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Histopathological effects of *S. xylosus* peptidoglycan in comparison to *E. coli* lipopolysaccharide in the urinary tract of mice

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Aim: The present work, among other previous studies done in our lab, aimed to highlight the histopathological effect of *S. xylosus* peptidoglycan in comparison to LPS of *E. coli*.

Materials and methods: One hundred and fifty urine specimens were collected from urinary tract infection patients visiting Baghdad hospitals. The histopathological effects of *S. xylosus* S24 peptidoglycan was studied in the urinary tract of female mice by injecting 5 animal groups at the following concentrations: 1000, 2000, 3000, 4000, and 5000 µg/mL. Another 5 groups were injected with 10, 25, 50, 75, and 100 ng/mL of *E. coli* (serotype 0128:B12) LPS.

Results: Ten isolates were confirmed to be *Staphylococcus xylosus*. Histopathological study showed different pathological changes caused by LPS and peptidoglycan, including shrinkage of glomeruli, congestion of blood vessels, and infiltration of inflammatory cells in the kidney, whereas dekeratinization and edematous dehydration of the epithelium were seen in the urinary bladder. Based on a comparison between histopathological changes caused by LPS and peptidoglycan, LPS induced changes of significantly higher severity than peptidoglycan.

Conclusion: Peptidoglycan of *S. xylosus* had remarkable histopathological effects on the renal system of mice in a dose-dependent manner. Nevertheless, these effects had less intensity than the effects of LPS of *E. coli.*

Key words: Lipopolysaccharides, peptidoglycan, pathogenicity, *Escherichia coli*, *Staphylococcus xylosus*

Introduction

Many bacterial components warn the immune system about the presence of bacterial invasion; therefore, they are considered key players in such invasion (1). Evidently, lipopolysaccharide (LPS) is not the only etiological factor responsible for septic shock syndrome (2). Peptidoglycan of gram-positive bacteria has the ability to activate leukocytes and trigger the release of proinflammatory cytokines, causing a systemic inflammatory response (3).

Infections caused by coagulase negative staphylococci have received much attention (4). Although *S. xylosus* is regularly isolated from food products, the environment, and human skin (5), Carrillo et al. (6) stated that *S. xylosus* is a newly emerging nosocomial pathogen. Moreover, this bacterium was the agent responsible for some serious infections like endocarditis, toxic shock syndrome, dermatitis, bacteremia, and urinary tract infection (UTI) (7). The capability of *S. xylosus* to initiate an infection could be attributed to its capacity to form a biofilm on different surfaces in addition to other virulence factors (8). Unlike *S. saprophyticus* less attention has been paid toward *S. xylosus* as a causative agent of UTI since its incidence was around 1%. However, in local previous studies this bacterium was isolated in a relatively high percentage of Iraqi patients who presented with UTI (9,10). Furthermore, *S. xylosus* was able to colonize the

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kidney and the urinary bladder of mice when injected intraurethrally (11).

Due to this increasing isolation percentage in Iraqi patients, we planned to investigate some of the potential pathological aspects of this species. Therefore, the present work, among other previous studies done in our lab, aimed to highlight the histopathological effect of *S. xylosus* peptidoglycan in comparison to LPS of *E. coli*.

Materials and methods

Specimen collection

One hundred and fifty mid-stream urine specimens were collected in sterile containers from patients who presented with UTI visiting Al-Yarmouk, Al-Za'frania, Madinat Altib, and Al-Karama hospitals in Baghdad.

Isolation and identification

All specimens were cultured on blood agar and incubated at 37 °C for 24 h. Thereafter, the grown colonies were transferred onto mannitol salt agar. Mannitol fermenter colonies were picked and transferred onto nutrient agar for a purity check and morphological study. Identification was performed according to Kloos and Schliefer (12) and Holt et al. (13). The biochemical tests were carried out in accordance with Frobes et al. (14). The API Staph system was employed to confirm the identification.

