EFFECT OF EXPERIMENTALLY-INDUCED RENAL ISCHEMIA/REPERFUSION INJURY ON RAT LIVER FUNCTION: ROLE OF ANGIOTENSIN II

By
Walaa Hassan Nazmy[*] and Nesreen Abdel-Twaab[**]
Departments of [*]Physiology and [**]Pathology,
El- Minia Faculty of Medicine

ABSTRACT:

Aim: The present study was designed to investigate the effect of experimentally-induced renal ischemia reperfusion (IR) injury on rat liver function. Also, to investigate the potential role of angiotensin II (Ang II), the final effector of rennin angiotensin system (RAS), in the pathogenesis of liver injury induced by renal IR.

Methods: 24 male albino rats were included in the present study. Animals were randomly divided into the following experimental groups (6 rats each): Sham-operated control group; in which rats were left freely wandering in their cages, Ischemia group; in which rats were subjected to renal ischemia for 60 min, IR-induced group; in which rats were subjected to renal ischemia for 60 min followed by 60 min reperfusion and finally, IR-losartan treated group; in which rats were given a single dose of losartan (a selective angiotensin II type 1 receptor blocker) at a dose level of (5 mg/kg), by oral gavage 24 and 1.5 hours before renal IR At the end of the experimental procedure, animals were sacrificed; serum was collected for determination of urea, creatinine, transaminases levels. Liver tissues were removed and used for determination of hepatic malondialdehyde (MDA) and tumor necrosis factor-α (TNF-α) contents or histopathological examination.

Results: In the present study, renal ischemia, either alone or followed by 1h reperfusion, caused significant alterations in both renal and liver function as evidenced by significant increases in serum urea, creatinine and transaminases as well as structural alterations in hepatic sections, which were more evident in IR group and were associated with a significant increase in hepatic MDA and TNF-α levels. Blocking of AT1 receptors by losartan abolished the injurious effect of renal IR on liver tissue as evidenced by the significant decrease in serum transaminases and improvement of histological changes, along with a significant decrease in hepatic MDA and TNF-α level.

Conclusion: Renal ischemia either alone or followed by 1h reperfusion caused injurious effect on liver histology and function, possibly via oxidative and inflammatory processes. In addition, these results suggest a possible role for RAS including its final product Ang II in the pathogenesis of liver injury induced by renal IR. So, pharmacologic blocking of RAS axis could be clinically useful to protect against remote organ injury including the liver, especially after post-ischemic renal insult.

KEYWORDS:

Ischemia reperfusion, Liver, TNF-α, Angiotensin II, ROS

INTRODUCTION:

Remote organ injury is a kind of oxidative damage that may be seen in various organs away from the tissue exposed to ischemia-reperfusion (IR) injury. The degree of damage depends

on the duration of the ischemia. However, it becomes apparent during the reperfusion period, which intensifies the damage by triggering an inflammatory reaction involving oxygen free radicals, endothelial factors, and leukocytes. Attracted and activated neutrophils migrate and adhere to the endothelium, causing release of proteases and free oxygen radicals[1].

Liver injury is one of the distant-organ damages induced by kidney IR. Acute renal failure associated with liver disease is an extensively encountered clinical problem of varied etiology and high mortality[2]. It is believed that IR injury induces an inflammatory response, which results in the formation of reactive oxygen species (ROS) that augments local tissue damage or affects organs remote from the site of IR[3].

Since the liver tissue represents one of the vascular beds into which ROS are delivered, it would be likely to manifest a number of toxic effects of these molecules. It has been suggested that dehydroepiandrosterone treatment has a beneficial effect on antioxidant defenses against hepatic injury after renal IR in rabbits, possibly by augmenting glutathione (GSH) levels and lowering malondialdehyde (MDA) production[4].

An important function of ROS is the regulation of cytokine gene expression[5]. Miyazawa et al.,[6] showed an influx of neutrophils and lymphocytes, not only in the clamped kidney, but also in the hepatic sinusoids concomitantly with liver dysfunction. These findings indicate that a systemic cellular immune response, including intermediate T cells, affects multiple organs during ischemic acute renal failure (ARF), which may play an important role in the development of multi-organ failure.

