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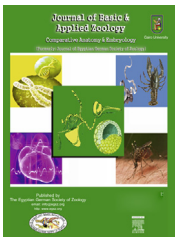

Effect of verapamil on some of the pro- and apoptotic factors during prenatal retinal differentiation of mice, *Mus musculus*

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KEYWORDS

Verapamil;
Cytochrome C;
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Abstract Verapamil is a calcium channel blocker that belongs to the phenylalkylamine group. It has been clinically used for various diseases such as combating hypertension, ischemic heart diseases, supraventricular antiarrhythmic and tycolysis. The study was conducted to investigate the effect of verapamil on selected pro- and apoptotic factors during prenatal retinal differentiation of mice at E14 and E17 of gestation. The pregnant females were classified into two groups, the first is the control and the second receives SC injection of repeated doses of verapamil (40 mg/kg) at the 7th day of gestation. The pregnant females were sacrificed at E14 and at E17 of pregnancy and their ocular regions were separated. The retina of mentioned ages were examined at histological and immunohistochemistry of Cytochrome C, Caspase-3 as a pro-apoptotic; Bak and TNFα R2 as apoptotic factors that engaged in proper normal development. The present findings revealed that verapamil-treatment exhibited comparative thinning of inner plexiform layer and reduction of nuclear in E14 and missing of the ganglion layer and comparative decrease of nuclear cells of E17 comparing to the control. Also, the expression of Cytochrome C, Caspase-3, Bak and TNFα R2 in the developing retina was obviously inhibited in verapamil-treatment at E17 compared to the control group. The study concluded that verapamil, as a calcium channel blocker, has the ability to alter the histology of the retina and suppress the studied markers resulting in disorganization of the eye during prenatal development.

Introduction

It is well known that calcium plays an integrated and crucial role in regulation of cellular movement and transport, electrical activation of excitable cells and various enzymatic reactions throughout the body (Rasmussen, 1986). Calcium is almost a universal intracellular messenger controlling diverse ranges of cellular processes such as gene transcription, muscle contraction, cell proliferation and activating different intracellular biochemical processes in response to extracellular stimuli (Bootman et al., 2001). Extracellular calcium has an important role in cell–cell adhesion in mammalian embryos during pre-implantation process. Also, intracellular free ionized calcium plays a key role in fertilization, oocyte maturation/and

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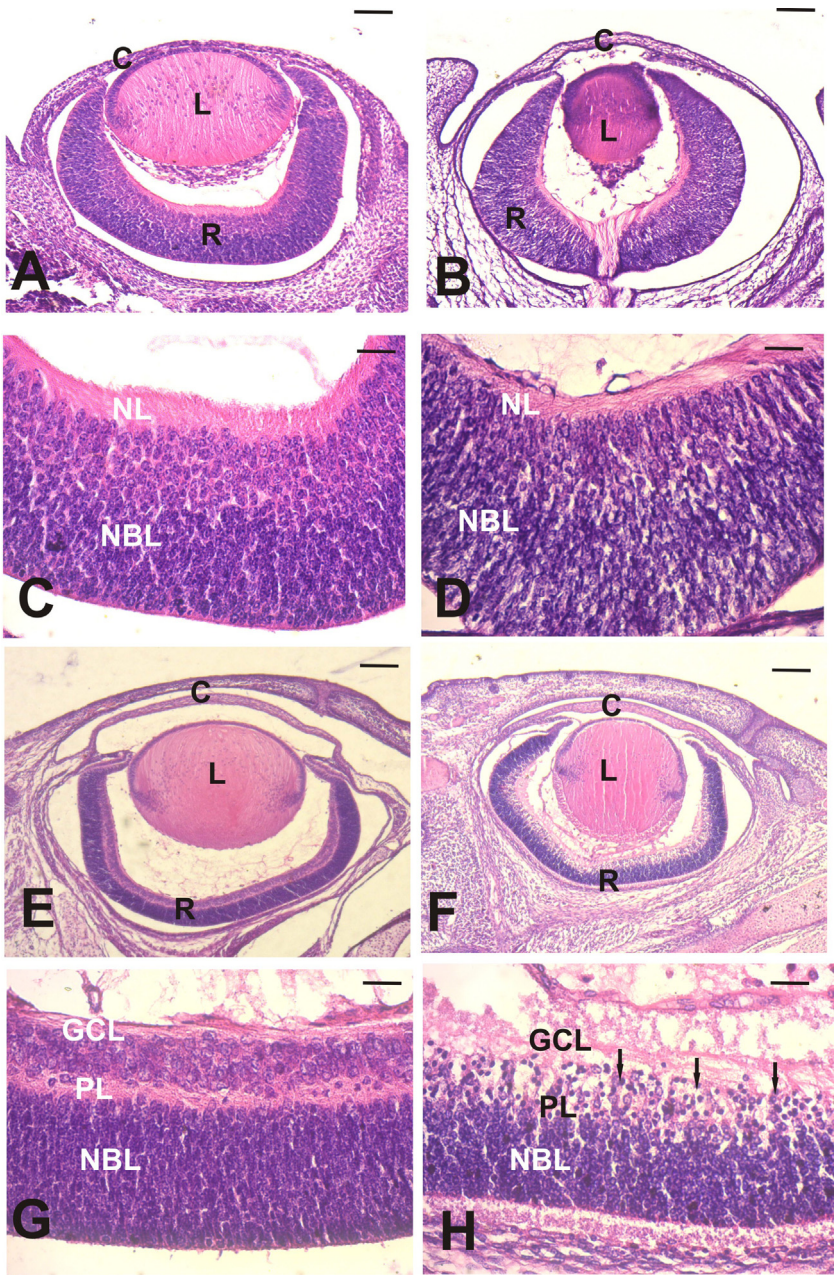

