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DENEYSEL DONUK YARALANMASINDA AZALMIŞ SERUM NİTRİK OKSİT DÜZEYLERİ: Ön Çalışma

DECREASED SERUM NITRIC OXIDE LEVEL IN EXPERIMENTAL FROSTBITE INJURY: A Preliminary Study

Mustafa TERCAN[1], Yusuf TÜRKOZ[2], Mustafa ÇEKMEN[3], İrfan ÖZYAZGAN[4], Cem EVEREKLİOĞLU[5], Mehmet BEKERİCİOĞLU[1]

[1]Department of Plastic and Reconstructive Surgery, School of Medicine, Gaziantep University, Gaziantep.
[2]Department of Biochemistry, Turgut Ozal Medical Center, İnonu University, Malatya.
[3]Department of Biochemistry, School of Medicine, Gaziantep University, Gaziantep.
[4]Department of Plastic and Reconstructive Surgery, School of Medicine, Erciyes University, Kayseri.
[5]Department of Oftalmology, School of Medicine, Gaziantep University, Gaziantep.

ABSTRACT: The pathogenesis of frostbite, a kind of ischemia-reperfusion injury, is not clear in spite of various researches. The present study was aimed to assess the determination of nitric oxide in frostbite injury. Frozen rabbit ears were used in this study. Serum nitric oxide levels were measured. Eight New Zealand albino rabbits were subjected to the study. The right ears of rabbits were immersed into ethyl alcohol 100 % at - 20° Celsius and the left ears were not subjected to the cold injury. Blood samples were taken from vein of the frozen ears at re-warming period and at the 4th hour. The control blood samples were taken from the left ears. Decreased nitric oxide levels were determined at the 4th hour in blood samples of the frozen ears ($p<0.05$).
These data suggest that nitric oxide may have an important role in frostbite.
[Key words: Nitric oxide, Frostbite injury, Ischemia-reperfusion]

INTRODUCTION

Pathogenesis of frostbite has not yet completely enlightened though lots of experimental study have been reported. Reperfusion injury has been blamed as one of the deleterious mechanism. Robson and Hegger have reported the high level of PGF_2 and TxB_2 in bulla fluid as vasoconstrictor and aggregan (1). Increased PGI_2 and TxB_2 metabolites in frozen tissue were reported recently (2). Various treatment modalities have been offered based on these mechanisms (3,4,5,6). But, none of them have useful enough to practical approach to frozen tissue. We investigated possible role of nitric oxide (NO) on the pathogenesis of frostbite injury. NO, first identified as an endothelium derived relaxation factor (EDRF), is now recognized as a regulatory of many mammalians cell and tissue functions. It is synthesized via the oxidation of arginine by a family nitric oxide synthesis (NOS), which are either calcium-dependent (constitutive) or calcium-independent (inducible). The endogenous production of NO plays a vital role in regulating physiological processes, e.g. blood vessel tone and neurotransmission. NO mediates vasodilatation and attenuates vasoconstriction in vitro. NO is a unique biologic messenger molecule. (7). NO mediated responses are impaired in animals and humans with chronic hypoxia (8,9,10).

MATERIAL AND METHODS

Eight New Zealand albino rabbits, weighing 1645-2415 gm, were subjected to the

study. Anaesthesia was achieved with Ketamin hydrochloride (80 mg/kg) and Xylasine (9 mg/kg) intramuscularly. After dry shaving, both ears were marked 8 cm proximally from the tip perpendicularly. The area beyond this line was drawn on transparent acetate. A thermocouple probe (Greisinger GTH 1160) was placed 2 cm away from the tip of ear. Temperatures were recorded before the procedures. Afterwards, the rabbits right ears were immersed in a container filled with 100 % ethyl alcohol at - 20 ° C up to the mark. Temperatures were recorded every 15 seconds throughout the procedure. The ears were removed from the ethyl alcohol 4 minutes later and left for thawing at room temperature spontaneously. Then, the temperatures were recorded every 1-minute during thawing and rewarming. The opposite ears were used as control. Rectal temperatures were also recorded during procedure.

