Experimentally Induced Diabetic Keratopathy in Albino Rats and the Possible Protective Role of Ginger

Manar A. Faried, Fouad K. Mansour, Ahmed S. Zolfakar, Wael B. El-Kholy

Anatomy and Embryology department, Faculty of Medicine, Menoufyia University
manarfaried@yahoo.com

Abstract: Background: Diabetes mellitus (DM) is increasing at an alarming rate and is considered as one of the main threats to human health in the 21st century in both developed and developing nations. Therefore, with a rapid increase in the prevalence of DM, ocular complications have become a leading cause of loss of vision in the world. Unfortunately, diabetic keratopathy (DK) has poor relation with regard to both clinical and research interest. So, this study was carried out to throw more light on the corneal complications in a streptozotocin (STZ)-induced rat model of DM and to understand the role of caspase-3 and vascular endothelial growth factor (VEGF) in the pathogenesis of DK and to explore the possible protective role of ginger. **Methods:** Seventy five adult male albino rats were divided into three experimental groups: control, diabetic, and ginger-treated diabetic. Diabetes was induced via a single intraperitoneal injection of STZ (50mg/kg body weight) and ginger was administered orally (500mg/kg body weight daily). Animals were grouped and sacrificed 4, 6 & 8 weeks after induction of DM. Body weight, blood glucose level, and glycated haemoglobin were measured. Corneal specimens were subjected to light microscopic study (histological, histochemical & immunohistochemical) in addition to electron microscopic study using transmission electron microscope (TEM). **Results:** Diabetic rats showed marked decrease in their body weight with highly significant increase in their blood glucose and glycated haemoglobin levels. Moreover, corneal sections of the diabetic rats revealed intense degenerative changes eg; epithelial and endothelial cytoplasmic vacuolations, stromal spacing and degradation of its collagen fibers, increased corneal thickness, increased glycogen content and increased expression of both caspase-3 and VEGF. In addition, electron microscopic evaluation revealed intracellular spaces between neighboring cells with degeneration of intracellular organelles. These damaging effects of DK were considerably ameliorated in ginger treated diabetic group. **Conclusion:** It could be concluded that caspase-3 & VEGF have played a pivotal role in the damaging effect of the DK induced by STZ and that ginger supplementation considerably mitigates STZ Induced DK.

[Manar A. Faried, Fouad K. Mansour, Ahmed S. Zolfakar, Wael B. El-Kholy. **Experimentally Induced Diabetic Keratopathy in Albino Rats and the Possible Protective Role of Ginger.** *J Am Sci* 2013;9(12):206-220]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 26

Key words: STZ-Cornea-Ginger-Caspase-3-VEGF

1. Introduction:

Diabetes mellitus (DM) is a multisystem disease with both biochemical and anatomical or structural consequences **(Khardori, 2011).** It represents a heterogeneous collection of metabolic diseases of very difficult etiopathogenesis characterized by the common feature of chronic hyperglycemia **(Rios and Fuentes, 2010).** This frequently results in severe metabolic imbalances and pathological changes in many tissues **(Ansari *et al.*, 2008).** Diabetes is a major threat to global public health, and the numbers of diabetic patients are rapidly increasing world-wide **(Ramudu *et al.*, 2011)**. According to a projection of the International Diabetes Federation (IDF), it was estimated that in 2011 there were 366 million people with DM, and this is expected to rise to 552 million by 2030 **(Whiting *et al.*, 2011)**. Egypt is one of the top ten countries in the world in relation to diabetes incidence and it is on rise**.** More than 11% of Egyptians suffer from diagnosed DM. The undiagnosed probably account for another percent, if not more **(Snouffer, 2011).** Egypt will have at least 8.6 million adults with DM by 2030 **(Shaw *et al.*, 2010).** Diabetes is the eleventh most important cause of premature mortality in Egypt, and is responsible for 2.4% of all years of life lost (YLL). Similarly, DM is the sixth most important cause of disability burden in Egypt **(Arafa and Amin, 2010).**

Previously, DK is not thought to represent a serious clinical or pathological entity and hence has been overlooked by both physician and scientist alike. Yet, with only investigation, it is obvious that many patients have visual loss secondary to DK **(Kaji, 2005).** Now it is considered as a serious vision-threatening disorder **(Zagon *et al.*, 2006)** and it is found in > 70% of patients with DM **(Saghizadeh *et al.*, 2005).** Diabetic complications appear to be multifactorial in origin but, in particular, the biochemical process of advanced glycation, which is accelerated in DM as a result of chronic hyperglycemia and increased oxidative stress, has been postulated to play a central role in these disorders **(Goh and Cooper, 2008).**

Recently, studies have verified that advanced glycation end products (AGEs) can induce apoptosis, in part, by increasing cellular oxidative stress **(Xiang *et al.*, 2006).** Furthermore, AGE interacts with several receptors and its binding to such receptors induces changes in cellular function, resulting in release of cytokines and growth factors **(Smit and Lutger, 2004).**

Unfortunately, many patients pay little attention to poor glycemic control until their vision becomes compromised. Therefore, to prevent diabetic ocular complications, prophylactic medical treatments are needed other than glycemic control **(Kakehashi, 2011).**

Worldwide studies have been done to make use of herbal medicine in different fields of medicine **(Nassiri *et al.*, 2009).** Ginger plant (Zingiber officinale) belongs to family Zingiberaceae **(Schwertner and Rios, 2007).** It continues to be used as an important cooking spice and herbal medicine around the world **(Kato *et al.*, 2006).**

Ginger has attracted much scientific attention during past few years as several pharmacological activities including antioxidant and anti-inflammatory properties have been documented for preparations of ginger **(Jarnes *et al.*, 2002)**. In the context of a need for developing and testing new antiglycating agents, several traditional medicines and some common dietary agents were evaluated and found that some spices principles have the potential to inhibit AGE formation under in vitro conditions. Among them, ginger was one of the agents that significantly prevented AGE formation in vitro **(Saraswat *et al.*, 2009)**.

2. Material and Methods

Chemicals:

Streptozotocin (STZ) was available in a powder form (1 gm) stored at -20Cº and was dissolved in distilled water. The form used in this study was a product of Sigma-Aldrich chemical Co., St. Louis. Mo, USA.

Ginger was available as tablets stored at temperature not exceeding 30Cº, each tablet contained 400mg ginger extract (*Zingber Officinal*e). The tablets were crushed and dissolved in distilled water**.** The form used in this study was a product of Arab Co. for Pharmaceuticals and Medicinal Plants "MEPACO-MEDIFOOD", Egypt.

Animals:

Seventy five adult male Sprague-Dawley albino rats, their average weight ranging from 180–200 grams, were used in this study. The rats were obtained from Animal House, Helwan, Egypt. Upon arrival, they were housed individually during the whole experiment in isolated cages at the room temperature under pathogen-free conditions to keep them in normal and healthy conditions with free access to a standard palletized diet and water. The rats were weighed and after one week of adjustment to the new environment, they were divided into experimental groups based on their body weight to attain approximately the same mean body weight in all groups.

Experimental design:

Induction of diabetes:

Diabetes was induced by a single intra-peritoneal injection of a freshly prepared STZ (50 mg/kg body weight) dissolved in 0.2 ml distilled water.

The blood sample was obtained from the retro-orbital venous plexus and blood glucose level was measured using One Touch Glucometer and strips. The rats were considered as diabetic if their blood glucose values reached above 250 mg/dl on day 3 after STZ injection. Rats with hyperglycemia (blood glucose > 250 mg/dl) were chosen for the study.

Animal grouping:

The animals were divided into three main groups:

Group I: Included forty five adult male albino rats and were further subdivided into three equal subgroups:

Subgroup Ia (plain control): Consisted of fifteen albino rats fed on standard diet, each of them was kept without any treatment all over the experimental periods and they were served as control group for all experimental groups.

Subgroup Ib (vehicle control): Consisted of fifteen albino rats. They were fed on standard diet and each rat received a single intraperitoneal injection of 0.2 ml distilled water "solvent of streptozotocin".

Subgroup Ic (ginger treated group): Consisted of fifteen albino rats. Each rat received ginger extract once daily orally by intragastric tube at a dose (500mg/kg/day) dissolved in 2 ml distilled water.

Five rats from each subgroup were anaesthetized with diethyl ether and then sacrificed at the same timing schedule corresponding to that of the diabetic control and ginger treated diabetic groups.

Group II (Diabetic group) (STZ treated group): Consisted of fifteen adult male albino rats. Diabetes was induced by a single dose of STZ by intraperitoneal injection. After diabetes confirmation test, the rats were further subdivided into three subgroups; IIa, IIb and IIc that were sacrificed 4, 6 and 8 weeks respectively after induction of DM.

Group III (Ginger treated diabetic group): Consisted of fifteen adult male albino rats. Diabetes was induced. After diabetes confirmation test, the rats received ginger extract once daily (500 mg/kg/day) orally by intragastric tube. The rats were further subdivided into three subgroups; IIIa, IIIb and IIIc

that received ginger daily and were sacrificed 4, 6 and 8 weeks respectively after induction of DM.

At the end of experiment and according to the previously mentioned timing schedule, the rats were weighed and blood samples were obtained from retro-orbital venous plexus for biochemical study.

The rats were anaesthetized lightly by diethyl ether inhalation and sacrificed by cervical dislocation. For light microscopic study, eyeballs were enucleated; fixed in 10% neutral buffered formaldehyde then was sagittaly sectioned into two halves. The specimens were processed and embedded in paraffin. Paraffin sections (3-5µm thickness) of the eye were cut and stained with different histological, histochemical and immunohistochemical stains.

