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Histopathological study of the effect of sunitinib in treatment of retinal angiogenesis induced by VEGF 165 in rabbits'eyes

Shahad Salah Aldeen *, Mustafa Ghazi Alabbassi *.

** Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University/Iraq*

Shahad_salah@ymail.com

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Abstract:

Eye is one of the important sensory structures in the body which responsible for vision through its anterior and posterior chambers. Retinal angiogenesis is the development of new, abnormal blood vessels in the retina that considered the pathological feature of different ocular diseases such as diabetic retinopathy, the major cause of vision loss in the world. The diseases were diagnosed by different methods and the treatment includes anti-angiogenic drugs and surgical therapy. This study was designed by dividing twenty-four rabbits in to four groups each with six rabbits; control (PBS-administered) group, angiogenic (VEGF-administered) group, ranibizumab-treated group, sunitinib-treated group. The result showed that there is absence of angiogenesis in sunitinib-treated group, which were similar to ranibizumab-treated group when compared with angiogenic group. The result explains the anti-angiogenic activity of sunitinib due to its blocking effect on VEGFRs.one can conclude from this study that sunitinib can be used in treatment of retinal angiogenic diseases in future.

Key words: retinal angiogenesis, vascular endothelial growth factor, sunitinib, diabetic retinopathy, ranibizumab.

دراسة نسيجية لتاثير السونيتينب في علاج تولد الاوعية الدموية في شبكية العين الناجم من حقن عامل نمو بطانة الأوعية- ١٦٥ في عيون الأرانب

شهد صلاح الدين*

مصطفى غازي العباسي *

*** فرع الادوية والسموم،كلية الصيدلة/الجامعة المستنصرية**

الخلاصة:

العين هي عباره عن عضو حسي مهم مسؤوله عن البصر من خلال اقسامها الامامي والخلفي.تكون الاوعية الدموية الجديدة في شبكية العين هو حالة مرضية في امراض العين المختلفة مثل اعتلال الشبكية السكري والذي يؤدي الى العمى.هذا المرض يمكن تشخيصه بطرق مختلفة بالاضافة الى الفحص النسيجي .العلاج يكون من خلال الادوية المضادة لتكون الاوعية الدموية والعمليات الجراحية الخاصة. صممت هذه الدراسه بتقسيم اربعه وعشرين ارنب على اربع مجاميع كل واحدة تحوي 6ارانب: المجموعة الاولى وهي التي حقنت بمحلول الفوسفات المنظم الملحي ، المجموعة الثانية وهي التي حقنت بعامل نمو بطانة الاوعية١٦٥،المجموعة الثالثة وهي التي عولجت بالرانيبيزوماب والمجموعة الرابعة وهي التي عولجت بالسونيتينب. اظهرت نتائج الفحص النسيجي لشبكية العين، بعد فترة معينة، علاج تكون الاوعية الدموية في الشبكية امجموعة السونيتينب والمشابه تقريبا الى مجموعة الرانيبيزوماب مقارنة مع مجموعة تكون الاوعية الدموية في شبكية العين. هذه النتيجة توضح التاثير المضاد لتكون الاوعية الدمويه في شبكية العين للسونيتنب بسبب فعاليته بغلق مستقبلات عامل نمو بطانة الاوعية –أ.من هذه الدراسة يستطيع المرء باستنتاج ان السونيتنب ممكن ان يستخدم في المستقبل لعلاج امراض تكون الاوعية الدموية الشبكيه

الكلمات المفتاحية : تولد الأوعية في شبكية العين ، عامل نمو بطانة الأوعية الدموية، سونيتينب ، رانيبيزوماب، اعتلال الشبكية السكري..

Introduction:

Eye considered one of the most complicated, sensory organs in the body. It composed of three different layers; the outer layer includes cornea and sclera[1].The middle layer involves the iris, the ciliary body , and the choroid [2].

