

Gossypin Protects Against Renal Ischemia- Reperfusion Injury in Rats

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Abstract

Renal injury occurring as a result of renal ischemia-reperfusion may lead to renal failure or even death. The aim of this study is to investigate possible protective effects of Gossypin on tissue damage occurred due to ischemia-reperfusion in rat kidney tissue. A total of 48 male Wistar albino rats were used in the study. These rats were randomly divided into 6 groups equally (n = 8). The created groups were control (C), sham (S), ischemia-reperfusion (I/R), I/R + DMSO, I/R + 400 µg/kg gossypin and I/R + 4 mg/kg gossypin. In the rats of sham group, the right nephrectomy was performed. In the rats of other groups rather than sham, the left renal artery was clamped after performing the right nephrectomy. Gossypin was administered intraperitoneally before the reperfusion. 24 h reperfusion was applied to the left renal after 1 h of ischemia. TNF-α, IL-1β, IL-6 and IL-10 levels were measured with spectrophotometric methods in the kidney tissues after the procedures were completed. Apoptosis and inflammatory pathways were evaluated histopathologically using Caspase 3 and NF-κB antibodies. There was a statistically significant decrease in IL-1β and IL-6 levels of the gossypin groups compared to the I/R group (P<0.05). As the level of TNF-α was decreased in the gossypin administered groups compared to the I/R group although not statistically significant, the level of IL-10 was increased. In the present study, we aimed to show that gossypin in renal I/R model is effective on inflammatory process and apoptosis and that it can be used in routine treatment to decrease the damage in all reasons that may cause I/R. In addition, this study can shed light on the studies to be done in this field in the future.

Keywords: Renal ischemia reperfusion injury, Gossypin, Cytokines, Caspase-3

Gossypin Sıçanlarda Böbrek İskemi- Reperfüzyon Hasarına Karşı Korur

Öz

Böbrek iskemi reperfüzyonu sonucu meydana gelen renal hasar, böbrek yetmezliğine ve hatta ölüme neden olabilir. Çalışmanın amacı sıçan böbrek dokusunda iskemi-reperfüzyona bağlı oluşan doku hasarına karşı gossypinin olası koruyucu etkilerini araştırmaktır. Çalışmada toplam 48 adet Wistar albino cinsi erkek sıçan kullanıldı. Sıçanlar randomize ve eşit olmak üzere 6 gruba ayrıldı (n=8). Kontrol (C), sham (S), istemi-reperfüzyon (I/R), I/R + DMSO, I/R + 400 µg/kg gossypin ve I/R + 4 mg/kg gossypin grupları oluşturuldu. Sham grubunda arka bölge açılarak sağ nefrektomi yapıldı. Gossypin reperfüzyondan önce intraperitoneal olarak uygulandı. Sol renal artere 1 saat iskemi sonunda 24 saat reperfüzyon uygulandı. Prosedürler tamamlandıktan sonra böbrek dokularında spektrofotometrik metodlarla TNF-α, IL-1β, IL-6 and IL-10 seviyeleri ölçüldü. Apoptozis ve inflamatuvar yolaklar kazpaz-3 ve NF-κB antikorları kullanılarak değerlendirildi. Gossypin grupları I/R grupları ile kıyaslanınca IL-1β and IL-6 seviyelerinde istatistiksel olarak önemli derecede azalma tespit edildi (P<0.05). I/R grubu ile kıyaslandığında gossypin uygulanan gruplarda istatistiksel olarak anlamlı olmasa da TNF-α miktarlarına azalma tespit edildi. Mevcut çalışmada gossypinin I/R modelindeki inflamasyon aşaması ve apoptozis üzerine etkisini ve I/R neden olan tüm durumlarda oluşacak hasarı azaltmada kullanılabileceğini göstermeye çalıştık. Ek olarak, bu çalışma bulanda ileride yapılacak olan çalışmalara ışık tutabilir.

