Bovine Lactoferrin Attenuates Renal Damage Induced by Nicotine Toxicity by Blocking Nuclear Factor Kappa-B Signaling Mechanisms

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ABSTRACT

Smoking cigarette is a devastating factor that can lead to chronic renal disease. The current investigation was designed to explore the prophylactic effect of bovine lactoferrin (LF) against the inflammation, fibrogenesis and angiogenesis induced renal damage in rat exposed to nicotine toxicity. Nicotine was administered intraperitoneally at either low (0.5 mg/Kg body mass) or high (2.5 mg /Kg body mass) for thirty consecutive days. LF (50 mg/ Kg body mass) was administered intraperitoneally simultaneously with nicotine administration daily for thirty days. The data demonstrated injection of LF to rats treated with the small or the large dose of nicotine , markedly ameliorated the increases in the renal inflammatory markers namely interleukin -6 (IL-6(and C reactive protein (CRP), fibrogenic cytokine, transforming growth factor –β1 (TGF- β1), the angiogenic factor, vascular endothelial growth factor (VEGF), and transcription factor, nuclear factor kappa B (NF-kB). LF treatment also could ameliorate the alterations in the serum renal function markers (creatinine, urea and cystatin C) in rats subjected to nicotine toxicity.

In conclusion, the present result demonstrated that bovine LF could ameliorate the toxic effects of nicotine –induced renal damage by suppressing inflammation, fibrogenesis, angiogenesis and NF-κB activation.

Key Words: bovine lactoferrin, nicotine, interleukin -6, nuclear factor kappa B

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INTRODUCTION

Chronic renal disease (CRD) is a common hazard factor contributes to morbidity and mortality [1]. There is growing evidence that smoking/tobacco use has a serious impact on renal health and is considered one of the causes leading to CRD [2].

Nicotine is the most abundant toxic alkaloid present in tobacco smoke [3]. It has a principle role inducing renal dysfunction [4]. Nicotine is easily absorbed through the lungs into the bloodstream and distributed to different organs including kidney [5]. The devastating role of smoking in the induction of renal damage has been well established [6]. In kidney patients, smokings hasten the progression of kidney dysfunction to end-stage kidney failure [2] and can also increase the hazard of chronic kidney damage in healthy people [2, 6] Nicotine can directly promote the secretion of antidiuretic hormone (ADH), causing an elevation in urinary osmolality and a depletion in free water clearance [7]. Experimental study has been showed that long term exposure to nicotine resulted in a decrease in glomerular Filtration Rate [8]. Nicotine also has proangiogenic potential activity which responsible for the different forms of glomerular injury [9]. Also, it has been reported that nicotine overuse can promote an inflammatory

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reactions by inducing the generation of pro-inflammatory proteins, including nuclear factor kappa (NF- kB), cyclooxygenase II, IL-6, TNF-α, CRP, etc [10] whose over expression finally can cause tissue damage [11]. In CRD, high levels of inflammatory immune reactions may contribute to renal dysfunction [11].
Many natural compounds have been reported to possess anti-inflammatory effect and can directly protect against inflammatory tissue damage [12].
Lactoferrin (LF, known as lactotransferrin) is a natural iron-binding glycoprotein of the transferrin family. This natural protein is secreted by glandular cells and neutrophils. It is found mainly in milk, mucosal secretions and bodily fluids [13]. LF has multi-therapeutic potential impacts that are mediated through specific receptors present on the surface of many cells [14]. It has anti-inflammatory, immunomodulatory, anticancer [12], antioxidant [15], and renoprotective properties [16]. It has been stated that large amount of LF is produced by renal tissue which has an important role in innate immunity of this organ, thus protecting kidney against inflammatory injuries [17].
Although experimental studies have investigated the protective impact of LF on renal injury under the effect of some renotoxic agents, however its protective impact against renal disorder in response to chronic inflammation induced by nicotine exposure is still unexplored. The objective of this investigation was to explore the profound prophylactic efficacy of bovine LF against the activation of NF-kB signaling mechanisms (inflammation, fibrogenesis and angiogenesis) induced renal damage in rats in response to nicotine toxicity

MATERIAL AND METHODS

Chemicals

Nicotine hydrogen tartrate and bovine lactoferrin (LF) were bought from Sigma Company, St. Louis, USA.