The inner layer of the eye is the retina a complex, light sensitive tissue composed

of multiple, parallel layers of neurons that convert images into neuronal signals to be sent to the visual cortex by optic nerve for processing [3]. Retinal pigment epithelial (RPE) cells are located at the outer part of the retina, between the choriocapillaris and photoreceptors. These cells act as metabolic sensors; they produce proangiogenic factors that directly affect the tone of vessels [3]. The retina is provided by two blood suppliers: the central retinal artery and the choriocapillaris [4]. The aqueous humor (AH) is a colorless, transparent fluid which fill the area between the cornea and the lens [5]. The vitreous humor (VH) is a colorless, gel-like fluid presents in the posterior compartment of the eye, between the lens and the retina [6].

Neovascularization is controlled process of new blood vessels (BV) generation which involve two main mechanism that are [7]. vasculogenesis, the process of blood vessels development during embryonic growth, firstly Mesoderm cells first differentiate into endothelial precursors cells (EPC) (angioblasts) and then into endothelial cells (EC) that unit togethers to form primitive tubes that then expand. Subsequent blood vessel formation take place by angiogenesis[8]. There are two types of angiogenesis: sprouting and non – sprouting angiogenesis; sprouting angiogenesis

Involve steps of antigenic stimuli, Sprouting, Elongation and branching, Tubulogenic, lumen formation, and Anastomosis, Stabilization/regression[9]. non-sprouting or intussuscepted angiog-enesis which charac-terized by formation of intussuscepted pillars [10].

Angiogenesis is regulated by balance between proangiogenic factors (such as acidic fibroplast growth factor (aFGF) , basic fibroblast growth factor(bFGF) and vascular endothelial growth factors (VEGFs)) and antiangiogenic growth factors such as (angiostatin,endostatin)[11].

The major angiogenic promoter in physiological and pathological conditions is VEGF. VEGF are family of cytokines which include VEGF-A or VEGF, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). They involve in the process of vasculogenesis, angiogenesis and lymphangiogenesis. VEGF-A is a 45 kilo dalton heterodimeric heparin-binding protein. Alternative splicing of VEGF gene generates four isoforms, VEGF165, the predominant one, VEGF121, VEGF189, and VEGF206. Vascular endothelial growth factor also known vascular permeability factor (VPF), Stimulates endothelial cells growth , proliferation and vascular permeability, preserves the survival of endothelial cells (EC) and prevents their apoptosis[12]. VEGF-A binds to receptors tyrosin kinase (RTK)that are VEGFR-1 and VEGFR-2 and non- RTK that are neuropilin (NRP) receptors family(NRP‑1 and NRP‑2) as co-receptors[13].

Retinal angiogenesis is a pathological condition characterized by formation of abnormal, new blood vessels within the retina. It presented in several ocular diseases such as diabetic retinopathy (DR), retinal vein occlusion (RVO) and retinopathy of prematurity (ROP) that lead to loss of vision[14]. The most popular one is diabetic retinopathy.

Diabetic Retinopathy is the most popular, chronic, microvascular complication of diabetes mellitus which lead to vision loss among working peoples in developed countries. Diabetic Retinopathy occurs when there is sustained , chronic elevation of blood glucose level cause accumulation of glucose in the endothelial cells (ECs) of retinal microvessels .This results in activeation of several biochemical pathways include oxidative stress ,Aldose reductase and polyol pathway, Advanced glycation end products, activation of Protein kinase C, activation of Mitogen-activated protein kinase, Leukostasis and platelet activation ,Nuclear factor-kappa B (NF-κB), Inducible nitric oxide synthase Cyclo-oxygenases, Intracellular cell adhesion molecules ,Vascular endothelium growth factor , Interleukins-1-beta,andTumor necrotic factor-alpha[15]. Finally, they lead to functional and structural alterations in the

microvesseles and neuroglial parts of the retina include thickening of capillary basement membrane (BM), loss of pericyte and endothelial cell, breakdown of blood-retinal barrier (BRB) and leakage, acellular capillaries, and neovascularization [16].

