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The effects of *in vivo* and *ex vivo* various degrees of cold exposure on erythrocyte deformability and aggregation

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: This study aimed to investigate alterations in hemorheology by cold exposure, *in vivo* and *ex vivo*, and to determine their relationship to oxidative stress.

Material/Methods: Rats were divided into 2 *in vivo* and *ex vivo* cold exposure groups. The i*n vivo* group was further divided into control (AR), AC (4°C, 2 hours) and ALTC (4°C, 6 hours) subgroups; and the *ex vivo* group was divided into control (BR) and BC (4°C, 2 hours) subgroups. Blood samples were used for the determination of erythrocyte deformability, aggregation, and oxidative stress parameters.

Results: Erythrocyte deformability and aggregation were not affected by 2-hour *ex vivo* cold exposure. While 2 hour *in vivo* cold exposure reduced erythrocyte deformability, it returned to normal after 6 hours, possibly due the compensation by acute neuroendocrine response. Six hours of cold exposure decreased aggregation index, and might be an adaptive mechanism allowing the continuation of circulation. Aggregation of *ex vivo* groups was lower compared to *in vivo* groups. Cold exposure at various temperatures did not cause alterations in plasma total oxidant antioxidant status and oxidative stress index (TOS, TAS, OSI) when considered together.

Conclusions: Results of this study indicate that the alterations observed in hemorheological parameters due to cold exposure are far from being explained by the oxidative stress parameters determined herein.

key words: **cold • rat • red blood cell • deformability • aggregation**

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Background

Hypothermia is a condition in which an organism's temperature drops below that required for normal metabolism and body functions [1], and can be graded according to measured body temperature values. The term "mild hypothermia" usually indicates a body temperature of 33–36°C,whereas "moderate hypothermia" is 28–32°C, "deep hypothermia" is 16–28°C and "profound hypothermia" is <15°C [2,3]. Although variations in body temperature, under certain limits, can be compansated with homeostatic mechanisms regulated by the hypothalamus and endocrine system, cold exposure is also known to negatively affect physical and cognitive performance and increase the mortality and morbidity risk [4,5].

Hemorheological parameters such as red blood cell (RBC) deformability and aggregation play an important role in the maintenance of circulation. Alterations in these parameters in response to various pathological conditions have been demonstrated either to contribute the development of the pathological process, or help adaptation of the body to this situation [6–8]. *Ex vivo* studies investigating alterations in hemorheological parameters induced by hypothermia have shown that the viscosity of whole blood was higher at low temperatures (24°C, 32°C, 35°C, 1 minute) as compared with those at 37°C [9]. Studies exploring RBC hemodynamic behavior in common blood banking procedures have demonstrated decreased RBC deformability, increased RBC aggregation and adherence at 1–6°C after 2 weeks [10,11]. On the other hand, results of another study have shown decrease in RBC deformability (after 6 hours) and aggregation (12 hours later) when blood was stored at 4°C [12]. Effect of hypothermia on hemorheological parameters was also studied *in vivo.* Deveci et al exposed rats to progressively lower air temperatures gradually reduced from 20°C to 5°C over 4 weeks accompanied by a reduction in photoperiod in order to simulate seasonal change, and demonstrated that RBC transit time was faster in the acclimated rats, indicating an increase in RBC deformability following an 8-week acclimation period [13].

Molecules with unstable structures, called free radicals, may occur during physiological events in the body. In normal conditions, these molecules are compensated by antioxidant defense systems. However, if the formation of free radicals exceeds the capacity of the antioxidant defense system, it leads to a situation called oxidant stress [1]. Oxidant stress, especially free radicals caused by reactive oxygen species, leads to the oxidation of lipids, proteins and nucleic acids, causing them to become non-functional [14,15]. It was shown that exposure to hypothermia has different effects on oxidant stress, varying by length of cold exposure, internal temperature reached and ratio of heating-cooling period [1,16,17]. Although it is known that there is a relationship between oxidant stress and erythrocyte deformability [6,18,19], studies looking at erythrocyte aggregation show inconsistent results [7,20,21].