Figure 1 Photomicrographs of the developing eye at E14 of the control (A and C) and verapamil-treated (B and D) showing similarity of the developing structures. F and H eye of verapamil-treated embryos at E17 showing degenerated retina compared to the control (E and G). Cornea (C), lens (L), retina (R), neural layer (NL), neuroblastic layer (NBL), ganglion cell layer (GCL), plexiform layer (PL), inflammatory cells (arrows). H and E stain scale bar 10 μm (A, B, E, F); 50 μm (C, D, G, H).

activation of cortical granule exocytosis (Stein et al., 1990; Whitaker, 2008).

Verapamil, a calcium channel blocker given either *in vivo* or *in vitro*, has the ability to inhibit calcium uptake (Goligorsky et al., 1985). It belongs to the phenylalkylamines that are used clinically for combating hypertension, ischemic heart diseases, supraventricular tachyarrhythmias and tycolysis (Dong-Yun et al., 2010). It also an active antianginal, antiarrhythmic and antihypertensive that is used in the treatment of cardiovascular diseases (Johnston et al., 1981; Lüllmann et al., 2003) and improve abnormal left ventricular diastolic relaxation in patients with hypertrophic cardiomyopathy (Hanrath et al., 1980; Bonow et al., 1981; Lorell et al., 1980, 1982). Verapamil has also neuroprotective effect in several acute neurotoxicity (Liu et al., 2011) and nephroprotective effect against lead and cadmium toxicity (Lermioglu and Bernard, 1998; Zhang et al., 2013). In addition, verapamil has antiproliferative effect on the tumor cell both *in vivo* and *in vitro* (Schmidt et al., 1988; Jian et al., 2007). Moreover, verapamil was recently used in the treatment of waste and surface waters (Khan and Ongerth, 2004; Hummel et al., 2006; Batt et al., 2008). Hence, the study was conducted to evaluate the effect of insufficient calcium due to verapamil treatment in pregnant mice on the histogenesis of the retina and to evaluate the effect on Cytochrome-C,

