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CUPRINS

REVIEW
CORRELATION BETWEEN HSP AND DIFFERENT DISEASES

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ABSTRACT

Circulating heat shock proteins (HSP) has been shown to correlate in diseases or disorders in which there is destruction or damage to target tissues or organs, including cardiovascular diseases, a wide range of human cancers and numerous autoimmune disorders such as autoimmune arthritis, type 1 diabetes mellitus, atherosclerosis, multiple sclerosis, and other autoimmune reactions

Key words: heat shock proteins, cancer, neurodegenerative diseases, cardiovascular diseases

INTRODUCTION

Heat shock proteins are highly conserved proteins that, when produced intracellularly, protect stress exposed cells. Heat shock response is nature's device to protect cells against environmental and physiological stresses. Cells under stress either mount heat shock response and survive, or succumb to the stress and die (9). This phenomenon is conserved through evolution. The sequential molecular events in heat shock response are extensively characterized. Its typical features include drastic repression of normal transcription and translation pathways, and activation of heat shock gene family by heat shock factor (HSF) (9). Circulating HSP has been shown to correlate in diseases or disorders in which there is destruction or damage to target tissues or organs, including cardiovascular diseases. Higher circulating levels of HSP70 predict the future development of cardiovascular disease in established hypertensives and a recent study demonstrating a decrease in HSP60 and HSP70 with advancing age (6).

ROLES OF HSPS IN CANCER

Recently the roles of Hsps in cancer are attracting increasing attention and have accelerated the study of heat shock response mechanism. Hsp expression levels are high in various tumors, compared to normal cells. Overexpression of Hsps inhibits the apoptotic machinery and promotes the scavenge of the misfolded proteins in proteasome-mediated degradation (1, 9), possibly inducing the resistance to chemotherapy. The depletion of Hsp27, Hsp70 and Hsp90 using RNA interference or inhibitors, induces the cell growth arrest or cell death (4, 9). Thermotolerant cells induced by chronic heat shock stress showed the inhibition of JNK activation and the rapid deactivation in response to heat shock (9). Studies onthe up-and down-regulation of various Hsps, obviously suggest that Hsps expression is well associated with the resistance of cell death in various tumors. Therefore, the strategy to inhibit the expression and biological function of Hsps has great potential in cancer treatment. Hsp expression has been analyzed in relation to the histopathological characteristics of the tumor tissues (eg, tumor type, grade of differentiation), with the expression of other molecules (eg, estrogen receptors, c-myc, mutated p53), and with patient parameters like sex and age (5). In addition, levels of circulating Hsps and anti-HSP antibodies have been correlated with patient and tumor characteristics. Hsps are overexpressed in a wide range of malignant cells and tissues. The presence of Hsps and antibodies to the Hsps in the serum of cancer patients is still a new research area. Although it seems that autoantibodies to certain Hsps are of significance as tumor markers in osteosarcomas, ovarian cancer and others, at present, we need more studies to draw a clear conclusion on this important subject. The same applies to the study of the polymorphism of the Hsp70-2 gene. HSP expression becomes deregulated in cancer leading to elevated expression (3). Elevated HSP expression promotes cancer by inhibiting programmed cell death (Hsp27, Hsp70) and by promoting autonomous growth (Hsp90) and leads to resistance to chemotherapy and hyperthermia. Tumor HSPs have another property that can be exploited in therapy. They are immunogenic and can be used to form the basis of anticancer vaccines. Elevation in HSP levels may thus have competing effects in tumor growth, being required for tumor cell survival but conferring a hazard for cancer cells due to their immunogenic properties. This dichotomy is also reflected by the approaches used to target HSP in therapy. Hsp expression levels can help indicate the presence of abnormal changes during the process of carcinogenesis (in certain tissues). For example, Hsp27 is overexpressed in hyperplastic endometrium, and this protein appears as a marker of squamous metaplasia in the uterine cervix; Hsp10 and Hsp60 are related with the process of carcinogenesis of the uterine cervix and colon; and Hsp70 is associated with carcinogenesis of the oral epithelium and as a marker of early hepatocellular carcinoma. In oesophageal carcinomas, Hsp27 decreases during the carcinogenesis that ends in adenocarcinomas but increases during the carcinogenesis that ends in squamous carcinomas. Then, Hsps can be used as subrogate biomarkers of certain cancers. The increased transcription of HSPs in tumor cells is due to loss of p53 function and to higher expression of the proto-oncogenes HER2 and c-Myc, and is crucial to tumorigenesis (4). The HSP family members play overlapping, essential roles in tumor growth both by promoting autonomous cell proliferation and by inhibiting death pathways. Hsp expression correlates with the degree of differentiation in certain tissues. Hsps associated with higher differentiation are: Hsp27 and Hsp90

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in endometrial carcinomas, Hsp27 in squamous carcinomas (uterine cervix, oral epithelium), and Hsp27 as a marker of keratinocyte differentiation in the skin. In contrast, Hsps associated with poor differentiation are Hsp70 in cancers of the breast, ovary, and oral epithelium, Grp78 in lung carcinomas, and Hsp27 in astrocytomas. At present, we do not have a clear explanation for these disparities and associations. Hsp70 has been involved not only with poor tumor differentiation but also with increased cell proliferation (breast, uterine cervix, lung), lymph node metastasis (breast, colon), increased tumor size (uterine cervix), presence of mutated p53 (breast, endometrium), and higher clinical stage (oral, colon, melanoma). Several Hsps are coexpressed in cancer tissues; in addition, certain Hsps can be significantly associated with other molecules. For example, Hsp27 has been associated with ERα in female breast carcinomas and endometrial carcinomas, but this protein did not appear associated with ERα in male breast carcinomas, cervical uterine carcinomas, hepatocellular carcinomas, and meningiomas (tissues that may express ERα). It is interesting that Hsp27, which was first described as an estrogen-regulated protein, is significantly associated with ERα in the female breast and endometrium. These 2 organs are under strong estrogen and progesterone regulation. Doxorubicin (DOX) is a widely used antitumor drug, but its application is limited because of its cardiotoxic side effects (7). Hsp20 has been recently shown to protect cardiomyocytes against apoptosis, induced by ischemia/reperfusion injury or by prolonged beta-agonist stimulation. However, it is not clear whether Hsp20 would exert similar protective effects against DOX-induced cardiac injury. Actually, DOX treatment was associated with downregulation of Hsp20 in the heart. To elucidate the role of Hsp20 in DOX-triggered cardiac toxicity, Hsp20 was first overexpressed ex vivo by adenovirus-mediated gene delivery. Increased Hsp20 levels conferred higher resistance to DOX-induced cell death, compared to green fluorescent protein control. Furthermore, cardiac-specific overexpression of Hsp20 in vivo significantly ameliorated acute DOX-triggered cardiomyocyte apoptosis and animal mortality. Hsp20 transgenic mice also showed improved cardiac function and prolonged survival after chronic administration of DOX. The mechanisms underlying these beneficial effects were associated with preserved Akt phosphorylation/activity and attenuation of DOX-induced oxidative stress (7). Coimmunoprecipitation studies revealed an interaction between Hsp20 and phosphorylated Akt. Accordingly, BAD phosphorylation was preserved, and cleaved caspase-3 was decreased in DOX-treated Hsp20 transgenic hearts, consistent with the antiapoptotic effects of Hsp20. Parallel ex vivo experiments showed that either infection with a dominant-negative Akt adenovirus or preincubation of cardiomyocytes with the phosphatidylinositol 3-kinase inhibitors significantly attenuated the protective effects of Hsp20. Taken together, our findings indicate that overexpression of Hsp20 inhibits DOX-triggered cardiac injury, and these beneficial effects appear to be dependent on Akt activation. Thus, Hsp20 may constitute a new therapeutic target in ameliorating the cardiotoxic effects of DOX treatment in cancer patients. Hsp70 has been described as an important molecule in the assembly and trafficking of steroid receptors, and in breast cancer, Hsp70 has been found associated with ERα (5). It is of interest to mention that Hsp70 can increase ERα transcriptional activity and growth in MCF-7 breast cancer cells (11), which in turn may explain the increased cell proliferation found in breast tumor biopsy samples that express Hsp70 (5). In addition, Hsp70 has been associated and complexed with mutant p53 in cancer cell lines (5, 11). This association has been studied in several cancer tissues, and the results have shown this association in certain cases only.The expression of certain Hsps can be correlated with the carcinogenic process as well as with the degree of differentiation and cell proliferation, and moreover, they have been implicated in the regulation of apoptosis (5). The prognosis of a particular cancer patient is very important in the clinic to individualize cancer treatments, to plan the patient's follow-up, and to answer questions from the patient or relatives. Overtherapy with cytotoxic drugs can be avoided in cancer patients if they are correctly identified as having good prognosis and vice versa. Hsp27 expression has been associated with poor prognosis in ovarian, gastric, liver and prostate cancer, and osteosarcomas (5). In contrast, Hsp27 expression has been associated with good prognosis in endometrial adenocarcinomas, oesophageal cancer, and in malignant fibrous histiocytomas. Although there are fewer studies in other cancers, the data suggest that Hsp27 has no prognostic value in head and neck squamous cancer, bladder and renal cancer, and leukemia (except when associated with other markers). Hsp70 expression is correlated with poor prognosis in breast cancer, endometrial cancer, uterine cervical cancer, and transitional cell carcinoma of the bladder. This is consistent with the Hsp70 associations with poor differentiation, lymph node metastasis, increased cell proliferation, block of apoptosis, and higher clinical stage, which are markers of poor clinical outcome. In contrast, high Hsp70 expression was correlated with good prognosis in oesophageal cancer, pancreatic cancer, renal cancer, and melanoma. Hsp70 expression showed no correlation with prognosis in ovarian cancer, oral cancer, head and neck squamous cancer, gastric and prostate cancer, and leukemia (5). Hsp90 expression in cancer tissues and presence of autoantibodies to Hsp90 have been correlated with poor prognosis in breast cancer. In contrast, Hsp90 expression is associated with good prognosis in endometrial cancer. Loss of Hsp90 (and Hsp60) expression has been associated in bladder carcinoma with invasive recurrence risk. Hsp90 expression was of no prognostic value in ovarian and oral cancer (5).

ROLES OF HSPS IN NEURODEGENERATIVE DISEASES

One of the characteristics of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), familial amylotrophic lateral sclerosis (FALS) and poly Q disease is the formation of plaques/inclusion bodies which co-localize with various chaperones and components of the ubiquitinproteasome degradation system (5, 11). In AD, Hsp70 is found in the extracellular senile plaques and is believed to play a role in phagocytic digestion of amyloid plaques by microglia (5). The unusual extracellular accumulation of chaperones is suggested to be facilitated by calcium-induced interaction with lipid rafts (2). In AD intracellular myloid-β (Aβ) starts cellular dysfunction before it accumulates in extracellular plaques. Grp78 interacts with amyloid precursor protein (APP) and inhibits the secretion of Aβ40 and Aβ42, suggesting that Grp78 might retain APP in the endoplasmic reticulum and protect APP from β/γ-secretase cleavage into Aβ. Tau, a neuronal microtubule binding protein that contributes to microtubule stability in normal condition, is hyperphosphorylated in pathologic conditions and accumulates in the neurofibrillary tangles, which are regarded as a hallmark of AD. At the same time, Hsp27 is reported to bind to a hyperphosphorylated tau variant in human brain samples (13). Down regulation of Hsp70 and Hsp90 using RNA-mediated interference and Hsp90 inhibitor, geldanamycin, induced tau aggregation, and overexpression of these chaperones showed the opposite effect. This suggests that Hsp70 and Hsp90 maintain tau in a soluble, functional conformation and prevent tau aggregation (9). Expression in the brain of Hsp70 and Hsp27, is notable because both proteins are highly inducible in glial cells and neurons following a wide range of noxious stimuli including ischemia, epileptic seizure and hyperthermia (8). In the central nervous system, constitutive expression of Hsp27 is limited to many (but not all) sensory and motor neurons of the brain stem and spinal cord, while there is little or no constitutive expression of Hsp70. However, inducible expression of both Hsp70 and Hsp27 is present in many areas of the brain and retina and is associated with cellular resistance to a variety of insults. Molecular chaperones are involved in most neurodegenerative diseases, providing protection at several levels.

This shows that modulators of chaperone expression can have therapeutic benefits for neurodegenerative diseases.

Heat Shock Protein and Its Role in Cardiovascular Disease

The expression of HSP in the early stages of cardiovascular disease might result from one or a combination of factors. For example, risk factors for atherosclerosis such as hyperlipidemia, diabetes, smoking, and hypertension cause oxidative stress. Oxidative stress, in turn, may lead to the induction of HSP expression in vascular smooth muscle cells (6, 10). In addition, prior research indicates that circulating HSP70 levels predict the development of cardiovascular disease in subjects with established hypertension (6, 12) These authors suggest that HSP70 protects against or modifies the progression of atherosclerosis in this subject group. The evidence is suggesting a protective role of HSP70 for cardiovascular diseases.

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HSP SI CORELATIA CU DIFERITE BOLI

REZUMAT

S-a demonstrat ca exista o corelatie intre proteinele de stres din circulatie (HSP) si anumite maladii in care se observa distrugerea tesutului sau organului tinta. In aceste categorii de boli sunt incluse maladiile cardiovasculare, o larga varietate de cancere, numeroase boli autoimune cum ar fi artrita, diabetul zaharat de tip 1, ateroscleroza, scleroza multipla si altele.

Cuvinte cheie: proteine de stres, cancer, maladii neurodegenerative, boli cardiovasculare

MODERATE ALCOHOL CONSUMPTION AND THE OXIDANTS/ ANTIOXIDANTS IMBALANCE IN RAT PREGNANCY

MIHAELA RUDEANU, ADRIANA MURESAN, SIMONA TACHE, REMUS MOLDOVAN, DOINA DAICOVICIU, NICOLETA DECEA

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ABSTRACT

Alcohol induced oxidative stress (AIOS) and alcohol induced nitrosative stress (AINS) are linked to metabolism of ethanol, involving both mitochondrial and microsomal systems. Low ethanol intake is known to have a beneficial effect on cardiovascular disease; it attenuates hypertension and atherosclerosis. Chronic low ethanol intake confers its beneficial effect mainly through its quality to increase antioxidant capacity and lower advanced glycation end products. Moderate alcohol consumption determined significant decrease of MDA, PC and SH and did not significant increase DH in non pregnant rats. Moderate alcohol consumption determined significant increase of MDA, and PC and significant decrease of DH in prim pregnant rats. Moderate alcohol consumption determined significant increase of MDA, and PC and significant decrease of DH and SH in multi pregnant rats.

Key words: alcohol, oxidative stress, pregnancy, rat

INTRODUCTION

The alcohol (ethanol) is the most widely consumed drug worldwide. The immoderate alcohol consumption is associated with high levels of morbidity, mortality and antisocial actions. A high-dose chronic alcohol consumption induces: iron overhead, alcoholic skeletal and heart myopathy, chronic alcohol mediated liver disease, neurological disorders (14,4,7,12,8,6), alcohol-induced congenital malformations (17), neuro-endocrine alterations in adult offspring (5) and others liver and lung diseases (11,13).

The pathogenic mechanisms that lie between alcohol intake and morbidity involve multiple pathways. Recent studies indicate that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important role. ROS and RNS are able to cause various cellular injuries, such as DNA damage, lipid peroxidation and protein modifications (1,18).

Alcohol induced oxidative stress (AIOS) and alcohol induced nitrosative stress (AINS) are linked to metabolism of ethanol, involving both mitochondrial and microsomal systems. AIOS and AINS are the results of a combined impairment of antioxidant defenses (SOD, CAT, GSH/GSSG, vitamin E and A, Se) and the production of ROS and RNS (3).

Moderate or low chronic alcohol consumption is associated with a lower risk of all-cause mortality and morbidity (8). Low ethanol intake is known to have a beneficial effect on cardiovascular disease; it attenuates hypertension and atherosclerosis. Chronic low ethanol intake confers its beneficial effect mainly through its quality to increase antioxidant capacity and lower advanced glycation end products.