Diabetic Retinopathy are classified into two different stages: the first stage called non proliferative DR (NPDR); characterized by weakening of retinal blood vessels ,damaged and leaking fluid in to the retina lead to retinal swallowing result in hemorrhages, microaneurysms ,exudates and interretinal microvascular abnormalities Prolifertive diaberic retinopathy (PDR) is the progressive stage of DR ,characterized by developing a new blood vessels in the retina induced by retinal ischemia as a result of The microvascular changes that occur in NPDR [17].

Diabetic macular edema (DME) is the thickening of retina that occurs in the center of macula. It considered the major cause of blindness among diabetic as a result of retinal hypoxia which increase expression of (VEGF), VEGF in turn induce vascular permeability and the trigger the formation of abnormal and leaking new vessels ,increase release of inflammatory cytokines and lack the tight junction among endothelial cells causing damage of blood- retinal barrier and thus accumulation of fluid in different area in the retina[18,19].

Diagnosis of Retinal angiogenic diseases was accomplished by Fundus imaging technique like fundus photography, color fundus photography, and fluorescence angiography [20]. Measuring the concentration of different molecules changed during ocular diseases like diabetic retinopathy [21] and histological examination of retinal tissues.

Treatment of retinal angiogenic diseases includes surgical treatment which indicated for progressive stages that characterized by vitreous hemorrhage and tractional retinal detachment.fore example Laser photocoagulation and Vitrectomy[22] .

Anti-angiogenic drugs act by 3 main mechanisms [22] including drugs bind with VEGF directly for example, Pegaptanib aptmer (Macugen), Bevacizumab (Avastin), Ranibizumab (Lucentis), Aflibercept (Eylea). Drugs act by inhibition of VEGF synthesis: by using silencing RNA (siRNA) sequences, a double-stranded RNA that are capable of silence the VEGF gene due to mutual homology. siRNA sequences penetrate across cellular membranes, block post-transcriptional RNA process, and thus inhibit synthesis of VEGF. Drugs act by inhibiting VEGF signaling; through blocking RTK for all members of VEGF family for example, pazopanib and multi-kinase inhibitors, like sorafenib and sunitinib that are used for treatment of renal and hepatocellular cancer.

Corticosteroids are used for management of ocular inflammatory diseases due to their anti-inflammatory effect in addition to anti angiogenic as well as anti-permeability properties. Their therapeutic effects involve blood -retinal barrier stabilization, decreasing in exudation and inhibition of inflammatory stimuli through down regulation of different cytokines and proteases that are involve in proliferative diseases. Currently three different steroids are utilized intravitreally: dexamethasone, triamcinolone acetonide and fluocinolone acetonide when there is no response to anti VEGF therapy [23].

Sunitinib chemically is indolin-2-one analogs. It is one of tyrosine kinase inhibitors (TKI) that exerts its antiangiogenic and antitumor effects by blocking multiple receptor tyrosine kinases, like VEGF, PDGF receptors, colony stimulating factor receptor type 1 (CSF-1R), stem cell factor receptor, glial cell-line derived neurotrophic factor receptor (RET) and Fms-Like Tyrosine Kinase-3 (FLT3). Sunitinib was approved by FDA in 2006 as a drug for the treatment of metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (GIST) [24]. Sunitinib malate is given orally. Following

a single oral dose of sunitinib malate, peak plasma concentrations achieved between 6 and 12hr, with prolonged half-lives of approximately of 40–60 hr and 80–110 hr for sunitinib and its primary active metabolite, SU012662, respectively. Sunitinib and its metabolite (SU012662) are metabolized mainly by the cytochrome P450 enzyme, CYP3A4 [25]. Sunitinib is mainly eliminated with the feces (70-84%), with 16% of the administered dose excreted in urine. Sunitinib can be administered with or without food [26].

In this study, we aimed to investigate the antiangiogenic effect of sunitinib.