Antibiotic susceptibility test

All *S. xylosus* isolates were tested for their susceptibility toward amoxicillin (25 μg), cephalothin (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), and gentamicin (10 μg), following the procedure described by Bauer et al. (15). For quality control measures *S. aureus* ATCC 6538P standard strain was tested as well.

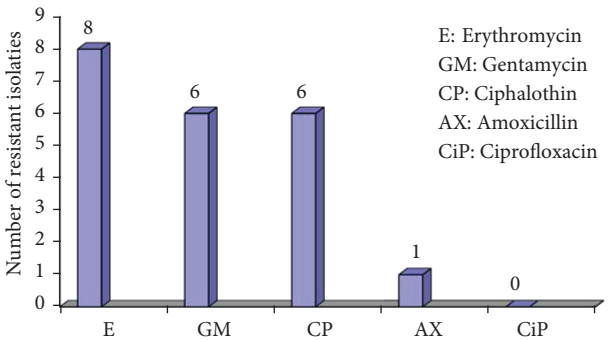

Figure 1. Antibiotic susceptibility of *S. xylosus* isolated from UTI patients.

Peptidoglycan extraction

The peptidoglycan of *S. xylosus* S24 was extracted following the procedure of De-Jonge et al. (16). Briefly, 1 L of culture was quickly chilled in an ice/ethanol bath until the temperature dropped below 10 °C. The cells were harvested by centrifugation for 10 min at 16,000 × *g* (4 °C) and subsequently transferred into 4% boiling sodium dodecyl sulfate (SDS). The cells were boiled for 0.5 h and the cell walls were then concentrated by centrifugation for 10 min at 30,000 × *g*. The walls were washed with water until no more SDS could be detected. Cell walls were broken with glass beads (0.2 mm) by vortexing at maximal speed at 4 °C for 15 min. The suspension was centrifuged at 2000 × *g*, and, after removal of the supernatant, the pellet was treated with glass beads as described above. The collected broken walls were centrifuged at 40,000 × *g* for 15 min, and the pellet was resuspended at 37 °C in 100 mM Tris-HCl (pH 7.5) with the addition of 20 mM $MgCl_2$, DNase, and RNase. DNase and RNase (Sigma) were added at a concentration of 10 μg/mL and 50 μg/mL, respectively, and the mixture was incubated for 2 h at 37 °C. The incubation was prolonged for another 2 h. Thereafter, the suspension was treated with trypsin (Sigma) in the presence

Table. Protein and carbohydrates concentrations in crude cell walls and peptidoglycan of *S. xylosus*.

Extract	Protein concentration (μg/mL)	Carbohydrate concentration (μg/mL)
Crude cell wall	197.5	16.600
Peptidoglycan	54.4	40.00

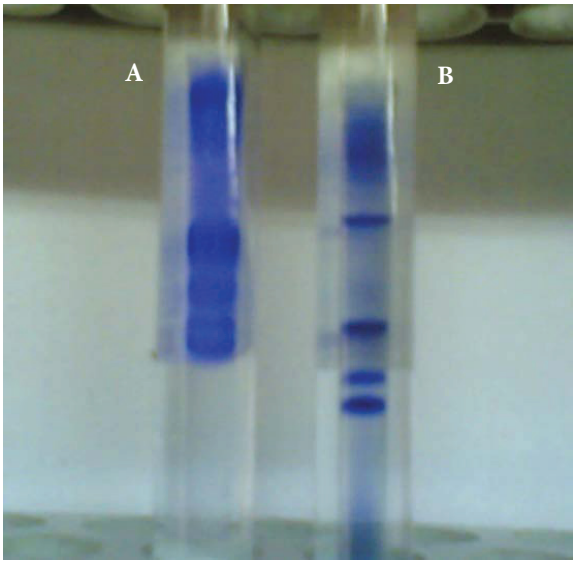

Figure 2. Gel electrophoresis of crude cell wall (A) and peptidoglycan (B) of *S. xylosus*.

of 10 mM $CaCl_2$ for 18 h. Autolytic enzymes were inactivated by boiling for 15 min in 1% SDS. The walls were collected by centrifugation as described above and washed twice with water, once with 8 M LiCl, once with 100 mM EDTA, and then twice with water before being washed with acetone. Finally, the broken walls were resuspended in aliquots of sterilized distilled water.