Experimental animals

Sixty male Wistar rats (160-190 g) were utilized for this investigation. The animals were bought from Experimental Animal Care Center, King Fahad Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Rats were housed in stainless steel cages at control conditions (20–22 °C, 60 % humidity and 12 hour dark / light cycle). Rats were supplied with balanced diet and tap water adlibtium for one week for acclimation. Animal handling was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care Committee at the King Abdulaziz University.

Experimental design

Animals were classified into 6 groups, ten rats in each group:
Group 1: Control animals
Group 2: Rats injected with bovine LF (50 mg / kg b .w. [18]. **Group 3**: Rats injected with nicotine small dose (0.5 mg / kg b .w [19].
Group 4: Rats injected with nicotine large dose (2.5 mg / kg b .w. [20].
Group 5: Rats intoxicated with small dose of nicotine and co-administered with LF (50 mg / kg b .w.)
Group 6: Rats intoxicated with large dose of nicotine and co-administered with LF (50 mg / kg b .w).
Nicotine hydrogen tartrate and bovine lactoferrin were dissolved in normal saline and then injected intraperitoneally simultaneously to animal groups (group 5 and 6) for 30 consecutive days. After 30 days of experimental period, the rats were starved overnight (12-14 hours), then the blood specimens were taken for serum segregation. Serum was isolated utilizing refrigerated centrifuge at 3000 rpm for ten minutes and utilized for biochemical serum analysis. After blood collection, all rats were sacrifice under light anesthesia and the renal samples were collected for biochemical analysis.

Biochemical Serum analysis

Determinations of renal damage biomarkers

Serum cystatin C, creatine and urea (biomarkers of kidney damage) were estimated using an automatic biochemical analyzer (ci16200, Abbott, USA).

Determination of inflammatory biomarker in renal tissue

IL-6 was estimated in renal tissue using rat-IL-6 sandwich enzyme-linked immunosorbent assay (ELISA) kit (ABCAM, ab119548, UK) depending on the manufacturer's instructions.CRP was estimatedutilizingrat CRP ELIZA kit(Elabscience, Houston, USA). TGF-β1 was measured utilizing quantitative TGF-β1 rat sandwich ELISA kit (MyBiosource , Southern California, San Diego, USA) The level of VEGF was determined quantitatively by sandwich ELISA assay kit (R&D Systems, UK) utilizing the manufacturer's instructions. VEGF level was calculated utilizing a calibration curve utilizing particular standards given by the manufacturer. NF-kappa-B (NF-kB) was estimated using rat ELISA kit (EIAAB products, East Lake Hi-Tech Development Zone, Wuhan China) following the manufacturer's instructions.

Statistical Analysis

Results are expressed as mean ± standard deviation (SD) of ten animals. The significant variations among data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test post-ANOVA. The differences among data were significant at $P<0.05$.

RESULTS

The effects of LF injection on the levels of inflammatory molecules IL-6 and CRP in the renal tissue of normal and nicotine intoxicated rats are shown in Figures 1 and 2 respectively. The data demonstrated that injection ofsmall (group 3) or large (group 4) dose of nicotine, significantly up-modulated the levels of these markers versus the control group (group 1, $P \leq 0.001$). These inflammatory indices were severely elevated in renal of rats injected with the large nicotine dosage. Co –administration of LF to rats injected with either the small (group 5) or the large (group 6) dose of nicotine, effectively ameliorated the increases in these molecules with respect to the nicotine untreated counterpart group ($P \le 0.001$).

Figure 3 shows the effect of LF on the level of fibrogenic cytokine, TGF-β1, in the renal tissue of rat groups injected with nicotine. The results illustrated that administration of the small or the large nicotine dose, dramatically caused an increase in this cytokine concentration in relation to normal rat group ($P \le 0.001$). Co administration of LF, markedly modulated the alteration in this cytokine compared with the intoxicated untreated counterpart groups.

