A Study on Therapeutic Use of Camel Milk with Metformin on Glycemic Level and Oxidative Stress in Alloxan® Induced Hyperglycemia in Rabbit Model

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INTRODUCTION

Diabetes mellitus characterized by hyperglycemia, is reckoned as the fastest growing global health disorder of 21st century. According to a surveillance report in Pakistan 26.7% of adults have diabetes thus making the estimated number of 33,000,000 cases living with diabetes (Azeem et al., 2022). Type 1 Diabetes (DMT-1) or insulin-dependent diabetes causes impairment of carbohydrate, protein, and lipid metabolism (Ozougwa et al., 2013). Moreover, it is assumed that oxidative stress associated production of oxygen reactive species exceeding the existing antioxidant defense system leads to metabolic disorder (Obi et al., 2016).

Currently, insulin used as a permanent monotherapy and its long-term use triggers array of hypoglycemia-associated consequences that might be confirmed as detrimental for the cardiovascular system. Thus, metformin (dimethyl biguanide), an oral antihyper-glycemic agent used as first-line treatment in insulin resistant diabetes gained the attention of the researchers and proved to be a useful adjuvant in DMT1 management (Triggle et al., 2022). It provides additional antioxidant protection by restoring the pancreas from oxidative stress-induced impairment in hyperglycemia (Obi et al., 2016). Pharmacokinetin studies suggest that metformin metabolic rate is non-significant and therefore eliminated intact in the urine. Since nephropathy is a common complication in hyperglycemic patients, understandably, long-term use of metformin leads to high plasma concentrations and metformin-associated lactic acidosis. Apart from its undesirable effects, clinically it is indicated in DMT1...
patients to benefit them by reducing their daily insulin dose requirements (Driver et al., 2018).

Therefore, it is imperative to investigate alternative remedies that justify scientific merit in alleviating symptoms of hyperglycemia. Camel milk is considered as an essential source of nutrients and bioactive constituents and thus provides nutrition and several health benefits to human beings (Redha et al., 2022). Camel milk became apparent as a therapeutic alternative in the management of DMT1 because it contains proteins that have many characteristics like pancreas secreted insulin. Camel milk protects the viability of its vital components by preventing coagulum formation in the acidic gastric environment and ensure rapid absorption via the intestine. Moreover, it has the potential to mimic the effects of indigenous pancreas-secreted insulin in terms of inhibition of gluconeogenesis in liver. Therefore, a study was planned to investigate the use of camel milk as a single therapeutic approach or as an adjuvant with metformin in alleviating glycaemia and oxidative stress in alloxan monohydrate induced hyperglycemia in the rabbit model.

MATERIALS AND METHODS

Experimental Design: Thirty-five female rabbits weighing 1400-1800g were purchased from local market. The rabbits were kept at the animal facility of the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad to acclimatize to the conventional environment. The trial was conducted by observing the guidelines provided by the Directorate of Graduate Studies and the Institutional Animal Ethical Committee. Fodder and water provided ad libitum. The basal diet provided to the study animals comprised of starch (65%), corn oil (10%), casein (15%), cellulose (5%) and vitamins (5%) (Peterson et al., 1971).

Alloxan® (Applichem, USA) 170mg/kg was injected intravenously to induce hyperglycemia in rabbits. The glycemic level was estimated with the help of a FreeStyle Optimum Neo Meter (Abbott Labs, Ltd). The animals having a glycemic level of >250mg/dL, were selected for the trial. The normal (negative control group) was injected with normal saline. The weight was recorded for each rabbit on weekly basis. The hyperglycemic animals were divided into four groups with seven animals in each group as following:

**Group 1:** Normal healthy animals (Negative control group).

**Group 2:** Hyperglycemic animals (Positive control group without treatment).

**Group 3:** Hyperglycemic animals were orally administered metformin (Glucophage®) @100mg/kg/day.

**Group 4:** Hyperglycemic animals were orally administered camel milk @40ml/kg/day.

**Group 5:** Hyperglycemic with combined oral treatment with camel milk @40ml/kg/day and metformin (Glucophage®) @100 mg/kg/day for sixty days.

