Histological Influence of Pembrolizumab on the Cornea of Adult Male Albino Rats, and the Efficacy of Topically Applied Axitinib with and without High Albumin Level in Tears

Original Article

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ABSTRACT

Introduction: Immune checkpoint inhibitor (ICI) therapy has revolutionized the treatment of a wide variety of malignancies. The toxic effects of ICIs can manifest themselves in a variety of organs, including the cornea.

Aim of the Work: The current research aimed to analyze the corneal adverse impacts of pembrolizumab and the efficacy of topically applied axitinib alone or with albumin.

Materials and Methods: Rats were classified into four groups: control group, pembrolizumab treated group (3mg/kg, i.p, 3 days per week for 4 weeks), Pembrolizumab +Axitinib (0.5 mg/ml two drops every 8 h for 4 weeks) and Pembrolizumab +Axitinib+ albumin (5 % of serum albumin). Animals were anesthetized and sacrificed at the end of experiment. Both animal eyes were removed for histological, immunohistochemistry, electron microscopic analyses and morphometric studies.

Results: Pembrolizumab treatment resulted in epithelium loss, desquamation, as well as vacuolated cytoplasm with pyknotic nuclei and a significant apparent decrease in epithelial and total corneal thickness. The area percentage of irregular collagen was declined, with spacing, neovascularization, and cellular infiltration, as well as an uneven damaged endothelium surface. Ultra structural changes in the corneal epithelium revealed damaged mitochondria with pyknotic nuclei and cytoplasmic vacuoles. Desmosomal connections were disrupted in the majority of cells, resulting in an increase in intercellular space. Collagen fibers were found to be extensively separated from keratocytes containing shrunken nuclei and vacuolated cytoplasm. Infiltration of eosinophils, mast cells, and lymphocytes, as well as stromal neovascularization was observed. Endothelial cells had mitochondrial degeneration and a thin Descemet's membrane. A substantial positive caspase-3, TNF-α, and VEGF immune response was observed in corneal epithelium, keratocytes, and corneal endothelial cells. Axitinib alleviated the ocular histological changes caused by pembrolizumab. Co-administration of albumin with axitinib enhanced results.

Conclusion: Although axitinib was demonstrated to attenuate immune-related corneal damage associated with pembrolizumab, albumin co-administration enhanced this response.

Received: 25 July 2021, **Accepted:** 09 September 2021

Key Words: Albumin, axitinib, corneal neovascularization, pembrolizumab.

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ISSN: 1110-0559, Vol. 46, No.1

INTRODUCTION

Targeted cancer therapy (TCT), also known as immune therapy, is a term that refers to innovative therapeutic methods that are designed to exclusively malignant cells by interfering with signaling pathways involved in carcinogenesis. While conventional chemotherapy, radiation, and surgery remain the gold standard of care, TCT is growing as a complementary or alternative strategy[1]. The principle of boosting the ability of the body's immune system to identify cancer cells has revolutionized therapy and elevated TCT to the forefront of modern medicine[2]. Over the last two decades, breakthroughs in understanding the molecular base and gene expression of cancer, as well as the development of new screening tools have resulted in advances in our understanding of the system of cancer development and the discovery of novel therapeutic strategies[3-4]. Recently, TCT, or immune therapy, is one of these innovative techniques that have recently been employed to treat a variety of cancer types. Through the use of particular molecules or antibodies, this therapy promotes the body's own immune system to attack cancer cells or to inhibit tumor growth and progression[5]. Immunotherapies include inhibitors of immunological checkpoints, therapeutic vaccinations, immune cell therapy, and therapeutic antibodies[6]. Immune checkpoint inhibitors (ICIs) are a fairly new category of immunotherapy agents that have been licensed for the treatment of a variety of cancer types. They include atezolizumab, avelumab, nivolumab, and pembrolizumab[7].

Pembrolizumab (Keytruda) is a humanized monoclonal antibody that represents a novel type of “targeted” cancer therapy. Pembrolizumab binds to and inhibits the

 DOI: 10.21608/ejh.2021.87471.1535

programmed death 1 (PD-1) receptor on the surface of T-cells. By inhibiting PD-1, T-cells are activated to hunt and destroy cancer cells. It's prescribed to treat melanoma, Hodgkin lymphoma, and lung cancer[8]. Despite the fact that these treatments are focused and successful in controlling tumor growth, they may have negative impacts on the digestive system, liver, skin, nervous system, heart, and eyes[9]. Patients on pembrolizumab suffered bilateral eye pain, conjunctival congestion, and dry eye[10]. Such inflammatory disorders and keratitis had a role in corneal neovascularization and opacities[10]. Axitinib (Inlyta) is a selective tyrosine kinase receptor inhibitor that is a novel, potent small molecule (TKI). It inhibited corneal angiogenesis at picomolar concentrations due to its potent and highly selective inhibitor of VEGF tyrosine kinase receptors[11]. It acts by inhibiting vascular endothelial growth factor (VEGF) from attaching to the VEGF receptor[1,2]. Axitinib was prescribed for the treatment of advanced metastatic renal cell carcinoma by the US Food and Drug Administration (FDA) and a number of other countries in 2012[12].

Human albumin has been shown to be a safe and effective as a therapeutic option for people suffering from dry eye syndrome[13]. Albumin is a ubiquitous soluble protein, represents for almost half of the total protein content in serum. It acts as a reservoir and carrier for medications and other small molecules[14]. Albumin, a necessary natural tear component (54 mg/L) for eye health, is a native protein to the ocular surface and therefore should be well tolerated when applied as eye drops. It is generated from plasma spills from conjunctival capillaries onto the ocular surface and combines with the tear film. In healthy eyes, albumin level in tears is typically low, but significantly increases in diseased eyes[15]. Tear albumin might thus be regarded as a non-specific measure of ocular surface integrity[13].

To our knowledge, no adequate research on pembrolizumab's harmful effects on the cornea has been documented and the majority of studies have been performed on small groups[16]. Thus, the goal of the proposed study was to analyze the corneal adverse effects of Pembrolizumab andthe efficacy of topically applied axitinibalone orin the context of elevated albumin levels in tears.

MATERIALS AND METHODS

Drugs and chemicals

Pembrolizumab (KEYTRUDA) (Schering-Plough Labo NV, Belgium), present in the form powder. A vial of powder contains 50 mg of pembrolizumab. Freshly dissolved in 2 ml distilled water, this solution contains 25 mg of pembrolizumab in 1 ml.

Axitinib was obtained from Sigma-Aldrich, St Louis, Missouri, USA, in the powdered form, 5 mg in glass bottle. Axitinib eye drops were prepared by dissolving the active ingredient axitinib powder in 10 ml saline to obtain the desired concentration (0.5 mg/ml)[17].

Human albumin 5% (ALBUMINEX 5%, contains 5 g per dl of human albumin in 250 mL (12.5 g) and 500 mL (25 g) glass vials) (Bio Products Laboratory USA) (BPL USA)

Animals

Fifty adult male albino rats weighing between 150 and 200 grams were employed in this research. They were housed in sanitary, well-ventilated cages. Throughout the trial, a healthy unrestricted access to diet and tap water were supplied. All areas of animal handling and management were conducted in accordance with the local ethical committee of Menoufia University's Faculty of Medicine.

Experimental design

The experimental animals were divided into four groups as follow:

Group I (control group) (20 rats): Rats were subdivided into four equal subgroups. (5 rats each); subgroup Ia: did not get treatment.

- **Subgroup Ib:** two drops of axitinib were topically administered to both eyes of rats of this subgroup, three times a day for 4 weeks[17].
- **Subgroup Ic:** Two drops of a 5% solution of human albumin were applied topically to both ocular surface of rats 4 times daily for 4 weeks[18].
- **Subgroup Id:** received axitinib and albumin in the same dose and duration of subgroups Ib and Ic respectively.

Group II (Pembrolizumab-treated group): included 10 rats, treated with freshly prepared Pembrolizumab at a dose of 3mg/kg[19], intraperitoneal (i.p) injection, three times per week for 4 weeks.

Group III (Pembrolizumab and Axitinib- treated group): included 10 rats, received pembrolizumab in the same dose and route of administration of group II and axitinib (0.5 mg/ml)[17], two drops of axitinib were applied topically to both ocular surface of rats three times daily for 4 weeks.

Group IV (Pembrolizumab + Axitinib and Albumin- treated group): contained 10 rats, administered pembrolizumab and axitinib at the same dose and route of administration of group III in addition ,rats were treated with 5 % solution of human albumin[18], two eye drops of albumin were topically administered for both eyes, four times daily for 4 weeks.

At the end of the experiment (4 weeks), animals were ether-anesthetized, sacrificed and tissues were fixed via animal perfusion. Both eyeballs were dissected from all groups. Histological, histochemical, immunohistochemical, and transmission electron microscopy examinations were performed on corneal specimens.

Histological and histochemical study

After immersing corneal specimens in 10% neutral-buffered formalin, they were washed, dehydrated, cleared, and embedded in paraffin. Hematoxylin and eosin (H&E), Mallory trichrome, and periodic acid Schiff (PAS) stain were used to stain sections of 4-6 µm thickness[20].

Immunohistochemical study for detection of

A. Activated caspase -3 (apoptotic marker) (purchased as anticaspase -3 rabbit polyclonal antibody, Thermo Scientic, Fermont, California, USA). The reaction was cytoplasmic and nuclear.

B. Tumor necrosis factor-α (TNF-α) (index of inflammation). The primary monoclonal antibody used was mouse anti-TNF-α (Santa Cruz Biotechnology, California, USA). The reaction was cytoplasmic.

C. Vascular endothelial growth factor (VEGF) (marker for neovascularization). Anti VEGF was a mouse monoclonal primary antibody (Thermo Scientific and Lab Vision Corporation, CA 94539, USA). The cellular staining pattern for VEGF was cytoplasmic.

