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Oxidant/Antioxidant Status in Pneumatic Tourniquet-Induced Ischemia-Reperfusion in Meniscal Patients Undergoing Arthroscopy

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In orthopedic surgeons, tourniquets are commonly applied to reduce blood loss and obtain a clear surgical field. When the tourniquet is released, excessive formation of reactive oxygen species (ROS) follows during reperfusion and oxygenation. Glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), Cu-Zn superoxide dismutase (Cu-Zn SOD), catalase (CAT), xanthine oxidase (XO) activities, glutathione (GSH) and malondialdehyde (MDA) levels were investigated before arthroscopy (Baseline), before tourniquet release (BTR), after 1 h tourniquet release (ATR) in patients in pneumatic tourniquet-induced ischemia-reperfusion injury in arthroscopy. Erythrocyte GSH level, GST, SOD, CAT activities was significantly lower in BTR and ATR periods than in baseline. Erythrocyte and plasma MDA levels and erythrocyte XO activity were significantly higher in BTR and ATR periods than in baseline. Erythrocyte GSH-Px activity were significantly higher in BTR and lower in ATR group than in baseline. Erythrocyte and plasma MDA levels, erythrocyte GSH level, GST activity were significantly higher in ATR period when compared to BTR group. However, erythrocyte SOD, GSH-Px, CAT and XO activities were significantly lower in ATR period when compared to BTR period. The results obtained in this study indicate significant changes in antioxidant defense system in the patients in pneumatic tourniquet-induced ischemia-reperfusion injury in arthroscopy.

Key Words: Glutathione, Glutathione peroxidase, Ischemia-reperfusion injury, Malondialdehyde, Superoxide dismutase, Tourniquet, Xanthine oxidase, Free radical.

INTRODUCTION

Re-introduction of oxygen to an ischemic or hypoxic tissue has been shown to cause additional damage to the tissue (reperfusion injury). ROS that damage cellular components and initiate the lipid peroxidation

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process are known to be responsible for the ischemia-reperfusion injury[1,2]. Although skeletal muscle is thought to be relatively insensitive to the deleterious effects of ischemia and subsequent reperfusion, injury can occur as a result of ischemia.

Free radical scavengers or antioxidant enzymes and molecules represent an important component of body defenses against such free radical mediated injury. Superoxide dismutase (SOD), the first line of defense against oxygen-derived free radicals, catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2). H_2O_2 can be transformed into H_2O and O_2 by catalase (CAT). Glutathione peroxidase (GSH-Px) is a seleno-protein, which reduces lipidic or non-lipidic hydroperoxides as well as H_2O_2 while oxidizing glutathione[3-5].

Xanthine oxidase (XO) is the last enzyme of purine catabolism. It catalyzes conversion of xanthine and hypoxanthine to uric acid and the production of superoxide anion radical $O_2^{\bullet-}$, which is potentially toxic to cellular structures[6].

Lipid peroxidation is one of oxidative conversion of polyunsaturated fatty acids to products such as malondialdehyde (MDA), which is usually measured as thiobarbituric acid reactive substances (TBARS) or lipid peroxides, which is the most studied, biologically relevant, free radical reaction[5,6].

To our knowledge, there is simultaneously no available data study on erythrocyte glutathione (GSH) and GSH-dependent enzymes (glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), XO, CAT, Cu-Zn SOD activities and erythrocyte and plasma malondialdehyde (MDA) levels in pneumatic tourniquet-induced ischemia-reperfusion injury in arthroscopy. Therefore, in the present study, we aimed to investigate erythrocyte GSH, GSH-dependent enzymes activities (GSH-Px and GST), XO, CAT, Cu-Zn SOD activity and erythrocyte and plasma MDA levels in the patients in pneumatic tourniquet-induced ischemia-reperfusion injury in arthroscopy.

EXPERIMENTAL

All patients were recruited into the study after obtaining their formal consent. Twenty (17 males + 3 females) patients comprised the study group with the age range being 18-56 years (mean ± SD, 37.4 ± 10.1). Mean arthroscopy time was 65 min (range: 50-85 min). No patient was receiving vitamin supplements. Arthroscopy was performed in patients with meniscus.

On arrival in the operative room automatic non-invasive arterial pressure and electrocardiograph and pulse oximetry monitoring (Model 64S, Hewlett Packard, Germany) were commenced and an infusion of lactated Ringer's solution was started at a rate of 10 mL/h. All patients' blood pressure, heart rate and oxygen saturation were measured and recorded at every

5 min for duration of surgical procedure. In any patient, hypertension (a decrease in systolic blood pressure of 30 % below baseline value), bradycardia, (heart rate < 50 bpm) and desaturation (SpO_2 < 90 %) was not observed. With the patient in the left lateral position, a lumbar puncture was performed at the L3-4 or L4-5 interspaces through a midline approach with a 25G Quincke needle. After free flow of cerebrospinal fluid was obtained, 3 mL % 0.5 hiperbaric bupivacaine (Marcain Spinal Heavy, Astra Zeneca, Turkey) was injected. Then, the spinal needle was withdrawn and the patient was positioned supine. After adequate anesthesia was observed, the tourniquet was applied at a pressure approximately twice the systolic arterial pressure.

