Investigation of the effects of intracameral carbachol on oxidative stress and apoptosis in rat corneas

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Abstract

Purpose: Our study aims to investigate the effects of intracameral carbachol on oxidative stress and apoptosis in rat corneas.

Methods: In this experimental study, 28 Wistar albino male rats were used. There were 9 rats in the carbachol group, 9 in the balanced salt solution (BSS) group, and 10 in the sham group. The carbachol group was injected with 0.01 cc of carbachol, and 0.01 cc of BSS was injected intracamerally into the BSS group. No drug was injected into the sham group, but the anterior chamber was entered with an empty syringe. Blood serum samples and corneas of rats were taken 1 week later. Caspase-3 and caspase-8 were immunohistochemically examined in rat corneal endothelial to investigate apoptosis. To determine oxidative stress in corneal endothelial tissue and serum, total antioxidant status (TAS), total oxidant stress (TOS), oxidative stress index (OSI), arylesterase (ARES), and paraoxonase (PON) levels were measured.

Results: The mean OSI values in the rat serum of the carbachol group were significantly lower than those of the sham and BSS groups (P = 0.05 and P = 0.004, respectively). The mean TOS value of the carbachol group's rat serum was significantly lower than the mean of the BSS group (P = 0.001). There was no significant difference in the mean TAS, TOS, OSI, ARES, and PON levels from the rat cornea of the carbachol, BSS, and sham groups (P > 0.05). All corneas in the carbachol group were caspase-3 negative, and a statistically significant difference was found among the sham, BSS, and carbachol groups (P = 0.002). No significant difference was observed among the sham, BSS, and carbachol corneas in caspase-8 staining (P = 0.094).

Conclusion: Our study demonstrates that intracameral carbachol is a safe intracameral drug. Carbachol reduced oxidative stress in rat serum compared to rats injected with BSS and the sham group. In addition, carbachol did not increase oxidative stress in rat corneas compared to the BSS and sham groups. Similarly, immunohistochemical examination showed that carbachol did not increase apoptosis in rat corneas and had a protective effect.

Keywords: Apoptosis; carbachol; Caspase-3; Caspase-8; cornea; oxidative stress.
Eye and cataract surgeries are among the most frequently performed surgeries worldwide. Intracameral drugs (e.g., antibiotics, anesthetics, mydriatics, and miotics) are commonly used during phacoemulsification surgery. In addition to their beneficial effects, these drugs used during surgery can cause anterior chamber reactions, increased intraocular pressure (IOP), corneal endothelial damage, and macular edema. Carbachol is a choline ester, a positively charged quaternary ammonium compound that is used to stimulate muscarinic and nicotinic receptors. Carbachol has a parasympathomimetic effect by binding to muscarinic acetylcholine receptors in the eye. The main parasympathomimetic effects in the eye are miosis and increased aqueous humor outflow.[1] Ideal miotics should not be toxic to the corneal endothelium. Intracameral carbachol (Miostat, Alcon Laboratories Inc., USA) is primarily used to induce miosis during cataract surgery. Intracameral carbachol helps stabilize the intraocular lens, provides post-operative IOP control, and reduces iris-related complications through miosis.[2] The cornea is a transparent structure that protects the eye from external injuries and infections. It also allows us to see clearly. Toxicity of corneal endothelial cells can lead to vision loss and blindness due to corneal edema. Free radicals damage essential cellular structures, such as proteins, lipids, and DNA. The body's total oxidant and antioxidant status levels can be easily and reliably measured.[3]

Apoptosis is programmed cell death that allows for the destruction of damaged cells in living organisms without harming other cells in the environment. Trauma induces apoptosis, displacing keratocytes, fibroblasts, and myofibroblasts. Apoptosis is essential for the proliferation, development, hemostasis, and wound healing of corneal tissue. The cells around the wound proliferate irregularly, leading to the accumulation of a fibrotic extracellular matrix. This extracellular matrix seals the wound site, protecting the underlying tissue from infection. Since apoptosis plays a role in the pathogenesis of cataracts, glaucoma, retinal detachment, keratoconus, and some eye diseases in ophthalmology, understanding the mechanisms that cause and control apoptosis can contribute to treatment efforts.[4]

