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Prevalence of infestations caused by certain parasite species in pre-school and school children in the area of the Pristina region

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Abstract

Introduction: Helminthiases represent a very serious problem to human health. Helminthiases take a dominant position in child pathology and, therefore, they represent a very serious medical and epidemiological problem. Intestinal parasitism represents a considerable problem in terms of the occurrence of illnesses in the population, especially in pre-school and school children. Social-economic conditions in which people live and work, general culture and especially the culture of health and ecological conditions of soil are extremely important and directly connected with the distribution and prevalence of intestinal parasites. Multicellular intestinal parasites are more prevalent in poor environments with a low standard of living and a low level of general and personal hygiene.

Materials and methods: Examinations were carried out at the laboratory of the Institute of Microbiology with the National Institute of Public Health of Kosovo in Pristina. The following methods were used during the laboratory tests: microscopic examination of perianal imprint and microscopic examination of feces.

Results: The study involved a total of 384 patients, 183 (47.7%) of whom were positive and 201 (52.3%) negative. Statistically significant difference among the examinees was established. Up to 47.7% of them were infested and they were mostly infested with *Enterobius vermicularis* (31.5%), followed by *Ascaris lumbricoides* (20.1%) and *Trichuris trichiura* (19.8%), while other parasites were considerably less prevalent, which is significantly different ($p<0.05$). The prevalence ratio of infestations caused by certain parasite species in the area of the Pristina region by municipalities suggests that the highest percentage of individuals infected with the observed parasite species was registered in the Urosevac municipality. Statistically significant difference among the examinees was established in relation to municipalities and parasite species ($p<0.05$). If we observe individually by parasite species and by municipalities, we can conclude that the highest prevalence of parasite *Ascaris lumbricoides* was registered in the Urosevac municipality with 41.6% of the total number of infested individuals, followed by the Glogovac municipality with 38.96%. The highest prevalence of parasite *Enterobius vermicularis* was registered in 50 infested individuals or 41.3% in the Urosevac municipality, followed by 35.54% in the Glogovac municipality.

Conclusions: Total percentage of infested individuals among pre-school and school children in the area of the Pristina region is considerable (47.7%); the examinees were mostly infested with *Enterobius vermicularis* (31.5%), followed by *Ascaris lumbricoides* (20.1%) and *Trichuris trichiura* (19.8%); the highest percentage of individuals infested with the observed parasite species was registered in the Urosevac municipality; the highest prevalence of parasite *Ascaris lumbricoides* was registered in the Urosevac municipality with 41.6% of the total number of infested individuals, followed by the Glogovac municipality with 38.96%; the highest prevalence of parasite *Enterobius vermicularis* was registered in 50 infested individuals or 41.3% in the Urosevac municipality, followed by 35.54% in the Glogovac municipality.

Key words: infestation, helminths, pre-school and school children, Pristina region.

1. Introduction

Helminthiases represent a very serious problem to human health. Helminthiases take a dominant position in child pathology and, therefore, they represent a very serious medical and epidemiological problem. Intestinal parasitism represents a considerable problem in terms of the occurrence of illnesses in the population, especially in pre-school and school children. Social-economic conditions in which people live and work, general culture and especially the culture of health and ecological conditions of soil are extremely important and directly connected with the distribution and prevalence of intestinal parasites. Multicellular intestinal parasites are more prevalent in poor environments with a low standard of living and a low level of general and personal hygiene.

External environment, favorable temperature, moisture and composition of soil make it possible for certain helminths to keep developing and existing and to continue their parasitic life in humans through eggs or larvae. The following things play the role of mediators in spreading of the infection: food, drinking water, waste waters, toilets, flies, dust in apartments and items handled by humans. The infection in humans is not only caused by a low standard of personal and general hygiene but also by poor sanitary conditions. Analyzing from the epidemiological point of view an epidemiological process of the chain of infection: etiological agent, external environment and human organism, the biggest problem is how to solve the influential relations, external environment. The problem of intestinal parasites is not only related to the incidence, it is also the influence on health-related and economic relations.

Helminths or worms belong to the group of cosmopolite parasites. Certain species infest humans. Climate factors, environmental conditions, as well as customs of the local population represent considerable elements that determine the occurrence and spreading of certain species. Infestation with helminths is especially evident in countries with a moist and warm climate of tropical and subtropical zones and, therefore, we find infestations with several species of intestinal or other helminths (polyhelminthiasis) in the local population quite frequently.

Helminths or worms (Greek *Helminth* – worm) are multicellular, free-living organisms in natural environments or they are adapted to the parasitic way of life. They can be found in humans, animals or plants. Infections – infestations with certain species are encountered in all parts of the world, especially in regions with a moist and warm climate, as well as in our region (1,2,3).

2. Materials and methods

Examinations were carried out at the laboratory of the Institute of Microbiology with the National Institute of Public Health of Kosovo in Pristina. The following methods were used during the laboratory tests: microscopic examination of perianal imprint and microscopic examination of feces.

3. Results

The study involved a total of 384 patients, 183 (47.7%) of whom were positive and 201 (52.3%) negative. The incidence of infestations caused by *Ascaris lumbricoides*, *Enterobius vermicularis*, *Trichuris trichiura*, *Strongyloides stercoralis*, *Taenia spp*., *Hymenolepis nana* in pre-school and school children in the area of the Pristina region was being observed.

No statistical significance ($p>0.05$) was registered in relation to the place of residence and sex of the examinees.

Statistically significant difference among the examinees was established. Up to 47.7% of them were infested and they were mostly infested with *Enterobius vermicularis* (31.5%), followed by *Ascaris lumbricoides* (20.1%) and *Trichuris trichiura* (19.8%), while other parasites were considerably less prevalent, which is significantly different ($p<0.05$).

Table 1. The prevalence of infestations by sex and age of the examinees

Sex	Positive / negative		Age		Total
			Pre-school	School	
Male	Pos. Neg.	Positive	54	48	102
		Negative	40	49	89
	Total		**94**	**97**	**191**
Female	Pos. Neg.	Positive	45	36	81
		Negative	53	59	112
	Total: pre-school/school		**98**	**95**	**193**
	Total: positive negative		**99 93**	**84 108**	**183 201**
	OVERALL		**192**	**192**	**384**
	p		p <0.05	p <0.05	

There is a statistically significant difference (X^2=12.121 p=0.00110) (X^2=12.620 p=0.00105)

Table 2. Age and sex structure of the infested examinees by municipalities of the Pristina region

Municipality	Age		Sex				Total	
	1-Pre-school 2-School		Male	%	Female	%	Number	%
Urosevac	Age	1.00	33	17.3	31	16.1	64	16.7
		2.00	34	17.8	31	16.1	65	16.9
	Total		**67**	**35.1**	**62**	**32.1**	**129**	**33.6**
Glogovac	Age	1.00	29	15.1	35	18.1	64	16.7
		2.00	31	16.2	32	16.6	63	16.4
	Total		**60**	**31.4**	**67**	**34.7**	**127**	**33.1**
Pristina	Age	1.00	32	16.7	32	16.6	64	16.7
		2.00	32	16.7	32	16.6	64	16.7
	Total		**64**	**33.5**	**64**	**33.2**	**128**	**33.3**
	OVERALL		**191**	**100**	**193**	**100**	**384**	**100**

There is no statistically significant difference (X^2=0.769) (p=0.9790)

Table 3. The prevalence of infestations caused by certain parasite species in the area of the Pristina region

Parasite	Positive		Negative	
	Number	%	Number	%
Ascaris lumbricoides	77	20.1	307	79.9
Enterobius vermicularis	121	31.5	263	68.5
Trichuris trichiura	76	19.8	308	80.2
Strongyloides stercoralis	5	1.3	379	98.7
Hymenolepis nana	27	7.0	357	93.0
Taenia spp.	2	0.5	382	99.5
2-parasite species	55	14.3	329	85.7
3-parasite species	35	9.1	349	90.9

There is a statistically significant difference (X^2=266.797) (p=0.00001)

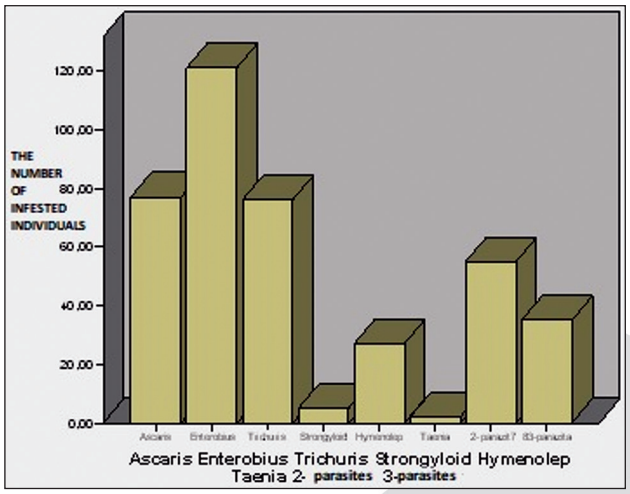

Chart 1. The overall prevalence ratio of certain infestations

Table 4 shows the trend of the prevalence of infestations caused by the abovementioned species of parasites in the positive examinees in the area of the Pristina region by municipalities.

The prevalence ratio of infestations caused by certain parasite species in the area of the Pristina region by municipalities suggests that the highest percentage of individuals infected with the observed parasite species was registered in the Urosevac municipality. Statistically significant difference among the examinees was established in relation to municipalities and parasite species ($p<0.05$). If we observe individually by parasite species and by municipalities, we can conclude that the highest prevalence of parasite *Ascaris lumbricoides* was registered in the Urosevac municipality with 41.6% of the total number of infested individuals, followed by the Glogovac municipality with 38.96%. The highest prevalence of parasite *Enterobius vermicularis* was registered in 50 infested individuals or 41.3% in the Urosevac municipality, followed by 35.54% in the Glogovac municipality.

