Effect of recreational scuba diving after a winter non-dive period on the oxidant / antioxidant status, SIRT1 and SIRT3 expression

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Abstract

The aim of this study was to examine effects of a first scuba dive after winter non-dive period on oxidative damage markers in erythrocytes and plasma, antioxidant system in peripheral blood mononuclear cells (PBMCs), as well as sirtuin 1 (*SIRT1*) and sirtuin 3 (*SIRT3*) gene expressions. For that purpose, 17 male recreational divers performed an immersion at a depth of 30 m for 30 min. Blood samples were collected immediately before and after diving, 3 and 6 h after diving. Erythrocyte lipid peroxidation measured by TBARS method was significantly increased immediately after diving, but returned to the baseline 6 h after diving, while no significant change was found for plasma TBARS and protein carbonyl derivates in both plasma and erythrocytes. Diving induced catalase (CAT), superoxide dismutase 2 (SOD2) and consequently total SOD activities in the PBMC samples (significantly increased immediately after diving, reached the maximum activities 3 h after diving, while 6 h after diving only CAT activity remained significantly increased). No significant change was observed for SOD1 activity and gene expression, as well as *SOD2* expression, while *CAT* and *SIRT1* expressions were slightly decreased immediately after diving and 3 h after diving. Interestingly, *SIRT3* expression was significantly increased 6 h after diving. The observed positive correlations between mRNA levels of antioxidant enzymes and sirtuins implicate their association. In conclusion, after winter non-dive period, activation of antioxidant defense was not sufficient to prevent oxidative damage, while *SIRT*3 upregulation provides a foundation for beneficial effect of recreational scuba diving.

Keywords: diving; hyperoxia; oxidative stress; sirtuin 1; sirtuin 3

Introduction

Recreational diving with the self-contained underwater breathing apparatus (scuba) has become very popular in the last 20 years, with millions of recreational divers worldwide. Contrary to the technical scuba diving, performed by professional divers at demanding depths using different gas mixtures, recreational or sports scuba diving implies diving to a depth of not more than 40 m, using only compressed air or nitrox (mixture of nitrogen and oxygen with no more than 40% oxygen), and never requiring a mandatory decompression stop [1]. In any case, scuba diving represents a special form of exercise-induced oxidative stress since the increased production of reactive oxygen species (ROS) is a result not only of a demanding physical activity but also of the exposure to low temperature and hyperoxia, which occurs due to exposure to the elevated pressure and breathing oxygen under increased pressure [2]. High production of ROS causes oxidative damage of the cellular structures such as lipids, proteins, and nucleic acids, and the accumulation of these damages can result in relevant changes in health status [3]. On the other hand, ROS have an important role as signaling molecules and their low-to-moderate increase plays multiple regulatory roles in cells, including the activation of antioxidant defense to suppress oxidative stress [3,4].

The mechanisms of the antioxidant enzyme activation and expression as well as their effectiveness, are still insufficiently understood. Sirtuin 1 (SIRT1) and sirtuin 3 (SIRT3), nicotinamide adenine dinucleotide (NAD) dependent deacetylases, due to their sensitivity to changes in the redox status and ability to regulate redox homeostasis [4], represent intriguing molecules that can be associated with the antioxidant response. Both SIRT1 and SIRT3 are associated with deacetylation of FOXO3a, a transcriptional factor which induces superoxide dismutase 2 (*SOD2*) and catalase (*CAT*) gene expression [4,5]. Other pathways that rely on SIRT1 to activate the antioxidant response

involve transcription factors p53 *via SOD2* and glutathione peroxidase 1 (*GPx1*) gene expression, and PGC1α *via SOD2* gene expression [5]. Additionally, SIRT3 has an important role in SOD2 activation [6], and by deacetylation of its numerous mitochondrial targets increases the activity of other antioxidant enzymes indirectly, by producing NADPH [7].

Current data on the effects of scuba diving on oxidative damage markers and antioxidant defense are controversial [8-13], but it can be explained by the different conditions in which studies were conducted including different depth and duration of hyperbaric exposure, gas mixture used, study population, sample type, and the method of determination. Consequently, in the field of recreational diving, there is a lack of studies dealing with this topic. The aim of this study was to examine the effects of recreational scuba diving after a winter non-dive period on erythrocytes and plasma oxidative damage markers and antioxidant system in peripheral blood mononuclear cells (PBMCs) immediately after diving, as well as 3 and 6 h after diving. The effect of scuba diving on *SIRT1* and *SIRT3* gene expression was also examined, with the aim to explore the possible linkage between their expressions and antioxidant defense.

