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RESEARCH ARTICLE

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HISTOPATHOLOGICAL STUDY ON THE TOXICITY EFFECTS OF SORAFENIB (MULTIKINASE INHIBITOR)ON THE ENDOCRINE AND EXOCRINEPANCREAS IN MALE ALBINO RATS

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A R T I C L E I N F O

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A B S T R A C T

Introduction and aim of the work: Sorafenib (Nexavar) is an oral inhibitor of multi-kinase proteins approved in 2005 for treatment of metastatic renal cell and advanced hepatocellular carcinoma. It causes many metabolic side effects, including diarrhea, hypertension, hand-foot skin reaction, and fatigue. This study aims to detect the histopathological changes of the rat pancreas under acute and chronic sorafenib treatment. **Methods:** The rats were divided into 3 groups. • Group 1: served as control (rats were orally administrated with ml of normal saline for a month. • Group 2: (acute group) Rats of this group were treated with the multikinase inhibitor sorafenib (60 mg/kg body weight/day) for 15 days by gavage. • Group 3: (chronic group) Rats of this group were treated with the multikinase inhibitor sorafenib (60 mg/kg body weight/day) for 30 days by gavage. Animals were sacrificed and specimens from the pancreatic tails were processed for histopathological, histochemical; by estimation of total carbohydrates, total mucine & collagen fibers and immunohistochemical studies by estimation of Anti-insulin antibody. **Results:**In treated animals, there were histopathological and histochemical alterations Immunohistochemical staining with anti-insulin antibody showed strong staining of the islets of treated animals with highly significant ((P<0.05)) increase than control rats. **Conclusion:** Sorafenib treatments caused pathological and toxic changes in the pancreas which need to careful using of this drug and may use of natural antioxidants will be useful.

INTRODUCTION

The pancreas is located on the right side of the abdominal cavity. The pancreas consists of a pale, elongated gland, situated in the interduodenal area and formed by ascending and descending duodenal loops. It is associated with the digestive system and is an exocrine as well as endocrine gland. The exocrine portion secretes basic electrolytes and digestive enzymes, whereas the endocrine portion secretes hormones such as insulin, glucagon, somatostatin, and pancreatic polypeptides (9, 19&32).

Tiwari AK, added that its hormones such as insulin regulate carbohydrate metabolism in the body and maintains passage of glucose across the cell membrane. Whereas its enzymes help in the digestion of carbohydrates, fats, and proteins (33). According to Singh V; chemotherapy is an essential component in the treatmentof cancer. While these therapies aim to inhibit cancerous tissue growth, both systemic and localized therapies are known to have adverse effects on normal tissues (29). Morgan C, detected that chemotherapy-induced pancreatitis is well documented.The onset of pancreatitis is variable and may range from hours to 1 month after drug administration (18).

One from this chemotherapy drugs is sorafenib. Sorafenib (Nexavar) is a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature .Sorafenib is an oral inhibitor of multi-kinase proteins approved in 2005 for treatment of metastatic renal cell carcinoma. It has also been approved for treatment of advanced hepatocellular carcinoma (17).

Ratain MJ, found that side effects associated with sorafenib include diarrhea, hypertension, hand-foot skin reaction, and fatigue (22).Where Van Erp NP, explained that sorafenib is metabolized primarily in the liver and undergoes oxidative metabolism mediated by cytochrome P450 3A4 isoform (CYP3A4), as well as glucuronidation mediated by uridine diphosphate glucuronyl transferase 1A9 (UGT1A9) (34).

In this respect, we aimed to investigate the toxicity of sorafenib on the endocrine and exocrine pancreas.

MATERIALS AND METHODS

Chemicals: sorafenib (Nexavar, 200 mg), was a kind gift obtained from the medical union pharmaceutical drug company (MUP), Egypt, imported from Germany.

Animals: Tow-month old (120 - 150 g body weight) male albino rats (*Rattus rattus*) were selected from animal house of National Research Center, Giza, Egypt. The animals were housed under controlled environment conditions (12 h light/dark cycle) at a temperature of 25˚C + 10˚C and

humidity of 60% + 5% and fed standard diet and water Ad libitum for the experimental period.

