The correlation between renal tissue oxidative stress parameters and TNF-α levels in an experimental model of ischemia-reperfusion injury in mice

Sıçanlarda iskemi-reperfüsyon modelinde böbrek dokusundaki oksidatif stres parametreleri ve TNF-α seviyelerinin ilişkisi

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BACKGROUND

To investigate the relationship between oxidative stress and pro-inflammatory response of the renal tissue at different ischemia times in I/R mice.

METHODS

Twenty-four male Swiss dormice were subjected to 30, 45 or 60 minutes of ischemia and then 60 minutes of reperfusion (Group 1 I 30/R60, Group 2, .I45/R60, and Group 3, I 60/R60 respectively). As markers of oxidative stress and antioxidant activity, levels of thiobarbituric acid reactive substances (TBARS), protein carbonyls and protein sulfhydryls (SH), tissue superoxide dismutase (SOD) and catalase (CAT) activities of the renal tissue and also renal tissue pro-inflammatory marker TNF-a levels were assessed.

RESULTS

The levels of TBARS and protein carbonyl rised in I30/R60 group (p=0.01). In I45/R60 group, levels of TBARS, protein carbonyls and TNF-α levels were significantly higher while SOD and CAT activities (p=0.01), and the levels of SH were significantly decreased (p=0.05). These findings were more relevant for I60/R60 group. Higher TNF-α levels correlated positively with higher levels of TBARS, and protein carbonyls and negatively with SOD, CAT and renal tissue SH (p=0.001).

CONCLUSIONS

In mice, oxidative stress after 45 minutes ischemia and 60 min reperfusion could induce pro-inflammatory cascade mediated through TNF-α.

Key Words: Renal ischemia- reperfusion, oxidative stress, TBARS, Superoxide dismutase, Catalase, sulfhydryl, carbonyl, TNF-α.

AMAÇ

Amacımız farklı iskemi ve ardından uygulanan reperfüzyon sürelerinde oluşan böbrek I/R modelinde oksidatif stres ile pro-inflamatuar yanıt arasındaki ilişkiyi araştırmaktı.

GEREÇ VE YÖNTEM

Yirmi dört erkek İsviçre fındık faresi 30,45,60 dakikalık sürelerde iskemi ve ardından 60 dakikalık reperfüzyona maruz bırakılmıştır (sırasıyla Grup 1, I30/R60; Grup 2, I45/R60; Grup3, I 60/R60). Oksidatif stres ve antioksidan aktivite belirteci olarak böbrek dokusunda tiobarbitürik tiyobarbitürik asit reaktif madde (TBARS), protein karbonil ve sülfidril (SH), doku süperoksit dismutazı (SOD) ve katalaz (CAT) aktivitesi çalışıldı. İskemi / reperfüzyona pro - inflamatuar yanıt olarak böbrek dokusu TNF-a düzeyleri araştırıldı.

BULGULAR

Oksidatif stres ve antioksidan aktivite belirteci olan TBARS ve protein karbonil düzeyleri I30/R60 grubunda artmıştır (p=0,01). I45/R60 grubunda ise TBARS ve protein karbonil ve TNF-a düzeyleri anlamlı derecede artarken SOD ve CAT (p=0,01) aktivitesi ve SH düzeylerinin anlamlı olarak azalmıştır (p=0,05). Bu bulgular I 60/R60 grubunda daha belirgindi. TNF-α düzeyindeki artışın oksidatif stres belirteçleri olan TBARS ve protein karbonil düzeyleri ile pozitif ve antioksidan aktivite belirteçleri olan SOD, CAT ve renal doku SH düzeyleri ile negatif ilişkisi olduğu belirlendi (p=0,001).

SONUÇ

Çalışmamızda fındık farelerinde 45 dakikalık iskemi ve ardından 60 dakikalık reperfüzyonun pro-inflamatuar süreçler zincirini TNF-α üzerinden indükleyebildiği sonucuna vardık.

Anahtar Sözcükler: Renal iskemi reperfüzyon, oksitadif stres, TBARS,, süperoksit dismutaz, katalaz, sülfidril, karbonil, TNF-α.