Blood samples were taken from frozen ears during the rewarming period and at the 4th hour after thawing. Control blood samples were taken from opposite ears.

Viability was evaluated on the frozen part of ears.

Nitrite analysis: Nitric oxide was quantified by measurements of the NO metabolite, nitrite, using the Greiss reagent as describe before (11). In short, serum (250 µl) was incubated at room temperature with 250 µl of substrate buffer (imidazole 0.1 mol/l, nicotinamide adenindinucleotide phosphate 210 µmol/l, flavineadenine dinucleotide 3.8µmol/l pH:7.6) in the presence of nitrate to nitrite. Excess reduced nicotinamide adenine dinucleotid phosphate, which interferes with the chemical detection of nitrite, was oxidized continuation of the incubation of 5 µg (1µl) of LDH (Sigma), 0.2 mmol/L (120 µl) pyruvate (Sigma) and 79 µl of water. Total nitrite was then analysed by reacting the samples with Greiss reagent (1% sulfanilamide, 0,1 % naphthalene-ethylene diamine dihydrochloride in 5 % H_3PO_4 spectroquant: Merck, Darmstadt, Germany). Reacted samples were treated with 500 µl of trichloroacetic acid (20%), centrifuged for 15 min at 8000 g and the absorbance at 548 nm. It was compared with that of $NaNO_2$ standard (0-100µmol/L).

Assessment of Viability: Necrotic areas of frozen tissue have become clear and detached in 20 days. Viable skin areas of the ear were drawn on the acetate that was prepared before the procedure. Both frozen part of ear and viable areas of them were drawn and cut on the cardboard bye using the acetate. Formed pattern of cardboards were weighed in balance by 0.0001 gm sensitivity so that percentile of viable part were determined (12).

Statistical Analysis: The related groups were compared by Wilcoxon signed sample test.

RESULTS

Rectal temperature of rabbits did not change throughout the experiment. Oedema and hyperaemia were seen on the first day of frostbite. Dry necrosis was seen at the 5th day after the frostbite. Necrotic part of the tissue detached after the 15th day. There was no difference between the groups ($p>0.05$). There were no statistically differences between the rewarming and control group as to NO levels ($p>0.05$). NO levels at the 4th hour were significant statistically ($p<0.05$)(Table-I). There was no correlation between the NO levels and rabbit weights.

DISCUSSION

Although the frostbite injury is not common, lots of experimental researches have been performed. Wheather-White and his colleagues have shown the viability of the frozen skin graft after placing on the healthy host tissue so that they believed the reversal of frostbite injury (13). In our study, we waited the re-warming of the rabbit ear after completion of the frostbite. We did not use the rapid re-warming in order to mimic clinical conditions. In fact, clinical cases are generally encountered during the re-warming period (14-15). Some studies were also performed in the

room temperature during the re-warming period on the frostbite injury (16).

In our study, NO levels decreased at the 4th hour according to the re-warming and control samples ($p<0.05$). Decreased NO levels and its relation with necrosis in the frostbite injury may be explained as follows.

NO synthesis is dramatically increased after IL-2 treatment (17,18). It is reported that hypothermia decreases the immune response by decreasing IL-1α and IL-2 (19). Decreased IL level is able to depend on the decreased NO level in frostbite injury.

Endothelial cells are the most sensitive cells to the frostbite injury (20). Endothelium originated NO is one of the important substances and maintains vascular blood flow and pressure (21,22). NO synthesis of endothelium may be decreased by frostbite.