To the best of our knowledge, no study has observed and compared changes in hemorheological parameters arising from *in vivo* and *ex vivo* exposure to cold at different temperatures. The aim of this study was to compare possible changes in hemorheological parameters due to *in vivo* and *ex vivo* exposure to cold at different temperatures, as well as to reveal its possible relationship to oxidant stress.

Material and Methods

Animals and experimental procedure

This study was conducted at the Pamukkale University Experimental Animal Unit. Sprague-Dawley rats, weighing 200–250 g were fed with standard diet and water ad libitum. A total of 35 rats were randomly assigned to 1 of 5 groups (n≅7 in each). Rats of the animal at room air (AR) group were kept in the laboratory (≅24°C) throughout the experimental period, whereas rats of the animal in cold (AC) group were kept in a refrigerator (≅4°C) for 2 hours in their cages. Their body and blood temperature was measured as ≅33°C and after this holding period. Rats of the AC group served as an *in vivo* mild hypothermia group. Rats of the animal in long-term cold (ALTC) group were kept in the refrigerator (≅4°C) for 6 hours in their cages until their body and blood temperature reached ≅24°C. At the end of the procedure, rats of these 3 groups were anesthetised with ketamine and xylazine intraperitoneally, and heparinized blood samples were harvested. Blood samples from the rats of the blood at room air (BR) and blood in cold (BC) groups were collected and stored either in the laboratory (BR group) or in a refrigerator (≅4°C, BC group) for 2 hours. Blood temperature was measured as ≅24°C for the BR group, whereas it was ≅11°C for the BC group. Blood collected from all experimental groups for the determination of hemorheological parameters (RBC deformability and aggregation) was used within 3 hours. Plasma samples for the measurement of parameters representing the total oxidant-antioxidant status were stored at –20°C until being used.

Animal handling during all experimental protocol was consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and approved by the Pamukkale University Ethics Committee of Animal Care and Usage.

RBC deformability measurements

RBC deformability (the ability of the entire cell to adopt a new configuration when subjected to applied mechanical forces) was determined by laser diffraction analysis using an ektacytometer (LORCA, RR Mechatronics; Hoorn, The Netherlands). The system has been described elsewhere in detail [22]. Briefly, a low Hct suspension of RBC in 4% polyvinylpyrrolidone 360 solution (MW 360 kD, Sigma P 5288, St. Louis, MO) was sheared in a Couette system composed of a glass cup and a precisely fitting bob. A laser beam was directed through the sheared sample, and the diffraction pattern produced by the deformed cells was analyzed by a microcomputer. On the basis of the geometry of the elliptical diffraction pattern, an elongation index (EI) was calculated for 9 shear rates between 0.3 and 30 Pascal (Pa) as: $EI = (L - W)/(L + W)$, where L and W are the length and width of the diffraction pattern, respectively. An increased EI at a given shear stress indicates greater cell deformation and hence greater RBC deformability. All measurements were carried out at 37°C.

Measurements of RBC aggregation

RBC aggregation was also determined by LORCA as described elsewhere [23]. The measurement is based on the detection of laser back-scattering from the sheared (disaggregated), then

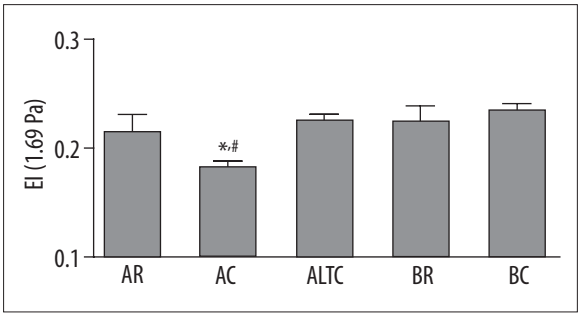

Figure 1. Red blood cell (RBC) elongation index (EI) values of all groups mesured at 1.69 Pascal (Pa) shear stress. AR: Animal at room air, AC: Animal in cold, ALTC: Animal in long term cold, BR: Blood at room air, BC: Blood in cold. Values are expressed as means ±SE. * p<0.05, difference from AR and ALTC groups; # p<0.01, difference from BR and BC groups.

