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REVIEW ARTICLE

IMMUNOSUPPRESSIVE EFFECT OF POLYCYCLIC AROMATIC HYDROCARBONS BY INDUCTION OF APOPTOSIS OF PRE-B LYMPHOCYTES OF BONE MARROW

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Summary: Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants, distinguished by genotoxic, hepatotoxic, nephrotoxic and immunotoxic effects. Especially secondary toxicity after bioactivation by microsomal monooxygenases (dependent on cytochromes P450) is characteristic of them. The immunotoxic effect is the result of very global impact on immunological reactivity of an organism and immunosuppression by induction of apoptosis of pre-B lymphocytes represents one of its particular forms. It has been proved that the effect of PAH is caused mostly by the following mechanisms: enzymatic induction by the way of activation of AhR (Aromatic hydrocarbon Receptor); alteration of cellular DNA; development of oxidative stress; increase in the concentration of intercellular calcium and decline of activity of NF-κB (Nuclear Factor – kappa B). Most sensitive to these changes are particularly B-lymphocytic precursors and pre-B lymphocytes. Intensity of entire manifestations is also considerably dependent on the presence and intensity of mechanisms of active or passive resistance of cells.

Key words: *Polycyclic aromatic hydrocarbons; Immunosuppression; pre-B lymphocytes; Apoptosis*

Introduction

PAH are ranked among ubiquitous environmental pollutants. They arise and get into life environment mostly by imperfect burning of fossil fuels, gases, wood but also a lot of other organic materials, especially tobacco (10). From a chemical point of view it is a very heterogeneous group of organic materials that are composed of at least two condensed benzene nuclei on which various substituents can be bound. PAH are considerably lipophilic. They enter an organism through the skin, by respiratory and gastrointestinal systems. Because of their physicochemical attributes they are able to get through the membranes very easily, without participation of active transporters (38).

Toxicity of polycyclic aromatic hydrocarbons

A much-discussed problem in connection with PAH is their toxicity for living systems. Primary toxicity (the toxicity PAH in the form in which they enter an organism) is relatively low. During biological experiments in rodents it has been proved that tissue damage with ultimate death have not been distinct until the quite high dose, calculated per kilogram of body weight (38). After the biotransformation to active metabolites (secondary toxicity) the toxicity of PAH rises very intensively and even at low doses it is possible to observe the disorders on sub cellular, cellular and organ levels. The damage of DNA – genotoxicity (and it implicates mutagenicity and potential carcinogenicity) – is among the most important toxic manifestations. Of organs, which are the most usually affected by PAH is necessary to mention liver (hepatotoxicity), kidney (renal toxicity) and immune system (immunotoxicity) (4,11,37,38)

Bioactivation of polycyclic aromatic hydrocarbons

In cytoplasm PAH induce a sequence of biological reactions whose products are molecules or molecular fragments with higher toxicity than the toxicity of the substances entering the reactions (bioactivation). The activity of the process mentioned above is different in different tissues in dependence on the expression of specific enzymes (11,16).

The metabolism of PAH is not basically different from the metabolism of any other xenobiotic. The first phase is the oxidation by one of the enzymes of a large family of cytochromes P450 which give a rise to phenols, dihydrodiols and epoxides. The second phase is their conjugation with sulfates, glutathione or glucuronic acid which gives a rise to water soluble conjugates which are excluded in urine.

The activity of cytochromes P450 seems to be necessary for biological effects of PAH because of the characte-

ristic electrophilic attributes of their metabolites. These attributes are given by the rise of an atom, missing an electron in its valence orbital (17). The electrophiles are very potent reactants which react with molecules rich in electrons – the nucleophiles – and create covalent adducts. Even the nuclear DNA is among these nucleophiles. Highly reactive free oxygen radicals, which are responsible for the development of oxidative stress, are the byproducts of oxidizing reactions mentioned above (28).

The group of cytochromes P450

Cytochromes P450 create a group of hemoproteins, which catalyze the oxidation of many endogenous molecules and xenobiotics. They are located predominantly in the endoplasmic reticulum of hepatocytes but they are also probably present in all the other cells (17). They have been subdivided into the families according to their structural characteristics. In human body there are mostly the families CYP1, 2 and 3. These are divided into the several subfamilies from which CYP1A1 (with substantial expression in different tissues), CYP1A2 (expressed mostly in a liver) and CYP1B1 are the most important enzymes for the metabolism of PAH. CYP1B1 is a recently described cytochrome, very strongly expressed in the cells of bone marrow (14). The expression of all those enzymes (mentioned above) is PAH inducible.

Immunotoxicity of polycyclic aromatic hydrocarbons

It is provable that immune system is damaged by exposure to PAH (21). This damage is expressed by immunopathological reactivity – as its gain (which can be clinically expressed as an allergy) or its dilution, which leads to the immunosuppression. The immunosuppression more or less generally affects single components of immune system with consequently increased susceptibility to infections or tumor proliferation. Mechanisms of the effect are different and in many cases very hardly definable and clinically provable. In addition, these mechanisms are usually combined to the final effect (5). The most important impacts include reduction of the thymus and the bone marrow cells due to the apoptotic death of precursor cells of T and B lymphocytes (40,22), alteration of antigen – receptor signaling, augmentation of another signaling pathways, interference with calcium metabolism (6) and impairment of the cytokine network by changes of level of some cytokines, particularly of interleukins 1 and 2 (38). According to some authors it is possible to observe the evident relationship between carcinogenity and immunosuppressive potential (4,11,37).

Molecular mechanisms of biological effects of PAH

As it was mentioned above, due to their lipophilic character, PAH are able to enter a biomembrane without a particular transporter. After the entry to the cytoplasm they start a lot of processes of which the activation of AhR (Aromatic hydrocarbon Receptor) (26), bioactivation of selected cytochromes, genotoxic effect of metabolites, development of the oxidative stress (28), impairment in the cellular homeostasis of calcium (7,32) and even a decrease in the activity of nuclear factor NF-κB (23) are essential for the eventual effect.

Functions of AhR, enzymatic induction

AhR is the intracytoplasmatic molecule belonging to the group of proteins/transcription factors Per/ARNT/Sim. In a resting phase, it is associated with stress protein Hsp90 and with molecule ARA9/XAP-2 (hepatitis B virus X-associated protein 2). Attachment of PAH is followed by release of AhR from the bond and its own translocation to the nucleus with the help of transporter ARNT (aryl/aromatic hydrocarbon receptor nuclear translocator) (22). In the nucleus, AhR interacts with nucleotides sequence called DRE (dioxin response elements; dioxin is a model ligand of AhR). DRE impulses regulate the group of genes, marked as AhR-gene battery. It consists of at least six genes which code enzymes that take part – more or less – in the metabolism of PAH, of which CYP1A1, CYP1A2 (28) and CYP1B1 (14) have to be stressed. The enzymatic induction by the activation of AhR mentioned above apparently modulates the reactivity of an organism to long-lasting exposure to PAH. However, it has been shown that it is not an essential variable for induction of apoptosis of preB-lymphocytes derived from the bone marrow and even more for apoptosis of experimental T- cell hybridoma (14,22,40).

Genotoxicity

Genotoxicity of PAH is understood as the ability of its active metabolites (particularly diols and epoxides) to damage the cellular DNA. The point mutations, DNA adducts formation, breaks of single-strand DNA and the chromosomal aberrations are induced by PAH (38). Predominant targets are the cells with active synthesis of DNA; it means the dividing cells, among them particularly lymphoid precursors. There can be a malignant transformation as well as an increased elimination of impaired cells by apoptosis, as a consequence of their genotoxic damage. It seems that in complex mixtures of PAH, occurring in the environment, the genotoxic effects are mostly associated with the content of benzo(a)pyrene (11).

Oxidative stress

Oxidative stress is defined as a damage of a cell or an organism by free radicals. The free radicals are highly reactive molecules or molecular fragments, containing unpaired electron in its valencial orbital. They usually rise as byproducts of oxidative reactions particularly in course of the respiratory chain (physiologically, 2–5 % of oxygen is reduced univalently on arise of free oxygen radicals, so-called ROS – reactive oxygen species) and in oxidation of substrates, e.g. PAH, by cytochromes P450 (28). Under normal cir-

cumstances there exists a balance between the production of ROS and activity of so-called scavengers (SOD, GPX, and catalase) and antioxidants (vitamins A, C, E, glutathione, methionin, cystein etc.). Only during their increased production (for example due to higher activity of cytochromes P450), they are responsible for the chemical degradation of numerous targets in the cell (9) and the positive transcriptial regulation of some genes, due to regulative motives from DNA, designated as ARE (antioxidants response elements) (28). The oxidative stress itself is very powerful inducer of apoptosis.

Interference with level of intercellular calcium

The deregulation of calcium homeostasis turns out to be a very important factor of toxicity of PAH metabolites (27). As it is proved below, the increased calcium ions concentration in cell is mediated by at least two mechanisms: first by its release from microsomes by the alteration of function of channel RyR1 (ryanodine receptor) (32) and second by the activation of protein kinase PTK with following activation of phospholipase C and mobilization of calcium which is dependent on production of inositoltriphosphate (IP3) (7).

Increase in the level of calcium is very stressful for the cell, especially for the risk of exhaustion of energetic reserves, dysfunction of microfilaments and the activation of hydrolytic enzymes (17). It is also a characteristic feature of the apoptosis.

Role of NF-κB transcription factor

The role of nuclear factor kappa B, as an important factor for survival of the cell, has already been discussed in association with its antagonistic effect on apoptosis. It has been proved that the nuclear factor kappa B is able to inhibit apoptosis of cells induced by specific extracellular proapoptotic stimuli TNF-α and IL-1α (2). Conditions associated with down regulation of activity of NF-κB lead to depression of life span of the cells. One of the examples is even the exposure to PAH. Mechanism mentioned above takes also part in the whole immunosuppressive effect, caused by apoptotic death of pre-B lymphocytes (23).

Apoptosis

Apoptosis can be defined as a particular form of genetically regulated cell death. In a life of an organism it has absolutely essential role to keep a tissue homeostasis, and an optimal number of functional cells. It represents the contrary to the mitosis. Physiologically, it occurs either regulated (elimination of the cells during embryonic development – programmed cellular death) or repressive, not regulated mechanism for removing of insulted cells either virally infected or malignantly transformed (unprogrammed cellular death) (35).

Under pathological conditions, in the case of its deregulation, apoptosis could be at the beginning and the development of illness (for example proliferation of malignantly transformed cell clone is associated with the loss of apoptotic potential or on the other hand, neurodegenerative diseases cause the excessive apoptotic death).

Apoptosis progresses with the highly conservative phylogeneticaly script. The basic principle of that script is a gradual activation of specific genes and proteins. In the end, there is a coordinated cleavage of cellular structures, which is energy dependent (29,30).

Phases of apoptosis

The cell in the course of apoptosis goes through these two phases: signaling and effector's one. During the signaling phase, numerous signals and information are processed in the cell to testify in or out cell's favor. They are either extracellular or intracellular. They can be either specific (TNFα, FasL, growth factors) or nonspecific (metabolism failure or DNA damage). Only after their summation – in the case of proapoptotic information prevalence – the cell enters the second phase. The activation of latent cystein proteases – caspases, is the hallmark of this process (12).

Characteristic signs of the process mentioned above are: the development of oxidative stress, which takes part in the degradation of some cellular organelles (9), the increase in intracellular calcium level and the increase in permeability of mitochondrial membranes (8).

4.1.1 Internal pathway of apoptosis, protein p53

Any change of internal environment represents a risk for keeping the integrity of the whole organism. As a most common example it is possible to stress the random mutation of genome, caused by fault in the replication of DNA or during the process of gene segments rearrangement. On this condition, the protective cell mechanisms are immediately activated. The cell cycle progress is stopped in attempt to repair the errors. If genetically defaults are irreparable, the altruistic suicide of cell by apoptosis is induced (13,33,39).

The gatekeeper of integrity of genome, which initiates the processes mentioned above, is p53 protein. It is a tumor suppressor protein with molecular weight 53 kDa. The p53 protein is bound to DNA by the area adjacent on C-end (rich on basic amino acids) where it is possible to detect the damaged area. In the same time that protein can immediately initiate the DNA reparation (13).

In the case that DNA damage exceeds the frame of easy reparation the expression of the p53 protein increases and it takes its function as a transcription factor. As a first step it is possible to detect the increase in the expression of p21 protein (blocker of cycline dependent kinase). This protein is able to stop the cell growth and division and also provides sufficient time for more effective reparation of DNA. The production of the other proteins (particularly so-called PIGs genes – p53 induced genes) which are functionally closely associated with development of the oxidative stress, followed by the entrance into apoptosis, increase immedia-

tely if genetic damage is irreversible. (13,20,28). It is being proved that the oxidative stress, is the key moment that decides about the fate of a cell.

Regulation of apoptosis

Integral part of either signaling or effector's phase is presented by the factors-proteins regulating apoptosis. These proteins block the intracytoplasmatic transduction of internal specific proapoptotic stimuli (proteins FLIPs and SODD) (35). Numerous proapoptotic (for example Bax) and antiapoptotic (for example Bcl-2) molecules belong to Bcl-2 family (8,15). Their right timed expression and mutual rate is also essential for normal development of B-lymphocytes in the bone marrow.

As it was mentioned above, some of the nuclear factors (particularly NF-κB) play an important role in the regulation of apoptosis.

Differentiation of B-lymphocytes in bone marrow

Precursors of B-lymphocytes are developed in the bone marrow by division of hematopoietic stem cells. They gradually differentiate through several stages – proB, preB and immature B-lymphocytes, which leave the bone marrow and settle secondary lymphatic tissues. During their development in bone marrow they create specific receptor for antigen (BcR), by the mechanism of the gene segments rearrangement and they become the immunocompetent cells. This process is essential for maturing of B-lymphocytes and it is tightly regulated.

BcR is a surface molecule with the immunoglobulin structure, which consist – similarly as secretory immunoglobulins – of two heavy and two light chains. An additional chain, which serves for stabilization in the membrane, two molecules marked as Igα (CD79a) and two molecules Igβ (CD79b) create also an integral part of BcR. That receptor is expressed at first as a pre-BcR and later in the form of BcR on the surface (25).

Role of microenvironment

The bone marrow microenvironment provides optimum conditions for maturation of B-lymphocytes. It produces growth factors which stimulate division and differentiation of B-lymphocytes (particularly IL-7 and CSF-1) (20) and inhibit their death by the apoptosis. In a case of any damage of the microenvironment, apoptosis can take part in inhibiting of the development and in eliminating of nonfunctional (and partly even autoreactive) clones of B lymphocytes (3).

Physiological importance of apoptosis for development of B-lymphocytes

It has been proved that BcR gene rearrangement generates a huge amount of errors leading to the both syntheses of nonfunctional BcR and syntheses of BcR with very high affinity to the self antigens. That's why it is necessary to ensure that during the development of B-lymphocytes there are several checkpoints where the mistakes can be removed or where insulted and dangerous cells can be eliminated (19).

Studies on the model of mouse bone marrow have proved that these points are at least two: 1) on the middle way from pro-B to pre-B lymphocyte when the first surface expression of newly synthesized heavy chain "μ" may happen and 2) at lymphocytes with low expression of sIgM (BcR), it means at immature but already immunocompetent cells (20).

The control of the errors and the elimination of the injured cells by apoptosis become at both checkpoints. Almost 70 % of cells die through this mechanism even in the first point which represents about 51 million of pre-B lymphocytes per day (20). In this content it is necessary to mention very important role of protein p53 (13,19).

The cells at the checkpoints show quite unique characteristics. The levels of proteins, which regulate the apoptotic changes, are variable. Production of Bcl-2 decreases and expression of the Bax protein increases. The ratio of Bcl-2/Bax is remarkable decreased but proapoptotic potential of these two precursors is increased (20). They are vulnerable and they easy yield to the exogenous apoptotic stimuli.

Pathological induction of apoptosis dependent on function of bone marrow

For the study of induction of apoptosis of pre-B lymphocytes a model system, based on the cellular structure of BMS2 (stroma cells of bone marrow) has been developed (14,22,23). On the basis of many studies, following conclusions could be drawn:

1) Apoptosis of pre-B lymphocytes is dependent on the cellular stroma. Only relatively high doses of PAH lead to the death of B-lymphocytes cultivated without presence BMS2 (22).
2) The bioactivation of PAH is important for induction of the apoptosis. Only the active metabolites of PAH (dihydrodiols and epoxides) are very potent inducers. This finding confirms the importance of cytochromes P450 (22).
3) From point of view of the general activity of cytochromes (in the association with apoptosis) basal expression of cytochromes plays more important role than their induction by the way of AhR. The activation AhR it-self is not sufficient for the immunosuppressive effects (22).
4) CYP1B1 seems to be the key enzyme that shows quite high activity especially in the stromal cells of bone marrow. Toxic metabolites created by presence of that enzyme are able to get into the cytoplasm of the lymphocytes and cause the genotoxic damage of DNA. From this point of view the role of the other enzymes is negligible (14).

5) The depression of activity of NF-κB and defects in the calcium metabolism take also part in the final effect (23).

From these facts we can draw out that the apoptosis of pre-B lymphocytes happen probably on the base of: 1) transduction of toxic PAH metabolites from the bone marrow stromal cells, 2) development of oxidative stress, 3) defects of calcium homeostasis and 4) depression of activity of NF-κB transcription factor in lymphoid precursors.

Pre-B lymphocytes are – due to their high proapoptotic potential and their replicate activity – the most sensitive to the effect of PAH.

Mechanisms of protection

Because of the considerable heterogeneity of the intensity in the clinical and laboratory manifestations of immunosuppressive effect of PAH it is necessary to discuss the most important modulating factors of individual reactivity of an organism. Besides the variable ability of induction of the enzymes from the group of cytochromes P450 (different response to activation of AhR), even the processes of detoxification of bioactivated metabolites, the proapoptotic potential of pre-B lymphocytes and some forms of the active cellular defense come to account.

Enzymatic equipment, detoxification

Highly reactive intermediate products that have an electrophilic or radical character rise during the metabolism of PAH. The cells have numerous enzymes and cofactors of enzymes that are able to reduce the toxic effect of PAH.

For the elimination of free radicals the cytoplasm reserves of antioxidants and so-called scavengers (enzymes which catalyze gradual conversion of free oxygen radicals to water) are the most important. Superoxid-dismutase (SOD), glutation-peroxidase (GPX) and catalase belong among the free oxygen radical scavengers (17).

For the detoxification of electrophiles the most important are next two enzymes: 1) glutathion-S-transpherase and 2) epoxid-hydrolase. Glutathion-S-transpherase (Gsta1) catalyzes their conjugation with nucleophilic glutathione. It is interesting that Gsta1 is prone to the enzymatic induction due to the both motives DRE (part of AhR-gene battery) and ARE (positive regulated by oxidative stress) (17, 28). The epoxid hydrolase catalyze trans-addition of water to epoxid group on arise of non-toxic alcohols. This is the way to reduce the risk of DNA damage by epoxides. It occurs in almost all tissues and its distribution is parallel with occurrence of cytochromes P450 (17).

Proteins regulating apoptosis

As it was mentioned, the fate of a cell is – in the association with apoptosis – dependent on many factors. The well-balanced co-expression of proteins from the Bcl-2 family, of which the principal representative is the protein Bcl-2 with the antiapoptotic effect, belongs among the most important factors. All the members of this group are connected by the common characteristic structural trait – BH1 and BH2 domains. These domains serve to mutual dimerisation and inactivation. By this mechanism, protein Bcl-2 (important inhibitor of apoptosis) can be for example discarded from the function by the proapoptotic protein Bax. It is the mutual rate of single members of this group that decide about survival or death of the cell under the various conditions (8,15).

It is worth mentioning that the protein Bax is prone to transcriptial induction by protein p53 (13). Except for another functions, protein Bcl-2 affects as an antioxidant and as a channel for calcium ions modulates their level in cytoplasm (8).

Active defense

Except for all mentioned mechanisms of passive defense of the constant intracellular environment (detoxification and elevation of the cellular life span by inhibition of apoptosis) there exist also effective means of active defense in a form of energy dependent efflux transport systems. In this consequence the importance of the expression and function of membrane protein P-gp (P-glycoprotein) is stressed (34).

P-glycoprotein

P-glycoprotein (P-gp, p170) is a membrane phosphoglycoprotein, from the ABC (ATP-binding cassette) family of transmembrane proteins. P-gp is expressed on the cell surface and it serves as on ATP dependent pump. Its function is to transport the xenobiotics from cytoplasm and thus reduce their concentration and their adverse effects. Expression P-gp was found on progenitor hematopoietic cells (CD34+), on peripheral lymphocytes and also on many other cells. Its function is to protect these cells against exogenic substances (18,24). Recently the importance of this protein increases in the association with undesirable aspects of its occurrence – increasing resistance of tumor cells against therapy by cytostatic drugs.

Because of the transport function of P-gp, various chemical compounds with aromatic circle(s) were intensively studied in the past (18). On the biological models it was proved that PAH are prone to efflux effect of P-gp (1) and that this effect can be even inducible by mechanism which is different from the way of activation of AhR (34, 36). It has been proved that variable ability to inducing the expression of P-gp can be understood as a negative predictive risk factor for the toxic effects of PAH (34). However, some works do not support this opinion (31).

Conclusion

PAH disturb immunological activity of an organism in a very complex way. Immunosuppressive effect by induction of apoptosis of pre-B lymphocytes is just one of many effects, resulted from immunotoxic influence of PAH. As it

was mentioned above, even here exists a considerable variability and individuality in sensitivity of the organism to the penetration of PAH. This sensitivity is given particularly by a) own primary and secondary toxicity of PAH and b) by active and passive resistance of the cells. It is impossible to forget also the very difficult context of mutual relationship between both particular parts of immune system and immune system as a whole and the other functional units and structures of organism. To evaluate the clinical importance of exposure to PAH, it is necessary to understand that during their lives people are exposed to very hardly definable and standardisable mixtures of xenobiotics.

The research on influence of compounds like PAH to an immune system is profitable and contribute to further understanding of individual reactivity.

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REVIEW ARTICLE

MICROELEMENTS AND INHERITED METABOLIC DISEASES

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Summary: In addition to the main groups of inherited metabolic diseases, including mitochondrial, peroxisomal and lysosomal defects, organic acidurias, porphyrias, defects of amino acids, saccharides and fatty acids metabolism, disorders of transport and utilisation of microelements have also been recognized. Recent findings concerning hereditary hemochromatosis (iron), Wilson and Menkes diseases (copper), molybdenum cofactor deficiency (molybdenum), defects of cobalamine synthesis (cobalt) and acrodermatitis enteropathica (zinc) are reviewed.

Key words: *Microelements; Inherited metabolic diseases*

Introduction

Inherited metabolic disorders in children are not common; nevertheless their cumulative incidence is estimated to be 1 in every 500 newborns. Besides others, this group of diseases includes also the defects of transport and utilisation of minerals and microelements. The last act as catalysts for many biological reactions within the body, including muscle response, transmission of messages through the nervous system, production of hormones, digestion and the utilisation of nutrients in food.

Of the total 118 elements, 40–50 of them found in man, only 20 are known to be essential for the structure and function of the human body, the effect of the others being either uncertain or harmful (pollutants). The mineral nutrients are usually divided into macroelements, the most abundant substances dissolved in the extracellular and intracellular fluids, and microelements with daily requirement less than 100 mg (21).

People can have deficient, adequate, or toxic intakes of any essential nutrient. Disturbances relevant to microelements might be caused by deficiency due to poorly balanced nutrition or by metabolic defects, both primary (inherited metabolic diseases) and secondary (digestive disorders and various chronic diseases).

Most common signs of the deficiency include growth retardation, nausea, diarrhoea, fatigue, muscle weakness, nervous and skin disorders, impaired immunity, difficulty in breathing, mouth and gum disorders (molybdenum), and smell and taste defects (zinc). The microelements supplied in higher concentration are toxic and the overdose symptoms are often similar to those following the element deficiency, e.g. nausea, vomiting, diarrhoea, dizziness, headaches, weight loss, shortness of breath. Considerable interactions in the process of the absorption have been described; e. g. high alcohol consumption leads to the higher urinary magnesium and zinc excretion and decreased zinc absorption, higher quantity of calcium in food reduces intestinal absorption of magnesium, iron, manganese and zinc, and antacids reduce iron and copper absorption (1).

Physiologically there is no need for extra purvey, but microelements supplementation is recommended under certain conditions, related to oxidative stress (smoking, exposure to chemicals, stress), digestive disorders, rapid growth (puberty), intensive exercise, ageing, pregnancy and lactation. Other indications might be special dietary regimes (diets rich in proteins, fats, carbohydrates, the vegan's or vegetarian's life style) and various secondary, but especially *primary metabolic defects of microelements* (22).

