Clinicopathological Studies on the Effect of Some Antidiabetic Substances in Rabbits

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ABSTRACT

Seventy clinically healthy white New Zealand rabbits divided into 4 main groups to evaluate the antidiabetic activity of glimepiride (amaryl) and propolis. Ten rabbits (gp.1) were used as negative control and untreated.The remaining sixty rabbits I/P injected with alloxan (50 mg / kg b.wt) freshly dissolved in sterile saline (50 mg/ml) and injected (I/V) with 10 ml of glucose 5% and supplied with 10% glucose in the drinking water for the first 24 h after the alloxan injection then after one week till induction of diabetes ,the diabetic rabbits were divided into 3 groups .Gp.(2) was kept without treatment allover the experimental period (+ ve control).Gp.(3) was treated with amaryl at a dose of (0.018 mg/kg b. wt daily for 2 months) orally. Gp.(4) was treated with propolis at a dose of (53.3 mg/kg b. wt daily for 2 months) orally.5 animals from each groups were slaughtered after one and two months Blood samples were collected into clean, dry, sterile and left to clot and serum was separated by centrifugation at 3000 rpm for 15 min.The obtained serum was used for determination of diabetic markers, lipid peroxidation (MDA) and antioxidant enzymes (CAT and SOD). Pancreas from all groups was collected after the 1st and 2nd months of treatment for histopathological examination.The results revealed the antihyperglycemic and antioxidant effect of amaryl and propolis on alloxan-induced diabetic rabbits. Both amaryl and propolis led to improvement of diabetic markers (blood glucose, serum fructosamine and insulin),in addition to significant increase of antioxidant enzymes activities (serum Catalase and Superoxide dismutase) and significant reduction in Malondialdehyde.

INTRODUCTION

Diabetes mellitus is a complex syndrome characterized primarily by the imbalance in blood glucose leading to hyperglycemia and a series of secondary complications caused by lack of insulin *(1)*. Abnormalities in lipid profile are one of the most common complications in diabetes mellitus, which is found in about 40% of diabetics. Diabetes induction causes an increase in the cholesterol, triglycerides, LDL and VLDL.The elevation of serum lipids is usually represents the risk factor for coronary heart disease *(2)*. Inspite of the presence of hypoglycemic agents in the pharmaceutical market (insulin, sulphonylureas, biguanides and thiazolidinediones) *(3)*, diabetes and its related complications continue to be a major medical problem. However search for new antidiabetic drugs continues.

Sulphonylureas have represented the backbone of non insulin dependant diabetes mellitus (NIDDM) therapy for more than 30 years. The insulinotrophic effect of sulphonylurea is augmented by glucose and they apparently increase beta cell sensitivity to glucose and non-glucose stimuli. Glimepiride has been developed for glycemic control in diabetic patients and represents the third generation sulphonylurea. It effectively inhibits the development of oxidative stress in diabetes by possessing a potent extrapancreatic effect on glucose metabolism and may directly stimulate glucose transport activity through phospholipid signaling pathway *(4)*.

Remedies from medicinal plants are used with success to treat diabetes, because of their perceived effectiveness, minimal side effects in clinical experience and relatively low costs *(5)*. Most of these plants have been found to contain substances like glycosides, alkaloids, terpenoids, and flavonoids that are frequently implicated as having antidiabetic effects. Plant drugs and herbal formulation are frequently considered to be less toxic and free from side effects than

synthetic one *(6)*. Propolis has attracted public interest since it is a natural product with many biological properties. It has been used since ancient times in folk medicine in many parts of the world. Propolis has shown pharmacological activities such as antioxidant, antiviral, antibacterial, antifungal, antiamoebic, anti-inflammatory and antidiabetic. Propolis exerts some of its anti-inflammatory and anti-infective properties through the inhibition of dihydrofolate reductase activity, which plays an important role in the rapidly dividing cells, such as bacteria or uncontrolled growing tissues like tumors *(7)*. More than 180 propolis constituents have been identified by gas chromatography-mass spectrometry (GC-MS). These compounds can be grouped as follows: free aromatic acids; flavonoids; benzyl, methylbutenyl, phenylethyl, cinnamyl, and other esters of these acids; chalcones and dihydrochalcones; terpenoids and others as sugars, ketones, and alcohols. No anatomical abnormality was observed after oral propolis administration, suggesting the absence of side effects after propolis treatment *(8)*.