Early detection of DK and the possible protective role of ginger by electron microscopic examination were done on the rats in subgroup IIa, IIIa and their corresponding control group i.e. rats that were sacrificed 4 weeks after induction of DM, one of both eyeballs that were enucleated was immediately immersed and injected at the corneoscleral junction with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3). After half an hour, the eye was cut carefully coronally. After another half an hour, the cornea was cut into about 1 mm^3 specimens and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3, for 3 hours at 4 Cº. The specimens were processed for semithin examined by light microscope, as a preliminary step, and then ultrathin sections examined by transmission electron microscope. TEM was done in Faculty of Medicine, Tanta University.

Methods

I- Quantitative study:

Quantitative study was performed on the control, diabetic and ginger treated diabetic groups. It included:

a. Estimation of the body weight:

The rats in each group were weighed at the end of the experiment according to the previously mentioned timing schedule and the mean body weight was calculated and subjected to statistical analysis.

b. Biochemical study:

bI): Estimation of blood glucose level:

Blood samples were obtained from the retro-orbital veins. The random blood glucose levels in all rats of each group were estimated at the end of the experiment according to the previously mentioned timing schedule using One Touch glucometer and strips and the mean blood glucose levels were calculated and subjected to statistical analysis.

bII): Estimation of glycated haemoglobin (HbA_1 %):

At the end of the experiment according to the previously mentioned timing schedule, blood samples were obtained from the retro-orbital plexus. The haemoglobin variants in heparinized full blood samples were separated on a cation-exchange resin column, and the percentage of glycated haemoglobin (HbA_1) was determined in all rats of each group by a spectrophotometric assay using Glycated Hemoglobin Kit. The mean glycated haemoglobin levels were calculated and subjected to statistical analysis.

II- Histological study:

IIa-Light microscopic study:

Preparation of paraffin sections:

The eyes were taken immediately and fixed in 10% neutral formaldehyde for 24 hours. The specimens were cut sagittaly then dehydrated in ascending grades of alcohol and xylol was used as a clearing agent. Impregnation was done in pure soft paraffin for two hours at 55Cº followed by embedding in hard paraffin.

Sections from these paraffin blocks were stained by:

***Histological (Hematoxylin & Eosin) and histochemical (Periodic Acid Schiff reaction) stains:**

Sections of about 3 µm thick were cut by microtome and stained by Hematoxylin & Eosin (Hx & E) and Periodic Acid Schiff reaction (PAS).

a. Hematoxylin and eosin stain (Hx & E) for routine histological examination.

b.Periodic Acid Schiff reaction (PAS) for detection of glycogen.

**** Immunohistochemical stains:**

Paraffin blocks prepared from specimens originally fixed in 10% neutral formaldehyde were cut into about 3µm thick sections and subjected to immunohistochemical studies. This was carried out using the peroxidase labeled streptavidin biotin method for detection of:

a. Caspase-3 "marker for apoptosis" expressed in the cytoplasm & nucleus using **Rabbit polyclonal caspase-3 antibody** (a product of Lab Vision, USA).

b. Vascular endothelial growth factor (VEGF) "marker for angiogenesis" expressed in cytoplasm, cell surface, extracellular matrix and nucleus using **Rabbit polyclonal VEGF antibody** (a product of Lab Vision, USA).

Morphometric study:

The parasagittal region of the cornea, near the midline, in five different stained sections obtained from five different rats (i.e. one section from each rat) was examined in each group.

Corneal epithelial thickness, stromal thickness and the whole corneal thickness were measured in pixels. Furthermore, number of caspase-3 and VEGF positive cells in corneal epithelium, stroma and endothelium were counted and the data were presented as percentage of the total number of cells.

This was performed using Image analyzer software (Image analyzer, Maryland, USA) in Anatomy and Embryology Department, Faculty of Medicine, Menoufyia University. The mean values were calculated per animal (n= 5) and the results were subjected to statistical analysis.

Statistical analysis:

Two types of statistical analysis were done:

Descriptive statistics: Mean (x) and standard deviation (SD).

Analytic statistics:

Kruskal Wallis test is a test of significance used for comparison between more than two groups.

Mann-Whitney test is a test of significance used for comparison between two groups.

The significance of data delivered from both tests was determined by the P value (probability of chance):

$P > 0.05 \rightarrow$ non significant (NS)

$P \leq 0.05 \rightarrow$ significant (S)

$P \leq 0.001 \rightarrow$ highly significant (HS)

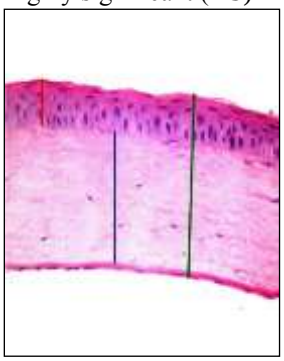

Figure (1): A photomicrograph showing measurement of the epithelial (red line), stromal (blue line) and whole corneal thickness (green line).

(Hx. & E. X 400).

Figure (2): A photomicrograph showing counting of caspase-3 positive cells within the corneal layers.

(Caspase-3 immunoreactivity X 1000).

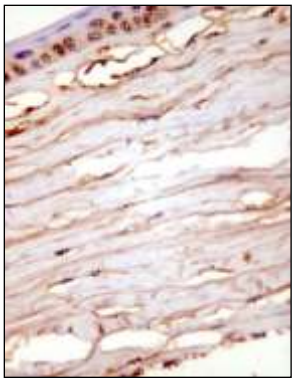

Figure (3): A photomicrograph showing counting of VEGF positive cells within the corneal layers.

(VEGF immunoreactivity X 1000).

IIb- Electron microscopic study

The samples taken from eyes were cut into about $1mm^3$ specimens. Tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3, for 3 hours at 4C° and routinely osmicated in 1% osmium tetroxide. After dehydration with graded ethanol series, the samples were embedded in Araldite. Semithin sections were stained with toluidine blue stain and were examined under light microscope as a preliminary step. Ultrathin sections were stained with lead citrate and uranyl acetate and were viewed under transmission electron microscope.

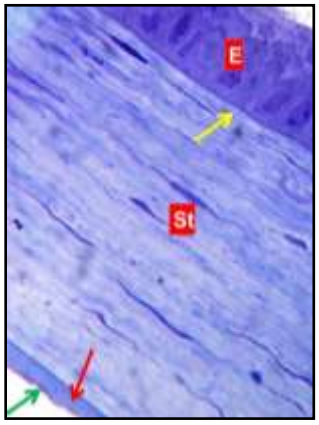

Figure (4): A photomicrograph of a rat corneal semithin section of the control group showing the different layers of the cornea; epithelium (E), Bowman's membrane (yellow arrow), stroma (St), Descemet's membrane (red arrow) and endothelium (green arrow).

(Toluidine blue X 1000).

3.Results:

Throughout the whole experiment, there was non significant difference between the different subgroups of group I. So, plain control was considered the reference one.

Body Weight:

Throughout the whole experimental periods, there was highly significant decrease in the body weight of the diabetic rats when compared with its corresponding control group. However, the ginger treated diabetic group showed highly significant increase in their body weight when compared with the diabetic non treated rats. Statistically, there was highly significant difference between the ginger treated diabetic group and its corresponding control one. The mean body weight 8 weeks after induction of DM was 252.12 ± 6.47, 110.38 ± 3.39 and 220.14 ± 3.25 for control, diabetic and ginger treated diabetic group respectively.

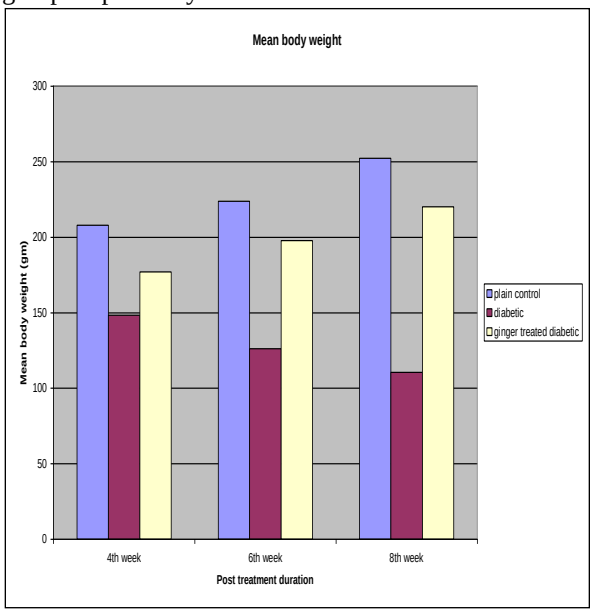

Chart (1): Column chart showing comparison of the mean body weight (gm) between the different experimental groups.

Blood Glucose & Glycated Haemoglobin Levels:

The diabetic rats revealed highly significant increase in their blood glucose & glycated haemoglobin levels 4, 6 & 8 weeks after induction of DM when compared with its corresponding control group. Treatment of the diabetic rats with ginger markedly ameliorated these effects but it can't reach the control levels.

The mean values for blood glucose level at the end of the experiment, 8 weeks after induction of diabetes, were 93.80 ± 9.31, 497.20 ± 8.78 and 213.60 ± 7.09 for control, diabetic and ginger treated diabetic groups respectively.

