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BIOCHEMICAL STUDIES ON THERAPEUTIC POTENTIAL OF SAPROPELIC MUD FROM TECHIRGHIOL IN ANKYLOSING SPONDYLITIS

ABSTRACT

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OBJECTIVES AND AIM OF THE PAPER

Ankylosing spondylitis (AS) is a complex disease, potentially debilitating, with an insidious onset and radiologic progression of sacroileitis after several years, having as consequences loss of working capacity because of invalidity, damage of health resources and of life quality. Pathogenesis of this condition is not completely elucidated. Yet immune mediated mechanisms involving human leucocyte antigen HLAB-27, cellular inflammatory infiltrations, proinflammatory cytokines, as tumor necrosis factor TNF-α and interleukin-10, as well as genetic and environment factors are playing an important role.

Until recently, therapeutic options for patients with AS at best have been able to reduce some of the symptoms of the disease. Many patients with AS have severe or progressive disease, which is responsible for significant direct and indirect socioeconomic costs. Traditional therapies including nonsteroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate and sulfasalazine, provide limited relief of symptoms. There is accumulating evidence that anti-tumor necrosis factor (anti-TNF) therapy is highly effective in AS, improving signs and symptoms of disease and quality of life, which may subsequently reduce the socioeconomic costs associated with the disease. However, further research is needed to demonstrate whether patients benefit from long-term therapy and whether radiologic progression and ankylosis can be slowed or halted.

Anti-TNF therapy is very costly (up to 13 000$ a year). Another major concern is that because these drugs are so new, long-term scrutiny for their possible side-effects is needed. TNF plays a key role in the body's defense against infection by promoting inflammation and helping cells repair themselves.

That's why these days we are witnessing a process of approaching to natural therapeutic factors, as helping therapy for many of the diseases for which modern medicine hasn't yet any remedy. Balneotherapy, no matter of the origin and composition of the natural factors used, has a holistic impact upon entire human body; the body is turned „upside down", all organs and systems are working „transiently" in a way that request all means of anti-entropic adjustment in order to maintain homeostasis of internal body.

Mud treatment is an ancient therapeutic method used for alleviate joint pain since antiquity. Use of mud for treating chronic rheumatic condition was done empirically over the years. In Romania few scientific studies are dating from '50 – '70 decades, when there were written approximately 50 papers, which substantiate entire activity with natural cure factors in our country and a monograph about biogenesis of Techirghiol Lake (ŢUCULESCU, 1965).

In our country were recently performed many studies, which tried to apply modern medicine research methods in order to demonstrate the effects of natural existing cure factors. In agreement with this, in Balneal and Rehabilitation Sanatorium from Techirghiol, within research department, were

performed histological studies on the impact of peloidotherapy upon skin (SURDU, 2006), biochemical studies on therapeutic effects of mud in chronic degenerative rheumatic diseases (MARIN, 2011). Other studies about the effects of mud treatment are ongoing, as well as studies about the possibility of getting some active biological products extracted from mud.

In this context I chose a research theme that stands in the crossroads of many fields – biochemistry, immunology and rheumatology – and I tried to find out the substrate of therapeutic effects (anti-inflammatory, improvement of oxidative stress, hormonal rebalance and improvement of gaseous exchanges in peripheral blood) of sapropelic mud from Techirghiol in patients with ankylosing spondylitis. From our daily medical practice I noticed an important improvement of disease outcome in these patients following spa cure, effect which lasts between 6 and 12 months. Functional prognosis of patients with AS has significantly improved after periodic repetition (every 6 months) of the spa cure to Techirghiol, has also decreased the consumption of symptomatic drugs. No wonder that a great percentage of patients with AS are coming back repeatedly in the resort for complex rehabilitation treatment, which includes specific natural factors (sapropelic mud and salted water).

Through this biochemical, immunological and rheumatological study I want to add a modest contribution to the elucidation of complex action mechanisms of mud in AS, respecting evidence-based medicine criteria and highlighting once again the invaluable therapeutic potential of sapropelic mud from Techirghiol, as helpful valuable therapy in chronic inflammatory rheumatism.

The objectives aimed in this study were:

1. Assessment of anti-inflammatory effect of sapropelic mud from Techirghiol in patients with AS. For this purpose I determined at different moments serum values of some markers of oxidative stress in AS patients treated with mud, considering the fact that there is a strong correlation between initiation of inflammatory process and reactive oxygen species. The following markers were measured: total anti-oxidative status (TAS), superoxide dismutase (SOD), glutathione reductase (GR), reduced glutathione (G-SH).

2. Assessment of anti-inflammatory effect of sapropelic mud from Techirghiol in patients with AS. The most important step in order to assess anti-inflammatory potential of mud was determination of proinflammatory cytokines, being shown their role in pathogenesis of chronic inflammatory rheumatisms, inclusive in AS. So I have determined dynamically: tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6). In order to complete the study of effects of mud therapy upon inflammatory syndrome in AS, I have measured certain biochemical hematologic parameters: leucocyte number, erythrocyte sedimentation rate after 1 and 2 hours and alkaline phosphatase.

3. Assessment of the effects of sapropelic mud from Techirghiol upon hormonal status in patients with AS, as I tried to demonstrate its complex action on endocrine balance. For this reason I

have measured plasma levels of cortisol and thyroid-stimulating hormone (TSH) in different moments of the therapy.

4. Assessment of the impact of mud therapy on gaseous exchanges and acid-base balance in patients with AS. For this I have determined dynamically the following parameters from peripheral blood: oxygen binding capacity (O_2CAP), oxygen content of hemoglobin (O_2CT), partial pressures of oxygen and carbon dioxide from blood (pO_2, pCO_2), saturation in oxygen of peripheral blood ($SO_2\%$), total carbon dioxide (TCO_2), pH, bicarbonate ion and serum lactate.

CHAPTER 5
MATERIAL AND METHODS

I have performed an extensive study that included 111 patients with ankylosing spondylitis, divided in many study groups, admitted in sanatorium between 2006-2007.

1. For the study of oxidative stress markers in patients with AS there were formed different groups, by number of patients with this condition at the moments of enzymatic measurements.

Determination of superoxide dismutase was performed on a group of 18 patients, from which we took peripheral blood samples before initiation of treatment, after first mud application (first day), second day of treatment and in the end of spa therapy, after 12 days.

Determination of total anti-oxidative status and of glutathione reductase was performed on a group of 20 patients, from which we took peripheral blood samples before initiation of treatment, 24 hours after first mud application, after 5 days of therapy and in the end of the treatment (after 12 days). Many of the samples from day 2 coagulated and can't be used for biochemical measurements or statistical analysis.

For all 3 enzymes it was also determined hemoglobin level for every patient and calculation for enzymatic values was made in international units per milliliter and also in units per hemoglobin gram, in accordance with international standards. Both cases the samples were taken at Balneal and Rehabilitation Sanatorium from Techirghiol and processed at Military Hospital from Constanta. We used kits Randox Laboratories, Crumlin, U.K.

Reduced glutathione was determined in 10 patients with AS before and after 12 days of spa therapy with mud in the sanatorium.

2. Measurements of proinflammatory cytokines TNF-α, IL-1β and IL-6 were performed on a group of 18 patients. Blood samples were taken before and after balneal treatment, in order to assess anti-inflammatory effect of mud in patients with AS. ELISA technique was used for determination and samples were processed in Tody Analysis Laboratory from Bucuresti.

In this group of patients we also measured ordinary blood parameters (acute phase reactants, markers of bone homeostasis), for a more complete assessment of treatment, as: leucocyte number, erythrocyte sedimentation rate at 1 and 2 hours, alkaline phosphatase. All this samples were taken in the beginning and in the end of mud treatment.

3. Hormonal measurements were performed on a group of 20 patients. We watched the variation of plasma cortisol and TSH using ELISA technique, blood samples were taken before starting the treatment, 24 hours after the first mud application and in the end of cure. Because these patients were admitted in the summer time, I chose as type of treatment between warm mud application (mud baths or mud packing) and cold mud ointment on the shore of Techirghiol Lake, depending of the activity of disease and another associated factors. Analysis of the results was made between the above two groups.

4. Determination of markers for gaseous exchanges in peripheral blood was made on a group of 25 patients. Because immediate adaptive responses can be different from late responses (in the end of treatment), in this group we took samples before initiation of treatment, immediate after first warm application (mud bath or salted water bath), 24 hours after first application and in the end of 12 days cure.

In order to respect the principle of evidence-based medicine obtained data were recorded in tables, from which we get parameters of central tendency (mean, module, median) and dispersion parameters (standard deviation). Validation of studied assumptions was made by t-test Student and unifactorial version analysis (ANOVA), for which it was considered a significantly statistic relevance $p(t) < 0,05$ in order to reject null hypothesis formulated for every situation. The results were plotted.

The informed consent was obtained for all the patients and they were informed about the use of their data for research purpose.

Materials used in this study were the following:

- Sapropelic mud from the deposit on the bottom of Techirghiol Lake, saline mineral water from the lake;
- Facilities of the treatment department of the Balneal and Rehabilitation Sanatorium from Techirghiol, where the patients which formed the studied groups got spa therapy: mud storage and heating bunker, tables for mud packing and bath tubes, bedding (sheets, blankets, towels), thermometers for water, tap water showers, the beach specially arranged on the lake shore with valves for mud and tap water showers;
- Laboratory devices used for measurement: for biochemical determination we used automatic analyzer Beckman Coulter Synchron CX7, for hematologic determination we used automatic hematological analyzer Diagon D-Cell 60 and for determination of parameters od gaseous exchanges we used a device type CCEX 6 Nova Biomedical.

APPLICATION TECHNIQUES OF THE TREATMENT WITH SALTED WATER AND MUD FROM TECHIRGHIOL LAKE

Cold saline bath or immersion in the water of the lake is made progressively, both as duration (5-10 minutes first days up to 20-30 minutes in the end of the spa therapy) and number (one bath per day first 3-4 days up to 3-4 immersion sessions per day last days of treatment). The temperature of the lake water from which immersion is possible is more than 20ºC. Between 16-20ºC one can feel the water “cold as ice”, between 20-24ºC water is cold, but nice, and up to 24ºC the water is cool. Bathing in the lake water is a part of the therapeutic complex consisting in general progressive sun exposure, cold mud ointment and immersion in lake water. This therapeutic method is known as “Egyptian method” (SURDU, 2006).

Warm saline bath can be prescribed and performed on a daily basis, as a unique major hydrotherapy procedure or every other day, alternatively with mud application or with warm herbal

extract bath. The temperature of water for bath belong to thermo neutrality field, respectively around 37,5-38ºC. For hydro-kinetic-therapy in the swimming pool water must be heated around 35ºC, producing the feeling of "warm" (LUPU, 1956; SURDU, 2006).

The mud is used as cold ointment, warm mud bath and hyper thermic mud packing.

Cold mud ointment is a therapeutic complex characterized of successively hot - cold contrast. Cold mud ointment is performed in the summer time, on a specially designed beach, where fresh extracted mud from the natural deposit is brought daily or every other day. After a 15-20 minutes sun exposure, the patient applies on the body surface a mud layer of 1-1,5 cm thickness. Drying of the mud on the skin can take 15-30 minutes, depending of the environment temperature. Than the patient enters into the lake water in order to remove dried mud and performs active movements of all body segments. In the end of the procedure the patient takes a tap water shower. Duration of sun exposure is increasing daily (from 5-10 minutes first day up to 30-40 minutes in the end of the cure), as well as the number of lake immersions. The number of mud applications per day is constant. During the cure the patients must take only one mud application per day (TELEKI et al., 1984).