Materials and methods

Subjects

The study included 17 male recreational divers median age (range) 41 (30-52) years with body mass index (BMI), expressed as the median and interquartile range (IQR) 27.5 (24.9-28.4) kg m^{-2}. Prior to diving, divers were subjected to the medical examination that included medical history taking, blood pressure measurement, anthropometric measurements and laboratory tests. None of them had symptoms of any

acute or chronic disease. Subjects did not take any antioxidant supplements or another type of dietary supplements two months before the study and any medications or alcohol 48 h before and during the study. None of the subjects was a professional athlete and practiced scuba diving during the winter period. The study was designed in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the University of Zagreb Faculty of Pharmacy and Biochemistry, Croatia. Participants were informed of the study purpose and protocol and they all signed informed consent.

Experimental procedure

The experimental dive was performed on the Adriatic Sea coast in April 2016. The sea temperature was 16 °C at the surface and 14 °C at the bottom and the air temperature was between 16 and 20 °C. The divers used equipment consisting of wetsuits, dive computers and scuba apparatus, and the air was used as a breathing gas. Divers were diving in the group at a maximum depth of 30 m for a total time of 30 min and return to the surface was conducted without a decompression. After surfacing, divers were examined by the specialist of hyperbaric medicine and no one developed symptoms of decompression sickness.

Venous blood samples (18 mL) were collected in the vacutainers with EDTA (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria) immediately before diving (t_0), immediately after diving (t_1), 3 h after diving (t_2) and 6 h after diving (t_3). Fresh whole blood was used to isolate PBMCs, to obtain plasma and to purify erythrocytes.

Erythrocytes and plasma purification

Blood samples were centrifuged at 1000 x *g* for 15 min within 1 h after blood sampling and collected plasma was stored at -80 °C. The remaining erythrocyte phase was

washed 3 times with phosphate-buffered saline (PBS) and centrifuged as above. Erythrocytes were reconstituted and hemolysed with ice-cold distilled water in the same volume as plasma. Hemoglobin was measured in the hemolysed samples on the hematology analyzer Cell Dyn 1800 (Abbott, Illinois, USA) and aliquots were stored at -80 °C.

PBMC isolation

PBMC fraction was isolated according to instruction for Leucosep™ Tubes with the separation barrier (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria). Leucosep tubes were prepared by adding the separation medium (Histopaque 1077, Sigma, Milan, Italy) and by centrifugation at 1000 x *g* for 30 sec. After 1 h at the RT, blood was diluted with an equal volume of PBS, added in Leucosep tube and centrifuged at 1000 x *g* for 15 min. The PBMC layer was collected, washed twice with PBS and centrifuged at 300 x *g* for 10 min. The isolated PBMCs were counted on the hematology analyzer Cell Dyn Ruby (Abbott, Illinois, USA) and stored at -80 °C for gene expressions studies and for antioxidant enzyme activity assays.

Oxidative damage markers

Oxidative damage markers were determined in erythrocytes and plasma samples. Lipid peroxidation was assessed by measuring the formation of thiobarbituric reactive substances (TBARS), according to Ohkawa *et al.* [14]. Protein carbonyl derivates (PCD) were measured according to Vidovic *et al*. [15]. Briefly, after lipid removal, 1 µg of protein was loaded into Maxisorb wells (Sigma Aldrich, St. Louis, MO, USA), derivatized using 2,4-dinitrophenylhydrazine (DNPH) to DNP hydrazone and probed with rabbit anti-DNP primary antibody (Sigma Aldrich, St. Louis, MO, USA), followed by goat anti-rabbit secondary antibody conjugated to HRP (Jackson ImmunoResearch,

West Grove, PA, USA). The absorbance was measured at 450 nm and expressed as nmol carbonyl per mg of protein. Protein concentration in erythrocytes and plasma samples was determined using Bradford method [16].