Protein concentration was estimated during and after purification according to the method described by Lowry et al. (17). Carbohydrates were assayed following the method reported by Dubois et al. (18). Peptidoglycan purity was confirmed by polyacrylamide gel electrophoresis under nondenaturizing conditions.

In vivo study

Animals

Female white mice (*Mus musculus*) aged 7–8 weeks and weighing 21–24 g were obtained from the National Center for Drugs Supervision and Researches, Baghdad, Iraq. The mice were housed in plastic cages and fed ad libitum with a conventional diet. The animals were divided into 11 groups and injected as follows:

Groups 1–5 were injected with 20 µL of peptidoglycan extract at concentrations of 1000, 2000, 3000, 4000, and 5000 µg/mL, respectively.

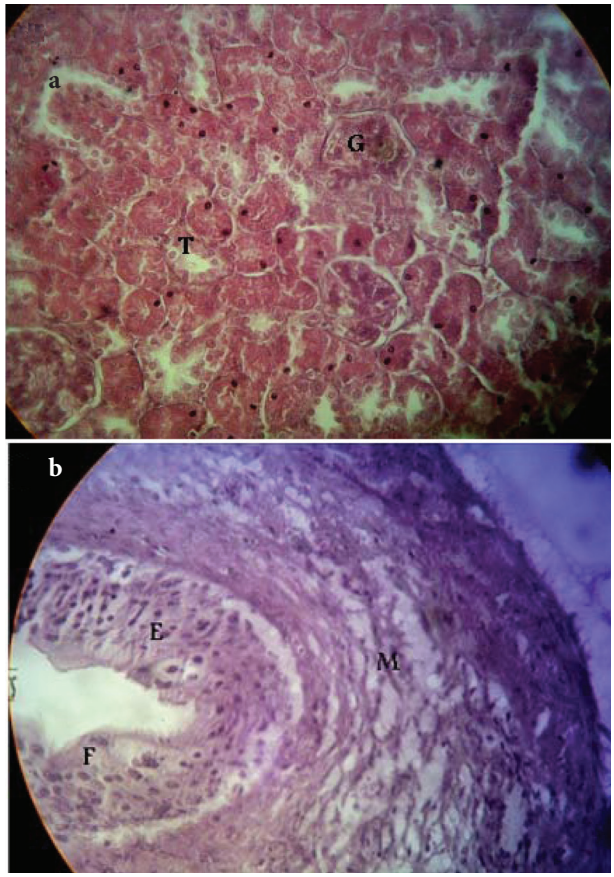

Figure 3. Cross section in mice kidney (A) and urinary bladder (B) shows the normal texture of them. G = glomerulus, T = tubules, E = epithelial layer, F = fat layer, M = mucosa layer. 400×. H&E.

Groups 6–10 were injected with 20 µL of *E. coli* serotype 0128: B12 LPS (purchased from Sigma, USA) at concentrations of 10, 25, 50, 75, and 100 ng/mL, respectively. Group 11 was injected with normal saline and, consequently, considered the control group.

Injection protocol

First of all the bladder was emptied of urine by pressing on the abdominal area. The urethra and surrounding area were sterilized with 75% ethanol and then a polyethylene tube (0.6 mm in diameter) was introduced into the urinary bladder via the urethra; the inoculums (20 µL) were injected by the aid of this catheter. Thereafter, the catheter was withdrawn immediately and the animals were returned to their cages with their lower end directed upward to avoid effusion of the inoculum outside (19).