Antihyperglycemic Agents: Fresh raw camel milk of Anmol Marecha Dodh® was collected in properly sterilized glass bottles and transferred to the animal house daily. The chemical analysis of camel milk constituents included water (86-88 %), fat (3.6-3.9 %), lactose (3.3-3.8%), protein (3.2-3.5%), Ash (0.7-1 %), zinc (0.51-0.56mg/100ml), calcium (110-114mg/100ml), sodium (0.3-0.55mg/100ml) and potassium (149-153mg/100m). Milk composition analysis was performed by National Institute of Food Science and Technology (NIFSAT), University of Agriculture Faisalabad and Punjab Livestock and Dairy Development Department, Lahore, Pakistan. Metformin (Glucophage®) 500 mg (Merck Marker Pvt. Ltd.) was purchased from local market.

**Sample collection:** Following completion of the study (60 days), blood was sampled from the jugular vein in anticoagulant (EDTA) coated vacutainers for hematological analysis. For serum collection, blood was collected in silica-coated gel containing vacutainers. Serum was harvested by centrifuging the blood samples at 3000 rpm for 10 minutes and stored at -20°C for analyses.

**Hematological analysis:** Hematological profile including total red cell count and white cell count, platelet count, hemoglobin (Hb) and packed cell volume (PCV) all were determined by using an automated hematology analyzer (Medonic, Sweden).

**Serum analysis:** Serum levels of glucose, aminotransferase (AST / ALT), urea, creatinine, uric acid, total protein, and albumin were estimated by following the protocol given by the manufacturers with commercially available kits (AST=Bioclin® Transaminase AST kinetic diagnostic kit; K048, Bioclin® Transaminase ALT kinetic diagnostic kit: K049, Bioclin® Total Protein Monoreagent Diagnostic Kit; K031, Bioclin®, Albumin Monoreagent Diagnostic Kit; K040, Bioclin® Uric acid Monoreagent Diagnostic Kit; K139, Bioclin® Creatinine Kinetic Diagnostic Kit: K067). Serum total oxidant status and total antioxidant capacity (TOS/TAC) were estimated by colorimetric method described by Erel (2005) after slight modifications (The oxidation reaction of the assay was enhanced, and precipitation of proteins was prevented).

**Serum insulin:** Serum insulin concentration was determined by ELISA (enzyme linked immunosorbent assay) using commercially available Elabscience® Rabbit INS (Insulin) ELISA® kit (Catalog No: E-EL-RB227496T).

**Collection of tissue samples:** All rabbits were euthanized after accomplishment of the study. Liver, kidneys, and pancreas were collected and immediately washed with 0.9% NaCl and fixed in 10% neutral buffered formalin. Tissue sections were prepared by paraffin embedding technique, sections of 5µm were taken and stained with Hematoxylin and Eosin (H&E). Slides were examined for observing degenerative changes (Suvarna et al., 2013).

**Statistical analysis:** Data were analyzed by one-way analysis of variance (ANOVA), followed by DMR test to estimate the statistical difference among the means of different treatment groups. Results were expressed as mean ± SE.
RESULTS

A significant decrease in bodyweight was recorded in all hyperglycemic rabbits when compared with normal healthy group. Weight gain was significant in G4 and G5 at the 8th week (P<0.05) as compared to G2 (Fig. 1).

Fasting glycemic level significantly increased (P<0.05) in G2. Significantly decreased fasting blood glycemic level was observed at (P<0.05) 4th week in G4 and G5 (Fig. 2). Significant increase in serum glycemic level was reported in G2 when compared with G1. A significant decrease in glycemic level was reported (P<0.05) in hyperglycemic rabbits.

Serum insulin level significantly decreased in the G2 (P<0.05) when compared with G1. Significant increase in insulin level (P<0.05) was observed in treated groups. Statistically insignificant difference in insulin level was recorded between G4 and G5 (Fig. 3). Serum GCK level was decreased at a significant level in G2. Serum GCK level increased significantly in G4 and G5 and restored serum level of GCK close to G1 (Fig. 4).