Antioxidant enzyme activities

PBMCs in PBS with protease inhibitors (Roche Diagnostics, Penzberg, Germany) were lysed using an ice-packed Potter–Elvehjem homogenizer (Braun, Biotech. Int., Germany) for 30 sec and centrifuged at 3000 x *g* for 15 min. Activities of CAT, total SOD (SOD), and isoforms CuZn-SOD (SOD1) and Mn-SOD (SOD2) were determined in supernatants. CAT activity was measured by the spectrophotometric method according to Aebi [17] based on the decomposition of hydrogen peroxide (H_2O_2). SOD activity was determined using RANSOD antioxidant kit (Randox, Crumlin, UK) according to the manufacturer's instructions. The activity of SOD2 isoform was determined under identical conditions with the addition of 3 mM KCN in the assay buffer for 30 min to inhibit SOD1 [18]. SOD1 activity was obtained by subtracting the SOD2 activity from the total SOD activity. Analyses were performed using Camspec M330 spectrophotometer equipped with M330 Camspec software package (Camspec LTD. Cambridge, UK). Cell count was calculated for each sample, and enzyme activity was expressed as IU per 10^6 cells.

RNA isolation and gene-expression analysis

Total RNA was extracted from 10 x 10^6 isolated PBMCs using Illustra^{TM} RNAspin Mini RNA Isolation Kit (GE Healthcare, Amersham, UK) according to the product manual and stored until analyses at -80 °C. The procedure included DNA digestion step. Quantity and purity of the extracted RNA were determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE,USA), and the integrity

was confirmed by formaldehyde-agarose-gel electrophoresis. Reverse transcription of total RNA was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with 1 µg of RNA per 20 µL cDNA reaction, according to the manufacturer's instructions. cDNA corresponding to 50 ng RNA was amplified for 40 cycles in a 25 µL PCR mix (TaqMan Gene Expression Master Mix, Applied Biosystems, Foster City, CA) that contained 1xTaqMan primers/probe mix, on 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). 2xTaqMan primers/probe mixes for mRNA of interest, as well as two reference genes, glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*) were available as inventoried validated assays (assay ID: Hs01009006_m1 for *SIRT1*, Hs00953477_m1 for *SIRT3*, Hs00156308_m1 for *CAT*, Hs00533490_m1 for *SOD1*, Hs00167309_m1 for *SOD2*, 4310884E for *GAPDH* and Hs99999903_m1 for *ACTB*), and cycling conditions were standard conditions recommended by the manufacturer for applied PCR mix and TaqMan primers/probes. The absence of potential gDNA contamination was assessed by including RNA isolate as a template control and the absence of PCR-product/cDNA contamination was assessed by including a no template control (NTC) in every run. All reactions were carried out in triplicate. The calculation of relative quantities *i.e.* relative mRNA levels of target genes of t_1, t_2 and t_3 samples of one individual in relation to t_0 sample of the same individual was performed based on the standard delta-delta-Ct method as recommended in Hellemans *et al*. [19], which includes normalization against average expression of the two reference genes *GAPDH* and *ACTB*.

Statistical analysis

Considering the small number of participants (less than 30), and according to the recommendation regarding sample size [20], only non-parametric tests were used for

testing and the data were presented as the median and interquartile range (IQR). A statistically significant difference between consecutive measurements was assessed using Friedman's test. In the case of a significant difference, Wilcoxon paired test was used for multiple comparisons with a Bonferroni's correction applied. The possible relationship between gene expressions in PBMCs was tested with Spearman's rank-order correlation. Statistical analyses were performed using MedCalc statistical software version 16.4.3 (MedCalc, Ostend, Belgium). The level of significance was set at $P < 0.05$.

Results

Oxidative damage markers

Effect of scuba diving on oxidative damage markers was assessed by measuring TBARS and PCD in erythrocytes and plasma of all participants (n=17). There were no significant differences between PCD values before and immediately after diving, as well as 3 and 6 h after diving, in either erythrocyte or plasma samples (Figure 1A, 1B). However, scuba diving significantly affected erythrocyte lipid peroxidation. Namely, the value of TBARS in erythrocytes was statistically increased immediately after diving (~44%, $P = 0.007$), but returned to the baseline 6 h after diving (Figure 1C). In plasma samples, there were no changes between TBARS value before and after diving (Figure 1D).

