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Serbian Journal of Experimental and Clinical Research, Medical Faculty, University of Kragujevac
Svetozara Markovića 69, 34000 Kragujevac, PO Box 124
Serbia
e-mail: sjecr@medf.kg.ac.rs
www.medf.kg.ac.rs/sjecr

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TOPICAL PELOID AND HERBAL EXTRACTS THERAPEUTIC EFFICACY ON ACNE

Zorica Potpara[1], Slavica Vucurovic[2], Dragica Bojovic[1], Nada Marstijepovic[3] and Slobodan Jankovic[4]
[1]Pharmacy Department, University of Montenegro, Podgorica, Montenegro
[2]Fontis Ltd., Podgorica, Montenegro
[3]Faculty of Metallurgy and Technology, University of Montenegro, Podgorica, Montenegro
[4]Medical Faculty, University of Kragujevac, Kragujevac, Serbia

EFEKTI LOKALNE TERAPIJE PELOIDOM I BILJNIM EKSTRAKTIMA NA AKNE

Zorica Potpara[1], Slavica Vučurović[2], Dragica Bojović[1], Nada Marstijepović[3] and Slobodan Janković[4]
[1]Univerzitet Crne Gore, Farmaceutski fakultet u Podgorici, republika Crna Gora
[2]Fontis Ltd., Podgorica, Crna Gora
[3]Fakultet metalurgije I tehnologije, Podgorica, Univerzitet Crne Gore
[4]Medicinski fakultet Univerziteta u Kragujevcu, Republika Srbija

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ABSTRACT

In spite of a variety of established therapeutic approaches to the treatment of acne vulgaris, up to 12.7% of patients cannot be cured using these methods. To overcome the limitations of established therapies, botanicals and natural mineral preparations are also used to treat acne. The aim of our study was to investigate the efficacy of topical preparations of peloid and medicinal plants from Montenegro in an acne therapy regimen.

The study design was a retrospective cohort study, with two cohorts defined by the type of topical preparation used: one cohort (n = 70) was comprised of the patients treated with Peloderm (a topical preparation containing both peloid and medicinal plants' extracts), and another cohort (n = 70) of the patients was treated with Antiacne (a topical preparation with only medicinal plants' extracts). Patients in both cohorts were treated for 18 months.

In both treatment groups, the FDA acne severity score improved gradually throughout the study visits. However, final FDA acne severity score (after 18 months of topical treatment) was significantly (T = 7.556, df = 1, p = 0.000) lower in the Peloderm group (1.0 ± 0.0) than in the Anitiacne group (1.8 ± 0.9).

Both topical preparations of peloid and selected medicinal plants from Montenegro, in ratios observed in this study, are efficacious and safe options for topical treatment of acne, with the peloid preparation demonstrating somewhat greater potency.

Keywords: *Peloid, acne vulgaris, topical therapy.*

SAŽETAK

Uprkos velikom broju utvrđenih terapijskih metoda za lečenje akni, čak 12.7% pacijenata ne može da se trajno izleči. Da bi se prevladala ograničenja utvrđenih terapijskih metoda, sve više se u lečenju akni koriste preparati dobijeni preradom lekovitih biljaka i minerala. Cilj naše studije je bilo ispitivanje efikasnosti lokalne terapije akni preparatima sačinjenim kombinovanjem peloida i lekovitih biljaka iz Crne Gore.

Studija je bila dizajnirana kao retrospektivna kohortna studija, sa dve kohorte definisane vrstom korišćenog lokalnog preparata: jednu kohortu (n = 70) su činili pacijenti lečeni Pelodermom (preparat za lokalnu primenu koji sadrži i peloid i ekstrakte lekovitih biljaka), a drugu kohortu (n = 70) pacijenti lečeni preparatom Antiakne (preparat za lokalnu primenu koji sadrži samo ekstrakte lekovitih biljaka). Pacijenti u obe kohorte su bili lečeni 18 meseci.

U obe studijske grupe, FDA skor težine akni se postepeno poboljšavao tokom lečenja. Međutim, krajnji FDA skor (posle 18 meseci lokalne terapije) je bio značajno (T = 7.556, df = 1, p = 0.000) niži u grupi sa Pelodermom (1.0 ± 0.0) nego u grupi sa Antiakne preparatom (1.8±0.9).

I peloid, i izabrane lekovite biljke iz Crne Gore, pripremljeni u obliku lokalnih preparata u odnosima korišćenim u našoj studiji, su efikasni i bezbedni u lokalnoj terapiji akni, pri čemu peloid ima nešto veći terapijski efekat.

Ključne reči: *Peloid, akne, lokalna terapija.*

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Correspondence to: Prof. Slobodan Jankovic, MD, PhD / Pharmacology Department, Medical Faculty, University of Kragujevac
Svetozara Markovica Street, 69, 34000 Kragujevac, Serbia / e-mail: slobodan.jankovic@medf.kg.ac.rs
tel. +381 34 306800 ext 1117 fax. +381 34 306800 ext 1117

INTRODUCTION

One of the most frequent skin diseases in adolescence is acne, which affects up to 85% of the population (1). A significant percentage of the affected adolescents continue to suffer from acne in adulthood, contributing to a population prevalence of 13% (2). Usually, adult patients suffer from the most severe forms of acne.

The mainstays of acne therapy are topical preparations with proven efficacy are benzoyl peroxide, antibiotics, azelaic acid, and retinoid. In patients with mild acne, topical preparations often suffice, but in those with moderate to severe acne, systemic therapy is necessary (3). Moderate acne is treated by systemic antibiotics (especially tetracyclines) and hormonal therapy (oral contraceptives or cyproterone acetate), but severe acne requires administration of oral isotretinoin. However, many patients are not cured by these therapies (up to 12.7%), and more are unsatisfied with the therapeutic results (4). There are also considerable adverse effects of systemic acne therapy, e.g. skin reactions with minocycline, interference with growth of bones and teeth with all tetracyclines, or depression and suicide attempts with isotretinoin (5).

To overcome the limitations of established standards of care, botanicals and natural mineral preparations are also implemented in acne therapy. A recent systematic review confirmed considerable efficacy for preparations containing Mahonia, tea tree oil, and Saccharomyces (6). However, although there were numerous attempts to use peloid preparations for the treatment of a wide spectrum of diseases (7), there are no published studies about its effects on acne. Montenegro is a coastal Mediterranean country, with some segments of coast being shallow, and contains mud rich with minerals. The aim of our study was to investigate efficacy of topical therapy of acne with preparations made by compounding peloid and medicinal plants from Montenegro.

MATERIAL AND METHODS

The preparations for use in topical acne therapy

The preparations used in our study were freshly prepared at Fontis Ltd. for each study participant. There were two study preparations for topical use: "Peloderm" and "Antiacne".

Peloderm is a galenic ointment consisting of peloid from the Ulcinj coast of Montenegro (2%) and propylene glycol extract from the following medicinal plants: Agrimonia eupatoria, Achilea millefolium, Plantago lanceolata, Matricaria chamomilla, and Foenegreci semen, prepared according to Pharmacopoeia Yugoslavica IV rules (20%), talc (4%), zinc oxide (12%) and stearin-type base (62%). The peloid used is mainly composed of SiO2, kaolin, kaolin 1T, halite and sylvite and has the following quantities of macro-, micro-elements, and ions: Al (26.19 mg/g), Ca (5.82 mg/g), Mg (13.60 mg/g), K (10.27 mg/g), Na (27.86 mg/g), Ti (1.34 mg/g), Fe (21.65 mg/g), Mn (0.46 mg/g), Si (7.43 mg/g), As (3.81 μg/g), Ba (82.75 μg/g), Cd (0.26 μg/g), Co (3.93 μg/g), Hg (0.02 μg/g), Se (<0.1 μg/g), Sr (18.72 μg/g), Cr (124.33 μg/g), Cu (22.89 μg/g), Ni (84.53 μg/g), Pb (39.25 μg/g), Zn (22.37 μg/g), chloride (61.68 mg/g), sulphate (32.53 mg/g), nitrate (0.41 mg/g), phosphate (0.84 μg/g) and trace quantities of carbonate and sulphide.

The Antiacne preparation is a galenic ointment consisting of: propylene glycol extract of the following medicinal plants: Calendula officinalis, Symphytum officinale, Achillea millefolium, Salvia officinalis, prepared according to Pharmacopoeia Yugoslavica IV rules (25%), zinc oxide (15%), titanium dioxide (1%), talc (4%) and a stearin-type base (55%).

Study population

The study population comprised patients suffering from acne who were treated at Fontis Ltd outpatient facility during the period from January 2007 to January 2010. The inclusion criteria were acne with any level of severity, absence of chronic comorbidities, and a treatment-free period of 30 days prior to study initiation. The exclusion criteria were concomitant comorbidities or topical therapy of acne, advanced age (>65 years), pregnancy, and breastfeeding.

Study design and sample size

We used a retrospective cohort study design with two cohorts defined by the type of topical preparation used, as follows: one cohort comprised of patients was treated with Peloderm, and the other cohort comprised of the patients treated with Antiacne. Treatment allocation was determined by prescriber preference, lacking conflicts of interest in this study. Taking into account expected effect size of 1.56 (the effect on acne was measured by a FDA-proposed five-category ranking system) (8): probability of type I error was 0.05, the power of the study was 95%, the treatment allocation ratio between cohorts was 1, and the number needed to treat calculated was 12 patients per group. However, we chose to include 70 patients per group. The study was approved by the Ethics Committee of Fontis Health Center.

Treatment protocol

After the first visit to Fontis outpatient facility, the patients received one of the topical preparations and were instructed to apply a thin layer twice daily (in the morning and in the evening) after washing their face. The patients were then followed up through 8 visits: after 15 days (visit 1), 45 days (visit 2), 75 days (visit 3), 90 days (visit 4), 120 days (visit 5), 180 days (visit 6), 12 months (visit 7), and 18 months (visit 8). During this 18-month period, the patients were not allowed to use other systemic or topical treatments for acne. At each visit, severity of acne was rated using the FDA-proposed five-category ranking system and recorded the patient's file.

The variables

Variables analysed were obtained retrospectively from the patients' file. The primary outcome variable of the study was severity of acne, rated by the FDA-

proposed five-category ranking system. The following possible confounders were assessed: previous systemic or topical antibiotic treatment of acne, duration of previous antibiotic use, previous topical therapy of acne other than antibiotics, previous systemic therapy with isotretinoin, previous hormonal therapy, other previous self-medication, smoking, occasional alcohol use, body mass index, sex, age, history of acne in family members, and chronic stress.

Statistics

The results were primarily described statistically with frequencies, measures of central tendency, and measures of variability. The difference in values of numeric variables among the study groups was assessed using the Student's T-test for independent samples, and the differences in frequencies of categorical variables' values were tested using the Chi-square test. All tests were two-tailed, and the confidence level for rejecting the null hypothesis was set to 0.05. All calculations were performed using the SPSS statistical software, version 18.

RESULTS

Prior to allocation to topical prescription therapy (at the first visit), the patients in the study cohorts differed with respect to the FDA acne severity score, body mass index, duration of previous antibiotic use, occasional alcohol use, and chronic stress, but other characteristics were similar. Baseline characteristics of the study cohorts (70 patients treated with Peloderm and 70 patients treated with Antiacne) are shown in Table 1.

In both treatment groups, the FDA acne severity score improved gradually throughout the study visits (Peloderm group: $F = 171.915$, $df = 8$, $p = 0.000$; Antiacne group: $F = 328.544$, $df = 8$, $p = 0.000$) (Figure 1). However, final FDA acne severity score (after 18 months of topical treatment) was significantly ($T = 7.556$, $df = 1$, $p = 0.000$) lower in the Peloderm group (1.0 ± 0.0) than in the Antiacne group (1.8 ± 0.9). An example of the treatment effect with Peloderm is shown in Figure 2.

None of the patients in either the Peloderm and Antiacne group experienced local or systemic adverse reactions to the study medications. All enrolled patients were fully compliant with their therapeutic regimens.

DISCUSSION

Both preparations for topical treatment of acne used in our study showed considerable efficacy, with excellent safety. However, final group results in patients using Peloderm were superior. The only two ingredients which are the same in both study preparations are extracts of Achillea millefolium and zinc oxide; notwithstanding this fact, many differences in preparations exist. Differences in preparations make it difficult to ascribe the observed positive effects of both preparations, and especially the difference between the effects, to any particular ingredient.

Parameter	Peloderm cohort (n=70)	Antiacne cohort (n=70)	Statistics
Age	20.7±4.4 years	21.0±5.3 years	T = -0.348 p > 0.05
Body mass index*	19.5±2.1	20.5±1.9	T = -2.982 p = 0.003
FDA acne severity score*	4.5±0.6	5.0±0.0	T = -7.490 p = 0.000
Sex	M/F = 52/18	M/F = 55/15	χ^2 = 0.357 p > 0.05
Previous systemic or topical antibiotic therapy of acne	55 (79%)	58 (83%)	χ^2 = 0.413 p > 0.05
Duration of previous antibiotic use (0 years / 1 year / 2 years / 3 years / 4 years*	0 / 10 / 38 / 8 / 14 (0% / 14% / 54% / 12% / 20%)	16/ 0 / 40 / 11 / 3 (23% / 0% / 57% / 16% / 4%)	χ^2 = 33.643 p = 0.000
Previous topical therapy of acne other than antibiotics	61 (87%)	52 (74%)	χ^2 = 3.717 p > 0.05
Previous systemic therapy with isotretinoin	2 (3%)	1 (1.5%)	χ^2 = 0.341 p > 0.05
Previous hormonal therapy	22 (31%)	17 (24%)	χ^2 = 0.889 p > 0.05
Previous self-medication	57 (81%)	58 (83%)	χ^2 = 0.049 p > 0.05
Smoking	35 (50%)	31 (44%)	χ^2 = 0.459 p > 0.05
Occasional alcohol use*	28 (40%)	13 (19%)	χ^2 = 7.761 p = 0.005
Acne in family	45 (64%)	43 (61%)	χ^2 = 0.122 p > 0.05
Chronic stress*	35 (50%)	14 (20%)	χ^2 = 13.846 p = 0.000

Table 1. Baseline characteristics of the study cohorts. * indicates significant difference

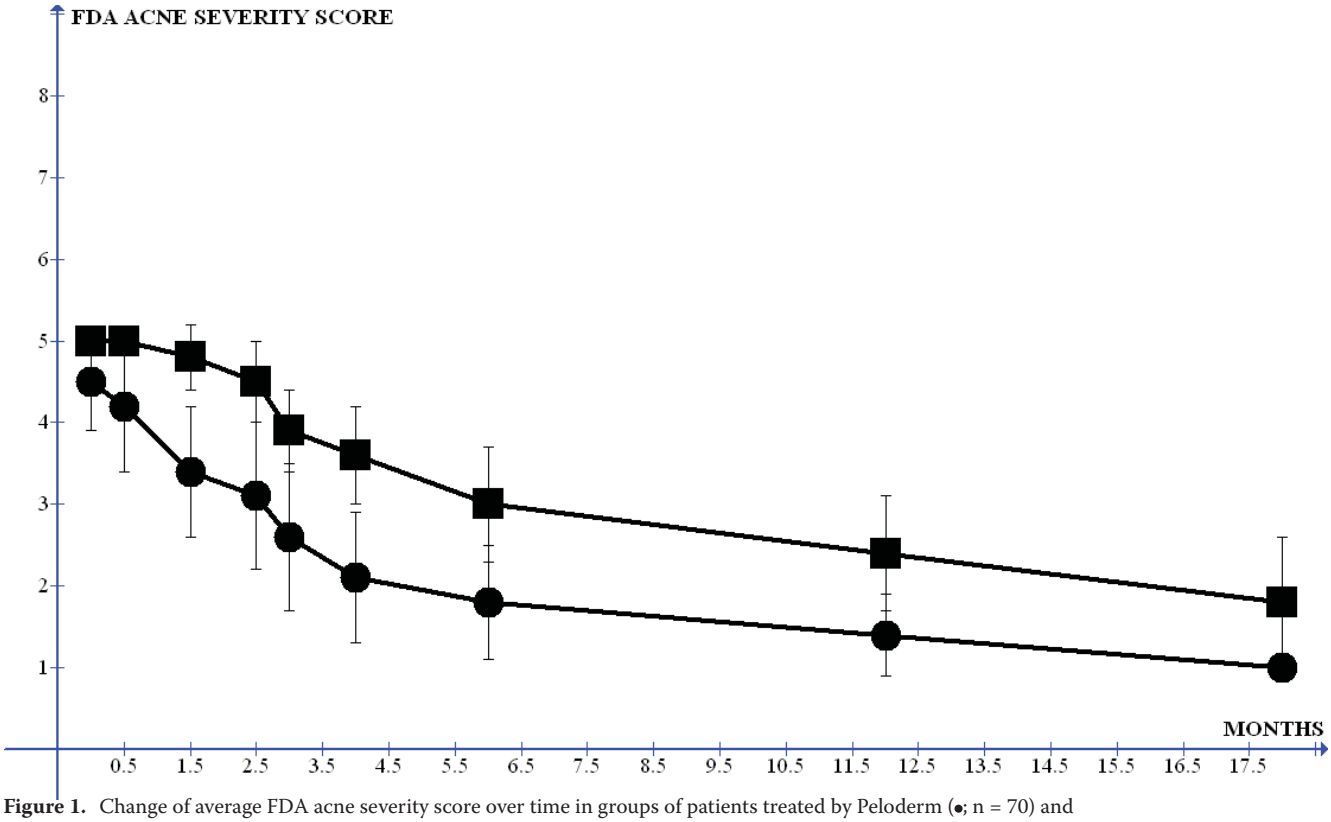
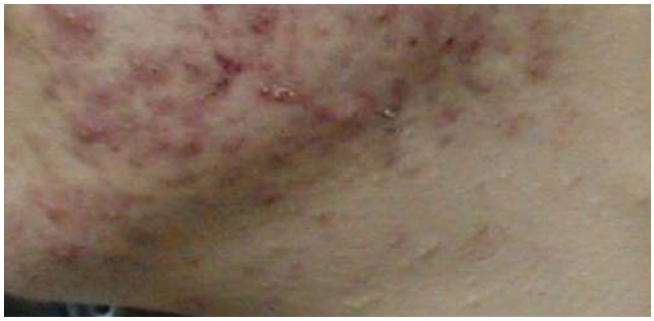


Figure 1. Change of average FDA acne severity score over time in groups of patients treated by Peloderm (•; n = 70) and by Antiacne (■; n = 70). Error bars = standard deviations.