OBJECTIVES

The aims of the present study are to follow the influence of moderate ethanol intake on the balance oxidants/antioxidants in non pregnant female rats and to study the influence of moderate ethanol intake on the balance oxidants/antioxidants in pregnant primipara and multipara female rats.

METHODS

Experimental groups were formed of Wistar female rats, which were used for analysis and divided into six groups (n = 10 rats/group), with a weight of 200-220 grams, as follows:

Group I – control non pregnant female rats
Group II – primipare females
Group III – multipare females
Group IV – non pregnant female rats exposed to alcohol
Group V – primipare females exposed to alcohol
Group VI – multipare females exposed to alcohol

The experiment was made in the Laboratory for experimental exploration at the Department of Physiology of "Iuliu Hatieganu" University in Cluj-Napoca in the period May-June 2008.

Animals were prepared for gestation using Ladosi method (10) adapted for rodents. The alcohol administration consisted in quantities of 0.5 ml 20% alcohol per animal/day by gavages.

Biochemical methods

Several parameters were identified in the serum, including: a) indicators of the oxidative stress (malonaldehyde, fluorimetric Conti method, 1991; carbonylated proteins, Reznick method, 1994), and b) indicators of the oxidative defense (hydrogen donor capacity, Janazewska method, 2002; content of sulphydril groups, Hu method, 1994).

The samples were analyzed in the Laboratory for experimental oxidative stress exploration at the department of Physiology of "Iuliu Hatieganu" University in Cluj-Napoca.

The blood to analyze was taken from the retro orbital sinus of the rats for the control group and the pregnant group (day 21-23), respectively.

Statistical analysis

Results were given as measures of central tendency and dispersion. The t-test

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for equal or unequal variances (the variances were tested with Levene test) was used for comparisons between two means of independent groups. The variables were normal distributed, the normal distribution were tested with Kolmogorov-Smirnov test.

Statistical significance was assessed with p less than 0.05 and two-tailed tests, computed using SPSS 13.0 and Statistica 7.0.

RESULTS

Alcohol exposure in **non pregnant females** (Group IV) (Table I and II) resulted in significant decrease of MDA, PC and SH (in comparison with non pregnant unexposed to alcohol females - Group I); DH values that didn't significantly differ from non pregnant unexposed to alcohol females (Group I).

In **primipare females** (Group V) (Table I and II) alcohol exposure induced significant increase of MDA (in comparison with primipara gestation females unexposed to alcohol - Group II), while the level of MDA was not significantly different from the level of MDA of non pregnant unexposed to alcohol females (Group I); significant increase of PC (in comparison with primipare females unexposed to alcohol - Group II), also increase PC in comparison with non pregnant unexposed to alcohol females (Group I); the values of SH were not significantly different from primipare females unexposed to alcohol (Group II); significant decrease of DH (in comparison with primipare females unexposed to alcohol - Group II), the level of MDA was not significantly different from the level of MDA of non pregnant females exposed to alcohol (Group IV).

In **multipare females** (Group VI) (Table I and II), the following were noticed: significant increase of MDA (compared with multipare females unexposed to alcohol - Group III); significant increase of PC (compared multipare females unexposed to alcohol - Group III), the level of PC was not significantly different from the level of PC of non pregnant females unexposed to alcohol (Group I); significant decrease of SH (compared with multipare females unexposed to alcohol - Group III), the level of SH was not significantly different from the level of SH of non pregnant exposed to alcohol females (Group I); significant decrease of DH (compared with multipare females unexposed to alcohol - Group III), the level of DH was not significantly different from the level of DH of non pregnant females exposed to alcohol (Group IV).

The groups exposed to alcohol (I, II, III) and the groups unexposed to alcohol (IV, V, VI) are summarized in Table I.

The p probabilities (Table II) show significant differences between the groups exposed and unexposed to alcohol.

Table II. The probability p between study groups for the indicators of antioxidants / oxidants scales

Groups	Statistical significance p between groups			
	MDA	PC	SH	DH
Group I _ Group IV	0.001	0.003	0.03	0.06
Group II _ Group V	0.004	<0.0001	0.14	0.002
Group III _ Group VI	0.04	0.003	0.001	0.02
Group I _ Group II	0.007	<0.0001	0.61	0.001
Group I _ Group III	0.00001	0.003	0.002	0.004
Group II _ Group III	0.0001	0.0004	0.0001	0.67
Group IV _ Group V	0.001	<0.0001	0.01	0.16
Group IV _ Group VI	0.42	0.002	0.01	0.29
Group V _ Group VI	0.0004	0.27	0.95	0.88
Group I _ Group V	0.36	0.04	0.62	0.62
Group I _ Group VI	0.0001	0.53	0.62	0.55
Group II _ Group IV	0.21	0.004	0.04	0.01
Group II _ Group VI	0.007	<0.0001	0.20	0.01
Group III _ Group V	0.0001	0.78	0.001	0.049
Group III _ Group IV	0.051	<0.0001	0.0003	0.01

Correlation between the indicators of antioxidants / oxidants scales

Our study set off acceptable correlation between MDA and PC in all studied groups (Table III). But, the correlation was in some groups positive, in others was negative. We can say that the correlation is between size (they grow/shrink in the same way), but there wasn't correlation between direction. The same affirmation we put in evidence between SH and DH (Table III).

Between PC and DH there was an acceptable correlation in 4 from 6 groups (Table III). We can say that PC and DH are significantly correlated (Table IV).

In Table III there is presented the coefficient of correlation Pearson r between the indicators of antioxidants / oxidants scales.

Table I. Descriptive measures of indicators for the antioxidants / oxidants scales in study groups

		Mean	St. dev.	St. error	Conf. interval of 95%				Mean	St. dev.	St. error	Conf. interval of 95%	
Group I	MDA	1.87	0.36	0.11	1.61	2.13	Group IV	MDA	1.27	0.35	0.11	1.02	1.52
	PC	1.58	0.38	0.12	1.31	1.85		PC	1.06	0.31	0.10	0.84	1.28
	SH	0.21	0.06	0.02	0.17	0.26		SH	0.08	0.16	0.05	-0.03	0.20
	DH	32.23	5.03	1.59	28.64	35.83		DH	36.01	3.32	1.05	33.64	38.38
Group II	MDA	1.45	0.24	0.08	1.27	1.62	Group V	MDA	2.06	0.54	0.17	1.68	2.45
	PC	0.66	0.22	0.07	0.50	0.82		PC	1.91	0.29	0.09	1.70	2.12
	SH	0.20	0.04	0.01	0.17	0.23		SH	0.22	0.03	0.01	0.20	0.24
	DH	42.06	6.12	1.93	37.68	46.44		DH	33.34	4.68	1.48	29.99	36.68
Group III	MDA	1.01	0.16	0.05	0.89	1.12	Group VI	MDA	1.17	0.16	0.05	1.05	1.28
	PC	1.09	0.22	0.07	0.93	1.25		PC	1.71	0.49	0.16	1.35	2.06
	SH	0.30	0.05	0.01	0.27	0.33		SH	0.23	0.04	0.01	0.20	0.25
	DH	40.85	6.43	2.03	36.25	45.45		DH	33.71	5.78	1.83	29.58	37.84

Table III. The coefficients of correlation Pearson r between MDA, PC, SH and DH in the study groups

The coefficient of correlation Pearson r	MDA – PC	MDA - SH	MDA - DH	PC -SH	PC - DH	SH - DH
Group I	0.41**	-0.01*	0.03*	-0.58***	-0.17*	0.48**
Group II	-0.50**	-0.21*	0.52***	0.35**	-0.33**	-0.42**
Group III	0.35**	0.15*	0.33**	0.02*	0.05*	-0.44**
Group IV	-0.75****	-0.06*	0.56***	0.17*	-0.61***	-0.49**
Group V	0.43**	0.49**	0.13*	0.05*	-0.32**	0.65***
Group VI	-0.25**	-0.35**	0.32**	0.47**	0.11*	0.51***
* inexistent or weak correlation , ** acceptable correlation, *** good correlation, **** very good correlation (Colton classification)						

The coefficients of correlation Pearson r between MDA, PC, SH and DH in all 6 studied groups are reproduced in Table IV.

Table IV. The coefficients of correlation Pearson r between MDA, PC, SH and DH in all 6 groups

Groups I-VI (n=60)	MDA - PC	MDA - SH	MDA - DH	PC -SH	PC - DH	SH - DH
The coefficients of correlation Pearson r	0.33	-0.04	-0.14	0.13	-0.49	0.05
p	0.01	0.79	0.29	0.31	0.0001	0.72

DISCUSSIONS

During the normal pregnancy an oxidative and nitrosative stress is produced affecting the balance O/AO, O is raised and AO is decreased (15).

The mechanism of SON production in the normal pregnancy are: maternal metabolism and rate of oxygen utilization at the mitochondrial level increase with consecutive production of oxygen radicals, the ischemia-reperfusion caused by increased O_2 consumption, and the physiological hypoxia which produces variation of the pO2 at the placental level.

The endothelial mechanical stress (2) can be explained as increased estrogen and choriogonadotropic hormone levels. Stimulation of the hormonal secretion has a pro-oxidant effect. The sources of SRO and SRN are: maternal, placental and vascular.

The ROS and RNS have **benefic effects** during gestation (16) due to the fact that they control cellular growing, differentiation and transformation into embryo and fetus ($O_2^{\cdot-}$, H_2O_2 (low quantities) ⟶ the regulation molecules ⟶ the transcription factors and gene expression modifications), as well as regulation of vascular tone in fetoplacental blood vessel ($O_2^{\cdot-}$, $NO^{\cdot-}$, $ONOO^-$). ROS and RNS are involved in metabolism of arachidonic acid with endoperoxides and PGI_2 formation, resulting in implications of GC and GMPc system.

The negative effects of SRO and SRN are could be differentiated into: a) cellular destruction and inflammation due to activation of neutrophiles with increase activity of myeloperoxidasis and NADPH oxidation, resulting in increased SRO and increased neutrophils aggregation to the endothelium, ischemia and injuries, and b) teratogen and toxic effects.

The chronic maternal alcohol intake in large quantities and the crossing over to placenta produces the fetal alcoholic syndrome and becomes a risk factor for the embryonic development, fetal development (teratogenic effects) and for the adult descendents (neuroendocrine, pulmonary, hepatic diseases and mental retardation).

The teratogen, neurotoxic and hepatotoxic effects of the maternal alcohol intake induced to the descendents, experimentally proven, can be due to direct peroxidative mechanism of the alcohol or indirect peroxidative mechanism of acetaldehyde (the alcohol metabolite) (11, 13).

Adverse oxidative effects of ethanol-exposed female rats can be ameliorated by antioxidative treatment vitamin E (α-tocopherol) (17).

The data in the literature have shown that antioxidant pretreatment with melatonin and U83836E does not ameliorate alcohol-induced Purkinje cell loss in the developing rat cerebellum (9).

Our experiments show that moderate intake of alcohol on witness non pregnant animals induces a reduction of SO.

Pregnancy in females induced a series of changes, depending on study group (Table II). For **primipare females** (Group II): significant decrease of MDA and PC compared with non pregnant females (Group I); the values of SH were not significantly different from the values of SH of non pregnant females (Group I); significant increase of DH compared with non pregnant females (Group I).

For **multipare females** (Group III): significant decrease of MDA and SH in comparison with non pregnant females (Group I) and, also for primipare females (Group II), significant decrease of PC in comparison with non pregnant females (Group I), but significant increase for the primipare females (Group II), significant increase of DH in comparison with non pregnant females (Group I), but no significant difference compared with primipare females (Group II).

CONCLUSIONS

Moderate alcohol consumption determined significant decrease of MDA, PC and SH and did not significant increase DH in non pregnant rats.

Moderate alcohol consumption determined significant increase of MDA, and PC and significant decrease of DH in primipare rats.

Moderate alcohol consumption determined significant increase of MDA, and PC and significant decrease of DH and SH in multipare rats.

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CONSUMUL MODERAT DE ALCOOL ŞI BALANŢA OXIDANŢI/ ANTIOXIDANŢI ÎN GESTAŢIE LA ŞOBOLANI

REZUMAT

Stresul oxidative (AIOS) şi nitrozativ (AINS) indus de alcool sunt corelate cu metabolismul etanolului, implicând atât sistemul mitocondrial şi microzomal. Aportul redus de etanol este cunoscut a avea un effect benefic în patologia cardiovasculară. Consumul cronic redus de etanol este benefic în principal prin calitatea lui de a creşte capacitatea antioxidantă şi reducerea produşilor finali de glicare. Consumul moderat de alcool a determinat scăderi semnificative de MDA, PC şi SH şi nu a crescut semnificativ DH la şobolanii negestanţi. Consumul moderat de alcool a determinat creşteri semnificative de MDA şi PC şi scăderi semnificative ale DH la şobolani în prima sarcină. Consumul moderat de alcool a determinat creşteri semnificative de MDA şi PC şi scăderi semnificative ale DH şi SH la şobolanii cu sarcini multiple.

Cuvinte cheie: alcool, stress oxidative, sarcină, şobolan

OXIDANT/ANTIOXIDANT RATIO: A PREDICTOR FOR A LOCAL POSTOPERATIVE WOUND EVOLUTION

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ABSTRACT

Surgical procedures often unbalance the organic systems, leading to a tendency to one or many others disequilibrium. Besides the operative intervention itself and many well-known clinical risk factors, including immobility, infections, a.s.o. there are various other perioperative factors that are being demonstrated to interfere with all the systems, of organism such as hypothermia, metabolic acidosis, fluid-coagulant imbalance, volume expanders, decreasing of antioxidant capacity of plasma and the receptivity to therapy. The first several hours after surgery there are marked increases to a hypercoagulable and hypofibrinolytic state and increasing of reactive 02 species (ROS). The levels of mediators released in this period, fluctuates rapidly and their degree of perturbation is dependent not only on the type, degree and duration of surgery, but also on the timing of blood collection. We have tried to introduce a new method of laboratory, research, proposed by Oczan E (12), to reveal the role of antioxidant capacity of plasma, in a dynamically evolution.

Key words: oxidants, wound healing, antioxidant capacity of plasma

INTRODUCTION

Early phase of reactivity, in post-anesthetic-surgical stress, to surgical patients, contributes significantly, to create the wound healing and the typology of morpho-clinical evolution. The interindividual variability response to surgical stimuli is profound, because of genomic variations within most of genes, in each patients, every gene encoding proteins, involved in signal transduction, in inflammatory process (Toll receptors), implicated in surgical wound extent and in individual cellular response to injury. Clinical significance of genomic markers remain to be tested, and may be used to participate to elect the strategy of therapeutic management into the future. Many authors (14,15) consider that individual genotype is implying and is strongly influenced by the balance between the two systems [oxidants (O) / antioxidants (Ao)], which has a crucial role upon the cellular cycle and cellular homeosthasis, both, interacting and inter-influencing the wound healing. Because of these effects (9,14,15) it is appreciated that to modulate the oxidative stress, or the antioxidant capacity of serum, become one of the best therapeutic manner, for obtaining an efficient wound healing. It is known that the oxidants/ antioxidants systems

are interactive, so the potent free radical reactions, starting with OH_, do not end in only a one-step reaction; generally, they continue, even forming a free radical chain reaction, and antioxidants prevent the prolongation of this kind of reactions. To show the antioxidant responses of the antioxidant components of serum, against the ascendant potent free radical reactions, is one of the major pathophysiological mechanism of organism defense, which could be included through the therapeutic objectives of surgical patients' management.

Aims of the study were the following: 1.To evaluate the kind of wound healing (per primam, per secundam, a.s.o.) in postoperative period, to surgical patients, who received antioxidants, associated to conventional therapeutic schema; 2. To observe if the reactive oxigen species (ROS) increase production in operated region and could induce, at a distance, into the healthy ("naïve") vascular endothelium, the same alterations; 3. To evaluate the quality of a novel parameter offered, by using an indirect performant and automated bioquantitative assay, proposed by Oczan Erel (12); 4. To estimate the quality of tissular response, during the period of wound healing process, correlated to O/Ao equillibrium, before the appearance of any perturbation, at a distance, and may be to block this one, by means of a locally therapy.

