Comparative Study between Enoxaparin and Unfractionated Heparin on Septic Renal Deterioration Induced by *Escherichia coli*: Their Impact on Protein C and Expression of iNOS and TLR4

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Abstract: Acute renal injury is an important cause of mortality in a significant proportion of patients with severe sepsis. We have investigated in the current study the possible protective effects of unfractionated heparin vs the low molecular weight heparin (enoxaparin) in an *Escherichia coli* induction model of renal sepsis in rats. Twenty four male Wistar albino rats were divided into normal control and septic group receiving *Escherichia coli* suspension which was further subdivided into non treated (control) and two septic groups pretreated with either enoxaparin (1.5 mg/kg b.wt. I.P.) or unfractionated heparin (7.5 mg/kg b.wt. I.P.). All groups were sacrified after 3hrs. Our results demonstrated that the perfusion flow rate significantly increased ($p<0.05$) by both enoxaparin and unfractionated heparin, while the basal perfusion pressure significantly improved ($p<0.05$) only by enoxaparin. Biochemical analysis revealed that mRNA expression of inducible nitric oxide synthase and Toll like receptor 4 quantified by real-time PCR and assessment of renal function through measurement of serum urea and creatinine significantly decreased only in enoxaparin pretreated group ($p<0.05$). Unfractionated heparin and enoxaparin significantly improved protein C level ($p<0.05$), however, there is significant difference between enoxaparin and unfractionated heparin ($p<0.05$). Histological examination showed that enoxaparin induced marked attenuation of renal inflammation as compared to unfractionated heparin. Therefore, it is regarded that the use of enoxaparin has a renoprotective beneficial benefit over unfractionated heparin in septic condition through its direct effect on protein C level and the expression of both inducible nitric oxide synthase and Toll like receptor 4.
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1. Introduction

Sepsis is the most common cause of acute kidney injury (AKI) in critically ill patients. Septic AKI was associated with greater derangement in hemodynamic and laboratory parameters which has been considered to be independent risk factor of death (Majumdar, 2010).

Toll-like receptors (TLRs) are a family of receptors positioned as a first line of innate defense by recognizing pathogen-associated molecular patterns as well as endogenous signals of tissue injury. These receptors initiate the innate immune response and regulate the adaptive immune response to infection or tissue injury (Uematsu and Akira, 2006).

TLR4, is the receptor for the endotoxin lipopolysaccharide (LPS) which is the major component of the outer membrane of Gram-negative bacteria causing Gram-negative sepsis (Hoshino *et al.*, 1999). As a first step, the innate immune system recognizes the endotoxin LPS (Barton, 2008). Recognition of LPS requires its binding to CD14, a membrane-linked receptor, and its association with the TLR4 (Takeda *et al.*, 2003). This leads to activation of the cytosolic transcription factor nuclear factor-kappa B (NF-κB). Activated NF-κB moves from the cytoplasm to the nucleus, binds to transcription sites and induces activation of an array of genes encoding for acute phase proteins, inducible nitric oxide synthase (iNOS), coagulation factors, proinflammatory cytokines, as well as enzymatic activation of cellular proteases (Sánchez-Lemus *et al.*, 2009).

iNOS has been incriminated in endotoxemia-related acute renal failure (ARF) by inhibiting renal endothelial nitric oxide synthase (eNOS) which is an important determinant of the renal response to endotoxemia (Wang *et al.*, 2006). Furthermore, a local induction of iNOS, which is constitutively expressed in the kidney, in particular in the medulla and proximal tubules, may be the cause of peroxynitrite-related tubular injury and cytotoxicity as a result of local formation of reactive oxygen and nitrogen species during systemic inflammation (Heemskerk *et al.*, 2006).