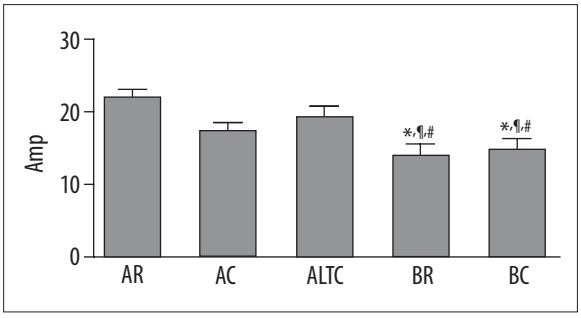

Figure 2. Amplitude (Amp) of red blood cell (RBC) aggregation values of all groups. AR: Animal at room air, AC: Animal in cold, ALTC: Animal in long term cold, BR: Blood at room air, BC: Blood in cold. Values are expressed as means ±SE. * p<0.001, difference from group AR; ¶ p<0.05, difference from group AC; # p<0.01, difference from ALTC group.

unsheared (aggregating) blood, performed in a computer-assisted system at 37°C. Back-scattering data were evaluated by the computer and the aggregation index (AI), aggregation half-time (t 1/2), which shows the kinetics of aggregation, and the amplitude (AMP), which is a measure for the total extent of aggregation, were calculated based on the fact that there is less light back-scattered from aggregating red cells. The hematocrit (Hct) of the samples used for aggregation measurements was adjusted to 40% and blood was fully oxygenated.

Measurement of plasma total oxidant status

The total oxidant status (TOS) of plasma was measured using a novel automated colorimetric measurement method for TOS developed by Erel [24]. In this method, oxidants present in the sample oxidize the ferrous ion–odianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules (lipids, proteins) present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equiv/L).

Measurement of plasma total antioxidant status

The total antioxidant status (TAS) of plasma was measured using a novel automated colorimetric measurement method for TAS developed by Erel [25]. In this method the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of a plasma sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the plasma, preventing the color change and thereby providing an effective measure of the TAS of the plasma. The assay results are expressed as mmol Trolox equiv/L.

Calculation of oxidative stress index

The ratio of TOS to TAS is referred as the oxidative stress index (OSI). The OSI is calculated according to the following formula [26]: OSI (arbitrary unit) = TOS (μmolH_2O_2 Equiv. /L)/TAS (mmol Trolox Equiv./L).

Statistical analysis

Results are expressed as means ± standard error (SE). Statistical comparisons between groups were done by one-way ANOVA followed by theTukey post-test, with p values <0.05 accepted as statistically significant. All analyses were carried out with the computerized SPSS 10.0 program (Statistical Package for Social Sciences, SPSS Inc).

Results

RBC deformability (assessed as the elongation index EI) for the RBCs of all experimental groups was measured at 9 shear stresses between 0.3 and 30 Pa, and EI values measured at 1.69 Pa (Figure 1). RBC deformability of animals in the cold (AC) group was found to be lower compared to the other groups p<0.05 compared to animals at room air (AR) and animal in long-term cold (ALTC) groups and p<0.01 compared to blood at room air (BR) and blood in cold (BC) groups.

The amplitude (Amp) of RBC aggregation, which is a measure for the total extent of aggregation, is shown in Figure 2. Amp of blood at room air (BR) and blood in cold (BC) groups were decreased compared to that of animal at room air (AR, p<0.001), animal in cold (AC, p<0.05) and animal in long-term cold (ALTC, p<0.01) groups. Figure 3 shows that the aggregation index (AI) of animal in long-term cold (ALTC), blood at room air (BR) and blood in cold (BC) groups were decreased compared to animal at room air (AR) and animal in cold (AC) groups. On the other hand, the RBC aggregation half-time (t 1/2) of the blood at room air (BR) group was statistically significantly higher compared to the animal at room air (AR) and animal in cold (AC) groups (p<0.01), and the blood in cold (BC) group was higher compared to the animal in cold (AC) group (p<0.05) alone (Figure 4). The increments in Amp and AI of aggregation are in aggreement with the decrement in t 1/2 and, considered together, indicate an increase in erythrocyte aggregation.