Moreover, the mean values for glycated haemoglobin level at the end of the experiment, 8 weeks after induction of diabetes, were 5.16 ± 0.36, 12.08 ± 2.13 and 7.90 ± 0.54 for control, diabetic and ginger treated diabetic groups respectively.

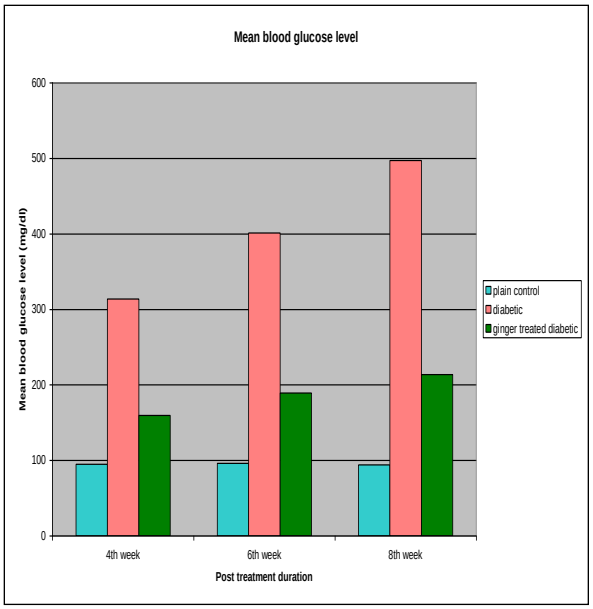

Chart (2): Column chart showing comparison of the mean blood glucose level (mg/dl) between the different experimental groups.

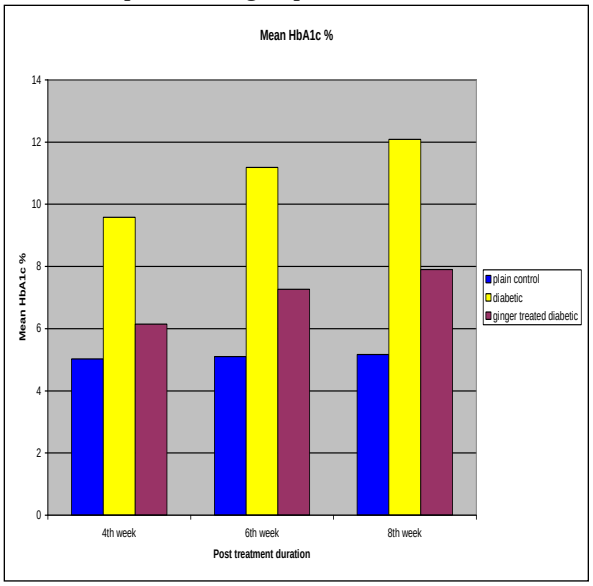

Chart (3): Column chart showing comparison of the mean glycated haemoglobin level (%) between the different experimental groups.

Light Microscopic & Morphometric Results:

Generally, regarding the group I, there were no histological differences between its various subgroups. In addition, using Kruskal Wallis test, there was non significant morphometric differences between subgroup Ia, Ib or Ic and even between the sections derived either 4, 6 or 8 weeks after the induction.

Hematoxylin and Eosin Stain (Hx & E):

Light microscopic examination of Hx & E

stained corneal sections of the control group showed non keratinized stratified squamous epithelium, stroma, Descemet's membrane and endothelium

Concerning the diabetic group, light microscopic examination of Hx & E stained corneal sections of diabetic rats sacrificed 4 weeks after induction of DM showed beginning of disturbance of normal corneal architecture in the form of cytoplasmic vacuolation in the corneal epithelial & endothelial cells in addition to focal areas of separation in the stromal collagen fibers. These effects became intense with progression of the disease. Rats that sacrificed 6 & 8 weeks after induction of DM revealed exfoliation of the surface epithelium with appearance of newly formed blood vessels in addition to intense stromal separation, marked epithelial and endothelial cytoplasmic vacuolations that may extend to areas of complete loss of the endothelial cells. On the other hand, ginger treated diabetic group showed more or less normal appearance of corneal epithelium, stroma and endothelium 4 weeks after the induction with mild cytoplasmic vacuolations and slight stromal separation 6 & 8 weeks after induction of DM.

Morphometrically, the diabetic group showed gradual progressive increase in the corneal thickness. Mann Whitney test possessed significant increase in the epithelial thickness, highly significant increase in the stromal and whole corneal thickness 4 weeks after induction of DM. It also showed highly significant increase in the epithelial, stromal and whole corneal thickness 6 & 8 weeks after the induction.

On the other hand, the diabetic rats treated with ginger showed highly significant decrease in the epithelial, stromal and whole corneal thickness when compared with that of diabetic non treated rats. Moreover, there was no statistically significant difference in the epithelial, stromal and even the whole corneal thickness between the ginger treated diabetic group and control group 4 weeks after induction of DM. Six weeks after induction of DM, there was non significant difference in the epithelial and whole corneal thickness with significant difference in the stromal thickness when compared with that of control group. However, highly significant difference in the epithelial, stromal and whole corneal thickness was observed 8 weeks after induction of DM when compared with its corresponding control group.

At the end of the experiment, 8 weeks after induction of DM, the mean epithelial thickness was 94.46 ± 6.32, 147.56 ± 11.73 and 112.41 ± 10.90, the mean stromal thickness was 346.94 ± 14.71, 667.70 ± 40.08 and 397.72 ± 8.47 however the mean whole corneal thickness was 449.09 ± 13.70, 827.63 ± 27.18 and 505.55 ± 13.32 for control, diabetic and ginger treated diabetic group respectively.

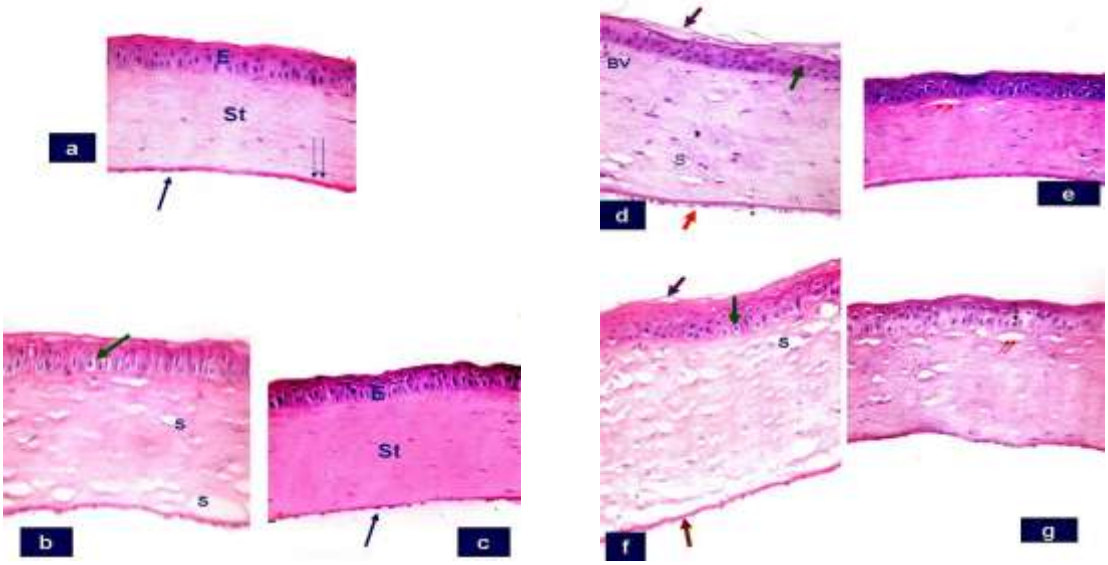

Figure (5): a: Corneal sections of control group showing normal epithelium (E), stroma (St), Descemet's membrane (double blue arrows) and endothelium(blue arrow). **b, d & f** : **Diabetic group sacrificed 4, 6 & 8 weeks after the induction respectively** showing cytoplasmic vacuolations of the epithelial cells (green arrows), exfoliation of surface epithelium (violet arrows), separation of collagen fibrils (s), newly formed blood vessels (BV), vacuolated endothelial cells (red arrows) and even areas of complete loss of the endothelial cells (brown arrow)**. c, e & g: Ginger treated diabetic group sacrificed 4, 6 & 8 weeks after the induction respectively** showing more or less normal histological appearance of the corneal epithelium (E), stroma (St) and endothelium (blue arrow) 4 weeks after induction of DM with mild epithelial cell vacuolation (black arrow) and slight separation of collagen fibrils (double red arrows) 6 & 8 weeks after induction. **(Hx. & E. X400)**

Table (1): Showing comparison of mean whole corneal thickness (in pixels) between plain control, diabetic and ginger treated diabetic groups at different experimental durations.

Post induction duration	plain control (X±SD) N=5	diabetic (X±SD) N=5	ginger treated diabetic (X±SD) N=5	Mann Whitney test	P value
	Whole corneal thickness				
4th week	448.72 ± 13.50	527.36 ± 31.68	457.79 ± 6.13	•5.11	<0.001 HS
				••4.82	<0.001 HS
				•••1.37	>0.05 NS
6th week	449.16 ± 13.04	689.73± 27.42	478.15 ± 19.31	•17.72	<0.001 HS
				••14.11	<0.001 HS
				•••2.78	>0.05 NS
8th week	449.09 ± 13.70	827.63 ± 27.18	505.55 ± 13.32	•27.81	<0.001 HS
				••23.79	<0.001 HS
				•••6.61	<0.001 HS

X: mean **SD**: standard deviation **HS**: highly significant **NS**: non significant

•: comparison between plain control and group II. ••: comparison between group II and group III.

