Original Article

Histological Study on the Effect of Canagliflozin-Metformin HCl Extended - Release (Invokamet XR) on the Liver of Normal and Experimentally Induced Diabetes Mellitus in Adult Male Albino Rats

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ABSTRACT

Background: diabetes mellitus represents a global health problem. It is considered the third greatest killer due to its complications. Invokamet XR is a recent single oral combination of canagliflozin plus metformin HCL used in treatment of type 2 diabetes mellitus (T2DM).

Objective: to evaluate the effect of Invokamet XR on the liver of normal and experimentally induced T2DM in adult male albino rats.

Materials and Methods: thirty five adult male albino rats weighing 160-180g were categorized equally into five groups. (G1) control group; (G2) Invokamet XR group treated orally with 200 mg/kg b.w/ rat /day; (G3) buffer control group, given a single intraperitoneal (i.p.) injection of 0.1M sodium citrate buffer (0.5 ml/rat); (G4) diabetic group and (G5) diabetic group treated with Invokamet XR. T2DM was induced in G4 and G5, by a single i.p injection of nicotinamide (NA) (110 mg/kg b.w) followed by streptozotocin (STZ) (35 mg/kg b.w) 15 minutes later. At the end of the experiment (3 weeks) blood was collected for biochemical analysis of AST, ALT & ALP enzymes. Liver samples were taken for light microscopic examination. The body weight and fasting blood glucose level, in addition to histomorphometrical and statistical analysis were done.

Results: the present study revealed that T2DM induced a significant increase in liver enzymes, loss of the normal histological structure as; congestion and dilatation of the blood vessels; mononuclear cell infiltration; hepatocyte vacuolation with pyknotic nuclei. Invokamet XR induced slight improvement in these changes in the diabetic animals.

Conclusion: Invokamet XR has a potent anti-hyperglycemic effect but it has a slight protective action on the biochemical and histological changes induced by T2DM that might be due to an improvement in the blood glucose level and a reduction in the complications associated with diabetes.

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Key Words: Diabetes, invokamet XR, light microscopy, liver.

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INTRODUCTION

Diabetes mellitus is a metabolic syndrome characterized by high blood glucose level, disturbances of carbohydrate, protein and fat metabolism, and classical symptoms of polyuria, polydipsia and polyphagia. It causes hyperglycemia resulting from insufficient insulin secretion, insulin action or both[1]. Type 2 diabetes mellitus results from insulin resistance, a condition in which cells fail to use insulin well[2]. Diabetic people are also at increased risk of other diseases such as; heart, peripheral arterial and cerebrovascular disease, obesity, cataracts, erectile dysfunction, and nonalcoholic fatty liver disease (NAFLD). They are also at increased risk of some infectious diseases. Diabetes might cause serious complications, including diabetic ketoacidosis, hyperosmolar hyperglycemic state, or even death[3,4].

The liver is a vital organ that plays a major role in maintaining normal blood glucose level by regulating the processes of de novo glucose production (gluconeogenesis) and glycogen breakdown (glycogenolysis), thus controlling the levels of hepatic glucose production[5]. Several pathways have been identified to cause liver damage in diabetic patients. The main cause of hyperglycemia is insulin resistance and the predominant causative factor compensatory hyperinsulinemia[6].

Invokamet XR is a recent single oral combination of canagliflozin and metformin hydrochloride (HCl) extended release approved by the U.S. Food and Drug Administration (FDA) in 2016. It is the first sodium glucose co-transporter-2 (SGLT-2) inhibitor (canagliflozin) plus metformin that is indicated for the treatment of patients with T2DM who are unable to achieve sufficient glycemic control to their maximally tolerated metformin as separate tablets[7]. Canagliflozin works on the kidney to promote the loss of glucose in the urine, whereas metformin decreases the

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production of glucose in the liver and improves the body's response to insulin; thus, this new combination improves, glycosylated hemoglobin, haemoglobinA1c (HbA1c) levels. In addition, canagliflozin combined with metformin results in greater reduction in body weight and systolic blood pressure compared with metformin alone[8]. Invokamet XR is not recommended in patients with T1DM or for the treatment of diabetic ketoacidosis. In addition, Invokamet XR is not associated with weight gain or increased risk of hypoglycemia[9]. Therefore, it has been chosen to perform this study.

AIM OF THE WORK

This study was designed to evaluate the effect of Invokamet XR on the liver of normal and experimentally induced T2DM in adult male albino rats.

MATERIALS AND METHODS

Materials

Drugs

- Streptozotocin (STZ) was used in this study for induction of T2DM. The drug was purchased from Sigma–Aldrich Company (St. Louis, Mo, USA) in the form of yellow powder, which was freshly dissolved in 0.1M sodium citrate buffer at PH 4.5[10].
- Nicotinamide (NA) was used to protect the β cells of pancreas from the hazardous effect of STZ in induction of T2DM. It was purchased from Sigma–Aldrich Company (St. Louis, MO, USA) in the form of white powder, dissolved in distilled water just before use[10].
- Canagliflozin-Metformin HCl (Invokamet XR) is a recent oral anti-diabetic drug, given to the patients suffering from T2DM. It was purchased from Janssen Pharmaceuticals Companies (Titusville, NJ: Janssen Pharmaceuticals, Inc. U.S.A). The daily human dose of the drug is two tablets; each one contains 150 mg Canagliflozin and 1000 mg Metformin HCl[9].

Experimental animals and design

Thirty-five adult male albino rats, weighing 160-180 g. were used in this research. They were purchased from Helwan breeding farm, Helwan, Egypt, housed in laboratory-slandered cages. All animals had free access chow diet and plenty of fresh tap water with continuous cleaning. They were acclimatized for one week prior to initiation of the experiment in the laboratory of Histology, Faculty of Medicine for girls, Al Azhar University. All the ethical protocols for animal treatment were followed according to the Research Ethics Committee (FMG-IRB), Faculty of Medicine (Girls), Al-Azhar University. Animals were categorized into equal five groups, seven rats each:

The control group (G1): animals were left without treatment and then scarified after 21 days from zero time.

Invokamet XR group (G2): animals were given Invokamet XR orally for 21 successive days from zero time (3 days after the induction of diabetes) in a dose of 200 mg/kg b.w according to Paget and Barnes[11], dissolved in 1ml distilled water for each rat.

Buffer control group (G3): animals were given single i.p injection of sodium citrate buffer (vehicle of streptozotocin) in a dose of 0.5 ml /rat.

Animals of both the fourth and fifth groups were subjected to induction of T2DM by i.p injection of NA (110 mg/kg b.w /rat) dissolved in 0.5 ml distilled water, followed by i.p injection of freshly prepared STZ (35mg/kg.b.w/rat)15 minutes later to overnight fasted animals. The induction of diabetes was achieved after 72 hours of STZ injection by estimation of fasting blood glucose (FBG) level, which was considered zero time. Only rats with FBG level above 200 mg/dl were considered diabetic[10].

Diabetic group (G4): seven diabetic animals were left without treatment.

Diabetic treated group (G5): the remaining seven diabetic animals were treated orally with Invokamet XR for 21 successive days from zero time (200mg/kg.b.w/rat/day) dissolved in 1ml distilled water.

Methods

- Estimating the body weight: each rat in all groups was weighed at the beginning of the experiment (zero time) and every week until the end of the experiment.
- Monitoring of fasting blood glucose level: FBG for the rats of all groups was estimated at the beginning of the experiment (zero time) then weekly until the end of the experiment (3weeks). The blood samples were collected from the tail vein of the overnight fasted rats by milking it. Estimation of FBG level was performed using a glucometer (One Touch Select Mini Blood Glucose Monitoring System, Life Scan Europe)[12].
- Blood collection and preparation: at the end of the experiment, on the day 21 from zero time, the overnight fasting animals were anaesthetized by intramuscular injection of 0.4 ml of ketamine hydrochloride "ketalar" (Parke-Davis, S.A. Barcelona-Spain). Blood was obtained from the retro-orbital venous plexus by capillary tubes from all rats, to determine alanine aminotransferase (ALT); the aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities. Blood samples were transferred into plain centrifuge tubes and allowed to clot at room temperature, then the samples were centrifuged at 4000 round per minute (rpm) for 10 minutes on a digital centrifuge (Biofuge 200, Bosch Medical Systems, Corinth, U.S.A.) to separate the serum from the clot. The resultant serum samples were stored frozen at -20ºC. Frozen sera were thawed and well mixed and all reagents were allowed to attain room temperature prior to assay.

Histological techniques

Light microscopic study: after 21 days from zero time, the abdomen of anaesthetized rats was opened by a median incision; small pieces from the right lobe of the liver were obtained, fixed in 10% neutral buffered formalin and processed for paraffin thin sections (5 um thickness). Sections were stained with hematoxylin and eosin (H&E) for detecting the general structure[13]; Mallory's trichrome stains, for staining the collagen fibers and periodic acid Schiff's (PAS) reaction, for detection of glycogen content of the hepatocytes[14].

Morphometrical studies: they were carried out using a computerized image analysis system (Primo star, Zeiss, China) at the Histology Department, Faculty of Medicine for Girls, Al Azhar University. Cytophotometric measurements were carried out on 3 sections for each specimen. In each of these 3 sections the measurements were carried out on 3 randomly chosen fields at x 200 magnification. Consequently, 9 area percentages were obtained for each animal. Then the mean was calculated for the 63 area percentages values obtained for each group. Data were computerized for statistical analysis. This was used to evaluate the area percentage of collagen fibers in Mallory's trichrome and glycogen in PAS reaction in all the studied groups.

Statistical studies: the body weight; fasting blood glucose level; liver function tests enzymes (AST, ALT and ALP) and area percentage of both collagen fibers and glycogen were statistically analyzed to evaluate the differences between the different studied groups using SPSS software version 22. The statistical significant was determined by one way analysis of variance (ANOVA) and repeated measures ANOVA for differences between the means of the different groups, followed by the post hoc test to compare the parameters in the different groups with each other. All data were expressed as mean ± standard deviation (±SD). Results were considered statistically significant when the probability (P) value $P \leq 0.05$ and highly significant when $P \leq 0.01$, while it was non-significant at $P > 0.05$[15].

RESULTS

Results of light microscopic examination

Hematoxylin and eosin stained sections of normal and buffer control adult male albino rats' liver were similar, showed normal hepatic structure. A thin capsule of fibrous connective tissue enclosing the liver covered with simple squamous epithelium (Plate1, A). Multiple classic hepatic lobules, each formed of plates of hepatocytes arranged in cords, radiating from the central veins to the periphery of the lobules. The cell cords were separated by blood sinusoids. The hepatocytes were polygonal in shape and their sides appeared in contact, either with the blood sinusoids or with the neighboring hepatocytes. They had eosinophilic cytoplasm and central large vesicular nuclei with prominent nucleoli, where each nucleus containing one or two nucleoli. Few hepatocytes were binucleated. Endothelial cells were seen lining the wall of the central veins and the blood sinusoids (Plate1, B). Normal portal tract areas were also seen contained branches of hepatic artery, portal vein and bile duct, which were lined by simple cubical epithelial cells (Plate1, C).