Antioxidant enzyme activities

Due to unsuccessful PBMCs isolation from the sample of one participant, antioxidant enzyme activities were analyzed in PBMC samples of 16 participants. Scuba diving to

the depth of 30 m for 30 min significantly affected CAT and SOD2 activities, and consequently total SOD activity (Figure 2A, 2B, 2C). Immediately after diving, CAT, SOD and SOD2 activities were significantly increased (CAT ~184%, $P < 0.001$; SOD2 ~72%, $P = 0.002$; SOD ~47%, $P = 0.003$, respectively). Their activities reached maximum values 3 h after diving (CAT ~194%, $P < 0.001$; SOD2 ~196%, $P < 0.001$; SOD ~202%, $P < 0.001$, respectively). Six hours after diving, CAT activity remained significantly increased (~91%, $P = 0.005$), while SOD and SOD2 activities dropped almost to the values before diving. The similar time course changes were also observed for SOD1 activity, but without statistically significant change (Figure 2D).

Antioxidant enzyme mRNA levels

To investigate whether only one dive is sufficient to induce the gene expression of antioxidant enzymes, the level of *CAT*, *SOD1*, and *SOD2* mRNA were estimated before and after diving, as well as 3 and 6 h after diving in PBMCs of 16 divers. No significant change in *SOD1* and *SOD2* mRNA levels was observed as a consequence of one single dive, although a slight decrease was present (Figure 3A, 3B). For *CAT* mRNA level, the statistically significant decrease was found immediately after diving (~19%, $P = 0.005$) and 3 h after diving (~36%, $P = 0.007$), while statistically significant change was not found 6 h after diving (Figure 3C).

SIRT1 and SIRT3 mRNA levels

Given the numerous roles of SIRT1 and SIRT3 in the activation of antioxidant defense, the effect of scuba diving was also examined on *SIRT1* and *SIRT3* mRNA levels in PBMC samples of 16 divers. Immediately after diving *SIRT1* mRNA level was ~25% lower than before diving ($P = 0.005$), 3 h after diving was ~34% lower than before

diving (P=0.007), while 6 h after diving the change did not reach significance due to sample-to-sample variation (Figure 4A). An interesting finding was the increase of *SIRT3* expression 6 h after diving (Figure 4B), when the *SIRT3* mRNA level was ~41% higher than the level before diving ($P < 0.001$), ~82% higher than the level immediately after diving ($P < 0.001$) and ~53% higher than the level noted 3 h after diving ($P < 0.001$).

Correlation between gene expressions in PBMCs

For a better understanding of the PBMCs antioxidant response to the diving session, the correlation between antioxidant enzymes mRNA levels and sirtuins mRNA levels was assessed. Positive correlations were found between *CAT* expressions and both *SIRT1* and *SIRT3* expressions at all time points. A positive relationship was also observed between *SOD2* and *SIRT1* expressions immediately after diving and 3 h after diving, between *SOD2* and *SIRT3* expressions immediately after diving, while *SOD1* expressions correlated only with *SIRT1* expressions both 3 and 6 h after diving (Table 1).

Discussion

To the best of our knowledge, this is the first study to examine oxidative status in recreational divers after a winter non-dive period. Our results have shown that the dive with compressed air to the depth of 30 m for the total time of 30 min induces an antioxidant response that was not sufficient to prevent oxidative damage. Lipid peroxidation in erythrocytes, determined by the TBARS method, was most elevated immediately after diving, while 6 h after diving returned to the baseline. Taking into

account that erythrocytes and plasma are considered the most sensitive blood fractions for oxidative damage [21] and since we found no difference in plasma TBARS level and also in carbonylated proteins level in either plasma or erythrocytes, peroxidative damage in erythrocytes appears to be the most pronounced response to oxidative stress caused by scuba diving. It is obvious that erythrocyte membranes are the most vulnerable to ROS overproduction, which could be the reason for the decrease of erythrocyte number after diving, reported in our previous work [22].