MATERIALS AND METHODS

Our clinical study was made upon: healthy subjects, volunteers who gave us, their acceptance and surgical patients from the Plastic and Reconstructive Surgery, from the Hospital nr.1 Craiova, UMF Craiova. The study was approved by Ethics Commitee of UMF Craiova. The study included: 60 patients, age: 40-60 years, submitted to a mild anesthetic surgical stress, divided into four groups: group A:10 cases(17%), control, healthy volunteers, group B: 28 cases (48%): with localised focus of bacterial infection, or infectiouse complications, at risk, group C: 12 cases (20%): several with multi-organic-dysfunctions (MODS) and failure (MOF), group D:10 cases (17%), with SIRS: 6 cases: septic shock (2 cases) and death (2 cases). For critically ill patients the scores: SOFA and APACHE, were calculated. From each group we have determined, from blood (serum) the values of circulating proinflamatory cytokines (IL1, TNF alfa), C reactive protein (CRP), procalcitonin (PCT), coagulabillity status of plasma, leukocytes, thrombocytes number, total antioxidant response (TAR), or total antioxidant capacity of plasma (TAOP). The study was approved by Ethical Committee of University of Medicine and Pharmacy and Hospital no.1 of Craiova.

Technique

Chemicals: Ferrous ammonium sulfate, ortho-dianisidine dihydrochloride (3-3V-dimethoxybenzidine), vitamin C (L(+) ascorbic acid), bilirubin, uric acid, reduced glutathione (GSH), (F)-catechin, 5,5V-dithiobis-(2-nitrobenzoic acid)

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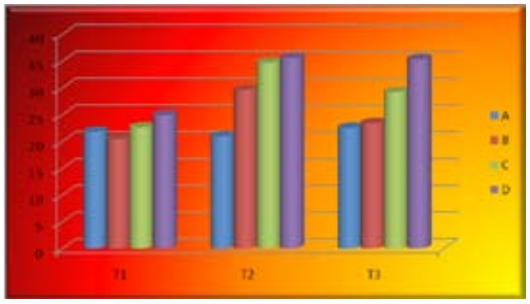

Fig.1. Plasma total peroxide µmol. H2O2.($p < 0.001$)

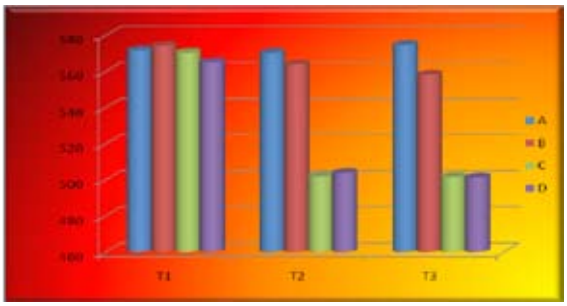

Fig.2. Plasma TAOP: µmol.Trolox equiv/l ($p<0.001$)

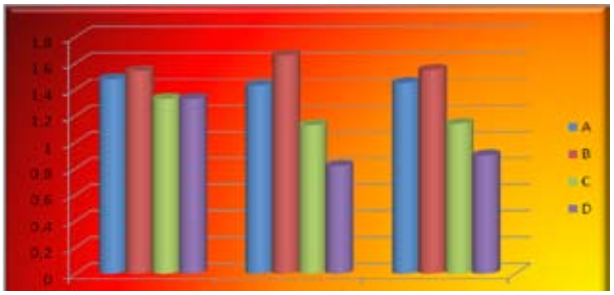

Fig.3. TAR: mmol.Trolox equiv/l

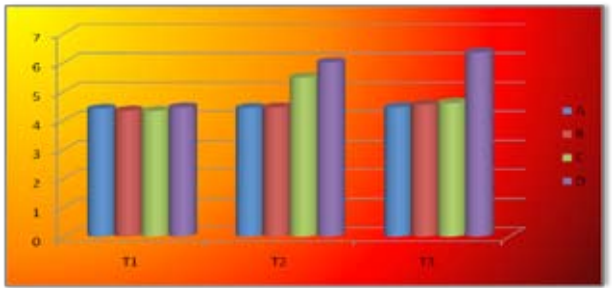

Fig.4. OSI ($p<0.001$)

(DTNB), ethylenediaminetetraacetic acid (EDTA), 2,2V-azino-bis (3- ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulfate, glucose, ribose, saccharose and sodium citrate were purchased from Sigma Co. and Merck Co. The watersoluble analogue of vitamin E (Trolox; 6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid) was from Sigma- Aldrich Chemical Co. All chemicals were ultra pure grade and type I reagent grade deionized water was used. Venous blood samples obtained were collected into tubes (and heparinized tubes) and serum (and plasma) was separated from cells by centrifugation at 1500 _ g for 10 min. Serum (and plasma) samples were run immediately or stored at _80jC. Antioxidants: stock solutions (1.0 mM) of ascorbic acid, glutathione and (F)-catechin were separately prepared in saline solution (0.9% NaCl). Uric acid and solid bilirubin were dissolved in 10 mM NaOH solution. Trolox was dissolved in phosphate buffer (10 mM, pH 7.4). Apparatus: A Cecil 3000 spectrophotometer with a temperature controlled cuvette holder (Cecil), and an Aeroset automated analyzer (Abbott). Assay principle of the method: a standardized solution of Fe2+–o-dianisidine complex reacts with a standardized solution of hydrogen peroxide by a Fenton-type reaction, producing OH_. These potent ROS oxidize the reduced colorless o-dianisidine molecules to yellow-brown colored dianisidyl radicals at low pH. The oxidation reactions progress among dianisidyl radicals and further oxidation reactions develop. The color formation is increased with further oxidation reactions. Antioxidants in the sample suppress the oxidation reactions and color formation. This reaction can be monitored by spectrophotometry. Assay calibration: the suppression of the color formation is calibrated with Trolox, which is widely used as a traditional standard for TAR measurement assays, so the results in this assay are expressed as in terms of millimolar Trolox equivalent per liter. Oczan Erel (12).

RESULTS

Morphological and clinical aspects of surgical wounds showed the three kinds of healing: per primam, per secundam and local complications associated with systemic bad evolution.

Cellular edema was the most important clinical sign, which was correlated to a lower TAR level. We observed the appearance of the resistance to antibio-or/ and corticoid therapy, in T2 period to cases from C, D groups; the last group, non- receiving to antioxidant postoperative exogenous therapy, didn't receive antioxidants, before intervention.

TAR and TAOP values were different in control, face to group B, receiving "per os" fruits, vegetables, rich in antioxidant polyphenolic compounds, in the first 4- 12 hours, postoperative. The total antioxidant capacity of group B was little increased, face to group A. For C-D groups, a great potent free radical reactions were dominant, an implication of plasma antioxidant capacity to assure the balance between oxidants/antioxidants, was associated to a low TAR serum level. (Figures 1, 2, 3, 4)

PCT values rise earlier, before CRP and CK, and have a high specificity, being a true marker for patients with multiorgan dysfunctions and failure. We appreciated that the increasing of plasma concentrations of CRP is associated to minor infections, but do not adequately reflected the severity of infections.

Hipercoagulability was common, in postoperative status in 70% of cases, from B, C and D groups. We observed also, great fluctuations of thrombocytes count, in group D, and considered the decreasing of their value as a dilutional thrombocytopenia, not as a direct effect of oxidants upon the leukocytes, thrombocytes and/ or erhytrocytes.

DISCUSSION

After tissue injury, produced by programmed trauma (surgical act) or activation of inflammatory defence reaction, the endothelial cell properties may be reversed. The endothelium properties of endothelial cells are due to three types of activities: a) control of vaso-regulation, by the release of vasomotor components as endothelin ET-1 prostacyclin (PGI2) and nitrite oxide (NO); b) anti-thrombotic, anticoagulant properties, such as the capture and degradation of thrombogenous substances (ADP, 5-HTP) and through the effects of active products on platelets; c) anti-adhesive properties, exerted by endothelial cells, express adhesion molecules, that can be modulate by mechanical or biochemical stimulations. Variations in local shear and oxidative stress may also modify the secretion of vasomotor substances and the postoperative locally evolution could take any way. Reactive oxygen species (ROS) are produced in metabolic and physiological processes, and oxidative reactions occurred in organisms, remove them via enzymatic and non-enzymatic antioxidative mechanisms. In intra-and postoperative periods, the tissues'incisions

create the conditions for apoptosis and also, to shift the oxidative/ antioxidative balance, toward the oxidative status. ROS are responsible for the oxidative injury of biomolecules, indirectly, by producing OH_ via Fenton reaction and/or iron-catalyzed Haber–Weiss reaction. Oxidized molecules have the capacity to form new radicals, leading to radical chain reactions that consume the antioxidant capacity of plasma. Decreasing of antioxidant molecules generates the disequilibrium oxidants/ antioxidants and creates the conditions to amplify the intracellular force of contraction that could dislocate the cytoplasm from the cell membrane. The intercellular gaps and cross bridging disappear, so the hydrostatic and osmotic gradient direct the circulation of water, through extracellular to intracellular space and in opposite sense, making possible a massive cellular death and blocking the wound efficient healing. This status of irreversibility and dezorganisation could be explained by means of the activation of an autocrin and paracrin mechanism from the altered endothelial cells (EC), which is transferred and extended at a distance.

Our results sustain that the mechanism generating the local and at a distance cellular destruction, is represented by the alteration of antioxidant capacity of circulatory components, differentiated, from one region to another. Because the measurement of different antioxidant molecule separately, is not practical and antioxidant effects of them are additive, actually, total antioxidant response (TAR) of a sample is measured and the result obtained is defined and recognized by the following names: total antioxidant capacity, total antioxidant activity, total antioxidant power, total antioxidant status. By means of the new parameter proposed (12), it could be evaluated the capacity of tissues to resist to injury or to be sensible to the applied therapy. Understanding the basic mechanisms underlying the locally wound healing failure, suggests that efficient therapies have to be developed, and applied early, after pathophysiological principles, before the appearance of the irreversible changes, and to reduce the death rates. Rau B and Steinbach G (13) sustain the predictive power of TAOP and PCT, and consider these tests to be almost equal, to that of fine needle biopsy-as the gold standard.

CONCLUSIONS

The novel parameter obtained by using an indirect performant and automated bioquantitative assay, proposed by Oczan Erel, is sensible, and may reflect the real tissular response, during the period of wound healing process.

Correlated to a local O/Ao disequilibrium, before the development of edema, or in the first 4 hours postoperative, TAOP could be used to select an adequate therapy, to block a bad evolution of wound.

The two periods of endogenous antioxidants rising level (at 4 and 24 hours postoperative), showed the favorable role of the adaptive mechanism of the organism sustaining the favorable effect of surgical intervention.

Early prognosis about the wound evolution, elaborated in immediate postoperative period, associating the exogenous antioxidants to conventional therapeutic schema, could prevent the wound evolution toward non-healing.

The final conclusion is those that to use the combinative therapies, according with the pathophysiological mechanisms, and especially with their pivotal expression: the lowering of TAR.

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RAPORTUL OXIDANȚI/ANTIOXIDANȚI: UN PREDICTOR AL EVOLUȚIEI SPRE VINDECARE A PLĂGII POSTOPERATORII

REZUMAT

Actul chirurgical este definit ca un stres sau o trauma programata, efectuata in favoarea pacientului, cu acordul acestuia. Postoperator, reactia de aparare a organismului fata de trauma se insoteste de tendinta la dezechilibrare a homeostaziei celulare, care se reflecta in alterarea procesului de vindecare a plagii chirurgicale. In afara interventiei chirurgicale, care stimuleaza direct apoptoza, sunt activati o serie de factori, care interfereaza cu subsistemele organismului, pe unele dintre aceste, putandu-le deregla: termoreglarea, echilibrul fluido-coagulant, oxidanti/antioxidanti, acido-bazic, etc. Se stie ca in primele ore postoperator, se produce o stare de hipercoagulabilitate si cresterea producerii speciilor reactive de O2. Nivelul mediatorilor eliberati in aceasta perioada, este oscilant iar gradul perturbarii este dependent nu doar de durata si intensitatea actului operator dar si de momentul si de tehnicile de recoltare a probelor de sange. Studiul nostru a incercat sa introduca o metoda performanta de laborator, cu ajutorul careia sa se evidentieze nivelul seric al capactitatii oxidante a serului apreciindu-se si rolul central al acestei valori, in cadrul reactiei de aparare tisulara, postinjurie.

Cuvinte cheie: oxidanti, vindecarea plagii, capacitate antioxidanta a plasmei

CORRELATIONS BETWEEN OXIDATIVE STRESS AND ENDOTHELIN-1 LEVELS IN PATIENTS WITH RHEUMATOID ARTHRITIS

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ABSTRACT

BACKGROUND. Rheumatoid arthritis (RA), a systemic chronic autoimmune inflammatory disease, is associated with excessive cardiovascular mortality, which cannot be explained by the traditional risk factors alone. There is growing evidence that inflammation plays an important role in the initiation and progression of atherosclerosis. One of the hypotheses incriminates systemic inflammation in inducing early endothelial dysfunction. Rheumatoid arthritis is also associated with an overproduction of oxygen reactive species and there is data about their involvement in the decrease of endothelial nitric oxide synthase (eNOS) activity. PURPOSE. We decided to investigate the endothelial function and the levels of vascular oxidative stress in patients with RA, in order to highlight the fact that the overproduction of reactive oxygen species (ROS) is involved in the development and progression of atherosclerosis in these patients as well as in the excessive cardiovascular mortality. METHODS. Twenty RA patients with a mean age of 50.7, with ACR 50 response to DMARD'S by the ACR criteria, were enrolled. They had a mean disease duration of 6.5 years and had no traditional cardiovascular risk factors except the age. Twenty healthy age matched volunteers were enrolled to form the control group. Pulse wave velocity (PWV) was measured in all subjects. Serum levels of endothelin-I, lipid peroxides (LP), carbonylated proteins (CP), ceruloplasmin (C) and donor hidrogen ability (DHA) were determined in all subjects. RESULTS. Results (mean±SD) in patients with RA and healthy patients, respectively, were as follows: PWV was significantly increased ($P<0,001$), correlated with a significantly increased production of endothelin-I ($P<0,001$), LP ($P<0,01$) and CP ($P<0,001$) and a significant decrease of serum antioxidant capacity: C ($P<0,01$) and DHA ($P<0,001$). CONCLUSIONS. Patients with RA have an altered endothelial activity and increased oxidative stress levels which aggravate the endothelial dysfunction, and this should be a powerful enough reason to initiate a long-term, efficient therapy, in order to reduce cardiovascular mortality.

Key words: Rheumatoid arthritis, endothelin-I, oxidative stress, endothelial dysfunction, pulse wave velocity.

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic disease characterized by persistent inflammation of multiple joints. RA is associated with an increased mortality from cardiovascular causes, which exceeds that of the general population (1, 2, 3). Recent reports demonstrate that endothelial function is reduced in RA patients with high inflammatory activity (4, 5, 6). Although the underlying mechanisms for endothelial dysfunction in RA are poorly understood, it is postulated that systemic inflammation may be involved in the early vascular damage (7, 8, 9). In support of this hypothesis, anti–tumor necrosis factor (anti-TNF) therapy has been shown to improve endothelial dysfunction in patients with active RA (6, 10).

The endothelium plays a pivotal role in the regulation of vascular tone and structure through the release of various vasoactive agents, such as vasodilators and vasoconstrictors. It has been recognized that alterations of endothelial function are involved in the development and progression of atherosclerosis and its clinical complications (11).