The aim of the present work was to evaluate the efficacy of Glimepiride (Amaryl) and Propolis as antidiabetic substances by some biochemical, parameters and histopathology.

MATERIAL AND METHODS

Experimental rabbits

A total of seventy white New Zealand apparently healthy rabbits of average 500 gm body weight were obtained from the animal house, Faculty of Veterinary Medicine, Zagazig University. Animals were kept under hygienic conditions, housed in metal cages and fed on balanced ration and water *ad–libitum*. The animals were acclimatized for one week before starting the experiment.

Glimepiride (Amaryl): 20 tablets each tablet contains 1 mg glimepiride.It was produced by Sanofi-aventis Egypt s.a.e under licence of Sanofi-aventis/Germany.

Propolis: propolis powder (Ethanolic extract 70% Dosic Imp. &Exp.Co., Ltd.)

Alloxan: It was obtained from Sigma Chemical Co. St. Louis, MO, USA. All the used chemicals were of the analytical grade.

Experimental design

Animals group ,treatment and time of sampling are shown in Table (1).

Table 1. Experimental design

Group	No. of rabbits	Treatment			sampling
		alloxan (50 mg / kg b.wt)	amaryl (0.018 mg/kg b. wt)	Propolis (53.3 mg/kg b. wt)	
Gp.(1) (- ve Control)	10	-	-	-	After 1st and 2nd months from starting treatment
Gp.(2) (Placebe gp.)	20	+	-	-	
Gp.(3) Diabetic treated with amaryl	20	+	+	-	
Gp.(4) Diabetic treated with propolis	20	+	-	+	

+ means group received the substance.
- means group didn't received the substance.

Sampling

A- Blood Samples

Blood samples were collected from the marginal ear vein . The blood samples were collected into clean, dry, sterile and labeled centrifuge tubes. The blood was allowed to flow smoothly into the tubes, left to clot and serum was separated by centrifugation at 3000 rpm for 15 min. The clear supernatant serum was aspirated gently by Pasteur pipettes. The obtained serum was transferred to dry, sterile and labelled stoppered vials for determination of diabetic markers, lipid peroxidation and antioxidant enzymes *(9)*.

B- Tissue specimens

Five animals from each group were slaughtered after 1st and 2nd month post-treatment. Pancreas from all groups was collected for histopathological examination.

Measurements of diabetic markers:

Glucose was determined by glucose RTU kits of BioMerieux according to *(10)*.Insulin concentration was determined in serum by radioimmunoassay method using a commercially available DSL-1600 insulin kit (Diagnostic Systems Laboratories, Inc., USA). Insulin values were expressed as µlU/ml. *(11)*.Fructosamine was determined by nitro blue tetrazolium method (**12**)using kits of Quimica Clinica Aplicada S.A. (QCA).

Measurements of antioxidant enzymatic activities

Catalase *(13)*.Superoxide dismutase activity *(14)* and MDA *(15)* were measured.

Histopathological examination

Pancreas from all groups was collected after the 1st and 2nd months of treatment for histopathological examination *(16)*.

Statistical analysis

The obtained data were analyzed using F-test *(17)*. Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a.

RESULTS AND DISCUSSION

Uncontrolled diabetes leads to increased hepatic glucose output. First, liver glycogen stores are mobilized and then hepatic gluconeogenesis is used to produce glucose. Insulin deficiency also impairs non hepatic tissue utilization of glucose. In particular, insulin stimulates glucose uptake in adipose tissue and skeletal muscle. Reduced glucose uptake by peripheral tissues leads to a reduced rate of glucose metabolism. The combination of increased hepatic glucose production and reduced peripheral tissues metabolism leads to elevated plasma glucose levels and weight loss which are the most seen symptoms of diabetes *(18)*.Gp.(2) (alloxan induced diabetic rabbits) showed highly significant increase in plasma glucose and fructosamine levels in addition to highly significant decrease in serum insulin level as shown in Table 2, these results previously obtained by *(19, 20)*. Our observations in this study correlates with the previous research findings, in that the blood glucose levels significantly increased in alloxan untreated diabetic rabbits due to the effect of alloxan which induces diabetes by damaging the insulin secreting cells of the pancreas leading to hyperglycemia *(21)*. Our results confirmed by the histopathological results of pancreas which showing severe vacuolar degeneration and necrosis of the β-cells in the islets of Langerhans .In some cases, the islets of Langerhans were atrophied, destructed and replaced by fibrous connective tissue (fig.1 and 2). Glimepiride (amaryl) treated group (gp.3) showed highly significant decrease in the plasma glucose and fructosamine with significant increase of serum insulin level toward the control range. A decline of blood sugar level following glimepiride treatment observed in the present study is in total agreement with earlier workers *(22)*. Sulphonylurea bind to specific receptors on beta cells resulting in closure of potassium ATP channels and subsequently open calcium channels leading to an increase in cytoplastic calcium that stimulates insulin release *(23)*.There is much controversy about the mode of action of sulphonylurea and specifically whether they lower blood glucose through extra pancreatic