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RESULTS

Renal failure as a consequence of ischemia/reperfusion disorders is of particular relevance to transplantation[1] coronary bypass surgery[2] aortic cross-clamping[3], shock[4] accidental/iatrogenic trauma[5] and sepsis[6] and remains a major cause of morbidity and mortality (30%)[7] among patients in intensive care units. Several studies revealed that an inflammatory response induced by ischemia is largely responsible for functional organ failure and organ damage.[8,9] The acute inflammatory response initiated by I/R is characterized by the induction of a pro-inflammatory cytokine cascade.[10,11]

Tumor necrosis factor alpha (TNF-α) is a main indicator and also found to be a triggering factor in apoptosis and inflammation. Previously, the presence of detectable TNF-α plasma levels and histological damage and increased neutrophil sequestration in the liver as well as TNF-α mediated remote pulmonary injury were demonstrated.[12]

The plasma membrane of the stimulated neutrophils produce reactive oxygen species (ROS)[13,14] whereas the specific granules contain microbicidal peptides, proteins and enzymes.[15] The oxidative burst results in a sequential production of ROS and the antioxidant activity, and protects the individual organs such as renal tissue from the effects of these sequential production of ROS. However, the balance between oxidative stress and antioxidant activity may change during the process of the diseases. When oxidative stress and inflammation trigger each other, dysfunction of any organ system as well as renal tissue may ensue.[8,9,12-15]

The aim of this study is to obtain the levels of oxidative stress and antioxidant activities in different durations of renal I/R injury and to evaluate the correlation between oxidative stress markers and TNF-α.

MATERIALS AND METHODS

Twenty-four male dormice weighing 35-40 g purchased from Selcuk University, Experimental Research Laboratories (Konya, Turkey) were included in this study. Animals were housed in standard laboratory cages and were allowed free access to food and water till 12 hours before the surgical procedure.

Experimental design. This study was designed as an experimental, randomized, controlled trial with blind assessment of the outcome. All procedures mentioned were approved by the local ethics committee. The study protocol was designed in accordance with 1996 revised form of The Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Unnecessary animal suffering was avoided throughout the study. The mice were randomly assigned into one of the 4 groups listed below:

Sham group (n=6): Mice underwent same surgical procedure as animals in study groups except for the clamping of renal pedicle. Groups 1 (n=6), 2 (n=6), and 3 (n=6) were exposed to ischemia:- reperfusion for various periods of time (30-60, 45-60, 60-60 minutes respectively)

Surgical procedure. The mice were anesthetized with 100 mg/kg ketamine (Ketalar amp, EIP, Istanbul, Turkey). A rectal probe was placed to monitor the body temperature. A heating pad was applied until animals recovered from anaesthesia in order to keep the body temperature at 39°C. Under aseptic conditions, a 1-cm-long midline abdominal incision was made. After the exposure of the right kidney, ischemia was induced by applying a non-traumatic vascular clamp to the left renal pedicle for 30, 45, 60 minutes in Group 1, 2, 3 respectively. After 3 minutes the kidney was inspected for signs of ischemia; the wound was covered with cotton soaked in sterile phosphate buffered saline. After removal of the clamp, the left kidney was inspected for restoration of blood flow for 60 min and mice in all groups were sacrificed and left kidney was harvested and stored for further analysis.

Tissue Homogenization. A portion of each renal tissue was homogenized. Homogenization was performed in 1:10 (w:v) 0.1 M potassium phosphate buffer (pH=7.4) with a Ultra Turrax homogenizer (IKA T18 basic, Wilmington NC, USA). After centrifugating the homogenates at 10000 rpm, and + 4°C for 10 min, the supernatants were removed and analysed.

Biochemical Analysis. The supernatants of homogenized tissue samples were analyzed to determine tissue concentrations of thiobarbituric acid reactive substances (TBARS), protein carbonyl and protein sulfhydryl (SH) as well as superoxide dis-

mutase (SOD) and catalase (CAT) activities. Biochemical analyses were also realized in blind manner, and the results were reported in relation to the code numbers of the samples. The end product of lipid peroxidation, TBARS, were measured using thiobarbituric acid method so as to assess the degree of renal tissue lipid oxidation.[15] To evaluate the potential for oxygen radical scavenger activity in renal tissue, protein SH groups were measured spectrophotometrically using Ellman's reagent This spectrophotometric analysis is based on the interaction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) with the thiol-disulfide.[15] Protein oxidation in the renal tissuewas assessed with a colorimetric assay that measured protein carbonyl content, through the interaction of the supernatant with dinitrophenylhydrazine, as described by Levin et al.[18] Renal tissue protein SH and carbonyl contents were expressed in relation to the tissue protein concentration that was measured using the Biuret method.[19] Activities of superoxide dismutase (SOD) and catalase (CAT) were measured as further indicators of tissue antioxidant activity against oxygen radicals, using the methods described by Winterbourn et al. and Beutler, respectively.[20,21] TBARS, SH and carbonyl levels were expressed in terms of the protein concentration of renal tissue samples, micromoles per mg protein (μM/mg protein), whereas SOD and CAT activities were expressed as units per mg of protein (U/mg protein). TNF-α levels were determined by using a commercially available mouse ELISA kit (MedSystems Diagnostics GmbH, Vienna, Austria) and the results were expressed as picogram per mg of protein (pg/mg protein).