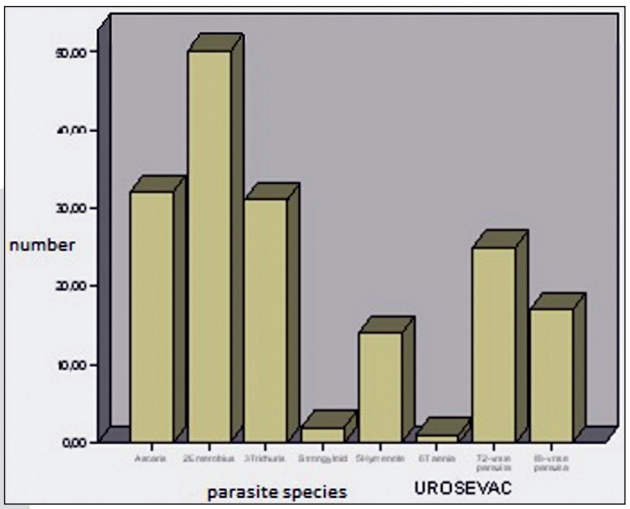

Chart 2. The prevalence ratio of infestations in the area of Urosevac

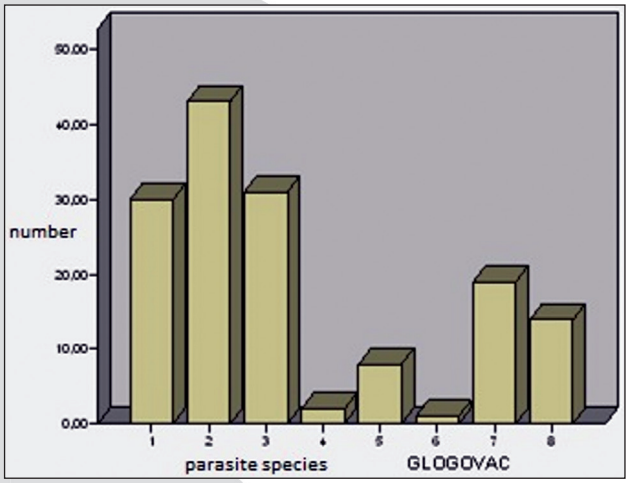

Chart 3. The prevalence ratio of infestations in the area of Glogovac

Table 4. The prevalence of infestations by municipalities of the Pristina region

Parasite	Municipality						Total	
	Urosevac	%	Glogovac	%	Pristina	%	Number	%
Ascaris lumbricoides	32	41.6	30	38.96	15	19.48	77	20.05
Enterobius vermicularis	50	41.3	43	35.54	28	23.14	121	31.51
Trichuris trichiura	31	40.8	31	40.79	14	18.42	76	19.79
Strongyloides stercoralis	2	4.0	2	40.00	1	20.00	5	1.30
Hymenolepis nana	14	51.85	8	29.63	5	18.52	27	7.03
Taenia spp.	1	50.00	1	50.00	0	-	2	0.52
2-parasite species	25	45.45	19	34.55	11	20.00	55	14.32
3- parasite species	17	48.57	14	40.00	4	11.43	35	9.11
p <0.05								

There is a statistically significant difference ($X^2=24.268$) ($p=0.0460$)

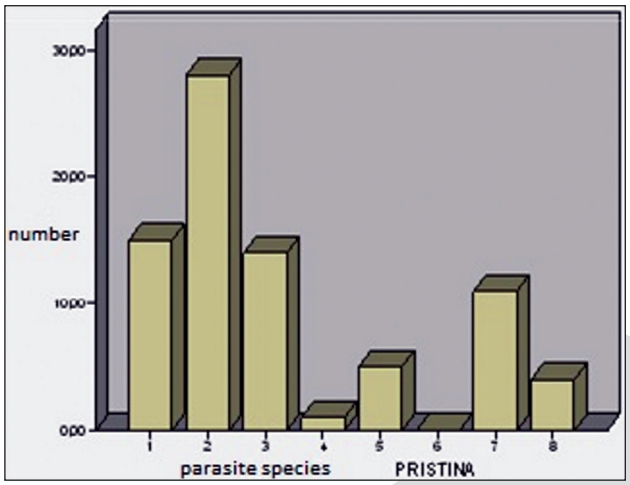

Chart 4. The prevalence ratio of infestations in the area of Pristina

4. Discussion

Parasitoses caused by multicellular intestinal parasites represent a very serious medical and public health problem to the health of humans, especially children. In order to understand the degree and the intensity of infestation with multicellular intestinal parasites in the examined area, it is necessary to get familiar with some of the factors that make it possible for them to keep spreading and existing. The Pristina region, where infestation with multicellular intestinal parasites in pre-school and school children was being examined, is a densely populated area with 252 inhabitants per square kilometer. Therefore, social-economic conditions in which people live and work, general culture and especially the culture of health, ecological factors and climate conditions are equally important and directly connected with the distribution and prevalence of multicellular intestinal parasites.

General features of the Pristina region and the Pristina municipality itself include the unfavorable sanitary-hygienic living conditions, unresolved problem of sanitarily safe water supply for a part of the population, as well as unsanitary disposal of waste. Rural population is mostly supplied with water from draw-wells, natural sources, cisterns and watercourses. Tapped springs are quite rare and they serve for washing clothes etc. Draw-wells are mostly near the houses and business facilities, so there is a high probability that residues from the surrounding surfaces are rinsed off into them. Pumps are positioned in a similar way as draw-wells and their surroundings, just like surroundings of draw-wells, does not meet the construction and maintenance requirements.

Continuous existence of helminthiases in the area of the Pristina region is a result of combination of several factors (customs, way of life, hygiene habits, nutrition, soil, climate, biological features of helminths etc.). Results of the previous studies of infestation of the examined pre-school and school children in the area of the Pristina region suggest a wide distribution of infestation with intestinal helminths (4). In our study, the highest percentage (47.7%) refers to infestations with *Enterobius vermicularis*, which means that the results of this study confirmed enterobiasis as the most frequent of all helminthiases, which is in accordance with data from the literature that enterobiasis is one of the most frequent helminthiases that occurs in humans. According to data from the literature, the infestation percentage is different in different parts of the world, depending on who did the study, what method was used and where the examinations were carried out. It is extremely important to undertake sanitary-preventive measures and to permanently educate expert staff capable of diagnosing these curable conditions in an adequate manner. The prophylaxis includes systematic examinations of children and adults in groups with respect to all family members of the infected individuals, as well as undertaking the measures of personal hygiene and healthcare education (5).

5. Conclusions

Total percentage of the infested individuals among pre-school and school children in the area of the Pristina region is considerable (47.7%).

The examinees were mostly infested with *Enterobius vermicularis* (31.5%), followed by *Ascaris lumbricoides* (20.1%) and *Trichuris trichiura* (19.8%).

The highest percentage of individuals infested with the observed parasite species was registered in the Urosevac municipality.

The highest prevalence of parasite *Ascaris lumbricoides* was registered in the Urosevac municipality with 41.6% of the total number of infested individuals, followed by the Glogovac municipality with 38.96%.

The highest prevalence of parasite *Enterobius vermicularis* was registered in 50 infested individuals or 41.3% in the Urosevac municipality, followed by 35.54% in the Glogovac municipality.

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Cytogenetic properties of novel immunomodulator met-enkephalin/tridecactide in comparation with pulse treatment of relapse in relapse remitting multiple sclerosis

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Abstract

Introduction: Immunomodulation with peptides aims to achieve long-term remission in patients with multiple sclerosis (MS) without adverse effects characteristic for immunosuppressive therapy. The combination of met-enkephalin and tridecactide in ratio 5:1 (Enkorten®, Farmacija d.o.o Tuzla), has been developed as a new generation of immunomodulatory treatment. According to available data from cytogenetic *in vitro* evaluations, there is a clear confirmation of the positive effects of met-enkephalin on normalization of the number and type of serious chromosomal aberrations, such as ring and dental chromosomes (Stambuk et al, 1998), but there is a lack of data about efficacy of combination of two endopeptide substances. Peripheral effects of α-MSH on the immune system include "anti-cytokine" activity. This study will aim to give an answer as to how much actually the combination being tested exerts cytogenetic effects at the time of relapse, but also after the end of the treatment period.

Methods: Randomized, two-arm, parallel interventional study was designed to evaluate cytogenetic effects of treatment of relapse of RRMS with a combination of met-enkephalin and tridecactide under the brand name Enkorten^{Ò} (ENK), Farmacija d.o.o., or pulse corticosteroid therapy.

Results: The study has not detected statistically significant difference in the frequency of structural chromosomal aberrations in RRMS patient's cultures prior to treatment relapse between two treatment groups, but difference was statistically significant compared to healthy volunteers. Statistically significant reduction of the number of aberrations was found after the ENK application compared to pulse therapy that we have detected could suggest synergistic effect of met-enkephalin and tridecactide. Findings of cultures that have been performed after the application of treatment show the reduction of all aberrations, and most of all the occurrence of bicentric chromosomes and quadradial figures. In addition, the statistically significant difference in the incidence of types of aberrations before and after therapy regardless of the type of therapy applied and the aberrations that were most commonly appearing in the culture prior to treatment are chromatid breaks and acentric fragments, but there is also the phenomenon of bicentric chromosomes and quadradial figures. The chromosomes that were most often involved in aberrations in the samples of patients with RRMS and it was identified that chromosome number 2 was involved, and from the other identified chromosomes in the aberration, more precisely defined chromosomes were grouped A and C.

Key words: cytogenetic, met-enkephalin/tridecactide , immunomodulators, multiple sclerosis

Introduction

Immunomodulation with peptides aims to achieve long-term remission in patients with multiple sclerosis (MS) without adverse effects characteristic for immunosuppressive therapy. The combination of met-enkephalin and tridecactide in

ratio 5:1 (Enkorten®, Farmacija d.o.o Tuzla), has been developed as a new generation of immunomodulatory treatment.

Immunomodulatory effects of the both components, met-enkephalin and tridecactide, that is by chemical composition deacetylated and deaminated a-melanocyte stimulating hormone (a-MSH), are already known to the scientific-research community, but their synergistic action is a novelty (Konjevoda et al. 2001). The therapeutic effect of this combination was confirmed in clinical research of Enkorten® and today the drug is successfully used in the treatment autoimmune diseases such as MS. The effects of both peptides include analgesia, antipyretic and antioxidant activity, and anti-inflammatory activity without most of the undesirable effects of steroid and nonsteroidal anti-inflammatory drugs, still mostly represented in the treatment of immune mediated diseases (Mulabegović i Rakanovic-Todic, 2008). The mechanism of the anti-inflammatory effect of a-MSH, tridecactide being considered as the carrier of the neuropeptide combination activity, is not fully defined, but it is known that its repeated central administration inhibits peripheral inflammation, which may be consistent with the neuroendocrine modulation of the immune response (Lipton et al, 1991; Lipton i Catania, 1997; Lipton et al, 2001).

