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Protective Effects of *Ganoderma lucidum* on the Changes Made in the Retinal Damage Induced by Traumatic Head Injury

Efectos Protectores de *Ganoderma lucidum* sobre los Cambios Producidos en el Daño Retinal Inducido por Lesión Traumática en la Cabeza

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SUMMARY: Head trauma affects the optic nerve visual function and visual acuity. As a result of head trauma occurring in the retina of the various biochemical, histological and immunohistochemical effects were investigated. The protective effect of *Ganoderma lucidum* was evaluated on the damage to the retina of the rats. Sprague–Dawley rats were subjected to traumatic brain injury with a weight-drop device using 300 g-1 m weight–height impact. Thirty rats were divided into three groups as group 1 control, 2 group trauma, 3 group trauma+*Gonoderma lucidum* (20 mL/kg per day via gastric gavage) *Ganoderma lucidum* was administered for 7 days after trauma.All rats were decapitated 5 days after the induction of trauma, and the protective effects of *Ganoderma lucidum* in retina were evaluated by histological, immunohistochemical and biochemical analyses. The antioxidant effect of *Ganoderma lucidum* on the cellular degeneration extracellular matrix and retinal barrier in retina after head trauma was investigated.

KEY WORDS: Head trauma; Retina; *Ganoderma lucidum*; Rat.

INTRODUCTION

The fungi *Ganoderma lucidum*, a traditional Chinese medicine is recorded in Chinese materia medica treatise Shen Nong Materia Medica. *G. lucidum* spores (GLS) have high concentration of *G. lucidum*, and have been used to strengthen body resistance, enhance and improve function of the nervous system, regulate immune system, stimulate anti-tumor activity, and eliminate fatigue (Zhou *et al*., 2012). Oral administration of *Ganoderma lucidum* has been shown to significantly reduce both cerebral infarct area and neuronal apoptosis in the ischemic cortex (Zhou *et al*.). Recent studies have demonstrated the neuroprotective effect of *Ganoderma lucidum* to reduce oxidative stress *in vitro* (Zhao *et al*., 2005), to induce neuronal differentiation (Cheung *et al*., 2000), and to prevent the harmful effects of the exterminating toxin Ab in Alzheimer's disease in cultured rat neurons (Lai *et al*., 2008). VEGF is known to play a crucial role in the pathogenesis of diabetic macular edema by promoting blood-retinal barrier (BRB) disruption and exacerbated vascular leakage (Zhang *et al*., 2008). A previous study has shown that systemic neutralization of VEGF in mice resulted in substantial neural retinal cell death (Saint-Geniez *et al*., 2008). Damage to the optic nerve (ON) causes immediate shearing of and induces secondary swelling in a proportion of retinal ganglion cell (RGC) axons,accompanied by subsequent RGC degeneration (Sarkies *et al*., 2004). Matrix metalloproteinase-2 (MMP2), one of the most ubiquitous members of the matrix metalloproteinase family, cleaves collagen type IV of the extracellular matrix maintaining equilibrium between matrix synthesis and degradation, thus providing a critical role in the cell integrity and cell survival (Malemud, 2006). Clinical and experimental studies have shown increased expression of MMP2 in diabetic subjects (Das *et al*., 1999). In this study; the antioxidant effect of *Ganoderma lucidum* on the cellular degeneration extracellular matrix and retinal barrier in retina after head trauma was investigated.

MATERIAL & METHOD

The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication no. 85-23, revised 1996). All experimental protocols were approved by the Dıcle University Animal Care and

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Use Committee. Male Sprague-Dawley rats (300–350 g) were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature (23±2 °C) and relative humidity (65–70 %) were kept constant. The animals were anesthetized by an intraperitoneal injection of 5 mg/kg xylazine HCl (Rompun, Bayer HealthCare AG, Germany) and 40 mg/kg ketamine HCl (Ketalar, Pfizer Inc., USA), and were allowed to breathe spontaneously. A rectal probe was inserted, and the animals were positioned on a heating pad that maintained the body temperature at 37 °C. The widely used diffuse brain injury model described by Marmarou *et al*. (1994) was used. Briefly, a trauma device which works by dropping a constant weight from a specific height through a tube was used. A weight of 300 g was dropped from a 1 m height, which can induce mild trauma, as shown by Ucar *et al*. (2006).

