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Protection of rat renal vitamin E levels by ischemic-preconditioning

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Abstract

Background: During renal transplantation, the kidney remains without blood flow for a period of time. The following reperfusion of this ischemic kidney causes functional and structural injury. Formation of oxygen-derived free radicals (OFR) and subsequent lipid peroxidation (LP) has been implicated as the causative factors of these injuries. Vitamin E is known to be the main endogenous antioxidant that stabilizes cell membranes by interfering with LP. The present study was designed to examine the role of ischemic-preconditioning (repeated brief periods of ischemia, IPC) in prevention of renal injury caused by ischemia-reperfusion (IR) in rats.

Methods: IPC included sequential clamping of the right renal artery for 5 min and release of the clamp for another 5 min for a 3 cycles. IR was induced by 30 min ischemia followed by 10 min reperfusion. Four groups of male rats were used: Control, IPC, IR and IPC-IR. Vitamin E, an endogenous antioxidant and as an index of LP, was measured by HPLC and UV detection in renal venous plasma and tissue. Renal function was assessed by serum creatinine and BUN levels. Renal damage was assessed in sections stained with Haematoxylin and Eosin.

Results: In the IR group, there was a significant decrease in vitamin E in plasma and tissue compared to a control group (p,0.05). In the IPC-IR group, vitamin E concentration was significantly higher than in the IR group (p,0.01). The results showed that 30 min ischemia in the IR group significantly (p,0.05) reduced renal function demonstrated by an increase in serum creatinine levels as compared with the control group. These results in the IPC group also showed a significant difference with the IR group but no significant difference in serum BUN and creatinine between IR and IPC-IR group were detected. Histological evaluation showed no structural damage in the IPC group and an improvement in the IPC-IR group compared to IR alone.

Conclusions: In this study, IPC preserved vitamin E levels, but it could not markedly improve renal function in the early phase (1–2 h) of reperfusion. IPC may be a useful method for antioxidant preservation in organ transplantation.

Background

In recent years, numerous studies have been conducted on the deleterious effects of reperfusion (R) following ischemia (I) in different organs including the kidney [1] in which OFR are known to be the probable cause of tissue injury and the organ's dysfunction [2]. There are also

many reports about the usefulness of antioxidant drugs [3] as well as the application of methods such as ischemic preconditioning (IPC) in the prevention of oxidative stress induced-injury [4].

Considering the discrepancy mentioned in previous reports, studies have attempted to use different preconditioning methods to investigate various aspects of IPC effects on the kidney.

Vitamin E is known to act as a chain breaking antioxidant that prevents the propagation of free radical reactions. It is proved to be effective in preventing lipid peroxidation and the other radical-driven oxidative events.

The present study was conducted to evaluate the impact of IPC (sequential brief cycles of ischemia-reperfusion) on renal oxidative stress status by measurement of vitamin E levels in vivo in rat plasma & renal tissues. Renal function was evaluated by serum creatinine and BUN measurements.

Methods

Chemicals and solutions

Alpha (α)-tocopherol and α-tocopherol acetate were used as external and internal standards respectively. Butylated hydroxy toluene (BHT), sodium dodecyl sulfate (SDS), HPLC – grade reagents (acetonitrile, methanol, ethanol and hexane) were purchased from Merck (Darmstadt – Germany). Collagenase and protease K were from Roche (Germany).

Instruments

Reversed – phase high – performance liquid chromatography (HPLC) was from Waters – Millipore Associates Inc. (Milford – mass), including a solvent – delivery pump (600 E), a Nova pack C18 guard column, a Nova pack stainless steel column filled with ultra sphere octa desylsilyl (ODS) 3.9 × 300 mm)(4 μm), a UV-detector (486), and an integrator (746). The UV-3100 spectrophotometer was used to calculate the concentration of vitamin E (α-tocopherol) standards at 292 nm. BUN and creatinine concentrations were analyzed by a spectrophotometer using a 704 Hitachi analyzer at 520 for BUN and 505 nm for creatinine.

