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## Original Research Paper

### EFFECTS OF THE TOLL-LIKE RECEPTOR 4 INHIBITOR BAICALIN ON THE KIDNEY OF LPS-CHALLENGED MICE

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#### ABSTRACT

**Background:** Lipopolysaccharide (LPS) is recognized by the innate immune system via Toll-Like Receptor 4 (TLR4) whose activation leads to the production of numerous immunoregulatory molecules and reactive oxygen species. This study was designed to investigate the effects of baicalin (TLR4-inhibitor) on LPS-induced renal oxidative and immunological changes of mice.

**Methods:** Male Swiss mice were injected with LPS (1 mg/kg; i.p.) and the effects of pretreatment with TLR4 inhibitor (baicalin 50 mg/kg; i.p.) on LPS-induced renal failure and kidney pathology were examined 3 or 24 hours post LPS injection. Plasma concentrations of urea, creatinine and lactate dehydrogenase (LDH) activity as well as kidney contents of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-10 were assessed. Oxidative stress as well as the RNA expression of *neutrophil gelatinase-associated lipocalin (NGAL)* and *inhibitor of nuclear factor-kappa B (NF- $\kappa$ B) alpha (I $\kappa$ B $\alpha$ )* in the kidney were also evaluated.

**Results:** LPS markedly increased plasma urea and creatinine levels as well as LDH activity. Furthermore, LPS augmented renal malondialdehyde and IL-10 levels as well as caspase-3 activity. However, it diminished the reduced glutathione and IL-1 $\beta$  levels; besides, it inhibited superoxide dismutase and catalase activities in the kidney. Histopathologic studies backed the previous observations. Baicalin pretreatment significantly ameliorated LPS-induced alterations and suppressed acute kidney injury (AKI) by modulating *NGAL* and *I $\kappa$ B- $\alpha$*  mRNA levels.

**Conclusion:** The present study suggests that baicalin has potential beneficial role in sepsis prevention and its associated renal derangements.

**Keywords:** Baicalin, Gene expression, Interleukins, LPS, Oxidative stress.

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#### INTRODUCTION

Septic shock is one of the most common causes of acute kidney injury (AKI).<sup>1</sup> It was observed that acute renal failure resulted in 45% mortality compared with 70% mortality when it is combined with sepsis.<sup>2</sup> Therefore, it is crucial to reveal the precise mechanisms involved in development of AKI. Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria that is involved in the

pathogenesis of sepsis-induced AKI.<sup>3</sup> LPS is commonly employed for investigating mechanisms of sepsis-related conditions<sup>4</sup>, where alterations of immunity as well as proinflammatory conditions were suggested as possible culprits.<sup>5</sup> Toll like receptor 4 (TLR4) is considered as the critical component of the LPS receptor complex.<sup>6</sup> Furthermore, TLR4 was suggested to recognize endogenous molecules

that are exposed during cellular injury and extracellular matrix remodeling.<sup>7</sup> Consequently, TLR4 activation may also be involved in signaling during tissue injury. *In vivo* LPS can cause endotoxic shock by inducing massive release of proinflammatory cytokines and chemokines from immune and non-immune cells that may be entirely mediated by TLR4. Activation of TLR4 leads to the nuclear translocation and activation of *nuclear factor-kappa B* (*NF-κB*)<sup>8</sup> that causes enhanced expression of inflammatory cytokines and chemokines important in the recruitment of neutrophils and macrophages.<sup>9</sup>

Induction of TLR signaling in response to infectious and non-infectious cellular damage may be involved in renal disease. Binding of bacterial wall components by TLR activates tubular epithelial cells and resident interstitial macrophages.<sup>10</sup> Activation of TLR4 on tubular epithelial cells has been shown to stimulate the *NF-κB* pathway in response to oxidative stress<sup>11</sup> and leads to secretion of chemokines<sup>12</sup>, indicating a role for these TLRs in the initiation of phagocyte influx and immune activation during acute tubular necrosis. Baicalin (7-glucuronic acid, 5, 6-dihydroxyflavone) is one of the major flavonoids isolated from the dry roots of *Scutellaria baicalensis* Georgi that was shown to have anti-allergic and anti-inflammatory<sup>13</sup>, antioxidant<sup>14</sup>, anti-tumor<sup>15</sup>, anti-viral<sup>16</sup> as well as anti-apoptotic activities.<sup>17</sup> The effect of baicalin in LPS-induced AKI is unknown. The aim of the current study was to examine whether pretreatment with baicalin had a protective effect on LPS-induced AKI in mice and explore the possible underlying mechanisms.

## MATERIAL AND METHODS

### Drugs and Chemicals

LPS from *Escherichia coli* 0111:B4, baicalin, thiobarbituric acid, vanadium III chloride, Ellman's reagent, reduced glutathione (GSH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the highest analytical grades commercially available.

### Animals

Male Swiss mice, weighing 20-30 g, were used in the study. Animals were maintained under controlled conditions (25 ± 1°C, 55% relative humidity, 12 h lighting cycle), and fed standard chow and water ad libitum throughout the experimental period. All procedures were approved by the Ethics Committee for Animal Experimentation and were carried out in accordance with the Guide for Care and Use of Laboratory Animals (US National Institutes of Health, Publication No. 85-23, revised 1996).

