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SYNERGISTIC EFFECT OF METFORMIN AND GRADED DOSES OF ZINC ON ALLOXAN-INDUCED DIABETIC MALE WISTAR RATS

Nwaedozie, C. E.
Department of Biochemistry, Federal Polytechnic Oko, Anambra State, Nigeria.
Email: chiazo.nwaedozie@federalpolyoko.edu.ng
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ABSTRACT

Diabetes depletes zinc, a trace element vital for managing diabetes by improving insulin sensitivity, function, and antioxidant protection. This study investigated the synergistic effect of metformin and graded zinc doses on alloxan-induced diabetic male Wistar rats. Diabetes was induced with alloxan monohydrate (120 mg/kg). Rats were divided into six groups (n=10): Group A (normal control), B (diabetic, untreated), C (diabetic, treated with 100mg/kg metformin), and Groups D, E, & F (diabetic, treated with 100mg/kg metformin plus 100, 200, and 400mg/kg zinc, respectively). Groups D-F were pretreated for 14 days before diabetes induction, with treatments continuing for 14 days post-induction. A significant ($p<0.05$) decrease in blood glucose levels was observed across groups A-F (87.63, 325.73, 257.80, 213.18, 204.42, and 170.70 mg/dl, respectively). Treatment also reduced key liver and kidney serum biomarkers. Liver markers (direct bilirubin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, and alanine transaminase) and kidney markers (urea and creatinine) were significantly lowered in the metformin-zinc groups (D-F) compared to the diabetic controls. The study reveals that co-administration of metformin (100 mg/kg) and zinc (100-400 mg/kg) reduces blood glucose and protects the liver and kidney in alloxan-induced diabetes. The results demonstrate a dosage-dependent ameliorative effect of zinc in synergy with metformin.

Keywords: Alloxan-Induced Diabetes, Blood Glucose, Metformin, Synergistic Effect, Zinc

1.0. INTRODUCTION

Type 2 diabetes mellitus (T2DM), a prevalent chronic condition manifested by increased levels of blood sugar (hyperglycemia) affecting over 400 million people globally, is driven by genetic and environmental factors. It is characterized by a total or partial decrease of insulin synthesis or secretion by β-cells of the pancreas, in addition to its inability to produce typical physiological effects (Ke *et. al.,* 2022).

Diabetes is linked to long-term organ damage, dysfunction, and failure, particularly in the eyes, kidneys, nerves, heart, and blood vessels (American Diabetes Association, 2023). With age, obesity, and a lack of physical activity, the chance of acquiring type 2 diabetes rises. Diabetes is linked to an increased risk of morbidity and mortality due to its consequences, which are majorly caused by the accumulation of Advanced Glycation End-products (AGEs).

All living species require zinc, which is the second most prevalent trace element after iron (Maret, 2013). Zinc exists as a divalent cation (Zn^{2+}) and is not redox-active under physiological conditions, which explains why it is involved in a wide range of physiological functions (Maret, 2013).

Zinc is an essential trace element that functions as a co-factor in the formation, storage, and release of insulin by the pancreas (Farooq, 2019). It serves three key biological functions: catalysis, structural support, and regulatory control. It is a structural component of many proteins in cellular signaling pathways, including growth factors,

cytokines, receptors, enzymes, and transcription factors, and is required for their biological function (Chasapis *et al*., 2011). Both physiologically and in diabetes mellitus, zinc plays an important role in the manufacture and action of insulin. Insulin action and insulin receptor tyrosine kinase activity appear to be stimulated by zinc (Chabosseau and Rutter, 2016). Zinc has been proven in certain trials to help patients with diabetes control their blood sugar levels (glycemic control). It plays a vital role in insulin action, and zinc supplementation could theoretically reduce the onset of diabetes in persons with insulin resistance. Reduced levels of zinc can impair the pancreas's ability to synthesize and secrete insulin and diminish glucose uptake by peripheral cells, leading to insulin resistance, a hallmark of diabetes mellitus (Tamura, 2021).

Apart from streptozotocin, alloxan is one of the most common drugs used to induce diabetes mellitus (Lenzen, 2008). It destroys the pancreatic beta-cells. As a result, alloxan-induced diabetes is a useful model for studying insulin-dependent diabetes. Metformin is a standard antidiabetic drug that affects blood glucose levels through three mechanisms: reducing hepatic glucose production, restricting intestinal glucose absorption, and enhancing insulin action in peripheral tissues. It was also found to enhance insulin secretion from beta cells.

Metformin has proven to be safe and effective and is usually well tolerated. There are various brands of metformin and users of these drugs suffer from health-debilitating diabetic complications such as neuropathy, nephropathy, and retinopathy. Thus, this study aimed to use zinc as a micronutrient adjuvant to the first-generation drug (metformin) to determine the dose-dependent concentrations of the supplement for ameliorating diabetic effects. This study examined the potential synergistic effects of zinc and metformin supplementation on oxidative stress and glucose metabolism in male Wistar rats with alloxan-induced diabetes, potentially leading to improved clinical outcomes.

2.0. MATERIALS AND METHODS

2.1.1 Animals

A total of 73 healthy male wistar rats weighing 110–130g were used for the investigation. Until the day of sacrifice, the animals were housed, supervised, and cared for in the animal house. The animals were separated into six (6) groups with a total of ten (n=10). Sixty (60) rats were used for the main study while thirteen (13) rats were used for toxicity investigation.

2.1.2 Experimental design

The animals were split into six groups, each with ten animals.
Group A: Non-diabetic rats (Control)
Group B: Diabetic rats (Untreated)
Group C: Diabetic rats (100mg/kg metformin)
Group D: Diabetic rats (100mg/kg metformin and 100mg/kg zinc),
Group E: Diabetic rats (100mg/kg metformin and 200mg/kg zinc),
Group F: Diabetic rats (100mg/kg metformin and 400mg/kg zinc).
They were fed a regular Pellet diet with unlimited water and fasted for roughly 16 hours before the experiment. Water was withdrawn from the experiment while fasting was continued. Groups D-F were pretreated for two weeks prior to diabetes induction and then treated for two weeks following diabetes induction.