According to available data from cytogenetic *in vitro* evaluations, there is a clear confirmation of the positive effects of met-enkephalin on normalization of the number and type of serious chromosomal aberrations, such as ring and dental chromosomes (Stambuk et al, 1998). Peripheral effects of α-MSH on the immune system include "anti-cytokine" activity. The suggested mechanism of anti-inflammatory activity of α-MSH is inhibition of pro-inflammatory cytokines (TNFα, IFN g and IL-2), but also the synthesis of anti-inflammatory cytokines, especially IL-10 (Weiss et al, 1991; Bhardwaj et al., 1996). Given the scientific fact that peptides derived from proopiomelanocortin are found in many tissues such as lymphocytes, monocytes, macrophages, their pharmacodynamic effects in these tissues would be logically expected. Also, since tridecactide copy the amino acid sequence 1-13 of adrenocorticotropic hormone (ACTH 1-13), it is presumed to behave in the sense of stimulation of the adrenal cortex. To date, it is known that the whole molecule (ACTH 1-39) and ACTH 1-24 possess both corticotropic and neurotropic activity, which is used in the treatment of neuro-immunologic diseases such as MS (Hol et. al. 1995, Darlington et. al.1996).

Met-enkephalin is one of the endogenous opioid peptides widely distributed in the central nervous system (CNS), and one of its peripheral activities is likely to be achieved by the stimulation of peripheral blood lymphocyte via OP1 (δ) receptors. Opioid peptides are suggested to act as growth factors that control cell proliferation and differentiation, and also participate in the healing process, tissue regeneration, and immune response (Zagon et al., 2000).

Cytogenetic research findings using *in vitro* methodology of peripheral blood cell cultures of blood samples taken from patients with remitting-relapsing MS (RRMS) with application of both Enkorten(R) neuropeptide components, either separately or together, establish disappearance of serious chromosomal aberrations such as ring chromosomes and chromosome fragmentations, pointing again to the protective effect of the neuropeptide combination (Rakanovic-Todić et al., 2013; Rakanovic-Todić et al., 2014; Rakanovic-Todić et al., 2015;). The same studies find the chromosome 14 as the most commonly involved in translocations, and Chataway et al (1998) found it to be a potent carrier of genes responsible for MS pathogenesis.

This study will aim to give an answer as to how much actually the combination being tested exerts cytogenetic effects at the time of relapse, but also after the end of the treatment period.

Materials and methods

Randomized, two-arm, parallel interventional study was designed to evaluate cytogenetic effects of treatment of relapse of RRMS with a combination of met-enkephalin and tridecactide under the brand name Enkorten® (ENK), Farmacija d.o.o., or pulse corticosteroid therapy.

Patients

This study included patients aged 18-60 years with clinically confirmed diagnosis of RRMS in accordance with the McDonald Diagnostic Criteria (2010). Subject that fulfilled the following ex-

clusion criteria were not selected for study participation: not confirmed diagnosis of MS, progressive form of the disease, pregnancy or planning a pregnancy, breastfeeding, severe mental disorders (severe depressive episode, psychotic disorders), refractory epilepsy, refusing to sign inform consent form at the entering the study.

Study treatments

Patients who met all inclusion and exclusion criteria were randomized into two study treatment arms, to receive either ENK or pulse corticosteroid therapy. The control group was matched by age and sex.

Patients treated with ENK undergone the following dosage regimen:

- During the first week three doses of 12 mg (2 mg of tridecactide and 10 mg of met-enkephalin) for three consecutive days were applied.
- During the second week three doses of 12 mg were administered, but every other day and
- During the third week three doses of 6 mg (1 mg of tridecactide and 5 mg of met-enkephalin) were administered every other day.

Patients treated with pulse corticosteroid therapy received 1000 mg of IV methylprednisolone once a day, and continued with an oral administration of prednisolone at a dose of 100 mg on the first day, 60 mg on the second and 20 mg during the third day of treatment.

Cytogenetic evaluation

Blood samples were analyzed on structural and numerical chromosomal aberrations at the Center for Human Genetics of the Faculty of Medicine, University of Sarajevo. Samples were collected at two points: at the time of relapse and after the treatment whether it is on 21st (+1) day of receiving the combination of met-enkephalin and tridecactide or fourth (+1) day of receiving high dose of methylprednisolone.

A full blood samples were cultivated following methods and techniques described by Moorhead et al. (1960) with the incubation of cultures on 37° C during 48 hours.

Colchemid stock solution (0.1 ml) was added 90 minutes before completion of the incubation period. PB MAX medium in an amount of 5 ml was used for all cultures. Microscopic analysis of chromosomal aberrations in PBL was done after Giemsa staining, with three hundred mitoses per subject scored. To determine mitotic index, the lymphocyte percentage in mitosis was detected in 300 lymphocytes.

Statistical analysis

Data were statistically analyzed in computer software SPSS (Statistical Package for Social Sciences©, version 17.0, SPSS Inc, Chicago, Illinois, USA). Comparison between mean values of group was performed by Mann-Whitney U test for variables without normal distribution. In order to test the difference between groups, we used a non-parameter test for related samples, two-tailed *Wilcoxon Ranks Test.* P values less than 0.05 were considered statistically significant.

Ethical aspect

Informed consent was obtained from all patients at the study enrollment. The study was approved by Local Ethic Committees of Faculty of Medicine University of Sarajevo.

Results

The study included a total of 78 patients with RRMS (56% women). The mean age of the subjects involved in the study was 39.038±10.92 years.

Chromosomal aberrations

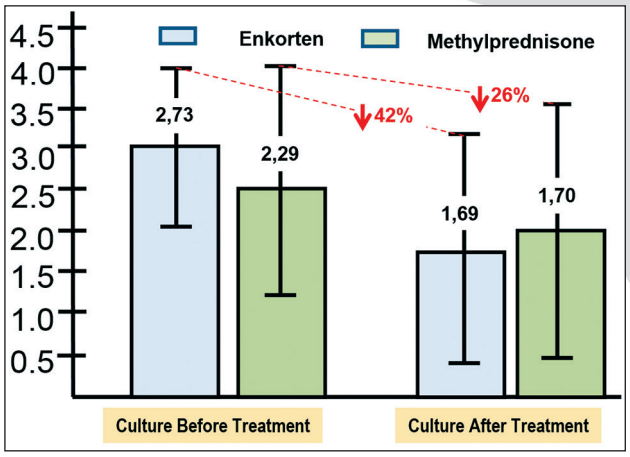
Number of detected structural chromosomal aberrations in the culture A, taken at the moment of patient admission (moment of disease relapse), and culture B, taken after the treatment, are presented in the table 1. The number of aberrations detected in the cultures A of the patients with RRMS ranged from 0 to 5, with the average number of 2.72 for a group treated with ENK vs. 2.29 in a group randomized on pulse corticosteroid therapy.

Table 1. Number of detected structural aberrations per culture for pre-treatment (A) and post treatment cultures (B)

Number of aberrations per culture	Before the treatment (culture A)			After the treatment (culture B)		
	ENK treatment	Pulse therapy	Control group	ENK treatment	Pulse therapy	Control group
0	0	2	17	3	3	17
1	4	5	7	11	7	7
2	7	2	8	5	2	8
3	8	3	3	3	2	3
4	4	4	2	2	3	2
5	1	1	0	0	0	0
Total	24	17	37	24	17	37

The statistically significant difference in the number of detected aberrations was reported between the patients with RRMS and healthy volunteers, as tested with the Mann-Whitney U test ($p < 0.005$, $Z = -4.284$, $p < 0.005$, $Z = -2.742$ respectively), while statistical significance between two groups of patients with RRMS that were subsequently randomized to ENK and pulse therapy was not established ($Z = -0.719$, $p = 0.472$).

The maximum number of detected aberrations per culture in a control group (healthy volunteers) was 4. Also, the maximum number of detected aberrations per culture of patients with RRMS after the treatment was 4. Greater decrease of the average number of total aberrations could be noted for the group receiving ENK (1.69), compared to the less prominent reduction in patients receiving pulse therapy (1.7) as shown on graph 1.



Graph 1. Mean values of number of structural aberrations per culture per culture for pretreatment (A) and post treatment cultures (B) according the treatment group

Multivariate analysis was performed to test differences between number of aberrations before and after the treatment, either with ENK or pulse corticosteroid therapy. Group of patients treated with ENK showed more complex basic profile and frequencies of chromosomal aberrations ($p<0.05$, F 11.143, Partial Eta Squared 0.231).

Wilcoxon signed rank test was used for testing pre-treatment and post-treatment differences in the number of detected aberrations. There was statistically significant reduction of the number of aberrations after treatment with ENK ($Z= -2.901^{a}$, $p< 0.05$), while no significant difference is detected between cultures A and B after treatment with pulse therapy ($Z= -1.348^{a}$, $p= 0.178$).

Types of detected chromosomal aberrations are shown in Table 2. Culture B shows general reduction and reduction of combined aberrations in both groups of subjects.

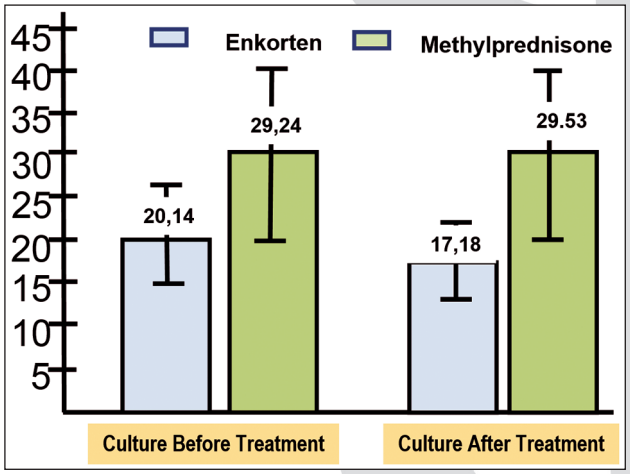
The chromosome identified with G band as the most commonly involved in aberrations in patients with RRMS was Hr2. This finding applies on both cultures (A and B), independently of the treatment application.

Mitotic index was evaluated as a percent of lymphocytes in mitosis on 300 lymphocytes (graph 2).

The mean value of lymphocyte counts did not change in patients receiving pulse therapy ($Z = -255b$, $p = 0.776$) compared to lower lymphocyte counts in subjects receiving the investigated drug ($Z = -1.913a$, $p = 0.056$).