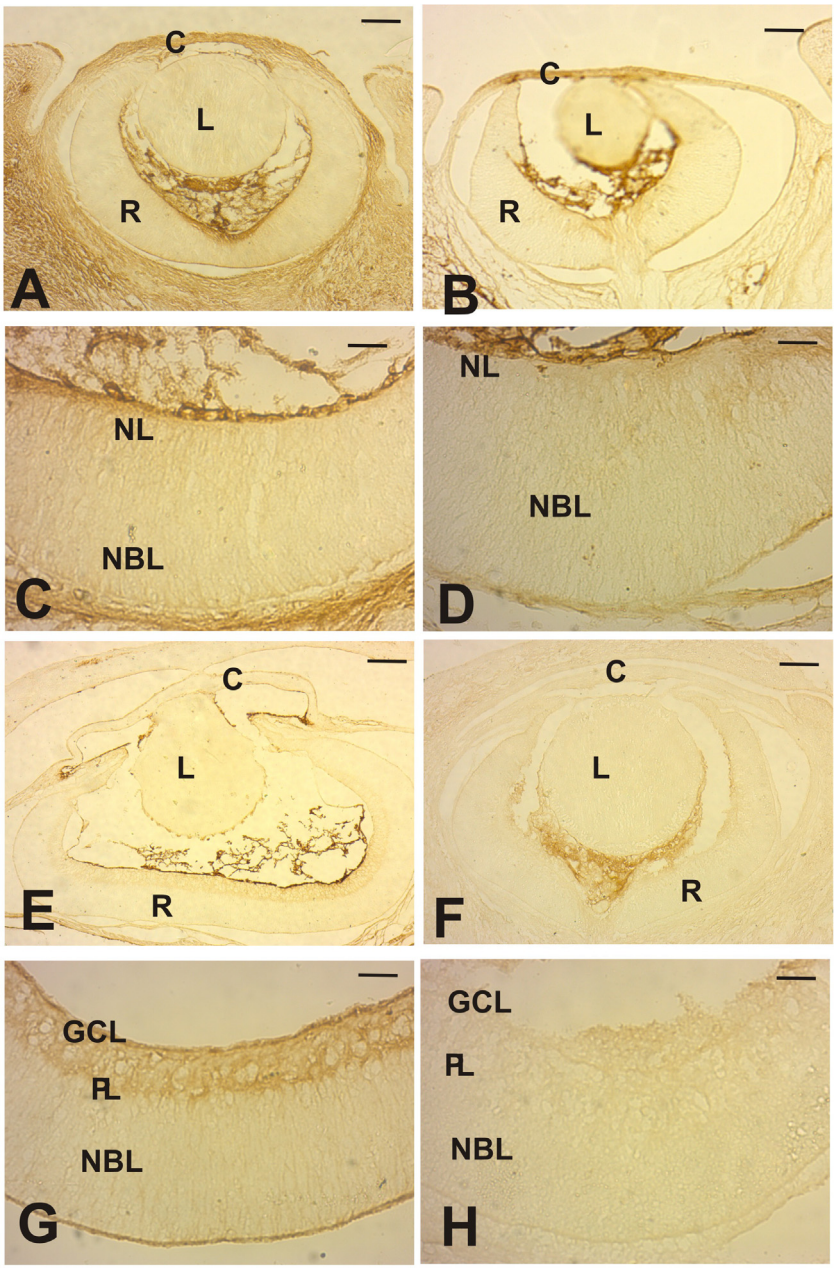

Figure 2 Photomicrographs of immunoreactivity of cytochrome C during eye development showing downregulated expression of control from E14 to E 17 that was restricted to the ganglion layer and the basement membrane of the neuroblastic layer (A, C, E, G). Verapamil treated embryos show the same expression at E14 (B and D), downregulation at E17 that disappeared in the ganglion layer and in the basement membrane of the neuroblastic layer of the retina (H), arrows, compared to the control. Cornea (C), lens (L), retina (R), neural layer (NL), neuroblastic layer (NBL). Scale bar 10 μm (A, B, E, F); 50 μm (C, D, G, H).

Caspase-3 as pro-apoptotic factors; BAK (an apoptotic member of Bcl2 family) and TNFα R2 receptor those incorporated in normal developmental processes during embryogenesis.