Renin-angiotensin system (RAS) is well known for its regulation of blood pressure and fluid homeostasis. Besides vasoconstriction on the cardiovascular system, angiotensin II (Ang-II), the final effector of RAS, is known to exert several effects, such as increased expression of adhesion molecules[7] and cytokines[8], and generation of ROS[9].

In gastrointestinal system, Ang-II reportedly constricts the gastric vasculature through AT1 receptor stimulation[10] and is involved in gastric damage[11]. Furthermore, Nakagiri et al.,[12] found a link between Ang-II activation and ROS production in gastric IR injury thus, raising the possibility for the involvement of Ang-II in the pathogenesis of IR injury in other tissues.

Therefore, the aim of the present study was to investigate the effects of experimentally-induced renal IR injury on rat liver function. Also, to explore the potential role of Ang-II in the pathogenesis of liver injury induced by renal IR.

MATERIALS AND METHODS:

Experimental design

Twenty four male albino rats weighing 250–300g were included in the present study. Animals were housed at room temperature with normal light/dark cycles and were fed a standard diet of commercial rat chow and tap water *ad libitum.* Animals were randomly assigned into one of the following experimental groups (n = 6):

1. *Sham-operated control group*; in which rats were left freely wandering in their cages.

2. *Ischemia group*; in which rats were subjected to renal ischemia for 60 min.
3. *IR-induced group*; in which rats were subjected to renal ischemia for 60 min followed by 60 min reperfusion.
4. IR-losartan treated group; in which rats were given a single dose of losartan (a selective AT1 receptor blocker) at a dose level of (5 mg/kg), by oral gavage 24 and 1.5 hours before being subjected to renal IR[13].

Induction of experimental renal IR

Renal IR was done according to a previous method described by Kelly[3]. In brief, rats were placed on a warming pad and anesthetized with pentobarbital sodium (60 mg/kg i.p) and supplemental doses were given if required. Body temperature was maintained at 37 ± 1^{o}C. A midline incision was performed and the renal arteries were carefully separated from around the tissues. Then, the renal arteries were occluded by non-traumatic micro-vascular clips for 60 min, followed by 1 h reperfusion. Occlusion was approved visually by color change of the kidney to a paler shade and reperfusion by blushing. Sham-operated animals underwent identical surgical treatment, including isolation of both renal arteries. However, artery occlusion was not performed.

At the end of the experimental procedure, animals were sacrificed; serum was collected for determination of urea, creatinine, transaminases levels. Liver tissues were removed and used for either determination of both hepatic MDA (markers of lipid peroxidation) and tumor necrosis factor-α (TNF-α) contents or histopathological examination.

Biochemical assay

Serum concentrations of urea, creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined by comercially available kits (Biodiagnostic, Egypt) following the manufacturers' instructions.

Hepatic malondialdehyde (MDA) assay

A commercially available kit (Biodiagnostic, Egypt) for colorimetric determination of hepatic MDA content was used as previously described [14] and based on the reaction of thiobarbituric acid (TBA) with MDA in acidic medium at temperature of 95^{o}C for 30 min to form TBA reactive product, the absorbance of the resultant pink product can be measured at 534 nm using a spectrophotometer (Spectronic 2000; Bausch & Lomb, Rochester, NY, USA).

Hepatic TNF-α assay

Hepatic TNF-α concentrations were measured by ELISA (Rat TNF-α ELISA kit, Biosource, USA). 100 mg of liver tissue were homogenized in 1 ml phosphate buffer solution (PBS) that contained antiproteases (0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA and 0.05% Tween 20). The samples were then centrifuged for 10 min at 3000 r/min and the supernatant was used for ELISA.

Histological procedures

Liver samples were immediately fixed in 10% neutral formalin for 24 h then changed to absolute ethanol for dehydration and embedded in paraffin. Sections were stained with hematoxylin and eosin for microscopical examination and were evaluated for the presence of cong--estion, cellular degenerative changes,

cytoplasmic vacuolization and leukocyte infiltration.

STATISTICAL ANALYSIS:

Data were represented as mean ± standard error of the mean (m ± SEM). Statistical analysis was performed using Graph pad Prism 5 software and significant difference between groups was done by one-way ANOVA followed by Tukey-Kramar post hoc test for multiple comparisons with a value of $P \leq 0.05$ considered statistically significant.

