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Renoprotective effect of camel milk in pediatric diabetic ketoacidosis: A focus on TLR-4/MAPK axis ☆

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1. Introduction

The incidence of diabetes mellitus and its complications is increasing worldwide affecting both adults and children. Hyperglycemic crises, namely diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic state are among acute diabetic complications that most commonly occur even before the first diagnosis of type I diabetes mellitus (T1DM) at young age. DKA often presents as a triad of uncontrolled hyperglycemia, metabolic acidosis and elevated levels of ketone bodies. It has recently been shown that the incidence of DKA varies between 14.7% (Denmark) to 79.8% (Saudi Arabia) [1]. Due to severe clinical and biological impairments, added to the treatment associated complications (cerebral edema, acute respiratory distress syndrome, hypokalaemia, and hypophosphatemia), DKA is being seen as a life-threatening condition [2]. It causes an inflammatory state with acute systemic elevations in activated lymphocytes, and cytokines among which are IL-1β, IL-6, IL-8 and IL-10 [3].

Among the factors that play a role in the pathogenesis of nephrotoxicity specifically acute kidney injury (AKI) is the inflammatory process. In this pathological process, the released macrophages are induced by the inflammatory milieu, reflected by the flux of pro-inflammatory cytokines [4]. Such cytokines include tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). Both oxidative stress and elevated levels of pro-inflammatory cytokines have been shown to participate in the development of nephrotoxicity [5].

Toll-like receptors (TLRs) are a class of pattern recognition receptors of the innate immune system. Activation of these receptors is capable of stimulating an inflammatory response. Up to the current moment, TLRs are classified into 13 subtypes. Among these subtypes, TLR-4 has been shown to be responsible for initiating a pro-inflammatory cascade. It has also been reported that chronic inflammation caused by activation of TLR-4 is associated with the progression of diabetic nephropathy. Signaling of the TLR-4 results in translocation of nuclear factor kappa B (NF-κB) into the nucleus, that in turn initiates the production of a number of pro-inflammatory cytokines among which are IL-1β, IL-6 as well as TNF-α, through activating a number of pro-inflammatory genes [19]. Moreover, TLR-4 has previously been reported to activate p38MAPK pathway, further mediating the NF-κB hyperinflammatory responses [6–8].

Circulating endotoxins, known with their capabilities of activating TLRs' responses, have been reported to be elevated in type 1 diabetes mellitus [9]. Previous studies have shown that elevated levels of hemoglobin A1c (HbA1c) is associated with increased TLR-4 expression in monocytes. Such a finding

Abbreviations: DKA, Diabetic ketoacidosis; T1DM, Type I diabetes mellitus; AKI, Acute kidney injury; TNF-α, Tumor necrosis factor-α; IL-1β, Interleukin-1β; IL-6, Interleukin-6; TLR, Toll-like receptors; NF-κB, Nuclear factor kappa B; HbA1-c, Hemoglobin A1c; KIM-1, Kidney injury molecule-1; CM, Camel milk; SOD, Superoxide dismutase; ANOVA, Analysis of variance

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suggests there is a link between inflammation and metabolic control of the metabolic syndrome-associated disorders. Such metabolic disorders represented by and not limited to hyperglycemia and dyslipidemia, have been depicted to predispose to diabetic nephropathy in patients with diabetes mellitus [10,11].

Among the potential early biomarkers of AKI are lipocalin-2, and kidney injury molecule-1 (KIM-1). Early detection of kidney impairment, before the increase of serum creatinine, is important for timely initiated therapy and recovery from AKI [12]. Lipocalin-2 is a member of the lipocalin family largely expressed in activated neutrophils and tubular epithelial cells in response to inflammation. It plays an important role in innate immunity. Accumulating evidence also has established both urinary and plasma lipocalin-2 levels as predictive biomarkers for AKI, and their outcomes in varied groups of patients including pediatrics and adults [13]. On the other hand, serum KIM-1 has recently been demonstrated to represent a potentially useful sensitive and specific diagnostic and prognostic marker for kidney injury, and its level was reported to be significantly increased in both AKI and chronic kidney diseases [14].