Of the ingredients in the study preparations, only zinc oxide and Calendula officinalis have been evaluated for effects on acne. Zinc oxide together with chloroxylenol in the same preparation showed the same efficacy on acne and better local tolerability in a clinical trial, when compared with 5% benzoyl peroxide cream (9). It seems that the beneficial effect of zinc oxide in acne therapy could be explained by antiinflammatory properties of zinc, which suppresses cytokine-induced NO production in keratinocytes (10). Calendula officinalis was tested for treatment of acne as the only ingredient of a homeopathic topical preparation, with "good" results in a series of patients, which were not objectively evaluated (11). Salvia officinalis was tested for antimicrobial activity in vitro on 29 different aerobic and anaerobic bacteria and yeasts, but no effect was observed (12).

A



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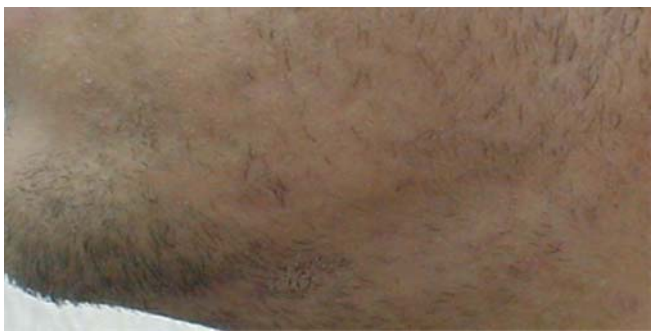

Figure 2. Photographs of the affected skin area in a patient before (A) and after (B) treatment with Peloderm topical preparation.

Since zinc oxide with established anti-acne effect was a common ingredient of both Peloderm and Antiacne preparations, at least some part of their observed efficacy in this study has to be explained by the beneficial effect of zinc. However, Peloderm was more effective than Antiacne, suggesting beneficial effects of ingredients other than zinc, especially of peloid, which was not part of Antiacne preparation.

Although previously not tested in patients with acne, peloid preparations have considerable potential beneficial effects when applied topically in this patient population. It shows antimicrobial activity on a variety of bacteria in vitro (13). Both inhibitory and stimulatory effects on some human and bacterial enzymes, like oxidoreductases (lactate dehydrogenase, malate dehydrogenase, etc.), were demonstrated in another in vitro study (14). Analytical studies (15) showed that the process of maturation of peloid is important for its potential therapeutic effects; mature mud is especially rich with organic components, such as phospholipids, phytosterols, and terpenes, which can affect human and bacterial regulatory molecules.

The main limitation of our study was its observational character, precluding testing of single compound preparations, containing only one of potentially active ingredients at a time. The observed beneficial effects on acne of two complex preparations with multiple ingredients are difficult to discern; however, we still can conclude that topical preparations of both peloid and selected medicinal plants from Montenegro, in ratios specified in this study, are effective and safe options for local treatment of acne, with the peloid preparation having somewhat greater potency.

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NUTRITION STATUS BASED ON MID UPPER ARM CIRCUMFERENCE AMONG URBAN, POOR PRE-SCHOOL CHILDREN IN NORTH 24 PARGANAS, WEST BENGAL, INDIA

Samiran Bisai [1, 2]
[1]Department of Anthropology, North Eastern Hill University, Shillong, Meghalaya.
[2]Department of Anthropology, Vidyasagar University, Midnapore, West Bengal.

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ABSTRACT:

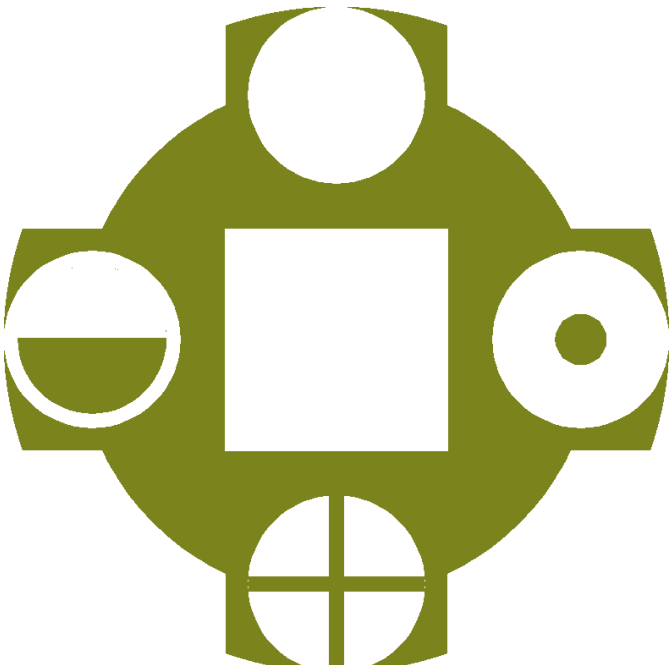
The study of nutritional status based on mid upper arm circumference (MUAC) among pre-school children in India is very limited. Therefore, a study was carried out from February to June 2006 in three municipal wards of the North 24 Parganas district, West Bengal, India, to determine the nutritional status based on MUAC among urban, poor pre-school children. Undernutrition was defined based on age- and sex-specific MUAC cut-off values as recommended by the World Health Organization (WHO) in 1995 and 2007. A total of 899 children, 57.5% boys and 42.5% girls, aged 1-5 years were measured randomly and included in the present analysis.

The overall proportion of undernutrition was 77.8%, of which 52.9 and 24.9% children were moderately and severely undernourished, respectively, using WHO 1995 MUAC cut-off values. Similarly, the rate of undernutrition was 69.8%, of which 43.9 and 25.9% children were moderately and severely undernourished, respectively, when the WHO 2007 MUAC cut-off points were used. The prevalence of undernutrition was significantly higher among boys than girls when using either of the cut-off values. Overall, about 9% and 7% of boys and girls, respectively, were overestimated as undernourished by the WHO 1995 cut-offs, as compared to the WHO 2007 cut-offs.

In conclusion, the overall prevalence of undernutrition among these children was very high, indicating a critical situation. Therefore, respective authorities should take initiatives to utilize low-cost methods such as MUAC for identifying children at risk for acute malnutrition at an early age. Such studies may assist policymakers in the formulation of appropriate measures to combat child undernutrition at the national level.

Keywords: *urban, poor, pre-school children, undernutrition, arm circumference.*

Running title: *Nutrition status of children based on arm circumference*



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Correspondence to: Dr. Samiran Bisai, Department of Anthropology, Vidyasagar University
Midnapore- 721102, West Bengal, India., Email: samiranbisai@yahoo.com

INTRODUCTION

Despite the economic development of the country, India retains one of the highest rates of child undernutrition in the world. The high level of child undernutrition not only increases morbidity and mortality in later life but also reduces the economic development and productivity of the county. Therefore, researchers from various disciplines worldwide constantly attempt to determine the prevalence of child undernutrition using different methods. Anthropometry is a widely accepted, low-cost technology for defining the nutritional status of children (1). However, the standard against which the nutritional status of the sample population should be determined remains controversial (2). Recently, the World Health Organization (WHO) developed age- and sex-specific mid upper arm circumference (MUAC) cut-off points to determine child undernutrition (3). MUAC is a comparatively simple measurement, particularly for screening children in emergency situations. The main advantage of MUAC is its simplicity, particularly for screening children in emergency situations. When compared with standard anthropometric indices, MUAC is a valuable, low-cost technology applicable at the village health worker level (4). It requires no scales, measuring devices or anything; it takes very little time and is easy to learn and perform by an unskilled worker (5).

It has been well documented that, compared with the weight-to-height index, MUAC has a very high specificity (6) and appears to be a better predictor of child mortality than the weight-to-height index (7). However, little information exists regarding the prevalence of undernutrition based on MUAC among preschool children in India (8-10) and West Bengal (11-13). Given this context, the aim of the present study was to determine the nutritional status of urban, poor pre-school children from the North 24 Parganas district, West Bengal, India, using the WHO (1,3) recommended age- and sex-specific MUAC cut-off points and to later compare the rates of undernutrition as identified by the two WHO recommended age- and sex-specific MUAC cut-off points at different time points.

METHODS

To determine the nutritional status based on MUAC, a cross-sectional study was carried out from February to June 2006 in three municipal wards of the Barasat and Madhyamgram municipalities in the North 24 Parganas district of West Bengal. The minimum sample size is (n=872) calculated following the standard formula: (4 x p (1-p) / d2), based on a 28.6% prevalence of undernutrition based on MUAC (11), with a relative precision of 3%. The study involved a random survey of lower socioeconomic status children. The vast majority of the households contained low-wage daily manual labourers. However, the study area was purposely selected. A trained investigator obtained information on the age, sex, weight, height and MUAC of all children. The ages of the children were noted from their parents or sometimes calculated using local events, which could be dated and linked to important points in their life history. The respective institutional ethical committee approved the study protocol, and informed consent was obtained from the parents of each child.

The nutritional status of the children was assessed by anthropometric measurements following standard techniques (14). MUAC was measured using a nonstretchable fibre tape to the nearest 1 mm. Nutritional status of the children was assessed using the following scheme:

Normal:	≥ 2 sd
Undernutrition:	< -2 sd
Moderate undernutrition:	< -2 sd to -3 sd
Severe undernutrition:	< -3 sd

Where, sd refers to the age- and sex-specific WHO (1,3) standard deviations of MUAC. The -2 sd and -3 sd of age- and sex-specific cut-off points are given in ***table 1*** and ***table 2.***

Age (years)	Boys		Girls	
	Moderate (-2 sd)	Severe (-3 sd)	Moderate (-2 sd)	Severe (-3 sd)
1	13.2	11.9	12.6	11.4
2	13.6	12.2	13.4	12.0
3	13.8	12.4	13.6	12.2
4	14.1	12.6	13.9	12.4
5	14.2	12.6	14.1	12.5

Table 1. The WHO (1995) recommended age- and sex-specific cut-off points for MUAC (cm).

Age (years)	Boys		Girls	
	Moderate (-2 sd)	Severe (-3 sd)	Moderate (-2 sd)	Severe (-3 sd)
1	12.5	11.6	12.4	11.1
2	13.0	12.0	12.7	11.7
3	13.5	12.5	13.3	12.2
4	13.7	12.7	13.6	12.5
5	14.0	12.9	14.0	12.8

Table 1. The WHO (2007) recommended age- and sex-specific cut-off points for MUAC (cm).

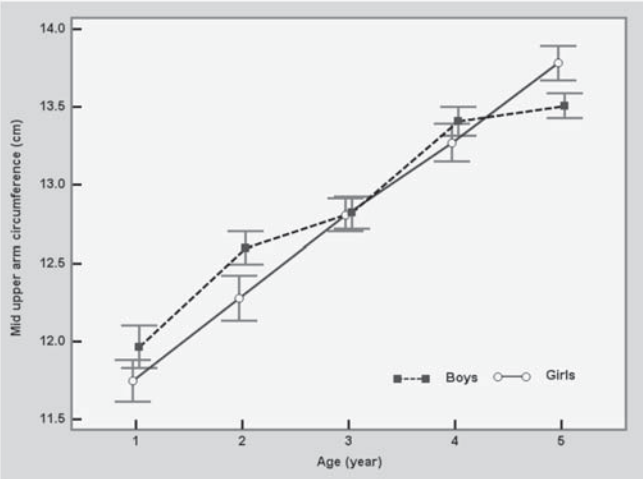
All statistical analyses were performed using EPI6 statistical software. One- and two-way analyses of variance (ANOVAs) were used to test the age and sex differences of the mean MUAC values. Odds ratios were measured using standard formulae to compare the risk between groups. The proportion test was employed to compare the prevalence of undernutrition in different groups. Moreover, p-values less than 0.05 were considered statistically significant.

RESULTS

A total of 899 children, 57.5% boys and 42.5% girls, aged 1-5 years old were measured and included in the present analyses. The age-sex distributions of mean MUAC values are presented in **figure 1**. The results of the one-way ANOVA reveal that the mean MUAC increased with age in boys (F=33.80, p<0.001) and girls (F=39.05, p<0.001). On the other hand, the two-way ANOVA results show that, except for sex, age (F=73.444, p<0.001) and the age-sex interaction (F=2.453, p<0.05) had a significant effect on MUAC. However, the mean MUAC was significantly lower in boys than in girls at age 5 (t=2.41, p<0.05).

The overall (age and sex combined) proportion of undernutrition was 77.8%, of which 52.9% and 24.9% children were moderately and severely undernourished, respectively, using the WHO 1995 (1) MUAC cut-off points (**table 3**). About 55% and 50% of the boys and girls, respectively, were moderately undernourished. The proportion ranged from 45.7 to 62.3% for boys and 38.5 to 56.7% for girls. Nearly 26% of the boys and 24% of the girls were severely undernourished; the proportion ranged from about 16% to 43% for boys and 7.7% to 38.5% for girls. It is noteworthy to mention that the proportion of severe undernutrition significantly decreased with age

Figure 1. Age-sex distribution of MUAC (means ± SEM) of the studied children.



in both sexes. The proportion of undernutrition was significantly higher in boys compared with girls (81.0 vs. 73.3%, x2=7.62, df=1, p<0.01).

The overall (age and sex combined) proportion of undernutrition was 69.8%, of which 43.9 and 25.9% children were found to be moderately and severely undernourished, respectively, using the WHO 2007 (3) MUAC cut-off points

Age (years)	Boys				Girls			
	n	Severe undernutrition	Moderate undernutrition	Normal	n	Severe undernutrition	Moderate undernutrition	Normal
1	54	42.6	48.1	9.3*	47	38.3	38.5	23.4*
2	100	33.0*	51.0	16.0	65	38.5*	44.6	16.9
3	92	37.0	45.7*	17.4	82	28.0	52.4*	19.5
4	114	16.7	62.3*	21.1*	84	19.0	48.8*	32.1*
5	157	15.9*	60.5	23.6*	104	7.7*	56.7	35.6*
Total	517	25.9	55.1	19.0*	382	23.6	49.7	26.7*
Age and sex combined undernutrition: Severe=24.9%, Moderate=52.9%, Total=77.8%								

Table 3. Assessment of nutritional status of the studied children based on MUAC (WHO 1995). *Significant sex differences; p<0.05.

Age (years)	Boys				Girls			
	n	Severe undernutrition	Moderate undernutrition	Normal	n	Severe undernutrition	Moderate undernutrition	Normal
1	54	33.3*	38.9	27.8*	47	21.3*	44.7	34.0*
2	100	25.0	48.0*	27.0	65	29.2	38.5*	32.3
3	92	43.5*	35.9*	20.7	82	28.0*	47.6*	24.4
4	114	19.3	43.9	36.8	84	22.6	38.1	39.3
5	157	26.1*	47.8	26.1*	104	15.4*	49.0	35.6*
Total	517	28.2	43.9	27.9	382	22.8	44.0	33.2
Age and sex combined undernutrition: Severe= 25.9%, Moderate= 43.9%, Total= 69.8%								

Table 4. Assessment of nutritional status of the studied children based on MUAC (WHO 2007). *Significant sex differences; p<0.05.

Age (years)	Boys (Undernutrition)			Girls (Undernutrition)		
	WHO 1995	WHO 2007	Difference	WHO 1995	WHO 2007	Difference
1	90.7	72.2*	18.5	76.8	66.0*	10.8
2	84.0	73.0*	11.0	83.1	67.7*	15.4
3	82.7	79.4	3.3	80.4	75.6	4.8
4	79.0	63.2*	15.8	67.8	60.7**	7.1
5	76.4	73.9	2.5	64.4	64.4	0.0
Total	81.0	72.1*	8.9	73.3	66.4**	6.9

Table 5. Comparison of the prevalence (%) of undernutrition as assessed by the two WHO recommended MUAC cut-off values. Significant difference: *p<0.01, **p<0.05.

(**table 4**). About 28% of the boys and 23% of the girls were severely undernourished, with the proportion ranging from about 19.3% to 43.5% for boys and 15.4% to 29.2% for girls. It is noteworthy to mention that the proportion of severe undernutrition decreased with age in both sexes. On the other hand, the proportion of moderate undernutrition was similar in both boys and girls. The proportion of undernutrition was higher in boys (72.1%) than in girls (66.8%).

Table 5 presents the comparison of the proportion of undernutrition (severe + moderate) as assessed by the two different WHO (1,3) recommended MUAC cut-off points. The proportion of undernutrition was significantly higher in both sexes when the WHO 1995 MUAC cut-off points were used for the assessment of undernutrition compared with the WHO 2007 cut-offs. Overall, about 9% and 7% of the boys and girls, respectively, were overestimated as undernourished by the WHO 1995 cut-off points, with the difference ranging from 2.5% for five year olds to 18.5% for one year olds in boys and 0% for five year olds to 15.4% for two year olds in girls.

DISCUSSION

Child nutritional status is well recognized as one of the important indicators of economic development of a country. In the last few decades, undernutrition has become a major public health problem in the developing world. Therefore, in 2000, the United Nations adopted child undernutrition as one of the eight Millennium Development Goals (15).

The age-combined prevalence of undernutrition based on the WHO 1995 cut-offs (boys = 81.0%; girls = 73.3%) and the WHO 2007 cut-offs (boys = 72.1%; girls = 64.4 %) was higher in boys than in girls. Whereas the prevalence of undernutrition was overestimated by the earlier cut-offs than the later ones in both sexes, overall the rates of undernutrition using both cut-offs were 77.8 and 69.8%, respectively. Compared with the general Indian population (**Table 6**), the prevalence of undernutrition among the preschool children of the present study was higher than those reported among preschool children from Punjab (10), Orissa (8,9), Kolkata (11), Hooghly (12) and Nadia (13). This study clearly indicated that the nutritional status of these pre-school children was critical with very high rates of undernutrition in both sexes.

More important, the application of the new WHO cut-offs are valid for developing countries like India, which were developed based on multinational samples of children with minimum constraints of growth. The WHO recommended that these new standards be used for the surveillance of nutritional status in all countries and compared the prevalence of data collected before the release of the new standards (16). However, county-specific standards are more appropriate for comparing health-compromised children, especially in India where the problem is more pronounced for undernutrition than overnutrition (17).

Table 6. Comparison of the overall prevalence (%) of undernutrition among the preschool children based on MUAC. * Significantly lower than the present study; p<0.05. **Significant difference; p<0.001.