Solutions

The concentrated stock solutions of α-tocopherol (5 mM) and α-tocopherol acetate (15 mM) were prepared in 100 % ethanol and stored at -20°C, and protected from light. They were discarded when the spectrophotometric absorbance showed a decrease in concentration [5]. Five % EDTA solutions were made in order to prevent blood coagulation in the syringe. Collagenase and protease solutions were made by adding 10 ml of cold PBS (phosphate buffered solution) to 500 mg of collagenase powder and 200 mg of protease powder respectively. They were mixed gently and stored at -20°C until use. SDS-ethanol-BHT solution was made by adding 1 ml of a 20% SDS-H2O stock solution to 19 ml of ethanol, vortex mixed and stored at room temperature for up to 5 days. BHT (0.1% W/V) was added to it immediately before use [4]. The mobile phase was made by mixing methanol, acetonitrile, and hexane in the following ratio (85: 10: 5, v/v/v). BHT solutions were prepared in ethanol (0.125%) and in hexane (0.025%) [5].

Experimental procedures

In this study, 4 groups consisting of control (sham-operated), ischemia-reperfusion (IR), ischemic preconditioning (IPC), and IPC-IR were used. Each group included 7 male Sprague-Dawley rats with the weights of 220–300 g. Rats had free access to water and food. Ketamine HCl (50 mg/kg) and chlorpromazine HCl (25 mg/kg) were injected intraperitoneally for anesthesia. The surgical protocol was approved by Tehran University of Medical Sciences Ethical Committee.

Control group

After anesthesia, these animals had only an abdominal incision. At 70 min (for temporal adjustment with other groups), two blood samples were collected from the renal vein. One sample was centrifuged at 3000 g, 10 min, for serum creatinine and BUN measurements and was analyzed immediately after collection. The other blood sample was centrifuged at 3000 g for 20 min, then aliquots of plasma were transferred into 2 ml test tubes and stored at -20°C until further analysis. After separating capsules and fats, the right kidneys were halved, one half was washed in cold PBS and stored at -20°C until vitamin E measurements were obtained, and the other half was fixed in 10% formalin for histological evaluations.

IR group

30 min after anesthesia and surgery, the right renal artery was clamped for 30 min (long ischemia) and declamped for 10 min (reperfusion). At 70 min blood was collected and kidneys were taken.

IPC group

40 min after anesthesia and surgery, the right renal artery was clamped for 5 min and declamped for 5 min consequitively for a total of 3 cycles. At 70 min blood was collected and kidney was taken.

IPC-IR group

Immediately after surgery, 3 cycles of sequential 5 min renal artery occlusion followed by 5 min reperfusion were induced before a 30 min sustained ischemia and 10 min

reperfusion. At 70 min blood was collected and kidney was taken.

Histology

For all kidneys, twenty four hours after formalin fixation (10% phosphate-buffered), the fixative was changed to 70% ethanol. Tissues were embedded in paraffin using routine methods. Paraffin-embedded renal sections (4 μm) were stained by hematoxyline and eosin. Histopathology for all kidneys was scored per section in at least 10 randomly selected non-overlapping fields at × 400 magnification of the sections. The results were scored as the percentage of the damaged tubules: 0, no damage; 1, areas of tubular damage less than 25%; 2, similar changes 25–50%; 3, more than 50%. Loss of brush borders from proximal tubules, cellular vocuolation, pyknosis and cell death and presence of luminal cast materials were used as evidence of tubular damage. Morphological examination was performed "blind" to the treatment.

Sample preparation procedures

Vitamin E extraction from plasma: The procedure was carried out according to Arnaud in 1991 [5]. BHT was used to prevent lipid peroxidation. All extraction steps were performed under dim light.

