



# Bovine Lactoferrin Ameliorates Cardiac Muscle Damage Caused by Nicotine Toxicity by Suppressing Inflammatory Signaling Pathway

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## ABSTRACT

The relationship between nicotine exposure and cardiovascular events is well documented. This investigation aims to study the protective impact of bovine lactoferrin (LF) in preventing inflammation, fibrogenesis and angiogenesis induced cardiac muscle damage in rats under the effect of nicotine toxicity. Nicotine was injected to rats intraperitoneally at either a low (0.5 mg /kg b.w) or a high (2.5mg/kg b.w.) dose for 30 consecutive days. LF (50 mg / kg b.w.) was co- injected intraperitoneally with nicotine administration daily for thirty days. The data showed that injection of LF to rats injected with the low or the high dosage of nicotine , markedly ameliorated the increases in the cardiac inflammatory indices, interleukin -6 (IL-6) and C reactive protein (CRP), the fibrogenic cytokine, transforming growth factor - $\beta$ 1 (TGF-  $\beta$ 1), the angiogenic factor, vascular endothelial growth factor (VEGF), and nuclear factor kappa B (NF- $\kappa$ B). LF treatment also could ameliorate the increases in the serum cardiac function markers namely creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) in both rat groups exposed to low and high nicotine dose.

In conclusion, the present result demonstrated that bovine LF could attenuate the toxic effects of nicotine -induced cardiac muscle damage by suppressing inflammation , fibrogenesis, angiogenesis and NF- $\kappa$ B activation.

**Key Words:** Bovine lactoferrin, nicotine, creatine phospho kinase , nuclear factor kappa-B

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## INTRODUCTION

Smoking is considered one of the principle causes for the incidence and the advancement of cardiovascular disease [1], including atherosclerosis, myocardial hypertrophy, coronary artery disease and fibrosis [2, 3]]. Nicotine is the most poisonous element in cigarette smoke. . Nicotine is easily absorbed through the lungs into the bloodstream and distributed to different organs [4].

Inflammation is a key mechanism contributing to nicotine -induced cardiac diseases [5]. Some authors demonstrated that tobacco smoke can cause chronic inflammatory reactions which play a major role in atherogenesis and acute ischemic illness [5]. Macrophages respond to nicotine exposure by producing

proinflammatory mediators which activate neutrophils and additional macrophages, resulting in inflammation and tissue damage [6]. Concomitantly, neutrophils produce free radicals , elastase, matrix metalloproteinases, and inflammatory proteins, such as TNF-a, IL-6 and IL-1b [7]. These mediators can cause collagen deposition and tissue fibrosis [8]. Also, it has found that inflammatory mediators can promote fibrosis in damaged organs via stimulation of different signaling mechanisms , including NF- $\kappa$ B [9].

An atherosclerotic plaque is formed due to adhering of activated monocytes to the site of injured endothelial cells, transformed into macrophages and then foam cells. Inflammation takes place when the activated

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macrophages release inflammatory mediators that induce burst of the plaque, causing narrowing of blood vessels and thrombosis [10]. Leukocytosis, inflammatory cytokines, C-reactive protein (CRP), and fibrogenic cytokines are strong inflammatory indicators of cardiovascular disorders seen in smokers [10]. Clinical study has reported that elevation of serum CRP concentrations in smokers with respect to nonsmokers [11]. High level in CRP has been found to be related to oxidative stress [12]. Over-generation of inflammatory proteins and oxidative stress are the principle cause of cardiovascular diseases [13]. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is considered the most potential fibrogeic factor involved in tissue fibrosis. TGF- $\beta$ 1 induces the phosphorylation of Smad2 and Smad3, resulting in elevated collagen I and III deposition in tissues [14]. Clinical studies have documented promotion of the TGF- $\beta$ 1/Smad pathway in smokers [15].

Many natural compounds have been reported to possess anti-inflammatory effect and can directly protect against inflammatory tissue damage [16].

Lactoferrin (LF) is multifunctional natural protein (80 kDa, transferrin family), found in milk, tears, saliva, pancreatic juice and gall [17]. LF is a part of the natural immunity and indirectly participate in acquired immunity [18]. It displays multi-therapeutic activities, including antimicrobial anti-inflammatory, immunomodulatory, anticancer [16], antioxidant [19], renoprotective and hepatoprotective properties [20-21].