2.1.3 Preparation of drugs and zinc solutions

The drugs (alloxan and metformin) and zinc solutions were prepared by dissolving all solute in distilled water.

2.1.4 Induction of Experimental diabetes

The mice were fasted for 16 hours before being given an intraperitoneal dose of 120 mg/kg body weight of alloxan to induce diabetes (Olak-Białoń *et al.,* 2024). To address the immediate hypoglycemia that could arise after 2 hours, 10% glucose was given orally (Olak-Białoń *et al.,* 2024). To assess the onset of diabetes, fasting blood glucose was measured 48 hours after alloxanisation (Olak-Białoń *et al.,* 2024). The therapies were then given for two weeks after the diabetes was induced and the fasting blood sugar levels were measured. However, the rats were given zinc for two weeks prior to the induction of diabetes to see if it could increase the animals' immune and possibly postpone the onset of diabetes.

2.1.5 Measurement of weight

The weights of the rats were measured on weeks 0, 1, 2, 3 and 4 respectively using a weighing scale.

2.2 METHODOLOGY

2.2.1 Acute toxicity (LD50) study of Zinc

Ibrahim *et al.,* (2013) approach was utilized to calculate the LD50. The zinc solution was given orally to three groups of four rats at doses of 10mg/kg, 100mg/kg, and 1000mg/kg body weight, and the animals were watched for 24 hours. The zinc solution was given orally to three groups of one rat each in the second phase, at doses of 1600mg/kg, 2900mg/kg, and 5000mg/kg, respectively, and the rats were watched for 24 hours. The toxic dose was determined from the mortality rate after treatment, and the therapeutic dose was selected thereafter.

2.2.2 Determination of blood glucose levels

All blood samples were taken from the rats' tail veins at 0, 1, 3, 5, 7, and 10 day intervals. The glucose oxidase method of Beach and Turner (1958) was used to determine fasting blood glucose levels with a digital glucometer (Accu-Chek Advantage, Roche Diagnostics, Germany), and the results were expressed in mg/dL.

2.2.3. Assessment of liver function

2.2.3.1. Determination of Aspartate Aminotransferase (AST)

The method of Reitman and Frankel (1957) was used to assay for the activiy of aspartate aminotransferase. Test, Test Blank, Standard and Standard Blank each received 0.5ml of ALT and AST substrates respectively. This was incubated at 37°C for 5 minutes. Following that, 0.1ml of serum was added to Test, 0.1ml of pyruvate standard was added to Standard, and 0.1ml of distilled water was added to the test and standard blanks, respectively. They were combined and incubated for 30 minutes at 37°C. Following the incubation, 5.0mL of 2,4 dinitrophenylhydrazine (DNPH) was added to each tube, stirred, and incubated at room temperature for 20 minutes (25 °C). After zeroing the Hinotek Spectrophotometer Model no. S23A with the blank, 5.0mls of 0.4N NaOH were added to all of the tubes, and they were read at 546nm (Reitman and Frankel, 1957).

2.2.3.2. Determination of Alanine Aminotransferase (ALT)

For alanine aminotransferase, 0.1 mL of sample was pipetted into a test tube and 0.5 mL of solution R1 (buffer containing phosphate buffer, l-alanine and α-oxoglutarate) was added, mixed and incubated for 30min at 37°C. Then, 0.5 mL of solution R2 (2,4-dinitrophenylhydrazine) was added and mixed and then allowed to stand for 20 min at 20–25°C. Finally, 5.0 mL of NaOH was added, mixed and after 5 min the absorbance was read at wavelength of 546 nm using Hinotek Spectrophotometer Model no. S23A (Reitman and Frankel, 1957).

2.2.3.3. Determination of Alkaline Phosphatase (ALP)

Serum ALP activity was determined according to the standard method recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Schumann *et al.,* 2011). Alkaline buffer (1.0 mL) and phenyl phosphate substrate (1.0 mL) were added to the Test, Blank, and Standard test tubes. At 37°C, they were incubated for 3 minutes. After that, 0.1ml of serum, 0.1ml of phenol standard, and 0.1ml of distilled water were added to the test, standard, and blank, and incubated for another 15 minutes at 37°C. After incubation, 1.0ml of 0.5NNaOH, 1.0ml of 0.5NNaHCO3, 0.1ml of 4-amino antipyrine, and 0.1ml of potassium ferricyanide were added to each tube, mixed, and read immediately after zeroing the Hinotek Spectrophotometer Model no. S23A with a blank at 510nm wavelength.

2.2.3.4. Determination of Bilirubin

Procedure for Total Bilirubin (T Bil)

Total bilirubin was determined using the method of Jendrassik and Grof (1938).The sample blank and sample tubes were filled with 200µl of sulphanilic acid. The sample tube was then filled with 50µl of sodium nitrite. The caffeine was then added to both the sample blank and the sample tube, totaling 1000µl. In addition, 200µl of the sample was placed in both the sample blank and sample tubes. The tubes were combined and left to stand at 20–25°C for 10 minutes. The titrate was then added to both the sample blank and sample tubes, totaling 1000µl. After mixing the tubes and incubating them at 25°C for 5-30

minutes, the absorbance of the sample blank was measured at 578nm (Aja *et al.*, 2020).

2.2.3.5. Procedure for Direct Bilirubin (D BIL)

Direct bilirubin was determined using the method of Jendrassik and Grof (1938). Three test tubes were labeled Test (T), Blank (B) and Standard (S). The reaction mixtures were prepared by pipetting 0.1ml of serum and 0.5ml of Diazoreagent in test tube (T), 0.1 serum and 0.5ml of distilled water in test tube (B) and 1.0ml of distilled water and 0.5ml of Diazoreagent in test tube(S). The tubes were thoroughly mixed and left to stand at room temperature (20-25°C) for exactly 10 minutes. After 10 minutes, 1.0 mL of the Color Developer (Alkaline Tartrate) was added to all three tubes. This step stabilized the color and enhanced the final hue. After 5 minutes, the absorbance of the sample was measured against the sample blank at a wavelength of 546nm.