Vitamin E extraction from renal tissues: The procedure was a modification of Peng's method [6,7]. After addition of PBS, 50 mg of frozen renal tissue was minced for 30 seconds. Then collagenase was added and the sample was homogenized on ice with a homogenizer for 45 seconds. After addition of protease, 250 μl of the sample was transferred into a 10 ml test tube. In order to precipitate tissue proteins, 250 μl of a 0.1% SDS-ethanol-BHT solution was added and centrifuged at 7000 g for 5 min in a refrigerated centrifuge. 250 μl of upper hexane layer was transferred to another test tube and dried under nitrogen gas. The dried extract was stable for at least 2 days at -20°C. The importance of this method was its ability to extract more micronutrients from a small piece of tissue.

Statistical analysis

The mean and standard error for each group was used to compare groups with a SAS program using one way analysis of variance. To show the difference among groups, student – Newman Keuls multiple comparison tests were applied.

Results

The amount of vitamin E in renal tissue and venous plasma was measured in all groups and the following results were obtained. Retention times for vitamin E were the same in all groups (demonstrated as an area under the curve). The amount of vitamin E extracted from the right renal venous plasma in IR group indicates a significant decrease compared with other groups (control, IPC, and IPC-IR, P,0.05). Vitamin E level in the IPC-IR group did not show any significant differences from the control group, but it was significantly higher than that in the IR group (P,0.01; Figure 1A).

The amount of vitamin E extracted from the right renal tissue in the IR group showed a significant decrease compared with other groups (Control, IPC and IPC-IR) (P,0.05), whereas the amount of vitamin E in the IPC and IPC-IR groups did not show significant difference from control group, but it had a significant increase in respect to the IR group (P,0.05; Figure 1B).

Thus in both renal tissue and venous plasma, vitamin E levels in the IPC-IR group did not show any significant differences from the control group, but it was significantly higher than the IR group (P,0.05, Figure 1A &1B).

BUN was not significantly different among the groups. Creatinine in the IPC group was not significantly different from the controls. Although creatinine was significantly (P,0.05) higher in the IR group comparing with control group but it was not significantly different from the IPC-IR group (Figure 2).

Sections from control group showed normal histology. Sections from the IPC alone kidneys were comparable with controls, although some brush border loss and cellular vacuolation was seen. In sections of IR kidneys, glomeruli displayed no significant changes detectable by light microscopy, but there were marked tubular damages. Complete loss of brush borders, extensive tubular casts and debris and tubular dilatations were observed (Figure 3). The IPC-IR kidneys showed a preservation of tissue histology (p,0.05).

Histological evaluations revealed that the tissue sections of IPC group were normal comparing to controls. The structural damage in the IPC-IR group was lower than that of the IR group (Figure 3).

Discussion

The most common cause of acute renal failure is renal ischemia which results in renal dysfunction, a decrease in high energy phosphate supply (ATP), an increase in free intracellular calcium concentration, activation of membrane disintegrative processes, and the formation of endogenous toxins. Therefore, comprehension of the nature of various mechanisms involved in cellular and tissue damage can provide new therapeutic ways for treatment and prophylaxis of ischemic injury [8]. In ischemia, accumulation of hypoxanthine as a result of ATP degradation, causes the generation of highly reactive oxygen free