Figure 4 illustrates the effect of LF on the concentration of VEGF (angiogenic index) in renal tissue of control and nicotine intoxicated rat groups. The data showed that injection of animals with the small or the large dosage of nicotine, significantly caused an elevation in concentration of this factor in comparison to control group ($P \le 0.001$). The alteration in this angiogenic marker was obvious in rat group injected with the large nicotine dosage. Co injection of LF to rats exposed to small or the large dose of nicotine markedly decreased the concentration of this marker when compared to the intoxicated counterpart group ($P \le 0.001$).

Result in Figure 5 shows the effect of LF on the level of renal transcription factor, NF-kB in rats injected with nicotine. The data revealed that rats subjected to the small or the large nicotine dosage significantly increased the level of NF-kB compared with control rats ($P \le 0.0001$). The alteration in this factor was pronounced in renal of rats injected with the large nicotine dosage. Co injection of LF for 30 consecutive days, effectively suppressed the increase in this transcription factor with respect to intoxicated counterpart group ($P \le 0.001$).

Data in Table 1 reveals the serum concentrations of renal function indices (creatinine, urea and cystatin c) in normal and different experimental nicotine intoxicated groups. The results illustrated that injection of small or large dosage of nicotine, caused marked elevation in these markers versus normal rats. Injection of rats with LF simultaneously with either nicotine dose, significantly reduced the concentrations of renal function markers versus nicotine intoxicated counterpart group ($P \le 0.0001$).

Non-significant changes were observed in all studied markers in rat group treated with LF only (G2) compared with normal control.

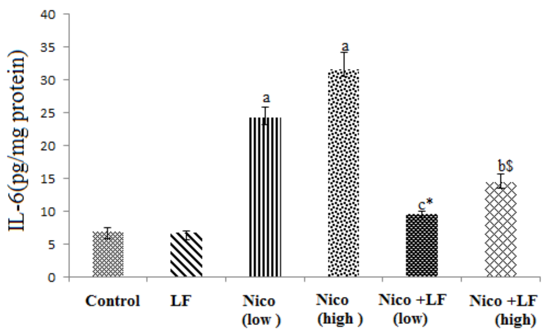

Fig 1: Effects of LF on renal IL-6 in rats intoxicated with the small or the large nicotine dosage .Values are expressed as mean ± S.D. (n=10) , $^{a}P \le 0.001$, $^{b}P \le 0.01$, $^{c}P \le 0.05$ with respect to the control group, $^{*}P \le 0.001$ versus small nicotine intoxicated group, $^{\$}P \le 0.001$ versus large nicotine intoxicated group.

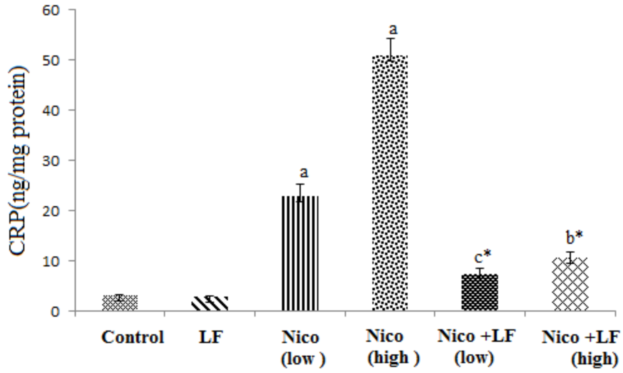

Fig 2: Effects of LF on the level of CRP in the rat kidneys administered the small or the large nicotine dosage .Data are represented as mean ± S.D. (n=10), $^{a}P \le 0.001$, $^{b}P \le 0.01$,$^{c}P \le 0.05$ compared with the control group, $^{*}P \le 0.001$ versus the small nicotine intoxicated group, $^{\$}P \le 0.001$ versus the large nicotine intoxicated group.