### Experimental Design

Mice were randomly allocated into five groups (n=8). **Group 1 "normal"**: received the vehicle (10 % DMSO/saline, *ip*). The remaining animals received LPS (1 mg/kg; *ip*)<sup>18</sup> and were classified into: **Group 2 and 3** were sacrificed 3h post LPS administration; **Group 2 "LPS-3h"**: received LPS, **Group 3 "Baicalin+LPS-3h"**: received baicalin plus LPS; **Group 4 and 5** were sacrificed 24h post LPS administration; **Group 4 "LPS-24h"**: received LPS, **Group 5 "Baicalin+LPS-24h"**: received baicalin plus LPS. Baicalin was administered as three doses (50 mg/kg; *ip*)<sup>19</sup> at 48, 24 and 3h prior to LPS administration.

### Analysis of Blood Samples

Blood samples were collected via retro-orbital sinus into heparinized tubes under mild ether anesthesia. Plasma was separated by centrifugation at 3000 rpm for 15 min at 4°C, divided into several aliquots and stored at -20°C till determination of urea, creatinine and LDH using commercial kits (Stanbio, San Antonio, TX, USA).

### Analysis of Tissue Samples

After blood collection, mice were immediately decapitated under mild ether anesthesia and kidneys were removed, rinsed in ice-cold saline, blot-dried and weighed. The left kidney was divided into two sections: one used for quantitative PCR analysis and the other used for histological investigation. The right kidney was homogenized in ice-cold saline to make 10% homogenate that was centrifuged at 12,000 rpm at 4°C for 30 min and used for measuring the rest of the biochemical parameters.

### Estimation of Oxidative Stress Markers

Lipid peroxidation was quantified as malondialdehyde (MDA) according to Uchiyama and Mihara<sup>20</sup> and was expressed as nmol/g wet tissue. GSH content was quantified according to the method of Beutler *et al.*<sup>21</sup> and expressed as µg/g wet tissue. Nitric oxide (NO) content was quantified indirectly as total nitrate/nitrite (NO<sub>x</sub>) according to the method of Miranda *et al.*<sup>22</sup> and expressed as µmol/g wet tissue. Superoxide dismutase (SOD) and catalase (CAT) activities were determined according to the method of Marklund and Marklund<sup>23</sup> and Góth<sup>24</sup>, respectively and expressed as U/mg protein. Homogenate supernatant protein content was determined according to Lowry *et al.*<sup>25</sup>

### Estimation of Caspase-3 Activity

Caspase-3 activity (apoptosis marker) was determined using ApoAlert caspase-3 colorimetric assay kit (USA) and expressed as U/mg protein.

### Estimation of Inflammatory Biomarkers

Kidney contents of IL-1β and IL-10 were assayed using mouse IL-1β and IL-10 ELISA kits (R&D Systems, Minneapolis, USA) and expressed as ng/g wet tissue.

### Real-Time Quantitative PCR

Total RNA was isolated using RNeasy mini kit (Qiagen, CA, USA) and the purity of obtained RNA was evaluated by the 260/280 ratio. Equal amounts of RNA were used to prepare cDNA using QuantiTect Reverse Transcription kit (Qiagen, CA, USA). cDNA was used for quantifying the expression of *neutrophil gelatinase-associated lipocalin (NGAL)*, *inhibitor of NF-κB alpha (IkBa)* and *high-mobility group protein B1 (HMGB1)* genes by real-time PCR using Rotor-Gene SYBR Green kit (Qiagen, CA, USA) with Rotor-Gene Q system (Qiagen, CA, USA). β-actin was used as the housekeeping reference gene. Primers sequences are shown in Table 1. The relative expression of target genes was obtained using comparative CT (ΔΔCT) method and presented as fold change using the  $2^{-\Delta\Delta CT}$  formula.<sup>26</sup>

### Histopathological Assessment

Kidney samples were kept in 10% formal saline for 24 h, dehydrated in ethanol and embedded in paraffin. Sections were cut at 4 µm thicknesses and H&E-stained. All processing and assessment of specimens were performed by an experienced pathologist blinded to the study groups. A semiquantitative scoring of tubular injury adopted from Nomura *et al.*<sup>27</sup> was conducted for each of three variables: tubular dilatation/flattening, tubular casts, and tubular degeneration/vacuolization. For each animal, 4 high-power fields (HPF) were examined at random. A score of **0**, < 5%; **1**, 5–33%; **2**, 34–66%, and **3**, > 66% of the tubules were affected. The average score of all three variables were summed to generate a total injury score for each animal.

### Statistical Analysis

Data were presented as means ± SE. One way analysis of variance (ANOVA) was used for comparison different groups followed by Tukey–Kramer multiple comparisons test using GraphPad Prism 5. Differences were considered statistically significant at  $p < 0.05$  for all tests.

## RESULTS

### Renal Function Tests

LPS caused marked nephrotoxicity as indicated by 2 fold increases in plasma creatinine level as well as nearly 2 and more than 3 fold increase in plasma urea level 3h and 24h post LPS administration, respectively as compared to normal group (Fig. 1a and b). Baicalin pretreatment considerably decreased creatinine level by 24% in LPS-3h group and normalized its level in LPS-24h group parallel to normalizing urea level in both LPS groups (Fig. 1a and b).