Materials and Methods

Materials used in this study are (Recombinant human VEGF 165 (R and D system,USA), ketamin (kepro, Holland), xylazine (kepro, Holland), Sodium dihydrogen phosphate powder (Riedel-Dehaen AG/Hannover, Germany), Sodium chloride powder (Sinopharm Co.Ltd. ,China) Di-sodium hydrogen phosphate powder (Fluka ,Switzerland), Ethyl alcohol 70%(Activecosmetics,China),Chloramphenicol eye drop (Amman pharmaceutical industries, Jordan), Ranibizumab 10mg/ml (Novartis ,France), Sunitinib malate powder (Molekula, UK),10% formalin, , paraffin, digital microscope, microtom, slides and its covers, stains (eosin and hematoxylin).

Twenty-four Dutch-belted rabbits weighing (1.3-2.7 Kg) were involved in the present study. They were handled according to the ethics committee in the College of Pharmacy/Mustansiriyah University. Rabbits were maintained in animal house of College of Pharmacy/Mustansiriyah University in iron cage and kept healthy for three weeks under appropriate conditions of temperature and ventilations. They were fed on fresh diet, controlled pellets and fresh water. they were divided in to four groups as following:

Group 1 (negative control group): (n=6) they were injected intravitreally in their right eyes by 0.1ml of phosphate buffer saline solution (PBS).

Group 2 (positive control group): (n=6) they were injected intravitreally in their right eyes by 0.1ml of VEGF165.

Group 3 (ranibizumab treated group): (n=6) they were injected intravitreally in their right eyes by 0.05ml of ranibizumab.

Group 4 (sunitinib treated group): (n=6) they were injected intravitreally in their right eyes by 0.1ml of sunitinib malate solution.

Induction of retinal angiogenesis: Lyophilized VEGF-165 was dissolved in 5ml solution of sterile, freshly prepared PBS and 0.1% human plasma albumin then stored at 2-8C^0 until administration of solution of VEGF -165 within 7dayes as illustrated in data sheet from R and D system company. 0.1ml of VEGF165 injected intravitreally to the right eyes of rabbit at 3.5–4.0 mm behind the limbus [27,28] , through specific syringe after anesthetized with intramuscular injection of ketamin 25mg/kg and xylasin 5mg/kg. After 1 week, the rabbits were sacrificed, and their eyes were enucleated, then stored in 10% formalin for histological study.

Administration of sterile phosphate buffer saline

After anesthetized with intramuscular injection of ketamin 25mg/kg and xylasin 5mg/kg 0.1ml of sterile PBS was injected in to right eyes of rabbits by intravitreal injection. Then after 1week, the rabbits were sacrificed for eyes enuculation and storing the eyes in 10% formalin for histological examination.

Administration of sunitinib malate solution

After the rabbits were anesthetized, they were injected intravitreally by a single dose of (0.1ml) of freshly prepared sunitinib malate solution with concentration of 12.5mg/ml into the vitreous humor of their right eyes [29], via needle of gauge 27G connected to syringe with size

of 1 ml. Then, the rabbits were kept for 29 days (similar to standard treatment with ranibizumab which is given single dose /month) before they sacrificed, and their affected eyes were enuculated and kept in 10% formalin for histological examination.

Administration of standard treatment

After anesthetized with intramuscular injection of ketamin 25mg/kg and xylasin 5mg/kg Rabbits were injected intr-avitreally by single dose of 5unit (0.5mg /0.05ml) of ranibizumab solution.The rabbits were maintained for 29 day [30], then they sacrificed and their eyes were stored in 10%formalin for histological study.

Histological examination

A-**Retinal tissue preparation and staining**

paraffin embedded method was used. According to Bancroft and Steven, the steps of Paraffin method include:

1. **Fixation:** fix of rabbit's eyes in 10% formaldehyde for hardening the tissue.
2. **Sectioning (tissue dissection):** Cross sectional cut was done.
3. **Dehydration and infiltration** were done for the tissue via using histokinette Shandon device.
4. **Embedding and solidification** of the tissue into hard paraffin cube.
5. **Section the tissue using microtome.**
6. **Slide preparation**: Labeled slides were used, by using a diamond pen for labeling.
7. **Placing the sections on slides**: By using water bath with clean water at 45 °C, a thin section of paraffin was taken after cutting by microtome and gently placed it then observe when the wax is melted, and the wrinkles are disappeared, a clean glass slide was dipped in water under the section and then pulled the slide.
8. **Slides clearing**: The paraffin was removed before staining so that only the tissue remains adhered on the slide because if paraffin present, it would prevent the stain from reaching the tissue.
9. **Slide staining**: Two different dyes (Eosin and Hematoxylin) were applied for slides staining which allow clear observation to the histological features.
10. **Mounting permanent cover slide**: Few drops of DPX (a mixture of distyrene, a plasticizer, and Xylene) a synthetic r-esin used for histology were added. It dries quickly and preserves stain, after that cover the slide with slide cover.

B- Microscopically study:

A digital microscope system with Leica DM4000 B LED was used to exame slide of retinal tissues from 5 area (corners and the center) at magnification power of X 100.

Results:

Examination of retinal tissues was done by digital microscope using hematoxylin and eosin staining revealed apparently normal looking of retinal structure in the right eyes of the normal rabbit. Figure (3-1)

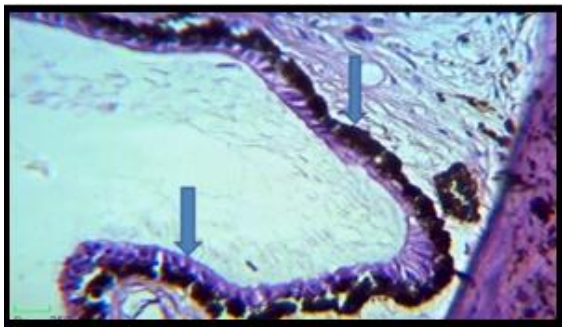

Figure (3-1): section of rabbit's normal retinal tissue under digital microscope of magnifying power x100 using H&E stains

Retinal tissue examination of control group (PBS-administered group) showed nearly normal looking group under digital microscope, figure (3-2).

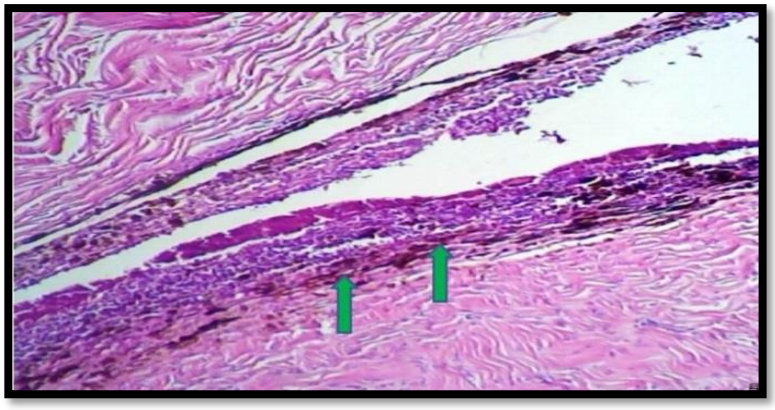

Figure (3-2): nearly normal appearance of retinal tissue slice under digital microscope of magnifying power x100 using hematoxylin and eosin stain (Green arrow: Nearly normal looking retinal tissue)

Figure (3-3): nearly normal appearance of retinal tissue slice under digital microscope of magnifying power x100 using hematoxylin and eosin stain (Green arrow: Nearly normal looking retinal tissue)

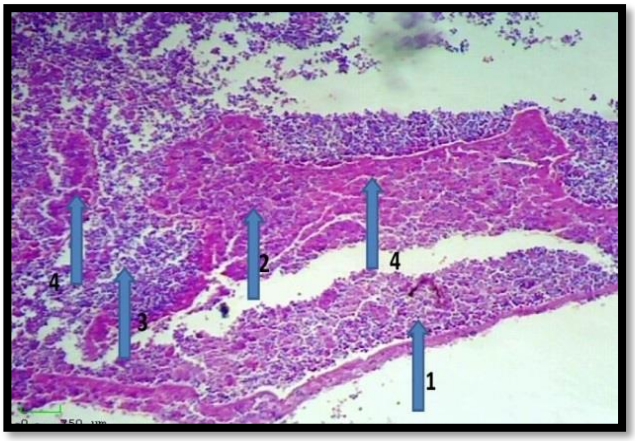

Figure (3-3): section of retinal tissue of angiogenic group with hematoxylin and eosin stain under digital microscope of magnifying power x100 which shows the following:(1. Necrosis of tissue.2. Tissue destruction.3. Inflammatory cells.4. Angiogenesis.).