Table 2. Total number of detected structural aberrations in cultures A and B per group

Type of aberrations	Before the treatment (Culture A)			After the treatment (Culture B)		
	ENK	Pulse therapy	Healthy Control	ENK	Pulse therapy	Healthy Control
NO aberrations				0	1	0
Acentric fragment (ACE)	6	0	6	6	5	6
MINUTE	0	3	6	2	2	6
ACE &CHROMOSOME BREAK (CB)	3	1	3	2	0	3
CHROMOSOME BREAK	6	2	3	5	2	3
MIN & CB	1	1	0	-	-	-
ACE, BIC, MIN, CB	1	1	0	-	-	-
ACE & MIN	3	3	1	6	2	1
ACE, MIN,QUADRADIAL	1	0	0	0	1	0
ACE, MIN,CB	2	3	0	0	2	0
ACE, QUADRADIAL	1	0	0	0	0	1
HR LOM, BIC, ACE	0	1	0	0	1	0



Graph 2. Mean values of mitotic index in culture A and B for both treatment groups

Discussion

Chromosomal aberrations are a standard parameter of the drug genotoxicity assessment.

More frequent appearance of chromosomal aberrations was noted in patients with MS (Štambuk et al 1998; D'Alessandro et al, 1990; Rakanović-Todić, 2013), what is in accordance with our findings. Our study has not detected statistically significant difference in the frequency of structural chromosomal aberrations in RRMS patient's cultures prior to treatment relapse between two treatment groups (2.62±1.09 vs. 2.29±1.57 for patients randomized on treatment with ENK and pulse therapy respectively), but difference was statistically significant (p<0,05) compared to healthy volunteers (1.08±1.23).

Statistically significant reduction of the number of aberrations after the ENK application compared to pulse therapy (Z= -2.901[a], p= 0.04; Z= -1.348[a], p= 0.178) that we have detected could suggest synergistic effect of met-enkephalin and tridecactide (Mulabegović N, 2009). Similar findings reported Štambuk et al, (1998) for in-vitro application of met-enkephalin alone. Another in-vitro research conducted by Rakanovic-Todic et al (2015) has not detect statistical significance after the application of combination of met-enkephalin and tridecactide, while these controversy in results may be due to a methodological difference in the length of incubation of PBL cultures after the test compound applications (72 hours).

It should be noted that, in contrast to the previous "in vitro" (Rakanović-Todić, 2015) research where certain numerical chromosomal aberrations (polyploidia) were noticed, they were not found in our study, except in one isolated case when after pulmonary therapy the observed phenomenon of endoreduplication, which could be interpreted by the technical artefact created in the process of preparation and treatment of the sample, as suggested by Zimonjic (1990).

We also reported a statistically significant difference in the incidence of types of aberrations before and after therapy regardless of the type of therapy applied (Chi 2 = 25.943, p = 0.001), and

the aberrations that were most commonly appearing in the culture prior to treatment are chromatid breaks and acentric fragments, but there is also the phenomenon of bicentric chromosomes and quadradial figures. Ring chromosomes are not seen in any culture. Findings of cultures that have been performed after the application of treatment show the reduction of all aberrations, and most of all the occurrence of bicentric chromosomes and quadradial figures.

The findings of our paper coincide with the research Štambuk et al (1998) and Rakanović-Todić et al. (2015) where the most commonly observed phenomenon is chromatid breaks and acentric fragments.

During the research, attempts were made to identify the chromosomes that were most often involved in aberrations in the samples of patients with RRMS and it was identified that chromosome number 2 was involved, and from the other identified chromosomes in the aberration, more precisely defined chromosomes were grouped A and C. Comprehensive literature which analyzes gene locuses on particular chromosomes, tries to give a better and clearer insight into the pathogenesis of multiple sclerosis. To date, there are several correlation certificates for certain genetic loci on chromosome 2 (usually 2q33) (Favorova et al 2006), but the target zones on chromosomes 17, 19, 5, 3, 6, 16 (International Multiple Sclerosis Genetic Consortium 2005; Hauser & Godin 2005)

The suppressive effects of αMSH on lymphocyte cycle and reduction of the number of aberrant clones of lymphocytes has been suggested in previous research also (Catania et al. 2000, Štambuk et al 1998). We haven't detected statistically significant change in mitotic index by ENK or pulse therapy (Z= --1.913[a], **p= 0.056**; Z= -.285[b], p= 0.776, respectively), with borderline tendency for ENK that should be explored further. The slight trend of reduction of mean value (from 20.14 to 17.38) was notable after the treatment with ENK, while after the pulse therapy mean value is almost the same (29.23 and 29.54 respectively).

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The science behind Corps Lignea Celulift: an evidence-based review on its mechanism of action

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Abstract

There is growing interest in using naturally occurring compounds as prospective therapeutic agents in human populations. In this regard, a significant correlation between dietary intake and various health benefits has been shown in scientific research data generated throughout the world. Evidence from numerous *in vitro* and *in vivo* studies has confirmed the benefits of dietary supplements. This evidence-based review discusses the main current data available and assess the science behind Corps Lignea Celulift, a new Brazilian dietary supplement.

Key words: Corps Lignea Celulift dietary supplement, health, *in vitro*, *in vivo*.

Introduction

A paradox in metabolism is that, while aerobic organisms require oxygen for their existence, oxygen is also a highly reactive molecule that can cause a lot of damage. Reactive oxygen species (ROS), physiological metabolites resulting from respiration such as the superoxide anion radical ($O2°^{-}$), the hydrogen peroxide (H_2O_2), or the hydroxyl radical (HO°), are very unstable and rapidly damage other substances including DNA, membrane lipids and proteins (DAVIES, 1995).

However, a complex network of antioxidant metabolites and enzymes work together to prevent this oxidative damage. ROS also have useful cellular functions, such as redox signaling. Thus, the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level (RHEE, 2006).

Oxidative stress is defined as an impaired balance between ROS production and antioxidant defenses, resulting in the accumulation of oxidative products. It is involved in many diseases, such as inflammatory or cardiovascular diseases (GRIENDLING; ALEXANDER, 1997). The removal of free radicals by exogenous antioxidant compounds could thus be an effective precautionary measure against various diseases. Several kinds of supplements containing antioxidant compounds have been described, including those with metabolites such as selenium or vitamins as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPx), which have longer lasting effects because of a lower rate of exhaustion.

One of the most critical oxidative radicals, the $O2°^{-}$ is biologically significant because of its capacity to generate other more reactive species such as HO° (MCCORD, 2000). The best physiological defense against $O2°^{-}$ is the enzyme superoxide dismutase, which converts two superoxide anions into one molecule of hydrogen peroxide and one of oxygen (FRIDOVICH, 1995; HASSAN; SCANDALIOS, 1990).

Superoxide dismutase and the antioxidant potential

In 1968, McCord and Fridovich characterized the SOD enzyme from bovine erythrocytes, by asking what could dissociate $O2°^{-}$ produced by xanthine oxidase into oxygen and H_2O_2 (NORDMANN; RIBIÈRE, 1991). Today, several common forms of SOD are known: they are oligomeric proteins cofactored with copper and zinc, or manganese. SOD subunits exhibit a two-domain structure: one domain contains α-helices and the second is composed of both α-helices and β-sheets. The metal binding site occurs between these two domains and the ligands of the metal ions are composed of histidine and aspartate side-chains (BEYER; IMLAY; FRIDOVICH, 1991).

In mammals, there are 3 isoforms of SOD, produced by distinct genes: copper/zinc SOD (Cu/Zn-SOD), a homodimer of 32 kDa, localized in the

cytosol or mitochondrial inter-membrane space; manganese SOD (Mn-SOD), a homotetramer of 88 kDa, localized in the mitochondria (matrix and inner membrane), and Cu/Zn form (EC-SOD), an extracellular tetrameric glycoprotein of 135 kDa (BEYER; IMLAY; FRIDOVICH, 1991; FRIDOVICH, 1995; FARACI; DIDION, 2004).

Some bacteria contain an iron SOD (FeSOD), others Mn-SOD, and some contain both (8). In plants, 3 isoforms are present: mitochondrial Mn-SOD, a Cu/ZnSOD in the cytosol and the chloroplasts and a Fe-SOD in the chloroplasts (HASSAN; SCANDALIOS, 1990; SCANDALIOS, 1997).

The main role of SODs in all aerobic organisms is to neutralize the $O2^{\circ-}$ produced in the cytosol, mitochondria and endoplasmic reticulum of cells. Several studies regarding the effects of SOD on animals were carried out by Emerit at the end of 70's. In 1983, he initiated the first human study in a case of fibrosis. Then, Edeas studied the effects of the bovine SOD on AIDS (1993).

Finally, the antioxidant enzyme was used in several therapeutic studies with promising results, as reviewed by Nordmann and Ribiere (1991). Use of SODs mimetics and the study of SOD-overexpressed models have also been developed in order to better understand SODs effects and their mechanisms of action.

There are numerous studies regarding the administration of SODs in very different pathological situations, raising the question of how a single molecule could have so many possible applications. Our group decided to carry out a review of the SOD-based dietary supplement Corps Lignea Celulift on these very heterogeneous results, in order to attempt to answer the following questions: What pathological situations can be improved by the administration of SODs? What mode of administration is efficient? What hypothesis could explain its mechanism of action?

Molecular basis of cellulite

One of the fundamental causes for the appearance of cellulite is related to adipocyte hypertrophy, that is to say, the increase in the size of fat cells [Rawlings, 2006]. This is partly due to a disturbance in lipid metabolism with inhibition of lipolysis, *i.e.* fat degradation (ROSSI, VERGNANINI, 2000). Even if cellulite should not be confused with obesity, some authors have observed that cellulite was more pronounced in obese women (MIRRASHED *et al*., 2004]. This worsening of cellulite with overweight, and the correlation between Body Mass Index (BMI) and severity of cellulite, reflects the expansion of adipose tissue in the dermis when the volume of fat is increased (GOLD, 2012).

These changes in adipocyte metabolism lead to an increased production of Reactive Oxygen Species (ROS) and a decrease in antioxidant defenses, leading to oxidative stress (FURUKAWA *et al*., 2004; LAY *et al*., 2014). Siems*et al.* (2005) showed anincrease in oxidative damage to lipids and proteins in women with cellulite.

Further studies have shown that antioxidant compounds capable of removing ROS, can improve lipid metabolism (GORINSTEIN*et al*., 2006; YANG *et al*., 2006]. Pina-Zentella*et al*. (2012) have shown that taurine stimulates the process of lipolysis in adipocytes. As the first line of antioxidant defenses, SOD could also be efficient in inhibiting adipocyte hypertrophy. Carillon *et al.* (2013) have reported stimulation of lipolysis in obese hamsters orally supplemented with SODB, a dried melon juice highly concentrated in SOD.