### Tissue Damage Biomarkers

Parallel to the observed deterioration in renal function markers, LPS markedly increased kidney *NGAL* gene expression by 170 fold as well as plasma LDH activity by about 2 and 8 fold in LPS-3h and -24h groups, respectively as compared to normal group (Fig. 2a and b). These LPS-induced elevations in injury markers was

reflected in the significantly increased kidney total cumulative histopathological score in both LPS-3h and -24h groups by 4 and 2.67 fold, respectively as compared to normal group (Fig. 2c). However, baicalin pretreatment decreased kidney *NGAL* gene expression in LPS-3h group by 68 %, almost normalized LDH activity in LPS-3h group and greatly attenuated it in LPS-24h group (Fig. 2a and b), and decreased histopathological score in LPS-24h group by 50% (Fig. 2c).

### Oxidative Stress Biomarkers

LPS significantly augmented kidney MDA content of LPS-3h and -24h groups by 1.4 fold plus NO content in LPS-3h and -24h groups by 2 and 3.5 fold, respectively meanwhile significantly diminished kidney GSH content in LPS-3h group by 36% along with SOD activity in LPS-3h and -24h groups by 33% and 36%, respectively and CAT activity in LPS-3h and -24h groups by 21% and 28%, respectively as compared to normal group (Fig. 3). Baicalin pretreatment normalized these parameters in both LPS-3h and -24h groups (Fig. 3).

### Cytokines and Inflammatory Biomarkers

LPS significantly increased kidney IL-10 content in LPS-3h group by 1.7 fold; while it decreased IL-1 $\beta$  content in LPS-3h and -24h groups by 13% and 26%, respectively as compared to normal group (Fig. 4a and b). Also, LPS decreased kidney *HMGB1* gene expression in LPS-3h and -24h groups by 37% and 68%, respectively; however, it increased *IkB- $\alpha$*  gene expression in LPS-3h group by 10 fold and decreased it in LPS-24h group by 87% as compared to normal group (Fig. 4c and d). Interestingly, baicalin pretreatment caused further elevation in kidney IL-10 level in LPS-3h group by 50% and triggered additional decrease in IL-1 $\beta$  content by 42% and 30% in LPS-3h and -24h groups, respectively (Fig. 4a and b). Furthermore, baicalin pretreatment did not modulate *HMGB1* expression level; yet, it increased *IkB- $\alpha$*  gene expression in LPS-3h and -24h groups by 23 % and 2.4 fold, respectively (Fig. 4 c and d).

### Apoptosis Biomarkers

LPS significantly elevated kidney caspase-3 activity in LPS-3h and -24h groups by 2 and 3 fold, respectively as compared to normal group. Baicalin pretreatment normalized caspase-3 activity in both LPS groups (Fig. 5).

### Histopathological Examination of Kidney

Kidneys from normal mice showed healthy architecture composed of glomeruli embedded among numerous tubules: the proximal convoluted tubules lined with pyramidal cells and the distal convoluted tubules lined with cuboidal cells (Fig. 6a). Kidneys from LPS-3h and -24h groups showed degeneration of tubular lining epithelium (score 1 and 2, respectively) and tubular dilatation (score 2 and 0, respectively) (Fig. 6b and c). Baicalin pretreatment prevented LPS-induced degeneration in the tubular epithelium in LPS-3h and -24h groups as well as tubular cells vacuolization (score 0 and 1, respectively), dilatation or casts (score 0) (Fig. 6d and e).

### DISCUSSION

In the present study, mice treated with LPS exhibited AKI as illustrated by increased plasma creatinine and urea levels parallel to elevated kidney LDH activity, *NGAL* expression and total cumulative histopathological score.<sup>28</sup> The observed LPS-induced functional impairment and tissue damage may be attributed to augmented oxidative stress and apoptosis.<sup>29</sup> *NGAL* gene is a well-established early marker of AKI<sup>30</sup> whose expression was previously shown to be enhanced in cortical tubular epithelia following LPS administration.<sup>31</sup> In the current investigation, LPS triggered oxidative stress as reflected in increased kidney MDA and NO levels coupled with decreased antioxidants: GSH content, SOD and CAT activities. It has been known that oxidative stress plays an important role in the development of LPS-induced AKI<sup>32</sup> Baicalin pretreatment abolished the observed oxidative stress indicating that baicalin have efficient antioxidant properties as previously demonstrated by Gao *et al.*<sup>14</sup> and Shin *et al.*<sup>33</sup>

Recognition of LPS by TLR4 initiates signaling pathways that induce production of

proinflammatory (e.g. IL-1 $\beta$ ) and anti-inflammatory (e.g. IL-10) cytokines. The balance of pro-inflammatory and anti-inflammatory cytokines plays a pivotal role in keeping the host homeostasis.<sup>34</sup> Stress-exposed organs activate their defense systems to cope with stress which is known as preconditioning.<sup>35</sup> Differential regulation of inflammatory and anti-inflammatory cytokines offer an effective strategy for preventing undesired inflammatory reactions. LPS triggers the release of anti-inflammatory cytokines to balance and control the inflammatory response<sup>36</sup>, which may explain the observed increase in IL-10 and suppressed IL-1 $\beta$  production. *In vivo*, IL-10 has been reported to attenuate macrophage-induced glomerular injury<sup>37</sup> and inhibit production of a variety of proinflammatory cytokines by monocytes and neutrophils. In the current work, we were the first to report that baicalin pretreatment augmented LPS-induced increase in the production of IL-10 as well as decrease in IL-1 $\beta$ . Thus, the use of baicalin improved the observed preconditioning, which represent an added value for this agent in protection from LPS-induced AKI.