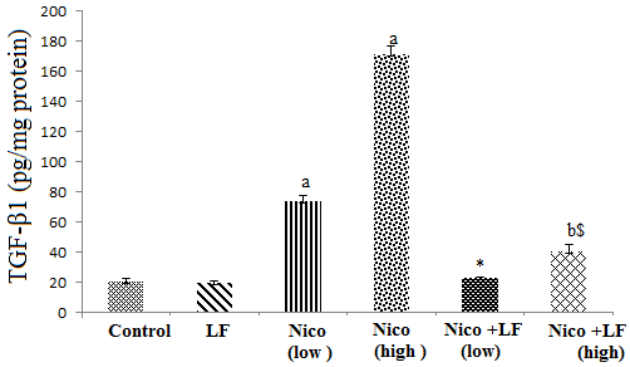

Fig 3: Effects of lactoferrin (LF) on the level of TGF-β1 in the rat kidneys intoxicated with the small or the large nicotine dose. Values are calculated as mean ± S.D. (n=10), $^{a}P \le 0.001$,$^{b}P \le 0.01$, in comparison with the control group, $^{*}P \le 0.001$ with respect to small nicotine intoxicated group, $^{\$}P \le 0.001$ with respect to large nicotine intoxicated group.

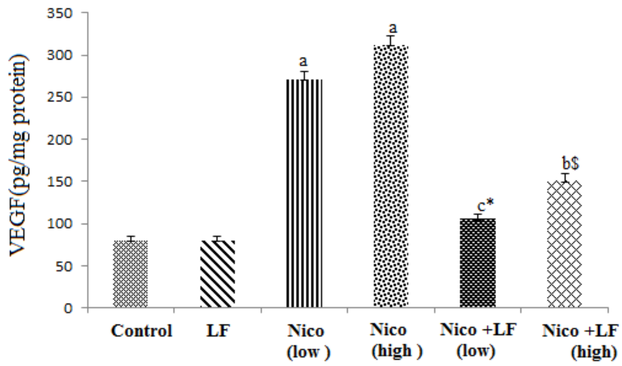

Fig 4: Effects of lactoferrin (LF) on the level of VEGF in the rat kidneys intoxicated with the small or the large

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nicotine dosage. Values are represented as mean ± S.D. from 10 rats, a $P \le 0.001$, b $P \le 0.01$, compared with the control group, * $P \le 0.001$ versus the small nicotine intoxicated group, $P ≤ 0.001 compared with high nicotine intoxicated group.

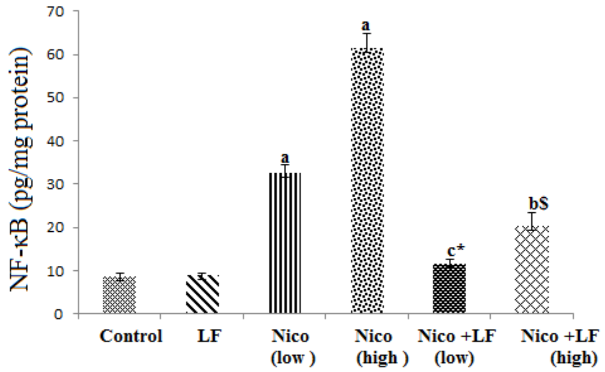

Fig 5: Effects of lactoferrin (LF) on the level of NF-kB in the rat kidneys intoxicated with the small or the large nicotine dosage. Results are expressed as mean ± S.D. (n=10), $^{a}P \le 0.001$, $^{b}P \le 0.01$,$^{c}P \le 0.05$ versus the control group, $^{*}P \le 0.001$ versus the small nicotine intoxicated group, $^{\$}P \le 0.001$ versus the large nicotine intoxicated group.