•••: comparison between group III and plain control group.

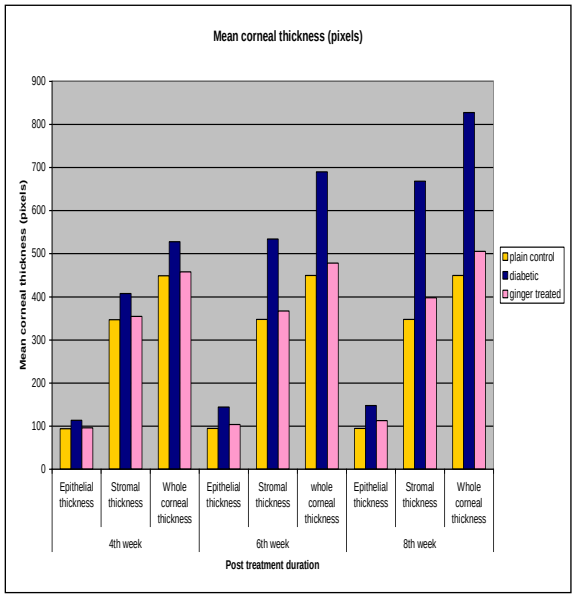

Chart (4): Column chart showing comparison of the mean epithelial, stromal and whole corneal thickness (pixels) between the different experimental groups.

PAS reaction:

PAS reaction revealed increase glycogen content in the corneal stroma of the diabetic rats with strong positive reaction in the Descemet's membrane especially 8 weeks after induction of DM and these effects were considerably improved in ginger treated diabetic group.

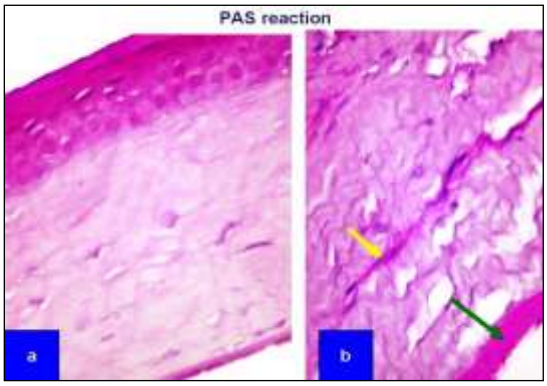

Figure (6): PAS reaction (X1000) **a:** control group. **b**: diabetic group 8 weeks after induction of DM showing intense positive PAS reaction within corneal stroma (yellow arrow) and Descemet's membrane (green arrow).

Caspase-3 immunohistochemistry:

The diabetic group at the different durations possessed positive immunoreaction to caspase-3 within the cells of the corneal epithelium, which appeared first within the basal cell layer, in addition to the stromal and endothelial cells. Mann Whitney test revealed highly significant increase in caspase-3 positive cells in the diabetic non treated group than that of the control group. Moreover, ginger administration to the diabetic rats caused highly significant decrease in its expression that still higher than that of the control group. This indicated that apoptosis through activation of caspase-3 played a pivotal role in early DK. The mean percent of caspase-3 positive cells in the diabetic group 4 weeks after induction of DM was 76.20 ± 8.21 for epithelial cells, 78.17 ± 5.97 for stromal cells and 83.70 ± 5.83 for endothelial cells. However, that of its corresponding ginger treated diabetic group was

19.93 ± 2.89 for epithelial cells, 14.32 ± 2.01 for stromal cells and 52.83 ± 9.03for endothelial cells.

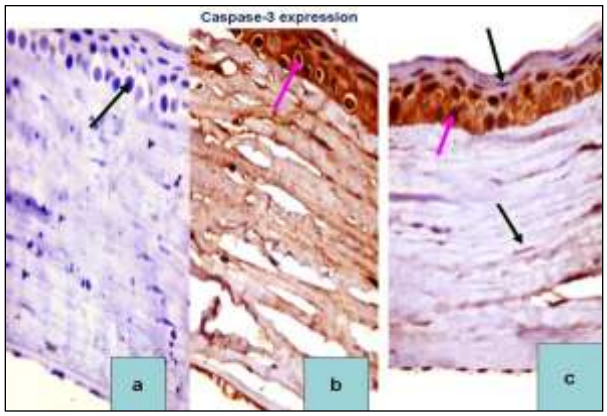

Figure (7): Expression of caspase-3 immuno-histochemistry 6 weeks after induction of DM (X 1000) **a:** control group with negative expression (green arrow). **b:** diabetic group with intense positive immunoreaction (pink arrow). **c:** ginger treated diabetic group with some positive cells (pink arrow) and others showing negative expression (green arrows).

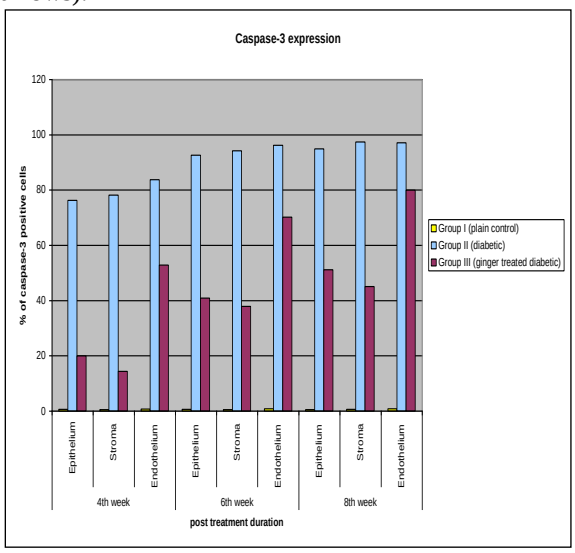

Chart (5): Column chart showing comparison of the mean percent (%) of caspase-3 positive cells between the different experimental groups.

VEGF immunohistochemistry:

Progressive increased expression of VEGF in the diabetic group was noted at the various durations with a highly significant increase in the number of positive cells than that of the corresponding control group. Furthermore, ginger treated diabetic group possessed a highly significant decrease in its expression that still higher than that of control group. This indicated that VEGF played a role in early DK. The mean percent of VEGF positive cells in the diabetic group 4 weeks after induction of DM was 47.34 ± 10.58 for epithelial cells, 38.90 ± 9.73 for stromal cells and 36.67 ± 4.75 for endothelial cells. However, that of its corresponding ginger treated diabetic group was 10.29 ± 1.33 for epithelial cells, 7.16 ± 1.66 for stromal cells and 6.73 ± 0.95 for endothelial cells.

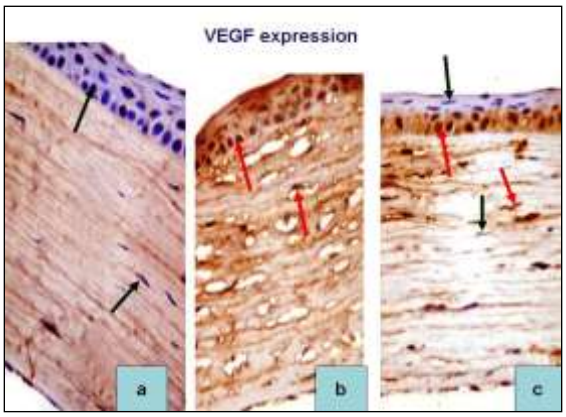

Figure (8): Expression of VEGF immuno-histochemistry 8 weeks after induction of DM (X 1000) **a:** control group with negative expression of most cells (green arrow). **b:** diabetic group with intense positive immunoreactionn (red arrows). **c:** ginger treated diabetic group with some positive cells (red arrow) and others showing negative expression (green arrows).

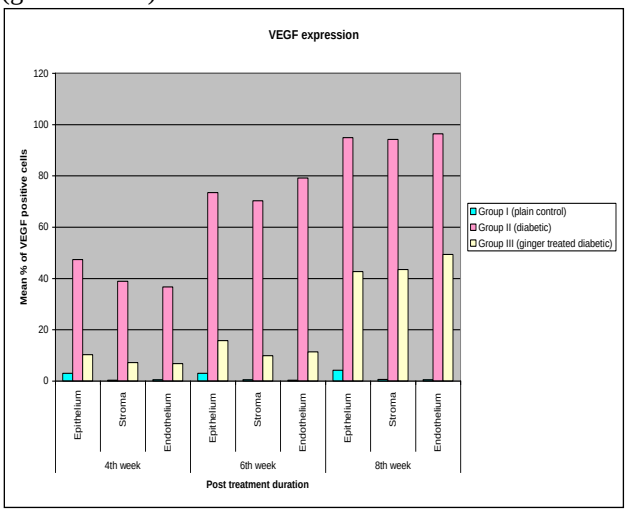

Chart (6): Column chart showing comparison of the mean percent (%) of VEGF positive cells between the different experimental groups.

Transmission Electron Microscopic (TEM) Results:

TEM examination of the rat corneal sections of the control group showed the epithelial cells with numerous mitochondria. Electron dense desmosomes in between the epithelial cells and hemidesmosomes above the basement membrane were noted. The stromal cells, squeezed in between the collagen

fibrils, had euchromatic nucleus with clumps of heterochromatin and long narrow cytoplasmic process. Moreover, the collagen fibrils were noted to be formed several lamellae of regularly organized collagen fibrils. The Descemet's membrane appeared as a homogenous acellular structure. In addition, the endothelial cells had a flat nucleus and many mitochondria.

