The possible therapeutic effect of Mesenchymal stem cells and their exosomes on experimentally induced diabetic retinopathy in rats: Histological and immunohistochemical study

Submitted in fulfillment for MD Degree in Basic Medical Science (Histology and Cell Biology)

by

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2020
وعلمت ما لم تكن تعلم وكان
فضل الله عليك عظيما

صدق الله العظيم

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Heba Elsayed
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<td>Blood retinal barrier</td>
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INTRODUCTION

Diabetes mellitus is a heterogeneous metabolic disorder characterized by the presence of hyperglycemia due to impairment of insulin secretion, defective insulin action or both. The chronic hyperglycemia of diabetes is associated with relatively specific longterm microvascular and macrovascular complications (Punthakee et al., 2018).

Diabetic retinopathy (DR) is the leading cause of blindness in the working-age adult population and one of the leading causes of vision loss in the elderly. Since the prevalence of diabetes mellitus is on the rise globally, the prevalence of diabetic retinopathy and associated vision loss is expected to rise as well (Park et al., 2017).

As the mammalian retina has limited self-regenerative nature, visual impairment due to retinal degeneration is difficult to treat. Much effort has been applied to the development of cell replacement therapies to functionally restore and replace lost or damaged tissues or organs that lack intrinsic tissue regenerative responses. Stem cells are a type of cell with high self-renewability and differentiation capability, which are mostly favored to be used as a candidate for cell replacement therapy (Ding et al., 2017).

Mesenchymal stem cells (MSC) are a subset of stromal cells ubiquitously found in almost all tissues in the body and migrate into the nervous system in response to injury. They can be isolated from cord blood, Wharton’s jelly, the placenta, bone marrow, teeth, and adipose tissue, which makes them favorable for autologous transplantation (Çerman et al., 2016).
Exosomes are specialized membranous 50–150 nm sized vesicles released from living cells to the extracellular microenvironment. Studies have found that the cellular immunity, angiogenesis, and regenerative effects in cell therapy mediated by mesenchymal stem cells (MSCs) and some other cells are implemented by the exosomes released from these cells, which provides the possibility of “cell-free cell therapy”. Therefore, exosome therapy has shown great application potential from oncology to regenerative medicine. (Liu and Su, 2019). MSC-derived exosomes (MSC-Exosomes), a type of microvesicle released from MSCs, are now recognized as natural vehicles involved in intercellular communication by carrying functional proteins and RNAs to recipient cells and playing therapeutic roles (Yu et al., 2016).
HISTOLOGY OF THE RETINA

The eye is a highly specialized organ for perception of form, light, and color. The eyeballs are located in protective cavities within the skull called orbits. Each eye contains a protective cover to maintain its shape, a lens for focusing, photosensitive cells that respond to light stimuli, and numerous cells that process visual information. The visual impulses from the photosensitive cells are conveyed to the brain via the axons in the optic nerve. Each eyeball is surrounded by three distinct layers (Fig.1): The outer tough layer is the sclera, an opaque layer of dense connective tissue, internal to the sclera is the middle vascular layer (uvea), and the innermost sensory lining of the most posterior chamber of the eye is the retina (Eroschenko, 2017).

The retina develops with two fundamental sublayers from the inner and outer layers of embryonic optic cup:

- **The outer pigmented layer** is a simple cuboidal epithelium attached to Bruch’s membrane and the choroidocapillary lamina of the choroid. This heavily pigmented layer forms the other part of the dual epithelium covering the ciliary body and posterior iris.

- The inner retinal region, the **neural layer**, is thick and stratified with various neurons and photoreceptors. Although its neural structure and visual function extend anterior only as far as the ora serrata, this layer continues as part of the dual cuboidal epithelium that covers the surface of the ciliary body and posterior iris (Mescher, 2018).
Fig. (1): The sagittal section of an eye shows the interrelationships among the major ocular structures, the three major layers or tunics of the wall, important regions within those layers, and the refractive elements (cornea, lens, and vitreous). (Mescher, 2018).

Two regions or portions that differ in function are recognized in the neural retina; 

- **The non photosensitive region (nonvisual part):** located anterior to the ora serrata, lines the inner aspect of the ciliary body and the posterior surface of the iris.
• **The photosensitive region (optic part)** lines the inner surface of the eye posterior to the ora serrata except where it is pierced by the optic nerve. The site where the optic nerve joins the retina is called the optic disc or optic papilla. Because the optic disc is devoid of photoreceptor cells, it is a blind spot in the visual field. The fovea centralis is a shallow depression located about 2.5 mm lateral to the optic disc. It is the area of greatest visual acuity. The visual axis of the eye passes through the fovea. A yellow-pigmented zone called the macula lutea surrounds the fovea. In relative terms, the fovea is the region of the retina that contains the highest concentration and most precisely ordered arrangement of the visual elements (*Ross and Pawlina, 2019*).

### Layers of the Retina:

Ten layers of cells (Fig. 2) and their processes constitute the retina. For convenience, neurons and supporting cells in the retina can be classified into four groups of cells:

• **Photoreceptor cells**: The retinal rods and cones

• **Conducting neurons**: Bipolar neurons and ganglion cells

• **Association neurons**: Horizontal, centrifugal, interplexiform, and amacrine neurons

• **Supporting (neuroglial) cells**: Müller's cells, microglial cells, and astrocytes.

The specific arrangement and associations of the nuclei and processes of these cells result in the retina being organized in ten layers that are seen with the light microscope. (*Ross and Pawlina, 2019*).

The ten layers of the retina, from outside inward, are:
1. Retinal pigment epithelium (RPE).
2. Photoreceptor layer (PRL).
4. Outer nuclear layer (ONL).
5. Outer plexiform layer (OPL).
6. Inner nuclear layer (INL).
7. Inner plexiform layer (IPL).
8. Ganglion cell layer (GCL).
9. Optic nerve fibers layer (NFL).
10. Inner limiting membrane (ILM).

1) **Retinal pigment epithelium (RPE):**

The retinal pigment epithelium is composed of hexagonal monolayer of epithelial cells that extends anteriorly from the margins of the optic nerve to the ora serrata where it is continuous with the pigmented layer of the ciliary body. Apically (towards the rods and cones), the cells bear long (5–7 µm) microvilli that contact, or project between, the outer segments of rods and cones. Near the apices, adjacent RPE cells are joined by numerous tight junctions to form the outer blood–retinal barrier. The base of the RPE has numerous invaginations (to increase surface area) associated with mitochondria. The RPE is highly pigmented with melanin, particularly in the central macular area (Denniston and Murray, 2018).

The RPE has numerous functions: it metabolizes vitamin A, forms the outer blood-retina barrier, phagocytoses photoreceptor outer segments, absorbs the scattered light in the retina by melanin pigment, and actively transports fluid out of the subretinal space to maintain adhesion of the neurosensory retina (Zeiss et al., 2018).
2) **Photoreceptor neurons: Rods and cones (PRL):**

The retina contains approximately 120 million rods and 7 million cones. They are not distributed equally throughout the photosensitive part of the retina. The largest density of cones is detected in the fovea centralis, which corresponds to the highest visual acuity and best color vision. The highest density of rods is outside the fovea centralis, and their density steadily decreases toward the periphery of the retina. Rods are not
present in the fovea centralis nor at the optic disc, which is devoid of any photoreceptors *(Ross and Pawlina, 2019)*.

Each rod or cone cell (Fig. 3) consists of a cell body containing the nucleus, and of external and internal processes, an inner fiber and spherule. The cell body (lying in the external nuclear layer) gives off two ‘fibers’, inner and outer. The outer fiber passes outwards up to the external limiting membrane and becomes continuous with the rod process, or the cone process. The process itself can be divided into an inner segment, and an outer segment *(Singh, 2016)*.

**The outer segment** contains stacks of flat membranous disks harboring a photopigment. The disks are infoldings of the plasma membrane that pinch off as they move away from the modified cilium, the outer-inner segment connecting region.

**The inner segment** displays abundant mitochondria, involved in the synthesis of adenosine triphosphate (ATP), the Golgi apparatus, and rough and smooth endoplasmic reticulum. The modified cilium consists of nine peripheral microtubule doublets but lacks the central pair of microtubules. The terminal portion of the photoreceptors is equivalent to an axon forming synaptic contacts with cytoplasmic processes, neurites, of bipolar cells and horizontal cells.

There are three significant differences between rods and cones:

1. The outer segment is cylindrical in the rods and conical in the cones.

2. The rods terminate in a small knob or rod spherule, which contacts dendrites of bipolar cells and neurites of horizontal cells. The cones end in a thicker cone pedicle. The cone pedicle also synapses with bipolar and
horizontal cells. The synaptic ending of cones and rods, spherules and pedicles, contains a synaptic ribbon surrounded by synaptic vesicles.

3. Rods contain the photopigment rhodopsin. Cones contain a similar pigment called iodopsin. Rhodopsin operates during night vision. Iodopsin perceives detail and discriminates color (blue, green, and red) (Kierszenbaum and Tres, 2019).

Fig. (3): A representation of rod cell (A) and cone (B) (Cui et al., 2011)
The density of cones is greatest in the fovea (about 1.5 million/mm²). Their density decreases sharply in proceeding to the margin of the central area, but thereafter the density is uniform up to the ora serrata (about 5000/mm²). The density of rods is greatest at the margin of the central area (about 1.5 million/mm²). It decreases sharply on proceeding towards the margin of the central area. There are no rods in the foveola. The density of rods also decreases in passing towards the ora serrata (where it is about 30,000/mm²) (Singh, 2016).

3) Outer limiting membrane (OLM):

Although the term outer limiting membrane is still used, this structure is not a membrane. It appears as a line that separates the outer segments of the photoreceptor cells from their nuclei in the outer nuclear layer. It is a region of zonulae adherents between Müller glial cells and the photoreceptors. Distal to this, microvilli of Müller cells project into the interstices between the inner segments of the rods and cones (Gartner, 2017).

4) Outer nuclear layer (ONL):

It contains the nuclei of the retinal rods and cones. The region of the rod cytoplasm that contains the nucleus is separated from the inner segment by a tapering process of the cytoplasm (outer rod fiber). In cones, the nuclei are located close to the outer segments, and no tapering is seen. The cone nuclei stain lightly and are larger and more oval than rod nuclei. Rod nuclei are surrounded by only a thin rim of cytoplasm. In contrast, a relatively thick investment of cytoplasm surrounds the cone nuclei (Ross and Pawlina, 2019)
5) Outer plexiform layer (OPL):

The outer plexiform layer contains the cell processes and synaptic connections between the photoreceptor cells, bipolar neurons and horizontal cells (*Lowe et al., 2019*).

6) Inner nuclear layer (INL):

It mainly consists of cell bodies of bipolar cells. It also contains cell bodies of horizontal amacrine and Muller’s cells and capillaries of central artery of retina. The bipolar cells constitute the first order neurons (*Khurana, 2018*).

Bipolar cells and their processes extend to both the inner and outer plexiform layer. In the peripheral regions of the retina, the axons of bipolar cells pass to the inner plexiform layer where they synapse with several ganglion cells. Through these connections, the bipolar cells establish communication with multiple cells in each layer except in the fovea, where they may synapse only with a single ganglion cell to provide greater visual acuity in this region (*Ross and Pawlina, 2019*).

Two major classes of bipolar cells can be distinguished.; **Rod bipolar cells**, linked to rod spherules. **Cone bipolar cells**, linked to cone pedicles. Cone bipolar cells consist of two major classes: The midget cone bipolar cell (synapse with a single cone pedicle and a single axon that contacts a single ganglion cell). The diffuse cone bipolar cell (have wider input and output pathways) (*Kierszenbaum and Tres, 2019*).

Horizontal cells are inhibitory interneurons. Their dendrites and axons extend laterally within the outer plexiform layer, making synaptic contacts with cone pedicles and rod spherules, and, via gap junctions at the tips of their dendrites, with each other. Their cell bodies lie in the outer part of the inner nuclear layer. Three morphological types of
horizontal cell can be distinguished in the human retina (HI, HII and HIII). The dendrites of HI and HIII cells contact cones, and their axons terminate on rods. Both the axons and dendrites of HII cells synapse only with cones \textit{(Douglas and Lawrenson, 2016)}.

Amacrine cell's processes pass inward, contributing to a complex interconnection of cells. Their processes branch extensively to provide sites of synaptic connections with axonal endings of bipolar cells and dendrites of ganglion cells. Besides bipolar and ganglion cells, the amacrine cells synapse in the inner plexiform layer with interplexiform and other amacrine cells \textit{(Ross and Pawlina, 2019)}.

7) Inner plexiform layer (IPL):

The inner plexiform layer consists of synapsing nerve fibers. The axons of bipolar cells synapse with dendrites of ganglion cells; and both these processes synapse with processes of amacrine cells \textit{(Singh, 2016)}.

There are two types of synapses in this layer: flat and invaginated. Invaginated synapsis consists of an axon of a single bipolar cell and two dendrites of either amacrine cells or ganglion cells or one dendrite from each of two different cells, thus making a dyad. Also located within this synapse is a shortened version of the synaptic ribbon which contains neurotransmitter \textit{(Gartner, 2017)}.

8) Ganglion cell layer (GCL):

The human retina contains 0.7–1.5 million ganglion cells, the output neurons of the retina. Their dendrites synapse with processes of bipolar and amacrine cells in the inner plexiform layer. Ganglion cell bodies, together with displaced amacrine cells, form the ganglion cell layer of the retina. Ganglion cell axons, which form the nerve fiber layer on the inner
surface of the retina, run parallel to the surface of the retina, and converge on the optic nerve head where they leave the eye as the optic nerve. Axons of ganglion cells are surrounded by the processes of radial glial cells and retinal astrocytes, and are almost always unmyelinated within the retina, which is an optical advantage because myelin is refractile (Douglas and Lawrenson, 2016).

9) Nerve fiber layer (NFL):

The optic nerve is formed by the axons arising from the retinal ganglion cell layer, which form the nerve fiber layer of the retina. It passes out of the eye through the cribiform plate (lamina cribrosa) of the sclera, a sieve-like structure. In the orbit the optic nerve is surrounded by a sheath formed by the dura, arachnoid and pia mater, continuous with that surrounding the brain. It is bathed in cerebrospinal fluid (CSF). The central retinal artery and vein enter the eye in the center of the optic nerve. The extraocular nerve fibers are myelinated; those within the eye are not (James et al., 2016).

10) Internal limiting membrane (ILM):

It is the innermost layer and separates the retina from vitreous. It is formed by the union of terminal expansions of the Muller’s fibers, and is essentially a basement membrane (Khurana, 2018).

Specialized Regions of the Retina:

Ora Serrata It is the anterior termination of the retina where it is continuous with the epithelium of the ciliary body.

Macula Lutea (Yellow Spot) It is an area 1.5 mm in diameter situated at the posterior pole, about 3 mm to the temporal side of the optic disc.
**Fovea Centralis** It is a small depression in the center of the macula. The cones predominate in this area. It is the most sensitive part of the retina (*Jogi, 2016*).

**Retinal glial cells:**

There are three types of retinal glial cells (Fig. 4): Radial Müller cells, astrocytes and microglia. Müller cells form the predominant glial element of the retina; retinal astrocytes are largely confined to the ganglion cell and nerve fiber layers; and microglial cells are scattered throughout the neural part of the retina in small numbers.

The nuclei of Müller cells are located in the inner nuclear layer. The cytoplasmic processes extend to the outer and inner limiting membrane. The inner limiting membrane represents the basal lamina of the Müller cells and serves to separate retina from the vitreous body. The cytoplasmic processes of Müller cells fill the spaces between photoreceptors and bipolar and ganglion cells (*Kierszenbaum and Tres, 2019*). Müller cells are the most abundant retinal glial cell, and span the width of the neural retina. Müller cell processes are associated with blood vessels and neurons, to maintain the blood-retinal barrier and to provide structural support. They also play a prominent role in neurovascular coupling (*Moran et al, 2016*).

The cell bodies of retinal astrocytes lie within the nerve fiber layer and their processes branch to form sheaths around ganglion cell axons. The close association between astrocytes and blood vessels in the inner retina suggests that they contribute to the blood–retinal barrier. Retinal microglia are scattered mostly within the inner plexiform layer. Their radiating branched processes spread mainly parallel to the retinal plane, giving them a star-like appearance when viewed microscopically from the
surface of the retina. They can act as phagocytes, and their number increases in the injured retina (*Douglas and Lawrenson, 2016*).

![Diagram of retinal glial cells](image.png)

**Fig. (4):** Retinal glial cells (*Cunha-Vaz et al., 2014*).

### Vessels of the Retina:

The retina receives its blood supply (Fig. 5) from two sources. The choriocapillaris immediately outside Bruch’s membrane, supplies the outer third of the retina, including the outer plexiform and outer nuclear layers, the photoreceptors, and the retinal pigment epithelium. While, branches of the central retinal artery, supply the inner two-thirds of the retina. The foveola is supplied entirely by the choriocapillaris and is susceptible to irreparable damage when the retina is detached. The retinal blood vessels have a nonfenestrated endothelium, which forms the inner blood-retinal barrier, whereas the endothelium of choroidal vessels is fenestrated. The outer blood-retinal barrier lies at the level of the retinal pigment epithelium (*Eva and Augsburger, 2018*). In the human retina, the vasculature consists of a central retinal artery that branches into four intraretinal arterioles that feed into a capillary bed. The retinal capillary
bed is organized into three layers, the superficial, intermediate, and deep vascular plexuses \((Moran \ et \ al., \ 2016)\).

Fig. (5): Schematic illustration of the ocular vasculature. The outer nuclear layer (ONL) is avascular, relying on the choroidal blood supply. The inner nuclear layer (INL) is vascularized by the deep and intermediate vascular plexuses. The ganglion cell layer (GCL) is vascularized by the intermediate and superficial vascular plexuses. NFL, nerve fiber layer; RPE, retinal pigment epithelium. \((Chen. \ J \ et \ al.,2016)\).