Immunohistochemical staining was carried by avidin-biotin peroxidase method Sections of cornea were incubated for one hour at 4°C with 1: 100 dilution of the primary antibody. After that, the sections were then counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted. Negative controls lacked the primary antibody. Tonsils were used as positive control in case of caspase-3, colon was positive control in case of TNF-α while kidney was positive control for VEGF[21].

Electron microscopic study

In phosphate buffer, the eyes were fixed for 1 hour with 1% glutaraldehyde and 4% paraformaldehyde. Every eye ball's anterior segment were sliced, dehydrated, and embedded in epoxy resin after being post-fixed in one percent osmium tetroxide. Ultrathin retinal sections (80-90 nm) were obtained and stained with uranyl acetate and lead citrate. The ultrastructure of the tissues was examined using a Jeol electron microscope[22]. TEM processing and analysis were done at the Unit of Electron Microscopy, Faculty of Medicine, Tanta University

Morphometric and Statistical analysis

All quantitative data was collected using the "Leica Qwin 500 C" image analyzer automated data processing system Ltd. (Cambridge, England). The mean total corneal thickness/um and the mean corneal epithelial thickness/ pixels of all groups, were measured in H&E stained sections using the distance parameters in the interactive measurement menu, with 10 random non-overlapping fields evaluated using 10 objective lenses. The mean area percentage (%) of collagen in Mallory stained sections and the optical densities of Caspase-3, TNF-α and VEGF immunoreactivities were determined within 10 fields at magnification of 200 of each rat using a Leica DML B2/11888111 microscope supplied with a Leica DFC450 camera. The measured variance was estimated using the software version K1.45 of Image J in anatomy department, faculty of Medicine, Menoufia University. The data was expressed as mean standard deviation, and significant differences between groups were determined using the Student t-test and variance test study. A P value of less than 0.01 was deemed statistically significant[23].

RESULTS

Light microscopic findings

H&E

Control subgroups underwent histological investigation and revealed the same morphologic results. H&E -stained sections of the control group demonstrated the cornea's characteristic histological nonvascular structure that composed of five distinct layers. The corneal epithelium was stratified, squamous non-keratinized and consisted of many layers of cells resting on a thin basement membrane. The deepest cells, the basal cells, stand in a palisade-like manner; these are columnar with rounded heads and flat bases. The wing cells, the second epithelial layer, were polyhedral cells that were convex anteriorly and capped the basal cells. The next two or three layers were also polyhedral and became wider and increasingly flattened towards the surface. The corneal epithelium appeared to be sitting on top of a non-cellular acidophilic layer termed Bowman's membrane. Corneal stroma (substantia propria) contained parallel lamellae of collagen fibers. Between the lamellae of collagen fibers were flattened fibrocytes called keratocytes with elongated spindle-shaped nuclei. There was a Descemet's membrane underneath the stroma, which showed as a thin uniform acidophilic layer. Finally, a single regular layer of flat cells with flat or oval nuclei (corneal endothelium) was identified on the posterior surface of Descemet's membrane (Figure 1a).

Treatment with pembrolizumab resulted in a localized loss and desquamation of the superficial corneal epithelium, as well as a significant apparent decrease in the epithelial and total corneal thickness (Table1, Histograms 1,2). Some epithelial cells showed intensily stained dark nuclei and vacuolated cytoplasm with underlying disrupted Bowman's membrane. The stroma exhibited widely spaced collagen fibers, neovascularization, and cellular infiltration. Additionally, an uneven endothelium surface and some disturbed endothelial nuclei with no nuclei were also found (Figure 1b).

Group III (Pembrolizumab and axitinib-treated group) exhibited almost identical results to those from the control group. However, some stromal fibers remained widely separated (Figure 1c). On the other hand, histological evaluation of corneal sections from the Pembrolizumab + axitinib and albumin-treated groups (group IV) revealed a

histological profile more or less similar to that reported in the control group (Figure 1d).

Mallory trichrome

A section of the cornea of control rats stained with Mallory trichrome revealed revealed blue regular compact, narrow consistently spaced bundles of collagen fibers with no spaces between them in the stroma (Figure 2a). The group treated with pembrolizumab had numerous significant gaps between unevenly dispersed few weakly stained collagen fibers (Figure 2b). Collagen area percentage was decreased significantly in this group opposed to the control (Table1, Histogram 3). In contrast, rats given pembrolizumab plus axitinib displayed a considerable reduction in the prevalence of some spaces between collagen fibers that were more or less consistently arranged (Figure 2c). Additionally, the group treated with pembrolizumab + Axitinib + Albumin displayed stroma with regular collagen bundles and tight gaps (Figure 2d), which was not significantly different from the control (Table1, Histogram3).

Histochemical findings

Control rat corneal sections stained with PAS demonstrated a strong positive PAS reaction with a distinctive magenta red coloring of Descemet's membrane and Bowmen`s membrane (Figure 3a). While sections from the group treated with pembrolizumab (group II) demonstrated a faintly week positive PAS reaction in the Descemet's membrane and apparently negative PAS reaction in Bowman's membrane (Figure 3b). On the other hand, group III sections exhibited a moderately positive PAS reaction of Descemet's membrane (Figure 3c). Whereas sections from group IV exhibited an apparently intense positive PAS reaction in Bowman's membrane and Descemet's membrane (Figure 3d).

Immunohistochemical staining

Caspase-3 immunostaining

Immunostained corneal sections of Caspase-3 demonstrated a negative immune response within the corneal cells in all layers of Group I (Figure 4a). In comparison to control, group II had a significantly stronger positive nuclear and cytoplasmic caspase-3 immune response in corneal epithelium, keratocytes, and corneal endothelial cells (Figure 4b). However, as contrasted to the control group, a significant moderate immunoexpression of Caspases-3 in corneal epithelium and mild keratocytes reaction was seen in group III (Figure 4c). Group IV demonstrated a negative immunoreactivity of the corneal epithelium and minimal caspase-3 immunoexpression of keratocytes (Figure 4d), with no statistically significant changes between groups IV and III (Table 1, Histogram 4).

TNF-α immunostaining

Sections from control group demonstrated that the corneal tissue had a negative immunological response (Figure 5a). Whereas the corneal epithelium and keratocytes from sections of pembrolizumab-treated (group II) demonstrated a significantly strong positive cytoplasmic expression of TNF-α (Figure 5b) compared to control group. In comparison to the control group, sections from group III (Pembrolizumab and axitinib-treated group) demonstrated a significant mild TNF- immunopositive reaction in the cytoplasm of corneal epithelium and keratocytes (Figure 5c). Additionally, sections from group IV (Pembrolizumab + axitinib and albumin-treated group) demonstrated a negative immunoreactivity of the corneal epithelium and keratocytes (Figure 5d), which was significantly different from group III but not from the control group (Table 1, Histogram 4).

VEGF immunostaining

Sections of cornea from control group stained with VEGF immunostaining revealed week reaction in superficial cells of corneal epithelium and negative immunoreactivity within corneal stroma (Figure 6a). However, in group II, a significant positive cytoplasmic immunoexpression was found in the corneal epithelium, endothelial cells lining new blood vessels, and keratocytes (Figure 6b), compared to the control. Group III demonstrated a non-significant cytoplasmic immunoexpression of VEGF in corneal epithelium and keratocytes (Figure 6c). Group IV corneal sections had negative immunoreactivity (Figure 6d).There was no significant disparity between GIII and GIV, or between the two groups and the control (Table 1, Histogram 4).

Electron microscopy

Electron microscopic analysis of rats in the control group revealed that the cornea had the well-known normal ultrastructure. The epithelium of the cornea looked to be composed of basal columnar cells with euchromatic rounded or oval nuclei, mitochondria, and RER. Numerous electron-dense hemidesmosomes connect the cells to the basal lamina, and multiple electron-dense desmosomes connect the neighboring cells (Figure 7). The intermediate cell layer is characterized by oval nuclei, mitochondria, and RER. Numerous electron-dense desmosomes linked the cells together with the presence of small intercellular spaces (Figure 8). The superficial layer's squamous cells featured flat nuclei; apical microvilli were connected by many electron-dense desmosomes (Figure 9). Corneal stroma was composed of regularly arranged lamellae of collagen bundles interspersed with keratocytes. In each lamella the collagen fibers were arranged in layers parallel with each other but cross approximately at right angles with the adjacent one. Keratocytes were elongated cells with an extended ovoid nucleus and a small amount of cytoplasm (Figure 10). A thick homogenous non-cellular electron-dense layer, Descemet's membrane, was identified adjacent to the stroma. It was lined by a single flattened endothelial cell layer with a flat nucleus and sparse cytoplasm (Figure 11).

The corneal epithelium (basal and middle cell layers) of the pembrolizumab-treated group (group II) contained irregular indented darkly stained nuclei with dilated perinuclear space, multiple large cytoplasmic vacuoles, and swollen degenerated mitochondria (Figures 12,13). Substantial disruption of desmosomal junctions and broadening of the intercellular space were also noticed (Figure 12). The superficial cell's nucleus shrank to a very electron dense size, with extensive cytoplasmic vacuolation (Figure 13). Keratocytes in the stroma had shrunken nuclei with condensed chromatin and cytoplasmic vacuoles (V), and they were widely separated from the collagen fibers (Figures 14,15). Furthermore, cellular infiltration of eosinophils, mast cells, and lymphocytes was observed between the disrupted collagen fibers. (Figure 15), as well as stromal neovascularization (Figure 16). Endothelial cells featured an oval nucleus and degenerated mitochondria, as well as a reduction in Descemet's membrane thickness (Figure 17).

Corneal sections from the group treated with pembrolizumab and axitinib (group III) revealed a basal epithelial cell having an euchromatic nucleus, mitochondria, and RER with widening of the intercellular spaces (Figure 18). Moreover, the corneal epithelium's superficial and intermediate epithelial cells appeared to be similar to those in the control group. There were, however, a few foci of cytoplasmic vacuolation and a small enlargement of intercellular spaces (Figure 19). The stroma contained regularly spaced collagen fibers (Figures 20,21), as well as fusiform keratocytes with an oval elongated nucleus and sparse cytoplasm (Figure 20). In Descemet's endothelial layer, an endothelial cell with an elongated euchromatic nucleus was present. Its cytoplasm contained swollen degenerated mitochondria (Figure 21).

