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Nephro-Pathological hallmarks for the investigation of metformin induced sub-acute toxicity in Wister rats

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Abstract
Metformin is an antidiabetic drug used to lower the blood sugar level in type 2 diabetic Patients. The chronic use of metformin for long duration may lead to nephropathy in humans and animals. This experimental study was conducted to assess the long term impact of Metformin on the structure and functionality of kidneys in rats. In this study, twenty adult male and female wistar rats were used for the investigation of Metformin induced sub-acute toxicity. The animals were randomly distributed into two groups and 10 animals are kept in each group. The first group served as the control and the second group received Metformin hydrochloride at a dose of 30 mg/kg body weight/day for a period of thirty consecutive days. All the animals were sacrificed at the end of study period in order to analyze the hematological, serological and the Nephropathological changes observed in the kidneys of rats. The results revealed that Metformin causes severe pathomorphological conditions in rats as evidenced by serum analysis and histopathology of kidneys.

Keywords: Renal dysfunction, histopathology, hematology, serology, metformin

Introduction
Diabetes is a chronic medical condition in which the body has high levels of glucose (sugar) in the blood. There are two main types of diabetes Type 1 diabetes and Type 2 diabetes. Type 1 diabetes occurs when the body's immune system attacks and destroys the cells in the pancreas that produce insulin, a hormone that regulates blood sugar levels. As a result, people with type 1. Diabetes need to take insulin injections or use an insulin pump to manage their blood sugar levels Type 2 diabetes occurs when the body becomes resistant to insulin or doesn't produce enough insulin to regulate blood sugar levels. Type 2 diabetes is often associated with lifestyle factors such as poor diet, lack of physical activity, and obesity. It can be managed through lifestyle changes such as diet and exercise, oral medications, and insulin therapy. There are also other types of diabetes, including gestational diabetes which occurs during pregnancy and usually resolves after delivery, and other forms of diabetes that can be caused by genetic or medical conditions. Diabetes can cause a range of complications if left unmanaged, including damage to the eyes, kidneys, nerves, and heart. It's important for people with diabetes to work closely with their healthcare team to manage their blood sugar levels and prevent complications. Metformin is a commonly used drug for the treatment of Type 2 diabetes which is responsible for lowering the blood glucose level by inhibiting gluconeogenesis in hepatocytes and decreases glucose absorption from the intestine Viollet *et al.* (2012) [1]. This helps to lower blood sugar levels and improve glycemic control in people with type 2 diabetes. Metformin was originally developed from glargine found in the plant Galega officinalis Bailey *et al.* (2004) [2]. In addition to its primary use in diabetes management, Metformin has also been found to have other potential benefits, such as improving insulin resistance Brown *et al.* (2008) [3], reduced expression of gluconeogenic enzymes Rena *et al.* (2017) [4], reducing the risk of cardiovascular disease and potentially even reducing the risk of some cancers Mobasher *et al.* (2021) [5]. It was recently used for the pre-clinical trials in patients suffering with COVID-19 Samuel *et al.* (2021) [6]. Metformin is usually taken orally in the form of tablets, and the dose is adjusted based on a person's blood sugar levels and response to the medication. Like all medications, metformin may cause side effects, but these are usually mild and include gastrointestinal symptoms such as nausea, vomiting and diarrhea. Metformin is generally considered safe and effective, and is often recommended as a first-line treatment for type

2 diabetes. However, it may not be suitable for everyone, and should be used under the guidance of healthcare professionals.

Metformin toxicity can occur in rare cases and is usually associated with an overdose of the medication. The symptoms of metformin toxicity can be similar to the side effects of the medication and may include Nausea, Vomiting, Diarrhea, Abdominal pain, Weakness, Fatigue, Muscle pain, Difficulty breathing, Dizziness and Loss of consciousness. In more severe cases, metformin toxicity can cause lactic acidosis, a rare but serious condition that can be life-threatening. Lactic acidosis occurs when there is a build-up of lactic acid in the blood, which can lead to metabolic acidosis, a condition in which the body's pH becomes too acidic. Symptoms of lactic acidosis include rapid breathing, abdominal pain, muscle weakness, and in severe cases, coma and death. Metformin toxicity is rare, but can occur in people with kidney or liver disease, those who take higher than recommended doses of the medication, or those who take other medications that interact with metformin. Besides its previous use, it was recently used for the treatment of cancer [5] and pre-clinical trials in patients suffering with COVID-19.

Based on the above listed biological effects of Metformin, this study was conducted to evaluate the role of long-term treatment with Metformin on the structure and functionality of kidney in experimental rats.

Materials and Methods

The details of experimental animals, experimental design and procedures employed to achieve the objectives of this study are described below:

Chemicals

All the chemicals and reagents utilized in this study were obtained from Erba. Metformin hydrochloride Manufactured by Merck were purchased from a Local Chemist in Srinagar district of Jammu and Kashmir.

Glass wares

All the glass wares used in this study were obtained from Borosil (India). The glass wares were properly cleaned and sterilized before use.

Plastic wares: All the plastic wares including microtips used in this study were obtained from Tarsons (India). The plastic wares were scientifically sterilized by autoclave prior to use.

Technical Programme

Experimental animals

A total number of twenty adult male and female wistar rats were used for the experimental purpose.