Experimental studies have evaluated the hepatoprotective and renoprotective impacts of LF against toxic agents, however its protective impact against cardiotoxicity is still unexplored. The aim of the current research was to study the beneficial prophylactic action of bovine LF against inflammation, fibrogenesis and angiogenesis induced myocardial damage in rats subjected to chronic exposure to nicotine toxicity.

## MATERIAL AND METHODS

### Chemicals

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

### Experimental animals

Sixty male Wistar albino rats (150-180 g) were utilized for the current investigation. The rats were bought from Experimental Animal Care Center, King Fahad Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Rats were housed in polypropylene cages at standard environment of temperature, humidity and 12 hour dark / light cycle. Rats were fed standard diet and provided tap water ad libitum for one week for acclimation. Animal handling was carried out according to the protocol supplied by the Experimental Animal Laboratory and accepted by the Animal Care Committee, the King Abdulaziz University.

### Experimental design

The rats were divided into 6 groups, each of ten rats:

**Group 1:** Control animals.

**Group 2:** Rats injected with bovine LF (50 mg/ Kg body weight ) [22].

**Group 3:** Rats injected with nicotine low dosage (0.5 mg /Kg body weight) [23].

**Group 4:** Rats injected with nicotine high dosage (2.5 mg/ Kg body weight ) [24].

**Group 5:** Rats intoxicated with low dose of nicotine and co-administered with LF (50 mg/ Kg body weight)

**Group 6:** Rats intoxicated with high dose of nicotine and co-administered with LF (50 mg/kg body weight).

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 animals were starved for about 14 hours, then the blood specimens were gathered for serum isolation. the sera were kept at -80°C till utilize for biochemical analysis. Animals were then sacrifice under light anesthesia and the cardiac samples were collected for biochemical analysis.

### Biochemical Serum analysis

#### Determinations of cardiac damage biomarkers

Serum CPK, LDH and ALP were estimated using an automatic biochemical analyzer (ci16200, Abbott, USA). Determination of inflammatory biomarker in cardiac muscles

IL-6 was estimated in cardiac muscles utilizing rat-IL-6 enzyme-linked immunosorbent assay (ELISA) kit (ABCAM, ab119548, UK) according to the instructions of manufacturer. CRP was measured utilizing rat CRP ELISA kit (Elabscience, Houston, USA). TGF- $\beta$ 1 was measured utilizing quantitative rat TGF- $\beta$ 1 sandwich ELISA kit (My Biosource, Southern California, San Diego, USA). The level of VEGF was determined quantitatively by sandwich ELISA assay ( R&D Systems, UK) depending on the instructions of manufacturer. VEGF concentration was calculated utilizing a calibration curve of particular standards given by the manufacturer. NF- $\kappa$ B was estimated utilizing rat NF- $\kappa$ B ELISA assay kit (EIAAB Products, East Lake Hi-Tech Development Zone, Wuhan China) following the instructions given by the manufacturer.

#### Statistical Analysis

Values are expressed as mean  $\pm$  standard deviation (SD) of ten rats. The significant variations among data were statistically analyzed utilizing 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 concentrations of the inflammatory proteins IL-6 and CRP in the cardiac muscles of normal and nicotine intoxicated rats are

shown in Figures 1 and 2 respectively. The data demonstrated that injection of small (group 3) or the big dosage (group 4) of nicotine, significantly up-regulated the levels of these indices with respect to the control group (group 1,  $P \leq 0.001$ ). These inflammatory proteins were dramatically increased in cardiomyocytes of rats exposed to the big nicotine dosage. Co-injection of LF to nicotine treated rats with the small (group 5) or the big (group 6) dosage, effectively reduced the increases in these molecules in relation to the intoxicated counterpart group ( $P \leq 0.001$ ).

Figure 3 shows the influence of LF on TGF- $\beta$ 1 content in the cardiac muscles of rat groups injected with nicotine. The data depicted that injection of rats with the small or the big nicotine dosage caused an increase in this cytokine concentration versus control rats ( $P \leq 0.001$ ). Co administration of LF, markedly attenuated the increase in this cytokine versus the intoxicated untreated counterpart group.