NO prevents the adhesion and aggregation of the platelet (23,24,25). In frostbite injury, both direct (cellular) and indirect injuries occur by means of thrombosis and ischemia. It is reported that circulation continues during the re-warming period (26,27,28). Then, vascular stasis occurs and vessels are occluded by thrombosis. Similar changes were reported ischemia-reperfusion injury. It is reported that the reperfusion of ischemic myocardium precedes the enhanced PMN adherence to the endothelial surface and decreases the NO release (29). Decreased NO may be responsible of tissue injury in frostbite by increasing the adherence of PMN cells. In the pathogenesis of the frozen tissue necrosis, low level of NO may be a contributor in addition to the increased aggregation and adhesion of platelet.

It is also reported that low level of NO has 3000-fold affinity to Hb more than O_2 (30). Decreased NO may cause tissue hypoxia that leads to tissue necrosis.

Nowadays, treatment of frostbite is based on the increased level of $PGF_2\alpha$ and Tx_{B2} in bulla fluid, which was shown by Robson and Hegger (1). Raine and his colleagues used anti-prostaglandin agents and stasis-inhibitors for four day in the frostbite injury (16). NO strongly interacts with the cyclo-oxygenase pathway and reduces the prostanoid synthesis and pressure effect of nitric oxide synthesis inhibitor (L-NAME) partly relies upon the vasoconstrictor effect of TxA_2 and PGH_2 (31).

Decreasing the adhesion of PNL on endothelium and count of leukocyte by means of PGI_2 has been reported (32,33).

First effect of PGI_2 is to increase the microcirculation in the frozen tissue. This hypothesis has been supported in ischemic flap model (34). It was shown that inhibiting adhesion and aggregation of PNL by using antibody minimised the frostbite injury by Mileski and his colleagues (35). It is important the count of PNL because of their contribution on producing free radicals, releasing protease and deleterious effects on healthy tissue (36,37). It was shown that exogenous intra-arterial injection of PGI_2 increased the survival of axial pattern flaps (38). In addition, TxA_2 synthesis is decreased by PGI_2 treatment (39).

Large amount of NO is syntheses by macrophages (40,41). Increased macrophages were reported in frozen tissue at the 3rd days of frostbite injury (2). Increased macrophage count in frozen tissue may be a salvageable mechanism of frostbite injury in order to increase NO synthesis.

In conclusion, NO may play an important role on the pathogenesis frostbite injury. We designed a new study to clarify which mechanism is essential on frostbite injury.

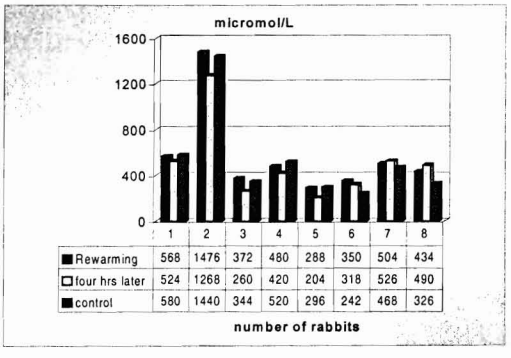

Table I: Serum nitric oxide levels (μmol/L)

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AUTHOURS:
M. TERCAN: Medical School of Gaziantep University, Department of Plastic and Reconstructive Surgery, GAZİANTEP.
Y. TÜRKOZ: İnonu University Turgut Ozal Medical Center, Department of Biochemistry, Malatya.
M. ÇEKMEN: Medical School of Gaziantep University, Department of Biochemistry, GAZİANTEP
İ. ÖZYAZGAN: Medical School of Erciyes University, Department of Plastic and Reconstructive Surgery, KAYSERİ
C. EVREKLİOĞLU: Medical School of Gaziantep University, Department of Oftalmology, GAZİANTEP
M. BEKERİCİOĞLU: Medical School of Gaziantep University, Department of Plastic and Reconstructive Surgery, GAZİANTEP.

ADRESS FOR CORRESPONDENCE:
Mustafa TERCAN, Medical School of Gaziantep University, Department of Plastic and Reconstructive Surgery, GAZİANTEP