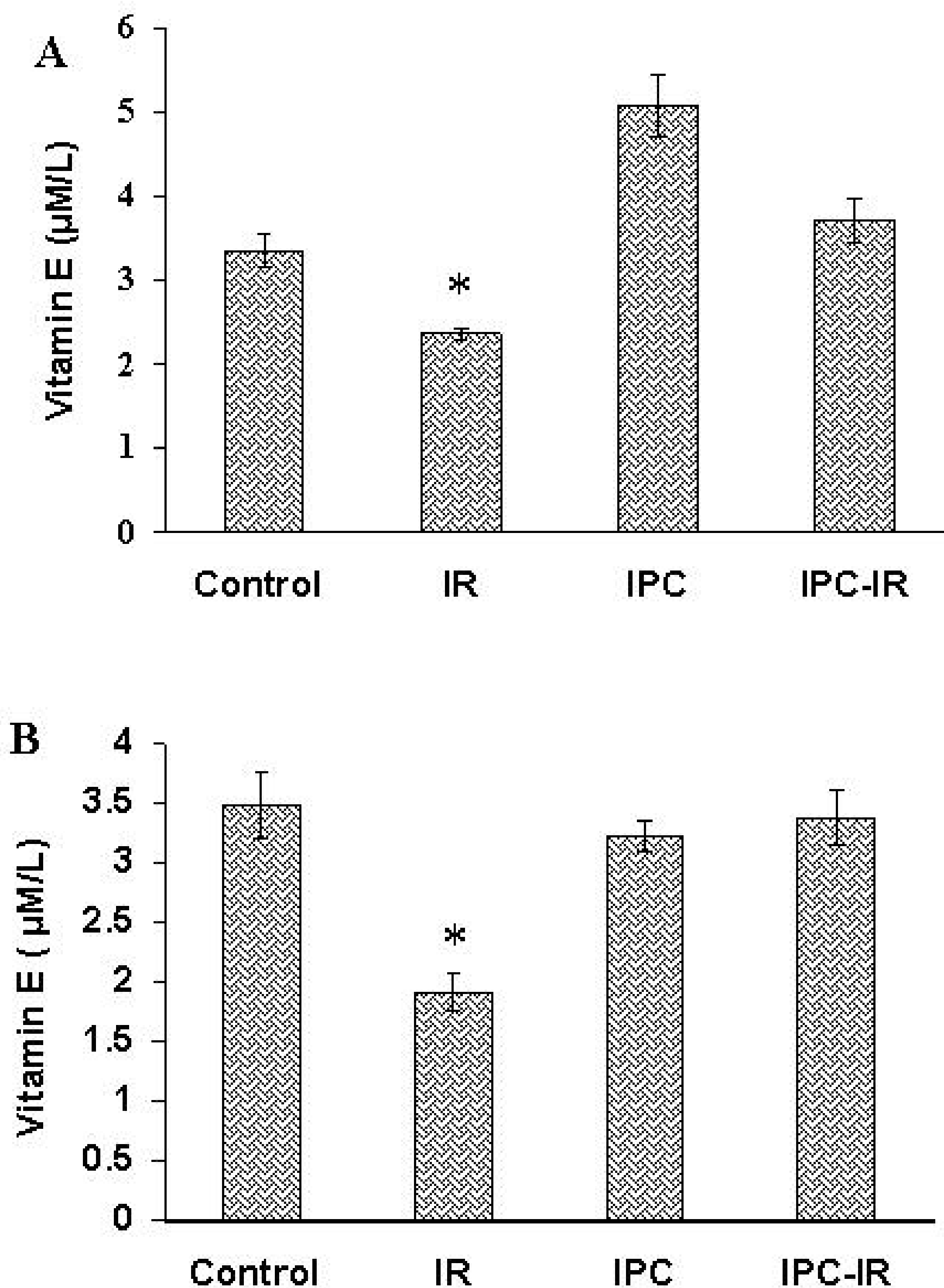

Figure 1
Vitamin E levels in plasma (A) and renal tissues (B) in different groups. The data are presented as mean ± SEM of each group. IR caused a significant reduction in vitamin E contents of the renal tissue and plasma. Induction of IPC before IR prevented this reduction (*$P < 0.05$ compared with the other groups). Ischemia-reperfusion, IR; Ischemic preconditioning, IPC; Ischemic preconditioning followed by ischemia-reperfusion, IPC-IR.