Free radicals are responsible for many injuries and induce oxidative stress and apoptosis. There are many oxidant and antioxidant substances in the body. Measuring oxidant and antioxidant molecules separately is not practical. Calculating the total oxidant–antioxidant status is easier and more reliable than calculating oxidant and antioxidant substances separately. Our study investigated the effect of intracameral carbachol immunohistochemically and pathologically. For this purpose, the total antioxidant status (TAS), total oxidant stress (TOS), oxidative stress index (OSI), arylesterase (ARES), and paraoxonase (PON) levels were investigated in rat serum and corneas. Apoptosis was analyzed through immunohistochemical examination of caspase-3 and caspase-8 in rat corneal endothelial.[5] In the literature, a few studies have investigated the anatomical, functional, specular microscopic, and physiological effects of intracameral miotic agents on the cornea. In these studies, intracameral carbachol was found to be an anatomically and physiologically safe drug on the corneal endothelium.[6-9]

Materials and Methods
Experimental Groups
Thirty adults male Wistar albino rats, 4–8 weeks old, and 180–200 g were used in our study. Three groups were formed: Sham, balanced salt solution (BSS), and carbachol. At first, there were 10 rats in each group. However, two rats were found dead in their cages: One rat in the BSS group on the 2nd day and one in the carbachol group on the 4th day. The study continued with 10 rats in the sham group, 9 in the carbachol group, and 9 in the BSS group. The rats were housed in standard cages containing up to five rats each at room temperature for 12 h during the day and 12 h at night. All experiments in this study were carried out according to the Laboratory Animal Care Principles approved by the Human and Animal Research Ethics Committee of Dicle University, Diyarbakır, Türkiye.

Intracameral Injection and Surgical Technique
All instruments were sterilized for this experimental study. For anesthesia, a mixture of 0.13 cc (13 mg/kg) xylazine (Rompun 2%, Leverkusen, Bayer AG, Germany) and 0.87 cc ketamine (Ketalar, Pfizer Ilaç, Istanbul, Türkiye) was used. Approximately 0.33 cc of intraperitoneal anesthesia was administered to each rat. The injections were administered using an operating microscope. The integrity of the rat eye was preserved during the application of BSS and carbachol to the anterior chamber. Intracameral drugs were distributed from the limbus at 12 o’clock with a 30-gauge insulin injector (Ayset Medikal Ürünleri Sanayi A.Ş., Adana, Türkiye) (Fig. 1). No drug was administered to the sham group, but the anterior chamber was entered with an empty syringe. The BSS group was administered 0.01 cc of BSS (Alcon Laboratories, Inc., Texas, USA). The drug group was administered 0.01% carbachol (Miostat, Alcon Laboratories Inc., Texas / USA). On the 7th day, under intraperitoneal anesthesia, the corneas of the rats were removed by making a
limbal incision with a 15° corneal knife. The samples were sent to the laboratory for histopathological and biochemical examinations. The abdomen was reached with a vertical 3 cm skin incision and a subcutaneous incision from the inguinal region. A blood sample of 1.5–2 cc was collected from the inferior vena cava. Decapitation of all rats was then performed. Pathological samples were transported in a container of 10% formaldehyde, and biochemical samples were transported in a dry transport container and stored at −70°C. The immunohistochemical method investigated apoptosis by staining the corneal endothelium with caspase −3 and caspase −8. TAS, TOS, OSI, PON, and ARES levels of oxidative stress factors were measured in the cornea and blood samples.

**Caspase-3 and Caspase-8 Staining**

Cornea tissue was painted with a standard streptavidin-biotin immunoperoxidase method and 4 mm paraffin blocks with caspase-3 antibodies (split; catalog no. PP 229 AA, Biocare Medical, Concord, CA, USA) (Fig. 2) and caspase-8 antibodies (clone: C502S catalog no. GTX59555, Gene Tex, Irvine, CA, USA) (Fig. 3). All samples were evaluated under a light microscope (Olympus Corp., Tokyo, Japan, Olympus BX51TF). Immunohistochemically stained materials were considered semiquantitatively (Table 1).

**TAS Measurement**

TAS was measured automatically using a new method developed by Erel using commercially available kits (Rel Assay Diagnostics Kit; RL0017, Mega Tip, Gaziantep, Türkiye). In the Erel method, potent biological and hydroxyl radicals are produced by mixing a solution of iron ions in Reagent 1 with hydrogen peroxide in Reagent 2. In this method, the Fe2+-o-dianisidine complex reacts with hydrogen peroxide, such as Fenton, to form an OH radical. The OH radical reacts with the o-dianisidin molecule at a low pH to form brown–yellow dianicidyl radicals. Participation of the formed dianicidyl radicals in oxidation reactions causes color formation. Antioxidants prevent these oxidation reactions and stop color formation. The color change was measured on an automatic spectrophotometric analyzer. The results were expressed as nmol Trolox Equiv./mg protein. This method mirrors the total antioxidant effect against strong free radical reactions. The technique has <3% error sensitivity.[10]