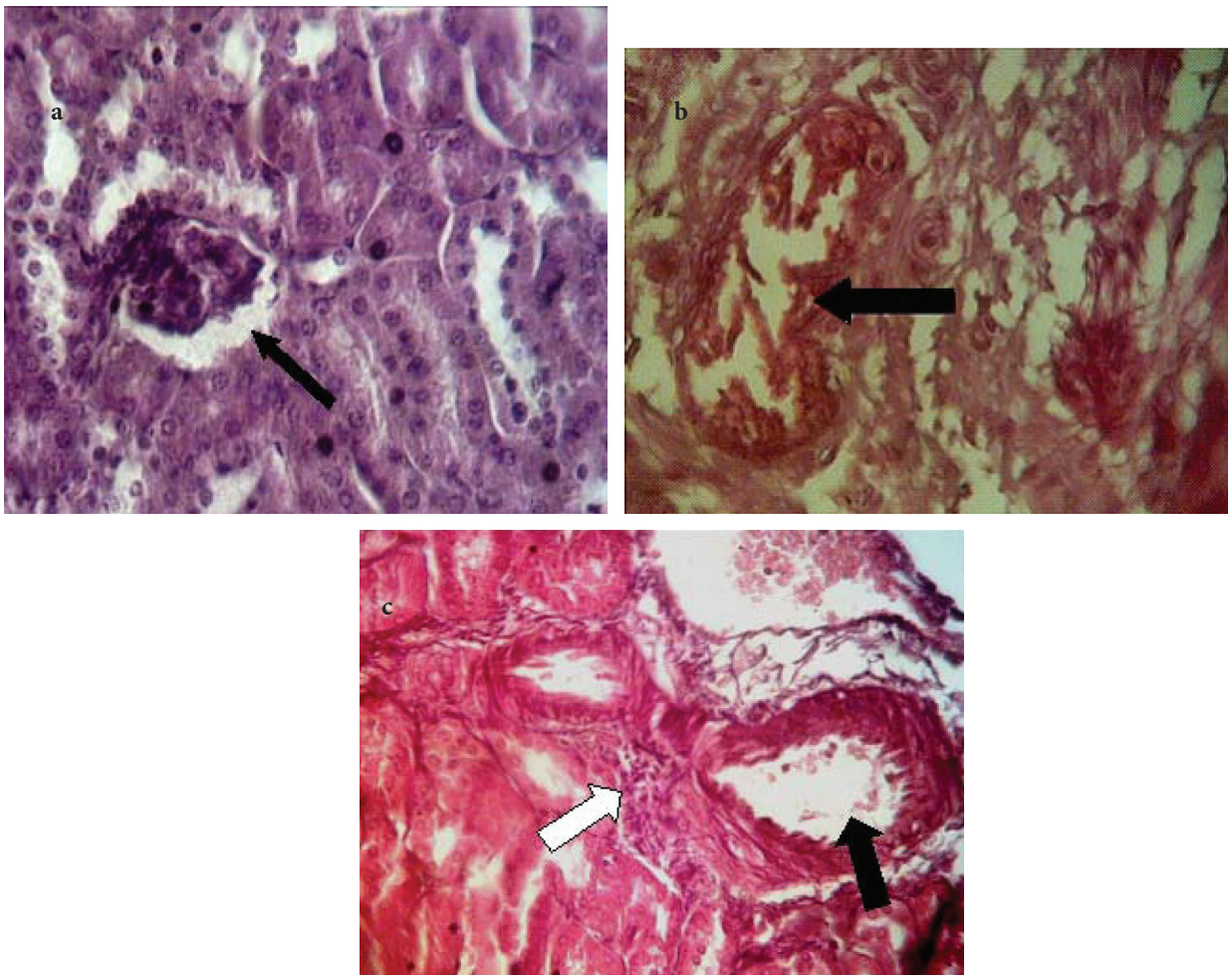

Figure 4. Cross section in mice kidney injected with a) 3000 μg/mL, b) 4000 μg/mL, and c) 5000 μg/mL of partially purified peptidoglycan of *S. xylosus* shows shrinkage of glomerulus (thin black arrow), hemostasis and congestion inside the blood vessel (thick black arrow), and infiltration of inflammatory cells (white arrow). 400×. H&E.