Blood samples were taken in vacutainer tubes with K_3-EDTA as anticoagulant (1/10, v/v), before arthroscopy (Baseline), before tourniquet release (BTR), after 1 h tourniquet release (ATR). They were centrifuged at 3000 x g for 15 min and plasma was removed by a Pasteur pipette. Then, erythrocytes were washed with 0.9 % NaCl solution three times and washed erythrocytes were hemolyzed by diluting with deionized water (50-fold). Hemoglobin (Hb) values of the samples were measured by a GEN-S counter hematology analyzer. The hemolyzate was kept in -80 ºC until biochemical determinations.

Biochemical measurements: MDA was determined on the basis of spectrophotometric absorbance measurement of the pink coloured product of the thiobarbituric acid-reactive substances complex[7]. Total thiobarbituric acid-reactive substances (TBARS) were expressed as MDA. Results are expressed as nmol/g Hb.

Cu-Zn SOD activity was detected by the nitroblue tetrazolium (NBT) reduction by $O_2^{\bullet -}$ generated by the xanthine/XO system[8]. Cu-Zn SOD activity is measured at 560 nm by detecting the inhibition of this reaction. By using a blank study in which all reagents present except the supernatant sample and determining the absorbance of sample and blank, the activity is calculated by difference. One Cu-Zn SOD unit is defined as the enzyme amount causing 50 % inhibition in the $NBTH_2$ reduction rate. Cu-Zn SOD activity was also expressed as U/mg Hb.

GSH-Px activity was measured according to the Paglia and Valentina method[9]. In this method, GSH-Px catalyzes the oxidation of glutathione in the presence of *tert*-butyl hydroperoxide. Oxidized glutathione is converted to the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to $NADP^+$. The reduction in the absorbance of NADPH at 340 nm is measured. By measuring the absorbance change per minute and by using the molar extinction coefficient of NADPH, GSH-Px activity is calculated. GSH-Px activity was expressed as IU/g Hb.

The assay principle for GSH is as follows: virtually all of the non-protein sulfydryl groups of erythrocytes are in the form of reduced glutathione. 5,50-Dithiobis (2-nitrobenzoic acid) (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl compounds to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration[10]. GSH level was expressed as μmol/g Hb.

GST activity was also assayed at 340 nm with 1,2-dichloro-4-nitrobenzene as substrate[11]. CAT activity was measured in hemolysates according to the Aebi method[12]. XO activity was determined by uric acid formation at 293 nm, as described[13]. Biochemical measurements were carried out using a CECIL CE 3041, (Cambridge, UK) spectrophotometer.

Statistical analysis: The findings were expressed as the mean ± SD. Statistical analysis was undertaken using the One-way Anova with post-hoc LSD test. A p value < 0.05 was accepted statistically significant. Statistical analysis was performed with Statistical Package for the Social Sciences for Windows (SPSS, version 10.0, Chicago, IL, USA).

RESULTS AND DISCUSSION

The results obtained in the study group were summarized in Table-1. As seen from Table-1, lipid peroxidation, measured by erythrocyte GSH level, GST, SOD, CAT activities was significantly lower in BTR and ATR periods than in baseline. Erythrocyte and plasma MDA levels and erythrocyte XO activity were significantly higher in BTR and ATR periods than in baseline. Erythrocyte GSH-Px activity were significantly higher in BTR and lower in ATR group than in baseline.

Erythrocyte and plasma MDA levels, erythrocyte GST activity were significantly higher in ATR period when compared to BTR group. However, erythrocyte SOD, GSH-Px, CAT, XO activities and GSH levels were significantly lower in ATR period when compared to BTR period.

Although skeletal muscle is thought to be relatively insensitive to the deleterious effects of ischemia and subsequent reperfusion, injury can occur as a result of prolonged ischemia. There have been some studies found in the literature concerning the local, systemic, metabolic and morphological effects of tourniquets on skeletal muscles[2,14,15]. For example, Concannon *et al.*[16] reported a significant increase in MDA production within 2 h in a rabbit model of tourniquet-induced skeletal muscle ischemia-reperfusion injury. We found that erythrocyte and plasma MDA level, which is an important indicator of oxidant stress, was significantly higher in both BTR and ATR periods than in baseline values. The present results for increase in MDA levels are in agreement with that obtained by Concannon *et al.*[16].