RESULTS:

- ***Changes in serum biochemical parameters (urea, creatinine, ALT, AST) in different experimental groups***

Both ischemia and IR groups showed significant increases in serum urea level (42.83±1.49 mg/dl and 63.33±4.57 mg/dl vs. 16.17±1.58 mg/dl, P≤0.05) compared to the sham control group. Serum creatinine level also showed significant increase in both groups (1.02±0.06 mg/dl and 1.27±0.05 mg/dl vs. 0.67±0.06 mg/dl, P≤0.05) compared to the sham control group. Loasartan pretreatment to IR group caused significant decrease in both serum urea and creatinine levels (33±4.89 mg/dl and 0.72±0.05 mg/dl vs. 63.33±4.57 mg/dl and 1.27±0.05 mg/dl, P≤0.05) compared to IR non treated group (table 1).

As regards Serum transaminases (ALT, AST), ischemia group showed significant increases in serum ALT and AST levels (102.2±7.48 U/L and 108.2±8.44 U/L vs. 55.17±4.49 U/L and 50.67±4.02 U/L respectively, P≤0.05) compared to the sham control group. In IR group, more significant increase in both serum ALT and AST levels was observed (178±6.20 U/L and 199.8±5.29 U/L) compared to either ischemia or sham control groups. Loasartan pretreatment to IR group caused significant decrease in both serum ALT and AST levels (74±5.96 U/L and 64.33±7.53 U/L, P≤0.05) compared to either ischemia or IR non treated groups (table 1).

Table 1: Changes in serum biochemical parameters (urea, creatinine, ALT and AST) in different experimental groups

Groups	Urea (mg/dl)	Creatinine (mg/dl)	ALT (U/L)	AST (U/L)
Sham	16.17±1.58	0.67± 0.06	55.17± 4.49	50.67± 4.02
Ischemia	42.83±1.49$^{\bullet}$	1.02± 0.06$^{\bullet}$	102.2± 7.48$^{\bullet}$	108.2± 8.44$^{\bullet}$
I/R	63.33±4.57$^{\bullet\circ}$	1.27± 0.05$^{\bullet\circ}$	178± 6.20$^{\bullet\circ}$	199.8± 5.29$^{\bullet\circ}$
I/R+losartan	33±4.89$^{\bullet}$*	0.72± 0.05°*	74± 5.96°*	64.33± 7.53°*

Data represent the mean ± SEM of observations from 6 rats. $^{\bullet}$: significantly different from Sham group; $^{\circ}$: significantly different from Ischemia group; *: significantly different from I/R group, P≤0.05; ALT; Alanine aminotransferase; AST; Aspartate aminotransferase.

- ***Changes in hepatic MDA and TNF-α levels in different experimental groups***

The level of liver MDA did not change significantly ischemia group. However, it was significantly increased in IR group (19.99±2.59 nmol/g tissue vs. 8.83±1.22 nmol/g tissue and 13.05± 1.14 nmol/g tissue, P≤0.05) compared to either sham control or ischemia

groups, respectively. On the contrary, pretreatment with losartan, caused a significant decrease in hepatic MDA levels (12.30±1.31 nmol/g tissue) compared to IR non treated group (Figure 1).

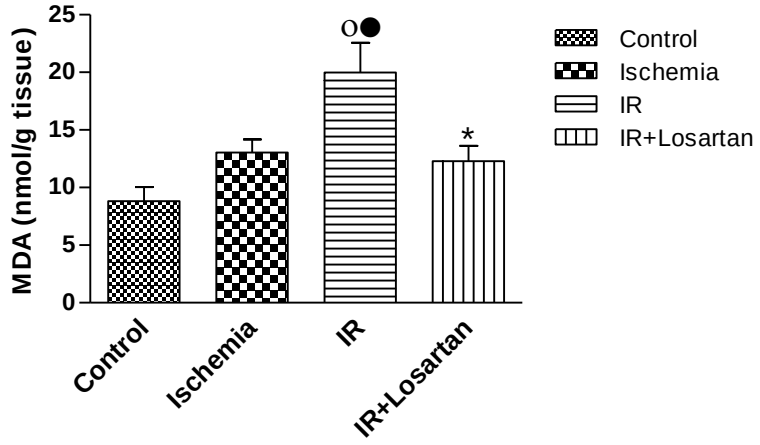

Fig. 1: Alterations in hepatic MDA level in different experimental groups. •: Significantly different from control group; °: Significantly different from ischemia group; *: Significantly different from ischemia/reperfusion (IR) group, p≤0.05.