Anahtar sözcükler: Renal istemi reperfüzyon hasarı, Gossypin, Sitokinler, Kaspaz-3

INTRODUCTION

Renal ischemic injury is a complex syndrome characterized by an accelerated cycle of inflammation, cell damage,

and persistent local ischemia caused by many cellular anomalies^[1]. Kidney is particularly sensitive to ischemia reperfusion (I/R) injury due to its high metabolism and vascular anatomy. Acute renal injury causes acute and



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chronic renal failure. The I/R injury is seen as secondary to trauma, shock, sepsis, renal transplantation, cardiovascular and urological surgery in intensive care units [2,3]. Renal I/R injury resulting in acute renal failure is a major clinical problem due to the high mortality rate [4,5]. Therefore, treatment strategies or therapeutics that prevent or reduce I/R-induced acute renal injury have clinical significance.

It was shown that increased production in reactive oxygen species (ROS) caused by I/R activates leukocyte infiltration in the kidney, and these infiltrated-leukocytes synergistically produce more ROS and cytokines that directly lead to renal damage [6]. ROS also inactivate antioxidant enzymes [7,8] and cause increase in activated neutrophils and cytokines [9,10] and contributes to the activation of apoptotic genes [11,12]. I/R increases level of pro-inflammatory cytokines such as nuclear factor kappa B (NF- κ B) which plays a role in the regulation of various genes involved in the acute phase inflammatory reaction [13], tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and decreases level of anti-inflammatory cytokines such as interleukin-10 (IL-10) [14-18]. Gossypin (Gos) (gossypin-8-O glucoside, 3,5,7,3,4-pentahydroxy-8-O-glucosylflavone) is a bioflavonoid naturally found in plants of the family Malvaceae, especially in *Hibiscus vitifolius* [19,20]. The ability of Gos to protect against various diseases has been proven in many studies [21-27]. In many studies, it was shown that Gos has antioxidant, anti-inflammatory and analgesic properties [19,23,28,29]. The aim of this study is to investigate the possible renoprotective effects of Gos as a new alternative to this damage by examining inflammation markers and apoptosis process molecules in the experimental renal I/R injury model by histopathological methods.

MATERIAL and METHODS

Chemicals

Gossypin (Biovision, USA) was dissolved in dimethylsulfoxide (DMSO, Amresco, Canada) and administered intraperitoneally at 400 μ g/kg and 4 mg/kg doses.

Approval of Ethics Committee and Center of Research

The study with Atatürk University Animal Experiments Local Ethics Committee approval (dated 19.04.2016, with decision no: 2/71) was conducted at the Atatürk University Experimental Animal Production and Research Center. All the procedures in the study were performed in line with the ethics committee protocol. During the course of the experiment, rats were conserved in 12 h light/12 h dark cycle at 20-22°C, and the ad libitum feeding of standard chow and normal tap water was performed in rats.

Experimental Animals and Creating Groups

A total of 48 male Wistar albino rats (12-16 weeks, 240-260 g) were used in the study. 6 groups with 8 rats in each

were randomly formed. Group 1 is the control group with performing no surgical intervention. The second group was sham group and back region of rats in this group were opened with the help of a bistoury, and the right renal pedicle was dissected by connecting with silk. The experimental model was performed over single kidney (left kidney). The third group was the I/R group. In third group, after similar application of the procedures of the second group, the left renal pedicle was clamped. After 1 h of ischemia, the clamp was opened and kidney was subjected to reperfusion for 24 h. The animals in the 4. (I/R + DMSO), 5. (I/R + 400 μ g/kg gossypin) and 6. (I/R + 4 mg/kg gossypin) groups underwent surgical procedures. And then, 300 μ L of DMSO to 4. group, 400 μ g/kg of gossypin to 5. group and 4 mg/kg of gossypin to 6. group were administered intraperitoneally before starting the reperfusion. At the end of the study, renal tissues in all groups were taken for necessary analyses.

Collection and Storage of Tissue Specimens After Sacrification

The experimental model in rats was performed under anesthesia formed with intramuscular administration of 75 mg/kg ketamine, 8 mg/kg xylazine. The kidney tissues of the sacrificed rats were divided in two, and one of the pieces was placed in a 10% formaldehyde solution for histopathological procedures and the other was stored at -80°C for biochemical analyses.

