Protective Role of Ginger on the Induced Diabetic Retinopathy in Adult Albino Rats

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Abstract: Diabetes mellitus (DM) is a global epidemic disease and the alarming increase in its prevalence is one of the major challenges to modern health care. Therefore, with a rapid increase in the prevalence of DM, ocular complications have become a leading cause of loss of vision in the world. Diabetic retinopathy (DR) is the most common complication in diabetic patients. Although it has long been considered a microvascular disease and that the blood-retinal barrier (BRB) breakdown is a hallmark of this disease, DR has also recently been viewed as a neurodegenerative disease of the retina. Therefore, this study was carried out, for the first time, to throw more light on the role of ginger extract on the retinal complications in a streptozotocin (STZ)-induced rat model of DM including its effect on both caspase-3 and vascular endothelial growth factor (VEGF) expressions. 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 sacrificed 4th, 6th & 8th week after induction of DM. Body weight, blood glucose level, and glycated haemoglobin were measured. Retinal specimens were subjected to histological (both light and electron microscopic), histochemical, immunohistochemical and quantitative studies. It was found that caspase-3 & VEGF have played a pivotal role in the damaging effect of the DR induced by STZ and that ginger supplementation considerably mitigates these damaging effects.
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Key words: Diabetic retinopathy-Ginger-Caspase-3-VEGF

1. Introduction:

Diabetes mellitus (DM), a life long progressive disease, is the result of body's inability to produce or use insulin to its full potential, and is characterized by high circulating blood glucose level **Kowluru and Chan (2007).** This disease 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 **Peppa and Vlassara (2005).** Diabetic retinopathy (DR) is the most severe ocular complication of DM and leads to visual loss in millions of patients worldwide as a consequence of both type 1 and type 2 DM **Saghizadeh *et al.* (2005).** The most frequent of these ocular complications are cataract, DR and neovascular glaucoma. Most cataracts can be treated safely by cataract surgery. However, DR is still a leading cause of visual loss and blindness in adults in most developed countries **Kakehashi (2011).** It is a duration-dependent disease that develops in stages; the incidence of retinopathy is rarely detected in the first few years of diabetes, but the incidence increases to 50% by 10 years, and to 90% by 25 years of diabetes **Kowluru and Chan (2007).**

In spite of all advances in the understanding of chronic diabetic complications, DR remains a significant clinical problem. The pathogenic mechanisms leading to the development of DR is indeed complex. Multiple interactive mechanisms may come into play leading to cellular damage and adaptive changes leading to the development of this devastating complication of DM **Chakrabarti (2007).**

As DR is a multifactorial disease, hyperglycemia is considered the major factor in its development **Kowluru (2010).** It results from a complex interplay between multiple pathogenic processes, developing in both retinal vasculature and the neural retina. Furthermore, tissue-specific overexpression or knock-down of specific genes creates the possibility of exploring the role of vascular and non-vascular mechanisms in the pathogenesis of DR **Obrosova *et al.* (2006).**

Diabetic retinopathy appears 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).** Moreover, AGEs interact 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 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 AGEs formation under in vitro conditions. Among them, ginger was one of the agents that significantly prevented AGEs 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 30ºC, each tablet contained 400mg ginger extract (*Zingber Officinale*). 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 research. 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:

DM 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 control group) (STZ treated group): Consisted of fifteen adult male albino rats. DM 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 $4^{th,}$ 6^{th} and 8^{th} weeks' respectively after induction of DM.

Group III (Ginger treated diabetic group): Consisted of fifteen adult male albino rats. DM 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 at the end of the 4^{th}, 6^{th} and 8^{th} 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 DR 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 at the end of the 4th week 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 sensory retina 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 C°. The specimens were processed for semithin and then ultrathin sections examined by light microscope and transmission electron microscope respectively. This 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 (blood analysis):

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 55C° followed by embedding in hard paraffin.

Sections from these paraffin blocks were stained by:

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

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

a. Haematoxylin 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 3 µm 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, nucleus, cell surface, and extracellular matrix using **Rabbit polyclonal VEGF antibody** (a product of Lab Vision, USA).

Morphometric study:

The same parasagittal region in five different stained retinal sections from five different rats (i.e. one section from each rat) were examined in each group. The whole thickness of the sensory retina was measured in pixels (Figure 1). Furthermore, caspase-3 and VEGF positive cells within the ganglion cell, inner nuclear and outer nuclear layers were counted (Figure 2) 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, Menoufia 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 (N.S)

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

$P \leq 0.001 \rightarrow$ highly significant (H.S)

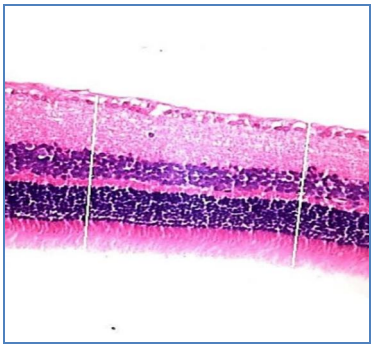

Figure (1): A photomicrograph showing measurement of the whole retinal thickness (white line).
(HX. & E. X 400).

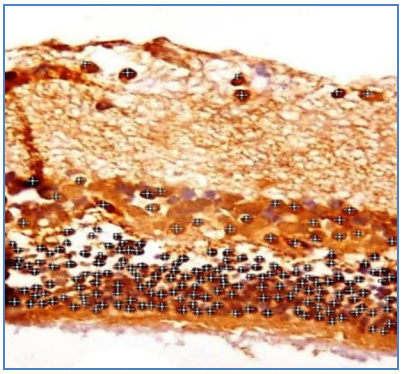

Figure (2): A photomicrograph showing the counting of the caspase-3 positive cells within the retina.
(Immunoreactivity to caspase-3 X 1000).

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^o 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 as a preliminary step (Figure 3) for selection of the areas needed for ultrathin examination. Ultrathin sections were stained with lead citrate and uranyl acetate and were viewed under transmission electron microscope.

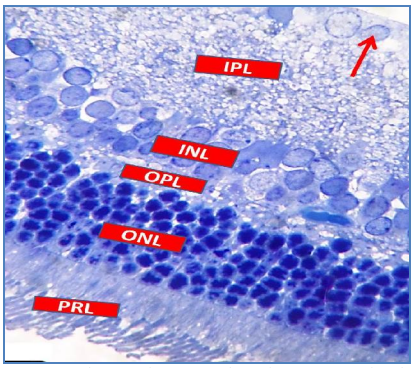

Figure (3): A photomicrograph of a rat retinal semithin section of the control group showing the photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (red arrow).
(Toluidine blue X 1000).

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.

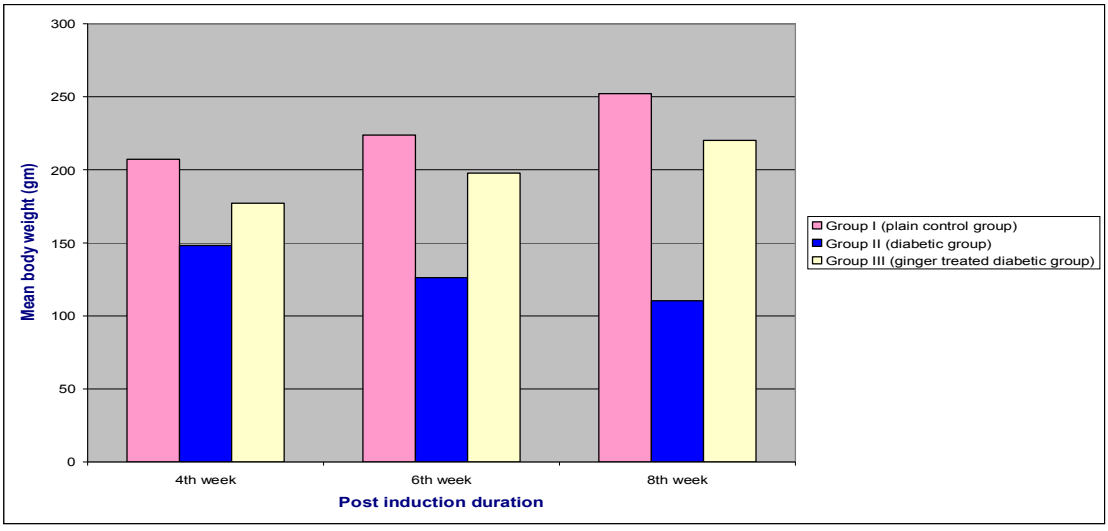

Chart (1): Column chart showing comparison of the mean body weight (gm) between the different experimental groups.