Studied area (district/state)	Number of children studied	Prevalence (%)	References
Punjab	6531	38.5*	Kaur et al., 2005
Khurda and Cuttack, Orissa	101	35.6*	Chakraborty et al. 2006
Cuttack, Orissa	292	29.1*	Mishra and Mishra, 2007
Kolkata, West Bengal	21	28.6*	Chatterjee and Saha, 2008
Hooghly, West Bengal	894	64.5*	Mandal and Bose, 2009
Nadia, West Bengal	2016	35.1*	Biswas et al., 2010
North 24 Parganas, West Bengal	899	**77.8 (WHO 1995) **69.8 (WHO 2007)	**Present study**

Therefore, regular surveillance in the form of nutritional surveys would be conducted at the village, block, state and national levels to monitor the nutritional and health status of children (16). Anthropometric examination is an almost mandatory tool in any research on health and nutritional condition in childhood, and the study of nutritional status is of great importance for understanding the social well-being in a population or country (18). Moreover, in community-based studies, application of MUAC appears to be a better predictor for assessment of childhood undernutrition than many other anthropometric indicators. Several researchers worldwide have used MUAC to identify children as having moderate and severe acute malnutrition for its simplicity (9, 19-21). Therefore, respective authorities should take initiatives to utilize low-cost methods like MUAC for identifying children at risk for acute malnutrition at an early age. Such studies would help policymakers to formulate appropriate measures to combat child undernutrition at the national level.

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PRE-EXERCISE SUPEROXIDE DISMUTASE ACTIVITY AFFECTS THE PRO/ANTIOXIDANT RESPONSE TO ACUTE EXERCISE

Dusica Djordjevic[1], Dejan Cubrilo[1], Vladimir Zivkovic[1], Nevena Barudzic[1], Milena Vuletic[1] and Vladimir Jakovljevic[1]
[1]Department of Physiology, Medical Faculty, University of Kragujevac, Kragujevac, Serbia

UTICAJ BAZALNE VREDNOSTI SUPEROKSID DISMUTAZE NA BALANS PRO/ANTIOKSIDANASA IZAZVAN AKUTNIM FIZIČKIM NAPOROM

Dusica Đorđevic[1], Dejan Čubrilo[1], Vladimir Živković[1], Nevena Barudžić[1], Milena Vuletić[1] and Vladimir Jakovljević[1]
[1]Katedra za Fiziologiju, Medicinski fakultet, Univerzitet u Kragujevcu, Kragujevac

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ABSTRACT

Superoxide dismutase (SOD), a first line of defence enzyme in red blood cells, has been commonly found to be influenced by chronic exercise and can be used to differentiate between well-trained subjects and controls. The aim of our study was to assess the differences in the pro-oxidant and antioxidant responses to acute exercise in subjects with different basal levels of SOD activity. For this study, 24 young handball players were subjected to a maximal graded exercise test, and blood samples were taken immediately before and after the exercise. The blood samples were used to determine the levels of superoxide anion radicals (O2-), hydrogen peroxide (H2O2), nitric oxide (NO, estimated by measuring nitrites NO2-), lipid peroxidation (estimated by measuring thiobarbituric acid reactive substances, TBARS), SOD activity and catalase (CAT) activity. Acute exercise induced statistically significant changes in all of the investigated biochemical parameters of redox homeostasis except O2-, and the most significant changes in these parameters were observed in the group of athletes with the lowest pre-exercise SOD activity. Significant correlations were found between the basal SOD activity and H2O2 and between basal SOD activity and NO (NO2-). These results suggest that pre-exercise SOD activity determines the effects of exercise on redox homeostasis.

Keywords: *oxidative stress, redox homeostasis, superoxide dismutase, athletes, exercise.*

SAŽETAK

Superoksid dismutaza (SOD), prva linija antioksidativnog zaštitnog sistema, enzim je koji je u prethodnim istraživanjima bio najpodložniji promenama usled uticaja trenažnog procesa. Ovaj enzim je takođe bio onaj po kom su se dobro utrenirani i netrenirani ispitanici istraživanja razlikovali. Cilj naše studije bio je utvrđivanje razlike u odgovoru parametara redoks ravnoteže na jednokratno fizičko vežbanje kod ispitanika sa različitom bazalnom aktivnosti SOD. 24 mlada rukometaša podvrgnuta su maksimalnom progresivnom testu opterećenja. Neposredno pre i nakon testa opterećenja ispitanicima su uzeti uzorci krvi, radi biohemijske analize parametara redoks ravnoteže – u plazmi je određivan nivo superoksid anjon radikala (O2-), hidrogen peroksida (H2O2), azotnog monoksida (NO, određivanog preko nitrita NO2-), indeksa lipidne peroksidacije (TBARS), a u eritrocitima aktivnost superoksid dismutaze (SOD) i katalaze (CAT). Jednokratno vežbanje izazvalo je statistički značajne promene svih praćenih parametara, osim O2-, a najznačajnije promene uočene su u grupi ispitanika sa najnižom bazalnom aktivnosti SOD. Značajnije korelacije pronađene su između bazalne aktivnosti SOD i H2O2, i SOD i NO (NO2-). Ovi rezultati navode nas na zaključak da bazalna aktivnost SOD određuje efekte vežbanja na redoks ravnotežu.

Ključne reči: *oksidativni stres, redoks ravnoteža, superoksid dismutaza, sportisti, vežbanje.*

Abbreviations used:
ADS - *antioxidative defence system;*
CAT - *catalase;*
GGSG - *oxidised glutathione;*
GPx - *glutathione peroxidase;*
GSH - *reduced glutathione;*
GXT - *graded exercise test;*
RBCs - *red blood cells;*
RONS - *reactive oxygen and nitrogen species;*
ROS - *reactive oxygen species;*
SOD - *superoxide dismutase;*
TBARS - *thiobarbituric acid reactive substances.*

Korišćene skraćenice
ADS - *antioksidativni odbrambeni sistem*
CAT - *katalaze*
GGSG - *oksidisani glutation*
GPx - *glutation peroksidaza*
GSH - *redukovani glutation*
GXT - *progresivni test opterecenja*
RBCs - *crvena krvna zrnca*
RONS - *reaktivne kiseonične i azotne vrste*
ROS - *reaktivne kiseonične vrste*
SOD - *superoksid dismutaza*
TBARS - *reaktivne supstance vezane za tiobarbituričnu kiselinu*

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Correspondence to: Vladimir Jakovljevic, MD, PhD / Associate Professor of Physiology / Medical Faculty, University of Kragujevac
Svetozara Markovica 69, P.P. 124, 34000 Kragujevac, Republic of Serbia, Tel.: +381 34 34 29 44, Fax.: + 381 34 30 68 00/ext 112, E-mail: drvladakgbg@yahoo.com

INTRODUCTION

Oxidative stress is a condition in which the cellular production of pro-oxidants exceeds the capacity of the antioxidant defence system (ADS) to render the pro-oxidants inactive (1). The generation of reactive oxygen and nitrogen species (RONS) occurs as a consequence of normal cellular metabolism, but excessive production of RONS, which appears to be induced by both psychological and physical stress, may contribute to pathological processes and diseases (2, 3).

Exercise training is associated with numerous health benefits (4, 5), but it can also be viewed as an intense physical stressor that could lead to increased cellular oxidative damage (6). It has been suggested that acute exercise induces oxidative stress in the blood (7-12), but that repeated exposure of the system to increased RONS production from chronic exercise training leads to an upregulation in the body's ADS (7, 8, 13, 14). This improvement in the ADS should provide adaptive protection from RONS during subsequent training sessions as well as during exposure to non-exercise related conditions (15).Because the levels of superoxide dismutase (SOD), a first line of defence enzyme in red blood cells (RBCs), were commonly found to altered by the influence of both acute and chronic exercise (17-19) and could be used to differentiate between well-trained subjects and controls (13, 20, 21), we hypothesised that the levels of SOD pre-exercise (basal) activity would determine the extent of oxidative stress induced by acute exercise. Thus, the aim of our study was to assess the differences in the responses to acute exercise in subjects with different basal levels of SOD activity.

MATERIAL AND METHODS

Subjects

The research was performed with a group of 24 male athletes who were all young handball players. All participants were healthy, used no medications or supplements, and were non-smokers. They were asked not to perform any heavy physical activity in the 24 h before the test and not to consume alcohol in the 48 h before the test. All participants and their parents gave written informed consent. The study was done in accordance with the Helsinki Declaration and approved by the Ethical committee of the Medical Faculty, University of Kragujevac.

Protocol

The research period started at 8 AM in the morning. After the participants filled in a standard sports medicine questionnaire and passed a standard sports medicine examination, a blood sample was taken from an antecubital vein. Body composition was estimated with the bioimpedance method using a *Biospace InBody 720* apparatus. Next, the athletes were subjected to a maximal graded exercise test (GXT) on a bicycle ergometer (*Kettler AX1*). The load was set to 2 W/kg and was increased every 3 min for 50 W; the subjects were instructed to ride at 60 rpm. We hypothesised that the maximal oxygen consumption (VO_2 max) was reached when the oxygen consumption reached a plateau (the time at which increasing the workload does not cause an increase in oxygen consumption) (22). Oxygen consumption was directly measured using a *Cosmed Fitmate Pro* apparatus. Immediately after finishing the GXT, another blood sample was collected.

Later, during the data analysis, the athletes were divided into three groups based on their basal SOD activity: 1) athletes with low basal SOD activity, 2) athletes with average basal SOD activity, and 3) athletes with high basal SOD activity. Group classification was performed according to tertiles that were generated from the basal SOD activity values of all subjects because there are not widely accepted cut-off values.

Biochemical assays

Blood samples were taken from an antecubital venule and placed into a Vacutainer test tube containing a sodium citrate anticoagulant. Blood was centrifuged to separate the plasma from the RBCs. Biochemical parameters, including superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), thiobarbituric acid reactive substances (TBARS, an index of lipid peroxidation), SOD and catalase (CAT) were measured spectrophotometrically.

Determination of antioxidant enzyme activities

Isolated RBCs were washed 3 times with 3 volumes of ice-cold 0.9 mmol/l NaCl, and hemolysates containing approximately 50 g/l haemoglobin (Hb) (prepared according to the protocol of McCord and Fridovich (23)) were used for the determination of CAT activity. CAT activity was determined according to Beutler (24). Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove Hb (25). Then 50 µl catalase buffer, 100 µl sample and 1 ml of 10 mM H_2O_2 were added together. Spectrophotometric measurements were obtained at 360 nm. Double distilled water was used as a blank probe. SOD activity was determined by the epinephrine method of Misra and Fridovich (26). Briefly, 100 µl of lysate and 1 ml of carbonate buffer were mixed, and then 100 µl of epinephrine was added. SOD activity levels were determined by spectrophotometric measurements at 470 nm.

Nitric oxide determination

NO decomposes rapidly and forms stable metabolite nitrite/nitrate products. Nitrite (NO_2^-) levels were determined as an index of NO production using Griess reagent (27). Briefly, 0.1 ml of 3 N perchloride acid (PCA), 0.4 ml of 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.2 ml of plasma were put on ice for 15 min and then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 was added. Nitrites levels were measured at 550 nm. Double distilled water was used as a blank probe.

Superoxide anion radical determination

Levels of the superoxide anion radical (O_2^-) were measured using a Nitro Blue Tetrazolium (NBT) reaction in Tris buffer with the collected plasma samples and measurements were obtained at 530 nm (28).

Hydrogen peroxide determination

The method for determination of H_2O_2 levels was based on the oxidation of phenol red in the presence of horseradish peroxidase (POD) (29). Briefly, 200 µl of sample with 800 µl of Phenol Red Solution (PRS) and 10 µl of POD were mixed (1:20). The H_2O_2 measurements were obtained at 610 nm.

Determination of lipid peroxidation by measurement of TBARS

The degree of lipid peroxidation in plasma was estimated by measuring TBARS. TBARS were measured by incubating plasma in 1% thiobarbituric acid (TBA) and 0.05 NaOH at 100°C for 15 min and then performing spectrophotometric readings at 530 nm. Double distilled water was used as a blank probe. A TBA extract was obtained by incubating 0.8 ml plasma and 0.4 ml trichloro acetic acid (TCA) on ice for 10 min and then centrifuging the sample for 15 min at 6000 rpm. This method was previously described by Ohkawa (30).

Statistics

Statistical analysis was performed using the statistical package *SPSS 10.0 for Windows*. The results are expressed as the means ± standard deviation in the text and in the tables or as the means ± standard error of the mean in the figures. After testing the distribution of the data with a Shapiro-Wilk test, the difference between the mean values from two related samples (i.e., before and after the GXT) were assessed by a Paired t-test or Wilcoxon test, whereas the difference between the mean values between the 3 groups gathered at a similar sampling time were assessed either by an ANOVA or a Kruskal-Wallis test. Correlation between the various variables was determined by a bivariate correlation, by using either Pearson's or Spearman's coefficient of correlation.

RESULTS

Demographic and clinical characteristics of the study subjects are shown in Table 1. The GXT induced changes in 5 out of the 6 investigated parameters of redox homeostasis (Table 2). Based on tertiles calculated from the basal SOD activity values of each subject, three groups were established, including athletes with low basal SOD activity (n=8; 291.0±141.9 U/g Hb x10^3), athletes with average basal SOD activity (n=8; 1508.9±440.9 U/g Hb x10^3), and athletes with high basal SOD activity (n=8; 4980.7±1417.3 U/g Hb x10^3).

A correlation was not found between the VO_2 max and the level of basal SOD activity (i.e., VO_2 max did not differ between the "basal SOD groups", P=0.823; correlation between basal SOD activity and VO_2 max, r=0.966). Correlations generated by comparisons of basal SOD activity with all investigated biochemical parameters before and after GXT are shown in Table 3. Statistical differences of all investigated biochemical parameters between the groups defined by basal SOD activity are shown in Table 4 and in Figures 1-6; these figures also show the significant changes of certain parameters in each group before and after GXT.

Characteristic	X±SD
Age (years)	16.1±0.6
Height (cm)	182.5±5.8
Weight (kg)	77.0±9.5
Body mass index	23.1±2.6
Fat (%)	11.3±4.4
Muscle (%)	50.4±2.5
Duration of sports engagement (years)	6.8±1.8
Maximal oxygen consumption (ml/kg/min)	46.7±6.1

Table 1. Demographic and clinical characteristics of the study group.

Parameter (X±SD)	Graded exercise test		*P*
	Pre-	Post-	
O_2^- (nmol/ml)	5.6±4.8	6.3±5.5	.466
H_2O_2 (nmol/ml)	2.8±1.6	4.5±2.8	**.001****
NO_2^- (nmol/ml)	2.2±1.5	3.0±0.8	**.037***
TBARS (µmol/ml)	0.1±0.1	0.5±0.3	**.000****
SOD (U/g Hb x103)	2260.2±2189.9	1353.2±2123.6	**.016***
CAT (U/g Hb x103)	11.0±8.5	5.2±4.1	**.002****

Table 2. Pre- and post-exercise values of the parameters of oxidative stress in all athletes.

Parameter	**SOD** Before GXT
O_2^- before GXT	-.135
O_2^- after GXT	-.129
H_2O_2 before GXT	**-.508***
H_2O_2 after GXT	**-.500***
NO_2^- before GXT	**.497***
NO_2^- after GXT	-.163
TBARS before GXT	.167
TBARS after GXT	-.286
CAT before GXT	-.275
CAT after GXT	.220
SOD after GXT	**.750****

Table 3. Correlation coefficient (r) of superoxide dismutase (SOD) activity before the graded exercise test (GXT) and the other redox parameters measured before and after the GXT.

Pre-exercise SOD activity group	X±SD		Test Razlika između 2 merenja
	Before GXT	After GXT	
O_2- (nmol/ml)			
Low (n=8)	6.8±5.5	6.0±5.6	*P*=1.000
Average (n=8)	3.5±2.4	5.5±4.0	*P*=0.154
High (n=8)	6.5±5.6	7.4±7.1	*P*=0.889
Test Razlika medju grupama	*P*=0.324	*P*=0.968	
H_2O_2 (nmol/ml)			
Low (n=8)	3.8±2.1	6.5±2.2	***P*=0.035***
Average (n=8)	2.9±1.2	4.2±2.6	*P*=0.090
High (n=8)	1.6±0.4	2.8±2.5	*P*=0.069
Test	***P*=0.012*** Low vs. high *P*=0.010** Average vs. high *P*=0.010**	***P*=0.047*** Low vs. high *P*=0.010**	
NO_2- (nmol/ml)			
Low (n=8)	1.0±1.1	3.3±0.9	***P*=0.002****
Average (n=8)	2.6±1.5	2.7±0.6	*P*=0.776
High (n=8)	2.8±1.3	2.9±0.8	*P*=0.798
Test	***P*=0.021*** Low vs. average *P*=0.027* Low vs. high *P*=0.008**	*P*=0.339	
TBARS (μmol/ml)			
Low (n=8)	0.12±0.10	0.61±0.53	***P*=0.018***
Average (n=8)	0.13±0.08	0.34±0.06	***P*=0.000****
High (n=8)	0.15±0.09	0.47±0.25	***P*=0.026***
Test	*P*=0.784	*P*=0.228	
SOD (U/g Hb x103)			
Low (n=8)	291.0±141.9	75.2±97.4	***P*=0.025***
Average (n=8)	1508.9±440.9	673.6±653.8	*P*=0.066
High (n=8)	4980.7±1417.3	3310.9±2757.8	*P*=0.198
Test	*P*=0.000** Low vs. average P=0.000** Low vs. high P=0.000** Average vs. high P=0.000**	***P*=0.000**** Low-average P=0.007** Low-high P=0.000** Average-high P=0.010**	
CAT (U/g Hb x10^3)			
Low (n=8)	12.6±7.8	3.9±1.7	***P*=0.020***
Average (n=8)	10.7±4.1	6.4±6.2	*P*=0.123
High (n=8)	6.5±1.6	5.8±2.9	*P*=0.423
Test	*P*=0.185	*P*=0.583	

Table 4. Pre- and post-exercise values of pro-oxidants and antioxidants in the groups of athletes defined by pre-exercise superoxide dismutase (SOD) activity.