The second main cause of the cellulite appearance is linked to the developmentof fibrosis of the connective tissue, as a result of the evagination of fat lobules in the dermis (PIERARD, 2005). Indeed, when subjected to pressure changes, fat cells must adapt their shape without changing their volume. The gradual accumulation of fats leads to the destruction of collagen fibers and the development of thickened and stiffened fibrous strands limiting the evagination of fat lobules. This process leads to an accumulation of connective tissue, which is characteristic of a localized fibrosis in areas affected by cellulite (stomach, thighs, flanks) (QUATRESOOZ *et al*., 2006). There is a close relationship between the change in oxidative status and fibrosis (SIEMS *et al*., 2005; LEONARDUZZI*et al*., 1997). One of the main mechanisms responsible for fibrosis is the release of ROS, such as superoxide anion ($O_2^{\circ-}$) and the hydroxyl radical ($OH^{\circ-}$) by inflammatory cells (DEL MAESTRO *et al*., 1980, CAMPANA *et al*., 2004). Due to their high instability, ROS will bind to adjacent structures and cause damage to the connective tissue and vascular network (DRAELOS, 2005).

The generation of oxidative damages has been reported in many cases of fibrosis observed in animal models but also in humans (POLI; PAROLA, 1997). Therefore, the use of antioxidants is a solution to reduce fibrosis. Siems*et al.* (2005) have demonstrated the correlation between the reduction of oxidative stress and improvement of the skin mechanical properties, leading to a smoother skin appearance. Thus, antioxidant enzymes such as SOD may be effective in preventing the accumulation of ROS and their effects on fibrosis. The anti- fibrotic properties of SOD have been extensively studied and are now well established (HOUSSET *et* al., 1989; LEFAIX *et al*., 1993; LEFAIX *et al*., 1996; VOZENIN-BROTONS *et al*., 2001; CAMPANA *et al*., 2004).

In the 1990s, SOD was even used as a drug in injectable form (Orgotein®, Pegorgotein®, and Ormentein®) to prevent and treat radiotherapy-induced fibrosis. Vozenin-Breton *et al.* (2001) have especially shown that the mechanism of action of SOD was correlated with the inhibition of the expression of the pro-fibrotic cytokine Transforming Growth Factor-β1 (TGF-β1) (MARTIN *et al*., 2000).

Prickly pear and the diuretic effect

Diuretics, either alone or in combination with other drugs, are valuable in the treatment of hypertension, congestive heart failure, pulmonary edema but, as for thiazides and furosemide, they are associated with severe side effects such as electrolyte imbalance, metabolic alterations, development of new-onset diabetes(LAHLOU*et al*., 2007). Hence, there is a need for new diuretics with lower potential for adverse effects, such as plant-based substances.

Opuntia ficus indica L. (a species in the cactus family *Cactaceae*) is a plant growing in dry, hot climates: northern Mexico, south-western United States, Africa, Mediterranean countries and Europe (DEFELICE, 2004). The cladodes (also called 'nopal') are used in the traditional medicine (PARK *et al*., 2001; FEUGANG *et al*., 2006; GALATI *et al*., 2007; LINARÈS *et al*., 2007; PANICO *et al*., 2007; SCHMITT *et al*., 2008; ZOURGUI *et al*., 2008).

The potential activities of the fruit (prickly pear) of *Opuntia ficus indica* have been explored more recently. The fruit has antioxidant activity and shows cytoprotective effects on gastric mucosa (GALATI *et al*., 2003), hepatoprotective effects (GALATI *et al*., 2005), protection of the endothelium (GENTILE *et al*., 2004), antiproliferative effects in various tumor cells (SREEKANTH *et al*., 2007) and diuretic effects (GALATI *et al*., 2002). Indeed, cultivars of prickly pear produce fruits of different colors, due to the combination of two betalain pigments, the purple-red betanin and the yellow-orange indicaxanthin. These two natural pigments present radical scavenging activities contributing to the antioxidant activity of prickly pear fruits that have been observed in *in vitro* (BUTERA *et al*., 2002; GALATI *et al*., 2003; TESORIERE *et al*., 2003; 2006; 2007; GENTILE *et al*., 2004; SREEKANTH *et al*., 2007), *in vivo* (GALATI *et al*., 2002; 2005) and in clinical studies (TESORIERE *et al*., 2004a; 2004b; 2005).

This ingredient is a dehydrated water extract of the fruits of the prickly pear cactus *Opuntia ficus indica* obtained by a process designed to preserve the nutritional and functional properties of the fruit. It's a standardized soluble amber-to-red powder naturally rich in betanin and indicaxanthin, indicaxanthin representing 65% to 85% of total betalains.

The diuretic action depends on stimulation of the urinary tract linked to the activation of neurohumoral mechanism, mediators of stimuli acting on glomerulus, tone acid on the pyelo-uretral peristaltis, effects due to the influence of electrolytes present in the plants. Knowing this, it is very important to limit any stress factor for animals that might influence the parameters measured and when several identical studies are performed, they must be performed in the same conditions and, if possible, during the same period of time in order to make valid comparisons, taking also into account the influence of seasons on the behaviour of animals.

Another important point which could explain the difference between the urinary excretion of sodium, potassium and uric acid observed in our experiment and the one of Galati *et al*. (2002) is the kinetic of the sodium and potassium dosage in urine. In our experiment, the analyses were performed every 24h whereas they were all performed 2h after treatment for Galati *et al*. (2002), showing a peak for sodium excretion 4h after hydrochlorothiazide ingestion which is clearly reduced 24h after the ingestion. In our experiment, the interval time applied for the characterization of the Na^+ and K^+ excretion could

mask an increase in the sodium and potassium excretion, induced by hydrochlorothiazide ingestion, occurring during the day because of a short-lived effect of the product on the natriuresis.

The mechanisms of diuretics, especially of vegetable source, are not clearly elucidated. Nevertheless, three main categories of diuretics can be distinguished depending on the target on the renal tubule: action on the proximal and distal convoluted tubules, on the thick limb of Henle's loop, and on the collecting tubule.

Prickly pear doesn't have an action on the thick limb of Henle's loop as the diuretics, acting on this target, induce an important natriuresis. Thiazides and derivates, as hydrochlorothiazide, can act on the proximal and distal tubules, inhibiting the NaCl re-absorption especially on the distal convoluted tubule. The effect of this type of diuretics on the proximal tubule is weak and, especially, short-lived on the natriuresis. The diuretic effect of prickly pear having no impact on the natriuresis and kaliuresis, we can suppose that its target is more oriented on the proximal tubules.

The pulp of prickly pear fruit is rich in potassium, between 900 and 2170 mg/kg according to Piga (2004), and other monovalent and bivalent cations might have a diuretic activity synergistically with potassium. The diuretic effect of prickly pear should be linked to an osmotic effect on the proximal tubule, but the possible implication of polar organic compounds is not excluded (GALATI *et al*., 2003; ABDALA *et al*., 2008; MARTÍN-HERRERA *et al*., 2008).

The oral administration of prickly pear at the end of seven days of consumption induced an antioxidant effect by the significant increase of blood globular levels of glutathione peroxidase. The improvement of the oxidative status implied several criteria such as enzymatic antioxidants (superoxide dismutase, glutathione peroxidise, etc.), low molecular weight antioxidants (vitamins C and E, gluthation, etc.), trace elements (selenium, copper, zinc) and biomarkers of the oxidizing stress (lipidic peroxides, oxidized proteins, oxidized low density lipoproteins (LDL) etc.). The results obtained with prickly pear on the improvement of the glutathion peroxidase level are in accordance with several previous studies performed on the antioxidizing properties of *Opuntia ficus indica* fruit.

Conclusion

The dietary supplementCorps Lignea Celulift is an innovative product for the Brazilian market, due to its antioxidant potential dependent on superoxide dismutase and its diuretic effect. These nuclei act synergistically for the treatment of cellulite.

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Urgent treatment to reduce mortality caused by sepsis in intensive care units

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Abstract

Introduction: Sepsis is a clinical syndrome that has physiologic, biologic, and biochemical abnormalities caused by a dysregulated inflammatory response to infection. Sepsis and the inflammatory response that ensues can lead to multiple organ dysfunction syndrome and death.

Aim: The current study was designed to examine the causes of sepsis and septic shock in our intensive care unit.

Methods: This was a retrospective study of septic patients, who were in Intensive care unit at the Clinic for Anesthesiology and Resuscitation at the University Clinical Center Sarajevo from July 1, to December 31, 2018. The following data were collected: age of patients, gender, weather they were surgery treated or not, cause of infection, duration of hospitalization and mortality rate.

Results: During the study period, 26 patients had sepsis or septic shock. Age ranged from 22 to 83 years. The most common was the endotoxic shock after abdominal surgery due to ileus and gangrene of the bowels. The most common manifestations of severe organ dysfunction were acute respiratory distress syndrome and acute renal failure. Mortality rate was high.

Conclusion: Identifying sepsis signs as soon as possible is the most important factor for starting effective treatment of sepsis and reducing mortality. Early treatment should begin within one hour according to the latest recommendations.

Key words: sepsis, shock, early treatment.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Sepsis is a clinical syndrome that has physiologic, biologic, and biochemical abnormalities caused by a dysregulated inflammatory response to infection. Sepsis and the inflammatory response that ensues can lead to multiple organ dysfunction syndrome and death.

A constellation of clinical, laboratory, radiologic, physiologic, and microbiologic data is typically required for the diagnosis of sepsis and septic shock. The diagnosis is often made empirically at the bedside upon presentation, or retrospectively when follow-up data return or a response to antibiotics is evident.

Societal guidelines place emphasis on the early identification of infected patients who may go on to develop sepsis as a way to decrease sepsis-associated mortality. The 2016 SCCM/ESICM task force have described an assessment score for patients outside the intensive care unit as a way to facilitate the identification of patients potentially at risk of dying from sepsis. A score ≥2 is associated with poor outcomes due to sepsis. The qSOFA score is easy to calculate since it only has three components, each of which are readily identifiable at the bedside and are allocated one point: respiratory rate ≥22/minute, altered mentation, systolic blood pressure ≤100 mmHg. The qSOFA score was validated as most useful in patients suspected as having sepsis outside of the intensive care unit (ICU).

Risk factors for sepsis include the following: intensive care unit admission, bacteremia, advanced age (≥65 years), immunosuppression, diabetes and obesity, cancer, community acquired pneumonia, previous hospitalization, genetic factors and multidrug-resistant infection.