Housing

The room was thoroughly cleaned and every part of the room was flamed. Next day white washing of the room was carried out and the room was further fumigated with a potassium permanganate and formalin. These experimental animals were kept in polypropylene cages under identical animal house condition and provided with water ad libitum. The animals were reared up to 2 weeks of age for acclimatization and are provided with bedding of husk and 12-hour light/dark cycles. The Environmental conditions were maintained at a temperature of 22 °C ± 2 °C and a relative humidity of 60% ± 10%. All the animals were reared only in cages from beginning up to the end of the experimental period.

Feeding

The rats were given drinking tap water and normal pelleted animal food ad libitum. The normal diet is composed of protein 21%, fats 3.2%, carbohydrates 68.2% and fibers 3.44%. The handling and treatments of rats were in accordance to guidelines of the National Institute of Health for laboratory animal care. The animals of each group had a separate feeding and watering trays.

Study Design

This study was conducted to evaluate the Nephropathological effect of metformin in wistar rats. In this study, wistar rats weighing between 170 and 200g were purchased from the animal house of CSIR Jammu. The animals were randomly distributed into two groups in which 10 animals are kept in each group. The wistar rats were given a period of two weeks for acclimatization before the experiment. The first group served as the control and the second group received metformin hydrochloride at a dose of 30 mg/kg body weight/day through oral gavage for a period of thirty days. The dose rate was selected on the basis of pervious literature Oluwatosin *et al.* (2012) [7]. The experimental protocol was approved by the institutional Ethics Committee at faculty of Veterinary Sciences, SKUAST-Kashmir vide no: FVSc/VCC-9/19/286-87.

Clinical observations: The experimental animals were monitored during the entire period of experiment for clinical signs. The weight of kidneys were noted after 10 days interval in sub acute intoxications.

Clinicopathological studies: For clinicopathological investigations, blood samples were collected from jugular vein of rats in sterile vials and heparin was added as anticoagulant for haematological studies. Rest of the blood was used for biochemical evaluation after separation of serum and stored at -20°C until used.

Hematological studies: The fallowing hematological investigation was carried out manually in this experiment

1. Hemoglobin
2. Total Erythrocyte count
3. Total leukocyte count
4. Hematocrit

Biochemical Analysis: The fallowing blood biochemical parameters were estimated by using Olympus biochemistry analyser with compatible kits from aspen chemicals.

1. Creatinine
2. Urea
3. Albumin
4. Globulin

Preparation of tissues: The tissue specimens of the kidneys were harvested from the rats and fixed in 10% formalin. The Paraffin blocks were prepared after completing the tissue processing in different grades of alcohol and xylene. The sections of about 5µm were prepared from paraffin blocks using microtome and then staining was done with the help of hematoxylin and eosin Lille *et al.* (1954) [8]. The tissue

sections are then visualized under a light microscope in order to observe cellular damage.

Statistical analysis

All data are presented as mean (M) ± standard deviation (SD). Unpaired t-Test was applied to determine the significant differences among different groups.

Results

Clinical Signs

The affected animals exhibited anorexia, dyspnea, lethargy, staggering gait and death. After 10 days of intoxication, there was decrease in feeding, polydipsia, severe depression, listlessness, reluctance to move, standing with sunken head and greenish-yellow diarrhea. A total number of five animals succumbed to intoxication during the experimental period. The onset of the symptoms was recorded on 7th day and reached its peak on 15th day of intoxication. There was a sudden drop in feed consumption and the animals appeared weak. The marked clinical signs appeared on 20th day when half of the intoxicated animals showed diarrhea, laboured breathing and distorted body appearance. At day 30, the intoxicated animals showed emaciation, depression and lameness. During this study period, the animals layed down with sternal recumbency for prolonged period of time. Also, the intoxicated animals showed the altered behavior such as reduced attentiveness, restlessness and hyper salivation. At later stage nervous signs were marked, which included ataxia, convulsions, dilation of pupil and respiratory depression. At the end of experimental period, the important clinical signs observed were dullness with closed eyes, loss of appetite, high rise of temperature, drowsiness and watery diarrhea leading to death. Before death, the animals fell suddenly revealing uneasiness, paddling of legs and unconsciousness. At the end of the study period, the intoxicated animals which survived for few days revealed retarded growth, diarrhea, muscle weakness, staggering gait and loss of response to stimulus prior to death.

Hematology

The results showed that the hemoglobin (Hb) level, total red blood cell (RBCs) counts, hematocrit (Hct) % and total white blood cells (WBCs) count did not significantly change between the groups under this study as shown in Table 1.

Table 1: Hematological parameters in different groups of rats.

Groups	Hb (g/dL)	RBCs (x106 /µL)	Hct (%)	WBCs (x103 /µL)
Control Group	10.31±0.43	9.62±0.53	41.3±2.6	11 ± 0.5
Treatment Group	10.70±0.21	9.93±0.32	40.7±2.3	11±0.4

Means of various Hematological parameters does not differ significantly

Serology

In this experiment, the animals in Metformin treatment group showed the highest level of urea creatinine and albumin in the serum as compared to the control group.