The animals treated with Invokamet XR showed moderate increase in the thickness of the fibrous connective tissue capsule enclosing the liver (Plate2, A). Nearly half of the animals had no structural changes in comparable with those of the normal controls (Plate2, B). The specimens collected from the remaining animals revealed many structural changes. Most blood sinusoids were dilated and congested although the central vein appeared either normal (Plate2, C) or markedly dilated with normal hepatocytes (Plate2, D&E). Focal areas of leucocytic aggregations (most probably lymphocytes) infiltrating between the hepatocytes and at the portal tract areas were present (Plate2, D-F). The portal tract areas had dilated congested portal veins, and many bile ducts lined by simple cubical epithelium (Plate2, G).

The diabetic animals revealed multiple structural changes in many parts. The thickness of the fibrous connective tissue capsule was apparently increased (Plate3, A). The majority of the hepatic lobules lost their normal hepatic architecture with dilatation and congestion of both the central veins and the blood sinusoids. It was noticed that, the thickness of the wall of some central veins were increased, surrounded by a layer of highly eosinophilic cells with deeply stained nuclei (Plate3, B-D). Structural degenerative process of variable severity took place in the cytoplasm and the nuclei of most hepatocytes. Patchy areas of highly eosinophilic cells appeared, while others were vacuolated. Some highly eosinophilic cells lost their boundaries and coalesced together, they appeared distorted with deeply stained nuclei (Plate3, E). Some cells were enlarged and ballooned and lost their normal shape with deeply stained nuclei and fragmented cytoplasm were seen while other cells appeared with fragmented nuclei, focal area of leucocytic aggregation, most probably lymphocytes was seen, in addition to many degenerative changes in multiple areas (Plate3, F&G). The portal tracts had dilated congested arteries, veins and many dilated bile ducts (Plate3, H).

The diabetic animals treated with Invokamet XR showed improvement in the histological structure when compared with those of the diabetic group. It showed irregular fibrous connective tissue capsule (Plate4a, A). The hepatic lobular architecture was preserved with more or less normal non-congested central veins. Although the central vein and the blood sinusoids of some hepatic lobules were still dilated and/or congested (Plate4a, B&C). Some hepatocytes regained their normal structure, containing eosinophilic cytoplasm and vesicular nuclei, while others still containing abnormal pale-stained vacuolated cytoplasm with either vesicular, condensed, ill-defined fragmented nuclei, or lost their nuclei. Some cells lost their boundaries and coalesced together. Ballooned cells with remnants of cytoplasm with either deeply stained or absent nuclei were still noticed (Plate4a, B, D& E). Some portal tracts appeared normal, except some areas contained dilated hepatic artery, portal

veins and normal bile ducts lined by simple cubical epithelial cells (Plate4a, E), other sections contained large dilated portal veins, hepatic arteries, dilated bile duct with many small ductules and lymphatics (Plate4b, F-I)

Mallory's trichrome sections of both normal and buffer control groups showed normal collagenous fibers distribution. A thin layer of corrugated collagenous fibers was noticed in the connective tissue capsule, around the central veins and at the portal tract areas (Plate5, A-C). The collagenous fibers of the normal animals treated with Invokamet XR appeared more thicker and corrugated when compared with the normal control animals, in the connective tissue capsule, around the central veins and at the portal tract areas (Plate5, D-F). In the diabetic group, the collagen fibers showed marked increase when compared with those of the control animals. It formed very thick corrugated collagenous bundles in the connective tissue capsule (Plate6, A), around the central veins and at the portal tract areas around the proliferated bile ducts (Plate6, B-F). While in the diabetic treated group, the collagen fibers in the connective tissue capsule and around the central veins showed slight decrease in density than the diabetic animals, but it was still more than the control animals. It consisted of moderately thick layer of dense corrugated collagenous fibers. However, increased density at the portal tract areas was still present (Plate7, A-C).

Periodic acid Schiff reaction (PAS) of both normal and buffer control animals showed that the glycogen content of the hepatocytes was high in the majority of the hepatocytes (Plate8, A).The majority of the specimens collected from the normal animals treated with Invokamet XR revealed slight decrease in glycogen content when compared with the controls. Some of the hepatocytes contained high glycogen content while it was moderate in others (Plate8, B). In the diabetic group, only one specimen showed high glycogen content in the majority of the hepatocytes, while it was negative in few cells around the dilated central vein (Plate8, C). Most of the hepatic lobules of the other specimens showed marked depletion in the glycogen content. The remaining showed either moderate or high glycogen content (Plate8, D&E).The diabetic treated group showed an increase in the glycogen content when compared to the diabetics, but it still lower than the normal. Some hepatocytes showed high or moderate glycogen content (Plate8, F).

Statistical Results

Body weights: at the end of the experiment, there were significant differences in the mean values of the body weights on comparing all groups with each other. The least recorded mean body weight (g) was among the diabetic animals followed by the diabetic ones treated with Invokamet XR, then normal rats treated with Invokamet XR. The highest value was recorded in the control group. The mean body weight of the diabetic group showed statistically significant decrease compared with that of the buffer control group. On the other hand, the body weight of the diabetic group treated with Invokamet XR showed statistically significant increase when compared to the diabetic group (Table 1 and Histogram 1).

Fasting blood glucose level: at the end of the experiment, there were significant differences in the mean values of FBG level (mg/dl) when comparing all groups with each other. It was significantly increased in the diabetic group when compared with the other groups. However, it revealed a highly significant decrease in the diabetic treated group when compared with the diabetic one (Table 2 and Histogram 2).

Biochemical analysis: at the end of the experiment, there were significant differences in the mean values of liver enzymes (IU/ L) on comparing all groups with each other. Our results revealed that AST, ALT and ALP were significantly increased in the diabetic group when compared with those of all the remaining studied groups. On the other hand, it showed statistically significant decrease in the diabetic group treated with Invokamet XR when compared with those of the diabetic group. The least mean values were recorded among the normal and buffer control animals (Table 3 and Histogram 3).

Morphometrical results

Mean area percentage of collagen fibers: the highest mean was recorded among the diabetic animals, followed by the diabetic ones treated with Invokamet XR then the normal rats treated with Invokamet XR. The least value was noted in the control groups (Table 4 and Histogram 4).

Mean area percentage of glycogen content: the highest mean was recorded among the control groups; while the least mean was recorded among the diabetic rats followed by the diabetic ones treated with Invokamet XR (Table 4 and Histogram 5).

Diabetes induced a highly significant increase in the mean area percentage of collagen, but a highly significant decrease in that of the glycogen when compared to the buffer group. On the other hand, in diabetic treated group a significant decrease in the mean area percentage of collagen fibers but a significant increase in that of the glycogen was noted in comparison with the diabetic group.

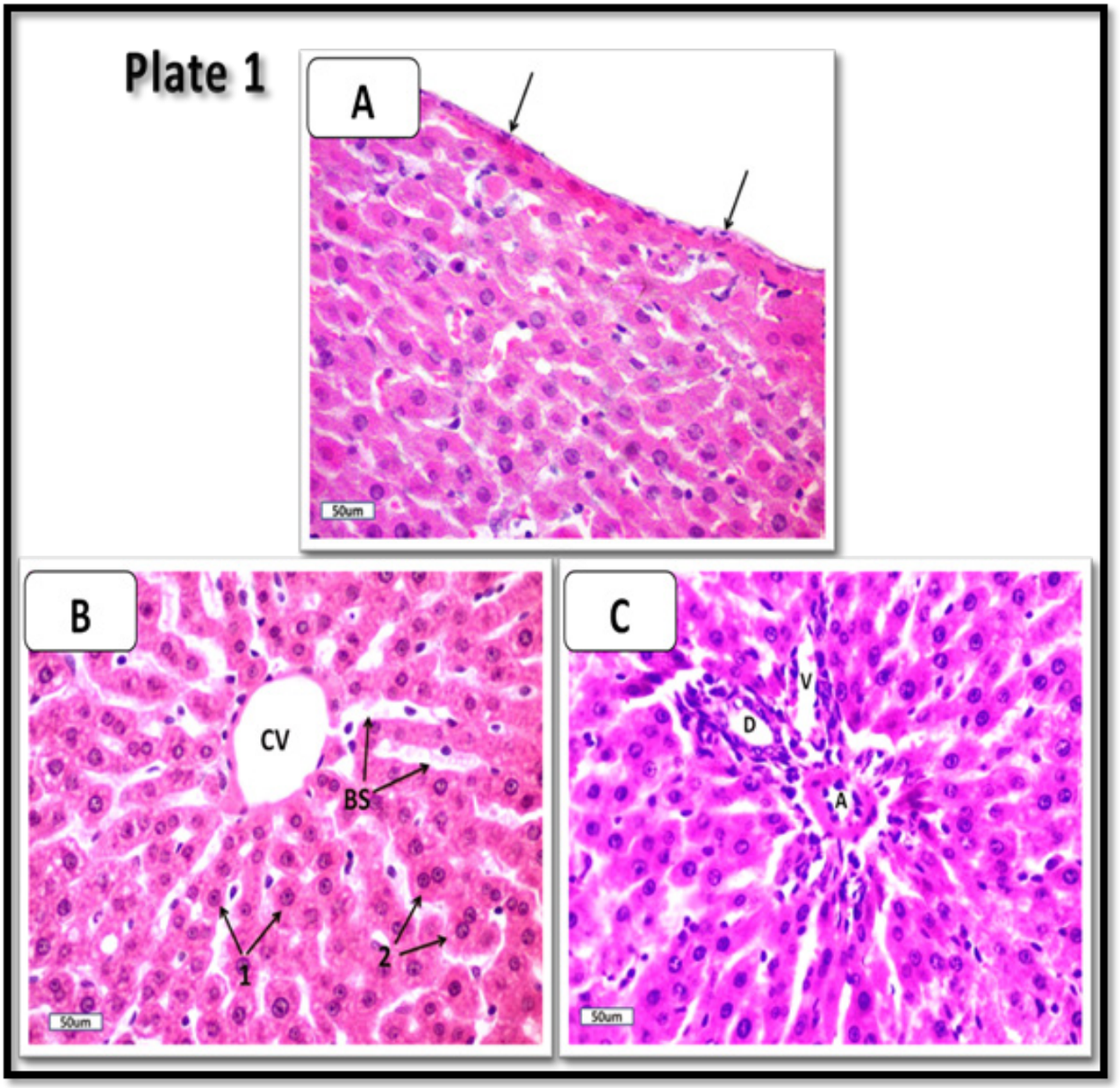


Plate 1: photomicrographs of liver sections of normal control group showing (A): thin fibrous connective tissue capsule lined by simple squamous epithelium (arrows). (B): cords of normal hepatocytes radiating from the central vein (CV) and separated by blood sinusoids (BS→), the hepatocytes contain eosinophilic cytoplasm and vesicular nuclei with prominent nucleoli (1→). Few binucleated cells are seen (2→). (C): portal tract area containing branches of hepatic artery (A), portal vein (V) and bile duct (D) lined with simple cubical epithelial cells.