Several such disorders have been described in detail, namely hereditary hemochromatosis (iron), Wilson's and Menkes diseases (copper), molybdenum cofactor deficiency (molybdenum), defects of cobalamine synthesis (cobalt) and acrodermatitis enterica (zinc).

Iron

Only about 10 % of iron (Fe) ingested is absorbed, animal sources being preferred. Acidic pH is necessary for Fe^{3+} reduction to Fe^{2+}. Iron interferes with zinc and cobalt absorption.

The toxic effect of Fe overload is due to oxygen free radicals formation, leading to symptoms like diarrhoea, vomiting, cardiac failure and hepatotoxicity. Anaemia, brittle nails, constipation, impaired brain function, short breath, and tastes for odd foods (clay) are the main signs of iron deficiency. Humans have no physiological pathway for iron excretion, so that the intestinal absorption must be well regulated.

Inherited metabolic defects are represented by a condition called *hereditary hemochromatosis*. High iron cumulation in many tissues (liver, pancreas, myocardium) due to its high intestinal absorption, exceeding the binding capacity for transferrin is very dangerous, because it leads to tissue damage.

The disease usually does not present itself until adulthood. The main signs include skin and other organ pigmentation, hemosiderosis, liver cirrhosis, hepatoma, carcinoma, diabetes, cardiac failure, arthritis and impotence, even if not all homozygotes manifest any symptoms. The cured patient has a normal life span, but the disease might be fatal if not diagnosed early or not treated aggressively.

The defect with an incidence 1:12 000 (more frequent in men) has an autosomal recessive trait. Two main (C282y and H63D) and other 15 rare mutations in the HFE (or HLA-H) gene on chromosome 6 expressed in duodenal crypts have been described since 1996. The other, recently described gene DMT1 (divalent metal transporter) and duodenal villi interact with the transferrin receptor. A distinct, non-HFE variant of the disease is juvenile hemochromatosis (impaired locus 1q). The other, mapped to 7q22, contains the gene encoding transferrin receptor 2 (3).

Pathogenesis: Impaired iron absorption is a cascade of events: defected HFE protein, giving a false signal of a low iron concentration in cells influences the DMT1 protein, stimulating the iron uptake from a diet. Iron binds in excess to transferrin, reaches portal veins and finely leads to the liver and other organ cells death. Diabetes mellitus is the major endocrine disorder associated with the disease: Pancreatic β-cells damage by iron deposits and/or lack of chromium (a metal essential for insulin's proper function) due to its competition with iron absorption in gut is the speculated cause of this complication.

Diagnostics are based on the higher serum iron and ferritin levels, increased transferrin saturation (used as a screening test, pathological level being >45 %), low iron-binding capacity, and especially DNA analysis.

Therapy based on the diet (uncooked sea-products, alcohol, minerals, and vitamin C restriction) or the chelating agents is usually not effective enough, so that phlebotomy or red cells apheresis must be used to prevent complications (450–500 ml of blood is removed every 1–2 weeks, until the ferritin level decreases to 10–20 mg/l) (3).

Copper

About 30 % of copper (Cu) ingested is absorbed in the stomach and intestine. This metal makes a part of many Cu-enzymes and plays an important role as an antioxidant, in iron transfer, and in haemoglobin synthesis. Copper deficiency may follow a higher intake of iron, molybdenum, zinc, milk or antacids in food and it manifests by symptoms such as weakness, diarrhoea and skin defects. Nausea, vomiting, dizziness, diarrhoea and headaches are the signs of copper overload.

There are two main inherited disorders of copper metabolism described so far, namely Menkes and Wilson's diseases.

Menkes disease results from impaired copper intestinal transport, caused by a deficit of extrahepatic Cu-ATPase.

Clinical symptoms of this neurodegenerative disorder are related to deficient levels of copper available, and include progressive mental deterioration, seizures, typical "facies" (cherubic, dolly face), peculiar (kinky, steely, brittle) hair, epilepsy, hypothermia, jaundice, bleeding, connective tissue abnormalities and skeletal changes. Death usually occurs in the first few years of life.

Pathogenesis: Copper deficit involves about 13 Cu-enzymes thus inducing diverse structural changes in hair, brain, bones, liver and arteries. Particularly deficient tyrosinase causes hair and skin depigmentations, and lysyl oxidase defect leads to splitting of arterial intima as a result of the defective elastin and collagen cross-linking. Monoamine oxidase and defective disulfide bonds in keratin are responsible for kinky hair; deficiency of cytochrome c oxidase leads to hypothermia, and decreased availability of ascorbate oxidase promotes skeletal deformities.

The incidence of this X-linked (males affected) defect runs about 1:300 000 (higher in Sardinia) with the most frequent mutations located on Xq12–q13.

Diagnostics are based on the low level of copper and ceruloplasmin in serum and high deoxypyridinoline in urine.

Therapy consisting in daily copper injections (physiological complex Cu-histidinate) is effective (more for the neurological than for the connective tissue abnormalities) only if started early, meaning between the ages of 3–13 years (4,19).

Wilson's disease (hepatolenticular degeneration) is the other defect of transport of copper, the deficient enzyme being specified as the liver Cu-ATPase. The disease presents itself as a chronic liver disease (jaundice, abdominal swelling and pain, cirrhosis, vomiting of blood), Keyser-Fleischer ring in cornea, haemolysis and renal defects. Neurological symptoms include coordination and speech defects with possible bulbar paralysis and may be combined with psychiatric disorders. The liver defects manifest between 6–18 years of age, the neurological signs occur after the age of 20 years. The first symptoms are often misdiagnosed as infectious hepatitis or infectious mononucleosis.

Wilson's disease, affecting about 1 in 30 000 people worldwide, is caused by gene ATP7B mutations on chromosome 13 (13q14.3) and transmitted as an autosomal recessive trait.

Pathogenesis: Deficiency of a copper-transporting ATPase protein leads to excessive cumulation of copper in the liver, basal ganglia and kidney, consequently to decreased excretion of copper in bile and low incorporation of copper into its transport-enzyme ceruloplasmin. Whether the primer is a low level of ceruloplasmin produced or it is the liver's inability to metabolise copper remains to be proved.

Biochemical findings include a high level of copper in the urine and in liver biopsy, normal or decreased copper and ceruloplasmin in serum, along with a low copper incorporation into ceruloplasmin. The disease is always fatal if not diagnosed and treated early. The lifelong therapeutic approach consists in copper exclusion form food (mushrooms, nuts, chocolate, dried fruit, fish, liver), in supply of zinc-acetate, the copper absorption blocator, or an application of chelating agents such as penicillamine, trientine or possibly tetrathiomolybdate, and liver transplantation in the end (5,19).

Molybdenum

Molybdenum (Mo) plays an important role as a part of Mo-pterine, cofactor of 3 enzymes: xanthine oxidase (XO, involves metabolism of purines), sulphite oxidase (SO, metabolism of cysteine) and aldehyde oxidase (AO, conversion of aldehydes to acids). The main role of the enzymes lies in detoxification and synthesis of intermediates.

A deficiency, usually due to the parenteral diet low in Mo, manifests as visual problems, tachycardia, headache, mental disturbances, and mouth and gum disorders. Symptoms of toxicity include diarrhoea, growth retardation, gout and copper deficit.

Inherited metabolic defects leading to individual enzyme deficiency might be isolated or combined: deficiencies of XO, SO and molybdenum cofactor are described. Although XO deficiency might be relatively benign, patients with isolated deficiencies of SO or molybdenum cofactor exhibit similarly severe abnormalities probably caused by the toxicity of sulphite and/or a deficit of inorganic sulphate available, especially in the brain. A defect of the third enzyme, AO is not combined with special symptoms.

Xanthinuria. Xanthine oxidase, catalysing the uric acid production from xanthine, is a complex of flavines, molybdenum, iron and sulphite cofactor.

Clinical symptoms of the enzyme deficit manifesting itself in early childhood include hematuria, nephrolithiasis (X-ray contrast), renal failure, arthropathy and myopathy, but as much as 50 % of homozygotes for the defect might be fully asymptomatic.

The disease, with a frequency 1 in 40 000 newborns, shows an autosomal recessive type of inheritance implying mutation on chromosome 2.

Diagnostics are based on low uric acid and high xanthine and hypoxanthine concentrations in the blood and urine.

Symptomatic therapy consists of low-purines diet, drinking regime, and lithotripsy (7).

Isolated deficit of sulphite oxidase leads to impaired metabolism of sulphur-containing amino acids (cysteine, methionine, homocysteine, taurine) in the liver, brain and kidney.

Clinical symptoms include lens subluxation accompanying seizures and diffuse neurological disease in infancy.

The cause of this rare genetic condition is a point mutation, causing very low enzyme activity due to changes of Mo-centrum geometry.

Diagnostics are based on a positive sulphite test (in fresh urine) and plasma amino acids, namely increased taurine and sulphocysteine. Mutation analysis can allow accurate prenatal screening (2).

Molybdenum-cofactor deficiency is a fatal disorder clinically manifesting shortly after birth with profound neurological and psychiatric problems, mental retardation, microscopic haematuria and ocular lens dislocation with Marfan-like habitus. This condition might be an easily missed cause of intractable seizures in the neonatal period.

The defect is an autosomal recessive disorder with genetic heterogeneity. A linkage of a defective gene to an 8–cM region on chromosome 6p21.3 was demonstrated.

The biochemical abnormalities include very low levels of uric acid in serum and urine (low XO activity) and low inorganic sulphate levels in urine (low SO activity). Elevated S-sulphocysteine and taurine (in plasma and urine), high excretion of xanthine, hypoxanthine and the oxidized derivative of a molybdopterin precursor (precursor Z) in urine are typical findings. A defect of terminal enzyme for converting precursor Z to active molybdopterin is supposed.

Molybdenum cofactor deficiency is probably frequently underdiagnosed due to the lack of specific clinical features. Screening of infants at risk for the presence of urinary sulphites and/or serum hypouricemia is rapid and inexpensive.

No specific therapy has been recommended so far. Prenatal diagnostics is available (17,18).

Cobalt

Cobalt (Co) is not absorbed in natural form, but makes a base of cobalamin (Cbl, vitamine B_{12}), which plays a role as a coenzyme of two important enzymes: methionin-synthase (cytosolic methyl-Cbl) and methylmalonyl-CoA-mutase (mitochondrial adenosyl-Cbl formation).

A deficiency of Cbl might occur under poor nutrition, especially in children of breast-feeding mothers on a B_{12}-deficient vegan's diet. Cbl toxicity appears as diarrhoea, fatigue and polycythemia.

Cobalamins are essential biological compounds structurally related to haemoglobin and the cytochromes. Although only microorganisms synthesize the basic cobalamin molecule, all mammalian cells can convert this into the coenzymes adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl).

Several genetic defects have been described, making two main groups: 1) disorders of Cbl absorption and transport, and 2) disorders of intracellular Cbl metabolism. These conditions are mostly inherited as an autosomal recessive trait with various mutations, total incidence is approximated as 0.8/100 000.

Pathogenesis is similar in all types of defects: deficiency of MeCbl and AdoCbl precursors leads to deficiency of the coenzymes causing defective enzyme reaction.

Ad 1) *Disorders of absorption and transport of cobalamin* may be caused by: a) gastric malabsorption (defect of Cbl release from proteins), b) deficiency of R-glycoprotein (which binds Cbl released in the stomach), c) deficiency of intrinsic factor (IF) or its receptor (cubilin), d) deficiency of transcobalamin II, or e) gut malabsorption (Imerslund-Gräsbeck syndrome).

Clinical symptoms include failure to thrive, lethargy, hypotonia, vomiting, developmental delay, megaloblastic anemia and progressive neuro(myelo)encephalopathy. Defects appear in toddlers or pre-school children, the defect of IF much sooner.

Diagnostics are based on a decrease of Cbl and higher levels of both methylmalonic acid and homocysteine in urine. Low transcobalamine II is typical for the type (d).

Therapy consists of daily application of hydroxy-Cbl (or cyano-Cbl) in a dose of 1 mg/day i.m., or the IF application in the type (c).

Ad 2) *Disorders of intracellular metabolism of cobalamin*. There exist several forms of Cbl intermediates giving names to individual subgroups of defects: a) Cbl-F defect – disorder of lysosomal Cbl release, b) Cbl-C and Cbl-D defects – cytosolic disorders of AdoCbl a MeCbl synthesis, c) Cbl-E and Cbl-G defects – disorder of MeCbl synthesis, and d) Cbl-A and Cbl-B defects – disorder of mitochondrial AdoCbl synthesis.

Clinical signs of the type (a) and (b) resemble the transport defects (low haemoglobin, trombocytopenia, anaemia, high homocystein and methylmalonic acid). Failure to thrive, growth retardation, hypo-/hypertonia, epilepsy, progressive encephalo/neuropathy and megaloblastic anemia are characteristic for type (c). The type (d) leads to methylmalonic aciduria, resembling the other type, caused by the methylmalonic acid-mutase deficiency (vomiting, coma, and death following by acidosis, ketosis, hyperammonaemia, hyperglycinemia, organic aciduria and hypoglycaemia. All defects manifest during the first months of life, sometimes even occuring neonatally.

High methylmalonic acid in urine is characteristic of types (a), (b) and (d), increased plasma homocysteine is typical for types (a), (b) and (c). Both Cbl and transcobalamine II are normal.

Application of 1 mg/day i. m. of hydroxy-Cbl (cyano-Cbl is less practical), betain and folate presents an effective therapy for those diseases.

Zinc

This metal, present in all tissues, plays an important role in cell growth and differentiation, immunity, taste and alcohol breakdown. It is an important component of insulin and a cofactor of multiple enzymes in metabolism of neurotransmitters and various hormones. Zinc (Zn) absorption in the upper intestine may be influenced by the presence of fibres (binding) and calcium (competition) in food.

Zinc deficiency, prevalent in population on cereal proteins, malabsorption conditions, liver cirrhosis and renal diseases, manifests itself as fatigue, brittle nails, infections, dwarfism and impaired growth, healing and fertility. An overload presents as nausea, vomiting and renal failure.

The only inherited metabolic defect connected with zinc recognised so far is called *acrodermatitis entheropathica*.

Clinical signs include skin rash, mucous lesions, total alopecia, diarrhoea, mood changes and irritability (but all of them are present in only 20% of the cases). Their intermittent occurrence and the vast range of other, also misleading symptoms, may cause a diagnostic problem.

Mutation of this autosomal recessive disorder was localised on chromosomal region 8q24.3.

Pathogenesis is still not clear: Defect of zinc transport in gut and other tissues leads to a defect of Zn utilisation resulting in decreased synthesis of prostaglandines. Other results suggest an intracellular defect in Zn metabolism rather then impairment of the absorption. The hypothesis that the exocrine pancreas modulates Zn absorption in the jejunum might help in explaning of the conflicting dates of this inherited defect. Transient acrodermatitis enteropathica due to a decreased Zn level in breast milk has been described in two children. Also bovine hereditary Zn deficiency with an absorption defect (Adema disease) has been recognized.

Low serum zinc and alkaline phosphatase activity are used as the diagnostic criterion. Zinc substitution (Zn-sulphate: 10 mg/kg/day) is effective in the therapy (14,15,16, 20).

Conclusions

Several other genetic defects derived from impaired utilization of trace minerals (selenium and manganese, e.g.) have been considered, but the pathogenesis is still not clear. As the number of known inherited metabolic diseases gradually increases, new defects of microelements will probably be recognised in the future. However, since the clinical picture is usually non-specific and the detection of their deficiency or accumulation requires application of special analytical techniques, the diagnostics might be rather difficult.

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ORIGINAL ARTICLE

TIME DEPENDENT APPEARANCE OF SELECTED APOPTOTIC MARKERS AND USEFULNESS OF THEIR DETECTION *IN VITRO*

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Summary: Many experiments have demonstrated that some cell lines are resistant to chemically induced apoptosis *in vitro*, and that apoptosis itself is far from being a homogenous phenomenon. Here we show that 10 μg/ml etoposide elicited only minor changes in Bowes human melanoma cells (temporary decrease in cell viability and proliferation, transient phospatidylserine externalization and caspase-3 activation), which weren't clearly capable to start apoptotic pathway in the entire treated population. On the other hand, potassium chromate at concentration of 150 μg/ml executed cell death bearing some features of apoptosis (cell blebbing, caspase-3 activation and cytoskeletal changes) but lacking or showing weakly others (DNA fragmentation and phospatidylserine externalization). Our results suggest that in detecting apoptosis several fault-proof detection systems are to be used to avoid misleading results and conclusions in each experimental setting.

Key words: *Melanoma; Caspase-3; Chromate; Apoptosis; Detection; Phospatidylserine; Etoposide; In vitro*

Introduction

The phenomenon of cell death, in particular apoptosis, has justifiably attracted numerous scientists both in basic as well as clinical disciplines. Due to this ongoing interest we have learned many useful details concerning its various forms, consequences and regulatory pathways (23). Beside other aspects associated with cell death, it is now chiefly regulatory pathways governing the cell entry into self-destructing process which inspire intensive studies, mainly due to the enormous clinical potential which modulation of these pathways holds (8,12,20,21).

To study individual apoptosis features and mechanisms, one has to determine whether studied cells are actually undergoing apoptosis or any other type of cell death. Furthermore, since it has become known that the initial (and frequently crucial) steps in apoptosis pathway may occur very quickly and discreetly, thereby often escaping our attention, efforts are to be paid to single out these events and reveal their timing. Until recently, researchers conveniently relied upon an established sequence of observable traits, and it seemed that apoptosis represents comparatively compact end point easily characterized by these universal features (morphological, biochemical and molecular) detectable in majority of *in vitro* and *in vivo* models. Nevertheless, with increasing number of experimental approaches and conditions where the course of apoptosis has been studied there appeared circumstances where the principle of universal appearance of apoptosis seemingly failed (15). Many studies have since shown that different cell lines display a wide heterogeneity in their genotypic and phenotypic makeup, and thereby they are predisposed to the heterogeneous behavior upon triggering of apoptosis pathway. In addition, as the apoptosis cascade and its regulation are quite intricate, individual apoptosis instigators employing variable mechanisms might provoke slightly diverse cellular responses (1,19). On the other hand, the need for a rapid and reliable method(s) for positive detection of apoptosis has prompted efforts to find such a test which would be applicable in most cases. During the past 20 years, approximately three groups of tests have been proposed and subsequently verified in detection of apoptosis. The first group includes the tests visualizing the changes in apoptotic membrane such as loss of its asymmetry, increased permeability, formation of blebs and activation of death receptors. In the second group there are assays exploring the apoptotic nucleus, i.e. morphological detection of chromatin shrinkage and clumping, visualization of the typical DNA fragmentation or measurement of subG1 DNA content in cells. The presence of various apoptosis-specific molecules (caspases, DNAses, cytosolic cytochrome c and so forth) along with measurement of functional state of some intracellular organelles and structures (mitochondria or cytoskeleton) represent the third group of tests (2,13,25,26).

The special place in the studies focusing on apoptosis is reserved for assays exploring various genes with which it is assumed nowadays lies the ultimate control of cell suicide process (7,20). Unlike the above-mentioned approaches, the gene-aiming tests are neither rapid nor easy to carry out however specific and valuable they prove to be. This is

probably the main reason why these tests are used only when other approaches have already hinted at apoptosis in the studied model.

In our previous works we have shown that different xenobiotics induce apoptosis in Hep-2 cell line (6,19). Despite general similarities, time course and particular appearance of dying cells upon treatment with these xenobiotics varied, and that is why we wanted to verify whether the time aspect and usefulness of selected apoptotic markers will be similar in alternative experimental system – Bowes human melanoma cell line.

Materials and Methods

Chemicals

Potassium chromate was purchased from Sigma-Aldrich (Prague, Czech Republic), dissolved in a serum-free Dulbecco's modified Eagle's medium – DMEM, (Sevapharm, Prague, Czech Republic), sterilized by ultrafiltration and kept at low temperature as 1.5 mol/l stock solution. Prior each experiment, the stock solution was diluted with DMEM to the final concentration of 150 μg/ml.

Etoposide (Vepesid inj., Bristol-Myers Squibb, New York, U.S.A.) was diluted from the original ampoules supplied by manufacturer in a serum-free DMEM to the tested concentration of 10 μg/ml.

WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was purchased from Boehringer Mannheim-Roche (Basel, Switzerland), DAPI (4', 6-diamidino-2-phenylindole), Triton-X was obtained from Sigma-Aldrich (Prague, Czech Republic). Annexin V-FITC was purchased from Bender MedSystems Diagnostics GmbH (Vienna, Austria). Monospecific antiserum for the detection of activated caspase-3 was obtained from New England Biolabs, Inc. (New England Biolabs, Beverly, U.S.A.). Secondary antibodies were from Molecular Probes, Inc. (Molecular Probes, Eugene, U.S.A.) and from EXBIO (EXBIO- Institute of Molecular Genetics, Prague, Czech Republic). All other chemicals were from Sigma-Aldrich (Prague, Czech Republic).

Cell line

Bowes human melanoma cell line (ATCC, No. CRL – 9607, Manassas, United States) was grown as adherent cell culture in DMEM PAN (Aidenbach, Germany) supplemented with 10 % fetal bovine serum (Aidenbach, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in an incubator at 37 °C and 5 % CO_2 atmosphere and were passaged two times a week using 0.05 % trypsin/EDTA.

Cell proliferation

Bowes cells in 200 μl of DMEM PAN medium containing 10 % fetal bovine serum were seeded in four 96 well microtiter plates (Nunclon, Roskilde, Denmark) with the first column of wells without cells (blank). After incubation (24 hours at 37 °C and in 5 % CO_2), cells were treated with 10 μg/ml etoposide and 150 μg/ml potassium chromate for 4, 6, 8, 10, 12, 18, 24, 48, 72 and 96hours. After each time interval, 100 μl of WST-1 (Boehringer Mannheim-Roche, Cat. No. 1 644 807) was added. The cells were further incubated for 2 hours in an incubator. The absorbance was read at 450 nm with 650 nm of reference wavelength by a scanning multiwell spectrophotometer Titertek Multiscan MCC/340 (Huntsville, U.S.A.). All experiments were repeated at least three times.

Time- lapse video microscopy

Observation of Bowes cells dynamic morphology after treatment with 10 μg/ml etoposide and 150 μg/ml potassium chromate was carried out as described elsewhere (19). In brief, 24 hours old cell cultures were treated with the above-mentioned chemicals, and after 20 minutes of temperature adjustment in an incubator were transferred to a 37 °C heated chamber under inverted microscope Olympus IX-70 (Olympus Optical CO, Ltd., Tokyo, Japan) equipped with a long-working distance condenser, a 20 x phase contrast lens and Mitsubishi CCD-100E camera (Mitsubishi Corporation, Tokyo). The recording was done with Mitsubishi video recorder HS-S5600 (Mitsubishi Corporation, Tokyo) using recording mode 480 for up to 96 hours. The resulting records were digitalized by means of Adobe premiere 6.0 program and analyzed.

Labeling of externalized phosphatidylserine with Annexin V-FITC

Bowes cells in cytospin chambers (Hettich, Tuttlingen, Germany) were centrifuged for 5 minutes at 500 rpmi (JOUAN, Nantes, France) at room temperature (RT), the medium was carefully aspirated and the cells were rinsed with phosphate saline buffer (PBS). To each chamber 1 ml of Annexin-binding buffer with 20 μl of Annexin V-FITC (A) and 20 μl of propidium iodide (PI) was added, and chambers were left at dark for 15 min. After second centrifugation (conditions see above), labeling medium was aspirated and 1 ml of Annexin-binding buffer was added for 1 min. Following third centrifugation the buffer was aspirated, cytospin chambers were disassembled and the cells on a slide mounted into a special anti-bleaching medium and examined under fluorescence microscope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa, Japan) equipped with digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 450–490 nm (emission filter 520 nm). The results were quantified by cell counting in 100 visual fields. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed. All the experiments were done in triplicate.

Nuclear chromatin fragmentation staining

Bowes cells in modified cytospin chambers (Hettich, Tuttlingen, Germany) were centrifuged for 5 minutes at 500

rpmi (JOUAN, Nantes, France), washed with cold PBS and air-dried. The cells were then labeled with DAPI (5 min) and mounted for examination. Examination was done under fluorescence microscope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa, Japan) equipped with digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 330–380 nm and emission filter 420 nm. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed. The results were quantified by cell counting in 100 visual fields. Experiments were done in triplicate.

DNA fragmentation assay

Isolation of DNA and investigation of its appearance in agarose gel was described elsewhere (19).