Thirty minutes after the trauma, rats were injected either saline or *G. lucidum* polysaccharides (GLPS) (400 mg/kg/day) via gastric gavage. The seventh day all the 30 animals were killed after TBI. Thirty rats were divided into three groups as group 1 control group, 2 trauma group, 3 trauma+*Gonoderma lucidum* group (20 mL/kg per day via gastric gavage) *Ganoderma lucidum* was administered for 7 days after trauma. Each group consisted of 10 animals. of these, six of them were used for biochemical and other four were used for the evaluation of histological analysis.

Determining the MDA Level and SOD, GSH-Px, and CAT Activity. The MDA level and SOD, GSH-Px, and CAT activity were determined in each retina and the average values of each group were calculated. The retina samples were prepared as a 10 % homogenate in 0.9 % saline using a homogenizer on ice according to weight. Then, the homogenate was sedimented at 2000 rpm for 10 min, and the supernatant was collected and diluted.The MDA levels were determined using the double heating method of Draper & Hadley (1990). MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Briefly, 2.5 ml of TBA solution (100 g/L) were added to 0.5 ml of homogenate in each centrifuge tube, and then the tubes were placed in boiling water for 15 min. After cooling with flowing water, the tubes were centrifuged at 1000 rpm for 10 min, 2 ml of the supernatant were added to 1 ml of TBA solution (6.7 g/L), and the tube was placed in boiling water for another 15 min. After cooling, the amount of thiobarbituric acid-reactive species (TBARS) was measured at 532 nm. The MDA concentration was calculated from the absorbance coefficient of the MDA–TBA complex. Data are expressed as nanomoles per gram (nM/g) wet tissue. The total (Cu/Zn and Mn) SOD activity was determined through the inhibition of nitrotetrazolium blue (NTB) reduction by the xanthine/xanthine oxidase system as a superoxide generator, as previously reported by Sun *et al*. (1988). Activity was assessed in the supernatant after 1.0 ml ethanol/chloroform mixture (5/3, v/v) and was added to the same volume of sample and centrifuged for 15 min at 3000 rpm. The production of formazan was determined at 560 nm. One unit of SOD was defined as the amount of protein that inhibited the rate of NTB reduction by 50%. Data were expressed as U/g protein.

The GSH-Px activity was measured by the method of Paglia & Valentine (1967). The enzymatic reaction in the tube that contained reduced nicotinamide adenine dinucleotide phosphate, reduced glutathione, sodium azide, and glutathione reductase was initiated by the addition of H_2O_2, and the change in absorbance at 340 nm was monitored by spectrophotometry. Data were expressed as U/g protein.

CAT activity was measured by the method of Cohen *et al*. (1970). The principle of the assay was based on the determination of the rate constant (s-1,k) of H_2O_2 decomposition. The rate constant of the enzyme was determined by measuring the absorbance change per minute. Data were expressed as k/g protein.The Lowry method was used to determine protein levels (Lowry *et al*., 1951).

Immunohistochemical staining. An antigen-retrieval process was performed in citrate buffer solution (pH 6.0) two times: first for 7 min, and then for 5 min in a microwave oven at 700 W. They were allowed to cool to room temperature for 30 min and washed in distilled water for 5 min twice. Endogenous peroxidase activity was blocked in 0.1 % hydrogen peroxide for 15 min. Ultra V block (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 10 min prior to the application of the primary antibodies (VEGF antibody, mouse monoclonal, 1/200, Santa Cruz Biotechnology, or MMP2 antibody, mouse monoclonal, 1/200, Santa Cruz Biotechnology) overnight. The secondary antibody (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 20 min. Then the slides were exposed to streptavidin-peroxidase for 20 min. Diaminobenzidine (DAB, Invitrogen, Carlsbad) was used as a chromogen. Control slides were prepared as mentioned above but omitting the primary antibodies. After counterstaining with Hematoxylene, washing in tap water for 5 min, and in distilled water for 2 × 5 min, the slides were mounted.

