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The Study of the Effects of Post Treatment with Rosmarinus Officinalis Aqueous Extract the First 48 Hours after Renal Ischemia/Reperfusion in Rats

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Abstract

***Background*:** Acute renal failure induced by ischemia / reperfusion (I/R) causes excretory functional disorders of nephrons. The purpose of this study is to evaluate the intake of oral rosemary extract (gavage) on hemodynamic changes and tissue damages caused by I/R.

***Materials and Methods*:** This study was conducted on 40 Sprague-Dawley male rats, composed of 4 groups (n=10) including control, sham, I/R+normal saline (I/R+NS) post-treatment and I/R+rosemary (I/R+RO) post-treatment. They were fed with 10 ml/kg of 8% aqueous extract or vehicle as gavages during the first 48 hours after I/R. To induce I/R, both renal artery and vein were blocked for 30 minutes followed by a reperfusion within 48 hours. Then urine and plasma samples were collected and histological study was also conducted after removal of both kidneys. Data were analyzed by SPSS-11.5 software. The one-way analysis of variance (ANOVA) and Duncan test were used for intergroup comparison and $p \leq 0.05$ was used as the significant level. The Kruskal–Wallis multiple comparison test was also used for doing histological analysis

***Results*:** Comparison of renal function between two groups of (I/R+RO) and (I/R+NS), showed a significant reduction in plasma creatinine (p=0.05), the blood urea nitrogen (p=0.05), absolute excretion of sodium (p=0.001) and an increase in absolute potassium excretion (p=0.01). Results of the histological study also indicated a significant decrease in the vascular congestion, Bowman's capsule space and oxidative stress in the group (I/R+RO) in comparison with (I/R+NS).

***Conclusion*:** Treatment with rosemary may be effective in the reduction of the functional and histological effects of I/R.

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Introduction

Acute Renal Failure (ARF) is a common clinical syndrome [1] which occurs with rapid and reversible decrease in the renal function within a period of several hours or several days [2]. The ischemia/reperfusion (I/R) model is the most common method for induction of ARF among experimental models [3] consisting of a mechanism by which it inflicts damage to tubular renal cells includes oxygen depletion and ATP that cause loss of brush borders of microvillies, cellular connections, displacement of integrins and Na^+-K^+-ATPase pump from the basal lamina to apical surface of the cells [4, 5] due to disintegration of the cytoskeleton. This issue causes changes in the polarity of tubular epithelial cells and all these alterations pave the way for the renal tubular obstruction [6]. ATP depletion causes the activation of proteases and phospholipases that cause oxidative damages following the reperfusion which has an important role in the pathogenesis of ARF. These damages occur in epithelial cells of tubular capillaries and especially in the outer medulla. After ischemia/reperfusion (I/R), the F-actin filaments of smooth muscle endothelial cells lose their organization [7, 8]. And these changes in the Cytoskeleton, destruction of cellular connections, ATP depletion and exposure to the oxidant agents are responsible for an increase in the paracellular permeability and back leak from the vascular bed to the surrounding tissue which causes swelling of the endothelial cells and impaired blood flow due to the compression of capillary and occlusion of the vascular lumen [9, 10]. Despite current medical treatments, adverse effects resulting from excessive and prolonged use of these drugs and the costs imposed on patients have led to an increased willingness towards alternative and traditional treatments.

Today, many studies have been conducted on the use of medicinal plants for the improvement of disorders developed following ARF [11]. Rosemary (*Rosmarinus officinalis*) is a native Mediterranean evergreen plant which is used in traditional medicine as a sedative and cardiotonic drug and for the treatment of coughing, fever, pain, flu and problems of stomach, liver and kidney [12]. The pharmacological activity of this plant includes antimicrobial, hyperglycemic and diuretic activities as well as antioxidant and anti-inflammatory properties and

is effective also on improving memory and protecting DNA [13, 14]. Clinical studies also show that oral intake of rosemary is effective on renal colic; dysmenorrheal and spasm-related pain relief [15].

Considering the existence of compounds with the above properties in aqueous extract of the rosemary leaves and mechanisms related to the excretory functional disorders of nephrons following ARF, the purpose of this study is to examine the effect of this extract on the improvement of functional and histological complications resulted by ARF from different aspects.