Table 2: Urea and Creatinine levels in the serum of rats in different groups

S.no	Group	Urea (mg/dl)	Creatinine (mg/dl)
1	Control	23.00±1.5 ^{a}	0.68±0.03^{c}
2	Treatment group	25.00±2.0 ^{b}	0.81±0.05^{d}

Means of various Kidney function parameters differ significantly

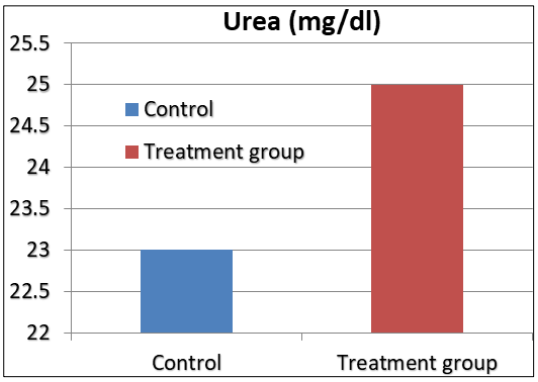

Fig 1: Effect of Metformin intoxication on Urea level (gm/dl) in rats

The graph represents increased level of serum urea in treatment group then control

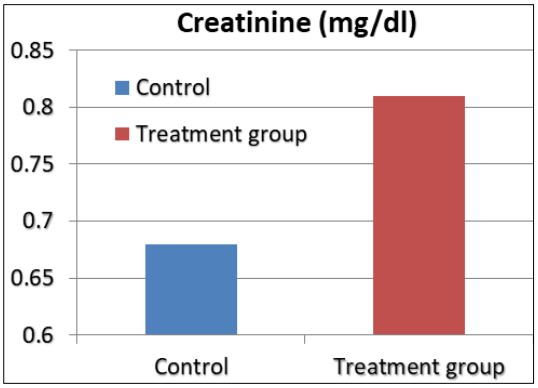

Fig 2: Effect of Metformin intoxication on Creatinine level (gm/dl) in rats

The graph represents increased level of serum creatinine in treatment group then control

Table 3: Albumin and total protein levels in the serum of rats in different groups

S.no	Group	Albumin (g/dl)	Globulin (g/dl
1	Control	3.23 ± 0.15 ^{a}	4.73±0.35 ^{c}
2	Treatment group	4.65± 0.34 ^{b}	5.98±0.52 ^{d}

Means of various Kidney function parameters differ significantly

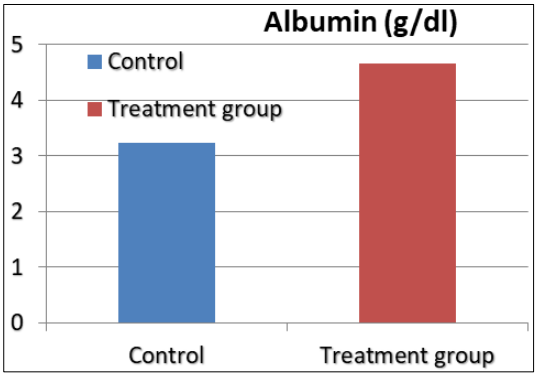

Fig 3: Effect of Metformin intoxication on Albumin level (g/dl) in rats

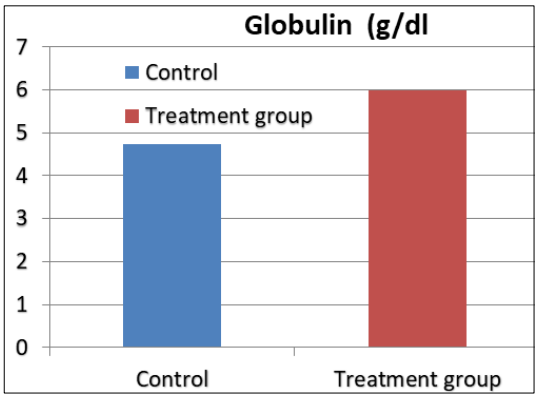

Fig 4: Effect of Metformin intoxication on Globulin level (g/dl) in rats

The graph represents increased level of serum globulin in treatment group then control

Pathomorphology

Kidneys: Grossly, the Kidneys of intoxicated rats were generally enlarged, cooked in appearance and revealed ecchymotic hemorrhages on the surface. The animals died during the first week of study period revealed swollen kidneys with petechiael hemorrhages on their surface. At the end of the study period, the kidneys of intoxicated animals were mottled with necrotic foci on their surface.

Histopathology of control group: The examination of renal cortex of the kidney sections of control rats showed normal appearance. The renal medulla is located beneath the renal cortex and consists of renal pyramids. These pyramids are cone-shaped structures that contain tubules which transport filtered urine from the cortex to the renal pelvis. The renal medulla also contains collecting ducts which plays an important role in urine formation. The glomerulus is enclosed by two layers of epithelium, surrounded by Bowman's capsule. The glomeruli are round and the proximal convoluted tubules were lined with simple epithelia. These epithelia had an acidophilic cytoplasm and the apex possesses abundant microvilli which formed a brush border. The distal convoluted tubules were lined with simple cuboidal epithelium. The kidney tissue is supported by a network of connective tissue, including collagen fibers and elastic fibers, which provide structural integrity to the organ.