Statistical analysis. was carried out using GraphPad Prism 4.0 software (GraphPad Software, 2003, San Diego, CA, USA). All data are presented as mean ± standard deviation (SD). Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p<0.05$ were considered as significant.

RESULTS

Biochemical Analysis

Tissue MDA levels. When the trauma group was compared with the control and GLPS groups, the MDA values were significantly increased ($p< 0.05$). Treatment with GLPS significantly decreased the MDA levels compared with the trauma group ($p< 0.05$). There were no statistically significant differences between the control and GLPS groups ($p= 0.816$).

Tissue glutathione peroxidase (GSH-Px) levels. Following TBI, tissue GSH-Px levels decreased significantly when both the control and GLPS groups were compared with the trauma group ($p< 0.05$). Treatments with GLPS significantly increased the tissue GSH-Px levels compared with the trauma group ($p< 0.05$). There were no significant differences between the control and GLPS groups ($p= 0.183$).

Tissue SOD activity. When the tissue SOD activities of the control and GLPS groups were compared with that of the trauma group, a statistically significant difference was observed ($p < 0.05$); these data showed that after TBI, tissue SOD activity was decreased. Treatment with GLPS significantly increased the tissue SOD activity ($p < 0.05$). There were no significant differences between the control and GLPS groups ($p = 0.430$).

Tissue CAT activity. Following TBI, tissue CAT activity decreased significantly when both the control and GLPS groups were compared with the trauma group ($p < 0.05$). Treatments with GLPS significantly increased the tissue CAT activity compared with the trauma group ($p < 0.05$). There were significant differences between the control and GLPS groups ($p < 0.05$). The biochemical results of the study are summarized in Table I.

In the section control retina, Normal appearance of the internal and outer nuclear layers and the normal organization of the retinal ganglion cell layer are seen. In the trauma group, degenerative changes were seen in some cells in the retina, the integrity of the inner and outer nuclear layer was also observed in the loss of the ganglion cells hypertrophy. The internal and external layers were regular, the nucleus rich in the pigmented cell layer was rich in chromate, and the ganglion layer cells showed mild hypertrophy. Cellular properties were observed to be preserved in gonoderma treated group.

In the section control retina, weak staining for VEGF was also found in the outer limiting membrane and retinal pigment epithelium, VEGF positive staining was observed in the inner limiting membrane, ganglion cell layer. In the trauma group, VEGF expression increased significantly in the ganglion layer. In the gonoderma group, VEGF positive expression was observed in the pigment epithelial layer and in the endothelial cells of the ganglion layer. MMP2 positive expression was observed in the membrane on the internal and external layers, on the basal membrane of the blood vessels and on the ganglion layer. The structural integrity of the extracellular matrix was observed to be preserved.

Table I. Biochemical results relevant to the study groups

	Control	*G. lucidum*	Trauma	Trauma+ *G. lucidum*
MDA (nmol/g)	20.54±1.14	20.41±0.62	34.69±0.93*	27.50±1.30^{+}
GSH-Px (U/g)	24.03±0.62	23.42±0.84	15.63±0.74*	21.19±0.99^{+}
SOD (U/g)	0.88±0.034	0.90±0.022	0.58±0.019*	0.80±0.012^{+}
CAT (K/g)	1.60±0.002	1.49±0.003*	1.19±0.012*	1.45±0.017^{+}

Values are represented as mean±SD. MDA: malondialdehyde, GSH-Px: glutathione peroxidase, SOD: superoxide dismutase, CAT: Catalase
* $p<0.05$, versus control
+$p<0.05$, trauma + G. lucidum versus trauma
Table I. Biochemical results relevant to the study groups.

