cocultures

MSCs and TAFs at passage P4 and P5 were cultured in 6-wells culture plates for 2 hours (adherence purpose), and tumor cells were added in different cellular ratios on top of MSCs/TAFs: 1:10, 1:20, and 1:50. Approximately 100,000 cells were cultured per well, and tumor cells were added in adequate proportions. Cells were grown in two types of media – tumor cells culture media and MSCs/TAFs specific culture media.

MTT-Based Toxicology Assay

Tumor-derived cells conditioned media was used to identify the influence on MSCs/TAFs proliferation capacity. An MTT based in vitro toxicology assay kit (Tox-1, Sigma) was used to determine proliferation rate of MSCs and TAFs at 24 and 48 hours. Trypan Blue cell counting and viability assessment were simultaneously performed at the same time intervals for better correlation of the results.

Cells were seeded at 2,000 cells/well in 96-well plates in quadruplicate and the average value of specific medium extinction was subtracted from the samples extinction read at 570/655 nm using a benchmark PR 2100 microplate reader from Bio-Rad (Hercules, CA, USA). Conditioned media was added in progressively diluted concentrations (100%-M1, 50%-M2, and 25%-M3) + 10% Fetal Calf Serum (FCS, PromoCell), so that to eliminate the influence of FCS lack in culture medium.

Cytokines and Chemokines Profile

ELISA analysis of supernatant was performed 2 weeks from the beginning of cocultures, or the next day from plating MSCs and TAFs using Quantikine Immunoassay kits (R&D Systems, Minneapolis, MN, USA) specific for IL-4, IL-10, IL-13, TGF-β1, TNF-α, IFN-γ, and VEGF. Supernatants of all MSCs, TAFs and cocultures were collected 24 hours after medium replacement, filtered through 0.22 µm strainers,

and stored at -70° C until analyzed in 96-well plates on the reader.

Immunocytochemical Analysis

Cells prepared for these analyses were grown in 4-well glass chamber slides, and 3-5 days from plating medium was removed, cells were washed, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and then investigated for expression of the proteins of interest, using for labeling the following antibodies: monoclonal mouse anti-swine Vimentin (clone V9), and monoclonal mouse anti-human Cytokeratin (clone MNF116), respectively. Cocultures of both cellular types, grown in 2 different media (tumor cells media and media specific to each cellular type) were also analyzed considering the same monoclonal antibodies.

Tumor cells were stained for specific tumor markers: anti-human estrogen receptor (ER) and andti-human p53, while cocultured cells were tested for maintenance of tumor specific markers in coculture after 2 weeks.

All primary antibodies were provided by DakoCytomation (Glostrup, Denmark) and tested for human specificity and cross-reactivity. Staining protocol continued with secondary biotinylated antibody binding, substrate addition, and hematoxylin counterstaining of the nuclei (LSAB2 System-HRP, Dako) following the manufacturer procedures. Microscopy analysis was performed on a Nikon Eclipse E800 microscope.

Statistic analysis

Statistic analysis was performed using Excell Microsoft Office 2003 (Microsoft Corporation) software. The central tendencies of the variables were expressed as a mean (M), and the dispersion ones as standard deviation (sd). In order to perform the statistic comparisons, „t"-Student test and the variance analysis (ANOVA) were used for continuous variables. Differences were considered significant for $p < 0.05$.

RESULTS

Morphological changes of co-cultured cells

Isolated bulk tumor cells were cultivated in suspension 6-wells culture flasks (Gibco) in tumor medium and grown for 1 week. Medium was replaced every 3 days with freshly prepared medium. Removal of dead cells and depletion of committed fraction lead to changed morphological aspect as well as decreased number of cells (Figure 3), suggesting that many of the tumor-isolated cells die in culture medium, or they are already committed towards other specific lineages.

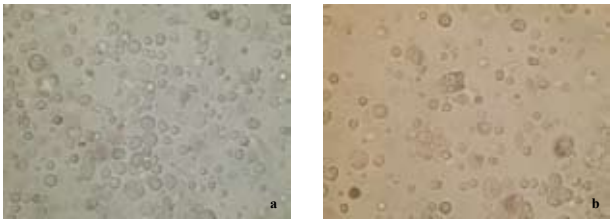

Fig. 3. Morphological aspect of tumor-isolated cells before (a) and after (b) removal of dead cells and committed cells. Cells were grown in suspension cultures and are heterogeneous, with rounded-shape morphology, large variations in nuclei size and cellular diameter.

When co-cultured together with MSCs or TAFs, direct contact between cells lead to isolated cellular clusters with round radial shape. Morphological changes in co-cultures aspect were induced after one week of co-culture, while in week 2 adherent cells started to detach from the culture plate (Figure 4). As long as we did not inactivate the underlying cells (MSCs or TAFs), the results could indicate either an increased proliferation rate of these cells and unfitted culture surface, or aggressive action from tumor cells against adhesion molecules or inhibition/stimulating processes targeting specific surface markers of these cells.

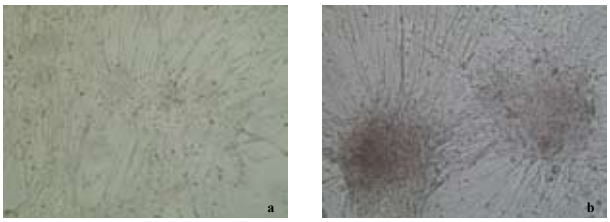

Fig. 4. Morphological aspect of cocultures (MSCs/TAFs and bulk tumor cells). Two types of media were used for cocultures: layer cells specific media and tumor cells specific media. Cellular behavior was similar using both type of media and underlying cellular substrate after 1 week (a) and 2 weeks (b), forming aggregates and starting to detach from the culture plate.

Immunocytochemical staining revealed increased Vimentin expression in co-cultured TAFs and MSCs, mainly when they were cultured in tumor specific media (Figure 5). Expression of Cytokeratin in tumor cells showed they can maintain an epithelial-like phenotype even in culture conditions (Figure 6).

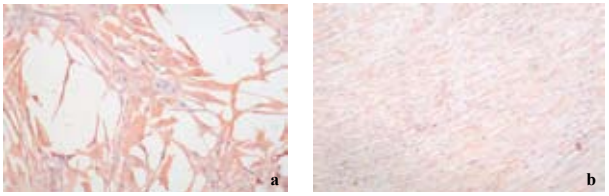

Fig. 5. Overexpression of Vimentin in MSCs cocultures: a. tumor-specific medium, b. MSCs specific medium. Different morphological aspects are underlined, MSCs specific medium being more beneficial for proper development of cocultures

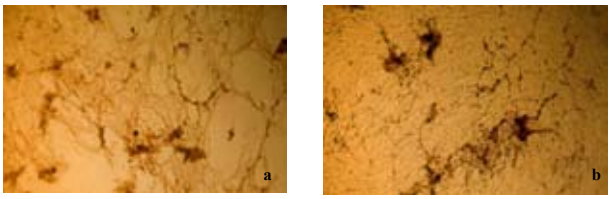

Fig. 6. Cytokeratin expression in TAFs cocultures: a. tumor-specific medium, b. TAFs specific medium. Morphological aspects suggest that FCS containing medium could prolong survival rates of cocultures by increasing the adherence of substrate cells.

Interestingly, tumors expressing estrogen receptor (ER) (50% of our tumor samples were positive for ER) maintained their expression in cocultures, in both types of experiments, showing that cocultures models could be a valid in vitro model for tumor progression and development (Figure 7). Presence of p53 was not detected by immunocytochemistry in primary tumors, nor in cocultures.

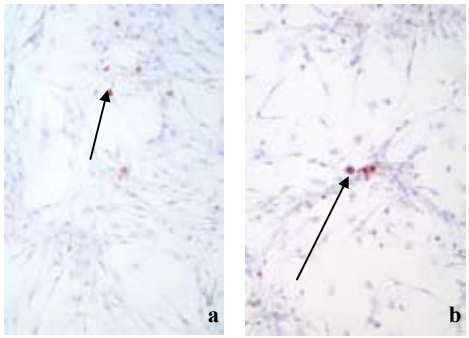

Fig. 7. Estrogen receptor (ER) expression in cocultures: a. MSCs cocultures, b. TAFs cocultures

Microenvironment-induced changes in cocultured cells

Conditioned medium decreased the MSCs proliferation rate, at all cellular densities, in a dose-dependent manner, and the results of the MTT assay were evaluated on a BioRad Plate Reader, subtracting the background absorbance at 590 nm from the specrophotometrically measured absorbance at 570 nm (Figure 8). TAFs proliferation rate decreased in a dose-dependent manner, but for values of 50% and 100% conditioned media, the cellular expansion reached a plateau phase.

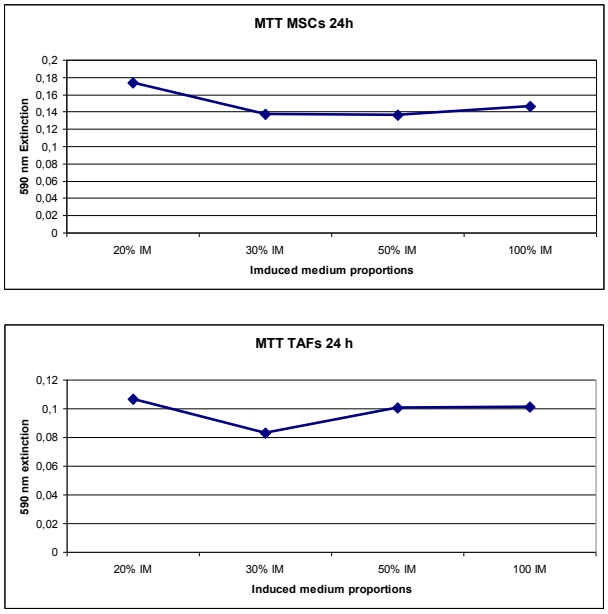

Fig. 8. MTT assay of MSCs and TAFs cultured in tumor conditioned media. In both cases the proliferation and expansion of cells decreases progressively, reaching a plateau (lag phase) for 50% and 100% conditioned medium

Cell culture supernatants were collected 24 hours from plating, normalized to cell number, filtered through 0.22 µm strainer, and stored at -80°C until analyzed. ELISA method revealed important differences in IL-10, IL-4 and TGF-β1 secretion, when comparing MSCs and TAFs, as well as their complementary cocultures. Secretion of cytokines with direct suppressive effect, such as IL-10 was significantly increased, thus suggesting the role of stromal cell in supporting and promoting tumor cell invasiveness and progression. TGF-β1 was determined as active form in cocultures supernatant, the entire amount of latent form being activated by the ELISA kit used for this assay, thus being impossible to determine the active amount ab initio.