Patients with suspected or documented sepsis typically present with hypotension, tachycar-

dia, fever, and leukocytosis (>12,000 microL^{-1}, or <4000 microL^{-1}). As severity worsens, signs of shock (eg, cool skin and cyanosis) and organ dysfunction develop (eg, oliguria, acute kidney injury, altered mental status). Importantly, the presentation is nonspecific such that many other conditions (eg, pancreatitis, acute respiratory distress syndrome) may present similarly.

Sepsis has a high mortality rate. Poor prognostic factors include the inability to mount a fever, leukopenia, age >40 years, certain comorbidities (eg, AIDS, hepatic failure, cirrhosis, cancer, alcohol dependence, immunosuppression), a non-urinary source of infection, a nosocomial source of infection, and inappropriate or late antibiotic coverage.

Early identification and appropriate immediate management in the initial hours after development of sepsis improves outcomes *(7−8)*.

Material and methods

This was a retrospective study of septic patients, who were in Intensive care unit at the Clinic for Anesthesiology and Resuscitation at the University Clinical Center Sarajevo in the period from July 1, to December 31, 2018. The subjects were both sexes, 22-83 years old. We included only those patients who had sepsis or septic shock. The following data were collected: age of patients, gender, cause of infection, weather they were surgery treated or not, length of hospitalization and mortality rate.

Results

During the study period, 26 patients had sepsis or septic shock. Age ranged from 22 to 83 years. The average age was 66.6 years (22 -83). The youngest patient was 22 years old. She had compromised immune system.

Analysis gender distribution shows that was more women (n=14, 53.8%). There is no statistically significant difference in gender distribution (figure 1.)

The most common locations for the primary infection include the ischemic bowel (n= 13, 50%), lungs (n=6, 23%), abdominal organs (necrotic pancreas: n=5, 19%, biliary tract n=2, 8%).

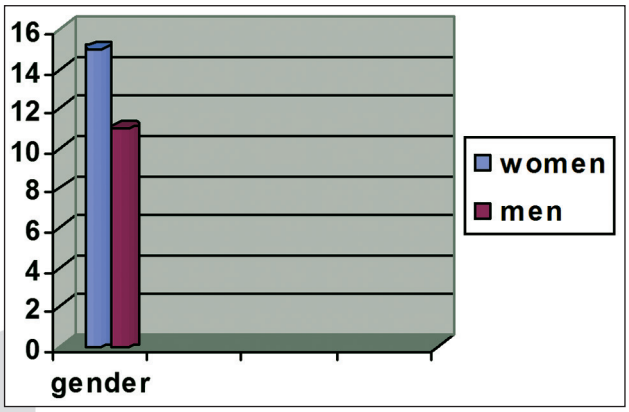

Figure 1. Gender distribution

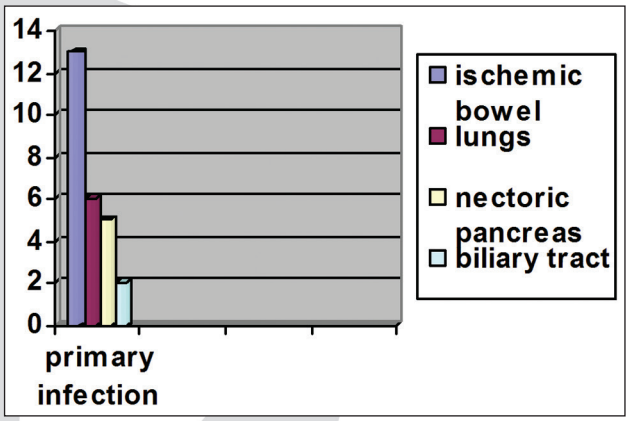

Figure 2. Primary infection

All patients had the most severe clinical form of the disease- septic shock.

In operatively treated group were 16 patients (61.5%) and in conservatively treated group were 10 patients (38.5%).

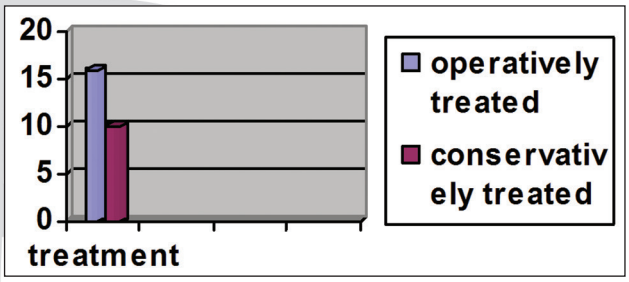

Figure 3. Treatment

The average lenght of hospitalization was 6.9 days (0-31).

Mortality rate was the highest on the second day after hospitalization in Intensive care unit. In the first day of hospitalization was 7.7% (n=2) and the second day after hospitalization was 19.2% (n=5).

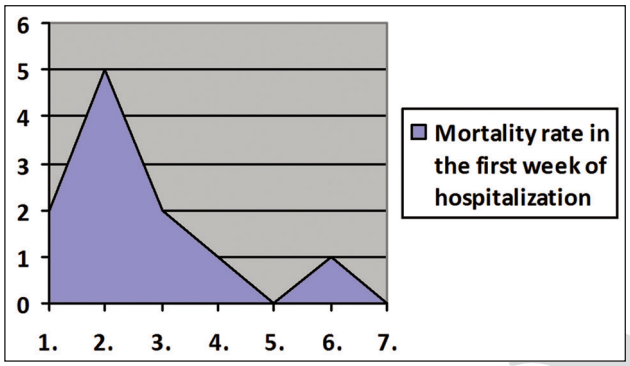

Figure 4. Mortality rate in the first week of hospitalization

The most common manifestations of severe organ dysfunction were acute respiratory distress syndrome and acute renal failure. Total mortality rate was high (77%).

Discussion

In our study, there were 53.8 % women and 46.2 % men. During the period from July 1, to December 31, 2018, we identified 26 patients with sepsis in Intensive care unit at the Clinic for Anesthesiology and Resuscitation at the University Clinical Center Sarajevo. The average age was 66.6 (22 -83). The site of infection in patients with sepsis may be an important determinant of outcome. In this study there were no patients with sepsis from a urinary tract infection. Sepsis from a urinary tract infection is associated with the lowest mortality rate. All patients had the most severe clinical form of the disease- septic shock.

The most common location for the primary infection in this study was ischaemic bowel. It can be reason for high mortality rate (77 percent). These results are similar to the results of the retrospective, multicenter cohort study of nearly 8000 patients with septic shock *(10)*. This study reported similar results with the highest mortality in those with sepsis from ischemic bowel (78 percent).

The high mortality rate in the first and second day of hospitalization (26.9%) suggested that the detection of sepsis was late. Early identification and appropriate immediate management in the initial hours after development of sepsis improves outcomes *(7−8)*. The guidelines state that these patients need urgent assessment and treatment. Clinicians need to begin treatment immediately, especially in patients with hypotension, rather than waiting or extending resuscitation measures over a longer period. More than one hour may be required for resuscitation to be completed, but initiation of resuscitation and treatment, such as obtaining blood for measuring lactate and blood cultures, administration of fluids and antibiotics, and in the case of life-threatening hypotension, initiation of vasopressor therapy, are all begun immediately.

Conclusion

Identifying sepsis signs as soon as possible is the most important factor for starting effective treatment of sepsis and reducing mortality. Early treatment should begin within one hour according to the latest recommendations. Urgent restoration of an adequate perfusion pressure to the vital organs is a key part of resuscitation. This should not be delayed.

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A technical-scientific review of Laminê: the first branded mix of the Brazilian dietary supplements market

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Abstract

Laminê is a branded mix, exclusive to Sweet-Mix, developed after years of studies, involving tests in the main national and international centers and research institutes. When administered orally, it presents scientifically proven benefits for hair, skin and nails, all approved by the Brazilian Sanitary Surveillance Agency. Laminê is a specific combination of functional ingredients that act synergistically in three biological nuclei: skin; hair and nails; antioxidant.

Key words: antioxidant; hair; Laminê; nails; skin; supplement.

Introduction

The skin protects the body from mechanical damage, microbial invasion, and radiation or UV light, as a barrier between the internal part of the body and the external environment. Thus, the skin plays an important role in the maintenance of homeostasis of the body by minimizing the moisture loss and regulating the temperature (BOELSMA *et al*., 2001).

Also, the skin is the outermost protective layer of the body which is continuously exposed to external stimulants such as environmental pollutants or ozone, radiation, UV light, and thus has been greatly under stress due to oxidative damages. Reactive oxygens aggressive to the cells are continuously produced in the body while the life goes on and they become the main culprit of the cell aging (JACKSON, 1999; RHIE *et al*., 2001).

Therefore, the roles of antioxidant for preventing the oxidation of skin cells have been concerned to maintain healthy and beautiful skin (BAE *et al*., 2006), and the development of products mainly applicable on the skin has been highlighted (BOGDAN; BAUMANN, 2008).

In the past, skin care and health management was treated by emphasizing skin troubles but in the present, the preventive program has come into the spotlight that integrates detailed skin analysis through skin care instruments, professional counseling, individualized care, dietary habits and exercise lifestyle (GIACOMONI, 2008).

Koyama *et al*. (2006) reported that collagen peptide intake increased the absorption rate of water of stratum corneum. However, skin health-related studies performed up to the present time are mostly about the influences of dietary habits and lifestyle on the skin (CHOI *et al*., 2003).

Considering the existence of problems related to skin health and skin condition which can be originated from the internal factors due to meals, it is necessary to study nutritional factors that affects skin condition, and develop new alternatives, such as Laminê, the first Brazilian branded mix.

Laminê is a specific combination of functional ingredients that act synergistically in three biological nuclei: hair and nails; skin; antioxidant.

The core of the hair and nails is made up of vitamins with high bioavailability were selected, pointing at the supplementation of essential nutrients for their strengthening, maintenance and pigmentation, ensuring healthy yarns and nails and providing well-being to consumers.

Aiming at the benefits of the skin, we apply the bioactive peptides of type I collagen, whose clinical effects result in the increase of the firmness and the elasticity of the skin, diminishing lines of expression and facial wrinkles.

The nucleus SOD corresponds to the antioxidant system of the formulation, whose activation occurs through a triad of organic minerals, also bioavailable, that provide protection to the reactive species of oxygen and free radicals, preventing and retarding the cellular aging.