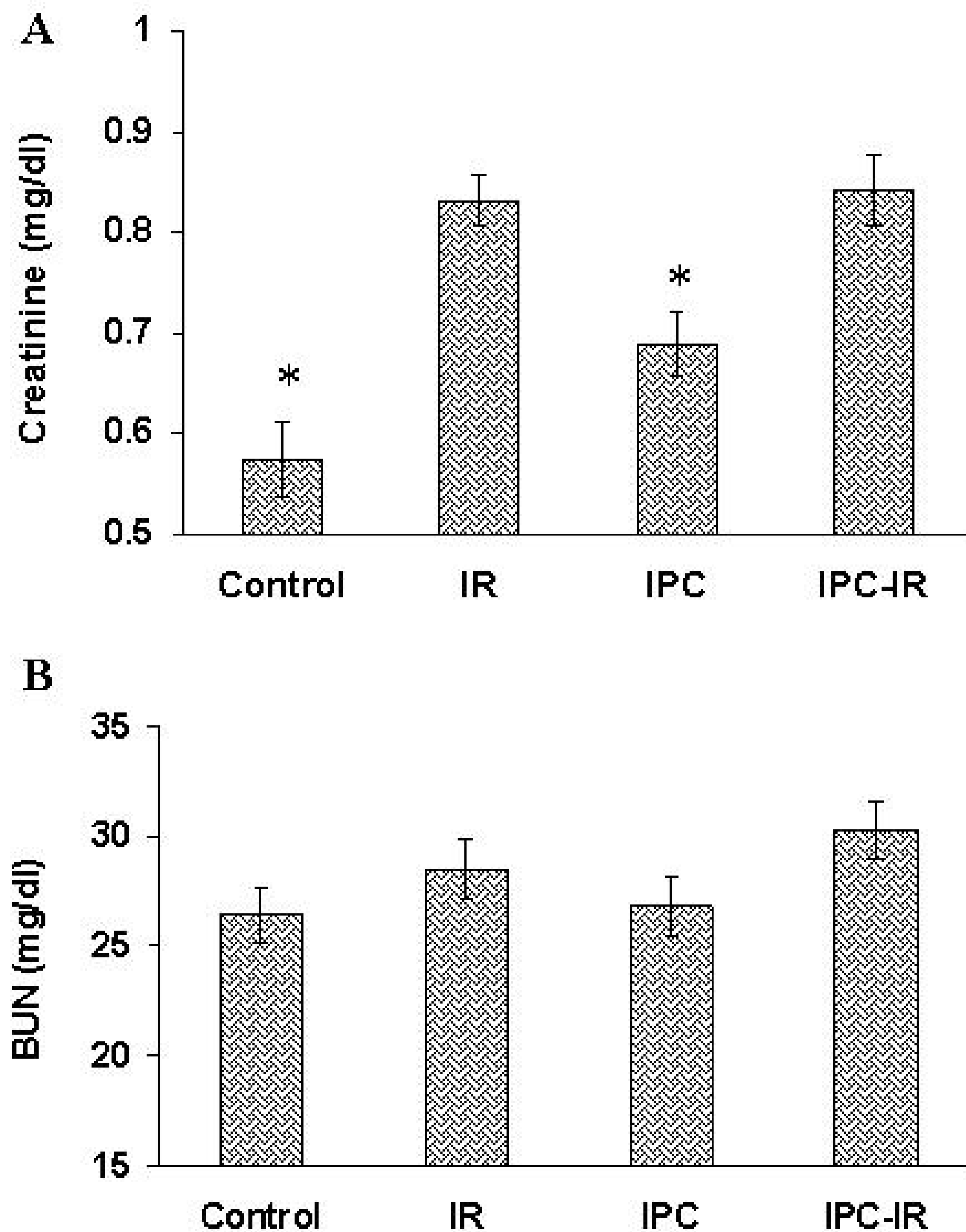

Figure 2
Alterations in serum creatinine (A) concentrations and BUN (blood urea nitrogen, B) in different groups. The data are presented as mean ± SEM. There was no significant difference in BUN among the groups. Creatinine in IR group was significantly higher than the control group. No significant differences were obsereved between the IPC-IR and the IR groups. *P < 0.05 compared with IR. Ischemia-reperfusion, IR; Ischemic preconditioning, IPC; Ischemic preconditioning followed by Ischemia-reperfusion, IPC-IR.