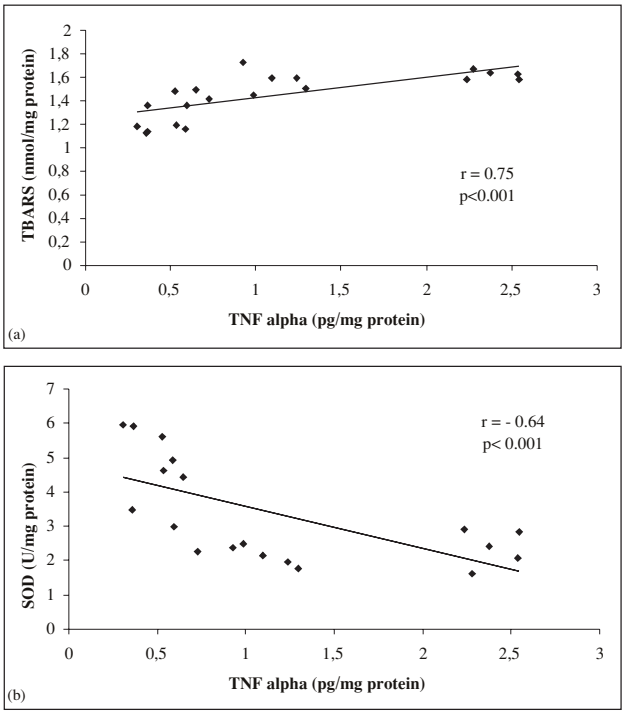
Statistical Analysis. The results were decoded and statistically analyzed. Data were expressed as the mean±standard error (SEM) of the means. Statistical analysis was performed using Kruskal Wallis and Mann Whitney- U test. Correlation and regression analysis were performed for TNF-α levels and SOD, CAT activities, TBARS and carbonyl levels. A p <=0.05 was considered to be significant.

RESULTS

Results are summarized in Table I. Oxidative stress has been initiated after 30 minutes of ischemia and 60 min of reperfusion and consistently became more significant after longer ischemic insult with the associated increased levels of TBARS and carbonyl levels when compared to sham-operated animals. In I45/R60 group, SOD and CAT activities were detectedly decreased when compared to sham-operated animals (p=0.01). Protein SH levels also decreased (p=0.05). On the other hand, TBARS and protein carbonyls increased significantly when compared with those in the sham-operated animals (p=0.01). TNF-α levels increased significantly in I45/R60 Group and reached maximum levels in I60/R60 Group (p=0.01). However SOD and CAT activities and protein SH levels were detected to be slightly decreased whereas TNF-α levels, as a marker of pro-inflammatory response slightly increased after 30 minutes of ischemia and 60 minutes of reperfusion. But no statistical significance was detected.

No statistically significant changes were observed in protein SH levels between groups exposed to different durations of ischemia. But in Group 2 and Group 3, SOD and CAT activities were detected to be decreased when compared with those

Figure 1: The correlation between TBARS and TNF-α levels (a), SOD activity and TNF-α levels (b). A positive correlation between TNF-α levels and TBARS levels was found(r = 0.75, p< 0.001) (a), whereas a negative correlation was observed between TNF-α levels and SOD activities (r = - 0.64, p< 0.001) (b).



found in Group I (p=0.05). On the other hand; in Group 3, TBARS and protein carbonyl levels were found to be significantly higher than those in Group 1 (p=0.01).In Group 3 TNF-α levels were significantly higher than those found in Group 1 and Group 2 (p=0.01).

There was a positive correlation among TNF-α TBARS (r = 0.75, p=0.001) (Fig 1-a), and carbonyl levels (r = 0.90, p=0.001) whereas a negative correlation was observed between TNF-α levels and SOD (r = - 0.64, p=0.001) (Fig 1-b), CAT activities (r = - 0.75, p=0.001), SH levels (r = - 0.71, p=0.001).

DISCUSSION

Activated neutrophils with their released substances protect the body and other organ systems from pathogens and adverse effects of inflammation, renal I/R conditions, hypovolemic shock or sepsis. ROS and TNF-α are related with activated neutrophils. Both of them are useful since they remove the pathıogens and necrotic debris. Nevertheless, they should be bufferred with anti-inflammatory cytokines and anti-oxidants. If this equlibrium fails, oxidative stress and deleterious effects of inflammation could proceed.[22]

The available evidence thus suggests a positive-feedback-cycle-type relationship between CFR and oxidative stress, each being capable of aggravating the severity of the other.[23] This cycle possibly operates relentlessly until complete loss of functional renal tissue. This cycle is fueled by the perpetuation potantial of the oxidative stress, via the modification of structural and functional macromolecules, including lipids, nucleic acids and proteins, and the activities of ROS. Superoxide anions, hydrogen peroxide, hypochlorous acid and singlet oxygen are the major ROS components.[24]