Tissue Homogenization and Determination of Biochemical Parameters

For biochemical measurements, 10% homogenate was formed by adding phosphate buffer to kidney tissues and then homogenized by centrifuging at 12.000 rpm for 1-2 min. on ice (IKA, Germany). Homogenized tissue samples were centrifuged at 5000 rpm for 30 min at +4°C to obtain supernatant. In the biochemical analysis of the groups, IL-1 β [Cat No: E-EL-R0012, Elabscience], IL-6 (Cat No: E-EL-R0015, Elabscience), IL-10 (Cat No: E-EL-R0016, Elabscience), TNF- α (Cat No: E-EL-R0019, Elabscience) levels were measured from supernatants using rat specific ELISA kits. Measurements were performed in accordance with kits' own protocols.

Histopathological Examination

Caspase-3, NF- κ B antibodies were used to investigate apoptosis and inflammatory pathways in the kidney tissues of the groups. Hematoxylin-eosin staining method was used to determine the damage levels.

Statistical Analysis

The IBM SPSS 20.0 package program was used in the analysis. Using the One Way ANOVA method for statistical analysis, $P < 0.05$ was considered statistically significant. Data were expressed as mean \pm standard deviation.

RESULTS

Cytokine concentration was measured after renal reperfusion of the kidney for 24 h. The TNF- α levels for each group are shown in Fig. 1A, there was no difference among the groups. Renal I/R caused a marked elevation of proinflammatory cytokines, IL-1 β and IL-6 in kidney tissue (Fig. 1B,C, respectively). In the groups treated with gossypin, the levels of these cytokines decreased. The level of IL-10, an anti-inflammatory cytokine, in renal tissue was significantly reduced in rats with renal I/R, and IL-10 levels were slightly increased in groups treated with Gossypin (Fig. 1D).

In Fig. 2, it is shown that the staining of the groups by the hematoxylin-eosin method. Differences and similarities between the groups have been expressed in various symbols.

Caspase-3 immunohistochemical staining of the study groups are shown in Fig. 3 and their evaluation is shown in Table 1. In Table 1, it is seen that there was a decrease in Caspase-3 immunopositivity of podocytes and tubule cells in the Gos groups compared to I/R group. NF- κ B immunohistochemical staining of the study groups are shown in Fig. 4 and their evaluation is shown in Table 2. In Table 2, it is seen that there was a decrease in NF- κ B immunopositivity of podocytes and tubule cells in the gos groups compared to I/R group.

DISCUSSION

Renal I/R injury occurs by reperfusion after reduced or discontinuation of blood flow to the kidneys, and causes acute renal failure. It is a common condition in many surgical procedures [30-32]. Acute renal failure caused by I/R injury is a serious health problem and, unfortunately,

there is no therapeutic or protective agent in this disease at the present. The aim of this study was to investigate the protective effects of GOS against I/R induced renal injury by some cytokine levels and histopathological analysis of NF- κ B and caspase-3 immunopositivity. Many studies have shown that gossypin is a flavonoid with strong anti-inflammatory and immunomodulatory properties [20,21,29]. However, the factors that mediate the anti-inflammatory effect of GOS remain unclear. Therefore, we tried to understand the effects of GOS on I/R-induced renal injury and the underlying anti-inflammatory mechanisms.

Initiation of reperfusion of GOS in ischemic tissue causes inflammatory reactions [33]. One of the most known intracellular signaling pathways of inflammatory responses is the NF- κ B signaling pathway [32]. In many studies, it was shown that NF- κ B is an important transcription factor during inflammatory process and ischemia reperfusion and NF- κ B activation is responsible for the activation of many proinflammatory cytokines such as interleukin-1 β , interleukin-6, tumor necrosis factor- α [34-36].