(H&E; all X400)

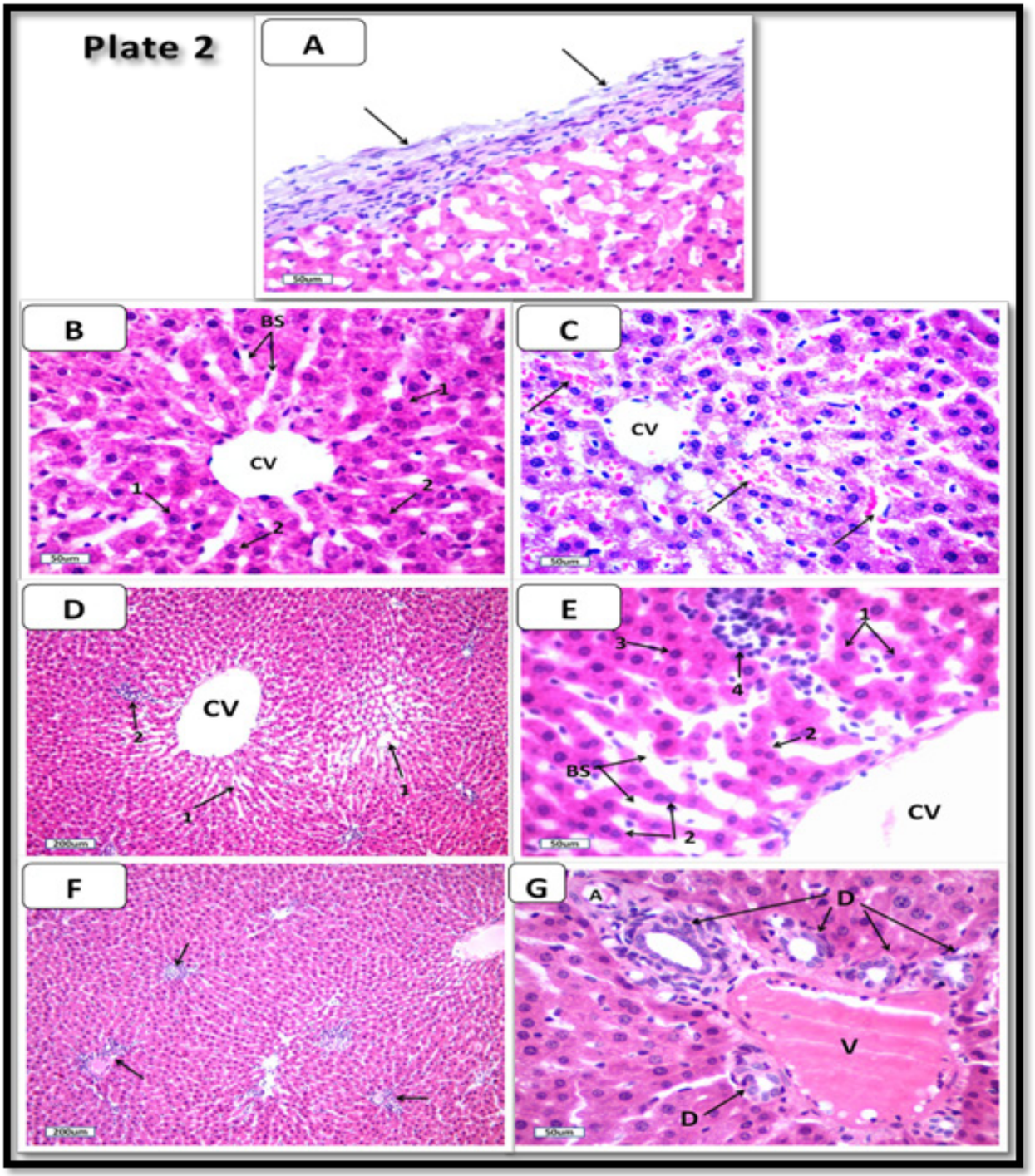


Plate 2: photomicrographs of liver sections of rats treated with Invokamet XR showing (A): moderately thick fibrous connective tissue capsule lined by simple squamous epithelium (arrows). (B): cords of normal hepatocytes radiating from the central vein (CV) separated by blood sinusoids (BS→), eosinophilic cytoplasm and the vesicular nuclei of the hepatocytes (1→); and few binucleated cells (2→).(C): loss of normal hepatic architecture and dilated congested blood sinusoids (arrows) with normal central vein (CV). (D): markedly dilated central vein (CV) and some blood sinusoids (1→) with some inflammatory cells in between the hepatocytes (2→). (E): a higher magnification of (D) showing a part of the dilated central vein (CV) and dilated blood sinusoids (BS→). Notice that the hepatocytes having either single vesicular nuclei (1→), or double nuclei (2→). Some hepatocytes contain highly eosinophilic cytoplasm and deeply stained nuclei (3→). Notice a focal area of leucocytic aggregations most probably lymphocytes infiltrating in between the hepatocytes (4→). (F): areas of leucocytic aggregations, most probably lymphocytes in close relation to many portal tract areas (arrows). (G): a portal tract area containing dilated congested portal vein (V), hepatic artery (A) and many bile ducts (D→) lined by simple cubical epithelium.

(H&E; all X400, except D& F X100)

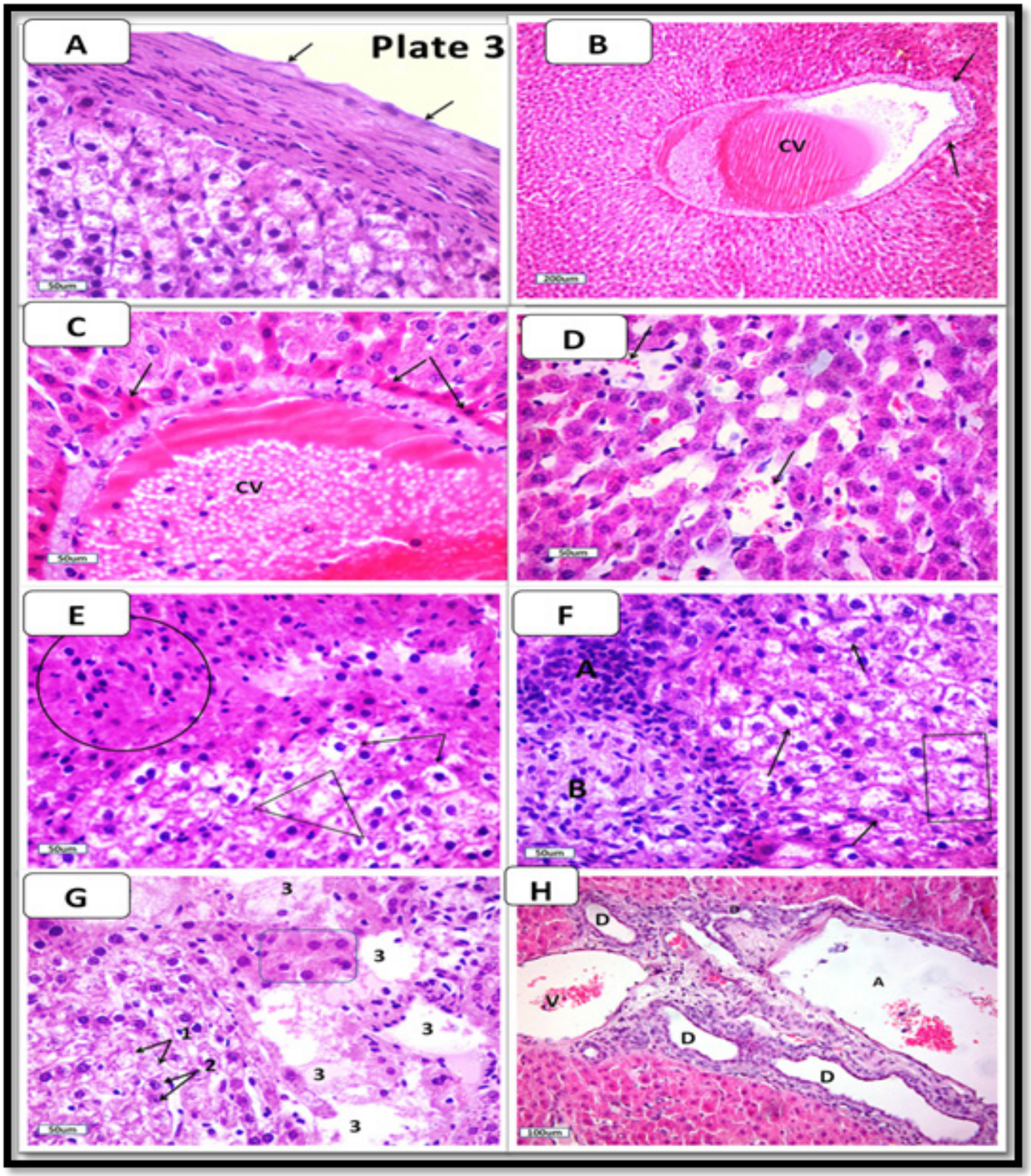


Plate 3: photomicrographs of liver sections of diabetic rats showing: (A) very thick fibrous connective tissue capsule (arrows). (B) marked dilatation and congestion of the central vein (CV) with apparent thickening of its wall (arrows). (C) a higher magnification of fig. (B) showing that the wall of the central vein (CV) is thickened and surrounded by highly eosinophilic cells with deeply stained nuclei (arrows). (D) dilated and congested blood sinusoids (arrows). (E) highly eosinophilic cells lost their boundaries and coalesced together and having deeply stained nuclei of variable sizes (circle). Notice, the ballooned vacuolated hepatocytes with remnants of cytoplasm and deeply stained nuclei (arrows), and other hepatocytes have remnants of cytoplasm and lost their nuclei (triangle). (F) loss of the normal hepatic architecture, focal area of leucocytic aggregation, most probably lymphocytes is seen (A), abnormal cells are seen bounded by leucocytes and have abnormal deeply stained nuclei (B), some cells enlarged, ballooned and lost their normal shape with deeply stained nuclei and fragmented cytoplasm (arrows), while other cells appear with fragmented nuclei ( ). (G) loss of the normal hepatic architecture. Notice a small area of eosinophilic cells (rectangle) and large area of vacuolated hepatocytes either free of nuclei (1→) or contain normal vesicular ones (2→). Multiple areas of degeneration (3) are also present. (H) a portal tract area containing dilated hepatic artery (A), portal vein (V) and many dilated bile ducts (D).