Warm mud bath is prepared using 10 kg of mud in 120-150 L of salted water from the lake. Application temperature is around 37ºC-38ºC, thermic neutrality point for mud being at 38ºC. The mud bath takes 20-25 minutes and temperature is maintained almost constant adding warm water mid-term of bath. Once the time expired, the patient takes a warm shower in order to remove the mud from the skin and a quick cold shower in order to avoid systemic vasodilatation. General mud bath is indicated once every two days, alternatively with salted warm bath (in the swimming pool or in the tube) or plants extract warm bath (ONOSE, 2000).

Hot mud general packing is prepared using 10-15 kg of mud heated at 42ºC-45ºC. The mud is smeared all over the body surface, from neck to toes. The patient is covered with a sheet and a blanket, gets a cold compress on his forehead in order to avoid a strong vasodilatation of cerebral vessels and remains like that for 30 minutes. After time expires, the mud is washed with a warm shower. The procedure ends with a short cold shower, in order to prevent irreversible vessels dilatation produced by heat. Mud packing is performed once every two days, alternative with a warm salted bath or with a bath containing plant extracts (SURDU, 2006; ONOSE, 2000).

Daily treatment applied to the patients admitted in Balneal and Rehabilitation Sanatorium from Techirghiol consists in hydro-thermo-therapy (warm mud applications alternatively with salted water baths in the swimming pool or in the tube) and a variable number of helpful procedures (electrotherapy, kinetic therapy, massage). We didn't change this regimen, because we really wanted to assess the effects obtained after specific treatment. Duration of the applied treatment is 12, 18, sometimes 21 days, depending of the disease and of the type of cure. To achieve the studied groups I selected the patients which underwent a balneal cure of 12 days.

For statistical analysis we used SPPS 12.0. We used Student tests for dependent and independent samples, Student test to compare mean with a specified value, as well as correlation analysis.

CHAPTER 6

RESULTS AND DISCUSIONS

6.1. EXPERIMENT 1

ASSESSMENT OF SOME MARKERS OF OXIDATIVE STRESS

AS is a progressive, chronic inflammatory disease with unknown etiology. Inflammation affects mainly the skeleton, peripheral joints and other internal structures. Physiopathological foundation of AS haven't yet been identified, but it seems that immunological mechanisms are involved. It has been shown that in AS many neutrophil's functions are emphasized; these stimulated functions of neutrophils mediate oxidative stress, which has an important role in AS pathogenesis (WENDLING et al., 1991; YAZICI et al., 2004).

Oxidative stress refers to the situation in which production of harmful oxidants exceeds body capacity to neutralize them. A decisive role of oxidative stress in inflammatory diseases, including AS, hasn't been yet fully elucidated. Though, reactive species of oxygen produced by activated neutrophils during inflammatory reactions play an important role in pathogenesis of many inflammatory diseases (KARAKOC et al., 2007; OZGOCMEN et al., 2003). Inflammatory reactions trigger oxidative stress, which leads to an increase in oxidants level and has as result decrease of anti-oxidants level. Anti-oxidant defense system is responsible for cell protection against potentially harmful effects of oxidant agents (WEISS, 1989).

Additionally, a small number of studies investigated the association between AS pathogenesis and oxidative stress. For example, OZGOCMEN et al. (2004) reported that oxidative stress and lipids peroxidation have been increased in patients with active AS without any treatment, but they didn't find any significant correlation between oxidants/anti-oxidants levels and activity of disease. With another study, KARAKOC et al. (2007) showed that acceleration of oxidant capacity and decrease of anti-oxidant capacity can be correlated with AS pathogenesis, but again didn't they find any significant correlation between anti-oxidant/oxidant parameters and degree of the condition activity. Aim of the study was to assess total anti-oxidant status (TAS), total oxidative status (TOS) and oxidative stress index (OSI) in patients with AS. Plasma level of TOS and OSI values were significantly increased, but plasma level of TAS decreased in patients compared with the control group.

The increase of oxidative metabolism of phagocytic system in AS was evidenced better with luminol test, suggesting activation of the myeloperoxidase system (WENDLING et al., 1991).

Since various functions of neutrophils are increased in AS, oxidative stress mediated by activation of neutrophils could have an important role in AS pathogenesis. In a study conducted by YAZICI et al. (2004) there were measured the activity of plasma myeloperoxidase (MPO), representing activation of neutrophils, and the biomarkers of oxidative stress, as advanced oxidation proteins products (AOPP) and thiol level. This is the first study which suggests formation of AOPP

mediated by neutrophils-MPO-hypochloric acid. Therefore, active neutrophils and chlorinated oxidants derived from neutrophils can be considered important factors in AS pathogenesis, being correlated with oxidative stress, especially with protein oxidation.

Radicals from human body as superoxide anion are considered a major cause of inflammation. Yet, their role in AS pathogenesis hasn't been clearly identified. In a study conducted by HO et al. (2000), the authors want to establish a relationship between AS and oxidative metabolism of phagocytes from integral blood. Predominance of phagocytes in the blood stream can be a determinant factor of AS onset.

The decrease of anti-oxidant status, which leads to generation of oxidative stress, can play an important role in AS pathogenesis (STANEK et al., 2010).

The results of the study conducted by OZGOCMEN et al. (2004) indicate that oxidative stress and lipids peroxidation are accelerated in untreated patients with active AS. The activity of serum catalase can be tightly connected with activity of the disease. Therefore, must be emphasized possible benefit of some therapeutic interventions which include high potential anti-oxidants; these will potentiate anti-oxidant defense mechanism and will decrease peroxidation.

It seems that certain mediators, as macrophage migration inhibiting factor (MIF) and interleukin-10 (IL-10), are involved in pathogenesis of some inflammatory diseases, including AS. Proinflammatory cytokines regulate the production of oxidative stress markers, as nitric oxide (NO) and malondialdehyde (MDA). Although in patients with AS has been reported the existence of oxidative stress and lipids peroxidation, association of AS with known markers of oxidative stress and with cytokines remains an uncertainty. Based on the results of the study conducted by KOZACI et al. (2010), MIF can be involved in pathogenesis of chronic inflammation from AS and, therefore, therapeutic aiming of MIF can be beneficial for preventing complications or for initiation of early treatment of the disease.

The objective of a study conducted by TÚNEZ et al. (2007) was to assess the effect of infliximab on oxidative stress in active chronic inflammatory diseases (RA, AS and psoriasis). Therapeutic effects were assessed after 6 weeks of treatment, as modification in the amount of lipids peroxidation products, in carbonyl protein groups, in the content of reduced glutathione, glutathione peroxidase, catalase and superoxide dismutase. The results of the study reveal that: (i) infliximab has anti-oxidant properties; (ii) patients with chronic inflammatory disease show increased levels of oxidative stress and (iii) oxidative stress is more intense in patients with active condition than in inactive patients.

Material and method

In order to assess oxidative stress in the patients with AS treated with mud we determined superoxide dismutase (SOD), glutathione reductase (GR), reduced glutathione (G-SH) and total anti-oxidant status (TAS), using kits Randox Laboratories, Crumlin, U.K. and the automatic analyzer

Beckman Coulter Synchron CX7. Blood samples were taken in Balneal and Rehabilitation Sanatorium Techirghiol and processed in Military Hospital from Constanta.

Determination of superoxide dismutase was performed on a group of 18 patients, from which we took peripheral blood samples before initiation of treatment, after first mud application (first day), second day of treatment and in the end of spa therapy, after 12 days.

Determination of total anti-oxidative status and of glutathione reductase was performed on a group of 20 patients, from which we took peripheral blood samples before initiation of treatment, 24 hours after first mud application, after 5 days of therapy and in the end of the treatment (after 12 days). Many of the samples from day 2 coagulated and can't be used for biochemical measurements or statistical analysis.

For all 3 enzymes it was also determined hemoglobin level for every patient and calculation for enzymatic values was made in international units per milliliter and also in units per hemoglobin gram, in accordance with international standards.

Reduced glutathione was determined in 10 patients with AS before and after 12 days of spa therapy with mud in the sanatorium.

Obtained data were statistically processed and plotted.

Inclusion criteria in the group were: correctly diagnosed patients with AS (following modified New York criteria), with active or inactive disease, without any drug treatment, admitted in sanatorium. Informed consent was obtained for all the patients and we guaranteed their privacy.

Exclusion criteria from the group were: patients with added respiratory, renal and dermatologic infections, decompensated cardiovascular diseases, patients with chronic renal, cardiovascular or hepatic diseases, which could influence oxidative stress.

Superoxide dismutase SOD (U/ml)

Superoxide dismutase (SOD), (E.C.1.15.1.1.) is often considered first defense line against oxidative stress, being the main scrubber from the cells. This enzyme exists in all cells with predominant aerobic metabolism and in optional aerobic bacteria.

SOD controls the level of superoxide anions, preventing initiation of forming reactions of the harmful radicals - hydroxyl and peroxinitrite (formed as a result of the reaction between superoxide anions and nitric oxide).

Because Levene test confirmed homogeneity of the variance ($p = 0,059 > 0,05$), one must read the values of Bonferroni test. From the results of Post-Hoc analysis (Bonferroni) we can see that:

1. There is a statistic significant increase between mean value of superoxide dismutase before initiation of the treatment (day 0) and after first mud application (day 1), while between initial moment and second day of treatment mean value of SOD don't increase significantly.
2. Mean value of superoxide dismutase is significantly increased in the end of treatment compared with any of the first days of therapy.

These conclusions are shown in figure 1.

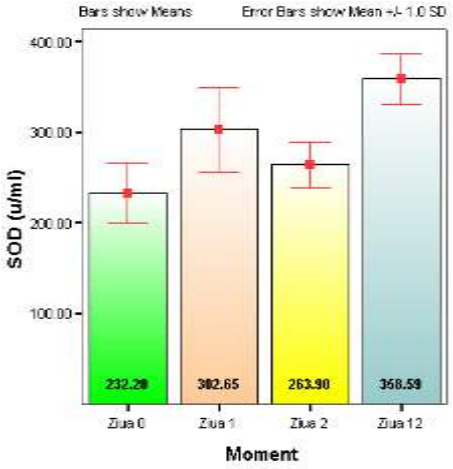

Fig. 1 Variation of SOD (U/ml)

Superoxide dismutase SOD (U/gHb)

In order to respect international research rigor, in all patients I determined concomitant the levels of hemoglobin and superoxide dismutase and I calculated also the level of SOD in units per gram of hemoglobin.

Because Levene test confirmed no homogeneity of variance ($p = 0,002 < 0,05$), one must read the values of Tamhane test. SOD level (U/gHb) has a similar variation with that of SOD (U/mL):

1. There is a statistic significant increase between mean value of SOD before initiation of treatment (day 0) and after first therapeutic application (day1).
2. SOD mean value is significantly increased in the end of treatment compared with any of the first days of therapy.

These conclusions are shown in figure 2.