The parameters showing the oxidant (TOS) and antioxidant status (TAS), as well as the oxidative stress index of

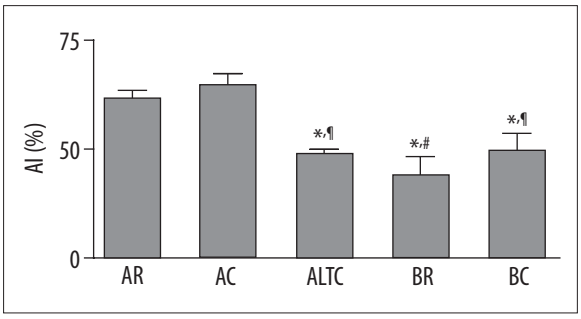

Figure 3. RBC aggregation index (AI) of all groups. AR: Animal at room air, AC: Animal in cold, ALTC: Animal in long term cold, BR: Blood at room air, BC: Blood in cold. Values are expressed as means ±SE. * p<0.05, difference from AR group; ¶ p<0.01, difference from group AC; # p<0.001, difference from AC group.

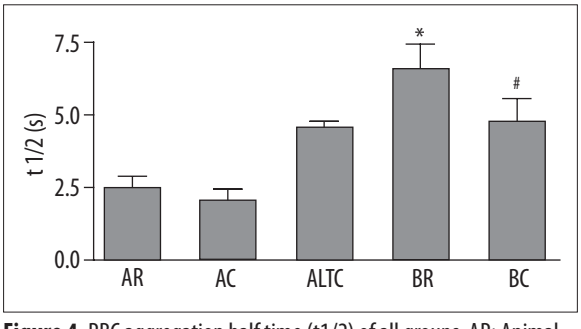

Figure 4. RBC aggregation half time (t1/2) of all groups. AR: Animal at room air, AC: Animal in cold, ALTC: Animal in long term cold, BR: Blood at room air, BC: Blood in cold. Values are expressed as means ±SE. * p<0.01, difference from AR and AC groups; # p<0.05, difference from AC group.

the groups, are presented in Figures 5, 6 and 7, respectively. Total oxidant status (TOS) of the blood at room air (BR) and blood in cold (BC) groups were decreased compared to animal in cold (AC, p<0.01) and animal in long-term cold (ALTC, p<0.001) groups. TOS of the animal in long-term cold (ALTC) group was highest of the groups (Figure 5). On the other hand, total antioxidant status (TAS) of the blood at room air group was lower compared to blood in cold (BC) and animal in long-term cold (ALTC) groups (p<0.05). The oxidative stress index (OSI), which was calculated for each animal as: TOS/TAS, although not statistically significant, was found to be higher for the animal in cold (AC) and animal in long-term cold (ALTC) groups.

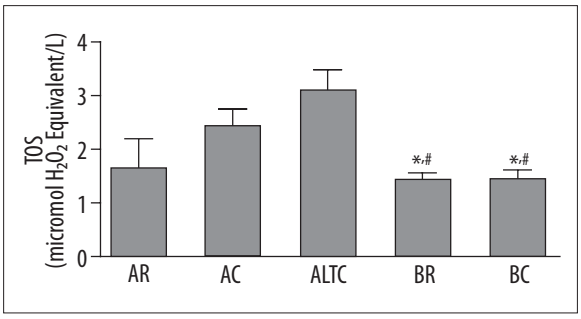

Figure 5. The total oxidant status of all groups. AR: Animal at room air, AC: Animal in cold, ALTC: Animal in long term cold, BR: Blood at room air, BC: Blood in cold. Values are expressed as means ±SE. * p<0.01, difference from AC group; # p<0.001, difference from ALTC group.