mechanisms other than stimulation of insulin secretion *(24)*. However, studies suggest that glimepiride has a potent extra pancreatic effect on glucose metabolism and may directly stimulate glucose transport activity through phospholipid signaling pathway *(25)*. Glimepiride lowers blood glucose effectively without much effect on fasting insulin levels. These results suggest that glimepiride lowers blood glucose not only by stimulating insulin secretion but also by its extrapancreatic effects. The pancreas revealed atrophy, edema and vacuolations of the islets of Langerhans (Fig.3). On the other hand, our data showed that treatment of the diabetic rabbits with ethanolic extract of propolis (EEP) in gp. (4) resulted in significant decrease in serum glucose, fructosamine with significant increase in serum insulin due to the potential ability of propolis to delay glucose release from maltose or disaccharides at the small intestinal membrane.Propolis may exert an antihyperglycemic effect through the inhibition of glucose production from dietary carbohydrates *(26)* or due to the potential antidiabetic activity of propolis for the treatment of insulin-insensitive diabetes by its ability to inhibit the expression of G6Pase . Oral treatment of STZ-induced diabetic rats with ethanolic extract of propolis (EEP) at a dose of 200 mg/kg b.wt ,daily for 5 weeks ameliorated alterations in the animal body weight as well as serum glucose, lipids profile by decreasing the serum glucose *(27)* on the other hand it has been reported that ethanolic extract of propolis (10 and 90 mg/kg b.wt/ P.O.) administration for seven days to STZ-induced diabetic rats did not influence the glycemia *(28)* .The difference in results may be due to difference in doses, durations and animal species. The pancreas showed some extent of atrophy of the islets of Langerhans. The exocrine pancreas revealed activated acini with escape of the zymogenic granules on the surrounding tissue (Fig.4).

Table 2. Glucose, Insulin and Fructosamine (mean values ± S E) in rabbits in gps. (1-4) after one and two months of treatment.

Periods / Groups	1st month			2nd month		
	Glucose mg/dl	Insulin µIU/ml	Fruct. µmol/l	Glucose mg/dl	Insulin µIU/ml	Fruct. µmol/l
Gp.(1) (-ve Control)	93.08 d ±2.04	15.67 a ±0.48	219.86 c ±7.39	96.56 d ±2.29	19.20 a ±0.83	220.41 c ±5.86
Gp.(2) (Placebe gp.)	346.40 a ±18.66	6.50 d ±0.37	444.89 a ±16.44	248.08 a ±9.43	9.89 d ±0.24	313.30 a ±6.25
Gp.(3) Diabetic treated with amaryl	200.18 c ±6.49	9.10 c ±0.23	310.79 b ±32.33	156.12 c ±11.70	11.62 c ±0.54	262.01 b ±4.54
Gp.(4) Diabetic treated with propolis	244.98 b ±21.05	11.81 b ±0.69	336.51 b ±11.54	188.22 b ±7.53	13.65 b ±0.50	310.45 a ±6.54

Much attention has been focused on the role of oxidative stress and it has been suggested that oxidative stress may constitute the key and common events in the pathogenesis of different diabetic complications *(29)*. In the serum of gp. 2 (alloxan diabetic rabbits), lipid peroxidation levels as evidenced by MDA determination increased significantly as compared to the control group during the experiment period. SOD and CAT activities were constantly and significantly decreased in alloxan diabetic rabbits as compared to normal rabbits as shown in Table 3. Similar finding was observed previously *(19)*. In our study; MDA (as an indicator of LPO) levels in diabetes group were found to be higher than those in control group, indicating increased free radical generation. Enzymatic antioxidant such as SOD and CAT are considered primary enzymes since they are involved in the direct elimination of reactive