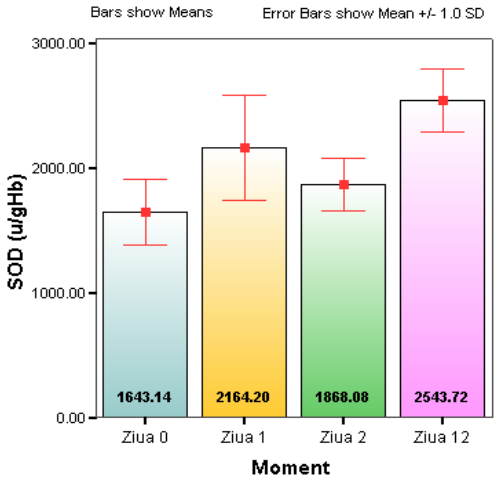

Fig. 2 Variation of SOD (u/gHb)

The level of superoxide dismutase increases statistically significant after the first mud application and very accentuated in the end of treatment. The increase of SOD level in patients with

AS treated with mud shows an improvement of anti-oxidant body capacity, given the fact that this enzyme has a positive role in ROS neutralization and NO inactivation.

Total antioxidant status TAS (U/l)

Because Levene test confirmed non-homogeneity of variance ($p = 0{,}001 < 0{,}05$), one must read the values of Tamhane test. From the results of Post-Hoc analysis (Tamhane test) we can conclude the following:

1. Total antioxidant status level decreases statistically significant day 5 compared with first determination (day 1).
2. Total antioxidant status level keeps decreasing during treatment, in the end being statistically significant decreased compared to day 1.
3. Total antioxidant status level decreased more pronounced first days of treatment, so there is not a statistic significant decrease between day 5 and final day of treatment.

These conclusions are shown in figure 3.

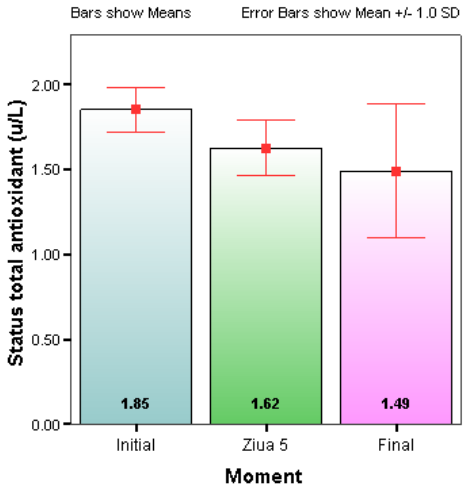

Fig. 3 Variation of total antioxidant status (U/l)

The decrease of total antioxidant status, more pronounced during first days of the treatment, could be a result of initial impact determined by the stress generated by the transfer in a medical facility, medical examination and initiation of the treatment. The impact of balneal cure generates also a metabolic stress, followed by an increased consumption of antioxidant factors. In the future would be interesting to assess TAS level after 2 weeks or even 1 month from the end of treatment, for a more accurate assessment of the impact of natural factors upon patient's body.

Glutathione reductase GR (U/l)

Glutathione reductase (GR) is essential for redox cycle of glutathione, which maintain adequate levels of cellular reduced glutathione. Oxidized glutathione is reduced in a sequence of reactions (GOLDBERG and SPOONER, 1983). GR from cells cytoplasm detoxifies toxic lipoperoxids resulted from the action of superoxide ions.

Because Levene test confirmed homogeneity of the variance (p = 0,192 > 0,05), one must read the values of Bonferroni test. Under these circumstances must be accepted the hypothesis that there are no significant differences between GR mean values corresponding to the three analyzed groups.

GR level decreased during the treatment, but not statistically significant, and remain in the physiological field. This conclusion highlights protective character of mud therapy upon oxidative stress: enzymes with positive role within this metabolism increase, such as SOD, or remain in the normal field, such as GR.

This conclusion is shown in figure 4.

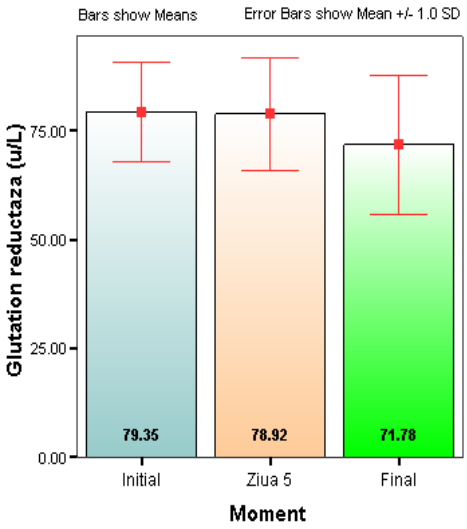

Fig. 4 Variation of glutathione reductase (U/l)

Glutathione reductase (U/gab)

Because Levene test confirmed homogeneity of the variance (p = 0,136 > 0,05), one must read the values of Bonferroni test. Under these circumstances must be accepted the hypothesis that there are no significant differences between GR mean values corresponding to the three analyzed groups.

This conclusion is shown in figure 5.

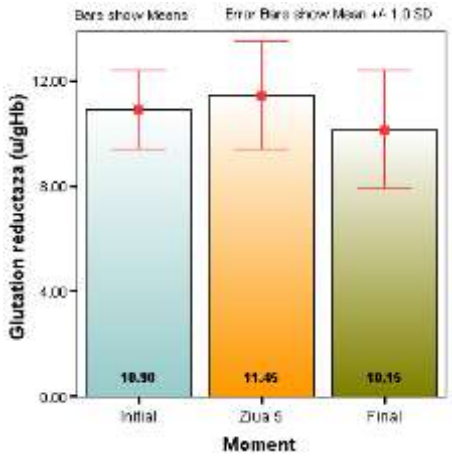

Fig. 5 Variation of glutathione reductase (U/gHb)

Glutathione reductase level is not significantly influenced by mud therapy. This conclusion highlights protective character of mud therapy upon oxidative stress: enzymes with positive role within this metabolism increase, such as SOD, or remain in the normal field, such as GR.

Reduced glutathione G-SH (mg/dl)

Reduced glutathione (G-SH) is one of the most important antioxidants which exist inside all cells, is a central constituent of the adaptive system, very sophisticated, for antioxidant defense (KIDD, 1997).

In order to find out if there are significant differences between mean values of G-SH before and after the treatment in patients from considered sample, one must apply t-Test for paired samples.

Because $p = 0{,}001 < 0{,}05$, it must be accepted the hypothesis that there are significant differences between mean values of reduced glutathione for the two different moments. Mean value of G-SH decreases statistically significant in the end of treatment.

This conclusion is shown in figure 6.

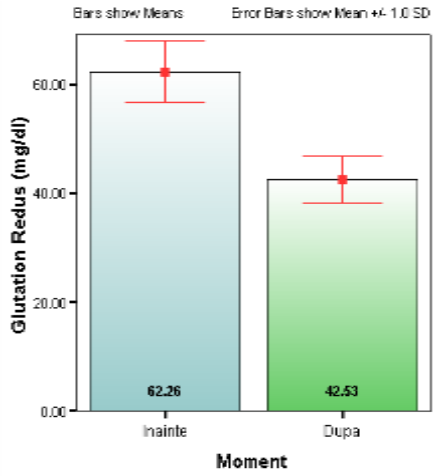

Fig. 6 Variation of reduced glutathione (mg/dl)

Statistical significant decrease of reduced glutathione in patients treated with mud suggests an increased consumption of G-SH in ROS neutralization reactions and indicates a normal response of cellular metabolism to oxidative stress triggered by the impact generated by spa therapy. In the future it would be interesting to assess, in a comparative study, the evolution of reduced glutathione and glutathione peroxidase, because first one is substrate for the enzyme's activity of hydrogen and peroxids reduction.

Discussions

All determinations made show a beneficial effect of mud therapy upon oxidative stress, suggested by the significant increase of SOD level and maintaining constant of GR level, enzymes with protective role against oxidative stress. On the other hand, significant decrease of G-SH level in the end of treatment and of TAS level, especially first days of treatment, but continuously till the end

of therapeutic cure, show the consumption of these factors induced by an additional stress, most likely determined by the impact of balneal factor upon human body and by the important syndrome of tissues reperfusion by opening arterial-venous shunts under the action of thermic and chemical factors of mud.

In the future it would be interesting to study long term outcome of TAS in patients with AS treated with mud, respectively following up oxidative stress parameters at 3, 6 and 12 months from the initiation of therapy.

6.2. EXPERIMENT 2

STUDY OF PLASMA CYTOKINES AND ASSESSMENT OF SOME BIOCHEMICAL AND ENZYMATIC MARKERS

6.2.1 Study of plasma cytokines

It's obvious that in chronic inflammatory diseases the levels of many cytokines are increased. Some lymphokines are systemically expressed in a variety of tissues; others are located in the affected tissue. In rheumatoid arthritis (RA) there is an increase of proinflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin-1 and 6 (IL-1, IL-6), granulocyte macrophage colony stimulating factor (GM-CSF), and IL-8. In addition, inhibitory cytokines IL-12 and IL-10 are also expressed in synovial spaces, significance of this imbalance being an intense subject of debate. In erythematous systemic lupus (LES) there is an abnormal expression of lymphokines, with a decrease of IL-2 and γ-interferon (IFN-γ) levels and an increase of IL-10 and IL-6 levels.

Cytokines play an important role in perpetuation and propagation of chronic inflammation in rheumatic diseases. The profile of inflammatory mediators can be different with the disease, but some common cytokines seem to be essential in inflammation, which leads to clinical anomalies. Inflammation was characterized by an obvious increase of TNF-α and IL-1.

Material and method

The studied group was formed by 18 patients admitted in Balneal and Rehabilitation Sanatorium from Techirghiol between July – August 2006, diagnosed with AS.

For assessing the impact of mud treatment upon patients with AS we made determination of proinflammatory cytokines TNF-α, IL-1β and IL-6, using ELISA method. Blood samples for these measurements were taken before initiation of treatment and in the end of cure; samples were processed in Tody Lab from Bucharest.

In the same group we also measured some biochemical parameters from peripheral blood, in order to complete assessment of effects of mud therapy upon inflammatory frame specific to AS and upon bone homeostasis. The following biochemical blood parameters were assessed: ESR by

Wintrobe method, leucocyte number by Coulter method – principle of volumetric electric impedance, alkaline phosphatase by colorimetric kinetic method.

Blood samples for these determinations were taken before the beginning of treatment, when patients came in the sanatorium and in the end of cure, after 12 days. For interleukin-1β we tried a comparison of the effects of warm mud applications (mud bath or packing) and cold mud applications. 14 patients from all 18 made cold mud ointments, the rest of them (4) made warm mud applications.

Because of the significant differences between high values of TNF-α and interleukin-6 in a small number of patients, we were bound from statistical analysis to separate the patients. We divided the patients into 2 groups for statistical analysis: one group of patients with normal values – Lot 1 (13 patients) and another group of patients with much higher values, that means with active disease – Lot 2 (5 patients).

Tumor necrosis factor α (pg/mL) - Lot 1

From the analysis of results of t-Test for paired samples, because $p = 0,295 > \alpha = 0,05$ (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of TNF-α (pg/mL) at the two moments for Lot 1.

This conclusion is shown in figure 7.