However, TEM evaluation of the corneal sections of the diabetic rats that sacrificed 4 weeks after the induction of DM showed the epithelial cells with degenerated swollen mitochondria. There were wide intercellular spaces with disrupted desmosomes in between the neighboring cells and interrupted hemidesmosomes of basement membrane. Regarding the stroma, some degenerated stromal cells appeared with chromatolysis of their nuclei and others were shrunken with darkly stained nucleus, mostly apoptotic cells. Most of the stromal cells revealed degenerated mitochondria. The stromal collagen fibrils showed areas of disorganization, fragmentation and even marked separation. Furthermore, newly formed blood vessels appeared within the stroma of that subgroup**.** The Descemet's membrane appeared to be thickened in this subgroup in comparison to the control group. In addition, the corneal endothelial cells were distorted in shape with irregular nucleus and vacuolation of their cytoplasm in addition to detection of degenerated mitochondria.

On the other hand, TEM examination of the corneal sections of the diabetic rats treated with ginger and sacrificed 4 weeks after induction of DM showed the corneal epithelium with normal mitochondria however degenerated mitochondria were also detected. There was absence of intercellular spaces in between the neighboring cells with normal appearance of the desmosomes. Moreover, the basement membrane appeared with more or less normal hemidesmosomes. The stroma showed more or less normal stromal cells while some cells revealed degenerated mitochondria in their cytoplasm. It also showed more or less normal distribution of collagen fibrils while focal areas of slight collagen fibrils disruption were also seen. The Descemet's membrane of this subgroup was thicker than that of control group. Regarding the endothelial cells, it appeared with flat nucleus and many mitochondria that were nearly similar to that of control group.

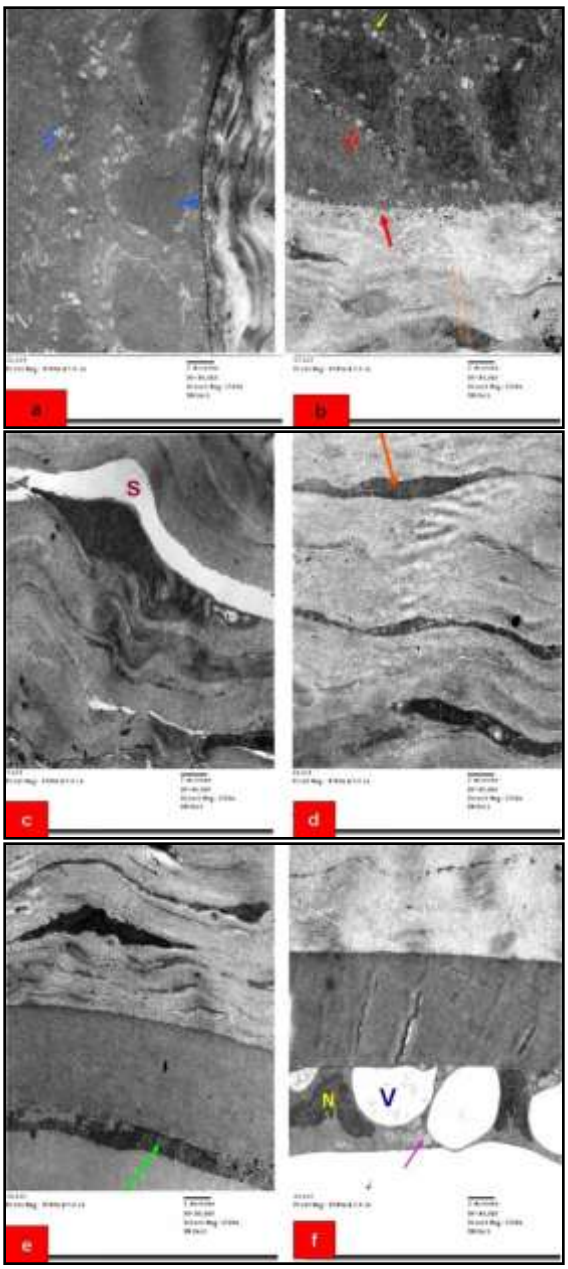

Figure (9): a, c & e: Electron-micrographs of corneal sections of the diabetic group showing interrupted hemidesmosomes (red arrow), wide intercellular spaces (double red arrows), degenerated mitochondria (yellow arrow), shrunken keratocytes (double orange arrows), spacing within the collagen fibrils (S), cytoplasmic vacuolation in the endothelial cells (V) with degenerated mitochondria (pink arrow) and irregular nucleus (N). **b, d & f:** Electron-micrographs of corneal sections of the ginger treated diabetic group showing more or less normal hemidesmosomes (blue arrow) with narrowing of intercellular spaces (double blue arrows), more or less normal keratocytes (orange arrow) and endothelial cells (green arrow). **(TEM X 1500).**

4. Discussion

Rats were particularly selected in this work because they were easily obtained, handled, controlled and tested. Moreover the rats seemed to mimic human. **Yang *et al.*, 2012** stated that the most widely accepted animal model for the evaluation of complications in DM was the STZ-induced diabetic rat as the lesions observed in the diabetic rats resembled the initial process that occurred in humans.

However alloxan and STZ are the most prominent diabetogenic chemical compounds in the experimental diabetes researches, STZ was particularly used in our research due to its ability to cause cellular necrosis and selective destruction of pancreatic islet beta cells as mentioned by **Zafar and Naqvi, 2010.** Furthermore, STZ had greater stability and long half-life than alloxan as reported by **Kumar, 2013.**

In this study, measurements of the diabetic rats' body weight revealed a highly significant decrease in their body weight in comparison with the control group. These results were in agreement with the results of **Obrosova, *et al.*, 2006.; Kusari *et al.*, 2007 and Gül *et al.*, 2008**. They contributed the decrease of body weight either to the increase of the urine output causing dehydration and loss of valuable fluids or to the breakdown of muscles caused by high blood sugar.

In the present study, treatment of the diabetic rats with ginger protected the rats against characteristic diabetic weight loss as there was highly significant increase in their body weight when compared with that of the diabetic non treated group although it still highly significantly different from that of control group. This was in agreement with **Thomson *et al.*, 2007** who noticed that ginger was effective in preventing the weight loss normally observed in diabetic rats and they attributed this effect to the hypoglycemic potential of ginger.

In this research, the blood glucose showed a highly significant increase in its level from the beginning till the end of the experiment in the diabetic rats. This was in agreement with all researches using STZ rat model eg., **Kowluru *et al.*, 2008.;** furthermore, there was a highly significant increase in the level of the glycated haemoglobin , a marker of protein glycation ,that was in accordance with **Sheykhzade *et al.*, 2000; Ramesh *et al.*, 2006 and Ansari *et al.*, 2008. Take *et al.*, 2006** referred the similar changes to the occurrence of reduction in the plasma insulin levels caused by selective irreversible necrosis in beta cells of pancreas.

In the ginger treated diabetic group through all studied durations there were highly significant decrease in both blood glucose level and glycated haemoglobin that didn't reach the level of the control group. These changes were in accordance with **Ansari *et al.*, 2008 and Arikawe *et al.*, 2012** who reported that there was decrease in these levels but not to normal level. This may be attributed to its hypoglycemic effect as mentioned by **Abdulrazaq *et al.*, 2012** who found that ginger was helpful for the treatment of DM through its effects on the activities of the glycolytic enzymes. In addition, **Ansari *et al.*, 2008** attributed the decrease in the level of glycosylated haemoglobin to the decreased level of blood glucose.

Meanwhile, only few studies focused on the effect of DM on the cornea concerning histological changes with references to some of mechanisms of pathogenesis

In this research there was a trial to explore the histological alterations in the DK and explore mechanisms that aided in the pathogenesis of DK.

The diabetic subgroups of the present study showed a significant increase in the thickness of corneal epithelium 4 weeks after induction of DM and highly significant increase 6 and 8 weeks after the induction. Similar results were observed by **Gül *et al.*, 2008** who stated that prolonged exposure of the corneal epithelial cells to high glucose levels might impair cellular behavior and cause epithelial thickness. Moreover, **Fabiani *et al.*, 2009** stated that dryness of the cornea occurred in the DK and led to increase in the corneal epithelial turnover and thickness.

Moreover, a highly significant increase of the mean stromal and even the whole corneal thickness was noted in the diabetic non treated group in this study as shown by our statistical analysis when compared with the control group. This was in agreement with the results of **Siribunkum *et al.*, 2001; Lee *et al.*, 2006; Gül *et al.*, 2008 and Kim *et al.*, 2011**.

Gül *et al.*, 2008 and Kim *et al.*, 2011 referred the increase in the stromal and corneal thickness to occurrence of corneal edema resulting from insufficient endothelial cell functions and tear film dysfunction.

This was in disagreement with **Take *et al.*, 2006** who observed focal narrowing in the epithelial layer in the diabetic rats and with **Yin *et al.*, 2011** who noted that there were no significant changes in the thickness of the diabetic corneas even up to 8 weeks after induction of DM.

In our study, the cornea showed many degenerative changes that began 4 weeks after the induction and became worse with the progression of the DM.

Concerning the histological examination of the cornea in the diabetic rats, the corneal epithelium in the present study showed many degenerative changes

such as prominent cytoplasmic vacuolations, seen by light microscopic study, with degenerated mitochondria as noticed by electron microscope. Moreover, there were wide intercellular spaces and interrupted desmosomes between neighboring epithelial cells with disruption of the hemidesmosomes in the epithelial basement membrane.