In the Middle East, camel milk (CM) has been consumed as an essential nutritional supplement with high energy and immunoglobulins content to help immune-deficient patients [15]. It has been regarded as superior to other types of milk since it is well tolerated. Moreover, it has several bioactive components with immunomodulatory properties among which are lactoperoxidase, and lysozyme [16,17]. Having lower content of fat, cholesterol and lactose along with higher minerals and vitamins as vitamin A, B2, C and E, actually characterizes CM from other ruminants' milk [18]. Moreover, CM is rich in secretory IgA and IgM, and contains several nanoantibodies with marked antibacterial and antiviral activities. Interestingly, CM is rich in lactoferrin, a protein with marked antioxidant and anti-inflammatory properties, in addition to **an insulin-like protein**, that is mostly responsible for its antidiabetic effect [19]. Previous reports have confirmed that CM exhibits protective and alleviating anti-inflammatory, antioxidant, and hypoglycemic properties against diabetes, diabetic nephropathy and alcohol-induced hepatic injury [20–22]. However, studies addressing its effect on DKA and its related complications have not been addressed and remain scarce. To this end, our study aims at investigating the potential alleviating effects of CM on DKA-induced inflammation, and further investigates its renoprotective activity in children with DKA by assessing the early injury kidney markers KIM-1 and lipocalin-2, together with an attempt to find out its possible underlying mechanism.

2. Methods

2.1. Study design and subjects

A randomized control trial that included 60 newly diagnosed children with T1DM and DKA (mean age; 8.78 ± 2.59 years) who were randomly recruited from the Diabetic Endocrine Metabolic Pediatric Unit, Abo El-Reesh Children Hospital, Cairo University, Cairo, Egypt. Recruitment started in September 2017 through February 2018. Children were equally randomized among two parallel groups using a computerized random number generator. Steps for random allocation sequence generation, enrolling the participants and assigning the participants to intervention were independently performed by three researchers. Patients with any critical complications like hypoglycemia, cardiovascular event, renal or acute infection were excluded from the study. Sample size was calculated based on fasting blood glucose levels. This number was increased so as to compensate for anticipated dropouts or possible differences in CM effect size due to different camel species. The control group consisted of 30 age and sex-matched healthy individuals recruited from outpatient clinic. Parents gave written informed consent and the study followed the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the Faculty of Medicine, Cairo University. Sample size was estimated based on an expected change in fasting blood glucose by about 15 mg/dL between CM plus insulin group versus insulin only group [23]. Accordingly, number of subjects per groups was calculated to be 21, at $\alpha = 0.05$ and a power of 80%.

The criteria for the diagnosis of T1DM with DKA are hyperglycaemia with blood glucose levels exceeding 11 mmol/L, heavy glycosuria (>55 mmol/L), ketonuria, metabolic acidosis (pH < 7.30) with a bicarbonate level of < 15 mmol/L and the presence of > 5% dehydration. The patients had no evidence of acute pancreatitis or acute/ chronic infection according to history and physical examination. Children diagnosed with DKA have been managed in the Pediatric Department under the care of the same pediatric diabetologist according to the protocol [24]. CM was collected from lactating healthy camels (*Camelus dromedarius*) without clinical manifestations of mastitis. The milk was collected into clean containers, pasteurized and refrigerated till distributed. Children have then been randomly divided among two age and sex matched groups so as to either receive CM plus insulin, or insulin alone. Insulin requirements were adjusted on weekly basis based on blood glucose levels. After a few weeks from starting the trial, the insulin requirement by the CM plus insulin children was almost lowered to its half (morning dose was reduced from 20 U to 10 U and the evening dosing from 30 U to 15 U).

2.2. Blood sampling

Fasting venous blood samples were collected (≈2 ml/subject) at base line and three months after consumption of raw CM on daily basis in addition to insulin, or alternatively insulin alone. The collected blood samples were divided into two portions; the first was used for serum separation, while the second was immediately transferred into heparinized tubes for isolation of peripheral blood mononuclear cells (PBMCs) for RNA extraction. Sera were prepared by centrifugation at 3500 × g for 10 min, and then stored at −70 °C for further use. At the time of analysis, serum samples were used to assess the levels of glucose, IL-1β, IL-6, IL-18, TLR-4, lipocalin-2 and KIM-1, as well as, superoxide dismutase (SOD).