All animals were kept in their cages without water for 24 h. After 4 days of injection they were sacrificed and the left kidneys and bladders were aseptically removed for histopathological study according to Bancroft and Steven (20).

Results and discussion

Isolation and identification

Out of 150 bacterial isolates, 61 (40.6%) isolates were identified as staphylococci. Forty-eight (78.7%) isolates were coagulase negative staphylococci. While 13 (21.3%) isolates were positive coagulase staphylococci, 10 (16.4%) isolates were identified as *S. xylosus*.

Antibiotic susceptibility

The results revealed that the 10 *S. xylosus* isolates varied in their antibiotic susceptibility. Erythromycin was the least effective antibiotic, whereas ciprofloxacin exhibited the highest efficacy (Figure 1). Moreover, *S. xylosus* S4 developed multidrug resistance toward all antibiotics under investigation; therefore, it was chosen for further experiments.

Al-Mathkhury et al. (10) confirmed that *S. xylosus* isolated from Iraqi patients who presented with UTI showed high sensitivity toward ciprofloxacin and high resistance toward erythromycin. Barger-Bächi (21) reported that CoNS developed resistance toward macrolides due to the erm gene.

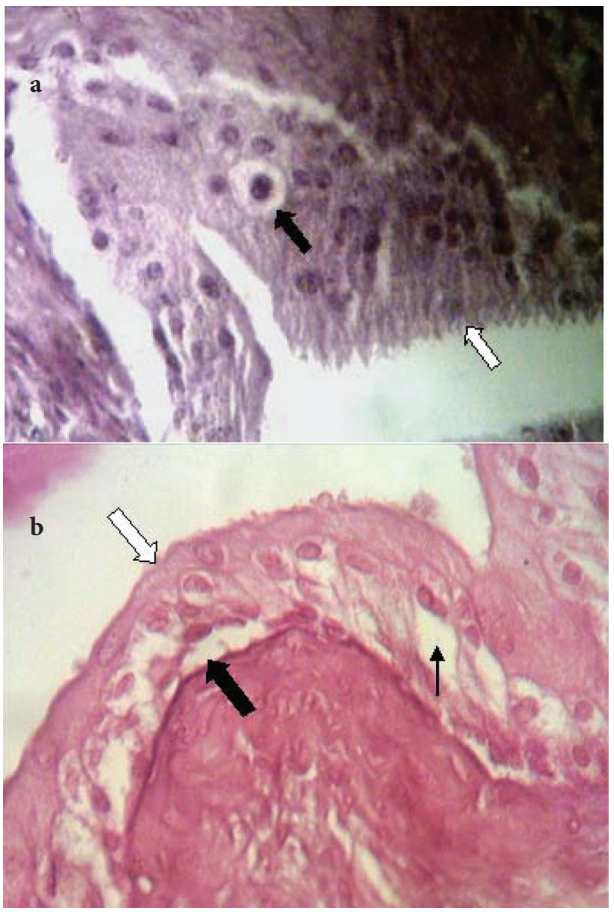

Figure 5. Cross section in mice urinary bladder injected with a) 4000 µg/mL and b) 5000 µg/mL of partially purified peptidoglycan of *S. xylosus* shows hydropic degeneration (black arrow) and absence of fat layer (white arrow) and increased space around the epithelial cells' nuclei (small black arrows). 400×. H&E.

As shown in the Table, at the end of the extraction process the protein concentration decreased while the carbohydrates concentration increased.

Figure 2 illustrates that the gel electrophoresis bands were reduced from 8 (crude cell walls) to 6 bands (peptidoglycan). These 2 results confirmed the efficacy of the extraction procedure (22).

Umenda et al. (23) reported that gel electrophoresis revealed 10 protein bands after extraction with SDS and proteolytic enzymes. Flaih et al. (24) stated that extracted *Streptococcus pneumoniae* peptidoglycan showed 4 bands while its crude cell wall developed 6 bands.