**Blood retinal barrier (BRB):**

The retina, as part of the central nervous system (CNS), has a blood–retina barrier (BRB), which is formed by tight junctions between adjacent endothelial cells, protects the neural tissue from various circulating components of the blood and enables the retina to regulate its own extracellular chemical composition. The interaction of blood vessels,
astrocytes and ganglion cells induces the expression of tight junction proteins, such as occludins, claudins and zonula occludens (ZO) proteins, which are important to maintain the BRB. Disruption has been linked to numerous diseases, such as stroke and brain tumors and can lead to edema (Altmann and Schmidt, 2018).
DIABETIS MELLITUS AND DIABETIC RETINOPATHY

Diabetes mellitus (DM) is one of the most prevailing non-communicable, metabolic disorder characterized by hyperglycemia resulting from defective insulin production, resistance to insulin action or both. Prolonged exposure to chronic hyperglycemia can lead to various ailments including both vascular and nonvascular complications. According to the International Diabetes Federation (IDF), more than 285 million people are already affected by DM worldwide, and this number is expected to rise to 439 million by 2030. (Reddy et al., 2018).

The majority of cases of diabetes can be broadly classified into 2 categories: Type 1 diabetes T1D and type 2 diabetes T2D. T1D is characterized by a total or near total loss of insulin production, and thus patients are insulin-dependent. In contrast, T2D is characterized by insensitivity to insulin, and the patients can be treated with other drugs along with insulin during the early phases of the disease (Homme et al., 2018). Metabolic disturbances associated with diabetes cause a vast number of complications ranging from cardiovascular and cerebrovascular diseases to neuropathy, retinopathy, nephropathy, and poor wound healing. These complications decrease the quality of life and can lead to death (Khamaisi and Balanson, 2017).

Diabetic retinopathy (DR) is a microvascular disease as well as a chronic inflammation and retinal neurodegeneration, which is not only influenced by local changes, but also by systemic metabolic and cardiovascular parameters. It involves microvascular changes as well as changes in the all major cell types of the retina. Degeneration, inflammation and vascular alternations occur and operate parallel and in close relation (Altmann and Schmidt, 2018).
According to the report of World Health Organization (WHO), the prevalence of DR is expected to increase and the number of people at the risk of vision loss is predicted to double by the year 2030 (Yu et al., 2015).

**DR classification:**

Based on their obvious manifestations during DR progression, microvascular lesions have been utilized as the major criteria for evaluating and classifying the retina in DR. However, diabetes-induced changes also occur in nonvascular cell types that play an important role in the development and progression of diabetic retinopathy. DR falls into 2 broad categories: the earlier stage of nonproliferative diabetic retinopathy (NPDR) and the advanced stage of proliferative diabetic retinopathy (PDR). Classification of NPDR is based on clinical findings manifested by visible features, including microaneurysms, retinal hemorrhages, intraretinal microvascular abnormalities (IRMA), and venous caliber changes, while PDR is characterized by the hallmark feature of pathologic preretinal neovascularization (Duh et al., 2017).

An important additional categorization in DR is diabetic macular edema (DME), which is an important manifestation of DR that occurs across all DR severity levels of both NPDR and PDR and represents the most common cause of vision loss in patients with DR. DME arises from diabetes-induced breakdown of the blood-retinal barrier (BRB), with consequent vascular leakage of fluid and circulating proteins into the neural retina causing abnormal retinal thickening and often cystoid edema of the macula (Zhang et al., 2014).
Risk factors of diabetic retinopathy:

Many risk factors for DR have been established. Poor glycemic control, raised blood pressure, duration of diabetes, and microalbuminuria or proteinuria are the main risk factors which initiates and then complicates this pathology. The likelihood of developing diabetic retinopathy is related to the duration of the disease (Aziz, 2018).

Pathophysiology of diabetic retinopathy:

The main causes of DR occurrence are immunology, metabolic, hormonal, blood rheological, hypoxic, genetic, and the other factors which cause lesions of walls in capillaries of eye retina and disturbances of vessels permeability. Chronic hyperglycemia dysregulates several biochemical pathways (hexosamine, aldose reductase, advanced glycation end-products, and protein kinase C) and leads to the accumulation of abnormal by-products that interfere with electron transfer through the cytochrome chain within the mitochondria. The resultant superoxides induce chronic inflammation by upregulating several chemokines and cytokines, including interleukin (IL)-1β, IL-6, interferon-inducible protein 10, intercellular adhesion protein-1, monocyte chemotactic protein-1, placental growth factor, and vascular endothelial growth factor (VEGF). Chronic inflammation activates retinal glial cells; damages capillary endothelial cells and pericytes, thereby producing neurodegeneration; and, together with advanced glycation end-products, thickens vascular basement membranes. Blood-retinal barrier breakdown enables albumin and water to pass into the retinal interstitium, resulting in the formation of diabetic macular edema (DME) (Stewart, 2017).
Fig. (6): Pathophysiology of diabetic retinopathy. Hyperglycemia instigates biochemical changes leading to vascular dysfunction which increases vascular permeability followed by macular edema and retinal neovascularization. (Shin et al., 2014).

Retinal ganglion cells (RGCs) are the earliest cells affected and have the highest rate of apoptosis. An elevated rate of apoptosis has been also observed in the outer nuclear layer, with a reduction in photoreceptors seen between the 4th and 24th weeks after diabetes onset. In addition to neuronal cell death, another early feature of diabetic-induced retinal neurodegeneration that has recently gained traction is glial cell activation and dysfunction. Müller glial cells (MGCs), the main glial cells of the retina, play a central role in retinal metabolism, making them highly sensitive to metabolic alterations such as those associated with diabetes. Consequently, the resident immune cells in the retina, called microglia, also become activated and start to produce pro-inflammatory mediators, exacerbating neuro-glial and vascular dysfunction (Rübsam et al., 2018).
Fig. (7): Schematic summary of hyperglycemia-induced changes of the neurovascular unit in the Wistar rat STZ-induced diabetes model (Hammes, 2018).

**Treatment of Diabetic Retinopathy:**

The risk of developing diabetic retinopathy can be reduced by early detection, timely tight control of blood glucose, blood pressure, and possibly lipids; however, clinically this is difficult to achieve (Tarr et al., 2013).

Current DR therapeutic strategies may include laser photocoagulation vitreoretinal surgery, and intravitreal injection of vascular endothelial growth factor (VEGF) neutralizing agents (e.g., ranibizumab) or corticosteroids, but these therapies have achieved only limited success (Gu et al., 2018). To date, no treatment has yet been developed to support regeneration of the damaged retinal vasculature as a result of long-term hyperglycemia. Cell-based therapies may be a feasible option for both preventing neurovascular damage and promoting regeneration of damaged retina (Kramerov and Ljubimov, 2016).
Cell Replacement Therapies and Retinal Tissue Engineering Approaches:

A more widely considered approach to improve the treatment of retinal disease is the possibility of retinal repair using cell replacement therapies. Therapies include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and retinal progenitor cells (RPCs), as well as RPE replacement. Many groups have successfully transplanted these cells into animal models and some therapies are even commencing into some clinical trials. To date, the main challenges of cell replacement therapy include; the delivery and integration of regenerative materials to the eye, overcoming the possibility of immune rejection and the guidance of neural growth to establish functional connections. The development of tissue-engineered constructs, such as scaffolds and smart biodegradable implants, may help to overcome some of these challenges by improving the delivery, integration and survival of transplanted cells (Gater et al., 2016).
STREPTOZOTOCIN

Chemical Properties:

Streptozotocin (2- deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose; STZ) is a monofunctional nitrosourea metabolite that was first isolated from Streptomyces chromogens. (Ragbetli et al., 2017). Streptozotocin has been used alone or in combination with other chemotherapeutic drugs for the treatment of colorectal carcinomas and other gastrointestinal cancers, but severe toxicity and myelosuppression were observed in most of the patients. It also has broad spectrum antibiotic activity and causes insulin dependent and independent diabetes (Tripathi and Verma, 2014).

![Chemical Structure of Streptozotocin](image)

Fig. (8): The chemical structure of streptozotocin. STZ (Sithole, 2009).

Mechanism of Action:

Streptozotocin prevents DNA synthesis in mammalian and bacterial cells. Streptozotocin is a toxic glucose analogue that enters the pancreatic cell via uptake by glucose transporter-GLUT2 (Glucose transporter 2). STZ is split into its glucose and methyl nitrosurea moiety. The latter is a powerful alkylating agent that induces multiple DNA strand breaks and damages pancreatic β-cells. So, it provides a predictable animal model of type 1 diabetes (T1D) (Tripathi and Verma, 2014).
**Route of Administration:**

A wide variety of dose schedules and routes of administration have been reported in inducing diabetes in rats with STZ. STZ is most commonly delivered by one of two routes, intraperitoneal or intravenous, although other methods including subcutaneous, intracardiac, and intramuscular delivery have been used in rodents.

Although intraperitoneal offers a quick and easy method of administration, accidental delivery into the bowel or sub-dermal space may result in increased moribundity or decrease in diabetogenic effect. Intravenous administration of STZ produces a more stable and reproducible model of diabetes than IP administration (*Goud et al.*, 2015).

**Triphasic blood glucose responses induced by Streptozotocin Injection:**

A triphasic response in blood glucose level occurs after streptozotocin administration.

- **In the first two hours** of STZ challenge, blood glucose rises. This transient hyperglycemia is due to inhibition of insulin secretion leading to hypoinsulinaemia and sudden breakdown of liver glycogen.
- **The second phase,** starting at about six hours after STZ dosing, is a hypoglycemic one, which may be severe enough to lead to death. This severe transitional hypoglycemia is produced by the flooding of the circulation with insulin as a result of toxin-induced secretory granule and cell membrane rupture.
- **The third phase,** that of permanent hyperglycemia, begins at about 10 to 12 hours after STZ administration. Structural alterations in pancreatic
beta cells (total degranulation) occur within 48 h after the administration of streptozocin and last for up to four months (Eleazu et al., 2013).
STEM CELLS

Stem cells are undifferentiated cells that have the ability of proliferation, regeneration, conversion to differentiated cells and tissue production. Stem cells may remain inactive for a long time till they enter cell division again (Varma et al., 2018).

Stem cells are unspecialized cells able to divide and multiply for extended periods of time. After division, the resulting cell can continue as a stem cell, like the parent stem cell or can differentiate to specialized cells. Both internal and external stimuli are important in this process. Internal stimuli are controlled by the cell’s genetic material. External stimuli are regulated by chemical factors secreted by other cells in the environment, by physical contact with neighboring cells, and by other molecules in the environment. (Öner, 2018).

Stem cells and regenerative medicine:

Regenerative medicine is defined as the process of replacing or regenerating damaged cells, tissues, and/or organs to restore normal function (Lee et al., 2015). Tissue damage and organ loss caused by degenerative diseases or neoplasia are still a great challenge to treat. End-stage organ failure is treated with transplantation procedures. In general, the results are relatively unsatisfactory in terms of immunosuppressive complications with exceeding numbers of failed organs and receding donor numbers (Shokeir et al., 2010).

“Cell therapy” can be defined as a set of strategies which use live cells with therapeutic purposes. The aim of such therapy is to repair, replace or restore the biological function of a damaged tissue or organ. Thus, the use of stem cells in cell therapy is being studied in several areas
of medicine (Yoland et al., 2014). Under the right conditions, or given
the right signals, stem cells can differentiate into many different mature
cell types such as heart cells, skin cells or nerve cells. It holds a great
potential for regenerative medicine especially in replacing cells in tissues
that hardly have intrinsic renewal capacity including the heart (Shah and
Shalia, 2014).

**Potency of stem cells:**

Potency of the stem cell specifies the differentiation potential i.e.,
the potential to differentiate into different cell types.

- **Totipotent** stem cells can differentiate into embryonic and
  extraembryonic cell types. Such cells can construct a complete, viable
  organism. These cells are produced from the fusion of an egg and sperm
  cell. The only totipotent cells are the fertilized egg and the cells produced
  by the first few divisions of the fertilized egg are also totipotent.

- **Pluripotent** stem cells are the descendants of totipotent cells and can
differentiate into nearly all cells, i.e. cells derived from any of the three
germ layers. These pluripotent cells are characterized by self-renewal and
a differentiation potential for all cell types of the adult organism. These
are true stem cells, with the potential to make any differentiated cell in
the body.

- **Multipotent** stem cells can differentiate into a number of nearly related
types of cells. For example, the bone marrow contains multipotent stem
cells that give rise to all the cells of the blood but not to other types of
cells. Adult Hematopoietic Stem Cells are multipotent. Adipose tissue is
another source of multipotent stem cells.
• **Oligopotent** stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells.

• **Unipotent** cells can produce only one cell type, their own, but have the property of self-renewal, which distinguishes them from non-stem cells. Such Unipotent cells include muscle stem cells (*Fathi and Zaminy, 2018*).

![Image: Lineage restriction of human developmental potency. (*Berdasco and Esteller, 2011*)](image)

**Fig. (9):** Lineage restriction of human developmental potency. (*Berdasco and Esteller, 2011*).

**Stem Cell Classification Based on Origin:**

Stem cells can be divided into three broad categories: embryonic (ESCs), foetal (FSCs) and adult stem cells (ASCs, among them mesenchymal stem cells – MSCs).
**Embryonic Stem cells:**

Embryonic stem cells (ESCs) are multipotent cells derived from the inner cell mass of the blastocyst; a stage of pre-implantation embryo, 4 days after fertilization. They generate the organism, whereas the surrounding trophoblast cells contribute to the placental chorion. These cells can differentiate into tissue of the 3 primary germ layers but can also be maintained in an undifferentiated state for a prolonged period in culture (*Zakrzewski et al., 2019*).

ESCs theoretically can be differentiated into almost all tissues in the human body. However, ESCs have limitation for use. The principal limitation is an ethical problem. Because ESCs are derived from the inner cell mass of a blastocyst, an early-stage embryo, isolating the embryoblast or inner cell mass results in destruction of the fertilized human embryo, which raises ethical issues. Although the source of the blastocyst was generally discarded material from *in vitro* fertilization clinics there is no consensus whether or not a human life at the embryonic stage should be granted the moral status of a human being. Other limitations are the risks of immunorejection and tumorigenesis (*Jung et al., 2012*).

**Fetal Stem Cells:**

FSCs are multipotent cells located in the foetal tissues and embryonic annexes. They have been subdivided into hematopoietic (blood, liver, bone marrow), mesenchymal (blood, liver, bone marrow, lung, kidney and pancreas), endothelial (bone marrow, placenta), epithelial (liver, pancreas) and neural ones (brain, spinal cord). Among FSCs the greatest potential use in regenerative medicine have stem cells found in foetal blood and in placenta because they are the easiest to harvest without harming the foetus (*Dąbrowska and Skopiński, 2017*).
**Adult Stem Cells:**

Adult or somatic stem cells are undifferentiated and found among differentiated cells in the whole body after development. The function of these cells is to enable the healing, growth, and replacement of cells that are lost each day. These cells have a restricted range of differentiation options. Among many types, there are; Mesenchymal stem cells are present in many tissues. Neural cells give rise to nerve cells and their supporting cells oligodendrocytes and astrocytes. Hematopoietic stem cells form all kinds of blood cells: red, white, and platelets. Skin stem cells form, for example, keratinocytes, which form a protective layer of skin. Induced pluripotent stem cells (IPSCs), a new category of stem cells has emerged that originate from somatic cells and can be reprogrammed by scientists back to their pluripotent stage. This was done by manipulating the expression of a set of genes. As somatic cells are coaxed into pluripotent cells in this category, they have been termed as “induced pluripotent cells” (*Zakrzewski et al., 2019*).

Because IPSCs are autologous, there is less risk of rejection and therefore, less need for immunosuppression. However, some IPSCs may trigger the T cell-mediated immune response due to their abnormal genetic composition. Furthermore, the high growth rate in vitro increases the risk of tumor formation. Tumor formation is believed to result from incompletely differentiated IPSCs (*Öner, 2018*).
Mesenchymal Stem Cells

The most widely accepted definition of an MSC was put by the International Society for Cellular Therapy (ISCT) in 2006 and includes three criteria: (i) In standard culture conditions, they are plastic adherent cells. (ii) They express CD105, CD73, and CD90, but no CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-II (human leukocyte antigen type II) surface molecules. (iii) They are able to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro. (*Denu et al., 2016*).

MSCs can be found abundantly in the adult tissues, such as bone marrow, adipose tissue, and dental pulp, as well as in the fetal tissues and fluids, including the umbilical cord-tissue and -amniotic fluid. In addition to their wide distribution, MSCs are capable of avoiding immune cell recognition, hence providing a potential platform for allogeneic and autologous cell transplants (*Ding et al., 2017*).

**Mechanism of action of MSCs in tissue regeneration:**

MSCs are of intense therapeutic interest because they represent a population of cells with the potential treat a wide range of acute and degenerative diseases (*Saeed et al., 2016*). The current enthusiasm surrounding the potential application of MSCs for therapeutic purposes is based on their multilineage differentiation capacity, their immunomodulatory properties, and their ability to secrete bioactive molecules:

*Therapeutic effect based on multilineage differentiation capacity:*

Recently, MSCs have appeared as a promising approach for regeneration of various tissues. It was originally thought that MSCs exert their therapeutic effect by migrating to sites of damage, engrafting, and
subsequently differentiating into desired cells for tissue regeneration. However, other studies have indicated that the therapeutic benefit of MSCs is attributable not only to their differentiation but also through factors they secrete (*Madrigal et al., 2014*).

* **Therapeutic effect based on immunomodulatory functions of MSCs:**

MSCs have been known to regulate the functions of immune cell from both innate immunity and adaptive immunity, that is, MSCs can suppress the proliferation, differentiation, and activation of T cells, B cells, macrophages, dendritic cells, and natural killer (NK) cells, especially when these immune cell responses are excessive. This immunomodulatory effect of MSCs on immune cells is exerted by the secretion of soluble factors such as prostaglandin-E2 (PGE2), indoleamine 2,3-dioxygenase-1 (IDO-1), nitric oxide (NO), transforming growth factor-β1 (TGF-β1), hepatocyte growth factor (HGF), and interleukin-1 (IL-1). (*Seo et al., 2019*).