*NF- $\kappa$ B* is a DNA binding protein that takes part in the regulation of multiple inflammatory responses by adjusting gene expression.<sup>38</sup> Its activity is regulated through interaction with I $\kappa$ B that sequesters it in an inactive form in the cytoplasm. Multiple stimuli can activate *NF- $\kappa$ B* signaling by degradation of I $\kappa$ B and release of the *NF- $\kappa$ B*, which translocates to the nucleus and regulates transcriptional activation of the target genes.<sup>39</sup> Therefore, the observed increase in *I $\kappa$ B- $\alpha$*  RNA expression level suggested enhanced inhibition of *NF- $\kappa$ B* activation with subsequent suppression of inflammatory mediators production. Such inhibition of *NF- $\kappa$ B* may partly explain the observed preconditioning. As a functional inhibitor of TLR4-mediated *NF- $\kappa$ B* activation, baicalin pretreatment augmented the increase in *I $\kappa$ B- $\alpha$*  expression, which also underscores the role of baicalin in enhancing the protective effect of preconditioning.<sup>40,33</sup>

*HMGB1*, a non-histone nuclear protein, belongs to the group of molecules known as alarmins.<sup>41</sup>

*HMGB1* can be passively released from necrotic cells or actively secreted by activated immune cells in response to inflammatory signals.<sup>42,43</sup> Excessive *HMGB1* release was found to play a crucial role in the pathogenesis of acute and chronic inflammation. Released *HMGB1* can bind to several cell surface receptors, including TLR4<sup>44</sup> which results in activation of *NF- $\kappa$ B* and increased cytokines production.<sup>45</sup> Therefore, the observed decrease in kidney *HMGB1* RNA expression level in both the LPS-3h and -24h groups may also contribute to diminished *NF- $\kappa$ B* activation that underscores the observed preconditioning. However, *HMGB1* expression was not affected by baicalin pretreatment suggesting that modulating *HMGB1* expression is not involved in baicalin renoprotective mechanisms.

Enhanced apoptosis is associated with the activation of caspase cascade. Caspase-3 is a downstream effector in this cascade that directly mediates apoptosis<sup>46</sup> and is regarded as a pivotal indicator of apoptosis during AKI.<sup>47</sup> LPS can induce caspase activation in the kidney via several possible mechanisms such as the generation of ROS<sup>48</sup> or stimulation of iNOS expression.<sup>49</sup> NO can activate caspases through its effects on mitochondria<sup>50</sup> or through generation of reactive nitrogen species.<sup>51</sup> We showed that baicalin pretreatment normalized both caspase-3 activity and NO production, suggesting that an anti-apoptotic activity may also contribute to the protective effect of baicalin against LPS-induced AKI.<sup>17</sup>

In summary, the present work is the first to show that baicalin pretreatment can significantly ameliorate LPS-induced AKI through reduction of oxidative stress, proinflammatory cytokines and apoptosis via inhibiting TLR4 pathway. Thus, interventions involving TLR4 can protect against LPS-induced kidney injury, which indicates that baicalin may represent an alternative treatment for preventing kidney injury in septic diseases.

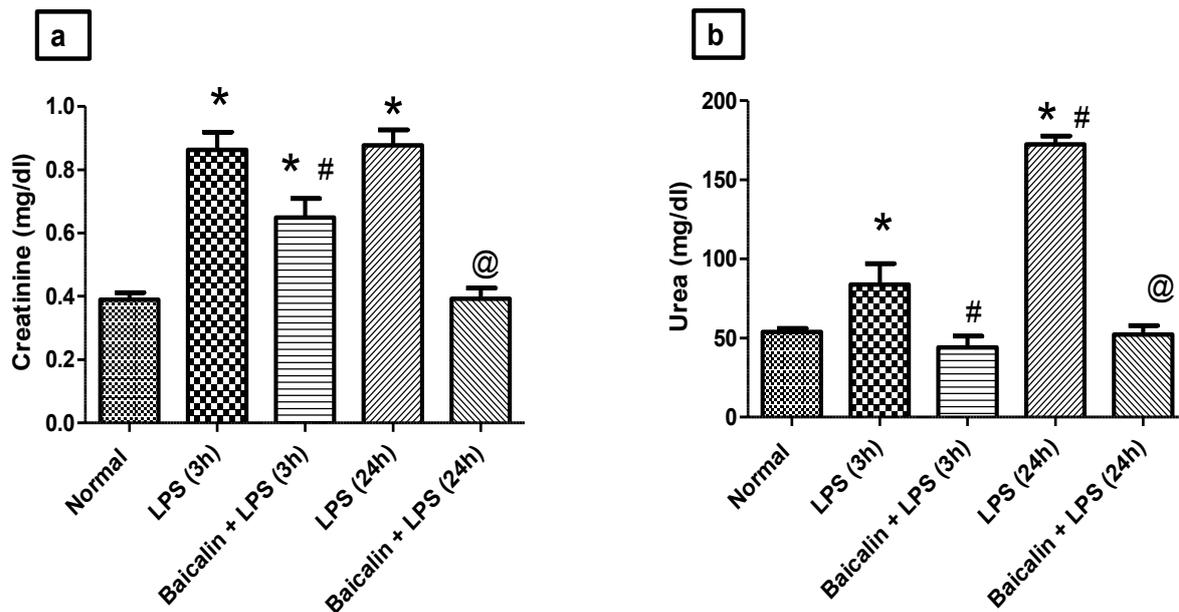
## ACKNOWLEDGEMENT

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Center, Giza, for histopathological investigations incorporated in this study.