In addition, digital microscope showed nearly complete absence of inflammation and angiogenesis in examination of retinal tissue of ranibizumab treated group, figure (3-4).

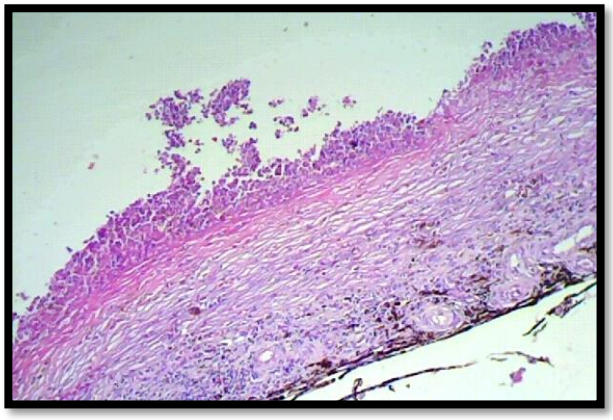

Figure (3-4): Section of retinal tissue of ranibizumab-treated group under digital microscope of magnifying power x100 using eosin and hematoxylin stains.

Furthermore, examination of retinal tissue of sunitinib-treated group revealed absence of angiogenesis with extensive reduction of inflammatory response, figure (3-5).

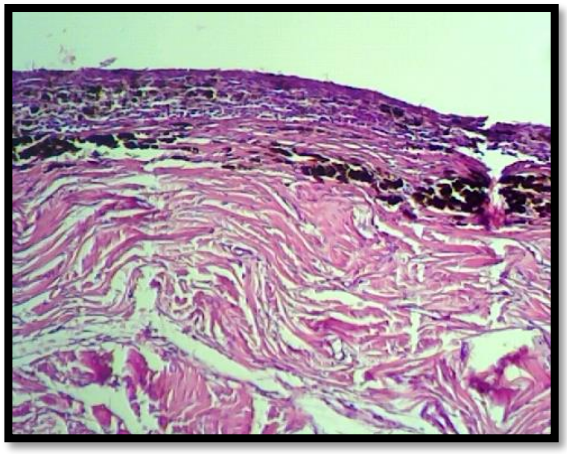

Figure (3-5): Section of retinal tissue of sunitinib –treated group under digital microscope of magnifying power x100 and using eosin and hematoxylin stains

Discussion:

Retinal angiogenesis is a pathological condition in the retina which associated with different retinal vascular diseases that lead to vision loss. The most important one among these diseases is diabetic retinopathy [31]. Proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME) considered the major causes of visual loss in patients with diabetic retinopathy. The main contributor to the development of abnormal blood vessels in PDR

and macular swallowing is an excessive production of VEGF [32] . Vascular endothelial growth factor-A (VEGF-A) is a pro angiogenic protein which stimulate neovascularization, vascular permeability and cell migration through activating its receptors (VEGFR-1, VEGFR-2) [13].