Although there are no studies that examined the effect of recreational scuba diving on the oxidative damage markers, there are some evidence on the effects of scuba diving with compressed air on oxidant/antioxidant status in professional divers [8-12]. Sureda *et al.* have shown no difference in protein and lipid oxidative damage markers in lymphocytes, erythrocytes and plasma immediately and 3 h after diving to the 40 m for 25 min with a decompression of 5 min [8,9]. Although diving in our study was performed at a lower depth (30 m), and the dive profile did not require decompression during the ascent, most likely explanation for the discrepancy between our and published result in the erythrocyte lipid peroxidation lays in the developed antioxidant system in professional divers that does not allow the occurrence of lipid degradation. However, after diving to the depth of 50 m for 35 min with a decompression stop of 9 min, the same authors have noted an increase of lipid and DNA oxidative damage markers in plasma [10]. Taken together, these findings suggest the importance of the depth and duration of hyperbaric exposure, which is reflected in the necessary decompression during ascent, on ROS overproduction and consequential damage. Interestingly, in professional police divers, the elevation of plasma TBARS level was observed immediately after diving at a lower depth (30 m for 30 min with a decompression of 3 min), as reported by Radojevic *et al*. [12].

Given the published studies on the effect of demanding exercise on the antioxidant response, antioxidant enzymes in lymphocytes (according to the isolation procedures, more correctly PBMCs) show a great adaptation to oxidative stress [21,23]. This is the reason why we used PBMCs for monitoring changes in antioxidant enzymes activities and gene expression levels. We showed that immersion to 30 m for 30 min has induced a fast response of antioxidant defense in PBMCs against ROS overproduction by the increase of CAT, SOD2 and consequently SOD activities. Although activities of CAT and SOD2 were increased immediately after diving, their activities reached maximum value 3 h after diving, indicating their prolonged action to remove ROS. Since SOD catalyses the formation of O_2^- to H_2O_2, while CAT is required for the decomposition of H_2O_2 to form water and oxygen, it is rational to assume that the reason for only CAT activity elevation 6 h after diving lies in their roles. The elevation of antioxidant enzyme activities was also recorded in previous studies conducted on professional divers. After diving to 40 m, an increase of lymphocyte CAT activity was observed but only 3 h after diving and glutathione peroxidase (GPx) activity immediately and 3 h after diving [8]. Unfortunately, SOD activity was not monitored in the mentioned study, and antioxidant enzymes in lymphocytes or PBMCs have not been determined in other studies conducted on professional divers. However, elevation of plasma SOD and CAT activities was also observed after diving to 40 and 50 m [9,10], while in erythrocytes this change was not recorded after diving to 40 and 30 m [9,12].

The significant increase of CAT and SOD2 activities recorded in our study was not accompanied by the increase of their mRNA levels in PBMC samples. Moreover, although with a slight change, level of CAT mRNA was statistically decreased immediately after diving (~19%) and 3 h after diving (~36%). The same pattern of changes in lymphocytes CAT expression and also activity was observed after running

bouts of 45 min in cold environment (10-12 °C), while the opposite direction of change in gene expression level was found after running in the hot environment (30-32 °C) in the same study [24]. Considering the complex regulatory mechanisms for gene expression that occur at both post-transcriptional and post-translational levels, the reason for CAT expression decrease remains elusive, but it seems that the environmental temperature participates in them. However, taking into account that the observed decrease in CAT mRNA levels was small, its biological significance should be considered with caution. On the other hand, some studies recorded the increase of antioxidant enzymes gene expression in lymphocytes or PBMCs after prolonged intensive exercise or longer period of regular physical activity [25-27]. Since in our study, the gene expression was assessed after only one scuba dive in the duration of 30 min, there is a possibility that consecutive diving in a longer period can cause the same response.

Antioxidant defense activation mechanisms are generally unknown. Even if the majority of data were derived from experimental findings in mice, the beneficial effects of oxidative stress and physical exercise in terms of sirtuins and ROS reduction have been adequately presented in several review articles [4,5,28,29]. However, there are limited data in the human model and lack of studies on sirtuins gene expression in blood fractions. In the present work we have shown that the oxidative stress provoked by immersion to 30 m leads to a slight decrease of *SIRT1* expression immediately and 3 h after diving and elevation of *SIRT3* expression 6 h after diving in PBMC samples. Contrary to our finding, after 3 h of intensive cycling, an increase in muscle *SIRT1* mRNA was observed in trained human subjects [30]. The reason for the discrepancy between the mentioned and our finding can only be speculated, but the main cause probably lies in a different type of sample or type and duration of exercise. Regarding