During sepsis, a broad array of soluble factors are altered including protein C (PC). Reduction in plasma levels of PC have been shown to be prognostic for sepsis and sepsis severity, and predictive of early death in a rat model of polymicrobial sepsis. Previous studies have shown that activated protein C (APC) exhibits anti-inflammatory properties and modulates endothelial function which contributes significantly to ARF during inflammatory diseases including septic shock (Gupta *et al.*, 2007).

Unfractionated heparin (UFH) has been used in treatment of severe sepsis and target organ injury induced by sepsis. Its protective role arises from double-action of anticoagulant and anti-inflammatory properties indicated by significant decrease in the production of interleukin-1α, tumor necrosis factor-α and interleukin-8 (Zarychanski *et al.*, 2011).

In the present study, we try to explore if the low molecular weight heparin (LMWH) enoxaparin has a more effective pharmacological action than UFH on acute renal injury during sepsis through the evaluation of chosen biochemical parameters which are mRNA expression of TLR4 and iNOS, protein C level and renal function. The results will be also confirmed by histopathological evaluation.

2. Material and Methods

Drugs and chemicals:

Heparin sodium was purchased from Amoun Pharmaceutical's Egypt (5000 IU/ml equal to 50 mg/ml). It is a sterile solution of heparin sodium derived from porcine intestinal mucosa, standardized for anticoagulant activity, in water for injection. Enoxaparin was purchased from Sanofi-aventis France and is 150 mg/ml concentration containing 15 mg enoxaparin sodium per 0.1 ml water for injection. All chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals:

Male Wistar albino rats (200–250g) of all groups were kept in controlled condition before the experiment with water *ad libitum*. Animal experiments (n=24) were carried out following the guidelines of the animal ethics committee of the institute.

Induction of sepsis: (Naaber *et al.*, 2000)

Viable *E. coli* (2×10^7/ml) was prepared by adding *E. coli* to saline until the solution turbidity reaches similar turbidity as a macferrline of 2×10^7/ml. Male Wistar albino rats were challenged with *E. coli* (1.5 ml) inoculated intraperitoneally to replicate a model of sepsis.

Experimental groups and parameters:

Twenty four male Wistar albino rats where divided into two main groups:

Group I (n=6): Normal control.

Group II (n=18): Septic group challenged with *E. coli*. This group was further subdivided into three equal groups as follows:

a- Control group.

b- Enoxaparin pretreated group (1.5 mg/kg b.wt. I.P.) (Iba *et al.*, 2009).

c- Heparin Na (UFH) pretreated group (7.5 mg/kg b.wt. I.P.) (Onda *et al.*, 1986).

All groups were sacrificed after 3hrs.

Measurement of basal prefusion pressure and perfusion flow rate: (Cox *et al.*, 1990 and David, 2004)

Rats were anesthetized (pentobarbitone-sodium, 50 mg/kg b.wt. I.P.) and one renal artery was cannulated via the aorta. The kidney was perfused at constant flow with Tyrode solution (37°C). The kidney was isolated by cutting the aorta, renal vein and ureter, and was placed in a chamber containing Tyrode solution at 37°C. Using isolated perfused organ apparatus, the perfusate was delivered into the renal artery and adjusted to achieve a constant pressure that was measured and outflow perfusate was collected to calculate the perfusion flow rate.

Biochemical assays:

Retro-orbital blood sample was collected from each rat after being anesthetized in a tube containing 3.2% sodium citrate solution for a final ratio of one volume sodium citrate to 9 volumes of blood for separation of plasma used in measurement of protein C level, then into a second tube without additives and blood was allowed to clot for separation of serum used in the assay of renal function. The abdomen was then opened and one kidney was excised. The kidneys excised from rats were stored immediately at -80°C until used in real-time PCR.