Materials and methods

Verapamil was obtained from Sigma Aldrich company USA. Adult female and male mice *Mus musculus* (weighting 25–30 g) were used. The animals were obtained from a closed random bred colony at Faculty of medicine, Assuit university, Egypt. The mice were maintained on food and water ad libitum and housed in groups in isolated cages. The animals were acclimatized for 2 weeks prior to usage. Females mice with regular estrous cycle were obtained after repeated vaginal examination. Estrous females were paired overnight with well proven fertile males. On the next morning, vaginal plugs were examined, when zero day of pregnancy was determined. The pregnant females were divided into two groups (n = 10), the first is the control, and the second group receives repeated SC injections of verapamil (40 mg/kg) at the 7th day of pregnancy. The selected period of drug administration at the 7th day up to the 17th day of gestation is considered as the most sensitive period of gestation to any adverse insult (Christian, 2001). Pregnant

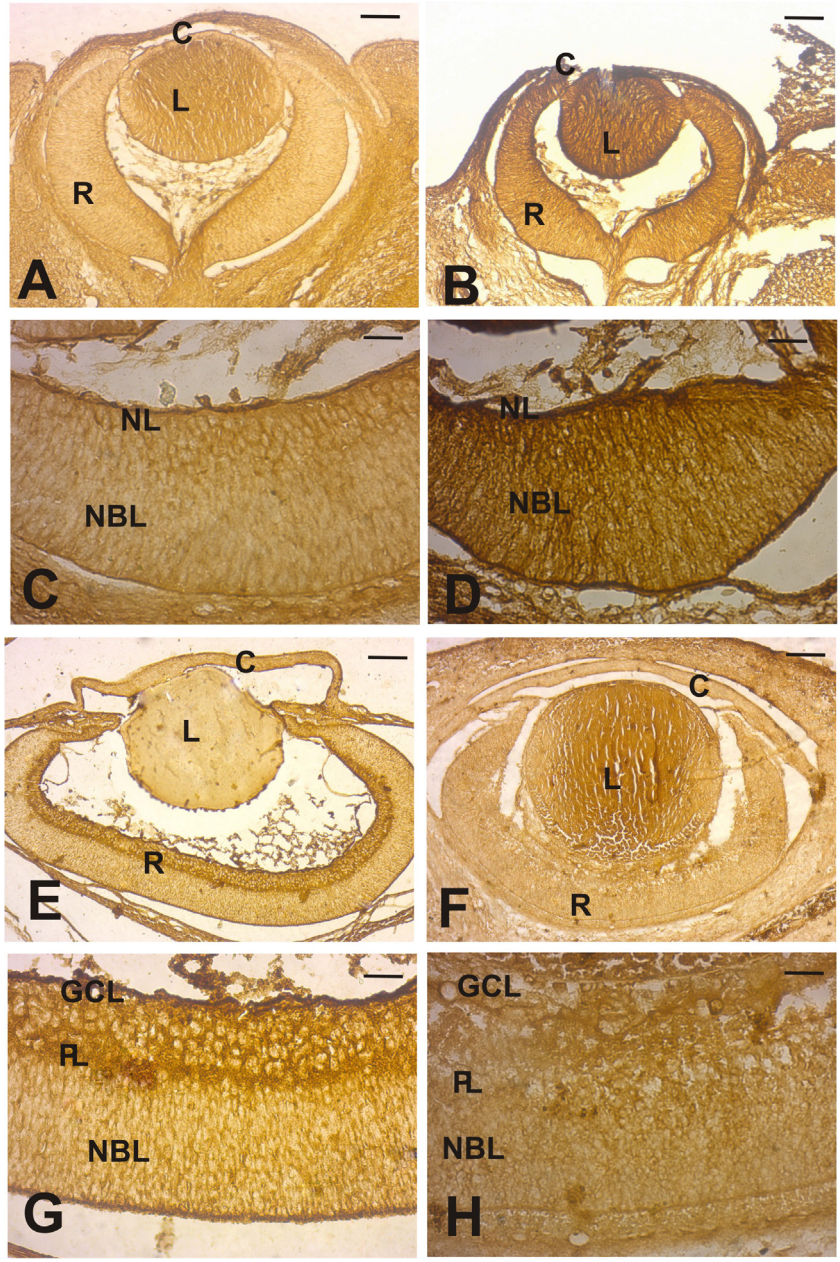

Figure 3 Photomicrographs of immunoexpression of caspase-3 during prenatal eye development in control at E14 (A and C) in all eye structures and retina that increased in verapamil treated embryos (B and D). Restricted intense expression was noted in the ganglion and the plexiform of control at E17 (E and G) that was inhibited in verapamil treated embryos of the same age (F and H). Cornea (C), lens (L), retina (R), neural layer (NL), neuroblastic layer (NBL), plexiform layer (PL), ganglion cell layer (GCL). Scale bar 10 μm (A, B, E, F); 50 μm (C, D, G, H).