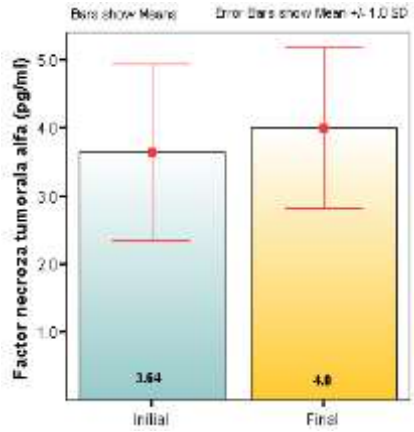

Fig. 7 Variation of tumor necrosis factor alpha Lot 1 (pg/mL)

As one can see from the chart above, TNF-α doesn't have significant variations for Lot 1 of patients treated with mud.

Tumor necrosis factor α (pg/mL) - Lot 2

Because $p = 0,001 < \alpha = 0,05$ (chosen significance level) we accept the hypothesis that there are significant differences between mean values of TNF-α (pg/mL) at the two moments for Lot 2 patients.

This conclusion is shown in figure 8.

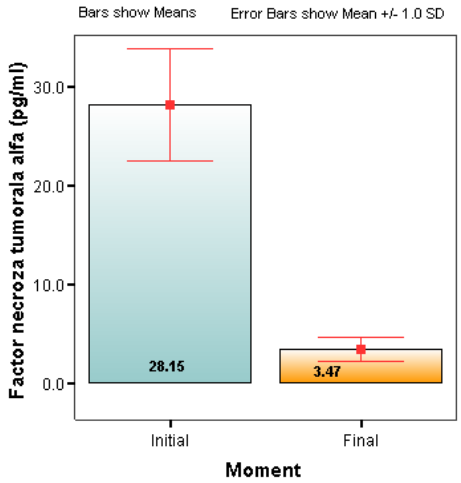

Fig. 8 Variation of tumor necrosis factor alpha Lot 2 (pg/mL)

Because of non-homogeneity of TNF-α values, it was not possible a unitary statistics. Cases were grouped as it follows: first lot included patients with normal values, with inactive disease and second lot included patients with high values of TNF-α, with active disease. TNF-α evolution in the patients treated with mud was different. Patients with physiological values didn't show changes of TNF-α value after the treatment. Contrary, in patients with active AS evolution of TNF-α value showed a statistically significant decrease after mud treatment up to physiological values. It follows that the decrease of TNF-α (an important proinflammatory factor) in patients with active AS train by itself an alleviation of the chronic inflammatory process specific to the disease, fact sensed by the patients as a marked improvement of pain syndrome and, respectively, an improvement of joint motion.

Interleukin-6 (pg/mL) - Lot 1

Because p = 0,301 > α = 0,05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of IL-6 (pg/mL) at the two moments for Lot 1. The IL-6 level shows a not statistically significant increase during the treatment in case of Lot 1, as it can be seen in figure 9.

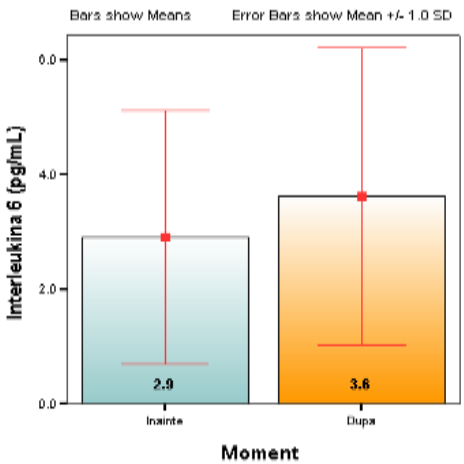

Fig. 9 Variation of interleukin-6 Lot 1 (pg/mL)

Interleukin-6 (pg/mL) - Lot 2

From the analysis of results of t-Test for paired samples, because p = 0,016 < α = 0,05 (chosen significance level) we accept the hypothesis that there are significant differences between mean values of IL-6 (pg/mL) at the two moments for Lot 2.

This conclusion is shown in figure 10.

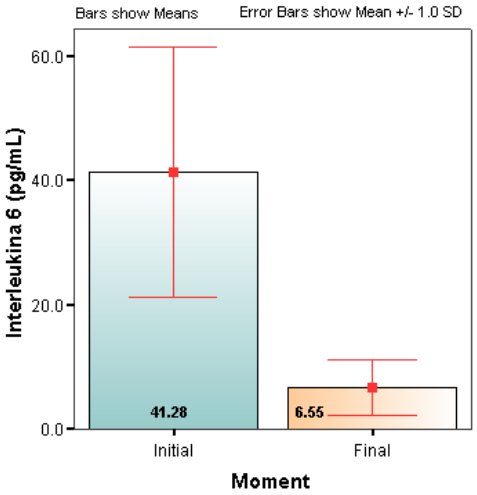

Fig. 10 Variation of interleukin-6 Lot 2 (pg/mL)

In case of IL-6, nonhomogeneous values asked for a different statistical approach, similar to TNF-α. So, the patients with values from normal field didn't show changes after treatment application. Contrary, the group of patients with high values of IL-6 showed after mud therapy a statistically significant decrease of these values up to normal, fact that explains once again alleviation of inflammatory syndrome in patients with active AS.

Interleukin-1ß (pg/mL)

Interleukin-1β has the role of stimulating production of ROS, but in certain conditions can stimulate the activity of glucose-6-phosphate-dehydrogenase, resulting NADPH + H^+, important in regeneration of reduced glutathione. IL-1β is also an activator factor of osteoclasts, stimulating bone destruction in inflamed joints.

For this parameter a comparison was made between the group of patients (4) which underwent warm mud baths (BN) and the group of patients (14) which underwent cold mud ointments on the lake beach (ON).

For BN: because p = 0,225 > α = 0,05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of IL-1β (pg/mL) at the two moments.

For ON: because p = 0,878 > α = 0.05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of IL-1β (pg/mL) at the two moments.

IL-1β, proinflammatory cytokine, doesn't show significant variations in patients with AS

treated with mud, no matter of the type of mud application. Yet, after the warm mud applications one can see a slight decrease of the IL-1β values, while in the end of cure through "Egyptian method" IL-1β values remain constant.

This conclusion is shown in figure 11.

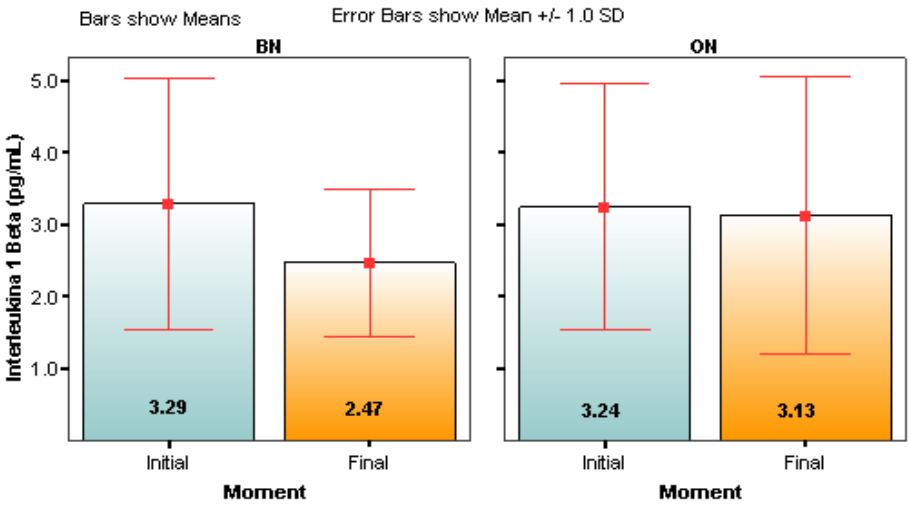

Fig. 11 Variation of interleukin-1ß (pg/mL)

Discussions

TNF-α evolution in the patients treated with mud was different. Patients with physiological values didn't show changes of TNF-α value after the treatment. Contrary, in patients with active AS evolution of TNF-α value showed a statistically significant decrease after mud treatment up to physiological values. That's why the decrease of TNF-α (an important proinflammatory factor) in patients with active AS leads to alleviation of the chronic inflammatory process specific to the disease.

In case of IL-6, nonhomogeneous values asked for a different statistical approach, similar to TNF-α. The patients with values from normal field didn't show changes after treatment application. Contrary, the group of patients with high values of IL-6 showed after mud therapy a statistically significant decrease of these values up to normal, fact that explains once again alleviation of inflammatory syndrome in patients with active AS.

IL-1β, a proinflammatory cytokine, doesn't show significant variations in patients with AS treated with mud, no matter of the type of mud application. Yet, after warm mud applications one can see a slight decrease of the IL-1β values, while in the end of cure through "Egyptian method" IL-1β values remain constant (PROFIR et al., 2011).

6.2.2. Assessment of some biochemical and enzymatic markers

For this group of patients we have also determined some biochemical and enzymatic markers for a more extended assessment of effects of mud therapy. The following parameters were measured before and after treatment: erythrocyte sedimentation rate (ESR) at 1 and 2 hours, leucocyte number,

alkaline phosphatase. The composition of the groups by type of prescribed mud application was maintained.

Erythrocyte sedimentation rate at 1 hour ESR (mm/1h)

For BN: because p = 0,483 > α = 0,05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of ESR (mm/1h) at the two moments.

For ON: because p = 0,399 > α = 0,05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of ESR (mm/1h) at the two moments.

ESR values at 1 hour are not significantly modified in patients with therapeutic application of warm or cold mud. One must consider the fact that initial mean value of ESR at 1 hour belongs to the normal field.

This conclusion is shown in figure 12.

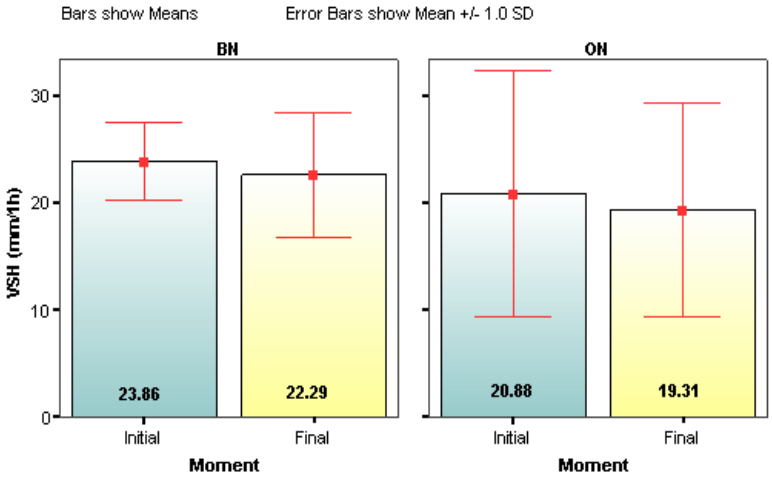

Fig. 12 Variation of ESR at 1 hour (mm/1h)

Erythrocyte sedimentation rate at 2 hours ESR (mm/2h)

For BN: because p = 1,00 > α = 0,05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of ESR at 2 hours at the two moments.

For ON: because p = 0,760 > α = 0,05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of ESR at 2 hours at the two moments.

Variation of ESR at 2 hours in patients with AS is either not influenced by the therapeutic applications with warm or cold mud; mean values of ESR remain in the physiological field in the end of treatment.

This conclusion is shown in figure 13.