Similarly, ischemia group did not show any significant change in liver TNF-α level but, in IR group, liver TNF-α level was significantly increased (594.3±63.81 pg/100mg tissue vs. 182.1±14.55 pg/100mg tissue and 274.4±23.21 pg/100mg tissue, P≤0.05) compared to either sham control or ischemia groups, respectively. Pretreatment with losartan, significantly attenuated the IR-induced increase in TNF-α level to 349.2±25.54 pg/100mg tissue, P≤0.05) compared to IR non treated group, but still significantly higher than the control level (Figure 2).

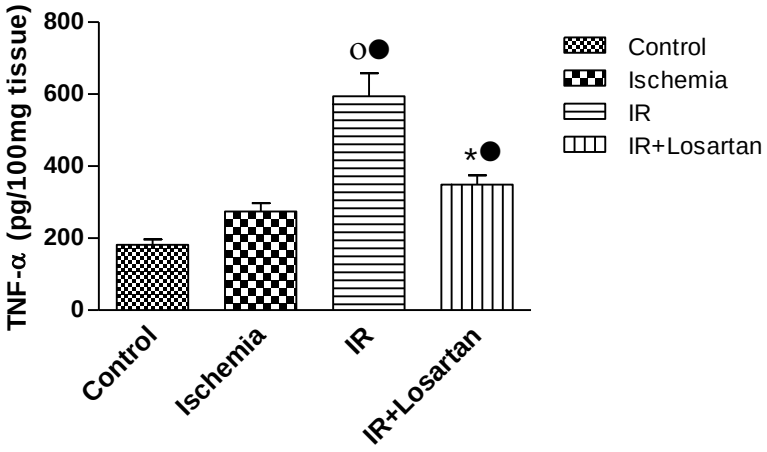

Fig. 2: Alterations in hepatic TNF-α level in different experimental groups. •: Significantly different from control group; °: Significantly different from ischemia group; *: Significantly different from ischemia/reperfusion (IR) group, p≤0.05.

- ***Histopathological changes in hepatic tissue in different experimental groups***

Liver sections were evaluated for the presence of congestion, cellular degenerative changes, cytoplasmic vacuolization and leukocyte infiltration. The sections from the sham-operated rats displayed minimal/no changes. In ischemia group, congestion was present, but there was no apparent evidence of cellular degenerative changes including cytoplasmic vacuolization or leukocyte infiltration. In IR group, congestion was more sever, vacuolezation was frequent, irregularity, pale nuclei, disintegrated cytoplasm, and infiltration of leukocytes were also seen. In losartan-pretreated IR group, histological changes were lessened showing less congestion, minimal vacuolization with absence of leukocyte infiltration (Figure 3).