Endothelial dysfunction is actually considered to be a reduced bioavailability of nitric oxide (NO), which is a major endothelium-dependent vasodilator. Endothelium dependent NO is also known to have other anti-atherosclerotic properties, including the inhibition of cell growth, leukocyte adhesion, and platelet adherence and aggregation. Regulatory mechanisms that control vascular NO bioavailability in pathophysiologic states are complex. It has been reported that vascular production of reactive oxygen species (ROS), such as superoxide (O^-_2), is increased in hypertension, atherosclerosis, or diabetes (12). O^-_2 reacts rapidly with NO, resulting in the formation of peroxynitrite ($ONOO^-$), which could lead to a loss of bioactivity of NO. The increased oxidative stress may affect the synthesis of NO. Vascular NO is mainly synthesized by endothelial NO synthase (eNOS) from the precursor L-arginine.

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However, previous data demonstrate that increased oxidative stress promotes a dysfunctional eNOS, which generates O^-_2 instead of NO (13). This dysfunctional enzyme is termed uncoupled eNOS.

Endothelins are potent 21 amino acid vasoconstrictor isopeptides produced in different vascular tissues, including vascular endothelium. Endothelin-I mediated vasoconstrictor tone increases with age and contributes to the pathogenesis of hypertension (14).

Our aim in this study was to bring new data concerning the correlation between ROS production and endothelin-I levels in the progression of endothelial dysfunction in patients with RA.

MATERIALS AND METHODS

Twenty RA patients with a mean age of 50.7, with ACR 50 response to DMARDs by the ACR criteria, were enrolled. They had mean disease duration of 6.5 years and had no traditional cardiovascular risk factors, except the age. Twenty healthy age-matched volunteers were enrolled to form the control group.

The subjects included in the study were divided into 2 groups:

-1st Group- Control group, 20 age-matched healthy patients;

-2nd Group- 20 patients with RA.

For all the subjects included in the study, endothelial dysfunction was assessed by measuring the pulse wave velocity (PWP) between common carotid artery and radial artery, by means of a COMPLIOR device. Serum levels of endhothelin-I (by ELISA method) were determined in all subjects. The oxidative stress was assessed using the following markers: lipid peroxides (LP, by Satoh method) (15) and carbonylated proteins serum levels (CP, by Reznick method) (16)- for the free radicals; ceruloplasmin (C, by Ravin method) (17) and hydrogen donor ability (HDA, by Hatano method) (18)- for the antioxidant capacity.

Statistical validation of our results was made by standard deviation method and student test. All the studies were conducted in accordance with the National Institute of Health and the experiments were carried out as per the Institutional Ethics Committee.

RESULTS

Figure 1 shows the PWP in patients with RA comparatively with the control group. A significant increase of PWP can be observed in patients with RA (the second group).

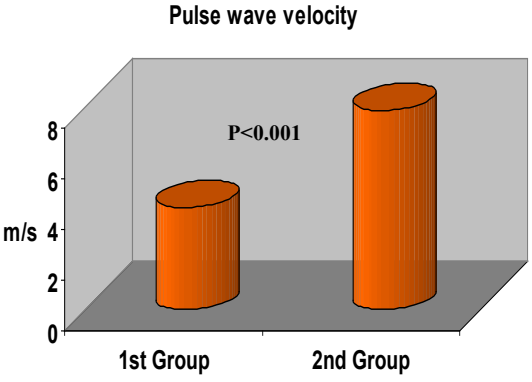

Fig.1. PWV was significantly increased (P<0.001) in the 2nd Group (7.89±0.98 m/s), as compared to the 1st Group (4.04±0.16 m/s).

Figure 2 shows a significant increase of endothelin-I serum levels in patients with RA (group number 2) comparatively to the control group.

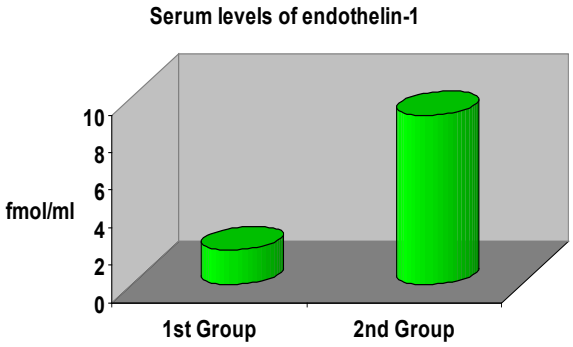

Fig.2. Serum levels of endothelin-I were significantly increased (P<0.001) in the 2nd Group (9.01±0.98 fmol/ml), as compared to the 1st Group (1.81±0.83 fmol/ml)

Figures 3 and 4 show the levels of free radicals: lipid peroxides and carbonylated proteins levels in the serum of control and experimental group. A significant increased oxidative stress was observed la pacientii cu RA.

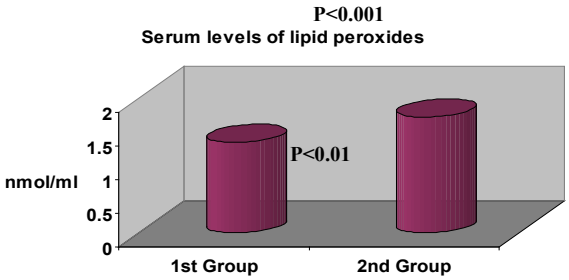

Fig.3. LP were significantly increased (P<0.01) in the 2nd Group (1.73±0.34 nmol/ml), as compared to the 1st Group (1.35±0.2 nmol/ml)

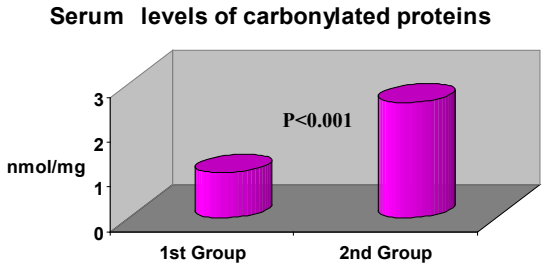

Fig.4. CP were significantly increased (P<0.001) in the 2nd Group (2.58±0.34 nmol/mg), as compared to the 1st Group (1±0.2 nmol/mg)

Figures 5 and 6 show the activities of ceruloplasmin and hydrogen donor ability of control and experimental groups. A significantly decreased oxidative stress was observed in RA patients.

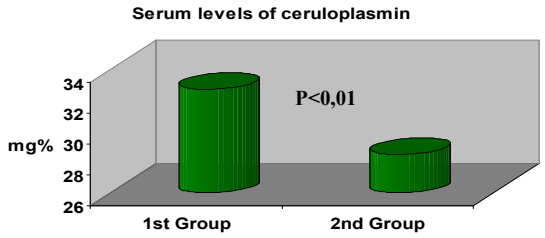

Fig.5. Ceruloplasmin was significantly decreased (P<0.01) in the 2nd Group (28.5±8.74 mq%), as compared to the 1st Group (32.73±4.9 mq%)

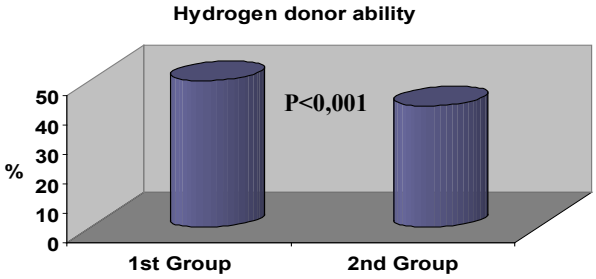

Fig.6. HDA was significantly decreased (P<0,001) in the 2nd Group (41.4±10.5 %), as compared to the 1st Group (50±1.5%)

DISCUSSIONS

Endothelial dysfunction has been associated with a number of pathophysiological processes. Oxidative stress appears to be a common denominator underlying endothelial dysfunction in cardiovascular diseases. Superoxide anion, the hydroxyl radical (·OH), H_2O_2, and peroxynitrite ($ONOO^-$) are produced in the vessels under both normal and stress conditions such as inflammation, injury and aging. Reactive oxygen species promote the contraction of vascular smooth muscle cells by facilitating the mobilization of calcium and increasing the sensibility of the contractile proteins to calcium ions (19).

Aging, hypertension, endothelial injury, as well as dyslipidemia (in particular hypercholesterolemia), smoking and increased body mass index are major risk factors for the development of atherosclerosis. They are all associated with increased oxidative stress levels and endhothelial dysfunction (20, 21, 22).

Endothelin-1 is the main endothelin generated by the endothelium and probably the most important in the cardiovascular system. Endothelin-I acts through specific receptors termed ET(A), represented only on smooth muscle cells and having the functions of growth promotion and contractions mediator, and ET(B), located both on smooth muscle cells, where they evoke contractions, and on endothelial cells, inducing relaxation through the production of the endothelium-derived relaxing factor nitric oxide. In physiological conditions endothelin-1 administration causes vasodilation and vasoconstriction at low and high concentrations, respectively (14).

There is substantial evidence supporting the fact that ROS and RNS (nitrogen reactive species) contribute to the initiation of RA lesions. These active toxic products can be produced through different mechanisms by the neutrophils, chondrocytes, and panos macrophages as well as by the xantin-oxidase in the synovial membrane through the hypoxia-reperfusion process (23).

Oxidative stress in the articulation becomes apparent both because of an increased production of ROS and RNS, and because of a decrease in the antioxidant activity, which cannot fight the oxidative aggression on the synovial liquid, synovial membrane and other articular components, including chondral and bone structures. There are mechanisms which can limit these processes, but they are perturbed and diminished by hypoxia and intrasynovial acidosis, which increase the formation of ROS and RNS.

Our study confirmed the fact that ROS and the concomitant decrease of the antioxidant capacity, correlated with an increased endothelin-I production may play an important role in the RA endothelial dysfunction.

CONCLUSIONS

Patients with RA have a significantly increased pulse wave velocity, as compared to the control group, reflecting the presence of endothelial dysfunction.

The serum level of endothelin -I is significantly higher in patients with RA comparatively to the control group, therefore contributing to the increase of endothelial dysfunction.

We have noticed a significantly increased generation of LP and CP and a decrease in the HDA and plasma level of ceruloplasmin, as markers of overproduction of reactive oxygen species in patients with RA.

Antioxidants will be an important option for a better management of endothelial dysfunction in patients with RA.

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CORELAȚII ÎNTRE STRESUL OXIDATIV ŞI NIVELUL ENDOTELINEI- I LA PACIENȚII CU POLIARTRITĂ REUMATOIDĂ

REZUMAT

INTRODUCERE. Poliartrita reumatoida (PAR), o boala inflamatorie cronica sistemica autoimuna, este asociata cu o mortalitate cardiovasculara excesiva, care nu poate fi explicata doar prin factorii de risc traditionali. Exista tot mai multe dovezi care sustin faptul ca inflamatia joaca un rol esential in initierea si progresia aterosclerozei. Una din ipoteze incrimineaza inflamatia sistemica in inducerea disfunctiei endoteliale. Poliartrita reumatoida este de asemenea asociata cu o supraproductie de specii reactive ale oxigenului (ROS, si exista informatii privind implicarea acestora in scaderea activitatii NO sintetazei endoteliale (eNOS). OBIECTIVE. Am decis sa investigam functia endoteliala si nivelurile stresului oxidativ vascular la pacientii cu PAR, pentru a evidentia faptul ca supraproductia de ROS este implicata in dezvoltarea si progresia aterosclerozei la acesti pacienti si, prin urmare, determina mortalitatea cardiovasculara excesiva intalnita in aceste cazuri. METODE. Au fost inclusi in studiu 20 de pacienti cu PAR avand o medie a varstei de 50,7 ani, cu raspuns ACR 50 la DMARD conform criteriilor ACR. Ei aveau o medie a duratei bolii de 6,5 ani si nu au avut alti factori de risc traditionali, cu exceptia varstei. Al doilea grup, grupul martor, a fost format din 20 de voluntari sanatosi. A fost masurata capacitatea undei de puls (PWV) la toti subiectii inclusi in studiu. Au fost determinate nivelurile serice ale endotelinei-I, ale peroxizilor lipidici (LP), proteinelor carbonilate (CP), ceruloplasminei(C) si capacitatea donarii de hidrogen (DHA) la toti subiectii. REZULTATE. Rezultatele (media±S) la pacientii cu PAR si, respectiv, la grupul martor, au fost dupa cum urmeaza: PWV a fost semnificativ crescuta (P<0,001), corelata cu o crestere semnificativa a productiei de endotelina-I (P<0,001), LP (P<0,01) si CP (P<0,001) si cu o scadere semnificativa a capacitatii antioxidante serice C (P<0,01) si DHA (P<0,001). CONCLUZII. Pacientii cu PAR au o activitate endoteliala alterata asociata cu o crestere a stresului oxidativ care agraveaza disfunctia endoteliala, iar acest lucru ar trebui sa fie un motiv suficient pentru initierea unei terapii eficiente pe termen lung, cu scopul de a reduce mortalitatea de cauza cardiovasculara.

Cuvinte cheie: Poliartrita reumatoida, endotelina-I, stres oxidativ, disfunctie endoteliala, velocitatea undei de puls.

PREVALENCE AND RESISTANCE TO ANTIBIOTICS IN ORAL VIRIDANS STREPTOCOCCI

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ABSTRACT

Aims: This purpose of this study was to determine the prevalence of species of oral viridans streptococci and their resistance to antibiotics.

Method: Isolation was carried out on blood agar 5%. The identification of the viridans streptococci species was made using API system (20 biochemical tests). Susceptibility to antibiotics was tested by disk-diffusion method Kirby-Bauer.

Results: Of the 54 samples collected from dental plaque, carious lesions and throat swab were isolated in 47 strains of viridans streptococci. Their distribution was: 29.78% *S. mitis*, 27.65% *S. mutans*, 14.89% *S. sobrinus*, 10.63% *S. oralis*, 8.51% *S. salivarius*, 4.25% *S. anginosus* and 4.25% *S. sanguis*. In terms of resistance to antibiotics, the order was: Ciprofloxacin (46.8%), Tetracycline (40,4%), Gentamicin (27.6%), Erythromycin (25,5%), Clindamycin (21,2%), Penicillin (17%), Doxycycline (15%) and Ampicillin (10,6%). It was not reported any resistant strain to Vancomycin.

Conclusions: Although viridans streptococi are part of the normal flora of the oral cavity which provides colonization resistance at this level, preventing colonization of other pathogenic species, they nevertheless represent a reservoir of the determinants of acquired resistance that can transmit these species (*S. pyogenes, S. pneumoniae and S. agalactiae*).

Keywords: dental plaque, cavities lesions, antibiotics resistance, viridans streptococci.

INTRODUCTION

Oral viridans group streptococci are part of the normal flora of the mouth which adheres to different surfaces providing oral mucosal colonization resistance and inhibiting bacterial colonization of other pathogens species. At this level, these microorganisms occur at the rate of 28% in dental plaque, 29% crevicular fluid, 45% on the tongue and 46% in saliva. Oral species viridans streptococci have a very different distribution in the mounth. Thus, Streptococcus salivarius prevails on the surface of tongue, while Streptococcus mitis is more prevalent at the mucosal of mouth. Streptococcus mutans and Streptococcus anginosus meet particularly in area teeth surfaces (8,9).

Dental plaque is initially colonized with Streptococcus sanguis and Streptococccus mitis, like supragingival dental plaque with Streptococccus gordonii and the subgingival dental plaque with Streptococcus anginosus and Strepococcus salivarius. Streptococcus mutans and Streptococcus sobrinus are the species most commonly isolated in carious lesions and dental plaque. The species often involved in infections in patients with neutropenia are Streptococcus oralis, Streptococcus mitis, Streptococcus salivarius and Streptococcus anginosus. Anyway their presence in the blood can sometimes be associated with severe infections like bacterial endocarditis especially in pacients with valvular prostheses where Streptococcus sanguis, Streptococcus mitis, Streptococcus mitis, Streptococcus oralis and Streptococcus gordonii are often isolated (2,6,11).

MATERIAL AND METHOD

54 samples have been collected from healthy children and young people aged between 4 and 18 years. The samples were collected in a private Orthodontics Pedodontics dental office in the period November 2007 - March 2008. Sampling was collected with sterile swabs, which subsequently were suspended in Stuart carriage medium. The samples were collected on the throat swab, supragingival dental plaque and dental caries.