In knockout mice, it is considered that NF- κ B plays an important role in reducing sensitivity to I/R injury, and NF- κ B-mediated inflammatory responses cause tissue damage [37]. In many I/R injury studies, levels of various proinflammatory cytokine such as TNF- α , IL-1 and IL-6 were reported to significantly increase during reperfusion [10,38,39]. IL-10, an anti-inflammatory cytokine [40,41], reduced the renal injury in mice by inhibiting inflammatory and apoptotic pathways [42]. In studies of gossypin, no information about IL-10 has been seen. In a nephrotoxicity model, it was shown that increased TNF- α , IL-1 and IL-6 levels in the kidney were reduced by gossypin administration [23]. It was shown that gossypin inhibited NF- κ B, in a culture study [29]. In parallel with this study, we determined that NF- κ B immunopositivity decreased in gossypin groups

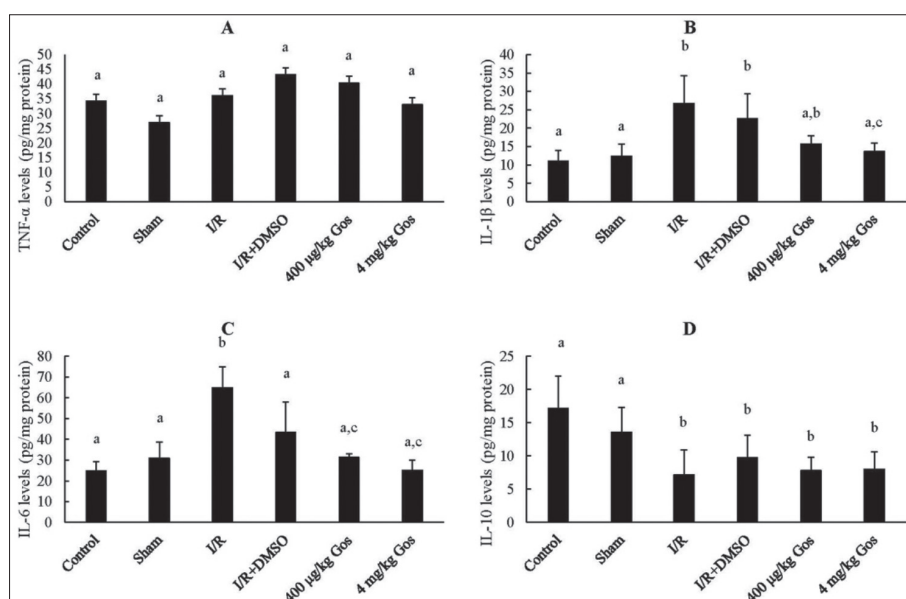


Fig 1. There was no significant difference between the groups in terms of TNF- α levels $P > 0.05$, IL-1 β and IL-6 levels b; $P < 0.05$, c; $P < 0.05$, IL-10 level b; $P < 0.05$