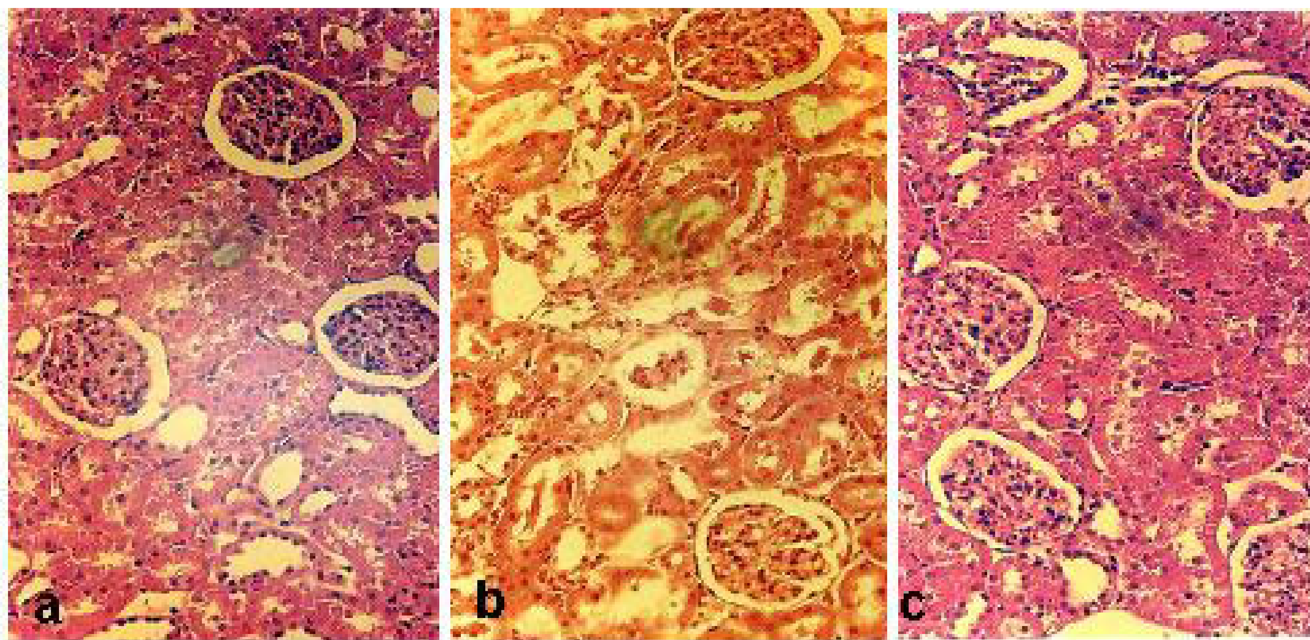

Figure 3
Histological evaluations of renal tissues. a, The normal histological characteristics of glomeruli and tubules of control group. There was minor changes in the IPC alone group (not shown). b, The major changes in tubules of IR kidneys including loss of nuclei, appearance of tubular debris and casts are remarkable. c, The IPC-IR kidneys showed a preservation of tissue histology by preconditioning before IR. All Haematoxylin and Eosin × 400.

radicals (OFR) during reperfusion [9]. Superoxide radical and its reduction products (hydrogen peroxide and hydroxyl radical) cause injury via lipid peroxidation of the mitochondrial and plasma membranes [10].

Naturally, tissues contain ample amounts of endogenous antioxidants including superoxide dismutase (SOD) to remove superoxide anions from the surroundings, catalase and glutathione peroxidase to inactivate hydrogen peroxide, and tryptophan, histidine, vitamins E and C to scavenge hydroxyl radicals (•OH) [11]. However, storage of endogenous antioxidants decreases gradually while reacting with free radicals, resulting in cell injury. There are reports about the gradual reduction of antioxidants in tissues such as the brain [12] and the heart [13], in which water-soluble antioxidants including ascorbate, glutathione and SOD are first oxidized to protect the cell membrane integrity. In this regard the lipid soluble antioxidants, ubiquinol and vitamin E, are consumed. Thus, vitamin E measurements, may, in addition to being an acceptable index of lipid peroxidation, be important as the ultimate defense barrier against IR insult [13].