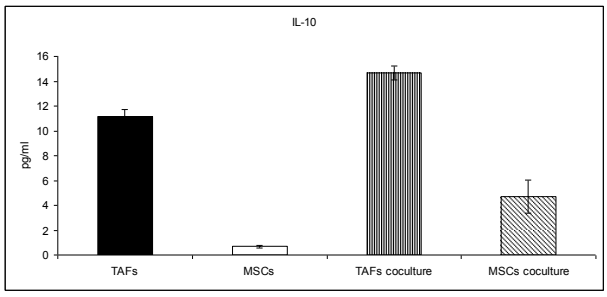

Fig. 9. Increased IL-10 secretion in ($p<0.001$) TAFs cocultures compared with both TAFs and MSCs.

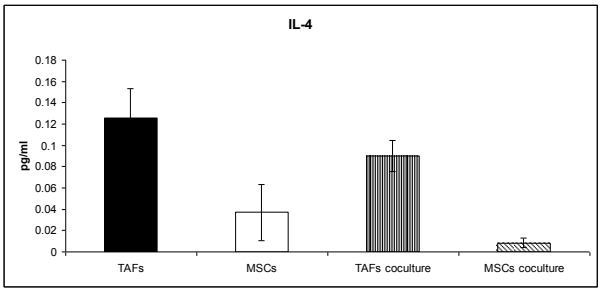

Fig.10. IL-4 secretion is decreased in both types of cocultures. Probably the effect of direct inhibition of immune response from the TAFs

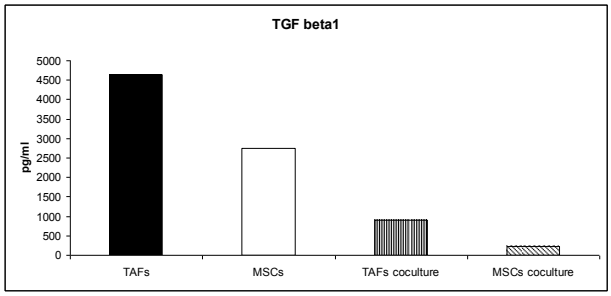

Fig. 11. TGF-β1 secretion in cocultures media is significantly decreased compared with both normal stromal cells and TAFs ($p<0.001$), consistent with its role in cellular apoptosis, proliferation inhibition and cell death

Our results indicate that cytokines profile is definitely modified within the cocultures, while compared with the MSCs or TAFs alone. IL-4 and TGF-β1 secretion is decreased, while IL-10 presented increased values in both coculture types, thus supporting the evidence that stromal cell compartment is required for maintenance and proliferation of tumor cells (Figures 9, 10, 11).

DISCUSSIONS

Soluble factors secreted by TAFs (and in lesser extent by MSCs) such as IL-10 are capable of inhibiting synthesis of pro-inflammatory cytokines like IFN-γ, IL-2, IL-3, TNF-α and GM-CSF produced by cells such macrophages and the type 1 T helper cells infiltrating the tumor. IL-10 also displays potent abilities to suppress the antigen presentation capacity of antigen presenting cells.

TGF-β1 is multifunctional peptide that controls proliferation, differentiation, and other functions in many tissues. TGF-β1 inhibits proliferation and can induce apoptosis in various cell types, but on the other hand, most human tumors produce this cytokine whose autocrine and paracrine actions promote cancer cell invasiveness and metastasis, epithelial to mesenchymal transition and regulates intratumoral angiogenesis [20]. Enhanced TGF-β1 production by TAFs may be a critical factor in tumor homeostasis not so much for the cancer cells directly, but for the TAFs themselves, via autocrine mechanisms, allowing them to proliferate and preserve their activated fibroblast behavior and supportive activity for the tumor. Moreover, TGF-β1 secreted by TAFs could suppress activation, proliferation and differentiation of the cytotoxic T lymphocytes, NK cells and macrophages recruited within the tumor, impairing immune surveillance and antitumoral immunity. The TGF-β-pathway is one of the major pathways altered in tumors, including breast cancer (12). Binding of TGF-β to the TGF-β receptor II (TβRII) activates the signal-transducing TGF-β receptor I (TβRI) that phosphorylates latent cytoplasmic transcriptional activators, Smad2 and Smad3. They then, together with Smad4, translocate into the nucleus

where they regulate TGF-β-dependent gene transcription (13, 14, 15). In breast cancer, TGF-β-signaling was shown to reduce growth of the primary tumor but also to promote metastasis, indicating that the apparent effect of TGF-β depends on its cellular context (16).

Both stromal cells types that we considered for our in vitro design had a profoundly altered morphological aspect, cells loosing the spindle shape, suffering destruction of cell membranes. Over-expression of Vimentin in co-cultured stromal cells may be a result of cellular injury induced by the tumor cells through their direct contact or different factors secreted into the microenvironment.

Relatively dose-dependent and time-dependent decrease of MSC/TAF proliferation after exposure to conditioned tumor medium may suggest that initial stroma-tumor microenvironment interaction may lead to drastic changes in MSCs or TAFs behavior. Long-term culture in conditioned media induces habituation of stromal cells, and even normal proliferation behavior.

We may also comment on tumor cells ability to maintain expression of tumor markers estrogen receptors - ER (breast cancer) in cocultured cells. Although initially the tumors presented low expression of these markers (50%), we were able to detect their presence in coculture conditions. There is evidence that estrogen, by regulating expression and secretion of angiogenic factors such as VEGF, can also stimulate angiogenesis (17,18), so that presence and maintenance of ER in coculture is required for formation of vessels and supportive role in tumor progression.

MSCs and TAFs can decrease in vitro proliferation of tumor cells, but their potential to inhibit tumor progression and development may be limited under the influence of different tumor aggressive factors secreted into the microenvironment, as well as by stroma-tumor cells cross talk.

Acknowledgements

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INTERACTIUNEA DINTRE MICROMEDIUL TUMORAL SI COMPARTIMENTUL STROMAL – Partea I

REZUMAT

Micromediul tumoral cuprinde celule imunocompetente si inflmatorii, celule endoteliale si fibroblasti. Toate aceste tipuri celulare prezinta o influenta graduala si etapizata asupra procesului carcinogenetic si aparitiei fenotipului malign. Este cunoscut faptul ca fibroblastii participa la reactiile imune in timpul distrugerii sau injuriei tisulare prin modularea mediului local celular si secretiei de citokine, ajustand cinetica si componentele infiltratului inflamator, precum si prin modularea statusului functional al celulelor imunocompetente. Scopul acestui studiu a fost investigarea rolului celulelor stromale in supresia raspunsului imun, ceea ce ar putea avea o influenta remarcabila asupra progresiei celulelor tumorale. Celulele stem mezenchimale (MSCs) si fibroblastii tumorali (TAFs) au fost folositi intr-un studiu comparativ pentru caracterizarea morfologica si imunofenotipica a interactiunilor dintre compartimentul stromal si celulele cu potential tumorigenic.

Cuvinte cheie: celule stem mezenchimale, fibroblasti tumorali, celule tumorale, micromediu

FROM INACTIVE CARRIER TO REACTIVATION OF HEPATITIS B VIRUS

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ABSTRACT

Background and aim: The natural evolution of the hepatitis B virus infection depends on the host immune system status. The aim of this paper is to evaluate time dynamics of the HBV-DNA viral load in HBsAg carriers in order to see how many of them remain in an inactive state and how many progress towards chronic hepatitis. Material and method: We included in our study 34 patients diagnosed initially as hepatitis B virus carriers for which we analyzed repeated HBV-DNA viral loads starting from 2 to 6 quantitative determinations, as well as levels for alanine aminotransferase (ALT). According to recent guides, we defined the upper limit of the serum HBV DNA viral load to be bellow 2.000 IU/ml (<10.000 copies/ml) in order to consider the patient an HBsAg carrier. Eco-guided liver biopsy was performed to those patients who developed chronic hepatitis and histologic activity index was taken into consideration.

Results: From the total number of 34 patients, 17 were women (50%) and 17 men (50%). The mean for age was 49.02 ±11.6 years (minimum 23 years, maximum 69 years). The average for the patient's follow up period was 35.47±3 months with a minimum of 14 months and a maximum of 118 months. Nine patients (26.47%) developed significant viral replication during this period of time: 4 women (44.4%) and 5 men (55.5%) after a medium period of 38.4 months. During the follow up period, the data showed that there was a weak positive association between the viral load value and the one of the serum ALT, though not statistically significant (Pearson correlation r=0.425, p=0.115).

Conclusions: Approximately 1 out of 4 hepatitis B virus carriers developed significant viral replication after an average follow up period of 3 years. The hepatitis B virus carriers should be evaluated periodically in order to discover a possible significant viral replication that could beneficiate from proper treatment.

Key words: hepatitis B virus carrier, reactivation, HBV-DNA viral load

INTRODUCTION

Hepatitis B virus (HBV) is a worldwide spread viral pathogen with multiple genotypes and molecular variants, leading to a great variety of stages for chronic HBV infection. Understanding the dynamic of chronic HBV infection is essential in the future management of HBV carriers. Four phases of HBV infection are described: immune tolerance, immune clearance, inactive carrier state and last but not least reactivation (28). If the disease is not controlled and the virus keeps replicating, complications such as cirrhosis and hepatocellular carcinoma can become inevitable.

It is known that in the Asia-Pacific region approximately 10% of people infected with HBV are actually in the carrier state. Approximately 25-40% of them will eventually die of liver disease (cirrhosis complicated or not with hepatocellular carcinoma with a death rate of 50% for male carriers and 15% for female carriers). That is why patients with chronic HBV infection must be continuously monitored and treated at the right moment, in order not to waste precious time (11,17).

