Gene expression of renal lactoferrin and glycemic homeostasis in diabetic rats with reference to the protective role of exogenous bovine lactoferrin

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Abstract

Background: This study is intended to clarify the influence of exogenous bovine lactoferrin (bLf) treatment on glycemic homeostasis, gene expression, and production of lactoferrin (Lf) in rats with experimentally induced diabetes.

Methodology: Fifty adult male rats were used; 15 rats were used as the normal control rats (group A) and were injected an intraperitoneal (I/P) with 1 ml of isotonic saline daily for 3 months. Thirty-five rats were administered I/P injection of 60 mg of streptozotocin per kilogram body weight to induce diabetes. Thirty streptozotocin-treated rats were selected and used as diabetic rats, and they were subdivided into 2 equal groups (15 rats each): the untreated diabetic group (group B) and treated diabetic group (group C). The rats in the diabetic group were administered an I/P injection of 1 ml of isotonic saline daily for 3 months, while rats in the diabetic treatment group were administered an I/P injection of 300 mg of bLf/kg body weight/day/3 months. At the end of the experiment, blood and renal tissue samples were collected from all rats. The levels of insulin, glucose, and lactoferrin in plasma were measured. Homeostatic model assessment for insulin resistance (HOMA-IR) was performed. The numbers of Lf-mRNA copies in renal tissue were assessed using quantitative RT-PCR. To measure Lf production in renal tissue, Western blot analysis was applied.

Results: The obtained data demonstrated that the treatment of diabetic rats with bLf maintained glycemic homeostasis at normal levels but increased the mRNA expression of renal Lf.

Conclusion: In diabetic rats, bovine lactoferrin treatment offers the potential for protection against incidences of insulin resistance (IR) by stimulating the suppressed expression and production of the *LTF* gene.

Keywords: Diabetes mellitus, Lactoferrin, Glycemic homeostasis, Gene expression, RT-PCR, Western blot analysis

Background

Lactoferrin (Lf) is a conjugated nonbinding protein in the transferrin family with pleiotropic biological and medical actions, including antineoplastic, immunomodulatory, and antimicrobial properties. A number of these properties do not seem to have connections with its capability for iron binding (Eizirik, Colli, & Ortis, 2009; Metz-Boutique et al., 1984). Lactoferrin includes two equal sections, the N-lobe and C-lobe, each comprising a single iron-active site.

While the N-lobe is believed to increase antimicrobial activities, the C-lobe undertakes many therapeutic activities that are still being investigated. It has previously been recognized that the C-lobe could potentially be used to treat diabetes, but the full elucidation of its exact function has not been undertaken (Sharma, Sinha,

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Kaushik, Kaur, & Singh, 2013). The C-lobe modulates inflammatory and immune responses through the down-regulation of numerous cytokines, interleukin 6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor A (Machnicki, Zimecki, & Zagulski, 1993; Pammi & Abrams, 2019). On the basis of its expression pattern and thus its biological properties, lactoferrin is one of the components of the innate immune system and works in the first line of immune defense (Brock, 1995).

The role of Lf in combating diabetes has been demonstrated empirically through the clinical outcomes of improved glycolytic equilibrium and dyslipidemia profiles, and anti-inflammatory effects. A number of experimental and clinical studies have reported these results for Lf (Agrawal, Tantia, Jain, Agrawal, & Agrawal, 2013). Hypolipidemic, hypoglycemic, and other anti-inflammatory outcomes undergo modulation through the TLR-4/NF-κB/SIRT-1 axis, a key signaling pathway that is well known to be an activator of inflammation transcription regulators (Fuentes-Antrás, Ioan, Tueen, Egido, & Lorenzo, 2014).

Teng (2006) reported that when nuclear receptors and transcription factors regulate gene expression, the joint action of multiple proteins is involved. Transcriptional activation takes place due to coregulation recruitment, binding with the response element of the promoter through nuclear receptors or transcription elements, and modification of chromatin granules. Although additional data regarding the general role and molecular mechanisms of Lf in gene regulation have recently been reported, there is very little available information regarding lactoferrin gene regulation in this field.