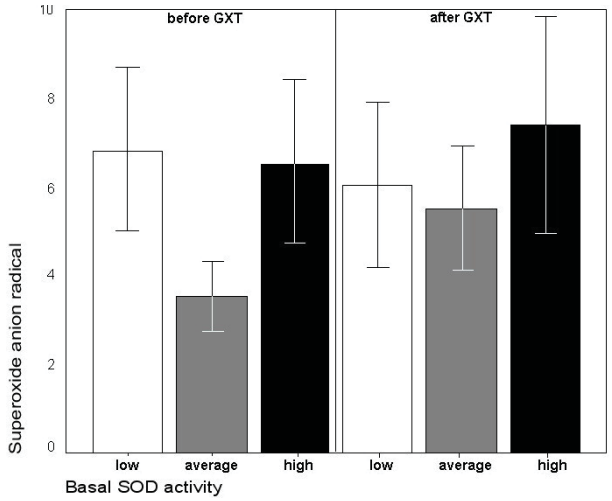

Figure 1. Levels of superoxide anion radical (nmol/ml) before and after GXT in the groups of athletes defined by basal SOD activity (results are presented as the means ± SE).

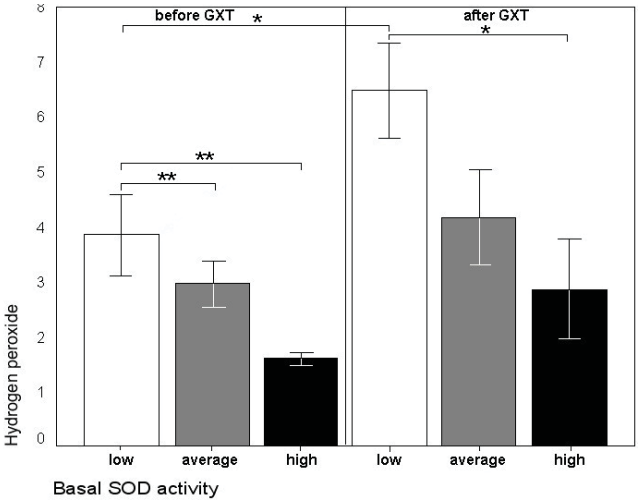

Figure 2. Levels of hydrogen peroxide (nmol/ml) before and after the GXT in the groups of athletes defined by basal SOD activity (results are presented as the means ± SE).

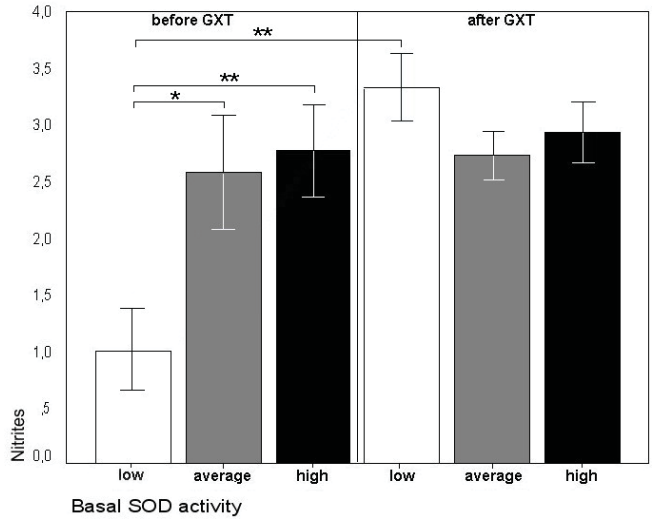

Figure 3. Levels of nitrites (nmol/ml) before and after the GXT in the groups of athletes defined by basal SOD activity (results are presented as the means ± SE).

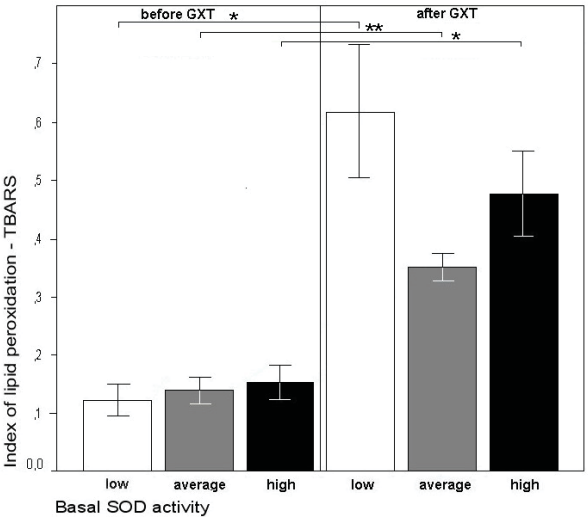

Figure 4. Levels of TBARS (μmol/ml) before and after the GXT in the groups of athletes defined by basal SOD activity (results are presented as the means ± SE).

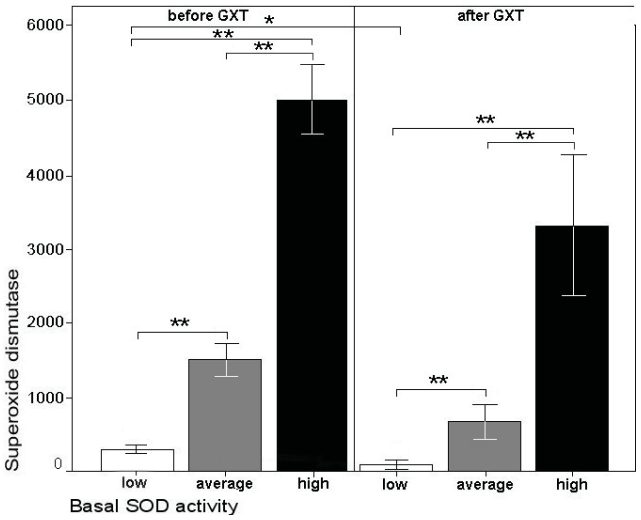

Figure 5. Activity of SOD (U/g Hb x103 nmol/ml) before and after the GXT in the groups of athletes defined by basal SOD activity (results are presented as the means ± SE).

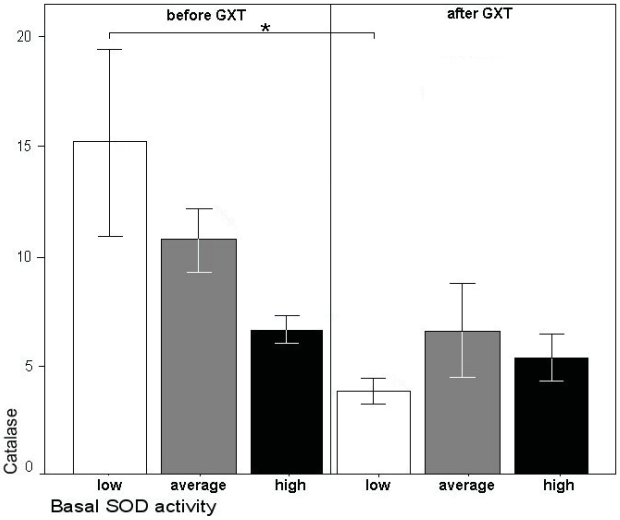

Figure 6. CAT activity (U/g Hb x103 nmol/ml) before and after the GXT in the groups of athletes defined by basal SOD activity (results are presented as the means ± SE).

Superoxide anion radical

No significant difference was found in the levels of O_2^- either between groups or between pre- and post-GXT measurements in any group.

Hydrogen peroxide

Significantly different values of H_2O_2 were observed between the groups both before (P=0.012) and after the GXT (P=0.047). Before the GXT, the group with high basal SOD activity had significantly lower levels of H_2O_2 than both the average and low basal activity groups (P=0.010 for both). In addition, after the GXT, there was a significant difference in the H_2O_2 levels between groups (P=0.047), but this difference was only between the groups with low and high SOD activity (P=0.010). The levels of H_2O_2 were significantly changed after GXT only in the group with low basal SOD activity (P=0.035).

Nitric oxide (nitrites)

NO_2^- differed between the groups in the samples collected before the GXT (P=0.021). The group with low basal SOD activity had lower levels of NO_2^- than both the average and high basal SOD activity groups (low vs. average, P=0.027; low vs. high, P=0.008). The levels of NO_2^- significantly changed after the GXT only in the group with low basal SOD activity (P=0.002).

Index of lipid peroxidation – TBARS

Lipid peroxidation did not differ between groups, but TBARS levels increased after the GXT in all groups (P=0.018, P=0.000, P=0.026 for low, average and high basal SOD activity groups, respectively).

Superoxide dismutase

SOD remained significantly different between the groups after the GXT (P=0.000) (low vs. average, P=0.007; low vs. high, P=0.000; average vs. high, P=0.010). SOD activities significantly changed after the GXT only in the group with low basal SOD activity (P=0.025).

Catalase

CAT activity did not differ between the groups, but there was a significant change observed between the CAT measurements before and after the GXT in the group with low basal SOD activity (P=0.020).

DISCUSSION

Oxidative stress has been suggested to play a primary or secondary role in the development of more than 100 acute and chronic human diseases (31-33). In sports medicine, it is thought that increased oxidative stress may be associated with chronic fatigue syndrome (34, 35) and might also be related to overtraining syndrome (8, 36-38). Overload training can lead to an impaired antioxidant defence and the absence of the anticipated adaptations to training (39) as well as distortion of the redox state balance (8). Furthermore, overload training can induce inflammation, which is related to oxidative stress (37). Thus, the importance of understanding the effects of exercise on redox homeostasis and finding a method to alleviate the extent of its harmful effects represents one of the most important goals in exercise physiology research.

Data on the acute effects of exercise on redox homeostasis in humans are controversial because of the many types of exercise and experimental conditions used in previous studies, which do not allow for comparisons between studies (9, 12, 17, 18, 40-50). The extent of oxidative stress induced by an acute bout of exercise depends on many factors, such as exercise mode, intensity, and duration and the participant's state of training, gender, age, and nutrition habits (7,52).

In our study, a maximal progressive exercise test induced significant changes in nearly all of the investigated parameters, which suggests that this kind of exercise is a potent oxidative stress inducer or that the ADS in our subjects was not able to efficiently resist the generated prooxidants. Variable results have been reported by previous studies that investigated redox homeostasis disturbance after some type of ergometer maximal test (46-51). Dekany, who explored the antioxidative status of basketball, handball, water polo and hockey players, formed his study groups based on their measured (increasing or decreasing) SOD activity with exercise (47). Tauler et al. reported that a maximal exercise test on a cycle ergometer produced no changes in the erythrocyte antioxidant enzyme activities of amateur sportsmen (48). Moreover, Podgorsky reported no change TBARS levels after a maximal incremental treadmill test (49). A study by Antoncic-Svetina et al. suggested that ergometry induced production of hydroxyl radicals and a systemic oxidative stress response in healthy subjects (50). Demirbag et al. demonstrated that treadmill exercise testing increased oxidants and decreased total antioxidant capacity, shifting the balance towards the oxidative state, but this stress was not enough to produce DNA damage (51). The only conclusion that can be drawn from these data is that additional, more homogenous studies are needed to clarify the influence of maximal ergometer tests on redox homeostasis.

During the analysis of the oxidative status of the athletes at rest, depending on the basal SOD activity of the group, it was noticed that there was a linear correlation between SOD and all other measured parameters, with the exception of O_2^-; however, this correlation was statistically confirmed only for SOD and H_2O_2 (negative correlation) and SOD and NO_2^- (positive correlation). The study subjects with the lowest basal SOD activity had the highest levels of H_2O_2 and the highest CAT activity, whereas their NO (NO_2^-) levels were the lowest. A negative correlation between SOD and CAT was not found to be statistically significant in this study, but we have observed a similar correlation in more than one of our previous studies (unpublished data). The correlation between SOD activity

and H_2O_2 levels could be explained by H_2O_2-induced inhibition of SOD activity; according to Blum and Fridovich, decreased SOD activity strongly suggests the presence of H_2O_2, which has been demonstrated to inhibit SOD activity in vitro (53). Regular exercise training not only improves antioxidant defence, but also improves endothelial function, i.e., it increases NO bioavailability (54, 55). Thus, subjects who had improved antioxidant defence, which was estimated by SOD activity, had higher levels of NO (NO_2^-). This finding explains the positive correlation between NO (NO_2^-) and SOD in subjects at rest. Another previously suggested phenomena, NO-mediated CAT inhibition (56, 57), is confirmed by our data; as shown in Table 4, the subjects who had the highest NO (NO_2^-) levels, the low basal SOD activity group, had the lowest CAT activity at rest. Exercise induced a significant rise in NO (NO_2^-) levels only in the low basal SOD group, which was accompanied by a significant decrease in CAT activity (the only significant decrease in CAT activity observed). Thus, at rest, the group with low basal SOD activity at rest had both the lowest NO (NO_2^-) and highest CAT activity levels. Upon the significant increase in NO (NO_2^-) levels, NO-mediated inhibition of CAT likely occurred, resulting in this group having the lowest CAT activity. Exercise also induced significant changes in H_2O_2 levels and SOD activities in this group, which was the only group, once again, in which significant changes of these parameters occurred. In addition, this finding supports the theory of H_2O_2-mediated SOD inhibition.

Our finding that TBARS levels were increased in all athletes, regardless of basal SOD activity, supports the presumption that this type of intensive exercise has great potential to induce oxidative damage. Additionally, recent data from other investigations have suggested a positive correlation between NO (NO_2^-) and TBARS (58). As indicated by the index of lipid peroxidation, it appears that RONS (i.e., NO/NO_2^-) induced damage of the cellular membrane. However, particular attention should be given when evaluating TBARS because it has been suggested that this measurement should be subject to caution due to potential malondialdehyde (MDA) overestimation (8); nevertheless, it has been accepted as a general marker of lipid peroxidation.

The finding of our study that in all study participants, 5 out of 6 investigated parameters of oxidative stress were changed due to an acute bout of exercise is important, but it is even more important that the statistical significance of this change has its roots in the group of athletes with the lowest basal SOD activity. This finding leads us to conclude that the pre-exercise SOD activity level determines the effects of exercise on redox homeostasis.

A limitation of our study is that we did not measure glutathione peroxidase or the ratio of reduced glutathione to oxidised glutathione, which should be a topic of further investigation. Finding correlations of these parameters of the redox state with SOD activity would help to further understand the influence of physical activity on redox homeostasis.

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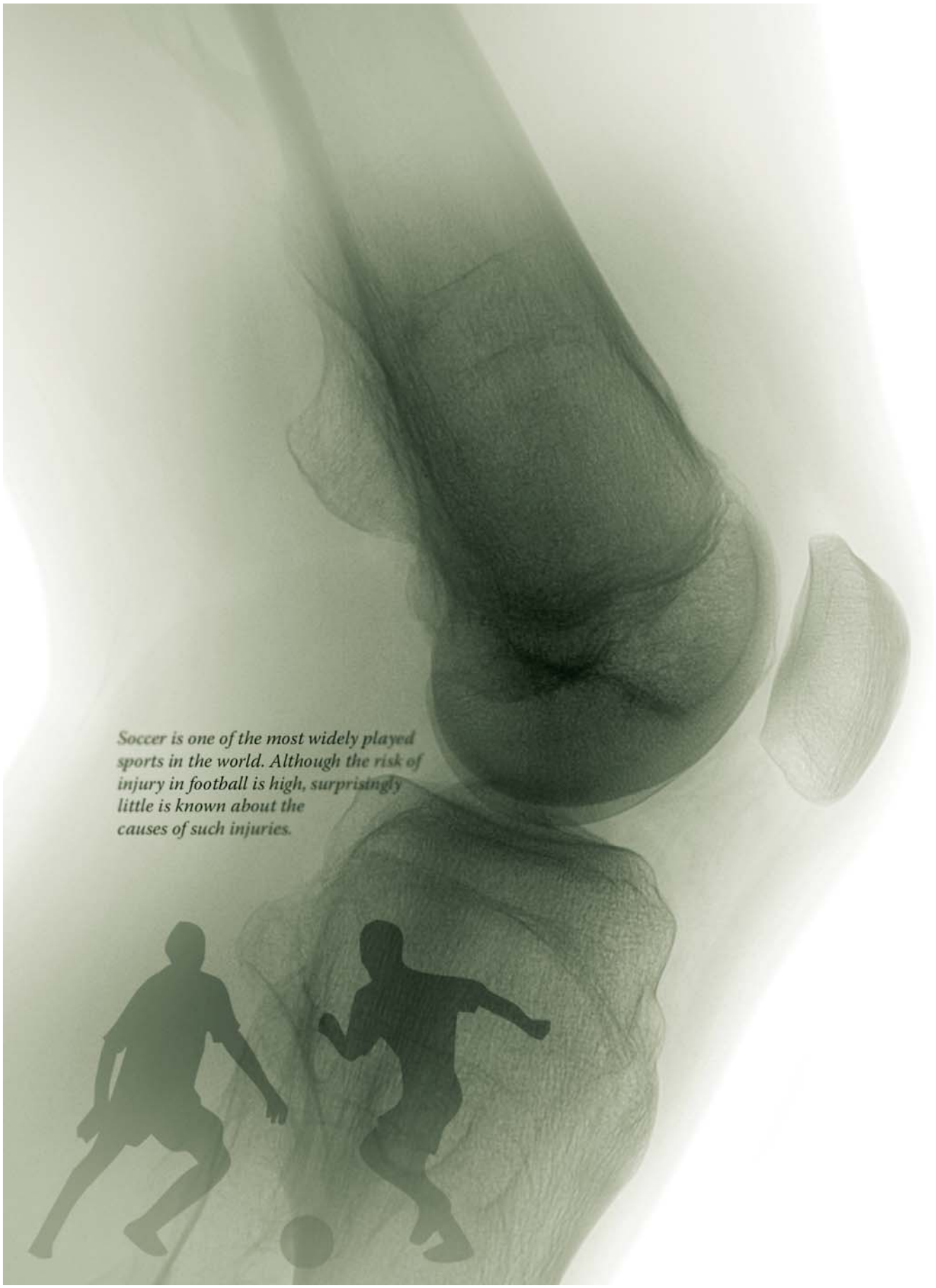
Soccer is one of the most widely played sports in the world. Although the risk of injury in football is high, surprisingly little is known about the causes of such injuries.