In highly endemic regions, such as Asia and Africa, 8-15% of the population has positive HBsAg mostly due to perinatal transmission and horizontal infection in childhood, while in low endemic areas, such as Western countries, the infection occurs mostly in adolescents and adults, due to high risk sexual behaviors, drug injections or insufficiently sterilized material (18).

Continuous discoveries are being made in order to have a better understanding of the natural evolution of HBV infection. It is well known that the host immune system status is essential for the further development of the disease. Not all patients go through all stages, some of them remain carriers all their life, and others develop chronic hepatitis with further progression to cirrhosis or hepatocellular carcinoma (3).

The aim of this paper is to evaluate time dynamics of the HBV-DNA viral load in HBsAg carriers in order to see how many of them remain in an inactive state and how many progresses towards chronic hepatitis.

MATERIAL AND METHOD

We performed a retrospective study in which we included 34 patients initially diagnosed as inactive HBsAg carriers, according to the following criteria: positive HBsAg for more than 6 months; negative HBeAg; positive HBe antibodies; persistently normal AST, ALT levels; serum HBV DNA <2.000 IU/ml (<10.000 copies/ml). Further on, we established two groups referring one that remained inactive carrier (25 patients) and another one that developed significant viral replication (9 patients).

Both HBeAg and HBe antibodies, as well as HBsAg were determined by using an Axsym Analizor, by means of MEIA Abbott method.

Each patient had repeated HBV-DNA viral load measurements (2 up to 6 quantitative determinations), during a maximum of 118 months follow-up period. The viral load was quantified by means of real-time PCR (m2000sp/m2000rt Abbott) with a detection limit of 51 copies HBV DNA/ml (1.71 log copies HBV DNA/ml). PCR assays allow the detection of very low serum HBV DNA levels and they are more reliable than hybridization techniques (24), so this particular technique is more frequently used nowadays.

According to recent guidelines, an arbitrary serum HBV DNA level of 2.000 IU/ml has been proposed to differentiate HBeAg-negative chronic hepatitis B from the inactive carrier state (14).

Alanine aminotransferase (ALT) is the most commonly used enzyme for the evaluation of liver disease status. In our group of patients the upper normal limit for ALT was 41 U/l, while for AST it was 38 U/l. All patients had serum ALT determined every 6 months and some of them underwent liver biopsy in cases of repeated increased ALT activity.

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All serum samples were processed in the same laboratory.

In some of the patients, liver biopsy was performed echoassisted using Menghini type modified needles, 1.4 and 1.6 mm in diameter. Only liver biopsy fragments with a length of at least 2 cm, including at least 8 portal tracts were considered adequate for pathological interpretation. The liver biopsies were assessed according to the Knodell score by a senior pathologist. The Knodell score is composed of four individually assigned numbers that summarize a single score reflecting the extent of liver inflammation and fibrosis. The first component (periportal and/or bridging necrosis) was scored from 0 up to 10. The next two components (intralobular degeneration and portal inflammation) were scored 0-4. The combination of these three markers led towards the following description:

0 = no inflammation

1-4 = minimal inflammation

5-8 = mild inflammation

9-12 = moderate inflammation

13-18 = marked inflammation.

The fourth component indicates the amount of fibrosis: 0=no fibrosis up to 4= cirrhosis. Each patient had signed an informed consent form before the liver biopsy.

Statistical analysis

Data were expressed as mean ± standard deviation, frequency; Pearson correlation coefficient was calculated and afterwards t-test was used to determine the significance of correlation between level of viral load and level of serum ALT. We used unpaired t-test for comparison the means between the two groups, as well as chi square test to compare percentages. A p value <0.05 was considered statistically significant. The statistical analysis was performed using specialized programs such as SPSS 10, Open Epi 2.3 and Microsoft Excel 2007.

RESULTS

Out of the 34 patients initially diagnosed as inactive HBsAg carriers, the distribution according to gender was equal: 17 women (50%) and 17 men (50%). The mean age was 49.02±11.6 years (minimum 23 years, maximum 69 years).

Referring to the follow-up period, our goal was to include in the study those particular patients who were monitored during an extensive period of time, in order to be able to detect a possible reactivation of the disease. For fulfilling that purpose, the mean follow-up period was 35.47±3 months, with a minimum of 14 months and a maximum of 118 months. Nine patients (26.4%) developed significant viral replication, 4 women and 5 men, after a mean follow-up period of 38.4 months. The rest of 25 patients remained inactive carriers during the entire follow-up period. There was no significant statistical difference between the mean age of the group who remained inactive carriers compared to the one who became replicative (p=0.64). Further on, the results will show the fluctuation of liver enzymes for all patients involved in this study.

All 9 patients presented ALT flares during their follow-up period. Moreover, when high viral replication occurred, concomitant increase of serum alanine aminotransferase (ALT) level was observed in 66.6% of cases (6 out of 9 patients) (Fig. 1).

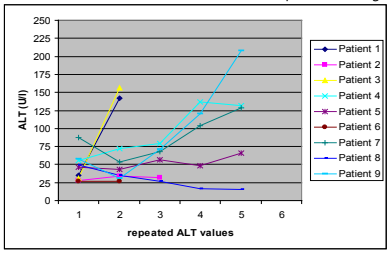

Fig. 1. ALT variation in patients with significant viral replication

The situation was slightly different for the 25 patients who remained inactive carriers according to repeated serum HBV DNA levels smaller than 2.000 IU/ml (<10.000 copies/ml). More exactly, 7 out of 25 patients (28%) had elevated ALT values at least once during their follow-up period (Fig.2). There were no significant statistical differences concerning the frequency of increased ALT levels between the two groups (p=0.0995).

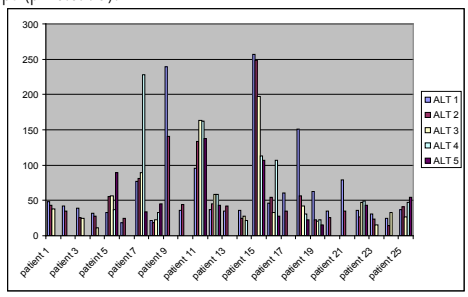

Fig. 2. Alanine aminotransferase variation in HBsAg carriers

We could not find a correlation between the initial viral load value and the one of the serum ALT (Pearson correlation r=0.003, p=0.99), but during the follow up period, the data showed that there was a weak positive association between the viral load value and the one of the serum ALT, though not statistically significant (Pearson correlation r=0.425, p=0.115).

Among patients initially diagnosed as hepatitis B virus carriers, 9 (26.4%) of them developed significant viral replication together with the increase of ALT values, though without a direct correlation between them. (Fig. 3). Liver biopsy was mandatory in order to be able to correctly quantify the damage of the liver.

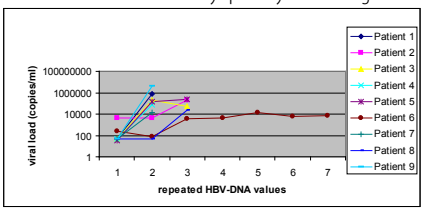

Fig. 3. Viral load variation in patients who developed significant viral replication

In our study, liver biopsy was performed in 77.7% (7 out of 9) patients in which reactivation occurred. No cases of cirrhosis were found. The mean value for fibrosis was 1.4±1.1 for those who developed significant viral replication, while the mean value for activity (HAI) was 8.8±3.2. (Table I)

Table I. Histologic activity index in patients who underwent liver biopsy

Knodell Score	Necro-inflammatory activity	Fibrosis
Patient 1	12	3
Patient 2	9	1
Patient 3	2	0
Patient 4	11	1
Patient 5	10	1
Patient 6	9	3
Patient 7	9	1

DISCUSSIONS

Hepatitis B virus carriers have three possible ways of evolving: they can either develop protective HBs antibodies together with HBsAg clearance, or remain in an inactive state, while some of them may develop chronic hepatitis together with its complications: cirrhosis and hepatocellular carcinoma.

The bright side of being an inactive carrier is that some of them eventually become HBsAg negative and develop protective HBs antibodies. The approximate HBsAg clearance is estimated to be 1%-2% per year in Western countries, where HBV infection is acquired during adulthood, and 0.05%-0.8% per year in endemic areas, where HBV infection is mostly acquired prenatally or in early childhood (21,8).

For a better understanding of the natural evolution of the disease, it would be appropriate to describe briefly its four phases.

Immune tolerance stage

HBV infection acquired perinatally or in early childhood has a tendency to evolve towards an initial tolerance stage characterized by: positive HBeAg, high DNA levels, but with normal ALT and minimal or no inflammation on liver biopsy (12). This is rarely the case of adult-acquired disease which usually evolves towards chronic hepatitis. Positive HBeAg is proven to function as an immunoregulatory protein that promotes chronicity (20). This may be a logical explanation for the high chronic HBV infection rate (90%) observed in babies infected by their HBeAg positive mothers. Published data proved that the immune tolerance stage can last 1 to 4 decades. During this phase, the rate of spontaneous or treatment induced HBeAg seroconversion is less than 5% (5).

Immune clearance stage

This phase is characterized by: positive HBeAg, high serum HBV DNA and ALT levels, accompanied by active inflammation and fibrosis in the liver. These flares of hepatitis may precede the disappearance of HBeAg and the appearance of HBeAg antibodies. Seroconversion is benefic for the patients' health status, as several studies had shown, being associated with biochemical and histological remission of inflammatory activity in most patients (19,7,25). Usually regression of fibrosis occurs several months or years after the HBeAg seroconversion. Both the duration of immune clearance phase and the frequency and severity of flares are responsible for the progression towards cirrhosis and hepatocellular carcinoma (16).

Inactive HBsAg carrier state

This stage is characterized by positive HBsAg for more than 6 months, negative HBeAg, positive HBe antibodies, serum HBV DNA < 2.000 IU/ml (<10.000 copies/ml), persistent normal AST, ALT levels, as well as by the absence of significant hepatitis on liver biopsy (22). Those patients are not candidates for treatment initiation, but they should be periodically monitored in order to discover a possible reactivation in an early phase, so that they should be treated properly and effectively.

Reactivation stage

Approximately one third of the inactive HBsAg carriers without serum reversion of HBeAg may develop an HBeAg-negative chronic hepatitis (27). It can occur either spontaneously or due to immune suppression in inactive carriers. Long term prognosis is poor.

