

The Effect of Intravitreal Bevacizumab on the Histological Structure of the Cerebral Cortex of Male Albino Rat

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Abstract: Background: Bevacizumab is one of the anti vascular endothelial growth factor (anti-VEGF) agents which has antiangiogenic and oedema-diminishing actions. It is used widely as an intravitreal solution to diminish new vessels formation and reduce the oedema of the ocular diseases. **Objectives:** to evaluate the safety of an intravitreal injection of bevacizumab on the cerebral cortex of rat. **Material and methods:** 20 animals were used in the experiment. They were divided into two groups 10 animals each. Group I: rats were injected with saline as a 5 uL single injection into the vitreous cavity of the left eyes. GroupII: rats were injected with 5 uL (25 mg/mL) of bevacizumab as a single injection into the vitreous cavity of the left eyes. Animals were sacrificed 4 days after injection. Brain was dissected and processed for light and electron microscopy. **Results:** Microscopic examination revealed various histological changes in the nerve cells. Some of them had pyknotic nuclei and acidophilic cytoplasm. This was associated with astrocyte gliosis as well as widening of both pericellular and perivascular spaces. Vacuolations of the neuropil was also observed. Others had irregular nuclei, numerous cytoplasmic vacuoles and dilated rough endoplasmic reticulum. Apparent decrease of free ribosomes was also observed. There was highly significant increase in the number of glial fibrillary acidic protein immunostained positive cells (astrocyte gliosis).

Conclusion: intravitreal injection of bevacizumab had toxic effect on the cerebral cortex of rat.

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1. Introduction:

Bevacizumab (avastin) is a humanized monoclonal antibody to VEGF (1). It is approved for use in colon cancer as anti-growth factor drug (2). It has been found that avastin caused significant improvement in both visual acuity and retinal morphology (3, 4). The ability of avastin to penetrate the neuroretina had been demonstrated in healthy monkeys and in rabbits (5, 6). The *in vitro* experiments had shown that avastin significantly reduced the proliferation of choroidal endothelial cells (7). Systemic administration of avastin had a significant risk of thromboembolism formation in patients with cancer (8). Several studies had not shown any evidence of ocular toxicity after the use of intravitreal avastin (9). On the other hand, there were some studies that reported some complications associated with intra-vitreous avastin injection such as retinal detachment, retinal pigment epithelial tear, acute vision loss and central retinal artery occlusion (10, 11). The serum concentration of avastin reaches a peak concentration after one day from its intravitreal injection (12). It can penetrate the blood-brain barrier and may affect the brain structure (13). It was found that avastin could reduce the brain angiogenesis and endothelial cellular proliferation (14).

There was little work on systemic complication of intravitreal injection so this study was carried to evaluate the effect of avastin on cerebral cortex. This might be of value in decision making regarding the safety of intravitreal avastin in choroidal and retinal neovascular disorders.

2. Material and methods:

Experimental design:

Twenty animals were used in the experiment. They were divided into two groups 10 animals each. The rats were anaesthetized by intra-peritoneal injection of ketamine (50mg/kg) and xylazine (8mg/kg) (volume ratio at 2:1) before the procedure. After intravitreal injection of the left eye, ophthalmic Tobrex ointment was applied on the rat eye to prevent infection.

GroupI (the control group): 10 rats were injected 5 microliter with saline as a single injection into the vitreous cavity of the left eyes.

GroupII: 10 rats were injected with 5 microliter (25 mg/mL) of bevacizumab as a single injection into the vitreous cavity of the left eyes.

Four days later the animals were sacrificed and the brain specimens were dissected and processed for further steps.

All experimental protocols were in compliance with University of Sohag Ethics Committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care.

Drugs:

Avastin (Roch Company) was obtained from a pharmacy, sohag governorate, Egypt.

Light microscopy

Specimens were taken from the frontal cortex of the brain of the control and avastin treated animals and were fixed in 10% formalin for H&E stain and immunohistochemical study.