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 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 ginger treated diabetic group and its corresponding control one. The mean body weight 8th week 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).

Blood analysis (Blood Glucose & Glycated Haemoglobin Levels):

STZ administration affected the levels of typical blood parameters characteristic for diabetes, which are also accepted tools in diabetes diagnostics (blood glucose & glycated haemoglobin), indicating high levels of glycosylation.

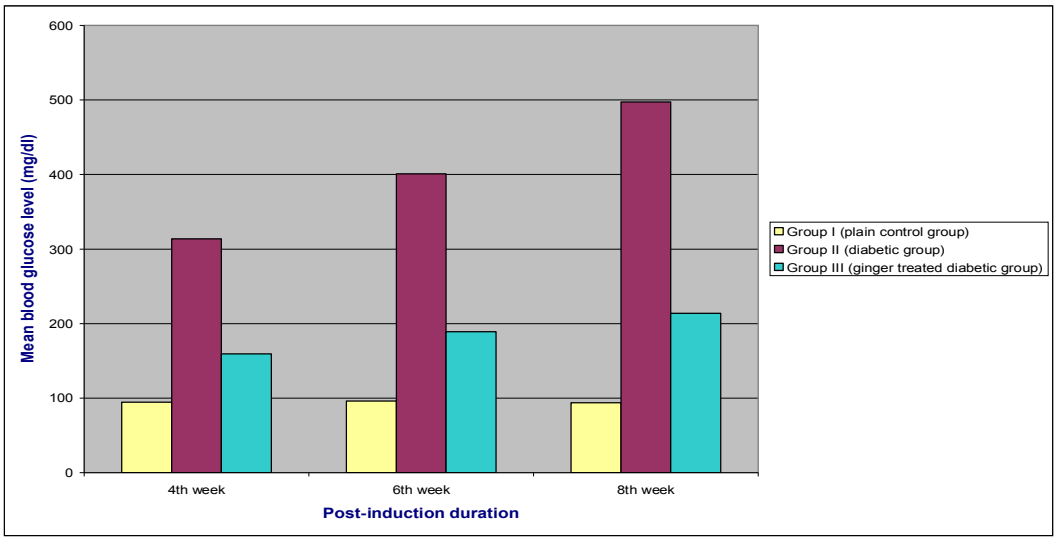

Chart (2): Column chart showing comparison of the mean blood glucose level (mg/dl) between the different experimental groups.

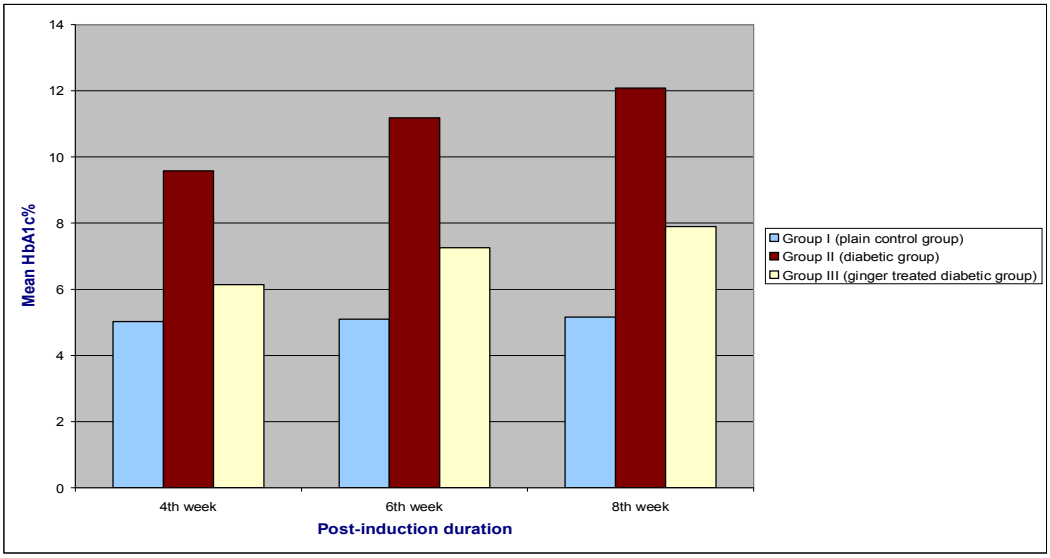

Chart (3): Column chart showing comparison of the mean glycated haemoglobin level (%) between the different experimental groups.

Blood glucose (Chart 2) and HbA1c (Chart 3) concentrations were highly significantly increased in untreated diabetic rats compared to control groups (p >0.001) 4th, 6th & 8th weeks after induction of DM. However, treatment of the diabetic rats with ginger markedly ameliorated these effects but it didn'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.

Haematoxylin and Eosin stain (Hx&E):

Compared with the control group (Figure 4), the diabetic group showed shrunken degenerated ganglion cells, wide spacing within the cells of the outer nuclear and inner nuclear layers in addition to disorganization of the photoreceptor layer 4th week after induction of DM (Figure 5-a). In addition, invasion of spindle shaped microglia cells within the inner plexiform layer, vascularization within the outer plexiform layer and marked destruction of the photoreceptor layer were detected 6th week after the induction (Figure 5-b). Moreover, marked damaging effects of DR were shown 8th week after the induction. In addition to the previous findings, areas of intense destruction that extended even to the degree of complete loss of the photoreceptor layer with disorganization of the retinal layers and intermingling between the cells of outer nuclear layer and that of inner nuclear layer and even disappearance of discrimination marks between the different layers was also revealed 8th week after the induction (Figure 5c - d). Throughout the whole experimental durations, obvious decrease in the sensory retinal thickness including most its different layers as outer nuclear & inner nuclear layers were observed especially 8th week after the induction.

On the other hand, ginger treated diabetic group showed considerable improvement in the retinal appearance and organization that became nearly normal either 4th or 6th weeks after the induction except for some degenerative changes that affected the ganglion cells 6th week after the induction. Moreover, 8th week after the induction of DM revealed good discrimination between the different layers of the sensory retina. However, slight degenerative changes in the ganglion cells, very mild widening of the intercellular spaces between the cells of the outer and inner nuclear layers and slight disorganization of the photoreceptor layer were observed 8th week after the induction (Figure 5 e- g).

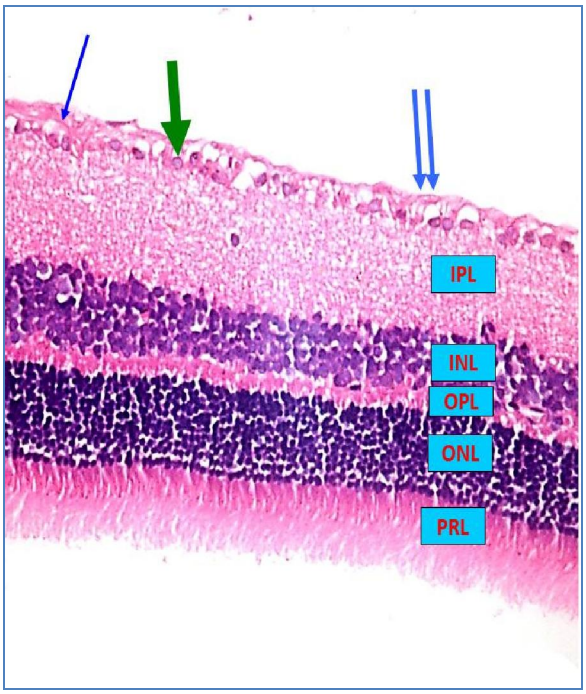

Figure (4): A photomicrograph of a rat retinal section of the control group showing the inner limiting membrane (double blue arrows, nerve fiber layer (blue arrow), ganglion cell layer (green arrow), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and photoreceptor layer (PRL). **(Hx & E X 400).**

Statistically, non-significant difference in the mean retinal thickness between the different subgroups of the group I was noted. Moreover, there was non-significant difference in the control group between the different experimental durations.