2.2.4 Assessment of kidney function

2.2.4.1 Determination of Urea

Serum urea concentration was measured by the Berthelot reaction using an enzymatic method, as described by Kaplan (1984). Serum (0.01 mL), urea standard (0.01 mL), and distilled water (0.01 mL) were introduced to the test, standard, and blank, respectively. Following these additions, 0.1ml of urea reagent 1 was added to each tube, mixed thoroughly, and incubated at 37°C for 10 minutes. 2.5mls of urea reagent 2 and 3 were added to each tube, mixed thoroughly, and incubated at 37°C for 15 minutes. Then, the Hinotek Spectrophotometer Model no. S23A was blanked with the reagent blank and read at 540nm.

2.2.4.2. Determination of Creatinine

Serum creatinine levels were measured using the Jaffe reaction, as described by Kaplan *et al.,* (1995). Reagent A and B (Alkaline solution and Picric acid solution) were mixed to make a functioning reagent. Reagent A (1ml) was combined with 1ml of Reagent B to make 2mls of working reagent for control and test for each determination. In the tube labeled standard, 0.1ml of standard serum was added, and in the tube labeled test, 0.1ml of test serum was added. The tubes were filled with 1ml of the working reagent. Within 30 seconds, the tubes were combined and the absorbance of the tubes was read and recorded. At exactly 2 minutes, the absorbances of the tubes were measured again. At a wavelength of 500nm, the tubes were read (Kaplan *et al.,* 1995).

2.3 Statistical Analysis

All data were calculated in triplicate and the differences between the groups were determined using ANOVA and the Waller-Duncan multiple test at p0.05. To compare the mean findings of the sample groups, one-way analysis of variance (ANOVA) was used to assess for statistically significant differences. The Statistical Package for Social Sciences (SPSS) version 17 was used as the statistical package. A p-value of less than 0.05 was considered statistically significant for all analyses.

3.0. RESULTS AND DISCUSSIONS

3.1 The result of acute toxicity (LD_{50}) of Zinc

Table 1: Showing the result of the acute toxicity of zinc in the dose range of 10 -5000, with the observation and the number of death (mortality)

Dose administered(mg/kg)	Observation	Mortality
10	**Normal**	**Nil**
100	**Normal**	**Nil**
1000	**Normal**	**Nil**
1600	**Slightly weak**	**Nil**
2900	**Slightly weak**	**Nil**

5000	**Weak**	**Nil**

3.2 The result of experimental animal weight

The results of the weight analysis are presented in figure 1 and 2 below. (The mean±SD weight and the percentage growth rate respectively).

The control group has the highest percentage increase in weight compared to the other groups.

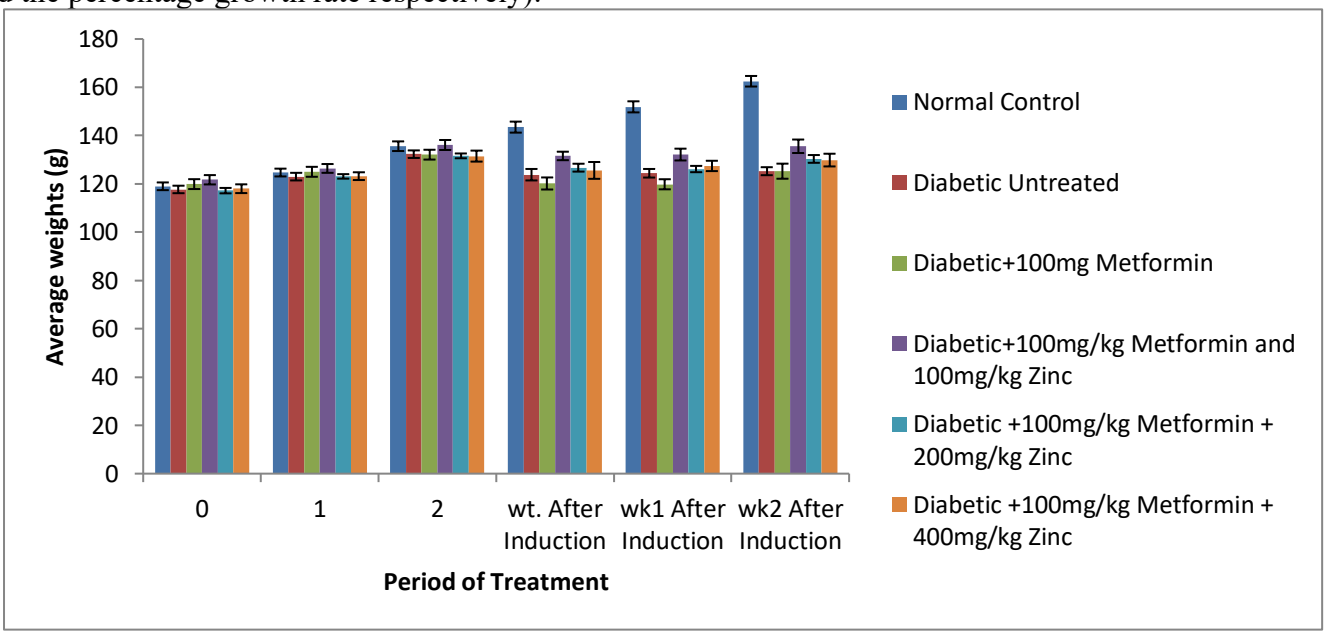

Figure 1. The effect of diabetes, Metformin and Zinc on the weight of alloxan-induced diabetic wistar rat. The Bars represent Mean±SEM