In vivo study

Peptidoglycan

Histopathological sections of the control group showed normal urinary tract texture (Figure 3). The results also revealed that injecting mice with 1000 and 2000 µg/mL of partially purified peptidoglycan of *S. xylosus* caused no pathological changes in kidney tissues, while injection with 3000 µg/mL caused mild histopathological changes represented by shrinkage of glomeruli (Figure 4a).

Furthermore, 4000 µg/mL of *S. xylosus* peptidoglycan caused hemostasis and congestion of the endothelial layer of blood vessels (Figure 4b). Moreover, severe changes were seen in sections of kidneys treated with 5000 µg/mL; in addition to previous changes, infiltration of inflammatory cells was also noted (Figure 4c).

Regarding the urinary bladder, injecting the mice with 1000, 2000, and 3000 µg/mL of partially purified peptidoglycan of *S. xylosus* did not lead to any pathological changes. The concentration 4000 µg/mL caused marked histopathological changes such as hydropic degeneration of epithelial tissue and loss of the fat layer (Figure 5a).

Additionally, Figure 5b shows histopathological changes due to injection of 5000 µg/mL of partially purified peptidoglycan of *S. xylosus* represented by hydropic degeneration of epithelial tissue and disappearance of the fat layer in addition to increased space around the epithelial layer nuclei.

Such histopathological changes could be attributed to immunological effects triggered by the interaction the peptidoglycan with receptors found on mononuclear cell surfaces and eventually lead to TNF-α and IL-1 release, and on macrophages to liberation of TNF-α (25). Moreover, it can stimulate polymorphonuclear leukocytes to induce autolytic enzymes and activation of mast cells to liberate histamine in addition to increasing blood vessel permeability. By means of its high molecular weight, peptidoglycan can induce acute and chronic immune responses (26).

In a previous study (10), similar changes were seen in mice injected with live cells of *S. xylosus* represented by glomerulus shrinkage, hemorrhage, congestion, vacuolation of blood vessels, and