SIRT3 expression, Mestre-Alfaro *et al*. showed that the lower environmental temperature (10-12 °C) during exercise increases *SIRT3* mRNA level in the human lymphocytes, while this effect was not found in the hot environment (30-32 °C) [24]. Since diving in our study was carried out at a similar environmental temperature, despite many differences in the type of exercise, their result is in accordance with our finding. The increase of *SIRT3* expression in relation to cold exposure was also observed in the animal model [31]. It was also shown that the regular aerobic training for 12 weeks and cycling exercise for 8 weeks may increase SIRT3 protein levels in the skeletal muscle in young and older participants [32,33]. Furthermore, interesting finding by Lanza *et al*. demonstrated that muscle SIRT3 protein expression decreases with age in sedentary adults, with no effect of age in trained adults [34]. Since in our study, one single scuba dive was enough to provoke *SIRT3* expression, it seems therefore that a longer practice period of diving may stimulate *SIRT3* expression to an even higher level. The increase of SIRT3 gene or protein expression, as well as activity can be a key step in the development of antioxidant defense in the frequent diving or exercise.

It should be mentioned that effects of SIRT3 on the so far discovered targets and binding partners make it a noble protein with protective and positive effects on health in general. Among the sirtuin members, SIRT3 has received much attention for its role in the aging process and many aging-related diseases such as diabetes, cardiovascular, neurodegenerative, respiratory and autoimmune diseases as well as cancer [35,36]. Therefore, the observed *SIRT3* upregulation in our study represents recreational scuba diving as a special form of hormesis in which low-to-moderate increase of ROS may have beneficial effects on health. Furthermore, the observed positive correlations between sirtuins mRNA levels and *CAT*, *SOD2* as well as *SOD1* mRNA levels indicate a close relationship between the expressions of these genes in human PBMCs. Models

of oxidative stress induced by exercise, particularly induced by scuba diving due to additional exposure to cold and hyperoxia, can be very useful in the translation of knowledge about sirtuins role in antioxidant response from the animal model to human model, in the most widely used sample, the blood sample.

Conclusion

Scuba diving represents a form of hormesis in which the border between positive and negative effects on health is very slippery. After the winter non-dive period the antioxidant defense was not powerful to prevent oxidative damage during diving to the 30 m for 30 min. Studies with the lower diving depth after the winter non-diving period could be useful for forming diving recommendations.

Elevation of antioxidant enzyme activities in PBMCs observed in our study represents an early and fast adaptation to the oxidative stress caused by scuba diving. Positive correlations between gene expressions of antioxidant enzymes and *SIRT1*, as well as *SIRT3* expression in divers' PBMC samples indicate the association of these genes that can be detected in human blood. Due to SIRT3 beneficial roles in human health, the increase of *SIRT3* gene expression recorded after only one single dive deserves attention in future research on the effects of successive diving on its expression. *SIRT3* upregulation can be a trigger towards hormetic response in recreational scuba divers.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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Table 1. Spearman rank correlation between mRNA levels of antioxidant enzymes and sirtuins in PBMCs

	Correlation coefficient	P
t_1 *CAT* mRNA vs. t_1 *SIRT1* mRNA	0.77*	< 0.001
t_2 *CAT* mRNA vs. t_2 *SIRT1* mRNA	0.76*	< 0.001
t_3 *CAT* mRNA vs. t_3 *SIRT1* mRNA	0.74*	< 0.001
t_1 *CAT* mRNA vs. t_1 *SIRT3* mRNA	0.74*	0.001
t_2 *CAT* mRNA vs. t_2 *SIRT3* mRNA	0.60*	0.015
t_3 *CAT* mRNA vs. t_3 *SIRT3* mRNA	0.75*	< 0.001
t_1 *SOD1* mRNA vs. t_1 *SIRT1* mRNA	0.48	0.060
t_2 *SOD1* mRNA vs. t_2 *SIRT1* mRNA	0.71*	0.002
t_3 *SOD1* mRNA vs. t_3 *SIRT1* mRNA	0.66*	0.005
t_1 *SOD1* mRNA vs. t_1 *SIRT3* mRNA	0.33	0.217
t_2 *SOD1* mRNA vs. t_2 *SIRT3* mRNA	0.41	0.116
t_3 *SOD1* mRNA vs. t_3 *SIRT3* mRNA	0.24	0.360
t_1 *SOD2* mRNA vs. t_1 *SIRT1* mRNA	0.55*	0.026
t_2 *SOD2* mRNA vs. t_2 *SIRT1* mRNA	0.54*	0.029
t_3 *SOD2* mRNA vs. t_3 *SIRT1* mRNA	0.21	0.444
t_1 *SOD2* mRNA vs. t_1 *SIRT3* mRNA	0.71*	0.001
t_2 *SOD2* mRNA vs. t_2 *SIRT3* mRNA	0.15	0.579
t_3 *SOD2* mRNA vs. t_3 *SIRT3* mRNA	0.28	0.289