2.3. Determination of serum parameters

For measuring FBG, a colorimetric enzymatic method with commercially available local kits (Cairo, Egypt) was used. IL-1β was measured using commercial IL-1β ELISA, while IL-6 was assessed using IL-6 ELISA kit, both of which are products of Anogen-YES Biotech Laboratories Ltd, Ontario, Canada. Assay range was 2–400 pg/mL for IL-1β and 7–2000 pg/mL for IL-6. IL-18 was measured using a commercial ELISA kit (CUSA-BIO Company, China) with detection range 31.25 – 2000 pg/mL. Lipocalin-2/NGAL Human ELISA kit (BioVendor Research and Diagnostic Products, Czech Republic) was used for the determination of serum lipocalin-2 level. Its limit of detection was 0.02 ng/mL. KIM-1 was analyzed by KIM-1 ELISA kit (Kamiya Biomedical Company, Seattle, WA, USA). Finally, human SOD was measured using commercial ELISA kit (Kamiya biomedical company, Seattle, USA).

2.4. Quantitative real time PCR

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood samples, and used for total RNA extraction with TRIzol reagent (Invitrogen, CA, USA) following the standard manufacturer's protocol. RNA purification was done by DNase1 (Amplification grade; Invitrogen, CA, USA) treatment. RNA was quantified on a Picodrop spectrophotometer (Picodrop, Hinxton, UK) at 260/280 nm wavelength. cDNA synthesis was carried out starting with 250 ng total RNA using random hexamer primers with high capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) as per manufacturer's instructions. qPCR was performed in the presence of SYBR Green fluorescent dye using Step One Real time PCR system (Applied Biosystems, CA, USA). Primers' sequences are presented in Table 1. The relative expression of target genes was obtained using the $\Delta\Delta C_T$ method as described previously by Livak and Schmittgen (2001) using beta-actin as a housekeeping gene [25].

2.5. Statistical analysis

All analyses were performed by original assigned groups. All results were expressed as means ± SD. Differences between groups were assessed by one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc multiple comparison test. Independent *t*-test was used to compare study parameters between the control group and DKA groups upon admission. The association between the parameters was determined using the Pearson's correlation coefficient. Categorical variables (gender) were analyzed using Chi-Square test. A P-value less than or equal to 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism Version 5 (GraphPad software Inc., CA, USA).

Table 1 – Primers sequences for real time qPCR.

Gene	5′-3′ Sequence
TLR-4	Forward AGGCAGCCATAACTTCTCCA
	Reverse GGTTGAGTAGGGGCATTTGA
NF-kB	Forward ACAACCCCTTCCAAGTTCCCT
	Reverse TGGTCCCGTGAAATACACCT
MAPK	Forward 5′-ATTGATTGAGCCGGGCGTGGTG-3′
	Reverse 5′-CACAAGGTAGAGATTACGGCCAC-3′
Beta-actin	Forward: CCT TCC TGG GCA TGG AGT CCT G
	Reverse: GGA GCA ATG ATC TTG ATC TTC

3. Results

The demographic data of the patients are illustrated in Table 2. The three groups were age, BMI and gender matched. Children with DKA have been admitted to the hospital with a significant reduction in pH relative to the control group (6.97 ± 0.94 versus 7.39 ± 0.043, respectively), and a concomitant elevation in lactate level (6.73 ± 1.24 versus 1.19 ± 0.31 mmol/L, respectively).

Diabetic ketoacidosis is a potentially life threatening condition, that resulted in a significant increase in the level of pro-inflammatory cytokines among which are IL-1β , IL-6 and IL-18 by about 6.4, 4.8 and 6.3 folds, respectively relative to the control group as shown in Table 3. Moreover, the DKA groups has shown a significantly higher level of early kidney damage biomarkers lipocalin-2 and KIM-1 by about 6–6.5 folds compared to the control, with a concomitant significant reduction in SOD level exceeding 50%, respective to the control group. This increase was significantly evident even after receiving insulin therapy throughout the study duration as depicted in Fig. 1.