Long term longitudinal studies on inactive HBsAg carriers have reported that 15% up to 24% of patients developed in time HBeAg negative chronic hepatitis; 20%-30% had moderate to severe inflammation, while up to 20% had advanced fibrosis or cirrhosis (15,9).

Fattovich G and al. performed a study on a cohort of 70 Caucasian patients observed during a median follow-up period of 25 years. The study showed that sixty-one (87%) patients underwent spontaneous HBeAg seroconversion. During a median period of 22.8 years after HBeAg seroclearance, 40 (66%) patients became inactive carriers, whereas the remaining 21 (34%) showed alanine aminotransferase elevation: one (1%) had HBeAg reversion, nine (15%) detectable serum HBV DNA but were negative for HBeAg, eight (13%) concurrent virus infection and three (5%) concurrent non-alcoholic fatty liver disease. Liver-related death occurred in 11 (15.7%) patients, caused by hepatocellular carcinoma in five and liver failure in six (9). Most patients with HBeAg seroconversion became inactive carriers with very good prognosis.

The REVEAL (Risk Evaluation of Viraemia Elevation and Associated Liver Disease)-HBV study group followed a cohort of 3653 HBsAg-positive patients in Taiwan (4). All of them acquired HBV infection perinatally. A baseline high HBV-DNA level > 10 000 copies/mL was associated with a significant increased risk of HCC (4) and with progression towards cirrhosis (13). Persistently high levels of viral replication, for up to four decades were correlated to highest risk for HCC/cirrhosis (1).

The viral evolution in inactive HBsAg carriers is usually benign. A long term follow-up (up to 18 years) of these carriers showed a very low risk of developing cirrhosis or hepatocellular carcinoma (2). Published data showed that the major factor for the development of HBV related hepatocarcinoma is the immune system (10). These patients must be periodically monitored by means of noninvasive methods such as abdominal ultrasound and transient elastography and also, by repeated viral load and AST and ALT measurements, since it is well known that multiple episodes of reactivation can lead to progressive liver destruction. It is extremely important for physicians to screen for HBV infection and to monitor liver disease progression in HBV carriers by using both serological and virological markers, so that effective treatment can be initiated early before the development of advanced liver disease (14).

According to literature, ALT levels can continue to increase without flare or have intermittent flares on a continuous elevation (22). It is crucial to be able to identify the patients that are no longer healthy carriers and who require proper treatment in order to have a chance of healing. Tai DI, Lin SM, Sheen IS et al. (26) studied the prognostic value of ALT in patients with chronic hepatitis B virus infection. A total of 4376 asymptomatic hepatitis B e antigen (HBeAg) negative, surface antigen (HBsAg) carriers with baseline ALT less than 2 times the upper limit of normal (ULN) were monitored with periodical ALT measurement during a period of three years. Baseline ALT level was normal in 3673 subjects and increased to abnormal level in 1720 (46.8%) cases. The incidence of liver cirrhosis, hepatocellular carcinoma and mortality was correlated with increasing maximal ALT level during follow-up, especially in those with maximal ALT of at least 2 times ULN, as compared to those who maintained normal ALT values. The conclusion was that persistently normal ALT values was associated with excellent long-term prognosis, while increased ALT levels of at least 2 times ULN was associated with increasing morbidity and mortality. ALT of at least 2 times ULN is therefore an appropriate threshold for anti-HBV therapy, whereas those with ALT 1 to 2 times ULN require liver biopsy for decision. Our study had also shown that increased ALT values were correlated with reactivation of the disease as demonstrated by the increase of HBV-DNA viral load.

A Greek study performed by George Zacharakis and al. revealed that 4 out of 195 (2%) patients initially classified as inactive carriers developed reactivation and had HBV-DNA viral load > 2000 IU/ml (29). The follow-up period was of maximum 12 years.

In the study performed by Chu and al. (6), a total of 1,965 inactive HBsAg carriers were evaluated (mean age 35.6 years; males: 1,076). During an 11.5 years mean follow-up, 314 carriers (13%) developed HBV hepatitis reactivation (ALT flares more than twice the upper limit of normal and positive HBV DNA by means of hybridization assays). The risk of HBV reactivation significantly correlated with advanced age when entering the study ($p<0.0001$) and male gender ($p<0.0001$). A total of 57 patients developed cirrhosis, the cumulative incidence being 15% after 25 years. The risk of cirrhosis significantly correlated with advanced age at entry ($p=0.004$) and HBV reactivation ($p<0.0001$). From the 1,651 carriers without HBV reactivation, 10 developed cirrhosis, and advanced age at entry was the only significant factor ($p=0.03$). From the 314 patients with HBV reactivation, cirrhosis developed in 47, the cumulative incidence being 8, 16, 27, and 46% at 5, 10, 15, and 20 years after reactivation. Male gender ($p=0.037$) and advanced age at reactivation ($p=0.006$) were the two independent risk factors. The conclusion of this study is that the so-called inactive carrier state cannot be generally viewed as an innocent, long-lasting condition with good prognosis, so that regular follow-up is necessary.

Another Greek study conducted by Papatheodoridis GV and al. (23) evaluated the severity of liver histology and the presence of histological indication for treatment in patients with HBeAg-negative chronic HBV infection focusing on those with low viremia and/or normal alanine aminotransferase. They included 399 patients with increased ALT and detectable serum HBV DNA (chronic hepatitis B patients) and 35

cases with persistently normal ALT and HBV DNA <2.000 IU/mL (inactive carriers). Histological indication for treatment was present in 17% (6/35) of inactive carriers due to moderate (stage 2) fibrosis without active necroinflammation. Minimal histological lesions were observed in the majority of HBeAg-negative patients with persistently normal ALT and low HBV DNA viral load, who may not require immediate liver biopsy and treatment but only close follow-up.

Our study showed that the mean value for fibrosis was 1.4±1.1 for those who developed significant viral replication, while the mean value for activity (HAI) was 8.8±3.2 showing a mild/moderate inflammation of the liver. Moderate fibrosis was found in 28.5% of patients. The beginning of an antiviral treatment is mandatory.

Our study had also its limitations such as: the small number of patients, as well as the absence of histology in "healthy" carriers.

CONCLUSIONS

In our study, approximately 1 out of 4 inactive HBsAg carriers (26.4%) developed significant viral replication after an average follow up period of 3 years (mean 38.4 months).

Inactive HBsAg carriers should be evaluated periodically by repetitive viral load assessment every 3-6 months, so that disease reactivation is diagnosed as soon as possible and a proper treatment is started.

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DE LA STAREA DE PURTĂTOR LA REACTIVAREA VIRUSULUI HEPATIC B

REZUMAT

Obiective: evoluția naturală a infecției cu virus hepatic B depinde de starea sistemului imun al gazdei. Scopul acestei lucrări este de a evalua dinamica în timp a viremiei HBV-DNA la purtătorii de AgHBs pentru a vedea câți dintre ei rămân în stare inactivă și câți progresează către hepatită cronică.

Material și metodă: Am inclus în studiu 34 pacienți diagnosticați inițial ca purtători de virus hepatic B la care am urmărit incărcătura virală pornind de la 2 până la 6 determinări cantitative, precum și nivelul alanin+aminotransferazei (ALT). Conform ghidurilor actuale, am definit starea de purtător ca fiind aceea în care limita superioară a viremie HBV DNA a fost mai mică decât 2,000 IU/ml (‹10,000 copii/ml). A fost efectuată puncție biopsie hepatică ecoghidată pentru a urmări indexul de activitate histologică la acei pacienți care au dezvoltat hepatită cronică.

Rezultate: Din totalul de 34 pacienți, 17 au fost femei (50%) și 17 bărbați (50%). Vârsta medie a fost 49.02 ±11.6 ani (minim 23 ani, maxim 69 ani). Perioada medie de urmărire a fost 35,47±3 luni cu un minim de 14 luni și un maxim de 118 luni. Nouă pacienți (26,47%) au prezentat replicare virală semnificativă: 4 femei (44,4%) și 5 bărbați (55,5%) după o perioadă medie de 38,4 luni. Pe timpul perioadei de urmărire s-a constatat un grad rezonabil de corelatie între valoarea viremiei si valoarea ALT serice, deși nesemnificativă statistic (r=0.425, p=0.115).

Concluzii: Aproximativ 1 din 4 purtători inactivi de virus hepatic B au dezvoltat replicare virală semnificativă după o perioadă medie de urmărire de 3 ani. Purtătorii de virus hepatic B trebuie monitorizați periodic pentru a putea surprinde o posibilă activare a replicării virale ce ar putea beneficia de pe urma unui tratament antiviral adecvat.

Cuvinte cheie: purtător inactiv de virus hepatic B, reactivare, viremie HBV-DNA

ELECTRODIAGNOSTIC OF THE NEUROMUSCULAR JUNCTION IN MYASTHENIA GRAVIS

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ABSTRACT

The electro diagnostic examination in patients with neuromuscular junction disorders requires a good comprehension on the physiology and pathphysiology of neuromuscular transmission. S.F.E.M.G. and R.N.S. are an essential part of the electro diagnostic study of the patients suspected by Myasthenia Gravis, being in particular useful in patients with the negative or uncertain pharmacological test (Tensilon) or minimal neurological findings, besides patients with seronegative Myasthenia Gravis. Also, the electro diagnostic of Myasthenia Gravis should be performed according to the patients' symptoms, medical and family history. Electromiography is a precious instrument in a complete and certain diagnostic of Myasthenia.

Key words: myasthenia gravis, electromyography, diagnostic tests

INTRODUCTION

Myasthenia Gravis is an autoimmune disease which results in muscle weakness, more pronounced during periods of physical activity and decreasing after periods of rest. The pathogenesis of Myasthenia is triggered by a defect in the transmission of nerve impulses to muscle. The immune phenomena are caused by the neurochemical mechanism in which acetylcholine auto antibodies attacks the receptor areas of the muscle, especially at the level of the neuromuscular junction.

Electromiography is an electrical recording of muscle activity that aids in the diagnosis of certain neuromuscular disease. As a neurophysiologic diagnostic this technique of investigation offers the most sensitive complete diagnostic test, but this fact doesn't exclude that is absolutely specific. Two major tests for Myasthenia Gravis diagnostic are used from electromyography techniques panel: Single Fiber Electromiography and Repetitive Nerve Stimulation.