Immunohistochemical study:

Paraffin sections of 4um thickness were immunostained using peroxidase-labelled streptavidin-biotin technique to detect glial fibrillary acidic protein (GFAP) in astrocytes. This stain is considered specific for the intermediate filaments fibrillary acidic protein which is found in astrocytes and not found in nerve cells and even other types of glial cells as oligodendroglia or microglia.

Staining procedure:

Formalin-fixed, paraffin-embedded tissue sections were done and mounted on coated glass slides. Sections were deparaffinized and rehydrated through descending grades of alcohols (100%, 90%, 80% and 70%) then put in distilled water for 5 min. Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide for 10 minutes using peroxidase blocking reagent. Antigen retrieval was done by boiling slides in citrate buffer solution (pH 6.0). The slides were microwaved at a high medium for 10 min. The sections were incubated with 1/50 of antiGFAP antibody at 4°C for 18–20h, washed and incubated with biotinylated secondary antibodies, and then with the avidin–biotin complex. Finally, sections were counterstained with hematoxylin, dehydrated, cleared, and mounted (15). GFAP-positive cells appeared brown and nuclei appeared blue. Negative control sections was done with omission of the Iry (antiGFAP) antibody. Universal kits and primary antibody (anti GFAP antibody) were purchased from Thermo fisher scientific company.

Morphometric and statistical analysis:

The number of GFAP-positive cells was counted per 5 high power fields (x400) in slides from each animal in each group using (CX21, Japan. Light microscope) in histology department, Faculty of Medicine, Sohag University. For statistical analysis independent "t" test was performed to compare the mean number of GFAP positive cells in avastin treated group with the control one. *P* value was considered significant when it is ≤ 0.05 .

2-Transmission electron microscopy:

Immediately after sacrificing animals, 10-12 small pieces were obtained from each animal and were fixed in 2.5% glutaraldehyde for 24 hours. Specimens were then washed in 3-4 changes of cacodylate buffer (pH 7.2) for 20 minutes in every change. Specimens were post fixed in cold 1% osmium tetroxide for 2 hours. They were washed in 4 changes of cacodylate buffer for 20 minutes each. Dehydration was done by using ascending grades of alcohol (30, 50, 70, 90, and absolute alcohol) 2 hours for each. Clearing was done in propylene oxide then they were embedded in Epon 812 using gelatin capsule. These samples were kept in incubator at 35 degree for one day, then at 45 degree for another day and at 60 degree for 3 days (16).

3. Results:

Control group (GI):

(1) Light microscope:

H &E stained sections examination revealed that, the cerebral hemisphere of the control rat was consisted of an outer cortex of gray matter and a sub-cortical region of white matter. The cortex contained neurons, glial cells and nerve fibers. The organizations of neurons in the cerebral cortex appeared as six layers; the molecular layer (fibrous with few nerve cell bodies), the external granular layer, the external pyramidal cell layer, the internal granular layer, the internal pyramidal cell layer and the multiform cell layer (Fig. 1). The neuropil contained neuroglia, nerve fibers and blood vessels with a narrow perivascular space. Cortical neurons had rounded vesicular nuclei with prominent nucleoli, slight basophilic cytoplasm and peripheral processes. Well-stained Nissel's granules in their cytoplasm were also observed (Fig. 2).

Immunohistochemical staining for GFAP showed positive staining in the cytoplasm of astrocytes and their processes. They appeared small with few short, thin processes. The positive reaction appeared as a brown coloration in the cytoplasm of astrocytes and their processes (Fig.3).

(2) Electron microscope:

Examination of ultrathin sections of the control group revealed that nerve cells had large euchromatic nuclei and prominent nucleoli. Their cytoplasm contained mitochondria, RER, lysosomes and numerous free ribosomes (Nissel's granules) (Fig.4). The glial cells had large euchromatic nuclei and their cytoplasm contained many organelles as mitochondria, RER, short filaments (Fig.5)

Avastin treated group (GII):**(1) Light microscope:**

In H & E stained sections, most of the neurons appeared shrunken with darkly stained pyknotic nuclei and surrounded by wide pericellular space. Blood vessels were surrounded by wide perivascular space compared with controls. Vacuolations of the neuropil were also noticed (Fig.6).