This was in agreement with **Kaji *et al.*, 2000** who noted corneal epithelial disorders with wide intercellular spaces and with **Papers, 2010** who noticed degenerated epithelial cells in addition to **Take *et al.*, 2006** who observed cytoplasmic vacuolations with mitochondrial cytolysis. They attributed these changes to the occurrence of disruption of normal cellular osmotic balance secondary to DM.

In addition, **Coughlan *et al.*, 2009** stated that the damaged mitochondria generated an excess of superoxide, which might mediate tissue injury in the DM. They hypothesized that AGEs led to increases in the cytosolic reactive oxygen species (ROS) which facilitated the production of mitochondrial super-oxide.

Moreover**, Kaji *et al.*, 2000** suggested that accumulation of AGEs in the corneal epithelial basement membrane had changed the molecular structure of basement membrane components and this led to decrease the adhesion and spreading of the corneal epithelial cells and to corneal epithelial disorders.

Furthermore, the detectable decrease in the hemidesmosomes of the epithelial basement membrane of the diabetic rats in our study, as seen by electron microscope, was in agreement with **Akimoto *et al.*, 2003** who reported that the hemidesmosomes in the epithelial basal cells were decreased in number in the DM. This may be attributed to alteration in the basement membrane components and integrins as a result of increase in their degradation by proteases elevated in diabetic corneas as suggested by **Agrawal and Tsai, 2003** and that in turn led to altered both basement membrane structure and function.

However, **Addy *et al.*, 2001** revealed that, in the DM, excess phosphorylation of integrins reduced its hemidesmosomes localization and decreased corneal epithelium attachment to the laminin, one of the major components of the basement membrane**.**

On the other hand, these findings were in disagreement with **Yin *et al.*, 2011** who revealed no detectable histological differences between hyperglycemic and normoglycemic rats.

Regarding the stromal damage observed in the DK in the present study, some stromal cells were degenerated with detectable chromatolysis of their nuclei and others had darkly stained nuclei, mostly apoptotic cells. Moreover, degenerated mitochondria were also seen. These results were in accordance with **Take *et al.*, 2006 and Kim *et al.*, 2011** who noticed degenerative changes in the stromal cells with presence of apoptotic stromal keratocytes. These changes may be attributed to the accumulation of AGEs as mentioned by **Shi *et al.*, 2013**. In addition, **Niiya *et al.*, 2012** suggested that the generation of intracellular ROS mediated cellular responses to AGEs.

Furthermore, **Gül *et al.*, 2008** revealed destruction of the intracellular organelles and referred these degenerative changes in the intracellular organelles to the formation of AGEs that led to protein cross – linking and caused destruction of intracellular organelles.

In the current work, the stroma showed disarrangement and even separation within its collagen fibrils. This was in agreement with **Jian *et al.*, 2003** and **Gül *et al.*, 2008. Hiromatsu and Toda, 2003** referred the occurrence of collagen free spaces in the cornea to presence of alteration in collagen biosynthesis in DM due to increased non enzymatic glycosylation of normally formed collagen. In addition, **Gül *et al.*, 2008** reported that the formation of AGEs led to protein cross – linking, changes in packing density and surface charge that resulted in appearance of wide spaced collagen.

This was in disagreement with **Take *et al.*, 2006** who noted intact collagen fibrils in the stroma of diabetic rats and they attributed these to continuity production of fibers by keratocytes lacking apoptosis.

Moreover, the stroma of the diabetic group in this study, showed invasion of newly formed blood vessels. This was agreed with **Chang *et al.*, 2001 and Zagon *et al.*, 2008.** They mentioned that the etiology of corneal neovascularization might be due to the presence of stromal edema in the diabetic rats proximal to the limbus which had been proposed as necessary to allow blood vessels into the usually compact stroma. However, **Pinto and Malucelli, 2002** postulated that the vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 were considered key factors in angiogenic response and this was confirmed in our research by increased expression of VEGF using immunohistochemical stain.

In the present work, using transmission electron microscope, there was apparent thickening in the Descemet's membrane. This was in agreement with **Take *et al.*, 2006.** They attributed these changes to failure of protocollagen assembly secondary to the increase in the non enzymatic glycosylation.

Regarding the endothelial cells, the present study revealed swollen endothelial cells with prominent cytoplasmic vacuolations that progress

even to areas of complete loss of endothelial cells in the diabetic group. This was in addition to presence of increased number of swollen degenerated mitochondria. These findings coincided with those of **Take *et al.*, 2006** who noted degenerative changes in the endothelial cells with vacuolated cytoplasm in the diabetic rats and with **Jian *et al.*, 2003** and **Papers, 2010** who revealed increase in number of swollen degenerated mitochondria.

Lee *et al.*, 2006 thought that the DM reduced the activity of Na+-K+ ATPase of the corneal endothelium and this caused these morphological and permeability changes. Moreover, **Akimoto *et al.*, 2008** stated that the high glucose levels in the DM caused increase in sorbitol and AGES accumulation in the cornea and that accumulation of these substances especially in the Descemet's membrane were responsible for the corneal endothelial abnormalities in DM.

In addition, **Sheng and Bullimore, 2007** stated that when some corneal endothelial cells die, the remaining cells could not divide fast enough to replace the dead cells.

PAS reaction in the corneas of the diabetic rats revealed increase in PAS reaction in the stromal collagen fibrils, lining of newly formed blood and within the Descemet's membrane. This indicated increased deposition of glycogen in the corneas of the diabetic rats. This was in agreement with **Sánchez-Chávez *et al.*, 2008** who examined the diabetic retinas and found increase in glycogen levels in diabetic retina and referred these changes to alterations in glycogen synthase regulation.

Concerning caspase-3 expression in the different diabetic subgroups of the current study, there was highly significant increase in the expression of caspase-3, a marker for apoptosis, which began to appear in the epithelial cells, mainly basal, and some stromal and endothelial cells 4 weeks after the induction of DM and this expression markedly increase with the progression of the disease. This indicated that apoptosis played an important role in the pathogenesis of DK. This was in accordance to **Take *et al.*, 2006 and Kim *et al.*, 2011** who noted apoptotic changes in the corneal epithelium, stroma and endothelium.

Kim *et al.*, 2011 attributed the apoptosis in cases of DK to the accumulation of AGEs and NF-κB (necrosis factor kappa beta) in the corneal tissues of diabetic rats. In addition, **Circu and Aw, 2010** postulated that the excessive production of ROS played an important role in apoptosis.

Furthermore, up to our knowledge there were no documented researches that explore the cause of increased expression of caspase-3 in the basal then intermediate and finally the superficial epithelial cell layers. This pattern of distribution may be attributed to accumulation of AGEs in the epithelial basement membrane.

In the current research, VEGF immuno-histochemial stain was highly expressed in the cornea of the different diabetic subgroups. This was in agreement with **Saghizadeh *et al.*, 2001** who stated that the diabetic corneas were unique in their increased VEGF mRNA levels. **Markowska *et al.*, 2010** demonstrated that galectin-3, a receptor of AGEs; mediated VEGF angiogenic response in DM. Moreover, **Basta, 2008** stated that AGEs-induced oxidative stress, fibrosis and inflammation accelerated the progress of diabetic vascular complications.

Generally, the damaging effects on the corneas of the diabetic rats attributed to many factors. **Papers, 2010** thought that oxidative damage might be engaged in the formation of DK. **Gül *et al.*, 2008** suggested that AGEs played an important role in the pathogenesis of DK. Moreover, **Shi *et al.*, 2013** postulated that AGEs induced human corneal cells apoptosis through generation of ROS.

AGE exerted its effect through interaction with its receptor (RAGE), which activated an array of various biochemical pathways as mentioned by **Sukkar *et al.*, 2012**. Moreover, **Sugamura and Keaney, 2011** added that ROS such as superoxide anion, hydroxyl radicals and hydrogen peroxide, initiated inappropriate or altered cellular signal transduction pathways and caused toxicity.

To ameliorate the damaging effect of DM on the cornea, ginger was used in this study. Up to our knowledge, there were no documented researches that used ginger in DK. In this work, it was noted that ginger considerably delayed the onset of DK in comparison to diabetic non treated rats along morphometric, histological, histochemical and immunohistochemical levels. This was in agreement with **Ramudu *et al.*, 2011** who found that injurious effect of DM on the kidney was recovered in the ginger-treated diabetic rats They attributed theses protective effects on the diabetic complication to the acceleration of anti-oxidant defense mechanisms. Thus, they thought that ginger might be used as a therapeutic agent in preventing complications in the diabetic patients.

As discussed before, because one of the major pathogenic mechanisms accelerating the onset of diabetic complication was increased level of AGEs, the potential effect of ginger to decrease the level of AGEs made it capable of causing delay in the commencement of DK. This was in agreement with **Saraswat *et al.*, 2010** who observed delay in the onset of diabetic cataract after ginger administration. They referred this improvement to the decrease in

AGEs and normalization of aldose reductase (ALR). They thought that the reduction of carbonyl mediated stress by ginger might be responsible for restoring ALR activity after feeding ginger.

Ginger treated rats in the current study showed restoration of more or less normal glycogen deposition, using PAS reaction, with apparent decrease in PAS reaction within the corneal collagen fibrils than that of the diabetic non treated rats. This was in agreement with **Abdulrazaq *et al.*, 2012** who noticed significant decrease in the kidney glycogen after ginger administration when compared with the diabetic controls through its effects on the activities of glycolytic enzymes.