Measurement of mRNA expression of iNOS and TLR4 by real-time PCR:

Total RNA was isolated from the homogenized rat kidney using TRI reagent (Sigma, MO, USA) according to the manufacturer's protocol. Using this protocol, the RNA exhibited an A260/A280 ratio of 1.8-2.1. cDNA was synthesized by reverse transcription of 2 µg of total RNA according to the manufacturer's instructions of the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) using oligo-dT primers. Quantitative real-time PCR was performed on LightCycler 1.5 system (Roche Applied System, Mannheim, Germany) using LightCycler-DNA

Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions and using the primers listed in table 1. The amplification was done under the following conditions: initial activation step was performed by heating at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C, annealing for 5 s at 55°C and extension for 10 s at 72°C. The individual targets for each sample were quantified by determination of the cycle threshold (Ct) and by use of calibration curves. The relative amount of the target was normalized with the housekeeping gene 18S rRNA and expressed as arbitrary units.

Table 1: Sequence of primers used in real-time PCR

Gene		Primer (5′–3′)	Gen Bank Accession Number
18S	Forward	GAGGCCCTGTAATTGGAATGAGTC	M11188
	Reverse	TCCCAAGATCCAACTACGAGCTTT	
iNOS	Forward	AGGCCACCTCGGATATCTCT	NM_012611
	Reverse	GCTTGTCTCTGGGTCCTCTG	
TLR4	Forward	AGCCGGAAAGTTATTGTGGTGGTG	NM_019178
	Reverse	CAACGGCTCTGGATAAAGTGTCT	

Assessment of renal function:
Serum urea and creatinine levels were assayed for assessment of renal function on an automated analyzer (Cobas c 111 Roche Diagnostics GmbH, Mannheim, Germany).

Protein C assay:
The protein C level (%) of the plasmas being tested was assayed by a colorimetric method using the STA-Stachrom Protein C kit, STA-Owren-Koller buffer, STA-System Controls (Diagnostica Stago, Asnieres, France) following the manufacturer's instructions and the analysis was done using the automated instrument STA Compact.

Histopathological examination: (Siaw *et al.*, 2008)
Kidney sample tissues were collected for histopathological assessment and stored immediately at -80°C until used. It was fixed in 10% (v/v) phosphate-buffered formalin for 24 hrs and then embedded in paraffin. The samples were sectioned (5 µm) using a microtome, stained with haematoxlin and eosine (H&E), and examined with light microscopy at x400 magnification.

Statistical analysis:
The data were analyzed using computer-based fitting program (GraphPad Prism**,** version 4, USA). The statistical tests used in this study were analysis of variance (ANOVA) followed by the Tukey's Multiple Comparison Test to determine differences between different groups. The results were expressed as mean ± SD. p values were considered significant at $p<0.05$.

3. Results

Effect of enoxaparin and UFH pre-treatment on basal perfusion pressure (mmHg) and perfusion flow rate (ml/g/min) in kidneys of male Wistar albino rats:

Regarding the changes in basal perfusion pressure, there was significant difference ($p<0.05$) between normal control and both septic control and septic group pretreated with UFH. Moreover, septic group pretreated with enoxaparin showed significant difference ($p<0.05$) with both septic group pretreated with UFH and septic control with no significant difference between septic group pretreated with enoxaparin and normal control ($p>0.05$).

As regards the perfusion flow rate, both enoxaparin and UFH induced significant improvement ($p<0.05$ vs septic control), indeed there was significant difference between enoxaparin and UFH pretreated groups ($p<0.05$) as shown in table (2).

Table (2): Effect of enoxaparin and UFH pre-treatment on basal perfusion pressure (mmHg) and perfusion flow rate (ml/g/min) in kidneys of male Wistar albino rats

Parameters	Normal control	Septic group		
		Control	Enoxaparin	UFH
Basal perfusion pressure (mmHg)	91.00±3.37	111.6±2.69*	96.29±0.57$^{\#\$}$	116.8±2.82*
Perfusion flow rate (ml/g/min)	5.46 ± 0.45	0.97 ± 0.064*	5.72 ± 0.43$^{\#\$}$	3.46 ± 0.176*$^{\#}$

The results were expressed as mean ± SD; n=6 per group Significant difference at $p<0.05$.
*$p<0.05$ vs normal control, $^{\#}p<0.05$ vs septic control, $^{\$}p<0.05$ vs UFH pretreated group.