ANKLE INJURIES IN SOCCER PLAYERS: A FOCUS ON AGE AND LEVEL OF COMPETITION

Nešić Miroslav[1], Milošević Oliver[1] and Čubrilo Dejan[1]
[1]Department of Physiology, Medical Faculty, University of Kragujevac, Kragujevac, Republic of Serbia

POVREDE SKOČNOG ZGLOBA KOD FUDBALERA FOKUS NA GODIŠTE I STEPEN TAKMIČENJA

Nesic Miroslav[1], Milosevic Oliver[1] i Cubrilo Dejan[1]
[1]Odsek za fiziologiju, Medicinski fakultet univerziteta u Kragujevcu, Republika Srbija

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APSTRAKT

Soccer is one of the most widely played sports in the world. Although the risk of injury in football is high, surprisingly little is known about the causes of such injuries. The aim of this study was to examine the incidence of ankle injuries in football players and to identify the potential risk factors for such injuries. A total of 73 injured players from 5 football teams of varying competitive levels participated in this study. The players were divided into two subgroups according to established age criteria (Group 1 ‹ 18 years old; Group 2 › 18 years old). Interestingly, there was no statistically significant difference between the number of injured athletes across the two age groups ($p < 0.05$). With respect to the type of activity that was being performed at the time of injury, the greatest number of injuries in both groups of athletes occurred during training periods. In relation to the type of terrain where the injury occurred, the highest percentage of injuries occurred on bumpy and slippery field surfaces. Interestingly, the most common mechanism by which a player became injured was the result of a stroke. Finally, according to clinical observations, there was no statistically significant difference in the types of ankle injuries that occurred between the two groups of athletes as a function of the level of competition at which the athletes were performing.

The results of our research indicated that the age of the athletes does not affect the probability of an injury occurring. The largest number of injuries occurred during training sessions that utilised bumpy and slippery field surfaces, where the main cause of ankle injury is kicking. Notably, distensions and distortions were the most frequently observed type of ankle injury.

Key words: *football, ankle, injury, age, competition level*

SAŽETAK

Fudbal predstavlja jedan od najčešće igranih sportova u svetu. Rizik povređivanja u fudbalu je visok, ali se malo zna o uzrocima povređivanja. Cilj našeg rada je bio ispitivanje učestalosti povreda skočnog zgloba i identifikacija faktora rizika za povrede u fudbalu.

U ovoj studiji je učestvovalo 5 fudbalskih timova različitih nivoa takmičenja. Ukupno je praćeno 73 fudbalera, sa dijagnostikovanim sportskim povredama, pri čemu su bili podeljeni u dve podgrupe u skladu sa kriterijumom starosti.

Nije uočena razlika između broja povređenih sportista u odnosu na godine starosti ($p < 0.05$). U odnosu na mesto povređivanja, najveći broj povreda u obe starosne grupe dešavao se u toku trenažnog perioda. U odnosu na vrstu terena, gde su se povrede dogodile, najveći procenat povreda se dogodio na neravnom i klizavom terenu. Prema mehanizmu povređivanja, najveći procenat povreda u obe starosne grupe nastaje kao posledica udarca. U odnosu na vrstu povrede skočnog zgloba na osnovu kliničkog nalaza u obe grupe sportista, nije uočena statistički značajna razlika u pojavi određenih vrsta povreda u odnosu na rang takmičenja ($X^2_e < X^2_t$).

Rezultati našeg istraživanja pokazuju da starost sportsita ne utiče na procenat povređivanja. Najveći broj povreda dešava se tokom treninga i to na neravnom i klizavom terenu, pri čemu je glavni uzrok povrede skočnog zgloba udarac. Distenzione distorzije predstavljaju najfrekventniji tip povreda skočnog zgloba kod sportista.

Ključne reči: *fudbal, skočni zglob, povrede, starost, nivo takmičenja*

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Correspondence to: Čubrilo D, MD, PhD / Research Fellow, Department of Physiology
Faculty of Medicine, University of Kragujevac, Svetozara Markovica 69, P.P. 124, 34000 Kragujevac, Republic of Serbia
Tel. +381 34 34 29 44, Fax. + 381 34 30 68 00/ext 112, E-mail: dejancubrilo@yahoo.com

INTRODUCTION

Soccer is one of the most widely played sports in the world (1,2) and is a sport that requires players to perform short sprints, rapid acceleration or deceleration, turning, jumping, kicking, and tackling (3,4). It is generally agreed that, through the years, the game has evolved to become a faster game, requiring a higher level of intensity and is characterised by more aggressive play than seen previously (2). Soccer, at its highest level, is a complex sport, and performance depends on a number of factors including a player's physical fitness, a player's level of technique, various psychological factors, and overall team tactics. Injuries and sequelae from previous injuries can also affect the players' ability to perform well in matches. During a 90-minute football match an elite player covers, on average, between 10 and 11 km (2,3,5). Although most of a player's movement during a match is at low or sub maximal intensity (6,2), it has been estimated that the mean work rate is at approximately 70% to 75% of the maximum oxygen uptake and is close to reaching the anaerobic threshold (6,7).

A very important problem associated with modern football matches is the possibility of an injury occurring during the competitive cycle. It can be reasonably assumed that the frequency of injuries suffered by players on a given team can significantly affect the overall performance of the team. In team sports such as football, the effect of injuries on team performance may be less obvious due to the fact that players can be replaced or strategically brought in to matches depending on the needs of the team. The study of Arnason and colleagues showed that there is a statistically significant correlation between the number of days of a player's absence due to injury and the overall success of the team (8). The importance of this problem becomes even more significant with decreased financial opportunities for replacing injured players. The injury risk in football is high, but little is known about the causes of injuries (9). Ankle injuries to football players have attracted particular interest from sports experts because of the high incidence of these injuries in young players, which are often severe and result in prolonged loss of training time (10). Understanding the individual risk factors for such injuries is important to serve as a basis to develop preventive measures. Notably, the data reported for epidemiological studies of the etiopathogenesis of ankle injuries in football players is somewhat contradictory (9, 10, 11).

The aim of our study was to monitor the incidence of ankle injuries in five football clubs in Kragujevac, Serbia, during three competitive football seasons (the 2004/2005 season, the 2005/2006 season, and the 2006/2007 season), with particular emphasis on severe injuries and factors that are associated with an increased injury rate.

PATIENTS AND METHODS

Five football teams from Kragujevac, Serbia, that compete at different skill levels (Republic, Zone, and Municipality levels) (Table 2) participated in this study during the 2004/2005, 2005/2006, and 2006/2007 seasons. Each coach selected the team's best players to participate in the study, which included a total of 380 athletes. This approach was used to test and follow a well-defined group of first-string players, that is, the players who were assumed to receive the most playing time in matches during the aforementioned seasons.

A total of 73 injured players were followed during this study. The players were divided into two subgroups according to the following criteria: Group I, which included players under 18 years old (N = 35), and Group II, which included players over 18 years old (N = 38) (Table 2). The players performed a series of testing procedures and completed a questionnaire about previous and recurrent injuries (type of injury, field conditions under which injury occurred, and severity of the injury) just prior to the start of the season to establish baseline information for potential injury risk factors.

The forms and questionnaires were administered by two of the manuscript's authors. The testing procedures included determining the peak O2 uptake and body composition of each player, analysing responses to a form and questionnaire to determine the types and number of previous ankle injuries, and obtaining information regarding the time and type of field surface where the new injuries occurred (type of terrain, injury mechanism, etc.).

Injuries were recorded prospectively throughout the season on a special form by a team of physical therapists, which was collected by one of the manuscript's authors once a month. During the same time period, the coaches recorded each player's overall training exposure, that is, the extent of player participation, for every training session (including the duration of each session) on a separate form.

The extent to which players competed in matches was also recorded. A player was defined as injured if he was unable to participate in a match or a training session due to an injury that occurred in a football match or during a training session. The player was considered injured until he was able to play a match or comply fully with all instructions given by the coach during a training session, including sprinting, turning, shooting, or otherwise playing football at full speed (12,13). The injury was diagnosed via a clinical examination performed by sports medicine specialists immediately after the injury occurred and where examination required the use of ultrasound of soft tissue injuries evaluated on the same day that the injury occurred by a radiologist

Ultrasound examination was performed using either a (Sono online Elegra, Siemens, Logic 500, GE Medical Systems) linear sound frequency of 7.5 to 10 MHz, a method involving longitudinal and transverse scans in the phase of relaxation and muscle spasms, or an examination of the tense and flaccid tendons. In all of the examined athletes, an overview of the contralateral limb was performed to reveal discrete changes that could potentially be overlooked.

A player's body composition was assessed by *Biospace In Body 720*, an apparatus that uses the Direct Segmental Multi-frequency Bioelectrical Impedance Analysis (DSM-

BIA) method (14). The parameters of interest in our study were weight, fat percentage and the body mass index (BMI).

The maximal oxygen uptake (VO2 max) was measured directly using the bicycle ergometer test (Kettler AX1) during a continuous and progressive increase in the load on the equipment (Fitmate Pro Cosmed, Italy).

Statistics

Statistical analysis was performed using the statistical package SPSS 10.0 for Windows. The results were expressed as the mean ± standard error. After testing the data distribution, the differences between the proportions of small groups were assessed using the X^2-nonparametric test. The level of statistical significance was set at $p < 0.05$.

To compare the average values of the observed numerical features between the two groups, the student's t-test for two independent samples was used. In addition, the Wilcoxon matched pairs test was used in cases in which the sample populations were relatively small with a significant dispersion of values. The significance level was set at $p < 0.05$.

RESULTS

The general parameters of injured athletes participating in our study are presented in Table 1. Athletes were of different ages and were categorised into the following two separate groups: Group 1 (under 18 years old) and Group 2 (above 18 years old). However, athletes in these groups had similar height, weight, BMI, and fat percentages. The percentage of athletes in Group 1 was 47.95%, and in Group 2 was 52.05% (Table 2). There were statistically significant differences between the aerobic capacity parameter VO2 max as measured between Group 1 and Group 2 athletes. Interestingly, injured athletes from Group 1 were shown to have lower VO2 max values.

Interestingly, there is no statistically significant difference between the number of injured athletes between the two groups. The highest percentage of injuries in Group 1 athletes was at the Republican competition level (45.71%). Regarding injury percentage, the order of magnitude is municipality > republic > zone. For Group 2 athletes, the highest injury percentage was observed at the Municipality competition level. The order of magnitude is municipality > republic > zone.

According to the time of injury occurrence, the greatest number of injuries in Group 1 athletes occurred during the training sessions (31.42%), and most of these injuries occurred between 16 and 30 minutes after the start of the session. The second most common time for injury to Group 1 athletes occurred during a competitive match with highest number of injuries occurring between 46 and 60 minutes after the start of the match (Figure 1). In Group 2 athletes, the highest number of injuries also occurred during training sessions (50%), with the largest number of injuries occurring between 61 and 75 minutes and 76 and 90 minutes after the start of the training session (Figure 2).

In relation to the type of field surface where the injury occurred, the highest percentage of injuries for both groups took place on a bumpy and slippery field surface (54% in Group 1 versus 68% in Group 2) (Figure 3).

Regarding the mechanism of the injury, the highest percentage of injuries in both groups of athletes was the result of a stroke (51% in Group 1 versus 44% in Group 2) (Figure 4).

The clinical findings indicate that there were no statistically significant differences between the types of injuries observed in both groups as a function of the level of competition ($p > 0.05$).

In the Group 1 and Group 2 athletes, the most common type of ankle injury was a distension (28.57% and 28.95%, respectively). The next most common type of injury for both groups investigated was an ankle rupture, followed by a contusion, a laceration, a luxation, and a fracture (Tables 3 and 4).

	Group 1 (n = 35)	**Group 2** (n = 38)	**t-test**
Age (years)	17.3 ± 0.2	24.4 ± 0.8	p < 0.001
Height (cm)	179.5 ± 9.5	184.7 ± 7.0	N.S.
Weight (kg)	81.6 ± 18.3	83.6 ± 10.7	N.S.
BMI (kg/m2)	22.6 ± 4.1	23.5 ± 2.7	N.S.
Fat (%)	13.3 ± 7.5	14.2 ± 5.2	N.S.
VO2 max (ml/min/kg)	39.6 ± 4.3	48 ± 3.1	p < 0.001

Table 1. Comparison of data obtained for the examined athletes. The results are presented as the mean ± SE; n = number of examinees.

Club	**Competitive level**	**Group 1**		**Group 2**		**Total**	
		n	**%**	**n**	**%**	**n**	**%**
FC Radnicki	**Republic**	16	45.71	10	26.32	26	35.62
FC Sumadija	**Municipality**	11	31.43	9	23.68	20	27.40
FC Vodojaza	**Municipality**	6	17.14	5	13.16	11	15.07
FC Arsenal	**Zone**	1	2.86	4	10.52	5	6.84
FC Slavija	**Zone**	1	2.86	10	26.32	11	15.07
Total		35	100.00	38	100.00	73	100.00

Table 2. Analysis of injured athletes who have participated at various competitive levels.

Competition level	Contusion	Distension	Laceration	Rupture	Luxation	Fracture	
						medial	lateral
Republic league	5.71	2.86	2.86	5.71	2.86	0	0
Zone league	5.71	14.82	5.71	8.57	5.71	2.86	0
Municipality league	5.71	11.43	5.71	8.57	0	2.86	2.86
Total	17.13	28.57	14.28	22.86	8.57	2	2.86

Table 3. Analysis of different types of ankle injuries observed at different levels of competition in Group 1 athletes. The results are presented as a percentage (%).

Competition level	Contusion	Distension	Laceration	Rupture	Luxation	Fracture	
						medial	lateral
Republic league	7.89	5.26	5.26	7.89	5.26	2.63	0
Zone league	5.26	7.89	7.89	2.63	5.26	2.63	2.63
Municipality league	2.63	15.79	2.63	2.63	2.63	2.63	2.63
Total	15.79	28.95	15.79	13.14	13.14	7.89	5.26

Table 4. Analysis of different types of ankle injuries observed at different levels of competition in Group 2 athletes. The results are presented as a percentage (%).

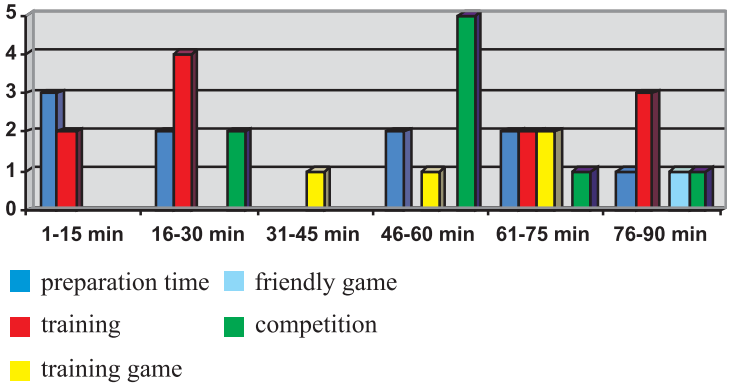

Figure 1. Place and time of injur in Group 1 athletes.

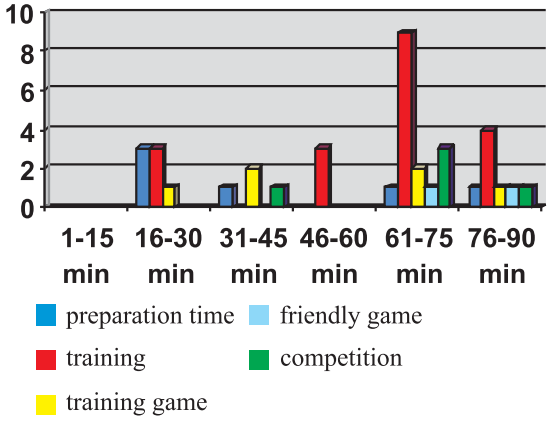

Figure 2. Place and time of injury in Group 2 athletes.

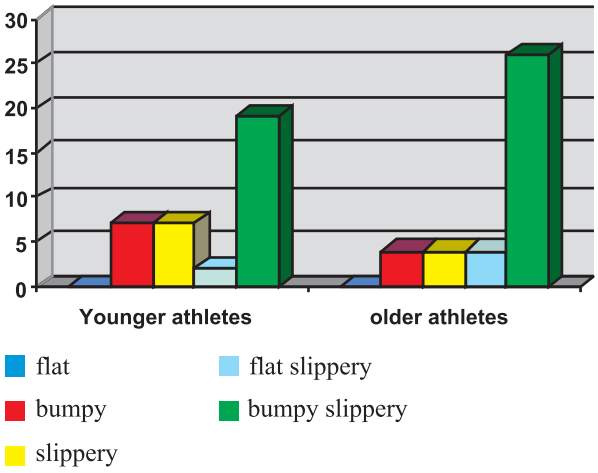

Figure 3. Type of terrain where injury occurred in Group 1 and 2 athletes.

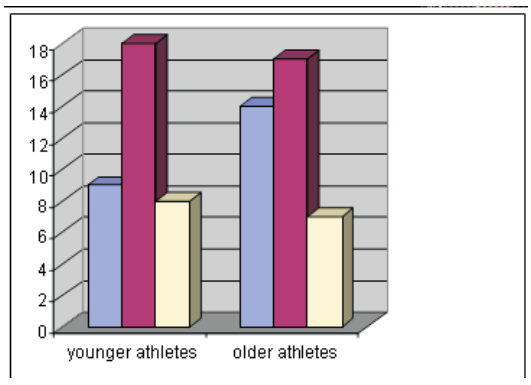

Figure 4. Incidence of different mechanisms of injuries in Group 1 and 2 athletes.

DISCUSSION

Football is a team sport that consists of simple rules without any significant material investments in equipment or space to play. The game is one of the most popular sports played today and is enjoyed by spectators in all nations, regardless of sex, age, or the level of skill of the spectator (15). Regarding the sport's level of intensity, football can be classified as an intermittently intensive team sport (16). A previously published study of the game has indicated that top-ranking players run roughly 10 to 12 km during a 90-minute match and that the athlete's intensity level approached the anaerobic threshold, which is approximately 80% to 90% of the athlete's maximum heart rate (17). For these reasons, it is evident that the players are expected to possess high levels of aerobic capacity and aerobic endurance. Analysis of the results obtained from studies of other elite players indicate that the maximum oxygen consumption for players range between 50 to 75 ml / kg / min for athletes on the field, while the values for goalkeepers is slightly lower, ranging between 50 to 55 ml / kg / min (18). Compared with results reported in the 1980s (20, 21), over the last 20 years there has been a trend in football players for an increasing aerobic capacity (19). This fact can be explained through the evolution of football into a profession, as well as improvements to the ongoing technical development and training process for athletes. On the other hand, the number of games played during the season, which is constantly increasing, in addition to changes to the rules have dictated a faster and more intense pace of the game. Analysis of the maximal oxygen uptake of the study's first-league players was determined as a function of the player's position (53.3 ± 1.9 ml / kg / min for the midfielders, 52.9 ± 4.4 ml / kg / min for the attackers, 51.8 ± 3.3 ml / kg / min for the defenders, and 50.5 ± 1.8 ml / kg / min for the goalkeepers) (22). Similar results have been reported by Djordjevic et al., who indicated a maximal oxygen uptake of 59.69 ml / kg / min (23).