Immunohistochemical staining for GFAP showed an apparent increase in the number of astrocytes which appeared larger with thick processes in comparison with controls (Fig. 7).

(2) Electron microscope:

Some neurons had rounded regular euchromatic nuclei with slight indentation. Their cytoplasm contained mitochondria with destructed cristae, vacuoles and apparent decrease in free ribosomes (Fig.8). Other neurons exhibited irregular shaped nuclei and large vacuoles (Fig.9). Some cells had small heterochromatic nuclei and electron dense cytoplasm. Their cytoplasm contained mitochondria with destructed cristae, RER and vacuoles (Fig.10).

Morphometric and statistical results:

The mean number of GFAP positive astrocytes was highly significant increase in avastin treated group compared to the control group (table 1 and histogram 1)

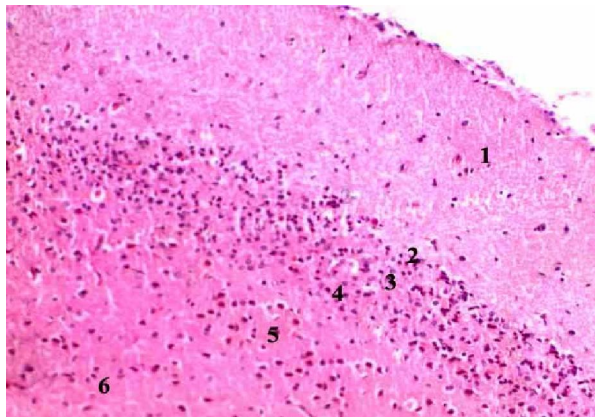


Fig. 1: A photomicrograph of a section from a control rat's cerebral cortex showing the six layers, which are clearly defined as: molecular (1), external granular (2), external pyramidal (3), internal granular (4), internal pyramidal (5), and multi-form, which is the deepest layer (6). (H&Ex200)

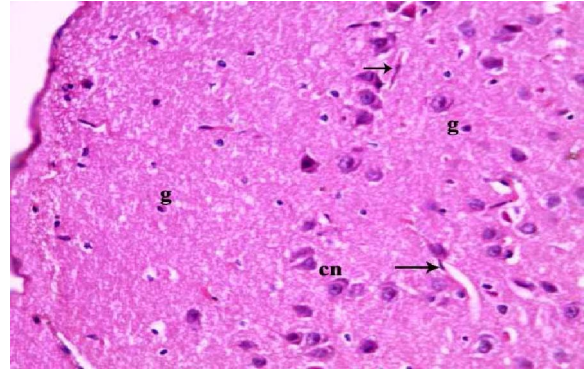


Fig. 2: A photomicrograph of a section from a control rat's cerebral cortex showing cortical neurons (cn) with rounded pale nuclei, basophilic cytoplasm and peripheral dendrites. Intercellular neuropils shows different types of neuroglia (g) and nerve fibers. Note: blood vessels with narrow perivascular spaces (arrows). (H&E x400)

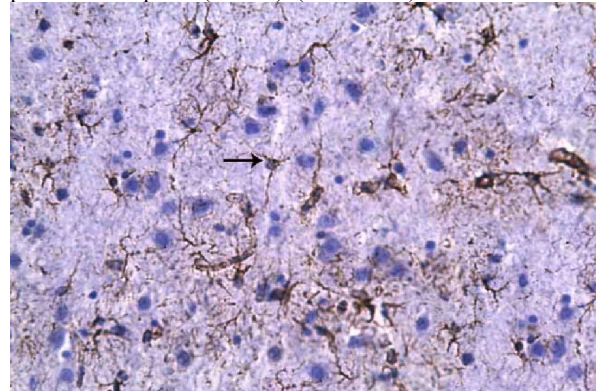


Fig.3: A photomicrograph of a section from a control rat's cerebral cortex showing astrocytes with few and thin processes (arrow). Note: the brown coloration in the cytoplasm of astrocytes and their processes. (GFAP immunostainedx400)