Effect of enoxaparin and UFH pre-treatment on mRNA expression of iNOS (iNOS/18S) and TLR4 (TLR4/18S) in renal tissues of male Wistar albino rats:

The acute septic effect of *E. coli* challenge induced overexpression of mRNA of iNOS and TLR4 in renal tissues of rats ($p<0.05$ vs normal control). The expression of mRNA of iNOS and TLR4 was significantly decreased in enoxaparin pretreated group ($p<0.05$ vs septic control) but was not significantly affected in UFH pretreated group ($p>0.05$ vs septic control) with significant difference between enoxaparin and UFH pretreated groups ($p<0.05$) as shown in figure (1).

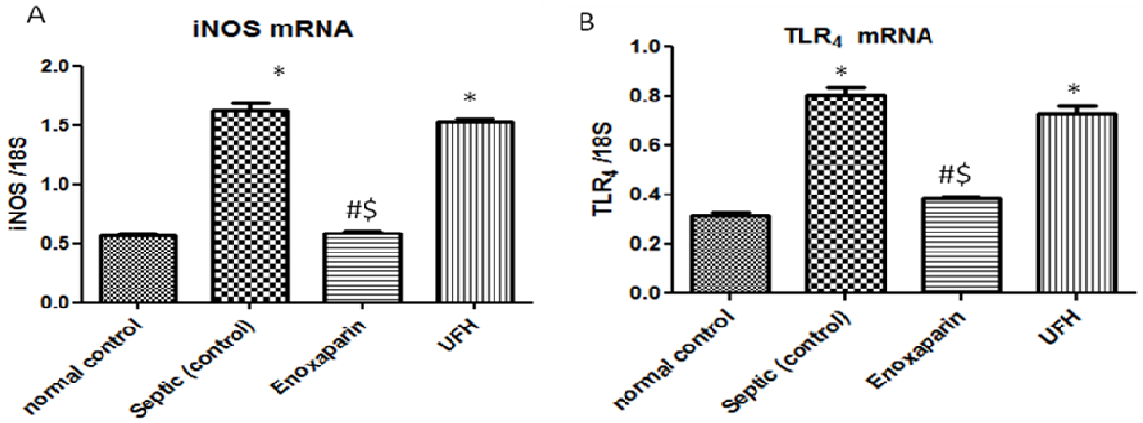

Figure (1): Effect of enoxaparin and UFH pre-treatment on mRNA expression of iNOS (iNOS/18S) and TLR4 (TLR4/18S) in renal tissues of male Wistar albino rats.
The results were expressed as mean ± SD; n=6 per group. Significant difference at $p<0.05$.
*$p<0.05$ vs normal control, $^{\#}p<0.05$ vs septic control and $^{\$}p<0.05$ vs UFH pretreated group.

Effect of enoxaparin and UFH pre-treatment on protein C level (%) of male Wistar albino rats:

There was significant difference ($p<0.05$) between normal control and both septic control and septic group pretreated with UFH. As regards enoxaparin and UFH, there was significant improvement ($p<0.05$ vs septic control) of protein C level (%) with significant difference between enoxaparin and UFH pretreated groups ($p<0.05$) as shown in table (3).

Table (3): Effect of enoxaparin and UFH on protein C level (%) of male Wistar albino rats

Parameter	Normal control	Septic group		
		Control	Enoxaparin	UFH
Protein C level (%)	14.34±0.22	13.50±0.08*	14.60±0.076$^{\#\$}$	13.92±0.17*$^{\#}$

The results were expressed as mean ± SD; n=6 per group. Significant difference at $p<0.05$.
*$p<0.05$ vs normal control, $^{\#}p<0.05$ vs septic control, $^{\$}p<0.05$ vs UFH pretreated group.