Immunocytochemical detection of activated caspase-3

Bowes cells in cytospin chambers (Hettich, Tuttlingen) were centrifuged for 5 minutes at 500 rpmi (JOUAN, Nantes) at RT, the medium was carefully aspirated and the cells were fixed with 1 ml of 4% formaldehyde. Cytospin chambers were disassembled and the cells on a slide were air-dried. The slides with cells were rinsed three times with phosphate saline buffer with Triton X (PBS-T) and then treated to skimmed milk for 30 min at RT. Next, a primary antibody was added to the cells and the slides were left overnight in a cultivation chamber MIST (The Binding Site Ltd., Birmingham) at 4 °C. The slides were then rinsed three times with PBS-T buffer, a secondary antibody (anti-mouse conjugated with Alexa 488) was added, and the cells were incubated in the cultivation chamber MIST for 90 min at RT. The specimens were optionally post-labeled with DAPI, mounted into a special anti-bleaching medium and examined under a fluorescence microscope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa) equipped with the digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 330–380 nm and emission filter 420 nm. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed. In all immunofluorescence experiments (see below), the system of immunological control was employed to avoid false positive or negative staining reactions. All the experiments were done in triplicate.

Immunocytochemical staining of F-actin

Bowes cells were cytospined, fixed with 1 ml of 2% paraformaldehyde and permeabilized with 1 ml of 0.1% Triton X solution. The cells were then stained with FITC conjugated phalloidin at concentration of 10 μg/ml for 30 minutes. After staining, the specimens were rinsed three times with cold PBS, post-labeled with DAPI and mounted into a special antibleaching medium. The localization and status of F-actin cables were examined under fluorescence microscope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa, Japan) equipped with digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 450–490 nm and emission filter 520 nm. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed.

Statistics

Statistical analysis was carried out with a statistical program GraphPad Prism. We used one-way Anova test with posttests Dunnet's or Bonferroni. Results were compared with control samples, and means were considered significant if $P<0.01$.

Results

Cell proliferation and time-lapse videomicroscopy. The growth of Bowes human melanoma cell line as assessed by colorimetric WST-1–based assay after treatment with 10 μg/ml etoposide and 150 μg/ml potassium chromate is shown at Fig. 1 and Fig. 2, respectively. When compared with control cultures, etoposide slightly slowed proliferation of Bowes cells in the interval of 24 hours of treatment, with longer treatment intervals (up to 96 hours) being no different from controls. Potassium chromate provoked a very quick decrease in Bowes cell viability as soon as 4 hours after the beginning of treatment but this trend didn't continue in the following 8 hours. The next significant reduction in cell viability occurred in the time interval 12–24 hours after the beginning of treatment.

Digitalized time-lapse videosequences of Bowes cells treated with 10 μg/ml etoposide revealed that with exception of a short period (about 8 hours of the treatment), where blebbing developed in some cells (approximately 5–8 % of cells in the visual field), the morphological appearance and behavior of Bowes cells did not undergo major alterations; i.e. cells showed migratory and mitotic activity and no features of cell damage (vacuolization, shrinkage or ballooning) were observable (Fig. 3 and 4). In the chromate treated cells, there appeared the first significant changes such as cell shrinkage and blebbing approximately 6 hours after the beginning of the treatment (Fig. 5) followed in the later time periods (18 hours of the treatment) by cell fragmentation yielding numerous spherical bodies (Fig. 6). After 24 hours, all treated cells were dead, with many of them remaining clumped together.

Labelling of externalized phosphatidylserine with Annexin V-FITC. Annexin V-FITC labeling of the cells reveals very early apoptotic changes. Furthermore, in combination with PI labeling it serves to distinguish the early apoptotic phases from the late apoptotic phases based on differing penetration rate of individual dyes. In our experiments, the number of A positive cells along with the number of both A and PI positive cells were followed during selected time intervals in treated and control cultures.

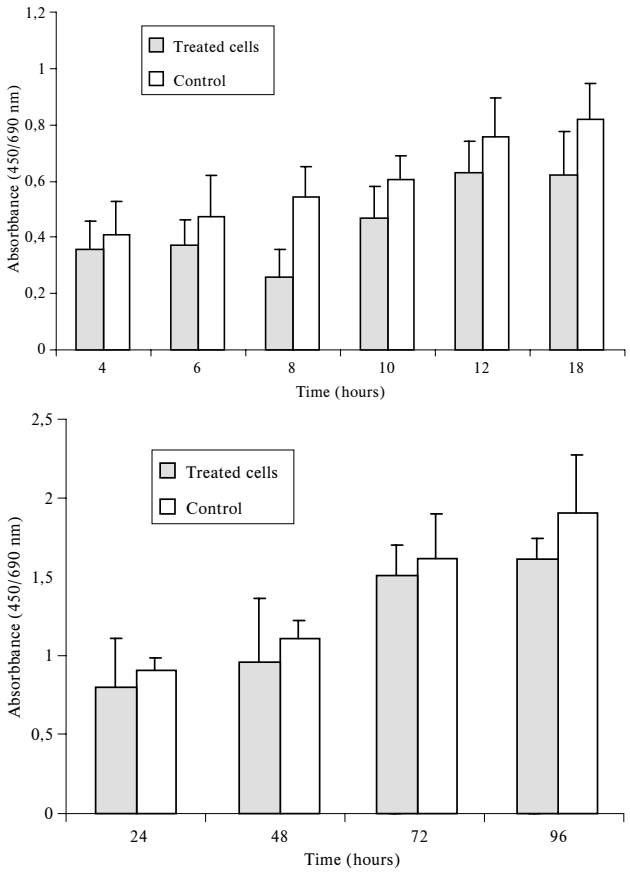

Fig. 1: Viability and proliferation of Bowes human melanoma cells exposed to 10 μg/ml etoposide during 96 hours as measured by colorimetric WST-1 assay. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

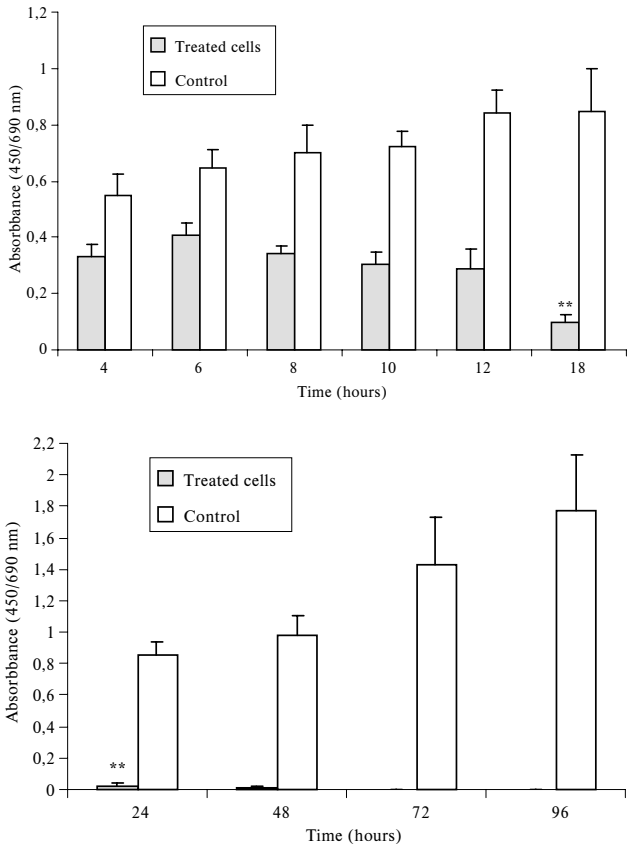

Fig. 2: Viability and proliferation of Bowes human melanoma cells exposed to 150 μg/m1 potassium chromate during 96 hours as measured by colorimetric WST-1 assay. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

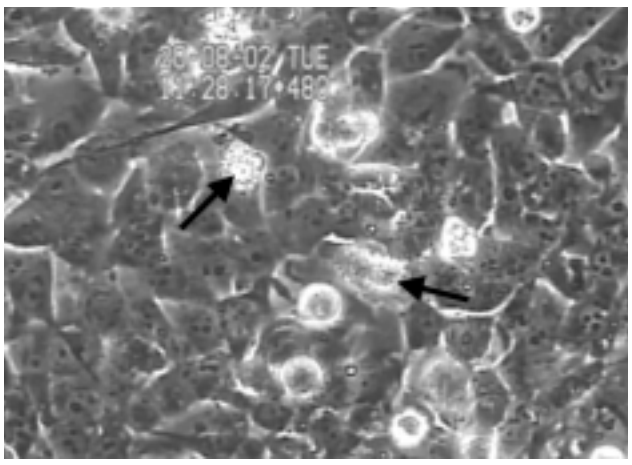

Fig. 3: Morphological appearance of Bowes human melanoma cells after treatment with 10 μg/ml etoposide during 8 hours. The arrows denote blebbing cells. The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

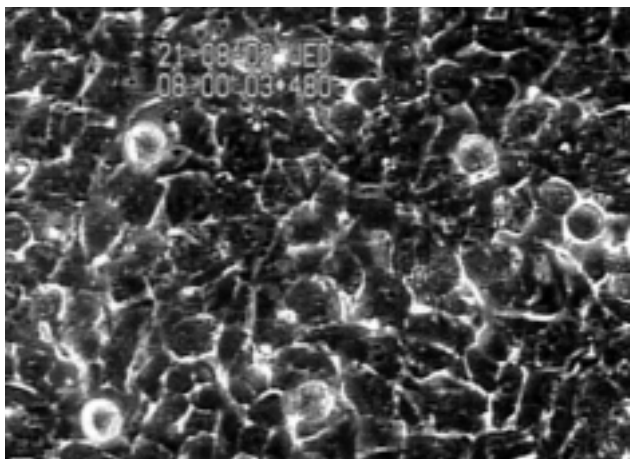

Fig. 4: Morphological appearance of Bowes human melanoma cells after treatment with 10 μg/ml etoposide during 24 hours. The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

In etoposide-treated cells, the maximum of A positive cells (15%) was observed at 96 hours of exposure. Between these intervals, a proportion of A-positivity oscillated, generally being no significant from control cultures (Fig. 7). A and PI positive cells were almost not present.

In chromate-treated cells, a significant proportion of A positive cells was detected at 8 hours of treatment (8 %), and the maximum (99 %) was reached at 24 hours of treatment as compared with control cells (Fig. 8). A and PI positive cells were found only at the very end of the treatment (data not shown).

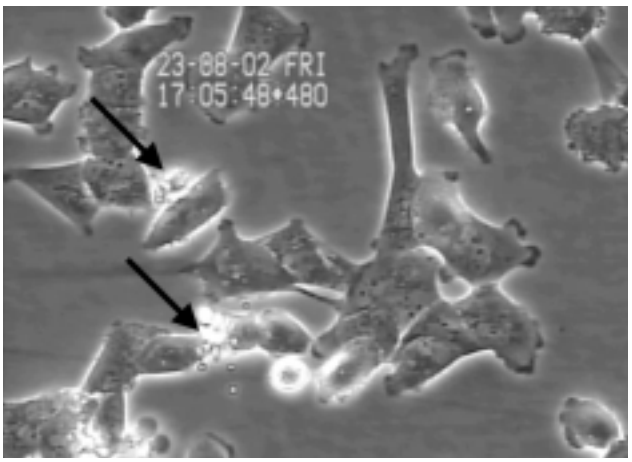

Fig. 5: Morphological appearance of Bowes human melanoma cells after treatment with 150 μg/ml potassium chromate during 6 hours. The arrows denote blebbing cells. The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

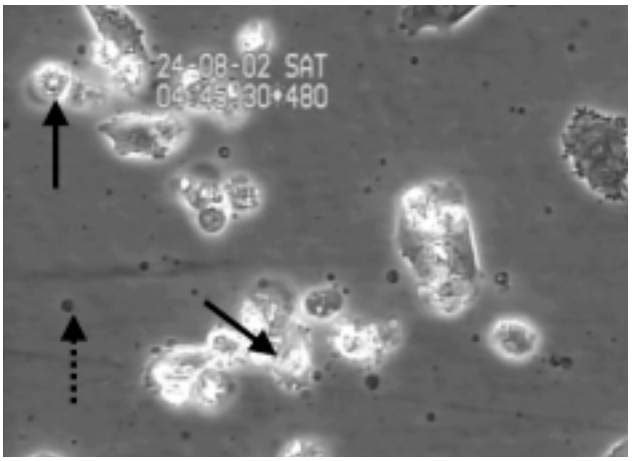

Fig. 6: Morphological appearance of Bowes human melanoma cells after treatment with 150 μg/ml potassium chromate during 18 hours. The arrows denote fragmenting cells. Visible are spherical structures originating from the fragmented cells (dashed arrow). The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

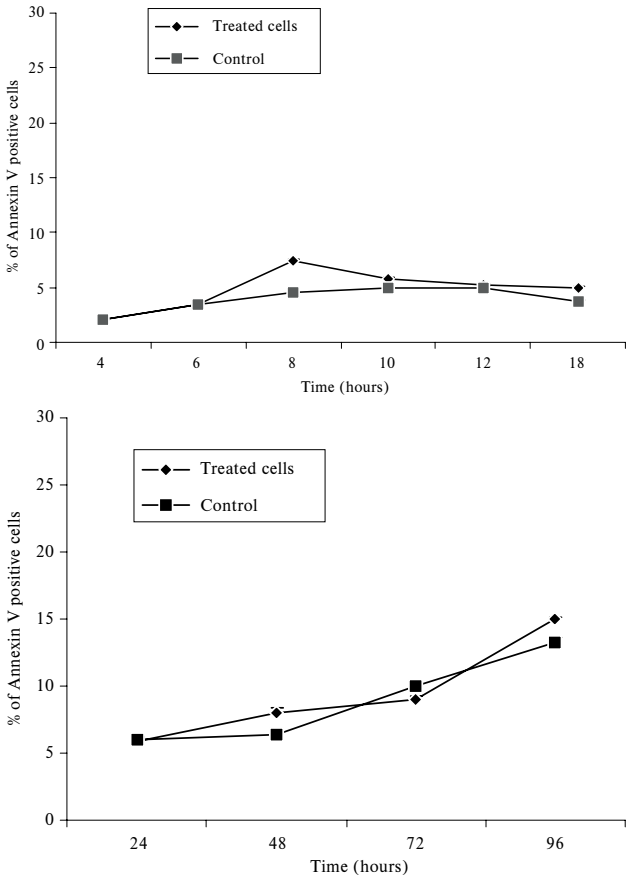

Fig. 7: Anexin V-FITC positivity of Bowes human melanoma cells exposed to 10 μg/ml etoposide during 96 hours. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

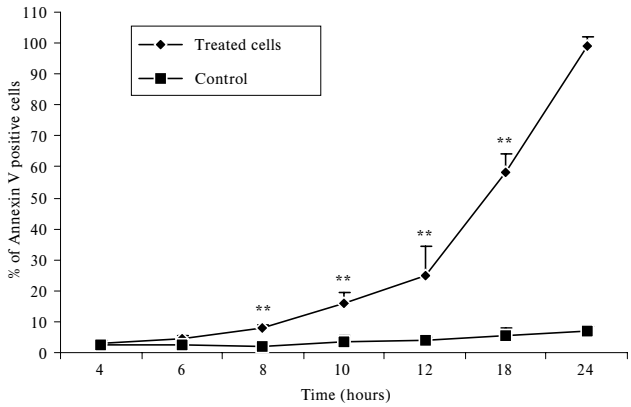

Fig. 8: Anexin V-FITC positivity of Bowes human melanoma cells exposed to 150 μg/ml potassium chromate during 24 hours. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

Nuclear fragmentation staining with DAPI. The distribution of the cells with fragmented nuclei during treatment with etoposide and potassium chromate is shown at Fig. 9 and 10. In etoposide treated cultures, very weak fragmentation was observed at 8 hours of treatment and next at 96 hours of treatment. In chromate treated cultures, the proportion of cells with marginalized and fragmented chromatin steadily raised to reach maximum at 24 hours.

Visualization of DNA fragmentation. The appearance of DNA of etoposide and chromate treated cells is shown at Fig. 11 and 12. At no probed treatment intervals specific internucleosomal DNA fragments were detectable.

Immunocytochemical detection of activated caspase-3. The conversion of inactive procaspase-3 into its active form (caspase-3) marks the beginning of the executory stage of apoptosis. The presence of caspase-3 in the cell is thus regarded as a point of no return in death cascade. We have detected caspase-3 positive cells by means of immunofluorescence staining.

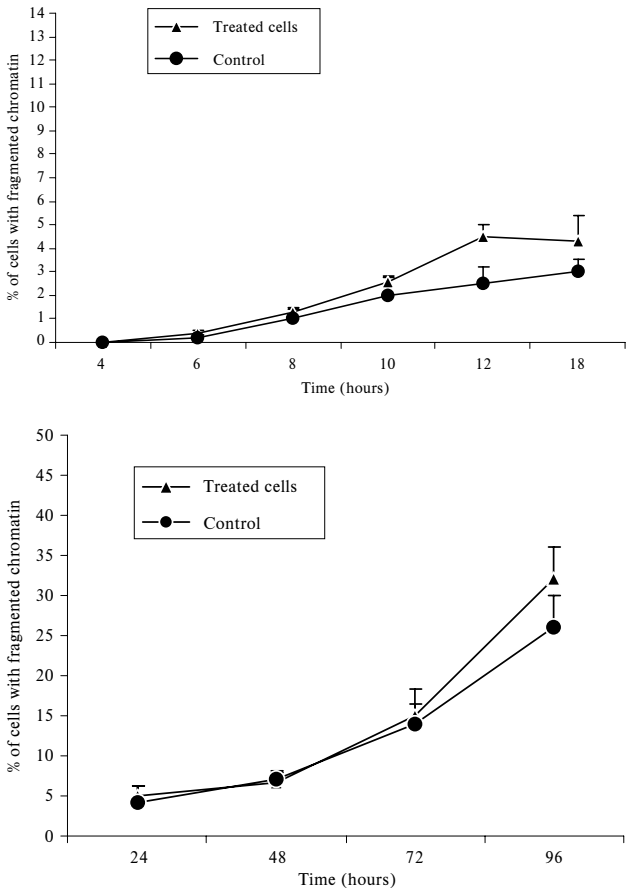

Fig. 9: Fragmentation of Bowes human melanoma cells nuclei exposed to 10 μg/ml etoposide during 96 hours assessed by nuclear specific dye DAPI. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

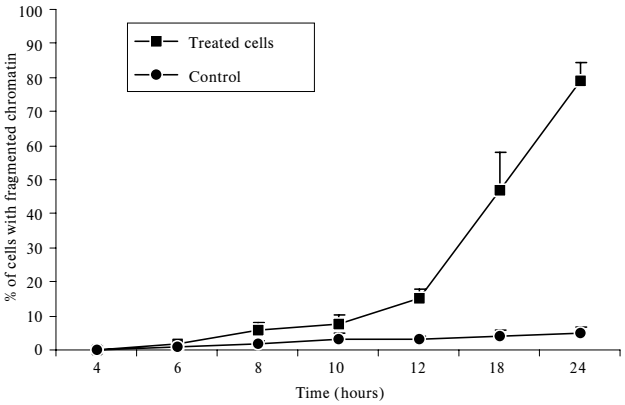

Fig. 10: Fragmentation of Bowes human melanoma cells nuclei exposed to 150 μg/ml potassium chromate during 24 hours assessed by nuclear specific dye DAPI. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

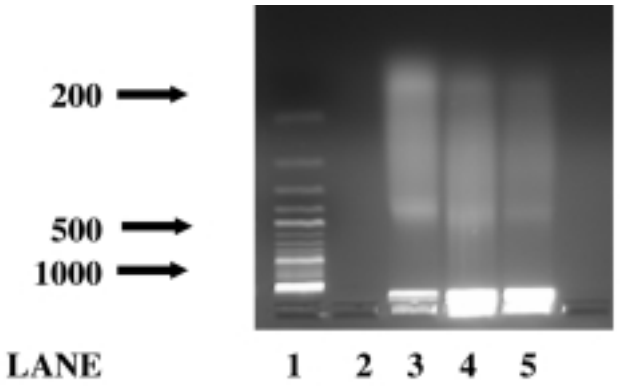

Fig. 11: The appearance of DNA from Bowes human melanoma cells treated with 10 μg/m1 etoposide at time intervals 8 (lane 3), 18 (lane 4), and 24 hours (lane 5). Size marker is shown in lane 1, negative control in lane 2.

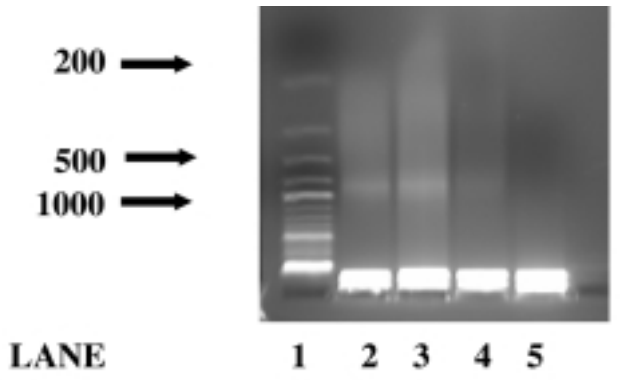

Fig. 12: The appearance of DNA from Bowes human melanoma cells treated with 150 μg/m1 potassium chromate at time intervals 8 (lane 2), 24 (lane 3), and 96 hours (lane 4). Size marker is shown in lane 1, negative control in lane 5.

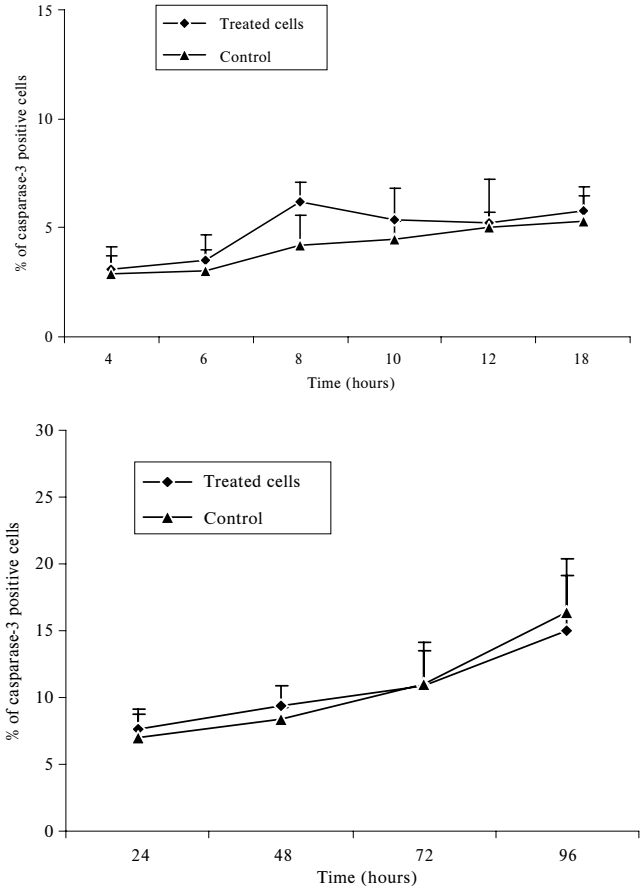

Fig. 13: Activation of caspase-3 in Bowes human melanoma cells exposed to 10 μg/ml etoposide during 96 hours as detected by immunofluorescence staining. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

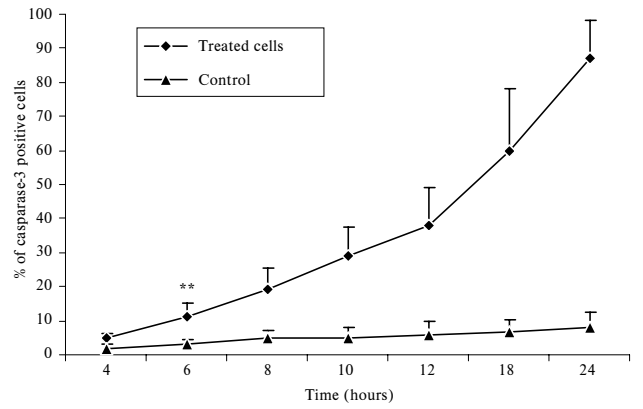

Fig. 14: Activation of caspase-3 in Bowes human melanoma cells exposed to 150 μg/ml potassium chromate during 24 hours as detected by immunofluorescence staining. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

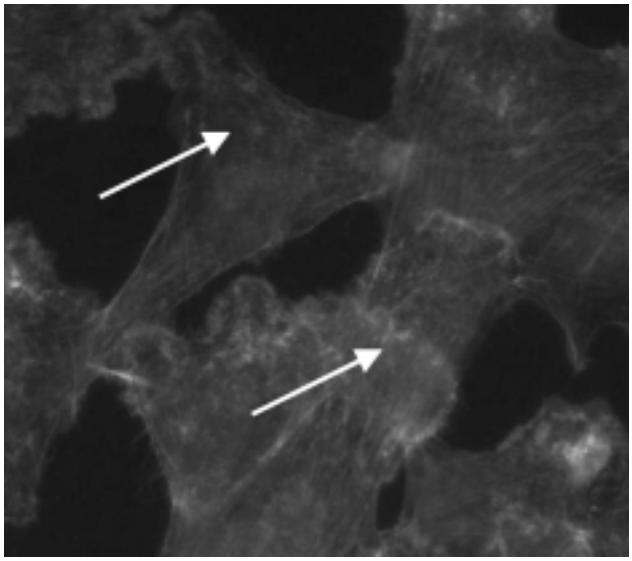

Fig. 15: Localization and arrangement of F-actin cables in adherent Bowes human melanoma cells. F-actin forms a continuous net spreading throughout the body of the cell (arrow), with its especial prominence in the ruffling edges (arrow). Immunofluorescence staining FITC-conjugated phalloidin. Nikon E 400, 400x.