* **Therapeutic effect based on paracrine secretions:**

MSCs produce and release a broad repertoire of growth factors, chemokines, and cytokines that modulate the action of adjacent cells. In fact, these secreted factors increase angiogenesis, reduce apoptosis and fibrosis, enhance neuronal survival and differentiation, stimulate extracellular matrix remodeling, restrict local inflammation, and adjust immune responses. In this way, MSCs directly or through paracrine secretion induce regeneration for rescuing injured cells, decreasing tissue injury, and finally accelerating organ repair (*Moravej et al., 2017*). MSC are able to pack trophic mediators into extracellular vesicles (exosomes). These extracellular vesicles cannot only transport proregenerative factors,
but also mRNA and microRNA but even mitochondrial components over a long distance (*Toh et al.*, 2018)

**Clinical Potential of MSCs:**

Due to their intrinsic properties and regenerative capacity, MSCs are considered to have therapeutic potential, which makes them a favorable candidate for cell-based therapies and tissue engineering applications. MSCs show a remarkable potential for the treatment of a number of diseases, including both immunological and non-immunological disorders. In particular, more than 756 clinical trials involving the use of MSCs are currently in progress. These include the treatment of different conditions such as: myocardial infarction, osteogenesis imperfecta, hematologic malignancies, graft-versus-host disease, Crohn’s disease, spinal cord injury, multiple sclerosis, and diabetes (for the healing of refractory wounds), without any reported serious adverse events (*Solarte et al.*, 2018).
**EXOSOMES**

The cell-secretome consists of all factors actively or passively released from cells and contains among others soluble proteins (e.g., cytokines, chemokines and growth factors), lipids, free nucleic acids and extracellular vesicles (EV). The latter can be further subdivided mainly based on their size, density, surface markers and origin into exosomes, microparticles and apoptotic bodies. *(Beer et al., 2017)*.

Exosomes are intraluminal vesicles which range in size from 40 to 150 nm and generated by fusion of multivesicular bodies (MVBs) with plasma membrane. A second EV subpopulation consists of microparticles (MPs) or microvesicles (also known as shed vesicles or ectosomes), which range in size from 150-1000 nm, originating from direct budding of the plasma membrane. A third EV subpopulation, is constituted by apoptotic bodies (ABs), which are larger vesicles (50-2000 nm) released from apoptotic cells that are rapidly engulfed by phagocytic cells. ABs are characterized by permeable membranes and the presence of fragmented nuclear DNA *(Yin et al., 2019)*.

![Fig. (10): Schematic representation of the mechanisms of formation of microvesicles, exosomes, and apoptotic bodies. *(Lawson et al., 2016)*.](image-url)
**Exosome Biogenesis:**

Exosomes originate from the inward budding of the endosomal membrane to form multivesicular bodies (MVBs) containing vesicles with a diameter of 30–120 nm, named as intraluminal vesicles (ILVs). MVBs fuse with the cell membrane releasing these vesicles in the form of exosomes. Once released into the extracellular space, exosomes can reach recipient cells and deliver their cargoes, which can induce intracellular signaling and affect the physiological or pathological status of the recipient cells (Zhang M. et al., 2019).

![Fig. (11): Schematic representation of the generation of exosomes (Zhang M. et al., 2019).](image)

**Contents of exosomes:**

Exosomes contain lipids, proteins, RNAs, and microRNAs (miRs). The lipid composition includes ceramide, sphingomyelin, phosphatidylcholine, and phosphatidylserine within the membrane bilayer and cholesterol inside the exosomes. Exosome-specific proteins include the ubiquitously expressed tetraspanins CD81, CD9, CD63, CD82,
HSP70 (heat shock 70-kDa protein), HSP60, and Alix (apoptosis-linked gene 2-interacting protein X); annexins and cytoskeletal proteins; and unique cargo proteins. Moreover, exosomes contain RNAs and miRs that can change depending on the exosome subtype, cell type, or disease state (Gartz and Strande, 2018).

**Micro RNA of exosomes:**

Recently, the existence of miRs in exosomes has been reported, suggesting that exosomes may serve as a vehicle for miR transfer and mediate intercellular communication. MiRs, a class of small non-coding RNAs (containing about 18–22 nucleotides), regulate gene expression on the posttranscriptional level by binding to specific mRNA and inducing their degradation and/or translational inhibition. MiRs are recognized to participate in a wide range of biological and pathological processes including the cell cycle, hematopoiesis, neurogenesis, aging, cancer, and cardiovascular disease (Gong et al., 2017).

**Sources of exosomes:**

Exosomes are released by several types of cells, including mast cells, dendritic cells, B lymphocytes, neurons, adipocytes, endothelial cells, epithelial cells and mesenchymal cells. Notably, tumor cells have been shown to produce and secrete exosomes in greater numbers than normal cells. Exosomes have been found in numerous body fluids, including blood, amniotic fluid, urine, malignant ascites, cerebrospinal fluid, breast milk, saliva, lymph, and bile, under both healthy and morbid conditions (Rashed et al., 2017).
Isolation of exosomes:

The most common method for isolating exosomes from biofluids relies on ultracentrifugation—a size-dependent method of separation that includes differential centrifugation steps reaching speeds of up to 200,000 × g to pellet exosomes from supernatant. However, this is a labor-intensive, time-consuming procedure. Variations on this method, such as adding a sucrose-gradient centrifugation step can increase purity and recovery rate (Chronopoulos et al., 2017).

Isolation by ultrafiltration is based on the exosome size. Exosomes can be enriched with a commercially available nano-membrane concentrator by centrifugation at 3000× g for 10–30 min. This method is less time-consuming than ultracentrifugation and does not require the use of specialized equipment. In addition to these traditional methods, exosome isolation kits and exosome precipitation solutions have been developed by various companies in recent years. These products provide convenient and efficient techniques to isolate exosomes (Yu et al., 2014).

Magnetic activated cell sorting technique (MACS) aims to pre-coat exosomes specific antibodies in magnetic beads, incubate them with exosomes together, then wash and re-suspend after ultracentrifugation. This method has high specificity, easy-to-operate, and guarantees the complete form of exosomes, but the beads antibody may affect the bioactivity of exosomes and the stability of subsequent experiments (Zhao et al., 2017).

Storage of exosomes:

After the isolation and characterization, exosomes for in vivo or in vitro applications must be frozen because they are unstable at room
temperature and 37 °C. Exosomes can be stored for 6 months at -20 °C without cryopreservative agents (Konala et al., 2016).

**Identification of exosomes:**

According to the proposal of the international society of extracellular vesicles (ISEV) in 2018, exosome identification methods include scanning electron microscopy, atomic force microscopy, nanoparticle tracking analysis, transmission electron microscopy, flow cytometric analysis, western blotting, and enzyme-linked immunosorbent assay (ELISA). Exosomes observed by transmission electron microscopy are usually double-membrane “cup-shaped” vesicles, with a concave side that are also in the correct size range. Specific marker molecules were found on the surface of exosomes, such as CD9 and CD63. (Liu and Su, 2019).

**Advantage of exosomes as therapeutic tool:**

It is advantageous to use exosomes concerning cell-based treatments. First, use of exosomes can avoid problems associated with the transfer of cells, which may already have damaged or mutated DNA. Second, most exosomes are small and can easily circulate through capillaries and cross barriers such as the cytoplasmic membrane and the blood/brain barrier, whereas the cells used in other cell-based therapies, such as MSCs, are too large to go through capillaries, and thus cannot get beyond first pass capillary beds, such as the lungs. Third, the level of MSCs in cell-based therapies may quickly diminish after transplant; however, exosomes can achieve a higher “dose” than the transplanted MSCs. Fourth, exosomes can also be utilized to tackle toxicity and immunogenicity problems resulting from such biomaterial treatments as nanoparticles (Wang et al., 2019).
Mechanisms of action:

Exosomes affect target cells through the following mechanisms: activation of certain signaling pathways by ligand–receptor interaction (without entrance), releasing their content through extracellular proteases-mediated cleavage and subsequent binding of contents to cell surface receptors, fusion to the cell membrane and releasing their content into the cytoplasm, and entering cells by endocytic mechanisms such as receptor-mediated endocytosis, phagocytosis, and micropinocytosis. Exosomes mediate many cellular activities such as angiogenesis and anti-angiogenesis, immunomodulation, anti-apoptosis and anti-fibrosis (Gomari et al., 2018).

Therapeutic applications of MSCs derived exosomes:

The MSC-derived exosomes ameliorated carbon tetrachloride-induced liver fibrosis, and conferred cyto-protective effects in models of necrotizing enterocolitis. In lung studies, the mouse MSC exosomes were effective in improving pulmonary hypertension, silicosis, and human MSC-exosomes improved endotoxin induced pulmonary edema, and cleared alveolar fluid from human lungs ex vivo. Other studies have shown that MSC-derived exosomes promoted re-epithelialization of cutaneous wounds by inducing epithelial cell proliferation and angiogenesis, activated collagen and elastin secretion by fibroblasts, and prevented myo-fibroblast formation thereby reducing scaring. The MSC-derived exosomes also promoted muscle regeneration and protected against experimental colitis (Phinney and Pittenger, 2017).
The use of Mesenchymal stem cells and their exosomes in the treatment of diabetic retinopathy

Regardless the great progress in medical research, there are still missing effective therapeutic protocols for the treatment of retinal degenerative diseases, and millions of people worldwide are waiting for a treatment option (Holan et al., 2017).

Stem cells are a type of cell with high self-renewability and differentiation capability, which are mostly favored to be used as a candidate for cell replacement therapy. In retinal degenerative diseases, research works have been focused on improving the cell recovery and regeneration of terminally-differentiated retinal neuronal cells through delivery of unmodified or modified stem cells by genetic, chemical, or mechanical manipulation (Worthington et al., 2016).

Collaborators proved effectiveness and safety of treatment for DM by use of SCs. So far, no therapy has been established yet that could support regeneration of the damaged vessels of retina resulting from a long-term hyperglycemia. Treatment using stem cells might become a feasible option for a purpose of both neurovascular damage prevention as well as promoting retinal damage regeneration, which is supported by the evidence from the recent studies by use of several types of stem cells. (Sych et al., 2017).

The ideal stem cell for cell replacement in eyes with retinal dysfunction would be a cell that can be expanded and easily directed to differentiate into the various cells of interest in the retina that may be affected by the pathologic condition. The ideal transplanted cell should have a long-term effect by integrating and surviving in the retina and restoring the neuronal activity within the retina. The cell should be stable...
and display limited proliferative potential after integration in the retina in order to minimize the risk of abnormal cellular proliferation and teratoma formation in the eye. *(Park et al., 2017)*

About two basic populations of cells are known as of today that contain progenitor cells which, under appropriate circumstances, may have therapeutic application in the treatment of retinal disease:

- **Tissue specific stem cells (Endogenous stem cells):**

  (1) Retinal stem cells that can give rise to photoreceptors and other retinal neurons; (2) Mueller/glia stem cells that can differentiate into retinal glia and/or neurons; (3) Retinal pigment epithelial (RPE) stem cells that can not only serve to replace diseased RPE but perhaps can also be stimulated to differentiate into photoreceptors.

- **Exogenous stem cells:**

  Endothelial, myeloid progenitor cells, adult stem cells, induced pluripotent stem cells (iPSC) and MSCs (of bone marrow and adipose tissue origin) that can contribute to the retinal vasculature and exert vasculo- and neurotrophic rescue effects *(Rajashekha, 2014).*

  Exosomes derived from mesenchymal stem cells play a major role in the paracrine effect of these cells. These exosomes are an attractive target for clinical research, as they can offer comparable therapeutic effects of mesenchymal stem cells without the potential adverse effects associated with cell therapy. In addition, exosomes are easy to obtain, store and administer, making clinical application feasible and appealing. *(Newton et al., 2017)*
**Fig. (12):** A schematic representation of Mesenchymal Stem Cells (MSCs) therapeutic strategies in retinal degenerative diseases. *(Ding et al., 2017).*
The aim of this study was to explore the possible therapeutic effect of bone marrow mesenchymal stem cell and their exosomes on experimentally induced diabetic retinopathy in adult male albino rats.
MATERIAL AND METHODS

A-Experimental animals:

Seventy-four rats were used in this work (Sixty-four adult male albino rats of weight range 250-300 grams for each and another ten young male rats average weight of 80 grams for each). They were obtained from the animal house, Moshtohor Faculty of Veterinary Medicine, Benha University. The animals were housed in special cages and fed with adequate available tap water and commercial diet. All Benha faculty of medicine ethical protocols for animal treatment were followed. The animal experimental protocol received approval from Institutional Animal Care Committee. Animal care was provided by laboratory animal house in pharmacology department, Benha faculty of medicine.

B-Drugs and chemicals:

- **Streptozotocin (STZ):** was purchased in a white powder (Sigma Chemical Co., St. Louis, MO, USA). The powder was stored at –20 °C. It was given as single intraperitoneal (IP) injection of freshly prepared STZ (60 mg/kg, dissolved in 0.1 M cold citrate buffer, pH 4.5) for each rat (*Ebrahim et al., 2018*).

- **Bone marrow mesenchymal stem cells:** The BMMSCs were prepared from the ten young rats in Stem cell unit, Central lab, Benha Faculty of Medicine, and injected intravitreally in a dose of 0.2ml solution containing 200×10^3 BMMSCs for each rat (*Çerman et al., 2016*).

- **MSCs derived exosomes labelled with PKH26:** Exosomes were prepared in the stem cell and molecular biology unit, central lab, Cairo
faculty of medicine. BMMSCs-exosomes were isolated from conditioned media of rat bone marrow derived MSC. They were labelled by fluorescent dye PKH26 and injected intravitreally in single dose of 0.5 ml for each rat at concentration (100 µg protein/mL) (Yu et al., 2016).

D-Induction of diabetic retinopathy (DR) model:

Type I diabetes mellitus (DM) was induced in overnight fasted rats by a single intraperitoneal (IP) injection of freshly prepared STZ (60 mg/kg, dissolved in 0.1 M cold citrate buffer, pH 4.5). After STZ injection, rats acquired drinking water containing sucrose (15 g/L) for 48 h, to lessen the early death due to insulin discharge from partially injured pancreatic islets. Seventy-two hours later, rats were checked for hyperglycemia, and those with fasting blood sugar more than 250 mg/dL were included in the study. Diabetic rats received long-acting insulin (2–4 U/rat) via subcutaneous injection to maintain blood glucose levels in a desirable range (350 mg/dL) and to avoid subsequent development of ketonuria. Maintenance of a diabetic state was confirmed by weekly tail-vein blood glucose measurements (Ebrahim et al., 2018).

C-Experimental design:

Sixty-four adult male albino rats were randomly divided into six main groups:

Group I (Control group; n= 24): The rats were further divided equally into three subgroups of 8 rats each:

Group Ia: The rats were left without intervention.

Group Ib: The rats were injected intraperitoneally with a single dose of 0.2 mL/kg body weight sodium citrate buffer (vehicle for STZ).
**Group Ic**: Rats received single intravitreal injection of 0.2 ml phosphate buffer saline (vehicle for Stem Cells and exosomes)

**Group II (affected group; n= 8)**:  
DM was induced according to *(Ebrahim et al., 2018)*. Rats were scarified at the end of the 8th week of the experiment to confirm histological changes of DR.

**Group III (DR + Stem Cells; n= 8)**: DM was induced as group II, then each rat was injected intravitreally with BMMSCs as a single dose of 0.2ml solution containing $200 \times 10^3$ BMSCs *(Çerman et al., 2016)*.

**Group IV (DR + exosomes; n=8)**: DM was induced as group II, then each rat was injected intravitreally with PKH26 labelled MSC-Exosomes at concentration (100 µg protein /mL) suspended in 0.5 ml PBS *(Yu et al., 2016)*.

**Group V (DR + Stem Cells + exosomes; n=8)**:  
DM was induced as group II, and then each rat was injected intravitreally with BMMSCs and PKH26 labelled MSC-Exosomes together intravitreally in the same doses as group III and IV.

**Group VI (Recovery group; n=8)**:  
DM was induced and rats were scarificed at the end of the experiment at the 12th week.

**Rats excluded:**
Six rats were excluded from the experiment due to failure of developing diabetes.

**Mortality rate:**
Five rats died (2 rats from group IV, one rat from group III and one rat from group IV). These rats excluded from the experiment.
E-Sampling:

Retinal specimens: Rats of groups II were sacrificed 8 weeks from the start of experiment, while rats of other groups were sacrificed 12 weeks from the start of experiment. The rats were anesthetized with sodium pentobarbitone 30mg/kg body weight (Viswanathan, 2011) after 12 h of fasting. The rats were fixed on an operating table, then, vascular perfusion fixation through the left ventricle was performed. Eyes were excised from all groups for obtaining samples.

Intravitreal injection of drugs:

Rats were anesthetized with 4% isoflurane. Prior to the injection, the eye was disinfected with topical povidone-iodine eye drops. Once the corneal reflex was abolished drugs were injected into the center of the vitreous humor, 0.5-mm posterior to the limbus, using a 30gauge needle (Filek et al., 2019).

Specimens were processed for:

I-Histological studies:
Paraffin sections of 5-7 µm thickness, mounted on glass slides for:
a- Hematoxylin and Eosin stain to examine the histological changes in the different groups.
a- Periodic Acid Schiff (PAS) reaction for demonstration of glycogen deposition in retina.

II-Immunohistochemical staining:
a- Immunohistochemical staining for VEGF.
b- Immunohistochemical staining for vimentin.
c- Mesenchymal stem cells by detection of CD105 marker.