Administration of CM concomitantly with insulin was able to significantly decrease the levels of the inflammatory mediators relative to the insulin only group by 31.5% for IL-1β, 42.5% for IL-6 and 25.7% for IL-18. However, it should be noted that despite the CM's ability to significantly decrease the level of these pro-inflammatory cytokines relative to the insulin group, their levels were still significantly higher than those of the control group at $P \leq 0.05$ as shown in Table 3. Moreover, CM was able to induce a significant reduction in the level of TLR-4 protein as well as its mRNA expression level. The reduction in the TLR-4 protein level approached almost 50%, and that of the mRNA level was about 83.5% lower than that of the insulin group as demonstrated in Table 3 and Fig. 2, respectively. CM together with insulin was able to abolish any significant difference in the mRNA levels of NF-kB as well as MAPK relative to the control as depicted in Fig. 2. Meanwhile, the levels of these two parameters were significantly higher in the insulin only group relative to the CM + insulin group (8.3 and 6.35 folds, respectively) as shown in Fig. 2.

Camel milk was able to significantly improve the level of SOD relative to the insulin group by 17%, however, it was not able to restore it back to normal. That is because the SOD level in the CM + insulin group was still significantly lower than the control group by about 31%.

Finally, it was demonstrated that the group of patients receiving CM in addition to insulin has significantly lower levels of both kidney injury markers lipocalin-2 and KIM-1 as compared to the group receiving insulin only by 16% and 14%, respectively. Once more, CM was not able to restore the level of these two biomarkers back to normal despite being able to significantly reduce them as demonstrated in Table 3.

Table 2 – Demographic data of enrolled patients.

Parameter/Group	Control pediatrics	Pediatrics on CM + insulin	Pediatrics on insulin only	P-value
Age (years) ± SD	9.83 ± 2.82	8.30 ± 2.93	9.27 ± 2.13	0.082
BMI (Kg/m^2) ± SD	17.12 ± 2.79	18.26 ± 2.86	18.17 ± 2.60	0.199
Gender (male/female)	19/11	16/14	17/13	0.727

Table 3 – The changes in lactate levels, inflammatory indicators (IL-1β, IL-6, IL-18), TLR-4 and SOD, in the investigated groups by the end of the trial.

Parameter	Control pediatrics	Pediatrics on CM + insulin	Pediatrics on insulin only
Lactate (mmol/L) ± SD	1.190 ± 0.31	1.487 * ± 0.19	2.690 * Ψ ± 0.38
IL-1β (pg/mL) ± SD	3.403 ± 0.88	11.78 * ± 1.60	17.21 * Ψ ± 1.77
IL-6 (pg/mL) ± SD	4.980 ± 1.07	11.38 * ± 1.34	19.78 * Ψ ± 1.85
IL-18 (pg/mL) ± SD	15.46 ± 2.87	57.16 * ± 12.04	76.91 * Ψ ± 6.24
TLR-4 (ng/mL) ± SD	1.320 ± 0.18	1.537 * ± 0.17	3.033 * Ψ ± 0.30
SOD (U/mL) ± SD	222.5 ± 14.90	153.1 * ± 11.94	130.8 * Ψ ± 8.07

Values are means ± SD.
* Significant difference from control group at $P \leq 0.05$.
Ψ significant difference from CM/insulin group at $P \leq 0.05$.