Minerals

Minerals, including zinc, copper, selenium, and manganese also have an important role in maintaining skin health. Zinc is an essential cofactor of numerous metalloenzymes. Its main function is to protect the skin against photodamage by absorbing UV irradiation, limiting penetration of radiation into skin (MITCHNICK *et al*., 1999). Co-treatment with zinc and vitamin C exhibits antimicrobial activity that helps to clear bacteria in acne (MITCHNICK *et al*., 1999). Moreover, zinc deficiency has been reported in patients with epidermolysis bullosa (FINE *et al*., 1989). Although patient with AD also showed a significant decreased level of zinc, zinc supplementation does not result in clinical improvement of AD (EWING *et al*., 1991).

Like zinc and vitamin C, copper with peptides also serves as an antioxidant, protecting skin from damage that is caused by an UV light-induced increase in free radical levels (PICKART *et al*., 2012). Furthermore, copper is known to stimulate the maturation of collagen, thus is critical in improving skin elasticity and thickness (PICKART, 2008).

While it also plays a role in melanin synthesis enables pigmentation of skin and hair, steely-hair syndrome (white and silver hair) is a severe multisystemic disease caused by copper deficiency/abnormal copper metabolism (MENKES, 1988).

Selenium protects the skin from UV irradiation induced oxidative stress by stimulating the activities of the selenium-dependent antioxidant enzymes, glutathione peroxidase and thioredoxin reductase, that are present in the plasma membrane of epidermal keratinocytes (BALAGOPALAKRISHNA *et al*., 1997; RAFFERTY *et al*., 1998).

Selenium also has been considered for treatment of psoriasis, which shows decreased glutathione peroxidase levels (JUHLIN *et al*., 1982; NAZIROGLU *et al*., 2012). Results from human studies showed that selenium supplementation lead to an increase in levels of glutathione peroxidase in patients with psoriasis, resulting in disease improvement (JUHLIN *et al*., 1982).

Since selenium deficiency has been detected in patients with recessive dystrophic epidermolysis bullosa, the level of selenium is one marker in this disease (FINE *et al*., 2008). Moreover, its deficiency is associated with an increased risk of skin cancer (MCKENZIE, 2000).

Zinc

Zinc is an essential mineral required by hundreds of enzymes and multiple transcription factors that regulate gene expression (OGAWA; KAWAMURA; SHIMADA, 2016). While the exact mechanism of action is unclear, one possibility centers on zinc's role as an essential component of numerous metalloenzymes important in protein synthesis and cell division (MACDONALD, 2000). Another possibility is zinc's role in the Hedgehog signaling pathway (RUIZ; ALTABA, 1999), a critical component in the pathways that govern hair follicle morphogenesis.

Zinc deficiency may be either inherited or acquired and may affect multiple organ systems. Patients may experience diarrhea, immunological effects, and delayed wound healing. Abnormalities in taste and smell may occur. Cutaneous effects include acral and periorificial dermatitis, while hair changes include TE and brittle hair (ST-JACQUES *et al*., 1998).

The autosomal recessive disorder, acrodermatitis enteropathica, results in decreased absorption of zinc, while acquired zinc deficiency may occur in malabsorption syndromes, such as inflammatory bowel disease (VALBERG *et al*., 1986) or following gastric bypass surgery. Other groups at risk include patients with malignancy, those with liver or renal dysfunction, pregnant women (CAULFIELD *et al*., 1998), and patients with alcoholism (DINSMORE *et al*., 1985). Drugs that can affect zinc levels include valproic acid (YILMAZ; TASDEMIR; PAKSU, 2009) and certain antihypertensives (BRAUN; ROSENFELDT, 2013).

Dietary risk factors include vegetarianism, as bioavailability of zinc is lower in vegetables than meat. Additionally, vegetarians typically consume more legumes and whole grains, which contain phytates that bind to zinc and inhibit absorption (HUNT, 2013).

Serum zinc, the most commonly measured index of zinc status, may be impacted by several variables, and the functional effects of deficiency may be observed before serum levels decrease below normal (MARET; SANDSTEAD, 2005).

Screening in those with risk factors is indicated, as hair loss due to zinc deficiency can be reversed. A case series demonstrated reversal of hair loss following oral supplementation in five patients with TE and zinc deficiency (KARASHIMA *et al*., 2012).

A study of 312 patients with AA, male pattern hair loss (MPHL), FPHL, or TE showed that all groups had statistically lower zinc concentrations as compared to 30 healthy controls (KIL; KIM; KIM, 2013). In patients with AA and low serum zinc levels, supplementation has been shown to have therapeutic effects (PARK *et al*., 2009).

Copper

Copper is an essential trace element for all biological organisms, from bacterial cells to human. Depending on the source of the biological material, copper content ranges from parts per billion to parts per million.

Copper deficiency has been linked to a variety to clinical signs, including pale coat, poor sheep, fleece quality, anemia, spontaneous fractures, poor capillary integrity, myocardial degeneration, hypomyelinization of the spinal cord, impaired reproductive performance, decreased resistance to infectious disease, diarrhea and generalized ill-health (TESSMAN *et al.*, 2001), causing severe economic losses.

Hypocuprosis is the second most widespread mineral deficiency effects on grazing animals. Many investigations concerning the mechanisms of copper activity in the body have dealt primarily with the distribution of copper in various tissues, the changes which occur in the blood after different conditions and the interrelationships between copper and various enzymes systems, vitamins and minerals.

Cerone *et al.* (2000) explained that copper is an essential trace element that has an important role in many physiological functions in nervous, hematological, cardiovascular, reproduction and immune systems.

Moreover, copper plays a significant role, being associated with specific proteins. The majority of the biological functions of copper are believed to be associated with copper's role as a ligand in the active site of metalloenzymes. Among the principal enzymes, ceruloplasmin (a plasma glycoprotein, may function as a

Copper transport and as an antioxidant), dopamine-β-monooxygenase (located in noradrenergic neurons and involved in conversion of dopamine to norepinephrine), cytochrome-c-oxidase (the terminal mitochondrial electron carrier), lysyl oxidase (responsible for oxidative deamination of peptidyl lysine), Cu-Zn-superoxide dismutase (a cytosolic protein that speeds up the dismutation of superoxide) and tyrosinase (located in melanocytes and involved in the conversion of tyrosine into melanin) and copper is needed for proper development of antibodies and white blood cells, in addition to antioxidant enzyme production (SHARMA *et al.*, 2005).

Copper deficient goats are more susceptible to be infected by infectious diseases and do not respond as well to the vaccinations, in addition, they tend to be less resistant to parasitic challenge. Goats receiving proper copper nutrition tend to be less susceptible to infections and have less severe infections when disease does occur.

One of the most visible signs of copper deficiency is change in hair color. Black animals develop a red tint and red animals become bleached and light colored. The coat becomes dull and animals may be slow to shed in the spring and in young animals, copper deficiency can result in diarrhea (SHALABY *et al.*, 2010).

Selenium

Selenium is an essential trace element that plays a role in protection from oxidative damage as well as hair follicle morphogenesis. Rats deficient in selenium display sparse hair growth (BATES *et al*., 2000), while knockout mice lacking specific selenoproteins exhibit progressive hair loss after birth (Sengupta *et al*., 2010).

Risk factors for deficiency include living in areas with low selenium soil content (particularly in parts of China, Tibet, and Siberia), long-term hemodialysis, HIV, and absorption disorders (KANEKURA *et al*., 2005).

There is research on selenium deficiency and alopecia in humans. One case report in a child described sparse hair, which improved after dietary supplementation (AMOR *et al*., 2010).

Manganese

Skin aging is characterized by atrophy, wrinkle formation, reduced tensile strength and impaired wound healing, with loss of the structural integrity and loss of the elastic and collagen fiber network due to dysfunctional fibroblasts (FISHER; VARANI; VOORHEES, 2008; QUAN *et al*., 2010). Dermal fibroblasts have therefore been used to model senescence in vitro (CRISTOFALO, 1993; SCHNEIDER; WLASCHEK, 2003; WLASCHEK *et al*., 2003) not only for the dermis, but also for other connective tissue rich organs.

Skin aging, among other changes, is characterized by a loss of collagen type I, collagen type III among other matrix constituents, dysregulated fibroblast-matrix interactions and impaired fibroblast interactions with organ parenchyma, mainly with organ-specific epithelial cells and muscle (WENK *et al*., 1999).

In human senescent skin, fibroblasts which develop a growth arrest, morphological and functional changes, increased ROS concentrations have been demonstrated in vitro and in vivo (SHIN *et al*., 2005) with an adaptive upregulation of the SOD on mRNA and protein level (BORLON *et al*., 2005), providing evidence for a common response phenotype of cellular senescence.

The upregulation of the manganese SOD in human fibroblasts has also been shown to be induced in a paracrine mechanism either via UV-irradiation (POSWIG *et al*., 1999) and/or the release of soluble factors (e.g., interleukin 1α, interleukin 1β, and tumor necrosis factor α) from keratinocytes (NADERI-HACHTROUDI *et al*., 2002).

In this case, when manganese SOD upregulation disturbs the balance of hydrogen peroxide (H_2O_2) level, the upregulation of H_2O_2 detoxifying enzymes ensures that an accumulation of H_2O_2 does not occur in the system. However, exclusive manganese SOD overexpression in vitro has earlier been shown to result in enhanced H_2O_2 concentration with activation of distinct signaling pathways and transcription factors, among them the heterodimeric AP-1 which enhance the transcription and activation of matrix-metalloproteinases among other genes and gene products (TREIBER *et al*., 2009).

The family of matrix-metalloproteinases (MMP) so far consists of at least 20 members with distinct, partly-overlapping substrate specificities for extracellular matrix proteins of the skin (HU *et al*., 2007). Imbalanced overexpression of manganese SOD resulted in enhanced H_2O_2 accumulation with the AP-1 dependent induction of interstitial collagenase (MMP-1) and degradation of interstitial collagen in the skin, a hallmark of skin aging (IBRAHIM *et al*., 2000).

Interestingly, there is indirect evidence that in aged skin, fibroblasts—both superoxide anion and H_2O_2 are increased (SHIN *et al*., 2005). In order to model the situation with an increase of superoxide anion, mice with deficiency of manganese SOD were generated. Mice with a homozygous deficiency of SOD in all organs die within postnatal day 8 to 18 due to cardiomyopathy and neurodegeneration (LEBOVITZ *et al*., 1996). Treatment with synthetic superoxide dismutase (SOD)-catalase mimetic (EUK) extends lifespan of mice with inactivated manganese SOD by 3-fold and attenuates mitochondrial defects (MELOV *et al*., 2001).