Figure 4 demonstrates the impact of LF on VEGF (angiogenic factor) content in cardiac muscles of control and nicotine intoxicated rat groups. The data showed that injection of rats with the small or the big dosage of nicotine, significantly caused an elevation in concentration of this factor with respect to control animals ( $P \leq 0.001$ ). The deviation in this angiogenic factor was obvious in rat group injected with the big nicotine dosage. Co injection of LF to rats exposed to either the low or the high dose of nicotine, significantly decreased the level of this marker in relation to the intoxicated counterpart group ( $P \leq 0.001$ ).

The impact of LF on the level of cardiac transcription factor, NF-kB in animals subjected to nicotine toxicity is depicted in Figure 5. The result showed that treatment of rats with the small or the big nicotine dosage, markedly increased the level of NF-kB versus control rats ( $P \leq 0.0001$ ). The alteration in this factor was pronounced in rats injected with the big nicotine dosage. Co injection of LF to rats treated with the small or the big nicotine dosage for 30 successive days, effectively inhibited the increase in this transcription factor when compared to intoxicated counterpart group ( $P \leq 0.001$ ).

Table 1 shows the activities of serum cardiac function enzymes (CPK, LDH and ALP) in control and different nicotine intoxicated groups. The data illustrated that injection of the small or the big dosage of nicotine, caused marked elevation in these markers with respect to control rats. Injection of rats with LF simultaneously with either nicotine dose, significantly reduced the concentrations of cardiac function markers versus nicotine intoxicated counterpart group ( $P \leq 0.0001$ ).

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

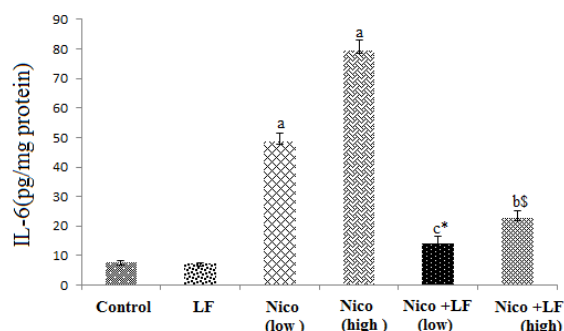


Fig 1: Effects of LF on cardiac IL-6 in the rats intoxicated with the small or the big nicotine dose. Data are represented as mean  $\pm$  S.D. (n=10),  $^a P \leq 0.001$ ,  $^b P \leq 0.01$ ,  $^c P \leq 0.05$  versus the control group,  $^* P \leq 0.001$  versus the small nicotine treated animals,  $^{\$} P \leq 0.001$  compared with high nicotine intoxicated group.

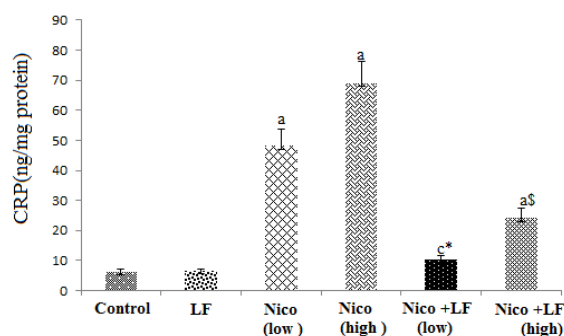


Fig 2: Effects of LF on cardiac CRP concentration in rats injected with nicotine. Data are expressed as mean  $\pm$  S.D. (n=10),  $^a P \leq 0.001$ ,  $^c P \leq 0.05$  with respect to the control group,  $^* P \leq 0.001$  versus the low nicotine dosage injected group,  $^{\$} P \leq 0.001$  versus the big nicotine dosage treated group.

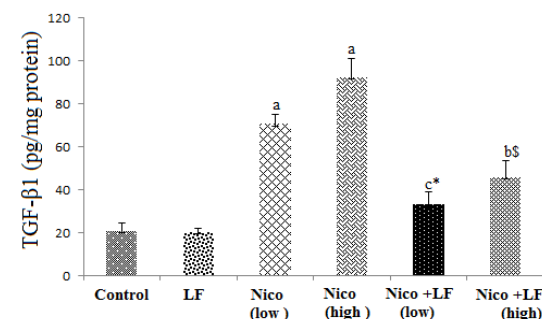


Fig 3: Effects of LF on cardiac TGF- $\beta$ 1 level in rats intoxicated with the nicotine. Results are represented as mean  $\pm$  S.D. (n=10),  $^a P \leq 0.001$ ,  $^b P \leq 0.01$ ,  $^c P \leq 0.05$  with respect to control rats,  $^* P \leq 0.001$  versus the low nicotine injected group,  $^{\$} P \leq 0.001$  versus the high nicotine injected group.