oxygen species (ROS) *(30)*. SOD is an important defense enzyme and scavenges O_2 – anion form H_2O_2 and hence diminishes the toxic effects due to this radical or other free radicals derived from secondary reaction *(31)*. CAT is a hemoprotein, which catalyzes the reduction of hydrogen peroxides *(32)* and known to be involved in detoxification of H_2O_2 concentrations *(31)*. Persistent hyperglycemia leads to increased production of free radicals *(33)*. The antioxidant enzymes such as SOD and CAT are known to be inhibited in diabetes mellitus as a result of non-enzymatic glycosylation and oxidation *(34)*. In our study, the activities of SOD and CAT decreased in diabetic rabbits as reported earlier *(29)* which could be due to inactivation caused by alloxan-generated ROS.Recently, diabetics and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes de novo free radicals generation *(35)*. Oxygen free radicals react with all biological substances; however, the most susceptible ones are polyunsaturated fatty acids. Reactions with these cell membrane constituents lead to lipid peroxidation. *(36)*. Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors *(30)*.

Diabetic rabbits treated with amaryl (gp. 3) showed significant increase of antioxidant enzyme activities (CAT and SOD) and decrease of serum free radical (MDA) might be due to less production of free radicals and lack of rise in lipid peroxidation (LPO) level *(37)*. Administration of glimepiride at a dose of 175 mg/kg b.wt orally daily for 4 weeks was enhanced the serum levels of catalase (CAT), superoxide dismutase (SOD) in streptozotocin (STZ)-induced diabetic rats *(38)*. Diabetic rabbits treated with propolis (gp. 4) showed significant increase of antioxidant enzyme activities (CAT and SOD) and decrease of serum free radical (MDA).The antioxidant effects of bee honey was attributed to its constituents of the most important antioxidant trace elements and to the antioxidant activity of its flavonoid compounds. Therefore bee honey has been suggested to be able to decrease the nitric oxide and lipid peroxidation. Similarly propolis was reported to decrease lipid perodxidation *(39)*.

It could be concluded that alloxan in low doses, produces an non-insulin dependant diabetes mellitus (NIDDM) like state, which can progress to a gradual recovery .Amaryl and propolis have antidiabetic and antioxidant activity. Although amaryl showed more antidiabetic activity but also propolis showed more antioxidant activity.

Table 3. Malondialdehyde (MDA), Catalase and Superoxide dismutase (SOD) levels (mean values ± S E) in rabbits in gps. (1-4) after one and two months of treatment.

Periods / groups	1st month			2nd month		
	MDA nmol/ml	Catalase kU/l	SOD U/ml	MDA nmol/ml	Catalase kU/l	SOD U/ml
Gp.(1)(-ve Control)	6.24 c ±0.32	52.43 a ±2.51	80.93 a ±3.23	6.22 c ±0.38	57.27 a ±2.48	84.07 a ±2.28
Gp.(2) (Placebe gp.)	28.80 a ±1.37	21.31 c ±0.38	41.60 d ±1.05	16.99 a ±0.81	45.15 c ±2.23	64.22 c ±1.74
Gp.(3) Diabetic treated with amaryl	13.97 b ±0.61	42.16 b ±1.54	59.32 c ±1.37	10.72 b ±0.33	50.83 bc ±1.68	75.69 b ±3.01
Gp.(4) Diabetic treated with propolis	11.96 b ±0.88	46.88 b ±1.64	74.24 b ±1.52	6.45 c ±0.41	54.90 ab ±1.65	86.38 a ±2.97

Means within the same column carrying different letters are significant at $P \leq 0.05$.