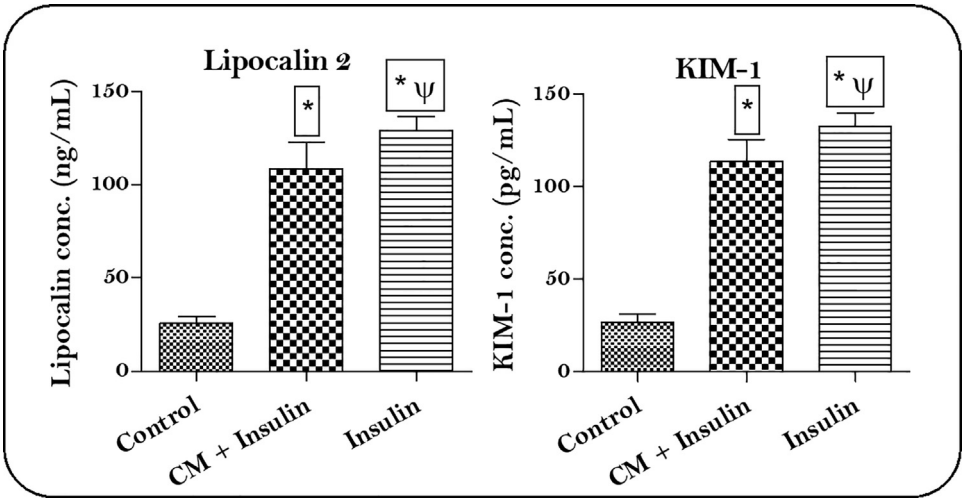

Fig. 1 – Protein levels of lipocalin-2 and KIM-1 in the three study groups as assessed by ELISA. * *Significant difference from control group at P ≤ 0.05. Ψ significant difference from CM/insulin group at P ≤ 0.05.*

The correlation analysis, illustrated in Table 4, revealed significant associations between serum lipocalin-2 and KIM-1 on one hand and the assessed inflammatory mediators (IL-1β, IL-6 and IL_18), as well as TLR-4 protein level on the other. Moreover, there was a significant positive correlation between both lipocalin-2 and KIM-1 and the expression levels of TLR-4, MAPK and NF-kB mRNA. Meanwhile, there was a significant negative correlation between the assessed early kidney injury biomarkers and SOD level.

4. Discussion

The findings of the current study highlight the ameliorative impact of CM on DKA-induced inflammatory responses and oxidative stress, which rationalize the renoprotective effect in pediatrics, by down-regulating TLR-4/MAPK/NF-κB signaling pathway. To the best of the authors' knowledge, this is the first study to report the potential renoprotective effects of CM in pediatric DKA.

Diabetic ketoacidosis is a severe complication of diabetes, and is the leading cause of hospitalization, morbidity, and mortality in children with type 1 diabetes [26]. AKI which implies a sudden worsening of the kidney's ability to function represents a common event among children [27]. Pediatrics with type 1 diabetes are always at a higher risk of DKA, as the glycemic control is this population is difficult to achieve [28].

Previous reports indicate that hyperglycemia and ketoacidosis independently induce changes in pro-inflammatory cytokines, as well as oxidative stress [29,30]. This rationalizes

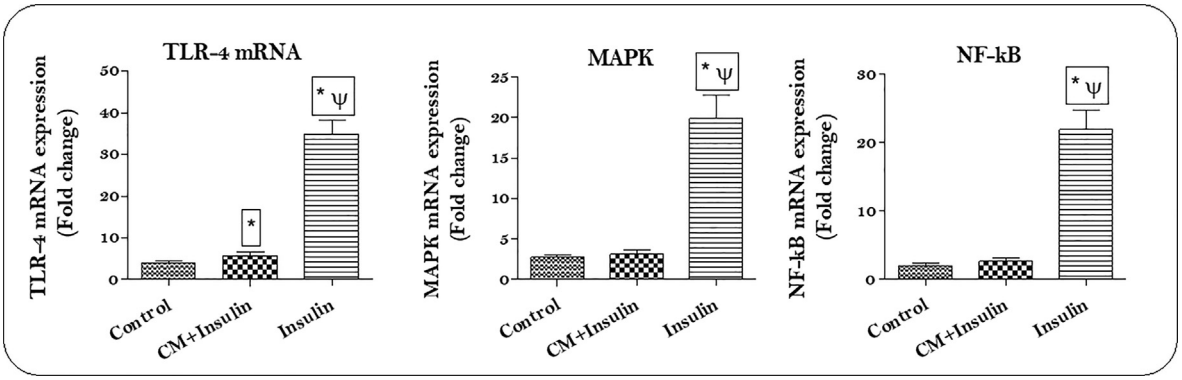

Fig. 2 – mRNA expression level of TLR-4, NF-kB and MAPK in the three study groups as assessed by quantitative real time PCR expressed as fold change. * *Significant difference from control group at P ≤ 0.05. Ψ significant difference from CM/insulin group at P ≤ 0.05.*