*Indicates a correlation at $P < 0.05$. Abbreviations: SOD - superoxide dismutase; CAT - catalase; PBMCs - peripheral blood mononuclear cells; t_1 - immediately after diving; t_2 - 3 h after diving; t_3 - 6 h after diving.

Figure 1. Effect of scuba diving on oxidative damage markers

Oxidative damage markers in plasma and erythrocytes were assessed by measuring the formation of thiobarbituric reactive substances (TBARS) and protein carbonyl derivates (PCD): before diving (t_0), immediately after diving (t_1), 3 h after diving (t_2) and 6 h after diving (t_3). Values are presented as median (interquartile ranges). The difference between consecutive measurements was assessed using Friedman test and the post-hoc analysis was performed using Wilcoxon test with the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered as significant. * Significant differences in relation to t_0 values. † Significant differences in relation to t_1 values. ‡ Significant differences in relation to t_2 values.

Figure 2. Effect of scuba diving on antioxidant enzyme activities

Activities of catalase (CAT), total superoxide dismutase (SOD) and isoforms SOD1 and SOD2 were measured in isolated peripheral blood mononuclear cells (PBMCs): before diving (t_0), immediately after diving (t_1), 3 h after diving (t_2) and 6 h after diving (t_3). Values are presented as median (interquartile ranges). The difference between consecutive measurements was assessed using Friedman test and the post-hoc analysis was performed using Wilcoxon test with the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered as significant. * Significant differences in relation to t_0 values. † Significant differences in relation to t_1 values. ‡ Significant differences in relation to t_2 values.

Figure 3. Effect of scuba diving on *SOD1*, *SOD2* and *CAT* mRNA levels.

Total RNA was extracted from isolated PBMCs, reverse transcribed and the relative amount of target mRNAs was determined by the quantitative real-time PCR, normalized to the average expression of two reference genes and expressed relative to the t_0 sample value of each individual. Values are presented as median (interquartile ranges). The difference between consecutive measurements was assessed using Friedman test and the post-hoc analysis was performed using Wilcoxon test with the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered as significant. * Significant differences

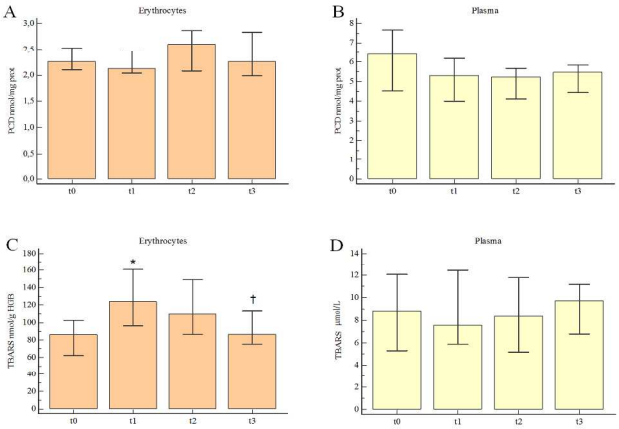
in relation to t_0 values. † Significant differences in relation to t_1 values. ‡ Significant differences in relation to t_2 values.

Abbreviations: SOD - superoxide dismutase; CAT - catalase; PBMCs - peripheral blood mononuclear cells; t_0 - value before diving; t_1 - value immediately after diving; t_2 - value 3 h after diving; t_3 - value 6 h after diving.

Figure 4. Effect of scuba diving on *SIRT1* and *SIRT3* mRNA levels.