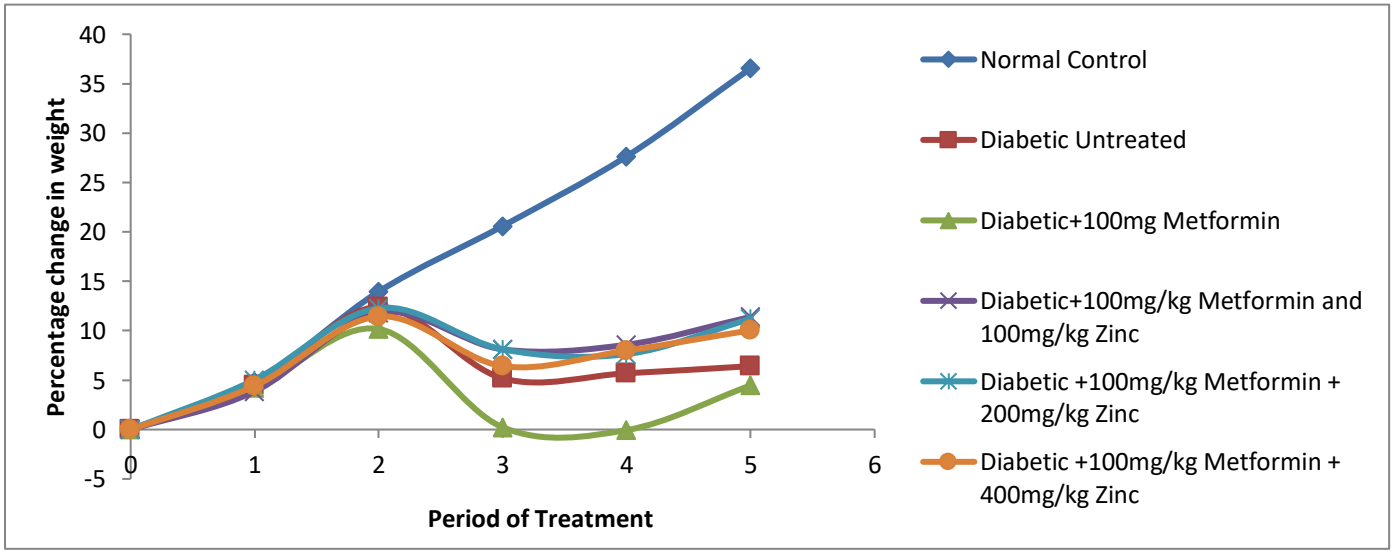

Figure 2: The percentage change in weight of the alloxan induced diabetic wistar rats.

3.3. The results of Blood Glucose Analysis

The results of the blood glucose analysis are presented in the figures 3 and 4 (the mean blood glucose level and the percentage rise in blood glucose respectively). Here the diabetic untreated has the highest percentage rise in blood glucose. The diabetic untreated group showed high percentage rise in blood sugar when compared the various groups. The administration of metformin and zinc showed a statistical significant change ($p<0.05$) on blood glucose level, when compared to the diabetic group.

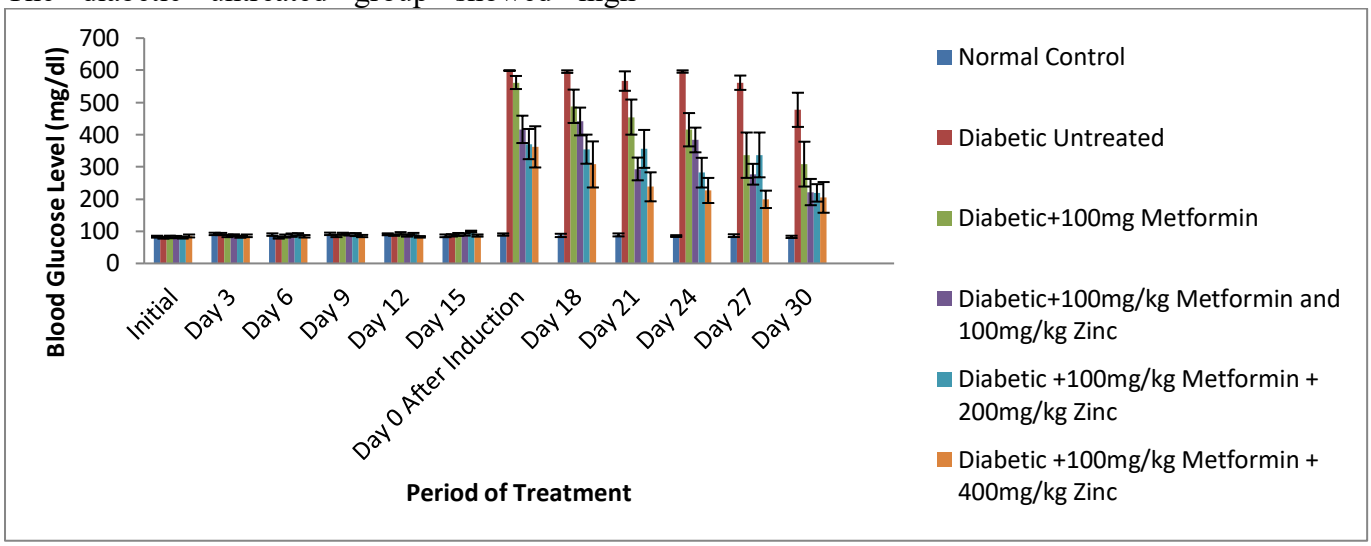

Figure 3: The effect of metformin and zinc on the glucose level of alloxan-induced diabetic wistar rats. Bars represent the Mean±SEM.