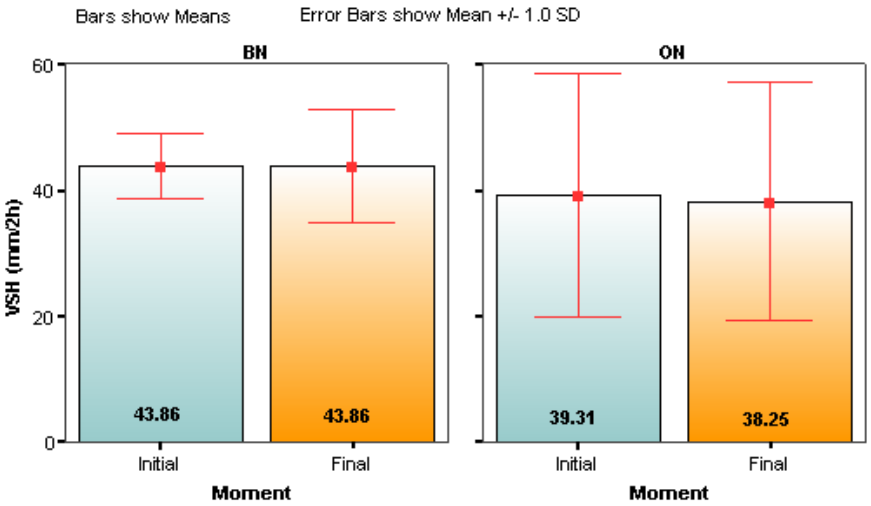

Fig. 13 Variation of ESR at 2 hours (mm/2h)

Leucocyte number (WBC)

For BN: because p = 0,229 > α = 0,05 (chosen significance level) we accept the hypothesis that there are no significant differences between mean values of leucocytes at the two moments.

For ON: because p = 0,04 < α = 0,05 (chosen significance level) we accept the hypothesis that there are significant differences between mean values of leucocytes at the two moments.

Leucocyte level decreases statistically significant after cold mud application and sun exposure, values remaining in physiological limits. The decrease of leucocyte number can be considered as a result of consumption because of an accentuated process of inflammation improvement within this therapy, because of complex immunological mechanisms triggered by the therapy with contrast factors.

This conclusion is shown in figure 14.

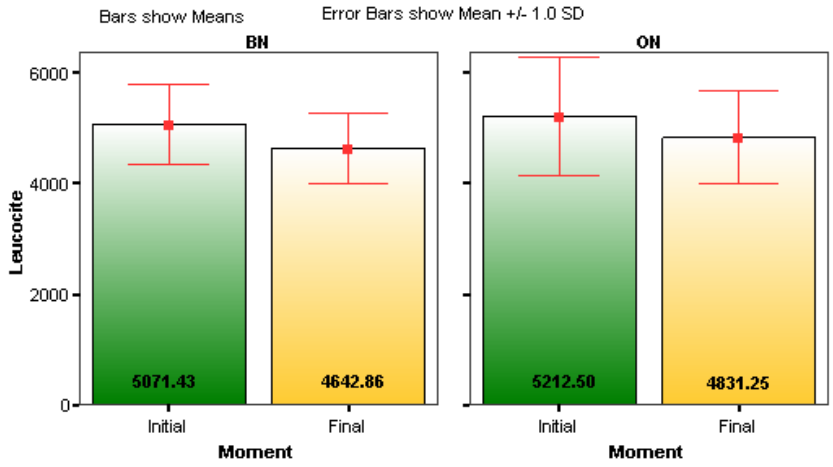

Fig. 14 Variation of leucocyte number

Alkaline phosphatase FA (U/L)

For BN: because p = 0,269 > α = 0,05 (chosen significance level) we accept the hypothesis

that there are no significant differences between mean values of FA (U/L) at the two moments.

For ON: because p = 0,043 < α = 0,05 (chosen significance level) we accept the hypothesis that there are significant differences between mean values of FA (U/L) at the two moments.

FA level decreases statistically significant after cold mud applications (figure 15). Given the important role of FA in bone metabolism and the fact that the patients with cold mud applications benefit also from sun exposure, can be explained the positive influence of cold mud ointment compared with warm mud bath. The decrease of FA values can be a sign of slowing the process of bone destruction in affected joints; therefore, cold mud therapy contributes on long-term in slowing down the progression of disease.

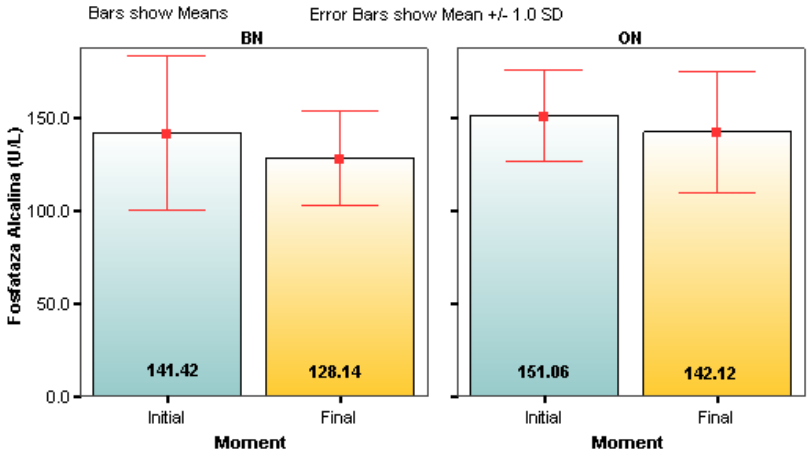

Fig. 15 Variation of FA level (U/L)

Discussions

ESR values at 1 hour are not significantly modified in patients with therapeutic application of warm or cold mud. One must consider the fact that initial mean values of ESR at 1 hour belong to the normal field.

Variation of ESR at 2 hours in patients with AS is either not influenced by the therapeutic applications with warm or cold mud; mean values of ESR remain in the physiological field in the end of treatment.

Leucocyte level decreases statistically significant after application with cold mud and sun exposure, values remaining in physiological limits. The decrease of leucocyte number can be considered as a result of consumption because of an accentuated process of inflammation improvement within this therapy, because of complex immunological mechanisms triggered by the therapy with contrast factors.

FA level decreases statistically significant after cold mud applications (figure 15). Given the important role of FA in bone metabolism and the fact that the patients with cold mud applications benefit also from sun exposure, can be explained the positive influence of cold mud ointment compared with warm mud bath. The decrease of FA values can be a sign of slowing the process of bone destruction in affected joints; therefore, cold mud therapy contributes on long-term in slowing down the progression of disease.

6.3. EXPERIMENT 3

ASSESSMENT OF HORMONAL STATUS

The inflammatory response is modulated in part by a bidirectional communication between the brain and the immune systems. This involves hormonal and neuronal mechanisms by which the brain regulates the function of the immune system and, in the reverse, cytokines, which allow the immune system to regulate the brain. In a healthy individual this bidirectional regulatory system forms a negative feedback loop, which keeps the immune system and central nervous system (CNS) in balance. Perturbations of these regulatory systems could potentially lead to either overactivation of immune responses and inflammatory disease, or oversuppression of the immune system and increased susceptibility to infectious disease.

Many lines of research have recently established the numerous routes by which the immune system and the CNS communicate. Evidence on involvement of these regulatory pathways in RA and other inflammatory diseases provide the reviews by EIJSBOUTS and MURPHY (1999), CROFFORD (2002) and IMRICH (2002).

There are two major pathways by which the CNS regulates the immune system: the first is the hormonal response, mainly through the hypothalamic–pituitary–adrenal (HPA) axis, as well as the hypothalamic–pituitary–gonadal (HPG), the hypothalamic–pituitary–thyroid (HPT) and the hypothalamic–growth-hormone axes; the second is the autonomic nervous system, through the release of norepinephrine (noradrenaline) and acetylcholine from sympathetic and parasympathetic nerves. In turn, the immune system can also regulate the CNS through cytokines.

The study conducted by KIRNAP et al. (2008), in which they stimulated HPA axis in patients with AS by low dose ACTH test (LDT), standard dose ACTH test (SDT) and insulin tolerance test (ITT), has concluded that both basal cortisol values and peak activity meanwhile stimulation were comparable with the values of the control group. In contrast with changes observed in other inflammatory diseases, such as RA or rheumatic polymyalgia, in patients with AS there are no apparent anomalies of the HPA axis activity. AS is an inflammatory condition, yet function of HPA axis in these patients is not affected.

Material and method

Hormonal measurements were performed on a group of 20 patients with AS. We watched the variation of plasma cortisol and thyroid-stimulating hormone (TSH) using ELISA technique; blood samples were taken before starting the treatment, 24 hours after the first mud application and in the end of cure. The patients in this group have received as treatment warm mud applications (mud baths or mud packing) or cold mud ointment on the shore of Techirghiol Lake, depending of the activity of disease and another associated factors. Analysis of the results was made between the following two

groups: Lot 1 composed from 10 patients with warm mud applications (BN) and Lot 2 composed from 10 patients with cold mud ointment (ON).

Cortisol (nmol/L)

p value following Levene test is p = 0,329 for BN and p = 0,069 for ON. Because both these values are higher than 0,05 (chosen significance level), we consider that dispersions within all three studied groups are homogenous for each type of treatment. For both therapeutic type of mud application we must read the values of Bonferroni test.

Under these circumstances, we accept the hypothesis that there are no significant differences between mean values of cortisol correspondent to all three analyzed groups, for any of the two types of applied therapy.

Cortisol shows different variation depending of the type of mud application: it decreases after cold mud application (ON) and increases during warm mud application (BN), but both variations remain in the normal field and are not statistically significant.

This conclusion is shown in figure 16.

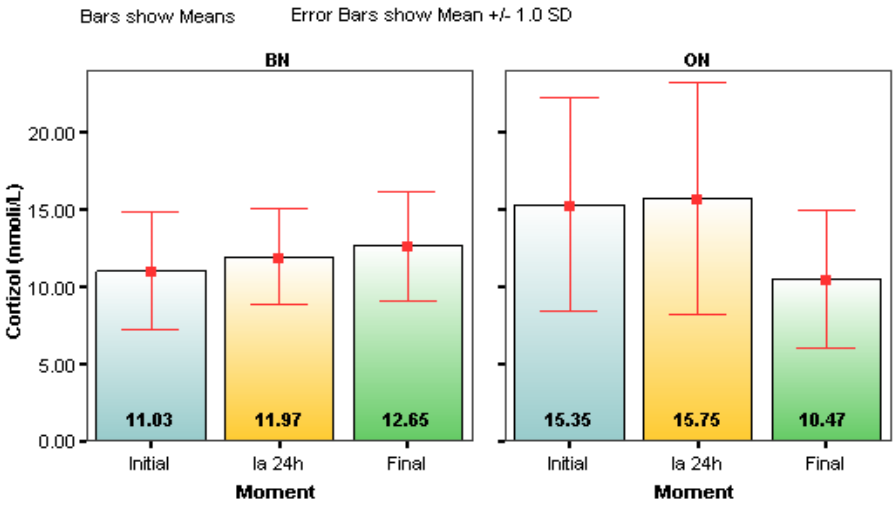

Fig. 16 Variation of plasma cortisol (nmol/L)

Thyroid-stimulating hormone TSH (U/mL)

In order to investigate modulation of secretion of hypophysal hormones since the first 24 hours, we determined variation of plasma TSH following the same scheme as for cortisol.