Experimental protocol: The rats were randomly divided into 3 groups of 12 animals each as follows:

- Group 1: served as control (rats were orally administrated with ml of normal saline for a month.
- Group 2: (acute group) Rats of this group were treated with the multikinase inhibitor sorafenib (60 mg/kg body weight/day) for 15 days by gavage.
- Group 3: (chronic group) Rats of this group were treated with the multikinase inhibitor sorafenib (60 mg/kg body weight/day) for 30 days by gavage.

Where the dose of sorafenib calculated according to the normal human dose; 400mg\day and converted to rat dose

Examinations

Rats of each group were sacrificed by cervical dislocation at the end of the experimental periodsn. Pancreas of each animal was obtained and fixed in buffered neutral formalin 10% solution for 24 hrs, dehydrated through alcohols, cleared in xylene and embedded in paraffin wax. Five-micrometer thickness paraffin sections were prepared and mount on clean slides. **For histopathological studies**, according to **(**Drury, R.A.B. and Wallington, E.A. such as sections were stained with Ehrlich.s hematoxylinand counterstained with eosin (6)**.For histochemical investigations,**the periodic acid Schiff's (PAS) technique of **Hotchkiss** was used for the detection of total carbohydrates; 1, 2 glycol group (12).Masson's trichrome stain was done for demonstration of collagen fibers according to Drury, R.A.B. and Wallington, E.A. (6).Alcianblue (AB) at pH 1.0 and 2.5 was used to determine weakand strong mucins, respectively. Meanwhile, AB at pH 2.5in combination with PAS staining (AB/PAS) was used forneutral and acid mucins. The combination of aldehyde(AF) and AB at pH 2.5 (AF/AB) was used to differentiatebetween sulfated and carboxylated mucins (2).

For immunohistochemical studies; other sections were de-paraffinized, placed on charged slides, and used for localization of **Anti-Insulin; Insulin Antibody**.

Anti-Insulin employed to stain the cells in an avidin–biotin-complex (ABC) immunoperoxidase technique. Specifically, the sections were incubated in 5% H_2O_2 (in methanol) solution for 10 min to block endogenous peroxidase activity and then incubated with primary **Anti-Insulin**rabbit monoclonal antibody (1:50 dilution in 1% bovine serum albumin solution; Pan-T Clone SP7, Thermo Scientific, Lab Vision, Fremont, CA) for 60 min at room temperature. After rinsing with phosphate-buffered saline (PBS, pH 7.4) to remove unbound primary antibody, the samples were incubated with diaminobenzidine (DAB) chromogenic solution for 5 min at 25°C. The sections were then counterstained with haematoxylin for 15 sec. **(**15**).**

For statistical analysis, each section was counted manually at high power (X400) after identifying at low power (x100). The representative areas with the highest concentration of stained cells were detected according to the recommendation of **Cohen and Hogan (1994); (4)**.About 1000 cells/slide were counted in each of five microscopic fields from well-labeled areas to determine the average of **Anti-Insulin**Labelling index. **Anti-Insulin** was expressed as number of labeled cells (positive for **Anti-Insulin**) as a percentage of the total number of cells counted in each specimen. All identifiable staining was regarded as positive.

Statistical analysis

The obtained results of **Anti-Insulin**expressed as mean + Standard Error (SE). They were also statistically analyzed by using the SPSS11 computer software program (ANOVA) analyses.

RESULTS

Histopathological results

The histological structure of the control pancreas of group 1 consisted of closely packed lobules of pancreatic acini. The acini are formed of pyramidal cells with basal nuclei and apical acidophilic cytoplasm. Islets of Langerhans were embedded within the exocrine portions and a cells located on the periphery (Figure. 1A). The present light microscopic study of acute group rats (group 2; rats treated with sorafenib for 15 days) revealed pathological changes of both exocrine and endocrine part of the pancreas represented by high congestion of pancreatic duct and a large irregularly shaped islet of Langerhans which looks that there is a sprouting of a new islet from a preexisting one. Some exocrine acini revealed focal acinar damage represented by cytoplasmic vacuolation and pyknotic nuclei of some acinar cells (Figure. 1B).

However the pancreatic sections of rats treated with sorafenib for 30 days (chronic group, group 3) showed more pronounced degenerative and necrotic changes, leading to wide architectural disruption. Few sections also showed decreased cellular density of the islets of Langerhans, with vacuolations in the islet and focal lymphocytic aggregation, i.e. chronic inflammatory infiltrate in the form of lymphocytes indicating immune response against β cells. Some of the sections showed significantly enlarged islets of Langerhans. Although the normal architecture was destroyed, the pancreatic acinar epithelium appeared disrupted. Interlobular ducts of the pancreas of this group appeared wider with irregular outline (Figure 1 C).