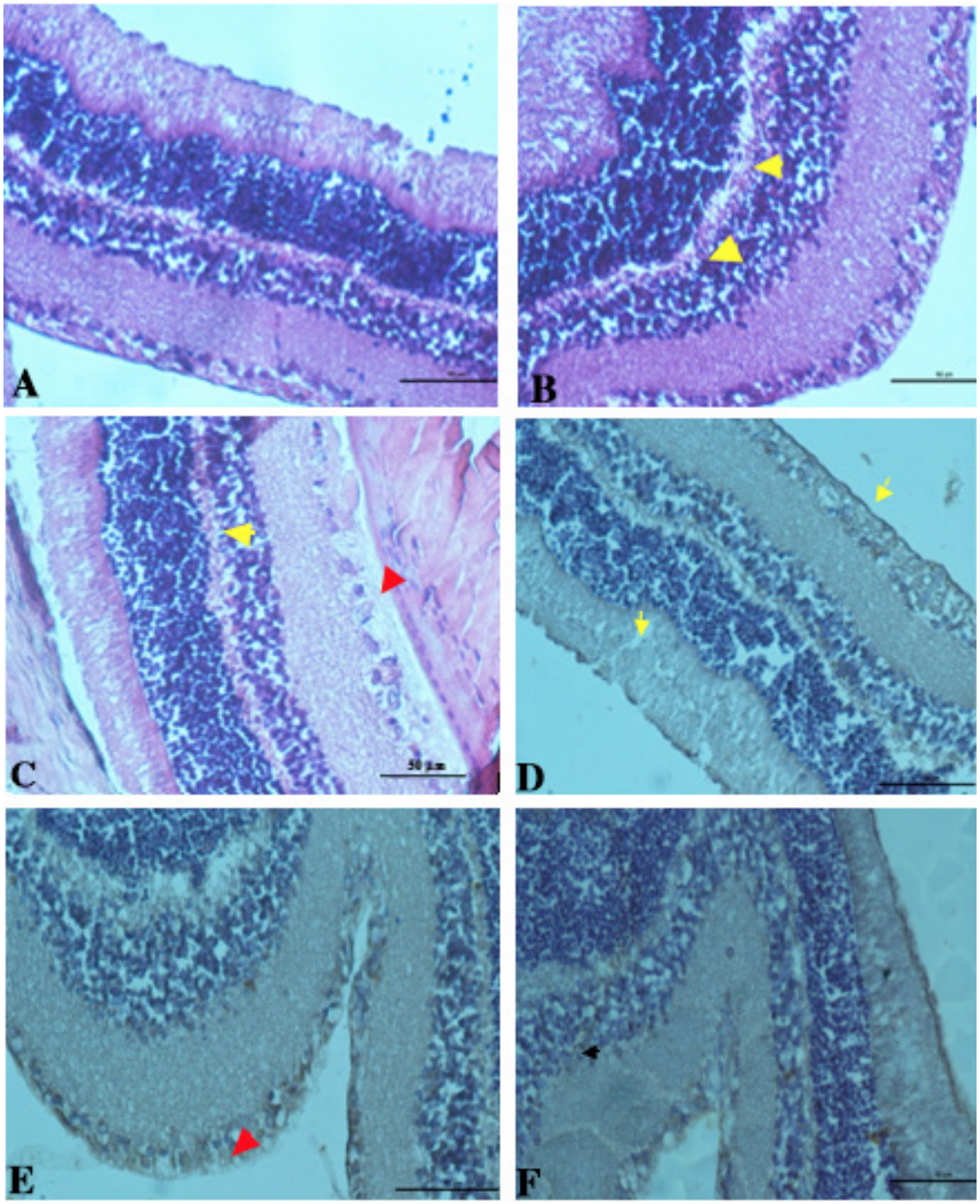

Fig. 1. A. Control group:Normal appearance of the internal and outer nuclear layers and the normal organization of the retinal ganglion cell layer.H-E staining Bar 50 μm B. Trauma group:Some degenerative changes in retinal layers and hypertropy in retinal ganglion cells are seen. Also separations of the cells are observed both in internal nuclear layer and outer nuclear layer (yellow arrow). H-E staining Bar 50 μm, C. Trauma+Gonoderma group; Internal and external layers regular(yellow arrow), pigmented cell layer nucleus rich in chromate and mild hypertrophy in ganglion layer cells (red arrow), D. Control group:Weak VEGF expression in the outer limiting membrane and retinal pigment epithelium (arrow), Positive VEGF expression in the inner limiting membrane, ganglion cell layer (yellow arrow),VEGF immunohistochemical