The present work revealed that ginger administration to the diabetic rats, throughout the different durations, significantly decreased the expression of caspase-3 in the cornea. Thus ginger had a protective effect on DK through its anticaspase-3 activity and thus anti-apoptotic effect. Up to our knowledge, there were no documented researches on the effect of ginger on caspase-3 activity in DK.

The ability of ginger to down regulate caspase-3 expression was in agreement with **El-Sharaky *et al.*, 2009** who clarified that ginger extract prior to bromobenzene administration resulted in decreased activation of caspase-3 in the liver of treated rats. As mentioned before, one of the mechanisms causing induction of apoptosis in the DK was increased production of AGEs and ROS. The ability of ginger to down regulate caspase-3 activity may be attributed to its antiglycation and antioxidant properties. **Ho and Chang, 2012** revealed that the capacity of spices to inhibit the formation of AGEs was attributable to its phenolic compounds. Moreover**, Dugasani *et al.*, 2010** mentioned that the shogaol, a component in ginger extract, exhibited the most potent antioxidant and anti-inflammatory properties in ginger, which could be attributed to the presence of alpha, beta-unsaturated ketone moiety.

However there were no documented researches on the effect of ginger on VEGF in the diabetic complications, the current study clarified that treatment of the diabetic rats with ginger alleviated the expression of VEGF that was markedly increased in DK. This was in agreement with **Kim *et al.*, 2005** who stated that [6]-gingerol inhibited VEGF induced proliferation of human endothelial and blocked capillary-like tube formation by endothelial cells in response to VEGF, and strongly inhibited sprouting of the endothelial cells in the rat aorta and formation of new blood vessels in the mouse cornea in response to VEGF. In addition, the role of ginger as VEGF inhibitors may be attributed to its antiglycation, antioxidant and anti-inflammatory properties.

As AGEs were implicated in the pathogenesis of diabetic complications, inhibiting the formation of AGEs and interfering with AGEs-mediated oxidative stress and inflammatory processes were two practicable strategies for developing a dietary adjuvant against diabetic complications as mentioned by **Ho and Chang, 2012.**

References

1. **Abdulrazaq, N.B.; Cho, M.M.; Win, N.N.; Zaman, R. and Rahman, M.T. (2012):** Beneficial effects of ginger (*Zingiber officinale*) on carbohydrate metabolism in streptozotocin-induced diabetic rats. Br J Nutr.; 108 (7): 1194-1201.
2. **Addy, A.; Motoi, O.; Luowei, L.; Mariana, G. and Adam, B. (2001):** Protein kinase Cδ-mediated phosphorylation of α6β4 is associated with reduced integrin localization to the hemidesmosome and decreased keratinocyte attachment. Cancer Research.; 61: 4591-4598.
3. **Agrawal, V. and Tsai, R. (2003):** Corneal epithelial wound healing. Indian. J. Ophthalmol.; 51: 5-15.
4. **Akimoto, Y.; Kawakami, H.; Yamamoto, K.; Munetomo, E.; Hida, T. and Hirano, H. (2003):** Elevated Expression of O-GlcNAc–Modified Proteins and O-GlcNAc Transferase in Corneas of Diabetic Goto-Kakizaki Rats. Invest Ophthalmol Vis Sci.; 44: 3802–3809.
5. **Akimoto, Y.; Sawada, H.; Ohara-Imaizumi, M.; Nagamatsu, S. and Kawakami, H. (2008):** Change in long-spacing collagen in Descemet's membrane of diabetic Goto-Kakizaki rats and its suppression by antidiabetic agents. Exp Diabetes Res.; 2008: 818341.
6. **Ansari, M.H.; Karimipour, M.; Salami, S. and Shirpoor. A. (2008):** The Effect of Ginger (*Zingiber officinale*) on Oxidative Stress Status in the Small Intestine of Diabetic Rats. Int J Endocrinol Metab.; 3: 144-150.
7. **Arafa, N.A.S. and Amin, G.E. (2010):** The epidemiology of diabetes mellitus in Egypt: Results of a National Survey. The Egyptian Journal of Community Medicine.; 28 (3): 29-34.
8. **Arikawe, A. P.; Oyerinde, A.; Olatunji-Bello, I.I. and Obika, L.F.O. (2012):** Streptozotocin diabetes and insulin resistance impairment of spermatogenesis in adult rat testis: central Vs local mechanism. Niger. J. Physiol. Sci.; 171–179.
9. **Basta, G. (2008):** Receptor for advanced glycation end products and atherosclerosis: From basic mechanisms to clinical implications. Atherosclerosis.; 196 (1): 9-21.
10. **Chang, J.; Gabison, E.; Kato, T. and Azar, D. (2001):** Corneal neovascularization. Opin Ophthalmol.; 12 (4): 242-249.
11. **Circu, M.L. and Aw, T.Y. (2010):** Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med.; 48: 749–762.
12. **Coughlan, M.T.; Thorburn, D.R.; Penfold, S.A.; Laskowski, A.; Harcourt, B.E.; Sourris, K.C.; Tan, A.L.; Fukami, K.; Thallas-Bonke, V.; Nawroth, P.P.; Brownlee, M.; Bierhaus, A.; Cooper, M.E.**

and Forbes, J.M. (2009): RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes. J Am Soc Nephrol.; 20 (4): 742-752.

13. **Dugasani, S.; Pichika, M.R.; Nadarajah, V.D.; Balijepalli, M.K.; Tandra, S. and Korlakunta, J.N. (2010):** Comparative antioxidant and anti-inflammatory effects of [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol. J Ethnopharmacol.; 127: 515–520.
14. **El-Sharaky, A.S.; Newairy, A.A.; Kamel, M.A. and Eweda, S.M. (2009):** Protective effect of ginger extract against bromobenzene-induced hepatotoxicity in male rats. Food Chem Toxicol.; 47 (7): 1584-1590.
15. **Fabiani, C.; Barabino, S.; Rashid, S. and Dana, M. (2009):** Corneal epithelial proliferation and thickness in a mouse model of dry eye. Exp Eye Res.; 89 (2): 166-1671.
16. **Goh, S. and Cooper, M.E. (2008):** The Role of Advanced Glycation End Products in Progression and Complications of Diabetes. The Journal of Clinical Endocrinology & Metabolism.; 93 (4): 1143-1152.
17. **Gül, M.; Emre, S.; Eşrefoğlu, M. and Vard, N. (2008):** Protective effects of melatonin and aminoguanidine on the cornea in streptozotocin-induced diabetic rats. Cornea.; 27 (7): 795-801.
18. **Hiromatsu, Y. and Toda, S. (2003):** Mast cells and angiogenesis. Microsc Res Tech.; 60 (1): 64-69.
19. **Ho, S. and Chang, P. (2012):** Inhibitory Effects of Several Spices on Inflammation Caused by Advanced Glycation Endproducts. American Journal of Plant Sciences.; 3: 995-1002.
20. **Jarnes, J.; Anderson, L.A. and Phillipson, J.D. (2002):** Herbal Medicines. 2nd edition. Pharmaceutical Press. London. PP. 243-249.
21. **Jian, C.; Jian-yan, H.; Fu-xing, T.; Zu-wen, G.; Song-bin, Z.; Yan-ping, W. and Chun-yun, W. (2003):** Changes of corneal ultrastructure in early diabetic rats. Chin J Pathophysiol.;19 (7): 942-945
22. **Kaji, Y. (2005):** Prevention of diabetic keratopathy. J Ophthalmol.; 89: 254-255.
23. **Kaji, Y.; Usui, T.; Oshika, T.; Matsubara, M.; Yamashita, H.; Araie, M.; Murata, T.; Ishibashi, T.; Nagai, R.; Horiuchi, S. and Amano, S. (2000):** Advanced Glycation End Products in Diabetic Corneas. Invest Ophthalmol Vis Sci.; 41: 362–368.
24. **Kakehashi, A. (2011):** Diabetic Ocular Complications in the SDT Rat. The Open Diabetes Journal.; 4: 37-40.
25. **Kato, A.; Higuchi, Y.; Goto, H.; Kizu, H.; Okamoto, T.; Asano, N.; Hollinshead, J.; Nash, R.J. and Adachi, I. (2006):** Inhibitory effects of *Zingiber officinale* Roscoe derived components on aldose reductase activity in vitro and in vivo. J Agric Food Chem.; 54: 6640–6644.
26. **Khardori, R. (2011):** type 1 diabetes mellitus in The European journal of endocrinology EJE. On line edition. http://ej-endocrinology.org /section-blog/27-cars.html
27. **Kim, E.C.; Min, J.K.; Kim, T.Y.; Lee, S.J.; Yang, H.O.; Han, S.; Kim, Y.M. and Kwon, Y.G. (2005):** [6]-Gingerol, a pungent ingredient of ginger, inhibits angiogenesis in vitro and in vivo. Biochem Biophys Res Commun.; 335 (2): 300-308.
28. **Kim, J.; Kim, C.S.; Sohn, E.; Jeong, I.H.; Kim, H. and Kim, J.S. (2011):** Involvement of advanced glycation end products, oxidative stress and nuclear factor-kappaB in the development of diabetic keratopathy. Graefes Arch Clin Exp Ophthalmol.; (4): 529-536.
29. **Kowluru, R.A.; Menon, B. and Gierhart, D.L. (2008):** Beneficial effect of zeaxanthin on retinal metabolic abnormalities in diabetic rats. Invest Ophthalmol Vis Sci.; 49 (4): 1645-1651.
30. **Kumar, N.T. (2013):** Review Article: Animal Models As An Experimental Tool In Diabetes Research. Journal of Drug Delivery & Therapeutics.; 3 (6): 152-154.
31. **Kusari, J.; Zhou, S.; Padillo, E.; Clarke, K.G. and Gil, D.W. (2007):** Effect of memantine on neuroretinal function and retinal vascular changes of streptozotocin-induced diabetic rats. Invest Ophthalmol Vis Sci.; 48 (11): 5152-5159.
32. **Lee, J.; Oum, B.; Choi, H.; Lee, J. and Cho, B. (2006):** Differences in corneal thickness and corneal endothelium related to duration in diabetes. Eye.; 20 (3): 315-318.
33. **Markowska, A.I.; Liu, F.T. and Panjwani, N. (2010):** Galectin-3 is an important mediator of VEGF- and bFGF-mediated angiogenic response. J Exp Med.; 207 (9): 1981-1993.
34. **Nassiri, M.; Khaki, A.; Ahmadi-Ashtiani, H.R.; Rezazadeh, S.; Rastgar, H. and Gharachurlu, S. (2009):** Effects of Ginger on Spermatogenesis in Streptozotocin-induced Diabetic Rat. Journal of Medicinal Plants.; 8 (31): 118-124.
35. **Niiya, Y.; Abumiya, T.; Yamagishi, S.; Takino, J. and Takeuchi, M. (2012):** Advanced glycation end products increase permeability of brain microvascular endothelial cells through reactive oxygen species-induced vascular endothelial growth factor expression. J Stroke Cerebrovasc Dis.; 21: 293–298.
36. **Obrosova, I.G.; Drel, V.R.; Kumagai, A.K.; Szábo, C.; Pacher, P. and Stevens, M.J. (2006):** Early diabetes-induced biochemical changes in the retina: comparison of rat and mouse models. Diabetologia.; 49 (10): 2525-2533.
37. **Papers, C. (2010):** Preliminary Experimental and Clinical Studies on Ocular Surface Disease in Diabetic Mellitus. China's Outstanding Master's Theses. Published online: www. http://mt.china-papers.com/2/?p=106505.
38. **Pinto, F. and Malucelli, B. (2002):** Inflammatory infiltrate, VEGF and FGF-2 contents during corneal angiogenesis in STZ-diabetic rats. Angiogenesis.; 5 (1-2): 67-74.
39. **Ramesh, B.; Saravanan, R. and Pugalend, K.V. (2006):** Effect of Dietary Substitution of Groundnut Oil on Blood Glucose, Lipid Profile, and Redox Status in Streptozotocin-diabetic Rats. Yale J Biol Med.; 79 (1): 9–17.
40. **Ramudu, S.K.; Korivi, M.; Kesireddy, N.; Lee, L.; Cheng, S.; Kuo, C. and Kesireddy, S.R. (2011):** Nephro-Protective Effects of a Ginger Extraction Cytosolic and Mitochondrial Enzymes against