Vascular endothelial growth factor receptor-1(VEGFR-1) is a receptor tyrosine kinase receptor (RTK) with

weak tyrosine kinase activity so that has weak angiogenic action. Vascular endothelial growth factor receptor-2 (VEGFR-2) is a receptor tyrosine kinase with stronger kinase activity than VEGFR-1. VEGFR-2 is the predominant receptor for induction of angiogenesis [33]
The results appeared development of retinal neovascularization (RNV) after about 1 week of intravitreal injection of -165.This explains that the injected VEGF bind with VEGFR-2 on endothelial cells result in dimerization and auto- phosphorrylation of the receptor. This results in activation of different intracellular signaling cascades that are involved in VEGF –induced endothelial cells proliferation, migration, survival and vascular permeability as following

Vascular endothelial growth factor –induced endothelial cell proliferation via VEGF-induced activeation of phospholipase C-gamma (PLCγ) that ultimately activates protein kinase C (PKC). PKC activate sphingosine kinase (SPK) which in turn activates RAS/RAF /ERK /MAPK signaling pathway that mediated VEGF-indu-ced endot-helial cell proliferation [33].In addi-tion, VEGF-induced act-iveation of Rho A (member of Ras-homology gene (Rho) family of small G-protein) causes stimulation of cell cycling and proliferation [34].

Vascular endothelial growth factor-induced endothelial cell migration through increasing production of mitochondrial reactive oxygen species (mtROS) by increasing mitochondrial metabolism. Mitoc-hondrial reactive oxygen species cause activation of Rac1 (member of Ras-homology gene (Rho) family of small G-protein). Activated Rac / PAK/ p38MAP signaling regulates migration [35]. In addition, formation of stress fiber through activated Src – induced RhoA activation and rear-angement of actin bundle also important for cellular migration [34] .

Vascular endothelial growth factor-induced endothelial cell survival by activation of protein kinase B (PKB) /Akt. Protein kinase B phosphorylates and inhibits the apoptotic activity of BCL-2 associated death promoter (BAD) and caspase 9. In addition, Akt stimulates the expression of antiapoptotic proteins like BCL-2 and the inhibitors of apoptosis (IAP) family mem-bers that block the aboptotic activity of caspase 3 and caspase 7[36] .

Vascular endothelial growth factor-induced vascular perm-eability through activation of PLCγ cause increase in intracellular free Ca^{+2} ion concentration or cause activation of Act. Both lead to activation of endothelial nitric oxide synthase and increase production of nitric oxide (NO)[36] . Furthermore, VEGF causes Src activation which results in phosphorylation of adherens junction vascular endothelial-cadherin (VE-cadherin) lead to internalization and dissociation of interendothelial adherens junctions [37].
In this study, the histology of retinal tissues for all groups was stained by hematoxylin and eosin stainig and examined under digital microscope.
It revealed that there were significant inflammation and angiogenesis in retina of right rabbit's eye of VEGF-treated group compared with normal retina of other eye rabbit and retina of rabbits treated with PBS.
This agrees with different experimental studies and theoretical rev-iews that explained the dramatic effects in the retinal structure and function for example retinal neo-

vascularization, breakdown of blood retinal barrier and vascular permeability lead to macular oedema; due to leukostsis and apoptosis of retinal neurons and endothelial cells because of up regulation of tumor necrosis factor alpha (TNFα). This resulted from activation of VEGFR expressed on endothelial and retinal cells via proangiogenic agent (VE GF). The activated receptor of VEGF cause stimulation of down-stream signaling pathways that lead to increase expression of different mediators included in development of inflammatory responses and generation of new, nonfunctioning blood vessels in the retina [38-40].

In addition, the microscopic examination of sunitinib-administered group showed that complete recovery from angiogenic lesion with significant reduction of inflammatory response that was nearly similar to that result from treatment group with standard therapy (Ranibizumab). This indicates that sunitinib suppresses VEGF-induced angiogenesis and inflammation as a result of its competing ATP on ATP-binding sites on VEGFR-2 which lead to inhibit kinase activity of the receptors and thus in activation of downstream signaling pathways that mediate action of VEGF and induction of RNV [39,40].

Up to our knowledge, this study considered the first research which investigates the therapeutic effect of sunitinib in treating retinal angiogenesis in *invivo* experimental model.

Conclusion:

From this current histopathological study one can conclude that sunitinib can be used for treating retinal angiogenesis so that it can be added in future to anti VEGF drugs that are used for treating retinal angiogenic diseases such as proliferative diabetic retinopathy and diabetic macular oedema, the major cause of blindness

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