III- Transmission Electron microscopic examination.
F- Isolation and culture of bone marrow mesenchymal stem cells (MSCs) (Baghaei et al., 2017):

BM-derived MSCs were prepared in the Stem cell unit, central lab, Benha faculty of medicine. Ten young rats were used to isolate the BMMSCs.

**Reagents:**

1) DMEM/F12 Media composed of:
   a- DMEM (Dulbecco’s Modified Eagle Medium) powder with high glucose, L-glutamine (GIBCO, USA).
   b- 10% Fetal Bovine Serum (FBS) (GIBCO, USA).
   c- 100u/ml Penicillin (Sigma).
   d- 100u/ml Streptomycin (Sigma).
   e- Sodium Bicarbonate NAHCO₃ (Sigma).

2) 0.25% Trypsin/1ml ethylenediaminetetraacetic acid (EDTA) (GIBCO, USA).

3) Phosphate Buffer Saline (PBS).

**Equipments for cell isolation & culture:**

b- Transmitted light and incident inverted microscopy (Axiovert 100-ZEISS).

b- Laminar flow cabinet (NUAIRE, Biological Safety Cabinet, Class II Type A/B3) equipped with UV light for decontamination.

b- Standard air CO2 incubator (Thermo).

cd- 40-mm nylon mesh filter (BD, Falcon, USA).

cd- 6- well and 24-well plastic cell culture plates (Costar, USA).

**Method:**

1- Ten young rats were sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from adherent soft tissue.

2- The tip of each bone was removed with a rongeur, and the marrow was harvested by inserting a syringe needle (27-gauge) into one end of the
bone and flushing with Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco). Repeat 2~3 times for each bone.

3-When all the marrows were obtained, resuspend the cells and pass the cell suspension through a 70μm cell strainer to remove the bone debris and blood aggregates.

4-The bone marrow cells were filtered through 40-um nylon mesh filters. After centrifugation at 2000 rpm for ten min, cells were plated into 6 well culture plates at a density of 25 X 106 cells per well in DMEM containing 15% fetal bovine serum (FBS; Sigma), 2mm L-glutamine (Gibco, USA), 100 u/ml penicillin (Sigma) and 100 u/ml streptomycin (Sigma).

5-Plate the four bones of one mouse in one well. This is considered as Passage zero (P0). The cells were kept in a humidified 5% CO₂ incubator at 37 °C for three days.

6-Non adherent cells Hematopoietic Stem Cells (HSCs) were removed after three days by changing the medium after washing two times with PBS.

7-The media were changed every three days until the adherent cells reach 70%-80% confluence, the cultured cells were splitted with 0.2 ml of 0.025% Trypsin/EDTA for two minutes at room temperature.

8-Plate Passage1 (P1) and Passage 2(P2) at density (2x10⁵) in 6 well culture plates until the adherent cells (MSCs) reached 70%-80% confluence. Split the cells with the same principle of splitting. The estimated time that the cells reached 70-80% confluence is three to four days.

9-Plate Passage3 (P3) at (3x10⁵) and Passage4 (P4) at (4x10⁵).

10-By counting the cells, passage 2(P2) proved to be the highest score. So, cells at (P2) used for the differentiation assay and transplantation.
**Splitting of cells**

1- Warm the DMEM/F12 medium to 37°C.
2- Prepare the trypsin: add 5ml EDTA/PBS to 100ul trypsin.
3- Remove the old medium and wash the plate with 6ml PBS two times.
4- Treat the cells with fresh prepared trypsin/EDTA. Watch under the microscope until the cells becomes rounded.
5- Knock the plate until cells becomes separated from the plate. Add DMEM/F12 media to stop the trypsin (If the cell density needs to be increased, remove a little of trypsin). Pipette up and down five times; make sure cells come out from a flat opening. Dissociate the cells well.
6- Count the cells: Mix the cells well, 10ul cells + 10ul 0.4% trypan blue. Add 10ul of the cells/trypan blue to haemocytometer. Count cells in four chambers (Total cell number should be above 100). Total number is divided by four, then times 2X10^4. The final result is the concentration of the cells (unit: number of cells/ml).

**Morphological identification of BM-derived MSCs:**

All cultures were examined using inverted microscope; Leica DM IL LED with camera Leica DFC295 (Leica microsystems CMS GmbH, Ernst-Leitz-Strabe 17-37, Wetzlar, D-35578, Germany). MSCs in culture were characterized by their adhesiveness and spindle shape by inverted microscope (*Khalilabadi et al., 2017*).

**Flow cytometric analysis:**

Flow cytometry was used to assess the immune profile of MSCs, using the standard for MSC Cells (Passeges 2-3) were harvested, pelleted and resuspended in 1% feotal bovine serum albumin (FBS), and counted. Each population containing 105 cells was used for flow cytometry. Cells were stained with directly PE (phycoerythrin) conjugated antibodies against, CD45, CD90, CD105 and CD34 (ebioscience, Germany). An appropriate isotype-matched control antibody named mouse IgG1 K Iso
control (ebioscience, Germany) was used in all analyses. Cells were analyzed on FACS flow cytometry using Cell Quest Software (Becton Dickinson, UK) (Baghaei et al., 2017).

G-Preparation of MSCs-derived exosomes (Bruno et al., 2012):

MSC-derived exosomes were obtained from the supernatant of MSCs, representing conditioned media. First, rat bone marrow-derived MSCs (BM-MSCs) were prepared in the stem cell and molecular biology unit, central lab, Cairo faculty of medicine. The MSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) without fetal bovine serum (FBS), but with 0.5% human serum albumin (HSA) (Sigma-Aldrich, St. Louis, MO, USA), overnight. The viability of the cells cultured overnight was more than 99%, as detected by trypan blue exclusion. Cells were plated at 4000 cells/cm² for 7 days. On day 7, cells were trypsinized, counted, and replated in expansion medium at a density of 2000 cells/cm² for another seven days (end of passage 1). The expansion was performed until the third passage. The conditioned medium was collected and stored at −80 °C. The medium was centrifuged at 2000g for 20 min to remove debris, and then ultracentrifuged at 100,000×g in a SW41 swing rotor (Beckman Coulter, Fullerton, CA, USA) for 1 hour at 4 °C. Exosomes were washed once with serum-free M199 (Sigma-Aldrich) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH = 7.4), and submitted to a second ultracentrifugation in the same conditions. Exosomes were stored at −80 °C for the experiment.

H-Electron microscopy for the exosomes: (Lu et al., 2017):

Exosomes obtained after differential centrifugation of conditioned cell-culture medium were suspended in PBS. Ten micrograms of exosome suspension were loaded onto formvar carbon-coated 200 mesh
copper grids for 10 min at room temperature. Excessive fluid was slightly drained with filter paper. Adsorbed exosomes were negatively stained with 1% phosphotungstic acid for 5 min. Finally, the air-dried exosome-containing grids were observed by transmission electron microscope (JEM-1400PLUS, Japan) operating at 100 kV.

I-Exosome Labeling with PKH-26 (Safwat et al., 2018).

PKH26 (Sigma-Aldrich, St. Louis, MO, USA) was used to confirm the exosome localization within the retinal tissue. The exosome pellet was diluted with PKH-26 kit solution to 1 mL, and 2 µL of fluorochrome was added to this suspension and incubated at 38.5 °C for 15 min. After that, 7 mL of serum-free HG-DMEM (high glucose-modified eagles medium was added to the suspension, then it was ultracentrifuged for second time at 100,000× g for 1 h at 4 °C. The final pellet was resuspended rapidly in HG-DMEM and stored at −80 °C for future injection in experimentally induced rats.

J-Perfusion fixation for electron microscopy specimen preparation (Viswanathan, 2011):

**Chemicals and Solutions:**

Paraformaldehyde (16%, EM grade)

glutaraldehyde (25%, EM grade).

**Flushing Solution:**

1 ml heparin (1000 units/ml), 1 ml 1% sodium nitrate, and 8.5 g NaCl in 1 L deionized H2O were used to flush blood from the circulation before perfusion with the fixative.

**Fixative Solution:**

710 ml deionized H2O, 250 ml 16% paraformaldehyde, 40 ml 25% glutaraldehyde, and 11.6 g NaH2PO4·H2O, pH adjusted to 7.2–7.4 constituted the fixative solution.
Whole-body vascular perfusion was administered by a cannula passed through the left ventricle. Buffered glutaraldehyde-formaldehyde fixative applied by gravitational force with the container placed approximately 1 m above the body of the animal.

**Steps of perfusion and tissue handling:**

1. Animals were anaesthetized with sodium pentobarbitone 30mg/kg body weight.
2. The heart was exposed.
3. The ascending aorta was cannulated with a catheter of 1mm diameter through an incision in the left ventricle.
4. The right auricle was incised for the outflow of fixative.
5. Perfusion with normal saline was done first to wash out blood.
6. Fixative was allowed to flow rapidly for a minute and then the flow was reduced to about 6ml/minute for 10 to 15 minutes (approximately 400 ml/animal).
7. Proper fixation was indicated by the loss of trembling movements, hardening of organs and stiffening of limbs.
8. Thereupon, both eye balls were excised, immersed in the same fixation medium, and transferred to a vial containing fixative until further processing. Each eye ball was cut at the level of ora serrata to separate the cornea, then the retina was cut approximately 0.5-mm slices using a vibratome to produce small pieces and further fixed with the same fixative for 2 hours.

N.B. Exposure of glutaraldehyde to blood without prior clearing of the system will cause coagulation of blood and result in the failure of the perfusion.
**I-Histological studies:**

1-Haematoxylin and Eosin; *(Suvarna et al., 2013):*

**Fixative:** Neutral buffered formalin solution.

**Section:** Paraffin sections cut at six micrometers.

**Solutions:**

1. **Mayer's hemalum:**
   - Haematoxylin 1.0 gm.
   - Alluminium Sulphate 50 gm.
   - Citric Acid 1 gm.
   - Sodium Iodate 0.1gm.
   - Water 750 ml.

2. **Eosin:**
   - Eosin Yellowish 2.5 gm.
   - Water 495 ml.
   - Glacial acetic acid 0.5 ml.

3. **Acid Alcohol:**
   - Alcohol 95% 500ml.
   - Concentrated Hydrochloric Acid 5ml.

The Hx was dissolved in alcohol before adding the other ingredients. The stain was left to ripen for some weeks in a cotton plugged flask which was exposed to sunlight and air in a warm place.

Eosin Y is readily soluble in water, so 1% aqueous solution of Eosin was prepared.

**Technique:**

1. Sections were brought down to water.
2. The sections were stained in Erlich's Hx for five minutes.
3. Blued in tap water for three minutes.
4. Stained in 1% aqueous eosin solution for one minute.
5. Washed in water.
6. Dehydrated in alcohol.
7. Cleared in xylol and mounted in Canada Balsam.

**Results:**

Nuclei → Blue.

Cytoplasm → Pink.

### 2-PAS (Periodic Acid Schiff) Staining Protocol ((Suvarna et al., 2019):

**Description:**

This technique is appropriated to indicate the presence of carbohydrates in tissues. As basement membranes are rich in carbohydrates, it is a useful technique to point out basement membranes. The reaction is based on oxidation that periodic acid induces in carbon–carbon ligation bonds, forming aldehydes that react with acid fuchsin, which in turn combine with pararosanilin that becomes magenta.

**Fixation:** 10% formalin.

**Section:** paraffin sections at five um.

**Materials:**

1. Schiff’s reagent:

Dissolve one g of basic fuchsin in 200 mL of boiling distilled H2O. Cool the solution to 70°C and add two g of sodium metabisulphite. Cool to room temperature. Add 10 mL of 1 HCl. Leave for 24 h in the dark. Add two g of activated charcoal, mix, and filter. Store at 4°C in a dark flask. Solution should be clear or light yellow. Stable for four-five months.

(Test for Schiff reagent: Pour 10 ml of 37% formalin into a watch glass. To this add a few drops of the Schiff reagent to be tested. A good Schiff reagent will rapidly turn a red-purple color. A deteriorating Schiff reagent will give a delayed reaction and the color produced will be a deep blue-purple.)
Material and Methods

2-0.5% Periodic Acid Solution:
   Periodic acid ------------------ 0.5 g
   Distilled water ----------------- 100 ml

Staining Method:
1. Dewax sections and bring to water.
2. Incubate in 1% periodic acid for 10 min.
3. Rinse in distilled water, three times.
4. Cover with Schiff’s reagent for 10 min (Sections become light pink color during this step).
5. Wash in running tap water (Immediately sections turn dark pink color).
6. Counterstain in Mayer's hematoxylin for one minute.
7. Rinse in distilled water for five minutes.
8. Dehydrate through ethanol.
9. Clear in xylene and mount.

Results:
*Glycogen and other carbohydrates: stain magenta.
*Nuclei: stain blue.

II-Immunohistochemical studies:

1-Vascular endothelial growth factor (VEGF) Antibody Staining Protocol (Van der Loos et al., 2010):
Description:
   VEGF (vascular endothelial growth factor) is a homodimeric, disulfide-linked glycoprotein. It is one of the most important growth and survival factors for endothelium. It plays a critical role in angiogenesis, vasculogenesis and endothelial cell growth through induction of endothelial cell proliferation and blood vessels permeabilization. VEGF bind to three tyrosine-kinase receptors, VEGFR-1, VEGFR-2 and VEGFR-3 which are expressed almost exclusively in endothelial cells.
i- **Primary Antibody of VEGF:** It is goat polyclonal antibody.

ii- **Detection system (Lab Vision Neomarker U.S.A.):**

- Labelled-Streptavidin-Biotin, also known as Streptavidin-Biotin Amplification which reacts with rat primary antibodies.
- Horse-radish Peroxidase enzyme.
- Chromogen.

**Counterstain:** Reagent: Mayer's Hematoxylin

**Reagents supplied:**

- **Reagent 1A:** one dropper bottle of ready-to-use serum blocking solution, 10% non-immune serum (goat).
- **Reagent 1B:** one dropper bottle of ready-to-use biotinylated secondary antibody.
- **Reagent 2:** one dropper bottle of ready-to-use streptavidin-peroxidase conjugate.
- **Reagent 3A:** one dropper bottle of concentrated substrate buffer.
- **Reagent 3B:** one dropper bottle of concentrated chromogen.
- **Reagent 3C:** one dropper bottle of 0.6% hydrogen peroxide.
- **Reagent 4:** one dropper bottle of ready-to-use Hematoxylin solution.
- **Reagent 5:** one dropper bottle of ready-to-use Histomount.

**Reagents & material required:**

- Xylene, ethanol and absolute methanol.
- Distilled water.
- 30% hydrogen peroxide.
- 10 ml phosphate buffered saline, pH 7.5.
- Humidity chamber (moist chamber for the incubation procedures).

iii- **Staining protocol:**

1- Sections were mounted on poly L-lysine coated slides or on positive-charged slides.
2- Paraffin sections were deparaffinized with xylene for one hour, and then rehydrated in descending grades of ethanol, and then rinsed in water.

3- Endogenous peroxide quenching was done by immersing slides in 30% peroxide solution for five minutes to reduce non-specific background staining.

4- Slides were washed two times in PBS for five minutes each.

5- Serum blocking solution were added to each slide, incubated for 30 minutes, then drained and blotted off the slides (but not rinsed) to block non specific background staining.

6- Two drops of ready-to-use goat polyclonal antibodies were applied to each section and then incubated for one hour at room temperature.

7- Sections were washed three times in PBS two minutes each.

8- Two drops of biotinylated secondary antibody were applied to each section at room temperature for 15 minutes and then rinsed well with PBS three times for two minutes each.

9- Two drops of streptavidin-proxidase were applied to each section at room temperature for 15 minutes and rinsed well with PBS three times for two minutes each.

10- Two drops of chromogen were applied to sections for 15 minutes.

11- Sections were washed in distilled water then counterstained by Mayer's hematoxylin.

12- Sections were dehydrated in ascending grades of ethanol and cleared in xylene. Two drops of Histomount were added to each slide, and mounted with a clean cover slides.

**iv-Results:** Staining Pattern: Nuclear membrane, cytoplasmic and cytoplasmic membrane of endothelial cells.
**Material and Methods**

**v-Control slides:**

a) **Positive Control for VEGF:**

Positive control tissue is indicative of correctly prepared tissues and proper staining techniques. Specimen of human ovary carcinoma labeled with Anti-VEGF polyclonal antibody showing brown cytoplasmic reaction in blood vessels endothelial cells. Fig. (13).

b) **Negative control:**

Retinal specimen was processed in the previously mentioned sequence but treated with buffer solution instead of the same concentration of primary antibody in every run. Fig. (14). Omission of the primary antibody gave no staining results.

![Image](image-url)  

**Fig. (13).** A photomicrograph of a section of Formalin-fixed and paraffin embedded: human ovary carcinoma labeled with Anti-VEGF polyclonal antibody showing brown cytoplasmic reaction in blood vessels endothelial cells.

(VEGF +ve immunostaining X 400)
Fig (14): A photomicrograph of section from retina of an adult male albino rat. It exhibit -ve immunostaining for VEGF.