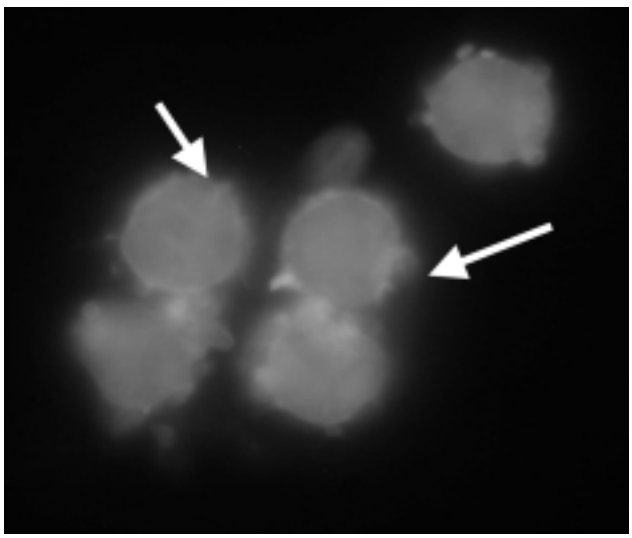

Fig. 16: Localization and arrangement of F-actin cables in apoptotic Bowes human melanoma cells after the treatment with 150 μg/ml potassium chromate for 8 hours. F-actin architecture shows different arrangement, with major localization points being blebs (arrow). Immunofluorescence staining FITC-conjugated phalloidin. Nikon E 400, 400x.

In etoposide-treated cells, the proportion of caspase-3 positive cells transiently increased at 8 hours of treatment but after this time interval again decreased to the level observed in control cells. Positively staining Bowes cells were again detectable in significant numbers at 96 hours of treatment (Fig. 13).

In case of chromate treatment, cells started to stain positively for caspase-3 as soon as 4 hours after the beginning of treatment, with significant increase after 6 hours of treatment. The proportion of positive cells steadily grew and reached its maximum at 24 hours of treatment (Fig. 14).

Immunocytochemical staining of F-actin. In adherent Bowes cells F-actin molecules localize to the cytoplasm and ruffling edges of the plasma membrane (Fig. 15). Treated cells regrouped F-actin cables and accumulated them in the blebbing plasma membrane (Fig. 16). There was no qualitative difference in distribution pattern of F-actin cables after treatment with both chemicals.

Discussion

The studies into appearance, dynamics and regulation of apoptosis induced *in vitro* have revealed many useful facts as well as peculiarities about this type of cell death and together with *in vivo* studies contributed to the better understanding of this phenomenon and its role in many pathological situations. Over past decades many research teams have been trying to find certain universal hallmarks of apoptosis which would be easily obtainable, reliable and unequivocally interpretable. Nowadays, after long and strenuous work, it is generally known that there are very few features that may be deemed universal to all apoptotic models (2). Thus in practice, it seems necessary to employ several methodological approaches to verify whether a particular study model we employ does not produce erroneous results in turn incorrectly interpreted. Several reviews and studies focusing on most commonly used techniques in detection of apoptosis came to the same conclusion (13,26). This conclusion appears to be of particular importance, especially when an *in vitro* model (cell line) which is resistant to standard apoptosis inducers is investigated.

In our experiment, we wanted to find out about dynamics of selected apoptotic markers and usefulness of their detection in Bowes human melanoma cells treated with established proapoptogenic compounds – etoposide and potassium chromate. The examined markers and intervals of their analyses were chosen to embrace the entire spectrum of possible apoptotic features in time with respect to the standardly used techniques.

Etoposide is a derivative of podophyllotoxin and topoisomerase II inhibitor (3,13). It introduces DNA strand breaks, and by interfering with expression of several apoptosis regulating genes triggers apoptotic pathway (7). Its efficiency has been documented in various cell lines and currently it is being used in treatment of hematological malignancies (13,21). The standard etoposide concentration of 10 μg/ml temporarily decreased Bowes cell viability and induced limited apoptosis during 8 hours of treatment (Fig. 1). These effects were of a transient nature as in the longer time intervals exposed cells grew and behaved normally (Fig. 1 and 4) as compared with control cultures. The individual assays revealed that blebbing and caspase-3 activation were detectable concurrently while there was no detectable chromatin and DNA fragmentation. These results support previous findings which proved that melanoma cells are often resistant to chemotherapy. This resistance has been attributed either to direct loss of the p53 protein, an important cell cycle regulator and apoptosis promoter, or to a mutation of the p53 effector Apaf-1 (24). Inhibition of topoisomerase II might therefore operate via this pathway and that would explain only a weak apoptotic response in our model. Moreover, when melanoma cells are treated with factors activating different signaling pathways such as interferon β or prostaglandin A1, growth inhibition and apoptosis are observed (9,18).

Considering the fact that etoposide functions only in S phase of the cell cycle, our observation of its maximal effect at 8 hours of treatment coincides with proliferative profile of Bowes melanoma cells (Fig. 1). On the other hand, this cytostatic effect did not continue in the later time intervals (24–96 hours) which would be partially explicable with the already mentioned p53–based resistance and cell line- specific growth properties and metabolism. The detected apoptotic markers at 96 hours of treatment (phosphatidylserine externalization) should be in this case attributed to natural apoptosis occurring in a culture after reaching confluency and depleting nutrition sources. Here it is interesting to note that changes in the plasma membrane composition were not detected prior to or simultaneously with other apoptotic markers at 8 hours of treatment where a number of cells started blebbing and expressed activated caspase-3 (Fig. 13). In this case it would actually mean that phosphatidylserine externalization was somehow suppressed or did not accompany the process of apoptosis which casts a suspicion on the universality of this hallmark.

Potassium chromate at concentration of 150 μg/ml is a representative of hexavalent chromium compound that has been identified as a potent apoptosis instigator activating several different signaling apoptosis pathways (22). In melanoma Bowes cells, it produced a set of morphological, biochemical and molecular changes which were detectable as soon as 4 hours after the beginning of treatment (Fig. 5), and culminating in time interval between 12–18 hours (Fig. 6). Morphologically, cells displayed all the known characteristics (blebbing, shrinkage, fragmentation) with exception of preceding nucleolar brightening suggesting that transcription process was not probably altered prior to other changes. Curiously, phosphatidylserine externalization was not significantly increased in treated cells until at approximately 8 hours of treatment when marked caspase-3 activation had already been present. This finding seemingly contradicts other authors who claim that changes in the

plasma membrane structure and molecular composition as revealed by Annexin V based staining belong to the first harbingers of programmed cell death (2,10,16). Still, the whole thing is probably more complex since there are reports evidencing phosphatidylserine externalization as a change accompanying other processes than apoptosis (17).

Despite the clear presence of activated caspase-3 and fragmented nucleus along with F-actin filaments rearrangement (Fig. 10 and 16) in dying cells we were not able to detect the specific DNA laddering after treatment with both etoposide as well as potassium chromate (Fig. 11 and 12). The absence of DNA fragments may be explained by several factors such as inactivity or absence of DNAases, high intracellular zinc content preventing fragmentation or transient nature of the process itself (5). Furthermore, some cell lines have been documented as being devoid of DNA fragmentation as well as other characteristic features upon undergoing apoptosis (4,11,14) and this may well be the case of Bowes human melanoma cells.

Conclusions

Bowes human melanoma cells represent a useful model for studying programmed cell death and its modulation *in vitro* with respect to clinical importance of malignant melanoma.

Etoposide at concentration of 10 μg/ml slowed proliferation and induced only transient and limited changes in this cell line. These changes appeared at 8 hours of treatment and may be characterized by simultaneous plasma membrane blebbing and caspase-3 activation, with longer treatment intervals (up to 96 hours) producing no observable or detectable changes. The observed resistance of Bowes human melanoma cell line to etoposide may be due to an alteration of the p53-based apoptosis pathway in these cells.

Potassium chromate at concentration of 150 μg/ml induced apoptosis in Bowes human melanoma cells. Analyses of the appearance of individual apoptotic markers proved that Annexin V based detection of phosphatidylserine externalization was delayed in comparison with caspase-3 activation or other morphological changes affecting the plasma membrane, cytoplasm and cytoskeleton. Despite the fact that apoptotic nuclei underwent characteristic degradation, no DNA specific fragmentation was evidenced at any examined treatment intervals.

Our results suggest that in a given model as well as in any other studies where apoptosis is to be detected, a combination of tests must be applied to rule out the possibility of misleading results. Out of utilized markers, the least reliable proved to be detection of externalized phosphatidylserin and DNA fragmentation which are, in our opinion, very liable to deviations. On the other hand, caspase-3 activation and nuclear degradation tested very consistently in this as well as other experimental models. Also, we recommend that whenever it is possible one should use conventional morphological methods based on microscopy (for instance time-lapse videomicroscopy) to have a feedback visual control of the studied model.

Acknowledgement

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ORIGINAL ARTICLE

ANTIACETYLCHOLINESTERASE ACTIVITY OF CYCLOSPORINE – A COMPARISON OF SINGLE AND REPEATED ADMINISTRATION AND EFFECT OF 7-METHOXYTACRINE

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Purkyně Military Medical Academy in Hradec Králové: Department of Toxicology,

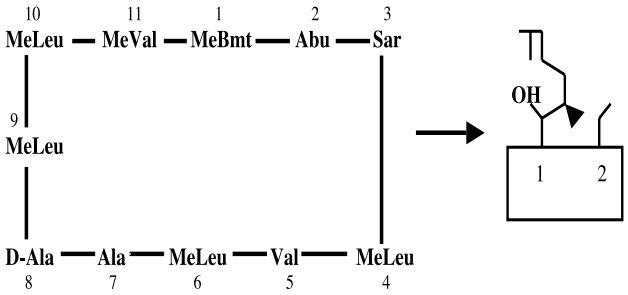
Summary: The aim of this work is a comparison of single and repeated peroral administration of cyclosporine (CsA) and the interaction of repeated administration of CsA and 7-methoxytacrine (MEOTA) on the activity of acetylcholinesterase (AChE) in the frontal cortex, hippocampus, septum, and basal ganglia in rats. Both single and repeated administration of CsA diminished the activity of AChE in the frontal cortex, septum and basal ganglia, while the enzyme activity in the hippocampus was diminished only in the case of repeated CsA, as well as repeated CsA + MEOTA administration. Repeated administration of CsA led to a further augmentation of anticholinesterase activity only in the frontal cortex and – in a lesser extent – in the basal ganglia. No augmentation of AChE activity was observed in the hippocampus and septum.

Key words: *Acetylcholinesterase; Brain parts; Cyclosporine; 7-Methoxytacrine; Rat*

Introduction

Cyclosporine (cyclosporine A, CsA) is a monopolar, cyclic polypeptide consisting of 11 amino acids (Fig.1). CsA mainly exhibits immunosuppressive activity. Moreover, it was experimentally as well as clinically demonstrated that CsA acts not only as an immunosuppressive agent but also as a drug which has beneficial effects on cells subjected to a variety of injurious conditions and was used as a useful agent for reducing cell damage (6, 14, 19). CsA, e.g., has been found to protect against dopaminergic depletion and to reduce cell death in animals models of stroke, cardiac arrest-induced and traumatic brain injury (12, 14). A membrane fluidizing effect of CsA enables to speculate on possibly increased penetration of some compounds through the cell membranes and organ barriers due to CsA (7). Indeed, CsA has been found to increase vincristine and vinblastine transport across the endothelial cell monolayer (3).

L-carnitine, a natural component of the mammalian tissue, is also capable to increase penetration of some chemical groups or drugs through biological barriers. We previously demostrated an augmenting effect of repeated administration of L-carnitine on the anticholinesterase activity of 7-methoxytacrine (MEOTA) namely in the frontal cortex and septum (9). We therefore attempted to establish the ability of CsA to influence the antiacetylcholinesterase activity of 7-methoxytacrine (7-MEOTA).



MeBmt – 4-[(E)-2-butenyl]-4,4N-trimethyl-L-threonin; Abu – L-aminomáselná kyselina; Sar – sarkosin; MeLeu – N-methylleucin; Val – L-valin; Ala – L-alanin; D-Ala – D-alanin; MeVal – N-methylvalin

Fig. l: Schematic representation of CsA molecule.

Material and Method

Male Wistar albino rats, weighing 200–230 g, were purchased from Biotest Ltd., Konárovice, Czech Republic. Animals were maintained in a air-conditioned room (22±1 °C and 50±10 % of relative humidity) with 12/12 day/night standard conditions and free access to standard chow and water. The directions of the Council of the European Communities (86/609/EEC) on animal care have been duly maintained. Handling of experimental animals was performed under the supervision of the local Ethical Committee.

Animals were divided into 4 groups with 6 in each. The study design was as follows:

1. Control group was administered only saline in a single dose in an amount of 0.1ml/100g p.o. and removal of the brain after 30 min.
2. Control group was administered of MEOTA in a single dose of 100 mg/kg i.m., removal of the brain after 30 min.
3. Administration of CsA in a single dose of 45 mg/kg p.o., removal of the brain after 30 min.
4. Repeated administration of CsA in three consecutive doses of 45 mg/kg p.o. separated by 24 hour intervals, removal of the brain 30 min after the last administration of the drug tested.
5. Repeated administration of CsA in three consecutive doses of 45 mg/kg p.o. separated by 24 hour intervals, application of MEOTA in a dose of 100 mg/kg i.m. after last CsA administration, removal of the brain after 30 min.

CsA was dissolved in olive oil, MEOTA was dissolved in saline, all doses tested were applied in an amount of 0.1 ml/100 g.

Animals were killed by decapitation and the following parts were prepared according to a previously described procedure: the frontal cortex, hippocampus, medial septum, and basal ganglia, respectively.

Acetylcholinesterase (AChE) activity in the homogenates (1:10) of the selected brain parts was determined using the method of Ellman el al. (4)as described elsewhere (1). Acetylthiocholine was used as the substrate and the results obtained were expressed as the number of nanomoles of the substrate hydrolyzed/min/100 mg wet weight tissue at 22 °C.

Acetylthiocholine was obtained from Lachema, Brno (Czech Republic). CsA was purchased from Sigma-Aldrich, St. Louis (USA).

Statistical significance was determined with the use of Student's test for independent samples and differences were considered significant when $p < 0.05$. Statistical analyses were performed on a PC using the programme Statistica 98 edition.

Results

CsA generally exerts inhibitory effect on the AChE activity in the brain parts chosen in comparison with the control (i.e., the „saline") group 1. Moreover, no differences between the single and repeated administration were observed in the frontal cortex and septum, while the enzyme activity in the hippocampus was diminished only in the case of repeated (group 4) and combined CsA + MEOTA (group 5) administration. On the contrary, the single administration of CsA was more efficacious in comparison with repeated and combined ones in the basal ganglia, irrespective of statistical signifigance of groups 3–5 with control group 1 (Fig. 2).

A further augmentation of inhibitory activity of MEOTA following repeated administration of CsA was observed in the frontal cortex (see statistical significance between control „MEOTA" group 2 and CsA + MEOTA group 5 in Fig. 3). Lesser extent of this augmentation (compare „MEOTA" group 2 vs. group 4 and „MEOTA" group vs. group 5) was observed in the basal ganglia. No augmentation was observed in the hippocampus and septum.

Discussion

Besides well-known side effects of CsA as, e.g., nephrotoxicity and hepatotoxicity, numerous side effects of central origin are observed (11, 14, 20). They include almost all categories, i.e., motoric, sensitive, affective, and cognitive functions. Some of them, such as confusion, seizure, spasticity, paresis or ataxia, could be attributed to acetylcholine system dysbalance. CsA exerts a protective effect on the density of muscarinic acetylcholine receptors following experimental brain ischemia (13) and also enhances a spontaneous acetylcholine release after a brief tetanus (16). Gaudry-Talarmain and Moulian (8) demonstrated an invol-

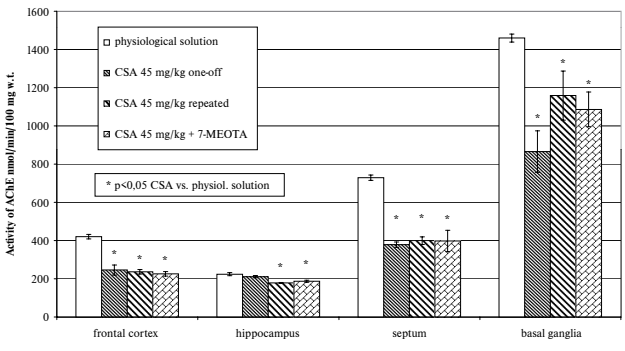


Fig. 2: Effect of CsA on AChE activity in the selected parts of the rat brain. (More detail description of control group 1 and experimental groups 3–5 see in text).

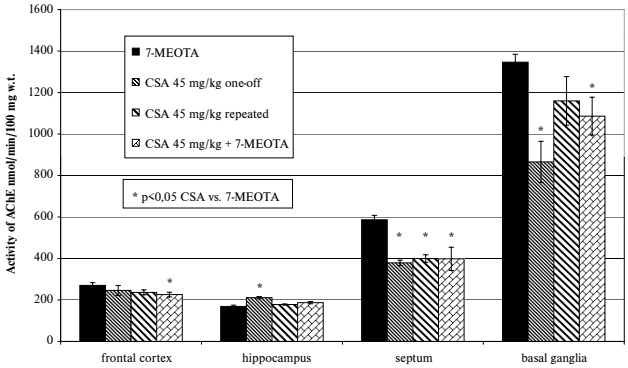


Fig. 3: A comparison of anticholinesterase activity of MEOTA and CsA. (More detail description of control group 2 and experimental group 3–5 see in text).

vement of CsA in the presynaptic mechanism of acetylcholine release in Torpedo synaptosomes. A prolongation of succinylcholine-induced neuromuscular blockade following CsA was also demonstrated (10). Famiglio et al. (5) observed epileptiform electroencephalographic (EEG) activity following CsA administered intraperitoneally in rats. Behavioural changes observed during abnormal EEG pattern were obviously subtle, animals were often still or slightly rocking. However, possible involvement of cholinergic mechanism in these findings is only a subject of speculation.

Borlongan et al. (2) proved the most relevant evidence concerning the interaction of CsA and the central cholinergic transmission in the brain. They described an enhancement of the septal choline acetyltransferase immunoreactivity in Wistar rats. The involvement of the septal region in the central effect of CsA is in good agreement with our observation.

We demostrated at least the same (in case of the frontal cortex and septum), or even higher (in case of the basal ganglia) inhibition efficacy of CsA in comparison with MEOTA. Moreover, the dose of MEOTA used in our experiments is relatively high, it corresponds to LD_{20} (1). The enhancement of the anticholinesterase activity of MEOTA following repeated administration of L-carnitine has been previously demonstrated. L-carnitine augmented this activity in the frontal cortex and septum, and – in case of higher doses used – also in the basal ganglia (9). On the contrary, the extent of the augmentation of anticholinesterase activity of MEOTA following CsA was less pronounced. It was apparent only in the frontal cortex, and – under some circumstances – in the basal ganglia. This finding confirms previously published results supporting the higher sensitivity of AChE in the frontal cortex to the effect of tacrine and its derivatives (9). However, the above mentioned differences between L-carnitine and CsA in relation to the anticholinesterase activity of MEOTA suggest different mechanisms of this interaction.

According to the most accepted opinion CsA acts through binding with the immunophilin cyclophilin, the complex CsA/cyclophilin inhibits calcineurin (16, 17). Calcineurin has been shown to be localized throughout the brain including septum and hippocampus and it is important for nitric oxide (NO) metabolism and nuclear import of transcription factors (2, 16). There is also evidence about the involvement of calcineurin in the synthesis of AChE (18). The possible link CsA – calcineurin – synthesis of AChE claims to explain the above described changes in the AChE activity after CsA.

Ackowledgement

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ORIGINAL ARTICLE

THE IMPAIRMENT OF SPATIAL MEMORY FOLLOWING LOW-LEVEL SARIN INHALATION EXPOSURE AND ANTIDOTAL TREATMENT IN RATS

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Summary: 1. To study the influence of antidotes on low-level sarin-induced impairment of cognitive functions, the rats were exposed to three various low concentrations of sarin (LEVEL 1–3) for 60 minutes in the inhalation chamber. In addition, one group of rats was exposed to LEVEL 2 of sarin repeatedly. 2. Testing of cognitive functions was carried out using the Y-maze evaluating learning and spatial memory. The correct averse behavior of sarin-exposed rats in the Y-maze was tested several times within four weeks following sarin inhalation exposure and antidotal treatment to look for any cognitive impairments. 3. The results were compared to the Y-maze performance of sarin-exposed rats without antidotal treatment and control rats exposed to pure air instead of sarin with or without antidotal treatment. While antidotal treatment was able to eliminate a short-term deficiency in the Y-maze performance in rats exposed to the LEVEL 1 of sarin, a significant decrease in the Y-maze performance in rats exposed to sarin at the LEVEL 2 and 3 was only shortened. Sarin-induced spatial memory impairments in rats exposed repeatedly to sarin at the LEVEL 2 was also shortened when rats were treated following each sarin inhalation exposure. 4. The findings confirm that antidotes currently used for nerve agent poisonings are beneficial for the treatment of rats singly or repeatedly exposed to non-convulsive symptomatic or even clinically asymptomatic concentrations of sarin.

Key words: *Sarin; Low-level inhalation exposure; Spatial memory; Rat; Antidotal treatment*

Introduction

The potential for the exposure to highly toxic organophosphorus compounds (OPs), called nerve agents, exists on the battlefield (e.g. Iran-Iraq war) as well as in a civilian sector as a threat by a terrorist group (e.g. Tokyo subway incident – 16) or as an accident as part of current demilitarization efforts. The irreversible binding to and subsequent inactivation of acetylcholinesterase (AChE, EC 3.1.1.7) in the central as well as peripheral nervous system allowing accumulation of acetycholine (ACh) and excessive stimulation of postsynaptic cholinergic receptors is generally believed to be the major mechanism of poisoning. The overstimulation of central cholinergic system is followed by the activation of other neurotransmitter systems including glutamate receptors leading to the increase in extracellular levels of the excitatory amino acid glutamate, a major excitotoxin mediating central neurotoxicity of OPs (14,24). Signs of acute toxicity with extensive AChE inhibition also include autonomic dysfunction (e.g. excessive salivation, lacrimation, urination and defecation), involuntary movements (e.g. tremor, fasciculation) and respiratory dysfunction (12,26). Acute exposure to nerve agents also produces psychiatric symptomatology that includes depression, antisocial thoughts, irrationality, emotional lability and anxiety (23).

OP-induced cholinergic effects are usually manifested immediately following high-level exposure (12,26), nevertheless, there are numerous studies in both humans and animals showing that survivors of high-level OP exposure can experience subtle but significant long-term neurological and neuropsychological outcomes that are detectable months or even years following the recovery from acute poisoning (3). In addition, behavioral alterations and impairments of cognitive functions were found following acute exposure to OPs in the absence of any classic signs of cholinergic toxicity (10,21).

The current standard antidotal treatment for poisoning with OPs usually consists of combined administration of anticholinergic drugs (preferably atropine) and oximes (preferably pralidoxime, obidoxime and HI-6). Anticholinergic drugs block effects of overstimulation by accumulated ACh at muscarinic receptors sites while oximes repair biochemical lesions by dephosphorylating the enzyme molecule and restoring activity (7,12). Nevertheless, very little is known about possible beneficial effects of antidotal treatment in the case of exposure to low-level, clinically asymptomatic concentrations of OPs. The purpose of

this study is to find out whether standard antidotal treatment of poisonings with OPs is able to influence low-level sarin-induced long-term adverse effects on cognitive functions, especially spatial memory, in rats.

