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**Original Research Article**

# Effect of diagnostic ultrasound on corneal apoptosis in rats

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## Abstract

***Purpose:*** *To investigate the effect of diagnostic ultrasound on corneal apoptosis in rats.*
***Methods:*** *24 male rats were randomly divided into 4 groups: control group, 10, 20 and 30 min group. The eyeballs of rats were irradiated continuously for different time lengths by Siemens ACUSON S2000 color Doppler ultrasound diagnostic instrument. 24 hours later, the animals were killed and the corneas were taken for Tunel apoptosis detection. The apoptosis rates of corneal epithelial cells, stromal cells and endothelial cells were calculated.*
***Results:*** *Apoptotic cells were detected in corneal epithelial cells, stromal cells and endothelial cells of normal rats. There was no significant difference between the 10 min group and the control group (P>0.05). The apoptosis rate of 20 min and 30 min groups was significantly higher than that of the control group. With the extension of irradiation time, the apoptosis rate of corneal epithelial cells, stromal cells and endothelial cells increased.*
***Conclusion:*** *20 min of rat eyeball irradiated by diagnostic ultrasound can increase the apoptosis of corneal cells, and the apoptosis is aggravated with the prolongation of ultrasound irradiation time.*

***Keywords:*** *Cornea; Ultrasonography; Apoptosis; Epithelial cells*



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## INTRODUCTION

Ultrasonic diagnosis has been used in China for many years, and the field of application and the level of diagnostic technology have been continuously improved with the development of science and technology. The main principle of ultrasonic diagnosis is to observe different ultrasonic reflections from different tissue structures of the human body. Observe and analyze by transforming abstract reflected waves into concrete images [1-2]. Ultrasound was the first to be used in A-ultrasound. With the development of technology, B-ultrasound was invented in 1950. At present, the widely used B-ultrasound examination also has some shortcomings. Some studies have shown that the clarity and resolution of B-ultrasound results need to be improved. The moving probe and dynamic human tissue, as well as the poor conduction of ultrasound in the air, make it difficult to guarantee good image results. And poor image results bring some difficulties for clinical diagnosis [3]. Color Doppler ultrasound is based on the existing black-and-white B-ultrasound plus color Doppler, which can observe

moving objects, and when used in human body, colored blood flow signals can be obtained, which provides a certain basis for improving clinical diagnosis. Ultrasonic diagnostic technology is a non-interventional, economical, practical, repeatable and widely adaptable examination method. Some studies have shown that ultrasonic vibration can change the permeability of cell membrane and improve the state of cell ischemia and hypoxia. However, whether continuous ultrasound irradiation has some damage to the human body remains to be further studied [4-5].

Since the concept of apoptosis was put forward by Kerr, it has become the research focus of tumor etiology and pathology. The understanding of apoptosis has been deepened gradually, and the molecular mechanism of apoptosis has been understood more and more thoroughly. However, it is also found that this process is far from as simple as originally thought, but contains complex regulatory mechanisms [6-8]. It has been reported that ultrasound can inhibit proliferation and promote apoptosis by activating p38 MAPK signal transduction [9]. However, there are still many problems or even key problems that have not been understood. In recent years, remarkable progress has been made in apoptosis signal transduction pathway, biochemical response mechanism of apoptosis and gene regulation of apoptosis.

Some studies have shown that the mechanism of ultrasonic diagnosis may be related to the changes of nuclear size and acoustic characteristics, which are related to cell death, especially the form of apoptosis [10]. Through the preliminary study of the effect of apoptosis on corneal cells caused by ultrasound irradiation time, this experiment preliminarily shows the role of apoptosis in the metabolism of normal cornea and the occurrence and development of some keratopathy, and attempts to study the changes of corneal cell apoptosis after diagnostic ultrasound irradiation in rats in order to find the safe range of diagnostic ultrasound examination.

## EXPERIMENTAL

Experimental animal grouping and model specimen preparation: 24 healthy male SD rats (Shanghai Shrek Animal Experimental Co., Ltd.), aged 28-30 days and weighing 65-85 g, were randomly divided into 4 groups: control group, 10, 20 and 30min groups. The instrument was Siemens ACUSON S2000 color Doppler ultrasound diagnostic instrument, the probe frequency was 7.5MHz, ultrasound output power 6.8mW, and the spatial and time average sound intensity ($I_{SATA}$) was 3.4mW/cm$^2$. In the experiment, the probe was directly contacted with the eyeballs of rats, fixed and irradiated continuously, and the rats were irradiated with 0, 10, 20 and 30min respectively according to different groups. 24 hours after the irradiation, the rats were anesthetized with 3% pentobarbital sodium 40mg/kg intraperitoneally. After anesthesia, the eyeballs of rats were carefully removed and fixed in 4% paraformaldehyde for 48 hours. The fixed tissues were washed with running water for gradient dehydration, and dehydrated in ethanol of concentrations of 50%, 70%, 85%, 95% and 100% successively for 1 hour each time. After dehydration, the tissue was immersed in a 1:1 mixture of ethanol and xylene for 1.5 h, then immersed in a xylene solution to remove ethanol from the tissue, followed by three paraffin waxes for 1 h each. First, add an appropriate amount of melted paraffin wax into the paraffin metal embedding box, then put the eyeballs into the embedding box, and add paraffin liquid to immerse the eyeballs, and place on ice for them to cool and solidify. Turn on the constant temperature water bath pot and set the temperature to 40 ℃, fix the embedded wax piece on the slicer, adjust the thickness of the slicer to 3 μm, uniform speed was required when slicing, and gently clip the cut wax slice into lukewarm water with tweezers, pick up the wax slice with glass slides, gently absorb the excess water with filter paper, and then bake the slides in an oven at 65 ℃ for about 2 hours.

