ABSTRACT

Purpose: To evaluate the effect of intravitreal (IV) Matrine (Mat) injection on retinal endothelial cell proliferation (REP), retinal morphology, and apoptotic activity in hyperoxia induced (HIR) in-Vivo ROP- mouse model using C57BL/6J.

Methods: Total of 48 C57BL/6J mice were divided in 6 group each including 8. Group A and B-S exposed to room air were negative control groups; group C, D-S, E and F exposed to 75% ± 2% oxygen from postnatal day (PD) 7 to PD 12 comprised HIR groups. Group C and D-S were control groups, group E and F were low and high dose IV-Mat injected groups. Group A and C received no IV injection, B-S and D-S received 1 μL IV sterile 0.9% NaCl injection, E and F received 0.78 μg and 6.25 μg IV-Mat injection respectively. After enucleation, we measured REP by counting neovascular tufts in cross-sections and examined histological, ultrastructural changes via light and transmission electron microscope. Apoptosis was detected using terminal deoxynucleotidyl transferase-mediated nick end-labeling.

Results: No REP was detected in negative control groups. REP levels were significantly different between low- and high-dose Mat groups. REP levels were significantly lower in the Mat groups, compared to the control groups. The incidence of mitochondrial dysmorphology and apoptosis were lower in groups receiving Mat compared to the control groups.

Conclusion: Mat seems to suppress REP and has anti-apoptotic activity in the HIR mouse model.

Keywords: Retinopathy of prematurity, in-vivo-ROP-Mouse-Model, Matrine, Neovascularization, Apoptosis.

INTRODUCTION

Retinal vein occlusion, hypertensive retinopathy, diabetic retinopathy as well as retinopathy of prematurity (ROP) may associated with retinal ischemia and retinal neovascularization (RNV).1 Local and systemic inflammation and vascular endothelial growth factor (VEGF) have a potential role in development of RNV.

The hyperoxia, induced by hyperventilation of preterm infants, may induce an enrichment of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the retina, resulting in arrest of the development of neuroretinal retina as well as vaso-obliteration, and eventually hypoxia and ischemia of retinal tissue.2

Standard treatment of ROP is laser photocoagulation of avascular zone in Type-I ROP. Intravitreal anti-VEGF injection is predominantly used in cases with Type-I in Zone-I or posterior Zone-II ROP or aggressive posterior-ROP (AP-ROP) is preferred treatment option but long term systemic side effects of intravitreal anti-VEGF treatment hasn’t been fully elucidated.3

Despite timely and adequate treatment, the treatment can fail in some cases.2, 4 Anti-VEGF treatment is able to suppress the angiogenesis completely and it is assumed that non-vascular cell types such as neuron, Müller cells, ciliary body, RPE cells, photoreceptors and choriocapillaris are involved in the genesis of ROP. Hence, there is a need for effective treatment with minimum side effects and well-defined doses, treatment duration and mechanism of action.2

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Matrine (Mat) is a naturally occurring alkaloid extracted from the herb *Sophora flavescens*. It has been demonstrated to exhibit anti-inflammatory properties and inhibits cell invasion in a number of cancer cell lines. Furthermore, Mat has anti-oxidative, anti-fibrotic, anti-proliferative and anti-apoptotic effects. Zao et al. have described different ways of anti-apoptotic effect. In an ischemia-reperfusion (I/R) injury model using Newborn Sprague-Dawley rats, Mat showed cardio-protective effects by reducing infract size, improving neurological parameter, attenuating apoptosis, oxidative stress and mitochondrial dysfunction.

In our study, we evaluated the effect of IV Mat injection on retinal endothelial cell proliferation (REP), retinal morphology, and apoptotic activity in HIR in vivo mouse model of ROP using C57BL/6J.

**MATERIAL AND METHODS**

All experimental procedures were approved by the Committee on Animal Care of Baskent University. All procedures were carried out in accordance with the Association of Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Vision Research.

**Experimental protocol**

In our study, we used the in vivo mouse model of hyperoxia-induced ROP established by Smith et al. Mat (Matrine 5319 Sigma Aldrich; Germany) was dissolved in 0.9% NaCl and Mat doses of 0.78 µg for 6.25 µg were prepared for IV injection in to the corneoscleral junction of the right eye.