Methods

Male albino Wistar rats weighing 170–190 g were purchased from VÚFB Konárovice. They kept in an air-conditioned room with light from 07:00 to 19:00 h at room temperature in cages (370x570x200 mm, 10 animals/cage) and allowed to access to standard food and tap water ad libitum. The rats were divided in eight groups of ten experimental animals per group (four group were treated with antidotes following sarin inhalation exposure, other four groups were treated with saline instead of antidotes following sarin inhalation exposure) and two control groups (N = 10) exposed to pure air instead of sarin with or without antidotal treatment. Handling of the experimental animals was done under supervision of the Ethics Committee of the Medical Faculty of Charles University and Purkyně Military Medical Academy.

Sarin was obtained from Zemianské Kostolany (Slovak Republic) and was 98.5 % pure. The oxime HI-6 of 98 % purity was synthesized at the Department of Toxicology of the Military Medical Academy, Hradec Králové. The purity was analysed using a HPLC technique. All other chemicals of analytical grade were obtained commercially and used without further purification.

The experimental rats were exposed to various low concentrations of sarin for 60 minutes in the inhalation chamber while control rats were exposed to pure air for 60 minutes in the same inhalation chamber. The chamber is a box (300x400x250 mm) made from enamel metal with sarin applicator where the rats are placed in the cage (185x310x65 mm, 3 rats/cage). Three concentration of sarin were chosen:

- clinically and laboratory asymptomatic concentration (0.8 μg/L) with a non-significant inhibition of erythrocyte AChE by 10% – LEVEL 1
- clinically asymptomatic concentration with a significant inhibition of erythrocyte AChE by 30% (1.25 μg/L) – LEVEL 2. This concentration was used for a single (LEVEL 2) or repeated (three times, each other day) exposure (LEVEL 2R). After the third exposure, a significant inhibition of erythrocyte AChE by 48 % was measured.
- non-convulsive symptomatic concentration with a significant inhibition of erythocyte AChE by 50% (2.5 μg/L) – LEVEL 3

The erythocyte AChE activity of sarin-exposed rats was measured in another experiment immediately following the inhalation exposure by the same laboratory using Ellman spectrophotometric method (9). The development of sarin-induced AChE inhibition during the testing of cognitive functions was not followed. In the case of 60 minute inhalation exposure in the same experimental conditions, the lethal concentration of sarin for rats was 4.48 μg/L and the convulsive concentration of sarin for rats was 3.25 μg/L.

One minute following the end of inhalation exposure, four groups of experimental rats and one group of control rats were treated intramuscularly (i.m.) with atropine and the oxime HI-6 at human relevant doses corresponding to 2 % of their LD_{50} value. In the case of repeated sarin exposure, the rats were treated with atropine and HI-6 following each sarin inhalation exposure. Other four group of experimental rats and one group of control rats were treated with saline instead of antidotes.

Cognitive functioning was tested using a Y-maze with averse motivation by a strong electric footshock, evaluating learning and spatial memory (11). The Y-maze is a fully automated apparatus used for the study of behavior of laboratory rats. It is a plastic box consisting of a square start area (285x480 mm) separated by a plexiglass sliding door from two trapezoid, black and white arms – choice area (140x324 mm). The grid-floor in the start and choice area is electrifiable. The animals are placed on the start area and after a couple of seconds, electric footshocks (60 V, 50 Hz, duration 0.5s) are applicated at 5 second intervals. The rats try to avoid the shock by escaping to one of two arms. In the case of moving of a rat to wrong (dark) arm, the rats fail to avoid further footshock. The animals were taught spatial discrimination with the preference of black or white arm in the Y-maze. The latency to enter the correct arm was measured and the number of wrong entries was counted. Before inhalation exposure to sarin, the rats were trained to avoid footshock by moving to correct (white) arm in the Y-maze. It usually tooks 4 weeks of training to reach the criterion which was 80% or more correct averse behavior (moving to the correct arm) within less than 1.5 second. During the training, 10 sessions (two trials/session) per week lasting 4 minutes were realized. The exposure started the day after the animals had reached this criterion. The spatial memory was tested 1 hour, 2 hours, 1 day and 1 week following the sarin inhalation exposure and then, once a week till the end of the fifth week following the exposure. The latency time to enter the correct arm by sarin-exposed treated rats and the number of entry errors were compared to the values obtained from sarin-exposed non-treated rats and the control rats exposed to the pure air instead of sarin.

Analysis of variance (ANOVA) with Bonfferoni's corrections for multiple comparisons was used for the determination of significant differences between experimental and control values (1). The differences were considered significant when $P < 0.05$.

Results

The results of the influence of various sarin concentrations on the Y-maze performance of rats following the single inhalation exposure and the influence of antidotal treatment with atropine and the oxime HI-6 on low level sa-

rin-induced impairment of spatial memory are shown in Figure 1–3. While treated as well as non-treated control rats did not show any changes in the rapidity of spatial discrimination in Y-maze following their exposure to the pure air in comparison with the values obtained before the exposure, a spatial memory of rats exposed to LEVEL 1 of sarin was significantly influenced for a short time ($P < 0.05$). On the other hand, experimental rats exposed to LEVEL 1 of sarin, treated with atropine and the oxime HI-6, did not show any significant increase in the reaction time following the exposure and antidotal treatment (Fig. 1).

The significant increase in the reaction time of rats exposed to LEVEL 2 of sarin was also demonstrated for a short time ($P < 0.05$). The antidotal treatment was only able to shorten, not eliminate a significant decrease in Y-maze performance of rats exposed to LEVEL 2 of sarin (Fig. 2).

The rats exposed to LEVEL 3 of sarin showed a significant decrease in Y-maze performance for a relatively long time (until the third week following the exposure) ($P < 0.05$). When the experimental rats were treated with atropine and the oxime HI-6, the sarin-induced impairments of spatial memory was markedly shortened (until the second hour following the exposure and treatment) (Fig. 3). In contrast to the reaction time, the number of entry errors of experimental rats was not different from the number of entry errors of control rats exposed to pure air, regardless of the sarin concentration used for the inhalation exposure of experimental rats.

The results of Y-maze performance of rats repeatedly exposed to LEVEL 2 of sarin compared to control values are given in Figure 4. While the first exposure to LEVEL 2 brought only short-time increase in the reaction time of sarin-exposed rats, the second and the third exposure to the same sarin concentration caused a significantly higher and longer spatial memory impairments compared to rats singly exposed to LEVEL 2 of sarin. The decrease in Y-maze performance of repeatedly exposed rats lasted until the end of

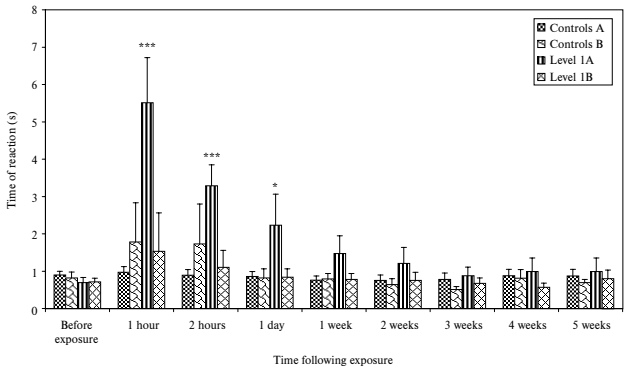

Fig. 1: The alteration of the Y-maze performance in rats singly exposed to LEVEL 1 of sarin and treated with atropine and the oxime HI-6, where A – without antidotal treatment, B – with antidotal treatment. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

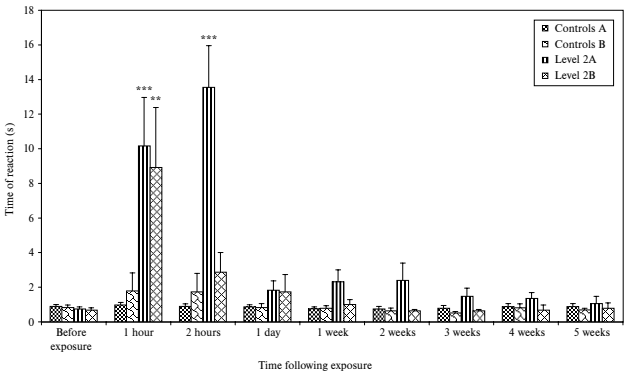

Fig. 2: The alteration of the Y-maze performance in rats singly exposed to LEVEL 2 of sarin and treated with atropine and the oxime HI-6. Symbols and statistical significance – see Fig. 1.

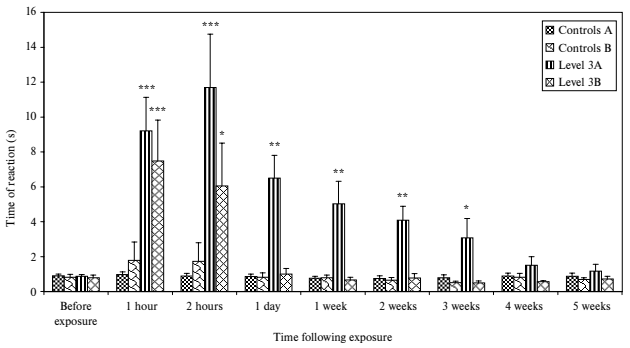

Fig. 3: The alteration of the Y-maze performance in rats singly exposed to LEVEL 3 of sarin and treated with atropine and the oxime HI-6. Symbols and statistical significance – see Fig. 1.

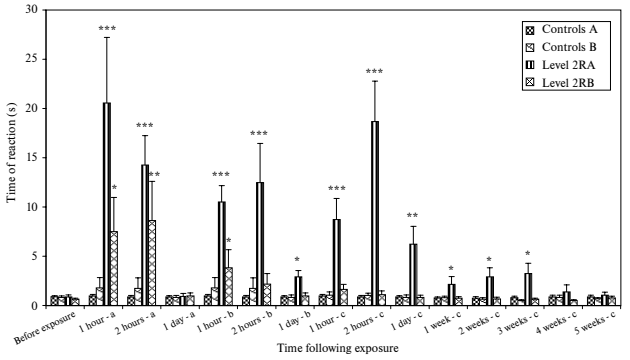

Fig. 4: The alteration of the Y-maze performance in rats repeatedly exposed (a – the first exposure, b – the second exposure, c – the third exposure) to LEVEL 2 of sarin and treated with atropine and the oxime HI-6. Symbols and statistical significance – see Fig. 1.

the third week following the last exposure to sarin. When sarin-exposed rats were treated with antidotes following each sarin exposure, the first and second exposure to LEVEL 2 only brought short-term increase in the reaction time of sarin-exposed rats ($P < 0.05$) and the third exposure to the same sarin concentration did not cause any spatial memory impairment (Fig. 4). The number of entry errors of rats repeatedly exposed to LEVEL 2 of sarin did not differ from control values.

Discussion

Acetylcholine is important in the modulation of various brain functions, including learning and memory, attention, arousal and the control of locomotor activity (2,8). Therefore, a shift in the cholinergic activity produced by OPs leads to functional changes in humans and animals, which may be observed as cognitive dysfunction (6,25). Impairment of cognitive functions, especially incapacitation of learning and memory, belongs to the most frequent central signs of acute OP poisoning (12,13). In addition, the adverse effects of OP compounds on cognition functions, such as learning and memory, may persist for quite some time after termination of toxicant exposure. The results from several studies have demonstrated the presence of OP-induced learning impairments several days after the behavioral signs of OP toxicity have subsided (4,5,13). The chronic exposure to OP agents can also result in specific long-term cognitive deficits even when signs and symptoms of excessive cholinergic activity are not present (18,19). Epidemiological studies showed alterations in cognitive functions, impaired memory and concentrations in humans after chronic low-dosage occupational exposure to organophosphorus insecticides (17,25). In addition, a behavioral alteration indicative of an increase in anxiety and impairment of cognitive functions, especially spatial memory, in the absence of any classic signs of overstimulation of cholinergic nervous system has been demonstrated after acute low-dosage exposure of experimental animals to OPs (10,21). Thus, the significant, clinically manifested AChE inhibition in the central nervous system leading to the neuronal degeneration of some brain regions including hippocampus, associated with the spatial learning and memory, is not necessary for the clinically manifested cognitive impairments. These findings correspond with earlier published data about neurological and neurophysiological outcomes detectable months or even years following recovery from acute OP poisoning (22, 28).

Our data clearly demonstrate that low-level sarin-induced decrease in Y-maze performance of rats is significantly influenced by the administration of standard antidotal treatment consisting of atropine and the oxime HI-6 shortly following sarin inhalation exposure. The administration of antidotes significantly shortened low-level sarin-induced impairment of spatial memory but they were not able to eliminate the increase in the reaction time in Y-maze. While non-treated rats singly exposed to sarin at LEVEL 3 or repeatedly exposed to sarin et LEVEL 2 showed long-term impairment of spatial memory lasting till the end of the third week following the inhalation exposure, rats treated with atropine and HI-6 suffered from sarin-induced impairment of cognitive functions very shortly (till 2 hours following the exposure) regardless of the concentration of sarin or number of sarin exposures.

It is rather difficult to explain the therapeutic effects of standard antidotes if they are administered following the exposure to OPs at levels that do not cause clinical signs of overstimulation of postsynaptic cholinergic receptors because their efficacy consists in blocking the effect of accumulated ACh at muscarinic receptor sites (atropine) and reactivating OP-inhibited AChE (HI-6). However, the ability of tested antidotes to shorten long-lasting impairments of spatial memory induced by low-level sarin inhalation exposure may be due to other antidotal mechanisms based on direct antimuscarinic and ganglion blocking actions, restoration of cholinergic transmission, retardation of the formation of the aged inhibitor-enzyme complex and inhibition of ACh release (20,27).

Although our findings are difficult to extrapolate directly to human low level exposure to OPs, they indicate that antidotes currently used for nerve agent poisonings seem to be beneficial for the antidotal treatment of humans exposed to low-dosage of OPs because they are able to shorten OP-induced chronic decline of memory functions observed, for example, in first responders exposed to sarin in the Tokyo subway attack (15). Therefore, the antidotal treatment of OP-exposed humans seems to be rational regardless of the dosage of OPs.

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ORIGINAL ARTICLE

DIAGNOSTIC VALUE OF C-REACTIVE PROTEIN IN PATIENTS WITH ANGIOGRAPHICALLY DOCUMENTED CORONARY HEART DISEASE

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Summary: Aim: The aim of this study was to evaluate the diagnostic value of serum C-reactive protein (CRP) level measurement in predicting coronary artery disease (CAD) that can be shown angiographically. Methods: CRP levels were determined in the blood of 198 patients (patients group, PG) with angiographically documented coronary artery disease and compared with that of 85 patients (control group, CG) who had a clinical indication for coronary angiography but have no angiographically determined coronary artery stenosis, as well as with that of 41 healthy volunteers as a healthy control group (HG) who did not have any complaint and did not have coronary angiography. CRP levels were measured 24 hours prior to angiography in PG and CG patients, and in the morning after not having eaten for same time. Any coronary artery stenosis or plaque formation was defined as CAD. Severity of the disease was assessed by both the number of diseased vessels (0 to 3) and the degree of stenosis (<50 % mild, 50–70 % moderate and >70 % severe). Results: Receiver Operating Characteristics (ROC) curves of CRP in angiographically documented CAD group showed a diagnostic value of 0.659 in female patients, followed by 0.542 in male patients, in predicting CAD. CRP levels were found to be significantly different between groups, higher in PG (6.2 ± 0.86 mg/L) than those of CG (3.7 ± 0.92 mg/L) and HG (0.854 ± 0.2 mg/L) ($p<0.05$). CRP levels were not associated with the number of diseased vessels, neither with the degree of the occlusion ($p>0.05$). Multiple logistic regression analysis after adjustment for the established coronary risk factors showed CRP as an independent discriminating risk factor for CAD. Conclusion: It is concluded that CRP measurement has a value in predicting the presence of angiographically documented CAD. However, CRP levels were not associated with the degree or severity of CAD.

Key words: *C-Reactive Protein; CRP; Sensitivity; Specificity; Diagnosis; Coronary Artery Disease; CAD; Angiography*

Introduction

Many lines of evidence, ranging from in vitro experiments to pathological analysis and epidemiological studies show that atherosclerosis is an intrinsically inflammatory disease.

Over the last few years, C-reactive protein (CRP) assays have been tested in a series of large-scale prospective clinical studies which demonstrated the importance of this marker in predicting risk of heart attack, stroke and peripheral vascular diseases. It is increasingly recognised that CRP may not merely represent an indication of inflammation but may also, because of its known functional properties, be actively involved in the initiation or perpetuation of local inflammatory reactions (11,26). It is known that CRP is a ligand for specific receptors on phagocytic leukocytes, mediates activation reactions on monocytes and macrophages, and activates complements. There is now overwhelming evidence that an inflammatory outburst is associated with acute coronary diseases such as myocardial infarction (MI) and unstable angina pectoris. Although, all of the potential triggers of inflammation are not fully known, and cytokines, oxidized lipoproteins, and local (arterial) and distant infections (gingivitis, bronchitis) have been implicated, the circulating CRP, an acute phase reactant produced by the liver in response to interleukin-6 (a cytokine induced by interleukin-1 and tumor necrosis factor-alpha), has the most consistent relationship to the future risk of CAD, under diverse clinical settings (4,25,27).

It is possible that CRP contributes to pathogenesis of CAD with different effect at every stage of progression. Production of CRP, a nonspecific reaction to various stimuli including infection, tissue damage and smoking may contribute to the initiation and progression of coronary artery disease. Acute phase proteins and CRP are stimulators of tissue factor production from mononuclear cells, an important initiator of coagulation. It also interacts with LDL and damaged membranes to activate the complement system. Results of various studies suggest that CRP levels not only reflect the presence of subclinical atheroma but

also the likelihood of clinical disease development (3,6,27). In normal healthy individuals, CRP is a trace protein with a range of up to 5 mg/L. 90 % of apparently healthy subjects have levels of less than 3 mg/L, and 99 % of them have that level less than 10 mg/L. There is no difference between levels in healthy men and healthy women, and no variation with age.

Many questions are still unanswered, including whether CRP is a culprit molecule or simply a marker, and the cut-off points and specificity of the methods are still debated. Drugs and other treatments have little or no affect on CRP production unless they also affect the disease process which is responsible for induction of CRP synthesis. It is thus of great interest that the serum level of CRP has been found to be higher in subjects having no complaint but at greater risk of developing MI, stroke and peripheral vascular disease (4,27).

The aim of this study was to compare CRP concentration in patients with CAD, documented by coronary angiography, with that of patients with normal angiograms but having complaints related to ischemic heart disease, as well as with that of normal healthy individuals, to determine the diagnostic value of CRP levels in the progression of CAD. We also aimed to study the association of this marker with the number of diseased vessels and the degree of stenosis.

Material and Methods

The study was designed to evaluate the serum CRP levels of individuals of three different groups. 198 patients were included in the Patient Group (PG) who had documented CAD proven by coronary angiography. The Control Group (CG) consisted of 85 patients with clinical indications for angiography, but normal coronary angiogram. Fortyone healthy subjects were included in the Healthy Group (HG).

Although the healthy subjects did not have coronary angiograms, they underwent a comprehensive physical examination by a physician, and completed the World Health Organisation standards Rose questionnaire on chest pain, and answered other questions about their past medical history. None of the individuals in the healthy group had angina or a prior history of CAD. All of them had normal electrocardiograms based on the Minnesota Coding Criteria.

All Coronary Angiographies were performed in the same centre. Patients diagnosed with acute coronary syndrome six months prior to the study were excluded.

All patients were monitored for somatic illness throughout the investigation period and excluded if symptoms of infection or systemic illness were present (rheumatic disease, chronic liver disease, renal disorder, cancer, etc). Additional exclusion criteria included the use of statins and other medications that could affect CRP concentrations.

Severity of the disease was assessed by counting both the number of diseased vessels (0 to 3) and the degree of the stenosis (<50 % mild, 50–70 % moderate and >70 % severe) (13).

Prior medical history, personal characteristics and habits were obtained by the questionnaire.

The presence of obesity, diabetes mellitus, and hypertension were also assessed. Obesity was defined as body mass index (BMI) greater than 27.8 kg/m^2 as proposed by the National Institute's of Health consensus statement. Diabetes was defined as fasting blood glucose >126 mg/dL or diagnosis of diabetes needing diet or drug therapy. Hypertension was defined as resting systolic blood pressure >140 mm Hg and/or diastolic blood pressure>90 mm Hg (17). Smoking was defined as being a current smoker and smoking at least 10 cigarettes per day for at least ten years.

Standard Blood Sampling

After an eight-hour fast venous blood samples were collected into vacuum tubes in the morning time on the day before the angiographic procedure on the PG and the CG patients. Blood was centrifuged at 3000 g for 10 minutes at 4 °C. After separation, the aliquoits were frozen at –70 °C until analysis.

Measurements

Serum CRP concentrations were determined with BNA 100 Nephlometer (Dade Behring, Germany) according to the manufacturer's instructions. The assay was linear from 0.175 to 230 mg/L, calibrators and controls (R-CL-I) were supplied by the manufacturer. Specifications of intra assay and inter assay coefficients of variation of CRP assay were assessed from quality control data of the laboratory which were 3.4 % and 6.2 %, respectively. The levels of glucose, urea, creatinine, CK, CK-MB, LDH, cholesterol, trygliceride, high-density lipoprotein cholesterol (HDL-C) both in PG and CG were analyzed by BM/902 (ROCHE Diagnostic Systems, Germany). Low-Density lipoprotein cholesterol (LDL-C) was calculated using Friedewald formula if tryglicerides were <400 mg/d (9).

Statistical Analysis

Values are expressed as mean ± SD for normally distributed variables, and log mean CRP serum levels at 20th and 80th percentiles are given.

Because the distribution of CRP is rightward skewed, values derived from log-transformed means were used as means for this variable throughout the study; these values virtually coincided with median values. A general linear model was used to consider case control differences after adjustment for other factors and the area under the ROC curves were used for comparison of the diagnostic values of different analyses (including PG and CG groups using angiography as the highest accepted standard). Optimal cut-off levels, sensitivity and specificity of CRP were selected based on ROC curves.

Results

No statistically significant difference was noted in HDL-C levels among the three groups. However, age, diabetes, hypertension, BMI, total cholesterol and trygliceride levels were significantly different in three groups (Table 1). Smokers consisted of 47 % in the PG, 16% in the CG, and 37 % in HG (Table 1).

CRP levels were different in the studied groups, and these differences remained statistically significant after correction (ANOVA) for other risk factors (Table 2). CRP levels were not related to the number of diseased vessels and degree of occlusion ($p>0.05$) (Table 3).

CRP levels were significantly higher in the PG (6.2 ± 0.86 mg/L) than in the CG (3.7 ± 0.92 mg/L) and the HG (0.85 ± 0.2 mg/L) ($p<0.05$) (Figure 1, Table 2). The number of subjects with concentrations of CRP ≥10 mg/L comprised of HG, CG and PG groups were 0.2 %, 9 %, and 11 % in the HG, CG and PG respectively.

Optimal cut-off levels of CRP providing the maximum efficiency were found in female and male patients with CADs of 6.9 mg/L and 6.4 mg/L, respectively (Figure 2). The area under the ROC curve was 0.659 in females and 0.542 in male patients (Figure 3 and 4 respectively). ROC curve based specificity of CRP levels (38 % for male, 49 % for female) was rather lower than the sensitivity of CRP levels (76 % male, 78 % for female) (Table 4).

A significant difference was seen in the gender-related difference of CRP concentrations in the PG (Table 5), and these differences were statistically significant after correction with ANCOVA for age, blood pressure, BMI, smoking, glucose, lipids concentration, number of diseased vessels and degree of occlusion.

CRP did not correlate with other continuous variables such as total cholesterol, (r: 0.095, p: 0.15), triglycerides (r: 0.07, p: 0.21), BMI (r: 0.09, p: 0.17), HDL-c (r: -0.087, p: 0.243), LDL-c (r: 0.07, p: 0.283), glucose (r: 0.06, p: 0.389).

Tab. 1: Baseline patients and normal controls characteristics (mean ±SD).

	Healthy Control Group (HG) n: 41	Control Group (CG) n: 85	Patient Group (PG) n: 198
Age (year)	33.9 ± 1.3*	52.2 ± 9	57.3 ± 6
Gender (%)	20 F/ 21 M(0,95)	46F/39 M(1.17)	47F/151M(0.32)
Current smoker (%)	37	16	47
Cholesterol (mg/L)	1736.8 ± 47.0*	2221.8 ± 69.0	2151.2 ± 41.0
LDL-C (mg/L)	1100 ± 40.7**	1314.7 ± 62.4	1313.5 ± 33.8
HDL-C (mg/L)	396.6 ± 16.4	424.3 ± 20.5	394.1 ± 10.5
Triglycerides (mg/L)	1200.2 ± 80.5*	2574.6 ± 224.7	2287.3 ± 85.9
Cholesterol/HDL-C	4.6 ± 0.2**	6.2 ± 0.5	5.9 ± 0.1
Diabetes (%)	3 (7.3)***	9 (10)	24 (12)
BMI (kg/m^2)	24 ± 4.1***	27.1 ± 3.5	27.6 ± 3.8
Hypertension (%)	10 (24)***	41 (48)	96 (48)
CRP (≥10 mg/L, n, %)	1 (0.2)*	8 (9)	22 (11)

*p<0.001 HG versus CG,PG
**p<0.005 HG versus CG,PG
***p<0.01 HG versus CG,PG

Tab 2: Serum CRP concentrations (mean ± SD)*.