**TOS Measurement**

The Erel method uses a colorimetric method based on oxidizing iron ions in samples into ferric ions through oxidant molecules. TOS levels were measured with Özcan Erel kits (Rel Assay Diagnostics Kit; RL0024, Mega Tip) using the Erel method. The results were written as μmol H2O2 Equivalent/L.[11]

**OSI Calculation**

OSI was expressed as a percentage of TOS to TAS:

<table>
<thead>
<tr>
<th>Caspase staining assessment</th>
<th>Negative staining</th>
<th>Poor staining</th>
<th>Medium staining</th>
<th>Severe staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative staining</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Poor staining</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Medium staining</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Severe staining</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>
Calculation of OSI values according to TOS and TAS values.

**PON Level Measurement**

PON is a lipophilic, hydrophobic, and antioxidant enzyme associated with high-density lipoprotein cholesterol. The PON activity was measured using the Rel Assay Diagnostics Kit (RL0031, Mega Tip). The PON enzyme hydrolyzes the paraoxon (O, O-diethyl-O-nitrophenyl phosphate) substrate and eventually forms a colored p-nitrophenol product. The absorbance of the formed colored p-nitrophenol was measured at 412 nm in kinetic mode, and the enzyme activity was expressed as U/L. [12]

**ARES Level Measurement**

Serum ARES activity was measured with the Rel Assay Diagnostics Kit (RL0055 Mega Tip). The serum ARES level was determined by measuring the phenol level formed after the phenylacetate reaction. The molar extinction coefficient of phenol was 4000 M-1 cm-1. The results were expressed as kU/L. [13]

**Statistical Analysis**

Statistical analyses were performed using the SPSS version 18 (SPSS Inc., Chicago, IL, USA) computer program. Numerical data related to oxidative stress were given as the mean (maximum-minimum value). Oxidative stress groups were compared using a One-way analysis of variance test. Comparisons of caspase-3 and caspase-8 levels between groups were performed with the $\chi^2$ test. P < 0.05 was accepted as statistically significant. P < 0.01 was considered statistically significant, while P < 0.001 was considered statistically significant.

**Results**

A total of 30 Wistar albino rats, 4–8 weeks old, and 180–200 g were used in our study. The number of subjects each in the sham, BSS, and carbachol groups was randomly determined as n = 10. However, two rats were found dead in their cages: One rat in the BSS group on the 2nd day and one rat in the carbachol group on the 4th day. Thus, our study was completed with 28 rats.

**Findings Related to Oxidative Stress in Rat Serum**

OSI values in the rat serum of the carbachol group were significantly lower than those of the sham and BSS groups (P = 0.05 and P = 0.004, respectively). In the rat serum, the TOS values of the carbachol group were statistically lower than those of the BSS group (P = 0.001). No statistically significant difference was found in the rat serum between the carbachol and BSS groups’ TAS, PON, and ARES values. In addition, no statistically significant difference was found between the carbachol and sham groups’ TAS, TOS, OSI, ARES, and PON values (Table 2).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham (n=10)</th>
<th>BSS (n=9)</th>
<th>Carbachol (n=9)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAS (mmolTrolox*equiv/L)</td>
<td>0.075 (0.05–0.1)</td>
<td>0.09 (0.02–0.16)</td>
<td>0.075 (0.05–0.1)</td>
<td>0.954</td>
</tr>
<tr>
<td>TOS (mmol H2O2equiv/L)</td>
<td>8.65 (7.05–10.25)</td>
<td>7.75 (5.32–10.19)</td>
<td>8.37 (5.78–10.97)</td>
<td>0.256</td>
</tr>
<tr>
<td>OSI (Arbitrary Unit)</td>
<td>11.46 (7.19–15.73)</td>
<td>18.45 (4.10–32.8)</td>
<td>12.57 (7.55–17.59)</td>
<td>0.273</td>
</tr>
<tr>
<td>PON (unit/litre U/L)</td>
<td>3.80 (2.01–5.6)</td>
<td>3.41 (0.57–6.26)</td>
<td>4.05 (1.9–6.2)</td>
<td>0.691</td>
</tr>
<tr>
<td>ARES (U/L)</td>
<td>4.05 (1.9–6.02)</td>
<td>3.45 (2.1–4.8)</td>
<td>4.46 (3.08–5.85)</td>
<td>0.051</td>
</tr>
</tbody>
</table>

TAS: Total antioxidant level; TOS: Total oxidant level; OSI: Oxidative stress index; PON: Paraoxonase and ARES: Arylesterase. Mean (minimum-maximum).
Findings Related to Oxidative Stress in Rat Cornea
No statistically significant difference was found in the TAS, TOS, OSI, PON, and ARES levels in the corneas of the three rat groups (Table 3).