Although therapies for type II diabetes mellitus (T2DM) are available, there are still challenges regarding fluctuations in glucose levels and insulin resistance (Golden & Sapir, 2012). As the aims of new therapies are to improve insulin resistance and mitigate both the short- and long-term effects of T2DM, this research is intended to examine the influence of exogenous bovine lactoferrin administration to rats with experimentally induced diabetes in terms of renal *LTF* gene expression and glycemic homeostasis.

Materials and methods
Chemicals
Chemicals were purchased from Sigma-Aldrich. Immunoblotting antibodies (anti-rat Lf, anti-β-actin, and horseradish peroxidase-conjugated secondary antibodies) were purchased from Biotechnology, Sigma, and Jackson Laboratory (USA), respectively. Additionally, proteinase inhibitor cocktails were obtained from Sigma-Aldrich (USA), and nitrocellulose membrane pore size 0.2 μm (PS 0.2 μm) were supplied by Thomas Scientific (USA). The Pierce BCA kit was purchased from Thermo Fisher Scientific, Inc. (Rockford, USA).

Animals
This study was undertaken at the Department of Biology, University College, Al-Jumum, Umm Al-Qura University, Makkah, Saudi Arabia, between June and August 2019, using 50 male Sprague-Dawley rats with an average weight of 200 g. These animals were kept at regulated temperatures of 24 ± 1 °C on a 12-hour day/night cycle (light between 7 a.m. and 7 p.m.). They had unlimited water and standard food with a calorific value of 3.45 kcal/g; the food comprised 51% nitrogen, 6.6% ash, 4.5% fiber, 4.6% fat, and 24.9% protein. Fifteen rats were used as controls. Diabetes mellitus (DM) was induced in the other 35 rats through intraperitoneal (IP) injections of streptozotocin at 60 mg/kg of body weight. Blood samples were tested for hyperglycemia (> 300 mg/dL) to verify the presence of diabetes 48 h after injection. Thirty rats with glucose levels higher than 300 mg/dL were regarded as diabetic. The analysis procedures employed by this research were approved by the ethical committee of the institution.

Experimental groups
The rats in this experiment were split into the following three groups: group A, controls (n = 15) orally administered 1 ml of isotonic saline each day for 3 months; group B, diabetic rats (n = 15) orally administered 1 ml of isotonic saline each day for 3 months; and group C, diabetic rats (n = 15) orally administered 300 mg of bovine lactoferrin per kilogram of body weight (Radiance Nutritional Company, New Zealand) daily for 3 months (Hegazy, Salama, Mansour, & Hassan, 2016).

Sampling and measurements
Determination of plasma glucose, insulin, and lactoferrin levels
At the end of the experimental period (3 months), morning fasting plasma samples were collected from each group of rats. Glucose analysis was undertaken using the glucose oxidase method (Trindler, 1969). Insulin levels were assessed using ultrasensitive insulin ELISA to quantify the concentration of insulin protein in plasma samples (ALPCO, USA). Insulin resistance (IR) was determined through the HOMA test by using the following equation: insulin (fasting level in μU/mL) × glucose (fasting level in mmol/L)/22.5 (Antuna-Puente et al., 2011). For each group, the plasma Lf concentration was assayed by employing an enzyme-linked immunosorbent serologic assay kit (Assaypro EL2011-1, St. Charles, USA). A monoclonal

antibody specific for rat Lf was precoated on a microplate. The intra- and interassay coefficients of variation were 4.8% and 7.3%, respectively. The cross-reactivity was less than 1%.

Renal tissue homogenate

Directly after blood sampling, the rats were anesthetized with ether and killed by cervical dislocation. The kidneys of all rats were immediately removed and washed with PBS to remove excess blood. Weighed samples were collected from all the kidneys and homogenized (Omni (TH) homogenizer, MSC, Ireland) in PBS to provide 20% homogenate that was stored at − 70 °C until needed for the extraction of RNA.