Pembrolizumab + axitinib and albumin-treated group (IV) had similar electron microscopic findings to the control group. The epithelium of the cornea (basal, intermediate, and superficial cells) seemed to be nearly normal (Figures 22,23,24). Collagen fibers were shown to be arranged in a regular pattern (Figure 25). Descemet's membrane and endothelial cells with oval nuclei, RER, and mitochondria were observed in their normal state (Figure 25).

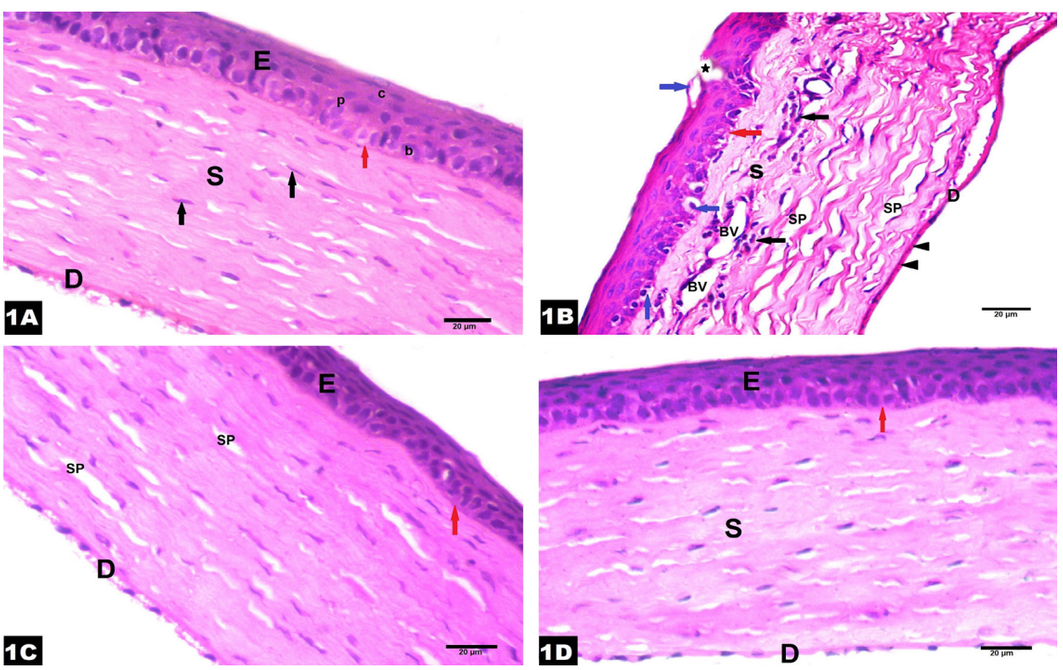


Fig. 1: A section of the cornea of (A) Group I Showing a stratified squamous non-keratinized epithelium (E) [basal columnar cells (b), intermediary polygonal cells (p), and superficial squamous cells (c)] with a Bowman's membrane (B) beneath the surface (red arrow). The stroma (S) is composed of bundles of acidophilic collagen fibers that are consistently arranged, with keratocytes (black arrows) in between. Descemet's membrane (D) and a single flattened endothelial cell layer (D) can be observed. (B): Group II demonstrates focal loss and desquamation of the corneal epithelium (star), with apparent focal decrease in thickness. Some epithelial cells with deeply stained nuclei and vacuolated cytoplasm (blue arrows), and disrupted Bowman's membrane (red arrow). The stroma (S) with widely separated collagen fibers (SP), neovascularization (BV), and cellular infiltration (black arrow). Endothelial surface irregularity (D) and degenerated karyolitic endothelial nuclei (arrow head) are seen. (C):Group III showing intact epithelium (E) and Bowman's membrane (red arrow). Some stromal fibers are still widely separated (SP). The Descemet's membrane and the endothelium appear regular and continuous (D). (D):Group IVshowing normal corneal epithelium (E) with smooth free surface and intact Bowman's membrane (red arrow). Stroma (S) shows regular collagen fibers with narrow spaces and normal keratocytes. The Descemet's membrane and the endothelium (D) appeared normal. (H&E X 400)

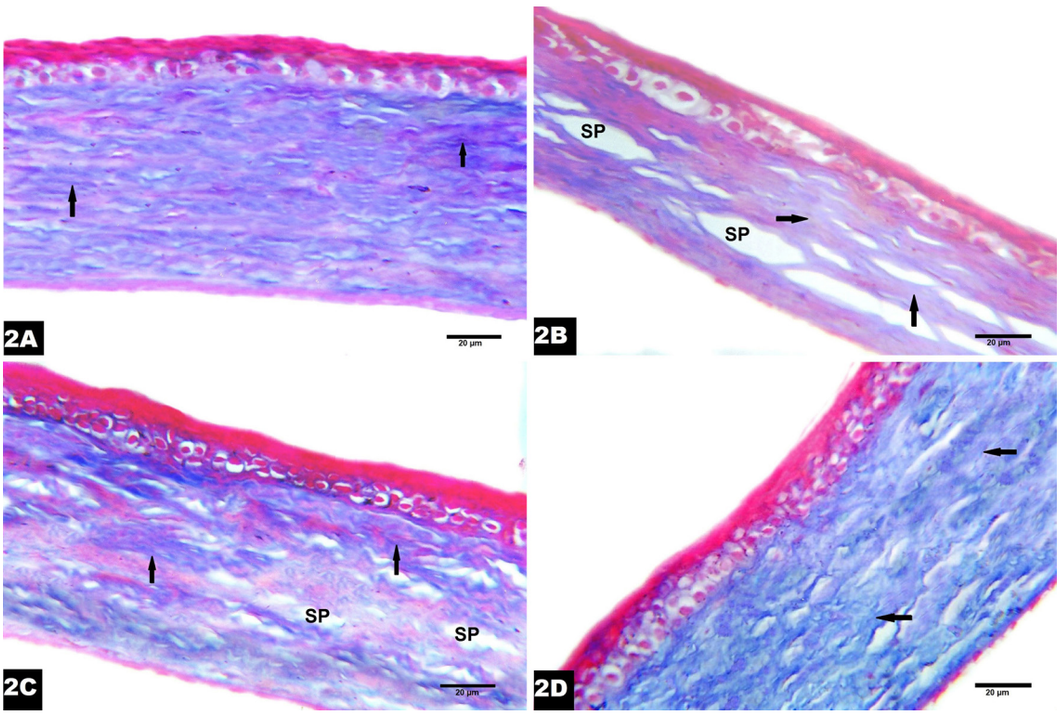


Fig. 2:A section of the cornea from (A): Group I, demonstrating consistently arranged bundles of collagen fibers (arrows) with tight spacing. (B): Group II has a few faintly stained bundles of collagen fibers (arrows) that are unevenly dispersed with large wide spaces (SP) in between. (C): Group III with more or less regular collagen fiber distribution (arrows) and few spaces (SP) in between. (D): Group IV stroma with regular collagen bundles (arrow) and narrow spaces in between. (Mallory trichrome X 400)

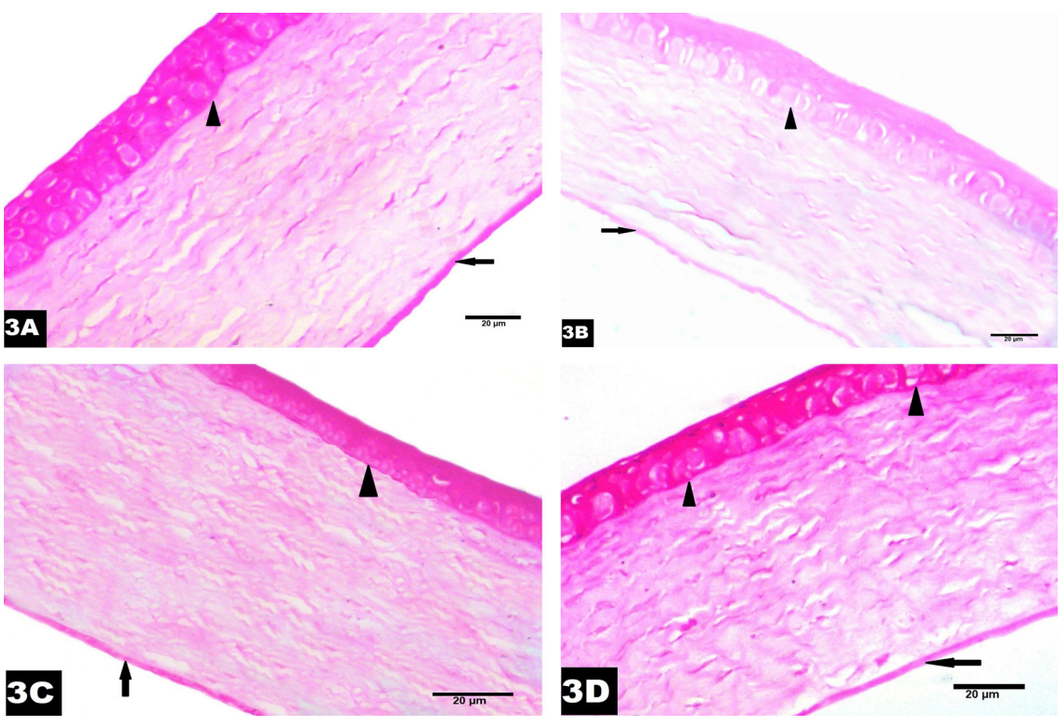


Fig. 3:A sections from the cornea of (A): Group I showing a strong positive PAS reaction of Descemet's membrane (arrow) and Bowman's membrane(arrow head) (B): Group II revealing a weak positive PAS reaction in Descemet's membrane (arrow) and apparently negative PAS reaction in Bowman` membrane (arrow head) (C): Group III showing a moderate positive PAS reaction of Descemet's membrane (arrow) and Bowman` membrane (arrow head) . (D): Group IV recording a strong positive PAS reaction in Descemet's membrane (arrow) and Bowman` membrane (arrow head). (PAS X 400)