Table 1: Serum biomarkers of renal tissue damage in normal and nicotine – treated different groups

Paramete	Contr	LF	Low nicoti	High nicoti	Low nicoti + LF	High nicoti +LF
Creatinin mg/dl	0.368 0.022	0.364 0.013	1.74± 0.06^{a}	2.5± 0.19 ^{a}	0.402± 0.015	0.53± 0.012^{c}
Urea mg/dL	15.7± 1.13	14.8± 1.0	36.7± 0.96 ^{a}	44.6± 3.9 ^{a}	18.6± 1.9*	25.2± 1.3$^{b\,\$}$
Cystatin mg/L	0.46± 0.015	0.45± 0.03	0.73± 0.072^{a}	0.97± 0.015^{a}	0.57± 0.015^{b}	0.7± 0.025^{a}

Results are represented as mean ± S.D. (n=10), $^{a}P \le 0.001$, $^{b}P \le 0.01$,$^{c}P \le 0.05$ with the control group, $^{*}P \le 0.001$ in comparison with small nicotine intoxicated animals , $^{\$}P \le 0.001$ versus with large nicotine intoxicated animals.

DISCUSSION

It has been found that nicotine overuse can induce an inflammatory response in body vital organs including kidney [6, 11].

The present work aims to to explore the beneficial renoprotective impact of bovine lactoferrin (LF) against the activation of NF-kB signaling mechanisms (inflammation, fibrogenesis and angiogenesis) caused renal damage in rats subjected to nicotine toxicity.

The results showed that injection of the small or the large dosage of nicotine to rats caused marked elevations in the immuno-inflammatory proteins (IL-6 and CRP) in the renal tissue of intoxicated rats, with respect to normal rats. This impact was pronounced in animals intoxicated with nicotine large dose compared with ones injected with the low dose. Our results are confirmed by many workers who have been stated that nicotine abuse can elicit an inflammatory response by promoting the generation of pro-inflammatory proteins, including IL-6 and CRP [11, 21-22] Some authors demonstrated that exposureto nicotine cause an increase in IL-6 mRNA expression, suggesting that nicotine may affect the immunological and acute-phase responses, causing the over-production of IL-6 [23] Also,clinical investigations have documented that increases in the concentration of CRP and inflammatory cytokines are accompanied with the exposure to nicotine [22, 24]. C-reactive protein is an acute phase protein, generated during inflammation [25]. It is produced principally by liver cells, but it can also be synthesized by other types of cells, suggesting that localized inflammation can promote CRP overproduction [26]. Production of IL-6 by different immune cells (granulocytes and macrophages) is considered as a primary regulator of CRP at damage sites. This cytokine joins to receptors of cell surface and induce cascade of intracellular signaling, which causes the stimulation of many transcription factors for promoting the expression of CRP by tubular renal cells [25]. There are growing evidences indicate that renal disease and renal dysfunction are related to renal inflammation caused by elevated CRP levels [12, 26].

Administration of LF to rats intoxicated with the small or the large nicotine dosage, markedly down-modulated the increase in both IL-6 and CRP with respect to rats intoxicated with nicotine counterpart group, indicating its anti-inflammatory potential action. This agent was more effective in down-modulating these inflammatory proteins in rats injected with the small nicotine dosage.

The result of the current study is confirmed by previous publication demonstrated that LF can inhibit the production of inflammatory cytokines, including IL-6 in human mononuclear cells in vitro [27].

TGF-β1 is another cytokine produced in rat renal tissues in response to exposure to low or high dose of nicotine presented in the current study. The up-regulation of this cytokine in renal tissue under the effect of nicotine may consider one of the indicators of renal damage. TGF-β1 is a multi-functional profibrotic cytokine produced in CRD, which induces several pathophysiological mechanisms. It is generated by different types of kidney cells and exerts its pathological effects via different signaling mechanisms [28]. In kidney diseases, TGF-β is increased and stimulates kidney cells to generate extracellular matrix proteins, causing glomerulosclerosis and tubulointerstitial fibrosis [28]. TGF-β can cause drastic pathophysiological changes in various types of kidney cells, leading to apoptotic cell death and hypertrophy, which ultimately result in renal failure [28, 29]. Administration of LF to rats intoxicated with either the low or the high nicotine dose, markedly down-modulated the elevation in TGF-β1 in rat renal tissue compared with rats intoxicated with nicotine counterpart group, indicating its anti-fibrogenic beneficial impact. The protective role of LF against inflammatory cystic fibrosis caused bronchial cell damage was previously documented [30].