Sixteen newborn C57BL/6J-type mice exposed to room air (21% oxygen) were included in the 2 negative control groups; 8 mice in group A (n=16 eyes) and 8 mice in group B-S received 1 µL of intravitreal sterile NaCl 0.9% (n=8 eyes).

Thirty-two newborn C57BL/6J-type mice comprising the HIR group were exposed to 75% ± 2% oxygen from PD 7 to PD 12 with their nursing mothers. On PD 12, the HIR mice were returned to room air and randomized in to 4 test groups of 8 mice each. The mice were deeply anaesthetized via an intraperitoneal (IP) injection of ketamine hydrochloride (40mg/kg) and xylazine hydrochloride (5mg/mL).

Group C served as the HIR control (receiving no injection). Group D-S was the injection control group receiving 1 µL of 0.9% NaCl. Group E received 0.78µg IV-Mat (the low-dose IV-Mat group), and group F received 6.25µg IV-Mat (the high-dose IV-Mat group).

All injections were performed on PD 12. On PD 17, the mice were sacrificed and the eyes were enucleated to evaluate the rate of REP. The mice with body weight ranging from 6.3 to 7.5 mg on PD 17 were included in the study.

Enucleated eyes were examined via light microscopy (LM; n=4 eyes) and transmission electron microscopy (TEM; n=4 eyes) by 2 independent observers. We evaluated apoptosis using the terminal deoxynucleotidyl transferase-mediated nick-end-labeling (TUNEL) technique.

**Light microscopy - Examination (LM)**

The LM (OLYMPUSBX51, Germany) evaluation of the enucleated eyes was performed after the eyes were fixed with 4% formalin and embedded into paraffin. We used the cross-section preparation methods established by Smith et al and Hammesetal as follows.

Hematoxylin and periodic acid-Schiff–stained axial sections (6 µm) were used for the quantitative analysis of neovascularization and morphologic evaluation. A total of 1.5 sections of equal length, each 18 µm apart, were used to quantify retina. Vascular cell nuclei on the vitreal side of the inner limiting membrane. The results are given as number of cell nuclei per 6 µm section per eye. We also evaluated the retina for morphologic changes such as cystic degeneration, hypocellularity or loss of the nuclear layer in each group. For the quantitative assessment of retinal neovascularization, endothelial cell nuclei counts are shown as the mean ± standard deviation.

**Transmission electron microscopy - Examination (TEM)**

For TEM examination, all tissues were fixed in phosphate buffer containing 2.5% glutaraldehyde for 2–3 hr; then they were post-fixed in 1% osmium tetroxide (OsO4) and dehydrated in a series of graded alcohols. After passing through propylene oxide, the specimens were embedded in Araldyte CY 212, 2-dodecenyl succinic anhydride (DDSA), benzylidethyl amine (BDMA) and dibutylpytalate. Semithin sections were cut and stained with 1% Toluidine blue and examined with a light microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with LEO 906E transmission electron microscope. TEM was used especially to evaluate the ultra-structural mitochondrial morphology of the inner (IPL) and outer photoreceptor layers (OPL) in each group.

The TEM and LM assessment of retinal tissues were performed by two independent observers in blinded fashion.

**Terminal deoxynucleotidyl transferase mediated nick end labelling (TUNEL) - Evaluation**

Enucleated eyes were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections of the retina (6 µm
thick) were obtained, starting from the optic disc. The third or fourth sections after the optic disc were taken for evaluation and stained with hematoxylin-eosin. One section per eye was evaluated. Terminal deoxynucleotidyl transferase-mediated nick end labeling staining was performed with a kit (In Situ Cell Death Detection Kit, AP, Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer’s instructions. Two independent, blinded observers performed LM (OLYMPUS BX51, Germany) to look for TUNEL-positive cells in randomly selected fields on each slide at 100· magnification (oil immersion). Apoptotic TUNEL-positive cells were measured in 10 randomly selected fields on each slide.