However, the diabetic group showed highly significant decrease in the mean retinal thickness when compared with its corresponding control group either 4th, 6th or 8th weeks after the induction.

On the other hand, diabetic rats treated with ginger possessed a highly significant difference in the mean retinal thickness when compared with that of diabetic group. Furthermore, there was non-significant difference regarding the mean retinal thickness between ginger treated diabetic group and the control group 4th and 6th weeks after induction of DM and highly significant difference 8th week after the induction.

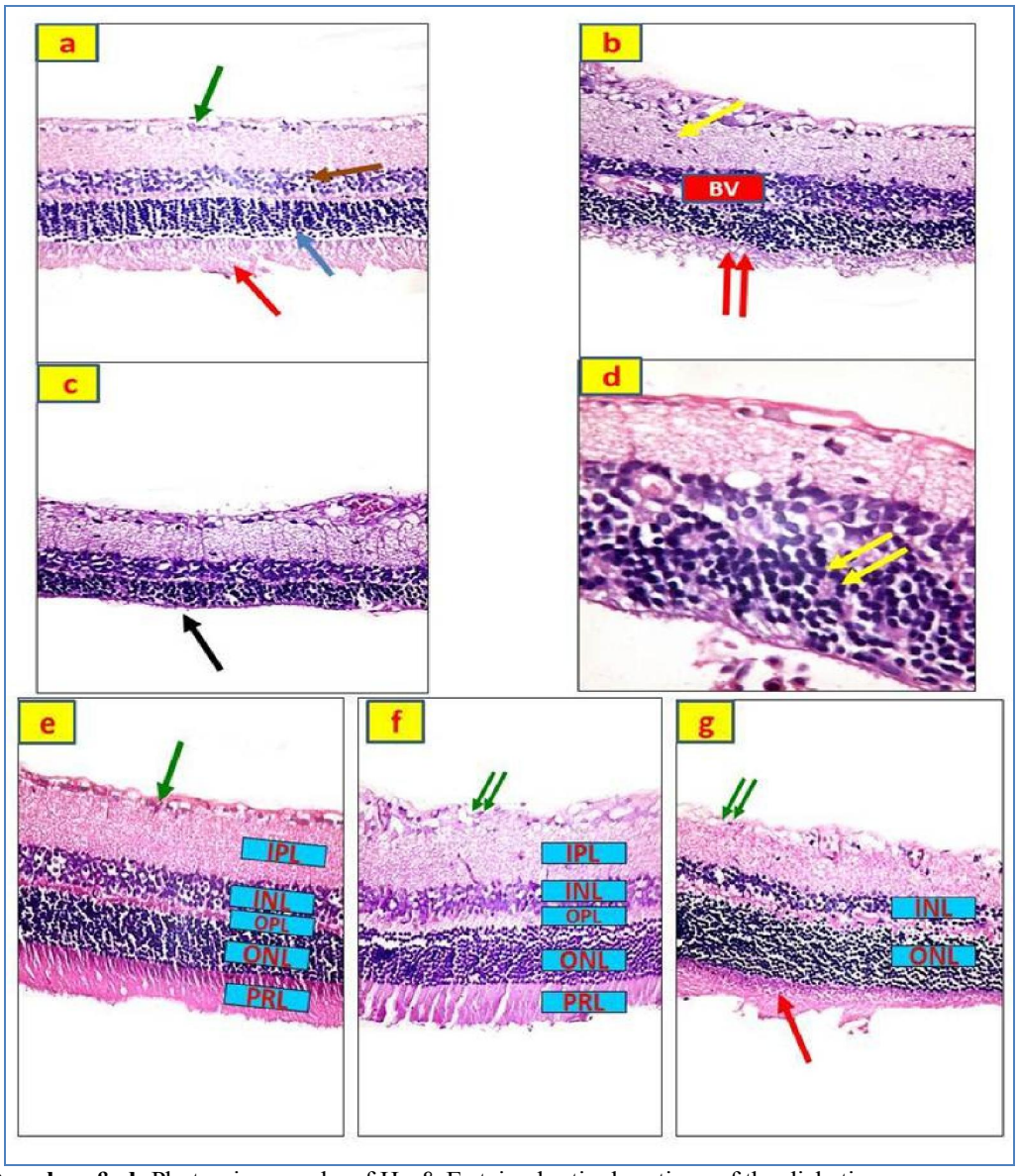

Figure (5): a, b, c & d: Photomicrographs of Hx & E stained retinal sections of the diabetic group:
a) Retinal section of the diabetic rat sacrificed 4th week after induction of diabetes showing shrunken degenerated ganglion cells (green arrow), wide spacing within the cells of the outer nuclear (blue arrow) and inner nuclear (brown arrow) layers in addition to disorganization of the photoreceptor layer (red arrow). **(Hx & E X 400).**
b): Retinal section of the diabetic rat sacrificed 6th week after induction of diabetes showing invasion of spindle shaped microglia cells (yellow arrow) within the inner plexiform layer, vascularization within the outer plexiform layer (orange arrow) and destruction of the photoreceptor layer (double red arrows). **(Hx & E X 400).**
c): Retinal section of the diabetic rat sacrificed 8th week after induction of diabetes showing areas of complete loss of the photoreceptors (black arrow). Note: marked decrease in the retinal thickness including outer nuclear and inner nuclear layers. **(Hx & E X 400).**
d): Retinal section of the diabetic rat sacrificed 8th week after induction of diabetes showing intermingling between the cells of outer nuclear layer and that of inner nuclear layer (double yellow arrows). **(Hx & E X 1000).**
e, f & g: Retinal sections of the diabetic rats treated with ginger and sacrificed 4th, 6th & 8th weeks after induction of diabetes respectively. Retinal sections of the rats sacrificed either 4th or 6th weeks after the induction showing nearly normal inner plexiform (IPL), inner nuclear (INL), outer plexiform (OPL), outer nuclear (ONL) and photoreceptor (PRL). Ganglion cell (green arrow) layer is apparently normal 4th week after the induction however 6th and 8th week after the induction ganglion cells are slightly shrunken and degenerated (double green arrows). Very mild spacing within the outer nuclear (ONL) & inner nuclear (INL) layers with slight disorganization of the photoreceptor layer (red arrow) is also noted 8th week after the induction.**(Hx & E X 400).**

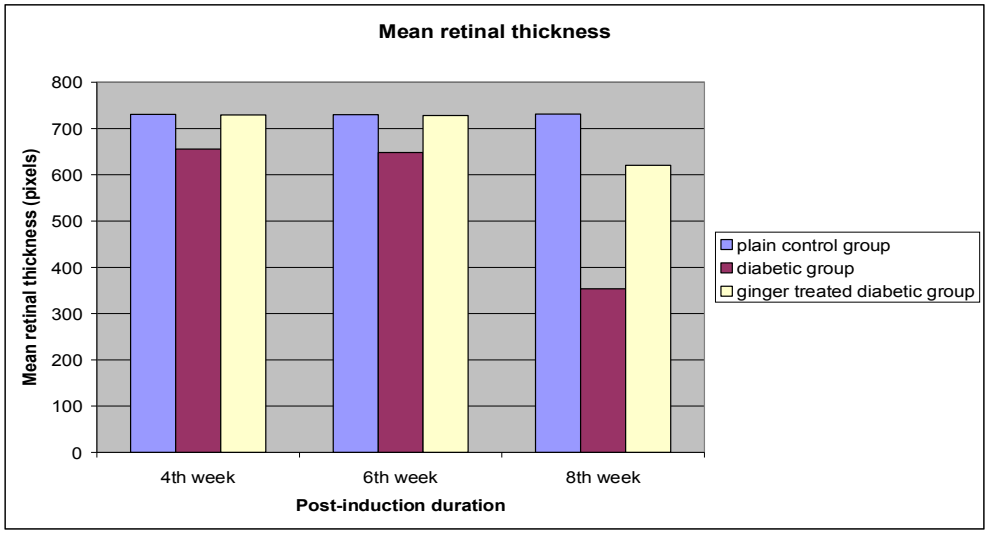

Chart (3): Column chart showing comparison of the sensory retinal thickness (in pixels) between the different groups.