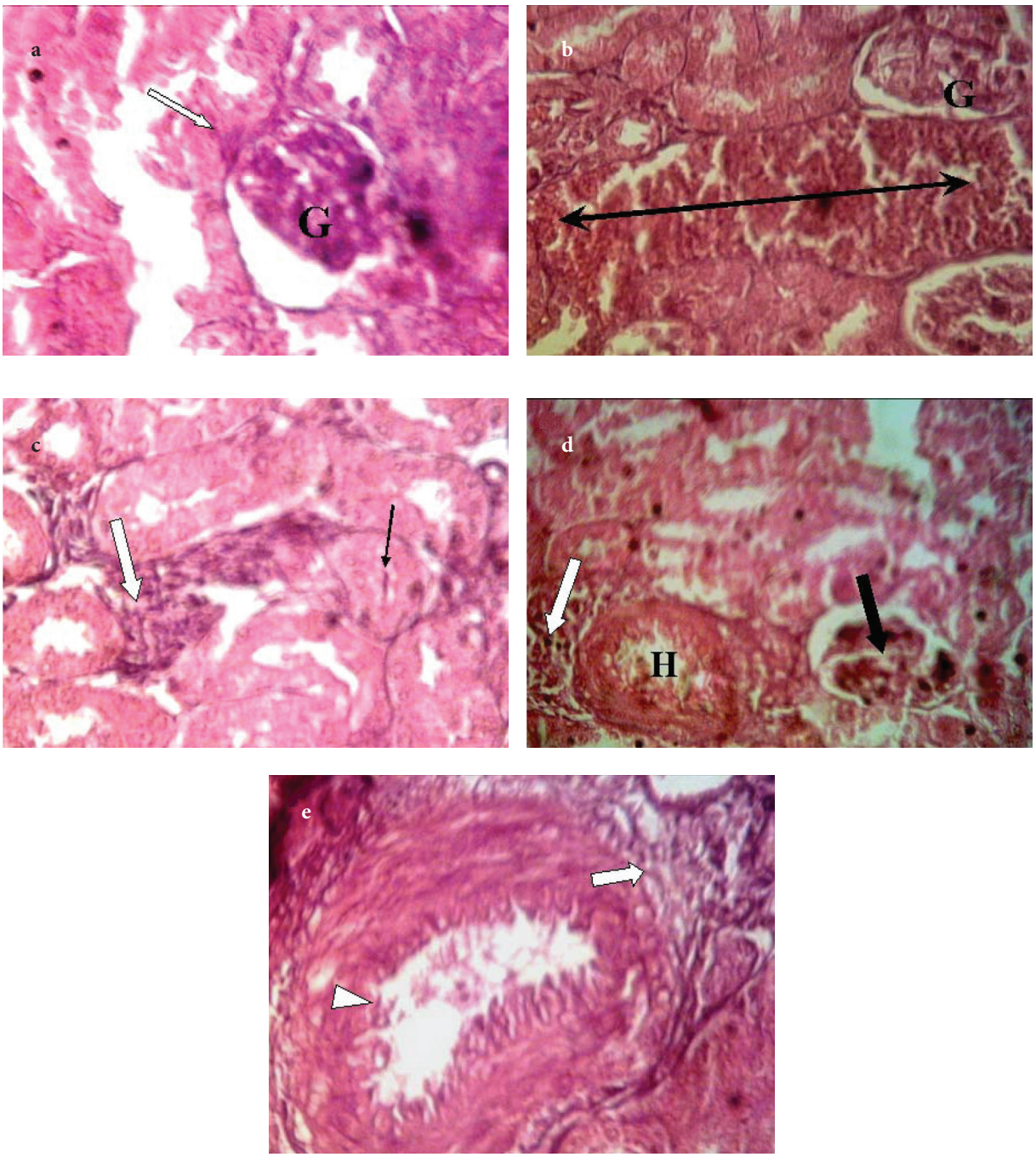

Figure 6. Cross section in mouse kidney injected with a) 50 ng/mL, b, c, d, and e) 100 ng/mL of *E. coli* (serotype 0128:B12) LPS shows infiltration of inflammatory cells (white arrow), shrinkage of glomerulus (G), hemorrhage (double-headed arrow), obstruction of tubules (thin black arrow), hemostasis inside blood vessel (H) and segmental corpuscle degeneration, hemostasis (black arrow), and vacuolation in the blood vessel (white triangle). 400×. H&E.

infiltration of inflammatory cells in kidney tissue. The urinary bladder suffered from hydropic degeneration, dekeratinization, and infiltration of inflammatory cells.

LPS of *E. coli* (serotype 0128:B12)

When the mice were challenged with 10 and 25 ng/mL of *E. coli* (serotype 0128:B12) LPS, kidney and