(H&E; all X400, except (F, G) X100& (H) X200)

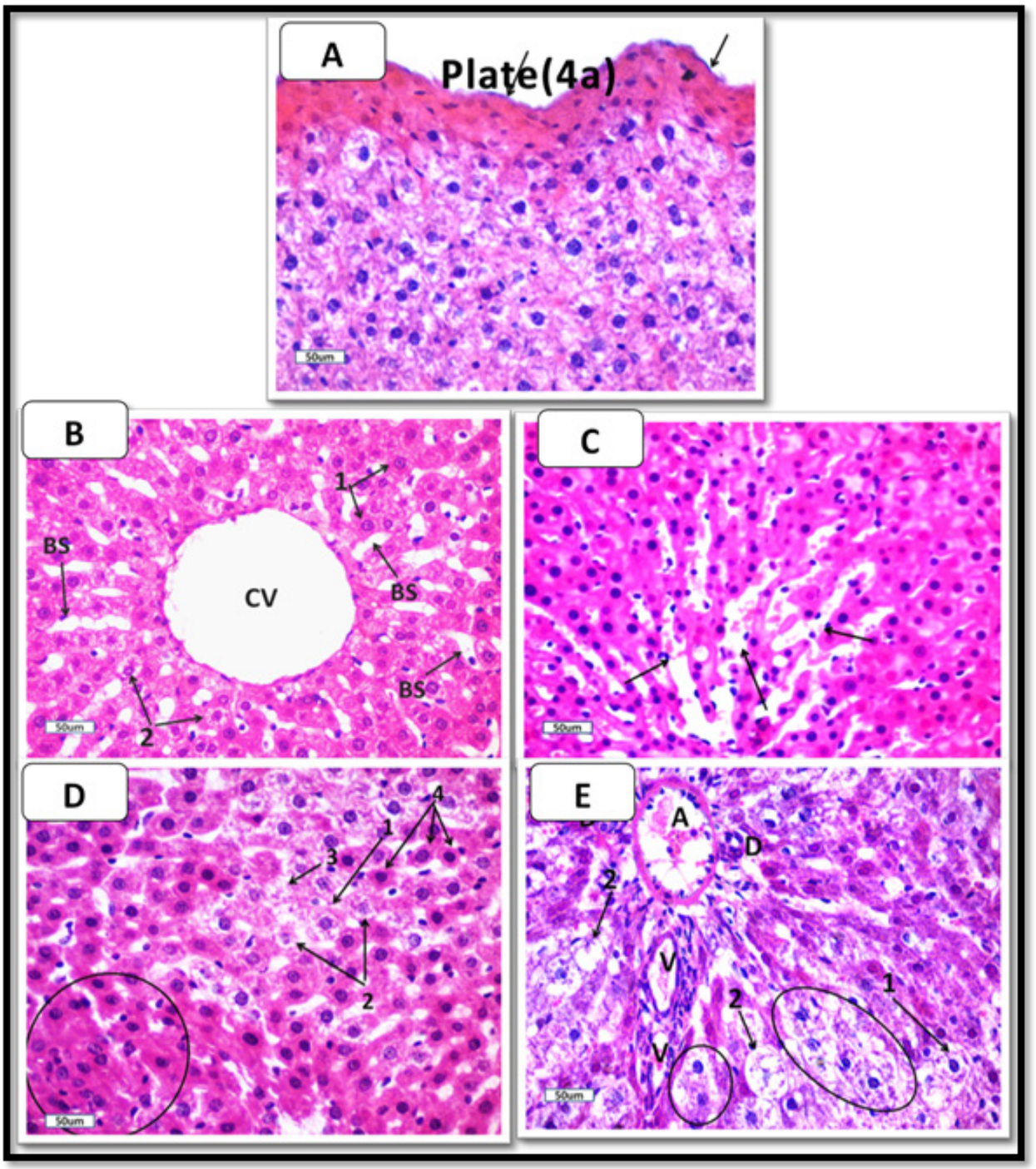


Plate 4a: photomicrographs of liver sections of the diabetic group treated with Invokamet XR showing (A) moderately thick and irregular fibrous connective tissue capsule (arrows). (B) cords of normal hepatocytes radiating from the markedly dilated central vein (CV) and separated by blood sinusoids (BS→). Notice that the majority of the hepatocytes contain eosinophilic cytoplasm and vesicular nuclei with prominent nucleoli (1→). Few cells contain vacuolated cytoplasm (2→). (C) many dilated and congested blood sinusoids (arrows). (D) patchy area of enlarged pale stained vacuolated hepatocytes with remnant cytoplasm and the nuclei either pale stained (1→), fragmented (2→) or absent (3→). Some hepatocytes fuse with each other with highly eosinophilic cytoplasm and lost their boundaries (circle). Notice, the distorted highly eosinophilic cells with deeply stained nuclei (4→). (E) a portal tract area containing dilated hepatic artery (A), portal veins (V) and bile ducts (D), some hepatocytes are ballooned with remnants of cytoplasm and the nuclei either deeply stained (1→) or absent (2→) and the fused cells with no cell boundaries (oval shape).

(H&E; all X400)

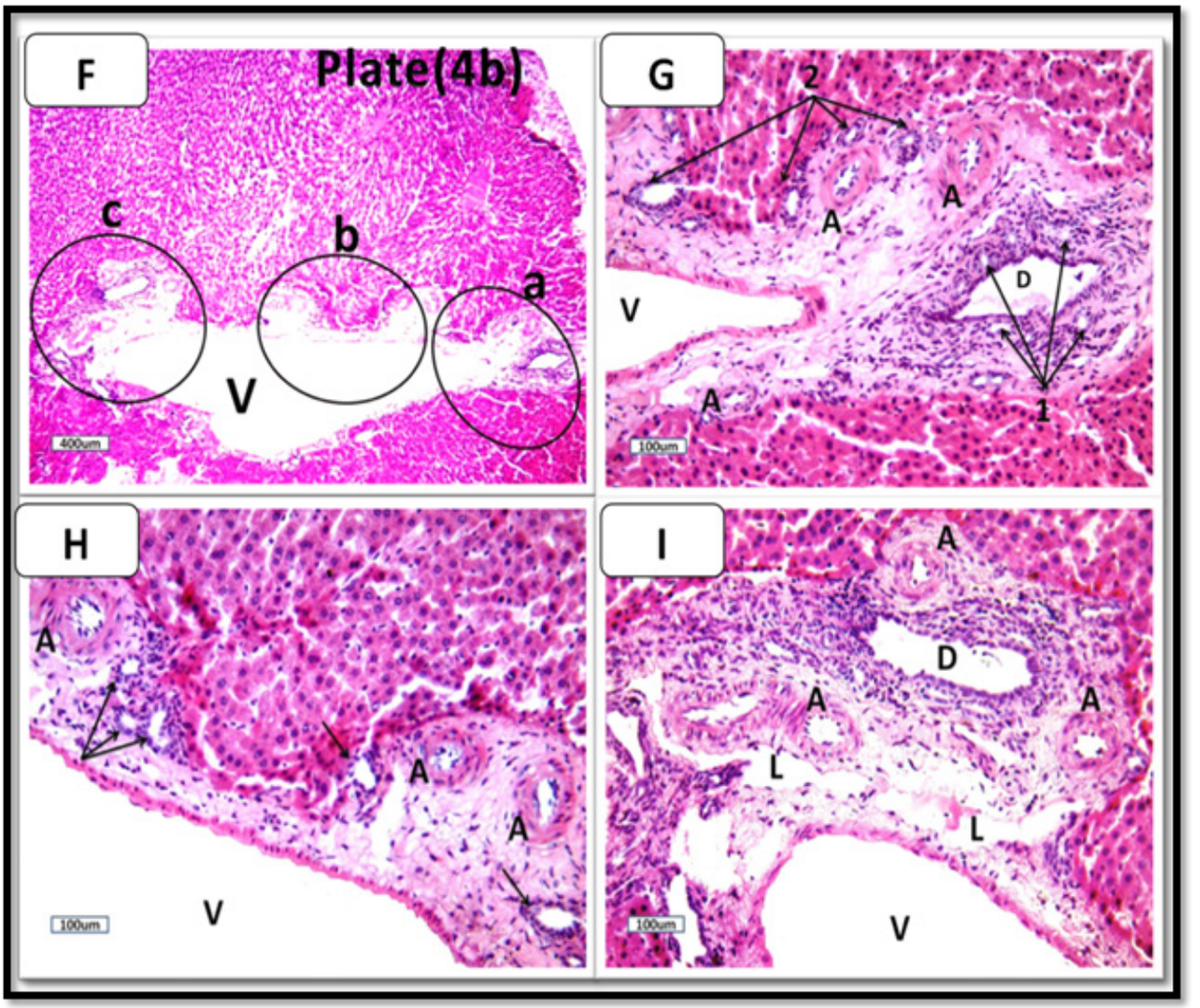


Plate 4b: photomicrographs of liver sections of the diabetic group treated with Invokamet XR showing (F) a portal tract area containing a large dilated vein (V), multiple hepatic arteries and multiple bile ducts (a, b and c). (G) a higher magnification of the area (a) of figure (F), showing dilated irregular bile duct (D), multiple ductules (1→), many arteries (A), many small ducts (2→) and a part of the dilated vein (V). (H) a higher magnification of the area (b) of figure (F), showing dilated irregular bile duct (D) surrounded by multiple ductules (1→), many arteries (A), many small ducts (2→) and a part of the dilated vein (V). (I): A higher magnification of the area (c) of figure (F), showing a dilated duct (D), multiple hepatic arteries (A), a part of the dilated vein (V) and lymphatics (L). (H&E; F X50 &G-I X200)