Histopathology of Metformin treatment group

Kidneys: Histopathologically, the animals sacrificed at the end of first week revealed moderated lesions in kidneys characterized by variable degree of congestion, degeneration of glomeruli, necrosis and sloughing of renal tubular epithelium (Fig1)**.** In most of the cases, the succumbed animals revealed diffuse hemorrhages, degenerated epithelial lining of renal tubules, glomerular congestion and interstitial nephritis featured by infiltration of mononuclear cells comprising macrophages (Fig2)**.** In severe cases, destruction of both parietal and visceral layer of glomerulus was evident in the affected animals. At places, the tubular epithelium was atrophied. Besides in a few cases, necrotic changes were more prominent in perivascular areas and were occasionally associated with damaged basement membrane and infiltration of mononuclear cells. The Cellular degeneration of convoluted tubules was characterized by cellular swelling with indistinct cell boundaries, rarefied nucleus and narrowing of tubluar lumens. The microscopic examination of kidney tissue sections further revealed marked degeneration in the cortical tubular epithelium with increased eosinophilia and granulation in cytoplasm with intact basement membrane. The animals scarified during second week revealed glomerular atrophy, dilatation of renal tubules, fragmentation of glomeruli associated with nephritis which was frequently noted in this study period (Fig3). The renal medulla of the affected animals revealed degenerative changes in the epithelium of renal tubules, peritubular fibrosis, hemorrhage and interstitial nephritis (Fig4). In addition to this, the collecting tubules revealed necrosis of epithelial lining, interstitial hemorrhages and cortical tubular degeneration characterized by cellular swelling. The affected animals also reveal severe renal tubular degeneration, congestion and glomerular nephritis (Fig5). In most of the cases, the Metformin intoxicated rats revealed dilated renal tubules and destruction of both parietal and visceral layers of glomerulus (Fig6). A few animals in this study period revealed loss of nuclei and indistinct cell boundary of renal tubular lining of epithelial cells with nephrotic changes which were more severe in subcapsular regions. Moreover there was cellular infiltration in the glomerulus with increased Bowman's space observed in the affected kidneys of Wister rats (Fig7).

The kidneys of the Metformin intoxicated rats sacrificed at the end of third week revealed severe degeneration, congested glomerulus and destruction of epithelial lining of renal tubules (Fig8). The kidneys of the affected animals reveal hemorrhage, necrosis and severe cellular infiltration in the renal tissue section which were observed throughout the study period (Fig9). In this study, frequently breaks in the basement membrane of proximal convoluted tubules and epithelial desquamation of collecting ducts were also evident. Few cases also revealed focal mononuclear cellular infiltration with marked nephrosis in the cortico-medullary junction along with focal hemorrhages and peritubular fibroplasia. Nephritic changes were more severe in animals which died during third week of the study period and were characterized by interstitial nephritis and cellular swelling of epithelial lining in proximal convoluted tubule, distal convoluted tubule and collecting ducts (Fig10). In most of the cases, microscopic examination revealed dilatation of renal tubules, congested blood vessel and vacuolar changes in the epithelial lining of convoluted tubules with focal interstitial nephritis characterized by mononuclear cell infiltration (Fig11). The renal tubules in the cortical and medullary region of affected kidneys revealed degenerative changes characterized by necrosis and peeling off epithelial linning from the renal tubules. The microscopic examination of the renal tubules also revealed hemorrhage and accumulation of eosinophilic hyaline mass in the lumen of tubules with necrosis (Fig12). Occasionally areas of coagulated necrosis, cytolysis in the periphery of glomeruli, vacuolar change and protenacious casts in the renal medullary tubules were also evident.

The animals sacrificed at the end of fourth week of the study period revealed congested blood vessel, marked denudation of renal tubular epithelium and thickening of the wall of the blood vessel (Fig13).Some cases also reveal medullary hemorrhages, nephrosis, mononuclear cell infiltration and atrophied glomerulus (Fig14). In addition to this, the kidneys also revealed vascular congestion, nephritic changes and increased Bowman's space (Fig15). The Convoluted tubules of the affected kidneys further revealed marked nephrosis,

lysis, denudation of epithelial lining, Neutrophilic infiltration in the intersitiium (Fig16). In addition to this, the affected kidneys also shows severe necrotic changes in the renal tubules with glomerular congestion (Fig17). The renal tubules continued to show cellular swelling with increased eosinophilia and cytoplasmic granularity throughout the study period. The Glomerulus of the affected kidneys revealed atrophy, hypocellularity, congestion, focal area of necrosis and degenerated epithelial lining of the renal tubule which were more severe at the end of study period (Fig18).

Histopathology of Kidneys of Wister rat intoxicated with Metformin

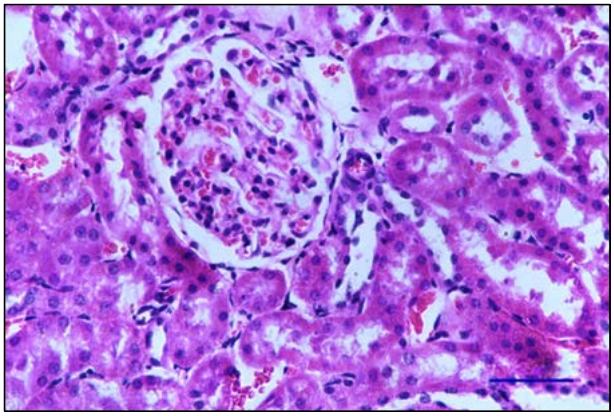

Fig 5: Photomicrograph revealing necrosis and sloughing of renal tubular epithelium (H&E 10X)