Ankle injuries are one of the most common injuries observed in young football players and are often severe, typically resulting in prolonged loss of training time. This has potential far-reaching implications, both on and off the field. The results reported herein indicated that injured players from both groups (Group 1 and 2) showed significantly lower aerobic capacity compared to values obtained in studies performed by other researchers investigating a sample of elite senior players from Serbia (24) and a separate sample of football players (25). Upon comparison of the injuries to players analysed herein on the basis of age, there was no statistically significant difference regarding the incidence of such injuries. In the literature there are conflicting reports regarding the effect of age as a risk factor for sports injuries (9). The results reported by Dvorak et al. and Chomiak et al. are in agreement with the results of our research (26, 27), as opposed to other reports indicating that older players are more prone to ankle injuries (28, 29). In both of this study's experimental groups, the largest number of injuries was observed at the Municipal competition level. In the younger group of athletes (Group 1), the highest percentage of ankle injuries occurred during training, specifically at the very beginning of the training period (first 30 minutes). These data suggest the need for a serious reconsideration of the training methods and possible adjustments to the intensity load of the training sessions. In contrast to the Group 1 athletes, the Group 2 athletes are also predominantly injured during training, although unlike the results of Group 1, these injuries occurred predominantly between 60 and 75 minutes after the start of training. These data are in agreement with the information provided by the practitioners of sports for the senior competition level as well as with published research reports from other scientists. It is for this reason that a large number of physicians engaged in these types of senior selection focus of prevention focuses on precisely this element of training. By monitoring the causes of injuries, one can determine the highest percentage of injury in both groups as a function of etiological factors.

In contrast to our results, research performed by Cloke et al. shows an increased incidence of injuries occurred during competitive matches and in contact situations (10).

For this reason, it may be necessary to impose preventative measures, such as introducing proprioceptive steps (30) in the regular physical training regimen, in order to reduce the incidence of ankle injuries (31, 32). A significantly higher number of injuries occurred in both groups of athletes evaluated in our study when athletes competed on bumpy and slippery field surfaces. Similar data were reported by Junge and Dvorak in the course of monitoring the incidence of injured players (31). These data can be used as guidelines for coaching staff interested in implementing strict procedures for monitoring all external factors that could significantly affect an athlete's risk for sports injuries in an effort to reduce the loss of playing time on the field. Our results indicate that the most common type of ankle injuries observed in both groups was a distension/distortion. The fact that tissue rupture is the second most commonly observed injury supports the notion that the under-developed functional and dynamic stability of the joints of young athletes may become overloaded during intense training. Thus, there is a need to introduce proprioceptive training and flexibility training in order to improve motor control and increase the strength of the muscle-tendon-ligament apparatus (32, 33).

CONCLUSION

Coaches and medical support teams should pay more attention to jump and power training and should strive to implement preventive measures by providing adequate rehabilitation time for athletes with previous injuries to increase the overall team success.

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CANCER STEM CELLS: A MYTH OR REAL TARGET

Snezana Matic[1]
[1]Center for Molecular Medicine, Faculty of Medicine, University of Kragujevac, Serbia

KANCERSKA STEM ĆELIJA, OD MITA DO STVARNOSTI

Snežana Matić[1]
[1]Centar za Molekulsku Medicinu, Medicinski Fakultet, Univerzitet u Kragujevcu, Srbija

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ABSTRACT

Ever since the 17th century, determining which cells are able to produce tumours has been a key question in cancer biology. The answer seemingly lies somewhere between the postulates of the stochastic and hierarchical hypotheses, which is to say that hierarchical order exists in both normal tissue and tumours, that both stem cells and differentiated cells (after dedifferentiation) can give rise to several cell lineages, and that both stem cells and mature cells can mutate. It has been found that tumours of various types contain a small percentage of chemotherapy- and radiotherapy-resistant tumour cells, which are long-lived and capable of self-renewal, much like normal stem cells. These cells, which are capable of regrowing tumours, were named "cancer stem cells" (CSCs). According to recent findings, CSCs are genetically and phenotypically similar to normal stem cells and represent the only cell population within tumours that can completely regenerate the original tumour following transplantation. They are the only malignant cells that can be grown as spheres in non-adherent, serum free cultures (a unique characteristic of stem cells). Normal stem cells and CSCs both reside anchored in specifically organized microenvironments, or niches, which modulate their behaviour and determine their destiny. CSCs are highly metastatic and resistant to conventional tumour therapies.

Conclusion. *CSCs and normal tissue stem cells share most of their signalling and self-renewal pathways. The only way to overcome CSCs may be to discover their unique regulatory mechanisms and attempt to block these pathways using targeted therapies. Given this conclusion, additional investigation should be performed.*

Key words: *cancer stem cells, isolation, stem cell niche*

SAŽETAK

Još od sedamnaestog veka, ključno pitanje u vezi sa biologijom kancera je: koje ćelije su izvor tumorskih ćelija? Izgleda da se odgovor nalazi negde izmedju postulata stohastičke i hijerarhijske hipoteze, što znači da i u normalnom i tumorskom tkivu postoji ćelijska hijerarhija; i stem ćelije i diferentovane ćelije (nakon dediferencijacije) mogu dati različite ćelijske linije; i stem ćelije i zrele ćelije mogu mutirati. Pronađeno je da različite vrste tumora sadrže mali procenat hemio i radiorezistentnih ćelija, koje imaju karakteristike dugoživećih ćelija i ćelija sa kapacitetom za samoobnavljanje, poput normalnih stem ćelija. Ove, tumorobnavljajuće ćelije su nazvane kancerske stem ćelije. Prema podacima skorijih istraživanja, kancerske stem ćelije su genetski i fenotipski slične normalnim stem ćelijama i predstavljaju jedinu ćelijsku populaciju tumorskog tkiva koju je moguće transplantirati, i iz koje se, nakon transplantacije, može potpuno rekonstituisati tumor iz kog su dobijene. Kancerske stem ćelije su jedine maligne ćelije koje je moguće kultivisati u neadherentnim kulturama, u odsustvu seruma, kao sfere (jedinstveno obeležje stem ćelija). Stem ćelije i kancerske stem ćelije se nalaze pričvršćene u specifično organizovanom mikrookruženju, niši, koja modulira njihovo ponašanje i određuje njihovu sudbinu. Kancerske stem ćelije poseduju visok metastatski potencijal i rezistenciju na konvencijalnu antitumorsku terapiju.

Zaključak. *Kancerske stem ćelije i stem ćelije normalnog tkiva, dele većinu signalnih puteva. Možda je jedini način da se kancerske stem ćelije savladaju, terapija usmerena na jedinstvene regulatorne mehanizme ovih ćelija. Iz tog razloga je neophodno nastaviti istraživanja u ovom pravcu.*

Ključne reči: *kancerske stem ćelije, izolacija, niša stem ćelija*

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Correspondence: Snezana Matic, Center for Molecular Medicine, Medical Faculty, University in Kragujevac, Svetozara Markovica Street 69, 34000 Kragujevac, Serbia, tel: +381 64 303 53 98

INTRODUCTION

Determining the cell type with the capacity for carcinogenesis is a central concern in cancer biology. There are two hypotheses that attempt to address this issue: the stochastic model proposes that specific events in a tumour cell population have the potential to transform any tumour cell into a tumour-initiating cell, while the hierarchy model proposes that a limited number of cells, termed cancer stem cells (CSCs), are capable of initiating a heterogeneous tumour (1). The latter hypothesis proposes that a small subset of cells is responsible for the initiation, proliferation and metastasis of a tumour. Furthermore, these cells are resistant to radiotherapy and many chemotherapeutical agents and are the basis for tumour regrowth in patients with relapsed disease following therapy (2). It is widely accepted that CSCs are equally capable of arising from either mutated early stem cell progenitors (through the acquisition of epigenetic and genetic changes required for tumourigenicity) or mutated mature, more differentiated cells (through dedifferentiation/trans-differentiation) (3). Given their potential role in tumourigenesis, CSCs are important targets for therapy. The ability to specifically target pathways that are dysregulated in cancers raises the hope of developing therapies with enhanced specificity and decreased toxicity (4). The CSC hypothesis has played an essential role in our understanding of carcinogenesis, and in the development of new approaches for cancer prevention and the treatment of advanced disease.

DEFINITION OF A STEM CELL

Stem cells are cells with the capacity to self-renew (symmetrical division) and to generate daughter cells capable of downstream differentiation into several cell lineages to form all of the cell types that are found in mature tissue (asymmetrical division) (5). There are essentially two types of stem cells: embryonic (pluripotent stem cells with the potential to give rise to any cell of the organism) and adult (multipotent stem cells with multilineage potential that can give rise to any cell from a particular tissue or organ). Adult stem cells can be further divided into stem cells responsible for tissue renewal (cells from the bone marrow, skin, or intestine, which are continually active), and stem cells responsible for tissue repair (satellite cells of muscle, or putative liver stem cells, which are inactive until required in response to environmental factors) (6).

All stem cells have the following unique characteristics: a) they are present in very small numbers within specific tissues; b) they express specific cell surface markers, which enables their isolation; c) they, and their progeny, are organized hierarchically within their tissue of origin; d) they may be phenotypically homogeneous but functionally heterogeneous; e) they are mitotically quiescent; f) they give rise to all the terminally differentiated cell types within a tissue; g) they can self-renew to give rise to new, functionally identical stem cells; h) they have cell fusion properties; and i) they are long lived cells (7, 8, 9).

DISCOVERY OF CSCs

In the 17th century, Georg Ernst Stahl, a German chemist and physician, speculated that cancers contain self-propagating seeds that often remained in the body after surgery (10). In the 19th century, Rudolf Virchow, a German anthropologist and doctor (cited as the first person to recognize leukaemia cells), observed that each cell stems from another cell ("*Omnis cellula e cellula*"), which provided the basis for the concept that cancer is a disease that originates from an immature cell (11). At the same time, Julius Friedrich Cohnheim, a German pathologist and Virchow's assistant, proposed the theory of "Embryonal rests". He postulated that excess germ cells from embryonic development subsequently develop into cancers and possibly link the origin of life to its end. He also proposed that cancers retained the embryonic capacity for cell division and unrestrained growth (12). In 1961, Till and McCulloch provided the first experimental models that suggested the existence of normal blood stem cells. Their reports were based on a novel method for the detection and enumeration of multipotent hematopoietic stem cells, and another method for the determination of the number of hematopoietic stem cells required to restore blood production (13). In 1963, Bruce and Van Der Gaag demonstrated that, analogous to normal bone marrow stem cells, only a minority of malignant blood cells could form colonies in the spleen of a mouse (14). In 1967, Fialkow et al. showed a clonal origin of chronic myelocytic leukaemia (CML) (15). In 1990, Fialkow's studies on CML and acute leukaemia provided the first conclusive evidence that a single progenitor cell can give rise to replicating clones that sequentially acquire additional mutations and create a tumour. The data from these studies suggested that a pluripotent stem cell is initially transformed, and that this transformed cell then produces malignant clonal progeny (16). In 1997, John Dick and colleagues characterised acute myeloid leukaemia (AML) stem cells and showed that only a small subpopulation of leukemic cells was capable of initiating leukaemia upon serial transplantation in the NOD/SCID mouse model. These cells, designated SCID leukaemia-initiating cells (SL-IC), express immunophenotypic markers that distinguish stem cells (e.g., $CD34^{+}CD38^{-}$), suggesting that the initial transformation event occurred in a stem cell rather than a committed progenitor cell (7, 17). Following the example of the leukaemia studies, investigators have also isolated cells with stem cell-like features from solid tumours. One of the first such studies was conducted in 2003, when Al-Hajj et al. reported that the $CD44^{+}CD24^{-/low}$ cell fractions from metastatic pleural effusions and a primary invasive breast carcinoma had significantly higher tumourigenic potential than the $CD44^{+}$/-$CD24^{+}$ cell fractions when injected into the mammary fat pad of female NOD/SCID mice (18). In the same year, the Dirks group, using the neurosphere culture

technique, discovered cancer stem cells in the $CD133^+$ fractions of brain tumours of different phenotypes (19). Continuing their investigations, in 2004, the same group reported the functional identification of human brain tumour CSCs (20). By measuring the ability of cells to form tumours in the brains of NOD/SCID mice, they found that only the $CD133^+$ cell fraction was capable of regrowing the original brain tumour. In contrast, the $CD133^-$ cell fraction failed to form tumours, even when 1000 times as many cells were injected into the brains of mice (20). These findings have been followed by the isolation of potential CSC fractions from other solid tumours, including lung cancers ($Sca\text{-}1^+CD34^+Lin\text{-}$) (21), ovarian carcinomas ($CD44^+CD117^+$) (22), prostate cancers ($CD44^+/\alpha_2\beta_1^{hi}/CD133^+$) (23) and colon cancers ($CD133^+$) (24).

Taken together, these data suggest that the CSCs share similar markers with the progenitors from their original tissue, as predicted by Virchow's law: *Omnis cellula e cellula*.

STEM CELL AND CANCER STEM CELL NICHES

Stem cells reside in a specialized supportive microenvironment, or niche, which differs depending on the tissue type (25). The niche serves as a physical anchoring site for stem cells, and interactions between the cells and the extracellular matrix (E-cadherin, β-catenin, integrins) in the niche play a major role in controlling their behaviour. These microenvironments contain several extrinsic factors and developmental regulatory signalling molecules (e.g., Hh, Wnts, BMP, FGF and Notch), which control stem cell number, differentiation and fate determination. Under normal conditions (at least in the hematopoietic, intestinal, and hair follicle systems), the niche inhibits stem cell proliferation and growth (promoting a quiescent state), and this maintenance of the delicate balance between proliferative and antiproliferative signals is a cornerstone of tissue homeostasis (25-27). In flies, genetic ablation of the germ line stem cell niche results in a loss of stem cells (28). In mice, increasing the size of the niche leads to an increased number of hematopoietic cells (29). There is evidence that the niche microenvironment can induce stem cells from nearby daughter cells if the stem cells are depleted (30). It has been shown that deregulation in the mammary gland stem cell niche leads to abnormal expression of TFFα, resulting in the development of breast cancer (31). Though the niche may act to maintain stem cells in a quiescent state for decades, these cells are highly dynamic once activated: an embryo develops from a single cell in 9 months, the intestine regenerates rapidly and constantly, and the liver recreates itself within a few days after partial hepatectomy (32). It therefore appears that stem cells niches may represent microenvironments that control tumourigenesis.

Studies using spontaneously arising tumours in rodents have shown that the number of cells required to successfully transplant a tumour into a syngeneic recipient depends on the specific location and tissue environment of the transplant, and on whether heavily irradiated tumour cells (feeder cells) are injected together with the viable tumour cells (the Ravesz effect) (33). The kidney capsule has been shown to be a highly receptive site for tumour cells, and the induction of inflammation at the injection site can modify the efficacy of tumour cell transplantation (34). Recent studies have demonstrated that efficiency of tumour cell transplantation can be increased when tumour cells are injected with Matrigel (a basement membrane-like substance containing growth factors) (35, 36). The mouse teratocarcinoma model provides a fascinating framework for studying the contribution of the cellular microenvironment to oncogenesis. It is possible to derive normally-functioning cells from teratocarcinoma cells, after the latter are introduced into a normal blastocyst environment (37). Experiments have demonstrated that Rous sarcoma virus causes tumour formation when injected into the wings of adult chickens but does not do so when injected into chick embryos. Viral particles were found to be expressed in most organs of the infected embryos but were not tumourigenic. However, if the infected embryos were dissociated and placed in culture, extensive transformation occurred within 24hr (38). Several studies have identified extracellular signals from the microenvironment as being potentially oncogenic. Inappropriate expression of different metalloproteinases (MMPs) leads to a loss of the tissue microenvironment and the generation of tumours (39). Studies involving the genetic manipulation of stromal cells have indicated that mutations in neighbouring cells, rather than in the tumour cells themselves, can serve as the initial basis for tumour formation (40). Stromal cells have been shown to acquire unique chromosomal rearrangements relative to the tumourigenic epithelium in some mammary carcinomas (41), and the higher incidences of cancer in carriers of certain heritable diseases have been shown to be due to stromal defects (42). Experiments with the HMT-3522 luminal epithelial cell line (isolated from a reduction mammoplasty) have shown that tumourigenic cells can retain their aberrant genome but revert to a normal phenotype if tissue polarity is restored. These cells were used to derive mutated S1 cells that do not have tumour-forming potential when injected into NOD/SCID mice or cultured in 3D laminin-rich basement membrane. Extensive passaging of S1 cells in the absence of EGF was able to derive a non-polarized T4-2 cell population that could form tumours in mice. Analysis of these T4-2 cells has shown that they have a number of altered signalling pathways: EGFR, MAPK, PI3 kinase and β1 integrin were highly active, and PTEN was downregulated. Treating T4-2 cells in laminin-rich 3D gels with blocking antibodies or pharmacological agents that reduce signalling through these key pathways causes formation of phenotypically normal acinus-like structures (i.e., the cells become less tumourigenic) (43). It is possible that tumour therapies that disrupt the stem cell niche, through ablation of the surrounding differentiated cells, could lead to the subsequent death of the cancer stem cells (44, 45).