Quantitative reverse transcription-PCR (RT-qPCR) assay

The numbers of messenger RNA (mRNA) copies of lactoferrin *LTF* gene (ACCE: KT006756) was derived through quantitative reverse transcription (RT-PCR) in RNA concentrates of renal tissue homogenates from each rat group. For RNA purification, we used the RNeasy Mini Kit (QIAGEN, Germany) in accordance with manufacturer protocols. A detection system (The StepOnePlus™ Real-Time PCR, Thermo Fisher, USA) and a specific diagnostic kit (SNP, i-DNA Biotechnology Pte Ltd., Singapore) were employed to perform RT-PCR assays for the determination of the specific quantities of rat Lf, in accordance with manufacturer instructions. Real-time complementary DNA (cDNA) amplification was performed in duplicate using the fluorescent dye SYBR Green. Target *LTF* gene amplification was normalized with the expression levels of ATP synthase 5 subunit O (ATP5O), which has no associations with the test variables under consideration in this research. The sequences of the primers used in RT-qPCR were as follows: for rat Lf, forward primer 5′-GAACCGTACT TCAGCTACTCTG-3′ and reverse primer 5′-CTCATA CTCGTCCCTTTCAGC-3′; for ATP5O, forward primer 5′-GCGATGCTTCAGTACCTCTG-3′ and reverse primer 5′-TGGCATAGCGACCTTCAATA-3′.

Western blot analysis

Renal tissue samples were minced on ice with scissors and then homogenized using the pestle of a glass Teflon homogenizer in 20 mL of RIPA lysis buffer (150 mM NaCl, 25 mM Tris-HCl [pH 7.4], 0.1% SDS, 40% Nonidet P, and 1 mM EDTA) that contained 1% (w/v) proteinase inhibitor cocktail (104 mM aprotinin, 80 mM AEBSF, 3.6 mM betain, 2.1 mM leupeptin, 1.4 mM E-64, and 1.5 mM pepstatin A). The tissue homogenates were then sonicated using a Sonic Dismembrator FB505 (Fisher, New Boston, USA) set at 15 for a minimum of three 5-s bursts prior to centrifugation for 40 min at 14,000 rpm and 48° (Beckman Coulter Microfuge R,

Schaumburg Il). The supernatants were removed and stored at − 70 °C until required. A Pierce BCA kit was used to assess the total protein.

Protein extracts (55 μg) were separated by SDS-PAGE (10%) and then electroblotted into a 100% Triton-free nitrocellulose membrane (PS 0.2 μm). Anti-rat Lf antibody (1:2000, 78 kDa band), anti-β-actin antibody (1: 40000, 42 kDa band), and horseradish peroxidase-conjugated secondary antibodies (1/10000) were employed for immunoblotting. To confirm equal loading, β-actin protein expression was quantified. Five micrograms of purified rat lactoferrin was used as a standard. Equal loading was confirmed through determination of β-actin protein expression.

Statistical analysis

For quantitative data, the data are represented as the mean ± SE. One-way analysis of variance (ANOVA) was employed to assess variations between groups, with a p value of 0.05 or lower being regarded as having statistical significance. The statistics were analyzed using SPSS version 14.0 for Windows.

Results

Plasma glucose, insulin, and lactoferrin levels and HOMA-IR index

The mean values of lactoferrin, HOMA-IR index, insulin, and glucose in all three test groups of rats are shown in Table 1. The analysis of the data showed that there is a significant decrease in plasma glucose levels ($p \leq 0.05$) in treated diabetic rats compared to untreated diabetic rats; however, the plasma glucose level of treated diabetic rats was significantly greater than that of the control rats. There was a demonstrable decrease in insulin levels in both treated and nontreated rats with diabetes compared to the control rats ($p \leq 0.05$). The lactoferrin levels were significantly lowered in untreated diabetic rats and significantly increased in treated diabetic rats ($p \leq 0.05$). HOMA-IR showed a significant increase in untreated

Table 1 Plasma levels of glucose, insulin, and lactoferrin and the HOMA-IR index in the renal tissue of control rats, untreated diabetic rats, and diabetic rats treated with Lf (300 mg/kg) for 3 months

Parameters	Control	Diabetic	Diabetic + lactoferrin
Glucose (mmol/L)	5.22 ± 0.80	24.25 ± 1.20[a]	8.35 ± 0.90[a]
Insulin (μU/mL)	20.40 ± 2.17	7.75 ± 0.15[a]	15.50 ± 1.60[a]
Lactoferrin (ng/mL)	155.45 ± 6.50	38.23 ± 2.40[a]	195.15 ± 11.20[a]
HOMA-IR index	4.73 ± 0.90	8.35 ± 0.12[a]	5.75 ± 0.85

Values are mean ± SE
[a]Significantly different than the corresponding control value ($p \leq 0.05$)

diabetic rats ($p \leq 0.05$), with no significant difference between treated diabetic rats and the controls.