Mean (±SE) values of hematological parameters including RBC count, Hb conc, PCV, WBC count, and platelets count in all groups were recorded (Table 1). All the parameters were significantly decreased (P<0.05) as observed in the G2 but not in G1. A non-significant difference was recorded in the RBC count in G3 and G4. Insignificant difference was observed in Hb levels of G2 and G3. Significantly different values were recorded for PCV between G3 and G4. A significant difference in platelet count (P<0.05) was recorded in G2 and G5.

The levels of liver and kidney function biomarkers is presented in Table 1. Significantly increased values of kidney function biomarkers were recorded in G2 when compared with G1. In G3 and G4 ALT and AST values decreased significantly. A significant decrease in urea level (P<0.05) was observed in all treated groups.

Decreased level of total protein in hyperglycemic groups restored significantly. Total protein level significantly increased (P<0.05) in G3 and G4. Albumin level increased significantly in G3 and G4. A significant increase was observed in G5 as compared to G3 and G4 (Table 1).

Alloxan® induced hyperglycemia increased total oxidative stress in all groups as compared to G1 (Table 1). Total oxidative stress reduced at a significant level (P<0.05) in all treated groups.

Alloxan® induced hyperglycemia decreased TAC in all groups but not in normal healthy animals (Table 1). Total antioxidant capacity increased significantly in treated groups (Table 1).

Histological examination of liver in G2 revealed that central vein was encircled by bi-nucleated and disrupted hepatocytes (Fig. 5II). The liver section of G3 and G4 showed restoration in arrangement of hepatocytes (Fig. 5III&IV). The results revealed more significant improvement in G5 with restoration of histological structure close to normal tissue (Fig. 5V). Histological study of kidneys in G2 revealed tubular damage along with shrinkage of urinary space and infiltrated by inflammatory cells in glomeruli (Fig. 6. II). In G3 and G4 tubules and kidney corpuscles restored close to normal (Fig. 6. III&IV) but more significant nephron protective effect was
Fig. 5: I Histomicrograph of rabbit liver tissue section G1 showed the normal radiating pattern (H) around the central vein (CV) with normal sinusoidal cords (S). II Histomicrograph of rabbit liver tissue G2 showed congestion and disrupted radiating pattern of hepatocytes (H) and sinusoidal cords (S) around the central vein (CV) with binucleated hepatocytes (bn). III Histomicrograph of rabbit liver tissue G3 showed hepatocytes (H) structure exhibiting restoration of hepatic pattern, congestion and hemorrhages (HM). IV Histomicrograph of rabbit liver tissue section G4 showed restoration of normal hepatocytes (H) structure and radiating hepatic pattern around the central vein (CV) with least congestion and hemorrhages (HM). V Histomicrograph of rabbit liver tissue section G5 showed restoration of normal hepatocytes (H) structure and radiating hepatic pattern around the central vein (CV). (H&E 100X)

Fig. 6: I Histomicrograph of rabbit kidney tissue section G1 revealed normal tubular (T) structure with normal glomerulus (G). II Histomicrograph of rabbit kidney tissue section G2 revealed disrupted tubular pattern with infiltration of inflammatory cells in glomerulus (G). III Histomicrograph of rabbit kidney tissue section G3 revealed restoration of glomerulus (G) and renal tubules (T) and recovery of cellular structure. IV Histomicrograph of rabbit kidney tissue section G4 revealed restoration of disrupted renal tubules (T) and glomerulus (G). V Histomicrograph of rabbit kidney tissue section G5 revealed significant restoration of disrupted renal tubules (T) and glomerulus (G). (H&E 100X)

Fig. 7: I Histomicrograph of G1 rabbit pancreas tissue section revealed normal endocrine structure (b) with normal lobulation and exocrine section (ex). II Histomicrograph of G2 rabbit pancreas tissue section revealed degeneration of endocrine structure (b) and shrinkage of exocrine section (ex) and lobulated septa. III Histomicrograph of G3 rabbit pancreas tissue section revealed restoration of endocrine (b) and exocrine (ex) section of pancreas. IV Histomicrograph of G4 rabbit pancreas tissue section revealed restoration of disrupted endocrine (b) section. V Histomicrograph of G5 rabbit pancreas tissue section revealed significant restoration of endocrine (b) section (H&E 100X).
observed in G5 (Fig. 6V). Histological findings of pancreatic tissue in G2 showed damaged parenchyma and in G1 with normal histological structure (Fig. 7. I&II). Significant improvement was observed in pancreatic section of G4 and in G5 with restoration of small sized islets of Langerhans (Fig. 7V).