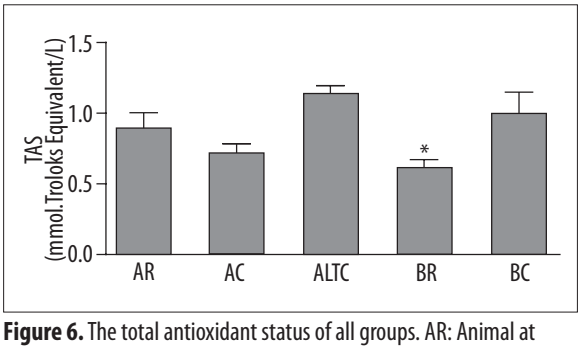

Figure 6. The total antioxidant status of all groups. AR: Animal at room air, AC: Animal in cold, ALTC: Animal in long term cold, BR: Blood at room air, BC: Blood in cold. Values are expressed as means ±SE. * p<0.05, difference from BC and ALTC groups.

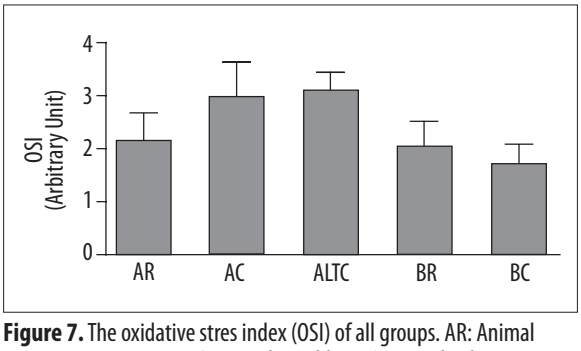

Figure 7. The oxidative stres index (OSI) of all groups. AR: Animal at room air, AC: Animal in cold, ALTC: Animal in long term cold, BR: Blood at room air, BC: Blood in cold. Values are expressed as means ±SE.

Discussion

Studies looking at the effect of cold on hemorheological parameters can be roughly divided into 2 parts; changes that happen due to cold exposure of living things (*in vivo*) and changes that happen due to cold exposure after the cells are taken outside of the body (*ex vivo*). *In vivo* studies usually observe changes arising from reactions in the body triggered by cold stress. *Ex vivo* studies, however, aim to observe effects of cold on the preservation of cells (eg, banked blood) or directly on cells (e.g., cell cultures). In this study, effects of *in vivo* and *ex vivo* cold exposure on hemorheological parameters, at various temperatures, were researched and compared to each other. It was determined that 2-hour *ex vivo* cold exposure did not have a significant effect on erythrocyte deformability; however, it was found that 2-hour *in vivo* cold exposure caused a high level of reduction in erythrocyte deformability, which was statistical significance.

Increase in noradrenaline and cortisol secretion due to cold has previously been demonstrated [4,27]. Stress hormones such as catecholamines and cortisol may be responsible for the hemorheological alterations during stress by an indirect effect on lipids [28]. The reduction observed

in RBC deformability in the 2-hour *in vivo* cold exposure group (mild hypothermia) may be related to the acute neuroendocrine-mediated response of the body, rather than a direct effect of cold on erythrocytes. This can explain our result of unaltered erythrocyte deformability in *in vitro* cold exposure groups. Although a more severe hypothermia was created (body and blood temperature 24°C, deep hypothermia), absence of alterations in RBC deformability in the 6-hour cold exposure group suggests that the body's reaction to stress emerges as an acute response, which is compensated within hours. Additionally, the absence of alterations in erythrocyte deformability in this group may be explained by the hemolysis of erythrocytes with altered morphology and decreased deformability caused by cold exposure over time [10].

In an *ex vivo* study by Berezina et al., in which banked blood was prepared according to standard procedures, it was demonstrated that RBC deformability at 4°C decreased significantly starting on the 14th day. The reduction in erythrocyte deformability was suggested to be related to abnormal shape changes in erythrocytes, which is also supported by electron microscopy findings that this develops in days, due to cold stress [29]. Uyuklu et al., using human blood, showed that there was no change in RBC deformability during 24 hours, measured as El max at 4°C; however, shear stress required for half-maximal deformation (SS1/2) increased after 8 hours. Increase in SS1/2 indicates reduction in erythrocyte deformability [12]. In our study, which is consistent with results from the previous study, the absence of a statistically significant change in erythrocyte deformability can be related to the shortness of the duration of exposure to *ex vivo* cold.