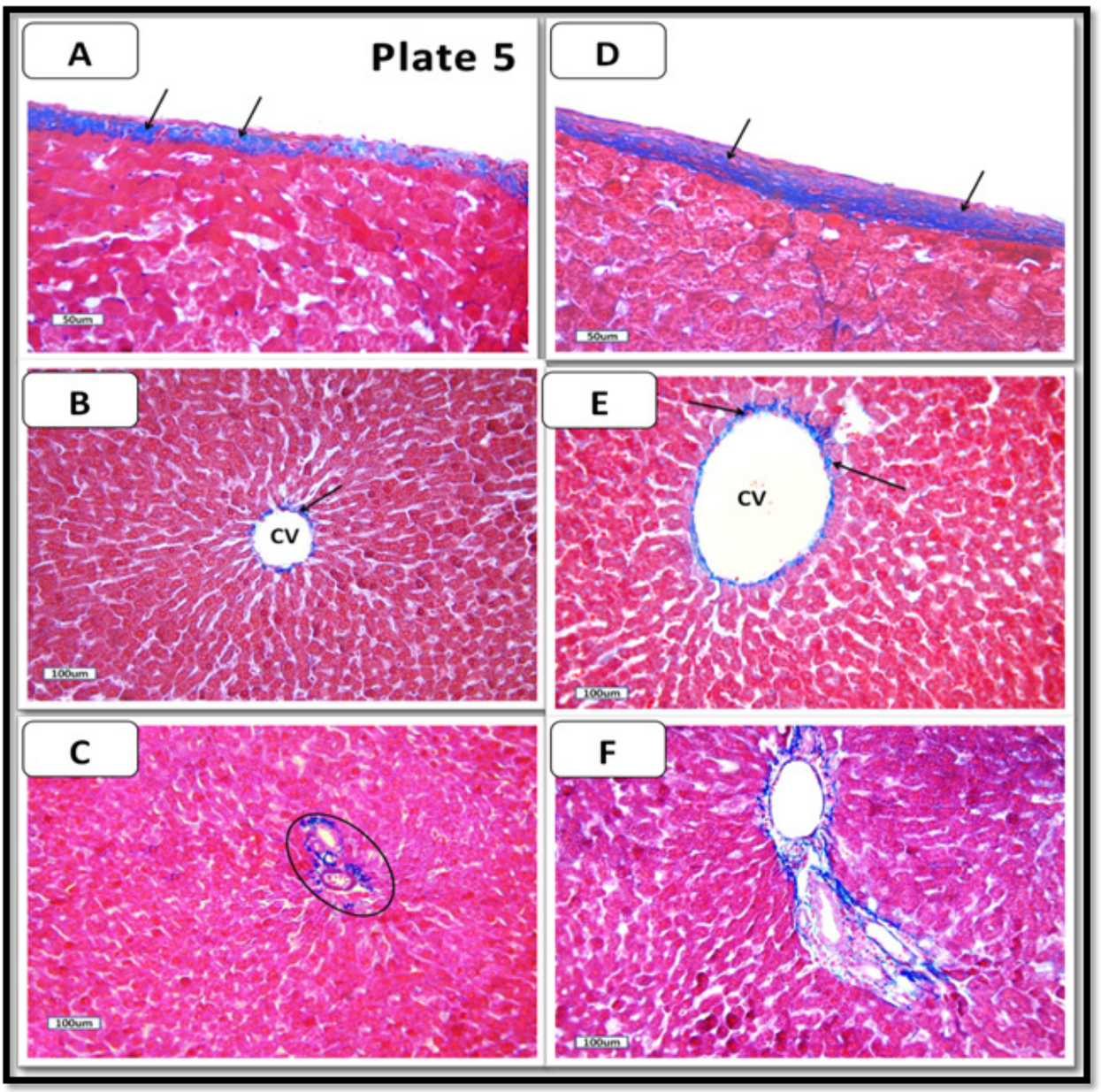


Plate 5: photomicrographs of liver sections in rats of normal control group (A- C) showing: a thin layer of corrugated collagenous fibers in the connective tissue capsule (arrows). Normal density and distribution of collagen fibers around the central vein (CV) and the portal tract (circle). (D-F) in normal group treated with Invokamet XR showing moderately thick layer of dense corrugated collagenous fibers in the connective tissue capsule, around the central vein (CV)(arrows) and at the portal tract area.

(Mallory's trichrome stain; all X200, except A&D X400)

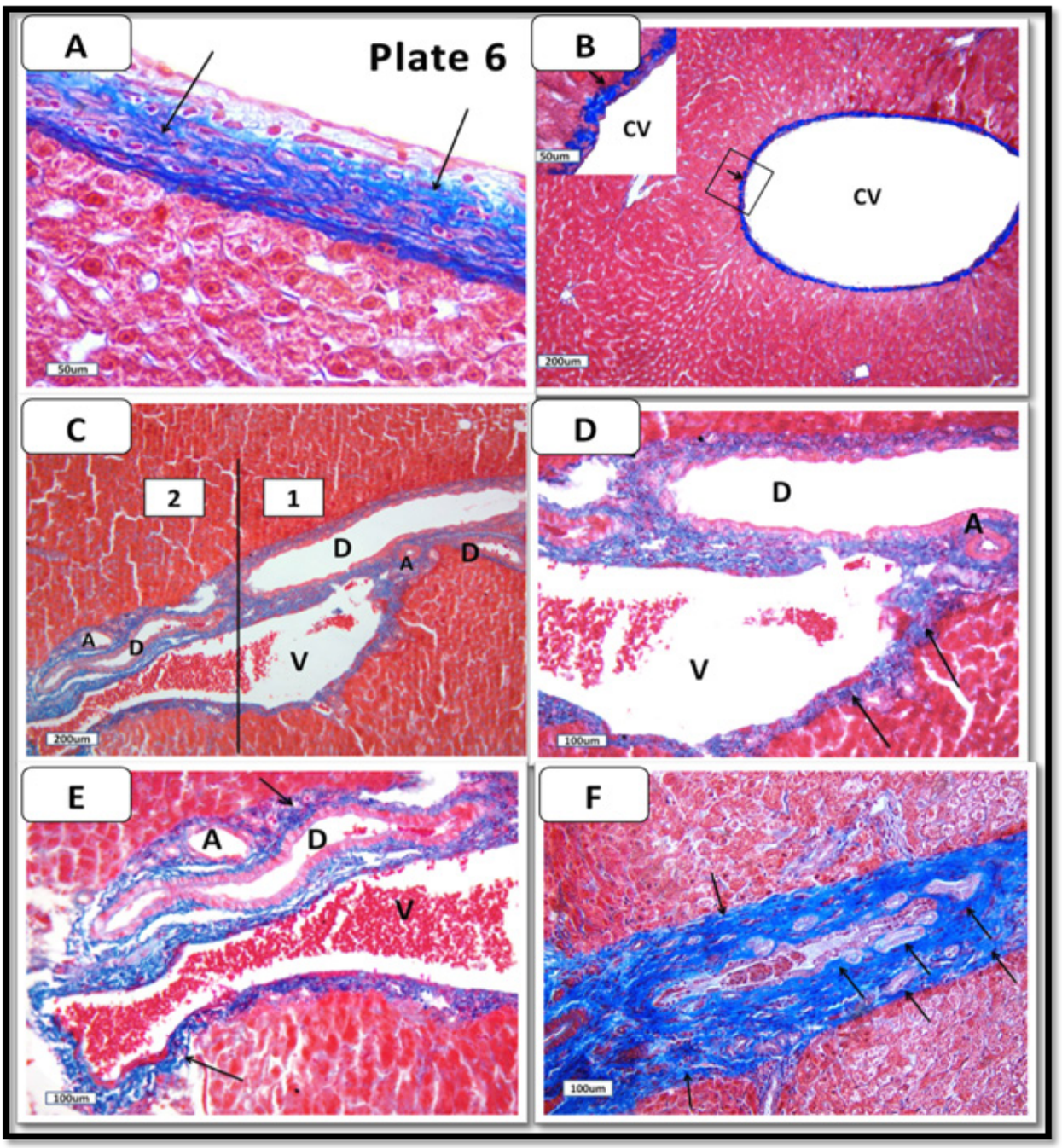


Plate 6: photomicrographs of liver sections of the diabetic group showing: (A) very thick layer of dense corrugated collagenous bundles in the connective tissue capsule (arrows). (B) dense corrugated collagenous fibers around the very dilated central vein (arrow) (C-F) at the portal tract areas that containing hepatic arteries (A), portal vein (V) and many bile ducts (D) (arrows). (D&E) higher magnification of area 1&2 in (C).

(Mallory's trichrome stain; A& the insert X400, B&C X100 and D-F X200)

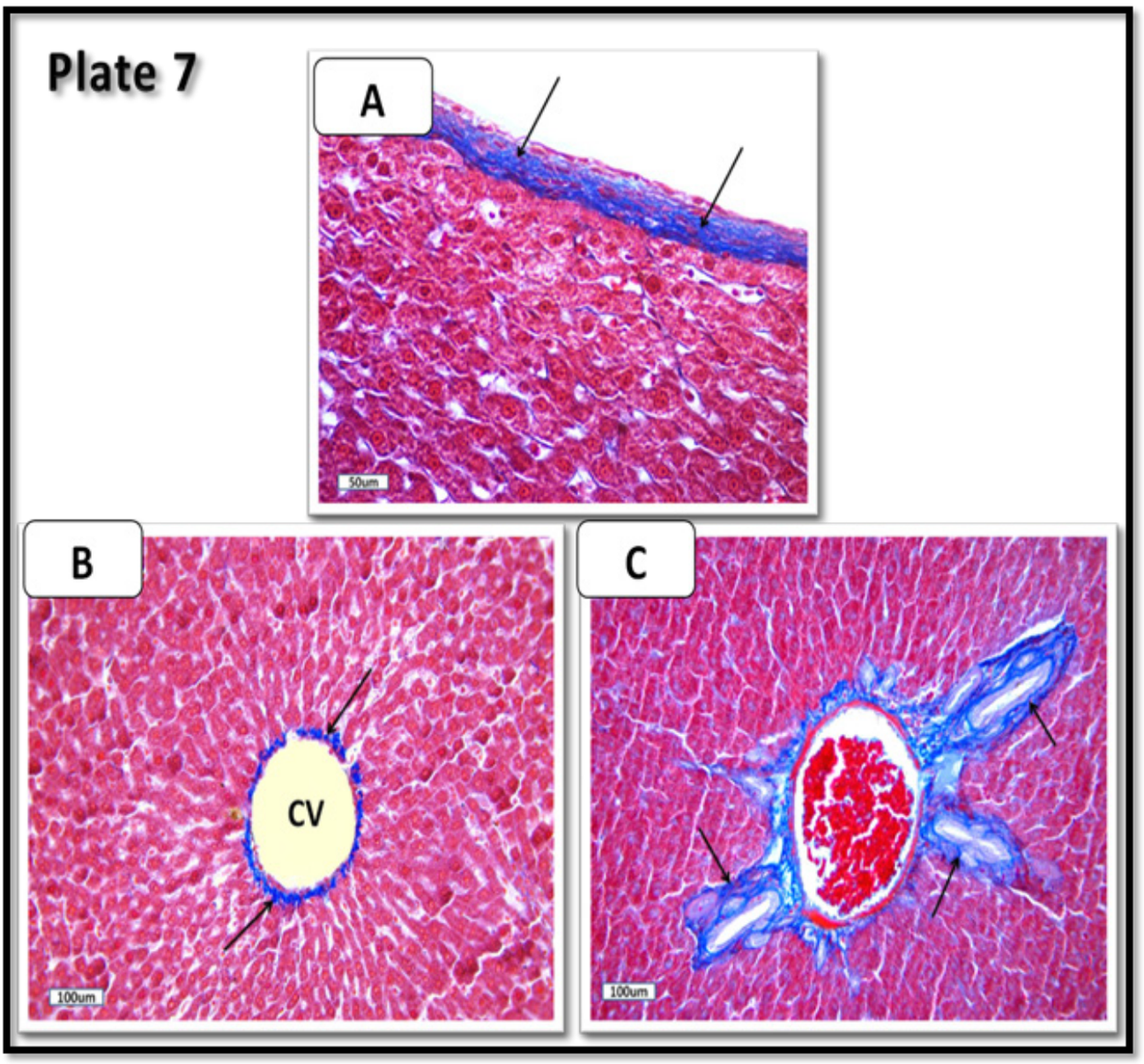


Plate 7: photomicrograph of sections of the diabetic group treated with Invokamet XR showing: (A) moderate thick layer of dense corrugated collagenous fibers in the connective tissue capsule (B) around the central vein (CV) and (C) at the portal tract area around the highly proliferated bile ducts (arrows).
(Mallory's trichrome stain; (A) X400; (B&C) X200)