Repetitive nerve stimulation shows a decremented response that is characteristic for myasthenia, the decrement being most pronounced in proximal muscles. Single fiber electromyography, is much more sensitive.

Repetitive nerve stimulation

It is used to diagnose Myasthenia Gravis and Lambert Eaton Myasthenic Syndrome. The recordings of the electrical stimulation are performed with surface electrodes. The intensity of stimulation must be supramaximal. The selected muscle will be chose in respect with the patient symptoms. In Myasthenia Gravis, axial and proximal body muscles are predominantly affected by a bulbar involvement. For E.M.G. we can select a muscle with moderate dysfunction degree. It is not easy to find a muscle like this, especially in myasthenia with ocular involvement. It is desirable to test a facial or a proximal muscle in these patients or in the patients with moderate symptoms. Among proximal muscle, most used is the deltoid. In this case, the electrical stimulation must be done in the supraclavicular fossa. In ocular myasthenia, the facial muscle is easy to stimulate too.

The recordings are acquired after orbucularis oculi stimulation being performed by placing the active electrode at the level of inferior eyelid, and the reference electrode placed on the wing of the nose. For nasalis muscle the active electrode is put on the wing of nose too. The facial nerve is stimulated under the ear. The standard protocol of stimulation says that the normal muscle may present a decrement of 8% percentage. A decrement up to 10% percentage is considered to be abnormal and pathologic. In order to confirm the fact that a resulting decrement after a repetitive stimulation is caused by the neuromuscular junction pathology, we can repeat the electromyographyc test after anti-cholinesterase administration (edrophonium or neostigmine) which leads to the remission of decrement.

Single fiber electromyography is a special technique discovered by Ekstedt and Stalberg, which offer information concerning the structure and function of the motor unit. In present S.F.E.M.G. is used in the diagnostic of the neuromuscular transmission defects. The "jitter" measurement is considered the most accurate electrophysiological method with higher sensibility.

Physiology of the Repetitive Nerve Stimulation

When repetitive stimuli are applied, at the level of neuromuscular junction, two different processes take place.

1. The pre-synaptic depression: the progressive decreasing of the acetylcholine clusters from synaptic endings. These are manifested trough a progressive reduction of the synaptic potentials, by a decreasing number of acetylcholine quanta that is released along with each nervous impulse, as measure of the acetylcholine depredation of clusters is instantly available. An increased synthesis and mobilization partially compensates this phenomenon.

2. Facilitated release of acetylcholine may be progressive as a consequence of the calcium ions that are present at the level of the nervous ending. This phenomenon is electrophysiologically translated by increasing amplitude of the compound muscle action potential area, after supported physically effort.

Tested muscles

In Myasthenia Gravis, predominantly affected is the bulbar musculature of the members (axial and proximal). Proximal muscles-frequently the deltoid muscle is tested. The electrical stimulation is performed into supraclavicular fossa. The active electrode is placed on the muscle and the reference electrode on the acromion. The voluntary activity will be attained by the arm abduction against resistance at an angle of 45-90°.The stimulation causes discomfort and activation of other muscles is practically inevitable. The trapezium muscle – is easy to be stimulate, the produced pain being much smaller. The spinal accessory nerve is stimulated at the half of sternocleidomastoidian muscle, on his posterior brink. The brachial

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biceps is stimulated with the pacient placed in clinostats, with the arm in abduction at 90°. The musculocutaneous nerve is stimulated in axial. The anconeus muscle is relatively easy to stimulate, less painful and with rough and ready sensitivity. In these situations, the patient is asked to stand in ortostatism, with forearm in pronation and sustained on examination table. The radial nerve is stimulated at the level of lateral intermuscular septum, at 1-2 cm upper to the lateral epicondilus. The active recording electrode is placed on the muscle, at three width of digital finger by olecran, in the top of an isosceles triangle, his basis being the line between olecran and medial epicondilus. The reference electrode is placed on the dorsal face of the forearm. Muscle activation is made by the elbow extension against resistance. Easy to be stimulated are facial muscles too. The electrical recordings for orbicularis oculi muscle is made by placing the active electrode at the level of inferior eyelid and with the reference electrode placed on the ala of nose.

For nasalis muscle the active electrode will be placed at the level of the ala of nose, on the right side. The facial nerve is stimulated under the ear. Easier to stimulate is the A.D.M. muscle, with distal stimulation of hand joint of ulnar nerve.

Single fiber electromyography

Technically speaking, S.F.E.M.G. consists in the extracellular recording of the individuals action potentials (with a needle-specific electrode) of the muscle fiber. In normal subjects sarcolema activated threshold will determine the mean variation of the neuromuscular transmission. The normal jitter is variable at the level of different synapses of the same muscle, even the same motor unit. Also, the medium values of jitter are variable on several muscles.

In Myasthenia Gravis the retrenchment of the post synaptic responsivity, decrease the amplitude of end plate potentials, which leads to a prolonged time in order to reach the discharge threshold. The jitter can be determined either of muscle voluntary activation or electrical stimulation of the tested muscle. At voluntary activation of muscle the activations potentials are recorded from two muscle fibers of the same motor unit. The first fiber is used as "trigger", that serve as reference in order to measure prior in time, the interval variability up to the second muscle fiber activity. The needle electrode is placed in the muscle that is in a mild syncopation, near to the motor endplate. The position in which two single fiber potential is recorded will be searched. The first potential will represent the "trigger" of the signal acquisition. Then, the variability of inter-potential interval (jitter) will be measured, after 50-100 successive discharged impulses.

For the majority of muscles, the normal range of jitter is situated between 5-55µs. The first sign of the neuromuscular transmission defect/impairment is increasing of jitter. When the jitter is up to 70µs, a neuromuscular block will appear.

In order to illustrate the things above said, in following images we can see the aspect of normal R.N.S (Fig.1) and S.F.E.M.G. (Fig.2) (with jitter) and also characteristic for Myasthenia.

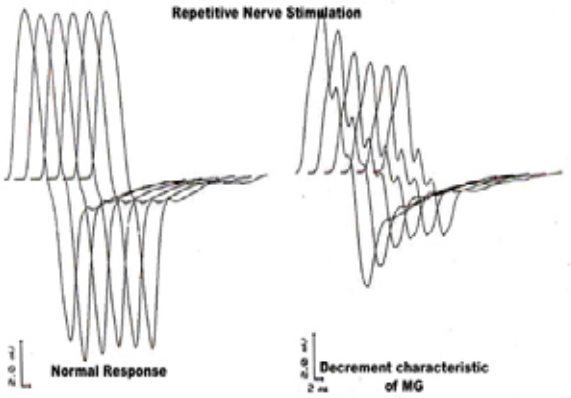

Fig.1. The normal aspect compared with the characteristic aspect of decrement in Myasthenia during R.N.S

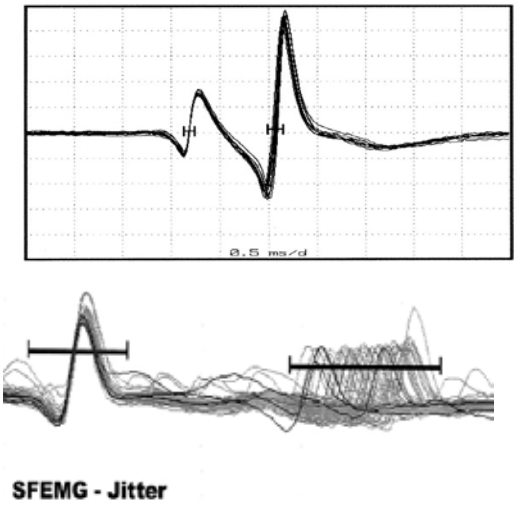

Fig.2. Normal and myasthenic aspect of jitter during S.F.E.M.G. examination

MATERIAL AND METHODS

We chose to present three clinical cases of which one is normal, without signs of neuromuscular impairment (control) in order to have criteria for comparison. The patients were directed to electromyography exam as a complementary test to confirm the diagnostic, besides the blood test for anti-acetylcholine receptor antibodies.

The electromyography test was performed with a Neropack 9100 portable electromiograph. The R.N.S. was performed wit surface electrodes and S.F.E.M.G. with needle electrodes. The characteristic electromyographic routs images of the clinical cases taken as examples are illustrated and interpreted in the section results of the article.

Case 1. A.L; sex F, 48-years-old. The electromyogram was performed by stimulating the right facial nerve and nasalis acquisition. (Table I, II, III; Fig. 3, 4)

Results: At the repetitive nerve stimulation exam (3Hz) of the right facial nerve and nasalis acquisition, there are no significant recordings changes of the compound muscle action potential. The electromiographic route above illustrated is normal in the examined area.