females were sacrificed at E14 and E17 of gestation. The use of experimental animals in this study was conducted under the guidance of the basic standards in the care and use of laboratory animals, which has been prepared and published by the institutional animal care committee. Eyes at E14 and E17 of the control and treated groups were fixated in Caryno's fixative, dehydrated in ethyl alcohol, cleared in methyl benzoate and processed for sectioning. 5μ thick sections were stained with hematoxylin and eosin for general histology (Drury and Wallnigton, 1976).

For immunohistochemical investigation, sections were mounted on Superfrost/Plus glass slides. The slides were deparaffinized in xylene, rehydrated and retrieved for re-antigenicity using 10 mM citrate buffer at pH 6 in 100 °C for an hour (Buchlowalow and Bocker, 2010). Sections were incubated with specific primary antibody against anti-Caspase 3 (Rabbit-polyclonal antibody, NeoMarkers, Fremont. CA, USA) at dilution (1:100); anti-TNFα-R2 (Rabbit polyclonal, spring, Bioscience, USA) at dilution (1:100);-Cytochrome C (mouse monoclonal antibody, Santa Cruz, Biotechnology, INC., Germany) at dilution (1:100) and anti-BAK (rabbit polyclonal antibody, Santa Cruz, Biotechnology, INC., Germany) at dilution (1:100), for three hours at room temperature. Sections were then washed using phosphate buffer and incu-