Table I. Distribution of samples collected by age group

Collected samples	Age			Total
	4-8 years	8-12 years	18 years	
Throat swab	6	6	6	18
Dental plaque	6	6	6	18
Dental caries	6	6	6	18
Total	18	18	18	54

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The samples were processed in the Laboratory of Bacteriology Department of UMF "Victor Babes" Timisoara, and in the private laboratory for medical analysis of Timisoara.

In the first day, the swabs shown areas with sheep blood agar 5%, which were then incubated at 37° C for 24h in 5% CO_2. The next day colonies suspected viridans streptococci were prick out in the Columbia agar +5% sheep blood sectors and then after 24h at 37° C in 5% CO_2 were identified the species taken in the study.

Identifying the oral viridans group streptococci has been made on the morpho-tinctorial character (gram positive in chain coccal bacteria), cultural character (colony with small dusty area haemolysis alpha-green) and metabolic character, using API 20 strep galleries (bio Merieux): 20 biochemical tests – making the reading at 4h after incubated at 37°C (1, 5).

Subsequently, the viridans streptococci species isolated were tested for anti-bacterial susceptibilities. For interpretation of the antibiogram testing of viridans streptococci strain disk diffusion by the method Kirby-Bauer and breakpoints procedures were used according to NCCLS (National Committee for Clinical nLaboratory Standard) standard and criteria. The viridans streptococci strains were tested against 9 antibiotics: Penicillin (P), Ampicillin (AM), Erythromycin (E), Clindamycin (CM), Tetracycline (TE), Doxycycline (DO), Gentamicin (GM), Ciprofloxacin (CIP) and Vancomycin (VA) (3).

RESULTS AND DISCUSSION

From the 54 samples collected were isolated a number of 47 strains of viridans streptococci belonging to 7 species: ***S. mitis, S. mutans, S.anginosus, S. oralis, S. salivarius, S. sanguis*** and ***S. sobrinus.***

Most strains of viridans streptococci were isolated as expected, in dental plaque: 23 strains (48.93%) and then throat swabs 16 strains (34%) and a lower number of carious lesions, only 8 strains (17.02%).

From the pharyngeal swabs, it were isolated 8 strains of viridans streptococci representing 17.02% of total strains isolated from the 35 children taken into the study.

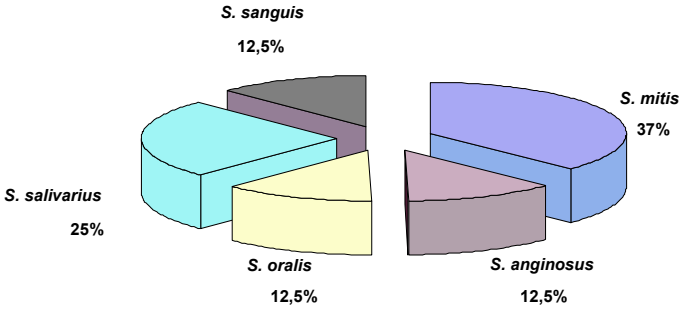

Fig. 1. Distribution of viridans streptococci ssp. isolated from throat swabs

In terms of age group, most strains of viridans streptococci isolated from throat swabs were from the children aged 4-8 years: 3 strains represented by ***S. mitis,*** 2 strains ***S. salivarius*** and which 1 strains by ***S. oralis, S. anginosus*** and ***S.sanguis.***

In dental plaque were isolated 23 strains of viridans streptococci representing 48.93% of all strains (47 strains).

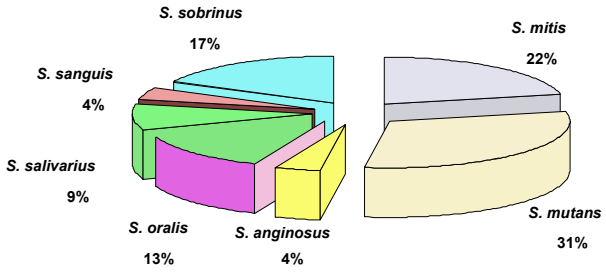

Fig. 2. Distribution of viridans streptococci ssp. isolated from dental plaque

Most viridans streptococci were isolated from children aged between 8-12 years and 18 years younger (8 strains). Large numbers of viridans streptococci isolated from these age group is explained by a greater amount of dental plaque present at the oral cavity and possibly impercipient hygiene from this young compared with other children where the parents probably education in the process of bringing their say.

Table II. Prevalence viridans streptococci in samples collected

Viridans streptococci ssp..	Evidence collected						TOTAL strains	
	Throat swab		Dental plaque		Dental caries			
	No strains	Frequency (%)	No strains	Frequency (%)	No strains	Frequency (%)	No strains	Frequency (%)
S. mitis	3	37.1	5	21.73	6	37.25	14	29.78
S. mutans	-	-	7	30.43	6	37.25	13	27.65
S. anginosus	1	12.5	1	4.34	-	-	3	4.25
S. oralis	1	12.5	3	13.04	1	6.25	5	10.63
S. salivarius	2	25	2	8.69	-	-	4	8.51
S. sanguis	1	12,5	1	4,34	-	-	2	4.25
S. sobrinus	-	-	4	17.39	3	18,75	7	14.89
Total viridans streptococi ssp.	8	100	23	100	16	100	47	100

In the carious lesions were isolated but only 16 strains of viridans streptococci, representing 34.04% of all strains isolated (47 strains).

A number of studies appreciates that have identified 10 areas that are in good correlation with viridans streptococci mainly ***S. mutans***, of wich 5 are in the hindquarters interproximale face and 5 in the vestibular.

Also other tests have shown that approximal and interproximale regions and fissures areas is a rich reservoir of ***S. mutans,*** because in these areas are less affected by the methods of oral hygiene.

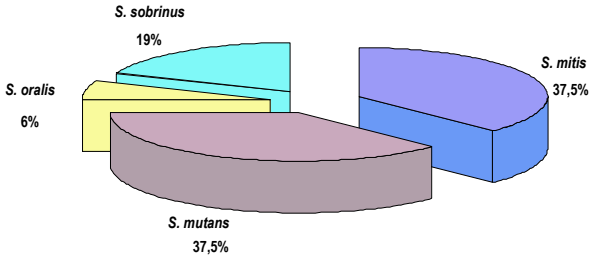

Fig. 3. Distribution of viridans streptococci ssp. isolated from dental caries

After antibiograms for the 47 strains of viridans streptococci isolated and identified in samples collected in the past to determine sensitivity to antibiotics in the 9 to do the testing.

Viridans streptococci resistance to antibiotics is a phenomenon acquired and is transmitted by determinants of resistance (plasmids), mostly as a consequence of the administration of antibiotics.

Resistance of 47 strains of viridans streptococci isolated to antibiotics has been tested in the following order: Ciprofloxacin (46.8%), Tetracycline (40.4%), Gentamicin (27.6%), Erytromycin (25.5%), Clindamycin (21.2%), Penicillin (17%), Doxycycline (15%) and Ampicillin (10.6%). To Vancomycin was not reported any resistant strain.

Among viridans streptococci isolated ***S. mitis*** presented the greatest resistance. In order of frecquency were followed ***S. oralis*** and ***S. mutans.***

Viridans streptococci are considered bacteria with low virulence. They don't posses endotoxins and not secret any exotoxins. They are very sensitive to the litic actions of serum and lysosomal enzymes. Although some species secret proteolytic enzymes, these are not responsible for the production of infection.

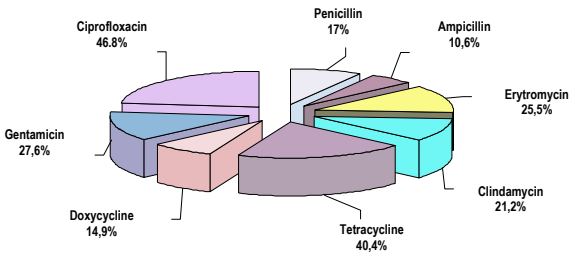

Fig. 4. Viridans streptococci resistance to antibiotics

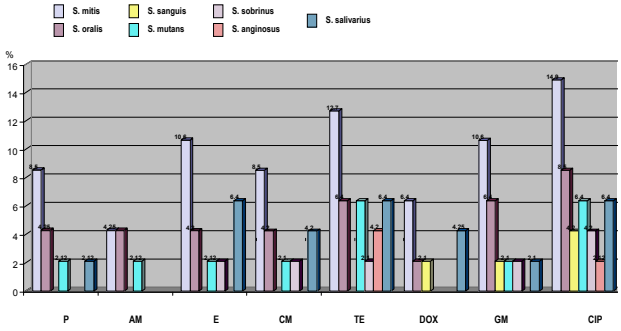

Fig. 5. Viridans streptococci species resistance to antibiotics

Infections caused by viridans streptococci usually are the result of penetration and their accidental spread in the body, especially to people susceptible or immunocompromised. Only their virulence factor is represented by their ability to adhere to heart valve offense leading to the installation endocarditis presence dextran extracellular playing a particular role in this case.

Clinical observations have shown that bacteremia consecutive dextran production by viridans streptococci increased incidence of endocarditis compared with streptococci non-production dextran. Studies have sown that dextran produced by strains of viridans streptococci increases their ability to adhere to heart valve in vitro and bring on endocarditis at the experience animals (2).

On the other hand, the production of dextran modifies the response to medical therapy. Thus endocarditis caused by strains of streptococci producing dextran is more resistant to treatment with Penicillin, compared with the non-production of dextran. Studies have shown that the experimental treatment endocarditis with Penicillin associated with dextranase increase the rate of sterilization in the valve than treatment only with Penicillin (4,12).

Also very effective to treat severe infection with strains has proved a combination of beta-lactams (Penicillin) with aminoglycosides (Gentamicin or Streptomycin). A number of other beta-lactams such as cephalosporins in vitro have showe a similar activity on Penicillin to the viridans streptococci. Ceftriaxone in particular (IIIrd generation cephalosporins) has proved extremely effective on viridans streptococci involved in the etiology of serious infections: endocarditis, meningitis or septicemias (7,10).

Other antibiotics that have proven effective in vitro on viridans streptococci: Vancomycin, Teicoplanin and Imipenem, while: Tetracycline, Chloramphenicol, Erytromycin, Clindamycin and fluoroquinolones have veritable activity which is exemplified in our study.

CONCLUSIONS

Even if viridans streptococci are part of normal flora of the oral cavity, great importance should be given to this group of bacteria because of their involvement in the etiology of dental cavity, gingivitis and other periodontal diseases, but especially in the endocarditis.

Monitoring of resistance to antibiotics is important to differentiate the therapeutical failure from a reinfection. For example in the case of endocarditis:

Therapeutical failure: in patients with recurrent endocarditis who were not treated properly during the acute episode, the same strain is isolated,

reinfection: another strain is isolated.

Viridans streptococci resistance to antibiotics can be transferred through the determinants of resistance acquired a plasmid in may pathogenic species such as ***Streptococcus pyogenes, Streptococcus pneumoniae and Streptococcus agalactiae.***

Special attention should be given to oral hygiene, which influences the incidence and severity of the disease at this level (dental caries, periodontal diseases, gingivitis and stomatitis), and the distance complications, especially in patients with risk.

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PREVALENȚA ŞI REZISTENȚA LA ANTIBIOTICE A STREPTOCOCILOR VIRIDANS ORALI

REZUMAT

Scop. Acest studiu a urmărit determinarea prevalenței speciilor de streptococi viridans orali şi rezistența lor la antibiotice..

Metodă. Izolarea s-a efectuat pe geloză sânge 5% la 37°C în atmosferă deCO_2, iar identificarea speciilor de streptococi viridans s-a făcut cu ajutorul sistemului API (20 teste biochimice). Sensibilitatea la antibiotice s-a testat prin metoda difuzimetrică Kirby-Bauer.

Rezultate. Din cele 54 probe recoltate din placa dentară, carii dentare şi exudate faringiene, s-au izolat 47 tulpini de streptococi viridans: 29,78% S. mitis, 27,65% *S. mutans*, 14,89% *S. sobrinus*, 10,63% *S. oralis*, 8,51% *S. salivarius*, 4,25% *S, anginosus* şi 4,25% *S. sanguis*. Sub aspectul rezistenței la antibiotice ordinea a fost: Ciprofloxacin (46,8%), Tetraciclină (40,4%), Gentamicină (27,6%), Eritromicină (25,5%), Clindamicină (21,2%), Penicilină (17%), Doxiciclină (15%) şi Ampicilină (10,6%). La Vancomicină toate tulpinile de streptococi viridans au fost sensibile.

Concluzii. Deşi streptococii viridans fac parte din flora normală a cavității orale unde asigură colonizarea de rezistență la acest nivel, împiedicând colonizarea altor specii mai patogene, ei reprezintă totuşi un rezervor de determinanți ai rezistenței dobândite ce se pot transmite la aceste specii (*S. pyogenes, S. pneumoniae, S. agalactiae*).

Cuvinte cheie: placa dentara, carii dentare, rezistenta la antibiotice, streptococi viridans

PATTERNS OF RESISTANCE TO ANTIMICROBIAL AGENTS OBSERVED IN ACINETOBACTER SPP. STRAINS ISOLATED FROM INTENSIVE CARE UNIT

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ABSTRACT

Background: The present study was conducted in order to study the resistance to antimicrobial agents of Acinetobacter spp. strains among clinical isolates from patients hospitalized in the Intensive Care Unit (ICU) wards of Clinical County Hospital Timisoara. **Methods:** A total of 60 Acinetobacter spp. strains were isolated from patients hospitalized in the ICU wards. Strains were isolated from specimens such as: bronchial aspirate, wound secretions, blood, cerebrospinal fluid, central venous catheter, urine, sputum. All samples were inoculated on blood agar supplemented with 5% sheep blood and lactose agar (AABTL, MacConkey). The plates were incubated aerobically for 24 hours. Smears were made from samples and stained by the Gram's stain. Acinetobacter spp. strains were identified using Vitek 32 automated system, with GNI (Gram Negative Identification) cards, and susceptibility testing was performed using GNS (Gram Negative Susceptibility) cards.
Results: Between June 2006 and July 2007, a number of 60 Acinetobacter spp. strains were isolated, mainly from bronchial aspirate (48.33%). The most frequent encountered β-lactam resistance phenotype was addition of mechanisms (43.33%); resistance to gentamicin-amikacin was expressed in 31.67% of all Acinetobacter spp. strains; 73.33% strains were resistant to quinolones. Meanwhile, 51.37% of all strains presented carbapenem resistance, aztreonam resistance reached 76.67%, and trimethoprim/sulfamethoxazole was resistant in 75% cases.
Conclusions: Acinetobacter spp. strains have predominated in bronchial aspirates, mainly because the specific of ICU procedures. Multdrug-resistance(MDR) was expressed for β-lactam antimicrobials, carbapenems, aminoglicosides, quinolones and others. The number of MDR Acinetobacter spp. strains is growing worldwide, making prevention, surveillance and control programs very necessary.
Key words: Acinetobacter spp, Intensive Care Unit, resistance phenotypes, antimicrobials.

INTRODUCTION

Species of the genus ***Acinetobacter*** are strictly aerobic nonfermentative gram-negative bacilli, catalase-positive and oxidase-negative (7). ***Acinetobacter*** spp are widely distributed in nature. They are able to survive on various surfaces (both moist and dry) in the hospital environment, thereby being an important source of infection in debilitated patients; it is an oportunistic germ that causes nosocomial infections, especially in the Intensive Care Units, where the number of immunocompromised patients is high (5). The role of the ***Acinetobacter*** spp strains in the etiology of nosocomial infections has increased in the last three decades and outbreaks of ***Acinetobacter*** infections were raported globally (3,9). ***Acinetobacter*** spp. strains are frequently encountered in the Intensive Care Units, mainly because of the increasing number of invasive diagnosis and treatment procedures used in the ICU in the latest years and because of this microorganism's capacity to survive long periods in the environment and on the hands of medical staff (6). The risk factors for nosocomial ***Acinetobacter*** spp. infection are represented by previous hospitalisation, the immunocompromised patient, mechanical ventilation, cardiac or respiratory insufficiency, previous infections and antibiotic treatment and the presence of central or vesical catheters (4). There are differences between susceptibility to antimicrobials of ***Acinetobacter baumannii*** strains and other species of the genus, ***A.baumanii*** being the most resistant.