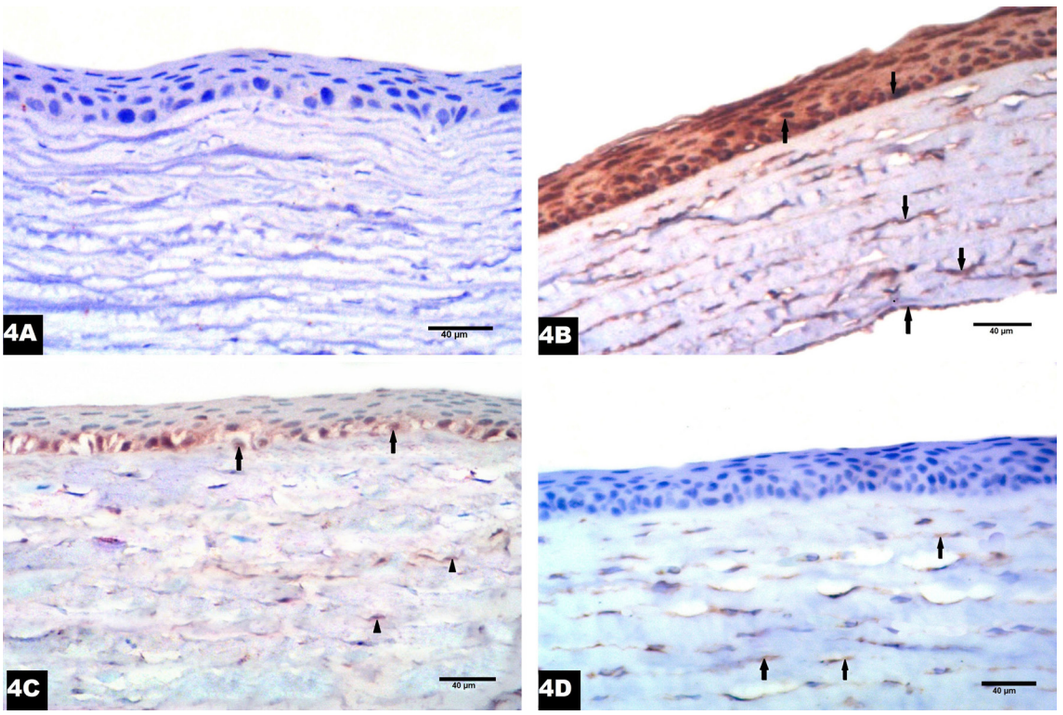


Fig. 4: A sections from the cornea of (A): Group I showing negative immunoreactivity within the corneal tissue. (B):Group II revealing a strong positive caspase-3 immune reaction in corneal epithelium, keratocytes and corneal endothelial cells (arrows). (C): Group III showing a moderate immunoexpression of Caspases-3 of corneal epithelium (arrow) and mild reaction of keratocytes (arrow head). (D):Group IV demonstrating negative immunoreactivity of corneal epithelium and mild caspase-3 immunoexpression of keratocytes (arrows). (Caspase-3 X 200)

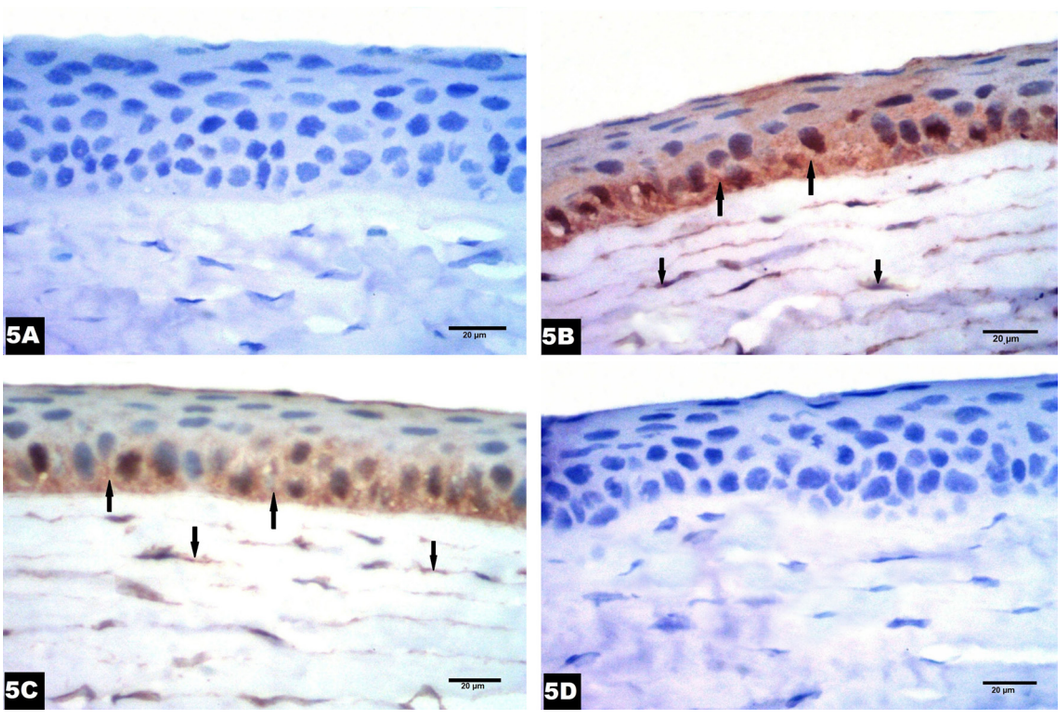


Fig. 5: A sections from the cornea of (A): Group Ishowing negativeimmune reaction within the corneal tissue.(B):Group II demonstrating strong positive cytoplasmic immunoexpressionin the corneal epithelium and keratocytes(arrows). (C): Group III revealing moderate immunoexpression of TNF-α in the cytoplasm of corneal epithelium and keratocytes (arrows). (D):Group IVillustrating negative immunoreactivity of corneal epithelium and keratocytes.(TNF-α immunostaining X400)

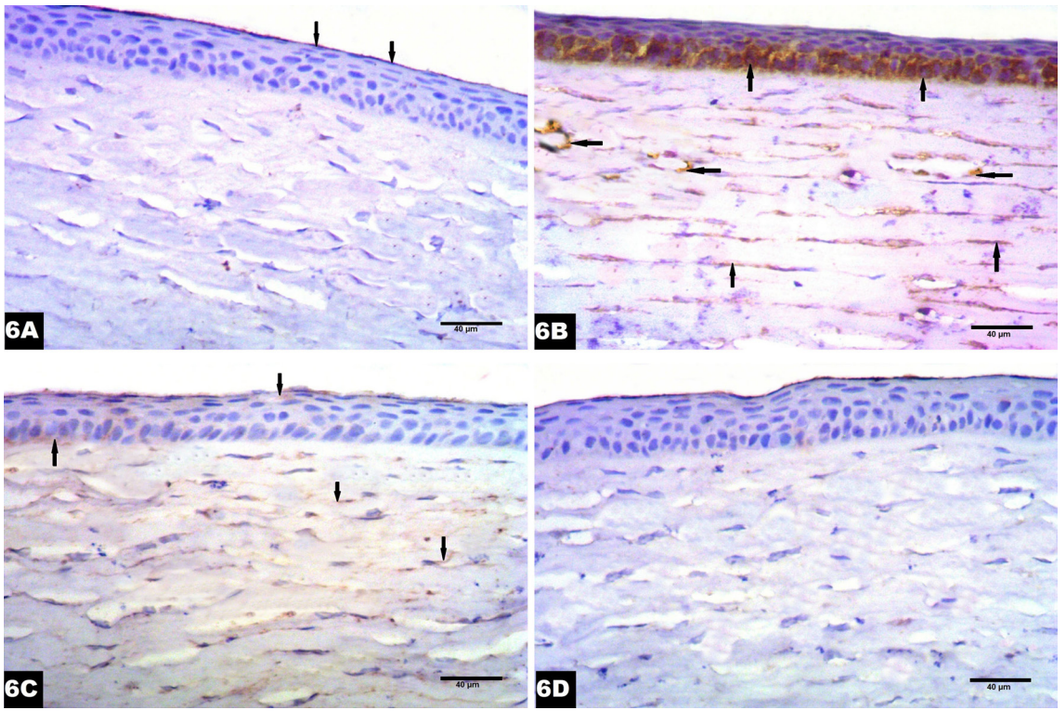


Fig. 6: A sections from the cornea of (A): Group I showing negative immune reaction of VEGF in the corneal stroma and week expression in superficial cells of corneal epithelium (arrow).(B):Group II strong positive cytoplasmic immunoexpressionin the corneal epithelium, endothelial cells lining blood vessels and keratocytes(arrows). (C): Group III revealing week cytoplasmic immunoexpression of VEGF in corneal epithelium and keratocytes (arrows).D): Group IV demonstrating negative VEGF immunoreactivity of corneal epithelium and keratocytes. (VEGF immunostainingX200)

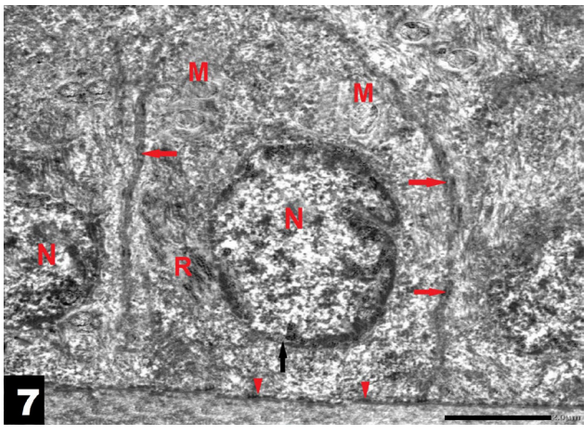


Fig. 7: An ultrathin section of a rat's cornea from control group showing corneal epithelial basal columnar cells having euchromatic rounded nuclei (N) with normal nuclear membrane (black arrow), mitochondria (M), and RER (R). The cells are linked to the basal lamina by several electron-dense dotted hemidesmosomes (arrowheads) and to one another by multiple electron-dense desmosomes (red arrow). (TEM X 4000)