Materials and Methods

This experimental study was conducted on 40 male rats of Sprague-Dawley race, in the weight range of 250-300g, provided from animal breeding center of Arak University of Medical Sciences. They were kept in 12 h light and 12 h dark conditions at the temperature range of 23±2˚C and they were provided with standard food and water *ad libitum*.

This study was conducted observing all the ethical codes of working on experimental animals approved by Ministry of Health and Medical education. Ischemia-reperfusion (I/R) model was also used in order to induce ARF. Initially, after weighing the rats, they were intraperitoneally (i.p) injected with 60 mg/kg pentobarbital (Sigma, UK). After shaving hair on abdomen, a longitudinal incision was cauterized on the surface of abdomen. Then kidneys were occluded for 30 minutes after observing the right and left kidneys using a clamp special for the renal arteries and veins and immediately after the expiry of the aforesaid period and removal of the obstruction, the area under surgery was sewn with 3-zero silk. This study was designed and conducted in four groups (n=10) as follows:

A) Control group: The rats did not receive any vehicle or drug during the experiment and they were treated with normal diet.

B) Sham group: Anesthesia, surgery, viewing and touching the renal artery and vein without their clamps and also receipt of 3ml/d drug vehicle for 48 hours immediately after surgery was performed as gavage in this group.

C) I/R post-treatment and (I/R+NS) vehicle recipient group: Normal saline was given as gavages immediately after induction of ARF and during the initial 48 hours after reperfusion

D) I/R post-treatment and rosemary (I/R+RO) recipient group: 8% aqueous extract was received as gavages immediately after ARF induction and during the initial 48 hours after reperfusion.

In all groups, rats were placed in metabolism cages in order to collect urine and after urine collection; its amount was measured using Gravimetric method. Then blood pressure was measured using Power Lab system (AD Instruments, Australia) device after anesthesia and opening the abdomen and separating the left renal artery and vein, flow meter with a specific probe (Model T402,USA) was placed around the artery and renal blood flow (RBF) was measured as ml/min through connection to the flow meter.

Blood sampling Alpha from the aorta was performed using a chilled heparinized syringe, and then plasma was separated after centrifugation at 3000 rounds per minute (rpm). After removing both kidneys and determining the weight of each sample, one of them was kept in 10% formalin for doing histological studies and the other was frozen in liquid nitrogen and kept in -20ºC for biochemical study and measurement of antioxidant capacity by Ferric Reducing Antioxidant Power (FRAP) and malondialdehyde (MDA). Measurement of [Na^+], [K^+], [Cr], and BUN were performed in urine and plasma samples, then values of absolute ($U_{Na}V^0$) and relative sodium (FE_{Na}) and potassium (U_KV^0 and FE_K) excretion were calculated. Creatinine clearance was calculated for determination of GFR.

Rosemary herbs were provided from the farm of medicinal plants at Arak University and after separation of the sample leaves and drying them in the shade and the powder preparation, about 8 grams of powdered plant was weighed and boiled in 100 ml distilled water for 5 minutes and was infused for 10 minutes. Cooled and passed through a filter paper, a clear solution was obtained, then the solution brought to the volume of 100 ml and each animal was gavaged with 3 ml/d of it for 48 hours [16].

Measurement of biochemical parameters of malondialdehyde (MDA): In order to determine the extent of damage caused by oxidative stress as the final product of lipid peroxidation, MDA index was used and its rate in tissue sample was measured using Ohkawa method, details of which were mentioned in previous studies [17].

Ferric Reducing Antioxidant Power (FRAP): FRAP measurement has been considered as one of the most common methods for the total antioxidant activity evaluation since 1996. This method is based on the ability of tissue fluids in reducing the ferric ions (Fe^{3+}) to the ferrous ions (Fe^{2+}) in the presence of a substance called Tripyridyl-S-Triazine (TPTZ). Details of its methodology have been mentioned in previous studies [18].

Histopathologic evaluation of kidneys: In order to prepare tissue samples for doing light microscopic studies, kidneys were divided into two parts fixed in formalin 10% solution (Merck, NJ, USA). Then 5 µm sections were made after tissue passage using routine histological method and molding in paraffin and samples were stained with hematoxylin and eosin. In histopathological examination of the cortex incisions, inner and outer medullas were studied separately.

Cortex were examined in terms of the status of renal bodies, number of red blood cells inside glomerule and cellular damages as well as the status of lumens of tubules; and the inner and outer medulla were also examined in terms of cellular damages of tubules, vascular congestion and proteinaceous casts.