Acinetobacter spp. strains have became resistant to the vaste majority of antibacterials available, including aminoglycosides, quinolones, and extended spectrum β-lactam antimicrobials (9). The majority of strains is resistant to cephalosporines, while resistance to carbapenems is often encountered. These multidrug-resistant (MDR) strains are in some cases susceptible only to polymyxines (colistin and polymyxin B), which were not used for a few decades and have more toxic effects than commonly used antimicrobials (8).

MATERIAL AND METHODS

The study was conducted at the Department of Microbiology of University of Medicine and Pharmacy "Victor Babes", Timisoara. Between June 2006 and July 2007, a total of 60 ***Acinetobacter*** spp. strains were isolated from the patients admitted to the ICU wards of Clinical County Hospital Timisoara. Patients included were from both sexes and were all over 15 years old. Prior to inclusion in this study, they have all been hospitalised for at least 48 hours, a requirement to define nosocomial infection. The only exclusion criterion was those patients who were on antibiotic therapy.

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Specimens such as bronchial aspirate, sputum, wound secretion, blood, urine, cerebrospinal fluid were collected. All samples were inoculated on blood agar supplemented with 5% sheep blood and lactose agar (AABTL and MacConkey agar) (6). The plates were incubated aerobically for 24 hours. Smears were made from samples and stained by the Gram's stain. Isolation and identification of strains were performed in the Microbiology Laboratory of the Hospital. Strains were identified using Vitek 32 automated system (bioMerieux, France) using GNI (Gram Negative Identification) cards, and the susceptibility tests were performed using GNS cards (Gram Negative Susceptibility) with the determination of Minimum Inhibitory Concentration (MIC) for the following antimicrobials: amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, gentamicin, imipenem, netilmicin, pefloxacin, piperacillin, piperacillin/tazobactam, ticarcillin, ticarcillin/clavulanate, tobramicin, trimethoprim/ sulfamethoxazol.

The age of the patients ranged from 15 to 91 years (Fig. 1). There were 38 males (63.33%) and 22 females (36.67%). There were 29 specimens of bronchial aspirate, 11 specimens of wouns swabs and six blood cultures (Table I).

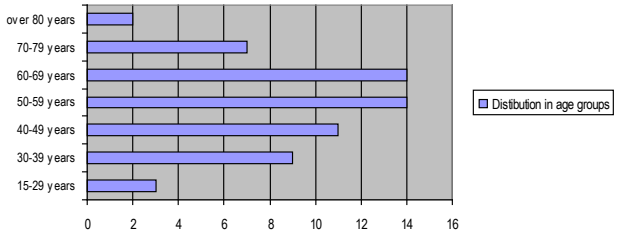

Fig. 1. Distribution of patients in various age groups

Table I. Samples isolated from patients

Samples	No.	Percentage
Bronchial aspirate	29	48.33%
Wound secretion/swab	11	18.33%
Blood	6	10.00%
Cerebrospinal fluid	4	6.67%
Central venous catheter	4	6.67%
Sputum	3	5.00%
Urine	3	5.00%
Total	60	100%

RESULTS AND DISCUSSIONS

In the same period, a total of 23 strains of ***Acinetobacter*** spp. were isolated from other Surgical Units of the Clinical County Hospital Timisoara (Plastic Surgery – 11 strains, Surgical Clinics I, II and III – 7 strains, Orthopedy – 2 strains, Urology – 2 strains, Neurosurgery – 1 strain), compared to those 60 strains of ***Acinetobacter*** spp. isolated from the Intensive Care Unit (Fig. 2).

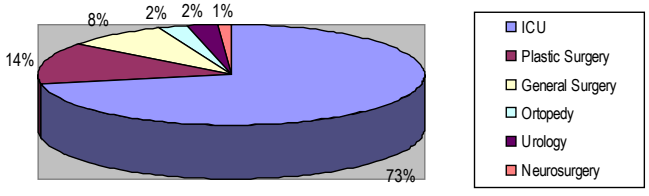

Fig. 2. *Acinetobacter* spp. isolates from different Surgical Units of Clinical County Hospital Timisoara (June 2006 – July 2007)

Out of the 60 Acinetobacter spp stains isolated from ICU patients, 57 strains (95%) were identified as Acinetobacter calcoaceticus baumannii complex, and 3 strains (5%) as Acinetobacter lwoffii. The total number of polymicrobial samples was 22, A. baumanii being associated with other Gram-negative germs (ex. Klebsiella pneumoniae, Providencia stuartii), but mostly with Staphylococcus aureus strains (in 19 cases). Staphylococcus aureus strains associated with A.baumanii was methicillin sensible (MSSA – 7 strains, 36.84%) or methicillin-resistant (MRSA -12 strains, 63.16%) (Fig.3). These A.baumanii - MRSA associations were 100% isolated from bronchial aspirates. Acinetobacter spp. strains showed resistance to different classes of antimicrobials: extended spectrum β-lactams, carbapenems, quinolones, aminoglycosides.

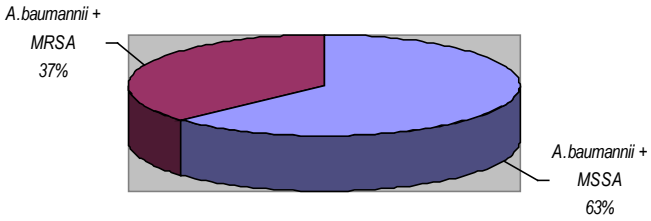

Fig. 3. Association of Acinetobacter baumannii with Staphylococcus aureus
MSSA – methicillin-susceptible Staphylococcus aureus; MRSA – methicillin-resistant Staphylococcus aureus

The resistance to β-lactams (2) was revealed by the presence of Acinetobacter spp. penicillinase-producing strains (6.67%) or cephalosporinase-producing strains (10%); 18 Acinetobacter spp. strains were penicillinase- and cephalosporinase-producing strains, and the vaste majority (26 strains, representing 43.33%) were β-lactam resistant through addition of mechanisms; only 5% of the strains were β-lactam susceptible (three strains out of the 60 isolated) (Table II).

Table II. β-lactam resistance phenotypes in *Acinetobacter* spp.

Resistance phenotypes	Number of strains	Percentage
Susceptible	3	5.00%
Penicillinase	4	6.67%
Cephalosporinase	6	1.00%
Penicillinase + cephalosporinase	18	30.00%
Addition of mechanisms	26	43.33%
Total	60	100%

Strains of ***Acinetobacter*** spp. presented susceptibility to aminoglycosides (2) in only 6.67% of all strains, resistance phenotypes most encountered were GA (resistance for gentamicin and amikacin – 19 strains, 31.67%), followed by GTA (resistance for gentamicin, tobramicin and amikacin – 15 strains, 25%). Resistance to all 4 aminoglycosides tested (GNTA phenotype – resistance to gentamicin, netilmicin, tobramicin and amikacin) was described in 7 ***Acinetobacter*** spp. strains (11.67%) (Table III).

Table III. Aminoglycoside resistance phenotypes in *Acinetobacter* spp.

Resistance phenotypes	Number of strains	Percentage
Susceptible	4	6.67%
G	4	6.67%
A	5	8.33%
N	1	1.67%
GA	19	31.67%
GT	1	1.67%
GN	1	1.67%
GTA	15	25.00%
GNA	1	1.67%
GTN	2	3.33%
GNTA	7	11.67%
Total	60	100%

Quinolone resistance [2] is frequently encountered in ***Acinetobacter*** spp strains. 44 strains (73.33%) showed ciprofloxacin and pefloxacin resistance, while only 4 strains (6.67%) remained susceptible to quinolones (Table IV).

Table IV. Quinolone resistance phenotypes in ***Acinetobacter*** spp

Resistance phenotypes	Number of strains	Percentage
Susceptible	4	6,67%
Ciprofloxacin resistance	7	11,67%
Pefloxacin resistance	5	8,33%
Ciprofloxacin + Pefloxacin resistance	44	73,33%
Total	60	100%

31 strains of ***Acinetobacter*** spp. (51.67%) were resistant to carbapenems, and 46 strains (76.67%) were resistant to aztreonam. Resistance to trimethoprim/sulfamethoxazol was encountered in 45 ***Acinetobacter*** spp. strains (75%).

Literature data show an increased and alarming number of multidrug-resistant ***Acinetobacter*** spp. strains (1,3). In Europe, studies such as SENTRY, MYSTIC and ESAR (European Surveillance of Antibiotic Resistance) confirmed the increasing number (9). Although antimicrobial resistance of ***Acinetobacter*** spp. appears to be increasing across Europe, it is difficult to estimate accurately the extent of this emerging problem, partially because the published susceptibility data are based on different methods. A reference method for susceptibility testing and MIC breakpoints should be established to better monitor trends of resistance. Surveillance of antimicrobial resistance, the study of resistance mechanisms, the use in medical practice of antimicrobials for which ***Acinetobacter*** spp. is still susceptible (colistin, azithromycin, doxycycline, rifampin) (1,8), the development of new drugs, and the prevention of the spread of multiresistant strains, are all important measures required to control the impact of these multiresistant bacteria [4].

CONCLUSIONS

A total of 60 ***Acinetobacter*** spp. strains were isolated from patients hospitalised in the Intensive Care Unit of Clinical County Hospital Timisoara between June 2006 – July 2007. The most frequent encountered species was ***Acinetobacter baumannii*** (95%), both from monomicrobial and polymicrobial samples (mainly bronchial aspirates). The most frequent association of germs was represented by ***A.baumannii*** + MRSA (63.16%).

The number of ***Acinetobacter*** spp. strains isolated in the same period from the other Surgical Units of this hospital was 23, compared to the 60 strains isolated from ICU; this situation is due to the specific procedures of this Unit.

Acinetobacter spp. strains presented multidrug-resistance (including β-lactams, carbapenems, aminoglycosides, quinolones) in high percentage.

One alternative in current treatment of nosocomial ***Acinetobacter*** spp. infections is represented by other antimicrobials for which ***Acinetobacter*** spp. strains are still susceptible (colistin, azithromycin, doxycycline, rifampin).

Multidrug-resistant ***Acinetobacter*** spp. strains are isolated with increasing frequence in healthcare facilities worldwide, and the treatment implies high economic costs. The development of innovative control strategies is needed in order to limit the spread of these pathogens.

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MODALITATI DE DOBANDIRE A REZISTENTEI LA AGENTII ANTIMICROBIENI INVESTIGATE LA TULPINILE DE ACINETOBACTER SPP. IZOLATE DIN UNITATILE DE TERAPIE INTENSIVA

REZUMAT

Scopul acestei lucrari este studierea sensibilitatii tulpinilor de *Acinetobacter* spp la chimioterapicele antiifectioase, tulpini izolate de la pacienţi internati în Clinica de Anestezie si Terapie Intensiva a Spitalului Clinic Judetean de Urgenta Timisoara in perioada iunie 2006 – iulie 2007.

Metoda: S-au izolat 60 tulpini de *Acinetobacter* spp. de la pacienti internati in Clinica de Terapie Intensiva. Tulpinile au fost izolate din diverse produse patologice reprezentate de: aspirate bronsice, secretii din plaga, sange pentru hemoculturi, urină, sputa, lichid cefalorahidian. Toate produsele patologice au fost insamantate pe agar cu 5% sange de berbec si pe medii lactozate (AABTL, MacConkey). Placile au fost incubate in aerobioza cu 10%CO_2 timp de 24 ore. S-au efectuat frotiuri colorate Gram din produsele patologice. Tulpinile au fost identificate cu ajutorul analizorului automat Vitek 32 folosind carduri GNI (Gram Negative Identification), iar testarea sensibilitatii la chimioterapice antiinfectioase s-a realizat cu ajutorul aceluiasi analizor automat, folosind carduri GNS (Gram Negative Susceptibility).

Rezultate: In perioada iunie 2006 – iulie 2007, s-a izolat un numar de 60 tulpini de Acinetobacter spp. in special din aspirate bronsice (48,33%). Fenotipul de rezistenţă la β-lactamine cel mai frecvent intalnit a fost prin acumulare de mecanisme (43,33%); rezistenta la aminoglicozide s-a exprimat cel mai frecvent sub forma fenotipului GA (rezistenta la gentamicina-amikacina, 31,67%), iar la quinolone rezistenta s-a instalat la 73,33% din tulpinile de *Acinetobacter* spp. Rezistenta la carbapeneme a fost de 51,37%, la aztreonam 76,67%, iar la trimetoprim/sulfametoxazol de 75%.

Concluzii: Tulpinile de *Acinetobacter* spp. au predominat în aspirate bronsice, in special datorita specificului Clinicii de Terapie Intensiva. Multirezistenta la chimioterapice antiinfectioase s-a exprimat in cazul β-lactaminelor, carbapenemelor, aminoglicozidelor, quinolonelor si a altor clase de chimioterapice antiinfectioase. Numarul tulpinilor de *Acinetobacter* multirezistente este in continua crestere, facand astfel foarte necesare programele de control, prevenire si supraveghere a tulpinilor multidrug-rezistente (MDR).

Cuvinte cheie: *Acinetobacter* spp, Terapie Intensiva, chimioterapice antiinfectioase, fenotipuri de rezistenta.

ACINETOBACTER STRAINS ISOLATED FROM NOSOCOMIAL INFECTIONS

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ABSTRACT

Aims: The aim of our study was to determine the prevalence of *Acinetobacter* strains, isolated from patients hospitalized in Intensive Care Unit (ICU), and their resistance phenotypes.

Methods: Identification of germs was performed by the API system (ID 32 GN) and susceptibility tests by disk-diffusion tests (CLSI standards). We categorized these strains according to their phenotypic patterns.

Results: In our study undertaken over a period of six months (Jan-June 2008) from 482 samples (bronchoalveolar fluids, wound secretions, urines, blood, etc.) we isolated 466 microbial strains with nosocomial potential, from which, 28 strains (6%) were *Acinetobacter* spp. The percentage of multidrug resistant isolates showing resistance to two or more antibiotics was 82.14% (23 strains).

Conclusions: *Acinetobacter baumannii* is responsible for nosocomial infections, especially pulmonary and urinary tract infections. These infections are difficult to eradicate in part because of the multiple resistance of this organism to antibiotics.

Keywords: nosocomial infections, resistance phenotype, *Acinetobacter*

INTRODUCTION

Members of the genus ***Acinetobacter*** are ubiquitous, free living, small aerobic Gram negative coccobacilli that prefer moist environment (4). They are usually considered to be opportunistic pathogens, and recently have been reported to cause a number of outbreaks of nosocomial infections in hospitalized patients like septicaemia, pneumonia, wound sepsis, endocarditis, meningitis and urinary tract infection (UTI) (8,12). Interpreting the significance of isolates from clinical specimens is often difficult, because of the wide distribution of ***Acinetobacter*** in nature and its ability to colonise healthy or damaged tissue (6).

The genus ***Acinetobacter*** comprises 17 validly named and 14 unnamed (genomic) species. Some unrelated (genomic) species have common designations, while some other species seem to be congruent but have different names. The knowledge of the biology or ecology of acinetobacters at species level is limited. This is due to the fact that identification of acinetobacters at species level is difficult. A phenotypic species identification system has been described and a variety of genotypic methods has been explored and applied to investigate the diversity or phylogeny in the genus. These methods include high resolution fingerprinting with AFLP, PCR-RFLP with digestion of PCR amplified sequences, and analysis of various DNA sequences. Of these, AFLP analysis and amplified 16SrRNA ribosomal DNA restriction analysis have been validated with large numbers of strains of all described species. Nucleotide sequence based methods are expected to be the standard for identification in the near future (2).