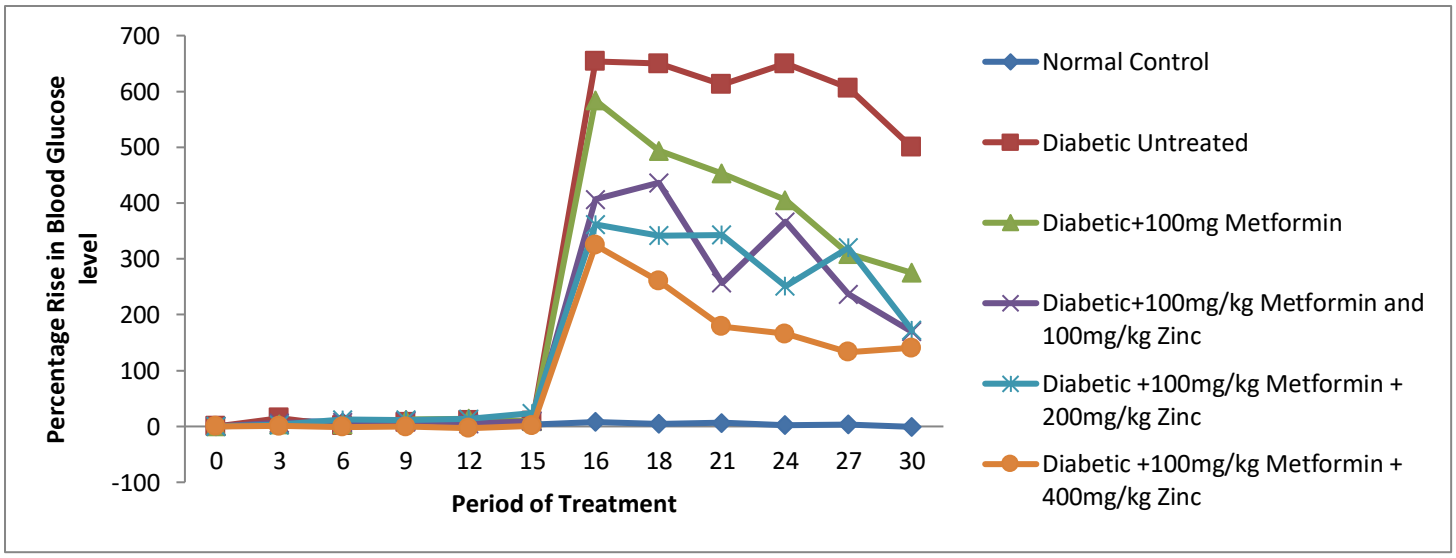

Figure 4: Percentage Rise in blood glucose

3.4. The results of the impact of the treatment on the liver

The results of the impact of the experiment treatments on the liver are presented in figures 5 (Direct bilirubin and Total bilirubin) and 6 below (Alkaline phosphatase, Aspartate Aminotransferase and Alanine transaminase).

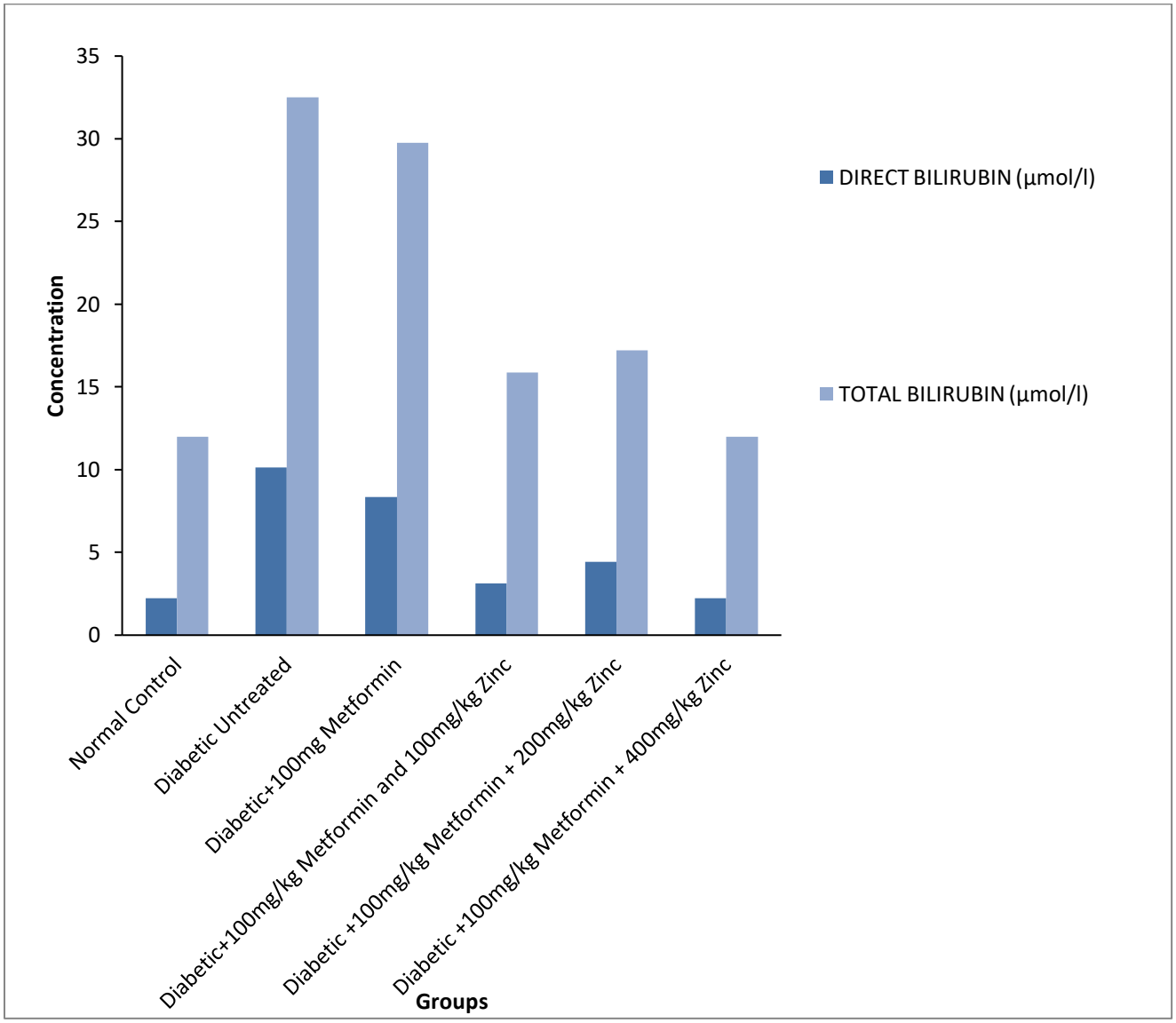

Figure 5. The effect of diabetes, metformin and zinc on the serum bilirubin concentration of alloxan-induced diabetic wistar rats.