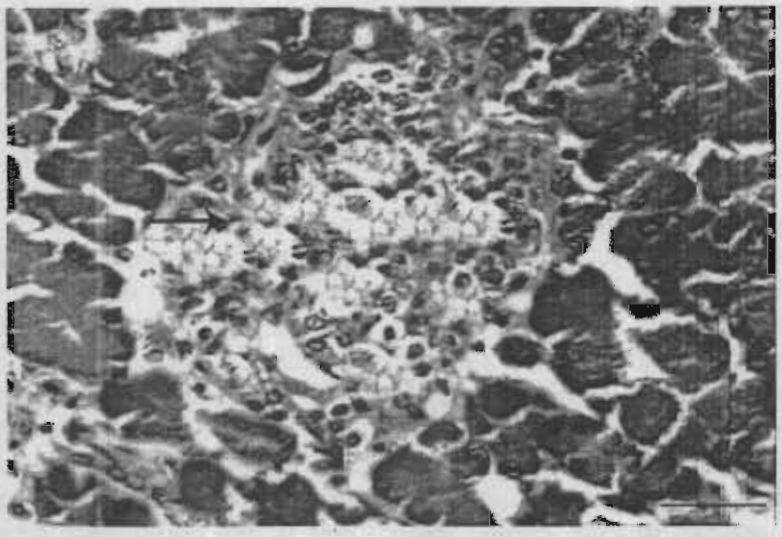

Fig. 1. Photomicrograph of the pancreas section of rabbit of gp. (2) showing severe vacuolar degeneration in the β-cells in the islets of Langerhans (arrow), HE (Bar = 100 µm).

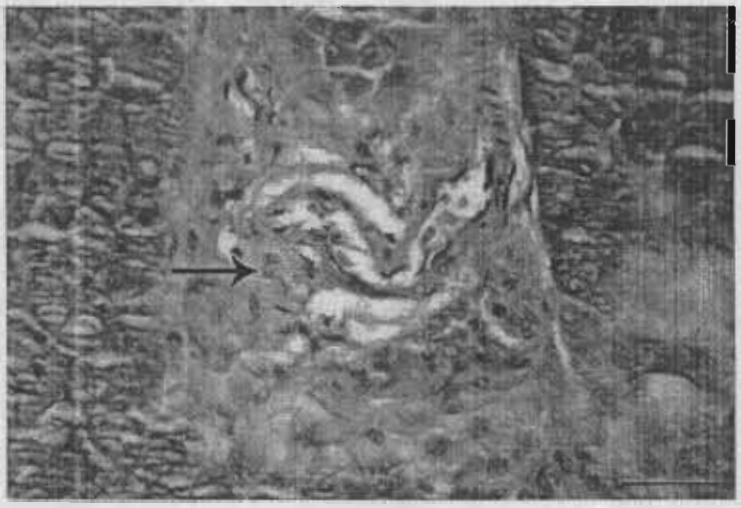

Fig. 2. Photomicrograph of the pancreas section of rabbit of gp. (2) showing atrophy and fibrous connective tissue replacing the islets of Langerhans (arrow), HE (Bar = 100 µm).

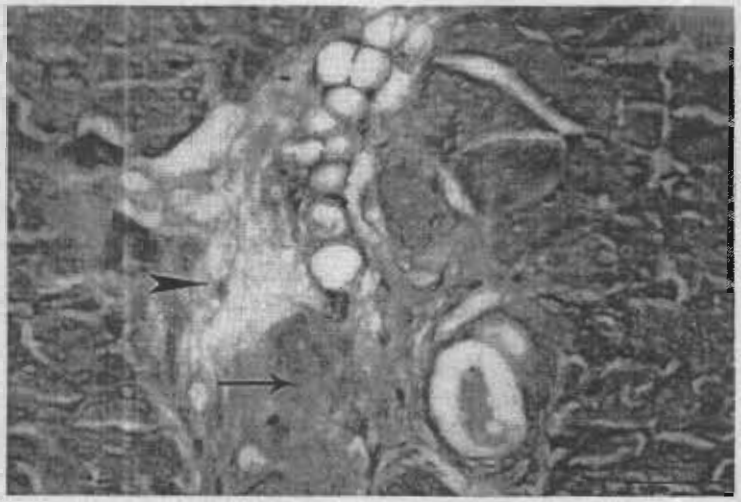

Fig. 3. Photomicrograph of the pancreas section of rabbit of gp. (3) showing atrophy (arrow), edema (arrowhead) and vacuolations of the islets of Langerhans, HE (Bar = 100 µm).