**Table 1: Primers Sequences**

Genes	Primers Sequences
$\beta$ -actin	For 5'-CTAAGGCCAACCGTGAAAAG-3' Rev 5'-ACCAGAGGCATACAGGGACA-3'
NGAL	For 5'-CCATCTATGAGCTACAAGAGAACAAT-3' Rev 5'-TCTGATCCAGTAGCGACAGC-3'
I $\kappa$ B $\alpha$	For 5'-ATGAAGGACGAGGAGTACGAGCAA-3' Rev 5'-TCTCTTCGTGGATGATTGCCAA-3'
HMGB1	For 5'-TGGGCGACTCTGTGCCTC-3' Rev 5'-GCCTCTCGGCTTTTATAGGATC-3'



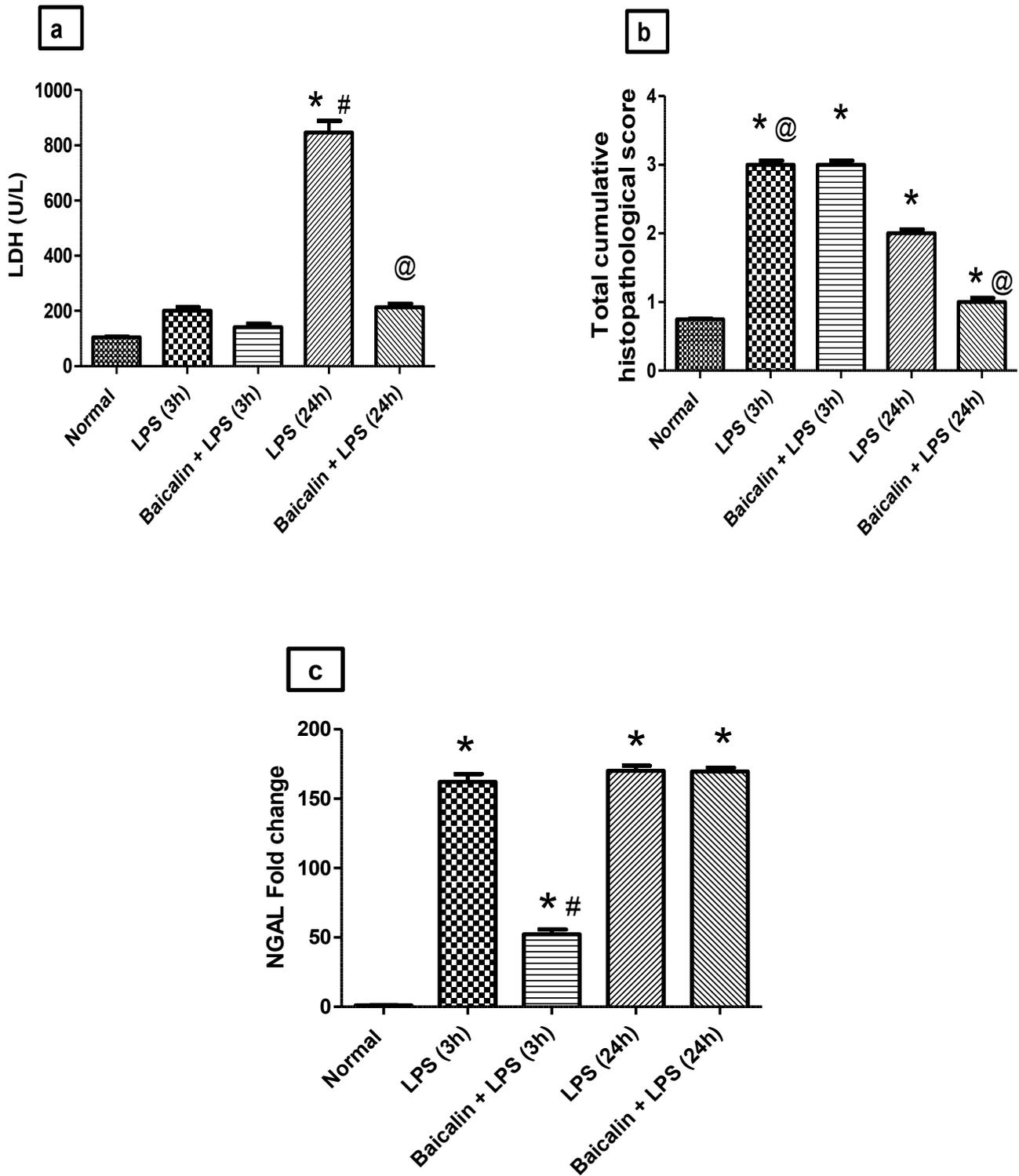
**Figure 1: Effect of baicalin pretreatment on plasma levels of (a) creatinine and (b) urea of LPS-treated mice**

Data are expressed as mean  $\pm$  SE (n = 6-8).