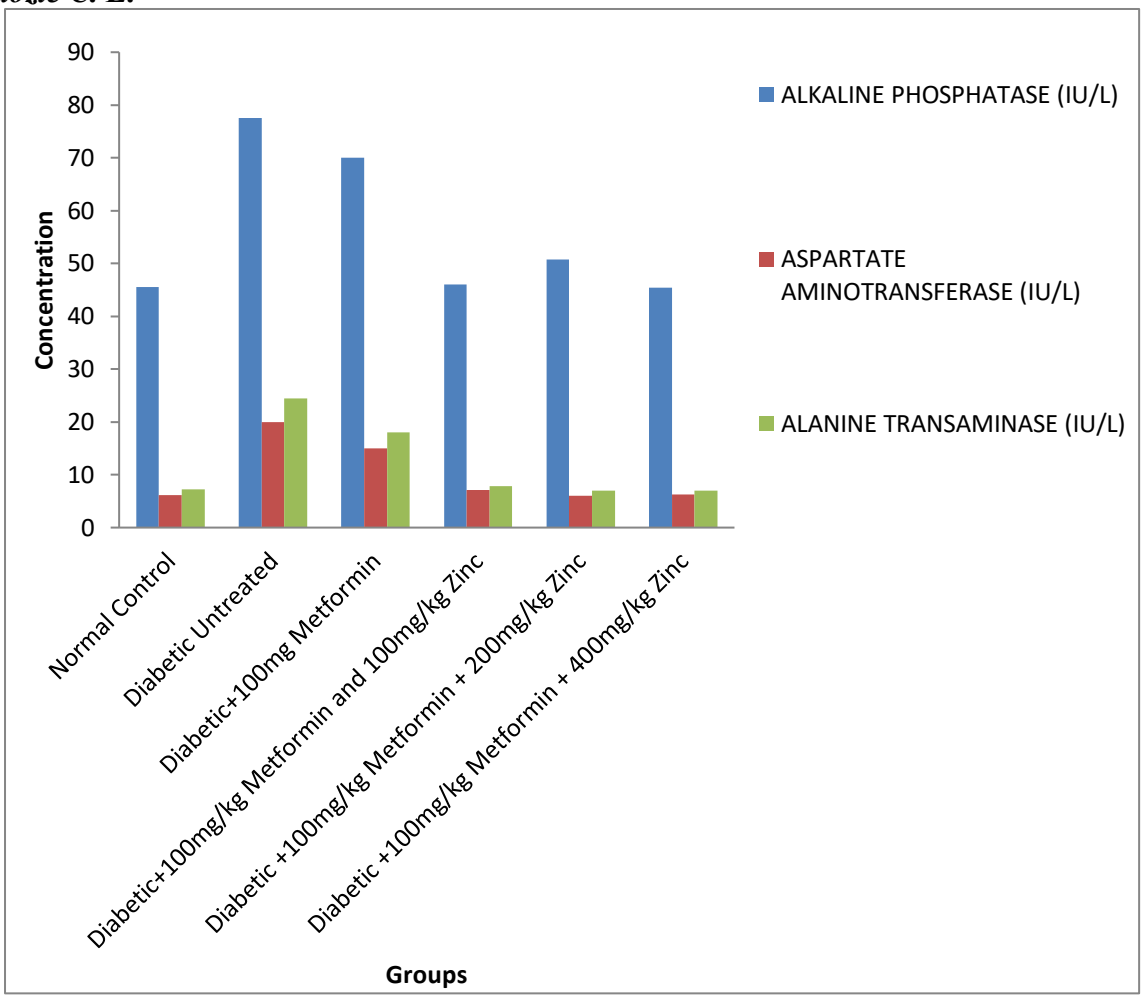

Figure 6. The effect of diabetes, metformin, and zinc on the serum Alkaline phosphatase, Aspartate aminotransferase, and Alanine transaminase of an alloxan-induced diabetic Wistar rats.

3.5. The results of the impact of the treatment on the Kidney

The results of the impact of the experimental treatment on the kidney are presented in the figures 7 and 8 below (The serum creatinine and Urea levels respectively). From figure 7 below, there is no statistical difference ($p>0.05$) between the control and zinc treated groups, but there is a significant difference between the zinc treated groups and the diabetic untreated group. There is no statistical difference ($p>0.05$) between the control and zinc treated groups, but there is a significant difference between the zinc treated groups and the diabetic untreated group as shown in figure 8 below.