Another hemorheological parameter measured in our study was RBC aggregation. Erythrocyte aggregation is affected by erythrocyte membrane components and factors related to the plasma [30]. We showed that 2-hour *in vivo* cold exposure (mild hypothermia) does not cause any changes in erythrocyte aggregation. While deep hypothermia (ALTC), generated *in vivo*, does not cause a statistically significant alteration in aggregation amplitude (Amp) and aggregation half-time (t 1/2), it did cause a significant level of reduction in the aggregation index compared to AC and the control group. Where (AMP) is a static parameter measuring total extent of aggregation, t 1/2 is regarded as a kinetic factor in relation to the speed of aggregation. Al, however, can be evaluated as a function of both static and kinetic factors [23]. While the body and blood temperature decreased to ≅33°C in the AC group, in the ALTC group body and blood temperature decreased to ≅24°C. Advanced decrease in body and blood temperature may have caused the reduction in Al. In this study, since the rats were not kept in the refrigerator for a longer time than were rats in the *in vivo*, ALTC group, the results of our study do not show what kind of a change deeper hypothermia causes in erythrocyte aggregation.

Our findings indicate that *ex vivo* cold exposure does not cause any alterations in erythrocyte aggregation in 2 hours. Findings from Uyuklu et al indicate that *ex vivo* RBC aggregation at 4°C decreased only after 24 hours, which is consistent with our results [12]. In our study, in both *ex vivo* groups (BR and BC), aggregation amplitude and aggregation index, measured by LORCA (Laser assisted optical rotational cell analyzer), were lower compared to the AR and AC groups, and aggregation half-time (t 1/2) was higher. Results from the *ex vivo* groups, when the changes in aggregation parameters are considered together, indicate a reduction in erythrocyte aggregation. Uyuklu et al have showed that in human blood, erythrocyte aggregation at room temperature decreased 6 hours after the blood was drawn, measured as M index using a photometric aggregometer [12]. While their result is consistent with ours, in our experimental design it occurred much earlier. This difference may be due to different methods used and their sensitivity levels.

Berezina et al, using banked blood prepared according to standard procedures, demonstrated a reduction in erythrocyte aggregation starting on the 21st day; however, this alteration disappeared after the erythrocytes were washed [29]. In another study, also using banked blood prepared according to standard procedures, RBC aggregation was shown to decrease over time, although no alteration was observed in this parameter when erythrocytes were preserved in their own plasma [11], and the authors suggested that this result was due to factors related to the plasma, especially a change in fibrinogen concentration [11]. In an *in vivo* study using pigs, Martini et al reduced body temperature of the animals to 32°C (moderate hypothermia), and showed a decrease in fibrinogen concentration as degradation in plasma fibrinogen increased [31]. In our study, *in vivo* and *ex vivo* blood temperatures of all groups except AR and AC were found to be less than 32°C. Although plasma fibrinogen concentrations were not measured, based on findings from Martini et al., one can claim that the reduction in Al in ALTC, BR and BC groups may be due to the decrease in plasma fibrinogen concentration.