Table I. Repetitive Nerve Stimulation (case 1)

Muscle / Train	Ampl. mV	d Ampl1 %	d Ampl2 %	Fac Ampl %	Area mV*ms	D Area1 %	D Area2 %	Fac Area %	Rate pps	Time
R NASALIS										
Baseline	1.8	2.1	3.3	100	4.9	-0.3	-3.6	100	3	0:00:00
Facilitation	1.8	0.9	-.2	102	5.1	-3.1	-10.3	104	3	0:01:24
@0.30	1.8	2.7	4.3	99.2	4.6	-0.1	4.4	94.6	3	0:01:29
@1.00	1.8	-1.4	-0.1	95.9	4.7	-4.6	-2.7	95	3	0:01:34
@2.00	1.8	-0.5	-2.4	101	4.8	-2.8	-9.1	97.6	3	0:01:39

Table II. Needle EMG (case 1)

EMG Summary Table									
	Spontaneous					MUAP			Recruitment
	IA	Fib	PSW	Fasc	H.F.	Amp	Dur.	PPP	Pattern
R. DELTOID	N	NONE	NONE	NONE	NONE	N	N	N	N
R. FIRST D INTEROSS	N	NONE	NONE	NONE	NONE	N	N	N	N
R. TIB. ANTERIOR	N	NONE	NONE	NONE	NONE	N	N	N	N
L. VAST LATERALIS	N	NONE	NONE	NONE	NONE	N	N	N	N
R. TONGUE	N	NONE	NONE	NONE	NONE	N	N	N	N
L. FIRST D	N	NONE	NONE	NONE	NONE	N	N	N	N

Table III. Repetitive Nerve Stimulation (case 1)

Muscle / Train	Ampl mV	d. Ampl1 %	d. Ampl5 %	Per Ampl %	Area mVms	D. Area1 %	D. Area5 %	Per Area %	Rate pps	Time
R.NASALIS										
Baseline	1.2	-0.3	2.5	100	3.6	-1	-0.5	100	3	0.00:00
Facilitation	1.2	1.2	0.7	98.4	3.6	0.1	-1.9	99.2	3	0.02:55
@0:30	1.2	-0.9	-1.2	101	3.6	0,1	-5.7	99.4	3	0.03:00
@1:00	1.2	-0.3	-0.7	101	3.5	-0.8	-0.5	97.3	3	0.03:04
@2:00	1.2	-0.8	-1.2	102	3.6	-1.2	-3	100	3	0.03:09

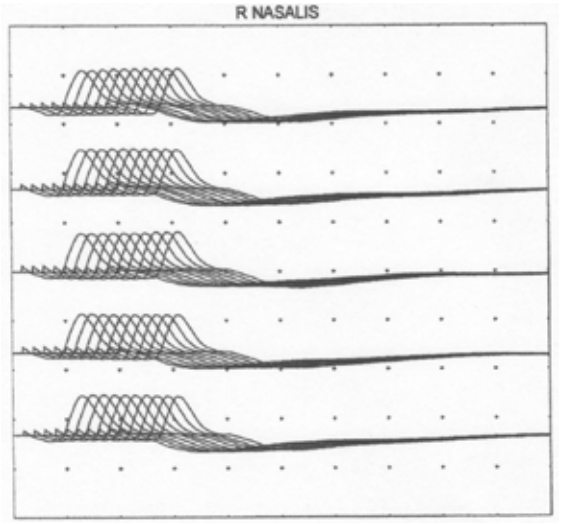

Fig.3. The EMG route after RNS (3Hz) (case 1)

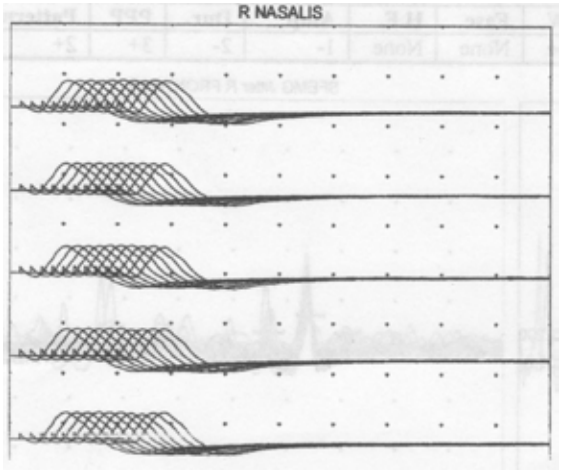

Fig.4. EMG aspect after RNS (3Hz)(case 1)

Case 2. F.K; sex F, 12-years –old. The electromyographic test in this case was performed by slowly repetitive electrostimulation of the right cubital nerve and ADM acquisition of currents (Fig.5, Table IV).

Results: At the slowly repetitive nerve stimulation (3Hz), of the right cubital nerve and ADM acquisition, an" U-shape" decrement is present, after a significant voluntary post activation. The electromyographic route is compatible and characteristic for Myasthenia Gravis.

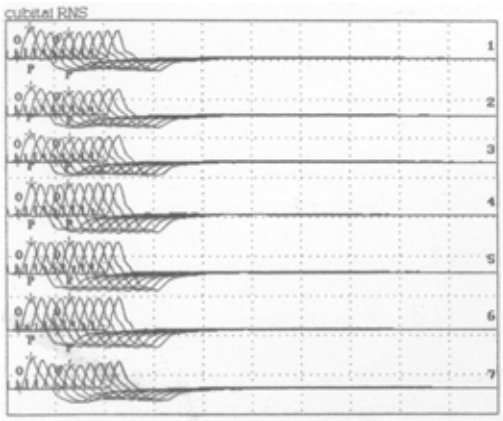

Fig.5. Repetitive nerve stimulation of CNS with a significant voluntary post activation (case 2)

Table IV. RNS Report. Nerve: Cubital. Muscle: ADM (case 2)

Trial	Side	Amp 1 (mV)	Amp 5 (mV)	Amp Diff %	Area 1 (mVms)	Area 5 (mVms)	Area Diff %
1	Right	7.80	6.14	-21.3	22.30	15.90	-28.7
2	Right	7.00	5.71	-18.5	20.70	15.49	-25.2
3	Right	7.04	5.79	-17.8	21.20	16.15	-23.8
4	Right	8.06	7.77	-3.5	19.80	20.02	1.1
5	Right	8.04	7.92	-1.4	21.45	20.45	-4.7
6	Right	7.89	7.78	-1.4	21.44	20.52	-4.3
7	Right	7.87	5.86	-25.6	22.46	15.63	-30.4

Case 3. S.G; sex F, 54-years-old,which besides the myasthenia was suspected by amyotrophic lateral scrlosis,reason for why the Single Fiber Electromiograpgy was performed, in right EDC muscle.Repetitive nerve stimulation was made too. (Fig.6, 7; Table V, VI)

Results: The electromyographic route in this context, show at the exam of repetitive nerve stimulation (3Hz) of right cubital nerve and ADM data acquisition, a decrement of 16%, with significant improvement in conditions of effort, followed by post activation exhaustion. A severe decrement was reported (45%). The single fiber electromyography, performed on EDC muscle, reveal an increased jitter with frequently blockage. The electromyographic route pleads for Myasthenia Gravis.

Table V. RNS Report. Nerve: Cubital. Muscle: ADM (case 3)

Trial	Side	Amp 1 (mV)	Amp 5 (mV)	Amp Diff %	Area 1 (mVms)	Area 5 (mVms)	Area Diff %
1	Right	7.80	6.14	-21.3	22.30	15.90	-28.7
2	Right	7.00	5.71	-18.5	20.70	15.46	-25.2
3	Right	7.04	5.79	-17.8	21.20	16.15	-23.8
4	Right	8.06	7.77	-3.5	19.80	20.02	1.1
5	Right	8.04	7.92	-1.4	21.45	20.45	-4.7
6	Right	7.89	7.78	-1.4	21.44	20.52	-4.3
7	Right	7.87	5.86	-25.6	22.46	15.63	-30.4

Table VI. SFEMG of right EDC (case 3)

Run	Samples	Blocks	MCD (µs)	MSCD (µs)	IFI (ms)	Freq (Hz)	FibDens
1	36	20	1044	136.6	1.92	12.3	3
Mean	36.0	20.0	1044	136.6	1.92	12.3	0.0
Std Dev	0.0	0.0	0.0	0.0	0.00	0.0	0.0

Ch	Hicut	Locut	Gain (µV/div)	Sweep (ms/div)
1	3000	100.0	100.0	1.0

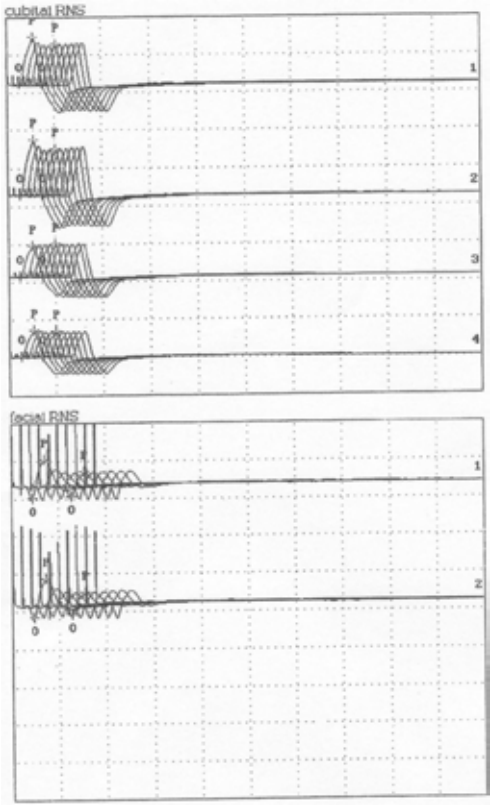

Fig.6. RNS after 20 seconds of required effort (case 3)

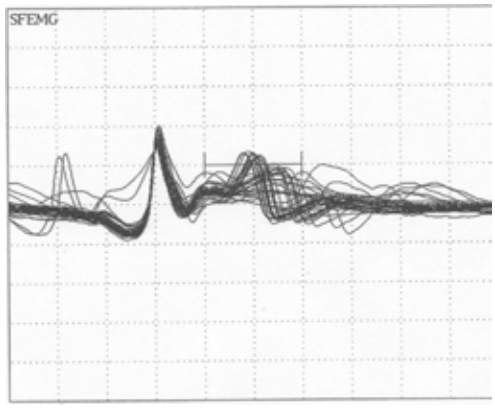

Fig.7. SFEMG on EDC muscle (case 3)

CONCLUSIONS

When myasthenia is already confirmed, with an MDS lower score (and the myasthenic deficit is also small), then the diagnosis must be made with certainty. Obviously, the positive response to the pharmacological test(with miostin) makes clear the evidence of myasthenia, but through electromyographic exam is important to see what kind of neuromuscular block is present: presynaptic or postsynaptic

Electromiography is useful for confirming the diagnosis of myasthenia, when is performed in the time of a myasthenic burst, in the territory of the neighboring muscle with an important deficit and within 12 hours after anticholinesterase administration.

An EMG performed outside the mysthenic burst or under the anticholinesterase effect, could offer a normal route and a false guidance in order to put the diagnosis. Even if the electromyographic route is normal doesn't mean that the diagnosis is excluded.