	HG n: 41	CG n: 85	PG n: 198
**CRP (mg/L)	0.85 ± 0.2	3.7 ± 0.92	6.2 ± 0.86

*(mean ± SD denote values derived from log transformed)
**p<0.001 PG versus HG
**p<0.05 PG versus CG
**p<0.01 CG versus HG

Tab. 3: Serum CRP and severity of coronary artery diseases.

	n	CRP (mg/L) (mean ± SD)*
One vessel	38	7.3 ± 3.7
Two vessel	54	5.2 ± 3.1
Three vessel	106	6.2 ± 1.9
<50 % **	26	5.4 ± 4.9
≥50 % and <70 % **	17	5.2 ± 3.1
≥70 % **	155	6.2 ± 1.9

*(mean ± SD denote values derived from log transformed)
**Degree of occlusion

Tab. 4: Optimal cut-off levels and the associated specificity, sensitivity, diagnostic value of CRP levels for the diagnosis angiographically documented CAD.

	Cut-off level (mg/L)	Specificity (%)	Sensitivity (%)	Diagnostic Value
Male CRP	6.4	38	76	0.542
Female CRP	6.9	49	78	0.659

Tab. 5: Gender related difference of CRP concentrations in the patient group (mean ± SD)*.

	Male n: 151	Female n: 47	
CRP (mg/L)	6.1 ± 1.7	7.2 ± 3.6	p<0.05

*(mean ± SD denote values derived from long transformed)

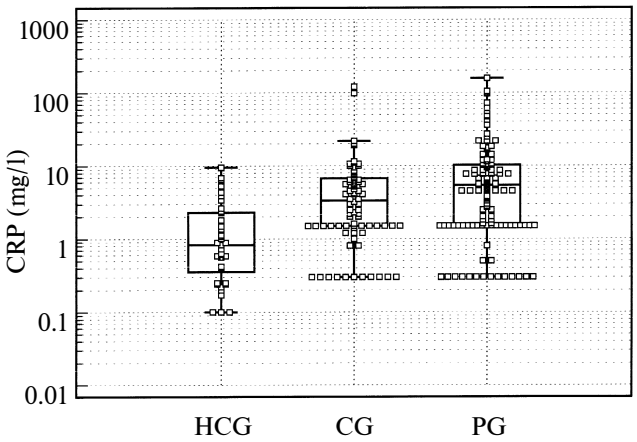

Fig. 1: Distribution of serum CRP concentrations in PG, CG and HG groups. The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line represents the median.

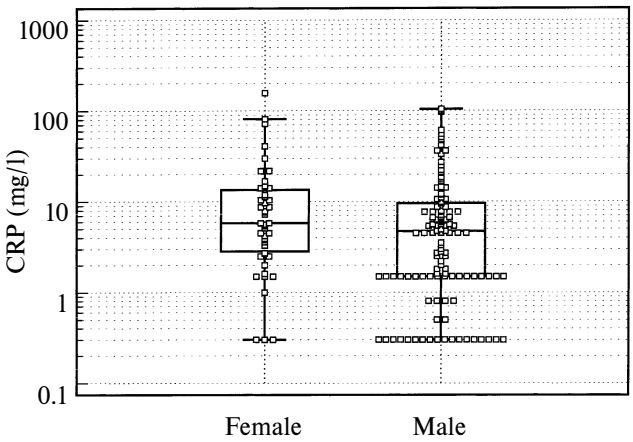

Fig. 2: Distribution of serum CRP concentrations in PG, male and female groups. The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line represents the median.

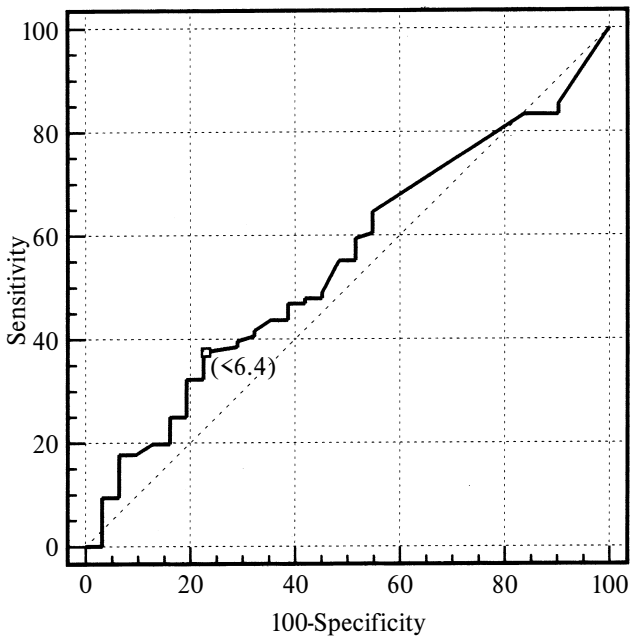

Fig. 3: ROC curve of CRP and optimal cut-off points in male patients.

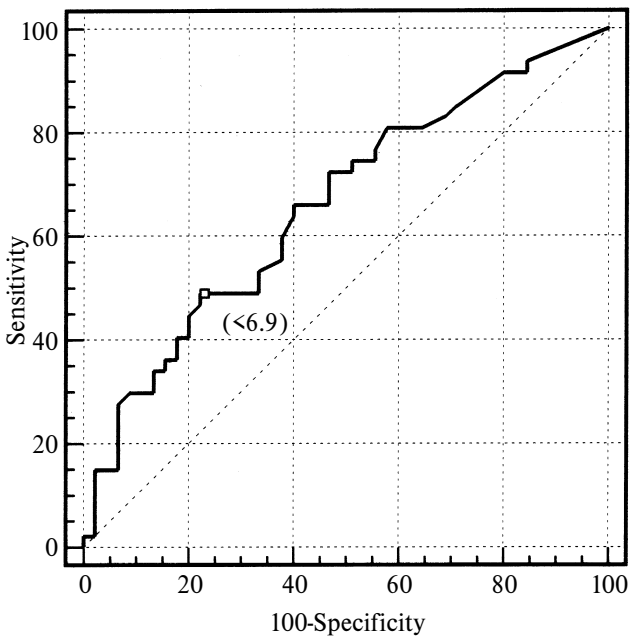

Fig. 4: ROC curve of CRP and optimal cut-off points in female patients.

Discussion

The data from our study demonstrated that the serum concentrations of CRP were increased during atherosclerotic process. However these increases failed to reflect the severity of the CAD. Our results also confirm the existing relationship between C-reactive protein levels and the presence of coronary artery disease documented by coronary angiography. The small area under the ROC curve suggest a possible presence of CAD in angiography. It should be pointed out that the differences seen between groups in CRP concentrations remained highly significant after the adjustment for age, smoking, hypertension, diabetes mellitus, BMI, total cholesterol, LDL-C and triglycerides.

Despite changes in lifestyle and the use of new pharmacological approaches to lower plasma cholesterol concentrations, cardiovascular disease continues to be the principal cause of death in Europe, USA and most of Asia. Half of the heart attacs occur in people with normal cholesterol levels (13). Although hyprecholesterolemia is important in approximately 50% of the patients with CAD, other risk factors need to be taken into consideration (21).

CRP is a sensitive marker for systemic inflammation. Recent work from Pasceri et.al. indicates that CRP itself might be involved in the pathogenic response. In their study they suggested that CRP could induce adhesion molecule expression in endothelial cells via its effect on monocyte chemoattracting protein 1. Regardless of the CRP's precise mechanism for inflammatory effects in the vasculature, perhaps the most relevant is the prospect of inflammation as a therapeutic target (18).

The peak plasma CRP values are strongly associated with post-infarct morbidity and mortality (17). In a meta-analysis of 7 prospective studies, elevated serum CRP concentration was shown to predict future risk of CAD (10). C-reactive protein levels below the conventional upper normal limit of 10 mg/L have been associated with a 2-to-3 fold increase in risk of myocardial infarction, ischemic stroke, peripheral artery diseases and CAD (18,21). Several prospective studies have shown that healthy people with higher serum CRP levels, though usually within the normal reference range, are at an increased risk of developing MI, stroke or peripheral vascular disease (1,5,14,22).

Rifai et al. demonstrated higher plasma concentration of CRP in men with angiographically documented CAD (20). Similarly, Haideri et.al. found an association of elevated CRP levels with angiographically documented coronary artery disease. They also reported that CRP concentration was positively related to the number of diseased vessels and associated with the degree of stenosis of coronary arteries (12). In this study, although CRP levels were found to be increased in patients with angiographically documented CAD, no correlation could be demonstrated between the CRP concentration and severity of CAD. These findings are in agreement with the results of Rifai and colleagues. We also found that, not with standart the normal coronary angiograms, the CG patients had elevated CRP levels, which has not been reported before (12,20).

It has been reported in previous studies that there was no sex-related difference nor association between the CRP levels and age in healthy individuals (7,15,16). However Onat et al. reported that gender is a significant independent determinant of CRP levels in a healthy population in western Turkey (17). In the NHLBI Family Heart Study, CRP values were associated with age, family type, and gender (8). Our study showed that CRP levels in male individuals were higher than that of females in HG individuals. ROC curves (figure 3,4) showed a low level of sensitivity and specificity in the present study, and showed uselessness to diagnose angiographically significant CAD. The area under the ROC curve was 0.542 in males in comparison to 0.659 in females in PG ($p<0.05$). To best of our knowledge these data were not reported in the literature so far.

One possible explanation of the increased CRP levels in patients with a clinical indication for cardiac catheterisation but with normal angiograms is the association of CRP levels with ongoing atherosclerosis. The increase in CRP levels in these individuals may reflect the diffuse atherosclerotic process in the vascular system rather than the degree of the localised obstruction.

In one study a cut-off value of 15 mg/L was chosen on the basis of ROC curves in patients with unstable angina and myocardial infarction (7). Recommended reference limits for serum CRP levels appeared to be insufficient for discriminating subjects with and without CAD because of considerable overlaps. In contrast to these findings, we found optimal cut-off levels for ROC curves to be 6.4 mg/L for males, and 6.9 mg/L for females with CAD. However, the potential limitations of this study warrant consideration. Although CRP was an independent discriminator between the PG and the CG patients, there was considerable overlap in CRP concentrations between the two populations. The presence of occult or ongoing coronary artery disease in the CG could not be excluded and may have attenuated the differences among the two groups, whereby the diagnostic value of CRP in CAD may be underestimated.

Conclusions

In conclusion, serum CRP level measurement appears to have an important diagnostic value in predicting the presence of angiographically detectable coronary artery disease. However, the higher levels of serum CRP seem not to be associated with the degree or severity of coronary artery disease.

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ORIGINAL ARTICLE

SOME RECRUITMENT ASPECTS OF POPULATION PHOTOSCREENING OF AMBLYOGENIC FACTORS AT CHILDREN YOUNGER ONE YEAR

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Summary: The aim of the study was to evaluate the keystone role of paediatric general practitioners (PGPs) in our system of photoscreening of amblyogenic factors at children younger one year. The parental involvement on the participation of their children at photoscreening was also analysed. From June 2000 to February 2001 we have performed 780 photoscreening tests of children aged from 3 months to 31 months (mean age 9.7 months). The photoscreening test was voluntary. Parents was invited to visit screening center by PGP's recommendation and by offering the Invitation/information brochure. The brochure was distributed by 56 paediatric general practitioners during obligatory examinations at 5., 8. resp. 12. month of age from June to November 2000. Based on the questionnaire (return rate 89 %) we documented following data: the number of children of particular age in the care of PGPs was 2060, 1458 Invitation/information brochures was distributed by PGPs. PGPs attended for more children at particular age distributed relatively less number of brochures if compared to PGPs with lower number of relative children. The willingness to the photoscreening programe participance of parents of children belonging to PGPs attended our educational seminary exhibited independence on distance between PGP office and our department in the strong contrast to families belonging with PGPs not participating on educational programme. Our further effort on elevating the percentage of children population participation on screening must be focused on education of PGP with large number of children at particular age and on ensuring the issuing of brochures or other forms of invitation at PGPs' offices.

Key words: *Photoscreening; Amblyogenic factors; Recruitment strategy; Population-based survey*

Introduction

We are seemingly entering into a photoscreening era in first year of thirth millenium (16). An increasing research effort in late 90' have been directed to further development of this method for early detection of vision problem potentials, preferably an anisometropia causing ambylyopia, in infants. The American Academy of Ophthalmology states that screening is perhaps the most important factor in the ocular health of infants and children (2). The development and dissemination of knowledge about effective detection of children at risk could be found among the goals of the SAVP Programme (Strabismus, Amblyopia and Visual Processing) of the National Advisory Eye Council (U.S. Department of Health and Human Services) (26). Such an importance of screening is based on the impact of the facts like that obtained from the National Eye Institute's population-based Visual Acuity Impairment Survey pilot study, the amblyopia is the leading cause of monocular vision loss in the age group of 20–70 years (21).

An ample evidence was colected on the methodology of fotoscreening during last years. On the contrary, only a minority research projects was focused on the organisational aspects accelerating effectivity of photoscreening systems. It is important to consider a wide range of issues when developing screening strategy, including the purpose of the program, how the program will be implemented, the population being served, and the potential impact on individuals. The participation by the target population is clearly a key element in the success of any mass screening programs (12). Not only in photoscreening, but similarly at other screening programs the involvement of the people invited was lower than expected. Since the effective and affordable recruitment methods are essential for achievement of screening goals (19,22), these are recently studied extensively.

The majority of our knowledge related to the recruitment strategies are derived from mammography screening and programs for early detection of colorectal carcinoma. The most of analysed systems was based on the mailing of invitations to screening (10,11,17). The influence of formal properties of such an invitation was analyzed. Whereas aggressiveness of message details, or a family physician's or higher authority's signature on the letter had no impact on compliance. A letter invitation for a routine mammogram

at a specific time resuled in an rate of compliance 3–fold higher than the baseline in the city of Haifa (Israel) (20). A policy of active patient refusal was effective in increasing HIV screening among pregnant women in a large urban obstetric clinic population (24). A personal recruitment strategies (i.e.: invitation letters with or without specified appointment times, either alone or with a follow up letter or telephone call to nonattenders) were more cost-effective than public strategies (i.e. local newspaper articles, community promotion, promotion to physicians) at Australian mammography screening study. The most cost effective personal strategy was an invitation letter without specified appointment time, followed by a second letter to nonattenders (13). The provision of the program information brochure (17), and a visits of mobile screening van (1) represents other studied minor approaches. As an ineffective recruitment strategies for screening mammography appeared letterbox drops and invitations for friends (25). Neither telephone intervention (telephone outcall or advance invitation plus outcall) had a significant effect (9). Currious approach of an scratch lottery ticket for the screening attenders did not improve the compliance with the programm too (25).

The aim of our study was to evaluate some aspects of the keystone role of paediatric general practitioners (PGPs) in our system of photoscreening of eye disorders at infants under one year. The wide agreement exists there on the significant importance of the role of PGPs at the effectivity of recruitment systems. General practice and its associated primary care services are the final common pathway for the delivery of most screening programm (23) and the involvement of general practitioners in test distribution was revealed as an essential to reach satisfactory participation (7,8,12). According to our knowledge of relative literature, nothing is known about the effect of incorporating the PGP to the photoscreening system focused on early identification of visual risk factors. Photoscreening programmes were generally focused on regular paedo-ophthalmology offices visitors or participants were recruited by sending an appointments according to the Community Medical Child Health register (5). We have analysed the effect of professional education of PGPs and the effect of size and location of PGPs' offices on recruitment effectivity. The parental involvement on participation of their children at photoscreening was also analysed.

Matherial and Methods

Photoscreening methodology

Photoscreening tests are performed by Ortoptic Section, Ophthalmology Department, Hospital Litomyšl, Czech Republic, since September 1999. Photoscreening is based on the excentric photorefraction principle described by Bobier and Braddick (6). Photoscreening photography are obtained to colour negative film Konica 100 ASA by camera composed of Yashica 109 multiprogram body (Kyocera Corporation, Optical Equipment Group, Tokyo, Japan), Sonnar electric 200/2.8 lens (Carl Zeiss, Jena, Germany), custom made distance ring of axial lenght 56 mm and mini top electronic flash unit Unomat F 140 (Unomat GmbH & Co. KG, Reutlingen, Germany). Photography of both eyes was obtained at dimmed room from distance of 80 cm (constant distance between eyes and lens aperture was justified by narrow depth of field at 2.8 f-number). Pupiles were not arteficially dilated by mydriatics. Photoscreening images were evaluated by modified method of crescend width (15). First, all the negatives images was wieved in slide viewing magnifier. Positive photography were made from suspect images. Final decision was gained by double observation of the photography of suspect image. All estimation was done by one specialist.

Photoscreening organisation

Evaluated population based photoscreening program ran at Ortoptic section, Ophthalmology Department, Litomyšl Hospital (photoscreening center) from June 2000 to February 2001. The keystone role in the children recruitment to photoscreening assumed paediatric general practitioners (PGPs) located at the area of concern (see below). Educational seminary focused on photoscreening of amblyogenic factors was organized for engaged PGPs (see below). Referral instructions were mailed to all engaged PGPs together with Invitational/informational brochures for parents (see below). PGPs were asked to offer all parents of 6–9 months old children with Invitational/informational brochures during obligatory examination and recommend them to participate on the photoscreening program. The lists of infants which parents was offered by the brochures was collected from PGPs at the end of program.

Target populations

PGPs from area of concern: 56 PGP were co-operating at photoscreening program.

Parents of infants aged 6–9 months at the time of the program: parents of 2080 infants in continuous care of engaged PGP from area of concern.

Area of concern

The program was opened for infants from Ortoptic Subdepartment, Litomyšl Hospital health care area. This represents city districts from north-eastern part of Bohemia: Brandýs nad Orlicí, Červená Voda, Česká Třebová, Hlinsko, Choceň, Jablonné nad Orlicí, Jevíčko, Králíky, Lanškroun, Letohrad, Litomyšl, Polička, Proseč, Skuteč, Svitavy, Ústí nad Orlicí, Vysoké mýto, Žamberk. The total population of the area is approximatelly 250.000 inhabitants.

PGP education seminary

Before start of the program, all PGPs were offered by the educational afternoon seminary focused on eye diseases and on photoscreening of amblyogenic factors in early infacy. 33 PGP participated on the seminary (58.9 % of PGPs engaged in the program).

Invitational/informational brochure for parents

The eight paged Invitational/informational brochure: Visual Illnesses in Eearly Infancy was issued to serve as the invitation to particiption at the photoscreening program. Information about photoscreening procedure, methodology and evaluation as well as general information related to the most frequent eye diseases and amblyogenic factors in infancy was presented in intelligible form to parents. The information related to importance of early detection of amblyogenic factors and heredity of eye diseases (squinting, amblyopia, refractive errors) were emphasized. The same structure of information complemented by interactive FAQs option was offered at www.lit.cz/strabismus.

Effectivity indices

(1) The number of 6–9 months aged infants belonging to each engaged PGP, (2) the number of Invitaional/informational brochures issued to parents by each engaged PGP and (3) the number of performed photoscreening test at patients of each engaged PGP were collected from PGP's and our documantation. As a value of PGPs' involvement on the programme we used the ratio of (2)/(1) and the ratio of (3)/(2) served us as a value of parental involvement.

Results

Within the period of photoscreening program 1458 Invitation/information brochures were issued by PGPs to the parents of appropriatelly aged infants. 780 of this children underwent the photoscreening procedure. It represents 53.5 % of infants invited to photoscreening by issued brochures and 37.9 % of all population of children at particular age. 105 infants was reffered to standard paedo-ophthalmologic examination on the consequence of their screening test failure. It is 13,5 % suspect results of all photoscreening tests performed in the frame of the program and 5,0 % of infants of concern.

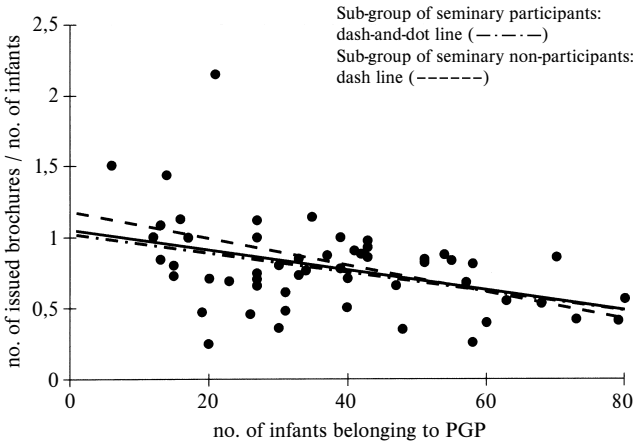

Fig. 1: Dependence of the relative number of distributed brochures (indice of PGP's involvement) on the nuber of infants belonging to the PGP

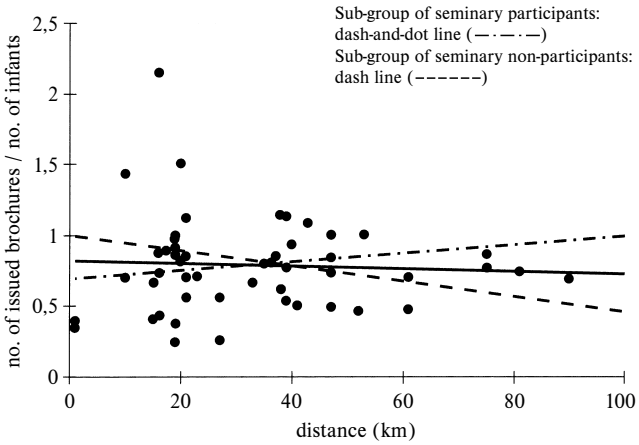

Fig. 2: Dependence of the relative number of distributed brochures (indice of PGP's involvement) on the distance between photoscreening center and the PGP's office

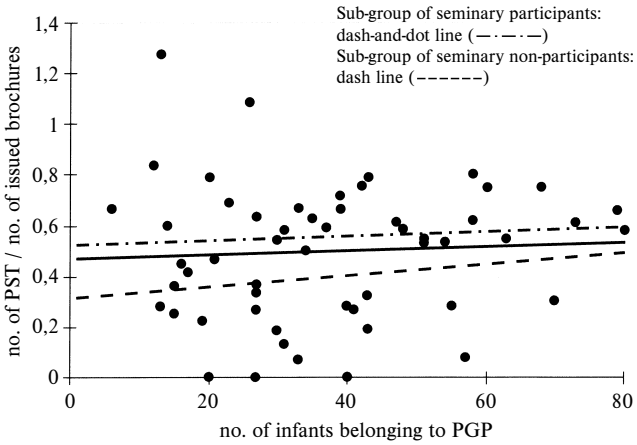

Fig. 3: Dependence of the relative number of photoscreening tests (indice of parental involvement) on the nuber of infants belonging to the PGP

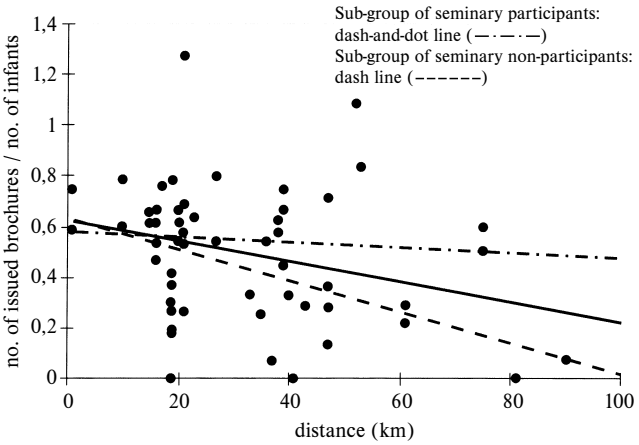

Fig. 4: Dependence of the relative number of photoscreening tests performed (indice of parental involvement) on the distance between photoscreening center and the PGP's office

The evaluation of the influence of the number of infants of particular age in the care of each PGP to the involvement of the PGPs on the programme is illustrated by the Fig. 1. No signifficant changes were revealed at the subgroup of PGPs participating, resp. not participating at the educational seminary.

The dependence of the same parameter on the distance between the PGPs' offices and photoscreening center illustrates Fig. 2. The same regression at the group of PGPs participated resp. not participated at the education seminary is demonstrated at Fig. 2 by dash-and-dot, resp.dash lines.