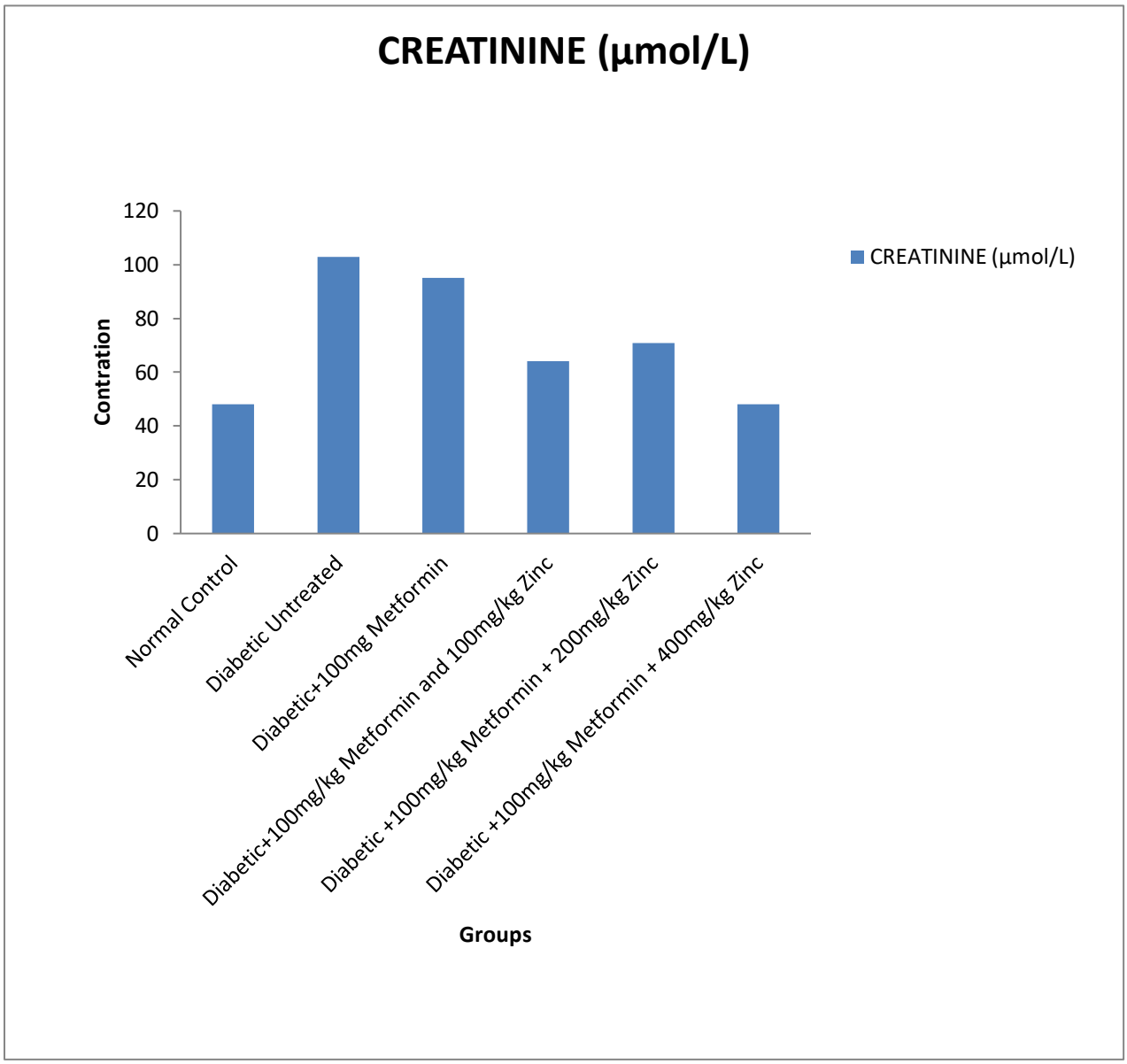

Figure 7. The effect of diabetes, metformin and zinc on the serum creatinine concentration of alloxan induced diabetic wistar rats.

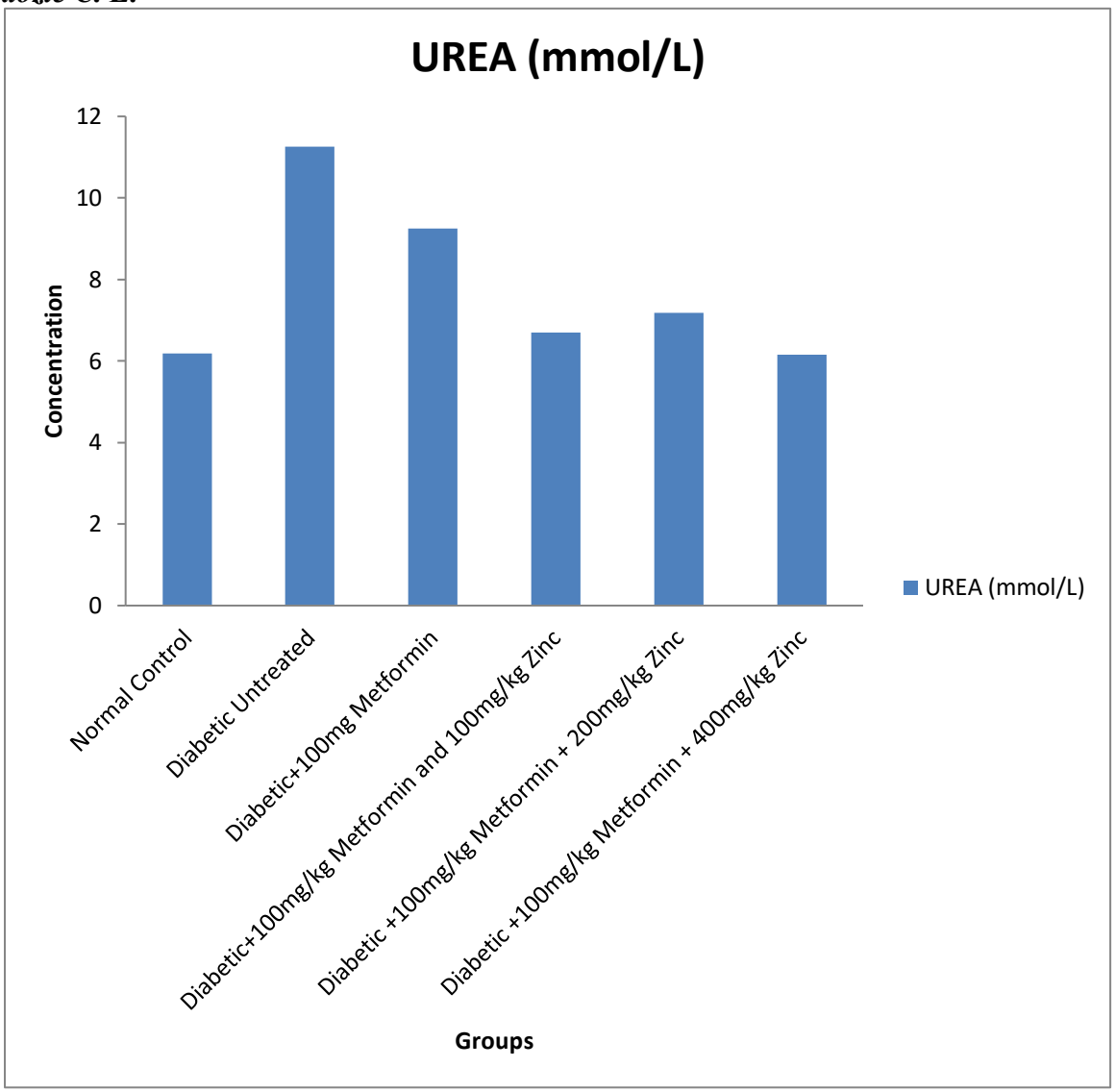

Figure 8. The effect of diabetes, metformin and zinc on the serum urea concentration of alloxan induced diabetic wistar rats.