Findings Related to Apoptosis in Rat Cornea
In the carbachol group, all rat corneas were stained caspase-3 negative. A high statistically significant difference was found between the carbachol group and the sham and BSS groups (P = 0.002) (Table 4). No statistically significant difference was found among the carbachol, sham, and BSS groups regarding caspase-8 staining (P = 0.094) (Table 5).

Discussion
All intracameral drugs can cause anterior chamber reactions, increased IOP, corneal endothelial damage, and macular edema. Ideal miotics should not be toxic to the corneal endothelium. Many studies have been conducted on the effect of intracameral carbachol on post-operative IOP, but limited studies have focused on the toxic effects of intracameral carbachol on the eye. The effects of intracameral miotic agents on the cornea have been investigated anatomically, functionally, specular microscopically, and physiologically. In these studies, intracameral carbachol was found to be an anatomically and physiologically safe drug for use on the corneal endothelium. In our study, carbachol did not increase oxidative stress and apoptosis.

Demir et al. showed that intracameral carbachol used during phacoemulsification in 96 patients did not affect the anatomical macular thickness. Liou et al. investigated the effect of intracameral lidocaine and carbachol on the corneal endothelial in rabbits using specular microscopy. They found that intracameral carbachol did not damage the rabbits’ corneal endothelium. Vaughn et al. investigated the effects of 1% acetylcholine chloride and 0.01% carbachol on the corneal endothelial of rabbits under a specular microscope. Under a specular microscope, corneal endothelial cells exposed to 1% acetylcholine for 15 min did not show any anatomical or physiological changes. Corneal endothelial cells exposed to 0.01% carbachol for 15 min showed changes in physiology during the 1st h and then returned to normal. No cellular changes were observed in the electron microscope. Vaughn et al. found that carbachol had no effect on corneal thickness.

Olson et al. showed that carbachol and commonly used intraocular medications had no significant effect on specular microscopy and the corneal endothelial in cats. Akiyama et al. investigated the effects of acetylcholine, carbachol, and mannitol on corneal endothelial function in rabbits. They found that carbachol caused a small increase in corneal thickness, while acetylcholine reduced corneal thickness. In their study, they found that acetylcholine is harmless to the endothelium, while carbachol has a harmful effect. In another study, Akiyema et al. examined the effects of carbachol and acetylcholine on corneal endothelial pumping activity in rabbits. They found that 1% acetylcholine stimulated the endothelial electrical potential difference, and the addition of 0.003% carbachol abolished the stimulus effect of acetylcholine.

Yee et al. investigated the in vitro effects of acetylcholine and carbachol on bovine corneal endothelial cells. In their study, bovine endothelial cells exposed to acetylcholine for 30 min experienced necrosis and degeneration, while those treated with carbachol did not show any morphological changes. Roszkowska et al. investigated the effect of intracameral acetylcholine and carbachol on extracapsular cataract extraction. They found no change in corneal thickness but found a statistically significant decrease in the number of endothelial. Using a specular microscope, Birnbaum et al. showed that perfusion with 0.01% carbachol for 15 min caused a significant increase in corneal thickness, and reperfusion with Ringer’s bicarbonate solution for 75 min caused a return to normal values. Yee et al. found that 0.01% carbachol chloride is less toxic to the corneal endothelium than 1% acetylcholine chloride. In another study, epinephrine and benzalkonium chloride were found to be toxic to the corneal endothelium, while intraocular miotic agents were found to be non-toxic.

Table 4. Caspase-3 staining results

<table>
<thead>
<tr>
<th>CASPASE-3</th>
<th>Sham</th>
<th>BSS</th>
<th>CARBACHOLA*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative staining (-)</td>
<td>5</td>
<td>6</td>
<td>9</td>
<td>P=0.002</td>
</tr>
<tr>
<td>Poor staining (+)</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Medium staining (+++)</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Severe staining (+++)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