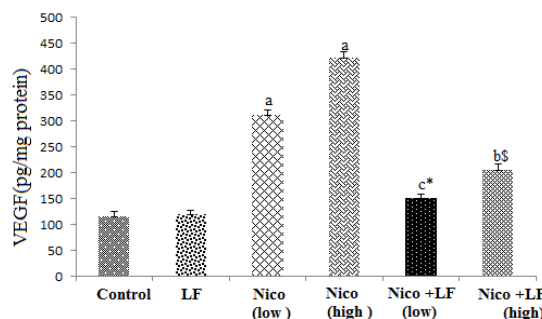


Fig 4: Effects of LF on cardiac VEGF level in rats treated with nicotine. Data are represented as mean  $\pm$  S.D. (n=10),  $^a P \leq 0.001$ ,  $^b P \leq 0.01$ ,  $^c P \leq 0.05$  versus the control animals,  $^* P \leq 0.001$  versus with low nicotine dosage treated group,  $^{\$} P \leq 0.001$  with respect to the high nicotine dosage exposed group.

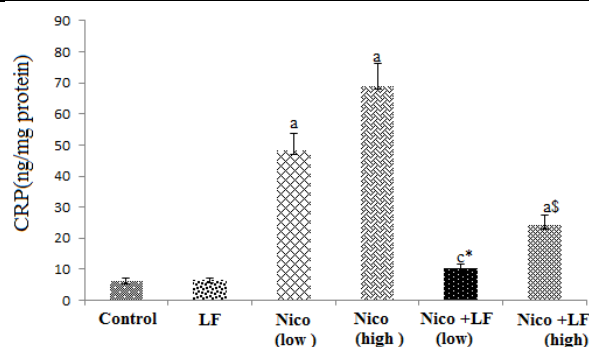


Fig 2: Effects of LF on cardiac CRP concentration in rats injected with nicotine. Data are expressed as mean  $\pm$  S.D. (n=10), <sup>a</sup>  $P \leq 0.001$ , <sup>c</sup>  $P \leq 0.05$  with respect to the control group, <sup>\*</sup>  $P \leq 0.001$  versus the low nicotine dosage injected group, <sup>§</sup>  $P \leq 0.001$  versus the big nicotine dosage treated group.

**Table (1):** Serum cardiac damage biomarkers in normal and nicotine – treated different groups

Parameters	Control	LF	Low nicotine	High nicotine	Low nicotine + LF	High nicotine +LF
CPK U/L	123.99 $\pm$ 4.05	125.18 $\pm$ 5.7	234.1 $\pm$ 5.4 <sup>a</sup>	505.12 $\pm$ 26.9 <sup>a</sup>	147.7 $\pm$ 8.6 <sup>c*</sup>	188.2 $\pm$ 7.5 <sup>b§</sup>
LDH U/L	202.06 $\pm$ 7.3	197.53 $\pm$ 7.9	309.22 $\pm$ 10.4 <sup>a</sup>	400.6 $\pm$ 12.78 <sup>a</sup>	220.7 $\pm$ 8.6 <sup>c*</sup>	255.7 $\pm$ 10.7 <sup>b§</sup>
ALP U/L	42.33 $\pm$ 3.05	37.3 $\pm$ 2.52	151.0 $\pm$ 4.5 <sup>a</sup>	204.66 $\pm$ 5.5 <sup>a</sup>	58.45 $\pm$ 3.6 <sup>c*</sup>	110.5 $\pm$ 10.45 <sup>a§</sup>

Data are represented as mean  $\pm$  S.D. (n=10), <sup>a</sup>  $P \leq 0.001$ , <sup>b</sup>  $P \leq 0.01$ , <sup>c</sup>  $P \leq 0.05$  versus the control group, <sup>\*</sup>  $P \leq 0.001$  versus the low nicotine injected group, <sup>§</sup>  $P \leq 0.001$  versus the high nicotine injected group.

## DISCUSSION

Cigarette smoking have been contributed to the increased risk of cardiovascular disease which is characterized by strong inflammation via activation of inflammatory signaling [10].