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Abbreviations and defining terms

E.M.G. electromyography
M.D.S. mysthenic deficit score
R.N.S. repetitive nerve stimulation
S.F.E.M.G. single fiber electromyography
JITTER mean variation in interpotential interval between single-fiber action potentials of the same motor unit

A.D.M. abductor digiti minimi (muscle)
E.D.C. extensor digitorum communis(muscle)
S.P.E. (sciaticus popliteus external (nerve)
T.A tibial anterior (muscle)
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ELECTRODIAGNOSTICUL JONCTIUNII NEUROMUSCULARE IN MIASTENIA GRAVIS

REZUMAT

Examinarea electro-diagnostica a pacientilor care prezinta afectarea jonctiunii neuromusculare necesita o buna intelegere a fiziologiei si fiziopatologiei transmiterii neuro-musculare.

S.F.E.M.G. si R.N.S. sunt o parte esentiala a studiilor electro-diagnostice in cazul pacientilor suspectati de miastenia gravis, fiind in mod particular utile in cazul acelor pacienti cu teste farmacologice negative sau incerte (Tensilon), in cazul pacientilor cu afectare neurological minimala si pentru pacientii seronegativi pentru miastenia gravis. De asemenea, electro-diagnosticul miasteniei gravis ar trebui efectuat in concordanta cu simptomatologia pacientilor, precum si cu istoricul medical si antecedentele heredocolaterale ale acestora. Electromiografia este un instrument pretios pentru stabilirea si certificarea diagnosticului de miastenie.

Cuvinte cheie: miastenia gravis, electromiografie, teste diagnostice

LIPOPEROXIDES –MARKERS OF OXIDATIVE STRESS IN THE NEWBORN WITH PERINATAL ASPHYXIA

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ABSTRACT

Background: Oxidative stress was included in the pathogenesis of asphyxia and ischemic hypoxic encephalopathy in the last years because of certain particularities of the central nervous system, which increase the vulnerability in the oxygen reactive species.

Objective: The study aimed to identify lipoperoxides in the newborns with perinatal asphyxia.

Materials and Methods: The study group included term newborns with asphyxia at birth, in the period January 2005- June 2006, in the Department of Neonatology of the 1st Gynecology Clinic, Cluj-Napoca, Romania. Lipoperoxides were measured in dynamics on the first and third days of life. The results were processed with the STATISTICA program.

Results: The study group included 11 term newborns of 38.91±0.70 weeks of gestation and 3391±380.90g body weight respectively. The term newborns presented: mild asphyxia 45.45%, moderate asphyxia 19.19% and severe asphyxia 36.36%. The oxygen concentration that was necessary on the first day of life was of 30.18±10.06% as compared to that of the third day, which was of 24.82±4.98% (p=0.0258). The level of MDA lipoperoxides was higher on the third day (4.72±1.97nmol/ml) than that on the first day (4.46±2.09nmol/ml).

Conclusions: Lipoperoxides represent a marker for oxidative stress in the newborn with perinatal asphyxia, which is a major source of morbidity, mortality and neurological consequences in the surviving cases.

Key words: asphyxia, lipoperoxides, newborn

INTRODUCTION

Perinatal hypoxic stress is a frequent cause of morbidity, mortality and of neurological sequelae in surviving cases suffering from neonatal asphyxia (3,6,12,15).

The hypoxic - ischemic brain lesion begins with the insult and continues during the rehabilitation period after reperfusion (6,17,18).

Oxidative stress has been lately considered as part of the pathogenesis of asphyxia and of perinatal AIE because of certain CNS particularities, which increase its vulnerability to ORS as follows: cell membranes rich in polyunsaturated fatty acids and shortage of antioxidants in the brain, especially in catalase and superoxide dismutase (SOD). Brain regions, which are rich in iron, during low antioxidative defense, highlight the discharge of iron with small molecular mass, leading to hydroxyl radical and lipid peroxidation (10,11).

Oxygen reactive species act upon biomolecules causing their degradation. The ORS effect upon these biomolecules can be identified by indirect methods (1,2,4). Focus was laid on the study of the ORS effect upon fats in asphyxia as the lipoperoxides are the markers most widely used to identify oxidative stress in the newborn during the first week of life.

All these theoretical considerations justify the study of lipoperoxides in neonatal asphyxia. The objective of this study was to identify lipoperoxidation in the term newborn with various types of asphyxia.

MATERIALS AND METHODS

The study group included 11 term newborns with asphyxia of different degrees of severity. The control group included 20 healthy term newborns. In order to study oxidative stress in asphyxia at birth, lipid peroxidation was studied by identifying malonilaldekide (MDA). The MDA is the most important aldekide, which results from lipid peroxidation.

Two measurements from the venous blood were made in all newborns of the study group, on the first and third day respectively. In the newborns of the control group only one measurement was made on the first day of life.

The dosing method used in this study was Satoh's method, whose testing is based on the reaction of an MDA molecule with two molecules of tiobarbituric acid, which results in a pink production with 530 nm absorption. The absorption intensity is directly proportional to the MDA quantity in the sample. The results are expressed in nmols MDA/ml of serum. The normal level in adults is of 1.29±0.37 nmol/ml.

Along with the dosing of lipoperoxides, the Astrup parameters were also measured on the first and third days of life, considering that in the neonatal period the precipitating conditions for ORS occurrence are: acidosis, hypoxia, hyperoxia and infections. Asphyxia and AIE constitute frequent pathological conditions associated with hypoxia and hyperoxia following oxygen therapy.

The Pearson and Spearman correlations, simple linear regressions, comparisons of initial and final measurements (on the first day and the third day respectively) were used for data processing. The Student Test was used in pairs of the study group for the data following a normal distribution and the Mann-Withney Test was used for the data not following a normal distribution.

RESULTS

The study group included eleven term newborns. Asphyxia severity was assessed according to the Apgar score at 5 minutes. Thus, three types of asphyxia were found:

1. Mild – Apgar score 7
2. Moderate –Apgar score between 6 and 4

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3. Severe – Apgar score between 3 and 1

The study group included eleven term newborns, four female newborns [36.36%, IC 95% (9.92-71.90)] and seven male newborns [54.565, IC 95% (28.10-80.99)](Figure 1).

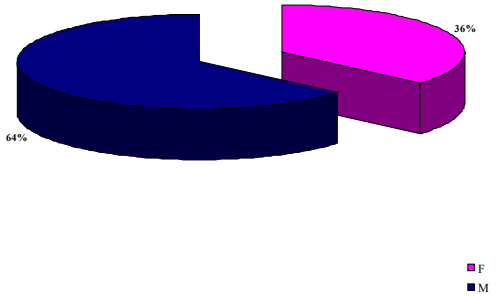

Fig.1. Gender incidence in term newborns with asphyxia

The mean gestational age of term newborns from the study group was of 38.91weeks with IC 95% (38.44-39.38), and the mean weight was of 3397 g with IC 95% (3141-3653).

The variation of the Apgar score in the study group of term newborns is shown in Table I.

Table I. Variation of Apgar score at 1 minute and 5 minutes in the term newborns

APGAR Score	1'			5'		
	Absolute incidence	Relative incidence	IC 95%	Absolute incidence	Relative incidence	IC 95%
1	3	27.27	(9.92-62.81)	1	9.09	(0.83-44.63)
2	2	18.18	(0.83-53.72)	3	27.27	(9.92-62.81)
3	1	9.09	(0.83-44.63)	0	0.00	n.a.
4	1	9.09	(0.83-44.63)	0	0.00	n.a.
6	2	18.18	(0.83-53.72)	2	18.18	(0.83-53.72)
7	2	18.18	(0.83-53.72)	5	45.45	(10.25-49.75)
Total	11	100		11	100	

The incidence of the various types of asphyxia in the group of term newborns is shown in Table II.

Table II. Asphyxia distribution in the term newborns

Type of asphyxia	Absolute incidence	Relative incidence	IC 95%
Mild	5	45.45	(9.92-62.81)
Moderate	2	18.18	(0.83-53.72)
Severe	4	36.36	(5.25 -44.75)
Total	11	100	

Cerebral hemorrhage was present in 2 cases and AIE in 4 cases of the study group.

The parameters were analyzed in pairs of the study group by using the Student Test. The results are shown in Table III.

Table III. Parameters analysis within the study group

	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p
FiO_2 – 1st day	30.18182	10.05801						
FiO_2 – 3rd day	24.81818	4.97631	11	5.3636	7.03239	2.5296	10	0.029891
pH – 1st day	7.34364	0.06435						
pH – 3rd day	7.34000	0.03633	11	0.0036	0.05714	0.2111	10	0.837085
pCO_2 – 1st day	33.33636	7.20878						
pCO_2 – 3rd day	36.01818	4.56307	11	-2.6818	7.62992	-1.1658	10	0.270767
pO_2 – 1st day	53.50909	14.83951						
pO_2 – 3rd day	54.62727	4.91001	11	-1.1182	15.56643	-0.2382	10	0.816504
SaO_2 – 1st day	91.01818	2.80208						
SaO_2 – 3rd day	89.89091	3.22655	11	1.1273	3.80029	0.9838	10	0.348415
MDA – 1st day	4.46091	2.09444						
MDA – 3rd day	4.71727	1.97025	11	-0.2564	1.03491	-0.8216	10	0.430469

MDA =lipoperoxides; FiO_2 = FiO_2 = concentration of oxygen that newborns were exposed to; pCO_2 = partial pressure of carbon dioxide; pO_2 =partial pressure of oxygen; SaO_2 =oxygen saturation. p=test significance,StDev=standard deviation

Table data analysis revealed a significant difference between the FiO_2 value on the first and third days ($p<0.05$). The correlation for the quantitative variables in the studied term newborns is presented in Table IV.

Table IV. Pearson correlation for the quantitative variables in the studied term newborns

GA = gestational age; W = weight; MDA = lipoperoxides; FiO_2 = concentration of oxygen that newborns were exposed to; pCO_2 = partial pressure of carbon dioxide; pO_2 =partial pressure of oxygen; SaO_2 =oxygen saturation.