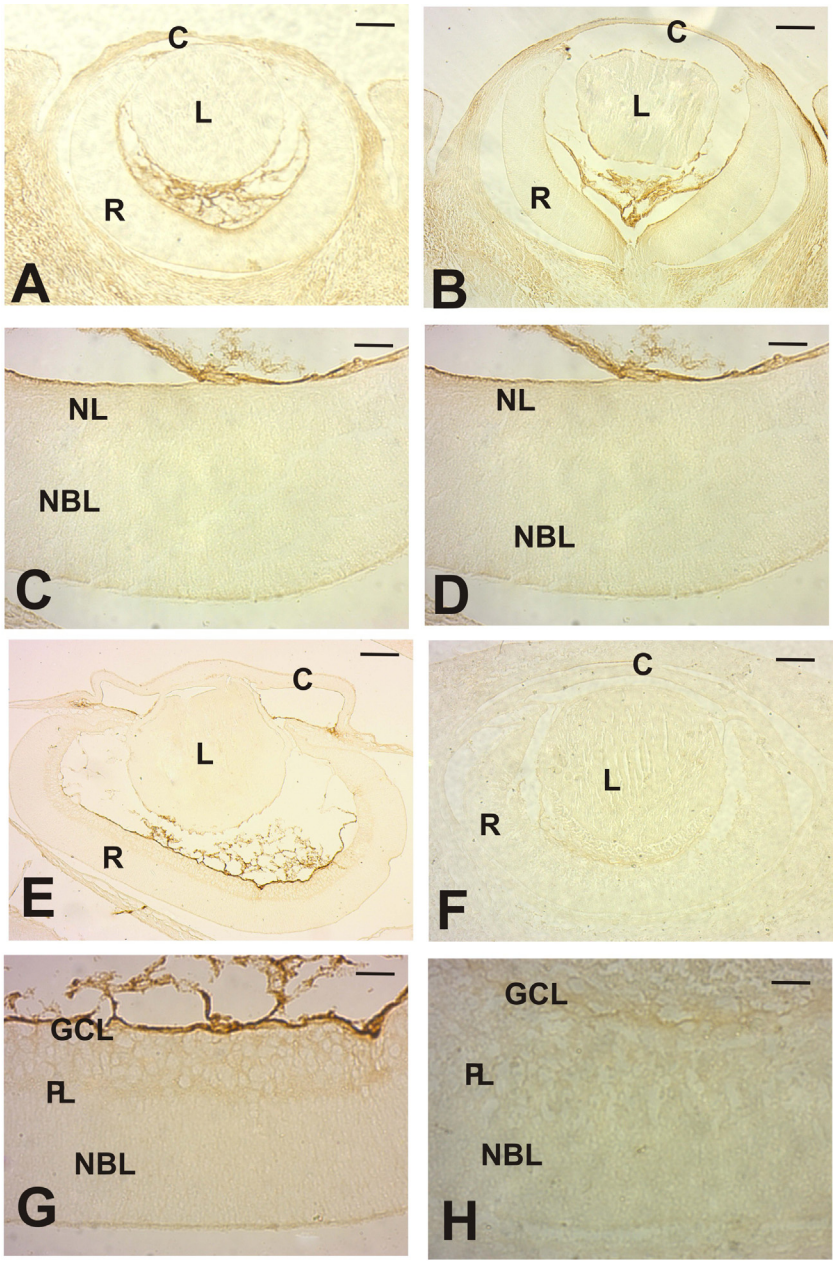

Figure 4 Photomicrographs of the immunostained sections of the developing retina at E14 (A and C) and at E17 (E and G) of the control showing restricted expression of BAK at the developing ganglion cell layer (GCL). Restricted expression of BAK at the neural layer in E14 (B and D) and inhibition of BAK expression at E17 (F and H) was noted in verapamil treated embryos (arrows). Scale bar 10 μm (A, B, E, F); 50 μm (C, D, G, H).

bated with secondary antibody (Biotinylated Goat Anti-polyvalent HRP DAB detection system, Spring Bioscience, USA). Then sections were washed with phosphate buffer and then visualized with chromogen solution that contained 0.05% 3′,3′-diaminobenzadine. Stained sections were mounted with DPX mounting media and investigated under a light microscope.

Results

At E14-old embryos the eye has a well developed cornea, lens and retina with differentiated neural and neuroblastic layers (Fig. 1A and C). At E17, ganglion cell layer and a plexiform layer were differentiated in addition to the remaining neuroblastic layer (Fig. 1E and G). In verapamil treatment, the histological structure of the developing eye at E14 is similar to that of control (Fig. 1B and D). At E17, the effect of verapamil was clearly manifested. The inner surface of the neuroblastic layer was infiltrated with inflammatory cells with de-differentiation of either the ganglion or the plexiform layers, also there was comparatively missing of ganglion cells and an apparent reduction of nuclear cell densities (Fig. 1F and H). In control E14, over-expression of cytochrome C was detected at the periphery and inner margin of the nuclear layer (Fig. 2A and C), however verapamil-treatment possessed comparatively less