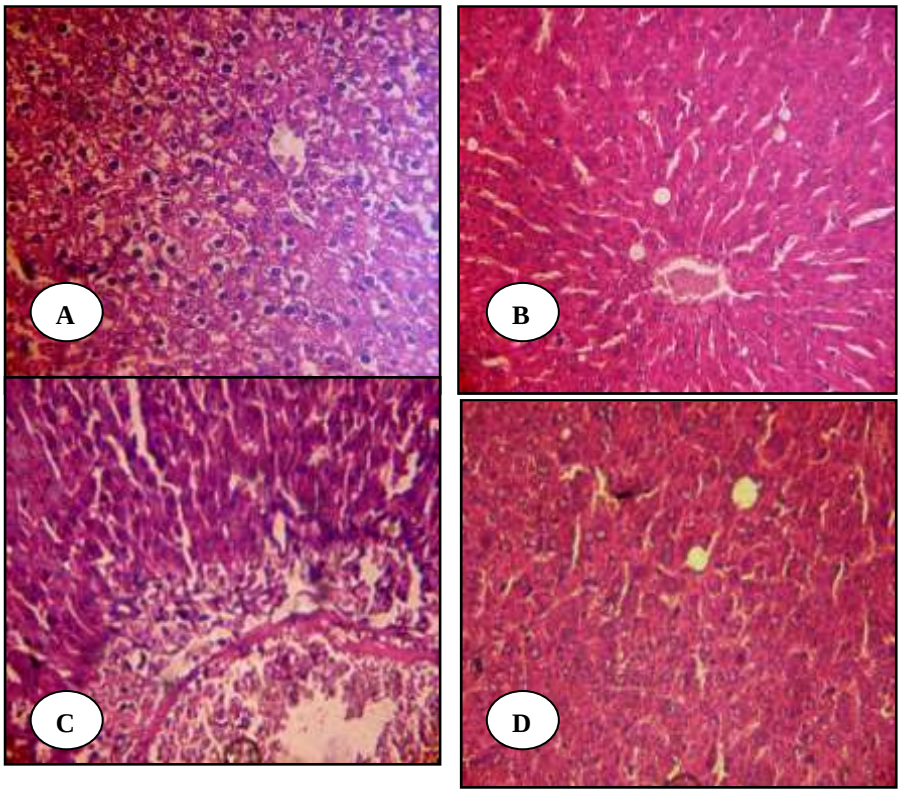

Figure (3): Hematoxylin and eosin-stained sections of rat liver. A: Sham-operated group: showed normal hepatic lobular architecture and normal hepatocytes with granulated cytoplasm and small uniform nuclei. B: Ischemia group: Showed congestion in the central vein with minimal cytoplasmic vacuolization. C: IR group: Showed marked congestion, pale nuclei with frequent vacuolization and leukocyte infiltration. D: losartan group: showed less congestion, minimal vacuolization but no leukocyte infiltration (x 400).

DISCUSSION:

Acute renal ischemic injury continues to be associated with a high mortality rate. Renal IR injury occurs in many clinical situations, such as transplantation, partial nephrectomy, sepsis, hydronephrosis, or elective urological operations. Although most research in this area has focused on the renal response to this injury, recent

work has suggested that renal injury may affect and also be regulated by the extra-renal organs including the liver[15,2].

In the present study, the changes in hepatic function, histology, MDA and TNF-α level were examined after induction of rat renal ischemic injury.

60 minute-period of renal ischemia was selected in the present study based on a previous study by Kadkhodaee et al.,[16] who reported that a minimum of 45-60 min ischemia is needed to study the effects of renal injury on the liver as a remote organ. So, the longer duration of renal ischemia was chosen to be certain of hepatic affection.

In the present study, renal ischemia, either alone or followed by 1h reperfusion, significantly attenuated the renal function as evidenced by significant increases in serum urea and creatinine levels. Liver function was also affected in both groups, as evidenced by a significant increase in serum transaminases (ALT, AST) and structural alteration, as evidenced by the histological changes in liver sections. However, these changes were more evident in IR group and were associated with a significant increase in hepatic MDA and TNF-α level, indicating the development of oxidative stress and inflammatory response. Findings which are in agreement with Serteser et al., (2002), who reported some changes in hepatic TNF-α levels and oxidation products after renal IR injury in mice[17].

Although ischemia can damage cells directly, liver cells have defense mechanisms to protect against such insults if the ischemic time is relatively brief [18], which was not the case in the present study, most probably due to the longer period of renal ischemia. However, even if the liver cells survive the ischemic insult, reintroduction of blood flow (reperfusion) often leads to cellular damage[19]. The hypothesis is that this second "hit" to the liver during reperfusion is mediated predominantly by inflammation and generation of ROS[20].

In support of this hypothesis, some authors have suggested that mobilization of the complement cascade stimulates Kupffer cells, which mediate hepatocyte damage after limb IR injury[21,22]. Furthermore, Hoke et al., (2007) demonstrated that acute absence of kidney function results in pulmonary injury independent of renal ischemia, and highlighted the critical role of the kidney in the maintenance of serum cytokine balance and pulmonary homeostasis[23]. Moreover, Park et al., (2011) reported an improvement in liver and intestinal protection in mice treated with neutralizing antibodies or mice deficient in TNF-α after renal ischemic injury[24], suggesting a critical role of proinflammatory cytokines namely, TNF-α in mediating hepatic injury induced by renal IR.

It has been previously reported that renal IR affects liver inflammatory status with subsequent hepatic injury, possibly via increased renal production or impaired clearance of mediators of tissue injury, namely proinflammatory cytokines such as TNF-α[24]; findings which were confirmed in the present study by the significant increase in hepatic TNF-α level observed in IR group.