Neural-endocrine reactivity, enzymatic and metabolic changes within the endocrine glands after mud therapy are different with the secretory type of the gland, with the functional stage of gland and are correlated with the type of therapeutic application. Following mud action, there is a harmonic stimulation within every gland, in the sense of increasing enzymatic and synthesis activity, but with maintaining of each gland specificity (ZIRRA et al., 1964).

p value following Levene test is p = 0,079 for BN and p = 0,247 for ON. Because both these values are higher than 0,05 (chosen significance level), one can consider that dispersions within all

three studied groups are homogenous for each type of treatment. Because Levene test confirmed homogeneity of variance, we must read the values of Bonferroni test.

In accordance with these results, for BN there is a statistically significant increase of TSH in the end of the treatment compared with the initial moment. In case of cold mud applications there are statistically significant differences between the final moment and both first day and second day (after first mud application). The increases of TSH level after mud applications in AS patients, no matter of the mud application type, show the stimulation of HPT and HPA axes.

This conclusion is shown in figure 17.

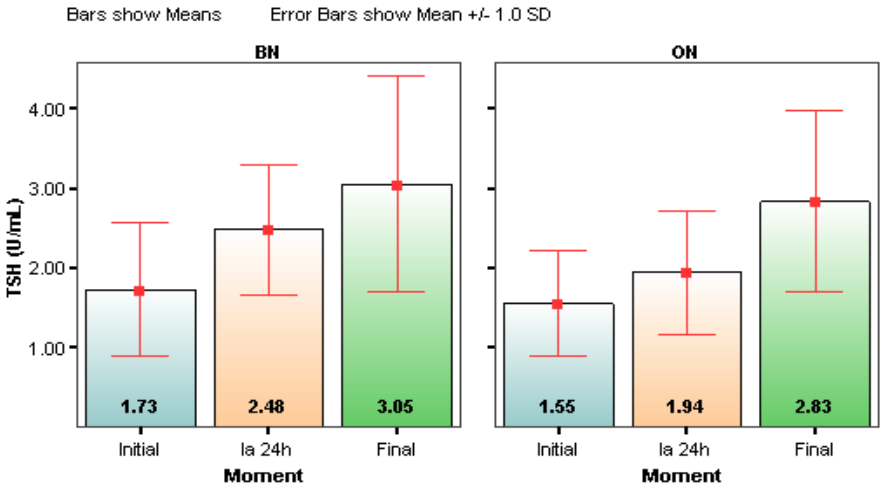

Fig. 17 Variation of TSH (U/mL)

Discussions

As a result of optimization of HPA axis activity after mud therapy in AS patients, we can see an optimization of plasma cortisol basal levels, but without statistically significant increases of them. These results are concordant with those from medical literature.

As for thyroidal activity after peloidotherapy, there are statistically significant increases of plasma levels of TSH in the end of treatment, no matter of the mud application type. In the end of cure there is an improvement of inflammation, resulting into consecutive stimulation of HPT axis (PROFIR et al., In print 2013).

6.4. EXPERIMENT 4

ASSESSMENT OF PARAMETERS OF GASEOUS EXCHANGES IN PERIPHERAL BLOOD AND OF ACID-BASE BALANCE

Material and method

We determined the parameters of acid-base balance and of gaseous exchanges in peripheral blood in a group of 25 patients with AS, treated in Balneal and Rehabilitation Sanatorium Techirghiol between November – December 2006. In this group we took samples before initiation of treatment,

immediate after first warm application (mud bath or salted water bath), 24 hours after first application and in the end of 12 days cure. We used the device CCEX 6 NOVA BIOMEDICAL. We have determined dynamically the following parameters from peripheral blood: oxygen binding capacity (O_2CAP), oxygen content of hemoglobin (O_2CT), partial pressures of oxygen and carbon dioxide from blood (pO_2, pCO_2), saturation in oxygen of peripheral blood ($SO_2\%$), total carbon dioxide (TCO_2), pH, bicarbonate ion ($HCO3^-$) and serum lactate.

Patients were divided into 2 group as it follows: 15 patients underwent a warm mud application (mud bath - BN) as initial procedure, the rest of 10 patients underwent as initial thermic therapy procedure a warm salted bath (BS). For all patients treatment included also electric therapy, massage and individual kinetic therapy for 20-30 minutes. We obtained from all patients their informed consent and we respected their privacy.

Oxygen binding capacity O_2CAP

p value following Levene test is p = 0,874 for BN and p = 0,086 for BS. Because both these values are higher than 0,05 (chosen significance level), one can consider that dispersions within all four studied groups are homogenous for each type of treatment. Because Levene test confirmed homogeneity of variance, we must read the values of Bonferroni test.

The results of ANOVA test are: F = 3,296; p = 0,027 < α = 0,05 for BN and F = 0,841; p = 0,477 > α = 0,05 for BS. Under these circumstances, we accept the hypothesis that mean values of O_2CAP are significantly different for at least two from studied groups only in case of BN.

Because Levene test confirmed homogeneity of the variance (p = 0,874 > 0,05) for BN, we must read the values of Bonferroni test. From the results of Post-Hoc analysis (Bonferroni) for BN we can see a statistically significant variation (p = 0,02 < 0,05) of O_2CAP mean values in the end of treatment compared with the initial moment.

For BS ANOVA test confirmed non-homogeneity of variance and so we must read the values of Tamhane test. This case there are no significant differences between any of studied groups.

This conclusion is shown in figure 18.

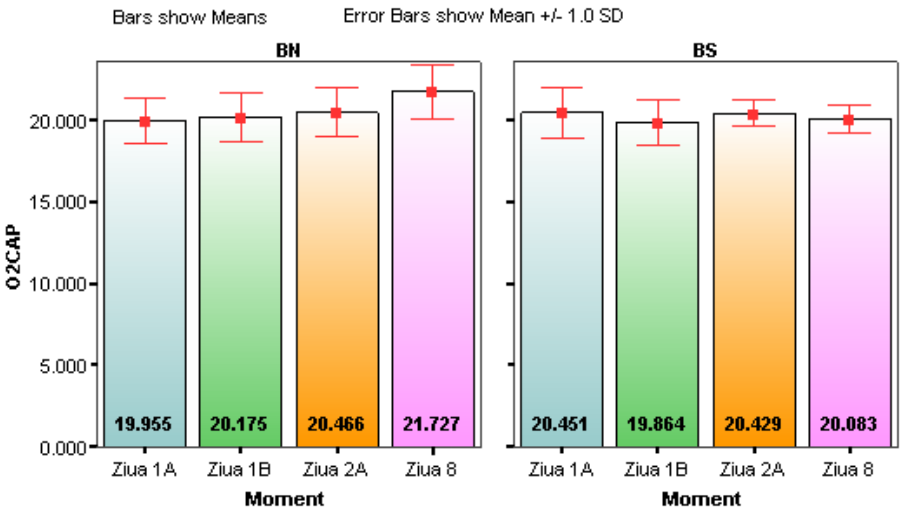

Fig. 18 Variation of O_2CAP

From the charts above we can see the significant increase of mean values of oxygen binding capacity in the end of treatment with BN, which can explain improvement of muscle metabolism by increase of local perfusion and, consecutive, increase of resistance to effort. We can see also a slight increase of O_2CAP value in the end of treatment, compared to day 1 after first mud application, but this variation is not statistically significant. As for BS, there are no significant variations during the treatment, which leads to the conclusion that not only the thermal factor play a role in regulation mechanisms of gaseous exchanges, but probably some chemical constituents within mud.

Oxygen content of hemoglobin O_2CT

p value following Levene test is p = 0,004 for BN and p = 0,02 for BS. Because both these values are smaller than 0,05 (chosen significance level), we can consider that dispersions within all four studied groups are non-homogenous for both type of treatment. Because Levene test confirmed non-homogeneity of variance for both therapeutic schemes, we must read the values of Tamhane test.

From the results of Post-Hoc analysis (Tamhane) for BN we can see a statistically significant variation of O_2CT mean values even the first day of treatment, after first mud application, but between initial and final moment there are no significant variations.

For BS, there are no statistically significant differences of O_2CT mean values during the treatment between any of studied groups.

This conclusion is shown in figure 19.

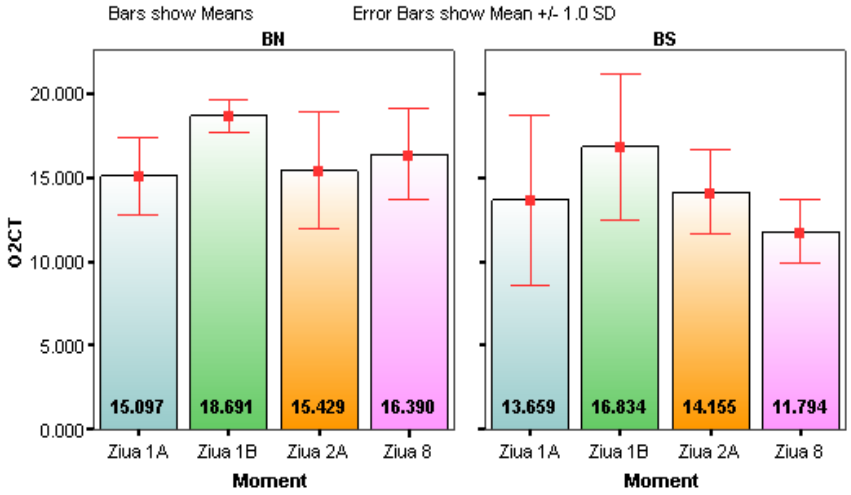

Fig. 19 Variation of O_2CT

In case of BN we can see a statistically significant increase of oxygen content of hemoglobin even the first day, after first mud application, but this is not maintaining till the end, when values return near to baseline. In case of BS we can see an increase of mean values of O_2CT immediately after first saline bath, but not statistically significant; final values decrease compared with initial values, but still not significant. Instead, there is an almost significant decrease ($p = 0,051$) of O_2CT values in the end compared with day 1, after first saline application.

Partial pressure of carbon dioxide pCO_2 (mmHg)

p value following Levene test is $p = 0,286$ for BN and $p = 0,004$ for BS. Because p value for BN is higher than 0,05 (chosen significance level), we can consider that dispersions within all four studied groups are homogenous. In case of BS, where p value is less than 0,05, dispersions within the four groups are non-homogenous.

Because Levene test confirmed homogeneity of variance for BN, we must read the values of Bonferroni test. As we can conclude from the results of Post-Hoc analysis, there are no statistically significant variations of pCO_2 during the treatment with mud baths.

In case of BS, Levene test confirmed non-homogeneity of variance, that's why we must read the values of Tamhane test. We find that even in this context there are no significant differences between studied groups.

This conclusion is shown in figure 20.