Alloxan produces a large drop in insulin release by destroying beta-cells in the Islet of Langerhans and thereby generating hyperglycemia (Johnson and Chen, 2022). The current study found that the Alloxan-induced diabetic rats that were left untreated (Group B) showed a remarkable elevation of blood glucose levels compared to the control group (Group A). In contrast, diabetic rats treated with only metformin showed a significant decrease in blood glucose levels. Then, diabetic rats treated with metformin and Zinc (Groups D, E and F), demonstrated an improved glycemic control with values close to control rats compared to the rats treated with metformin alone (Group C). This result suggests that Zinc supplimentation which is dosage dependent, plays a vital role in the reduction of blood glucose level. The study proposed that this effect may be due to the ability of zinc to renew the activity of beta cells, stimulation of insulin release, and decreasing the blood glucose level (Khan and Smith, 2023). These observations affirm the fact of the synergistic therapeutic effect of Zinc and Metformin against Type 2 Diabetes mellitus-induced hepatic insulin resistance and β-cell function improvement (Khan and Smith, 2023).

The liver is an essential organ for maintaining blood glucose levels, metabolic functions, storage, detoxification, and excretion (Hall, 2020). From the results, it has been established that the increase in Bilirubin levels and serum activities of transaminases and ALP in T2D-induced rats may be due to hepatic injury linked with Alloxan resulting in leakage of these enzymes from the liver into plasma (Obasi and Ogugua, 2021). In figure 5, there is no significant difference ($p>0.05$) between the control and

metformin + zinc treated groups, but there is high level of bilirubin in the untreated and solo-metformin groups with a significant difference ($p<0.05$).There is no statistical difference ($p>0.05$) between the control and metformin + zinc treated groups, but there is a significant difference between the metformin + zinc treated groups and the diabetic untreated and solo-metformin groups as shown in figure 6. The current study has shown that treatment of Type 2 diabetes mellitus with the combined therapy of metformin and zinc restored the activities of ALT, AST, and ALP toward normal levels, and bilirubin levels returned to near normal levels, more than with a solo-metformin therapy. These results could be due to zinc's role in protecting the integrity and function of liver tissues, as well as its radical-scavenging activity (Kang, 2025). Zinc's protective properties are attributed to its ability to prevent collagen accumulation in the liver and its important physiological role in regulating cell structure and function (Sidhu *et al*., 2004). Lechu *et al.,* (2021) also identified that zinc supplementation prevented hepatic damage in a Type 2 Diabetic Mouse model.

According to the findings, metformin and zinc had a protective effect on liver and kidney function in the metformin/zinc-treated group compared to the untreated group, with an increase in serum biomarkers. Serum levels of creatinine and urea were markedly elevated in untreated rats (group B), which are clear signs of renal dysfunction. The observed renal dysfunction in the Alloxan-induced rats was reversed by treatment with Zinc and/or Metformin monotherapy as well as combined-therapy. There was a remarkable decrease in the levels of creatinine and urea in rats treated with zinc and metformin (groups D, E, and F) compared to the rats treated with metformin alone (group C). The reduced levels of creatinine and urea with zinc supplementation observed in this study support previous findings that Zinc reduces the rate of renal damage (Bakr, 2025). Supplementing with Zn decreased the rate of renal damage in comparison to the control groups by activating metallothionein, a cysteine-rich protein that interacts with Zn and iron to reduce ROS and, in turn, the oxidation process (Özcelik *et al.*, 2012). This implies that these two substances may have a synergistic anti-oxidative impact when used together. This effect may be related to the complementary ways in which these two chemicals scavenge free radicals and strengthen antioxidant defense systems. Diabetes is closely linked to oxidative stress, where elevated reactive oxygen species (ROS) production contributes to the pathogenesis of the disease (Deanfield *et al.,* 2017). Because diabetes may be mediated, at least in part, by oxidative stress, which can harm the heart, vascular system, kidneys, retina, and peripheral nerves, zinc plays an important role in cellular antioxidative defense (Bonnefont-Rousselot, 2004).

The current study also looked at the effects of diabetes on animal weight. The higher weight gain in the metformin/zinc-treated groups (C, D, E, and F) compared with the untreated group (B) suggests that the animals were recovering and that zinc, in combination with metformin, helped alleviate the effects of diabetes in alloxan-induced male Wistar rats. Aaishwarya and Prajakta (2021) also reported a decrease in blood sugar level and recovery in body weights in diabetic rats treated with metformin and other micronutrients (Selenium, Chromium picolinate, Vanadium pentoxide,and Magnesium sulphate) compared to those treated with metformin alone.

4.0. CONCLUSION

In conclusion, the present study has revealed that the administration of metformin (100 mg/kg b w) and Zinc (100, 200 &400 mg/kg b w) reduced blood glucose levels and are protective of the liver and kidney by the reduction in the serum biomakers in alloxan-induced hyperglycemia in Wistar rats. The combination of zinc and metformin is an effective and safe alternative treatment and provides additional benefits over metformin alone. The combined-therapy, which have separate mechanisms for enhancing glucose metabolism and lowering oxidative damage, could provide a novel therapeutic approach to alleviating diabetes consequences. The use of this synergy implies the possibility of establishing more effective therapy procedures that enhance glucose metabolism while lowering the risk of diabetes-related organ damage. Future studies

should include clinical studies to confirm these findings in humans and discover the best dosing regimens.

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