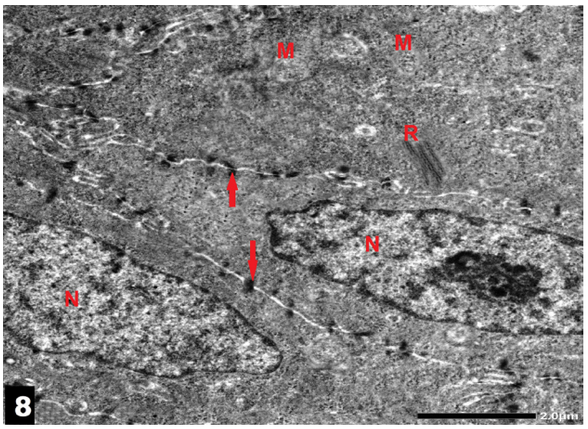


Fig. 8: An ultrathin section of a rat's cornea from control group revealing cells of the middle layer of the corneal epithelium with oval nuclei (N), mitochondria (M), and RER (R). The cells are connected to each other by many electron-dense desmosomes (arrows) with narrow intercellular spaces. (TEM X 4000)

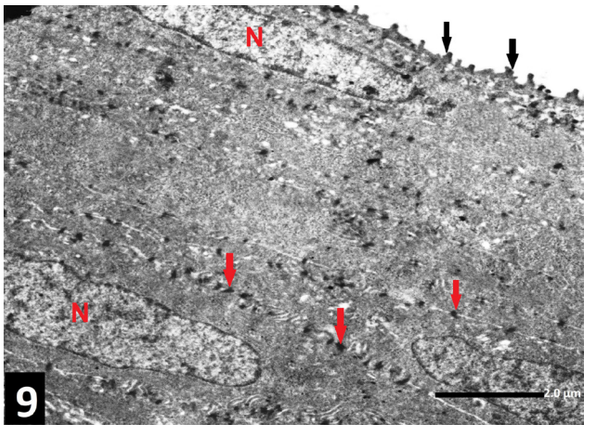


Fig. 9: An ultrathin section of a rat's cornea from control group exhibiting squamous cells of the superficial layer with flat nuclei (N) and numerous electron-dense desmosomes (red arrows) and microvilli (black arrow). (TEM X 4000)

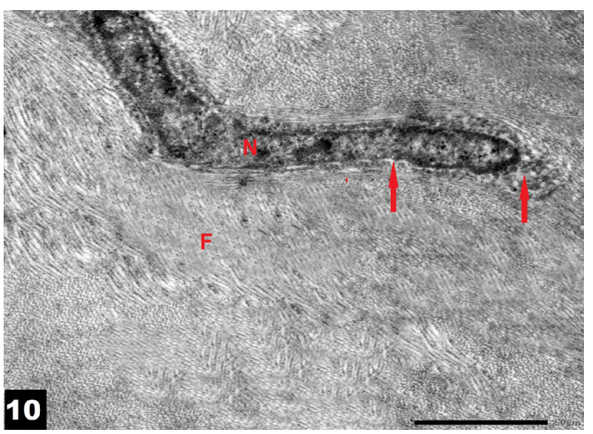


Fig. 10: An ultrathin section of a rat's cornea from control group showing the stroma with regularly distributed collagen fibers (F) and a spindle-shaped keratocyte with an elongated oval nucleus (N) and sparse cytoplasm (arrow).(TEM X 4000)

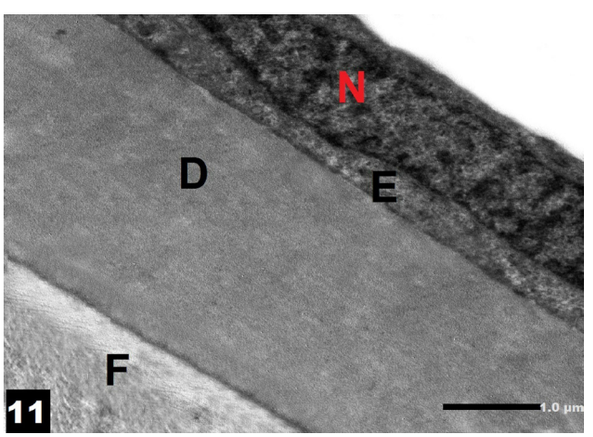


Fig. 11: An ultrathin section of a rat's cornea from control group displaying Descemet's membrane, acellular homogenous electron-dense layer (D) lined by a single endothelial cell layer (E), with a flat nucleus (N) and sparse cytoplasm, is seen. Note of the presence of the stroma's regularly ordered collagen fibers (F). (TEM X 14600)

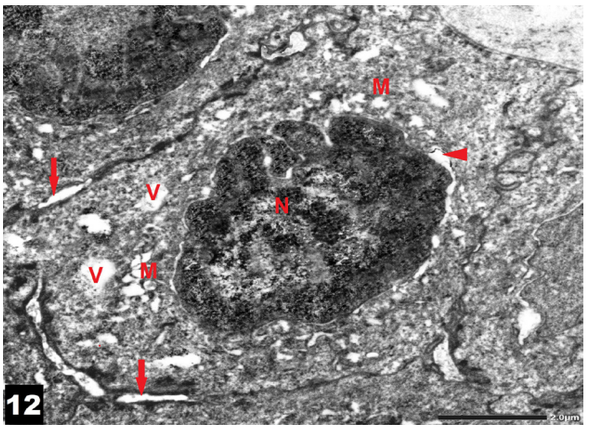


Fig. 12: An ultrathin section through the cornea of a rat from Pembrolizumab-treated group reveals a basal cell of corneal epithelium with an irregularly indented darkly stained nucleus (N) with dilated perinuclear space (arrow head), multiple large cytoplasmic vacuoles (V), and swollen degenerated mitochondria (M). There is a partial loss of desmosomal connections and an expansion of the intercellular spaces (arrow). (TEM X 4000)

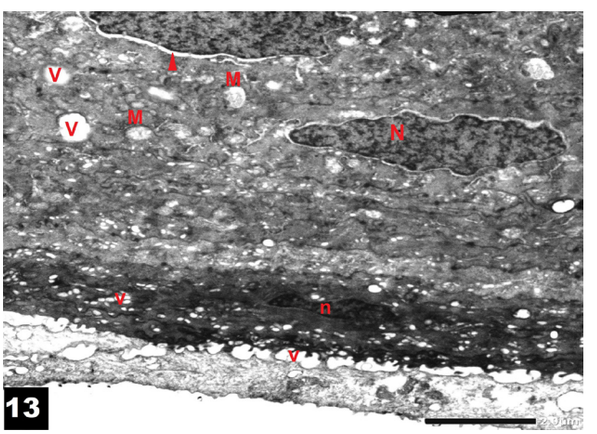


Fig. 13: An ultrathin section of the cornea of a Pembrolizumab-treated rat shows dark irregular nuclei (N) with dilated perinuclear space (arrow head), degenerated mitochondria (M), and cytoplasmic vacuoles (V). The superficial cell's shrunken dark nucleus (n) with severe cytoplasmic vacuolation (V) was noticed. (TEM X 4000)

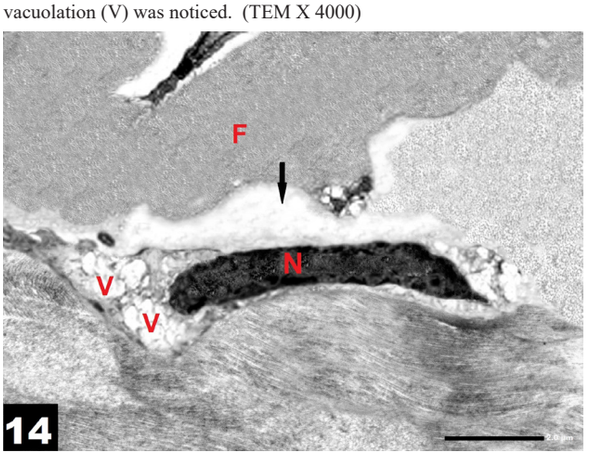


Fig. 14: An ultrathin section through the cornea of a rat from Pembrolizumab-treated group, revealing the stroma including a keratocyte with a shrunken darkly stained nucleus (N) and cytoplasmic vacuoles (V). The keratocyte is extensively separated (arrow) from the stromal collagen fibers (F). (TEM X 4000)

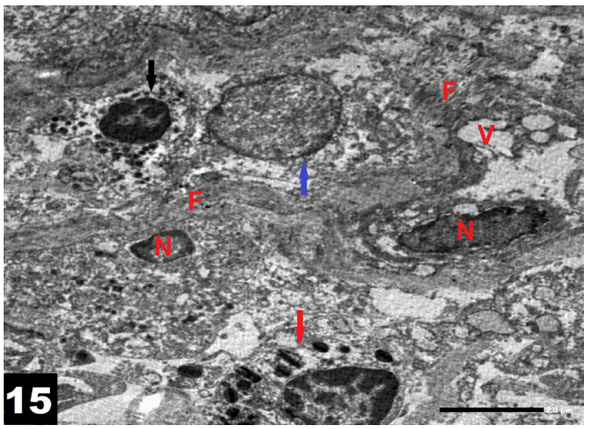


Fig. 15: An ultrathin section of a rat's cornea from Pembrolizumab-treated group shows stromal infiltration with mast cells (black arrow), eosinophils (red arrow), and lymphocytes (blue arrow) dispersed amongst disrubbed collagen fibers (F). Keratocytes with shrunken darkly stained nuclei (N) and vacuolated cytoplasm are seen (V). (TEM X 4000)