Streptozotocin (STZ)-Induced Diabetic Complications in Rats. Chinese Journal of Physiology.; 54 (2): 79-86.

41. **Rios, M.S. and Fuentes, J.A. (2010):** Classification of diabetes, criteria for diagnosis in Type 2 diabetes mellitus. Chapter (1). Elsevier, Espana. pp 1-12.
42. **Saghizadeh, M.; Chwa, M.; Aoki, A.; Lin, B.; Pirouzmanesh, A.; Brown, D.J.; Ljubimov, A.V. and Kenney, M.C. (2001):** Altered expression of growth factors and cytokines in keratoconus, bullous keratopathy and diabetic human corneas. Exp Eye Res.; 73 (2): 179-189.
43. **Saghizadeh, M.; Kramerov, A.A.; Tajbakhsh, J.; Aoki, A.M.; Wang, C.; Chai, N.; Ljubimova, J.Y.; Sasaki, T.; Sosne, G.; Carlson, M.R.J.; Nelson, S.F. and Ljubimov, A.V. (2005):** Proteinase and Growth Factor Alterations Revealed by Gene Microarray Analysis of Human Diabetic Corneas. Invest. Ophthalmol. Vis. Sci.; 46 (10): 3604-3615.
44. **Sánchez-Chávez, G.; Hernández-Berrones, J.; Luna-Ulloa, L.B.; Coffe, V. and Salceda, R. (2008):** Effect of diabetes on glycogen metabolism in rat retina. Neurochem Res.; 33 (7): 1301-1308.
45. **Saraswat M.; Reddy, P.Y.; Muthenna, P. and Reddy, G.B. (2009):** Prevention of non-enzymatic glycation of proteins by dietary agents: prospects for alleviating diabetic complications. Br J Nutr.; 101: 1714–1721.
46. **Saraswat, M.; Suryanarayana, P.; Reddy, P.Y.; Patil, M.A.; Balakrishna, N. and Reddy, G.B. (2010):** Antiglycating potential of *Zingiber officinalis* and delay of diabetic cataract in rats. Mol Vis.; 16: 1525–1537.
47. **Schwertner, H.A. and Rios, H.A. (2007):** High performance liquid chromatographic analysis of 6-gingerol, 8- gingerol, 10- gingerol and 6- shogaol in ginger – containing dietary supplements, spices, teas and beverages. J. Chromatography B.; 856: 41-47.
48. **Shaw, J.E.; Sicree, R.A. and Zimmet, P.Z. (2010):** Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes research and clinical practice.; 87 (1): 4–14.
49. **Sheng, H. and Bullimore, M. (2007):** Factors affecting corneal endothelial morphology. Cornea.; 26 (5): 520-525.
50. **Sheykhzade, M.; Dalsgaard, G.T.; Johansen, T. and Nyborg, N.C. (2000):** The effect of long-term streptozotocin-induced diabetes on contractile and relaxation responses of coronary arteries: selective attenuation of CGRP-induced relaxations. Br J Pharmacol.; 129 (6): 1212–1218.
51. **Shi, L.; Yu, X.; Yang, H. and Wu, X. (2013):** Advanced glycation end products induce human corneal epithelial cells apoptosis through generation of reactive oxygen species and activation of JNK and p38 MAPK pathways. PLoS One.; 8 (6): e66781. doi: 10.1371/journal.
52. **Siribunkum, J.; Kosrirukvongs, P. and Singalavanija, A. (2001):** Corneal abnormalities in diabetes. J Med Assoc Thai.; 84 (8): 1075-1083.
53. **Smit, A.J. and Lutgers, H.L. (2004):** The clinical relevance of advanced glycation end products (AGE) and recent developments in pharmaceutics to reduce AGE accumulation. Curr Med Chem.; 11 (20): 2767-2784.
54. **Snouffer, E. (2011):** Zero Healthcare and Top 10 Diabetes World Ranking Make More Trouble for Egypt. Web site: http://www.diabetes24-7.com/?p=611.
55. **Sugamura, K. and Keaney, J.F. (2011):** Reactive oxygen species in cardiovascular disease. Free Radic Biol Med.; 51 (5): 978–992.
56. **Sukkar, M.B.; Ullah, M.A.; Gan, W.J.; Wark, P.A.; Chung, K.F.; Hughes, J.M.; Armour, C.L. and Phipps, S. (2012):** RAGE: a new frontier in chronic airways disease. Br J Pharmacol.; 167 (6): 1161-1176.
57. **Take, G.; Karabay, G.; Erdogan, D. and Duyar, I. (2006):** The ultrastructural alterations in rat corneas with experimentally-induced diabetes mellitus. Saudi Med J.; 27 (11): 1650-1655.
58. **Thomson, M.; Al-Amin, Z.M.; Al-Qattan, K.K. and Ali, M. (2007):** Hypoglycemic effects of ginger in mildly and severely diabetic rats. The FASEB Journal.; 21: 103.104.
59. **Whiting, D.R.; Guariguata, L.; Weil, C. and Shaw, J. (2011):** IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract.; 94 (3): 311-321.
60. **Xiang, M.; Yang, M., Zhou, C.; Liu, W. and Qian, Z. (2006):** Protective effect of puerarin on diabetic retinopathy in rats. Mol Biol Rep.; 6: 1129-1133.
61. **Yang, Y.; Mao, D.; Chen, X.; Zhao, L.; Tian, Q.; Liu, C. and Zhou, B. (2012):** Decrease in retinal neuronal cells in streptozotocin-induced diabetic mice. Mol Vis.; 18: 1411–1420.
62. **Yin, J.; Huang, J.; Chen, C.; Gao, N.; Wang, F. and Yu, F.S. (2011):** Corneal complications in streptozocin-induced type I diabetic rats. Invest Ophthalmol Vis Sci.; 52 (9): 6589-6596.
63. **Zafar, M. and Naqvi, S. (2010):** Effects of STZ-Induced Diabetes on the Relative Weights of Kidney, Liver and Pancreas in Albino Rats: A Comparative Study. Int. J. Morphol.; 28 (1): 135-142.
64. **Zagon, A.S.; Sassani, J.W. and Mclaughlin, P.J. (2006):** Insulin Treatment Ameliorates Impaired Corneal Reepithelialization in Diabetic Rats. Diabetes.; 55 (4): 1141-1147.
65. **Zagon, I.; Klocek, M.; Griffith, J.; Sassani, J.; Komáromy, A. and McLaughlin, P. (2008):** Prevention of exuberant granulation tissue and neovascularization in the rat cornea by naltrexone. Arch Ophthalmol.; 126 (4): 501-506.