TBARS are indicators of the oxidative stress, since they are generated from the breakdown of lipid peroxyl radicals.[25] Another indicator of oxidative stress is the carbonyl content of proteins which are the oxidation products of specific amino acid residues. On the other hand, the SH groups of proteins like albumin, are considered to represent 'antioxidant' activity by functioning as 'sacrificial' antioxidants in extravascular spaces.[26]

In addition, the activities of SOD and CAT enzymes also contribute to oxygen-radical-scavenger-activity. Kidney tissue contains 2 types of SOD, namely cytoplasmic and mitochondrial SOD. Renal subcellular organelles also contain CAT and peroxizomes. SOD transforms the initial ROS produced (i.e. the superoxide anion) into the less reactive molecule, hydrogen peroxide (H_2O_2), which is more stable than superoxide, but still capable of damaging cell membranes. CAT acts on H_2O_2 and converts it to water. If this conversion fails, H_2O_2 can lead to the emergence of two other ROS, and, via the interactions between ionic chloride and H_2O_2 (hypochlorous acid), and also between hypochlorous acid and H_2O_2, (singlet oxygen).

Thus, increased TBARS and protein carbonyl content, the loss or oxidation of protein SH groups, and decreased SOD and CAT activities, all provide physiologically relevant estimates of oxidative stress in tissues. These parameters were used as the markers of oxidative stress in renal tissue in our experimental study.

The role of TNF-α in septic shock in both experimental models[7,27,28] and clinical medicine[29] has been a subject of great interest. TNF-α appears to be involved in many types of inflammatory processes and has recently been detected in specific disease states.[1-9,29] In some cases, an increased level of TNF-α has been correlated with the severity of the disease.[7,27-29] Intravenously injected recombinant TNF-α is known to cause pulmonary hemorrhage and edema.[6,27] Colletti et al demonstrated that TNF-α is produced after lobar hepatic I/R. They also reported that subsequent pulmonary neutrophil sequestration and hemorrhagic edema were completely abolished with the pretreatment of neutralizing anti-TNF-α anti-serum.[12] TNF-α is also established as the main mediator of renal I/R injury and associated with endotoxemia.[30] Consequently inflammatory response is orchestrated by cytokines and TNF-α is one of the main pro-inflammatory cytokines.[6,12,26-30]

Under the view of these considerations we hypothesized that the duration of ischemia might effect the oxidative stress and correlated pro-inflammatory cytokines in I/R model in mice.

We found that the markers of oxidative stress such as TBARS and carbonyl content of proteins

increased after 30 min of ischemia and 60 min of reperfusion while anti-oxidative activity markers such as SOD, CAT activities and SH levels of renal tissue proteins gained importance when ischemia time reached to 45 minutes.

Transplantation, coronary bypass surgery, aortic cross-clamping, and shock may result in I/R abnormalities which can induce inflammatory responses.[1-16,26-30] This response characterised among by the induction of a pro-inflammatory cytokine cascade. Our study may be the first one addressing the determination of this relation between oxidative stress and pro-inflammatory cytokine (i.e. TNF-α) responses in I/R model with different durations of ischemia. Thirty minutes of ischemia resulted in the increase of oxidative stress with increased levels of TBARS and protein carbonyls. But the changes in the levels of TBARS and protein carbonyls have been correlated with the induction of pro-inflammatory cascade after 45 minutes of ischemia and 60 minutes of reperfusion while they were not correlated with this cascade after 30 minutes of ischemia and 60 minutes of reperfusion. Forty five minutes of ischemia and 60 minutes of reperfusion significantly changed the oxidant - anti-oxidant balance negatively. Pro-inflammatory cytokine response induced with TNF-α was more relevant for I60/R60 models.

Takada[8], Vedder[9] and their associates showed that inflammatory responses induced by ischemia are largely responsible for the functional organ failure and organ damage. It seems to be more rational to remain under 45 minutes of renal ischemia with I/R conditions like transplantation, coronary bypass surgery, aortic cross-clamping, shock, trauma and sepsis in order to prevent the induction of inflammatory cascade and oxidative stress.

Our findings suggest that 45 minutes of ischemia in renal tissue and oxidative stress might induce pro-inflammatory cascade through TNF-α. The increase in TNF-α levels correlated positively with oxidative stress markers like TBARS, and protein carbonyls, and negatively with the alterations in the levels of markers of antioxidant activity like SOD, CAT and renal tissue protein SH after 45 minutes of renal ischemia in I/R murine model.

In conclusion, the oxidative stress induced by ischemia lasting 45 minutes or longer in renal I/R models could be correlated with the induction of pro-inflammatory cascade mediated by TNF-α.

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