Histochemical results

PAS staining showed positive thick basement membranes of the islets capillaries of treated animals of acute group (group 2; rats treated with sorafenib for 15 days) (Figure 1 E) but highly positive reaction of treated animals of chronic group treated with sorafenib for 30 days (Figure 1 F), compared with the mild positive relatively thin basement membranes of the islets capillaries of control animals of group 1 (Figure 1 D)

By Masson trichrome stains, the control pancreas of group 1 showeda normal structure characterized by the presence of delicate collagen fibers in the septa and around the pancreatic acini. The collagen fibers were seen to incompletely surrounding the islets and around the blood capillaries between the endocrine cells of the islets (Figure 1 G).

Pancreas sections of acute group rats (group 2; rats treated with sorafenib for 15 days) showed atrophy in the islands of Langerhans cells associated with dense collagen fibers around the acini (Figure 1 H).

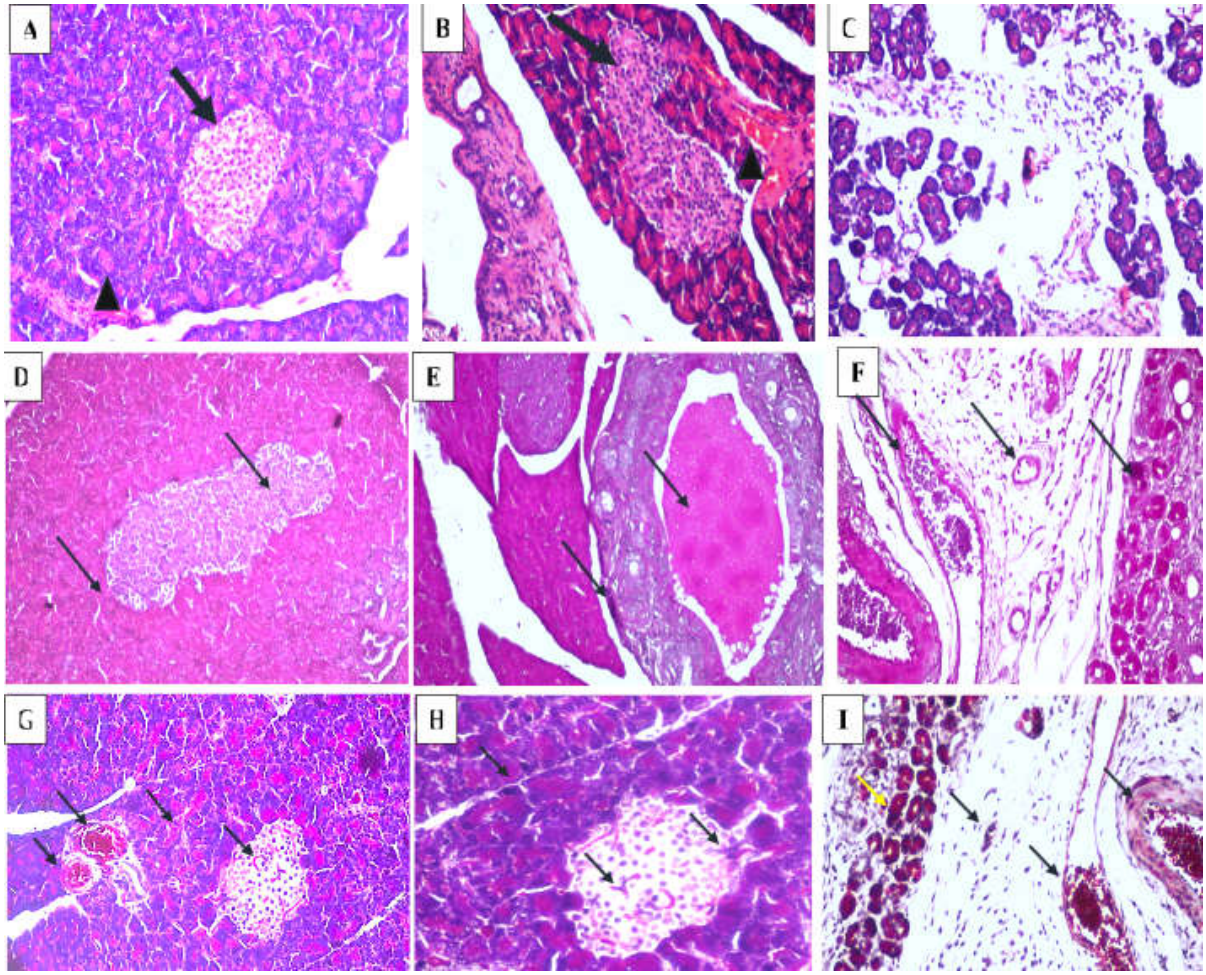

Figure 1 (A) A photomicrograph of the pancreas of a control animal group 1 showing; a rounded islet of Langerhans (arrow) with an adjacent regularly rounded interlobular duct and the pancreatic lobules (head of arrow)(***HE stain, ×400.***). **(B)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 15 days as acute treatment group 2 showing; a large irregularly shaped islet of Langerhans. It looks that there is a sprouting of a new islet (arrow) from a preexisting one in addition high congestion of interlobular duct (head of arrow) (***HE stain, ×400.***). **(C)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 30 days as chronic treatment group 3 showing; high degeneration and atrophy of islet Langerhans and pancreatic lobules in addition high necrosis of interlobular cells (***HE stain, ×400.***).**(D)**A photomicrograph of the pancreas of a control animal group 1 showing mildly positive basement membrane of the capillary network **(↑)** within an islet of Langerhans and pancreatic lobules (***PAS stain, ×400.***). **(E)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 15 days as acute treatment group 2 showing; a relative increase in the positivity of the basement membrane of most blood capillaries (↑) within a large elongated islet of Langerhans and pancreatic lobules (***PAS stain, ×400.***).**(F)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 30 days as chronic treatment group 3 showing; erosion of Langerhans and high positivity of the basement membrane of blood capillaries and pancreatic lobules (↑) (***PAS stain, ×400.***).