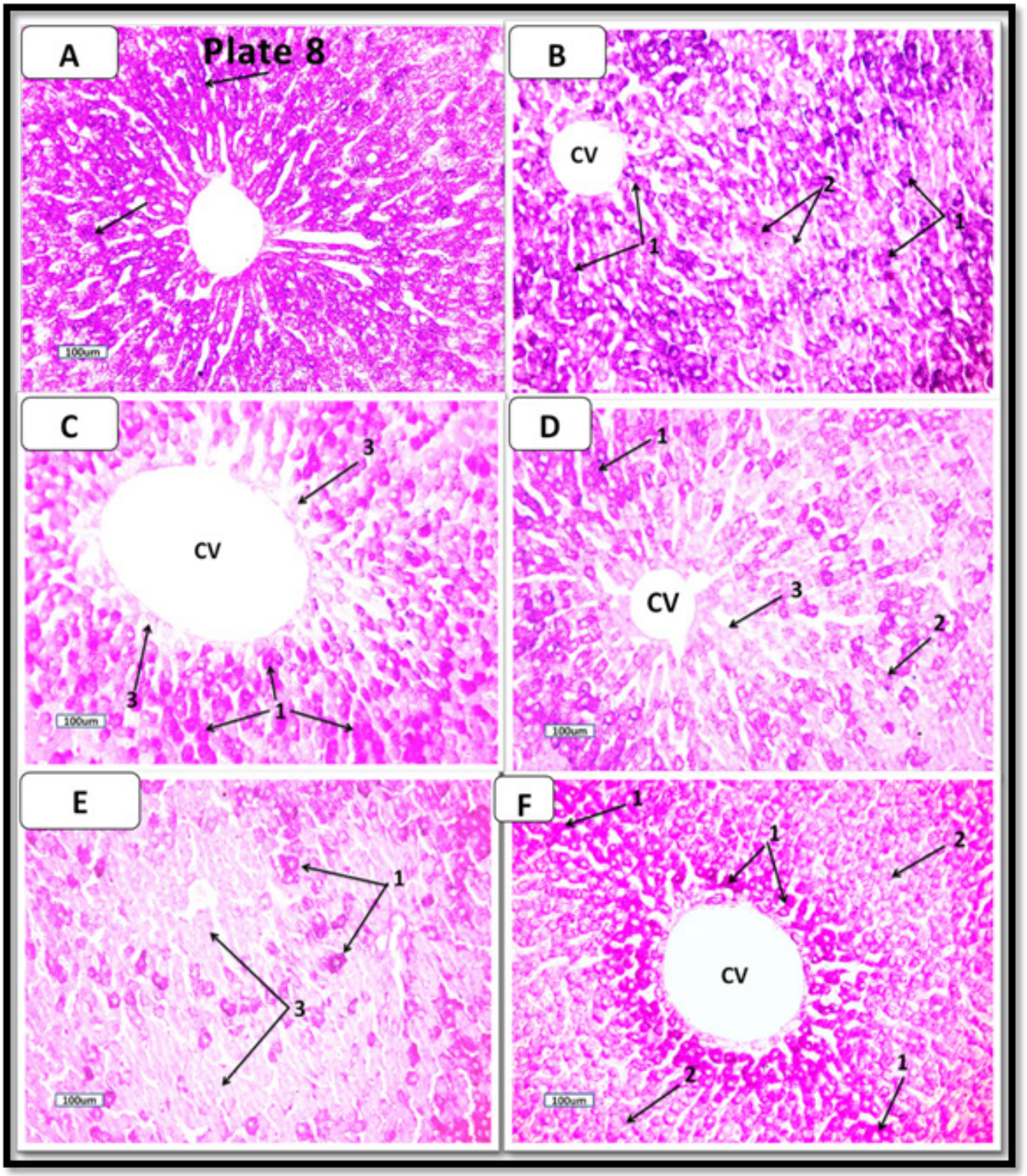


Plate 8: photomicrographs of liver sections of the all groups: (A) normal control group showing that the majority of the hepatocytes contain high glycogen content (arrows). (B) normal group treated with Invokamet XR showing that some of the hepatocytes contain high glycogen content (1→) while it is moderate in others (2→). (C-E) the diabetic group showing variable reactions in different sections, it is either high (1→), moderate (2→) or negative (3→). (F) diabetic group treated with Invokamet XR showing also variable reactions.

(PAS reaction; all X200)

Table 1: Comparison between the body weights (g) of all the studied groups along the time of the experiment

Studied Groups \ Time	Starting Point Mean ± SD	After one week Mean ± SD	After two weeks Mean ± SD	After three weeks Mean ± SD
The control group	170.0±9.5	177.8±9.8	194.4±12.6	205.7±13.3
Invokamet XR group	173.3±11.2[a]	171.1±11.2[a]	168.3±11.4[*a]	167.2±11.4[*a]
Buffer control group	172.2±6.7[a]	186.1±9.3[a]	201.1±11.7[a]	210±10[*]
Diabetic group	171.1±7.8[b]	166.6±8.6[**b]	156.2±8.5[**b]	148.8±9.3[**b]
Diabetic group treated with Invokamet XR	171.1±9.3[bc]	166.7±15.0[*bc]	163.3±15.0[**b*c]	161.7±13.2[**b*c]

*: Statistically significant (*P value* ≤ 0.05)
* *: Highly statistically significant (*P value* ≤ 0.001)
SD: Standard deviation
a: Compared to the normal control group
b: Compared to the buffer group
c: Compared to the diabetic group

Table 2: Comparison between the fasting blood glucose levels (mg/dl) of all the studied groups along the time of the experiment

Studied Groups \ Time	Starting Point Mean ± SD	After one week Mean ± SD	After two weeks Mean ± SD	After three weeks Mean ± SD
The control group	93.0 ± 14.6	102.2±2.5	102.0±12.8	99.1±3.3
Invokamet XR group	109.1 ± 40.9[a]	102.1±40.8[a]	96.89±14.9[a]	78.2±23.4[*a]
Buffer control group	92.6 ± 25.1[a]	106.0±10.8[a]	91.0±9.1[*a]	74.0±9.0[*a]
Diabetic group	511.7 ± 91.4[**b]	525.0±119.2[**b]	423.8±144.2[**b]	443.5±130.8[**b]
Diabetic group treated with Invokamet XR	473.4±114.2[*bc]	169.7±78[*b**c]	102.2±23[b**c]	93.7±25[b**c]

• Range of fasting blood glucose level in normal rats (70-110 mg/dl)[16]
*: Statistically significant (*P value* ≤ 0.05)
* *: Highly statistically significant (*P value* ≤ 0.001)
SD: Standard deviation
a: Compared to the normal control group
b: Compared to the buffer group
c: Compared to the diabetic group

Table 3: Comparison between the levels of Liver functions test enzymes of all the studied groups (IU/L) at the end of the experiment

Studied Groups \ Parameter	ALT Mean ± SD	AST Mean ± SD	ALP Mean ± SD
Normal control group	12.3 ± 1.8	17.0± 2.29	121.1± 3.8
Normal treated with invokamet XR	25.0±3.9[*a]	23.0±4.8[*a]	154.7±13.8[*a]
Buffer control	13.3±1.5[a]	22.6±0.52[a]	117.7±4.9[a]
Diabetic untreated group	35.0±7.6[**b]	66.4±16.9[**b]	205.2±31.4[**b]
Diabetic treated with invokamet XR	30.0±1.2[**b*c]	44.7±12.7[**b*c]	179.0±15.3[**b*c]

*: Statistically significant (P value ≤ 0.05)
* *: Highly statistically significant (P value ≤ 0.001)
SD: Standard deviation
a: Compared to the normal control group
b: Compared to the buffer group
c: Compared to the diabetic group
Ranges of normal liver enzymes of the rat (AST, ALT and ALP) are (50- 150), (10 - 40) and (30 - 130) IU/L respectively[17]
ALT: alanine aminotransferase
AST: aspartate aminotransferase
ALP: alkaline phosphatase

Table 4: Comparison between the mean values of area percentage of collagen and glycogen of all the studied groups

Parameter / Studied Groups	Mean area percentage of the collagen Mean ± SD	Mean area percentage of glycogen Mean ± SD
The control group	1.71±0.80	25.81±7.55
Invokamet XR group	2.20±0.97[a]	24.24±7.41[a]
Buffer control group	1.80±0.49[a]	25.30±5.24[a]
Diabetic group	10.69±3.77[b**]	17.74±4.17[b**]
Diabetic group treated with Invokamet XR	4.65±1.73[b*c*]	21.00±6.78[bc*]

*: Statistically significant (*P value* ≤ 0.05)

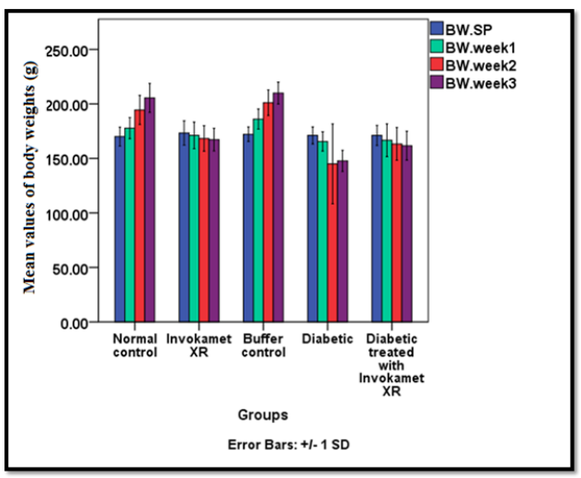
* *: Highly statistically significant (*P value* ≤ 0.001)