(VEGF-ve immunostaining X 400)

2-Vimentin Staining Protocol (Satelli and Li, 2011):
Description: This monoclonal antibody reacts with the intermediate filament protein vimentin present in cells of mesenchymal origin like fibroblasts and Schwann cells.

i-Primary Antibody: Vimentin mouse monoclonal antibody.

ii-Detection system (Lab Vision Neomarker U.S.A.):
- Labelled-Streptavidin-Biotin, also known as Streptavidin-Biotin Amplification which reacts with rat primary antibodies.
- Horse-radish Peroxidase enzyme.
- Chromogen.
iii- Steps:

1. Paraffin section or frozen section to water and rinse in PBS for 2x2 min.
2. Antigen Retrieval: perform antigen retrieval with Epitope Retrieval Solution if necessary.
4. Primary Antibody: incubate sections in primary antibody at appropriate dilution for 1 hour at room temperature or overnight.
5. Rinse in PBS for three or two mins.
6. Peroxidase Blocking: incubate sections in peroxidase blocking solution for 10 minutes at room temperature.
7. Rinse in PBS for three or two mins.
8. Secondary Antibody: incubate sections in Biotinylated secondary antibody in PBS for 30 minutes at room temperature.
9. Rinse in PBS for three or two mins.
10. Detection: incubate sections in ABC-Peroxidase Solution for 30 minutes at room temperature.
11. Rinse in PBS-Tween 20 for three or two mins.
13. Rinse in PBS for three or two mins.
15. Rinse in running tap water for two-five minutes.
16. Dehydrate through 95% ethanol for 1 minute, 100% ethanol for 2x3min.
17. Clear in xylene for 2x5 min.
18. Coverslip with mounting medium.
iv- **Results:** Staining Pattern: Cytoplasmic brown staining.

v- **Control slides:**

a) **Positive Control for vimentin:**
positive control tissue is indicative of correctly prepared tissues and proper staining techniques. Specimen of human liver cancer tissue stained with anti-vimentin showing cytoplasmic brown staining Fig. (15).

b) **Negative control:**
Retinal specimen was processed in the previously mentioned sequence but treated with buffer solution instead of the same concentration of primary antibody in every run. Fig. (16). Omission of the primary antibody gave no staining results.

**Fig (15):** A photomicrograph of a section in human liver cancer tissue stained with anti vimentin showing cytoplasmic brown staining.

(Vimentin immunostaining X 400)
**Material and Methods**

**Fig (16):** A photomicrograph of a section from retina of an adult male albino rat. It exhibit -ve immunostaining for vimentin.

(Vimentin -ve immunostaining X 400)

**3-CD105 immunostaining: (Castrechini et al., 2010):**

**Primary Antibody:** Ab-1 monoclonal antibody, Clone QBEnd/10, Lab Vision Corporation laboratories, CA 94539, USA

**Steps:**
The immunohistochemical staining were performed on paraffin-embedded tissue sections. Briefly, the sections were deparaffinized using xylene and ethanol. Antigen retrieval was achieved by boiling the tissue slides with 0.01 M citric buffer in a microwave for 5 min. Hydrogen peroxide (0.5%) was applied at room temperature (RT) for 10 min to quench the endogenous peroxidase activity. After blocking for 10 min with 10% horse serum-Tris buffer for 20 minutes at RT, the sections were incubated with the primary antibody at the dilution noted above at RT for
2 h and, thereafter, with the secondary antibody for two hours. Slides stained only with a non-specific IgG were used as negative controls. The nuclei were counterstained with hematoxylin.

**Results:**
The brown staining of nuclei, cytoplasm or both was considered a positive staining.

**III-Transmission electron microscopic studies:**

**Preparation of the specimens for transmission electron microscopy (Ayub et al., 2017):**
Specimens were processed in EM unit, Tanta faculty of medicine.

**Fixation:** Perfusion fixation.

**Post perfusion immersion:**
It is common to place the small pieces of tissue in a vial containing the same fixative for additional two hours to ensure that the fixation is adequate.

**Post Fixation:** The specimens were post fixed in 1% osmic acid for one hour. Then, the tissues were washed in phosphate buffer for two changes, half an hour each.

**Dehydration:**
This was carried out in a series of ascending grades of alcohol according to the following schedule:
- Alcohol 50% 10 minutes to rinse out osmium.
- Alcohol 70% 10 minutes.
- Alcohol 80% 10 minutes.
- Alcohol 95% 10 minutes.
- Alcohol 100% two changes 10 minutes each.
Clearing and infiltration:
Clearing was performed in propylene oxide for 20 minutes at room temperature. Infiltration was then done by using equal parts of propylene oxide and Epon 812. The specimens were left in the infiltration medium for overnight.

Embedding:
The specimens were embedded in gelatin capsules filled with fresh (Epon 812). The capsules were kept in oven at 60°C for 48 hours to allow polymerization.

Semi-thin sectioning:
After trimming the blocks, semi thin sections were cut at one um with a glass knife. Sections were picked on glass slides and dried on hot plate for 30 minutes. They were stained with Toluidine blue dissolved in 1% borax. Excess stain was washed with distilled water and sections were mounted in polymount and examined to choose the desired areas for ultrasectioning.

Ultra-sectioning:
Ultrathin sections 80 nm in thickness were obtained from the selected area using glass knife. They were picked up on copper grids.

Staining:
The grids were stained with saturated solution of uranyle acetate for 20 minutes and lead nitrate for 10 minutes. They were washed in between with distilled water to remove the excess stain.

Electron micrograph and micrographic processing:
Grids were examined and electron micrographs were taken using transmission electron microscope JOEL (JEM-100 SX, Akishima, Tokyo, Japan) in, Electron Microscope unit, Tanta faculty of medicine, Tanta University.
**Morphometric study and statistical analysis:**

The mean optical density of PAS reaction staining, vimentin and VEGF immuno-expression were done.

**Slide imaging and digitizing:**

Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 0.5X photo adaptor, using 40X objective. The result images were analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for object counting and analysis. Two slides from each rat were prepared, 5 random fields from each slide were analyzed with total 10 images.

Data were analyzed using Statistical Package for Social Science software computer program version 23 (SPSS, Inc., Chicago, IL, USA). Data were presented in mean and standard deviation. One-way Analysis of variance (ANOVA) followed by post-hoc and tukey was used for comparing different groups. $P$ value less than 0.05 was considered statistically significant.
RESULTS

BMMSCs Characterization and Tracking:

MSCs were identified in culture by inverted microscope as adherent spindle shaped cells between rounded cells at 3rd day (Fig.17) and day 14 (Fig.18). Also, BMMSCs were identified by flow cytometric analysis showing that they are positive for CD90 and CD105 and negative for CD34 and CD45 (Figs. 19 and 20). MSCs, also were detected in the retinal tissues by CD105 immunohistochemical study (Fig.21).

MSCs- Exosomes Characterization:

A transmission electron microscopic examination of purified exosomes demonstrated their characteristic spheroid double-membrane bound morphology with a diameter of 90-100 nm (Fig.22).

Exosomes labelled with PKH26 fluorescent dye were detected in retinal tissues as strong red fluorescence (Fig. 23).
Fig. (17): An inverted microscope micrograph at day 3 from primary culture of MSCs showing few adherent spindle shaped cells (black ↑), appeared between the rounded non adherent refractile cells (white ↑).

(X200)
**Fig. (18):** An inverted microscope micrograph on day 14 from primary culture of MSCs showing more adherent spindle shaped cells (black↑) between rounded non adherent cells (white↑).

(X400)
**Fig. (19):** A photomicrograph of a section in rat retina of group III (DM for 8 weeks with injected stem cells) showing positive CD105 expression (arrow) in the outer plexiform layer (OPL), inner nuclear layer (INL) and ganglion cell layer (GCL).

*(CD 105 immunostaining X 400)*
A) Fig. (20): Flow cytometric analysis images of MSCs showing that they are positive for CD90 (A) (98.8%) and CD105 (B) (98.3%).

B)
Fig. (21): Flow cytometric analysis images of MSCs showing that they are negative for CD 34 (A) (1.11%) and CD 45 (B) (4.73%).
Fig. (22): A transmission electron micrograph of exosomes demonstrated their characteristic spheroid double-membrane bound morphology with a diameter of 90-100 nm (black ↑).

(X2000)
**Fig. (23):** A fluorescent microscope photograph showing homing of exosomes labelled with PKH26 dye in retinal tissue (↑).

 *(X1000)*
H & E Stain results:

Examination of all subgroups of the group I (control group) showed similar histological architecture. Therefore, results of subgroup Ia were used to represent this group.

**Group I** (control group) showed retinal pigment epithelial (RPE) layer, photoreceptor layer (PRL), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NFL) and inner limiting membrane (ILM) (Fig. 24).

**Group II** (affected group) revealed apparent decrease retinal thickness. The GCL revealed wide clear areas around darkly stained nuclei. Large dilated congested blood vessel appears in GCL. Focal widening of intercellular spaces of the inner nuclear layer and the outer nuclear layer. The nuclei of the inner nuclear layer (INL) appear small and darkly stained. Multiple congested blood vessels in the inner nuclear layer (INL) encroaching toward the outer plexiform layer (OPL) (Fig. 25).

**Group III** (DR + Stem cells) showed some ganglionic cells nuclei in the ganglion cell layer are still shrunken and pyknotic, with slightly diminished clear areas. New blood vessels in the inner nuclear layer were encroaching toward the outer plexiform layer. Focal widening of intercellular spaces between the cells of the outer nuclear layer (Fig. 26).

**Group IV** (DR + Exosomes) showed apparently increased retinal thickness, with more regularly arranged layers. Small new blood vessel was seen in the inner nuclear layer and the outer plexiform layer. (Fig. 27).
**Group V** (DR + Stem cells+ Exosomes) showed nearly normal retinal histological architecture. Ganglion cells appear with vesicular nuclei and small blood vessel was seen in the outer plexiform layer (Fig. 28).

**Group VI** (Recovery group) showed apparent marked reduction in the retinal thickness. The GCL revealed darkly stained pyknotic nuclei of ganglionic cells. The nuclei of the inner nuclear layer (INL) and outer nuclear layer (ONL) are small and darkly stained. Multiple dilated new blood vessel in the nerve fiber layer (NFL)and the ganglion cell layer (GCL). Widening of intercellular space between the cells of the outer nuclear layer (ONL). The choriocapillary layer can be seen with dilated congested blood vessels. (Fig. 29).
**Fig. (24):** A photomicrograph of a section in the retina of adult male albino rat of group I (control group) showing the normal retina consists of 10 layers from outward to inward; Retinal pigment epithelium (RPE) single layer of cells with oval nuclei (▲), the photoreceptor layer (PRL) with outer segments (o) and inner segments (i) of rods and cones, the outer limiting membrane (OLM), the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform (IPL), the ganglion cell layer (GCL) with large vesicular nuclei (arrows), the nerve fiber layer (NFL) and the inner limiting membrane (ILM).

*(H&E X400)*
**Fig. (25):** A photomicrograph of a section in the retina of adult male albino rat of group II (affected group) showing apparent decrease in retinal thickness. The GCL revealed shrunken nuclei (black↑) and wide clear areas (arrow heads). Focal widening of intercellular spaces of the inner nuclear layer (>) and the outer nuclear layer (white ↑). The nuclei of the INL appear small and darkly stained (angled ↑). Large dilated congested blood vessel appeared in GCL (*). Multiple congested blood vessels (red ↑) in the inner nuclear layer (INL) encroaching toward the outer plexiform layer (OPL).

(H&E X 400)
Fig. (26): A photomicrograph of a section in the retina of retina of adult male albino rat group III (DR + Stem cells) showing the GCL with darkly stained nuclei (black ↑) and clear areas (arrow head). Blood vessel in the GCL (*) and in the INL (red ↑) encroaching toward the outer plexiform layer. Focal widening of intercellular spaces between the cells of the ONL (white ↑).

(H&E X 400)
**Fig. (27):** A photomicrograph of a section in the retina of adult male albino rat of group IV (DR + Exosomes) showing apparently increased retinal thickness, with more regularly arranged layers. Some ganglion cells appear with euchromatic nuclei (**black ↑**), with slightly decreased intercellular spaces between the cells (**arrow head**). Small blood vessel (**red ↑**) can be seen in the inner nuclear layer (INL) and outer plexiform layer (OPL).

*(H&E X 400)*
**Fig. (28):** A photomicrograph of a section in the retina of adult male albino rat of group V (DR+ Stem Cell+ Exosomes) showing nearly normal histological architecture. Ganglion cells showing more vesicular nuclei (black ↑). Small blood vessel (red ↑) can be seen in the outer plexiform layer (OPL).

(H&E X 400)
**Fig. (29):** A photomicrograph of a section in the retina of adult male albino rat of group VI (Recovery group) showing apparent marked reduction in retinal thickness. The GCL revealed pyknotic nuclei (black ↑) of ganglion cells. The nuclei of the inner nuclear layer (INL) and outer nuclear layer (ONL) are small and darkly stained. Multiple dilated blood vessels (star) in the nerve fiber layer (NFL) and ganglion cell layer (GCL). Widening of intercellular spaces (white↑) between the cells of the outer nuclear layer (ONL). Part of the choriocapillary layer (Ch) can be seen with dilated congested blood vessels (blue ↑).

*(H&E X 400)*
PAS staining results:

Examination of all subgroups of the group I (control group) showed similar results. Therefore, results of subgroup Ia were used to represent this group.

**Group I** (control group) showed normal retinal layers with minimal purple-magenta color PAS stain areas especially in OPL and OLM (Fig. 30).

**Group II** (affected group) showed marked PAS staining in all layers of the retina indicating deposition of strong positive PAS material within the different retinal layers. Deposition of positive PAS material within the lining of newly formed blood vessel (Fig. 31).

**Group III** (DR + Stem cells) showed moderate PAS staining in layers of the retina especially in the INL, OLM and PRL (Fig. 32).

**Group IV** (DR + Exosomes) showed mild PAS staining in layers of the retina (Fig. 33).

**Group V** (DR + Stem cells + Exosomes) showed weak PAS staining in the nearly normal arranged layers of the retina (Fig. 34).

**Group VI** (Recovery group) showed intense PAS staining in all layers of the retina indicating deposition of strong positive PAS material within the different retinal layers. Deposition of positive PAS material within the lining of newly formed blood vessel in the ganglion cell layer (GCL), and in the vessels of choriocapillary layer (Ch) (Fig. 35).
Fig. (30): A photomicrograph of a section in the retina of adult male albion rat of group I (control group) showing normal retinal layers with minimal PAS stain (↑) especially in INL and OLM.

(PASX 400)
Fig. (31): A photomicrograph of a section in the retina of adult male albino rat group II (affected group) showing marked purple-magenta color of PAS staining (black ↑) in all layers of the retina.

(PASX 400)
Fig. (32): A photomicrograph of a section in the retina of adult male albino rat of group III (DR + Stem cells) showing moderate purple-magenta color of PAS staining (black ↑) in layers of the retina especially in the IPL, OPL and the PRL.

(PASX 400)
**Fig. (33):** A photomicrograph of a section in the retina of adult male albino rat of group IV (DR +Exosomes) showing mild purple-magenta color of PAS staining (black ↑) in the layers of the retina.

(PASX 400)
**Fig. (34):** A photomicrograph of a section in the retina of adult male albino rat of group V (DR + Stem cells + Exosomes) showing weak purple-magenta color of PAS (black ↑) staining in the nearly normal arranged layers of the retina.

(PASX 400)
**Results**

**Fig. (35):** A photomicrograph of a section in the retina of adult male albino rat group VI (Recovery group) showing intense PAS staining (black ↑) in all layers of the retina and within the basement membrane of newly formed blood vessel (green ↑) in the ganglion cell layer (GCL), and in the vessels (blue ↑) of choriocapillary layer (Ch).

(PASX 400)
Morphometric study: PAS stain

Table (1): Showing the mean optical density % and ±SD of PAS stain in all groups:

<table>
<thead>
<tr>
<th>VEGF (%)</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.21</td>
<td>5.20</td>
<td>3.20</td>
<td>1.80</td>
<td>0.65</td>
<td>12.31</td>
</tr>
<tr>
<td>±SD</td>
<td>0.03</td>
<td>0.87</td>
<td>0.53</td>
<td>0.30</td>
<td>0.11</td>
<td>2.05</td>
</tr>
</tbody>
</table>

Significant at P < 0.05: b,c,d,e,f; a,c,d,e,f; a,b,d,e,f; a,b,c,e,f; a,b,c,d,f; a,b,c,d,e

Data expressed as mean ±SD

SD: standard deviation

Test used: One-way ANOVA followed by post-hoc tukey

a: significance relative to Group I
b: significance relative to Group II
c: significance relative to Group III
d: significance relative to Group IV
e: significance relative to Group V
f: significance relative to Group VI

Histogram (1): showing the mean optical density of PAS reaction % in all groups.
**VEGF staining:**

Examination of all subgroups of the control group showed similar results regarding immunohistochemical examination. Therefore, results of subgroup Ia were used to represent this group.

**Group I** (Control group) showed negative immunoreaction for vascular endothelial growth factor (VEGF) in almost all retinal layers (Fig. 36).

**Group II** (affected group) showed markedly positive VEGF immunoreaction especially in NFL, GCL, INL and OPL of retina (Fig. 37).

**Group III** (DR + Stem cells) showed moderate positive VEGF immunoreaction in GCL and OPL of retina (Fig. 38).

**Group IV** (DR + Exosomes) showed mild positive VEGF immunoreaction in NFL and OPL of retina (Fig. 39).

**Group V** (DR + Stem cells + Exosomes) showed minimal positive VEGF immunoreaction in GCL and INL of retina (Fig. 40).

**Group VI** (Recovery group) showed intense positive VEGF immunostaining in NFL, GCL, INL and OPL of retina (Fig. 41).
**Results**

**Fig. (36):** A photomicrograph of a section in the retina of adult male albino rat of group I (control group) showing negative immunoreaction for vascular endothelial growth factor (VEGF) in almost all retinal layers.

*(Anti-VEGF immunostaining, ×400)*
**Fig. (37):** A photomicrograph of a section in the retina of adult male albino rat of group II (affected group) showing markedly positive VEGF immunoreaction (**black ↑**) especially in NFL, GCL, INL and OPL of retina.

(Anti-VEGF immunostaining, ×400)
**Fig. (38):** A photomicrograph of a section in the retina of adult male albino rat of group III (DR + Stem cells) showing moderately positive VEGF immunoreaction (black↑) in GCL and OPL of the retina.

(Anti-VEGF immunostaining, ×400)
Fig. (39): A photomicrograph of a section in the retina retina of adult male albino rat of group IV (DR + Exosomes) showing mild positive VEGF immunoreaction (black ↑) in NFL and OPL of retina.

(Anti-VEGF immunostaining, ×400)
**Fig. (40):** A photomicrograph of a section in the retina of adult male albino rat of group V (DR + Stem cells + Exosomes) showing minimal positive VEGF immunoreaction (black ↑) in GCL and INL of retina.