**(G)** A photomicrograph of the pancreas of a control animal group 1 showing; the stained periductal collagenfibers within the interlobular and interlobar connective tissue and islet of Langerhans (↑)(***Masson's trichrome stain, ×200***).**(H)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 15 days as acute treatment group 2 showing;the relative increase in periductal collagen fibers within theinterlobular connective tissue and the appearance of interacinar collagen fibers (↑)(***Masson's trichrome stain, ×400***).**(I)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 30 days as chronic treatment group 3 showing; H ighincrease in periductal collagen fibers within theinterlobular connective tissue and the appearance of interacinar collagen fibers (↑)with high erosion of Langerhans and pancreatic lobules (***Masson's trichrome stain, ×400***).

But Thickening and hypertrophy were detected in the media with swelling in the endothelium of the intima of the congested stromal blood vessels surrounded by collagen, dense collagen fibers around the acini with some vacuoles in the pancreatic sections of rats treated with sorafenib for 30 days (chronic group, group 3) (Figure 1 I).

Negative reaction of the pancreatic acini to Alcian Blue was recorded in both acute and chronic groups of treatment by sorafenib when compared to the mild positive reaction of control group. (Figures 2 B, C & A respectively).

Immunohistochemical results

Immunohistochemical staining with the **anti-insulin antibody**; revealed that the positive staining of the islets of control animals group 1 was concentrated mainly in their center (Figure 2 D). In treatedanimals, of acute group rats (group 2; rats treated with sorafenib for 15 days) and chronic group rats (group 3; rats treated with sorafenib for 30 days) there were obvious increase in the intensityof the positive reaction which seemed to occupy thewhole islet mass (Figures 2 F & H respectively). But at acute group 2 the intensity of the positive reaction was more condensed and pronounced than the chronic group 3. Moreover, the immunostaining of the islets themselveswas deep brown in treated animals (Figures 2 G & I respevtively) comparedwith the orange brown color observed in the control islets (Figure 2 E).

The mean optical density of **Anti-insulin antibody** expression for all groups presented in Table 1. There was a significant increase ($P<0.05$) in **Anti-insulin antibody**expression in Sorafenib-intoxicated, acute group 2 with mean value 52.70 $\pm$ 2.25compared with control group 1 with mean value 32.00 $\pm$ 0.97.There was a significant increase ($P<0.05$) in Sorafenib-intoxicated, chronic group 3 with mean value 41.60 $\pm$1.05 when compared with control group 1.