\* Significantly different from normal group at  $p < 0.05$ .

# Significantly different from LPS (3h) group at  $p < 0.05$ .

@ Significantly different from LPS (24h) group at  $p < 0.05$ .



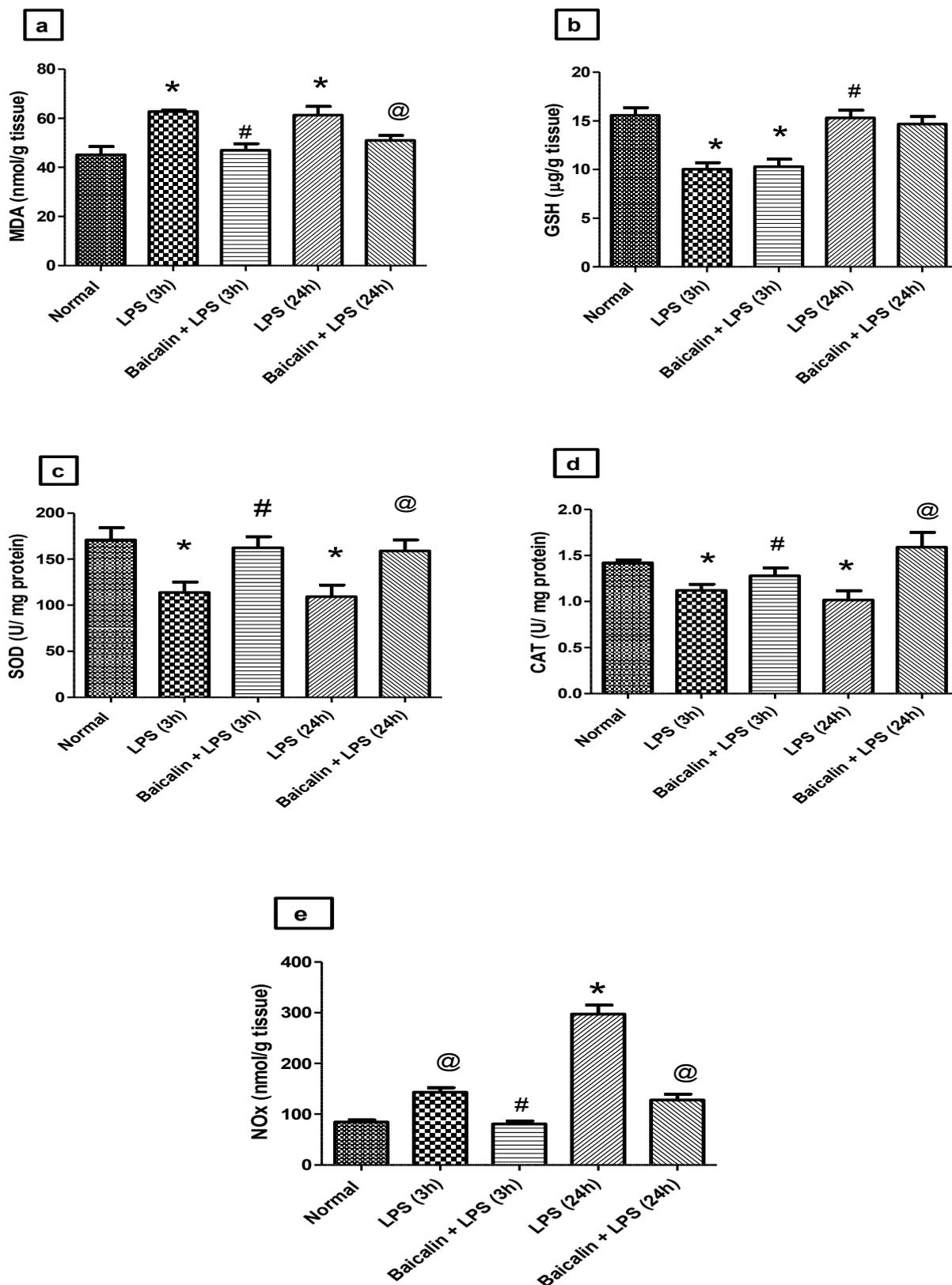
**Figure 2:** Effect of baicalin pretreatment on (a) plasma LDH activity, (b) total cumulative histopathological score and (c) gene expression of NGAL of LPS-treated mice

Data are expressed as mean  $\pm$  SE (n = 6-8).

\* Significantly different from normal group at  $p < 0.05$ .

# Significantly different from LPS (3h) group at  $p < 0.05$ .

@ Significantly different from LPS (24h) group at  $p < 0.05$ .