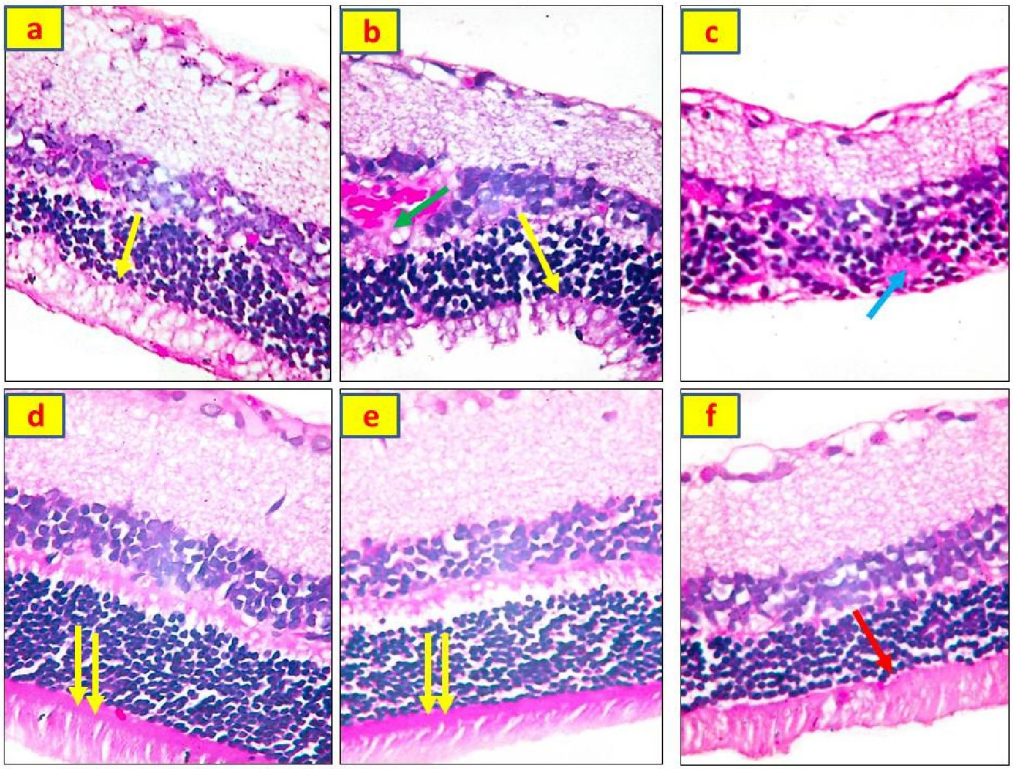

Figure (6): Photomicrographs of the rat retinal sections stained with PAS.

a, b & c: Retinal sections of the diabetic untreated rats that sacrificed 4th, 6th & 8th weeks after the induction respectively showing weaker positive PAS reaction within the outer limiting membrane & inner segment of the photoreceptor layer (yellow arrows), deposition of moderate positive PAS material within the lining of newly formed blood vessel (green arrow) and deposition of strong positive PAS material (blue arrow) within the different retinal layers indicating increased glycogen deposition. **(PAS X 1000).**

d, e & f: Retinal sections of the diabetic rats treated with ginger and sacrificed 4th, 6th & 8th weeks after the induction respectively showing stronger positive PAS reaction than that of the diabetic untreated rats, nearly similar to the control group, within the outer limiting membrane and inner segment of photoreceptor layer (double yellow arrows) either 4th or 6th weeks after the induction that become weaker 8th week after the induction with obvious decrease in glycogen deposition within the different retinal layers. **(PAS X 1000).**

Periodic acid schif (PAS) reaction:

The diabetic group showed weaker positive PAS reaction within the outer limiting membrane and the inner segment of the photoreceptor layer with deposition of moderate positive PAS material within the lining of newly formed blood. Moreover, deposition of strong positive PAS material, indicting glycogen deposition, within the retinal layers was noted especially 8th week after the induction (Figure 6 a-c). On the other hand, considerable decrease in PAS positive material within the retinal layers was noted 8th week after the induction (Figure 6 d-f)**.**

Caspase-3 immunohistochemical stain:

Negative immunoreaction to caspase-3 in the cells within the ganglion cell, inner nuclear and outer nuclear layers was observed in the retinal sections of the control group. These findings were nearly the same at the different experimental durations.

However the diabetic group showed strong positive immunoreaction in many cells, other cells showed negative immunoreaction 4th and 6th weeks after the induction. Moreover, intense positive immunoreactivity to caspase-3 in almost all the retinal layers was detected 8th week after the induction (Figure 7 a-c).

On the other hand, diabetic rats treated with ginger revealed large number of cells with negative immunoreaction however some cells still showed positive immunoreactions either 4th, 6th or 8th weeks after induction of DM (Figure 7 d-f).

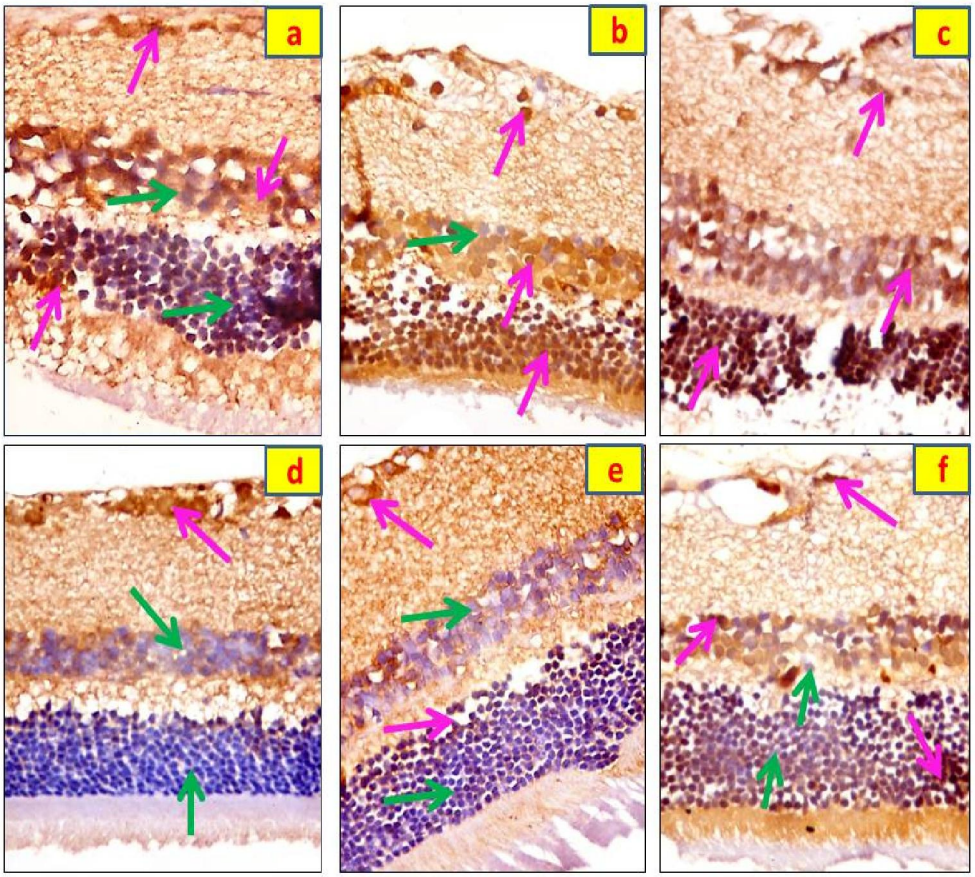

Figure (7): photomicrographs of caspase-3 expression in retinal sections.

a, b & c: Retinal sections of the diabetic untreated rats sacrificed 4th, 6th & 8th weeks after the induction respectively showing strong positive immunoreaction (pink arrows) in many cells, other cells reveal negative immunoreaction (green arrows) 4th and 6th weeks after the induction. Moreover, strong positive immunoreactivity to caspase-3 (pink arrows) in almost all the retinal layers is detected 8th week after the induction. **d, e & f:** Retinal sections of the diabetic rats treated with ginger and sacrificed 4th, 6th & 8th weeks after the induction respectively showing rats large number of cells with negative immunoreaction (green arrows) however some cells still show positive immunoreactions (pink arrows) either 4th, 6th or 8th weeks after induction of DM.