Oxidative stress and ROS production after reperfusion also seems to be a critical component in remote tissue injury[16]. There most likely are numerous sources of ROS during reperfusion. For example, reperfusion

itself leads to the generation of free radicals via enzymes such as xanthine oxidase[25]. Furthermore, intrinsic inflammatory cells (e.g.,macrophages) and recruited inflammatory cells (e.g., neutrophils) also generate ROS in liver[22]. These observations were confirmed, in the present study, by the significant increase in hepatic MDA level (as a marker of lipid peroxidation) in IR group, which was associated with marked alterations in hepatic function and histology. Thus, it is likely to say that oxidative stress and ROS production may also be implicated in the pathogenesis of hepatic damage in such condition.

In a trial to clarify the possible underlying mechanisms mediating the injurious effect of renal ischemia on liver function, a separate group was made in which rats were pretreated with losartan, a selective AT1 receptor antagonist, before being subjected to renal IR to examine whether the RAS, which is well known to be activated under ischemic conditions[13], may or may not be a possible mechanism of hepatic affection in such condition.

In the present study, pretreatment with losartan abolished the injurious effect of IR on the liver tissue as evidenced by the significant decrease in serum transaminases and improvement of histological changes. This was also accompanied with a significant decrease in hepatic MDA and TNF-α level, indicating attenuation of oxidative stress and inflammatory process. Thus, these results suggest a possible role for RAS including its final product Ang II in the pathogenesis of liver injury induced by renal IR.

It has been proposed that the RAS may contribute to inflammation in target organs, including the liver. However, most work investigating such a role has been in models of chronic tissue damage (e.g., renal and hepatic fibrosis). Although some studies have suggested that the RAS is involved in some models of acute inflammation in liver[26], the potential mechanisms by which this system may contribute to inflammation and damage are unclear. In the present study, losartan pretreatment blocked the IR-induced increase in hepatic TNF-α level. RAS inhibition also had profound protective effects on subsequent liver damage and recruitment of inflammatory cells (as evidenced by absence of leukocytic infiltration in hepatic sections; findings which are in agreement with those of Bataller et al.,[27].

Ang II has been shown to stimulate expression of proinflammatory cytokines[28], adhesion molecules (e.g., intercellular adhesion molecule-1 [ICAM-1][7] and to activate proinflammatory transcription factors (e.g., activator protein-1 and nuclear factor κB)[29]. Furthermore, Harrison et al.,[30] reported that Ang II can lead directly to increased superoxide production via nicotina-mide adenine dinucleotide phosphate oxidase in macrophages. In the liver, stellate cells have been shown to respond to Ang II to produce proin-flammatory and profibrotic cytokines and chemokines and reactive oxygen species[27]. So, blocking the action of Ang II by losartan would be expected to lessen the severity of liver damage induced by renal IR, mostly via suppression of inflammatory cytokines namely, TNF-α, findings which were confirmed by the data of the present study.

In addition to preventing the inflammation and subsequent damage, blocking of AT1 receptors by losartan also protected against oxidative stress

and ROS production induced by renal IR, as evidenced by the significant decrease in hepatic MDA levels compared to IR non treated group. This protective effect was observed, not only in the biochemical analysis, but also in hepatocytes that appeared undamaged by histological assessment. Taken together, these data clearly show that losartan had an antioxidant effect against hepatic injury under such condition.

A broader definition of antioxidants includes not only therapies that directly intercept free radicals, but also those that prevent the formation of these species[31]. In this context, it is likely to say that the antioxidant effect of losartan, observed in the present study could be a consequence of the prevention of the infiltration and activation of inflammatory cells by losartan rather than a direct free radical scavenging effect[13].

In conclusion, the data of the present study clearly demonstrated that renal ischemia per se or followed by 1h reperfusion caused detrimental changes in liver histology and function (which was more evident after reperfusion), possibly via oxidative stress and inflammatory cytokines, which ultimately leads to attenuation in hepatic defense mechanisms and subsequent damage. Activation of RAS could be a possible mechanism mediating that effect. Therefore, there might be a rational for pharmacological blockade of RAS to protect against remote tissue injury, in that case the liver, especially following renal ischemic insult.