### TUNEL staining

The TUNEL kit is provided by Roche (Roche). The paraffin slices of the above special treatment were dewaxed to water, washed twice with PBS, and then repaired in Proteinase K working solution for 30 minutes at 37 ℃. After antigen repair, 1% TritonTMX-100 was used to penetrate the membrane for 5 minutes, and then washed once with PBS. Seal the wet box with 1% BSA at room temperature for 30 minutes, wash it with PBS for 5 minutes after the seal, add TUNEL-reaction mixture and incubate at 37 ℃ for 1 h. After incubation, wash with PBS for 3 times and stained with DAB. After dehydration and transparency, seal with neutral gum, and the apoptosis of cells could be observed under light microscope. The specimens in the negative control group were only treated with labeling solution without enzyme treatment, and the other steps were the same as above. Apoptosis was observed and counted under light microscope. 10 high power microscopes (400 ×) were selected for each slice, count the numbers of normal cells and apoptotic cells, and calculate the percentage of apoptosis.

### Statistical analysis

The apoptosis rates of rat corneal epithelial cells, stromal cells and endothelial cells were calculated and analyzed by chi-square test. SPSS 22.0 software was used for statistical analysis of the data. The difference was considered statistically significant when $p < 0.05$.

## RESULTS

### Effect of diagnostic ultrasound on apoptosis rate of rat corneal epithelial cells

According to the statistics of the results of TUNEL staining, we found that there were normal apoptotic cells in normal rat corneal epithelial cells, corneal stromal cells and corneal endothelial cells to some extent. After diagnostic ultrasound irradiation, there was a significant difference in the number of apoptotic cells in rat corneal epithelial cells at different irradiation time lengths. The apoptosis rate of corneal epithelial cells was the highest in the 30min group. Of the apoptosis of corneal epithelial cells stained by TUNEL, the statistical results showed that there was no significant difference in the apoptosis rate of corneal epithelial cells between the control group and the 10min irradiation group (P>0.05; see Fig 1). The apoptosis rate of corneal epithelial cells in 20min group and 30min group was significantly higher than that in control group (P<0.01, P<0.001). With the extension of irradiation time, the apoptosis rate of corneal epithelial cells increased significantly.

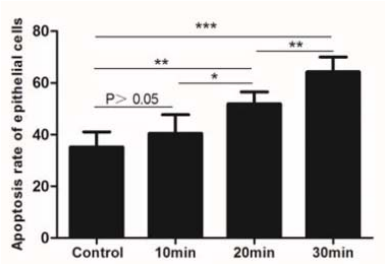


**Figure 1:** Apoptosis rate of corneal epithelial cells in rats exposed to ultrasound at different time points. *$p<0.05$; **$p<0.01$; *** $p<0.001$

### Effect of diagnostic ultrasound on apoptosis rate of rat corneal stromal cells

The apoptosis rate of rat corneal stroma cells (see Fig 2), TUNEL staining results showed that there was also apoptosis in corneal stroma cells under normal physiological conditions, there was no significant difference between the control group and the 10 min group (P>0.05); There was significant difference between the 20min group and the control group (P<0.05). The apoptosis rate of corneal stromal cells in the 30min group was the highest, which was significantly different from that in the control group (P<0.001). With the extension of irradiation time, the apoptosis rate of corneal stromal cells increased significantly.

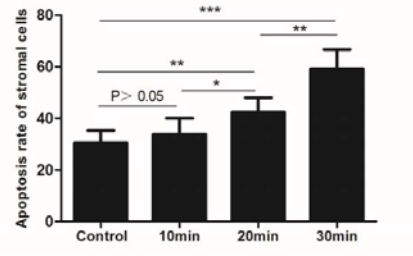


**Figure 2:** Apoptosis rate of corneal stromal cells in rats exposed to ultrasound at different time points. * *$p<0.05$; **$p<0.01$; ***$p<0.001$*

### Effect of diagnostic ultrasound on apoptosis rate of rat corneal endothelial cells

The effect of diagnostic ultrasound on the apoptosis rate of rat corneal endothelial cells (see Fig 3). According to the analysis of the results of TUNEL staining, we found that there was normal apoptosis in rat corneal endothelial cells, and there was no significant difference in the apoptosis rate between the control group and the 10min group (P>0.05). The apoptosis rate of corneal endothelial cells in the 20min group was significantly higher than that in the control group.