The extent of the resulting damage was graded on the basis of computing percentage, so without impairments is considered as zero, 1-20% damage is considered as the grade 1, 21-%40 damage is considered as the grade 2, 41-

60% damage is considered as the grade 3, 61-80% damage is considered as the grade 4 and 81-100% damage is considered as the grade 5. Results were reported as the mean and standard error (Mean±SD) and one-way analysis of variance (ANOVA) followed by Duncan test were used by SPSS-11.5 statistical software for inter-group comparison, while considering ($p \leq 0.05$) as a significant level. Also Kruskal–Wallis multiple comparison test was used for histological analysis [19].

Results

Table1 indicate that none of the groups show any significant change in the mean value of systolic blood pressure (P_{SYS}). After 30 bilateral renal obstruction (vessel occlusion) followed by a 48h reperfusion, various plasma factors in ischemic group showed a significant difference between group (I/R+NS) $[Cr]_P$, $[BUN]_P$ (which has the average blood flow) and the sham group; but each of them approached to the baseline. (OSM_u) and 0 U_KV^o in the (I/R+NS) group show 38% and 35.5% reduction respectively compared to the sham group.

$U_{Na}V^o$, FE_{Na} and FE_K in (I/R+NS) group showed 157%, 5 times and 44% increase respectively compared to the sham group. Comparison of the results related to the hemodynamic factors were reported as mean±SD and significant level was expressed as p=0.001, p=0.01, p=0.05. Results of the histopathological studies show that no tissue damage (grade 0) was found in sham group and in (I/R+NS) group, as hemodynamic studies also showed, tissue changes has greatly been improved compared to the initial phases of ischemia elimination, so that the space of Bowman's Capsule showed a smaller increase (grade 1) compared to the sham group (grade 0).

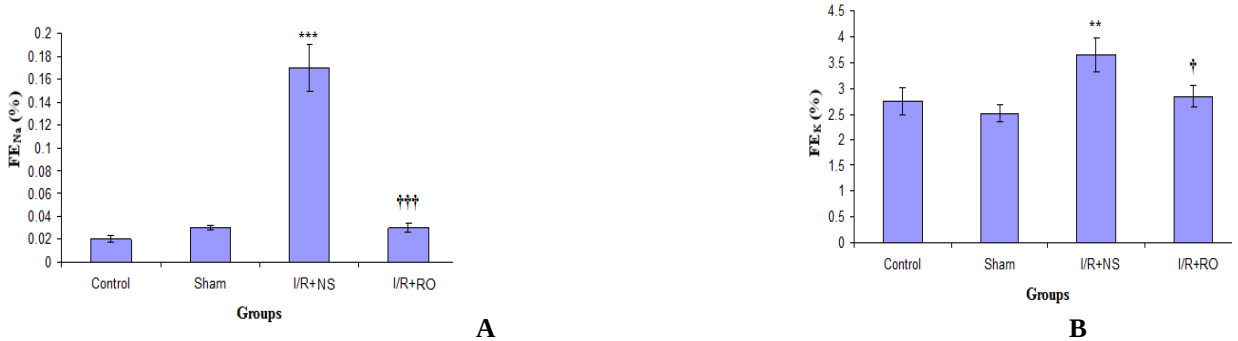
Also the vascular congestion (grade 2) and somewhat exfoliated cells (grade 1) were observed, but a large number of proteinaceous casts (grade 3) were found compared to the sham group (grade 0). In (I/R+RO) group, no changes in the glomerule and the space of Bowman's capsule (grade 0) were found in the cortex and no significant difference was found between (I/R+RO) and (I/R+NS) groups as far as the number of proteinaceous casts (grade 2) in the medulla and exfoliated cells (grade 1) in the cortex and medulla of kidney are concerned.

In the group of rosemary, vascular congestion (grade 1) was less than that in the (I/R+NS) group. Then plasma creatinine values ([Cr]p), plasma urea nitrogen (BUN), absolute sodium excretion ($U_{Na}V^o$), absolute potassium excretion (U_KV^o), urine osmolarity (OSM_u), creatinine clearance (Ccr), mean arterial flow and systolic blood pressure (P_{SYS}) within 48 hours of reperfusion followed by 30 minutes of bilateral renal ischemia among the rats belonged to the (I/R+RO) group were compared to the relevant values after orally intake of 10 ml/kg normal saline by (I/R+RO), (I/R+NS) and the sham groups; then obtained values were reported as Mean±SD.