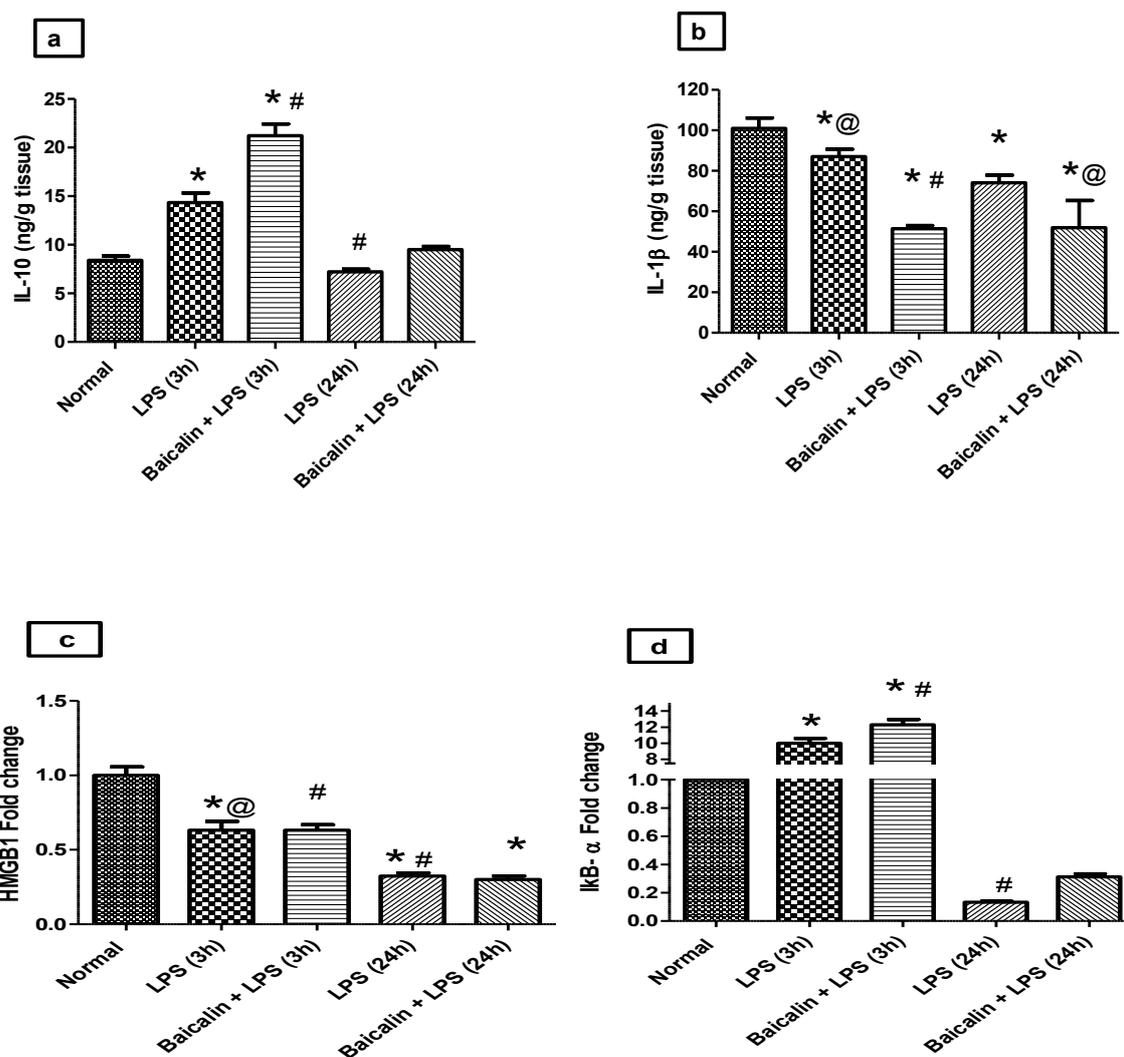
**Figure 3:** Effect of baicalin pretreatment on kidney contents of (a) MDA, (b) GSH and activities of (c) SOD and (d) CAT as well as (e) NO<sub>x</sub> content of LPS-treated mice

Data are expressed as mean ± SE (n = 6-8).

\* Significantly different from normal group at  $p < 0.05$ .

# Significantly different from LPS (3h) group at  $p < 0.05$ .

@ Significantly different from LPS (24h) group at  $p < 0.05$ .



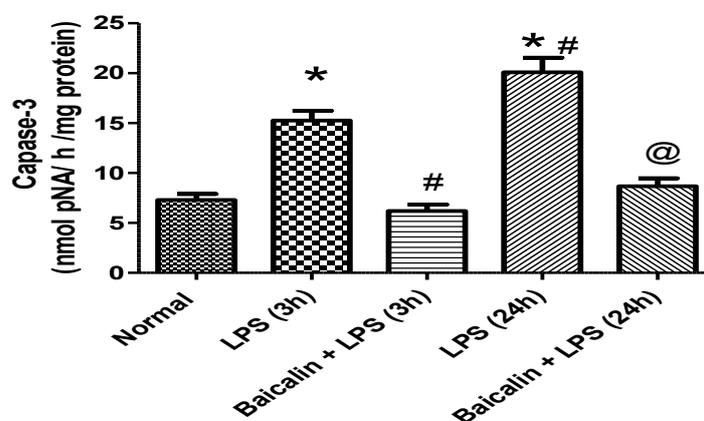
**Figure 4:** Effect of baicalin pretreatment on kidney contents of (a) IL-10, (b) IL-1β as well as gene expression of (c) HMGB1 and (d) IκB-α of LPS-treated mice

Data are expressed as mean ± SE (n = 6-8).