RT-PCR quantification of lactoferrin mRNA and Western blot analysis
Figures 1 and 2 present the outcomes of Western blot analysis and quantitative RT-qPCR analysis for the control, untreated diabetic, and treated diabetic rats. The data show the gene expression of Lf in the diabetic rats, and the results indicate that the mRNA and protein expression of Lf was significantly decreased in untreated diabetic rats compared to control rats ($p \leq 0.05$). Compared with both untreated diabetic rats and control rats, the diabetic rats treated with Lf showed significant increases in the expression level of Lf protein and qPCR products ($p \leq 0.05$).

Discussion
This research demonstrates counteractive increases in blood Lf alongside improvements in glycemic parameters in diabetic rats, as shown in Table 1. The researchers attempted to detail the association between exogenous lactoferrin administration and glucose homeostasis parameters, including insulin and fasting glucose levels, in diabetic rats. Additionally, the chances of insulin resistance were calculated via the HOMA-IR index.

Insulin levels were significantly increased, and blood glucose was decreased (but remained greater than the control levels) by lactoferrin administration; the fact that the HOMA-IR index remained normal excluded the influence of IR. It was demonstrated that lactoferrin causes hypoglycemic effects through both an insulin-like influence and insulin-sensitizing properties (Gupta, Kono, & Evans-Molina, 2010). Compared to the control condition, exogenous Lf treatment significantly increased insulin levels in blood. Research has shown that diet is a

significant factor that influences insulin signaling and similar functions, including decreased oxidative stress, increased glucose disappearance rates, and increased insulin receptor expression in mature adipocytes (Moreno-Navarrete et al., 2013).

Furthermore, it has been demonstrated that Lf combats diabetes via anti-inflammatory actions, ameliorating dyslipidemia, and maintaining glucose homeostasis. Numerous experimental and clinical studies have found a similar antidiabetic influence (Agrawal et al., 2013), but the precise pathway has never been fully explained. The hypoglycemic and concomitant anti-inflammatory effects are modulated by the TLR-4/NF-κB/SIRT-1 axis, a key signaling pathway that activates the transcription of inflammatory regulators (Fuentes-Antrás et al., 2014; Mohamed & Schaalan, 2018). Furthermore, cell-based research showed that Lf significantly decreases the levels of intracellular reactive oxygen species (ROS) in a dose-dependent manner and protected against oxidative stress (El-Desouky, Osman, Shams Eldin, & Emaraa, 2017).
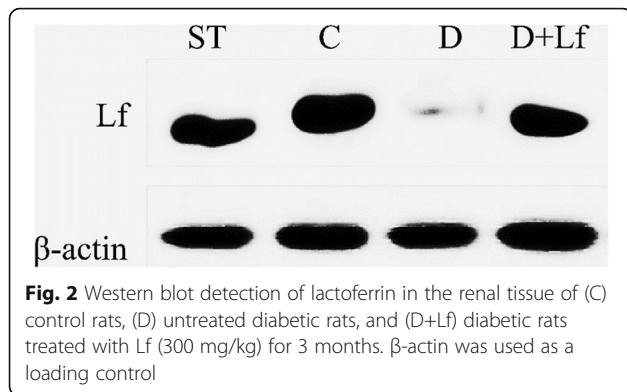
RT-PCR quantification of lactoferrin mRNA and Western blot analysis
The findings demonstrated a significant increase in the protein and mRNA levels of Lf in the renal tissue of rats treated with bLf compared to that of untreated diabetic rats and control rats. Recent reports suggested that Lf can be transported through partial membranes into certain cell nuclei and that it can bind to specific sequences of DNA, causing transcriptional activation (He & Furmanski, 1995). It has been suggested that Lf is able to bind to DNA (Garre, Bianchi-Scarra, Sirito, & Ravazzolo 1992) and that it is capable of mitigating oxidative stress (El-Desouky et al., 2017).