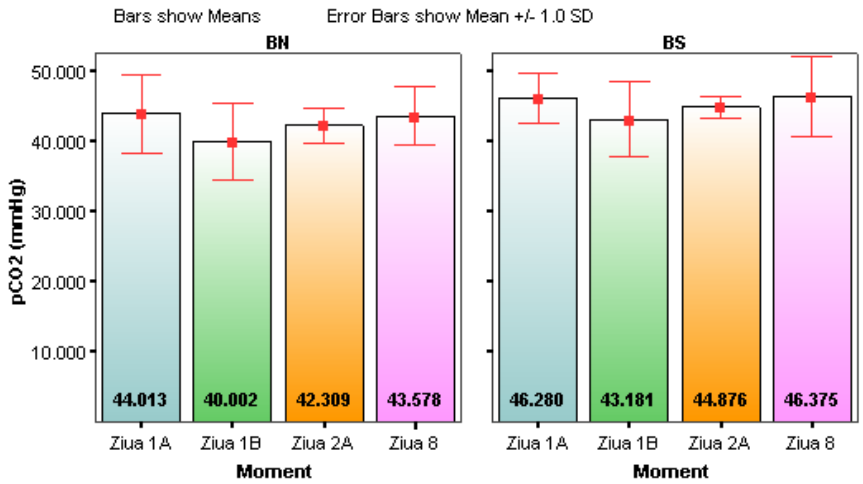

Fig. 20 Variation of pCO_2 (mmHg)

As one can see in the charts above, there is a more accentuated decrease of pCO_2 right after the first mud application, but not statistically significant, after that pCO_2 level begins to increase slightly close up to initial value in the end of treatment. In case of saline baths, pCO_2 values have non-significant variations during therapy, it maintain almost constant throughout the balneal cure.

Partial pressure of oxygen pO_2 (mmHg)

p value following Levene test is p = 0,327 for BN and p = 0,001 for BS. Because p value for BN is higher than 0,05 (chosen significance level), we can consider that dispersions within all four studied groups are homogenous for this type of application an non-homogenous in case of BS, where p value is less than 0,05.

Because Levene test confirmed homogeneity of variance for BN, we must read the values of Bonferroni test. From analysis of results of Post-Hoc test, we can see that there are statistically significant variations of pO_2 mean values both after the first mud application and between day 1 and day 2. There is also a significant decrease of pO_2 values in the end of treatment compared to day 1 after mud procedure.

In case of BS, Levene test confirmed non-homogeneity of variance, that's why we must read the values of Tamhane test. As it results from results analysis there are no significant variations of mean values of pO_2 during treatment with saline bath as initial procedure.

This conclusion is shown in figure 21.

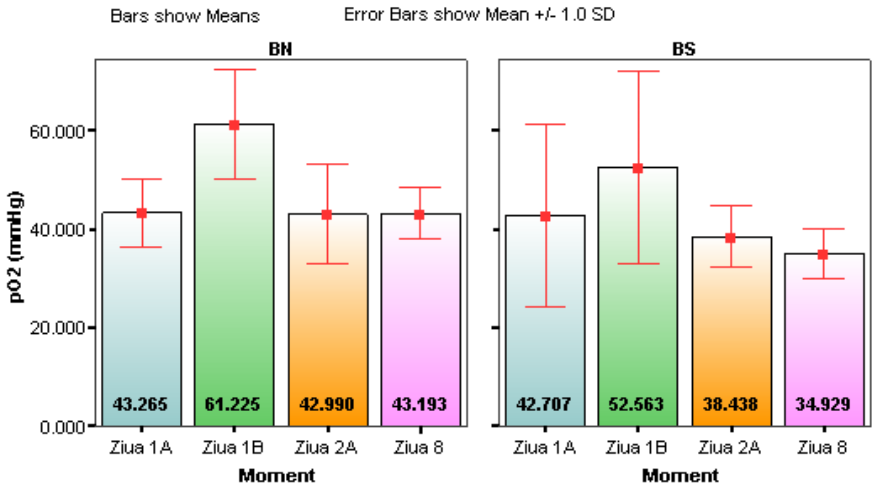

Fig. 21 Variation of pO_2 (mmHg)

There is a significant increase of mean values of pO_2 after the first warm mud application, as well as a significant decrease of partial pressure of O_2 in day 2, close up to values comparable with the initial values, which are maintaining even in the end of cure. We can conclude that the first warm mud application is a critical moment as link of complex mechanisms of action of mud, felt also by the patient as a slight emphasis of pain first days of treatment.

Saturaţia în oxigen a sângelui periferic SO_2 (%)

p value following Levene test is p = 0,026 for BN and p = 0,084 for BS. Because p value for BS is higher than 0,05 (chosen significance level), we can consider that dispersions within all four studied groups are homogenous for this type of application. In case of BN, where p value is less than 0,05, we consider that dispersions within all four groups are non-homogenous.

Because Levene test confirmed non-homogeneity of variance for BN, we must read the values of Tamhane test. From analysis of results of Post-Hoc test, we can see that there are statistically significant variations of SO_2 mean values after the first mud application, which are not maintaining in the end of treatment.

Because Levene test confirmed homogeneity of variance for BS, we must read the values of Bonferroni test. Concordantly with the results obtained in case of warm mud applications, one can see also statistically significant variations of SO_2 only after the first saline bath as initial procedure.

This conclusion is shown in figure 22.

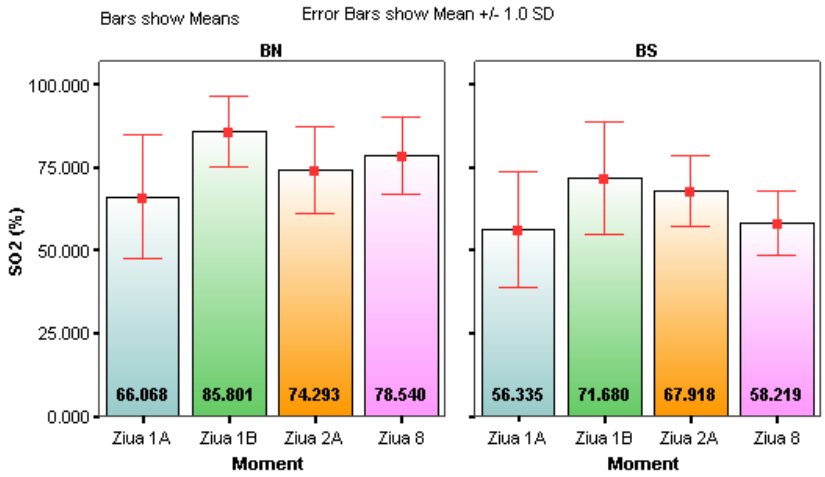

Fig. 22 Variation of SO_2 (%)

In case of warm mud applications there is a statistically significant increase of mean value of O_2 saturation in peripheral blood first day after the procedure, followed by a slight decrease the second day and a slight increase in the end of therapy. In case of saline bath as initial procedure, there is also a statistically significant increase of mean value of SO_2 after the first procedure, after which there is a slow decrease close up to initial value in the end of treatment. Again one can see significant changes of parameter only after first warm application (mud or salted water).

Total blood carbon dioxide TCO_2

p value following Levene test is p = 0,866 for BN and p = 0,600 for BS. Because p value for both therapeutic application types is higher than 0,05 (chosen significance level), we can consider that dispersions within all four studied groups are homogenous.

Because Levene test confirmed homogeneity of variance for both type of application, we must read the values of Bonferroni test. In this context, we can see that there are no significant differences between studied groups for any of the thermic procedures.

This conclusion is shown in figure 23.

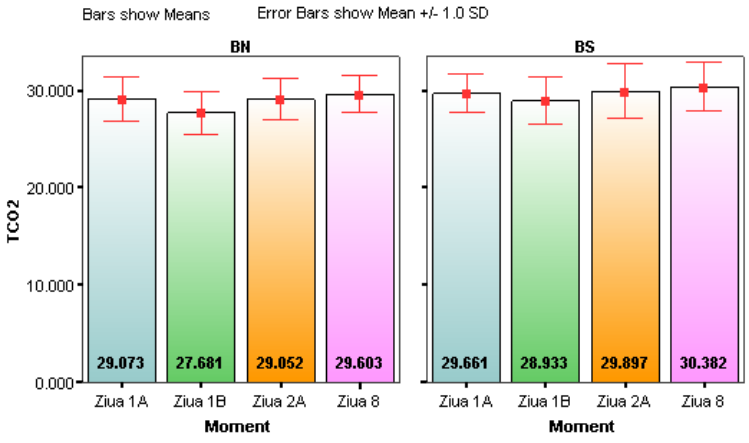

Fig. 23 Variaţia TCO_2

As one can see from the charts above, in case of both BN and BS there are no significant variations of mean value of total carbon dioxide during the treatment. It can be seen a slight decrease of values after the first warm application, after which the values are slowly increasing up to initial value.

Blood pH (7,35-7,45)

Blood pH is a constant of great importance for the activity of some enzymatic systems. pH value is maintained in the normal field (7,35 – 7,45) by complex physical-chemical mechanisms of buffer systems and by physiological mechanisms of respiratory and renal systems. Decrease of pH value less than 7,35 produces metabolic acidosis and increase of pH higher than 7.45 produces metabolic alkalosis.

Plasma buffer system CO_3HNa/CO_3H_2 has a special biological value because bicarbonic anion (HCO_3^-) is the best H^+ acceptor and resulted CO_3H_2 can be rapidly eliminated by lungs as H_2O and CO_2.

p value following Levene test is p = 0,024 for BN and p = 0,851 for BS. Because p value for BS is higher than 0,05 (chosen significance level), we can consider that dispersions within all four studied groups are homogenous for this type of application. In case of BN, where p value is less than 0,05, dispersion within all four groups is non-homogenous.

For BN, because Levene test confirmed non-homogeneity of variance, we must read the values of Tamhane test. In this context we see that there are no significant differences of mean pH between groups.

Because Levene test confirmed homogeneity of variance for BS, we must read the values of Bonferroni test. From the analysis of Post-Hoc test, there are no significant variations of mean pH during treatment with saline baths.

This conclusion is shown in figure 24.

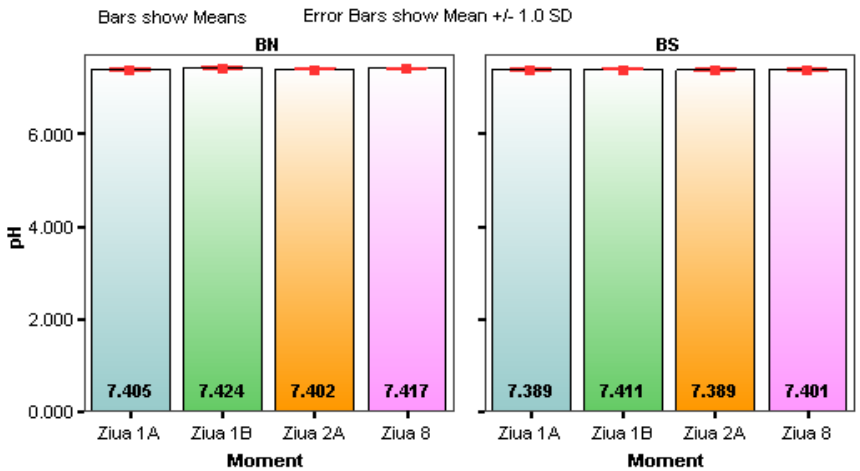

Fig. 24 Variation of pH

From the charts above, one can see that pH remain almost constant during warm applications, both of mud and of saline water, probably throughout intervention of buffer systems of human body.

Serum lactate LAC (mmol/L)

p value following Levene test is p = 0,005 for BN and p = 0,221 for BS. Because p value for BS is higher than 0,05 (chosen significance level), we can consider that dispersions within all four studied groups are homogenous for this type of application and non-homogenous in case of BN, where p value is less than 0,05.