During organ transplantation, the transplanted organ must remain out of the body with little or no blood perfusion for a period of time. Reperfusion of this ischemic organ causes the release of OFR, which are known to further exacerbate the primary injury induced by ischemia [14]. Excess generation of OFR and/or reduction in antioxidant levels have been implicated as the causative factors of oxidative injury. Since antioxidants can scavenge toxic oxygen metabolites, they may have an effective role in attenuating IR injury [15]. For many years, vitamin E has been known as a lipid soluble antioxidant which can stabilize polyunsaturated lipids against autooxidation. There is an overall agreement about the similar protective function of vitamin E in different tissues.

Free radicals react with polyunsaturated fatty acids (PUFA) in cell membranes, resulting in cellular destruction. It seems that vitamin E reacts with OFR preventing free radical chain reactions to protect the membranes [16]. Therefore, determination of changes in vitamin E levels in the plasma and tissues during IR may be important.

In the present study, ischemic preconditioning was shown to be protective in kidneys against following sustained ischemia to preserve the vitamin E levels. The amount of vitamin E in both renal tissue and plasma of the IR group demonstrates a significant decrease, when compared with the control group (P, 0.05), indicating accumulation of OFR during IR. The body uses its endogenous antioxidants such as vitamin E to fight with OFR. The amount of vitamin E in both renal tissue and venous plasma of the IPC-IR group did not show any significant difference from the control group, while it was higher than that in the IR group (P, 0.01 and P, 0.05 respectively). This suggests that vitamin E consumption may have been significantly less in the IPC group than that in the IR group. Due to the recycling property of vitamin E by vitamin C, possibly it has been regenerated and entered into the blood circulation.

Ischemia in the IR group significantly reduced renal function demonstrated by an increase in serum creatinine compared with the control group. Creatinine levels were not significantly different between the IR and IPC-IR group, perhaps because creatinine and BUN are not sensitive markers of mild alterations in renal function. It might have been more appropriate to measure GFR to estimate renal function, but in our model it was difficult to collect enough urine samples.

Since vitamin E is considered to be a major endogenous antioxidant against lipid peroxidation [17], reduction of vitamin E levels during IR in tissue and plasma could be due to its consumption during removal of free radicals in membranes, as reported by several studies in the other organs [17]. The effectiveness of IPC regimen often depends on the number [18] and duration [19] of cycles. In the present study, application of sequential cycles of IR and their durations seems to be suitable and could significantly prevent reduction in vitamin E levels in both tissue and plasma.

Although this IPC model could not highly improve renal function in the early phase of reperfusion, histology showed a trend to improvement in the IPC-IR group. The results are in agreement with Jefayri's study (2000) which reported no difference in creatinine levels while less dead renal cells were in the IPC-IR compared to the IR group alone [20].

Murry in 1986 showed that IPC caused no reduction in the amount of high energy phosphates in tissues [21] but surprisingly the amount of vitamin E in plasma of IPC group was significantly higher than that in the control group. This may indicate the possible rapid recycling of vitamin E from its mobile sources such as LDL, red blood cell membranes and other tissues such as the liver into the circulation.

Conclusions

This study suggests that IPC method might be used prior to or at the beginning of kidney transplantation to preserve endogenous antioxidant levels, but a decisive conclusion requires more research. This study also suggests that vitamin E could be a valuable criterion for evaluation of the effect of IPC in different tissues including kidney.

Competing interests

This study was supported by a grant from Tehran University of Medical Sciences.

Authors' contributions

1 – MK conceived, coordinated the study and edited the manuscript

2 – SA collected data and wrote the manuscript

3 – MF conceived and coordinated the study

4 – MZ assisted in collection of data and biostatistical work

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