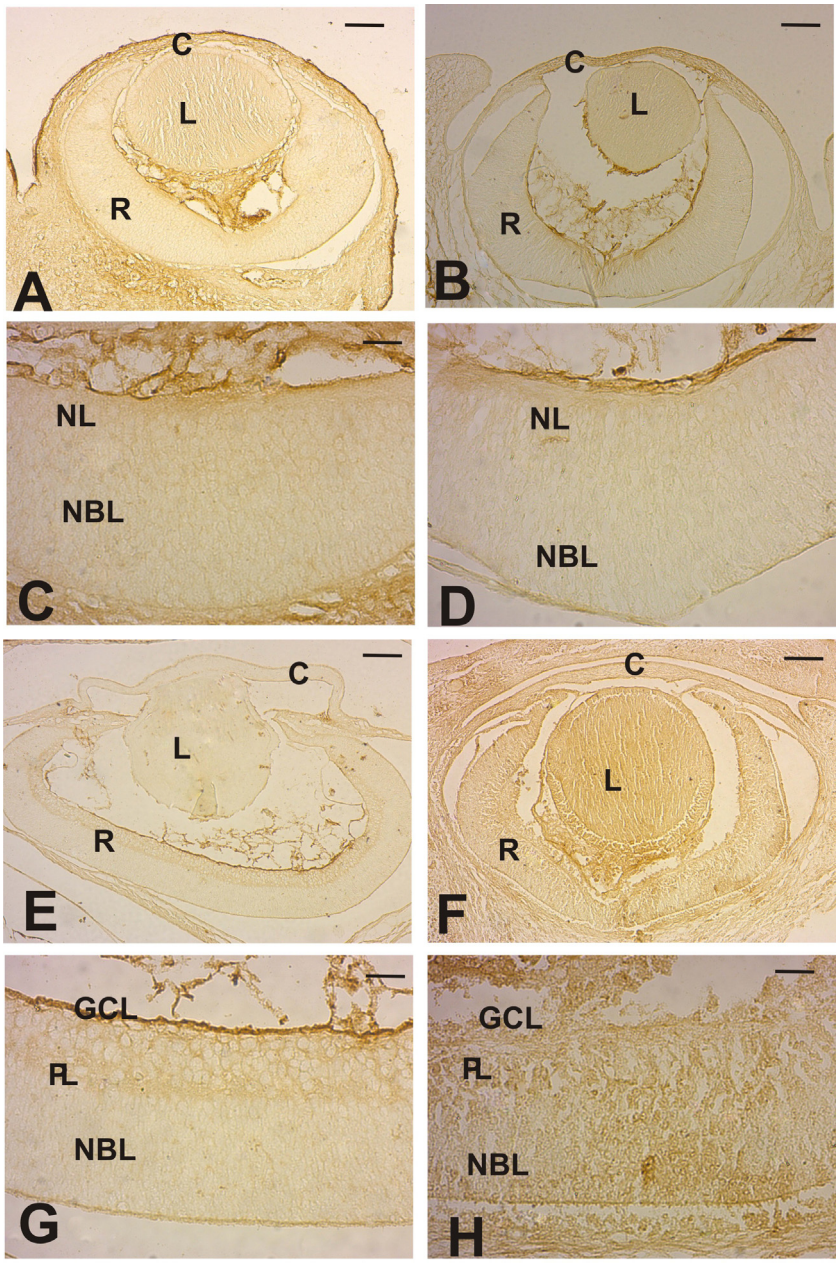

Figure 5 Photomicrographs of the immunostained sections of the developing retina at E14 (A and C) and at E17 (E and G) of the control showing restricted expression of TNFα R2 at the developing ganglion cell layer (GCL). Restricted expression of TNFα R2 at the neural layer in E14 (B, D) and inhibition of TNFα R2 expression at E17 (F and H) was noted in verapamil treated embryos (arrows). Scale bar 10 μm (A, B, E, F); 50 μm (C, D, G, H).

immunostaining in the mentioned regions (Fig. 2B and D). Comparing with control E17, the ganglion cell and inner margin of the nuclear layer showed decreased expression of cytochrome c (Fig. 2F and H). Concerning caspase3, verapamil-treatment at E14 (Fig. 3B and D) exhibited moderate immunostaining throughout the nuclear layer and was highly expressed in its peripheral and inner margins, comparing with the negative reaction in the control (Fig. 3A and C). In the developing retina, the expression is homogenous that was best detected in the basement membrane of the neuroblastic layer of the control (Fig. 3C) and increased in verapamil treated embryos (Fig. 3D). In older embryos at E17 of development, the expression is restricted to the differentiated ganglion and the plexiform layers of control embryos (Fig. 3E and G) that were inhibited in verapamil treated embryos (Fig. 3F and H) compared to the control.

The expression of both BAk and TNFα R2 are the least of the studied factors. The normal immunoexpression of BAK in the control group showed weak expression at E14 in NBL and NL (Fig. 4A and C) that gradually increases and best detected GCL at E17 (Fig. 4E and G). In verapamil treated embryos, similar expression was noted at E14 (Fig. 4B and D) while no expression was noted at E17 of verapamil treated embryos as compared to the control (Fig. 4F and H). TNFα R2 receptor expression was confined to the developing ganglion cell layer in the control embryo at E14 (Fig. 5A and C) and at

E17 (Fig. 5E and G). Verapamil treated embryos at E14 (Fig. 5B and D) didn't showed any change in expression while developing ganglion showed inhibition of the TNFα R2 receptor expression at E17 (Fig. 5F and H).