### DISCUSSION

In the current study, significant bodyweight loss was recorded in hyperglycemic rabbits but not in normal healthy rabbits. G4 and G5 have shown a significant increase in body weight (P<0.05) when compared with G2. Loss in body weight occurs due to uncontrolled diabetes mellitus. It may be a result of increased activity of lipolysis, glycogenolysis, and gluconeogenesis associated with loss of tissue protein that leads to muscle wasting and weight loss. The ability of camel milk to restore weight gain might be attributed to its hypoglycemic and hypolipidemic effects (Korish et al., 2020).

In the current study, glycemic level decreased significantly in all treated groups. These findings agree with studies reported in literature. A study by Sboui et al. (2010) reported a decrease in glycemic level after administration of camel milk at a dose rate of 500ml for thirty-five days to alloxan® monohydrate induced hyperglycemic dogs. A significant difference in the glycemic level of hyperglycemic dogs (10.88 ± 0.55 mmol/L) was recorded at the end of the experiment (5.77 ± 0.44 mmol/L). Since camel milk contains insulin-like nanoparticles in high concentration (52.03 U/I) that share features with human insulin. These nanoparticles are encapsulated in lipids and bypass the acidic gastric environment and rapidly absorb into the small intestine (Agrawal et al., 2004; Oselu et al., 2022).

A study by Moon et al. (2007) concluded that metformin works effectively in presence of insulin and restores insulin sensitivity in peripheral tissues. In the course of the study, it might be assumed that camel milk insulin like protein and metformin worked synergistically and significantly (P<0.05) reduced glycemic level in hyperglycemic rabbits.

In this study, camel milk and metformin significantly (P<0.05) improved glucokinase level in hyperglycemic animals. Glucokinase is a primary regulator of glucose metabolism. Its activity decreases under oxidative stress and its level has been restored due to suppression of oxidative stress after treatment with camel milk and metformin (Henquin, 2004). Insulin level was inversely proportional to serum glucose level in all the treated groups but not in G2. It is hypothesized that the presence of insulin mimicking nano-proteins in camel milk cause antihyperglycemic effect in the body (Hussain et al., 2020).

During the course of study treatment with camel milk ameliorated some disturbed hematological parameters in hyperglycemic rabbit model. Hyperglycemia associated anemia is correlated with increase in nonenzymatic glycosylation process in membrane of erythrocyte. Moreover, protein oxidation in the glycosylated membranes along with consistent hyperglycemia enhance production of lipid peroxides responsible for red blood cell lysis. The statistical decrease in the PCV in hyperglycemic rabbits and its normalizing with camel milk treatment strengthen its potential role in erythropoiesis in contrast to metformin. Results suggest that camel milk treatment elevated antioxidant levels and thus stabilized the integrity of red blood cell membrane. Antioxidants in camel milk down-regulate the non-enzymatic glycosylation and may prevent the breakdown of red blood cell membrane helpful in antagonizing the anemic effect of alloxan monohydrate (Erukainure et al., 2013). Metformin despite its antihyperglycemic and the antioxidant effect is correlated with vitamin B12 deficiency needed for effective erythropoiesis (Abdel-Moneim et al., 2019). Literature suggests that the defense mechanism of the body against various infections becomes naïve due to decrease in white blood cell activity attributed to hyperglycemic. In the present study, camel milk and metformin increased the lowered white cell count that might be responsible for stimulation of the body’s defense mechanism against infections in the hyperglycemic rabbits. Alloxan®-induced hyperglycemia increased platelet count in G2 while camel milk and metformin administration decreased platelet count close to normal reference value. Increase in platelet count was due to decrease in insulin’s anti-aggregating mechanism and the defect in endothelial production of prostaglandin (PGI2) and nitric oxide (NO) as stated by Vinik et al. (2001). Another presumption is the excessive deposition of the advanced glycosylation products that decrease fluidity of platelet membrane and lead to platelet hyperactivity. In the present study, glycemia and platelet count significantly decreased in treated groups.