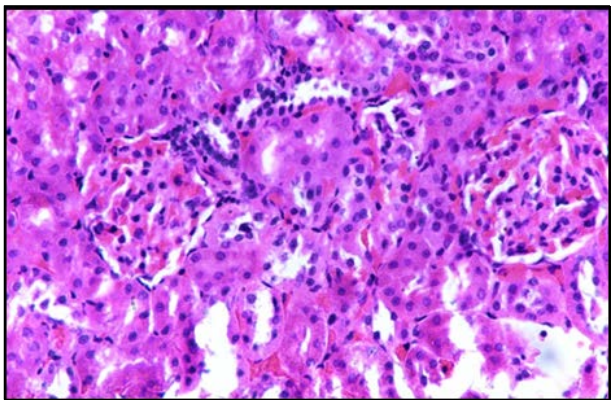

Fig 6: Photomicrograph revealing glomerular congestion and interstitial nephritis (H&E 10X)

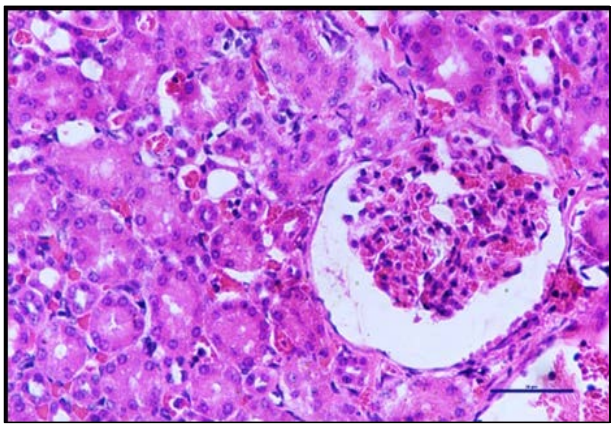

Fig 7: Photomicrograph revealing glomerular atrophy and dilatation of renal tubules (H&E 10X)

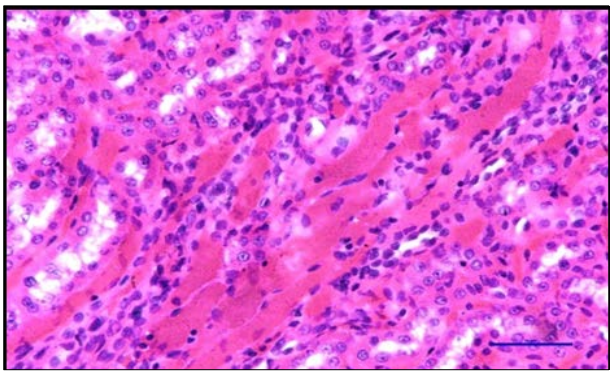

Fig 8: Photomicrographs revealing hemorrhage with severe cellular infiltration in the interstitium (H&E 10X)

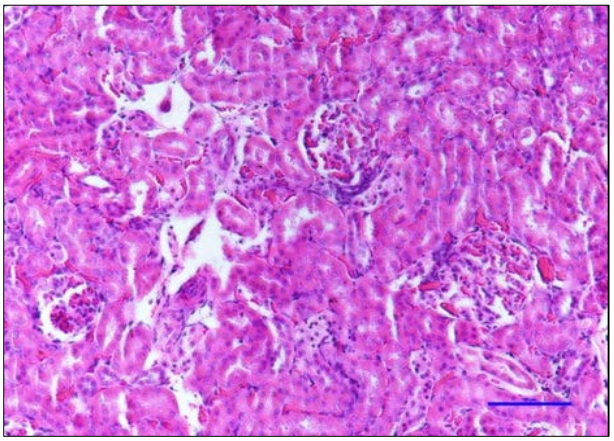

Fig 9: Photomicrograph revealing congestion and glomerular nephritis (H&E 10X)

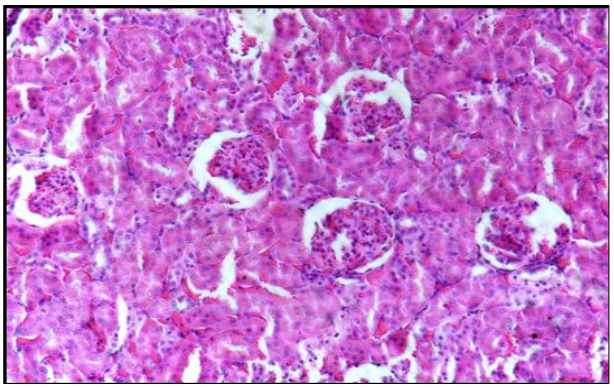

Fig 10: Photomicrograph revealing destruction of both parietal and visceral layers of glomerulus (H&E 10X)

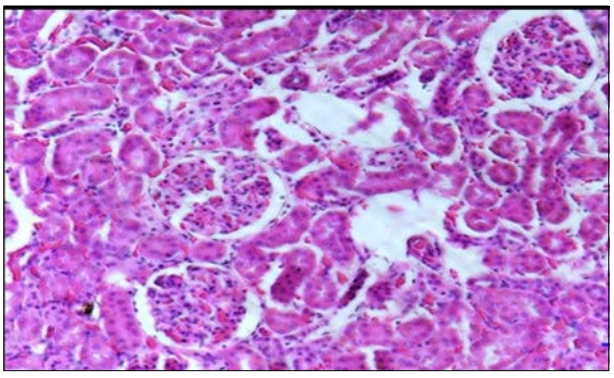

Fig 11: Photomicrograph revealing cellular infiltration in the glomerulus with increased Bowman's space (H&E 10X)