TABLE-1
MEAN ± SD OF GSH-Px, GST, CAT, SOD, XO ACTIVITIES, GSH, MDA LEVELS IN THE PATIENTS IN PNEUMATIC TOURNIQUET-INDUCED ISCHEMIA-REPERFUSION IN ARTHROSCOPY

	Baseline	BTR	ATR
p-MDA (nmol/mL)	7.90 ± 1.00	9.90 ± 1.3[c]	13.80 ± 2.8[d,f]
e-MDA (nmol/g Hb)	4.80 ± 1.10	5.80 ± 1.2[a]	7.20 ± 1.6[d,f]
SOD (U/mg Hb)	30.40 ± 10.30	21.90 ± 6.7[c]	14.60 ± 5.3[d,f]
GSH-Px (IU/g Hb)	55.50 ± 11.40	65.20 ± 12.8[b]	37.60 ± 10.0[d,h]
GST (U/g Hb)	22.50 ± 4.20	16.90 ± 3.5[d]	17.40 ± 5.4[d,g]
CAT (k/g Hb)	4.90 ± 1.00	4.00 ± 1.2[a]	3.30 ± 1.3[d,e]
XO (U/g Hb)	0.74 ± 0.16	1.21 ± 0.35[d]	0.95 ± 0.32[a,f]
GSH (μmol/g Hb)	132.40 ± 15.80	119.00 ± 12.0[b]	121.50 ± 16.3[d]

a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$, d: $p < 0.0001$, when compared to baseline group, e: $p < 0.05$, f: $p < 0.01$, g: $p < 0.001$, h: $p < 0.0001$, when compared to BTR group.
e-MDA: erythrocyte malondialdehyde, p-MDA: plasma malondialdehyde, Baseline: before induction of anaesthesia, BTR: before tourniquet release, ATR: after tourniquet release.

Damage from ROS has been documented in many different tissues after reperfusion, including skeletal muscle. Several studies have shown that ROS were produced during reperfusion of ischemia skeletal muscle and might be mediators of the resulting damage[2,14]. ROS are capable of reversibly or irreversibly damaging compounds of all biochemical classes, including nucleic acids, protein, free amino acids, lipids and lipoproteins, carbohydrates and connective tissue macromolecules. These species may impair cell activities such as membrane function, metabolism and gene expression. Propagation of this chain reaction results in a repeated chain reaction[6,17]. When the balance between ROS production and the antioxidative defense mechanisms is impaired, ROS levels may increase. When the ROS are not removed by natural scavengers, damage occurs through peroxidation of structurally important PUFAs within the phospholipid structure of the membranes[6,18]. Lipid peroxidation decreases both the fluidity and the barrier function of membranes, resulting in disturbances in structural organization, enzymic inhibition and possible cell death. In addition, lipid peroxides may inhibit protein synthesis, block macrophage function and alter chemotactic activity[6].

XO catalyzes the conversion of xanthine and hypoxanthine to uric acid with the production of $O_2^{\bullet-}$. In this regard, XO is a key enzyme between purine and free radical metabolism. There is growing evidence that $O_2^{\bullet-}$ generated by XO is primarily responsible for the cellular deterioration

associated with several conditions[6,19]. Grisham *et al.*[20] reported that reactive oxygen metabolites generated from XO and inflammatory leukocytes may play an important role in mediating mucosal injury during active episode of ulcerative colitis. Similarly, we found that an erythrocyte and plasma MDA level, which is an important indicator of oxidant stress, were significantly higher in both BTR and ATR periods than that of the baseline values. The erythrocytes cannot de novo synthesize XO. The blood flow increases to inflamed regions. Possibly, XO that was released by injured cells may be absorbed by erythrocytes, which may cause increased XO activity in erythrocytes.

SOD specifically quenches aberrant $O_2^{\bullet-}$. The role of SOD in tourniquet-induced ischemia-reperfusion injury is not yet clearly understood. It is possible that the decrease in SOD activity could be related to the increase in $O_2^{\bullet-}$ concentration produced by XO or from other sources. The decreased SOD activity will result in an increase in $O_2^{\bullet-}$ concentration[6]. SOD enzyme is the most important defense mechanism against the $O_2^{\bullet-}$ radicals producing in the cells. Increased $O_2^{\bullet-}$ radicals may be responsible for lipid peroxidation of cells membrane and increased MDA levels[21].

The statistically significant decreases in GST, GSH-Px and CAT activities and GSH level may show that the antioxidant system could not need the pneumatic tourniquet-induced ischemia-reperfusion injury in arthroscopy.

In conclusion, it is found that decrease in erythrocyte GHS level, GSH-Px, GST, CAT, SOD activities and increase in XO activity and erythrocyte and plasma MDA levels were present in patients exposed to the pneumatic tourniquet-induced ischemia-reperfusion injury in arthroscopy compared with the baseline values. The results suggest that oxidative stress in these patients causes the deficiencies in antioxidant enzymes and some other molecules, which arise as a result of enormous production of ROS in the system. Antioxidant supplementation may be given to improve the antioxidant defense system in such patients.

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