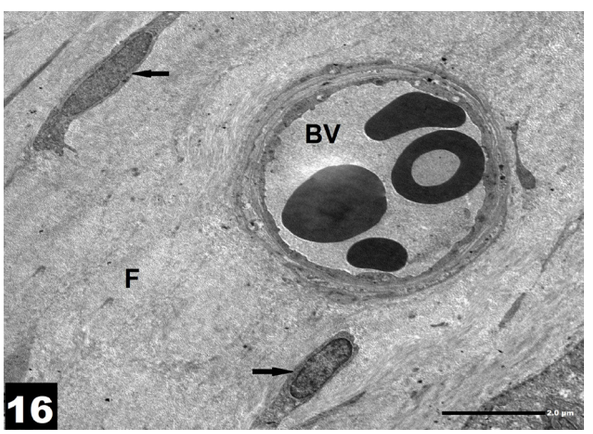


Fig. 16: An ultrathin section of a rat's cornea from Pembrolizumab-treated group shows the stroma with collagen fibers (F), keratocytes (arrow), and blood vessels (BV). (TEM X 4000)

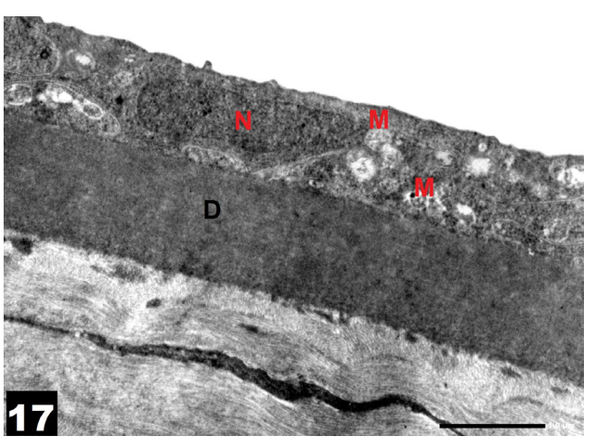


Fig. 17: An ultrathin section of the cornea of a rat from Pembrolizumab-treated group reveals endothelial cells with oval nuclei (N) and degenerated mitochondria (M). Notice oeof the obvious reduction in the Descemets membrane (D). (TEM X 14600)

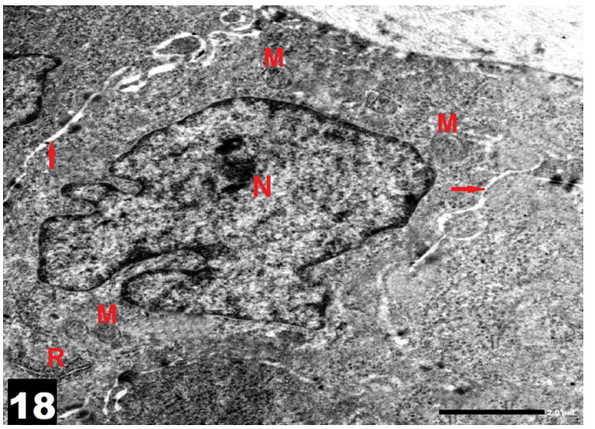


Fig. 18: An ultrathin section of the cornea of a rat treated with Pembrolizumab plus axitinib reveals basal epithelial cells with euchromatic nuclei (N), mitochondria (M), and RER (R). The intercellular spaces (arrow) are widening. (TEM X 4000)

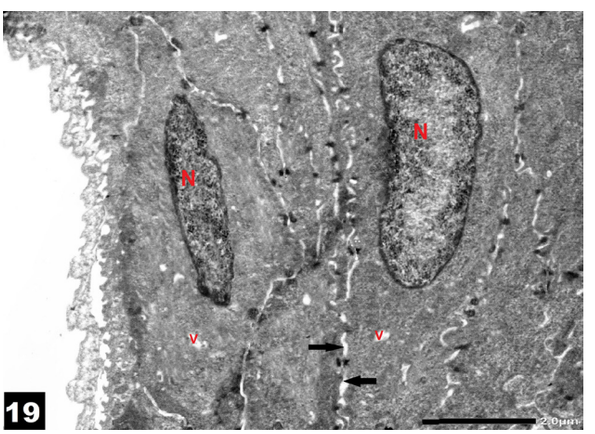


Fig. 19: An ultrathin section in the cornea of rat from Pembrolizumab and axitinib-treated group showing the superficial and intermediate epithelial cells of corneal epithelium, having regular euchromatic nuclei (N). Few focal cytoplasmic vacuolation (V) and slight widening of intercellular spaces (arrow) are present. (TEM X 4000)

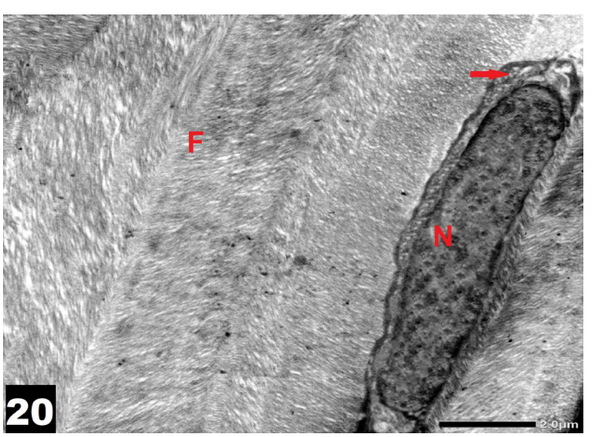


Fig. 20: An ultrathin section of the cornea of a rat treated with pembrolizumab plus axitinib reveals a stroma with regularly ordered collagen fibers (F) and a spindle-shaped keratocyte with an oval elongated nucleus (N) surrounded by sparse cytoplasm (arrow). (TEM X 4000)

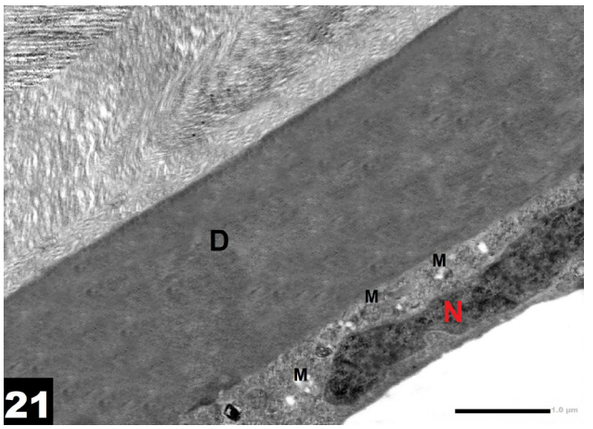


Fig. 21: An ultrathin section of the cornea of a rat treated with pembrolizumab and axitinib reveals stroma with consistently organized collagen fibers (F). Descemet's endothelial layer (D) contains an endothelial cell with an elongated euchromatic nucleus (N).The cytoplasm contains swollen degenerated mitochondria (M). (TEM X 14600)

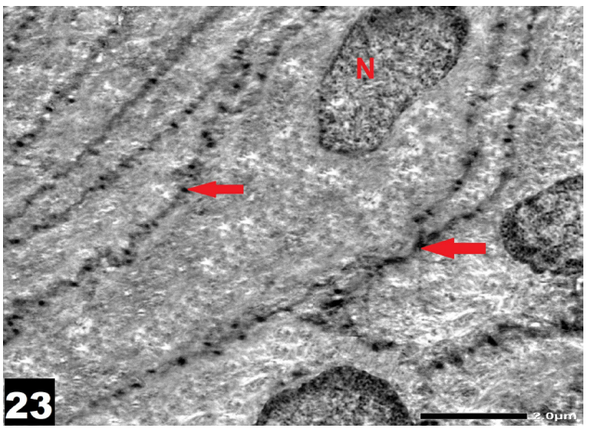


Fig. 23: An ultrathin section in the cornea of rat from pembrolizumab + axitinib and albumin -treated group revealing polygonal cells of the middle layer of corneal epithelium with oval nuclei (N). Numerous electron-dense desmosomes (arrows), connect cells together. (TEM X 4000)

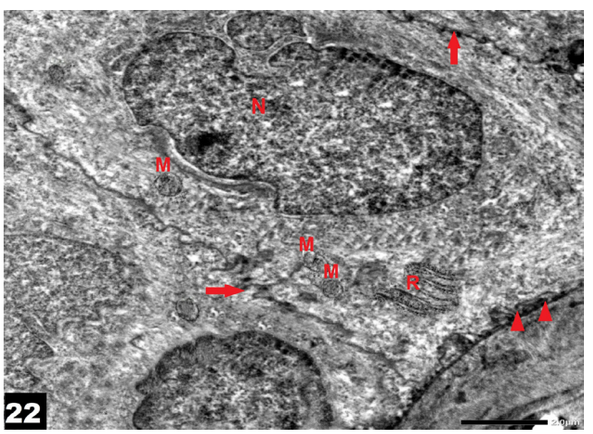


Fig. 22: An ultrathin section of the cornea of a rat from pembrolizumab +axitinib and albumin-treated group reveals basal cells of the corneal epithelium with oval euchromatic nucleus (N), mitochondria (M), and RER(R), are linked to the Bowman's membrane by hemidesmosomes (arrow head). The cells are linked together by several electron-dense desmosomes (arrow). (TEM X 4000)

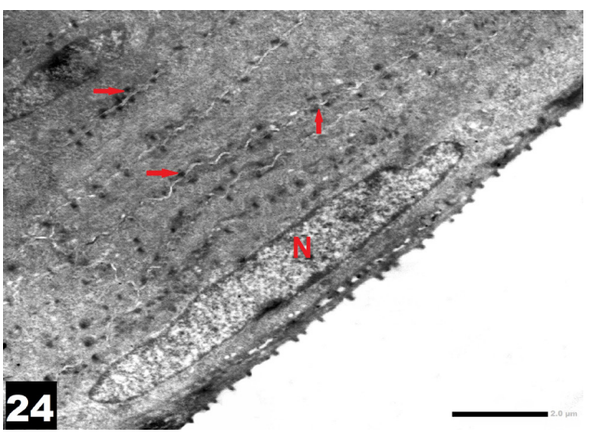


Fig. 24: An ultrathin section of the cornea of a rat treated with Pembrolizumab + axitinib and albumin reveals squamous cells of the superficial layer of corneal epithelium with flat nuclei (N). Multiple desmosomes connect the cells (arrows).(TEM X 4000)