The sole experience was harwested at two PGPs' offices. Regardless to our instruction to offer the Invitational/informational brochures personally by PGP, at this offices the brochures were simply laying on the table at waiting rooms. At both cases only one patient came and underwent the photoscreening test in spite of the fact that this PGPs took the care of 17, resp. 22 infants of appropriate age.

The ratio between the number of photoscreening tests performed to infants belonging to PGP and the total number brochures issued by this PGP was used as a general measure of the parental involvement on the performance of the photoscreening test at their infants. The relation of this ratio to the volume of PGPs' file of patient of particular age is presented at Fig. 3. The black line illustrates the regression on the whole group of PGPs. The interrupted lines represents the same dependece at sub-group of PGP participating (dash-and-dot line) and not-participating (dash line) on the educational seminary.

Fig. 4 represents the dependence of this parameter on the distance between PGPs' offices and photoscreening center. Dash-and-dot, resp. dash lines on Fig. 4 illustrates the same dependece at the sub-group of PGPs participating, resp. not-participating on the educational seminary.

Discussion

During the time period of our study, 1458 Information brochures was provided, 780 children underwent the photoscreening procedure. The gained attendance rate of 53.5 % is comparable to analogous parameters from other studies. Attendance rate at visual screening program (based on isotropic photoscreening method) at Cambridge, UK, varies from clinic to clinic between 62 % and 80 %, (5). In Victoria (Australia) only 55 % of the population with diabetes accessed eye care services at the recommended intervals (17). The participation rates at other, mainly oncologic, types of screening (i.e. colorectal carcinoma screening, cervical carcinoma screening, breast cancer screening, etc) varies widely from 45 % to 92 % (4,10,12,14,20). The ratio achieved at our study is remarkably lower than those from Atkinson and Braddick's screening programme at Cambridge (5). The possible explanation can be derived from the fact that the participation rates among some demographic sub-groups are substantially low (18). The overall participation rate at Haemocult screening for colorectal carcinoma performed by Herbert, et. al. (12) at county of Calvados, France, varied essentially according to the place of residence: from 65.5 % in urban areas and 48.9 % in intermediate areas to 27.7 % in rural areas. Our helath care area could be characterised as an intermediate area.

The our recruitment strategy was based on two pillars. The first was the paediatric general practitioners involvement (PGPs' involvement) and the second was the appropriatelly aged infant parents involvement (parental involvement).

The keystone role of the personal recommendation to participate on screening by general practitioner is widely accepted as an esencial prerequisit for reaching satisfactory effectivity of screening recruitment system (3,12). A significantly higher attendance rates was documented in one town that received the family physician involvement intervention compared with its matched town which received the community intervention (68 % vs. 51 % attendance rate) on the other study performed at rural communities in New South Wales, Australia (8). On the other study performed at the same region, the media/GP based campaign was associated with significant increase in attendances in all three regions (whereas the television media alone increase attendance only in one region and the media/letter based campaign in two regions respectively) (7). In the Haifa study of recruitment for mammography screening were having a health professional recommendation among the major predictors of compliance. Whereas aggressiveness of message details, or a family physician's or higher authority's signature on the letter had no impact on compliance (20). In spite of the fact that our research was not focused on the question whether the PGP recommendation attributes the attendance or not, we could support these literature data by the two ocassional obserwations. At two PGP's offices where the Invitational/informational brochures simply laiyng at waiting rooms (no PGP's recommendation) the attendance rates dropped to 6 %, resp. 4.5 %.

In our study the ratio between number of issued brochures by PGP and number of aprpriately aged children at the PGP's health care served as a measure of the PGP's involvement. Such an objective measure represents the effort, which was payed by each PGP engaged at study to provide all his/her patients by photoscreening at appropriate age.

One aspect we have studied at the behaviour of PGPs at recruitment process was the impact of number of children aged 6–9 months at practitioners regular care onto the PGP's involvement measure. There was revealed evident tendency of reduction of effort at PGPs with larger number of appropriately aged infants (see Fig. 1) at regular care. In a few cases of PGP with smaller number of respecticve patients, we registered the tendency to issue brochures even for older children (ratio above 1,0).

Bearing the importance of PGP recommendation in mind, we studied the influence of educational and informational policy to the co-operating practitioners. For further analysis of the PGPs involvement we divided them to the sub-groups of seminary participants and non-participants. It is important that invitation for seminary participation was mailed to all PGP, so that the sub-groups mentioned above are not divided randomly and obtained diferences between both sub-groups are resulting not only from participation at seminary but could reflect a previous attitude towards visual screening. The division of the group of PGPs according to participation at educational seminary does not reveal any difference between subgroups at the relation of PGPs involvement measure and the number of patients.

On the contrary to the sub-group of PGPs non-participating on the seminary, the evident tendency of increasing the relative number of issued brochures (indice of PGPs' involvement) with increasing distance between practitioner's office to the screening centre was documented at the sub-group of seminary participants (see Fig. 2). Such a tendency is highly appreciable because it outweights the opposite trend at parental involvement as described below.

The distance between PGPs' office and screening center does not play signifficant role at measure of PGP's involvement if related to the number of 6–9 months aged infants in regular care of respective PGP.

Practical conclusions related to the first pillar (PGPs' involvement):

1) To ensure the providing the Invitation/information brochure by PGP personally.
2) To focuse the encouragement of PGPs with large number of 6–9 months aged infants to issue the Invitation/information brochures more systematically.
3) To invite PGPs from more distant offices for participation at professional education dealing with various aspects of visual screening.

The second pillar of our recruitment system was parental involvement. There is no published research focused on this aspect of recruitment strategy, according to our best knowledge. Our analysis was focused on the estimation of influence of number of 6–9 months aged infants belonging to PGP and the distance from PGP's office to photoscreening center on the parental involvement. The educational and recruitment effectivity of the Invitational/informational brochure was not a subject of our study.

The ratio of the number of photoscreening tests performed as a consequence of the invitation at issued brochure to the number of this issued Invitational/informational brochures by PGP served at our study as a measure of involvement of families belonging to respective PGP on performance of visual screening at their children.

On the contratry to the decrease of the PGPs involvement with increase of number of infants belonging to respective PGP, the parental involvement demonstrated the opposite tendency. With increase of size of the PGP's health care area, the increase of parental involvement was recorded (compare Fig. 1 and Fig. 3). The similar steepness of the regression line was revealed in the sub-groups of parents belonging to PGPs participating resp. non-participating on the educational seminary (see dash-and-dot and dash lines at Fig. 3). Ony one difference between these two sub-groups was shift down of the regression line at sub-group of parents belonging to the non-participating practitioners. Such an observation confirms the superb importance of the form of PGPs' recommendation to participate on visual screening.

Second analysed feature of parental involvement was the influence of distance between screning center and the respective PGP's office on it. There were no records of infants' addresses obtained during photoscreening procedure. Because it is common to be registered at nearest PGP's office, the locations of respective PGPs' were considered as locations of infants homes with approppriate simplification. According to our hypothesis the willingness of families to participate at the visual screening process was the function of the distance between place of PGP's office and screening center. With increase of the distance we have recorded decrease of the measure of parental involvement (see Fig. 4.) Our observations are in good accordance to the sole findings of influence of reachability of screening center to attendance ratio. A negative impact of concerns on participation, althought only marginally significant, revealed by telephone interviews was observed among those who perceived it to be difficult to get to the health center where the breast cancer screening was provided by Health Maintenance Organisation at USA. (18). Dash-and-dot, resp. dash line at Fig. 4 represent the same relation but at the sub-groups of patients belonging to PGPs participating, resp. non-participating at the educational seminary. From comparisson of both lines, the difference at declination of the lines is visible. It could be hypothetised that PGPs participating at seminary are more effective at recommending the participation at screening, so that could outweight the negative influence of distance from place of living to the screening center. This influence of seminary participation is additive to the effect of seminary attendance to the relative number of issued brochures at more distant practitioners' offices (see dash-and-dot line at Fig. 2).

Practical conclusions related to the second pillar (parental involvement):

1) To ensure participation of PGPs from more distant offices for participation at professional education dealing with various aspects of visual screening.

Conclusions

Each of the screening centers must tailor recruitment to its individual catchment area. For intermediate (non-rural, non-purely-municipal). The keystone role of PGPs' recommendation to participate on visual screening could be ammeliorated by professional educaion preferably for practitioners from more distant offices and from offices with larger number of approppriatelly aged infants.

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ORIGINAL ARTICLE

MAXILLARY SINUS AUGMENTATION WITH DEPROTEINIZED BOVINE BONE (BIO-OSS®) AND IMPLADENT® DENTAL IMPLANT SYSTEM PART II. EVALUATION OF DEPROTIENIZED BOVINE BONE (BIO-OSS®) AND IMPLANT SURFACE

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Summary: The objective of this clinical study was to determine the predictability of endosseous implant placed in a maxillary sinus augmented with deproteinized bovine bone (Bio-Oss®). A total of 185 implants (109 titanium and 76 hydroxyapatite-coated) were placed in 77 patients representing 92 sinuses either a one- or two-stage surgical technique. A mixture of venous patient's blood and Bio-Oss® was used alone within 20 sinuses (Group 1), or in combination with autogenous bone within 72 sinuses (Group 2). Thirty-nine implants were placed in Group 1 and 147 implants were inserted in Group 2. The grafted sinuses were evaluated clinically and radiographically at second stage surgery. According to certain criteria, of the implants placed, only two titanium implants (1.08 %) failed with 98.91 % implant survival. There was no statistically variable difference for the use of hydroxyapatite-coated or titanium implants. The two failed implants were from Group 2. No clinical benefit has been achieved from the combination with autogenous bone ($P<0.05$). All the grafted sinuses were sufficient to place dental implants of at least 12 mm length (100 % graft success). The results of this short-term study support the hypothesis that Bio-Oss® can be a suitable material for sinus augmentation.

Key words: *Endosteal implant; Maxillary sinus; Augmentation materials; Xenograft; Sinus lift; Bio-Oss®*

Introduction

The use of dental implants in oral rehabilitation has become a standard of dental care. Unfortunately, replacement of missing teeth with implants in the posterior maxilla often associates with challenging problems. In most instances, the poor bone density of this region is compromised by sinus pneumatization and bone resorption, causing a lack of height for endosseous implants of adequate length to support occlusal loads. Successful implantation in this area calls special surgical techniques and procedures such as a maxillary sinus floor elevation (sinus lift operation). Sinus lifting is a type of inlay augmentation of the maxillary sinus in order to create more bone height in the edentulous lateral maxilla for the placement of dental implants of sufficient length. In-fracture of the lateral wall of the maxillary sinus provides access for sinus mucosa elevation and augmentation material placement (10,19). However, it has not been definitely determined: what is the best grafting material, the best implant surface (hydroxyapatite-coated versus titanium), and whether immediate or delayed implant placement is desirable.

A wide variety of grafting materials have been used to augment bone volume within the sinus including both block and particulate autografts, demineralized lyophilized human bone, xenografts, and resorbable and nonresorbable alloplast grafts. These materials have been used alone or in combination. Xenograft has witnessed a rebirth in popularity over the past decade. Its use was popular in the 1960s but fell into disfavor because of reports of patients developing autoimmune diseases after bovine-derived bone transplants. The re-introduction of these products to the marketplace in the 1990s comes after years of careful scientific evaluation and the development of methods to further deproteinate bone particles (6). The processing reduces the antigenicity, thus resulting in almost complete removal of the organic component of the bone, making it more acceptable to the host tissues. Bio-Oss® (Geistlich, Wolhusen, Switzerland) is a particular inorganic bovine bone matrix of calcium-deficient carbonate apatite. The biocompatibility of this bone graft was demonstrated by Dennissen et al (2). This material has excellent osteoconductive properties (7) and it has been investigated in several studies of guided bone regeneration and augmentation (23). Moreover, it has

been used for sinus floor augmentation both clinically (11,17) and experimentally (13). It is deproteinated by being heat-processed at 300s C for more than 15 hours so that all organic and possibly antigenic components are eliminated. After alkaline treatment, the material, consisting of hydroxyapatite (HA) and carbonate, is sterilized at 160s C; this leaves the crystalline structure (crystal size = 10 to 60 nm), with its high porosity, intact. The material is commercially available in three particle sizes (250 to 1,000 μm, 500 to 1,000 μm, and 1,000 to 2,000 μm) as well as in two different bone types (cortical or cancellous bone) (22). This material is similar to human cancellous bone both in its crystalline and morphological structure. In addition, the physical properties of Bio-Oss® granulate also approximate to the values for human bone tissue and the modulus of elasticity is similar to that of natural bone (21). The large-mesh, interconnecting pore system (75 % pores) of xenogenic bone substitute material facilitates angiogenesis and the migration of osteoblasts. Simultaneously, the inner surface becomes greatly enlarged (Bio-Oss®: 100 m^2/g, HA: 1–10 m^2/g), which is intended to influence positively the formation and inward growth of new bone, and thus the bonding between transplant material and bone (12).

The aim of the present investigation was to evaluate:

1. The clinical and radiographic results of sinus augmentation procedure performed with Bio-Oss®.
2. The clinical benefit of using hydroxyapatite (HA)-coated implants in this procedure.

Materials and Methods

Patient selection. The present study comprised 77 patients (36 men and 41 women) with severe atrophy of the maxillary alveolar process, as diagnosed by panoramic radiographs, who underwent sinus lift operation with Bio-Oss® in our center from January-1998 to March-2000 (Tab. 1).

Surgical technique. It was performed as reported in the first part of this publication (Fig. 1 a,b,c).

Augmentation material. We used a mixture of Bio-Oss® and venous patient's blood, with/without autogeous bone harvested from the maxillary tuberosity. The use of autogenous bone was only considered depended on the possibility to harvest that bone. The sinuses that were augmented only with Bio-Oss® mixed with venous patient's blood were classified as group 1 (20 sinus, 38 implants); while the other sinuses were considered as group 2 (72 sinuses, 147 implants).

Implant type. A total of 185 implants (Impladent®, Lasak, Czech Republic) were placed in these sinuses. They were two types either 109 titanium (109 implants) or HA-coated implants (76 implants). These implants were placed into the grafted sinus in one- or two-stage surgical procedure in the same protocol that was described in the first part of this publication. The types of the surgical procedure and the augmentation material are presented in Table 2.

Follow-up. All patients were given appropriate antibiotic treatment for 1 week beginning 1 hour before the surgery. Clinical evaluations were noted and radiographs were taken prior to sinus augmentation, 9 months after implantation and at yearly intervals thereafter. Implant mobility, at second stage procedure, was determined with the aid of a Periotest® (Siemens, Bensheim, Germany). The following were investigated and subjected to statistical analysis:

Tab. 1: Number of patients and sinus floor elevations.

	Number of patients	Number of sinus grafts
Unilateral sinus lift	62	62
Bilateral sinus lift	15	30
Total	77	92

Tab. 2: The type of the surgical procedure and the augmentation material.

	One-stage procedure	Two-stage procedure	Total
Venous patient's blood and Bio-Oss®	3	17	20
Venous patient's blood, Bio-Oss®, and autogenous bone	32	40	72

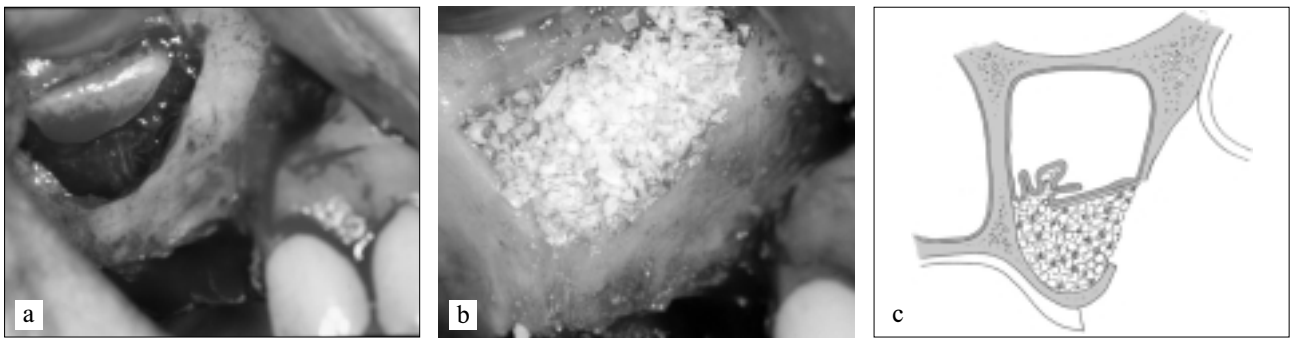

Fig. 1: (a) Inward and upward rotated trap-door of the right lateral maxillary sinus wall. (b) Space underneath lifted maxillary sinus mucosa and trap-door is filled with Bio-Oss® graft material. (c) Illustration demonstrates the surgical procedure- frontal section.

1. What was the healing time of the Bio-Oss®?
2. What was the failure rate of the implants?
3. What was the type of implant surface?
4. Was the graft height sufficient to place an implant of at least 12 mm?

Success criteria. At second stage procedure, the criteria for implant success were taken from the O'Roark and Wayne study published in the International Journal of Oral Implantology in 1991, in which success was defined as, "Survival: Any implant removed or one that will be removed because of any reason by experienced implantologist is a failure. The remainders are reported as percent survival" (21). A sinus augmentation was deemed successful if sufficient bone was generated to allow placement entirely in bone of an implant of at least 12 mm in length, such regeneration was assesses with panoramic radiographs.

Statistical study. Fisher's exact test was used statistically to compare our results ($P<0.05$).

Results

Postoperative complications. The most common complication during operation was sinus mucosa perforation (51.08 %). Postoperatively wound dehiscence occurred in 15.21 %, however, it healed spontaneously within a period of 3 weeks and did not influence the ultimate healing process in a negative fashion.

Surgical observations. When implants were placed 6 months after grafting, few particles of Bio-Oss® were present within the implant osteotomy.

Implant failure. Two titanium implants (1.08 %) were removed during the second stage surgery from the same sinus that was augmented in combination with autogenous bone. There was no clinical evidence of crestal bone loss around the survival implants (98.91 %) (Tab. 3). However, no statistically variable result for the use of HA-coated or titanium implants ($P = 0.513$), and no clinically benefits have been achieved from the combination of Bio-Oss® with autogenous bone ($P = 1,00$). At abutment connecting stage all of these implants appeared well integrated and they tolerated the torque force (35 N cm) applying to stretch the abutment's screws without pain feeling. Clinical evaluation of their stability using the Periotest® instrument (Siemens, Bensheim, Germany) showed positive results. All implants were loaded prosthetically at the time of the investigation.

Graft success. Radiological examination of the orthopantomogram at the evaluation period revealed no distinct changes in vertical graft height. In one sinus contained three implants, one of them was 10 mm implant because of insufficient bone height to place longer; however, because in the same sinus other two implants were placed 12 mm length so this augmented sinus was considered successful. The other grafted sinuses were sufficient in height to place implants of at least 12 mm length (100 % graft success) (Fig. 2 a,b).

Tab. 3: Implant clinical results.

	Successful implants	Failed implants	Survival percentage
Titanium implants	76	2	97,36 %
HA-coated implants	109	0	100,00 %
Total	185	2	98,91 %

Discussion and Conclusions

Bio-Oss® is chemically deorganified and undergoes a physiologic remodeling characterized by three phases. First, the particles are incorporated and surrounded by host bone. Second, the particles are resorbed by osteoclastic activity. Third, new bone is formed by osteoblasts and replaces the Bio-Oss® particles with dense lamella bone. The rate of conversion of dense lamellar bone is dependent on the cellularity, as well as local and systemic factors. However, in the literature, the resorption of this material has been the subject of controversy; it has been demonstrated in animal experiments where the material was placed in

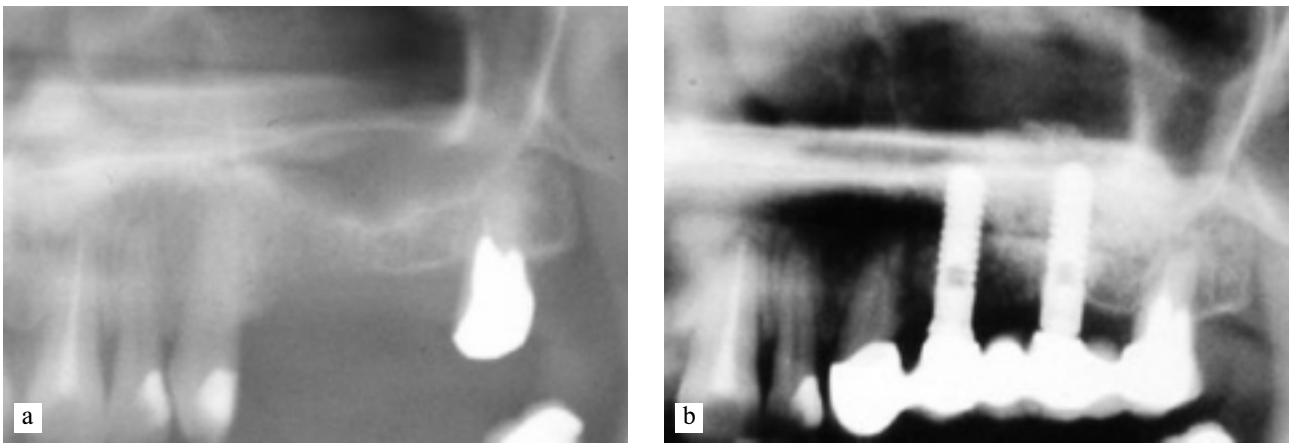

Fig. 2: (a) Radiograph before the operation demonstrates insufficient bone height to place proper implant length. (b) Sufficient bone height to place 14 mm implant length after sinus lift operation.

skull bone defects in rabbits (9). In human studies, Smiler et al (17) in clinical evaluations demonstrated that the formation of new bone by osteoconduction is a slow process taking several months, which can be expected to become established in humans after a time lapse of 1–5 years. On the other side, no overt signs of resorption of the Bio-Oss® particles were visible in Valentini et al's study (20). In Hallman et al's (5) study there were no signs of resorption or degradation of the Bio-Oss® particles. In other radiographic examination has been able to identify the presence of Bio-Oss® granulate even after a resting time of up to 7 years (15).

The primary method of long-term evaluation of sinus grafts has been implant survival in these regions (8,14). Hypothetically, if the graft is composing of good quality bone, the endosteal implant should be maintained in health. Of course, proper implant and prosthetic procedures are required for these implants (3). The results obtained in this study represent better results than other have been published at least for short-term study. Yildirim et al (21) used Bio-Oss® and placed simultaneously 38 BrÍnemark® implants. When the implants were uncovered, after an average healing phase of 6.8 months, 4 of the 28 implants had become loose. Thus, the resulting clinical survival rate, prior to prosthetic loading, was 89.5 %. In Sinus Graft Consensus Conference (1), implant survival was 80 % after 3 years in augmented sinus by autogenous bone and xenograft. It is noticeable that frequently a failed implant was observed early at the time of uncovering, within the first 3 weeks following uncovering, at abutment placement stage, before loading, or during the first year of loading. Thus, specific reasons for the lack of osseointegration were speculative (4). Moreover, our experiences with the sinus lift procedure with Bio-Oss® calls into question the current gold standard of exclusive use of autogenous bone in this procedure. The survival rate of 98.91 % that we achieved over the period of observation with Bio-Oss® is comparable to that was achieved with autogenous bone or some time better. In the Sinus Consensus Conference (1), implant survival after 3 years using autogenous bone graft was 94 %.

Bio-Oss® mixed with autogenous bone will be converted faster than Bio-Oss® alone. That is because the combination of Bio-Oss® with autogenous bone allows the achieving of the autogenous bone osteinductive properties. However, no clinical benefits have been obtained from this combination in our study. The addition of patient's intravenous blood to the mixture of augmentation material improves the material degradation by increasing the growth factors concentration. Moreover, the blood will infuse into the pores of the augmentation material, discouraging particle extravasations and making the graft mixture a more solidified mass.

Positive clinical results have been reported with the use of HA-coated implants in association with maxillary sinus augmentation (8,16). In the present study, HA-coated implants did not offer better results as titanium implants that can be interpreted to the high success of titanium implants too.

As conclusion, from this short-term study, it can be deduced, a positive features of Bio-Oss® as a material for sinus augmentation. For clinical use, both titanium and HA-coated implants can be considered predictable in this surgical procedure. Clinically, it is not important to add autogenous bone to the augmentation mixture especially if that will complicate the surgical technique. However, further follow-up of these patients is important to determine the long-term stability of both material and technique; and more number of patients is necessary to explain if there is relation between the surgical technique and the surgical procedure that we could not demonstrate.