Table 1. Comparison of the changes resulted from renal ischemia/reperfusion (I/R) on the plasma parameters and renal excretory function parameters and effects of rosemary on them

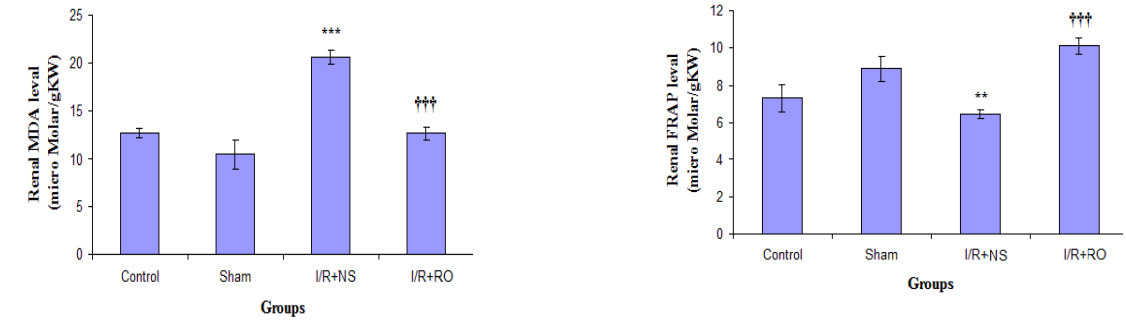
Groups	$[Cr]_p$ (mg dl-1)	$[BUN]_P$ (mg dl-1)	$U_{Na}V^0$(mmol/ min per kg)	U_KV^0(mmol/ min per kg)	$[OSM]_u$ (mOsm kgH2O-1)	C_{cr} (ml min-1 kg-1)	Mean Flow (mlit /min)	P_{SYS} (mmHg)
Control	0.65±0.04	17.62±0.6	0.5±0.05	1.36±0.1	1458±46.8	13.02±1.6	7.97±0.3	94.39±5.2
Sham	0.7±0.03	24.2±1.5	0.89±0.2	1.8 ±0.3	1486±47.2	19.19 ±3.2	8.41 ±0.4	81.96±5.6
I/R+NS	1.2 ±0.1***	60.2±4.8**	2.29 ±0.3 ***	1.16 ±0.1**	919±13.4***	9.56 ±0.9**	7.05 ±0.5*	86.33±6.5
I/R+RO	0.79 ±0.1†	32.77 ±3.7†	0.71±0.1†††	1.83 ±0.1††	1245±71.3†	16.69 ±0.9†	8.11 ±0.3	93±9.1

* p=0.05, ** p =0.01, *** p =0.001, significant difference with sham group
† p =0.05, † † p =0.01, † † † p =0.001, significant difference between I/R+NS and I/R+RO groups



* p=0.05, ** p=0.01, *** p=0.001, significant difference with sham group
† p=0.05, † † p=0.01, † † † p=0.001, significant difference between I/R+NS and I/R+ RO groups

Figure 1. Comparison of the values of a) relative excretion of sodium (FENa), b) relative excretion of potassium (FEK) within 48 hours of reperfusion following renal bilateral ischemia for 30 minutes among (I/R+RO) rats through orally intake of 10 ml/kg in (I/R+RO) group or normal saline in (I/R+NS) group or normal saline in sham group and values of which were reported as mean ±SD



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* p=0.05, ** p=0.01, *** p=0.001, significant difference with sham group
† p=0.05, † † p =0.01, † † † p=0.001, significant difference between I/R+NS and I/R+ RO groups

Figure 2. Comparison of the values of (a) Malondialdehyde (MDA) and (b) Ferric Reducing Antioxidant Power (FRAP) in (I/R+RO) groups through orally intake for 48 hours in amount of 10 ml/kg in (I/R+RO) group or normal saline in (I/R+NS) group or normal saline in sham group and values were reported as mean±SD