Total RNA was extracted from isolated PBMCs, reverse transcribed and the relative amount of target mRNAs was determined by the quantitative real-time PCR, normalized to the average expression of two reference genes and expressed relative to the t_0 sample value of each individual. Values are presented as median (interquartile ranges). The difference between consecutive measurements was assessed using Friedman test and the post-hoc analysis was performed using Wilcoxon test with the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered as significant. * Significant differences in relation to t_0 values. † Significant differences in relation to t_1 values. ‡ Significant differences in relation to t_2 values.

Abbreviations: SIRT - sirtuin; PBMCs - peripheral blood mononuclear cells; t_0 - value before diving; t_1 - value immediately after diving; t_2 - value 3 h after diving; t_3 - value 6 after diving.



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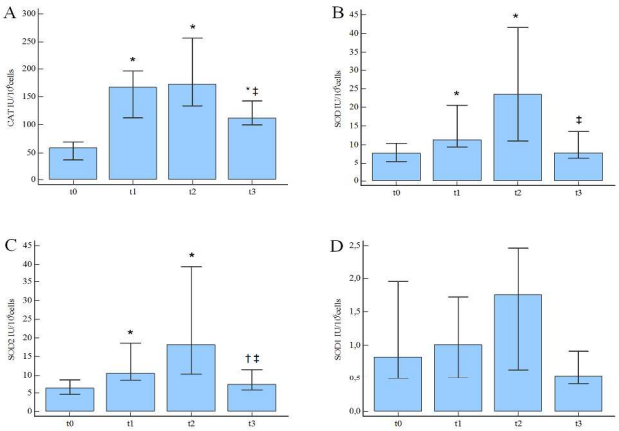

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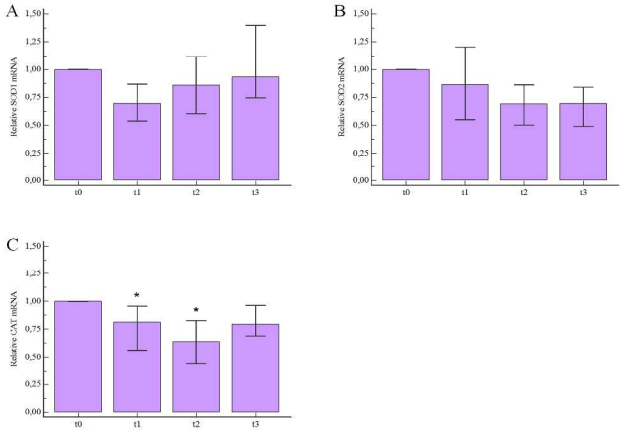

Figure 3. Effect of scuba diving on SOD1, SOD2 and CAT mRNA levels.
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Abbreviations: SOD - superoxide dismutase; CAT - catalase; PBMCs - peripheral blood mononuclear cells; t_0 - value before diving; t_1 - value immediately after diving; t_2 - value 3 h after diving; t_3 - value 6 h after diving.

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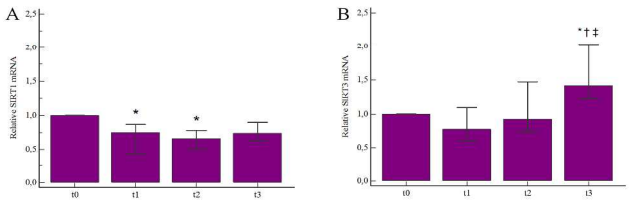

Figure 4. Effect of scuba diving on SIRT1 and SIRT3 mRNA levels.
Total RNA was extracted from isolated PBMCs, reverse transcribed and the relative amount of target mRNAs was determined by the quantitative real-time PCR, normalized to the average expression of two reference genes and expressed relative to the t_0 sample value of each individual. Values are presented as median (interquartile ranges). The difference between consecutive measurements was assessed using Friedman test and the post-hoc analysis was performed using Wilcoxon test with the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered as significant. * Significant differences in relation to t_0 values. † Significant differences in relation to t_1 values. ‡ Significant differences in relation to t_2 values.
Abbreviations: SIRT - sirtuin; PBMCs - peripheral blood mononuclear cells; t_0 - value before diving; t_1 - value immediately after diving; t_2 - value 3 h after diving; t_3 - value 6 after diving.

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