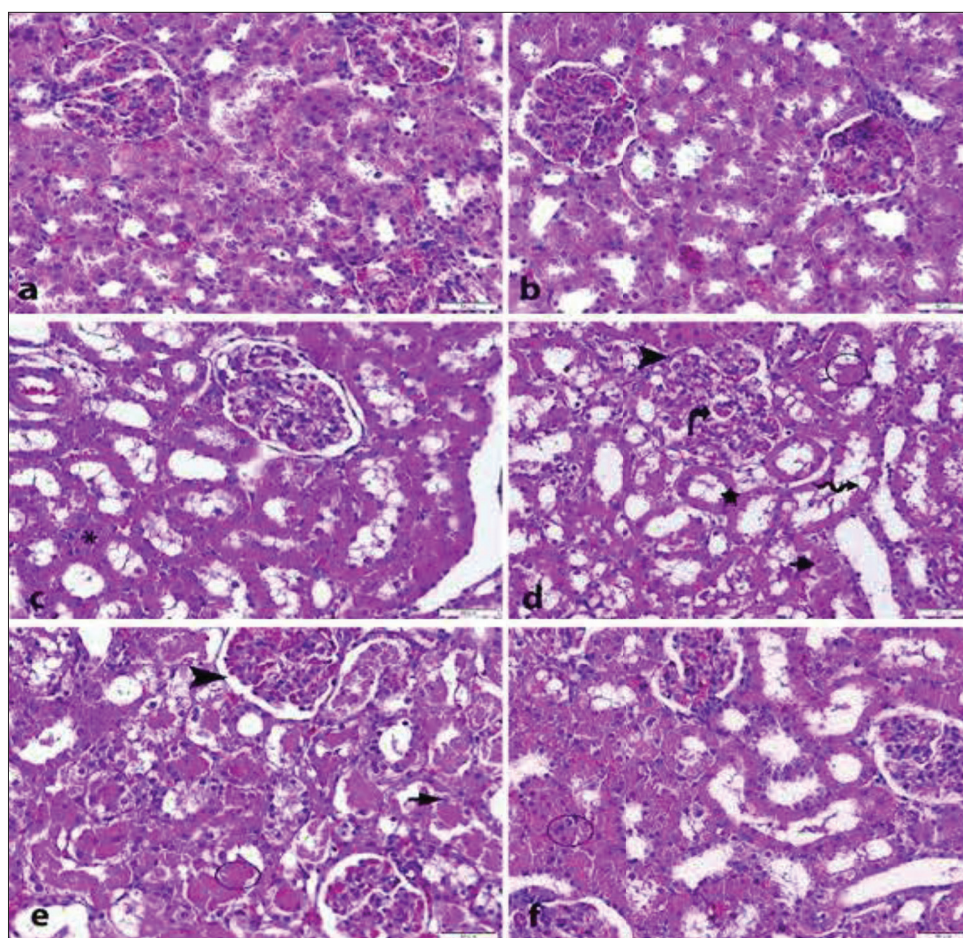


Fig 2. Hematoxylin-Eosin, a: Control Group; b: Sham Group; c: I/R + DMSO Group; d: I/R Group; e: I/R + 400 µg/kg Gossypin Group; f: I/R + 4 mg/kg Gossypin Group. Asterix: Increase in connective tissue; Arrowhead: irregularities in Bowman's capsule; Star: Cellular loss in tubules; Spiral arrow: Tube basal membrane defect; Thin arrow: Hypertrophy in proximal tubule cells; White arrow: Distal tubule pyknotic nucleus; Rotating arrow: Capillary dilatation; Circle: Hyalinization in Tubules- 20X (scale bar 50 µm)

Table 1. Caspase-3 immunopositivity

Immunoreactivity	Control	Sham	I/R	I/R+DMSO	400 µg/kg Gos	4 mg/kg Gos
Podocyte cells	-	-	+++	-	+	+
Tubule cells	-	-	+++	+	++	+

Expression levels; -: No immunopositivity; + Mild immunopositivity; ++ Moderate immunopositivity; +++ Severe immunopositivity

Table 2. NF-κB immunopositivity

Immunoreactivity	Control	Sham	I/R	I/R+DMSO	400 µg/kg Gos	4 mg/kg Gos
Podocyte cells	-	-	+++	-	++	+
Tubule cells	-	-	+++	+	++	+

Expression levels; -: No immunopositivity; + Mild immunopositivity; ++ Moderate immunopositivity; +++ Severe immunopositivity

compared to I/R groups. In our study, the level of TNF-α decreased in the gossypin administered groups although not statistically significant, compared to the I/R group. It was shown that there was a statistically significant decrease in IL-1β level of the gossypin groups compared to the I/R group. Irfan et al. reported that NF-κB and some

cytokine levels decreased in sepsis model similar to our results [21]. In statistical analysis, IL-10 levels were detected to be significantly decreased in the I/R group compared to the control group. IL-10 levels in gossypin administered groups increased, although not statistically significant, compared to I/R group. When viewed as a whole, it was