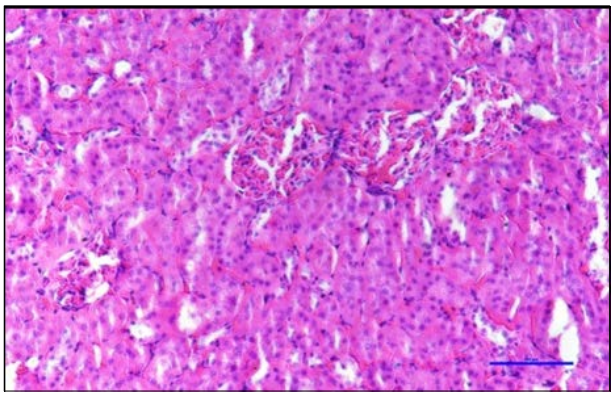

Fig 12: Photomicrographs revealing destruction of epithelial lining of renal tubules and congested glomerulus (H&E 10X)

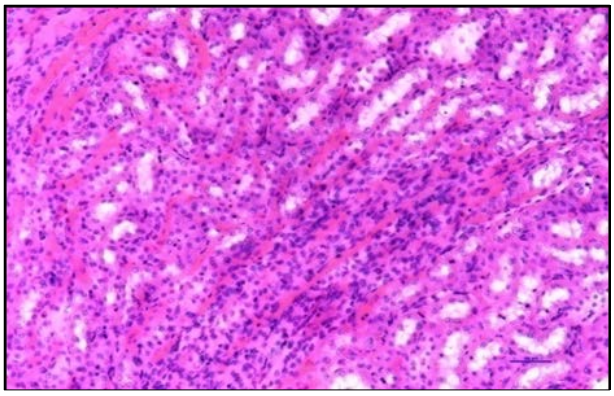

Fig 13: Photomicrographs revealing hemorrhage, necrosis and severe cellular infiltration in the renal tissue section (H&E 10X)

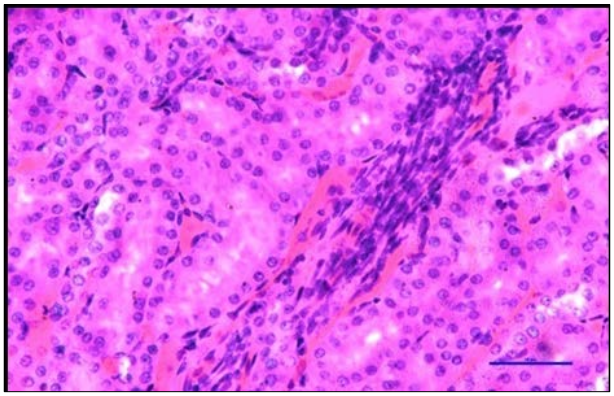

Fig 14: Photomicrographs revealing severe interstitial nephritis with tubular necrotic changes in the tissue section (H&E 10X)

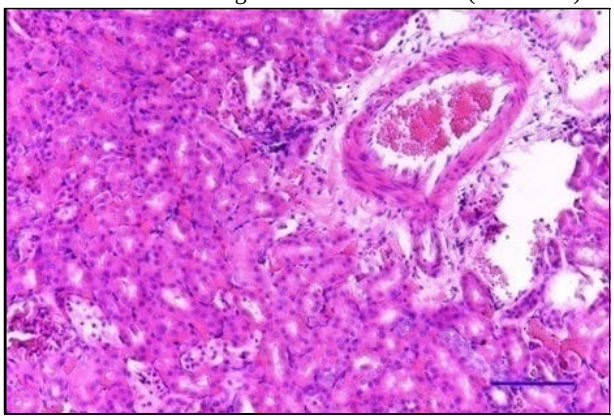

Fig 15: Photomicrograph revealing perivascular infiltration of neutrophils with congested blood vessel (H&E 10X)

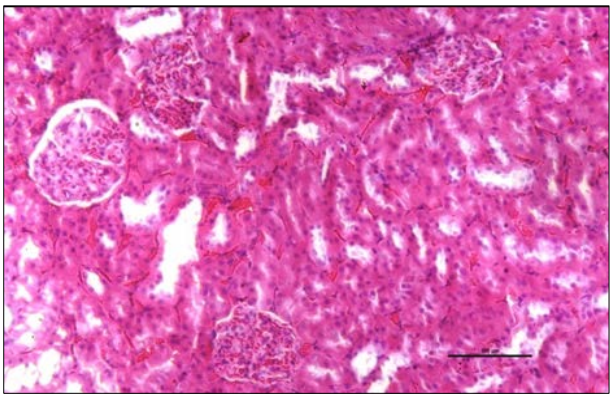

Fig 16: Photomicrographs revealing severe hemorrhage with necrotic changes in renal tissue section (H&E 10X)