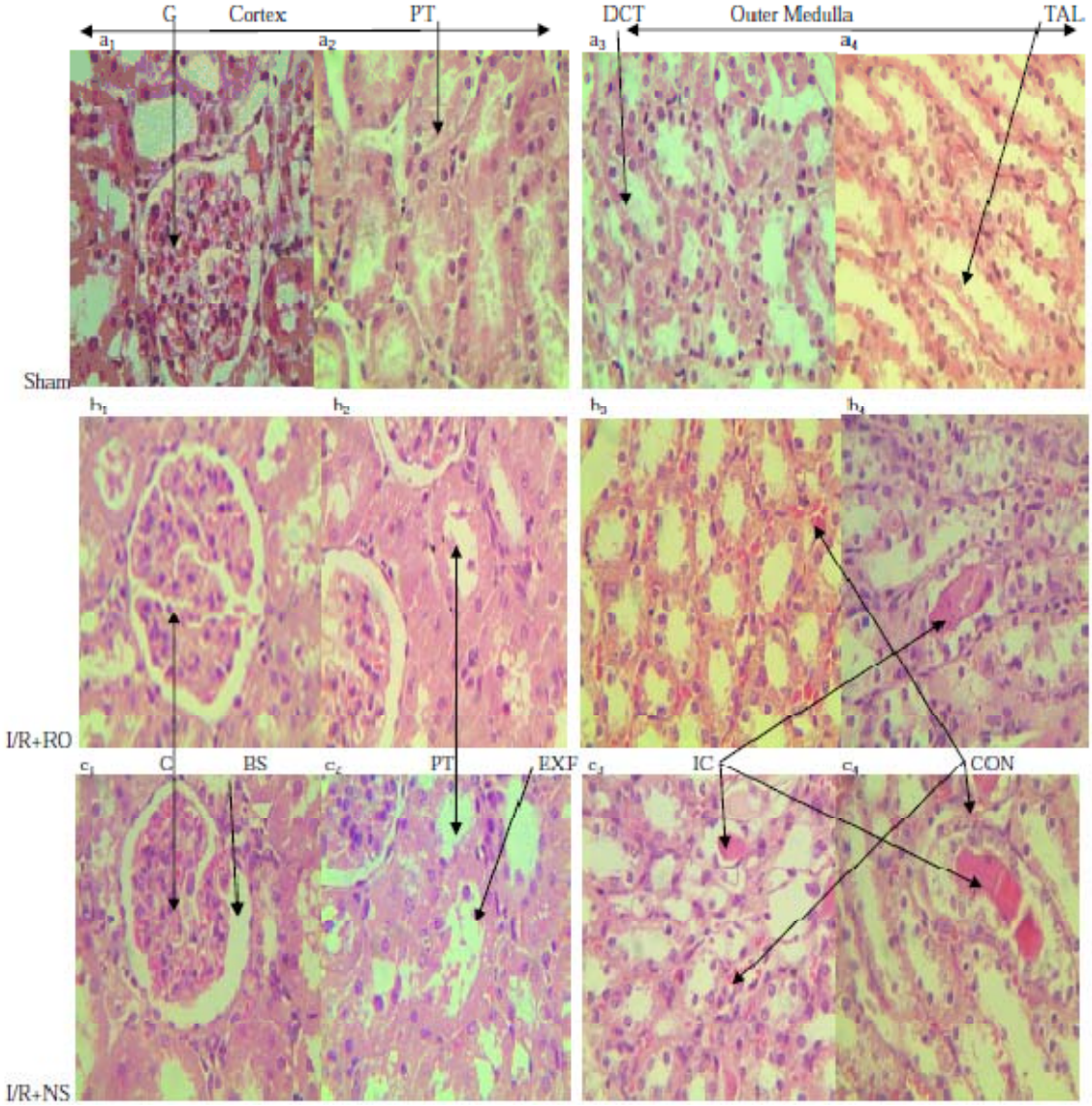

Figure 3. Images taken by the light microscope from the left kidney from cortex, (a_1, a_2) and outer medulla (a_3, a_4) for sham group, cortex (b_1, b_2) and outer medulla (b_3, b_4) for the (I/R+RO) group, cortex (c_1, c_2) and outer medulla (c_3, c_4) for the (I/R + NS) group, stained with Hematoxylin - Eosin and ×400 magnification
BS= Bowman's space; DCT= distal convoluted tubule; Exf= exfoliated cells; G=glomerulus; IC= intratubular cast; PT= proximal tubule; TAL= thick ascending limb of Henle's loop; CON=congestion