TECHNIQUES FOR ISOLATION OF CSCS

A critical issue in the investigation of CSCs is the isolation of sparing cancer cells, with an unlimited potential for growth, from tumours. There are a few techniques that are commonly used with aim of studying CSCs: a) the side population (SP) technique; b) isolation based on surface marker expression; c) the ALDEFLUOR assay; d) *in situ* detection; and e) the anchorage-independent cell culture technique (46). **The SP technique**, based on the ability of stem cells to exclude vital dyes, has been used for many years to isolate both normal and tumour stem cells from various organs and species (47, 48). Normal stem cells and CSCs express transmembrane transporters, such as the ATP-binding cassette (ABC) transporter ABCG2/BCRP1 (breast cancer resistance protein 1). These molecules allow stem cells to exclude dyes, such as Hoechst 33342 and Rhodamine 123, a property not found in differentiated cells, which remain positive for these dyes (as detectable by flow cytometry). However, functional studies using Hoechst staining are limited by the toxicity of this agent (49). **The expression of cell surface markers** has been widely used to isolate stem cells, but the choice of markers can vary greatly depending on the tissue or species. The following phenotypic marker combinations have been used in studies of breast CSCs: $CD44^+/CD24^{-/low}/Lin^-$ (the first characterised CSC fraction from a solid tumour) (18); $CD44^+/CD24^{-/low}/Lin^-/ALDH1^+$ (identified as being more tumourigenic than the former fraction) (50); and $CD44^+/CD24^{-/low}/Lin^-$, together with combinations of CD10, MUC1 and ESA markers (to determine the compartment from which the isolated cell originated) (51). Other phenotypic markers have been used to identify CSCs in other tissues: CD133 for brain (20, 52) and colon CSCs (1, 24, 53); the $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ phenotype for prostate CSCs (23, 54); the $Stro\text{-}1^+/CD105^+/CD44^+$ phenotype (together with activated STAT3, and Oct3/4 and Nanog expression) for bone sarcoma CSCs (55); CD44 and CD117 (c-kit) for ovarian carcinoma CSCs (22); CD20 for skin carcinoma CSCs (56) Sca-1/CD34 together with the negative expression of lineage markers for lung carcinoma CSCs (21, 57); and the $CD34^+/CD38^-/CD90^-/IL\text{-}3R^+/CD71^-/HLADR^-/CD117^-$ phenotype for AML CSCs (58) (CSC phenotypic markers are summarized in table 1). Taken together, these data suggest that CSCs often share similar markers with the progenitor cells of their original tissue. **The ALDEFLUOR assay** may fit the universality required for the reliable identification of CSCs across all species and tissues. It is based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH 1), a detoxifying enzyme responsible for the oxidation of retinol to retinoic acid. ALDH 1 may have a role in early stem cell differentiation. It has been shown that ALDEFLUOR positive CSCs are capable of differentiating into multiple lineages *in vitro* and have a higher capacity to engraft following transplantation *in vivo* (in comparison to ALDEFLUOR negative CSCs) (59, 60). *In situ* **detection** of CSCs has a future in routine clinical practice for patient treatment and prognosis evaluation. This technique makes it possible to detect ALDH 1 expression in formalin-fixed, paraffin-embedded tissue with immunostaining, while also performing double immunostaining using antibodies specific to CD44 and CD24 (60, 61). **The anchorage-independent cell culture technique** was adapted to grow cancer cells with the capacity for of independent growth under serum-free conditions (a property of stem cells). Cell culture under non-adherent conditions was initially adapted to normal breast tissue obtained from reduction mammoplasty. Human mammary stem and progenitor cells were able to survive in suspension and produce spherical colonies (mammospheres) composed of both stem and progenitor cells. To date, culturing cells under non-adherent conditions has been adapted for the cultivation of CSCs from various cancers, including breast cancers (mammospheres), bone sarcomas (sarcospheres) and brain tumours (neurospheres) (62, 63).

Table 1. Cancer stem cell markers in some tumours.

Type of tumour	Cancer stem cell marker	Reference
Breast cancer		
a) First characterised	$CD44^+/CD24^{-/low}/Lin^-$	18
b) More tumourigenic phenotype	$CD44^+/CD24^{-/low}/Lin^-/ALDH1^+$	50
c) Compartment markers	$CD44^+/CD24^{-/low}/Lin^-$, in combination with CD10, MUC1, or ESA	51
Brain cancer	$CD133^+/nestin^+$	20, 52
Colon carcinoma	$CD133^+$	1, 24, 53
Prostate carcinoma	$CD44^+/\alpha_2\beta_1^{hi}/CD133^+$	23, 54
Bone sarcoma	$Stro\text{-}1^+/CD105^+/CD44^+$ (with activated STAT3, and expression of Oct3/4 and Nanog)	55
Ovarian carcinoma	$CD44^+/CD117(c\text{-}kit)^+$	22
Skin carcinoma	$CD20^+$	56
Lung carcinoma	$Sca\text{-}1^+/CD34^+/Lin^-$	21, 57
AML	$CD34^+/CD38^-/CD90^-/IL\text{-}3R^+/CD71^-/HLADR^-/CD117^-$	58

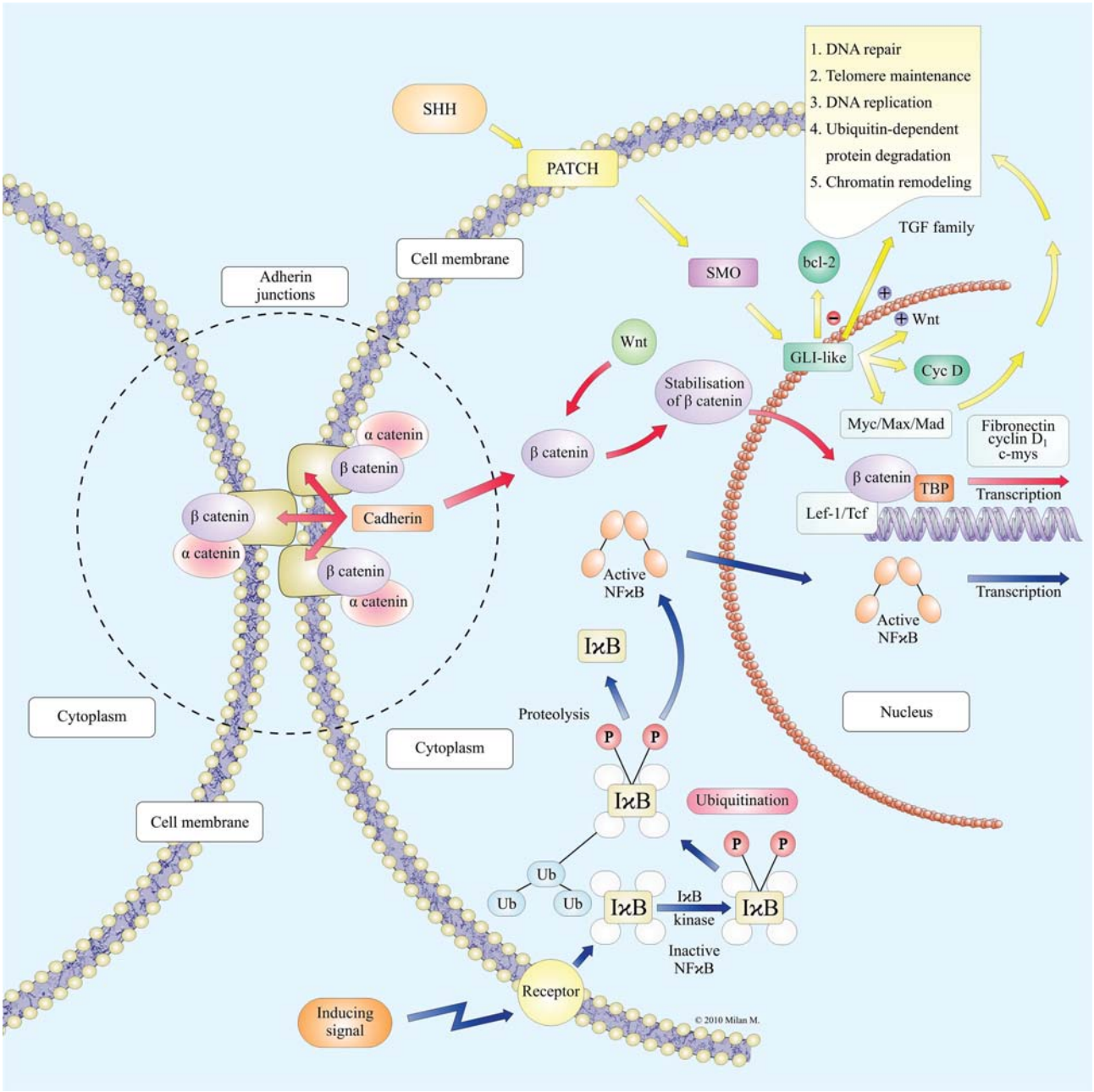

Figure 1. Signaling pathways in stem cells and CSCs.
Yellow arrow- signalling pathway through SHH.
Red arrow - β-catenin/Wnt signalling pathway.
Blue arrow – NFκB signalling pathway.

Abbreviations:
SMO – G-protein-coupled receptor (with the **PATCH** protein), receptor for sonic hedgehog **(SHH)** proteins.
Wnt – Wg (wingless) and Int ("wint"), involved in development of *Drosophila melanogaster*.
GLI-like – transcription factor.
β-catenin – cadherin subunit.
Myc/Mad/Max – group of transcription factors
LEF-1/Tcf – transcription factor.
NFκB – transcription factor.
IκB – inhibitor of NFκB
cycD – cyclin D- family of proteins controling cell cycle.
c-myc – transcription factor.
bcl-2 – antiapoptotic protein.

It should be noted that all of the above methods are still insufficient to separate these CSC populations, with high specificity, from the rest cells in the tumours. Thus, this field requires further investigation develop methods to precisely characterise and isolate CSCs.

MOLECULAR SIGNALLING PATHWAYS IN CSCs

Signaling pathways, such as Hedgehog (Hh), Wnt/β-catenin and Notch, that play a role in embryogenesis and organogenesis, also play a role in the maintenance of adult tissues by regulating the balance between stem cell self-renewal and differentiation (64). Because both normal stem cells and CSCs must renew themselves, it is reason-

able to assume that they share some molecular mechanisms that regulate this critical stem cell function. Mutation of the *SHH* (Sonic hedgehog) locus causes Gorlin's syndrome (65), whereas activation of *SHH* has been implicated in skin, breast and brain carcinogenesis (66, 67). The Wnt/β-catenin pathway is involved in the maintenance of normal intestinal epithelial cells and in regenerative processes during tissue repair. Wnt inhibitors retard hematopoietic reconstitution *in vivo*. Wnt signalling increases the expression of HoxB4 and Notch-1, both of which have been implicated in self-renewal. Wnt signalling also plays a role in blood diseases, colon (activating mutations of β-catenin, or inactivating mutations of APC) and breast carcinomas (β-catenin accumulation) (68). The Notch (Notch 1-4) signalling pathway is well conserved from nematodes to humans and regulates homeostatic processes in almost all tissues in organism. Notch mutations can cause T-cell acute lymphoblastic leukaemia and breast carcinoma (in the case of Notch 3 and 4). It was found that Bmi-1 (PCGF4), a member of the Polycomb-group protein family, is responsible for the self-renewal of hematopoietic stem cell (HSC), neural stem cells and leukaemia stem cells (LSCs) in mice (69). In mouse models, aberrant expression of Hox genes affects the proliferation and differentiation of HCSs. Overexpression of HoxB6 culminates in AML, suggesting that genes responsible for stem cell proliferation are directly involved in AML initiation (70). A schematic representation of the pathways involved in CSC and stem cell signalling are presented in figure 1.

RESISTANCE OF CSCS TO CHEMOTHERAPY AND RADIOTHERAPY

Residual CSCs may survive in a quiescent state for many years after cancer remission and result in later relapse and metastasis. Several intrinsic features of CSCs should make them less susceptible to chemotherapy and/or radiotherapy: a) the presence of efflux pumps; b) their relatively low proliferative activity and high levels of anti-apoptotic proteins (bcl-2 and survivin); c) the presence of ALDH, which metabolizes chemotherapeutic drugs such as cyclophosphamide; and d) CSC-derived VEGF and other angiogenic factors that help to maintain the stem cell niche (71- 74).

Recent studies of brain and breast carcinomas have implicated CSC radioresistance (75, 76). It was shown that CD133+ CSCs contribute to glioma radioresistance through preferential activation of the DNA damage checkpoint response and a higher capacity for DNA repair, compared with CD133- tumour cells. The radioresistance of CD133+ glioma stem cells could be reversed with a specific inhibitor of Chk1 and Chk2 checkpoint kinases, which are closely associated with cellular resistance to radiation, thereby providing a therapeutic advantage to reducing brain tumour occurrence (75).

These data make it clear that cancer therapy needs a more targeted approach. Most cancer drugs target signalling pathways, such as hedgehog (cyclopamine) or Wnt/β-catenin (imatinib) (77). Another way to combat CSCs is to force them to differentiate (as can achieved using trans-retinoic acid, TPA, DMSO, butyric acid, vitamin D and nerve growth factors) (78). There is evidence that certain cancer drugs accomplish their functions by directly blocking ABCG1 pumps (verapamil and cyclosporine) (79) or ABCG2 pumps (the natural compound fumitremorgin C) (80). The pumps mentioned above are unable to remove some substances that are tumorotoxic but not cytotoxic (such as the phytochemical sulforaphan from broccoli), which gives us hope for potential natural, specific antitumour therapies (81). It has been shown that it is possible to prevent primary tumour and metastasis formation in animal models by blocking the homing factor CXCR4 (82).

It is unlikely that there will be a single magic bullet. The future of cancer treatment may require individualized combinations of therapies targeting molecular pathways, perhaps those unique to the appropriate type of CSC.

CONCLUSION

CSCs appear to use the same self-renewal pathways as stem cells from normal tissues. Thus, a compelling approach for studying CSCs is to understand the biology of normal tissue stem cells in order to better characterise CSCs. Ideal CSC markers have yet to be identified, so novel methods to accurately identify and target these cells would represent a significant advance in cancer therapy. Future research must focus on establishing reliable criteria for the identification and isolation of CSCs, and finding ways to briefly disrupt CSC niches without damaging normal stem cell niches.

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HETEROTOPIC PREGNANCY AFTER BILATERAL SALPINGECTOMY IN AN IVF PATIENT

[1]Snezana Adamoska-Klisaroska, [1]Slobodan Lazarevski, [1]Mihajlo Popovic, [1]Klementina Kuzeska, [1]Aneta Anevska, [1]Ana-Marija Stefanovska
[1]Department for Assisted Reproduction, Clinical Hospital- Sistina, Skopje, R.Macedonia

VANMATERIČNA TRUDNOĆA POSLE OBOSTRANOG ODSTRANJENJA JAJOVODA KOD PACIJENTKINJE PODVRGNUTE VEŠTAČKOJ OPLODNJI

Snežana Adamoska-Klisaroska[1], Slobodan Lazarevski[1], Mihajlo Popović[1], Klementina Kuzeska[1], Aneta Anevska[1], Ana-Marija Stefanovska[1]
[1]Klinička bolnica Sistina, Odsek za asistiranu reprodukciju, Skoplje, Republika Makedonija

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ABSTRACT

Purpose: *To report a rare clinical case of a heterotopic pregnancy after in vitro fertilisation (IVF) was performed on a patient who had previously undergone bilateral salpingectomy.*

Methods: *A 32-year-old woman, suffering from mechanical infertility, underwent IVF. The patient had an extrauterine pregnancy a year prior to the IVF procedure and underwent a laparoscopic bilateral salpingectomy of the right fallopian tube due to the extrauterine gravidity and of the left fallopian tube due to hydrosalpinx.*

The IVF treatment resulted in a heterotopic pregnancy that involved an intrauterine and a cornual pregnancy, which were managed by performing a laparotomy and a resection of the tubal stump. This intrauterine pregnancy resulted in a term singleton delivery.

Conclusion: *Although extremely rare, every gynaecologist treating an IVF patient should consider the possibility of a cornual heterotopic pregnancy under circumstances where the patient previously underwent a bilateral salpingectomy.*

Keywords: *Bilateral salpingectomy, Ectopic pregnancy, Heterotopic pregnancy, IVF*

SAŽETAK

Cilj: *Da prikažemo redak slučaj heterotopne trudnoće sa in vitro fertilizacijom(IVF), kod pacijentkinje sa prethodnom bilateralnom salpingektomijom.*

Metod *- prikaz slučaja: 32 godišnja pacientkinja, zbog mehaničkog faktora steriliteta, tretirana je IVF metodom. Imala je vanmateričnu trudnoću pre godinu dana i tokom laparoskopije obe tube su otstranjene: desna zbog vanmaterične trudnoće, a leva zbog hidrosalpinksa.*

IVF tretman rezultirao je sa heterotopnom trudnoćom: intrauterinom i kornualnom trudnoćom sa rupturom i intraabdominalnim krvarenjem, zbog čega je urađena laparotomija i resekcija tubalnog stumpa. Intrauterina trudnoća rezultirala je sa terminskim rađanjem.

Zaključak: *Iako je mogućnost razvoja cornualne heterotopne trudnoće veoma retka kod pacijentkinje sa bilateralnom salpingektomiom, ginekolog koji radi IVF treba ovo uvek imati u vidu, kak o bi se na vreme otkrila i bezbedno po intrauterinu trudnoću tretirala.*

Klucne reči: *bilateralna salpingectomia, vonmatericna trudnoća, IVF, heterotopna trudnoća.*

Abbreviations

ART - *assisted reproductive technologies*
ET - *embryo transfer*
IVF - *in vitro fertilisation*

Skraćenice

ART - *asistirane reproduktivne tehnologije*
ET - *embrio transfer*
IVF - *in vitro fertilizacija*

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Correspondence: Dr. Snezana Adamoska Klisaroska / Department for Assisted Reproduction, Clinical Hospital-Sistina, Skupi BB, 1000 Skopje, R.Macedonia
Home address: Nikola Trimpare 7/ 5,1000 Skopje, R.Macedonia / Tel: +389 70 38 11 99, fax: + 389 2 3222 355, e-mail: adamoskas@yahoo.com

INTRODUCTION

A heterotopic pregnancy is defined as the simultaneous occurrence of an intrauterine and ectopic pregnancy. Duverney first described this diagnosis in 1708 upon analysing the findings from an autopsy performed on a patient, who died during an ectopic pregnancy (2). The incidence of this diagnosis was initially thought to be on the order of 1 in 30,000 pregnancies, but more recent reports have revised this incidence to 1 in 3,889 pregnancies (5,8). When implementing assisted reproductive technologies (ART), the incidence of heterotopic pregnancies may increase to 1 in 100 pregnancies (5,8). The higher rates of heterotopic pregnancies among ART patients are not surprising because multiple embryos are usually transferred during the ART procedure, thereby increasing the potential for multiple embryo implantations (in the uterus or elsewhere). Heterotopic pregnancies are an obstetric complication with potentially serious consequences. Due to the presence of a concurrent intrauterine gestation, the ectopic pregnancy may be more difficult to diagnose, and this delay in diagnosis may increase the risk for adverse maternal outcomes such as rupture, hypovolemic shock or the need for a blood transfusion. These complications may also jeopardise the integrity of the intrauterine pregnancy. The presence of acute abdominal pain, haemorrhagic shock and an intrauterine pregnancy are some of the symptoms that could be used to diagnose a heterotopic pregnancy.