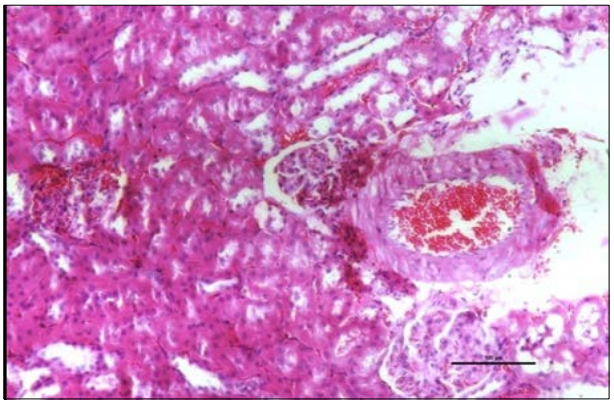

Fig 17: Photomicrograph revealing congested blood vessel with thickened blood vessel wall (H&E 10X)

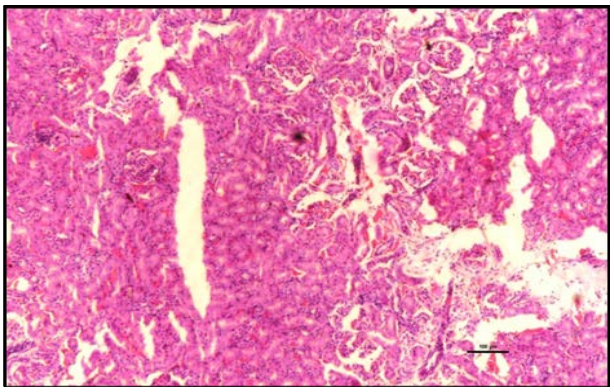

Fig 18: Photomicrograph revealing degeneration with atrophic changes in the glomerulus (H&E 10X)

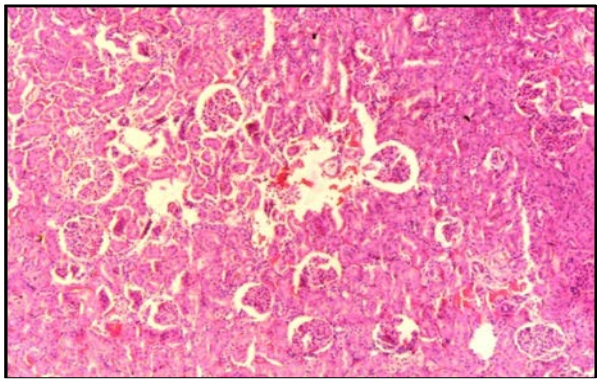

Fig 19: Photomicrograph revealing coagulative necrosis in the kidney tissue section with increased Bowman's space (H&E 10X)

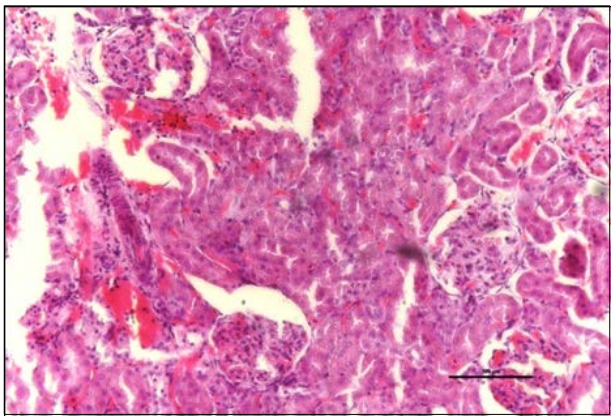

Fig 20: Photomicrograph revealing severe hemorrhage with Neutrophilic infiltration in the renal tissue section (H&E 10X)

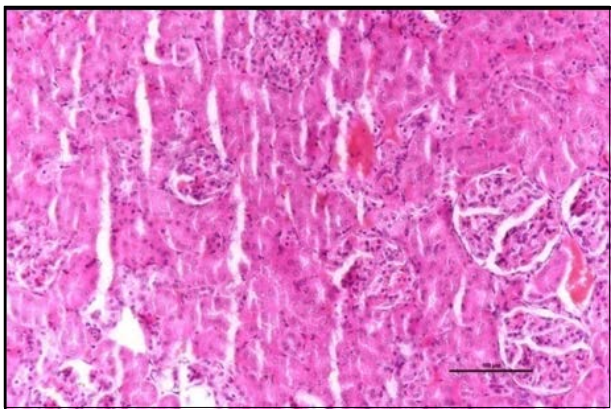

Fig 21: Photomicrograph revealing degenerated renal tubules with severe cellular infiltration in glomerulus (H&E 10X)

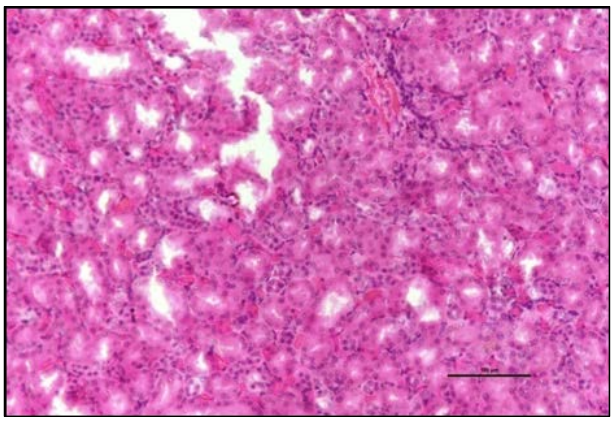

Fig 22: Photomicrograph revealing denudation of renal tubular epithelium with dilated renal tubules (H&E 10X)