Discussion

In this study, urine osmolarity in (I/R+NS) group decreased significantly (Table 1). Several studies show that during I/R, TAL (image c4) and proximal tubules (image c2) are among those main places prone to damage. Since countercurrent multiplier mechanism is one of the main systems used for urinary concentration and the existence of TAL is necessary for the performance of this mechanism, it has been determined that damages to these cells during ARF can be an important factor of defect in urine concentration [21, 22]. Wang et al. showed that following 30 minutes of renal artery obstruction and 48h reperfusion, decreased expression of Na^{+}-K^{+}-$2Cl^{-}$ carrier in the apical membrane during I/R reduced reabsorption of sodium and chlorine and loss of osmotic gradient in the renal medulla. This status is associated with the suppression of vasopressin activity, AQP2 and consequently defects in urine concentration during I/R process [23].

Furthermore, Sonoda et al. specified in their study that AQP1 expression about 6, 30 and 96 hours after induction of ARF was significantly decreased in all areas of cortex, outer and inner medulla [24]. But in the (I/R+RO) group, urine osmolarity is much closer to the base, so that, improvement of the proximal tubule (image b_2), reduction in proteinaceous casts and the elimination of vascular congestion in the TAL tubules) (images b_3 and b_4) confirm that this situation is due to the role of rosemary herb in the removal of oxygen free radicals. Several studies indicate that the highest rate of ROS production during I/R is occurred in the proximal tubule.

This study shows a significant increase in $[Cr]_P$ in the (I/R+NS) group compared to the sham group. Although this difference is significant; it shows to a large extent an improvement trend (Table 1) as compared to the initial phases after elimination of ischemia. In a study conducted by Wang et al., it was found that after 30 minutes, renal artery obstruction and 12 and 24 hours of reperfusion $[Cr]_P$ were increased, but it was sharply decreased 48 hours after the removal of occlusion. The results of this study suggest that although renal function is not yet fully modified 48h after reperfusion, it was significantly improved compared to that after 24 h of reperfusion [23].

This study also showed that although the level of plasma creatinine has not completely reached to the baseline, but has improved greatly compared to the sham group. In his investigation, Huiling Cao revealed that after 60 min of I/R creation and hemodynamic evaluation of renal function during 16, 24, 48 and 72 h of reperfusion, $[Cr]_P$ reached its highest level, 24 h after the removal of occlusion and this value was greatly reduced during reperfusion for 72 hours.

Creatinine clearance in group (I/R+NS) shows a significant reduction which was associated with an increase in $[Cr]_P$. After 30min renal artery obstruction and 24h reperfusion, Wang et al showed that the value of GFR, as an index or marker of renal function, greatly reduced 24hrs hours after 30min occlusion, but it was significantly increased after 48 hours of reperfusion [23].

Also the significant improvement in Ccr which is a marker for GFR is observed in this study. The process of recovery in renal blood flow in Group (I/R+NS) after 48 h of reperfusion which is one the factors effective in Ccr, confirms this status. The histological studies also show that vascular congestion of medulla (image c3) and vasoconstriction through the physical barrier and increase in the resistance to the passage of red and white blood cells reduce the renal artery blood flow, but this situation has dramatically improved after 48 hours of ischemia and also the reduced space of Bowman capsule (image c1) implies that there was found only 16.8% significant difference in the renal blood flow of (I/R+NS) group compared to the sham group.

Although there was not any significant difference in the renal blood flow between the groups of (I/R+RO), and (I/R+NS) (Table 1), but the improvement process was seen in renal blood flow, so that RBF (images b_1, b_3) was greatly improved in the (I/R+NS) group. This issue can be attributed to its anti-inflammatory and antioxidant properties. In a study conducted by Mengoni et al., it was found that carnosol and carnosic acid which are abundant in the rosemary herb decrease expression of IL-1β and TNF-α, selective inhibition of COX-2 and the migration of leukocytes. These studies also show that the carnosol contents of rosemary activate Nrf-2 factor which in turn inhibits the expression of inflammatory genes and it was cleared that the enzymes encoded by a gene associated with Nrf-2 cause protective effect against oxidative stress [26, 27].

The results of this study indicate that RBF in the (I/R+NS) group showed 16.18% significant decrease compared to the sham group (Table 1) and in a similar situation a 50% significant decrease was found in C_{Cr} value (Table 1).