In our study, when *ex vivo* groups are compared amongst themselves, it was observed that an oxidant stress indicator, plasma TOS level, did not change after 2 hours of cold exposure; however, plasma TAS level increased with cold exposure. Cold exposure did not have a statistically significant effect on oxidative stress index (OSI), a parameter obtained by evaluating TOS and TAS levels together. This finding shows that *ex vivo* cold exposure does not affect oxidative stress. We found no studies in the literature that looked at the effect of 2-hour *ex vivo* cold exposure on plasma TOS, TAS and OSI levels. In the *ex vivo* study by Dumasvala et al, using banked blood prepared as standard procedures, erythrocyte glutathione level on the 42nd day was lower compared to day 1, and oxidized glutathione/glutathione ratio had increased [32]. In another study using banked blood, donor blood stored at for 4°C showed no change in erythrocyte malondialdehyde (MDA), glutathion peroxidase (GSH-Px) and superoxide dismutase (SOD) levels on days 4, 7, 14, 21, 30 and 42; glutathion (GSH) level decreased significantly, starting in the 3rd week [33]. The difference between these 2 studies and ours may be due to our study being much shorter, the difference in parameters evaluating the antioxidant system, and the difference in standard procedures blood was exposed to while preparing banked blood.

When *in vivo* cold exposure groups in our study were compared with each other, a statistically insignificant amount of increase was found in plasma TOS levels, depending on the length and intensity of cold exposure. Alterations in

plasma TAS and OSI levels are not statistically significant. When these 3 parameters are considered together, it was observed that *in vivo* exposure to cold at different temperatures did not cause any alterations in these parameters. This finding suggests that alterations observed in hemorheological parameters, in *in vivo* cold exposure groups, cannot be explained by oxidative stress.

Gamez et al. lowered body temperatures of rats to approximately 26.8°C (moderate hypothermia) during 30 minutes of cold exposure at 4°C, and demonstrated that antioxidant parameters such as erythrocyte Cu-Zn-SOD, Catalase, GSH-Px and total plasma sulfhydryl groups were significantly lower and plasma thiobarbituric acid reactive substances (TBARS) were higher compared to the control group [1]. In our study, although the duration of cold exposure was longer, no statistically significant alterations were observed, possibly due to the difference in parameters used in analyzing the oxidant-antioxidant system. In order to determine total oxidant-antioxidant capacity, we measured TOS and TAS levels. These 2 parameters allow us to determine all antioxidant capacity indicators such as bilirubin, uric acid, vitamin C, polyphenols, proteins, and oxidant stress products (eg, lipid peroxidation products and protein-SH groups), which were analyzed separately and partially in other studies [34]. As far as we know, our study is the only one in the literature measuring the effect of cold exposure on oxidant and antioxidant systems using plasma TOS, TAS levels and OSI parameters. On the other hand, Gamez et al used anesthetised rats, while conscious animals were used in our study. There may be a difference in the relative contribution of the regulatory nervous system and the endocrine system in conscious and anesthetised animals [35].

Our results indicate that *ex vivo* cold exposure causes a statistically significant amount of reduction in plasma TOS levels, compared to *in vivo* cold exposure groups (AC and ALTC). This finding suggests the contribution of hypothalamus and endocrine system-mediated mechanisms, triggered by stress *in vivo*, in order to increase TOS levels [4, 27]. Findings from this study indicate that *ex vivo* cold exposure does not change plasma TAS levels compared to *in vivo* exposure groups. Similarly, in the BC group, although a decrease in OSI was determined, this reduction was not found to be statistically significant. These findings indicate that the observed difference in RBC aggregation between *in vivo* and *ex vivo* cold exposure groups cannot be explained by alterations in oxidative stress.

Conclusions

The findings of this study indicate that mild hypothermia, achieved by 2-hour *in vivo* cold exposure, causes a reduction in erythrocyte deformability; however, in the deep hypothermia group, achieved by increasing the time of cold exposure, erythrocyte deformability returns towards control levels, possibly due to compensation of acute neuroendocrine response formed against stress. While *in vivo* mild hypothermia does not affect RBC aggregation, deep hypothermia causes decreased aggregation index. This can be explained as an adaptive mechanism developed to facilitate blood flow, as in the case of deep hypothermia. In the *ex vivo* cold exposure groups, while erythrocyte deformability did not change in 2 hours, erythrocyte aggregation was found to be lower compared to *in vivo* groups. The results of this study indicate that alterations observed in hemorheological parameters due to cold exposure do not seem to be mediated by the oxidative stress parameters measured herein. Further studies are required to elucidate the mechanisms responsible for the effects of hypothermia on hemorheological parameters.

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