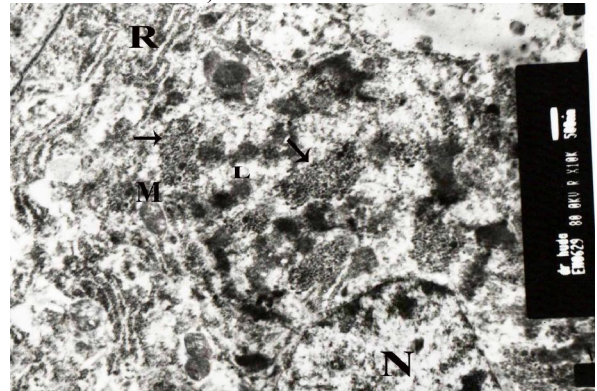


Fig.4: An electron photomicrograph from a control rat's cerebral cortex showing cortical neuron with euchromatic nucleus (N), the cytoplasm contains numerous rough endoplasmic reticulum (R), mitochondria (M), lysosomes (L) and numerous free ribosomes (arrow). (Uranyl acetate & lead citrate x10000)

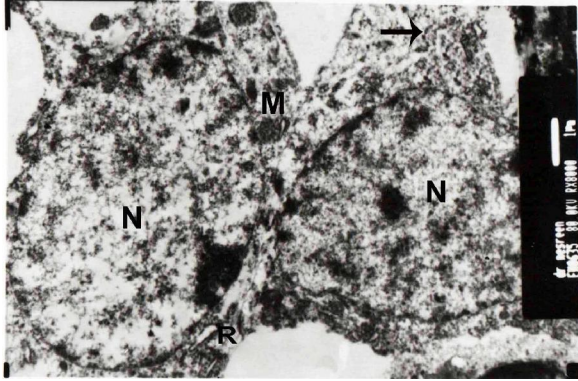


Fig.5: An electron micrograph from a control rat's cerebral cortex showing two adjacent glial cells have large euchromatic nuclei, RER (R), mitochondria (M) and short filaments (arrow). (Uranyl acetate & lead citrate x8000)

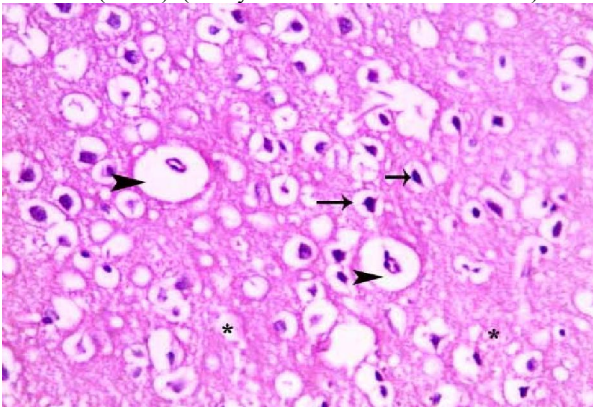


Fig. 6: A photomicrograph of a section from avastin-treated rat's cerebral cortex showing most of the neurons appeared shrunken. They contain darkly stained nuclei and surrounded by wide pericellular space (arrow). Blood vessels are surrounded by wide perivascular space (arrow head). Neuropil vacuolation is also seen (*). (H&Ex400)

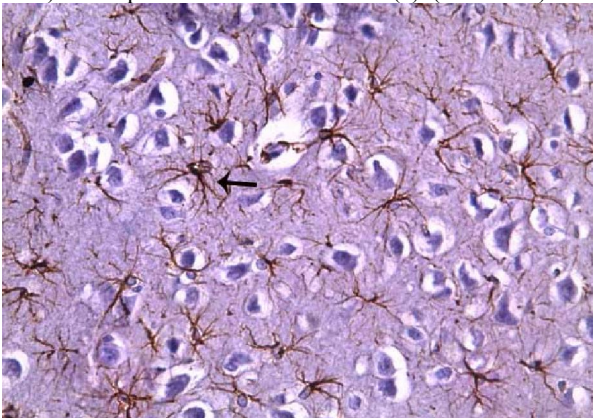


Fig.7: A photomicrograph of a section from avastin-treated rat's cerebral cortex showing apparent numerous astrocytes with thick and numerous (arrow). (GFAP immunostained x400).