In case of BN Levene test confirmed non-homogeneity of variance, so we must read the values of Tamhane test. From the analysis of this test results, we can see a statistically significant variation of mean values of serum lactate between first mud application and the end of treatment.

Because Levene test confirmed homogeneity of variance for BS, we must read the values of Bonferroni test. From the analysis of Post-Hoc test, there is a significant variation of mean values of lactate between initial and final moment of treatment with saline baths. There are also statistically significant variations between all intermediary moments (after the first saline bath, day 2) and the last warm application.

This conclusion is shown in figure 25.

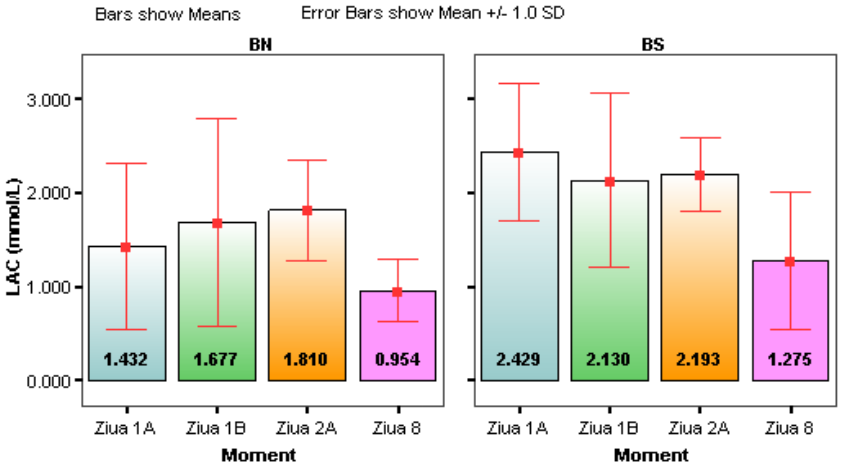

Fig. 25 Variation of serum lactate (mmol/L)

In case of BN there is a slight increase of mean values of lactate after the first mud application, followed by a significant decrease till the end of treatment. Variation of mean values of lactate during saline applications is different; there is a significant decrease in the end of therapy compared to any of the intermediary moments.

Bicarbonic ion HCO_3^- (mmol/L)

p value following Levene test is p = 0,872 for BN and p = 0,580 for BS. Because p value for both type of application is higher than 0,05 (chosen significance level), one can consider that dispersions within all four studied groups are homogenous for every type of therapeutic application.

Because Levene test confirmed homogeneity of variance, we must read the values of Bonferroni test. From the analysis of Post-Hoc test, there are no significant values of mean values of HCO_3^- during mud applications.

In case of initial saline applications, there are no statistically significant variations of bicarbonic anion during balneal cure.

This conclusion is shown in figure 26.

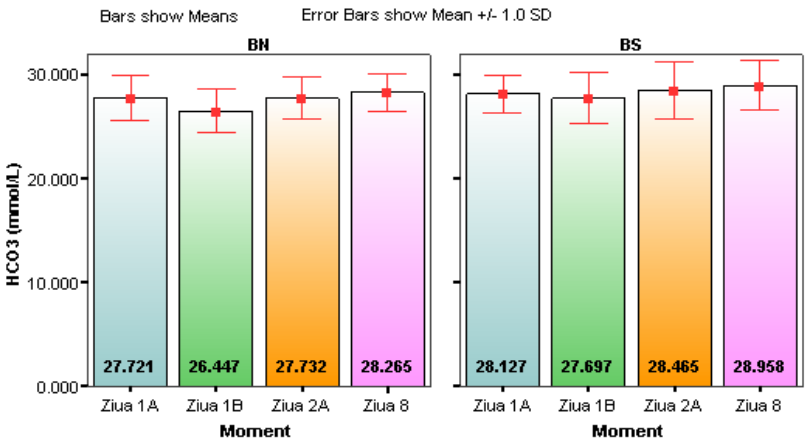

Fig. 26 Variation of bicarbonic anion (mmol/L)

From the charts above, one can see that there are no significant variations of bicarbonic ion values in patients with AS treated with mud and saline water; these values are maintaining relatively constant, after a slight decrease first day, till the end of balneal cure. This evolution is concordant with pH variation, emphasizing once more the efficiency of buffer systems in regulation of acid-basic balance during a stressful metabolic situation, such as peloidotherapy.

Discussions

From the analysis of results obtained after determination of gaseous exchanges parameters in peripheral blood of AS patients, one can see that there are statistically significant increases of pO_2 and SO_2, but only first day after mud application, slowly coming up to initial value in the end of treatment. Oxygen saturation in peripheral blood shows the same significant increase after the first saline bath, similar to initial mud application, probably by vasodilatation produced by thermic factor of mean of immersion. We can conclude that the first mud application represents a critical moment in regulation of neural-vegetative and endocrine balance. Because of pO_2 increase there is an improvement of oxygen diffusion in tissues, which leads to an increase of effort capacity of patients, alleviation of peripheral circulation of the limbs and insomnia. As consequence there is the possibility of early initiation of kinetics, the main link of rehabilitation programme, in order to prevent deformities produced by the disease.

As for pCO_2 and total blood CO_2, the values of these parameters show no significant variation after mud application. Though, there are slight decreases of the values first day of treatment, after mud bath, variations concordant with those of oxygen, then values come back up to initial value.

Blood pH and bicarbonic ion stay constant after mud treatment, probably by intervention of buffer systems activated by the stress of balneal cure.

The values of serum lactate increase after the first mud application and decrease statistically significant in the end of treatment. In case of saline baths there is a similar evolution, with accentuated decrease of values in the end of therapy (MARIN, PROFIR et al., 2011).

CONCLUSIONS

1. Anti-inflammatory potential of sapropelic mud from Techirghiol in patients with AS by assessment of some **markers of oxidative stress** was confirmed by the statistically significant **increase of superoxide dismutase level and by decrease of reduced glutathione and total antioxidant status** after the mud treatment.

SOD controls the level of superoxide anions, preventing initiation of forming reactions of the harmful radicals - hydroxyl and peroxinitrite (formed as a result of the reaction between superoxide anions and nitric oxide). Increase of SOD level after mud therapy can be associated with an improvement of inflammation and represents a positive response of the human body, considering the role of this enzyme in neutralization of ROS and inactivation of NO.

G-SH is an efficient detoxifier of ROS (peroxil lipid radicals, peroxinitrite and hydrogen peroxide), both by direct pathway and indirectly throughout enzymatic reactions. G-SH can conjugate NO forming S-nitrous-glutathione, which is split by thioredoxine system, resulting G-SH and NO. Interacting with glutaredoxine and thioredoxine (thiol proteins), G-SH plays an important role in regulation of redox homeostasis within cells. As for the antioxidant protective mechanism, G-SH is the substrate for glutathione peroxidase (G-SH is used to reduce hydrogen and organic peroxides to water and alcohol). The statistically significant decrease of reduced glutathione in patients treated with mud suggests an increased consumption of G-SH in neutralization reactions of ROS and probably in peroxidation reactions catalyzed by glutathione peroxidase. This variation of reduced glutathione indicates a normal response of cellular metabolism to oxidative stress triggered by the impact of balneal cure upon human body. Would be interesting in the future to establish, by prospective studies, if there are correlations between variation of reduced glutathione and glutathione peroxidase in patients with AS treated with mud, as well as following up of obtained effects.

The decrease of total antioxidant status, more pronounced during first days of the treatment, could be a result of initial impact determined by the stress generated by the transfer in a medical facility, medical examination and initiation of the treatment. The impact of balneal cure generates also a metabolic stress, followed by an increased consumption of antioxidant factors. In the future would be interesting to assess TAS level after 2 weeks or even 1 month from the end of treatment, for a more accurate assessment of the impact of natural factors upon patient's body.

Glutathione reductase level, an essential enzyme for redox cycle of glutathione and protective factor in the balance of oxidative stress, was not significantly influenced by mud therapy. This conclusion highlights protective character of mud therapy upon oxidative stress: enzymes with positive role within this metabolism increase, such as SOD, or remain in the normal field, such as GR.

2. Anti-inflammatory potential of sapropelic mud from Techirghiol in patients with AS by assessment of **proinflammatory cytokines** was confirmed by statistically significant **decrease of tumor necrosis factor (TNF-α) and interleukin-6 (IL-6)** values in patients which had initially increased values, possible being with active AS. The values of IL-1β (a marked proinflammatory factor), although show no statistically significant variation after mud therapy, indicate a slight increase in case of warm mud applications (bath or packing). The obtained results confirm anti-inflammatory action and recommend mud as a helpful therapeutic solution in treating AS.

Study of biochemical blood parameters and some enzymes after mud treatment showed that:

- **Leucocyte level decreases** statistically significant after cold mud application and sun exposure on the lake shore, values remaining in physiological limits. The decrease of leucocyte number can be considered as a result of consumption because of an accentuated process of inflammation improvement within this therapy, because of complex immunological mechanisms triggered by the therapy with contrast factors.

- **Alkaline phosphatase level decreases** statistically significant after cold mud applications. Given the important role of FA in bone metabolism and the fact that the patients with cold mud applications benefit also from sun exposure, can be explained the positive influence of cold mud ointment compared with warm mud bath. The decrease of FA values can be a sign of slowing the process of bone destruction in affected joints; therefore, cold mud therapy contributes on long-term in slowing down the progression of disease.

3. The asessment of **hormonal status** in patients with AS treated with mud showed an optimization of plasma cortisol levels and revealed also stimulation of HPA axis activity sustained by statistically significant **increase of TSH levels**. The increase of TSH level for both type of mud application (warm or cold) can be explained by improvement of inflammation, resulting into consecutive stimulation of HPT axis. Endocrine mechanism is implied also in induction of anti-inflammatory effects of mud therapy by modulation of HPA axis activity and by general endocrine rebalance, effects which last probably even after treatment.

4. Assessment of **gaseous exchanges parameters** in peripheral blood of patients with AS treated with mud showed a **statistically significant increase of pO_2 and SO_2 values,** but only after the first mud application, values coming up to initial ones in the end of treatment. Oxygen saturation of peripheral blood shows the same significant increase after the first saline bath, similar to initial mud application. We can conclude that the first mud application is a critical moment in regulation of neural-vegetative and endocrine balance of the body. Because of pO_2 increase, there is an improvement of oxygen diffusion in tissues, which leads to increase of the effort capacity of patients,

alleviation of peripheral circulation of the limbs and insomnia. As consequence there is the possibility of early initiation of kinetics, the main link of rehabilitation programme, in order to prevent deformities produced by the disease.

As for pCO_2 and total blood CO_2, the values of these parameters showed no significant variations after peloid application. Though, it can be seen slight decreases of values first day of treatment, after mud bath, variation concordant with pO_2 variation, after which values are coming up to initial ones.

We can see that pH and bicarbonic ion values remain almost constant during warm applications, probably throughout intervention of buffer systems of body mobilized by the stress produced by initiation of balneal cure.

Contrary, **values of serum lactate** increase after first mud application and **decrease statistically significant** in the end of treatment. The same evolution of lactate values can be seen in case of saline application as initial procedure, with marked decrease of values in the end of therapeutic cure.

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LUCRĂRI ELABORATE DE DOCTORAND

I. LUCRĂRI PUBLICATE IN EXTENSO ÎN REVISTE RECUNOSCUTE C.N.C.S.I.S.

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