Discussion

The present investigation revealed that verapamil (a calcium channel blocker) exerts histological damage and alters the expression of the pro- and apoptotic factors under investigation. Since earlier, calcium channel blockers are widely prescribed for therapeutic purposes and some have been recommended for use during pregnancy for defined indications; e.g. verapamil for treating fetal paroxysmal tachycardia (Wolff et al., 1980; Lilja et al., 1984; Truccone and Mariona, 1985) and during tocolytic therapy (Strigl et al., 1981). These therapeutic uses of calcium channel blockers are recommended at late pregnancy after organogenesis has already been completed (Stein et al., 1990). Calcium channel blockers have been shown in animal experiments to induce teratogenic effects and to increase the incidence of embryolethality in mammalian animals (Lee and Nagele, 1986; Stein et al., 1990; Robert et al., 2011; Uslu et al., 2013). In addition, verapamil causes malformations, edemas and reduced heart rate in the embryos, larvae and adult fish (Rottbauer et al., 2001; Shin et al., 2010; Steinbach et al., 2013). Verapamil inhibits glucose uptake in insulin-sensitive tissues such as adipocyte skeletal myocytes and cardiac myocytes (Khil et al., 1997; Whitehead et al., 2001;Tenharmsel et al., 2005). Verapamil also inhibits the glucose transport activity of GLUT 1 in a dose-dependent manner (Larry et al., 2010). These effects of verapamil are consistent with the retinopathy induced by verapamil treatment in pregnant animals of this study. The retinopathy of the retina can be attributed mainly to calcium disturbance induced by verapamil that may cause a decrease in cell adhesion and alteration of the ionic coupling of retinal cells during differentiation. In this context, verapamil was found to diffuse through the placenta resulting in low blood concentration of the developing embryo than in maternal blood (Solans et al., 2000). In addition, verapamil was also shown to induce miscarriages via inhibiting implantation at high doses (Uslu et al., 2013).

Immunohistochemical investigation revealed the expression of the pro-apoptotic factors (cytochrome c and caspase-3) in normal development of the retina at pre-natal stages at E14, E17 of gestation. Immunoexpression of these two factors reflect their role in normal development regardless the effect of verapamil on calcium uptake of the developing retina cells. Also, the expression of BAK and TNFα R2 in normal retinal differentiation however was restricted to the differentiated nerve fibers of the ganglion neural layer and also reflects their role in normal development regionally in contrast to the expression of the other two factors that coincides with the different regions of the developing retina. Of these factors, the pro-apoptotic factor (caspase-3) expression is the only of the studied factors that increased especially at E14 of prenatal development as a result of verapamil treatment that results in the damage and de-differentiation of the retinal layers. In normal development the role of both the pro-and apoptotic factors under investigation was reported (Liu et al., 1996; Tezel et al., 2001; Hahn et al., 2003; Zeiss et al., 2004; Abdul-Ghani and Megeney, 2008). This confirm the results obtained at E17 of the retinal development as a result of verapamil treatment since the ganglion and the plexiform layers were deformed in the retina of downregulated expression of all the studied factors. Programed cell death (apoptosis) is crucial for the normal development of the retina and it is a tight orchestrated event under control of genetic programs. Vecino et al. (2004) explain the typical signaling pathways which lead to cell apoptosis in the developing vertebrate retina that involved the studied markers and clarified their roles during retina development. From the other hand suppression of normal apoptosis in verapamil treatment was recorded in several studies (Zawadzki et al., 2008; Wyska, 2009; Mei-Ping et al., 2011). Alteration of normal apoptosis as well the normal development of the retina in the present study result from the downregulation as in the case of cytochrome c; BAK and TNF R2 or the elevation of caspase-3 expression in verapamil-treated embryos that exert a drastic effect on the ganglion cell layer in the present study. From the previous and the present studies it can be concluded that verapamil as a calcium channel blocker has the ability to alter the normal apoptosis during prenatal retinal differentiation of mice through downregulation of cytochrome c; BAK; TNF R2 and upregulation of caspase-3 that results in a drastic effect on the developing retina regardless the intra- or extracellular calcium ion concentration.

Conflict of interest disclosure

The authors declare no conflicts of interest.

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