staining Bar 50 μm, E. Trauma group: increase of VEGF expression in the ganglion layer. VEGF immunohistochemical staining Bar 50 μm, F. Trauma + Gonoderma group; VEGF-positive expression in the pigment epithelial layer and in the endothelial cells of the ganglion layer, VEGF immunohistochemical staining Bar 50 μm.

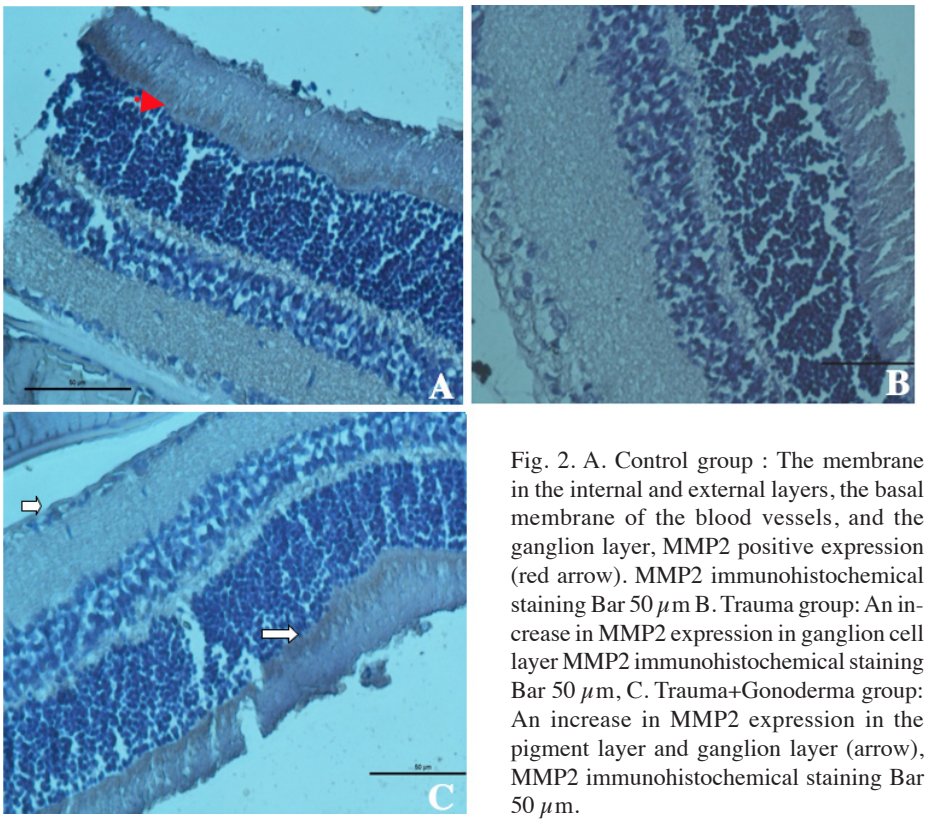

Fig. 2. A. Control group : The membrane in the internal and external layers, the basal membrane of the blood vessels, and the ganglion layer, MMP2 positive expression (red arrow). MMP2 immunohistochemical staining Bar 50 μm B. Trauma group: An increase in MMP2 expression in ganglion cell layer MMP2 immunohistochemical staining Bar 50 μm, C. Trauma+Gonoderma group: An increase in MMP2 expression in the pigment layer and ganglion layer (arrow), MMP2 immunohistochemical staining Bar 50 μm.