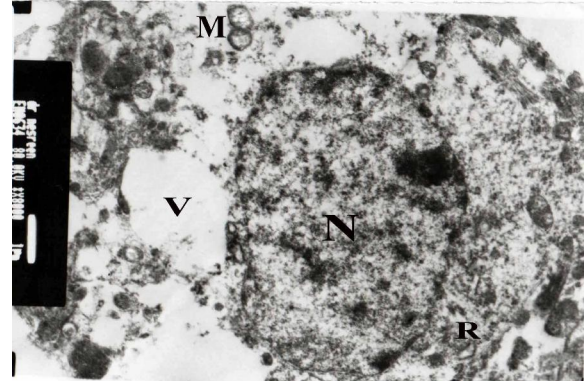


Fig.8: An electron micrograph from avastin-treated rat's cerebral cortex showing a cortical neuron with rounded slightly indented euchromatic nucleus (N) with prominent nucleolus. The cytoplasm contains mitochondria with destroyed cristae (M), dilated RER (R), apparent decrease in free ribosomes and large vacuoles (V). (Uranyl acetate & lead citrate x 8000).

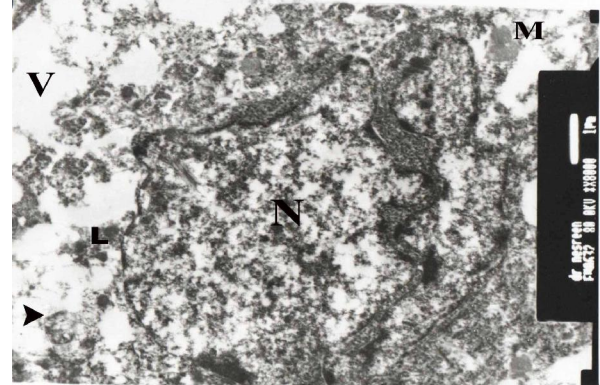


Fig.9: An electron micrograph from avastin-treated rat's cerebral cortex showing a cortical neuron contains irregular nucleus (N), lysosomes (L), mitochondria (M) some of them with destroyed cristae (arrow head) and numerous vacuoles (V). (Uranyl acetate & lead citrate x 8000).

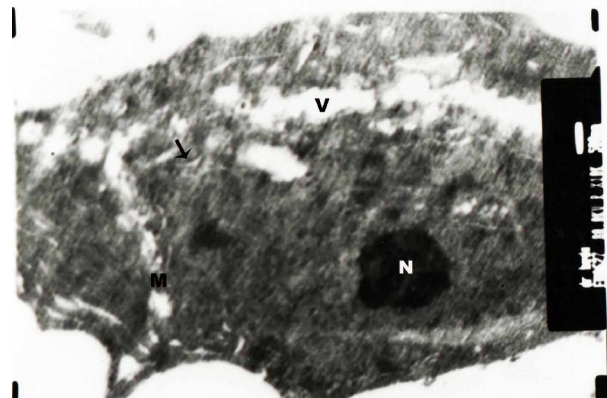
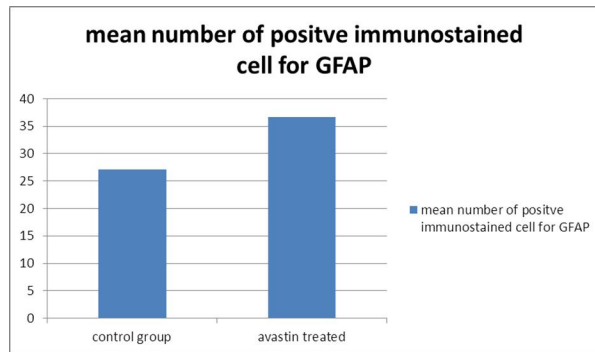


Fig. (10): An electron micrograph from avastin treated rat's cerebral cortex showing a cortical neuron with heterochromatic nucleus (N). The electron dense cytoplasm contains vacuoles (V), RER (arrow) and mitochondria with destroyed cristae (M). (Uranyl acetate & lead citrate x5000)

Table 1: showing the difference between the mean number of GFAP positive cells in control and avastin treated group

Groups	Control group	Avastin treated group
Mean number of positive stained cells	27.16±6.81	36.68±6.37
<i>P</i> value	<i>P</i> * < .001	

*P** highly significance

Histogram 1: showing the difference between the mean number of GFAP positive cells in the control group and avastin treated one.