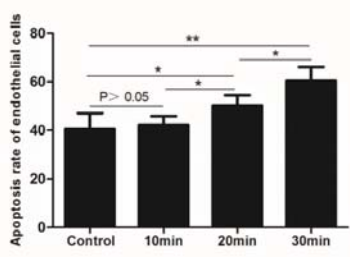


**Figure 3:** Apoptosis rate of corneal endothelial cells in rats exposed to ultrasound at different time points (P<0.05)

The apoptosis rate of corneal epithelial cells in 30min group was significantly higher than that in the control group (P<0.01). With the extension of irradiation time, the apoptosis rate of corneal endothelial cells increased significantly.

## DISCUSSION

Under normal physiological conditions, there is a certain degree of apoptosis in corneal cells. Some studies have shown that corneal epithelial

injury and the reorganization of surrounding keratinocytes are the main reason for inducing apoptosis. Some activated keratinocytes are transformed into myofibroblasts and changed in this process after the fibroblast stage [11]. Extracellular matrix, including collagen, accumulates in keratinocytes, resulting in corneal opacity and increased thickness [12]. Some studies have shown that diagnostic ultrasound irradiation time can increase corneal apoptosis, which may lead to corneal opacity, thinning and bulge [13]. Some studies have shown that the intensity of ultrasound and the duration of irradiation will cause changes in the morphology of corneal tissue, a decrease in the level of corneal collagen, and corneal cells will experience apoptosis for a period of time; injured corneal cells will cause corneal epithelial cell apoptosis due to the accumulation of inflammatory cells, and chronic epithelial cell injury will lead to stroma thinning and epithelial hyperplasia [14-16]. Other studies have shown that ultrasound-induced corneal cell injury is caused by apoptosis; ultrasound-induced free radicals can cause corneal endothelial cell damage [17-19]. Corneal endothelial cells are non-renewable, cells cannot regenerate, and the gap between senescent and apoptotic cells is supplemented by the expansion and migration of other endothelial cells. After the injury, the adjacent endothelial cells enlarged, expanded and migrated to cover the damaged area [20-22]. Our study shows that corneal apoptosis induced by diagnostic ultrasound can be avoided to some extent, and there is no statistically significant difference between the control group and the 10min group. However, the apoptosis rate of corneal epithelial cells increased significantly when the continuous irradiation of ultrasound was longer than 10min.

TUNEL staining is a method used to detect apoptosis. Under pathological stimulation, the DNA of cells is broken, which exposes 3'-OH. Under the action of terminal deoxynucleotidyl transferase, fluorescein labelled dUTP can be added to label apoptotic cells [23]. Through TUNEL staining, we found that normal rat corneal epithelial cells, stromal cells and endothelial cells have typical apoptosis, and there is a typical apoptosis phenomenon in the process of corneal epithelial cell self-metabolism. Our study showed that there was no significant difference in the apoptosis rate between the 10min group and the control group, but there was a significant difference in the apoptosis rate between the 20 min group and the 30min group compared with the control group. The increase of irradiation time of ultrasound diagnostic equipment also increased the apoptosis rate of corneal cells. The eyeball is a very sensitive organ in human tissue. Ultrasonic diagnosis is a diagnostic method in which ultrasonic testing technology is applied to the human body to understand the data and morphology of physiological or tissue structure, find diseases and make hints. It is a non-invasive, painless, convenient, intuitive and effective means of examination, and the influence of ultrasound irradiation is also the focus of attention [24-25]. In this study, we established a rat irradiation model to observe the effect of different irradiation time lengths on the apoptosis rate of corneal cells in rats. The results showed that the apoptosis rate of corneal cells increased with the increase of time after continuous ultrasound irradiation. There was no significant difference between the 10min group and the control group, but after the irradiation time was longer than 10 min, the number of apoptotic corneal cells increased and the apoptosis rate increased, which was higher than that in the control group and 10min group. When ultrasound is used to examine eyeball diseases, the duration of irradiation should be strictly controlled. Long-term continuous irradiation will increase the apoptosis of corneal epithelial cells, stromal cells and endothelial cells, resulting in further eyeball damage.

## CONCLUSION

The results of this study provide a theoretical basis for the standardized use of diagnostic ultrasound in clinical science. They also provide some ideas and directions for follow-up research. However, the mechanism of apoptosis is very complex and the mechanism of corneal apoptosis induced by ultrasound needs further investigation.

## DECLARATIONS

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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