Table 4 – Correlational analysis between serum lipocalin-2 and KIM-1 on one hand and the assessed parameters on the other.

Parameter	Lipocalin-2	KIM-1
IL-1β	*r* = 0.923 P < 0.001	*r* = 0.9312 P < 0.001
IL-6	*r* = 0.881 P < 0.001	*r* = 0.874 P < 0.001
IL-18	*r* = 0.929 P < 0.001	*r* = 0.946 P < 0.001
TLR-4	*r* = 0.698 P < 0.001	*r* = 0.696 P < 0.001
TLR-4 mRNA	*r* = 0.674 P < 0.001	*r* = 0.669 P < 0.001
MAPK	*r* = 0.643 P < 0.001	*r* = 0.637 P < 0.001
NF-kB	*r* = 0.652 P < 0.001	*r* = 0.646 P < 0.001
SOD	*r* = -0.934 P < 0.001	*r* = -0.938 P < 0.001

Pearson's correlation analysis, where P-values ≤ 0.05 are statistically significant.

why patients with hyperglycemic crises have been shown to have decreased levels of SOD, and significantly elevated levels of pro-inflammatory cytokines among which are IL-1β, TNF-α and IL-6 [31]. These findings coincide with ours where children admitted with DKA (assessed in terms of lactate and pH) had significantly elevated levels of the pro-inflammatory cytokines IL-1 β, IL-6 and IL-18, as well as lipocalin-2 and KIM-1, in addition to a significantly decreased level of SOD as compared to healthy children.

Both inflammation and oxidative stress are reported to be contributing factors for AKI and are associated with higher levels of renal tubular injury biomarkers [32]. More importantly, it has recently been reported that 64% of pediatric patients admitted to hospitals for DKA met the criteria for AKI, 65% of which have severe AKI (stage 2 or 3), suggesting that a large proportion of those children may experience intrinsic tubular injury [27]. In alignment with these studies, we were able to demonstrate that children presenting with DKA have significantly elevated levels of renal tubular injury biomarkers lipocalin-2 and KIM-1. These biomarkers were shown to be beneficial for identifying exposures that lead to early kidney injury and the increased risk of developing CKD [33].

Camel milk, previously reported to have anti-inflammatory, antioxidative and immunomodulatory effects [34], was able to provide a significant renoprotective effect when used in combination with insulin compared to insulin alone. This was evident by the significantly lower levels of lipocalin-2 and KIM-1, two of the best-studied biomarkers for AKI, in the CM treated group [33]. Camel whey proteins are some of the very few ingredients shown to modulate different immune cell functions, cytokine secretion, phagocytic activity and oxidative stress [35,36]. However, it should be noted that despite the significant ability of CM to reduce the levels of the aforementioned biomarkers, yet, it was not able to restore their levels back to normal.

Investigating the mechanism behind the renoprotective effects of CM, we were able to demonstrate that these effects have probably been modulated through inhibiting the TLR-4/MAPK/NF-κB signaling pathway, an important signaling pathway known to activate transcription regulators of inflammation [37] that are closely associated with renal tissue damage [38].

Toll-like receptors previously reported to have a positive correlation with interstitial macrophage infiltration and HbA1c levels, are known to represent basic elements in the innate immune system involved in the progression of the pro-inflammatory signaling pathways. Moreover, TLRs overexpression is evident in the human diabetic kidney. High glucose is reported to upregulate TLR-4 mRNA level and to activate NF-κB translocation in tubule cells. In addition, increased expression of TLR-4 in intrinsic renal cells and leukocytes has been implicated in various acute and chronic kidney diseases [10]. Thus, inhibition of TLR-4 could be a potential therapeutic target for kidney injury in diabetes and DKA through suppressing renal inflammation, presumably through inhibiting the NF-κB signaling pathway, a corner stone of inflammation in diabetic nephropathy [10].