Lf cloning has been accomplished with a number of types of livestock, and the Lf cDNA and genes in mice
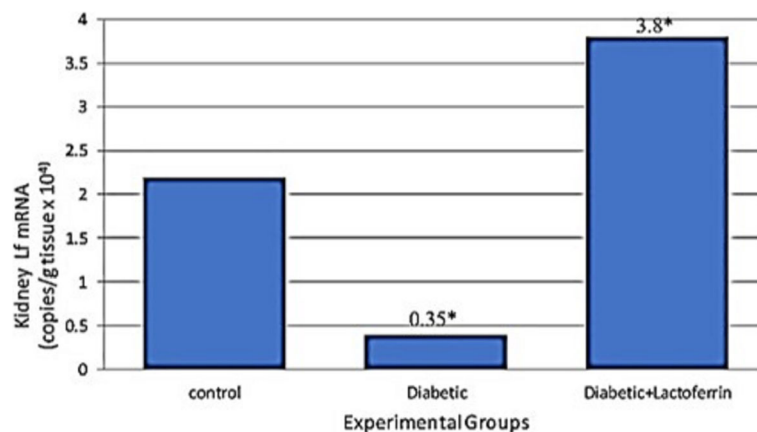
Fig. 1 Copies of Lf mRNA in the renal tissue of control rats, untreated diabetic rats, and diabetic rats treated with Lf (300 mg/kg) for 3 months. Asterisk indicates the significant variation from the control group at $p < 0.05$

Fig. 2 Western blot detection of lactoferrin in the renal tissue of (C) control rats, (D) untreated diabetic rats, and (D+Lf) diabetic rats treated with Lf (300 mg/kg) for 3 months. β-actin was used as a loading control

have been isolated and characterized in the laboratory (Cunningham, Headon, & Conneely, 1992). Furthermore, reports have suggested a number of regions of the lactoferrin gene and protein products have high sequence homology among a variety of species (Teng, 2002); polyclonal rabbit antiserum developed to target mouse or human lactoferrin may undergo cross-reactions with lactoferrin if introduced to samples from other types of rodents.

Recent research has demonstrated that the administration of lactoferrin to mice causes autopotentiated increases in the expression of the *LTF* gene. Decreases in lactoferrin mRNA levels associated with diabetes were also found in the adipose tissues of mice (Moreno-Navarrete et al., 2013); these results support the findings recorded in the current study.

It has been reported that Lf gene expression is inducible and constitutive and is specific to certain species, tissues, and cell types (Zheng, Ather, Sonstegard, & Kerr, 2005). The regulation of *LTF* gene expression is complicated, and this regulatory process responds to diet, cell developmental stage, pathogen attack, and hormone levels (Li, Limmon, Imani, & Teng, 2009). Notably, acute kidney injuries are linked to variations in the renal expression of the *LTF* gene (Famulski, Reeve, & de Freitas, 2013).

In the current research, decreased lactoferrin gene expression found in diabetic rats may be attributable to the excess production of inflammatory cytokines associated with diabetic hyperglycemia, e.g., TNF-α and IL-6, which can induce the production of C-reactive protein (CRP), an inflammatory protein, that causes dysfunction in renal endothelial cells (Ramasamy, Yan, & Schmidt, 2012). A significant amount of research has demonstrated that in diabetic rats, treatment with Lf significantly downregulated the increases in serum TNF-α and IL-6 cytokines (Zimecki, Dawiskiba, Zawirska, Krawczyk, & Kruzel, 2003). If this is the case, this offers an explanation for the stimulating effect of exogenous bLf treatment on *LTF* gene expression.

Conclusion

In diabetic rats, treatment with bLf allowed for the maintenance of normal glucose homeostasis, which reduced the incidence of IR. Increased Lf levels were shown to be associated with increases in renal Lf expression and protein production; this effect may have been caused by a decrease in the expression of inflammatory cytokines, although this requires further research. The outcomes demonstrated that Lf treatment has the potential to protect against the suppressed *LTF* gene expression and increased IR in DM.

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