**RESULTS**

**Quantification of retinal endothelial cell proliferation**

Hyperoxia-induced REP was evaluated by counting neovascular tufts in the sections [12, 13]. No REP was detected in the negative control groups A and B-S (Fig. 1A). In groups C and D-S (control groups), the retinal tissue presented numerous REP widening in to the vitreal space (Fig. 1B, arrow). In groups C and D-S, the mean ± SD number of REP was 71.75±2.6 and 72.1±4.5 per histological section, respectively; the difference between the groups did not reach statistical significance (p=1.0).

REP counts in group E (0.78μg /μL Mat IV) and group F (6.25μg /μL MatIV) were 9.0±0.1 and 4.1±0.5, respectively, which was a statistically significant difference (p=0.006) (Fig. 1C, D). Compared to the average of control group C, the REP counts were significant different in groups -A (p=0.0001), -E and -F (p=0.0001). If group E and group F were compared with group D-S, the results appeared similar.

Figure 4 demonstrates the mean REP counts in group A to group F.

**Fig. 1:** LM images. (A) LM image from group A representing negative controls as groups A and B-S. Mouse retina exposed to room air without endothelial cell nuclei. (B) LM image from group C representing HIR (receiving no IV injection) controls as groups C and D-S with multiple neovascular tufts (black arrows). (C) LM image represents IV-Mat-injected group (low-Dose). (D) represents IV-Mat injected high dose group. Hematoxylin and periodic acid-Schiff staining. Mat, Matrine; IV, intravitreal; LM, light microscopy.
**Light microscopy**

In all groups evaluated, morphological analysis of retinal layers using LM showed no signs of cystic degeneration, hypo-cellularity or loss of the nuclear layer in control and negative control groups (Figure 1A-B). Hypo-cellularity and loss of the nuclear layer in all retinal layers were detected irreducibly in group E and F (IV Mat injected groups) (Figure 1C-D).

**Transmission electron microscopy**

The IPL and OPL of the retina were analyzed for ultrastructural changes of the mitochondria in each group. In ultrathin sections, the negative controls (groups A and B-S) showed no changes in the mitochondria (Fig. 2A). Control groups C and D-S had mitochondria with well-preserved membranes, brindled matrices, and baked structures centrally arranged in the cristae encircled by swelling and a disarray of the cristae in the IPL (Fig. 2B). Mitochondrial damage was also observed in the Mat-injected groups (Fig. 2C). We described an aggrieved mitochondrion with a brindled matrix and baked structures centrally arranged in the cristae encircled by the swelling and disarray of the cristae, to verify the number of damaged mitochondria in the visual field with the same magnification (3250×) for each sample in all groups. Atypical mitochondrial counts (AMCs) as determined by defaced mitochondria were comparable between the negative control groups A and B-S (p = 0.7) and the control groups C and D-S (p = 0.8). A significant difference in AMC was detected between groups A and C (p = 0.001) and between group C and the Mat-injected groups E (p = 0.005) and F (p = 0.001). Statistically significant difference was noted between the high- and low-dose Mat groups (p = 0.018). Figure 5 demonstrates the mean AMCs in group A to group F.

**Evaluation of apoptosis using TUNEL Technique**

No significant difference was noted between the negative control (groups A and B-S, p = 0.6) and control groups (groups C and D-S, p = 0.8) when evaluating apoptosis in the outer (ONL) or inner nuclear layer (INL) in the TUNEL stained sections (Figure 3A).

No significant difference in apoptosis was noted when comparing group A with group E (p = 0.6) or group C with group E (p = 0.4).

Statistically significant differences in apoptosis were noted when we group F compared with groups A, C, and E (p = 0.018, p = 0.021, and p = 0.015, respectively) (Figure 3B).

Figure 6 showing the count of detected apoptotic cells [mean (SD)] in different study groups (A-F).