Effect of enoxaparin and UFH pre-treatment on renal function of male Wistar albino rats:

Estimation of serum urea and creatinine showed significant difference ($p<0.05$) between normal control and both septic control and septic group pretreated with UFH. As regards enoxaparin, there was no significant difference between enoxaparin pretreated group and normal control ($p>0.05$) with significant difference between enoxaparin and UFH pretreated groups ($p<0.05$) as shown in table (4).

Table (4): Effect of enoxaparin and UFH pre-treatment on the renal function of male Wistar albino rats

Parameters	Normal control	Septic group		
		Control	Enoxaparin	UFH
Serum urea (mg/dl)	31.31±1.97	85.79±5.46*	37.41±3.57$^{\#\$}$	82.51±6.2*
Serum creatinine (mg/dl)	0.35±0.033	0.77±0.034*	0.4±0.028$^{\#\$}$	0.7±0.061*

The results were expressed as mean ± S.D; n=6 per group. Significant difference at $p<0.05$.
*$p<0.05$ vs normal control, $^{\#}p<0.05$ vs septic control, $^{\$}p<0.05$ vs UFH pretreated group.

Effect of enoxaparin and UFH pre-treatment on the renal tissue of male Wistar albino rats:

The acute septic effect of LPS on kidney was examined according to Siaw *et al.*, 2008 showing mesangial proliferation with tubular dilatation, vacuolization, hemorrhage and inflammatory cell infiltration in fig. (2-B) in comparison to control kidney fig. (2-A). Enoxaparin fig. (2-C) shows marked attenuation of tubular dilation and inflammatory cell infiltration while, UFH fig. (2-D) shows mildly attenuated tubular dilation with very mild attenuation in glomerular mesangial proliferation.

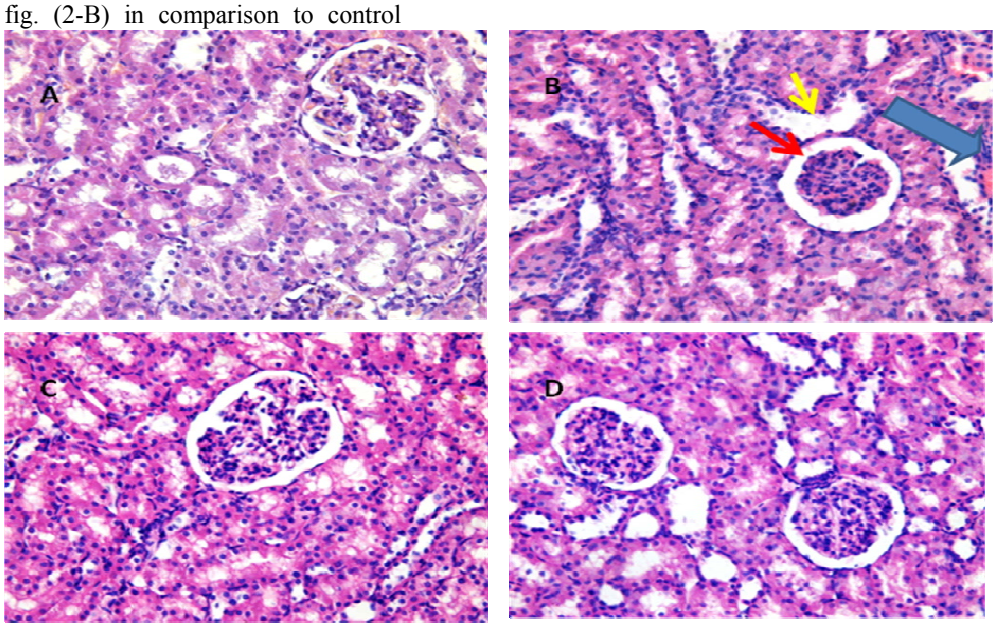

Figure (2): Photomicrographs show normal glomeruli (A) and tubular and mesangial proliferation ( ) in the kidney of LPS injected rat (B) with tubular dilatation, ( ) vacuolization and inflammatory cell infiltration with areas of hemorrhage ( ).