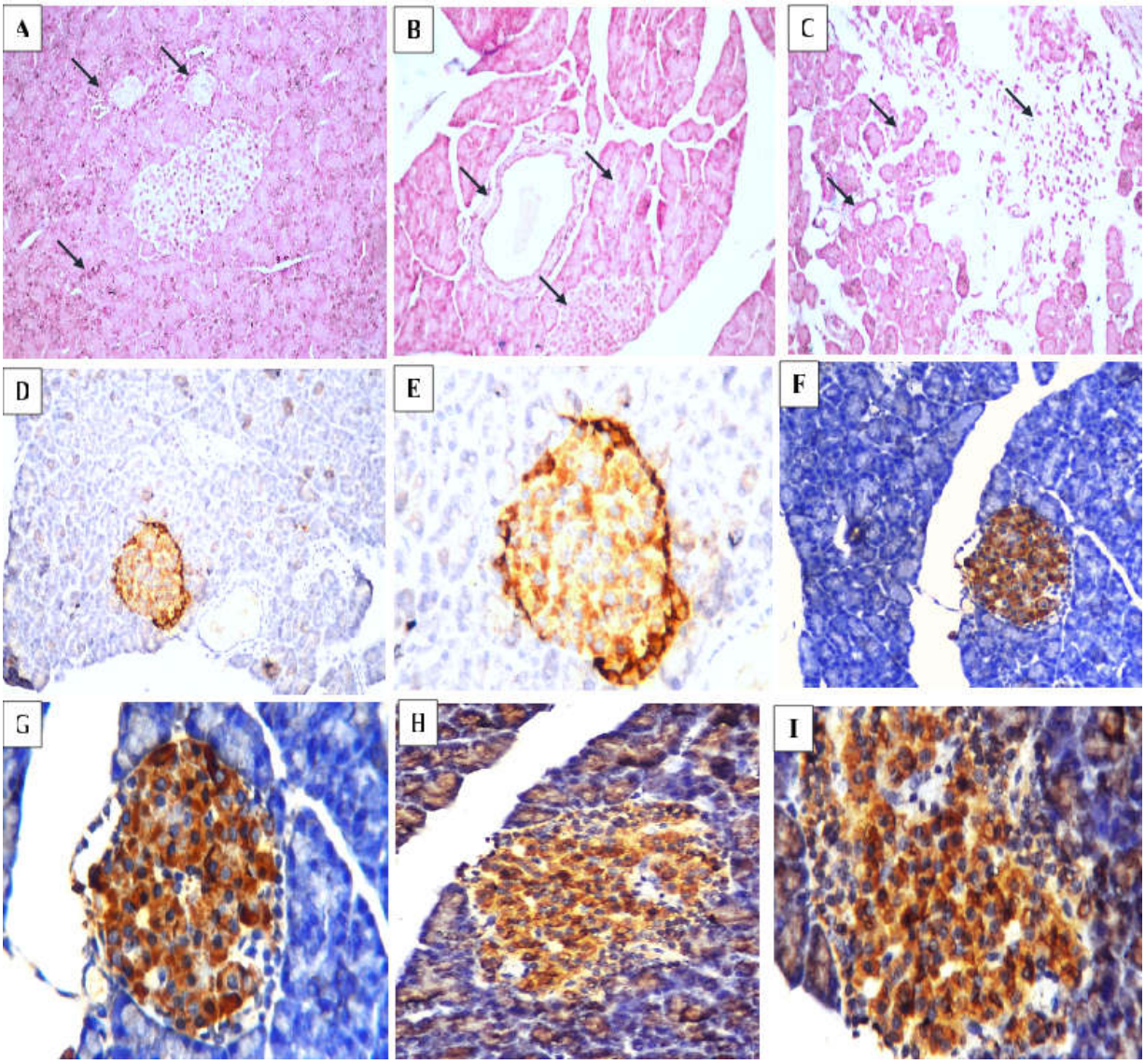

Figure 2 (A)A photomicrograph of the pancreas of a control animal group 1 showing; a mild positive reactionaround an adjacent regularly rounded interlobular duct and in-betweenthe pancreatic lobules (↑) (***Alcian blue stain, ×400.***). **(B)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 15 days as acute treatment group 2 showing; a negative reaction around the irregularly shaped islet of Langerhans and interlobular duct as well as pancreatic interlobules(↑)(***Alcian blue stain, ×400.***). **(C)**A photomicrograph of the pancreas section of rat treated with Sorafenib for 30 days as chronic treatment group 3 showing; high degeneration and atrophy of islet Langerhans and pancreatic lobules in addition high necrosis of interlobular cells with negative reaction of stain(↑)(***Alcian blue stain, ×400.***).**(D& E)**Photomicrographs of the pancreas of a control animal group 1 showing;the orange brown staining of the cytoplasm ofbeta-cells of an islet (***Immunostaining with anti-insulin antibody, ×200 & 400 respectively***).**(F & G)**Photomicrographs of the pancreas section of rat treated with Sorafenib for 15 days as acute treatment group 2 showing;deep brown staining of the cytoplasm of nearly all the cells of an islet (***Immunostaining with anti-insulin antibody, ×200 & 400 respectively***).**(H & I)**Photomicrographsof the pancreas section of rat treated with Sorafenib for 30 days as chronic treatment group 3 showing; erosion of Langerhans and high positivity of thedeep brown staining of the cytoplasm of the remaining cells of the islet (***Immunostaining with anti-insulin antibody, ×200 & 400 respectively***).

But **Anti-insulin antibody** expressions more pronounced increased at Sorafenib-intoxicated acute group 2 in mean value 52.70 ± 2.25when compared with Sorafenib-intoxicated chronic group 3 in mean value 41.60 ±1.05.

Table 1 Means ofAnti-insulin antibodyvalues recorded at different treatments

Parameters Groups	Mean + SE	P value
control, group 1	32.00 ± 0.97	--
Sorafenib-intoxicated, acute group 2	52.70 ± 2.25	< 0.05
Sorafenib-intoxicated, chronic group 3	41.60 ±1.05	< 0.05

DISCUSSION

Improving of therapy management and drug selection in cancer therapy depending on identifying predictive biomarkers of drug response. Sorafenib (Nexavar) is one from the oral multitargeted tyrosine kinase inhibitor with potent antiangiogenic activity. According to Escudier B, Sorafenib was first approved to treat renal-cell carcinomabut it is also active in other solid tumors, such as hepatocellular and thyroid carcinomas (8).

Ratain MJ, detected that there were side effects associated with sorafenib include diarrhea, hypertension, hand-foot skin reaction, and fatigue (22). Amar S and Li M, added that prolonged use of sorafenib may cause cumulative, serious, and life-threatening late toxic effects due to the long-term effect of antiangiogenic therapies on the microvessels also, sorafenib-associated with pancreatitis (1 & 16).