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Fig. 2: TEM images demonstrating ultra-structural morphology of the mitochondria. (A) represents negative control groups A and B-S. (B) represents control groups C and D-S. (C) represents IV-Mat injected groups. Magnification 3250×. Mat, Matrine; TEM, Transmission electron microscopy; IV, intravitreal.
**Fig. 3:** Light micrographs of retinas from C57BL/6J mice demonstrating apoptotic cells using TUNEL staining. A represents control groups C and D-S. B represents group E, and group F; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

**Fig. 4:** Figure 4 demonstrates the mean REP counts in groups A to F. Bar graphs showing the count of detected endothelial cell nuclei counts (mean [SD]) in different study groups (A-F) in the retina of C57BL/6J mouse.
Fig. 5: Figure 5 demonstrates the mean AMCs in group A to group F. Bar graphs showing the count of detected atypical mitochondrial counts (mean [SD]) in different study groups (A–F) in the retina of C57BL/6J mouse.

Fig. 6: Bar graphs showing the count of detected apoptotic cell counts [mean (SD)] in different study groups (A–F) in the retina of C57BL/6J mouse.
DISCUSSION

Angiogenesis — the process of new blood vessel growth — has an essential role in development, reproduction and repair. The HIR in the mouse, with vascular development similar to the human, with reproducible and quantifiable proliferative retinal neovascularization, is useful for studying pathogenesis of retinal neovascularization as well as medical intervention for ROP and other retinal vasculopathies.

Angiogenesis is tightly regulated by interaction of angiogenic cytokines. Numerous cytokines of IL-1 family have inflammatory effect acting on immune response and are closely associated with angiogenesis. Various factors are involved in the pathogenesis of ischemic retinopathy, whereas oxidative stress and inflammatory factors have undoubtedly important roles. Hypoxic injury may induce retinal ganglion cell (RGC) death in the neonatal retina mediated by excess production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by microglia.

Mat has anti-inflammatory activity with suppression of production of inflammatory mediators as TNF-alpha, IL-6, IL-23, IL-17 and IL-1. In HIR retinopathy model, Zhaou et al. revealed a prominent role for elevated IL-1b levels in choroidal involution associated with a subsequent progression of outer retinal dysfunction. In our study we could demonstrate that endothelial cell nuclei counts were reduced in the intravitreal Mat injected groups compared with control groups, whereas the effect was pronounced in the Mat-High dose group.

Compared with a potent anti-VEGF as bevacizumab, intravitreal injected Mat has moderate antiangiogenic effect in the mouse model of HIR using C57BL/6J.

Carmi et al. showed that, using C57BL/6J mouse in normoxic and hyperoxic conditions, neutralization of IL-1 in cell culture may able to abrogate angiogenesis and suppress VEGF level up to 85%. In the present study we did not verified the cytokines specifically.

In in vivo HIR model, retinal histomorphological analyses showed variations due to different experimental conditions. In our study, we exposed the C57BL/6J juvenile mouse to hyperoxia (75% oxygen) during PD 7 and PD12; then, the mice were returned to room air (21% oxygen) during PD12 and PD17 and on PD 17 enucleation was performed. Morphological analysis of retinal layers using LM revealed no sign of cystic degeneration, hypo-cellularity or loss of the nuclear layer in control and negative control groups (Figure 1A-B). Hyпо-cellularity and loss of the nuclear layer in all retinal layers were detected irreducibly in group E and F (intravitreal Mat injected groups) (Figure 1C-D). This may be due to a toxic effect on retina or due to the toxic effects of the doses used in our study. Two different doses of IV Mat were administered in our study. There is limited number of in vivo and/or in vitro studies involving ocular or intravitreal Mat administration. However, pharmacokinetic dose studies involving prospective administration of intravitreal matrine are warranted to determine effective doses and effect/side-effect profiles.

Returning to normoxia after a period of HIR induced a relative hypoxia in mice. In ultrathin sections, the negative controls (groups A and B-S) showed no changes in the mitochondria (Fig. 3A). Control groups C and D-S had mitochondria with well-preserved membranes, brindled matrices, and baked structures centrally arranged in the cristae encircled by swelling and a disarray of the cristae in the IPL (Fig. 3B). This finding suggests that the retina of the C57BL/6J mouse is squeamish to hyperoxia.