Discussion

Diabetes in animals is a chronic metabolic disorder that affects the regulation of blood sugar levels in humans and animals. Diabetes can occur in various animals, including domesticated animals such as cats, dogs and horses, as well as in wild animals. Diabetes in cats, also known as feline diabetes mellitus, is a relatively common endocrine disorder that affects the regulation of blood sugar levels in cats. It is similar to type 2 diabetes in humans, where the body becomes resistant to insulin. Diabetes in dogs, also known as canine diabetes mellitus, is a metabolic disorder and is similar to type 1 diabetes in humans, where the immune system attacks and destroys the insulin-producing cells in the pancreas, resulting in less insulin production and causation of diabetes. Diabetes in wild animals are rare as compared to domesticated animals. Wild animals are generally adapted to their natural environments and have specific physiological mechanisms for regulating their blood sugar levels. In some cases, wild animals with diabetes may require intervention, such as capturing and providing medical care. The conservation efforts must be taken to protect wild animals and their natural habitats, as well as minimizing human impact on their environments, can play a significant role in reducing the risk of diabetes and other health issues in wild animals.

Diabetes mellitus can occur in both pet rats and laboratory rats. The rats can develop diabetes due to insufficient insulin production or insulin resistance, resulting in high blood sugar levels. Some rats may develop diabetes spontaneously that is similar to type 1 diabetes in humans, where the immune system attacks and destroys the insulin-producing cells in the pancreas resulting in the hyperglycemia. In research settings, rats may be induced with diabetes to study the disease or test experimental treatments. This can be achieved through various methods, such as chemical toxins, genetic manipulation, or high-fat diets. These induced models of diabetes in rats can mimic type 1 or type 2 diabetes in humans, depending on the method used to induce the condition. The symptoms of diabetes in rats may include increased thirst and urination, weight loss, increased appetite, poor coat condition, lethargy, and changes in behavior or activity levels. Diabetic patients are more vulnerable to several complications such as nephropathy, retinopathy, and neuropathy Chen *et al.* (2011) [9]. Metformin is commonly used drug for the treatment of Polycystic ovary syndrome, Diabetic and prediabetic patients Ortega *et al.* (2014) [10]. Metformin have shown better control on diabetes as compared to chlorpropamide therapy Rafieian *et al.* (2013) [11]. Metformin have capacity to reduce the incidence of angina, stroke and sudden death in Diabetic patients Kim *et al.* (2015) [12]. With the lapse of time it has been realized throughout the world that these anti diabetic drugs may pose a source of toxicity to the humans and animals. The danger posed by these drugs lies in their residual effect and our ultimate goal should be to know the toxicipathological effect of metformin on kidneys of humans and animals.

In our study, the metformin was experimentally induced in rats in order to know the nephropathy in intoxicated animals. In this study, One group was kept as control and other treatment group in which metformin was given orally to the rats. The dose rate was selected on the basis of pervious literature and the route selected in this study was such as to simulate the natural condition in which animals are exposed for the toxicity under field conditions. Metformin is generally considered safe in clinical practice, with few gastrointestinal side effects such as nausea, dyspepsia, abdominal bloating, abdominal cramps and diarrhea Motta *et al.* (2009) [13].

Our results did not show a significant change among the groups in Hb level, total WBCs counts, total RBCs counts and Hct% value. This study clearly showed that the long-term treatment of Diabetes with Metformin did not cause any significant changes in the hematological parameters Muhammad *et al.* (2012) [14], Charity *et al.* (2019) [15], Fagbohun *et al.* (2020) [16].

Our study also investigated the efficacy of Metformin treatment on the kidney function test in rats. A significant increase in the level of urea and creatinine was observed in

intoxicated rats Lin *et al.* (2018) [17].

The hyperurecemia and increased creatinine level in metformin intoxicated rats clearly reflected that repeated use of metformin for prolonged period may induce marked renal dysfunction. This fact has been fully substantiated by pathomorphological observations of the kidneys i.e. congestion and atrophy of bowmans capsule, cellular infiltration, degeneration with accumulation of hyaline mass in tubular lumens Khadre *et al.* (2011) [18], Albasher *et al.* (2020) [19].

The kidneys of the metformin intoxicated rats on gross examination appeared enlarged and congested with varying degrees of hemorrhages. Hstopathologically, the kidneys of metformin toxicity group revealed vascular congestion and nephritis which were manifested with degeneration, desquamation of renal tubular epithelium, necrosis, neutrophilic infiltration, cellular swelling and vacuolar changes in the lining epithelium of convoluted and collecting tubules Adaramoye *et al.* (2012) [20]. Metformin causes histological alterations in the kidney tissues of intoxicated rats that exhibits atrophy and disorganization of the glomeruli, intratubular hemorrhages and disorganization of the renal tubules Al-amri *et al.* (2020) [21]

Conclusion

Collectively, the data of the present study showed that rats administered with metformin revealed an increase in kidney functions biomarkers accompanied with alterations in histological architectures of kidney tissues. Therefore, the prolonged treatment with metformin revealed a renal dysfunctions in experimental rats.

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