In this study, the histopathological examination of the pancreatic tissues and islets of sorafenibtreated animal's revealed signs of hyperplasia in the form of large sized

irregularly-shaped islets, hyper atrophy and necrosis. Hamsa TP & Kuttan G. and Owari M, mentioned that sorafenib treatment has been associated with the generation of free radicals and ROS, which produce oxidative stress where the free radicals generated in the tissues constitute the biochemical basis of the toxicity of sorafenib (11 & 21).Chandrasekara N and Shahidi F. explained that oxidative stress occurs when oxidative substances disturb the oxidant-antioxidant balance and cause oxidative damage to deoxyribonucleic acid, proteins, and lipids (3).

Srivastava A and Shivanandappa T demonestrated that sorafenib treatment can deplete the GSH level, which may be due to the direct conjugation of metabolites of sorafenib, acrolein with GSH, producing oxidative stress where the depletion of GSH lowers the cells' defense against injury induced by free radicals lead to necrotic cell death (30).

In this study, Masson's trichrome stain revealed ahighly significant increase in theperiductal collagenfibers within the interlobar and interlobular connectivetissue, and the appearance of interacinar collagen fibers of both acute and chronic treatment by srafenib.Riccillo FL,detected a similar observation in pancreas of old rats and was described as interstitial fibrosis which is composed of cystic pancreatic hyperplasia associated with increase in collagen fibers (23). Also Yasuda H., found in long-term diabeticdogs, atrophy, hyperplastic nodules, and interacinar and interlobular fibrosis were observed suggesting that insulinopenia may cause injurious effects on exocrine pancreas producing interacinar fibrosis (35)

PAS stain was used in this study to delineate thebasement membranes of the islets capillaries.An obviousand highly significant thickening of the capillary basementmembranes was noticed in the present study in treatedanimals. It was previously observed over two decades of research that hyperglycemia was the primary causal factor mediating basement membrane thickening. Where Roy S, explained that the major contributing factor to basement membrane thickening was the excess synthesis of its components and vascular basement membrane thickening is afundamental structural alteration of small blood vesselsin cases of diabetes (25). Also Stitt AW., said that it was considered the majorand earliest morphological characteristic of diabeticmicroangiopathy in many organs such as the retina, kidneys, muscle and skin of both diabetic humans anddiabetic animals (31).