(Immunoreactivity to caspase-3 X 1000).

Statistically, non-significant difference in the mean percent of caspase-3 positive cells between the different subgroups of group I either in the ganglion cell, inner nuclear or outer nuclear layers was noted. In addition, there was non-significant difference between the different experimental durations.

However, the diabetic group showed highly significant increase in the mean percent of caspase-3 positive cells within the ganglion cell, inner nuclear and outer nuclear layers either 4th, 6th or 8th weeks after induction of DM when compared with its corresponding control group (Chart 4).

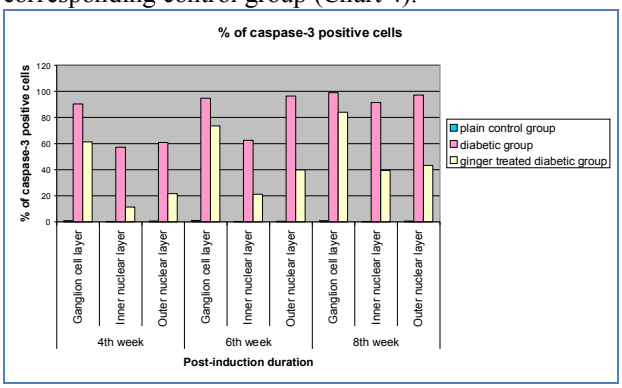

Chart (4): Column chart showing comparison of the % of caspase-3 positive cells within the different retinal layers between the different groups.

On the other hand, ginger treated diabetic group possessed highly significant decrease in the mean percent of caspase-3 positive cells in the ganglion cell, inner nuclear and outer nuclear layers at various durations when compared with its corresponding diabetic group. However, there a highly significant difference between ginger treated diabetic group and control group was also revealed (Cart 4).

VEGF immunohistochemical stain:

VEGF immunohistochemical staining of the retinal sections of group I revealed negative immunoreactivity in nearly all the cells of the ganglion, inner nuclear and outer nuclear layers however detection of very weak positive reaction in few cells within the inner nuclear cell layer was also noted.

The retina of the diabetic rats showed strong positive immunoreaction in some cells of the ganglion, inner nuclear and outer nuclear layers with detection of negative immunoreaction in other cells 4th week after induction of DM. Moreover, positive immunoreaction in nearly in all the retinal layers was seen 6th and 8th weeks after the induction (Figure 8 a-c).

However, ginger treated diabetic group showed negative immunoreaction in most of the cells with positive immunoreactions in other cells either 4th, 6th or 8th week after the induction (Figure 8 d-f).

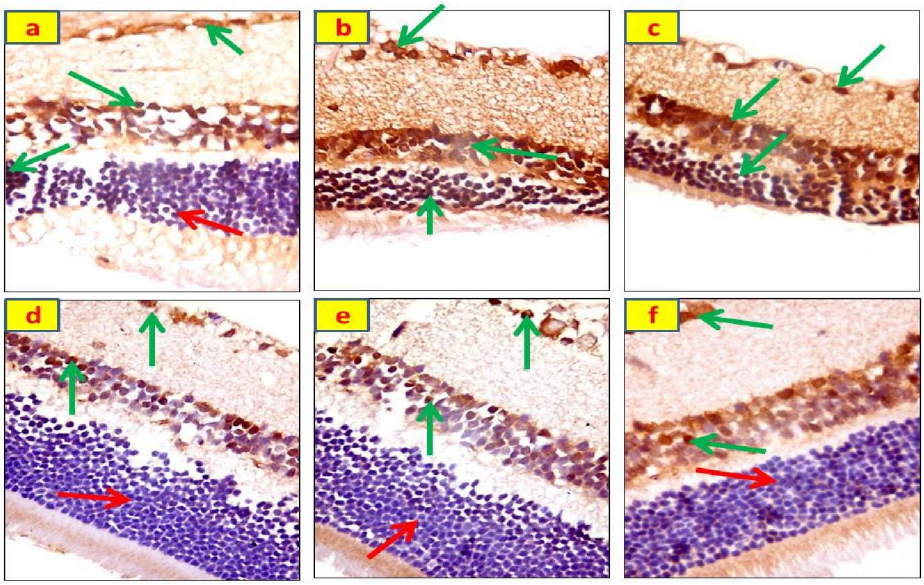

Figure (8): Photomicrographs of the expression of the vascular endothelial growth factor (VEGF) in retinal sections.

a, b & c: Retinal sections of the diabetic untreated rats sacrificed 4th, 6th & 8th weeks after the induction respectively showing strong positive immunoreaction (green arrows) in many cells, other cells still reveal negative immunoreaction (red arrow) 4th after the induction. Moreover, strong positive immunoreactivity to VEGF (green arrows) in almost all the retinal layers is detected 6th & 8th weeks after the induction. **d, e & f:** Retinal sections of the diabetic rats treated with ginger and sacrificed 4th, 6th & 8th weeks after the induction respectively showing rats large number of cells with negative immunoreaction (red arrows) however some cells still show positive immunoreactions (green arrows) either 4th, 6th or 8th weeks after induction of DM.
(Immunoreactivity to VEGF X 1000).

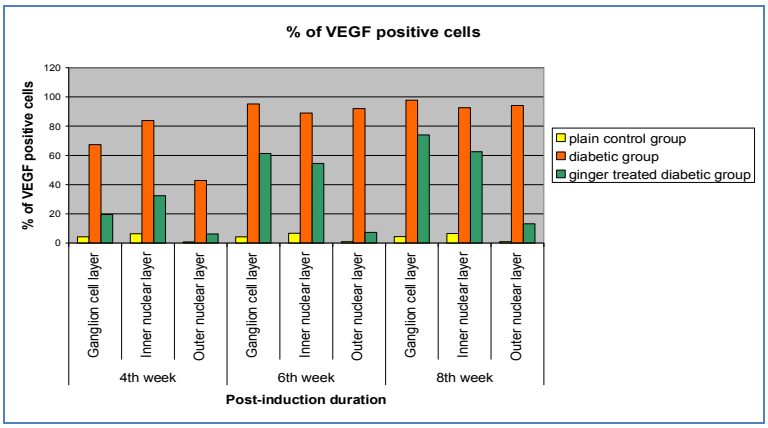

Chart (5): Column chart showing comparison of the % of VEGF positive cells within the different retinal layers between the different groups.