DISCUSSION

G. lucidum extract reduced the expressions of proinflammatory and cytotoxic factors from the activated microglia, and effectively protected the dopaminergic neurons against inflammatory and oxidative damage (Huang *et al*., 2012). Early degeneration of the endothelium by free radical damage, production of advanced glycation end-products (AGE) and hypersecretion of various basement membrane proteins may affect transport, permeability and integrity of tight junctions and the blood-retinal barrier (Cagliero *et al*., 1991). A low oxygen tension stimulates vascular endothelial growth factor (VEGF) synthesis and VEGF receptor expression in an attempt to revascularise the ischaemic retina. VEGF has been reported to stimulate vasodilation and leakage of water and large molecular weight proteins from blood vessels, resulting in edema (Bates & Harper, 2002). In the rabbit model of explosive blast injury to the spinal cord, co-existent apoptotic and necrotic changes in cells were reported (Wang *et al*., 2010). In the present study, we have localized VEGF protein expression to the ganglion cell layer, the inner nuclear layer. Increased gene and protein expression of VEGF was observed in the retinas of traumatic animals. VEGF expression may contribute to increased degeneration of retinal cells. MMPs are a family of proteolytic enzymes that degrade extracelluar matrix (ECM) proteins such as collagen and elastin and are essential for cellular migration and tissue remodeling under physiological and pathological conditions (Rajagopalan *et al*., 1996). The major sources of MMPs in the vessel are endothelial and vascular smooth muscle cells (Vincenti *et al*., 2001).

Robertson *et al*. (2013) observed that MMP-9 plays an important role in maintaining intraocular pressure, and due to extracellular matrix remodeling, leads to ocular hypertension and glaucoma. Structural changes were observed in retinal cells after head trauma due to the decrease in extra cellular matrix change in inner and outer limiting membranes. Reduction in vascular endothelial protein, together with structural changes in retinal vessels, has been shown to influence angiogenesis.

In the gonoderma-treated group, structural integrity was evident in the blood vessels and VEGF expression was increased. Structural changes in degeneration veins in retinal pigment cells following head trauma and loss of basal

membrane integrity are histopathologically significant. It has been observed that cellular degeneration is minimal in the treatment of *Ganoderma lucidum*, VEGF protein expression in vascular endothelial cells and integrity is preserved because MMP2 expression is positive in the basement membrane. It shows that the potential is useful as a *G. lucidum* for the treatment of eye disorders induced by traumatic head injury.

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RESUMEN: El traumatismo craneal afecta al nervio óptico en relación a su función y la agudeza visual. Se estudiaron los diversos efectos bioquímicos, histológicos e inmunohistoquímicos en la retina producidos por una lesión y trauma a la cabeza. En esta investigación se evaluó el efecto protector de *Gonaderin lucidum* sobre el daño a la retina de ratas. Ratas Sprague-Dawley fueron sometidas a una lesión cerebral traumática con un dispositivo de caída de peso usando un impacto de 300 g-1 m de peso-altura. Treinta ratas se dividieron en tres grupos: grupo 1, de control; grupo 2, trauma; grupo 3, de trauma + *Gonoderma lucidum* (20 ml / kg día, a través de una sonda gástrica). *Ganoderma lucidum* se administró durante 7 días después del trauma. Todas las ratas fueron decapitadas 5 días después. La inducción del trauma y los efectos protectores de *Ganoderma lucidum* en la retina fueron evaluados mediante análisis histológicos, inmunohistoquímicos y bioquímicos. Se investigó el efecto antioxidante de *Ganoderma lucidum* sobre la degeneración celular en la matriz extracelular y la barrera retiniana en la retina después del traumatismo craneal.

PALABRAS CLAVE: Trauma de cabeza; Retina; *Ganoderma lucidum*; Rata.

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