(Anti-VEGF immunostaining, ×400)
Fig. (41): A photomicrograph of a section in the retina of adult male albino rat of group VI (Recovery group) showing intense positive VEGF immunoreaction (black ↑) in NFL, GCL and INL and OPL of retina.

(Anti-VEGF immunostaining, ×400)
Morphometric study: VEGF

Table (2): Showing the mean area % and ±SD of VEGF expression of all groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>VEGF (%) Mean ±SD</th>
<th>Significant at P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2.43 ±0.41</td>
<td>b,c,d,e,f</td>
</tr>
<tr>
<td>II</td>
<td>10.14 ±1.69</td>
<td>a,c,d,e,f</td>
</tr>
<tr>
<td>III</td>
<td>8.82 ±1.47</td>
<td>a,b,d,e,f</td>
</tr>
<tr>
<td>IV</td>
<td>7.83 ±1.31</td>
<td>a,b,c,e,f</td>
</tr>
<tr>
<td>V</td>
<td>4.27 ±0.71</td>
<td>a,b,c,d,f</td>
</tr>
<tr>
<td>VI</td>
<td>14.79 ±2.47</td>
<td>a,b,c,d,e</td>
</tr>
</tbody>
</table>

Data expressed as mean ±SD
SD: standard deviation
Test used: One-way ANOVA followed by post-hoc tukey

Histogram (2): Showing the mean area % of VEGF expression in all groups.
**Vimentin staining:**

Examination of all subgroups of the control group showed similar results regarding histochemical examination. Therefore, results of subgroup Ia were used to represent this group.

**Group I** (control group) showed minimal vimentin immunoreactivity in the Muller cell end feet at the inner limiting membrane (ILM). Positively stained fine brown Muller's radial processes appear as long as filamentous structure extending in NFL, GCL, IPL, INL, OPL and ONL (Fig.42).

**Group II** (affected group) showed markedly positive vimentin immunostaining of muller cell bodies and their processes and observed throughout most retinal layers (Fig. 43).

**Group III** (DR + Stem cells) showed moderate positive vimentin immunostaining of muller cell bodies and their processes. (Fig.44).

**Group IV** (DR + Exosomes) showed mild positive vimentin immunostaining of muller cell bodies and their processes. (Fig.45).

**Group V** (DR + Stem cells + Exosomes) showed weak positive vimentin immunostaining of muller cell bodies and their processes. (Fig.46).

**Group VI** (Recovery group) showed intense positive vimentin immunostaining of muller cell bodies and their processes observed throughout most retinal layers especially in the NFL and GCL (Fig.47).
**Fig. (42):** A photomicrograph of a section in the retina of adult male albino rat of group I (control group) showing minimal vimentin immunostaining in the Muller cell end feet (arrow head) at the inner limiting membrane (ILM). Positively stained fine brown Muller's radial processes appear as long filaments (red ↑) extending in NFL, GCL, IPL, INL, OPL and ONL.

*(Anti-Vimentin immunostaining, x400)*
Fig. (43): A photomicrograph of a section in the retina of adult male albino rat of group II (affected group) showing marked vimentin immunostaining of muller cell bodies and their processes throughout most retinal layers (black ↑).

(Anti-Vimentin immunostaining, ×400)
**Fig. (44):** A photomicrograph of a section in the retina of adult male albino rat of the group III (DR + Stem cells) showing moderate positive vimentin immunostaining of muller cell bodies and their processes in NFL, INL, OPL and ONL (black ↑).

(Anti-Vimentin immunostaining, x400)
Fig. (45): A photomicrograph of a section in the retina of adult male albino rat of group IV (DR + Exosomes) showing mild positive vimentin immunostaining of muller cell bodies and their processes in NFL, IPL, INL and OPL (black ↑).

(Anti-Vimentin immunostaining, ×400)
**Fig. (46):** A photomicrograph of a section in the retina of adult male albino rat of group V (DR + Stem cells + Exosomes) showing weak positive vimentin immunostaining of muller cell bodies and their processes in NFL and OPL (black ↑).

(Anti-Vimentin immunostaining, ×400)
Fig. (47): A photomicrograph of a section in the retina of adult male albino rat of the group VI (Recovery group) showing intense positive vimentin immunostaining of muller cell bodies and their processes observed throughout most retinal layers (black ↑) especially in the NFL, GCL, IPL and OPL.

(Anti-Vimentin immunostaining, ×400)
Morphometric study: Vimentin

Table (3): Showing the mean area % and ± SD of Vimentin expression of all groups.

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>vimentin</td>
<td>Mean</td>
<td>0.87</td>
<td>3.51</td>
<td>2.73</td>
<td>1.81</td>
<td>1.43</td>
</tr>
<tr>
<td>±SD</td>
<td>a,c,d,e,f</td>
<td>0.14</td>
<td>0.59</td>
<td>0.46</td>
<td>0.30</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Data expressed as mean ±SD  
SD: standard deviation  
Test used: One-way ANOVA followed by post-hoc tukey  
a: significance relative to Group I  
b: significance relative to Group II  
c: significance relative to Group III  
d: significance relative to Group IV  
e: significance relative to Group V  
f: significance relative to Group VI  

Histogram (3): showing the mean area % of vimentin expression in all groups.
Electron Microscopic Results

**Retinal Pigment Epithelium (RPE) & Photoreceptor outer segment (POS):**

**Group I** (control group) showed Retinal Pigment Epithelial (RPE) cell and photoreceptors outer segments (POS). RPE was resting on Bruch's membrane with large euchromatic oval nucleus, basal mitochondria and long apical microvilli surrounding the outer segment of the photoreceptor. POS appeared as elongated, straight structures. Parallel lamellar structures of the outer segment of photoreceptors and Choriocapillary layer could be noticed (Fig. 48).

**Group II** (affected group) showed retinal pigmented cell with extensively destructed broken apical microvilli, and numerous large phagosomes. The photoreceptor outer segments were markedly disorganized and vacuolated with loss of normal orientation of their lamellar disc membranes. There were wide areas of complete loss of photoreceptors outer segments (Fig. 49).

**Group III** (DR + Stem cells) showed retinal pigmented cell resting on distorted Bruch's membrane and had large oval nuclei with some broken apical microvilli. Multiple photoreceptor outer segments were present. Some of them were with normal lamellar appearance and others with loss of normal orientation of their lamellar disc membranes and still showing vacuolations. There were areas of loss of photoreceptors outer segments (Fig. 50).

**Group IV** (DR + Exosomes) showed retinal pigmented epithelial cells resting on Bruch's membrane with oval large heterochromatic nuclei and apical microvilli. Multiple photoreceptor outer segments were slightly
separated by spaces. Some of these segments were with normal lamellar appearance, and others had vacuolation (Fig. 51).

**Group V** (DR + Stem cells+ Exosomes) showed nearly normal Retinal pigment epithelium resting on Bruch's membrane separating it from the Choriocapillary layer. RPE had large oval heterochromatic nucleus and many long apical microvilli enclosing the outer segments. The photoreceptors outer segments were with normal lamellar membranous discs (Fig. 52).

**Group VI** (Recovery group) showed retinal pigmented cell with shrunken nucleus that had areas of heterochromatin, vacuolated cytoplasm, distorted apical microvilli and large phagosomes. The photoreceptor outer segments were markedly distorted and shrunken. There were large areas filled with debris. Protruded some photoreceptors nuclei into the area of the outer segments could be seen (Fig. 53).
Fig. (48): A transmission electron micrograph of a section in the retina of adult male albino rat of group I (control group) showing a part of retinal pigment epithelial (RPE) cell and some photoreceptors outer segments (POS). RPE is resting on Bruch's membrane (thick white arrow) with large euchromatic oval nucleus (N), basal mitochondria (▲) and long apical microvilli (Mv) surrounding the outer segment of the photoreceptor. POS appears as elongated, straight structures. Parallel lamellar discs of the photoreceptors outer segments can be seen (*). Choriocapillary layer can be noticed (Ch).

(TEM, X2000)
Fig (49): A transmission electron micrograph of a section in the retina of adult male albino rat of group II (affected group) showing retinal pigmented cell (RPE) is resting on Bruch's membrane (thick white arrow) with extensively destructed broken apical microvilli (Mv), oval nucleus (N) with karyolysis (curved arrow) and large phagosomes (ph). The photoreceptor outer segments (POS) are disorganized and vacuolated (V). There are wide areas of lost photoreceptors outer segments (thick black arrow).

(TEM, X2000)
Fig (50): A transmission electron micrograph of a section in the retina of adult male albino rat of group III (DM + Stem cells) showing retinal pigmented cell (RPE) resting on distorted Bruch's membrane (thick white arrow). RPE has large oval nucleus (N) and broken apical microvilli (Mv). Some photoreceptors outer segments (POS) are present with normal lamellar appearance (*) and others with loss of normal orientation of their lamellar disc membranes (black ↑) and vacuolation (V). There are areas of loss of photoreceptors outer segments (thick black arrow)

(TEM, X2000)
Fig (51): A transmission electron micrograph of a section in the retina of adult male albino rat of group IV (DM + Exosomes) showing retinal pigmented cell (RPE) resting on Bruch's membrane (thick white arrow) with normal large oval nucleus (N) and apical microvilli (Mv). Multiple photoreceptors outer segments (POS) are present slightly separated by spaces (thick black arrow). Some of these segments have normal lamellar appearance (*), and others showing vacuolation (V).

(TEM, X2000)
Fig. (52): A transmission electron micrograph of a section in the retina of adult male albino rat of group V (DM + Stem cells + Exosomes) showing nearly normal Retinal pigment epithelium (RPE) resting on Bruch's membrane (thick white arrow) separating it from the Choriocapillary layer (Ch). RPE has large oval heterochromatic nucleus (N) and many long apical microvilli (Mv) enclosing the outer segments. The photoreceptors outer segments (POS) are nearly normal with lamellar membranous discs (*).

(TEM, X2000)
Fig (53): A transmission electron micrograph of a section in the retina of adult male albino rat of group VI (Recovery group) showing retinal pigmented cell (RPE) resting on Bruch's membrane (thick white arrow) with distorted apical microvilli (Mv), large phagosomes (ph), vacuolated cytoplasm (V) and oval nucleus (N) showing areas of heterochromatin (Hc). The photoreceptor outer segments (POS) are markedly distorted and shrunken. There are large wide areas (thick black arrow) filled with debris (<). Notice the protrusion of some photoreceptors nuclei (PN) into the area of the outer segments.

(TEM, X2000)
Results

Outer nuclear layer (ONL):

Group I (control group) showed outer nuclear layer with predominately rod cells. Their nuclei were more rounded, heterochromatic with highly condensed centrally located chromatin, and were surrounded by a thin rim of cytoplasm. The cells were tightly backed with no intercellular spaces. Outer plexiform layer could be seen, with many processes containing mitochondria (Fig. 54).

Group II (affected group) showed cells with severe cytoplasmic vacuolization. The cells were separated by intercellular spaces filled with debris, pyknotic nucleus could be seen (Fig. 55).

Group III (DR + Stem cells) showed many photoreceptor nuclei with heterochromatin. Wide areas separating the photoreceptor nuclei and many cytoplasmic vacuolations were noticed (Fig. 56).

Group IV (DR + Exosomes) showed many photoreceptor nuclei with heterochromatin. Little intercellular spaces were separating the cells (Fig. 57).

Group V (DR + Stem cells + Exosomes) showed nearly normal nuclei with heterochromatin surrounded by rim of cytoplasm, with no intercellular spaces between the cells. (Fig. 58).

Group VI (Recovery group) showed apparently decreased number of photoreceptor cell nuclei and many degenerated cells with severe cytoplasmic vacuolization. Some areas showed complete degeneration of photoreceptor nuclei and were filled with debris. Some other pyknotic nuclei were present. (Fig. 59).
Fig. (54): A transmission electron micrograph of a section in the retina of adult male albino rat of group I showing outer nuclear layer with the predominate rod cells (R). Their nuclei are rounded with highly condensed centrally located heterochromatin (*), and surrounded by a thin rim of cytoplasm (angled ↑). Notice the cells are tightly backed with no intercellular spaces. Small area of the outer plexiform layer (OPL) can be seen with many processes (Pr) containing mitochondria (M).

(TEM, X2000)
**Fig (55):** A transmission electron micrograph of a section in the retina of adult male albino rat group II (affected group) from the outer nuclear layer showing rod cells with dense nuclei (N) and cytoplasmic vacuolation (**black ↑**). The nuclei are separated by intercellular spaces (**thick white arrow**) that filled with debris (**>).** Pyknotic nucleus (**Py**) can be seen.

(TEM, X2000)
Fig (56): A transmission electron micrograph of a section in the retina of adult male albino rat of group III (DR + Stem cells) from the outer nuclear layer showing many photoreceptor nuclei (N) with heterochromatin (*). Wide area separating some nuclei can be seen (W). Many cells with cytoplasmic vacuolation (black ↑) are present.

(TEM, X2000)
Results

Fig (57): A transmission electron micrograph of a section in the retina of adult male albino rat of group IV (DR + Exosomes) from the outer nuclear layer showing many photoreceptor nuclei (N) with heterochromatin (*). Little intercellular spaces are separating cells (thick white arrow).

(TEM, X2000)
**Fig (58):** A transmission electron micrograph of a section in the retina of adult male albino rat of group V (DR + Stem cells + Exosomes) from the outer nuclear layer showing nearly normal photoreceptor nuclei (N) with heterochromatin (*) surrounded by thin rim of cytoplasm (angled arrow) with no intercellular spaces between cells. Process of muller cell can be noticed with electron lucent cytoplasm and different organelles (P).

*(TEM, X2000)*
Fig (59): A transmission electron micrograph of a section in the retina of adult male albino rat of group VI (recovery group) from the outer nuclear layer showing apparently decreased number of photoreceptor cells nuclei (N). Degenerated cells with cytoplasmic vacuolization (black ↑) can be seen. Some areas are showing complete dissolution of photoreceptor nuclei (star) and filled with debris (›). Some other pyknotic nuclei are present (Py).

(TEM, X2000)
**Inner nuclear layer (INL):**

**Group I** (control group) showed amacrine cells with large, pale euchromatic nuclei. Müller cells had dense nuclei and irregular outlines with prominent processes. The cell bodies of bipolar cells containing round nuclei. Normal retinal blood capillaries lined with endothelial cell could be observed (Fig. 60).

**Group II** (affected group) showed many cytoplasmic vacuolation and mitochondrial swellings in inner nuclear layer cells. Some amacrine cells had disintegrated cytoplasm filled with rough endoplasmic reticulum. Another amacrine cells showed large nuclei with heterochromatin. Bipolar cells showed shrunken nuclei with much peripheral heterochromatin. (Fig.61).

**Group III** (DR + Stem cells) showed amacrine cells had irregular shrunken nuclei with peripheral heterochromatin and their cytoplasm showed mitochondria with destructed cristae. The nuclei of some bipolar cells appeared irregular with dense areas. Few müller cells showed large irregular nuclei with dilated perinuclear membrane and markedly dilated RER. (Fig. 62).

**Group IV** (DR+ Exosomes) showed few amacrine cells had shrunken nuclei with areas of peripheral heterochromatin. Some Müller cells appeared irregular in shape with dark irregular nuclei and dilated nuclear envelope. Other Müller cells showed shrunken nuclei with indentations, dilated rough endoplasmic reticulum and slight mitochondrial swellings could be detected. The other amacrine and bipolar cells appeared nearly normal. (Fig. 63).
**Group V** (DR+ Stem cells + Exosomes) showed nearly normal Müller, amacrine and bipolar cells. Few bipolar cells revealed mitochondria with destructed cristae. Müller cells appeared with prominent processes. (Fig. 64).

**Group VI** (recovery group) showed degenerated amacrine and bipolar cells with decrease in the cell density. The degenerated cells were at various stages of disintegration and most of their cytoplasmic organelles were absent. The empty spaces left by degenerated cell bodies and processes were filled with debris. Müller cells appeared with irregular thin cytoplasmic processes. (Fig. 65).
Fig (60): A transmission electron micrograph of a section in the retina of adult male albino rat of group I from the inner nuclear layer showing amacrine cells (Am) with large and pale euchromatic nuclei (N). Müller cells (Mu) have dense nucleus and irregular outlines with prominent processes (P). The cell bodies of bipolar cells (Bi) show round nuclei (n). Normal retinal blood capillary (BC) lined with endothelial cell (e) can be observed.

(TEM, X2000)
Fig (61): A transmission electron micrograph of a section in the retina of adult male albino rat group II (affected group) from the inner nuclear layer showing many cytoplasmic vacuolation (V) and swollen mitochondria with destructed cristae (↑) in its cells. One amacrine cell (Am1) appears with disintegrated cytoplasm filled with rough endoplasmic reticulum (r). Another amacrine cell (Am2) showing large nucleus (N) with heterochromatin. Bipolar cell (Bi) showing shrunken nucleus (n) with peripheral heterochromatin. A Müller cell can be seen (Mu).

(TEM, X2000)
Fig (62): A transmission electron micrograph of a section in the retina of adult male albino rat of group III (DR+ Stem cells) from the inner nuclear layer showing some normal bipolar (Bi) and amacrine cells (Am1). Another amacrine (Am2) cell showing shrunken nucleus (N) with areas of peripheral heterochromatin (hc). One Müller cell (Mu1) appears irregular in shape with dark irregular nucleus showing dilated nuclear envelope (arrow head). Another Müller cell (Mu2) showing shrunken and indented nucleus (curved arrow) and phagocytic material and electron dense bodies (►►). Some mitochondria with destructed cristae can be detected (†). Bipolar cells (Bi) appear nearly normal.