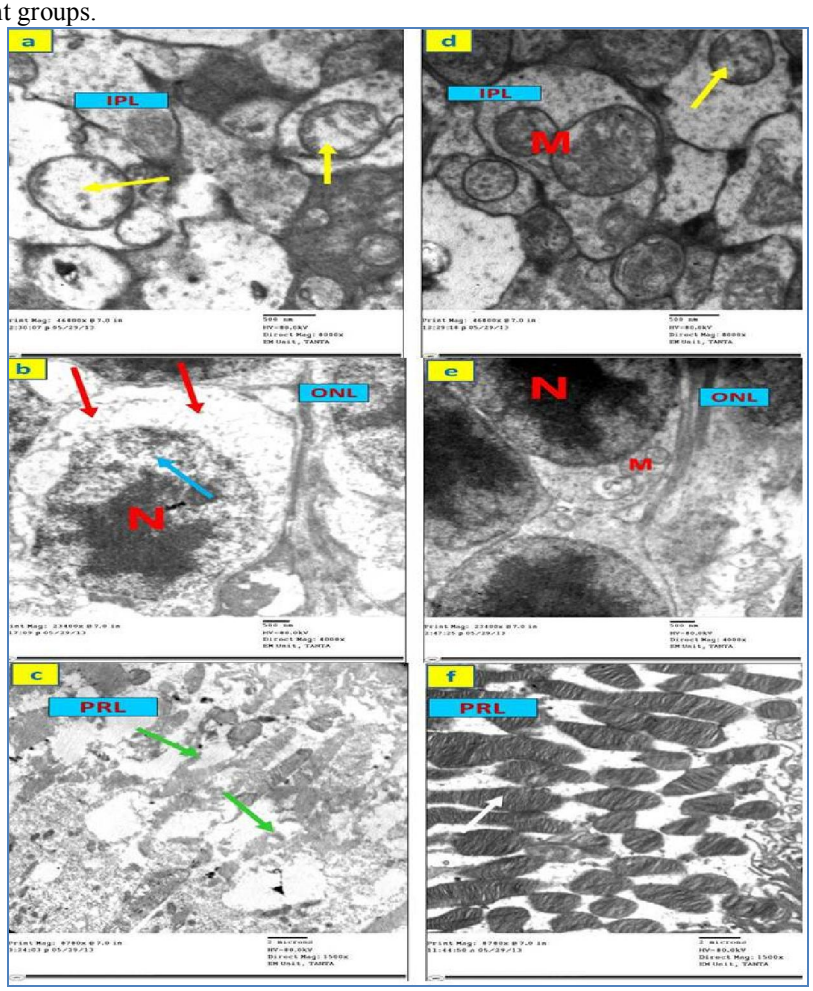

Figure (9): Photomicrographs of the retinal sections using transmission electron microscope (TEM).
a, b & c: Retinal sections of the diabetic untreated rats that sacrificed 4th week after induction of DM showing degenerated mitochondria with destructed cristae (yellow arrows) within the inner plexiform layer (IPL), less condensed chromatin (blue arrow) of the nucleus (N) in the outer nuclear layer (ONL) with vacuolated cytoplasm (red arrows) in addition to destructed degenerated (green arrows) photoreceptors (PRL). **d, e & f:** Retinal sections of the diabetic rats treated with ginger that sacrificed 4th week after induction of DM showing normal appearance of most of the mitochondria with normally arranged cristae (M) within the inner plexiform layer (IPL) whereas few mitochondria reveal slightly destructed mitochondria (yellow arrow), highly condensed chromatin of the nucleus (N) within the outer nuclear layer (ONL) with well-organized mitochondria (M). In addition, normally arranged photoreceptors (PRL) with slight spacing in-between were also detected.

Statistically, non-significant difference in the mean percent of VEGF positive cells either in the outer nuclear, inner nuclear or in the ganglion cell layer was noted between the different subgroup of the group I. In addition, there was non-significant difference between the different experimental durations.

However, the diabetic group revealed highly significant increase in the mean percent of VEGF positive cells in the outer nuclear, inner nuclear and ganglion cell layers when compared with that of control group either 4th, 6th or 8th weeks after induction of DM (Chart 5).

On the other hand, ginger treated diabetic group showed highly significant decrease in the mean percent of VEGF in the outer nuclear, inner nuclear and ganglion cell layers at the different durations when compared with that of diabetic group. Moreover, there was highly significant difference between ginger treated diabetic group and control group (Chart 5).

****Transmission electron microscopic results:**

The diabetic group sacrificed 4th week after the induction showed markedly degenerated mitochondria with destructed cristae in nearly all the retinal layers. In addition, some cells within the outer nuclear layer appeared with less condensed chromatin in their nuclei and rarified vacuolated cytoplasm. Moreover, marked destruction of the photoreceptor layer was also observed (Figure 9 a-c).

However, ginger treated diabetic group that sacrificed 4th week after the induction showed considerable improvement than that of diabetic untreated one. Normal organization of most mitochondria with few slightly degenerated ones was detected in nearly all the retinal layers. Moreover, the cells of the outer nuclear layer had highly condensed chromatin in their nuclei nearly similar to that of control group with normal structure of most of their mitochondria however; few degenerated destructed mitochondria were also detected. It also showed nearly normal appearance of the photoreceptor layer with slight spacing in-between (Figure 9 d-f).

4. Discussion:

Kusari *et al.* (2007) stated that DR is the most common complication of DM as it affects more than 90% of the diabetic patients and progresses to complete blindness in approximately 5%. Although DR is an angiopathy, neuroretinal changes should receive more attention in DR.

Rats were particularly selected in this work because they were easily obtained, handled, controlled and investigated. Moreover, the rats seemed to mimic human. This was in accordance with **Yang *et al.* (2012)** who stated that the most widely accepted animal model for the evaluation of the diabetic complications was the STZ-induced diabetic rats 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 experimental diabetes researches, STZ was particularly used in our research due to its ability to cause cellular necrosis and selective destruction of the pancreatic islet beta cells as mentioned by **Zafar and Naqvi (2010).** Furthermore, STZ had greater stability and longer half-life than alloxan as reported by **Kumar (2013).**

In this study, the 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 to that of diabetic non treated group although it was 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, blood glucose showed a highly significant increase in its level from the beginning till the end of the experiment in experimentally induced 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 glycosylated haemoglobin, a marker of protein glycation, that was in agreement with **Sheykhzade *et al.* (2000); Ramesh *et al.* (2006) and Ansari *et al.* (2008).** This may be attributed to its action in affecting insulin secretion causing diabetes mellitus as mentioned by **Take *et al.* (2006)** who referred the significant elevation in blood glucose level in STZ induced diabetic rats to the reduction in the plasma insulin levels caused by selective irreversible necrosis in beta cells of pancreas.

In the ginger treated diabetic group, through all the studied durations, there was highly significant decrease in both blood glucose level and glycated haemoglobin that didn't reach the level of control group. These changes were in agreement 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 diabetes through its effects on the activities of glycolytic enzymes.

Furthermore, **Li *et al.* (2012)** stated that ginger promoted glucose clearances in insulin responsive peripheral tissues and augmented insulin release which maintained blood glucose homeostasis. They added that there was evidence from in vitro studies that ginger extract and its pungent gingerol principles enhanced glucose uptake in cultured rat skeletal muscle cells. In addition, **Ansari *et al.* (2008)** attributed the decrease in the level of glycosylated haemoglobin to the decreased level of blood glucose.

As neurodegenerative changes occurred in the DR could be inevitable as mentioned by **Park *et al.* (2003)** and that it appeared even before the onset of visible vascular lesions as revealed by **Van Dijk *et al.* (2009)**. We therefore investigated the pathological changes in the neural retina, including neuronal and vascular changes, of a STZ-induced rat model of DM.

In the current study, there was a highly significant decrease in the thickness of the sensory retina with the progression of the disease. This was in agreement with **Park *et al.* (2003)** who attributed this decrease in the thickness to the degenerative changes that involved the sensory retina as a result of DM.

In the present study, several degenerative changes were noted in the retina of the different diabetic subgroups as early as 4th week after induction of DM and became worse as the DR progressed. These degenerative changes affected nearly all layers of sensory retina. This was in accordance with **Martin *et al.* (2004)** who reported that the histological changes to the inner retina seemed to be more severe. Moreover, **Kusari *et al.* (2007)** demonstrated significant decrease in the retinal functions within 4 to 5 weeks after STZ treatment.

These neuronal affections may be attributed to several pathological mechanisms potentially involved increased production of AGEs and elevated aldose reductase activity, which were related with oxidative stress and inflammation as suggested by **Shi *et al.* (2013).** Moreover, **Miranda *et al.* (2006)** considered the retina as the neurosensorial tissue of the eye that extremely rich in polyunsaturated lipid membranes. This feature made it especially sensitive to oxygen and/or nitrogen activated species and lipid peroxidation.

This was in disagreement with **Feit-Leichman *et al.* (2005)** who did not detect significant loss of retinal neurons in diabetic mice 8th week after induction of DM.

As regard the ganglion cell layer, the present work showed marked degeneration of the ganglion cells. **Feit-Leichman *et al.* (2005**) attributed this degeneration to the presence of apoptosis in ganglion cells and this was confirmed in this study by up-regulation of caspase-3 in the ganglionic cells of the diabetic rats.