Compared to the control groups, the number of AMC was significantly lower in the intravitreal Mat injected groups. However, the Mat-injected groups had more AMC counts compared with the negative control groups A an B-S. Mat may have dose depended protective effect against hyperoxia-induced retinal damage in the pigmented C57BL/6J mouse. The protective effect on mitochondria is pronounced in the high-dose Mat-group.

During oxidative stress, Mat is able to support survival of cells and reduces the endogenous mitochondrial ROS production and protects mitochondria for membrane dysfunction. Furthermore Mat seems to be able to influence the mitochondrial activity via modulation of Bcl-2/Bax-Interaction and inhibition of caspase-3 expression. In our study, we did not observed the redox-reactions and did not determined the proteins of intrinsic and extrinsic way of apoptosis but we were able to show via TEM analysis that (group C and D-S) the number AMC was higher in the hyperoxia induced groups compared to in intravitreal Mat injected groups (Group E and F).

In a study on Sprague-Dawley (SD) rats, Wang et al. detected that Mat treatment improved cardiac function in AGEs exposed hearts. Mat inhibited the disassociation of FKBP and RyR2, decreased the activity of RyR2, [Ca2+], apoptosis, expression levels of cytochrome c and active caspase 3 in vivo and in vitro. Mat attenuated AGEs induced cardiac dysfunctions by regulating RyR2 mediated calcium overload triggered myocardial apoptosis. Inhibited apoptosis was detected by TUNEL assay. Rats were pre-treated with Mat by intraperitoneal injection.
daily for consecutive 20 days at dosages of 50, 100 and 200 mg/kg/d. AGEs (1 mg/d) was administrated to rats via intraperitoneal injection for consecutive 20 days. Rats that were not injected with AGEs-BSA received control BSA. The Mat treatment significantly reduced the apoptosis in rat hearts exposed to AGEs in a dosage-dependent manner.8

Zhao et al. described using mouse I/R-model anti-apoptotic and anti-inflammatory effect of Mat and elevating the anti-oxidant activity by pretreatment of the mice with intraperitoneal injection of 30 mg/kg Mat.6 These data are in accordance with our results regarding anti-apoptotic impact of intravitreal injected Mat in C57BL/6J HIR-Mouse-Model using 0.78µg/mL or 6.25 µg/µL IV Mat. In our study we were able to demonstrate dose dependent anti-apoptotic activity with TUNEL technique.

Recent evidences suggested that prenatal, perinatal, and postnatal inflammation might contribute to a gradual increase in the risk for ROP.2 Cytokines such as IL-1β, TNF-α, and IL-6 act as primary initiators of inflammation following infection or tissue damage, although both pro- and anti-inflammatory properties have been observed.2

To our knowledge, this is the first study investigating the effect of intravitreal injected Mat in HIR-Mouse-Model. The imitation of our study was our addiction on manually counting the retinal cross sections of the nuclei of the micro-vessel cells invading the vitreous to assess retinal neovascularization. While other studies have used a similar cross-sectional approach that yields what is essentially a snapshot review, a full quantitative assessment of neovascularization would be possible via flat mounting the entire retinal tissue or with more precise software technique.11, 29 We did not evaluate the cytokines in particular. We used only two different Mat concentrations; dose response curves and investigations regarding cytological, morphological effects and side effect should have to be performed.

In spite of our limitations, we were able to demonstrate a moderate anti-angiogenic effect of Mat. Mat has anti-inflammatory and neutralizing activity on IL-1; thus, indirect suppressive on VEGF and VEGFR-2 expression. Furthermore Mat has anti-apoptotic effect in intravitreal injection in the HIR-Model using C57BL/6J mouse. High-Mat doses induced marked anti-proliferative and anti apoptotic effect.

Analyzing AMC counts using TEM and TUNEL-positive cells using LM enabled us to assess the possible protective effect of Mat against HIR damage in the pigmented C57BL/6J Mouse.

ROP is a multifactorial disease and inflammation which seems to have a key role in the pathogenesis, causing 100,000 new cases of childhood blindness annually.30, 31 Experimental and clinical studies for prevention and therapy of ROP should have to investigate and explore biomarkers, anti-angiogenic, anti-oxidative and anti-inflammatory effective agents.

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