\* Significantly different from normal group at  $p < 0.05$ .

# Significantly different from LPS (3h) group at  $p < 0.05$ .

<sup>@</sup> Significantly different from LPS (24h) group at  $p < 0.05$ .



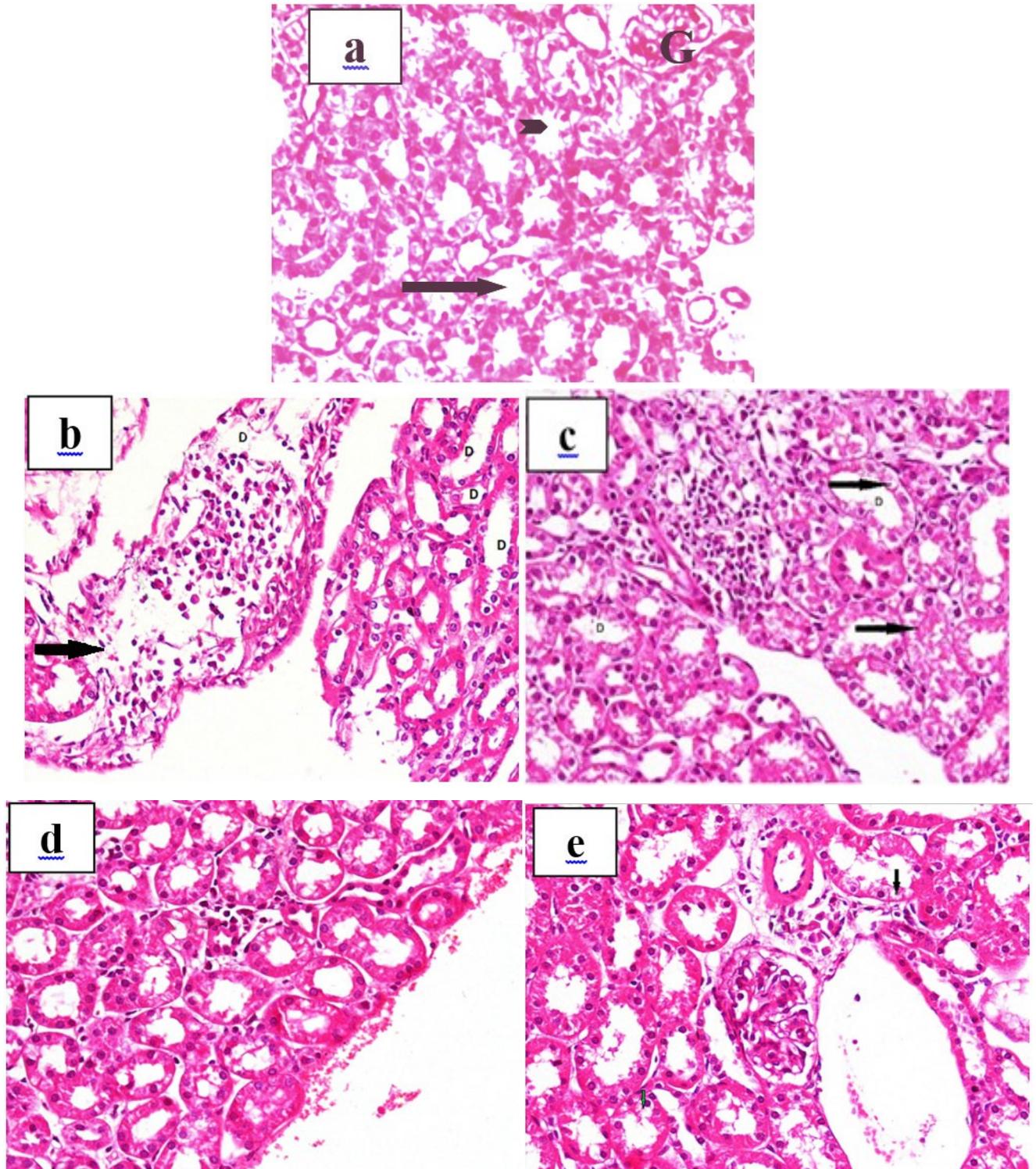
**Figure 5:** Effect of baicalin pretreatment on kidney caspase-3 activity of LPS-treated mice

Data are expressed as mean ± SE (n = 6-8).

\* Significantly different from normal group at  $p < 0.05$ .

# Significantly different from LPS (3h) group at  $p < 0.05$ .

<sup>@</sup> Significantly different from LPS (24h) group at  $p < 0.05$ .



**Figure 6:** Photomicrographs of mice kidneys sections stained with H&E (400x): (a) control group showing the normal architecture of renal tissue composed of a number of glomeruli (G) embedded among a great number of different tubules most prominently the proximal convoluted tubules (arrow head) and the distal convoluted tubules (arrow). (b) LPS-3h group showing tubular dilatation (D) and degeneration of lining epithelium (arrow) (c) LPS-24h group showing tubular vacuolization (black arrow) (d) baicalin-pretreated mice for LPS-3h group and (e) baicalin-pretreated mice for LPS-24h group showing minimal tubular vacuolization (black arrow)

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