Moreover, **Bui *et al.* (2009)** revealed that ganglion cell dysfunction could result from changes in the axonal transport mechanisms within the optic nerve of STZ induced diabetic rats.

Concerning the outer nuclear and inner nuclear layers of the retina in the present work, spacing between their cells with apparent rarified vacuolated cytoplasm were noted. In addition, there was apparent decrease in their number. This was in accordance with **Szabadfi *et al.* (2012)** who noticed degeneration of cones with decrease in the number of the cells in outer nuclear layer with degenerative changes in cells of the inner nuclear layer and with **Lu *et al.* (2003)** who found that the inner nuclear layers of the retina decreased from 3–4 rows to 2 rows, whereas the photoreceptor cell nuclei decreased from 8 rows to 3–6 rows that was confirmed in our morphometric results by decrease in the whole retinal thickness.

These changes may be attributed to increase in apoptotic cells in both outer nuclear and inner nuclear cells and this was in accordance with **Feit-Leichman *et al.* (2005)** who proved that there was induction of cell apoptosis and caspase-3 activation in the mice retinal neurons within days of DM induction and these findings also documented in this study by increased number of apoptotic cells in the nuclei of both outer and inner nuclear layers.

This was explained by **Li and Puro (2002)** who reported that diabetes- induced dysfunction of the glutamate transporter in retinal Muller cells was responsible for elevated retinal glutamate levels. **Ng *et al.* (2004)** noted that expression of the N-methyl-D-aspartate (NMDA)-type glutamate receptors was also up-regulated in the diabetic retina. **Kusari *et al.* (2007**) suggested that excessive stimulation by glutamate resulted in several types of neurodegenerative diseases.

Concerning the separation between the cells in outer and inner nuclear layers, **Brownlee (1992)** suggested that glucose irreversibly modified long-lived macromolecules by forming AGEs. He added that AGEs caused qualitative and quantitative changes in extracellular matrix components such as type IV collagen, laminin, and vitronectin. These AGEs-induced changes could affect cell adhesion, growth, and matrix accumulation.

In addition, however there was absence of apparent affection of the outer and inner plexiform layers in 4th week after induction of DM by light

microscopic study, there were degeneration and destruction of their mitochondria as seen by electron microscopic in early DR at 4th week after induction.

This was in accordance with **Aizu *et al.* (2002)** who speculated that the axon and the processes of retinal neurons were sensitive in DM. **Park *et al.* (2003)** added that degenerated mitochondria appeared in a few photoreceptor terminals and their post-synaptic processes in the outer plexiform layer, and in some axon terminals in the inner plexiform layer.

Kanwar *et al.* (2007) attributed the presence of degenerated mitochondria in DR to the occurrence of oxidative stress in cases of DM. Furthermore, **Liang and Godley (2003)** stated that the mitochondria were the major source of superoxide production and were subjected to direct attack of ROS causing damage to the mitochondrial components.

Kanwar *et al.* (2007) postulated that there was direct evidence that superoxide levels were significantly elevated in the mitochondria isolated from the retinas of diabetic mice and that the overexpression of mitochondrial super oxide dismutase (MnSOD) prevented diabetes-induced increases in mitochondrial superoxide. They demonstrated that the mitochondria were the source of increased superoxide because the mitochondrial electron transport system can be both the source and a target of excess ROS.

As mentioned before, the current study declared vacuolated rarified cytoplasm in the different layers of the retina of diabetic rats seen by transmission electron microscope especially the outer nuclear layer. This was in agreement with **Alvarez *et al.* (2010)** who attributed the presence of vacuolated cytoplasm within the diabetic retinas to the occurrence of hyperosmolarity state as a result of hyperglycemia.

In the present study, there were scattered small darkly stained nuclei between the cells of inner retinal layers most probably microglia especially at 6th and 8th weeks after induction of DM. This finding coincided with the results of **Zeng *et al.* (2008)** who stated that in cases of DR, microglial cells were markedly increased in number and activity in the inner retinal layers. Meanwhile, **Rungger-Brändle *et al.* (2000)** suggested that during early DR, microglial cells were closely opposed to ganglion cell bodies and axons and in association with blood vessels. This association could favor initial microglial activation by compromised neurons, with the release of free fatty acids being a likely inducer.

Moreover, **Rungger-Brändle *et al.* (2000)** thought that the damaged blood retinal barrier which was observed at 2 weeks of induced diabetes before glial reactivity might trigger this behavior. This suggested that glia were the early targets of vascular hyper-permeability. This was supported by our results coinciding with increased expression of VEGF in the diabetic retinas.

In addition, **Gardner *et al.* (2002)** mentioned that activated microglia released pro-inflammatory cytokines and chemokines, such as VEGF and tumor necrosis factor (TNF); it was likely that they further exacerbated retinal vascular permeability in diabetes.

On the other hand, **Zeng *et al.* (2000)** suggested that microglia might be involved in clearing of degenerated neuronal elements elicited by the induced DM. In addition, microglial cells in DR might also function as antigen presenting cells in inflammation of the retina and the neighboring tissues.

In the present study, neovascularization of the diabetic retina was shown 6th and 8th weeks after induction of DM. This was in agreement with **Hassan *et al.* (2012)** who noticed growth of new blood vessels within the retina of diabetic patients. They referred the occurrence of neovascularization to the extensive lack of oxygen in the retinal capillaries of diabetics. In addition, **Frank (2004)** mentioned that VEGF expression was enhanced by hypoxia which was a major stimulus for retinal neovascularization. This was confirmed in the current study by immunohistochemical staining of VEGF.

Neuronal degenerative changes in the present work included, disorganization of photoreceptors with apparent decrease in their number that progressed even to complete loss 8th week after induction of DM. This was in agreement with **Szabadfi *et al.* (2012)** who noticed degeneration with decrease in the length of photoreceptor layer in DR.

This may be attributed to apoptotic change in photoreceptor cells as suggested by **Park *et al.* (2003)** who clarified occurrence of apoptosis in a few photoreceptor cells at 4th week and that the number of apoptotic photoreceptors increased thereafter and they thought that the visual loss associated with DR could be attributed to an early phase of substantial photoreceptor loss. These apoptotic changes also confirmed in this research by detection of increased expression of caspase-3 immunohistochemical stain. In addition, **Van Eeden *et al.* (2006)** suggested that overexpression of VEGF in the retina resulted in photoreceptor degeneration and this was confirmed in this study by increased immunohistochemical expression of VEGF in the retinas of the diabetic rats.

On the other hand, **Kohzaki *et al.* (2008)** found that the photoreceptoral (a-wave) responses were not significantly reduced by STZ treatment.

Concerning PAS reaction, the diabetic group showed decreased PAS reaction within the inner segments of photoreceptor layer and the outer limiting membrane that indicated destruction in their components. Up to our knowledge, this was not documented in previous researches. This finding may

be explained by **Murakami and Yoshimura (2013)** who thought that the photoreceptor damage was represented by disruption of the outer limiting membrane.

Moreover, the current study showed increased deposition of PAS positive material within the retina of diabetic rats and in the lining of the newly formed blood vessel and this revealed increase in glycogen content in cases of DR. This was in agreement with **Sánchez-Chávez *et al.* (2008)** who noticed increase in glycogen content within the retina of diabetic rats. They attributed this increase in glycogen content to the increase in glycogen synthase activity between 50% and 100% in the diabetic retina by glucose-6-phosphorylase. Furthermore, **Tesseromatis *et al.* (2009)** suggested that DM resulted in the development of micro-vessel damage and that the abnormalities included accumulation of PAS positive material within the blood vessel was considered one of the histological markers of diabetic microangiopathy.

Regarding caspase-3 expression in the DR, the current study revealed that up-regulation of caspase-3, a marker of apoptosis, played a pivotal role in the pathogenesis of DR along the neuronal level. This was confirmed by increased expression of caspase-3 in the cells of outer nuclear, inner nuclear and ganglion cell layers that began 4th week after induction of DM and became more intense with progression of the disease.