The current work showed a significant elevation in the VEGF (angiogenic factor) in the renal tissue of rats subjected to either of the two dosages of nicotine. Our result is copped with [10] **who** documented the angiogenic potential impact of nicotine which may relate to renal damage. Overexpression of VEGF by chronic exposure to nicotine can induce thickening of glomerular basement membrane, accumulation of mesangial matrix, and infiltration of inflammatory immune cells, leading to glomerular injury [10]. It has been found that overexpression of different inflammatory tissue factors, cytokines, and chemokines promote the synthesis of this angiogenic factor by inflammatory immune cells [31] **.**Administration of LF to nicotine intoxicated rats, significantly reduced theelevation in VEGF in renal tissue of nicotine injected rats compared with rats intoxicated with nicotine counterpart group. This result may give a clue to the anti-angiogenicimpact of LF. Our result is confirmed by some authors have been demonstrated that LF could inhibit the growth of tumor by suppressing VEGF -induced angiogenesis in the rat [32].

Concerning with the effect of nicotine toxicity on the level of renal transcription factor, NF-κB, in rats, the result illustrated that a pronounced elevation in this factor in the renal of rats injected with either of the two nicotine dosages. This impact was more obvious in rats exposed to nicotine large dose. The production of NF-kB in response to nicotine exposure was confirmed [11] whose over production eventually contribute to tissue injuries and damage [33].

NF-kB is a pivotal inflammatory transcription factor that has a key job in the cellular signaling pathway for inflammation in various pathological conditions [34]. NF-kB signaling mechanism is shown to play a role in the renal injury caused under the effect different agents [35]. NF-kB activates several inflammatory genes resulting in cellular damage [34]. At resting normal state, NF-kB is inactive state by its binding with its specific inhibitor (IkB) in the cell cytosol. However, production of inflammatory cytokines activate the NF-kB signalling mechanism [35], after degradation of IkB [36]. The activated NF-kB enters the nucleus and stimulates the transcription of many genes such as IL-6, TGF-β1 and VEFG [37]. These proteins, in turn, stimulate many reactions, including nitric oxide (NO) overproduction, generation of free radicals, activation of apoptotic mechanisms and increased production of extracellular matrix (ECM) proteins, thus leading to renal damage [38]. Co administration of LF to rats exposed to the small or the large nicotine dosage, effectively down-modulated the increase in NF- kB, in renal tissue of intoxicated rats, compared with intoxicated counterpart group. Similar to this result, some authors have been reported thatprophylactic treatment of rats with LF markedlycorrected the increase in the level of NF- kB in nephrotoxicity induced rats [17]. The current result may suggest that the suppressing effects of LF on the expression of proinflammatory mediators (IL-6 and CRP), fibrogenic factor (TGF-β1) and angeiogenic factor (VEGF) were through its inhibitory effect on NF- kB activation.

The current work illustrated that the levels of creatinine, urea and cystatin C were markedly elevated in rats intoxicated with either of the two nicotine doses with respect to control rats, implying that the nicotine toxicity caused renal dysfunction. Our result is supported by [9] who reported that chronic exposure to nicotine resulted in decreased GFR. Clinically increasing in the level of serum creatinine, urea and cystatin C is considered as an index of a disorder in glomerular filtration rate (GFR) and renal injury [39]. Administration of LF to rat groups intoxicated with either nicotine dose markedly mitigated the kidney function markers, documenting its potential renoprotective impact [17].

CONCLUSION

The present study illustrated that induction of inflammatory mediators (IL-6 and CRP), the fibrogenic cytokine (TGF-β1) and the angiogenic factor (VEGF) in renal rats subjected to nicotine toxicity are collectively involved in renal damage which may relate to the activation of the transcription factor, NF-kB, signaling pathways. Prophylactic treatment with bovine LF could protect against nicotine -induced renal dysfunction in rats. The beneficial protective impact of LF may relate to its anti-inflammatory, and anti-fibrotic and antiangiogenic properties with down-regulation of NF-kB activation.

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