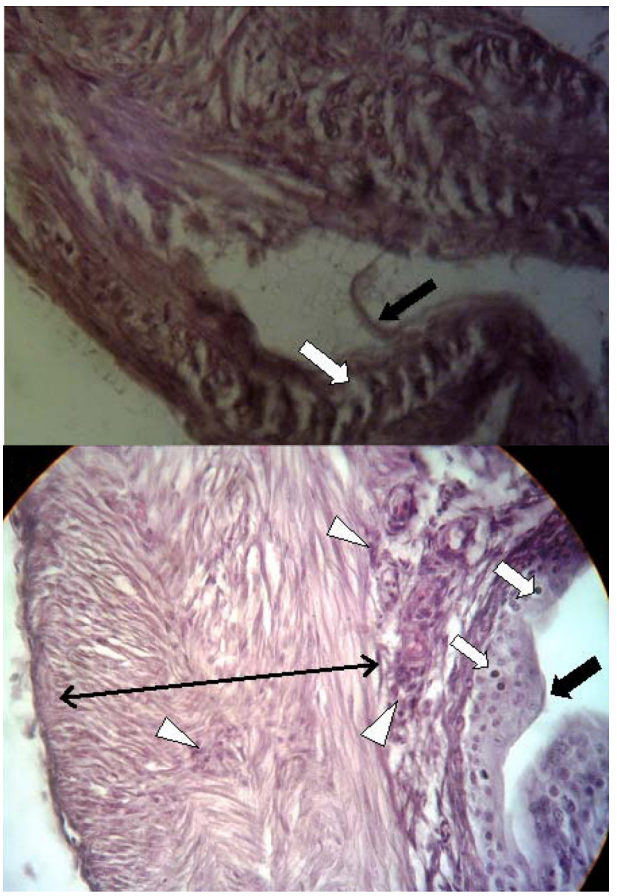

Figure 7. Cross section in mouse urinary bladder injected with a) 75 ng/mL and b) 100 ng/mL of *E. coli* (serotype 0128:B12) LPS shows partial loss of the fat layer (black arrow) and hydropic degeneration (white arrow), increase in thickness of the subepithelial layer (double-headed arrow), and infiltration of inflammatory cells (triangles) 400×. H&E.

urinary bladder cross sections shows no pathological signs. However, mild infiltration of inflammatory cells and shrinkage of glomeruli were noted in kidney tissues after injection of 50 and 75 ng/mL of LPS (Figure 6a).

More severe histopathological changes such as hemorrhage (Figure 6b), infiltration of inflammatory cells, obstruction of tubules (Figure 6c), segmental corpuscle degeneration, hemostasis (Figure 6d), and vacuolation (Figure 6e) of the blood vessels were seen in the kidneys of mice injected with 100 ng/mL of *E. coli* (serotype 0128:B12) LPS.

On the other hand, 10, 25, and 50 ng/mL of LPS had no effect on the urinary bladder, while 75 ng/mL caused partial loss of the fat layer and hydropic degeneration in the epithelial layer, as shown in Figure 7a.

The urinary bladder tissue of mice injected with 100 ng/mL of LPS developed serious pathological changes such as complete loss of the fat layer, hydropic degeneration in the epithelial layer, expansion of the subepithelial layer, and infiltration of inflammatory cells (Figure 7b).

All these changes in the tissues of the kidney and urinary bladder caused by LPS could be assigned to the immunopathological role of this molecule, which leads to activation of the coagulation pathway and aggregation of leukocytes, consequently to destruction of the endothelial layer, and eventually to organ failure. Nevertheless, the increase in LPS concentration causes a corresponding increase in symptoms and signs of inflammation (27,28).

Regarding all these findings, we can see clearly that the pathological effects are similar for *S. xylosus* peptidoglycan and LPS of *E. coli* (serotype 0128:B12) but they differ in terms of intensity.

Weidemann et al. (29) found that peptidoglycan, like LPS, was able to induce IL-6 and IL-1 production by mononuclear cells. However, dose-response experiments revealed that at least 3000 ng of peptidoglycan per mL was necessary for induction, whereas the optimal LPS concentration was 1 ng/mL.

LPS has been reported to increase leptin mRNA expression, as well as circulating leptin levels (30). Recently, MacKenzie et al. (31) stated that peptidoglycans derived from gram-negative bacteria (*E. coli* 0111:B4 and K12) are potent inducers of IL-1β and IL-6 gene expression and were equal to, or more potent than, crude LPS. On the other hand, peptidoglycans of gram-positive bacteria, DNA, RNA, and lipoteichoic acid were weak stimulators, while lipid A, lipoprotein, and ultrapure LPS were nonstimulatory.

In conclusion, peptidoglycan of *S. xylosus* demonstrates noteworthy pathological effects on the renal system of mice in a dose dependent manner. However, these effects have less intensity than the effects of LPS of *E. coli* (serotype 0128:B12). Much work is needed to shed light on these differences with respect to cytokine production, responses to the involved receptors, and above all their inflammatory response in vivo or ex vivo.

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