### Table 1: Comparative values of hematological and serum biochemical parameters of control and treatment groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Negative control group (G1)</th>
<th>Positive control group (G2)</th>
<th>Metformin treated group (G3)</th>
<th>Camel milk treated group (G4)</th>
<th>Camel milk + metformin treated group (G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (x10^12)/µL</td>
<td>6.34±0.05</td>
<td>3.59±0.19</td>
<td>4.28±0.09</td>
<td>5.76±0.16</td>
<td>6.13±0.16</td>
</tr>
<tr>
<td>Ht% (g/dL)</td>
<td>12.48±0.29</td>
<td>8.87±0.23</td>
<td>9.10±0.09</td>
<td>11.12±0.19</td>
<td>10.24±0.21</td>
</tr>
<tr>
<td>PV (%)</td>
<td>34.92±0.57</td>
<td>22.85±1.22</td>
<td>23.28±0.88</td>
<td>34.28±0.68</td>
<td>37.14±1.05</td>
</tr>
<tr>
<td>WBC (x10^3/µL)</td>
<td>9.22±0.27</td>
<td>6.95±0.17</td>
<td>8.30±0.05</td>
<td>8.77±0.18</td>
<td>8.84±0.23</td>
</tr>
<tr>
<td>Platelet count (x10^3/µL)</td>
<td>299.28±4.56</td>
<td>324.71±0.56</td>
<td>297.00±4.77</td>
<td>302.85±1.31</td>
<td>285.85±2.54</td>
</tr>
<tr>
<td>Serum Glucose (mg/dL)</td>
<td>125.81±3.33</td>
<td>327.15±0.10</td>
<td>244.07±2.13</td>
<td>230.13±2.63</td>
<td>224.42±1.80</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>25.32±0.76</td>
<td>34.04±0.78</td>
<td>29.50±1.63</td>
<td>29.87±0.76</td>
<td>24.21±0.68</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>28.05±0.68</td>
<td>41.89±1.09</td>
<td>32.99±0.85</td>
<td>34.45±0.16</td>
<td>29.11±0.49</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>17.80±0.22</td>
<td>87.82±0.99</td>
<td>38.98±0.70</td>
<td>39.33±0.83</td>
<td>37.66±0.70</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>1.59±0.02</td>
<td>3.24±0.15</td>
<td>2.00±0.22</td>
<td>1.81±0.22</td>
<td>1.76±0.02</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.80±0.13</td>
<td>3.78±0.14</td>
<td>2.11±0.07</td>
<td>1.80±0.13</td>
<td>1.54±0.20</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>6.47±0.13</td>
<td>4.52±0.10</td>
<td>5.74±0.20</td>
<td>5.94±0.14</td>
<td>6.46±0.03</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.33±0.10</td>
<td>1.75±0.04</td>
<td>3.29±0.03</td>
<td>3.23±0.15</td>
<td>3.91±0.17</td>
</tr>
<tr>
<td>TAC (mmol/L)</td>
<td>1.63±0.04</td>
<td>0.50±0.18</td>
<td>1.49±0.07</td>
<td>1.57±0.08</td>
<td>2.29±0.13</td>
</tr>
<tr>
<td>TOS (µmol/L)</td>
<td>4.41±0.28</td>
<td>31.28±4.89</td>
<td>9.12±0.67</td>
<td>8.02±0.72</td>
<td>6.08±0.23</td>
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Values are expressed as mean ± SE; n=7 animals in each group; Different superscripts on means ± SE along the same row indicate P<0.05.
In the course of study, increase in level of liver aminotransferase in hyperglycemic rabbits were due to the cytotoxic effect of alloxan monohydrate manifested as leakage of these enzymes from liver tissues and migration to blood circulation (Shakeel et al., 2022). Camel milk reduced leakage of enzymes at a significant level and our findings agreed with Khan et al. (2013). Liver enzymes significantly decreased (P<0.05) in G5 due to quench in oxidative stress status.