SD: Standard deviation

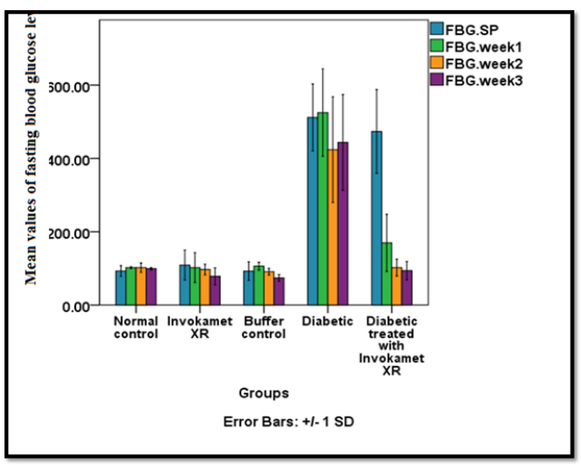
a: Compared to the normal control group

b: Compared to the buffer group

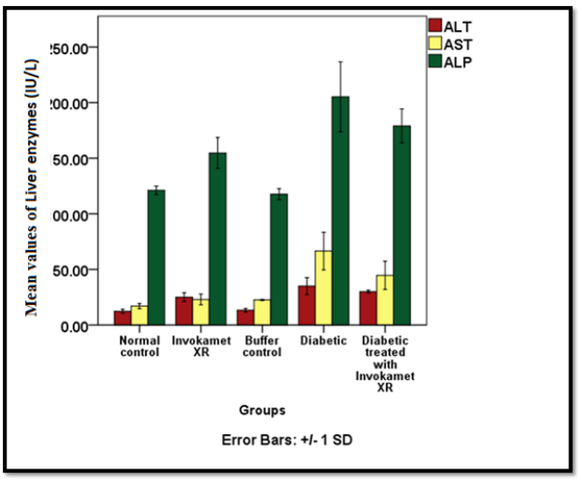
c: Compared to the diabetic group



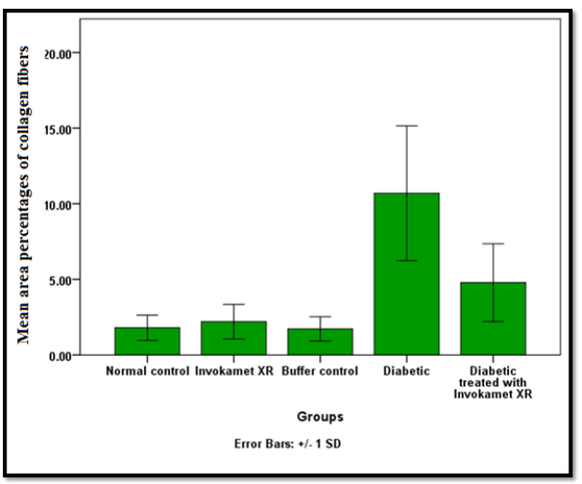
Histogram 1: Comparison between the body weights (BW) (g) of all the studied groups along the time of the experiment



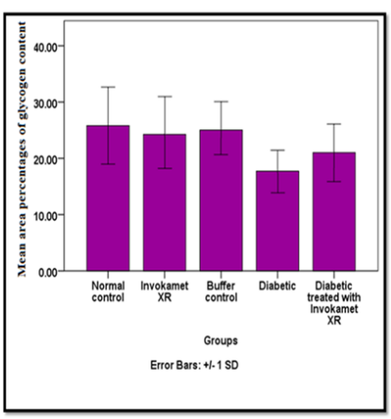
Histogram 2: Comparison between the fasting blood glucose levels (mgl/dl) of all the studied groups along the time of the experiment



Histogram 3: Comparison between the levels of Liver function tests' enzymes (IU/L) of all the studied groups at the end of the experiment



Histogram 4: Comparison between the mean area percentages of collagen fibers of all the studied groups



Histogram 5: Comparison between the mean values of area percentages of glycogen contents of all the studied groups

DISCUSSION

Diabetes is a growing public health and economic problem worldwide because of its possible complications. It is one of the leading causes of death[18]. Liver diseases are considered one of the leading causes of death in patients with diabetes[19]. This study aimed at determination of the effect of Invokamet XR on the liver of normal and experimentally induced diabetes mellitus in adult male albino rats, in a model of STZ-NA induced T2DM.

In the present study we used STZ-NA rat model in accordance to Okonkwo and Okoye[10] who stated that, streptozotocin injection caused β cells degeneration in rats; therefore, release of insulin by the pancreas was decreased resulting in hyperglycemia. Administration of streptozotocin and nicotinamide produced moderate hyperglycemia which has clinical similarities especially with respect to the insulin response to the glucose and as such streptozotocin nicotinamide (STZ-NA) is a method currently used to induce diabetes in animals that resemble non obese type 2 diabetes mellitus (DM) in man.

The marked decrease in the body weight in the diabetic animals, which was noticed in this study, was in agreement with Suganya *et al*.[20] and Ribola *et al*.[21]. They attributed that to degeneration of the fat cells and to destruction of structural proteins. The statistically significant increase in the mean body weight in the diabetic group treated with Invokamet XR, when compared to the diabetic group, may be due to a better control of hyperglycemic state by Invokamet XR, while in comparison to the buffer control group there was significant reduction in the mean body weight. These results are in agreement with Wilding *et al*.[22], Boyle & Wilding[23] and Ji *et al*.[24] who stated that canagliflozin had been associated with weight loss in T2DM compared with other anti- diabetic therapies.

The current study revealed that the mean body weight of the normal group treated with Invokamet XR showed statistically significant decrease when compared with that of the normal controls. This was in accordance with the findings of Hasnain *et al*.[25], Wang *et al*.[26] and Jarskog *et al*.[27]. They reported in their studies the effect of metformin in reducing the body weight in patients with diabetes mellitus, obesity, polycystic ovarian syndrome and Schizophrenia. Simes and Mac Gregor[28] added that there were multiple clinical trials currently investigating the effect of SGLT-2 inhibitors on obese individuals without diabetes. They were not effective enough as a monotherapy for obesity; but SGLT-2 inhibitors could potentially be effective in weight loss when combined with other drugs that reduce the consumption of food. Therefore, the reduction of body weight in our study in the diabetic treated group may be referred to the action of both metformin and canagliflozin.

In the present study, administration of Invokamet XR to diabetic adult male albino rats resulted in marked reduction of blood glucose level. This may be attributed to the hypoglycemic effect of Invokamet XR as mentioned by Fala[9] who reported that Invokamet XR (canagliflozin– metformin HCl combination) had a good glycemic control. Both agents lower glucose levels through different mechanisms. The present results also agree with Ribola *et al*.[29], Lentferink *et al*.[30] and Shiba *et al*.[31] who stated that the lowering of blood glucose level in patients used canagliflozin, is due to the reversible block of SGLT2 inhibitors. In addition, Inoue *et al*.[32] stated that canagliflozin stimulates fatty acid utilization, and reduces subcutaneous and visceral fat as well as hepatic fat in patients with T2DM and non-alcoholic fatty liver. Gabr & Mohammed[33] added that canagliflozin could be used as a supportive therapy in both types of diabetes because it showed the best results of lowering blood glucose and increasing insulin.

The observed increase in the liver enzymes in the diabetic group of the present study is in agreement with Okonkwo & Okoye[10]. They stated that AST and ALT reflects the concentrations of intracellular AST and ALT that have leaked into the general circulation and thus, serves as an indicator of hepatotoxicity. Rai *et al*.[34] and Makena *et al*.[35] recorded same results in diabetic animals. Komolkriengkrai *et al*.[36] added that the expression of ALP enzyme was increased when the bile ducts become blocked. Both AST&ALT are the main enzymes found in mitochondria of the liver. If liver damage is present, the enzymes are released into the bloodstream following liver cell death.

Improvement of the liver enzymes' levels that was encountered in the diabetic treated group is in agreement with Seko *et al*.[37] who reported that canagliflozin made a reduction of 30% or more in the baseline ALT level, which was associated with amelioration of the NAFLD activity score and progression of liver fibrosis in NAFLD patients. In addition, Leiter *et al*.[38] and Gautam *et al*.[39] found a significant reduction in serum AST and ALT with canagliflozin treatment of diabetic patients.

The liver sections of the normal and diabetic adult male albino rats' liver treated with Invokamet XR, showed dilatation and /or congestion of many central veins, blood sinusoids and portal veins. This agrees with Hawley *et al*.[40] and Uthman *et al*.[41]. They reported that canagliflozin had a direct vasodilator effect on diabetic patients. Sayour *et al*.[42] explained this finding. They stated that canagliflozin had a slight blood pressure lowering effect on healthy rats and enhanced endothelium-dependent vasorelaxation, which causes peripheral vasodilatation and decrease peripheral vascular resistance so reducing the expression of apoptotic and nitro-oxidative stress markers.

In the diabetic group, there was hepatic congestion and/or dilatation of the central veins, blood sinusoids and portal veins in many hepatic lobules. These results were in agreement with Rungseesantivanon *et al*.[43] who documented that nitric oxide (NO) is an important molecule that has many vascular functions. Diabetes induced inactivation of NO leading to vascular dilatation and diabetic vascular complications. Elmarakby & Sourour[44] and Al-Ani *et al*.[45] observed marked congestion of the central veins and hepatic sinusoids in diabetic rats' liver.

In the diabetic group, the increased hepatocyte acidophilia or vacuolation; nuclear pyknosis, degeneration or necrosis are in agreement with the results recorded by Oršolić *et al*.[46]. They observed that the liver sections of alloxan-induced diabetic mice showed several lesions as cytoplasmic eosinophilia and cellular vacuolation but with individual variability. Hassan *et al*.[47] and Elamin *et al*.[48] attributed the same results to inflammatory changes or ischemia and hypoxia. Elamin *et al*.[49] mentioned that the histopathological changes in STZ diabetic rats could be due to STZ or diabetes. The mechanism by which STZ and diabetes affect the liver could be through increased reactive oxygen species (ROS) production. Prolonged hyperglycemia increases ROS by autoxidation of glucose, which in turn leads to lipid peroxidation, and membrane damage, then cause hydropic degeneration.

In the present work, many cells appeared vacuolated in the diabetic group most probably due to fatty infiltration. This was in agreement with Oršolić *et al*.[46] who stated presence of variable size vacuole-like spaces in the hepatocytes of diabetic rats' liver. They referred that to increased quantity of fat inside the cells due to impaired metabolism of fatty acids. Panqueva[49] and Cao *et al*.[50] stated that vacuole formation in the hepatocytes is typical of apoptotic cells but the mechanism of vacuolation was not clear. Ipsen *et al*.[51] stated that hepatic steatosis reflects an imbalance between the uptake and synthesis of fatty acids by the liver and their oxidation and export as patients with T2DM have dyslipidemia. Hassan *et al*.[47] stated that cytoplasmic vacuolation was attributed to lipid peroxidation because of oxidative stress that damage cell membrane as well as membranes of cell organelles leading to increase in their permeability and disturbance of the ions concentrations in the cytoplasm and cell organelles.

The ballooned cells, which were noted in this research, are in agreement with Hassan *et al*.[47] and Shaker *et al*.[52] who attributed that to microtubular disruption and severe injury of the hepatocytes.

Our results revealed some improvement in the liver architecture of the diabetic adult male albino rats treated with Invokamet XR, where less vacuolation or deeply acidophilic hepatocytes were present. This was in agreement with Mookkan *et al*.[53] who found mild improvement in hepatic vacuolation in animals treated with metformin. In addition, Shiba *et al*.[31] stated that the lobular inflammation and hepatocyte ballooning reduced by canagliflozin treatment. Elamin *et al*.[48], Abd El-Motelb *et al*.[54] and Sindhuja *et al*.[55] indicated that metformin possesses antioxidant properties through reduction of ROS by inhibiting mitochondrial respiration and decreases advanced glycosylation end product, indirectly through reduction of the blood glucose and directly through an insulin-dependent mechanism.