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ORIGINAL ARTICLE

EVALUATION OF THE ACCURACY OF PANORAMIC RADIOGRAPH IN THE DEFINITION OF MAXILLARY SINUS SEPTA

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Summary: The purpose of this study was to determine the accuracy of panoramic radiography in identification of maxillary sinus septa. Out of 68 sinuses were radiographically examined using both panoramic and computerized tomographic radiographs (CT scan). Using CT scan, 24 (35.9 %) out of 68 cases maxillae showed at least one septum, 22 sinuses (32.3 %) showed one septum, whereas two sinuses (2.9 %) exhibited two septa. Panoramic radiograph led to a false diagnosis regarding the presence or absence of sinus septa in 18 of 68 sinuses (26.5 %). On the other hand, they gave negative diagnosis of sinus septa in 12 of 24 septa (50 %). There was fully agreement between the two methods (positive septa) only in 12 of 24 septa (50 %). We cannot depend on panoramic radiograph for the detection of sinus septa because it can lead to false or negative results.

***Key words**: Maxillary sinus; Underwood's septa; Panoramic radiography; CT scan*

Introduction

The maxillary sinus septa were first described by Underwood in 1910 and are thus also referred to as Underwood's septa (12). These septa are barriers of cortical bone and may be varied in number, thickness, and length and they even may divide the sinus into two or more cavities that may communicate (1,6,9).

Etiologically, sinus septa constitute partly congenital, partly acquired malformations. Congenital septa, also referred to as "primary septa", can develop in all maxillary sinus regions (e.g., the floor, the anterior wall) and evolve during the growth of the middle part of the face (4). In contrast, Vinter et al. (13) observed that atrophy of the maxillary alveolar proceeds irregularly in different regions and leaves bony 'crests' on the maxillary sinus floor. Therefore, incomplete septa on the sinus floor, also known as 'secondary septa', can be found because of different reasons. They can be a result of tooth loss and to various phases of maxillary sinus pneumatization. This assumption is also supported by the fact that the sinus floor anterior and posterior to the septum often was on different levels (6). Another explanation is that these septa act as a masticatory force carry struts during the dentate phase of life and seem to disappear slowly when teeth have been lost (1). In addition, they can act to strengthen the maxillary sinus structure (5).

For decades, these septa were considered clinically insignificant anatomical variations. However, detailed knowledge of maxillary anatomy has become increasingly important after the introduction of maxillary sinus lift elevation as a good solution to increase the posterior available bone in maxilla that increase the success rate of the dental implant. Such this knowledge allows more exact planning of invasive surgery and helps to avoid complications since the presence of sinus septa can cause difficulties during sinus lift procedure such as they can limit the creation of a window in the lateral sinus wall and elevation of a hinged door or making difficult to prevent perforation of sinus mucosa during elevation it from an alveolar recess containing septa (3,10) (Fig.1).

The aim of this study was to determine whether it is possible to differentiate the sinus septa clearly on panoramic radiographs.

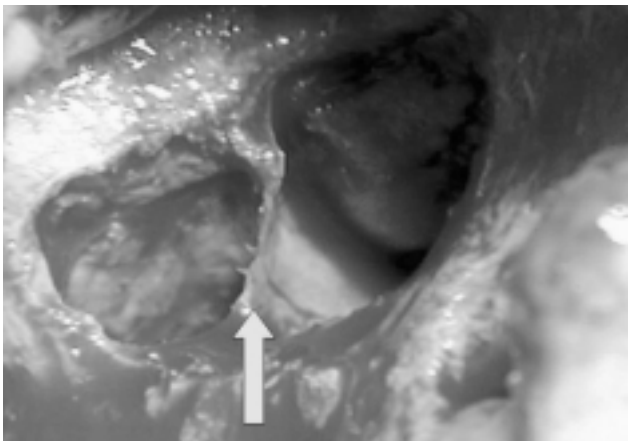

Fig. 1: Intra-operative view of a complete sinus septum that divides the sinus into two cavities (arrow).

Material and method

This study was based on 34 pairs of panoramic radiographs and computerized tomographic radiographs (CT scan) [Somatom Plus 4 (Siemens, Erlangen, feed and slice thickness: 1 mm; window width: 2600; middle position: 500 HU; 2 s, 500 mAs, image matrix: 512x512)] of non-selected adult patients (14 females and 20 males) who had been required both panoramic and CT scan radiographies for preoperative evaluation. The sinus septa were evaluated using mainly an axial plane and the coronal plane of section. The two sets of images were assessed independently by a single dental implantologist (SK) and then the data were confirmed and reviewed by some other authors. The incidence of sinus septa was evaluated using CT scan was compared with the data obtained from panoramic radiography. CT scan was considered as a standard parameter according to the data obtained from panoramic radiographies that were judged. Only those bone lamellae were considered as septa that showed a height of at least 2.5 mm. The term "false septa" indicate the septa that were proposed in panoramic radiograph but were not present in CT scan. We referred to the septa that were not noticed in panoramic but could be observed in CT scan as "negative septa". "Positive septa" defines the septa that were recognized in panoramic and were confirmed by CT scan projections. Mean values were compared using screening test.

Results

Using CT scan, sinus floor with at least one septum were observed in 24 sinuses (35.9 %) (Fig. 2, 3), 22 sinuses (32.3 %) showed one septum, whereas two sinuses (2.9 %) exhibited two septa. Panoramic radiographies led to false diagnosed septa in 18 of 68 cases (26.5 %). On the other hand, they gave negative diagnosis of sinus septa in 12 of 24 septa (50 %). There were positive septa only in 12 of 24 septa (50 %). Using the CT scan as a standard criterion, the sensitivity of screening test (positivity in panoramic for septa that are absent in CT) was 0.50 which means that there is only 50 percent chance for differentiate sinus septa using panoramic radiograph. The specificity of screening test (negativity in panoramic radiograph for septa that are present in CT scan) was 0.59 that means that there is 59 percent chance to find of negative sinus septa using panoramic radiograph (Tab. 1).

Tab. 1: The results of a screening test applied to the presence and the absence of sinus septa using both the radiographic projections.

Panoramic radiograph	**CT scan**	
	Septa presence	Septa absence
Septa presence	12	18
Septa absence	12	26

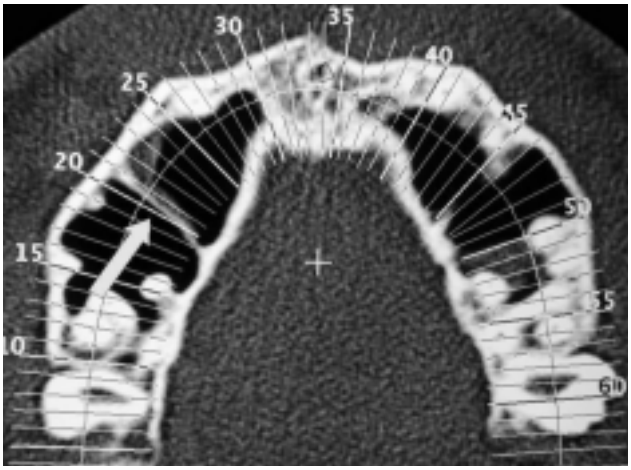

Fig. 2: Axial CT scan showing a complete, thin septum on one side of the sinus (arrow).

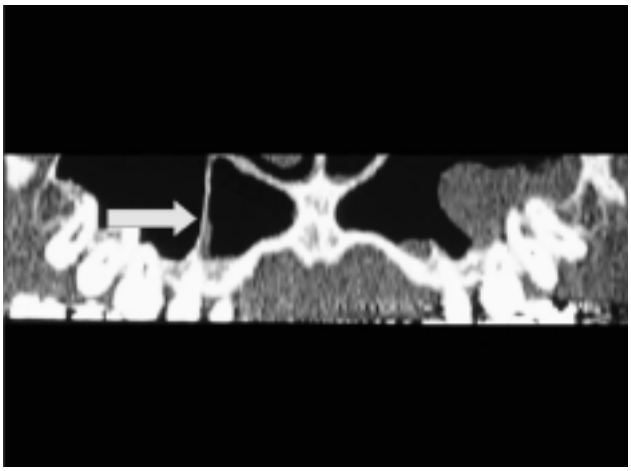

Fig. 3: Sagittal reconstruction corresponding to Figure 1, showing a complete septum of the sinus floor (arrow).

All septa showed frontal or largely frontal orientation (i.e., they were oriented in a buccopalatal plane). Sagittal septa, or septa that followed the arch of the alveolar process, were not observed. It was noticeable that the sinus floor anterior and posterior to the septum often was on different levels.

Discussion

It is difficult to compare between the panoramic radiographic data with the clinical one in relation to the presence of sinus septa because the septa are not always visible in the surgical site. The panoramic data, of this study, were compared to the CT scan that it the high-resolution imaging of delicate bone structures and that could be considered the method of choice for visualization of sinus septa (8).

According to literature, the incidence of sinus septa varies between 16 % and 58 % (1). Underwood (12) found 30

septa in 45 skulls (90 maxillary sinuses). Ulm et al (11) found nearly the same incidence (31.7 %) in anatomical specimens of atrophic maxillae. In clinically preselected population, Jensen and Greer (7) found anatomic variations in 15 out of 26 patients. Betts and Miloro (2) estimated the incidence of maxillary sinus variations to be about 20 %. The incidence of radiographically discernible sinus septa in non-preselected patients in this study (35.9 %) is quite similar to that in the above mentioned studies. Nevertheless, the value is high enough to attain significance in clinical routine.

Only bone lamellae were considered as septa when they showed a height of at least 2.5 mm. This criterion was taken from Ulm et al's study published in the International Journal of Oral & Maxillofacial Implants in 1995 (11). Thus was possible to exclude from the analysis uneven patches of the alveolar recess, which can be observed in any sinus floor.

The great number of the false diagnoses established by the use of panoramic radiograph indicates that it is not suitable enough for thorough evaluation of the sinus septa. The panoramic radiograph often demonstrates the maxillary sinus as multicompartmented, with radiopaque bone septa. This can be interpreted by the fact that the image of the maxillary sinus is found to be transversed by an occasional, or several, radiopaque lines in panoramic radiographs that can gives false septa. These lines can artifact corresponding in its lower half to the posterior surface of the zygomatic process of the maxilla, and in its upper half to the posterior surface of the frontal process of the zygoma. It should be distinguished from the vertical septa, reinforcement webs, or the posterior wall of the sinus. Moreover, the x-ray beam of panoramic radiographies is seldom directed tangentially to these structures that can lead to negative results. In contrast, modern CT scan and subsequent reconstruction allow high-resolution of delicate bone structures, it therefore can be considered the method of choice for visualization of sinus septa. The differences in results between CT and panoramic radiography were large, but probably cannot be attributed to interobserver variation, even though such variation may exist. Axial sections, as have been used in this study, run perpendicular to the orientation of such septa and can be considered the optimal sectional plane for visualization of these bony variations.

Conclusion

Comparatively to CT scan, panoramic radiograph cannot clearly differentiate the sinus septa from some different other anatomic structures resulting from superimpositioning effects. It can give false negative results.

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BRIEF COMMUNICATION

LOW DOSE KETOCONAZOLE THERAPY AND THYROID FUNCTIONS IN RATS

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Summary: To determine whether low dose ketoconazole (KTZ) has antithyroid action, we studied thyroid function tests in the 13 rats treated with KTZ (20 mg/kg twice daily) for thirty days. Serum triiodothyronine and thyroxine levels were decreased (P <0.05) and serum thyroid-stimulating hormone levels were mildly increased (P >0.05) at the end of treatment. Histopathological analysis of the thyroid glands demonstrated an increase in cylindrical cells in study group, but the epithelial cells were mainly cubical in control group. These findings showed that low dose KTZ had antithyroid effect in rats. The responsible mechanisms may be direct effect of the drug on thyroid gland.

Key words: *Ketoconazole; Thyroid gland; Rat*

Introduction

Ketoconazole (KTZ), a synthetic imidazole antifungal, is effective for superficial fungal infections, genital candidiasis and chronic mucocutaneous candidiasis, and has been used in immunocompromised patients and advanced prostatic carcinoma (4,9). High doses of the drug, however, can exert an inhibitory effect of gonadal and adrenal steroidogenesis in vitro and in vivo. This endocrine effect can be rapidly reversed by interrupting the administration of the drug (14). Its chemical structure, comprising several heterocyclic rings and electron donor groups, indicated that KTZ may possess antithyroid activity (2). Namer et al (12) reported that some patients with high doses of KTZ (1200 mg/kg/day) for breast cancer presented a biological hypothyroidism that required hormone replacement. Kitching (10) reported on two patients with severe mucocutaneous candidiasis and suggested that hypothyroidism might be a rare but not unlikely side-effect of KTZ therapy. In the previous study, we found that high dose of KTZ (60 mg/kg/ /day) had antithyroid effect in the rats (1). The purpose of this study was to determine whether low dose KTZ (20 mg/kg/day) had antithyroid effect in-vivo.

Material and Methods

KTZ in powder form (Bilim Pharmaceuticals, Istanbul) was suspended in 0.3% Noble agar and administered orally to a group of 13 rats, twice daily (20 mg/kg/0.1 ml dose) at 07^{00} and 17^{00} h for thirty days (5,8). A control group received a placebo solution for thirty days. Twenty-six rats in two groups were included in this study. Control group; and study group; 13 rats treated with oral KTZ (20 mg/kg twice daily) for thirty days. Blood samples were drawn from the jugular vein to determine serum triiodothyronine (T_3), thyroxine (T_4) and thyroid-stimulating hormone (TSH) levels at the initial and on the 30^{th} day of therapy. At the initial the rats were weighed. At the end of the treatment period, the rats were killed and weighed. The body weights were compared with those of the controls. Thyroid glands were removed and weighed, and compared with those of the controls. Cross-section of thyroid glands was cut and examined histologically after suitable fixation and staining. A single cross-section (including both lobes) was evaluated for each animal and > 20 follicles were measured by calculating the area enclosed by the apical surface of the follicular cells. The larger, more inactive, follicles along the periphery were excluded from the analysis. The activity of the gland was indicated by the appearance of the cells, and by the presence or absence of colloid. Hyperfunction was scored from + to ++++ as a function of the ratio of cylindrical to cubical cells (2,3,13).

– = normal activity (<5 % of cylindrical cells);
\+ = moderate activity (>5 % to % 50 of cylindrical cells);
++ = clear activity (>50 to 75 % of cylindrical cells);
+++ = strong activity (>75 to 90 % of cylindrical cells);
++++ = extra-strong activity (>90 % of cylindrical cells).

The concentrations of T_3 and T_4 in the serum were assayed using the radioimmunoassay (RIA) kit supplied by Diagnostic Products Corporation (Los Angeles, USA) and rat TSH with the RIA kit supplied by Amerlite Diagnostics Ltd. (Amersham UK). Statistical analysis was performed by Mann-Whitney U – Wilcoxon Rank Sum W test.

Results

The body and the thyroid gland weights of the animals are shown in Table 1. There was no statistically significant difference in the body weight and thyroid gland weight between the control and study group. In histopathological analysis of the thyroid gland, the epithelium was mostly cubical cells in control animals. In study group, cylindrical cells were increased in analysis of the thyroid glands. The hypothyroid state of the rats treated with KTZ was indicated by hyperfunction of the thyroid gland as indicated by the presence of cylindrical thyrocytes. However, there was no significant difference between the two groups ($P > 0.05$). Serum T_3, T_4, and TSH levels in the control group and on the 30th day of therapy of study group are shown in Table 2. Serum T_3, and T_4 levels on the 30th day of therapy were decreased in study group compared with control group ($P < 0.05$). Serum TSH levels were mildly increased at the end of treatment; however, there was not a significant difference between the groups for serum TSH levels ($P > 0.05$). Serum thyroid hormone levels (T_3, T_4, and TSH) at the initial of therapy in the study group were as follows; T_3: 0.0833 ± 0.0227 μg/dl (0.0462–0.1139 μg/dl), T_4: 5.26 ± 1.14 μg/dl (3.09–6.95 μg/dl), and TSH 1.54 ± 0.45 μU/L (0.99–2.42 μU/L). There was not a statistically significant difference for serum T_3, T_4 and TSH levels between the initial values of the therapy in the study group and control group ($P > 0.05$).

Discussion

The effects of KTZ on different endocrine systems such as testosterone synthesis and cortisol production have been reported in-vitro and in-vivo. However, there is little information available on its action on thyroid gland and thyroid cells. Kohan et al (11) reported that KTZ (1–50 μmol/l) slightly increased the basal iodide uptake but, at higher concentrations (75–100 μmol/l), it sharply decreased iodide uptake below the basal levels. The present work is the first report of KTZ action in a normal rat thyroid cell line. De Pedrini et al (6,7) studied the effect of KTZ on thyroid function in five patients with thyrotoxicosis, five patients with hypothyroidism and ten normal subjects treated with KTZ at high dose (600 mg/daily in three 200 mg oral doses) for four weeks. They found no inhibitory effect on the synthesis of thyroid hormones and thyroid function and they did not detect any antithyroid activity after administration of KTZ to the hypothyroid patients and ten healthy subjects.

Tab. 1: The weights of body and thyroid gland of the rats.

	First body weight (g) X ± SD	Last body weight (g) X ± SD	z	*P*
Control group	188.4 ± 40.3	184.6 ± 30.5	0.20	>0.05
Study group	212.7 ± 34.6	206.9 ± 33.3	1.8	>0.05
	Thyroid weight * X ± SD			
Control group	10.66 ± 1.60		-1.50	>0.05
Study group	12.19 ± 3.44			

* mg/100 g body weight

Tab. 2: Serum T_3, T_4 and TSH levels in the control group and on the 30th day of therapy in the study group.

Parameters	Control Group (n: 13)	Study Group (n: 13)	z	*P*
T3 (μg/dl)				
X ± SD	0.0721 ± 0.0199	0.0500 ± 0.0149	-2.89	0.003
(min-max)	(0.0312–0.1098)	(0.0301–0.0837)		
T4 (μg/dl)				
X ± SD	6.23 ± 1.63	3.31 ± 0.53	-4.13	0.00001
(min-max)	(3.47–9.11)	(2.50–4.17)		
TSH (μU/L)				
X ± SD	1.28 ± 0.41	1.55±0.57	-1.07	0.281
(min-max)	(0.61–1.87)	(0.88–2.71)		

The normal ranges of T3, T4 and TSH levels were as follows; T3 0.0204 –0.1447 μg/dl; T4 2.25–9.89 μg/dl; and TSH 0.25–2.05 μU/L.

In our study, there was mild decreased body weight and mild increased thyroid weight in the study group compared with control group. Comby et al (2) observed a fall in body weight with respect to controls, which may have been due to a loss of appetite induced by the drug, and they also observed thyroid weight increased, as in our study. Comby et al (2) suggested KTZ could inhibit 5'-desiodase, which is responsible for T_3 and T_4 des-iodation, so keeping T_4 levels higher than expected in the in-vivo study. Our previous and present studies showed that both high and low dose KTZ had antithyroid effect in the rats. The responsible mechanism may be direct effect of the drug on thyroid gland. Nonetheless, since it is well-known that the changes of total plasma can effect the capacity and affinity of plasma proteins for thyroid hormones and the food intake has a clear cut effect on conversion of T_4 to T_3, these factors may also responsible for our results.

The work has been carried out in Erciyes University Faculty of Medicine, Department of Pediatrics, Kayseri, Turkey

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BRIEF COMMUNICATION

YELLOW NAIL SYNDROME OR DIFFUSE LYMPHATIC NETWORK DISEASE

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Summary: We report a man aged 68 years old with pneumothorax and chronic bilateral pleural effusion in association with a history of yellow nails. The diagnosis of yellow nail syndrome based on yellow nails, lymphedema, chronic pleural effusion and intestinal lymphangiectasia.

Key words: Yellow nail syndrome; Pneumothorax; Chronic pleural effusion

The yellow nail syndrome (YNS), a combination of yellow discolored nails, lymphedema and pleural effusions, is a rare autosomal dominant disorder of obscure etiology (10). YNS is a rare clinical condition. During the last 35 years only about a hundred cases have been reported. We present the clinical findings of a man who suffered from undiagnosed bilateral pleural effusions and lymphedema for 27 years and was admitted to pulmonary department with pneumothorax.

Case Report

Three months ago, a 68–year-old patient was admitted to the emergency department with dyspnea, thoracic pain and chronic lower extremity edema. Clinical examination revealed bilateral effusions, left pneumothorax and yellow discoloration of the nails of the feet (Fig. 1). He told us that the yellow color of the nails and the peripheral edema and edema in eyelids first appeared 27 years ago. He did not ask for medical advice for 13 years, until he developed progressive dyspnea with gradual worsening of his exercise tolerance and bilateral pleural effusions in x-ray. Laboratory examinations in many general (Greek and German) hospitals revealed in addition to bilateral pleural effusions, a latent hypothyroidism thirteen years ago. Pleuroscopy revealed no abnormal findings. He was released with medicaments thyroxin and spironolactone. Due to normal thyroxin blood levels, he stopped ten months later using thyroxin. Thoracocentisis was used when dyspnea was worsening. He submitted during the last six years in three thoracocentisis and the last thoracocentisis took place nine months ago during his last hospitalization.

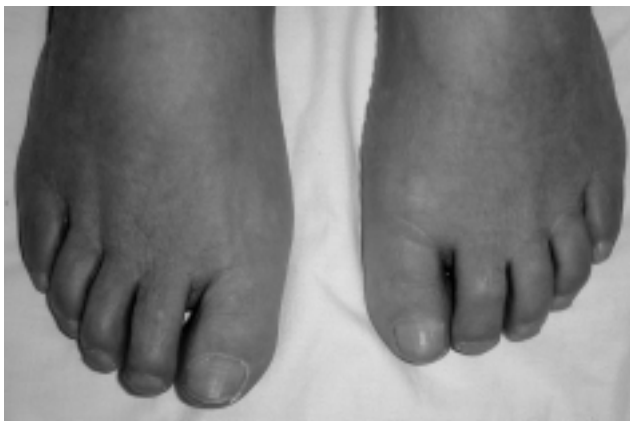

Fig. 1: Yellow nails in the lower limbs.

Our examinations showed normal hemoglobin level of 14.1 g/dl, normal leukocytes (8800/mm^3, 66% neutrophils) and ESR 45mm in the first hour. Albumin was reduced at 2.9 g/dl. All other biochemical agents were at normal levels. Immunoglobulins and thyroid function test were also at normal levels. Chest x-ray examination revealed left pneumothorax besides the bilateral pleural effusion. Billau system was used to control the pneumothorax successfully. Three paracentheses in the right hemithorax gave together about 4 l of fluid. The type of the liquid in both sides was exudative. Also gastroscopy and biopsy from jejunum were performed during his hospitalization. In some sections there were lymphangiectasis. When liquid stopped reproduction, we took off the Billau system. He was released without dyspnea and with advice to use diuretics.

Discussion

YNS was first described in 1964 by Samman and White (10). The full syndrome is characterized by rhino-sinusitis,

pleural effusion, bronchiectasis, lymphedema and dystrophic yellow nails (8,9). Individual manifestations of the syndrome may appear at different ages beginning from birth up to late adult life (2,11,12). Most patients develop YNS in early middle age and the overall male: female ratio seems to be 1:1.6 (9). YNS result from slow growth, possibly secondary to defective lymphatic drainage (7). Historically, nail changes were the first to be recognized. The slow rate of nail growth may be accompanied by color changes (pale yellow/green), onycholysis, and occasionally a distinct hump of the nail (10). Moreover, spontaneous clearing of the nail changes has been reported without resolution of the respiratory involvement (3). Pleural effusion appears to be a later manifestation. Our patient presented dyspnea due to pleural effusion eleven years later after the YNS onset. Pleural and pericardial effusions, chylous ascites and persistent hypoalbuminaemia can be explained by possible microvascullar permeability (1). Parietal lymphatic is the major route of pleural fluid exit. Fluid comes in the pleural cavity from the microvessels of systemic circulation (parietal pleura) and bronchial network (visceral pleura) (4,5). Dysfunction of lymphatic stomata is the reason of the pleural effusions in the YNS. Jejunal and duodenal biopsies may show the typical histological findings of intestinal lymphangiectasis, which may contribute to the pathogenesis of this syndrome (6).

YNS manifestations from respiratory system include bronchiectasis (14), which can be detected by high – resolution computed tomography (13). The cause of bronchiectasis is unclear, but again dysfunctional lymphatics are thought to play an important role with compromised drainage of secretions and local immune function (13). The reason of pneumothorax may be the persistent infection in bronchiectasis or the bad condition of pleura disturbance of the lymphatic system or to repeating thoracocentisis of the patients.

Chronic bilateral pleural effusion may be an index of suspicion of YNS. Dystrophic yellow nails especially in the lower limbs may appear many years before. Pneumothorax is an unusual and unclear sign of the syndrome. Disorder of the lymphatic system is the reason which could explain all the signs of YNS.

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