In our study a negative reaction of the pancreatic acini to Alcian Blue was recorded in both acute and chronic groups when compared to the mild positive reaction of control group. This is referred to the toxic and erotic effect of sorafenib to the pancreatic cells that led to prevent the protective effects and transportation of pancreatic secretion of neutral, acidic, and mixed mucosubstances in the epithelium of pancreas (14 &28).

In this study, immunohistochemical staining with anti-insulin antibody revealed increased amount and intensity of positive beta-cells within the islets of treated animals. They appeared deep brown in color and high significant instead of the orange brown color of the positive cells of the control.So this immunohistochemical investigations indicated the histochemical results by PAS, Masson's trichrome and Alcian blue staining. Where Schneider HM, indicated a similar finding observed in islets of diabetic patients and was attributed to the staining not only of the stimulated beta-cells but to the staining of the surrounding extracellular matrix as well. The staining of this matrix was attributed to the deposition of extracellular amyloid composed mainly of insulin and proinsulin (27).

A previous immunohistochemical studyof galactosemic rats indicated that there was an increasein labeling densities of collagen type IV and lamininin thickened basement membranes of retinal capillariesalong with the expression of interstitial collagens likecollagen type III and an abnormal collagen that weaklycross-reacts with antibody to collagen type I (5).Moreover, Nikolova G, mentioned that laminins were identified as endothelialsignals that promote insulin gene expression andproliferation of beta-cells (20).

According to Junqueira LC, it seems that islets hyperplasia usually results from beta-cell hyperplasia. Despite the fact that the pancreatic islets of Langerhans are multi-hormonal endocrine micro-organs that contain four principle types of hormone secreting cells, the predominant cell type is the insulin-secreting beta-cells (13). Previous studies reported that one possible pathomechanism for hyperglycemia induced by the sorafenib is the induction of insulin resistance (7).Scheen AJ, added that beside their major role in increasing insulin resistance, these drugs could markedly and selectively impair cholinergic-stimulated insulin secretion by blocking muscarinic M2 receptors, which could be one of the contributing factors to their higher risk for producing hyperglycemia and diabetes leading to inadequate compensation by the beta-cells (26).

Rother KI, indicted that there were two types of beta-cell hyperplasia, primary and secondary. Primary beta-cell hyperplasia is associated with hyperinsulinemia and hypoglycemia, while secondary hyperplasia is usually associated with insulin resistance and hyperglycemia (24). From the previous studies, it is suggested that the sorafenib - associated beta-cell hyperplasia observed in this study at acute group 2 treatment by sorafenib for 15 days is related to the secondary type, which is associated with insulin resistance and hyperglycemia. Where the anti-insuline antibody was significantly increased at this group than the control group with large sized irregularly-shaped islets.

Hyperplastic changesof the pancreatic islets were also experimentally observedin sand rat with obesity-diabetes syndrome. It wasobserved that the number of islets was higher thannormal (polynesia), with the islets themselves enlarged(macronesia) and double islets in the secretory ducts ofthe exocrine pancreas were frequent (10).Beta-cell plasticity is a unique property of these cellsto adapt their number and volume (beta-cell mass), and their function to the increased secretory demand linkedto insulin resistance.This may described the significant increase of anti-insulin antibody at chronic group 3 of sorafenib treatment for 30 days than the control group however there were high atrophy and cell degeneration at pancreas lobules and Langerhans at this group.

CONCLUSION

Collectively, the histopathological, histochemical and immunohistochemical changes observed in the endocrine and exocrine pancreas of the sorafenib (nexavar)-treated rats in

the present study were very similar to those occur in type 2 diabetes mellitus and highly oxidative reagent. For this reasons, it is important for patients under sorafenib treatment to be adequately monitored for the occurrence of glucose metabolism abnormalities at least at a three months interval during therapy. Also, a careful selection of patients is a good preventive measure before starting sorafenib treatment,avoiding those with high-risk factors for diabetes suchas obese patients or those with family history of diabetes. Also, the antioxidant natural products treatment with patient under sorafenib treatment may important to decrease the side effects of this drug.

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