In the current study, in the diabetic adult male albino rats' liver focal areas of leucocytic aggregation were observed in close relation to the central vein, portal tract and infiltrating the hepatic lobule. Tan *et al*.[56], Aboonabi *et al*.[57] and Alrashed & El-Kordy[58] recorded same results. They stated that the diabetic animals exhibit high oxidative stress due to chronic hyperglycemia that result in depletion of the antioxidant defense system and produce de novo generation of free radicals. ROS and lipid peroxidation cause direct damage to hepatocytes by destructing membranes, protein, and DNA which induce an inflammatory response and oxidative stress involves the secretion of cytokines, mainly tumor necrosis factor (TNF-α) and interleukin (IL-1). They attributed this to hepatocyte stress and/or damage that result in the release of signals that stimulate activation of other cells, particularly those of the innate immune system, including Kupffer cells, natural killer cells, and natural killer T- lymphocytes cells. These cells contribute to the progression of liver injury by producing pro-inflammatory mediators and secreting chemokines to produce further inflammatory cells to the liver.

Hassan *et al*.[47] and Bayomy *et al*.[59] explained that activated adipocytes (Ito cell or HSCs) secrete cytokines that attract inflammatory cells. Furthermore, many researchers had been reported that activation of HSCs might be closely related to the role of ROS and oxidative stress in stimulating the expression of proinflammatory and profibrotic molecules. All these factors contributed to the chronic inflammatory condition and the hepatocytes injury. This might initiate capillarization of the sinusoids. The latter was characterized by a progressive loss of fenestrae in the sinusoidal endothelial cells, in addition to development of a basal lamina, and deposition of collagen in the space of Disse. This might be accompanied by adhesion of leucocytes to the sinusoidal endothelium, followed by leucocytic infiltration into the hepatic parenchyma to form inflammatory foci.

In the current study, in either normal or diabetic animals treated with Invokamet XR, focal areas of leucocytic aggregation were also observed in some animals in close relation to the central vein, portal tract and infiltrating the hepatic lobule, but less than that in the diabetic group. This finding was not in agreement with Tahara *et al*.[60] and Kothari *et al*.[61] who mentioned that canagliflozin and Ipragliflozin reduced plasma and liver inflammatory markers in high-fat diet and STZ-NA T2DM mice and rats. Moreover, SGLT-2 inhibitors reduced leucocytosis induced by hyperglycemia and reduced inflammation and oxidative stress. In addition, Abd El-Motelb *et al*.[54] stated that metformin-treated diabetic animals showed a reduction in the proinflammatory cytokines. Metformin might show anti-inflammatory action through inhibition of advanced glycation products, which increase inflammation and ROS. This contradiction between our results and those authors may be due to the side effects of the combined drug on the liver or due to diabetes, which may need further studies.

Proliferation of bile ducts observed in the present study in diabetic adult male albino rats' liver at the portal tract areas,was in agreement with Hassan *et al*.[47]. They explained the proliferation of bile ducts as ductular reactions is characterized by an increase in the number of intrahepatic bile ducts that occur in response to insulin resistance,

impaired hepatocellular replication, and advanced stages of fibrosis, indicating progressive fibrosis.

In the present study, Mallory's trichrome stained sections of the diabetic animals showed significant increase in the collagenous fibers in the connective tissue capsule, around the central vein, perisinusoidal space and at the portal tract areas when compared with the control animals. Our results were in accordance with the finding of Elmarakby and Sourour[44] who observed that collagen fibers were increased in the liver of diabetic rats compared with the control animals. Khidr *et al*.[62] illustrated that lipid peroxidation induces oxidative damage to proteins and nucleic acids, which lead to increased collagen and ground substance formation. However, many authors have suggested that the fibrosis detected in diabetic liver is not related directly to diabetes, but it is due to an underlying genetic tendency rather than hyperglycemia itself and a liver vascular abnormality in the rat's strain. Hassan *et al*.[47] indicated that these findings might be attributed to activation of HSCs that leads to fibrosis. There is a cross-link between liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells. Healthy LSECs prevent activation of hepatic stellate cells and inactivate the activated ones. Before hepatic fibrosis, LSECs develop an altered phenotype called capillarization that loses the ability to prevent hepatic stellate cell activation and inactivate activated hepatic stellate cell. In addition, Komolkriengkrai *et al*.[36] and Mahmoud *et al*.[63] explained that hyperglycemia and hypoinsulinemia stimulate the proliferation of hepatic stellate cells and stimulates production of collagen, resulting in hepatic fibrosis. Connective tissue growth factor is produced by activated hepatic stellate cells and has been implicated in the development and progression of hepatic fibrogenesis, which transform into myofibroblast-like cells.

The diabetic adult male albino rats' liver treated with Invokamet XR showed partial decrease in the collagenous fibers around the central vein, perisinusoidal space and at the portal tract area when compared with those of the diabetic animals. This finding was explained by Kaji *et al*.[64] who stated that SGLT-2 inhibitors markedly inhibits liver fibrosis development in rats via suppression of hepatic Ito cells proliferation and collagen synthesis. Shiba *et al*. [31] mentioned that, canagliflozin reduce hepatic inflammation, fibrosis and nonalcoholic steatohepatitis.

In the present study, PAS stained sections of diabetic adult male albino rats' liver revealed that the majority of the hepatic lobules showed marked depletion of the glycogen content. Our results were in agreement with Ozougwu,[65] who stated that one of the functions of the liver is to maintain and control blood glucose with glycogenesis and glycogenolysis. Therefore, the hepatic glycogen content of the STZ treated animals was decreased. In addition, Komolkriengkrai *et al*.[36] and Khidr *et al*.[62] reported that in diabetes, the activities of glycogen synthase and hexokinase were diminished because of insulin deficiency, so glucose could not be transformed into glycogen; and glycogenesis was reduced and thus the amount of glucose increased. On the other hand, Makena *et al*.[35] showed that hepatic accumulation of fat and glycogen are common complications of diabetes mellitus, which were reported in 80% of patients with diabetes. Glycogen accumulates in patients with diabetes because of insulin deficiency that may increase synthase activity resulting in increased rate of glycogenesis and more glycogen accumulation. T1DM is not associated with fat accumulation if blood glucose level is well controlled. However, fat accumulation occurs in 70% of patients with T2DM regardless of blood glucose control.

The diabetic adult male albino rats treated with Invokamet XR showed that the liver glycogen content was increased than the diabetic ones but it still lower than normal. This finding was in agreement with Motshakeri *et al*.[66] who observed an increase in the glycogen content of the diabetic rats treated with metformin and stated that insulin regulates the entry of glucose into tissues and leads to glycogen storage.

Our morphometrical and statistical results confirmed the histological results. Either in the changes of mean area percentage of collagen fibers or in the glycogen content in the liver tissue among all the experimental groups.

CONCLUSION

In conclusion, our results revealed that Invokamet XR therapy for 3 weeks decreased the blood glucose level, the body weight, and the liver enzymes of the diabetic rats in addition to improving some histological changes in their liver. The drug has a potent anti-diabetic effect and consequently improves the other related (or dependent) metabolic processes. So, Invokamet XR represents a novel therapeutic approach in the management of type 2 diabetes but still need more studies to evaluate presence of major side effects on other organs.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة نسيجية على تأثير عقار كاناجليفلوزين وميتفورمين هيدروكلورايد ممتد المفعول (إنفوكاميت إكس آر) على كبد ذكور الجرذان البيضاء البالغة الطبيعية والمصابة بمرض السكري المستحث تجريبيا

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خلفية البحث: يمثل مرض البول السكرى مشكلة صحية عالمية حيث يعتبر السبب الثالث للوفاة بسبب مضاعفاته. إنفوكاميت إكس آر هو عقار جديد يؤخذ عن طريق الفم، مركب من كاناجليفلوزين بالإضافة إلى عقار الميتفورين هيدروكلورايد ممتد المفعول ويستخدم لعلاج مرض البول السكرى من النوع الثانى.

الهدف: تقييم تأثير عقار إنفوكاميت إكس آر على كبد ذكور الجرذان البيضاء البالغة الطبيعية والمصابة بمرض السكرى المستحث تجريبيا.

المواد والطرق: استخدم في هذا البحث خمسة وثلاثون ذكراً من الجرذان البيضاء البالغة التي تراوح وزنها بين ١٦٠ – ١٨٠جم ، تم تقسيمها بالتساوى الى خمس مجموعات وهى:المجموعة الأولى: المجموعة الضابطة الطبيعية ؛ المجموعة الثانية: مجموعة طبيعية عولجت بعقار إنفوكاميت إكس آر عن طريق الفم بجرعة ٢٠٠ ملليجرام/ كيلوجرام يومياً لكل جرذ؛ المجموعة الثالثة: مجموعة ضابطة منظمة تم إعطاؤها نصف ملل من محلول الصوديوم سيترات المنظم لكل جرذ مرة واحدة عن طريق الحقن فى التجويف البريتونى؛ المجموعة الرابعة: مجموعة مصابة بمرض البول السكرى؛ المجموعة الخامسة: مجموعة مصابة بمرض البول السكرى عولجت بعقار إنفوكاميت إكس آر. تم إحداث مرض البول السكرى من النوع الثانى فى المجموعة الرابعة والخامسة عن طريق حقن عقار ستربتزوتوسين ٣٥ مليجرام/ كيلوجرام داخل الغشاء البريتونى بعد حقن نيكوتيناميد ١١٠ ملليجرام/ كيلوجرام داخل الغشاء البريتونى بحوالى ١٥ دقيقة. فى نهاية التجربة،بعد ثلاثة أسابيع، تم تجميع عينات الدم للتحليل الكيميائى لإنزيمات الكبد. تم أخذ عينات من الكبد لفحصها بالميكروسكوب الضوئى. وقد تم قياس وزن الجسم ومستوى الجلوكوز الصائم فى الدم، هذا بالاضافة الى إجراء دراسة نسيجية قياسية مع التحليل الاحصائى.

النتائج: أظهرت الدراسة الحالية أن مرض البول السكرى من النوع الثانى أحدث زيادة كبيرة فى انزيمات الكبد مع فقدان النسيج الهيكلى الطبيعى مثل احتقان واتساع الأوعية الدموية ، تسلل الخلايا الإلتهابية، ظهورفجوات بالخلايا الكبدية مع تغلظ الأنويه. وقد أحدث إنفوكاميت إكس آر تحسنا ضئيلا فى تلك التغييرات النسيجية فى حيوانات السكرى.

الإستنتاج: نستنتج من الدراسة السابقة أن عقارإنفوكاميت إكس آر لديه تأثير قوى مضاد لارتفاع السكر بالدم ولكن لديه حماية طفيفة بشأن التغييرات البيوكيميائية والنسيجية التى يسببها مرض البول السكرى من النوع الثانى وقد يعزى ذلك إلى تحسين مستوى السكر فى الدم وتقليل المضاعفات المرتبطة بمرض البول السكري.