Study was proposed to prospect the beneficial cardioprotective impacts of bovine lactoferrin (LF) against inflammatory signaling caused myocardial damage in rats under the effect of nicotine toxicity

The values demonstrated that injection of small or big dosage of nicotine to rats caused increases in the inflammatory molecules (IL-6 and CRP) in the cardiac muscles of intoxicated rats, in relation to control group. This alteration was pronounced in rats intoxicated with nicotine big dose compared with ones injected with the low dose. These results are documented by some studies have documented that nicotine overuse can promote an inflammatory reaction by eliciting the generation of inflammatory proteins, including IL-6 and CRP [25-27]. Also, a study showed that treatment with nicotine cause an elevation in IL-6 mRNA expression, suggesting that nicotine may influence the immunological and acute-phase responses, causing the over-generation of IL-6 [28]. Also, clinical investigation has proved that increases in CRP concentration and inflammatory cytokines are contributed to the exposure to nicotine [29-30]. C-reactive protein is an acute phase protein, produced under the effect of inflammation [31]. Its high concentration in cardiac muscles may predict coronary heart disease [32]. CRP may have a role in the genesis of atherosclerotic lesion, since it reduces the expression of nitric oxide (NO) synthase and prostacyclin synthase, and binds LDL-C and promotes its uptake by macrophages, a key immune cells in atherogenesis. CRP also up-regulates the expression of adhesion molecules on endothelial cell. All these events are

contributed to atherogenic lesion [33]. In addition, it has been established that increased CRP level is related to the increased coronary cardiac disease, cardiac infarction, stroke and sudden cardiac muscle death [34].

IL-6, as an Inflammatory cytokine, is also has a fundamental pathogenic role in the development of cardiac diseases [35]. IL-6 plays an atherogenic impact via promoting the secretion of different molecules which cause proliferation and immigration of vascular smooth muscle cells (VSMCs), increasing production of adhesion molecules of endothelial-cell, inducing the secretion of different chemokines to activate macrophages at the damaged area and promoting their adhesion to endothelial cells [36-37]. An increase of IL-6 concentration in blood circulation has been contributed to the poor prognosis in subject with myocardial infarction [38]. Evidence showed that thrombosis caused by smoking is the major risk of myocardial infarction and sudden death [39].

Injection of LF to rats intoxicated with either nicotine dose, markedly reduced the elevation in both IL-6 and CRP with respect to rats intoxicated with nicotine counterpart group, indicating its anti-inflammatory potential action. LF was effective in reducing the levels of these markers in low nicotine intoxicated rats compared with intoxicated ones with high dose. The result of the current study is confirmed by previous investigation illustrated that LF can inhibit the production of inflammatory cytokines, including IL-6 in human mononuclear cells in vitro [40].

TGF- $\beta$ 1 is another cytokine produced in rat cardiac muscles in response to nicotine toxicity. The up-regulation of this cytokine in cardiac muscles in rats subjected to nicotine toxicity may consider one of the indicators of cardiac muscle damage. Experimental investigation on TGF- $\beta$ 1 signaling pathway has documented the important key roles of TGF- $\beta$ 1 in the incidence of cardiac fibrosis and cardiovascular



diseases (CVD) [41]. This cytokine is the most isoform found in the cardiac muscles [42]. Previous researches have demonstrated that stimulation of TGF- $\beta$ 1 is closely contributed to incidence of cardiovascular disorders, including hypertension [43], cardiac hypertrophy [44] cardiac fibrosis [45] and atherosclerosis [46], leading to cardiac failure. It has been reported that TGF- $\beta$ 1 promotes the generation of extracellular matrix (ECM) proteins and stimulates the formation of fatty streak lesion [47-48]. In addition, it has been reported that TGF- $\beta$ 1 reduces collagenase production, inhibits the degradation of ECM, triggers VSMCs to generate collagen, causing excessive ECM accumulation [49]. Over-production of TGF- $\beta$ 1 in transgenic animals causes interstitial cardiac fibrosis and cardiomyocytes hypertrophy [50]. Therefore, downregulation of the TGF- $\beta$ 1 production may consider as a potent therapeutic strategy to treat cardiac fibrotic damage. Administration of LF to rats injected with the small or the big nicotine dosage, significantly reduced the increase in TGF- $\beta$ 1 in rat cardiac muscles compared with rats intoxicated with nicotine counterpart group, indicating its anti-fibrogenic beneficial impact. The protective role of LF against cystic fibrosis caused bronchial cell damage was previously documented [51].