				Day1						Day3					
		GA	W	FiO_2	pH	pCO_2	pO_2	SaO_2	MDA	FiO_2	pH	pCO_2	pO_2	SaO_2	MDA
	GA	1.00													
	G	0.24	1.00												
Day1	FiO_2	0.17	0.65	1.00											
	pH	-0.63	-0.27	-0.12	1.00										
	pCO_2	-0.23	-0.20	0.26	0.07	1.00									
	pO_2	0.22	0.20	0.00	-0.45	-0.67	1.00								
	SaO_2	0.30	0.25	-0.31	-0.22	-0.32	-0.07	1.00							
	MDA	-0.27	0.24	0.22	0.14	0.00	0.20	-0.12	1.00						
Day3	FiO_2	-0.12	0.25	0.76	0.07	0.69	-0.37	-0.25	0.05	1.00					
	pH	-0.16	-0.29	-0.02	0.47	-0.09	0.10	-0.75	0.04	-0.17	1.00				
	pCO_2	0.16	-0.23	0.10	-0.49	0.22	0.01	-0.23	-0.48	0.09	0.13	1.00			
	pO_2	-0.49	-0.34	-0.51	0.65	-0.39	0.01	-0.10	0.27	-0.21	0.26	-0.57	1.00		
	SaO_2	-0.10	-0.39	-0.19	0.34	-0.07	-0.21	0.21	-0.26	-0.32	-0.10	-0.05	0.54	1.0	
	MDA	-0.15	0.29	0.05	-0.07	-0.01	0.28	0.06	0.07	-0.03	-0.16	-0.21	0.28	-0.09	1.0

The Spearman correlation test for quantitative and qualitative variables is

presented in Table V.

Table V. Spearman correlation for quantitative and qualitative variables

In order to study lipoperoxidation the MDA value registered on the first and third days in the studied term newborns was compared with the MDA value in the control group that consisted of healthy term newborns. The results are illustrated in Tables VI and VII.

	Day 1						Day 3					
	FiO_2	pH	pCO_2	pO_2	SaO_2	MDA	FiO_2	pH	pCO_2	pO_2	SaO_2	MDA
G	0.12	0.03	0.18	-0.30	-0.12	-0.24	0.46	-0.12	0.25	-0.45	0.00	-0.42
Apgar1	0.01	0.17	0.66	-0.45	0.08	0.30	0.26	-0.28	-0.56	0.35	0.28	0.11
Apgar5	-0.03	0.12	0.59	-0.45	0.27	0.19	0.21	-0.40	-0.40	0.24	0.29	0.03
Asphyxia	0.04	-0.06	-0.60	0.42	-0.23	-0.12	-0.25	0.42	0.35	-0.30	-0.32	-0.08
HG	-0.25	0.41	-0.40	0.30	-0.20	-0.20	-0.20	0.47	-0.21	-0.05	-0.05	-0.50
CH	-0.53	0.08	0.37	-0.45	-0.23	-0.45	-0.11	0.23	0.24	0.15	0.56	-0.52
Hbr	-0.44	-0.50	-0.23	0.35	0.47	-0.17	-0.71	-0.06	0.00	0.33	0.29	0.23
AIE	0.48	0.27	-0.18	0.12	-0.37	0.18	0.12	0.56	0.32	-0.55	-0.69	-0.36
UV	-0.48	-0.34	-0.06	0.12	0.73	0.06	-0.80	-0.16	-0.25	0.21	0.33	0.12
MV	0.12	0.03	0.18	-0.30	-0.12	-0.24	0.46	-0.12	0.25	-0.45	0.00	-0.42

MDA = lipoperoxides; FiO2 = concentration of oxygen that newborns were exposed to; pCO2 = partial pressure of carbon dioxide, pO2 =partial pressure of oxygen, SaO2 =oxygen saturation; G= gender, Apgar 1 = Apgar score at 1 minute, Apgar 5 = Apgar score at 5 minutes, HG = Iute Hypoxemic gravidarum; CH = cerebral hemorrhagy; AIE= acute ischemic encephalopathy; UV = phototherapy; MV =mechanical ventilation

Table VI. Comparison between MDA value on the first day in term newborns with asphyxia versus controls

	$Rank_{nnt}$	$Rank_{control}$	U	N_{nnt}	$N_{control}$	p
MDA D1	134.0000	362.0000	68.00000	11	20	0.086758

MDA D1 = MDA value on the first day; Rank Sum = sum of ranks; U=; N prem = sample size of prematures; sample size of controls

Table VII. Comparison between MDA values on the third day in term newborns with asphyxia versus controls

	$Rank_{nnt}$	$Rank_{control}$	U	N_{nnt}	$N_{control}$	p
MDA D3	147.5000	348.5000	81.50000	11	20	0.243656

MDA D3 = MDA value on the third day; Rank Sum = sum of ranks; U=; N prem = sample size of prematures; sample size of controls

The comparison between the MDA value in the studied group and normal values in adults is presented in Table VIII.

Table VIII. Comparison of MDA in term newborns with asphyxia and in adults

NNT	MDA day 1	MDA day 3
Sample median	18.12	0.00
Population median	1.29	1.29
Standard deviation in population	0.37	0.37
Sample size	11	11
Standard error of sample median	0.111559197	0.1115592
Parameter of Z test	150.8309113	-11.5633675
Significance threshold	0.05	0.05
P (test significance)	0.00E+00	0.00E+00

DISCUSSIONS

The MDA in term newborns with asphyxia was determined in order to study lipid peroxidation. Table II data analysis revealed that mild asphyxia prevailed (45.45%), followed by severe asphyxia (36.36%) and moderate asphyxia (18.18%). All newborns with severe asphyxia also presented AIE. In intranatal asphyxia ORS are released in the central nervous system and will contribute to the genesis of encephalopathy lesions (52,110,157,158). Oxygen reactive species are produced in the reoxygenation stage following perinatal asphyxia. The brain is at major risk of developing oxidative lesions since membrane lipids are rich in polyunsaturated fatty acids and the quantities of some antioxidative defense enzymes (catalase and superoxide dismutase) may be reduced. Some brain areas are rich in iron. Iron from cells is released under the action of cell lesions caused by asphyxia. Non-transferin bound iron generates hydroxyl radicals, which, in their turn, will trigger a chain peroxidation reaction that increases MDA levels in blood and urine (5,7,8). Cerebral hemorrhage, which occurred in two cases, was caused by obstetrical trauma.

In the study group oxygen therapy was applied at a median FiO_2 of 30.18% on the first day (95% CI (23.42-36.94)) as compared with a median FiO_2 of 24.82% on the third day (95% CI (21.48-28.16)). The oxygen supply required was low. On the third day the median value of the FiO_2 applied was close to the concentration of oxygen in air. The difference between the FiO_2 value on the first day and on the third day was statistically significant ($p = 0.0298$). Respiration improved rapidly after asphyxia thus reducing oxygen administration. The clinical picture was replaced by neurological manifestations once the vital functions became stable and the respiratory symptomatology improved (3,13,14,16).

The median value of the pH in the studied group was the same on the first and third days, with a median of 7.34 (95% CI (7.30-7.39)). The values of pCO2, pO2 and SaO2 did not register statistically significant differences ($p > 0.05$) on the first and third days.

The median MDA value in the studied newborns was higher on the third day (4.72nmol/ml; 95% CI (3.39-6.04)) as compared with the first day (4.46nmol/ml; 95% CI (3.05-5.87)). However, the difference was not statistically significant ($p > 0.05$). The more intense peroxidation process registered on the third day may be interpreted as a consequence of perinatal hypoxic stress, which is an important source of morbidity, mortality and neurological consequences in surviving newborns (109,117,120).

The comparison between the MDA in the control group and the MDA value in the studied term newborns with asphyxia was not statistically significant ($p >$

0.05) (Tables VI,VII). The comparison between the median MDA value in the studied newborns and the median MDA value in adults revealed significant lower values in adults ($p < 0.001$). Therefore the peroxidation process is more intense in newborns. This is generated by poor antioxidative defense mechanisms and circumstances that favor the production of oxidative stress: perinatal hypoxic stress, birth, the transition from intrauterine to extrauterine life and neonatal asphyxia.

CONCLUSIONS

1. The studied newborns presented all types of asphyxia: mild, moderate and severe. The mild form prevailed.

2. The studied newborns had other associated pathologies: cerebral hemorrhage (18%) and AIE (36%).

3. The control group consisted of 20 term newborns without associated pathology, with gestational age over 38 weeks and a mean weight of 3500 g.

4. Exposure to oxygen was significantly lower on the third day as compared to the first day ($p = 0.0298$) due to the rapid restoration of the vital functions and the replacement of respiratory distress with neurological distress (9,11,16).

5. There were no statistically significant differences between the values registered on the first and third day of life in the studied term newborns as far as pH (7.34; $p > 0.05$), pO_2, pCO_2 and SaO_2 values were concerned.

6. Lipid peroxidation reflected by the median MDA value was higher on the third day as compared with the first day. However, it was not statistically significant ($p > 0.05$) since the peroxidation process was more intense on the third day of life given the postasphyxia context.

7. The median MDA value in the newborns with asphyxia was significantly higher than normal values in adults.

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LIPOPEROXIZII – MARKERI AI STRESULUI OXIDATIV LA NOU-NASCUTII CU ASFIXIE PERINATALA

REZUMAT

Introducere: In ultimii ani, stresul oxidative a fost inclus in patogeneza asfixiei si a encefalopatiei ischemice hipoxice datorita anumitor particularitati ale sistemului nervos central, care duc la cresterea vulnerabilitatii la speciile reactive ale oxigenului. Studiul urmareste identificarea lipoperoxizilor la nou-nascutii cu asfixie perinatala.

Materiale si metode: Grupul de studio a inclus nou-nascuti la termen cu asfixie la nastere, din perioada Ianuarie 2005-Iunie 2006, din Departamentul de Neonatologie al Clinicii de Ginecologie Nr. 1, Cluj-Napoca, Romania. Lipoperoxizii au fost masurati in dinamica in prima si a treia zi de viata. Rezultatele au fost prcesate folosind programul computerizat STATISTICA.

Rezultate: Grupul de studio a inclus 11 nou-nascuti la termen la 38.91±0.70 saptamani de gestatie, avand greutate la nastere de 3391±380.90g. Nou-nascutii la termen au prezentat: 45.45% asfixie usoara, 19.19% asfixie moderata si 36.36% asfixie severa. Concentratia oxigenului necesar in prima zi de viata a fost 30.18±10.06%, comparative cu ziua a treia, cand oxigenul necesar a fost in concentratie de 24.82±4.98% (p=0.0258). Nivelul lipoperoxizilor MDA a fost

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