However, because routine identification in the clinical microbiology laboratory is not yet possible, they are divided and grouped into three main complexes:

Acinetobacter calcoaceticus-baumannii complex: glucose-oxidising nonhemolytic, (***A.baumannii*** can be identified by OXA-51 typing)

Acinetobacter lowffii: glucose-negative nonhemolytic

Acinetobacter haemolyticus: hemolytic

MATERIAL AND METHODS

In a period of six months (Jan-June 2008) we collected 482 samples from 444 patients hospitalized for at least 48 hours in the Intensive Care Unit`s of Clinical Emergency County Hospital Timisoara.

From 457 positive samples (bronchoalveolar fluids, wound secretions, urines, blood, peritoneal fluids, catheter tips) we isolated 466 microbial strains with nosocomial potential, from wich 28 were ***Acinetobacter*** spp. strains.

All the samples were inoculated on 5% sheep blood agar and MacConkey agar at 37°C for 24 hours.

The identification of germs was based on colonial appearance and biochemical characteristics. Identification of Gram negative germs was performed by the API system (ID 32GN/BioMerieux). ***Acinetobacter*** species differentiation was done on basis of glucose oxidation, haemolysis, growth at 37 and 44°C, susceptibility to penicillin and chloramphenicol discs.

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The sensitivity of isolated germs to antimicrobials was tested using standardised Kirby-Bauer technique (CLSI standards) with manual and automatic reading methods (Osiris-BioRad Laboratories). We categorized these germs according to their phenotypic patterns.

RESULTS

From a total of 466 isolates of various bacteria obtained from different specimens, 28 (6%) isolates belonged to the *Acinetobacter* spp. Species differentiation of the isolates showed a predominance of *A. baumannii* –complex at 92.85%, while *A. lowffii* showed an isolation rate of 7.14%.

The distribution of these strains from various specimens (bronchoalveolar fluids, wound secretions, urines, blood, peritoneal fluids, catheter tips) are as shown in Table I.

Table I. Clinical specimens showing isolation rates and antibiotic resistance

Specimen	Isolates	No./% of isolates	No./% MDR*
Bronchoalveolar fluids	A. baumannii A. lowffii	12/ 46.15% 2/ 100%	12 / 52.17% 2/ 8.69%
Wound secretions	A. baumannii	5/ 19.23%	4/ 17.39%
Urines	A. baumannii	6/ 23.07%	5/ 21.73%
Blood	A. baumannii	1/ 3.84%	0/ 0%
Peritoneal fluids	A. baumannii	1/ 3.84%	0/ 0%
Catheter tips	A. baumannii	1/ 3.84%	0/ 0%

*MDR: multidrug resistant

The percentage of multidrug resistant isolates showing resistance to two or more antibiotics was 82.14% (23 strains). The highest was seen in bronchoalveolar fluid and urine specimens at 60.86% and 21.73% respectively. Only 3 (10.71%) of isolates were sensitive to all antibiotics exception trimethoprim-sulphamethoxazole. Resistance phenotypes in *Acinetobacter* spp. are presented in Table II.

Table II. Resistance phenotypes in Acinetobacter spp. isolates

Antibiotics	Resistance phenotypes	No.	%
b-lactam antibiotics	wild phenotype	3	10.71%
	PASE	2	7.14%
	CASE	1	3.57%
	PASE+CASE	16	57.14%
	Mechanisms accumulation	6	21.42%
Aminoglycosides	wild phenotype	5	17.85%
	G	10	35.71%
	GT	10	35.71%
	GN	1	3.57%
	GTN	2	7.14%
Fluoroquinolones	wild phenotype	6	21.42%
	resistant phenotype	22	78.58%
Trimethoprim-sulphamethoxazole	wild phenotype	0	0%
	resistant phenotype	28	100%
Doxiciclyne	wild phenotype	22	78.58%
	resistant phenotype	6	21.42%
Chloramphenicol	wild phenotype	5	17.85%
	resistant phenotype	23	82.14%

Legend: PASE-penicillinase, CASE-cephalosporinase, G-gentamycine, T-tobramycine, Nt-netilmicin, A-amikacin

DISCUSSIONS

Studies on Acinetobacter in various countries (13) have shown a predominance of isolation from urine (21-27%) and tracheo-bronchial secretions (24.8-48.8%). In this study, respiratory secretions samples were predominantly received for bacterial diagnosis (50%).

A. baumannii is naturally resistant to first and second generation cephalosporins and at low levels to trimethoprim. Acquired resistance in this species is secondary to the presence of transposable elements or of plasmids (7,10). The percentage of b-lactam antibiotics resistance, in our study, was 89.29%, higher than in other European countries. In this study Acinetobacter strains were resistant in all cases to trimethoprim-sulphamethoxazole.

Aminoglycoside resistance in Acinetobacter spp. is common and due to enzymes which modify the antibiotics. 3'-Phosphotransferase types I and II, 2'-acetyltransferase, 3-acetyltransferase type I, 6'-acetyltransferase, 9-adenylyltransferase, 3",9-adenylyltransferase, and 2"-adenylyltransferase have been detected in this bacterial genus (1,3,5,9,10,11). Since it is inactivated only by the 6'-acetylating enzyme, amikacin remained until recently the most active aminoglycoside for infections due to Acinetobacter spp. We did not isolate any strains with amikacin resistance.

CONCLUSIONS

Acinetobacter baumannii showed predominance amongst the isolated species. Another identified species was Acinetobacter lowffii.

Acinetobacter baumannii is responsible for nosocomial infections, especially pulmonary and urinary tract infections. These infections are difficult to eradicate in part because of the multiple resistance of this organism to antibiotics.

Multi-drug resistant Acinetobacter nosocomial infection has emerged as an increasing problem in intensive care units. These infections are difficult and costly to treat. The analysis of risk factors and susceptibility pattern will be useful in understanding epidemiology of this organism in a hospital setup.

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TULPINI DE ACINETOBACTER IZOLATE DIN INFECȚII NOSOCOMIALE

REZUMAT

Scop: Determinarea prevalenței tulpinilor de *Acinetobacter* spp., izolate de la pacienți spitalizați într-o Secție de Terapie Intensivă, şi fenotipurile lor de rezistență.
Metode: Germenii au fost identificați utilizând galerii API (ID 32 GN), iar pentru testarea sensibilității am folosit metoda difuzimetrică (conform standardului CLSI). Ulterior, tulpinile au fost încadrate în fenotipuri de rezistență.
Rezultate: Din 482 produse patologice (aspirate bronşice, secreții de plagă, urini, sânge,etc.), recoltate timp de 6 luni (Ianuarie-Iunie 2008), am izolat 466 germeni cu potențial nosocomial, dintre aceştia, 28 de tulpini (6%) au fost reprezentate de *Acinetobacter* spp. Un număr de 23 de tulpini (82,14%) au fost catalogate drept tulpini multirezistente deoarece au prezentat rezistență la două sau mai multe antibiotice.
Concluzii: *Acinetobacter baumannii* este frecvent implicat în infecțiile nosocomiale, în special ale tractului respirator şi urinar. Aceste infecții sunt dificil de tratat în parte şi datorită multirezistenței acestor germeni.
Cuvinte cheie: infecții nosocomiale, fenotipuri de rezistență, *Acinetobacter*

PHOTODYNAMIC THERAPY WITH TMPP AND ZNTMPP IN TUMOR BEARING RATS

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ABSTRACT

Photodynamic therapy (PDT) is a relatively new and highly promising treatment for cancer. It uses porphyrin derivates (photosensitisers), which are being activated in the presence of light and generate reactive oxygen species. To improve this treatment new photosensitisers are being synthesized and tested.
The aim of this study is to assess the effect of PDT with 5,10,15,20-tetrakis (methoxyphenyl) porphyrins (TMPP) and zinc-5, 10,15,20-tetrakis (methoxyphenyl) porphyrins (Zn-TMPP) on serum levels of reactive oxygen species (ROS) in male rats with experimentally Walker carcinoma sarcoma 256.
The rats were randomly divided into groups as follows: group 1 - no treatment, group 2 - the tumors were only irradiated, group 3 - were given TMPP 10mg/kg peritumoral and irradiated, group 4 - were given Zn-TMPP 10mg/kg peritumoral and irradiated, group 5 - were given 5-aminolevulinic acid (5-ALA) and irradiated. Laser light (at fluence rate 100 J/cm^2 and pulse frequency rate 10 kHz) at 685 nm was delivered to tumors for 15 minutes following 24 h after drug administration.
Serum was analyzed for ROS levels: malondialdehyde (MDA), carbonylated protein (CP), the hydrogen donating ability and total SH groupings at 24 h after treatment.
The groups treated with TMPP and Zn-TMPP was compared with those given 5-ALA regarding the ROS levels in plasma.
The MDA levels were lower than those obtained with 5-ALA (TMPP: 1.37±0.13; ZnTMPP: 1.44±0.21 nmoles/mg protein vs. 3.37± 0.70 nmoles/mg protein, $p<0.01$).
Regarding the levels of carbonylated proteins PDT with TMPP and ZnTMPP showed significantly increased levels compared to the control group (TMPP: 1.08±0.39; ZnTMPP: 1.03±0.22 vs. control: 0.61±0.07 nmoles/mg protein; $p<0.01$).
The levels of SH groupings were increased after the 5-ALA treatment and also after TMPP and Zn-TMPP but they didn't reach statistical significance. Our results suggest that PDT with TMPP and ZnTMPP perturbs the normal redox balance and induces citotoxic effects.
Key words: Photodynamic therapy, ROS, TMPP, ZnTMPP

INTRODUCTION

Photodynamic therapy (PDT) is a relatively new and highly promising treatment for cancer. It consists of the administration of a photosensitizer, which is selectively retained by tumor cells and the subsequent irradiation with visible light in the presence of oxygen, which specifically inactivates neoplastic cells (10). Basically, two types of reactions can occur after photoactivation of the photosensitizer. One involves the generation of free radicals (type - I photochemical reaction) and the other the production of singlet molecular oxygen, (type II) as the main species responsible for cell inactivation. Evidence favors the role of the type - II process in cells, although the photodynamic process of the sensitizers on neoplastic tissues is still not well understood (11). Besides the direct effect on the tumor cells, PDT also destroys the malignant cell by indirect paths such as affecting the endothelial cells of the tumor vascularisation determining vascular damage leading to the blockage of the blood supply to the tumor (10). In addition to this direct killing process, tumor eradication also arises from an acute inflammatory response featured by an increased level of various mediators in the PDT-treated tumor area (IL-1 β, G-CSF, IL- 8, MIP–2) (2). To improve this treatment new photosensitizers are being synthesized and tested.

The tetrapyrrolic macro cycles have been used in PDT the last few years with promising results both in the treatment and detection of malignant tumors. The 5,10,15,20-tetrakis (4-methoxyphenyl) porphyrin is a tetrapyrrolic macrocycle with a high coefficient of absorption in visible light and with a long life of the triplet state. The methoxy substitutes offer important photophysics and photosensitizing properties, and also they increase the polarity of the compound but in the mean time they preserve its lipophilic character, which confers a good interaction with the tissue. The formation of metalloporphyrin complexes with Zn or Cd causes changes in the free-base photophysical properties by increasing the duration of the triple and of the quantity of singlet oxygen leading to an increased photodynamic effect (8). In previous studies, the photodynamic activity of 5,10,15,20-tetrakis(4-methoxyphenyl) porphyrin was studied in different biomimetic and biological media. This synthetic porphyrin and its complexes with metals are effective photosensitizers, and can be used as model compounds to investigate the theoretical and instrumental aspects of PDT (5).

Taking into account the promising results of the in vitro testing of these porphyirins, the aim of this study is to evaluate the presence of oxidative stress in the plasma by indirect methods caused by PDT with TMPP and ZnTMPP in male Wistar rats with experimentally Walker carcinoma sarcoma 256.

MATERIALS AND METHODS

Animal model

In this study we used male Wistar rats, 10 weeks old, weighing 180-200g. The animals were fed a standard diet and had water ad libitum. The environmental conditions were standard, with a temperature of 19-23°C and 12:12-hr light:dark

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cycle through the entire experiment. The PDT experiments were in agreement with The National Ethical Committee's Guidelines on Animal Welfare, Romania.

The Walker carcinoma-sarcoma 256 was obtained from tumor bearing rats offered by The University of Agriculture Sciences and Veterinary Medicine, Cluj-Napoca. The tumor was maintained in The Biobase of the Physiology Department of UMP "Iuliu Hatieganu", Cluj Napoca by successive inoculations at a 30 days period. The tumoral material was harvested in sterile conditions (Betadine was used to clean the tegument above the tumor) from the tumor-bearing rat; the rat was anesthetized prior to this procedure using xylazine and ketamine (2:1). The harvesting was done after the tegument and conjunctive fibers that form the tumor capsule were dissected. The harvested tumor was put in a Petri platelet and mixed with a sterile NaCl 0,9% solution and Penicillin G and was cut in small pieces ready for inoculation using a scalpel. Small bits of the tumor were inoculated subcutaneous in the right flank using a trochar. Prior to this procedure the rats were anesthetized and the teguments were disinfected using Betadine. At the site of inoculation a solution of Penicillin G was applied.

Light experiment

The photodynamic therapy experiments were carried out when the tumor reached a volume of 1cm^3 at a variable interval of 21- 30 days from inoculation. The animals were first anesthetized with a mixture of ketamine 90mg/kg and xylazine 10mg/kg and shaved. After 24h from the administration of the porphyrins, the tumors were irradiated using red light applied directly on the tegument above the tumor (λ=685 nm), at a light dose of 100 J/cm^2 and a medium power of 25 W with a frequency of 10Hz for 15 minutes. The light source was Therapeutic LASER Model D-68.

The animals were randomly divided in 5 groups each containing 10 animals:

group 1 (CONTROL) - no treatment;

group 2 (LIGHT) - the tumors were only irradiated;

group 3 (TMPP) - were given TMPP 10mg/kg b. w. peritumoral and irradiated;

group 4 (ZnTMPP) - were given Zn-TMPP 10mg/kg b.w. peritumoral and irradiated;

group 5 (5-ALA)- were given 5-aminolevulinic acid (5-ALA) 250mg/kg b.w., peritumoral and irradiated.

The animals were sacrificed after 24h from the irradiation and blood was harvested on EDTA.

Drug administration

For administration TMPP and ZnTMPP were dissolved just prior to use in PBS and the pH adjusted to 7 with NaOH 1N. The animals were injected with TMPP and ZnTMPP 10mg/kg b.w. in 0.75 ml solution, PBS was neutralized prior to use with a solution of NaOH 1N. The TMPP and ZnTMPP were synthesized by Prof. PhD Rodica Mariana Ion (ICECHIM Bucharest). All the solutions were protected from light with aluminum sheets. Five-aminolevulinic acid (purity 98%) was obtained from Sigma-Aldrich Inc. (Germany). 50 mg of 5-ALA chlorhydrate were dissolved in 0.75ml PBS. PBS was neutralized prior to use with a solution of NaOH 1N. Each animal received a dose of 250mg/kg b.w. of 5-ALA. Absolute ethanol, HCl, ethilacetate and n-butanol were purchased from Chimopar (Bucharest). Thiobarbituric acid and KH_2PO_4, Streptomycin, DNPH, guanidine chloral hydrate, 1,1-diphenil-pycrilhydrazile, dithiobisnitrobenzoic acid, Tris were purchased from Sigma-Aldrich Chemicals GmbH (Germany), EDTA-Na_2, trichloracetic acid from Merck KgaA Damstadt (Germany). All the reagents were of analytical grade.

To evaluate the presence of oxidative stress in the plasma we used indirect methods. These quantify the lesions produced by the reactive oxygen species on the organism's biomolecules, the so-called effect biomarkers. In our study we evaluated the products resulted from the oxidation of lipids by determining the malondialdehyde (1) with the fluorescence method and also the effects on proteins determining the carbonylated proteins (9). We also evaluated the antioxidant capacity by determining the hydrogen donating ability (4) and total SH groupings (3).