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تأثيرالاصابة بالقصور الشرياني الحاد للكلى واعادة الارتواء المحدث تجريبيا علي وظيفة كبد الفأر: دورالأنجيوتنسين 2

الهدف: يهدف البحث دراسة تأثيرالاصابة بالقصور الشرياني الحاد للكلى واعادة الارتواء المحدث تجريبيا علي وظائف الكبد في الفئران, والدور المحتمل للأنجيوتنسين 2 في آليات اصابة الكبد في مثل هذه الحالة.

طرق البحث: وقد استخدمت في هذه الدراسة 24 من ذكور الفئران البيضاء حيث قسمت عشوائيا إلى المجموعات التجريبية التالية (6 فئران في المجموعة):أولا المجموعة الضابطة، حيث تركت الفئران تجول بحرية في أقفاصها، ثانيا مجموعة القصور الشرياني الحاد للكلى، والتي أحدث فيها القصور الشرياني الحاد للكلى لمدة 60 دقيقة؛ ثالثا مجموعة القصور الشرياني الحاد للكلى مع اعادة الارتواء حيث تم احداث القصور الشرياني الحاد للكلى لمدة 60 دقيقة تلاه اعادة للارتواء لمدة 60 دقيقة أخرى, وأخيرا مجموعة القصور الشرياني الحاد للكلى مع اعادة الارتواء والمعالجة مسبقا بعقار اللوسارتان (كمضاد للنوع الأول من مستقبلات الأنجيوتنسين 2) بجرعة مقدارها(5 مجم / كجم)، بالتزقيم عن طريق الفم 24 و 1.5 ساعة قبل احداث القصور الشرياني الحاد للكلى واعادة الارتواء. وفى نهاية التجربة، تم قتل الفئران؛ حيث تم جمع عينات من الدم لقياس نسبة اليوريا، والكرياتينين، وانزيمات الكبد. كما أخذت عينات من أنسجة الكبد لقياس محتواها من الشوارد الحرة وعامل نخر الورم-ألفا, هذا بالاضافة الى الفحص المجهرى لأنسجة الكبد.

النتائج: وقد أظهرت نتائج الدراسة حدوث تغيرات دالة احصائيا في كل من وظائف الكبد والكلى عند التعرض للقصور الشرياني الحاد للكلى اما وحده أو مصحوبا باعادة الارتواء والواضحة من خلال زيادة دالة احصائيا في مستوى اليوريا و الكرياتينين وانزيمات الكبد في الدم, فضلا عن التغييرات المجهرية في قطاعات الكبد, وان كانت أكثر وضوحا في مجموعة القصور الشرياني الحاد للكلى مع اعادة الارتواء والتي كانت أيضا مصحوبة بزيادة دالة احصائيا في مستوى الشوارد الحرة ومعامل نخر الورم-ألفا في الكبد. كما أظهرت نتائج الدراسة التاثير الوقائي لعقار اللوسارتان ضد اصابة الكبد الناتجة عن القصور الشرياني الحاد للكلى مع اعادة الارتواء الواضحة من خلال الانخفاض الدال احصائيا في انزيمات الكبد وتحسن التغيرات المجهرية في

أنسجة الكبد، والذي ترافق مع انخفاض دال احصائيا في مستوى الشوارد الحرة ومعامل نخر الورم-ألفا في الكبد مقارنة بالمجموعة الغير معالجة.
الاستنتاج: نستخلص من هذه الدراسة التأثير السلبي للقصور الشرياني الحاد للكلى علي وظائف الكبد، وربما يعزى ذلك الى تنشيط عمليات الأكسدة والالتهابات, كما تشير نتائج الدراسة الى الدورالمحتمل للأنجيوتنسين 2 في حدوث اصابة الكبد في هذه الحالة. ولذلك فان التدخل الدوائى باستخدام مضادات الرينين-أنجيونسين قد يفيد في وقاية أعضاء الجسم البعيدة من الاصابة الناتجة عن التعرض للقصور الشرياني الحاد واعادة الارتواء, وخاصة للكلى.

الكلمات الدالة: القصور الشريانى الحاد واعادة الارتواء, الكبد, أنجيوتنسين 2, الشوارد الحرة, معامل نخر الورم-ألفا.