Corresponding to naturally-aged mouse skin, where the number of senescent cells increases (WANG *et al*., 2009) and the mitochondrial activity decreases, constitutive lack of SOD correlates with an increase in senescence-associated β-galactosidase expression, impaired mitochondrial complex II activity and increased nuclear DNA damage in the skin. Cellular senescence in manganese SOD analogous mice with decreased proliferation and increased terminal differentiation of keratinocytes may result in the observed thinning of the epidermis (VELARDE *et al*., 2012).

Niacin

Pellagra, due to a deficiency of niacin, results in the well-known triad of photosensitive dermatitis, diarrhea, and dementia. Alopecia is another frequent clinical finding (WAN *et al*., 2010).

Pellagra became rare in many developed countries after niacin (vitamin B3) fortification of food was introduced. Alcoholism is now considered the most common cause of pellagra in developed countries. Other causes include absorption disorders or drug-induced cases, such as with isoniazid (WAN *et al*., 2010).

Biotin and others complex B

Vitamins are essential for human health. Biotin, or vitamin B7/H, serves as a cofactor for carboxylation enzymes. In isolated sheep hair follicles, incubation in biotin-containing solutions resulted in increased DNA concentration and protein synthesis (GALBRAITH, 2010).

Symptoms of deficiency include eczematous skin rash, alopecia, and conjunctivitis (WOLF, 1993). One study of an infant fed with a formula lacking sufficient biotin content reported manifestations of periorificial dermatitis and patchy alopecia, both of which resolved with daily oral supplementation of biotin (FUJIMOTO *et al*., 2005).

Biotin deficiency is rare, as intestinal bacteria are typically able to produce adequate levels of biotin. Deficiency is seen in cases of congenital or acquired biotinidase or carboxylase deficiency, antibiotic use disrupting the gastrointestinal flora, and antiepileptic use. Deficiency can occur from excessive ingestion of raw egg whites due to binding by avidin (WOLF, 1993).

Biotin is found in multiple supplements marketed to consumers for hair loss. This marketing approach have been chosen as biotin has shown positive effects in the treatment of brittle fingernails and onychoschizia (ROGERS; AVRAM, 2008; COLOMBO *et al*., 1990).

In recent years there has been much interest in the importance of plasma homocysteine as a graded risk factor for cardiovascular disease. Homocysteine is a thiol-containing amino acid that arises as a product of the metabolism of the essential amino acid methionine (STAMPFER *et al*., 1992).

It is not incorporated into protein and therefore its concentration is regulated by the rate of its synthesis and metabolism. The main determinants of the homocysteine concentration in tissues and consequently in the circulation are genotype and diet. Homocysteine is metabolized through two main routes, transsulfuration, which is vitamin B6 dependent, and remethylation to methionine, which is folate, vitamin B12, and riboflavin dependent (BOUSHEY *et al*., 1995).

Supplementary vitamin B12 has modest homocysteine-lowering effects under certain circumstances, whereas reports of the effects of supplementary vitamin B-6 are inconsistent (SELHUB; MILLER, 1992).

Riboflavin has been largely ignored, despite the fact that FAD is a cofactor for methylenetetrahydrofolate reductase (EC 1.7.99.5), which metabolizes folate to the form used in homocysteine methylation (KANG *et al*., 1995).

A common mutation of methylenetetrahydrofolate reductase, for which 5% to 30% of different populations are reported to be homozygous, is associated with increased plasma homocysteine concentrations. Further evidence for a role of riboflavin in homocysteine homeostasis comes from a report of elevated homocysteine in the skin of riboflavin-deficient (LAKSHMI; LAKSHMI; BAMJI, 1990).

Certain combinations of B vitamins, such as Laminê, demonstrate a positive influence on human keratinocytes and fibroblasts. Vitamins especially promotes fibroblast migration, and a statistically significant induction of keratinocyte proliferation. Therefore, oral vitamin ingestion could benefit the physiologic wound healing process.

Vitamin C

Exposure to excess UV irradiation induces oxidative stress, impacting the genetic integrity of a living organism, including the skin (CHEN *et al*., 2012). While UVB (wavelengths 280-310 nm) directly damages DNA, UVA (320-400 nm) causes indirect DNA mutations by generating reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide (DOUBLE *et al*., 2002; CHEN *et al*., 2012).

Excessive exposure to UV irradiation is associated with photoaging and the development of skin cancer (CHEN *et al*., 2012). UV irradiation triggers the production of pro-inflammatory cytokines and growth factors (CHEN *et al*., 2012). These mediators increase expression of MMPs (MMP-1, -3, -8 and -9) *via* either activation protein-1 (AP-1) and/or NF-κB activation, resulting in degraded collagen and elastin in the skin (SARDY, 2009; CHEN *et al*., 2012).

Moreover, UV irradiation-induced ROS have been shown to suppress expression of transforming growth factor (TGF)-β, which is a signaling mediator to promote collagen formation (WALRAVEN *et al*., 2014).

These results indicate that an increase in production of ROS following exposure to UV irradia-

tion could degrade the structural integrity of skin by altering the collagen and elastin components in the dermis, causing skin aging characterized by deep wrinkles, coarse textures, telangiectasia, and pigmentation.

In addition, UV irradiation-induced ROS have been suggested as a mutagen in certain skin cancer; *e.g.*, squamous cell carcinoma (SCC) (HALLIDAY, 2005). ROS induces mutation of p53 gene, driving precursor lesions to malignancy (HALLIDAY, 2005).

But the mechanistic connection between ROS and SCC is still unclear. In this regard, vitamin C is a water-soluble, powerful antioxidant that has been shown to attenuate UV irradiation-mediated damages in the skin (STEWART *et al*., 1996; MCARDLE *et al*., 2002). Vitamin C significantly suppresses the UV light-triggered production of free radicals, protecting cells from oxidative stress (MCARDLE *et al*., 2002).

It has an additional role in wound healing by increasing pro-collagen and collagen synthesis (PETERKOFSKY, 1991; FISHER *et al*., 1996), which stimulate the formation of the skin barrier. In efficacy studies on human skin, vitamin C significantly increased epidermal moisture content, improving skin hydration (CAMPOS *et al*., 2008).

As noted earlier, scurvy is a disease caused by lack of vitamin C. Symptoms of scurvy in the skin include a thickening of the stratum corneum and spots of small subcutaneous bleeding (HODGES *et al*., 1971). In addition, cutaneous wound healing is delayed due to the scurvy-mediated decrease in mature collagen (ROSS; BENDITT, 1962).

Collagen

Collagen peptides are natural bioactive ingredients used in many nutricosmetic products, orally taken nutritional supplements which provide skin health and beauty benefits. Collagen peptides present as a mix of specific peptides of different length with high abundance of the amino acids hydroxyproline, glycine, and proline, which are produced by enzymatic hydrolysis of native collagen extracted from animal connective tissues (VERDIER-SEVRAIN; BONTE, 2007).

Hydroxyproline is unique to collagen and can be used analytically to differentiate collagen from other proteins. Collagen peptides are efficiently digested into di- and tripeptides, which are resistant to further intracellular hydrolysis (LIU *et al*., 2009). The peptides are transported across the intestinal mucosa by the transporter PEPT-1 (AITO-INOUE *et al*., 2007).

In humans, hydroxyproline containing di- and tripeptides have been shown to appear one hour after ingestion of collagen peptides at nanomolar concentrations in the blood (SHIGEMURA *et al*., 2014). Investigations using radioactively labeled collagen peptides have demonstrated that the absorbed peptides reach the skin (KAWAGUCHI, NANBU, KUROKAWA, 2012) and are retained in the tissue for up to 2 weeks (WATANABE-KAMIYAMA *et al*., 2010).

A growing body of evidence demonstrates the efficacy of collagen peptides to improve parameters of skin physiology in preclinical studies. Collagen peptides were shown to increase hyaluronic acid production in dermal fibroblasts (OHARA *et al*., 2010; OBA *et al*., 2013) and to improve skin barrier function by increasing the water content of the stratum corneum (TANAKA *et al*., 2009; OHARA *et al*., 2010; SHIMIZU *et al*., 2015).

Further, collagen peptides induce the synthesis of collagen on the mRNA and protein level (LIANG *et al*., 2010; ZAGUE *et al*., 2011) as well as the production of stronger collagen fibrils (Matsuda *et al*., 2006) promote growth of skin fibroblasts (SHIGEMURA *et al*., 2009) and induce fibroblast migration (POSTLETHWAITE; SEYER; KANG, 1978; ALBINI; ADELMANN-GRILL, 1986).

Conclusions

Micronutrients, including vitamins and minerals, are not only essential components of skin structure, but they also modulate multiple biological functions.

Although the importance of these micronutrients has been widely characterized, therapeutics utilizing such nutrients have been limited to antioxidants and stimulating wound healing.

In turn, Laminê presents scientifically proven benefits for hair, skin and nails, all approved by the Brazilian Sanitary Surveillance Agency, being a specific combination of functional ingredients that act synergistically in different biological nuclei.

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Abstract

In this paper the instructions for preparing camera ready paper for the Journal are given. The recommended, but not limited text processor is Microsoft Word. Insert an abstract of 50-100 words, giving a brief account of the most relevant aspects of the paper. It is recommended to use up to 5 key words.

Key words: Camera ready paper, Journal.

Introduction

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Instructions for the authors

Times New Roman 12 points font should be used for normal text. Manuscript have to be prepared in a two column separated by 5 mm. The margins for A4 (210×297 mm2) paper are given in Table 1.

Table 1. Page layout description

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Regular paper may be divided in a number of sections. Section titles (including references and acknowledgement) should be typed using 12 pt fonts with **bold** option. For numbering use Times New Roman number. Sections can be split in subsection, which should be typed 12 pt *Italic* option. Figures should be one column wide. If it is impossible to place figure in one column, two column wide figures is allowed. Each figure must have a caption under the figure. Figures must be a resolution of 300 DPI, saved in TIFF format, width 10 cm min. For the figure captions 12 pt *Italic* font should be used. (1)

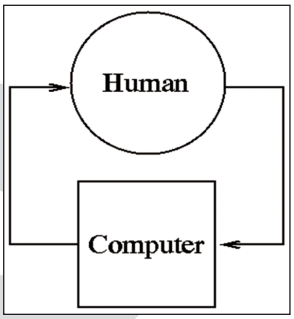

Figure 1. Text here

Conclusion

Be brief and give most important conclusion from your paper. Do not use equations and figures here.

Acknowledgements (If any)

These and the Reference headings are in bold but have no numbers.

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