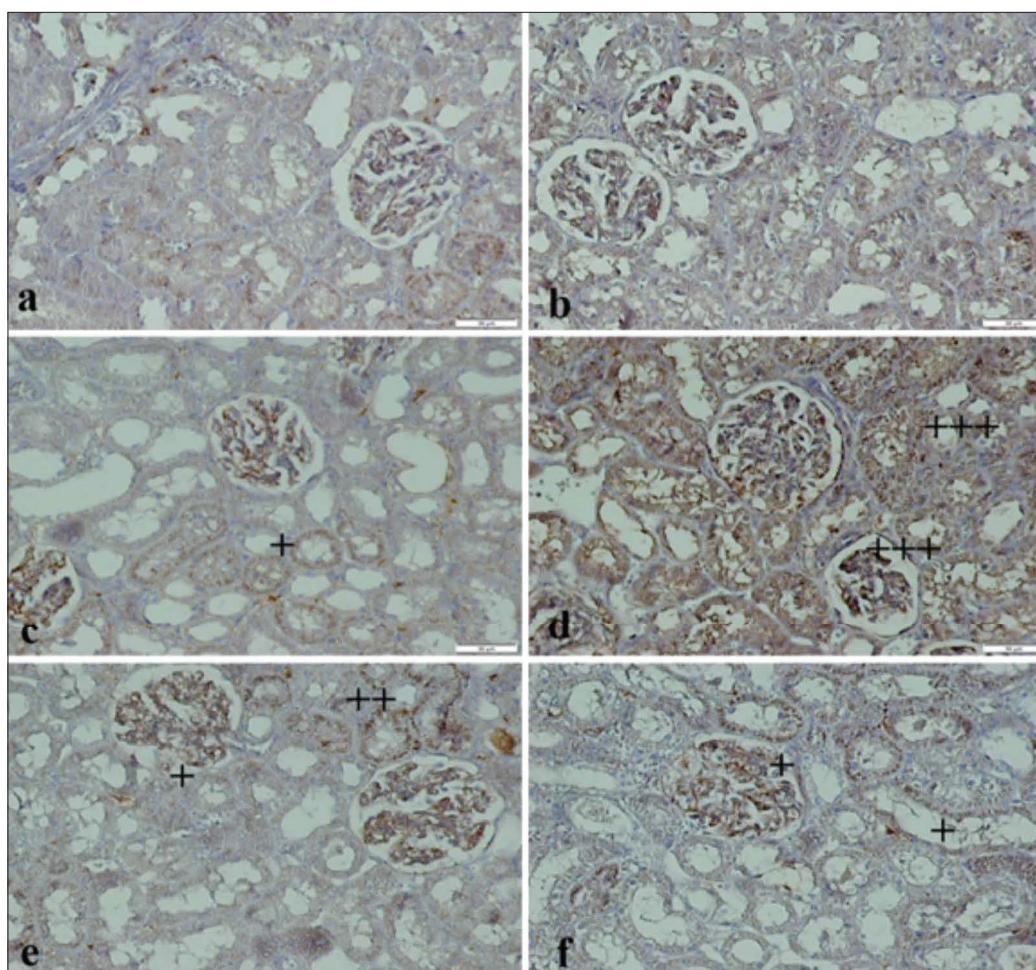


Fig 3. Caspase-3, a: Control Group; b: Sham Group; c: I/R + DMSO Group; d: I/R Group; e: I/R + 400 µg/kg Gossypin Group; f: I/R + 4 mg/kg Gossypin Group (scale bar: 50 µm)

shown that gossypin reduces the I/R injury by suppressing the inflammatory pathway.

Apoptosis is important for the development and homeostasis in many types of tissue [43]. Apoptosis is a programmed cell death caused by endogenous or exogenous factors. It eliminates abnormal or dead cells to maintain homeostasis. Apoptosis and necrosis are two main types of cell death during I/R injury, and more than half of the dead cells die of apoptosis during the first 24 h of reperfusion [44-46].