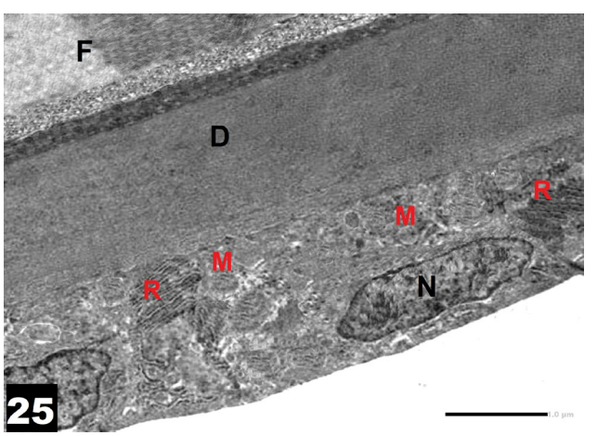
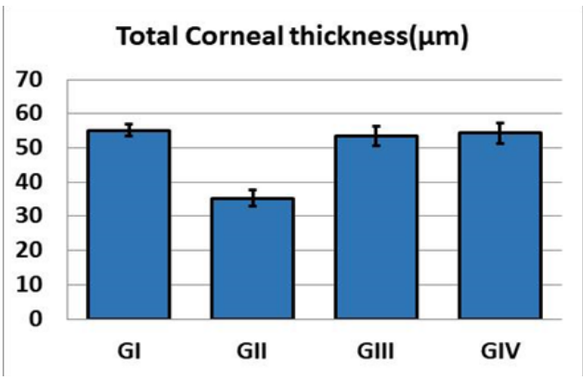


Fig. 25: An ultrathin section of the cornea of a rat treated with Pembrolizumab + axitinib and albumin reveals a typical homogenous Descemet's membrane (D) with an oval nucleus of endothelial cell (N). RER(R) and mitochondria are found in the cytoplasm (M).Regularly arranged collagen fibers(F) was observed. (TEM X 14600)

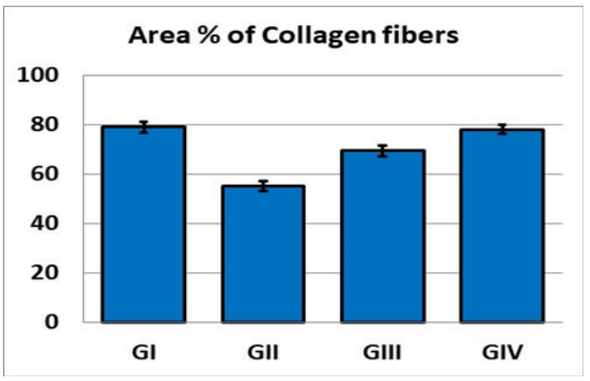
Table 1: Morphometric and Statistical analysis (The mean total corneal thickness, the mean corneal epithelial thickness, the mean area percentage (%) of collagen, and the optical densities of Caspase-3, TNF-α and VEGF immunoreactivities)

	$\bar{X} \pm SD$				*P-Value*
	GI	GII	GIII	GIV	
Total corneal thickness (µm)	55.2 ±1.8	35.3 ±2.35	55.4 ±2.83	54.3 ±2.9	P1= 0.0001 P2= 0.09 P3= 0.23 P4= 0.47
Total epith thickness (pixels)	115.8 ±2.4	72.9 ±3.1	112.2 ±4.66	114.4 ±2.1	P1= 0.0001 P2= 0.04 P3= 0.21 P4= 0.14
Area % of Collagen	79 ±2.2	55.1 ±1.97	69.5 ±2.17	78.1 ±1.8	P1= 0.0001 P2= 0.0001 P3= 0.07 P4= 0.0001
Optical density of Caspase-3	24.2 ±2.8	64.8 ±8.5	27.1 ±4	24.4 ±2.9	P1= 0.0001 P2= 0.003 P3= 0.7 P4= 0.002
Optical density of TNFα	12.5 ±1.26	26.6 ±4.76	16.1 ±2.23	12.8 ±1.23	P1= 0.0001 P2= 0.0001 P3= 0.59 P4= 0.0004
Optical density of VE GF	10.1 ±1.37	27.6 ±1.26	15.1 ±1.2	10.7 ±1.16	P1= 0.0001 P2= 0.0001 P3= 0.35 P4= 0.0001

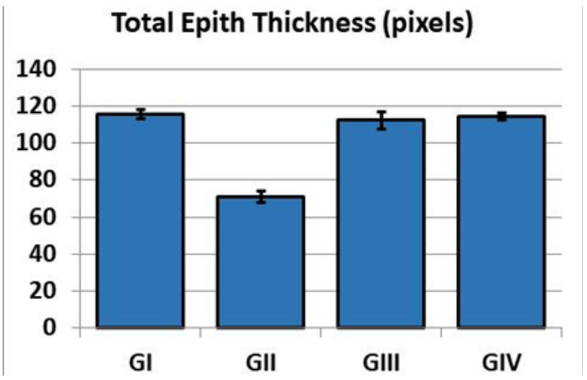
$\bar{X}$= the mean value. SD= the standard deviation.
P1= Group 2 compared to control
P3= Group 4 compared to control
Significant = (Pvalue≤0.001).
P2= Group 3 compared to control
P4= Group 5 compared to group 3



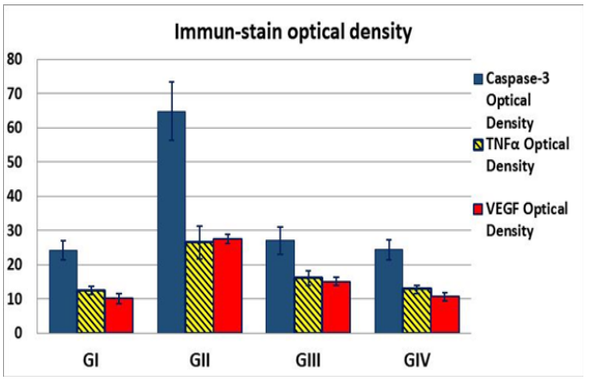
Histogram 1: Total corneal thickness (µm)



Histogram 3: Area percentage (%) of collagen fibers



Histogram 2: Total epithelial thickness (Pixel)



Histogram 4: Immun-stain Optical density

DISCUSSION

Targeted therapy and immune checkpoint inhibitors are frequently the final hope for individuals with advanced cancer. With increasing patient exposure to immunotherapy, the nature and degree of immune-related adverse reactions become more defined[24]. Pembrolizumab is a relatively recent biological medication and despite its efficiency, it requires close monitoring for safety[25]. To our knowledge, no adequate research on pembrolizumab's harmful effects on the cornea has been documented.

In the present study, histological changes accompanying treatment with pembrolizumab resulted in a significant apparent decrease in epithelial and total corneal thickness. The epithelial cells showed vacuolated cytoplasm, massively damaged mitochondria, pyknotic nuclei with more condensed chromatin and cytoplasmic vacuoles. Desmosome connections were largely disrupted, resulting in an increase in intercellular space. The superficial corneal epithelium exhibited localized loss and desquamation. Regarding the stroma, there was an evidence of irregularly spaced collagen fibers, neovascularization, and cellular infiltration with eosinophils, mast cells, and lymphocytes. Keratocytes were characterized by shrunken nuclei and cytoplasmic vacuoles and were entirely separated from collagen fibers, as well as an uneven endothelium surface and damaged karyolitic endothelial nuclei. The endothelial cells had degenerated mitochondria and a thin Descemet's membrane.

These findings corroborated those of Hsiao *et al*.,[10], who reported bilateral eye discomfort, congested conjunctiva, severe corneal erosions, and epithelial abnormalities in patients treated with pembrolizumab. According to another studies[26,27], pembrolizumab has been associated with uveitis, dry eye syndrome, ocular myasthenia, and inflammation of the eye. Weng and his colleagues[28] and other researchers[29] reported a deep corneal ulcer with stromal breakdown and a shallow ulcer in the opposite eye with a massive stromal cellular infiltrate in a patient treated with pembrolizumab.

The most often documented corneal adverse effect of Checkpoint inhibitor immune therapy is dry eye, which manifests as generic eye irritation and can be severe enough to result in corneal perforation[30]. There were also reports of orbital inflammation, episcleritis, blepharitis, peripheral ulcerative keratitis linked with pembrolizumab treatment[31]. Although data are scarce, a risk of ocular toxicity has been documented clinically[32] as patients treated with pembrolizumab suffered from ocular erosions[33]. The programmed death protein-1 is a crucial biological molecule that contributes to the protection of ocular tissues against T-cell-mediated injury[34]. Pembrolizumab is a monoclonal antibody that targets lymphocytes' programmed cell death protein 1 (PD-1) receptor. It specifically targets and inhibits the PD-1 protein on lymphocyte surface. By inhibiting PD-1, T lymphocytes are induced to seek out and destroy cancer cells. Pembrolizumab stimulates the immune system and can result in autoimmune-like symptoms[35]. Immune-related adverse effects are distinct toxicities generated by innate immunity activation. They can impact virtually any body organs. It was described in several trials as a secondary effect of immune checkpoint inhibitor (ICI) therapy[36]. Pembrolizumab's toxicity is dose-dependent, with a larger risk of adverse events at higher dose levels[37]. Parker and colleagues[38] described an ulceration of the cornea associated with nivolumab that seems to be immune-mediated, reflecting an inflammatory pathophysiology.