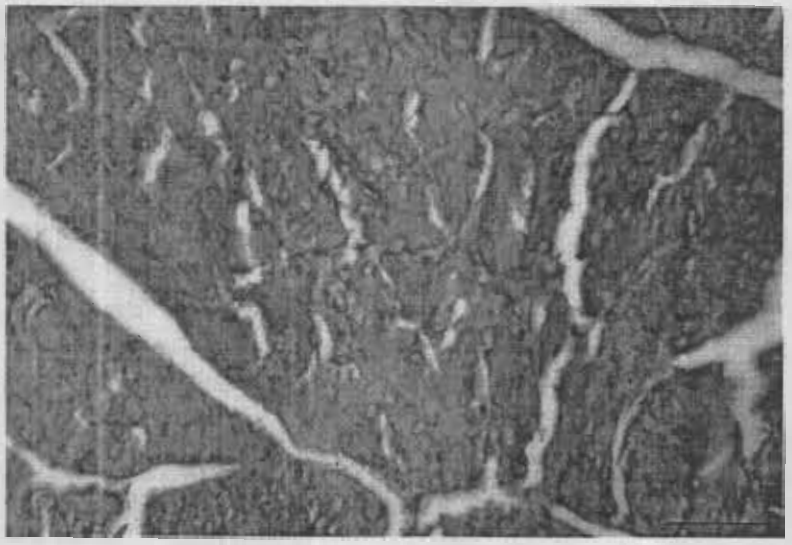

Fig. 4. Photomicrograph of the pancreas section of rabbit of gp. (4) showing activated acini with escape of the zymogenic granules on the surrounding tissue, HE (Bar = 100 µm).

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الملخص العربى

دراسات باثولوجية اكلينيكية على تأثير بعض المواد المخفضه لسكر الدم فى الأرانب

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أجريت هذه الدراسه لمعرفة تأثير الاماريل والبروبوليز فى علاج مرض البول السكرى وارتفاع السكر فى الدم الناتج عن استخدام اللالوكسان و اجرى البحث على 70 من الارانب وقد تم تقسيمها الى اربعة مجموعات :-

المجموعة الأولى :- مكونة من 10 ارانب تركت كضابطة للتجربة. **المجموعة الثانية :-** مكونة من 20 ارنب اعطيت اللالوكسان (50 ملجم/ كجم من وزن الجسم) عن طريق الحقن البريتونى

المجموعة الثالثة :- مكونة من 20 ارنب اعطيت اللالوكسان ثم عولجت بالاماريل بجرعه(0.018 ملجم/كجم من وزن الجسم يوميا عن طريق الفم يوميا لمدة شهرين). **المجموعة الرابعة :-** مكونة من 20 ارنب اعطيت اللالوكسان ثم عولجت بالبروبوليز بجرعه(53.3 ملجم/كجم من وزن الجسم يوميا عن طريق الفم يوميا لمدة شهرين). .

تم تجميع عينات الدم من المجموعات الأربعة بعد شهر وشهرين من بداية التجربة لاجراء التحاليل البيوكيميائية. ثم ذبحت الارانب فى نفس المدد السابقة وتم اخذ البنكرياس للفحص الباثولوجى أظهرت نتائج دلائل مرض السكرى زياده كبيره للغايه فى مستوى السكر والفركتوزامين بالاضافه الى نقص شديد فى مستوى الانسولين هرمون بعد شهر من حدوث مرض السكرى وقد حدث تحسن ذاتى بسيط فى هذه التغيرات بعد شهرين من حدوث المرض وهذه النتائج تم تأكيدها بالفحص الميكروسكوبي للتغيرات الباثولوجية للبنكرياس اما الارانب المصابه بالسكرى والتى عولجت بالاماريل والبروبوليز اظهرت نقص فى مستوى السكر والفركتوزامين وزياده فى مستوى الانسولين هرمون مقارنه بالمجموعه المصابه بالمرض والتى لم تعالج وهذه النتائج تم تأكيدها أيضا بالفحص الميكروسكوبي للتغيرات الباثولوجية للبنكرياس وقد أظهرت النتائج الخاصه بالمالونالديهيد والكاتاليز والسوبر اوكسيد ديسميوتاز زياده شديده فى مستوى المالونالديهيد مع نقص شديد فى مستوى الكاتاليز والسوبر اوكسيد(الانزيمات المضاده للاكسده) ديسميوتاز فى المجموعه الثانيه المصابه بمرض السكرى اما الارانب المصابه بالسكرى والتى عولجت بالاماريل و البروبوليز فى المجموعتين الثالثه والرابعه اظهرت تحسن فى هذه التغيرات مقارنه بالمجموعه المصابه بالمرض عن طريق نقص فى مستوى المالونالديهيد مع زياده فى مستوى الكاتاليز والسوبر اوكسيد ديسميوتاز (الانزيمات المضاده للاكسده) وكان التحسن اكثر وضوحا فى المجموعه الرابعه المعالجه بالبروبوليز عن الممجموعه الثالثه المعالجه بالاماريل.

وهذا يدل على مدى كفاءة الأماريل كمخفض للسكر فى حالات مرضى السكرى وايضا قدرة البروبوليز كمضاد للاكسده.