This reduction is likely due to the oxidative changes in the epithelial cells which decrease renal blood flow followed by the reduction in the urinary clearance through disrupting the balance of vasoactive agents and the dominance of the vasoconstrictive factors including angiotensin II and endothelin on dilator agents and there was not found any significant difference between the groups of (I/R+RO) and (I/R+NS) as far as renal blood flow is concerned, but an increase of 43% in the value of Ccr was observed.

This shows the effective role of rosemary in renal hemodynamic performance improvement through the elimination of ROS [28]. In his study, Mitsuhiro Wada found that aqueous extract of rosemary eliminates ROS stronger than the non-aqueous extracts [29]. In a study, Zahedi et al. found that mRNA levels of SSAT (Spermidine/spermine N-acetyltransferase) which is a diagnostic marker of tubule cell damages significantly increased following renal ischemia for 30 min, within 24 hours of reperfusion and reached to its basal level after 48 hours. This fact indicates that the peak damage of tubule cells is occurred 24 hours after ischemia and the improvement of tubules is realized within 48 hours of

reperfusion [30]. In this study, RBF largely returned to its normal condition after 30 minutes occlusion and 48 hours of reperfusion. This could be an indication of improvement in renal tubules, 48 hours after elimination of occlusion. Following the evaluation of systolic blood pressure within 24 hours of reperfusion, it was found that the value of P_{SYS} didn't have any significant difference between various groups and therefore indicates that the differences observed in the indices of renal function in the test groups were not resulted from the changes in arterial blood pressure (Table 1).

The results of this study showed the significant increase of 82% and 37% in FE_{Na} (Fig. 1a) and FE_K (Fig. 1b) respectively in the group (I/R+NS) compared to the sham group which confirms the increased severity of damages in the proximal tubule carriers, which is mainly due to the increased production of ROS [22]. In their study, Wang et al. also showed that the mRNA expression of NHE-3 and Na^+-K^+-$2Cl^-$ carriers which are responsible for the much of exchanges of sodium and potassium in the proximal tubule was decreased as equal to 78% and 82% respectively, after 30 min occlusion and 24 and 48 h reperfusion. Also Na^+-K^+-ATPase exchanger was decreased as equal to 10% and 15%, respectively that is responsible for the increased EF_{Na} and FE_K after renal ischemia.

Furthermore, loss of the polarity of epithelial cells and Na^+-K^+-ATPase carrier could be another cause of this problem. Also the significant reduction in the expression of carriers of Na^+-K^+-$2Cl^-$ within 48 hours reperfusion, despite significant improvements in GFR, caused severe excretion of sodium, chloride and water which are responsible for the diuretic phase of ARF which occurs during the recovery phase of ARF [23].

In this study, FE_{Na} and FE_K show 81% and 25% reduction respectively in (I/R+RO) group which demonstrates the effectiveness of rosemary herb on improving these damages. The level of MDA in this study (Fig 2-a) which is an index for the evaluation of oxidative stress showed an increase of 49.7 % compared to the sham group which indicates the existence of the oxidative stress condition due to the presence of ROS after realization of I/R condition. The level of MDA in (I/R+RO) group showed a reduction of 38.5% compared to the (I/R+NS) group which indicates the effectiveness of rosemary, as a strong antioxidant on strengthening the antioxidant system. Also the increase of 36% in the rate of FRAP (Fig 2) confirms this case.

In the study conducted by Tavafi on the effect of Rosmarinic acid on diabetic nephropathy in rats, it was found that the Rosmarinic acid can significantly improve glomerular nephropathy and reduction in the number of glomerule. It was also found that this compound can make lipid peroxidation in diabetic rats, the level of MDA of which had significantly increased, to be closer to the baseline. In a study conducted on the effect of rosemary on lipid levels of blood and the effects against lipid peroxidation in rats, Wu et al revealed that the rosemary herb, in 3 forms of essential oil, aqueous extract and powder can significantly reduce triglyceride levels in Hyperlipidemia rats. It was also found that dose-dependent rosemary in two forms of aqueous extract and powder can inhibit MDA, but essential oil of this herb does not have this effect [32]. Results from this study show that using aqueous extract of rosemary during the first hours after I/R may be effective on the reduction of the functional and tissue damages caused by ischemia – reperfusion.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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