Inflammatory conditions, keratitis, and autoimmune diseases all played a significant part in neovascularization of the cornea. Corneal epithelium and endothelium, and also inflammatory cells can release angiogenic cytokines such as vascular endothelial growth factor (VEGF) during inflammation. VEGF stimulates endothelial cell matrix metalloproteinase production that promotes vascular endothelial cell proliferation and migration into the corneal stroma and encourages blood vessel development[39]. Following epithelial ulceration, stromal infiltration by polymorph nuclear and lymph mononuclear cells occur producing collagenases and proteases, resulting in destruction of the corneal stroma. Neutrophils, mast cells, and eosinophils have been identified[40].

Significantly greater positive caspase-3, TNF-α, and VEGF immune response in corneal epithelium, keratocytes, and endothelial cells were detected in the pembrolizumab treated group of the current study. Others[27] confirmed the findings, reporting that pembrolizumab treatment resulted in ocular damage via eye dryness, which resulted in devitalized epithelial cells. Dry eye was reported to develop corneal damage[30]. PD-1 is necessary to protect corneal tissues from T-cell-mediated injury because it promotes T cell death in the cornea. By inhibiting PD-1, pembrolizumab suppressed the corneal immune system, allowing activated T cells expressing Fas to penetrate the cornea. Fas Ligand (FasL) and Fas are TNF-receptors and TNF family members, respectively. Corneal cells, notably the endothelium and epithelium, express FasL, a Fas receptor molecule. When Fas is ligated with FasL, a caspase cascade is initiated, resulting in initiation of apoptosis[41]. A number of disparate cells, including macrophages, T-cells, fibroblasts, as well as corneal endothelium, release VEGF family proteins[42]. The expression of the PD-L1 protein was reported to be substantially linked with the expression of VEGF[43]. Another study discovered a link between the levels of PD-L1 and VEGF in tumor tissue[44]. Tumor necrosis factor-α(TNF-α) is primarily produced in response to inflammatory processes by activated macrophages, T lymphocytes, and natural killer cells[45]. TNF- α is also produced by dry eyes[46].

Axitinib inhibits the tyrosine kinase receptors VEGFR-1-3 selectively (vascular endothelial growth factor receptor)[47]. Pembrolizumab plus axitinib-treated group demonstrated improvement of pathological changes that were essentially identical to those observed in the

control group. While some stromal fibers remained widely apart, their organization was more or less uniform. The group's ultrastructure was more or less normal. Caspases-3 expression was moderate in the corneal epithelium, together with a week keratocyte reaction, mild TNF- α and negative immunoexpression of VEGF in the corneal epithelium.

Axitinib topical therapy expedited epithelial repair and resulted in a considerable increase in both epithelial thickness and overall corneal thickness, which was comparable to pembrolizumab. These findings corroborated prior findings that anti-inflammatory effect of axitinib was demonstrated by reducing infiltrating cells, increased thickness of epithelium, and total cornea[48].

Due to the fact that inflammation is associated with the angiogenetic pathway[49], we evaluated axitinib's anti-inflammatory properties, a selective suppressor of VEGFR tyrosine kinases. Riquelme *et al*.[50] found that axitinib suppressed corneal angiogenesis in rabbits at various doses by inhibiting the vascular endothelial growth factor and platelet-derived growth factor (PDGF) pathways. Axitinib treatment led to a decline in VEGF expression. It had a high affinity for VEGFRs (VEGFR-1-3), blocking VEGF signal transmission by adhering to the VEGFR kinase binding site, keeping it in an inactive state. When applied topically, axitinib is an effective therapy for corneal neovascularization. Axitinib's anti-angiogenic activity may be enhanced by suppressing platelet-derived growth factor (VEGFR inhibitor)[51]. Proangiogenic factors such as VEGF, PDGF and TNF-α.Stimulate angiogenesis[52]. Previous research has demonstrated that inhibiting TNF-α can help prevent corneal angiogenesis[53]. As with our findings, Axitinib suppressed proinflammatory cytokines including TNF-α[54]. It has been reported that TNF- α induced apoptosis in corneal fibroblasts[55]. Axitinib had no effect on the potential of the mitochondrial membrane, leading in the initiation or maintenance of a persistent suppression in the G2 cell cycle phase and postponed apoptosis[56].

Albumin functions as a reservoir for therapeutics and other micro molecules. While albumin concentrations in tears are normally low in healthy eyes, they drastically increase in sick eyes[57].

The point of the study was to determine the efficacy of axitinib whether given topically alone or in the presence of significantly increased albumin levels in tears.

When albumin was added to pembrolizumab + axitinib, a histological and ultrastructural profile virtually identical to that of the control group was observed. The epithelium and stroma were found to be normal, with regular collagen bundles and tight gaps, as well as a normal Descemts membrane. Caspase-3, TNF-, and VEGF immunostaining were negative in the corneal epithelium and keratocytes.

Consistent with our findings, a case control report conclusively proven albumin eye drops can aid in the maintenance of the corneal epithelial layer's integrity after corneal ulcer and epithelial loss[15]. Serum albumin eye drops alleviated epithelial lesions in patients with incurable corneal epithelial keratitis[58]. Albumin was effective in treating dry eye by decreasing apoptosis and thereby reducing corneal eroding area[59]. Albumin was able to prevent apoptosis induced by caspase-3 activation in the corneal epithelium scraped off. Albumin eye drops reduced caspase activity and boosted the viability of conjunctival cells in rabbits. Corneal erosions in rabbits healed substantially faster when albumin was used[18]. The antioxidant action of albumin has been established[60]. A pharmacological formulation based on albumin may enhance absorption and antioxidant activity as an efficient eye protectant[61]. Anti-inflammatory effects were possessed by albumin[62]. Albumin inhibited the production of TNF- by pembrolizumab, which corroborated prior study demonstrating that suppressing pro-inflammatory cytokine TNF- alleviates dry eye disease[63].

CONCLUSION

It was concluded that pembrolizumab; which represents the last hope for patients with advanced cancer; induced immune-related corneal adverse effects evident histologically, biochemically, and immunohistochemically. So, it should be used with considerable precaution with regular monitoring of eye. Additionally, axitinib offers hope for reducing pembrolizumab's corneal side effects particularly when co-administered with Albumin. So, it is recommended to use eye drops of combined axitinib and albumin in patients treated with pembrolizumab.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير النسيجي لعقار بيمبروليزوماب على قرنية ذكور الجرذان البيضاء البالغة وفعالية أكسيتينيب المستخدم موضعياً مقابل أكسيتينيب في وجود ارتفاع مستوى الألبومين في الدموع

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مقدمة البحث: أحدثت العلاجات المناعية الموجهة (ICI) ثورة في علاج مجموعة متنوعة من الأورام الخبيثة.و يمكن أن تظهر التأثيرات الجانبية للعلاجات المناعية الموجهة في اعضاء الجسم المختلفة ، بما في ذلك القرنية.

الهدف من البحث: البحث الحالي لتحليل التأثيرات الجانبية لعقار بيمبروليزوماب على القرنية وفعالية أكسيتينيب المستخدم موضعياً وحده أو مع الألبومين.

المواد والطرق: تم تصنيف الجرذان إلى أربع مجموعات: المجموعة الضابطة ، المجموعة المعالجة بيمبروليزوماب (٣ مجم / كجم ، بالحقن البريتونى، ٣ أيام في الأسبوع لمدة ٤ أسابيع)، مجموعة بيمبروليزوماب والاكستينيب (٥مجم / مل نقطتان كل ٨ ساعات لمدة ٤ أسابيع) وبيمبروليزوماب + أكسيتينيب + الالبومين (٥٪ من ألبومين الدم). تم تخدير الحيوانات والتضحية بها في نهاية الدراسة (٤ أسابيع). تمت إزالة كلتا عينى الحيوانات لإجراء التحليلات النسيجية والكيمياء المناعية والمجهر الإلكتروني والدراسات الشكلية المورفومترية.

النتائج: نتج عن علاج بيمبروليزوماب انخفاض واضح في سمك القرنية الكلي وسمك الخلايا الطلائية. كما نتج فقدان الخلايا الطلائية للقرنية وتقشرها، وكذلك وجود فجوات سيتوبلازمية وانكماش انوية الخلايا. كما انخفضت نسبة ومساحة الكولاجين الذى اصبح غير منتظم ومتباعد. كما ظهرت الأوعية الدموية بالقرنية والتسلل الخلوي، بالإضافة لتلف خلايا بطانة القرنية وعدم استواءها وكشفت التغيرات الهيكلية الفائقة في خلايا القرنية الطلائية عن وجود ميتوكوندريا تالفة مع نوى متجمعة وفجوات حشوية. تعطلت اتصالات الخلايا عن طريق الدسموسوم في غالبية الخلايا، مما أدى إلى زيادة في الفراغات بين الخلايا. ووجدت ألياف الكولاجين مفصولة على نطاق واسع عن الخلايا القرنية التي تحتوي على أنوية منكمشة وسيتوبلازم مفرغ، كما لوحظ تسلل للخلايا الحمضية، والخلايا البدينة، والخلايا الليمفاوية، وكذلك وجود أوعية دموية. وظهرت الخلايا البطانية بها تنكس بالميتوكوندريا وغشاء ديسيميت الرقيق.كما لوحظ وجود استجابة مناعية إيجابية كبيرة لـ caspase-3 وTNF-α وVEGF في الخلايا الطلائية والخلايا القرنية والخلايا البطانية للقرنية. خفف أكسيتينيب من التغيرات النسيجية للعين التي يسببها بيمبروليزوماب. استخدام الألبومين مع الاكسيتينيب ادى الى تحسن النتائج.

الخلاصة: على الرغم من أن أكسيتينيب قد ثبت أنه يخفف من تلف القرنية المرتبط بالاعراض الجانبية المرتبطة بعقار بـبيمبروليزوماب، فإن الاستخدام المشترك للألبومين مع الاكستينيب قد عزز هذه الاستجابة.