A: a significant difference was found between the Carbachol and Sham groups in terms of caspase-3 staining in the χ² test (P=0.033). *: P<0.05, **: P<0.01, ***: P<0.001. BSS: Balanced salt solution.
In the literature, the effect of topical parasympathomimet- 
cics on corneal epithelial healing in rabbits was investigated.  
The findings showed that cholinergic agents, especially  
acetylcholine and pilocarpine, may have an important  
therapeutic role in the treatment of severe corneal epithe-

tial damage.[22] Our study determined that the OSI values  
in the rat serum of the carbachol group were statistically  
lower than in the sham and BSS groups, and the TOS val-

ues were statistically lower than in the BSS group. The  
lower serum TOS and OSI values in the carbachol group  
compared to the BSS group may be due to reasons such  
as decreased IOP of carbachol and reduced pupil diameter.  
All rat corneas were stained caspase-3 negative in the car-
bachol group, and a statistically high difference was found  
between the sham and BSS groups. This result shows that  
carbachol may have a protective effect on the corneal  
endothelium. The limitation of this study is that the study  
was conducted on a small number of rats, and it is not known  
by which mechanism carbachol may have a protective  
effect on the corneal endothelium and rat serum. This pro-
tective effect may be caused by carbachol-causing miosis,  
thus decreasing IOP or increasing aqueous flow in the post-
operative period.

**Apoptosis and Caspases**

Apoptosis plays a critical role in corneal tissue proliferation  
and wound healing. Cell death occurs in two different ways:  
apoptosis and necrosis. After cell death, unwanted or dam-
aged cells must be removed. Apoptosis is programmed cell  
death in living organisms that eliminates cells that have  
completed their task or have been damaged without dam-
aging other cells and structures. Apoptosis can be mea-
sured using biochemical, morphological, and molecular  
changes. Corneal wound healing begins because of trauma,  
including to the epithelium, endothelium, and stroma. In  
cases of epithelial abrasions, minor ocular surgery, or lim-
ited infection, limited keratocyte apoptosis occurs, and the  
epithelium or endothelium self-regenerates.[23] Apoptosis  
in the cornea is a complex event that restores corneal trans-
parency. Apoptotic changes in keratocytes can be detected  
by electron microscopy.[24]

Cytokines are essential in cell migration, proliferation, dif-
ferentiation, and fibrosis. The basement membrane was  
best detected by electron microscopy or immunohisto-
chemical staining.[25] One well-known biochemical fea-
ture of apoptosis is the activation of cysteine proteases.  
Caspases (cysteine-aspartic proteases) are proteolytic en-
zymes that control inflammation and death in the cell. Cas-
pases play an essential role in apoptosis. There are many  
types of caspases, such as caspases 1–16. The detection of  
caspase-3 and caspase-8 in cells is a necessary method for  
the detection of apoptosis.[26,27]

**Oxidative Stress and the Antioxidant System**

The human eye is exposed to oxidative stress due to sun-
light, surgery, or trauma. Oxidative stress is cell and tissue  
damage caused by free oxygen radicals in an organism. Ox-
idative stress plays a key role in many disease processes.  
Oxidative stress can cause apoptosis, necrosis, and cell  
death. As a result of the increase in oxidants, the decrease  
in antioxidants cannot be prevented, and the oxidative-an-
tioxidative balance shifts toward the oxidative direction.  
The tear film, cornea, and aqueous humor play key roles  
in combating free radicals.[11] There are many enzymatic  
and non-enzymatic antioxidants in the cornea.[28] The con-
centrations of different oxidants and antioxidants can be  
measured separately in laboratories, but measuring in this  
way is time-consuming and impractical. The Erel method  
is easy and reliable and can help us automatically calculate  
TOS and TAS.[11]

**Conclusion**

Carbachol reduces oxidative stress in the rat serum com-
pared to the BSS and sham groups. In addition, carbachol  
did not increase oxidative stress in rat corneas compared  
to the BSS and sham groups. Similarly, immunohistochem-
ical examination showed that carbachol did not increase  
apoptosis in rat corneas and had a protective effect. These  
results show us that carbachol is a safe drug for the eyes  
and has a protective effect.

**Ethics Committee Approval:** This study was approved by Dicle  
University Ethics Committee (11.03.2014 date; number 2014/09).

**Peer-review:** Externally peer-reviewed.

**Authorship Contributions:** Concept: Ö.F.Y., A.A., U.Ö.; Design:  
Ö.F.Y., U.Ö.; Supervision: Ö.F.Y., N.U.; Resource: Ö.F.Y., N.U.; Materia- 
als: Ö.F.Y.; Data Collection and/or Processing: Ö.F.Y., S.K.; Analysis  
and/or Interpretation: Ö.F.Y., S.K., N.A.; Literature Search: Ö.F.Y.;  

**Conflict of Interest:** None declared.

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