This was in agreement with **Gastinger *et al.* (2006)** who found evidence of neuronal alterations included the presence of apoptosis in the inner retinal layers of diabetic animals and with **Park *et al.* (2003)** who noticed apoptotic evidence in the diabetic retinas using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Moreover, **Feit-Leichman *et al.* (2005)** showed activation of caspase-3 in DR.

Kusari *et al.* (2007) suggested that excessive stimulation by glutamate resulted in several types of neurodegenerative diseases and this was in agreement with **Donello *et al.* (2001)** who reported elevated levels of vitreal glutamate in several pathologic conditions. The excitotoxicity of glutamate was the result of over-activation of NMDA-type glutamate receptor. Activation of NMDA receptors resulted in increases calcium permeability, which initiated calcium-dependent processes that led to cell death as mentioned by **Smith (2002).** Moreover, **Rosa and Rapoport (2009)** reported that excess Ca2+ stimulated Ca2+ dependent phospholipases which, in turn, promoted release of bioactive cytotoxic fatty acids into the neurons causing neuronal apoptosis.

In addition, **Kniep *et al.* (2006)** stated that AGEs as well as hydrogen peroxide as a result of high blood glucose level induced retinal neurons to reduce their intracellular pH and augmented their production of ROS which in turn led to apoptosis.

In our research, detection of VEGF expression within the retina of the diabetic rats was performed as it was considered not only as a proangiogenic marker but also a detector of the occurrence of vascular permeability and breakdown of the blood retinal barrier that characterized early stages of DR as mentioned by **Kusari *et al.* (2007).**

Increased expression of VEGF within the retina of diabetic rats either cytoplasmic or nuclear was revealed in this study from the 4th week after induction of DM and thereafter. This was in agreement with **Chung et al. (2005) and Kusari *et al.* (2007)** who noticed increased VEGF protein levels in the retinas and vitreous fluid of diabetic rats using enzyme linked immunosorbent assay (ELISA) kit and VEGF-mRNA by reverse transcription polymerase chain reaction (RT-PCR). In addition, **Ray *et al.* (2004) and Simo *et al.* (2006)** found that the VEGF was significantly elevated in the vitreous and aqueous fluids in the patients with either non proliferative or proliferative DR.

Kusari *et al.* (2007) stated that VEGF was produced by retinal pigment epithelium cells, ganglion cells, Muller cells, pericytes, and smooth muscle cells of human retina and choroids and it might act directly on endothelial cell tight junctions to decrease their protein content or to increase their phosphorylation, and either or both of these effects might increase paracellular permeability.

Xu *et al.* (2004) attributed the increased expression of VEGF in the DR to the presence of increased calcium concentration as a result of elevated glutamate which in turn stimulated protein kinase C (PKC) that was responsible for the up regulation of retinal VEGF proteins.

Furthermore, **Funatsu and Yamashita (2002)** found that various stimuli associated with DR had been reported to increase the expression of VEGF, including hypoxia, an elevated glucose concentration and activation of angiotensin II. In addition, **Hughes *et al.* (2007)** suggested that AGEs altered the expression of certain genes that affected cellular growth, differentiation, inflammation and angiogenesis, which were the basis of ischemic and proliferative DR.

Generally, DR is a complex disease that potentially involved increased production of AGEs and elevated aldose reductase (AR) activity, which are related with oxidative stress and inflammation as suggested by **Shi *et al.* (2013).**

Although, there have been major advances in the control of hyperglycemia through dietary changes, hypoglycemic agents, insulin, and islet transplantation, the management of long-term

complications of DM, such as blindness, remains serious problem to be dealt with as thought by **Saraswat *et al.* (2010).**

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).**

So, to ameliorate the damaging effects of DM on the retina, ginger was used in this study. Up to our knowledge, there were no documented researches that used ginger in DR.

In this work, it was noted that ginger markedly delayed the onset of DR in comparison to the diabetic untreated rats along morphometric, histological, histochemical and immunohisto-chemical levels.

In the present study, ginger ameliorated the damaging effect of DM on the retina along the morphometric and histological 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 and with **Shanmugam *et al.* (2010)** who noticed that ginger exhibited a neuroprotective effect on the brain of diabetic rats. They attributed these protective effects on diabetic complication to the acceleration of anti-oxidant defense mechanisms and down regulation of the malonaldehyde (MDA) levels to the normal levels in the diabetic rats. Thus, they thought that ginger might be used as therapeutic agent in preventing complications in diabetic patients.

As discussed before, because one of the major pathogenic mechanisms accelerating the onset of diabetic complications 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 DR. 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.

Saraswat *et al.* (2010) suggested that the ginger contained over 20 phenolic compounds. The major active principles included zingiberene, bisabolene, gingerols, and shogaols and that these agents exerted multiple actions like antiglycating, antioxidant, and antidiabetic /hypoglycemic properties that might provide a viable approach, either food based or pharmacological, in the treatment of diabetic complications.

In ginger treated rats in the current study, there was more or less restoration of normal glycogen deposition, using PAS reaction, in the sensory retina with apparent decrease in PAS reaction within the retinal tissues than that of diabetic non treated rats indicated that there was decrease in glycogen content in ginger treated diabetic rats. This was in agreement with **Abdulrazaq *et al.* (2012)** who noticed that ginger administration caused significant decrease in the kidney glycogen contents when compared with diabetic non treated rats.

Abdulrazaq *et al.* (2012) found that the activities of glucokinase, phosphofructokinase and pyruvate kinase in diabetic rats were decreased by 94, 53 and 61 %, respectively, when compared with normal controls; and that ginger significantly increased those enzymes' activities in STZ-diabetic rats. Therefore, they suggested that ginger was a potential phytomedicine for the treatment of diabetes through its effects on the activities of glycolytic enzymes.

The present work revealed that ginger administration to the diabetic rats throughout the different durations decreased the expression of caspase-3 within the retina of diabetic treated rats. Thus ginger had a protective effect on the DR 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 diabetic complications.

The ability of ginger to down regulate caspase-3 expression was in agreement with **El-Sharaky *et al.* (2009)** who declared 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 DR 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. This was in agreement with **Saraswat *et al.* (2010)** who noticed the antiglycating effect of ginger in the lens of diabetic rats and with **Nassiri et al. (2009)** who found that ginger administration increased the total antioxidant capacity in the testes of the diabetic rats.

Ho and Chang (2012) revealed that the capacity of spices, including ginger, 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.

However there were no documented researches, up to our knowledge, on the effect of ginger on VEGF in diabetic complications, the current study clarified that the treatment of the diabetic rats with ginger

alleviated the expression of VEGF that was markedly increased in DR. So, the protective effect of ginger against the diabetic vascular abnormalities may be through its ability to down regulate VEGF expression in the retina and thus it had anti-VEGF property.

This was in agreement with **Kime *et al.* (2005)** who stated that [6]-gingerol inhibited VEGF induced proliferation of human endothelial cells and strongly inhibited sprouting of endothelial cells in the rat aorta and formation of new blood vessels in the mouse cornea in response to VEGF.

As mentioned before that elevated expression of VEGF in diabetic non treated rats was as a result of increased levels of AGEs and subsequently increased oxidative stress and calcium concentrations. Thus the role of ginger as VEGF inhibitors may be attributed to its antiglycation and antioxidant anti-inflammatory properties as mentioned by **Saraswat *et al.* (2010)** and **Afshari *et al.* (2007)** who investigated the anti AGEs and antioxidant ability of ginger respectively.

In addition, **Verma *et al.* (2004)** suggested that gingerol, shogaol and other structurally-related substances in ginger had the ability to inhibit prostaglandin and leukotriene biosynthesis through suppression of 5-lipoxygenase or prostaglandin synthetase. Additionally, they could also inhibit synthesis of pro-inflammatory cytokines.

Moreover, decreased expression of VEGF in the retina of the diabetic rats treated with ginger may be due to the ability of ginger extract to decrease the level of calcium concentration. This was in agreement with **Ghayur and Gilani (2005)** who proved that ginger administration decreased the level of calcium concentration in hypertensive rats through blockade of voltage-dependent calcium channels.

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