The present study illustrated a marked elevation in the VEGF in the cardiac muscles of animals subjected to nicotine toxicity. Our result is copped with Parikh and Pollak [52] who documented the angiogenic potential impact of nicotine which may relate to cardiac damage. Some studies revealed that the production of different inflammatory proteins such as cytokines and chemokines, induces the expression of VEGF by different immune inflammatory cells [53]. Previous investigation stated that overexpression of VEGF can induce the expression of tissue factor on blood vessel endothelial cells [54]. Production of tissue factor is thought to have a key role in many organ dysfunctions in acute damage [54]. This may suggest that VEGF overexpression cause cardiac muscle damage or/and systemic organ dysfunction. Co-injection of nicotine treated rats with LF, significantly depleted the increase in VEGF in cardiac muscles of rats compared with rats intoxicated with nicotine counterpart group. This result may give a clue to the anti-angiogenic impact 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 (55).

Concerning with the effect of nicotine toxicity on the level of cardiac transcription factor, NF- $\kappa$ B, in rats, the result illustrated that a pronounced elevation in this factor in the cardiac muscles of animals exposed to either of the two dosages of nicotine. This effect was severe in rats intoxicated with nicotine high dose compared with ones injected with the low dose. The production of NF- $\kappa$ B in response to nicotine exposure was confirmed [27] whose over production eventually contribute to tissue injuries and damage [56].

NF- $\kappa$ B is an important transcription factor that participates in the cellular signalling pathway for inflammation in various pathological conditions [57]. It has been found a strong relation between NF- $\kappa$ B signalling mechanism and cardiac failure [58]. Activation of NF- $\kappa$ B pathway contributed to the development of vascular inflammation and

initiation and progression of atherosclerotic lesion formation [59]. NF- $\kappa$ B activates several inflammatory genes resulting in cellular damage [57]. At resting normal state, NF- $\kappa$ B is inactive state by its binding with its specific inhibitor (I $\kappa$ B) in the cell cytosol. However, production of inflammatory cytokines activate the NF- $\kappa$ B signalling mechanism [60]. The activated NF- $\kappa$ B enters the nucleus and stimulates the transcription of many genes such as IL-6, TGF- $\beta$ 1 and VEGF [61]. These proteins, in turn, trigger cascade of 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 cardiac damage [62, 58]. Co administration of LF to rats intoxicated with the small or the big nicotine dosage, effectively attenuated the deviation in NF- $\kappa$ B, in heart muscles of intoxicated rats, compared with intoxicated counterpart group. Similarly, some authors have been reported that prophylactic treatment of rats with LF markedly corrected the increase in the level of NF- $\kappa$ B in nephrotoxicity induced rats [21]. The current result may suggest that the suppressing effects of LF on the expression of proinflammatory mediators (IL-6 and CRP), fibrogenic factor (TGF- $\beta$ 1) and angiogenic factor (VEGF) were through its inhibitory effect on NF- $\kappa$ B signaling mechanisms.

The current study revealed that the levels of serum CPK, LDH and ALP were significantly increased in rats intoxicated with both nicotine doses in relation to control rats, indicating that the nicotine toxicity caused cardiac muscle damage. These biomarkers have prognostic value in predicting cardiovascular ailment, such as injury, or myocardial infarction [63-64]. Co injection of LF to rat groups injected with either nicotine dose, markedly ameliorated the cardiac function markers, implying its potential cardioprotective impact.

## CONCLUSION

The present investigation demonstrated that activation of the transcription factor (NF- $\kappa$ B) may has the major role in the expression of the inflammatory mediators (IL-6 and CRP), the fibrogenic cytokine (TGF- $\beta$ 1) and the angiogenic factor (VEGF), which are collectively involved in cardiac muscle damage caused by nicotine toxicity. Prophylactic treatment with bovine LF could protect against nicotine-induced cardiac dysfunction in rats. The potent protective effect of LF may correlate to its anti-inflammatory, and anti-fibrotic and antiangiogenic properties with down-modulation of NF- $\kappa$ B.

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