4. Discussion

Bacterial infusion models can approximate introduction of a single pathogen in a controlled manner, allowing reproducible infection. The rodent sepsis models of *E. coli* infusion developed renal microcirculatory failure, as evaluated by precise methods, including intravital two-photon video microscopy (*Doi et al.*, 2009).

Langenberg *et al*, (2005) showed that in experimental sepsis, renal blood flow was reported to be decreased in two-thirds of studies (62 %). In the present study, enoxaparin significantly improved both the basal perfusion pressure and the perfusion flow rate while UFH improved only the perfusion flow rate.

LMWH derivatives are characterized by low anti-coagulant activity and marked anti-inflammatory effects that allow for these molecules to be viewed as a new class of non-steroidal anti-inflammatory drugs (NSAIDs) with less interference with platelet and vessel wall interaction compared to that of UFH (Ceccarelli *et al.*, 2009).

The present study showed that *E. coli* challenge significantly increased expression of both TLR4 and iNOS mRNA quantified by real-time PCR ($p<0.05$ vs normal control) which is in agreement with Baumgarten *et al*, (2006), Uematsu and Akira, (2007) and Sánchez-Lemus *et al*, (2009). UFH did not significantly decrease their expression ($p>0.05$ vs septic control) which is in agreement with Brunialti *et al.*, 2006. On the other hand, enoxaparin administration significantly decreased their enhanced production ($p<0.05$ vs septic control) with significant difference between enoxaparin and UFH pretreated groups ($p<0.05$). These results indicated that enoxaparin has more anti-inflammatory effect in Gram-negative sepsis as compared to UFH through its direct effect on the expression of TLR4 which is responsible for recognition of the endotoxin LPS of Gram-negative bacteria and production of cytokines, chemokines and release of endogenous danger signals (Nieto *et al.*, 2009). Another evidence of the powerful anti-inflammatory effect of enoxaparin is its ability to decrease the expression of iNOS in this renal sepsis model thus decreasing the peroxynitrite-related tubular injury and cytotoxicity as evidenced by the improvement of renal function in enoxaparin pretreated group ($p<0.05$ vs septic control) which did not happen in UFH pretreated group ($p>0.05$ vs septic control) and histopathologically through the marked attenuation of tubular dilation and inflammatory cell infiltration

induced by enoxaparin in contrast to UFH which showed mildly attenuated tubular dilation with very mild attenuation in glomerular mesangial proliferation.

Previous studies have shown that APC exhibits anti-inflammatory properties (Gupta *et al.*, 2007). This study demonstrated that protein C level (%) was significantly increased in enoxaparin pretreated group ($p<0.05$ vs septic control) with no significant difference between enoxaparin pretreated group and normal control ($p>0.05$). Moreover, although UFH significantly increased protein C level (%), which is in agreement with Comp, (1984) and De Cristofaro *et al*, (1998), yet there was significant difference between enoxaparin and UFH pretreated groups. Therefore, enoxaparin exhibits potential higher anti-inflammatory effect as compared to UFH.

Biochemical analysis revealed that APC downregulated iNOS mRNA levels and nitric oxide by-products in the kidney (Gupta *et al.*, 2007). Therefore, the significant decrease of iNOS production in enoxaparin pretreated group may be either through direct mechanism or indirectly through protein C related effects.

On this basis, we can hypothesize that the use of enoxaparin in renal sepsis provides more renoprotective effect than UFH due to its higher anti-inflammatory effect through the decrease of TLR4 and iNOS mRNA expression and the increase of protein C level (%).

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