When the caspases that play a role in the later stages of the apoptosis pathway activated once, the effector caspase induces a series of hydrolysis reactions leading to the initiation of cell death [47]. Caspase-3 is an important marker of apoptosis [48-50] and leads to the initiation of cascades causing apoptosis [51]. It is widely accepted that Caspase-3 is an important protease and is an important effector substance involved in hydrolysis by acting alone or in association with apoptosis-related proteins [52,53]. We encountered only two cancer studies investigating the effect of gossypin on apoptosis in the literature and it was shown that gossypin increases apoptosis to destroy the cancer in these studies [29,54]. In the present

study, we determined that there was a decrease that is in caspase-3 immunopositivity of podocytes and tubule cells in the gossypin groups compared to I/R group, and we showed that gossypin has a renoprotective effect due to antiapoptotic properties by reducing the level of Caspase-3 in contrast to the effect observed in cancer.

As a result, treatment with Gossypin significantly reduced the renal injury caused by renal I/R. However, both treatment doses used in the study reduced cytokine levels and oxidative stress, suppressed apoptosis in kidney tissues.

CONFLICT OF INTEREST

None.

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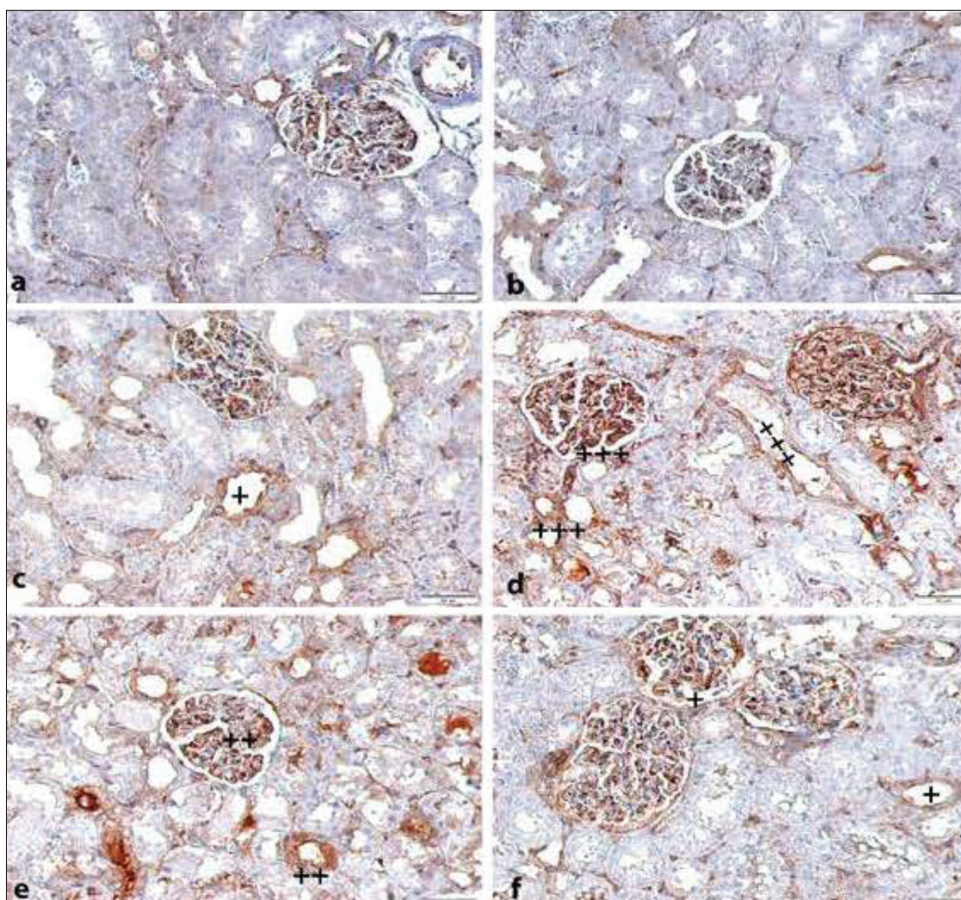


Fig 4. NF-κB, a: Control Group; b: Sham Group; c: I/R + DMSO Group; d: I/R Group; e: I/R + 400 µg/kg Gossypin Group; f: I/R + 4 mg/kg Gossypin Group

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