(TEM, X2000)
**Results**

**Fig (63):** A transmission electron micrograph of a section in the retina of adult male albino rat of group IV (DR+ Exosomes) from the inner nuclear layer showing an amacrine cell (Am) having normal nucleus (N). The cytoplasm showing mitochondria with destructed cristae (↑). Müller cell (Mu) showing large irregular dense nucleus with dilated nuclear envelope (arrow heads) and dilated RER (thick white arrow). Bipolar cell (Bi) appear with its nucleus (n).

(TEM, X2000)
Fig. (64): A transmission electron micrograph of a section in the retina of adult male albino rat of group V (DR+ Stem cells + Exosomes) from the inner nuclear layer showing nearly normal amacrine (Am) and bipolar cells (Bi) with little mitochondria showing destructed cristae (↑). Müller cell (Mu) appear with irregular outlines and prominent processes (P).

(TEM, X2000)
Fig (65): A transmission electron micrograph of a section in the retina of adult male albino rat of group VI (recovery group) from the inner nuclear layer showing amacrine cells (Am) and bipolar cell (Bi) with decreased cytoplasmic density and their organelles are mostly absent. The empty spaces left by degenerated cell bodies and processes (thick black arrow) are filled with debris (*) Müller cells (Mu) appear with irregular thin cytoplasmic processes (p).

(TEM, X2000)
Ganglion cell layer (GCL):

**Group I** (control group) showed ganglion cells had large rounded euchromatic nuclei with uniformly staining nucleoplasm and prominent nucleoli. Few mitochondria, Rough endoplasmic reticulum and scattered ribosomes were observed. Ganglion cell axon developed from a portion of the axon hillock was observed (Fig. 66).

**Group II** (affected group) showed degenerated ganglion cells with indented nuclei exhibiting highly convoluted sinuses and areas of electron-dense heterochromatin. Swollen mitochondria with destructed cristae and decreased matrix density could be noticed. The cytoplasm showed vacuolation (Fig. 67).

**Group III** (DR+ Stem cells) showed ganglion cells with oval slightly granular nuclei and swollen mitochondria with destructed cristae (Fig.68).

**Group IV** (DR + Exosomes) showed ganglion cells with large euchromatic nuclei. Some mitochondria appeared with destructed cristae (Fig.69).

**Group V** (DR + Stem cells + Exosomes) showed nearly normal ganglion cells with large oval euchromatic nuclei. The cytoplasm contains plenty of ribosomes, slightly dilated RER and slightly swollen mitochondria. Ganglion cell axon developed from a portion of the axon hillock (Fig.70).

**Group VI** (recovery group) showed ganglion cells with highly rarefied cytoplasm containing large vacuoles. Nuclei were granular and showing vacuolation with slightly dilated nuclear envelope. Areas of disrupted envelope could be noticed. The inner limiting membrane appeared abnormal and the inner plexiform layer showed destructed axons (Fig. 71).
**Fig (66):** A transmission electron micrograph of a section in the retina of adult male albino rat of group I (control group) from the ganglion cell layer showing ganglion cell having large rounded euchromatic nucleus (N) with uniformly staining nucleoplasm and prominent nucleolus (n). Few mitochondria (black ↑), Rough endoplasmic reticulum (arrow heads) and scattered ribosomes (r) are observed. Ganglion cell axon developed from a portion of the axon hillock (a). Part of inner plexiform layer (IPL) can be seen with many axons (X) containing mitochondria (zigzag arrow).

(TEM, X3000)
Fig (67): A transmission electron micrograph of a section in the retina of adult male albino rat of group II (affected group) from the ganglion cell layer showing ganglion cell with indented nucleus (N) exhibiting highly convoluted sinuses (star) and areas of electron-dense heterochromatin (white ↑). Swollen mitochondria (black ↑) with destructed cristae and decreased matrix density can be noticed. The cytoplasm shows vacuolation (V).

(TEM, X3000)
Fig (68): A transmission electron micrograph of a section in the retina of adult male albino rat of group III (DR + Stem cells) from ganglion cell layer showing ganglion cell with oval granular nucleus (N). The cytoplasm showed swollen mitochondria with destructed cristae (black ↑). Part of the inner plexiform layer (IPL) can be seen.

(TEM, X3000)
**Fig (69):** A transmission electron micrograph of a section in the retina of adult male albino rat of group IV (DR + Exosomes) from ganglion cell layer showing ganglion cell with large euchromatic nucleus (N). Some mitochondria appear swollen with destructed cristae (black ↑). Prominent normal Golgi apparatus was seen (G).

(TEM, X3000)
Fig (70): A transmission electron micrograph of a section in the retina of adult male albino rat of group V (DR+ Stem cells + Exosomes) from ganglion cell layer showing nearly normal ganglion cell with large oval euchromatic nucleus (N) and normal nuclear membrane (↑↑). The cytoplasm contains plenty of ribosomes (r), slightly dilated RER (arrow head) and slightly swollen mitochondria (black ↑). Ganglion cell axon developed from a portion of the axon hillock (a). Part of the nerve fiber layer (NFL) was observed.

(TEM, X3000)
**Fig (71):** A transmission electron micrograph of a section in the retina of adult male albino rat of group VI (recovery group) from the ganglion cell layer of the retina showing ganglion cell with highly rarefied cytoplasm (cy) containing large vacuole (*). Nucleus is granular (N) and karyolitic (K), slightly dilated nuclear envelope (↑↑). Area of disrupted envelope (d) can be noticed. The inner limiting membrane (ILM) was vacuolated and the inner plexiform layer (IPL) shows destructed axons (x).

(TEM, X3000)
DISCUSSION

Despite advances in medical health and technologies, the incidence of diabetes has reached epidemic proportions globally and thus diabetic complications are increasing throughout the world. Diabetes complications generally grouped into microvascular or macrovascular complications (Homme et al., 2018).

Diabetic retinopathy is one of the most substantial microvascular complications of diabetes mellitus, and is the most common cause of blindness in people under the age of 65 (Tawfik et al., 2019). All persons with diabetes are at risk of developing retinopathy, however, persons living with type 1 diabetes (T1D) have a higher chance of getting DR as compared to persons living with type 2 (Kashim et al., 2018). The retina faces a unique challenge in diabetes due to the combination of high metabolic demand and minimal vascular supply. This limits the retina’s ability to adapt to the metabolic stress of diabetes (Bhatwadekar et al., 2017).

Streptozotocin (STZ) was used in this study because it is considered the most widely accepted animal model for the evaluation of retinal complications in diabetes and this agreed with Jiang et al. (2016) who reported that STZ induces various biochemical and histological alterations closely resemble the initial process of diabetic retinopathy that occurs in humans. The mechanism of DM induction by STZ was explained by Aloud et al. (2018) who stated that STZ impairs insulin activity by selectively damaging pancreatic cells, leading to increased blood glucose levels. STZ enters β cells through carrier protein glucose transporter 2 (GLUT2) and impairs insulin secretion and action through alkylation of pancreatic β-cell DNA.
Group II (affected group) in the present study showed varying degrees of lesions occurred in almost all retinal nerve tissues in the 8th week of the DM. Animal model manifested decreased retinal thickness, disorganized ONL and INL cells, pyknotic ganglion cells, dilated congested new blood vessels, significant increase in PAS staining, VEGF and Vimentin expression. EM examination revealed vacuolated retinal pigment epithelium with nuclear heterochromatin and destructed microvilli, destructed outer segment of the photoreceptors, increased spaces that filled with debris between the nuclei of the ONL and the INL, necrotic ganglion cells (GC) with vacuolated cytoplasm, destructed mitochondria and indented nucleus. These results were in agreement with the results of Rong et al. (2018), Saxena et al. (2014), Yang et al. (2018) and Fan et al. (2014) as they reported.

These pathological findings explained by Roy et al. (2013) and Pearsall et al. (2019) who stated that in diabetic retinopathy, mitochondrial dysfunction, endoplasmic reticulum stress, and subsequent breakdown of cellular homeostasis begin early in diabetes and play a critical role in retinal cell death. Changes in mitochondrial morphology, mitochondrial membrane potential heterogeneity, oxygen consumption rate and protein misfolding are recognized. Mitochondrial dysfunction increases production of neurotoxic ROS, and also results in primary energetic deficits that are detrimental to retinal neurons.

Apoptosis of retinal neurons, which are further phagocytosed by glial cells, may also contribute to the appearance of empty spaces (Sadek et al., 2017). In addition, retinal edema contributes to separation between retinal cells due to dysfunction of the blood–retinal barrier and accumulation of fluid in the inner and outer retinal plexiform layers (Zhang et al., 2014).
Statistically increased PAS staining was due to increased advanced glycation end products (AGEs). AGEs bind to their multiligands, known as a receptor of advanced glycation end products (RAGE), which activates different kinase and NADPH oxidase leading increased levels of ROS. AGEs induce irreversible crosslinks in long-living extracellular matrix and upon binding to specific cellular proteins change the local concentrations of cytokines, growth factors, and other bioactive molecules and induces inflammation. Notably, the AGEs not only destroy insulin-producing cells but also develop insulin resistance (Değirmenci et al., 2019). AGEs accumulation has been observed in vascular cells, neurons, and glia (Sharma et al., 2012).

This group showed also statistically increased VEGF expression. During DR, neovascularization is caused by an imbalance of pro-angiogenic mediators and ischemia resulting in abnormal growth of new vessels, which interferes with light transmission. The major regulator and pro-angiogenic factor is VEGF, which was increased after hyperglycemia and hypoxia. An imbalance in the expression of VEGF is responsible for the increased neovascularization in DR (Altmann and Schmidt, 2018).

Retinal VEGF expression is markedly increased in diabetes. VEGF damages blood-retinal barrier through breaking endothelial tight junctions, increasing vascular permeability and injuring the endothelial cells. The consequences are leaky vessels and an accumulation of fluids and proteins. VEGF also activates the aggregation and adhesion of leukocytes so, inducing inflammatory response. The inflammation in turn stimulates further release of VEGF (Chen et al., 2017). In cases when the BRB is disrupted, the role of VEGF is emphasized, since it has 50000 times higher potency in induction of vascular permeability than histamine (Nisic et al., 2018).
Increased retinal vimentin expression in the Diabetic group indicated gliosis which explained by Çerman et al. (2016) who stated that in the early stages of STZ induced diabetes, the neuronal and glial alterations in the retina precede the typical vascular changes. Normally after about 6 weeks of induction of diabetes, Müller cell gliosis and neuronal deficits begin to become prominent. Vimentin is a biomarker of Müller cells. Overexpression of vimentin occurs during Müller cell gliosis, which is known to be increased in STZ induced diabetic retinopathy.

The majority of established therapies target quite advanced states of DR. It is desirable to develop strategies which target early phases in DR to delay or even prevent DR (Park, 2016). Stem cell therapy has become one of the most promising therapeutic strategies for DR with the development of modern medical technology in the field of gene and stem cell therapy (Paterniti et al., 2015). The eye is an ideal organ for stem cell therapy because of its relative immunological privilege, surgical accessibility, and its being a self-contained system. (Ouyang et al., 2016).

Group III (Stem cell group) in the present study showed slightly increased retinal thickness with some small congested blood vessels, significant decrease (P<0.05) in PAS staining, VEGF and Vimentin expression compared to group II. Also, there was some photoreceptors with normal lamellar disc membranes and some with destructed and vacuolated disks, spacing between ONL cells, slightly distorted mitochondria of INL cells and ganglion cells.

These results were well-matched and confirming the findings of previous study done by Çerman et al. (2016) conducted on an induced diabetic retinopathy rat model, whereby administration of BM-MSCs demonstrated selective protection against retinal gliosis, increased
vascular integrity, and retinal function. Also, another study by Ezquer et al. (2016) focused on the reactive oxygen species (ROS)-induced damage to the neurovascular unit of the retina, to find that retinal ganglion cells death, vascular leakage, apoptosis and inflammation were obviously downregulated when MSC were injected.

Moreover, Mathew et al. (2017) found that intravitreal administration of BMMSCs aided the survival of the retina in rats suffering ischemic damage and MSCs had an anti-apoptotic effect through decreased TUNEL and caspase-3 expression, attenuated inflammation by reducing levels of TNF-α, IL-1β, and IL-6, and preserved autophagy.

Fiori et al. (2018) also explained that, MSCs exert at least a dual role in DR. First, these seem to resume a pericyte function, acquiring perivascular localization and endothelial cell enwrapping. By this, MSCs appears to adopt pericytic regulatory functions, controlling survival and proliferation. Second, they secrete a variety of trophic factors which modulate the local adverse milieu by regulating oxidative stress, inflammation and integrity of the neurovascular unit. Also, (Galderisi and Giodano, 2014) added that the immune modulatory effect of mesenchymal stem cells may play an important role in tissue regeneration, especially in conditions such as diabetic retinopathy where inflammation has been shown to play a role in disease progression.

Many studies investigating MSC for DR showed an improvement in glucose metabolism. MSCs were also shown to absorb reactive oxygen species (ROS) via expression of sulfoxide reductase A, which may suggest a mechanism for their neuroprotective effect (Kramerov et al., 2016).
Peng et al. (2018) also found that neuroprotective growth factors such as brain-derived neurotrophic factor (BDNF), ciliary-derived neurotrophic factor (CTNF), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), and basic fibroblast growth factor (bFGF) were significantly increased in DR rats injected with MSCs.

But, one of the main concerns regarding MSC therapy for DR is the secretion of pro-angiogenic growth factors, mainly VEGF and platelet derived growth factor (PDGF), that might worsen the course of DR. VEGF is a potent angiogenic and vascular permeability factor that enhances endothelial cell proliferation and migration, and promotes angiogenesis in the retina (Shi et al., 2018).

However, Ezquer et al. (2016) reported that, depending on the microenvironment, MSCs could produce trophic factors that may modulate between a pro-angiogenic and anti-angiogenic environment. His team found that MSCs exerted cytoprotective action through secretion of potent anti-angiogenic factor, Thrombospondin Type-1 (TSP-1), which is also produced primarily from the RPE, choroid, and Müller glial cells. TSP-1 is a glycoprotein that has been found to modulate MSC functions including anti-angiogenic, anti-inflammatory, as well as immunomodulatory activities. All these results can explain the significant decrease in VEGF immunoreactivity in our results.

Moreover, Zou et al. (2010) also reported that, in animal models of retinal ischemia, bone marrow derived cells appear to play an important role in the formation of physiologic vessels in the retina and do not appear to contribute to pathologic retinal neovascularization.

In contrast to our results, Çerman et al. (2016) showed in his study increased expression of Vimentin after intravitreally transplanted BMSCs
which indicates that these cells may have been differentiated to Muller glia, as Vimentin is marker of Muller cells.

Also, **Gravina et al. (2017)** noticed that after the transplantation of MSCs into the vitreous, the cells could not be maintained for a long time and this was explained by **(Kazmi et al., 2019)** who showed that, the microenvironment under diabetic conditions is harsh for stem cells to survive or migrate to the targeted site and exert their reparative functions. Also, Limited synthesis of proteoglycans and glycosaminoglycans in the surrounding environment causes minimal proliferation and viability of MSCs in vivo. Moreover, the production of advanced glycation end products (AGE) inhibits proliferation of MSCs by activating apoptotic mechanism and ROS production.

Regardless the benefits of MSCs, one of the barriers to stem cell transplantation therapy is the difficulty of inducing incorporation of transplanted cells into the host cell structure, and this could be complicated by immune reaction **(Ludwig et al., 2019)**. Also, **Tassoni et al. (2015)** addressed the potential risks associated with cell injection as retinal glial responses (graft-induced reactive gliosis) upon intravitreally injected BM-MSC in rats. Furthermore, **Jian et al. (2015)** reported that inflammation and extensive reactive gliosis were accompanied by macrophage infiltration and retinal detachment upon BM-MSC intravitreal transplantation.

**Park et al. (2017)** reported that the paracrine trophic effects of MSCs on the damaged retina may represent a more viable approach to treat retinal dysfunction than direct cell replacement since most retinal disorders are associated with damage to more than one cell type in the retina and extensive remodeling often occurs in response to the damage.
So, if the trophic regenerative effects of mesenchymal stem cells can be replicated using exosomes secreted by MSCs, this non-cellular approach may be a safer method to achieve retinal regeneration. This is a promising new therapeutic area of on-going research (Park et al., 2017).

Exosomes represent a class of extracellular vesicles approximately 100 nm in size (diameter of 40–150 nm). MSC-Exosomes carry a complex cargo of protein, nucleic acid and lipids, contain abundant miRNAs, and transmit these contents into the recipient cells. They are the principal therapeutic agents that mediates the paracrine action of MSCs and supports their therapeutic abilities. They can offer comparable therapeutic effects of mesenchymal stem cells without the potential adverse effects associated with cell therapy. (Zhang W. et al., 2019).

The advantages of using exosomes instead of live cells are connected to their minimal immunogenicity (allowing an allogenic use), low inherent toxicity, and potentially lower risk for tumor formation. Moreover, because of their chemical composition and small size, exosomes may easily diffuse across the biological barriers reaching target cells (Zazzeroni et al., 2017).

The vitreous humor is predominantly comprised of collagen and hyaluronic acid along with a network of extended random coil molecules that fills in the meshes of the collagen fiber network (Le Goff and Bishop, 2008). Very interesting study by Mathew et al. (2019) showed that the MSC-Exosomes remained in the vitreous humor for up to 4 weeks after injection suggesting that the effect is due to binding to vitreous humor proteins and collagen I fiber network in a dose-dependent and saturable manner. As a result, the vitreous humor serves as a reservoir for release of EVs into the retina and this property could be used advantageously to prolong their availability to retinal cells and
minimizing the number of injections necessary to produce long-term effects.

Exosomes were isolated from BM-MSCs in this study and injected intravitreally in the rat's eyes of group IV (exosome group). Data showed moderate improvement in the retinal histology, significant decrease (P<0.05) in PAS staining, VEGF and Vimentin expression compared to group II. Additionally, there were slightly vacuolated and separated POS, slightly spaced nuclei in ONL, slightly destructed mitochondrial cristae of amacrine cells and vacuolated ganglion cells.