Statistical Analysis

The data were analyzed using the SPSS 16 program. They were tested for statistical significance using the Mann-Whitney test and the Kruskal-Wallis test, the criteria for statistical significance was $p<0.05$.

RESULTS

The groups that received PDT treatment with TMPP and ZnTMPP were compared to the groups that received 5-ALA and also with two control groups: one that received no treatment and the other one was only exposed to light without being given the photosensitisers.

Malondialdehyde (MDA)

Malondialdehyde is a marker that shows the effect of the reactive oxygen species on lipids. The values that were registered for TMPP and ZnTMPP are lower then those obtained by PDT with 5-ALA (TMPP: 1.37±0.13: ZnTMPP: 1.44±0.21 nmoles/mg protein vs. 3.37± 0.70 nmoles/mg protein, $p<0.01$). And they were also significantly lower then those obtained in the control group 2.41±0.18 nmoles/mg protein and only with light 2.35±0.17 nmoles/mg protein, $p<0.01$ (Fig.1).To appreciate if there is an overall difference between the value of MDA in this groups we used the Kruskal-Wallis test and we obtained a $p=0.001$, so the difference is statistically significant. When comparing the MDA values between the group that underwent PDT with TMPP and that with ZnTMPP we found that there was no difference between the two groups

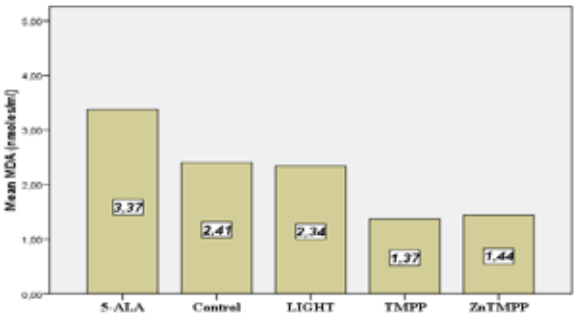

Fig.1. The mean level of malondialdehyde in the plasma of tumor bearing rats, = $p<0.05$ compared to the control group

Carbonylated proteins (CP)

The plasma levels of carbonylated proteins in PDT with TMPP and especially with ZnTMPP is lower than that of PDT with 5-ALA, but not statistically significant, but when compared to the control group the values are significantly higher (TMPP: 1.08±0.39; ZnTMPP: 1.03±0.22 vs. control: 0.61±0.07 nmoles/mg protein; $p<0.01$) (Fig.2). Also, the values of CP after PDT with 5-ALA are higher than those of the control group ($p=0.014$). The Kruskal-Wallis test showed a statistically significant difference between the 5 groups ($p=0.011$).

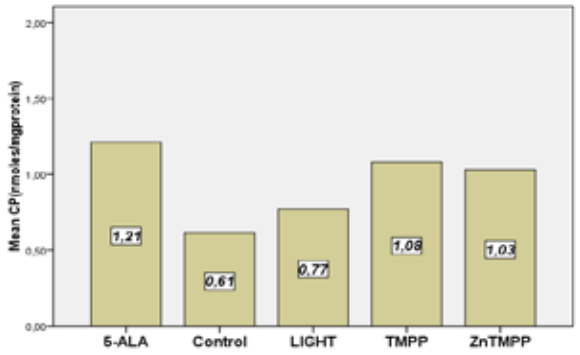

Fig.2. The mean level of carbonylated proteins in the plasma of tumor bearing, = p<0,05 compared to the control group

The total hydrogen donating ability (DH)

The antioxidant capacity evaluated using the test with DPPH shows an increase in all the groups compared to the control group. The values of DH are significantly higher in the groups treated with 5-ALA, TMPP and ZnTMPP compared to the control group (p<0.05) (Fig.3). And also the group that received PDT with ZnTMPP has a significantly higher value of DH compared to that treated with 5-ALA (p=0.009). The Kruskal-Wallis test showed also statistical significance with a p=0.004.

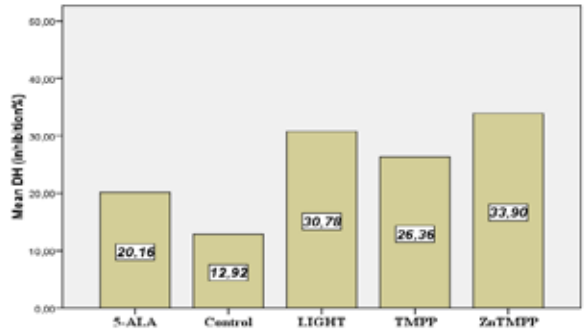

Fig.3. The mean level of the total hydrogen donating ability in the plasma of tumor bearing rats, = p<0.05 compared to the control group

4. The SH groupings

The levels of SH groupings were increased after the 5-ALA treatment and also after TMPP and ZnTMPP but they didn't reach statistical significance (Fig.4).

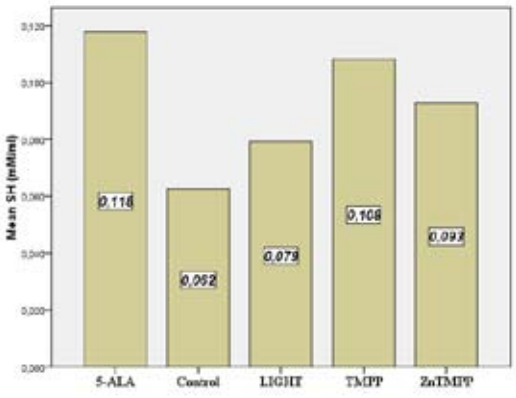

Fig.4. The mean level of total SH groupings in the plasma of tumor bearing rats

DISCUSSION

One of the challenges of PDT in the treatment of cancer is to find an ideal photosensitizer that should be a chemically pure drug, with a preferential uptake in tumors, rapid clearance, and a strong absorption peak at light wavelengths >630 nm (2). The PS that are now in use fail to reach the criteria for the ideal PS, so new drugs are being developed and some of them such as ZnTMPP and TMPP showed promising results when tested in vitro. In vitro, the survival curves of Hep-2 cells, treated with TMPP, were markedly dependent on the light wavelength ranges used for irradiation; also singlet oxygen (102) was found to be the main species responsible for cell inactivation (6).

We found that PDT with ZnTMPP and TMPP influence the oxidative stress parameters in plasma increasing significantly the level of carbonylated proteins (fig.2), which leads us to the conclusion that their antitumor effect is mainly due to the effect of these porphyrins on proteins. Surprisingly, when we look at the effect that these porphyrins have on the lipids by measuring the oxidative stress effects using MDA in fluorescence we observed that they are not affected byTMPP and ZnTMPP (fig.1).

We also evaluated the antioxidant capacity, we used the test with DPPH and we found higher levels than those obtained in the control group (fig.3).

Also in vitro studies showed that the cytotoxic effect in a human carcinoma cell line increases in the order: CuTMPP << TMPP < ZnTMPP approximately CdTMP (2). Our studies didn't reveal any statistical significant difference between TMPP and ZnTMPP regarding the effects of the oxidative stress on the serum parameters we evaluated.

To prove the efficiency of these porphyrins in vivo we compared the effects they have on the oxidative stress parameters in plasma with those obtained with 5-ALA. The second-generation photosensitizer 5-ALA received approval for the treatment of cancerous lesions in 1999. The use of 5-ALA PDT has several advantages over PDT with porfimer sodium: there is a more rapid clearance (limiting skin photosensitivity to 1–2 days), it can be applied topically for the treatment of skin cancer and orally for cancer in the oral cavity or digestive tract, and greater tumor selectivity is achieved. The disadvantage of 5-ALA is that it is strongly hydrophylic and therefore not able to enter cells easily. This led to the development of several alkyl esters of 5-ALA that do penetrate the cell easier (3).

Our results showed that 5-ALA is more effective than TMPP and ZnTMPP regarding the parameters that we studied, but although the methods we used offer a good overall image of the effects of the oxidative stress parameters they don't quantify the singlet oxygen generated in the tumor, they don't offer information on the intimate mechanism of cell destruction, and also they can be influenced by other compounds with similar structures. So before we decide on the efficiency of these porphyrins further in vivo studies should be conducted first to see if the dose and the incubation period are proper; by histological means we should evaluate the tumor necrosis and the effect of these porphyrins on tumor vasculature, knowing that an important role in PDT is played by the destruction of the vessels that offer blood supply to the tumor. Other tests that should be performed: specific tests to determine the cellular organites (mitochondria, lysosomes) affected by PDT with TSPP, to quantify the genes' expression that are induced by PDT cyclooxigenase 2, hemoxigenase 1, aldehiddehydrogenase, citocrome p450, RhoB, HSP27, HSP70, to quantify the level of singlet oxygen generated in the cells, the TUNEL test to determine the apoptosis in the tissues.

CONCLUSION

PDT with TMPP and ZnTMPP determines increased levels of protein carbonyls in plasma in Walker bearing rats. We found no difference between the rats treated with TMMP and those treated with ZnTMPP. The results obtained using 5-ALA

were better than those obtained with the two new porphyrins. We also observed an important increase in the levels of compounds that measure the antioxidant capacity (total antioxidant capacity and SH groupings); this may lead to a decrease in the damage done by the reactive oxygen species, namely in the values obtained for malondialdehyde.

Additional studies are necessary in order to establish the mechanisms, which lead to tumor destruction by PDT with these porphyrins.

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TERAPIA FOTODINAMICĂ CU TMPP ŞI ZNTMPP LA ŞOARECI PURTĂTORI DE TUMORI

REZUMAT

Terapia fotodinamică (PDT) este un tratament relativ nou şi foarte promiţător în cancer ce implică utilizarea derivaţilor de porfirine ca substanţe fotosensibilizante care, activate în prezenţa luminii, generează specii reactive ale oxigenului, citotoxice. Pentru a îmbunătăţi efectele acestui tratament s-a încercat testarea de noi substanţe fotosensibilizante.

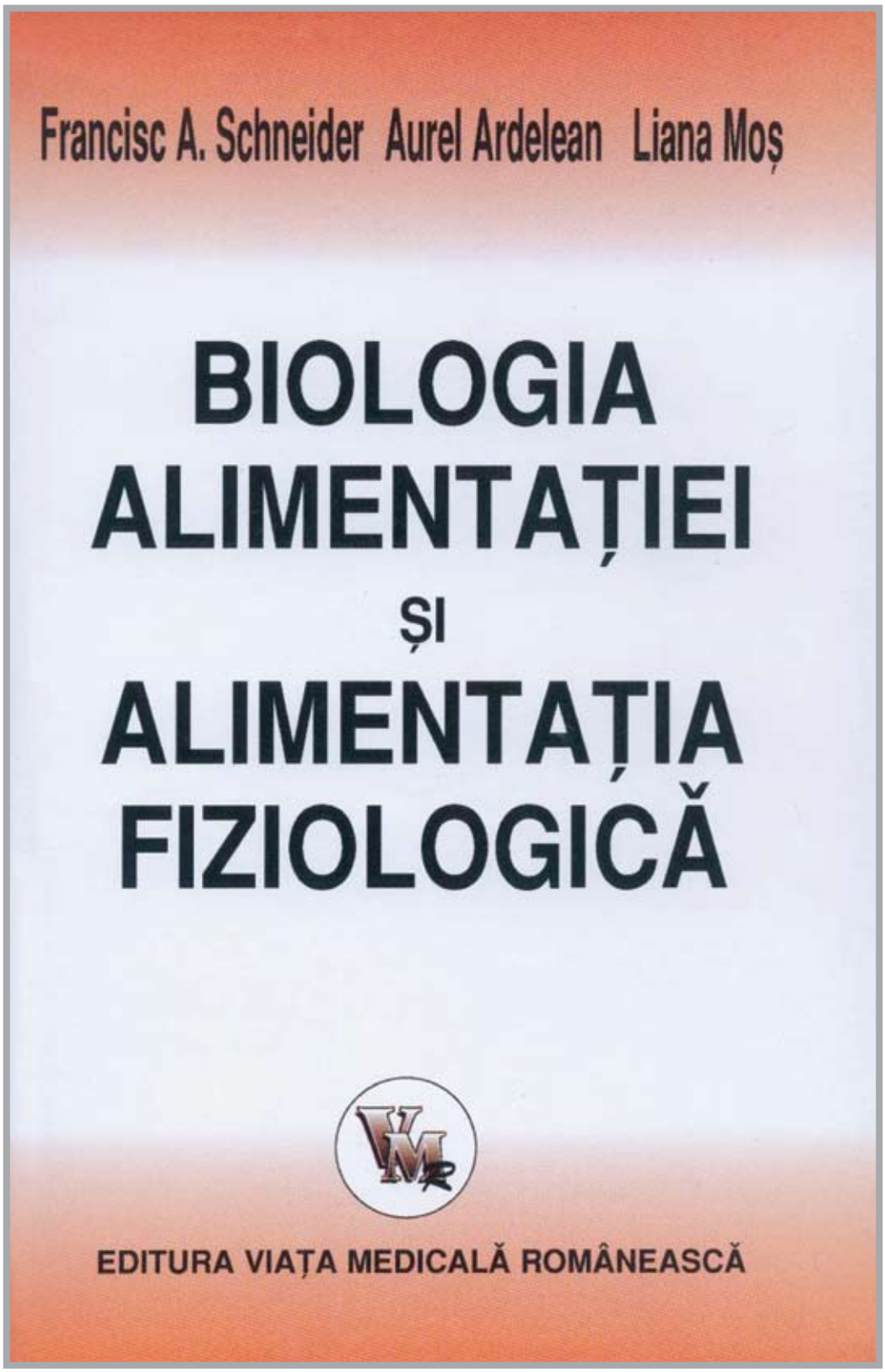
Scopul studiului a fost să evalueze efectele PDT cu 5,10,15,20-tetra(metoxifenil) porfirină (TMPP) şi zinc-5,10,15,20-tetra(metoxifenil) porfirină (ZnTMPP) asupra nivelelor serice ale speciilor reactive ale oxigenului (ROS) la şobolani masculi purtători de carcino-sarcom Walker 256.

Şobolanii au fost împărţiţi aleator în 5 grupuri după cum urmează: grupul 1- control, grupul 2- tumori iradiate, grupul 3–animale tratate peritumoral cu TMPP 10 mg/kgc şi apoi iradiate, grupul 4 – animale tratate peritumoral cu Zn MPP 10mg/kgc şi iradiate, grupul 5 – animale tratate cu acid 5- aminolevulinic (250 mg/kgc) şi iradiate cu lumină roşie aplicată direct pe tegumentul suprajacent tumorii (λ=685 nm), în doză de 100 J/cm^2, la o putere medie de 25 W şi frecvenţă de 10 Hz, timp de 15 minute la 24 de ore de la injectarea porfirinelor. La 24 ore după tratament s-au determinat din ser malondialdehida (MDA), proteinele carbonilate (CP), capacitatea de donor de hidrogen şi grupările SH totale iar rezultatele au fost comparate cu cele obţinute după PDT cu 5-ALA.

În PDT cu TMPP şi ZnTMPP nivelul MDA în ţesutul tumoral a fost mai scăzut comparativ cu 5-ALA (TMPP: 1,37±0,13; ZnTMPP: 1,44±0,21 nmoli/mg proteină faţă de 3,37±0,7 nmoli/mg proteină, $p<0,01$). În ceea ce priveşte nivelul proteinelor carbonilate concentraţia acestora creşte semnificativ comparativ cu lotul martor (TMPP: 1,08±0,39; ZnTMPP: 1,03±0,22 vs. control: 0,61±0,07 nmoli/mg proteină; $p<0,01$). Apărarea antioxidantă apreciată prin conţinutul în grupăril sulfhidril (SH) se modifică după tratamentul cu cele trei porfirine dar nesemnificativ statistic.

Rezultatele obţinute sugerează că PDT cu TMPP şi ZnTMPP modifică echilibrul redox şi determină efecte toxice asupra macromoleculelor din celule.

Cuvinte cheie: terapie fotodinamică, ROS, TMPP, ZnTMPP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