The current study shows that CM decreased DKA-induced inflammation by inhibiting the TLR-4/MAPK/NF-κB axis with

subsequent reduction in serum pro-inflammatory cytokines; IL-1, IL-6 and IL-18 in children previously exposed to DKA. These inhibitions were significantly greater than those shown in children receiving insulin only. Moreover, the reduction in pro-inflammatory cytokines was accompanied by a significant reduction in biomarkers of tubular kidney injury, KIM-1 and lipocalin-2, relative to the insulin treated group as well. This was further supported through the significant positive correlation demonstrated hereby between the levels of lipocalin-2 and KIM-1 on one hand and the investigated pro-inflammatory cytokines as well as TLR-4 protein level on the other. These results are in accord with a previous study reporting that pharmacological inhibition of TLR-4 conferred renoprotection in diabetic mice [10].

The reduction in TLR-4 level, in the current study, could be attributed to the fact that CM has anti-inflammatory as well as anti-oxidative properties [39,40], which was evident by the restoration of SOD levels towards normal values, with a statistically significant difference from the insulin group. Yet, it should be noted that SOD level in the CM + insulin group was still significantly higher relative to the control group. This could be interpreted in light of the fact that CM has a relatively high amount of the potent antioxidant vitamin C in addition to high amounts of antioxidant aminoacids [41]. The modulation of the TLR-4 pathway and the pro-inflammatory cascade, via inhibition of reactive oxygen species generation and through the direct use of anti-inflammatory agents, is previously reported [42,43]. The anti-inflammatory properties of CM are mainly attributed to the presence of lactoferrin, where previous studies have depicted that camel lactoferrin has immunomodulatory effects that are capable of reducing pro-inflammatory cytokines levels [44]. Similarly, and at the cellular level, camel lactoferrin was shown to modulate differentiation, activation, migration, proliferation and functions of immune cells using the signaling pathways, NF-κB and MAPK [40,45].

Though insulin is reported to have anti-oxidative, and anti-inflammatory effects in a number of metabolic diseases [46], the current work emphasizes the beneficial effects of introducing CM concomitantly with insulin to children with diabetes previously exposed to DKA, and its ability to better improve the assessed markers. The beneficial effects of the oxidative properties of CM on the level of the kidney injury biomarkers is further evidenced by the significant negative correlation between SOD level on one hand and serum lipocalin-2 and KIM-1 on the other.

5. Conclusions

In conclusion, CM ameliorates DKA-induced inflammatory responses and oxidative stress, and down-regulates TLR-4/MAPK/NF-κB signaling pathway in pediatrics. The use of CM in combination with insulin may be considered for protection against renal injury in pediatric population. Moreover, our results points out that TLR-4 may be a promising therapeutic target for AKI in pediatric DKA. At present, several TLR-4 antagonists have been developed, and their potential application in the treatment of inflammation is under investigation [47]. Whether these compounds can also exert an effective control over AKI in DKA warrants further studies. However, it should be noted that all our subjects were recruited from a single hospital in Cairo, which limits the generalizability of the study results. Hence, we recommend for the commencement of further trials with more heterogeneous subject recruitment sites so as to support the study results and help generalize our findings.

6. Declarations

6.1. *Ethics approval and consent to participate*

All subjects gave informed written consent via their parents (parental consent). The study followed the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the faculty of Medicine, Cairo University.

6.2. *Consent for publication*

Not applicable.

6.3. *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

6.4. *Competing interests*

The authors declare that they have no competing interests

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6.6. *Authors' contributions*

WM and MS contributed equally in the study design and conception. WM contributed in the acquisition of data. HM contributed in the study design, analysis and interpretation of data as well as in writing the manuscript. MS did a critical revision of the final version of the manuscript. All authors read and approved the final manuscript.

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