In the course of the study, serum total protein along with albumin decreased significantly in hyperglycemic animals. These findings were in compliance with the statement that reduction in serum total proteins and albumin might be due to decrease in amino acids uptake, low concentration of a variety of essential amino acids, glycogenic amino acids conversion to carbon dioxide and water, reduced synthesis of proteins due to a decrease in the amount and availability of mRNA and also reduction in ribosomal protein synthesis on account of insulin deficiency (Helal et al., 2012). In the course of the study, G3, G4, G5 revealed significant increase in total proteins and albumin concentration. This improvement in protein profiles is consistent with increased serum insulin level responsible for amino acid transportation and incorporation into proteins (Godill et al., 2005). Serum urea and creatinine can be used for screening of glomerular filtration rate, as an index of kidney function. These kidney function markers increased significantly (P<0.05) in hyperglycemic animals. It happens due to failure of the body to excrete the excessive metabolic end products of proteins. Use of camel milk and metformin significantly decreased kidney function biomarkers and enhanced the impaired renal function in line with findings reported in literature (Khan et al., 2013; Sang et al., 2021).

In the course of the study total oxidative and total antioxidant capacity status showed a significant difference in hyperglycemic animals. Under hyperglycemic conditions, ROS increase in various tissues and is involved in the development of macrovascular complexities. Excessive generation of ROS lead to simultaneous lipid peroxidation and a massive rise in the concentration of calcium in cytosol resulting in abrupt damage of insulin producing pancreatic β-cells by increasing oxidative stress. Increase in oxidative stress status decreases level of enzymatic and nonenzymatic antioxidants like, catalase, glutathione, superoxide dismutase and certain water and fat soluble vitamins which subsequently impair the metabolism and enhance multisystem damage in the body. Total oxidative status and total antioxidant capacity showed inverse relationship in G4 and G5. Similar findings were reported by Ashraf et al. (2022).

In the course of study, the histological findings of selected organs were in consistent with studies indicating that the variations in the activities of the antioxidants are correlated with significant changes in the cellular structure of these organs (El-Said et al., 2010). Histopathological changes in alloxan monohydrate induced hyperglycemia in rabbits revealed, hepatocellular degeneration, vascular congestion, necrosis, sinusoidal dilatation and distorted cell boundaries as stated in literature (Usman et al., 2018). Use of camel milk restored histological structure close to normal with no enlargement and distortion of central and portal vein. Congested glomeruli with reduced periglomerular space and hypercellularity were observed in hyperglycemic animals in the current study. Degenerative changes in the convoluted tubules in the cortex were also observed. Similar findings were reported in literature (Mir et al., 2013; Chen et al., 2022). Treatment with camel milk and metformin reduced periglomerular space close to normal structure. Necrosis in endocrine section of pancreas after alloxan monohydrate induced diabetes and restoration of small sized islets of Langerhans were observed in treated groups. Alloxan™ induce lysis of pancreatic β cells by impairment of redox potential, increased production of ROS and impairment in cellular calcium homoeostasis (Hussain et al., 2020). Since camel milk is good source of minerals and vitamins therefore it has potential for preventing the tissue injuries associated with the toxic diabetogenic agents (Abdel-Mobdy et al., 2023). Camel milk due to its antioxidant potential might be able to restore β cell damage and revive the damaged metabolic pathways by neutralizing ROS and leading to the subsequent increase in insulin secretion.

Conclusions: Considering the research findings, it has been found that camel milk has both antihyperglycemic and antioxidantizing qualities that can help to reduce oxidative stress and impaired erythropoietic system in hyperglycemic rabbits. Moreover, use of camel milk as an adjuvant with metformin may prove a rationale complementary therapeutic approach in alleviation of hyperglycemia and insulin related complications in diabetes Type1 and warrants further longer duration investigations.

REFERENCES