4. Discussion

Bevacizumab is a one of the anti-VEGF agents which have antiangiogenic and oedema-diminishing actions. It is widely used as intravitreal injection to diminish new vessels formation and reduce the edema of ocular diseases (17-20).

The present study was conducted to evaluate the effect of intravitreal injection of avastin on the cerebral cortex of rat.

It was found that avastin induced various histological degenerative changes in the brain. Some neurons appeared shrunken with pyknotic nuclei and acidophilic cytoplasm. Others had irregular nuclei, numerous vacuoles, some mitochondria with destructed cristae, and dilated RER cisternae in their cytoplasm.

These changes might be caused as a result of impaired blood perfusion and reduced neurovasculature of the cerebral cortex. Many studies supported this suggestion as they found that avastin led to decrease brain angiogenesis and endothelial cellular proliferation through its anti VEGF action (13, 14). This factor has a major role in maintaining perfusion of ischemic organs and mediating their vascular autoregulation. This might obtained through both modulation of nitric oxide vasodilator effect and maintenance of vascular architecture (21-23). Moreover, it was found that VEGF has an endothelial protective role. This could be obtained through prevention of the intravascular thrombocyte

aggregation and degranulation as well as maintaining vascular patency (23). Some authors added that this might occur after binding with platelet and activation of its FcγRIIa receptor (24). In accordance to these findings similar results of neuronal injury observed after brain hypoxia or ischemia (25, 26).

Furthermore the ultrastructural changes of the mitochondria in the present study might affect ATP synthesis (27). It was reported that inhibition of ATP synthesis could promote the glutamate release (28) which bind to N-methyl-D-aspartate receptors in the synapse. This mediates several processes like synaptic transmission, neuronal development and excitotoxic neuronal death (29). Many authors proved that toxic activation of excitotoxic neurotransmitter receptors by glutamate played a critical role in a number of brain injuries (30). This could affect the protein machinery unit of the cell which observed in the present work as apparent decrease in the free ribosomes and Nissel's granules and presence of numerous cytoplasmic vacuoles which might be dilated RER and or Golgi body.

In this study, there was a highly significant increase in the number of astrocytes that confirmed by immunostaining for GFAP in avastin treated group compared to the control one. This might be a reaction to the degeneration of the neighboring neurons (31). Astrocytes form a major glial cell population and play important physiological roles in brain functions. The cross-link between astrocyte and neuron occurs through the release of several neurotrophic factors that maintain CNS homeostasis. Moreover, astrocytes respond to various neurodegenerative processes by astrogliosis (32, 33). These activated astrocytes secrete different neurotrophic factors to improve the neuronal survival. It was found that rapid and severe activation led to an inflammatory response and neuronal death (34, 35). Similar results found in aged and hypercaloric rat (36).

In contrast to our findings some studied reported that single injection revealed no histological changes in the brain (37-39). Other studies reported that multiple injections were more susceptible for systemic complications than the single one. This conflict could be attributed to an inter-species difference in the volume of vitreous or in the dose injected. In addition to that, rodents are known to be fast drug metabolizers compared with humans (40).

On conclusion, the results of our study revealed accumulating evidences that intravitreal injection of avastin might be a risk factor for the cerebral cortex. It is recommended that the use of avastin should be restricted in treatment of severe retinal disease with decreasing the dose. Further studies must be done to determine either these changes are reversible or not.

Other studies should be done searching for the beneficial effect of using natural product to prevent or decrease the hazardous effect of avastin on the nervous tissue is another recommendation.

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