CASE REPORT

A 32-year-old female was admitted to the emergency department of our hospital with acute abdominal pain at 7 weeks of gestation after undergoing the IVF procedure. The patient's medical history included a pelvic inflammatory disease that was treated with antibiotics roughly ten years ago. The patient got married five years ago and had her first pregnancy three years ago. However, the aforementioned pregnancy was a right tubal extrauterine pregnancy that was treated with Methotrexate. A year ago she became pregnant for a second time, however, once again it was a right tubal extrauterine pregnancy and as such, a laparoscopy was performed. A bilateral salpingectomy was subsequently performed due to a tubal pregnancy within the right fallopian tube and a hydrosalpinx within the left fallopian tube. Due to the patient's absolute requirement for assisted reproduction (bilateral salpingectomy), she was referred to our department. Routine investigations, in accordance with our established protocol for IVF were performed. One cycle of an agonist protocol of ovarian stimulation was initiated. Three days after oocyte retrieval, three embryos at the 8-cell developmental stage were transferred into the patient. Fourteen days after embryo transplant, B-HCG testing was positive. Five days later, a transvaginal ultrasound was performed and an intrauterine gestational sac of 10 mm in diameter that presented a yolk sac was detected. Two weeks thereafter the patient was admitted to our hospital with symptoms of acute abdominal pain. She described her pain, which originated in the lower abdomen, as severe, sharp and constant. The pain was not associated with nausea, emesis, dyspnea or the patient's body position. The initial physical examination revealed a blood pressure of 100/60 mmHg, a heart rate of 94 beats per minute and a temperature of 36.8 °C. Upon palpation, the patient had tenderness in the right lower quadrant of the abdomen. Subsequent laboratory analyses showed blood reduction indicated by haemoglobin b (Hg b) levels of 93 gr/l and hematocrit (Hct) levels of 0.27. A vaginal ultrasonography (US) was performed that revealed an intrauterine pregnancy with an embryo of a cranio-caudal length of 15 mm (which is standard according to this stage of the pregnancy) with cardiac activity. Notably, a large amount of free fluid around the uterus and in the Morrison's Pouch (hepatorenal space) was detected. Due to the serious condition of the patient, she was taken to the operating room and an explorative laparotomy was immediately performed. During the laparotomy, we observed intra-abdominal bleeding (a total of 700 ml of blood was aspirated) from the rupture of right cornual portion of the uterus due to a coexistent heterotopic pregnancy. We identified and carefully removed the trophoblastic tissue. The cornual scar was closed by the use of Vicryl 1 stitches. The patient received a transfusion of 2 units of packed red blood cells and had a benign convalescence. Subsequent histological examination confirmed products of conception consistent with an ectopic pregnancy. The patient was discharged from the operating room on Postoperative Day 4. The testing of foetal vitality (performed using ultrasound) at Day 4 and 2 weeks after the operation, both showed normal progress of the intrauterine pregnancy. The patient had an uncomplicated intrauterine pregnancy and spontaneously delivered a 3360 g boy at term (38 weeks of gestation).

DISCUSSION

Heterotopic pregnancies are an exceptional occurrence when the pregnancy occurs spontaneously (1 case out of 7,000 to 30,000 pregnancies) (1, 2, 4). Its incidence is increased after a patient receives IVF-ET treatment, reaching a rate that is estimated to be approximately 1% of such pregnancies. Possible risk factors include a high number of transferred embryos, a transfer that occurs near the uterine horn, excessive pressure on the syringe during the transfer, or difficulties during the ET procedure (2, 6). Bilateral salpingectomy is likely to be another risk factor for cornual pregnancies (6,7). For nonsalpingectomised patients, the peri- and intratubular adhesions, which may or may not be related to endometriosis, are additional risk factors. (2, 4, 9)

Certain authors also consider the quality of the embryos and the hormonal milieu at the moment of transfer as possible causes of this unusual type of pregnancy (5, 8).

The development of a pregnancy in the uterine horn creates a high risk for organ rupture and is often extremely haemorrhagic due to the richness of the local vascularisation through the branches of the uterine and ovarian arteries. The therapeutic objective is simple: to interrupt the evolution of the ectopic pregnancy and preserve the intrauterine pregnancy. The most frequently described treatment is surgically based, via resection of the uterine horn by laparotomy or laparoscopy (10). The rate of live births is around 60%.

The choice to perform the laparotomy seemed to be the more reliable direction to pursue compared to laparoscopy in order to ensure a solid myometrial suture and complete haemostasis. In this case, the extrauterine pregnancy was located in the junction of the right tubal stump within the uterine horn and resulted in rupture and intra-abdominal bleeding. No other therapeutic alternative was possible. Due to the direct correlation between the number of embryos transferred and the chances of a heterotopic pregnancy, it would have been prudent, in this case, to transfer only two embryos to the uterine cavity. However, three embryos were transferred in our patient. The most important diagnostic method for assessing a heterotopic pregnancy is the high-resolution transvaginal ultrasonography (5). However, the sonographic diagnosis of an ectopic pregnancy in cases of heterotopic pregnancy is difficult to confirm due to the presence of a concurrent intrauterine gestational sac and hyperstimulated ovaries. Thus, women with heterotopic pregnancies are at significantly greater risk for hypovolemic shock and thus require a blood transfusion.

Another factor was the age of the patient (32 years). It appears to be important to limit the number of embryos transferred to a young woman to two embryos of good quality.

In conclusion, the appearance of a heterotopic pregnancy after an IVF-ET procedure remains a rare occurrence, particularly after performing bilateral salpingectomy. It is very important to diagnose this occurrence as soon as possible if the associated symptoms appear (e.g., vaginal bleeding or pain). It is also important to understand the necessity of a systematic exploration of the pelvis upon the first ultrasound scan of the pregnancy performed at 7 to 8 weeks of gestation, even if there are no apparent risk factors. Our patient was certainly at a higher risk to lose the intrauterine pregnancy due to the intra-abdominal bleeding. However, the patient was fortunate to have underwent an uncomplicated intrauterine pregnancy. Thus, the "gold standard" for treating patients with heterotopic pregnancy is still surgery (7, 10).

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9th EFIS-EJI TATRA IMMUNOLOGY CONFERENCE MOLECULAR DETERMINANTS OF T CELL IMMUNITY

Strbske Pleso, Slovakia, September 4-8, 2010

The 9th EFIS-EJI Tatra Immunology Conference was organized by Czeck, Slovak, Austrian and British Societies of Immunology and supported by European Federation of Immunological Societies and European Journal of Immunology.

There was 17 invited speakers and 80 submitted abstracts, of which 6 were selected for oral presentation and 74 for poster presentations. There was approximately 120 delegates attending the conference. The scientific programe was divided in 6 sessions covering basic immunological aspects of T cell immunity and its role in autoimmunity and tumor immunology.

The conference was opened by Radek Spisek (Charles University, Prague, Czech Republic). Several lectures were of interes for our research in Kragujevac.

The short talk was dedicated to the regulation of T cell response by dendritic cells. Diana Dubziak (Nikolaus-Fiebiger-Center of Molecular Medicine and Department of Dermatology, University Hospital of Erlangen, Germany) presented a novel mechanism by which dendritic cells induce prominent CD4 helper T cell or CD8 T cell response. Also, by antigen targeting of DCs it is possible to induce a de novo differentiation of peripheral antigen specific regulatory T cells. So, specific antigen presentation by DCs might influence the outcome of immune reactions.

Catherine Sautes-Fridman (Cordeliers Research Center, UMRS872, Paris Descartes University, Pierre and Marie Curie University, Paris France) presented the two faces of the tumor microenvironment in human lung cancers. She showed that immune cells are organized in tertiary lymphoid structures „Ti-BALT“ in lung tumors. The density of mature dendritic cells in T cell areas of BALT is in correlation with prolonged survival of patients with early-stage NSCLC. She also presented data about correlation of chronic inflammation with tumorogenesis, in particular she demonstrated the expression of TLR7 and TLR8 by tumor cells in human lung cancer in situ and cell lines. Transcriptional analysis of primary lung cancers suggests chronic stimulation of tumor cells by TLR ligand in situ. Also, she showed that stimulation with TLR7 and -8 agonists led to atypical NF-kB activation, upregulation of Bcl-2 expression, increased tumor cell survival and increased chemoresistance. She concluded that TLR signaling occuting during infection in lung cancer patients could directly favour tumor development.

Miodrag Lukic (Center for Molecular Medicine, Faculty of Medicine, University of Kragujevac, Serbia) presented the data about effects of ST2 and Galectin-3 deletion on T cell mediated immunopathology and tumor immunology. He showed that Gal-3 deletion attenuated EAE. Galectin 3 deletion is accompanied with reduced influx and survival of dendritic cells and macrophages in CNS and decreased proliferation of encephalitogenic T cells. Galectin 3 defficient dendritic cells induce Th2 cell polarization. Also his data showed that lack of IL-33/ST2 axis is accompanied with aggravated diabetes and hepatitis which is related to enhanced Th1 and Th17 cells. Furthermore, he showed that single injection of IL-33 significantly suppressed hepatitis induction in wild type mice (mice with normal IL-33/ST2 axis). In mouse model for breast tumor, ST2 deletion attenuated tumor growth and metastasis which is accompanied with enhanced cytotoxic capacity of NK cells. He concluded that IL-33/ST2 axis may downregulate some inflammatory autoimmune processes and anti-tumor immunity and therefore might be considered as a therapeutic target.

The title of our abstracts were ″Galectin-3 deletion enhances anti-tumor immunity in malignant melanoma model″ and ″Heme exposed IVIGs show enhanced downregulatory potential in EAE″, and they have been accepted for poster presentations at the 9th EFIS-EJI Tatra Immunology Conference. They were presented on Monday, September 06 th at The Congress Hall Foyer. The posters were presented during “interactive poster presentation” session, with Adrian Hayday, London, UK as chairperson.

This conference was a great success, especially for opportunity to exchange ideas with colleagues from different research groups.

Marija Milovanovic
Ivan Jovanovic

12th MEETING OF THE SOCIETY FOR NATURAL IMMUNITY NK 2010

Cavtat, September 11-15, 2010

The NK 2010 meeting was organized by Society for Natural Immunity and supported by European Federation of Immunological Societies and European Journal of Immunology.

More than 280 abstracts have been accepted, covering a wide range of topics. Over 450 delegates participated. The scientific sessions were divided into Keynote lecture and ten sessions covering "NK receptors", „NK activation and inhibition", „NK cell regulation", „NK cell development and subsets", „NK cell education, tolerance and memory", „NK cells and infection", „NK cells and cancer" and „Clinical applications".

The keynote lecture "Toll-like receptor signaling in infection and inflamation" was performed by professor Luke O Neill (Trinity College, Dublin) who is well known as an excellent researcher with a clear focus on the Toll-like receptor physiology.

Dr Adelheid Cerwenka (Innate Immunity, German Cancer Research Center, Germany) continued on the theme of NK cells, but with regard to the role of NK cells in antitumor immunity. The focus of this lecture was on a harness of NK cells against tumor following multiple strategies including: 1) enhancing NK cell function in tumor bearing hosts; 2) increasing the number of NK cells in tumor; 3) enhancing suscepbility of tumor cells to NK cell mediated killing and 4) counteracting suppressive forces tumor microenvironment. She reported that after subcutaneous injection of RMA-S tumor cells, NK cells infiltrating the tumor tissue show a less mature phenotype as compared to blood NK cells. When compared to blood NK cells, tumor infiltrating NK cells displayed a signature genes involved in cell inhibition. Also, Dr. Cerwenka talked about strategies to efficiently up regulate ligands for activating receptors on tumor cells in order to increase the visibility of tumor cells for NK cells. These data might provide the basis for novel design of NK cell based anti-tumor immune therapies.

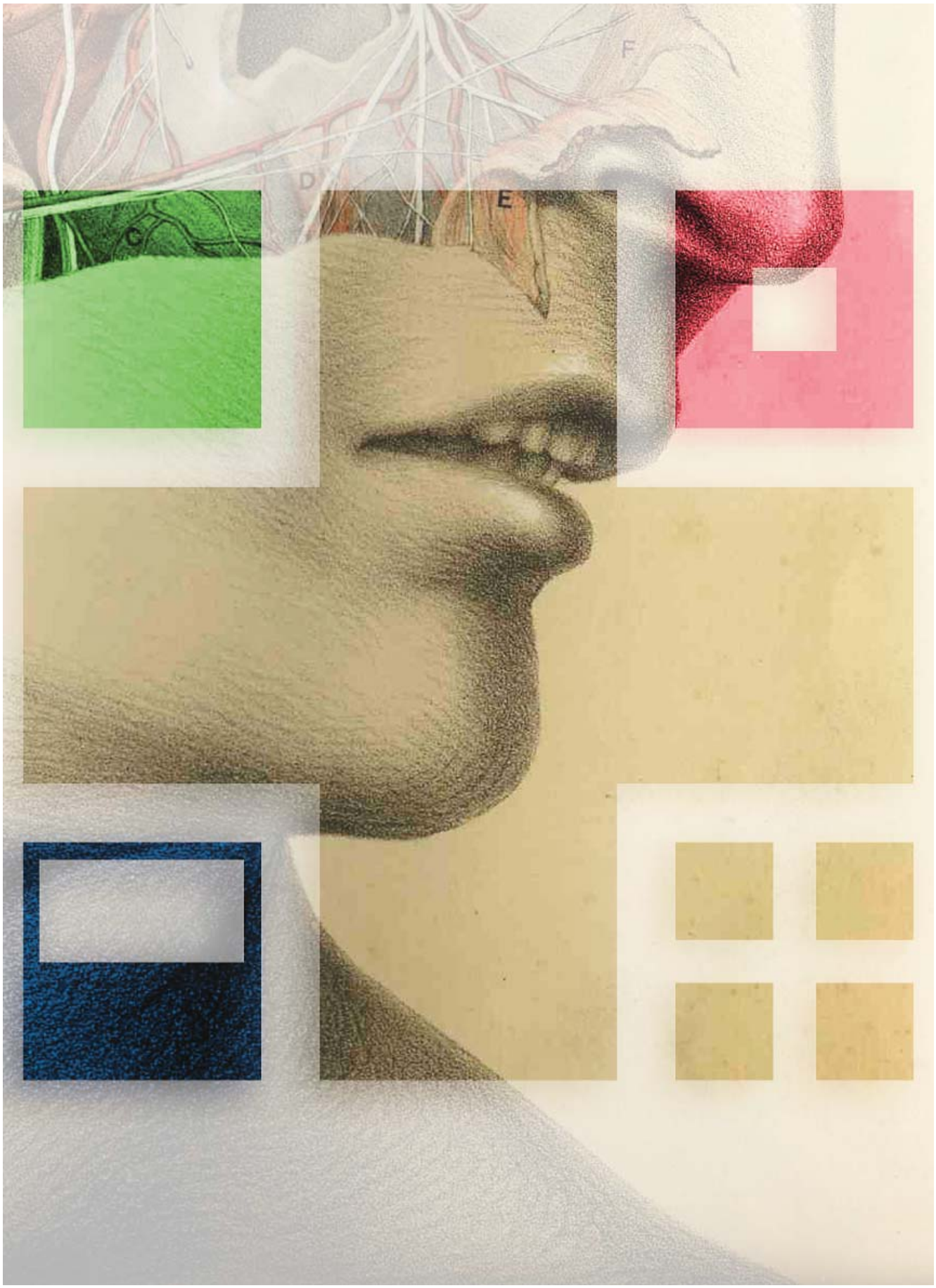
Professor Michael van Gelder (Internal Medicine, Academic hospital Maastricht, Netherlands) described for the first time that haploidentical spleen and bone marrow transplantation cures breast cancer bearing mice. Tumor inoculation in mice non-myeloablative conditioning (2 x 2Gy total body irradiation and 200 mg/kg cyclophosphamide) was followed by transplantation of spleen and bone marrow (BM) from haploidentical donors. Only 10 % of breast cancer bearing mice transplanted showed progressive tumor growth. Mice transplanted with spleen and BM cells from T-cell depleted donors did not develop tumors at all, in contrast to recipients of in vivo NK-cell depleted grafts. His report provides the first evidence that chemorefractory tumor cells are killed in vivo by alloreactive NK cells resulting in cure of breast cancer.

Jacques Deguine (G5 Dynamics of Immunes Responses, Institut Pasteur, France) described intravital microscopy in a Rae-1β expressing solid tumor in order to address how NKG2D engagement affects intratumoral NK cell dynamics. NKG2D ligand drove NK cell accumulation, activation and motility within the tumor. NK cells established mainly dynamic contacts with their targets, whereas CTLs formed stable contacts in tumors expressing their cognate antigen. In vitro, contacts between NK cells and their targets were cytotoxic but did not elicit sustained calcium influx, and CTL contact stability was critically dependent on extracellular calcium entry. He concluded that NK cells and CTLs can exert cytotoxic activity using remarkably different contact dynamics.

The title of our abstracts were ″Galectin-3 deletion enhances anti-tumor immunity in malignant melanoma model″ and ″Modulation of NK cell phenotype by deletion of ST2 receptor″, and they have been accepted for poster presentations at the 12th Meeting of the Society for Natural Immunity. They were presented on Sunday and Tuesday, September 12th and 14th at in Tihi salon and Bobara Hall. The posters were presented during "interactive poster presentation" session.

This meeting provided an excellent forum to discuss NK cell physiology from several different viewpoints, a lively exchange of ideas and opportunity to network with experts and colleagues from different backgrounds. We had an opportunity to discuss our data with leading experts in relevant fields (Professor Luke O Neill, Professor Stipan Jonjic and others).

Gordana Radosavljevic
Ivan Jovanovic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THE MEDICAL FACULTY KRAGUJEVAC
Svetozara Markovica 69, 34000 Kragujevac, SERBIA
P.O. Box 124
Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112
e-mail: sjecr@medf.kg.ac.rs

www.medf.kg.ac.rs