These findings were parallel with a recent report done by, He et al., (2018) who showed that MSCs-exosomes ameliorate retinal laser injury and concluded that, MSC-exosomes might be optimal candidates for intravitreal injection, which could potentially overcome the obstacles and risks related to stem cell transplantation therapy, such as possible long-term pathological differentiation, vitreous opacities, and poor preservation. Also, Moisseiev et al. (2017) study findings showed that intravitreal injection of human MSCs-exosomes was well-tolerated and significantly reduced the degree of retinal ischemia and neovascularization in Oxygen-induced retinopathy (OIR) model. Same results confirmed by Mead et al. (2018) in rat model of glaucoma, and by Shigemoto-Kuroda et al. (2017) and Bai et al. (2017) in autoimmune uveaitis model.

Safwat et al. (2018) study demonstrated that systemic, subcutaneous and intraocular administration of rabbit adipose MSCs exosomes can induce repair of diabetic retinal degeneration in a model of rabbits.

Consecutively, (Atienzar-Aroca et al., 2016) showed that MSCs-Exosomes could inhibit the neovascularization by inhibiting nuclear
factor β (NF-β) signaling and by downregulation of VEGF expression that might be influenced by vital proteins or RNAs encapsulated in MSC-Exosomes.

Angiogenesis, abnormal growth of retinal blood vessels, was associated with the severity of DR. It is not surprising that MSC-exosomes have both pro- and anti-angiogenic effects. These opposing effects reflect the influence of the tissue-specific microenvironment on the exosomal cargo signature of MSCs and their biological function on target cells (Rosenberger et al., 2019). Interestingly, MSC-Exosomes contain antiangiogenic miRNAs such as miR-16 and miR-100 that suppress angiogenesis by targeting vascular endothelial growth factor (VEGF) Pakravan et al. (2017). It was found that specific micRNAs, such as micRNA-17-5p, micRNA-126, micRNAs-221/222, and micRNA-296 negatively regulate angiogenesis by binding to the c-Kit receptor (Safwat et al., 2018).

In laser induced retinal injury, Yu et al. (2016) compared the effect of intravitreal injection of MSC-Exosomes and found that the protective effect of MSC-Exosomes on injured retina was equal to that of MSCs in limiting the damage extent, reducing apoptosis, and inhibiting inflammatory responses.

Exosomes promote specific interaction with targeted tissues/cells along with the disposal of unwanted proteins, antigen presentation, genetic exchange, immune responses, angiogenesis, inflammation, tumor metastasis, and spreading of pathogens or oncogenes (Peng et al., 2018).

These results suggested that transplantation of MSC-Exosomes might act as a putative therapeutic tool to protect the retina as reported by Wang et al., (2018). Also, Zhang, W. et al., (2019) hypothesized that
miR-126 transferred by MSC-Exosomes suppresses the hyperglycemia-induced retinal inflammatory response, and enhance vascular repair.

Group V (stem cells and exosomes group) in the present study showed nearly normal retinal histology. There was significant decrease (P<0.05) in PAS staining, VEGF and Vimentin expression compared to group II. Ultrastructurally, the retina appeared nearly normal except little vacuolation in INL cells and slightly swollen mitochondria of ganglion cells.

A comparable study by Aali et al., (2014) and his team indicated that co-administration of MSCs and their conditioned media could control the hyperglycemia in diabetic rats more than either group alone. Therefore, it could be assumed that there is synergistic effect between MSCs and supernatant of MSCs in pancreatic regeneration of diabetic animals. Also, a rat study by Chen K et al. (2016) demonstrated that combined MSCs and MSC-Exosomes delivery was superior in terms of brain protection and neurological recovery when compared with MSC transplantation or exosomes injection only.

Lou et al. (2017) explained that MSC-derived exosomes have a key role in mediating the capacity of MSCs to function as stromal support cells to maintain homeostasis within the tissue and respond to external stimuli. This role is particularly important when the homeostasis of the tissue microenvironment is disrupted by disease or injury; this, in turn, compromises normal tissue function.

Retinal samples of group VI that were taken 12 weeks after induction of diabetes in the present study showed no improvement in the histological and immunostaining results of the diabetic retinas indicating irreversibility of STZ induced diabetic retinopathy. This was explained by
Yang et al. (2018) who stated that STZ-induced type 1 diabetic rat model does not experience self-remission or recovery over time as a result of the STZ-induced permanent destruction of islet β cells.

After 12 weeks, group VI (recovery group) showed more worsen signs of degeneration. The ONL, INL and GCL were apparently thinner compared to control rats. There were more angiogenesis, significant PAS staining, VEGF and vimentin immunostaining. Ultrastructurally, there were highly vacuolated pigmented cells with destructed microvilli, destructed POS with large areas filled with debris, degenerated ONL cells, INL cells and ganglion cells.

Results of this study agreed with study done by Gong et al. (2013) who showed that the pathological and morphological changes of INL and ONL, thinning of the retinal layers, the number of new blood vessels and mRNA expression of VEGF were all increased in retinas of diabetic rats at both 3, 4 months after the development of diabetes, compared to two months after diabetes. Also, He et al. (2019) and Sadek et al. (2017), reported significant global thinning of the total retina due to a reduction in GCL, INL and ONL as a consequence of diabetes. Moreover, Li et al. (2014) compared the retinal sections results eight weeks and 12 weeks after development of diabetes and reported the same findings.

Chronic hyperglycemia results in the production of more reactive oxygen species that worsens the inflammation and induces more apoptosis of the retinal pigment epithelium and further progression of DR. Prolonged hyperglycemia also causes accumulation of advanced glycation end-products (AGEs) beneath the endothelial layer and changes the vascular structure, increases vascular stiffness (Değirmenci et al., 2019).
SUMMARY

Diabetic retinopathy (DR) is a common, potentially devastating microvascular complication of diabetes. It is a leading cause of acquired blindness among working-age people. Current therapeutic options, such as laser photocoagulation, corticosteroids, anti-vascular endothelial growth factor agents and vitrectomy, are limited by their considerable side effects. Therefore, developing novel, mechanism-based therapeutic strategies is highly desirable for clinical management of DR. Adult bone marrow derived mesenchymal stem cells (BM-derived MSCs) offer the potential to open a new frontier in medicine.

This work performed to evaluate the possible therapeutic effect of BMMSCs and their exosomes in the treatment of diabetic retinopathy.

*Ten young male albino rats were used to isolate the BMMSCs and their exosomes.

*Sixty-four adult male albino rats were randomly divided into six groups:

- **Group I (control group; n=24):** Twenty-four rats were divided equally into three subgroups; Ia, Ib and Ic.
- **Group II (affected group; n=8):** Each rat was received a single intraperitoneal injection of STZ (60 mg/kg body weight), freshly dissolved in 0.2 mL of citrate buffer (0.1 mol/L, pH 4.5). A week later, rats were fasted, and blood glucose levels were determined; the rats with blood glucose levels higher than 250 mg/dL were considered diabetic.
- **Group III (DR + stem cell; n=8):** Rats were treated as group II, then eight weeks after induction of diabetes, rats were injected intravitreally with a single dose of BMMSCs.
- **Group IV (DR+ exosomes; n=8):** Rats were treated as group II, then eight weeks after induction of diabetes, rats were injected intravitreally with single dose of PKH26 labelled MSC-Exosomes.

- **Group V (DR +stem cell + exosomes; n=8):** Rats were treated as group II, then eight weeks after induction of diabetes rats were injected with BMMSCs and PKH26 labelled MSC-Exosomes together intravitreally in the same doses as group III and IV.

- **Group VI (recovery group; n=8):** Rats were treated as group II and left for 12 weeks without treatment.

Rats of groups II were sacrificed 8 weeks after induction of diabetes. Rats of other groups (III, IV, V, VI) were sacrificed 12 weeks after induction of diabetes. Retinal samples were taken and processed for:

I-Histological studies:
Paraffin sections of 5-7 µm thickness, mounted on glass slides for:
- Hematoxylin and Eosin stain to examine the histological changes in the different groups.
- Periodic Acid Schiff (PAS) reaction for demonstration of glycogen deposition in retina.

II-Immunohistochemical staining:
- Immunohistochemical staining for VEGF.
- Immunohistochemical staining for vimentin.
- Mesenchymal stem cells by detection of CD105 marker.

III-Transmission electron microscopic examination.
The study revealed the following results:

- **Affected group** showed apparent reduced retinal thickness, ganglion cells were disorganized, disturbed and widely separated. Cytoplasmic vacuolation of the outer and inner nuclear cells and retinal pigment epithelium, pyknosis of most retinal cells with noticeable neovascularization. Immunohistochemical examination revealed marked PAS staining, significant VEGF and vimentin immunoreactivity. Electron microscopic examination confirmed the results.

- **Stem cell treated group** showed little improvement in diabetic retinopathy induced by STZ (less vacuolations, pyknosis, neovascularization, wide separation of some cells and significant decrease (P<0.05) in PAS staining and VEGF and vimentin immunoreactivity compared with group II.

- **Exosomes treated group** showed slightly disorganized retinal cells with cytoplasmic vacuolations, apparently normal mitochondria with different sizes and shapes, with significant decrease (P<0.05) in PAS staining and VEGF and vimentin immunoreactivity compared with group II.

- **Stem cells and exosomes group** showed nearly normally arranged retinal layers and the same ultrastructure of retina. There was a significant decrease (P<0.05) in PAS staining and also significant decrease in VEGF and vimentin immunoreactivity compared with group II.

- **Recovery group**: There was no improvement in samples taken after 12 weeks of STZ injection.
Conclusion

This work concluded that both BMMSCs and their exosomes can treat diabetic retinopathy, but their co-administration can give better results.
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Bone marrow-derived cells are differentially involved in pathological and physiological retinal angiogenesis in mice.
 يعتبر اعتلال الشبكية السكري هو أحد مضاعفات الأوعية الدموية الشائعة والدماغية الم̀تحلة لمرض السكري. وهو سبب رئيسي للع subtreeضات بين الأشخاص من هم في سن العمل. إن الخيارات العلاجية الحالياً، مثل التختير الضوئي بالليزر، والكورتيزونات، ومضادات عامل نمو بطة الأوعية الدموية، واستئصال الجسم الشرياني تعتبر محدودة بسبب أثارها الجانبية العالية، ولذلك، فإن تطوير استراتيجية علاجية مبنية على أطية العصار أمر مرهوب للغاية لإدارة السكرية لإعتلال الشبكية السكري. وتتوفر الخلايا الجذعية الوسيطة المأخوذة من النخاع العظمي للكبار القطرة على فتح حدود جديدة في الطب.

نفذ هذا العمل لإظهار التأثير العلاجي المحتمل للخلايا الجذعية الوسيطة المأخوذة من النخاع العظمي وحولصالاتها الدقيقة في علاج اعتلال الشبكية السكري.

- استخدم عشرة جرذان ذكور صغيرة لعزل الخلايا الجذعية الوسيطة وحويلصالاتها الدقيقة. وتم تقسم أربعة وستون من ذكور الجرذان البيضاء البالغة بشكل عشوائي إلى 5 خمس مجموعات:

  • المجموعة الأولى (مجموعة متشابهة) (26 جرذا): تم تقسيم أربعة وعشرون جرذا ذكر بالتساوي إلى ثلاث مجموعات فرعية 1أ، 1ب، و 1ج.

  • المجموعة الثانية (مجموعة متشابهة) (8 جرذان): تم حقن كل جرذ بعقار ستريپتوتوسين مرة واحدة داخل التجفيف البريتنوني من (60 مجم / كجم من وزن الجسم) من مذاب طازجاً في (6.0 مل من عازلة السَّترات 6.1 مول / لتر، ودرجة الحموضة 5.5). وبعد أسبوع، تم تحديد مستويات الجلوكوست في الدم؛ واعتبرت الجرذان التي لديها مستويات جلوكوست في الدم أعلى من 0.56 ملغم / دسُسلتر مصاب بالسكير.

  • المجموعة الثالثة (مجموعة الخلايا الجذعية) (8 جرذان): تم اعطاؤها عقار ستريپتوتوسين مثل المجموعة الثانية. ثم بعد 8 أسابيع من استخدام السكري، تم حقن الجرذان في صفق العين بجرعة واحدة من الخلايا الجذعية الوسيطة.
المجموعة الرابعة (مجموعة الحويصلات الدقيقة) (8 جرذان): تم اعطاؤها عقار ستريبيتوزوتوسين مثل المجموعة الثانية، ثم بعد 8 أسابيع من استحداث السكري، تم حقن الفئران في صفاق العين بجرعة واحدة من الحويصلات الدقيقة.

المجموعة الخامسة (مجموعة الخلايا الجذعية والحويصلات الدقيقة) (8 جرذان): تم اعطاؤها عقار ستريبيتوزوتوسين مثل المجموعة الثانية، ثم بعد 8 أسابيع من استحداث السكري، تم حقن الفئران في صفاق العين بجرعة واحدة من الخلايا الجذعية الوسيطة وحويصلاتها الدقيقة معا.

المجموعة السادسة (المجموعة المعافاة) (8 جرذان): تم اعطاؤها عقار ستريبيتوزوتوسين مثل المجموعة الثانية، وتم ذبحها بعد 12 أسبوع من استحداث السكري.

ذبحت جرذان المجموعة الأولى بعد 8 أسابيع من حقن المذيبات كلا على حدة. وذبحت جرذان باقي المجموعات بعد 12 أسبوعا من استحداث مرض السكري.

وأخذت عينات الشبكية وتم تجهيزها للاتي:

1- الدراسات النسيجية:

- مقاطع بارافين بسمك 7-5 ميكرن، مثبتة على شرائح زجاجية من أجل:
  - صبغة هيماتوكسيلين وإيوسين لدراسة التغيرات النسيجية في المجموعات المختلفة.
  - تفاعل حمض شيف الدوري لإظهار ترسب الجليكوجين في شبكية العين.

2- كيمياء الأنسجة المناعية:

- صبغة هستوكيمينائية مناعية لعامل نمو البطانة الوعائية.
  - صبغة هستوكيمينائية مناعية للفيمنتين.
  - الخلايا الجذعية الوسيطة عن طريق الكشف عن بروتين سي دي 105.

3- الفحص المجهر بالمجهر الإلكتروني.
المؤشرات النتائج التالية:

مجموعة ستريبتوزوتوزيس: أظهرت انخفاض ملحوظ في سمنة الخلايا، عدم انتظام واضطراب و انفصال واسع في الخلايا، و تجويفات ستوبلازمية للخلايا النموية الخارجية والداخلية، و تغطية في أنسجة مسام خلايا الشبكة، و اضطراب الأوعية الدموية و تراكم ذو دلالة إحصائية لصبغة بيرونيك في الخلايا الحاضمة و تفاعل بروتين عامل النمو الوعائي البطاني و بروتين الفيمينتين، وقد أكد الفحص بالمجهر الإلكتروني ما أظهره المجهر الضوئي.

مجموعة الخلايا الجذعية: أظهرت انخفاض ملحوظ في انتظام و انفصال واسع الواسع بين الخلايا، و تجويفات ستوبلازمية للخلايا النموية الخارجية والداخلية، و تغطية في أنسجة مسام خلايا الشبكة، و اضطراب الأوعية الدموية و تراكم ذو دلالة إحصائية لصبغة بيرونيك في الخلايا الحاضمة و تفاعل بروتين عامل النمو الوعائي البطاني و بروتين الفيمينتين، و مقارنة مع المجموعة الثانية.

مجموعة الخلايا الجذعية: أظهرت انخفاض ملحوظ في انتظام و انفصال واسع الواسع بين الخلايا، و تجويفات ستوبلازمية للخلايا النموية الخارجية و الداخلية، و تغطية في أنسجة مسام خلايا الشبكة، و اضطراب الأوعية الدموية و تراكم ذو دلالة إحصائية لصبغة بيرونيك في الخلايا الحاضمة و تفاعل بروتين عامل النمو الوعائي البطاني و بروتين الفيمينتين، و مقارنة مع المجموعة الثانية.

مجموعة الخلايا الجذعية و الحويصلات الدقيقة: ظهرت طبقات الشبكية غير منظمة قليلاً و ذات فجوات ستوبلازمية، و ميتوكوندريا ظهرت بأحجام و أشكال مختلفة، و فصل لبعض الخلايا، و انخفاض ذو دلالة إحصائية لتركم صبغة بيرونيك في الخلايا الحاضمة و تفاعل بروتين عامل النمو الوعائي البطاني و بروتين الفيمينتين، و مقارنة مع المجموعة الثانية.

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المجموعة المتعافية: أظهرت المجموعة السادسة عدم ظهور أي تحسن في العينات التي أخذت بعد 12 أسبوعًا من حقن ستريبتوزوتوزيس.
خلاصة

خَلَصَ هَذَا الْعَمَلُ إِلَى أن الخلايا الجذعية الوسيطة المسخرة من نخاع العظام وحويصلاتها الدقيقة يمكن أن تعالج اعتلال الشبكية السكري. و لكن عندما يتم إعطاء هم سويا يمكن الحصول على نتائج أفضل.
التأثير العلاجي المحتمل لخلايا الجذعية الوسيطة وحويصلاتها الدقيقة على اعتلال الشبكة السكرية المستحث تجريبياً في الجرذان
دراسة هستوسيميائية وعمرية مناعية
رسالة مقدمة توطني للحصول على درجة الدكتوراة في علم الأنسجة وبيولوجيا الخلية

اسم الباحثة
هبة السيد عبد الحليم بيومي
مدرس مساعد بقسم الأنسجة وبيولوجيا الخلية - كلية طب - جامعة بنها

المشرفون
الأستاذة الدكتوراه / أميمة كامل هلال
أستاذ الأنسجة وبيولوجيا الخلية
كلية الطب - جامعة بنها

الدكتورة/ نهلة العراقي العزب
أستاذ مساعد الأنسجة وبيولوجيا الخلية
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الدكتورة/ نسرى ابراهيم سالم
أستاذ مساعد الأنسجة وبيولوجيا الخلية
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2020