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Retinal Neovascularisation in Newborn Rats Submitted to Variations of Concentrations of Oxygene. Histopatological Aspects.

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Abstract. This study was conducted on two groups of newborn rats: a control group and an experimental group. The control group and was composed of seven newborn rats, which were placed at 4 hours after birth, in a pediatric incubator, together with their mother, in conditions of normoxia for 21 days. The experimental group consisted of seven newborn rats, which were placed at 4 hours after birth, in a pediatric incubator with their mother, in conditions of normoxia for 7 days, then five days in a daily alternating periods of hyperoxia (80%) and hypoxia (10%), and then nine days in conditions of normoxia. Slaughtering of the rats was performed on day 21 and the eyeglobes were harvested in order to perform histopathological examinations. Microscopic examination revealed a normal development of the retina in the control group. In the experimental group, on the surface of the retina, an exaggerated neovascularization both respects the number of vessels, as well as their size was observed. Excessive proliferation of blood vessels caused severe histo-architectural changes, irreversible and bilateral, with serious damage of the retinal function.

Keywords: hyperoxia, hypoxia, oxygene, rat, retina.

INTRODUCTION

Maturation of the human visual vascular system is made in the intrauterine life, in conditions of physiological hypoxia by two processes: vasculogenesis and angiogenesis. Despite of many experimental researches, knowledges about human retinal vascular development are incomplete. (Pau, 2008; Hughes, 2000).

Premature newbornes are born with an immature visual system, vulnerable to postnatal extrauterine conditions. Hyperoxia exposure, to compensate an unstable pulmonary status, initially cause a severe vasoconstriction and vaso-obliteration followed by an abnormal proliferation of retinal vessels when it returned to normoxia, neovascularization, with apparition of retinopathy of prematurity (ROP) (Albert, 2008). Despite the fact that ROP is considered a disease of retinal vascular development and the fact that in some cases it regress spontaneously with vascular repermeabilisation, although in adults with a history of ROP also neural dysfunction has been shown, due to structural retinal abnormalities that accompany vascular anomalies. (Fulton, 1995, 1996, 2001, 2005).

Direct relationship between oxygenotherapy and ROP was highlighted by Michaelson (1948), Campbell (1951), Ashton *et al.* (1954). Although O2 toxicity in living tissues has been noted much earlier, in 1770 by Priestley (1775) the mechanisms governing its spectacular effects began to be elucidated in the last quarter century. These destructive effects of hyperoxia are severe especially against endothelial cells from the developing systems: brain, lungs and eyes being particularly affected (Smith *et al.*, 1994; Halks, Miller *et al.*, 1986; Auge *et al.* 1995; Kondo *et al.*, 1996).

The retina is an excellent organ for studying neovascularization, whereas a gradual increase of blood vessels can be monitored by angiofluorography, ophthalmoscopy and histopathologic exams. Many evidences regarding normal and abnormal development of blood vessels in the retina were obtained from animal models, studies on human premature neonates being difficult. In some species, cats, dogs and rats, most part of the retinal vascularization develops after birth in conditions of normoxia. (Chan-Ling, 1990; McLeod 1996) Exposure to oxygen of animals neonates, which present an immature retina at birth, followed by normoxia, produces oxygen-induced retinopathy (OIR), similar to human ROP. (Penn *et al.*, 1994; Smith *et al.*, 1994).

Newborn rat model represents a solution to study normal and abnormal development of primary visual system of human subjects. The rat is born with closed eyes and it has an immature visual system. After birth, the rat visual system gradually matures, the eye opening being realised in 14 days. (Cairns, 1959). Complete maturation of vascular retina, neuroglial and visual system is realised at the end of the first month of life. Unlike other animals used in studying the pathophysiology of OHR (Chan-Ling, 1992; Smith, 1994; McLeod, 1996) newborn rat offers the next advantages: retinal vascularization occurs in the first two weeks postnatally, which enables an observation of vascular development (Connolly, 1988; Blanks, 1983); stage of development of retinal vasculature is comparable to that of a human premature in 4-5th month of pregnancy (Gyllensten, 1955), an extremely immature retina at birth comparable to that of a human fetus of 26 weeks (Ricci, 1990).Neovascularization is a process widely studied both in the ocular level, as it leads to decreasing of vision and even blindness (ROP, diabetic retinopathy, macular degeneration age-related degeneration,) and at non-ocular level, being involved in diseases such as sugar diabetes, tumors, ischemic diseases.

MATERIALS AND METHODS

Animals used in this study were white rats, Wistar line: newborn rats with a birth weight of about 10g. Experimental study was performed at the Department of Physiology of University of Medicine and Pharmacy Cluj-Napoca. They made two groups: one control group and one experimental. The control group consisted of seven newborn rats, which were placed at 4 hours after birth, in a pediatric incubator with their mother, in conditions of normoxia for 21 days. Conditions of incubation: temperature 23-24^{O}C, cyclic exposure 12 day/12 darkness using artificial white light 200 lux, feeding of rats being ensured by the milk of the mother ad libitum. The experimental group consisted in seven newborn rats, which were placed at 4 hours after birth, in a pediatric incubator with their mother, 7 days under conditions of normoxia, then 5 days in a daily alternating periods of hyperoxia (80% for 22.5 hours) and hypoxia (10% for 1hr) and 9 days again in conditions of normoxia. To achieve hiperoxia, mobile oxygen concentrator adapted to the incubator was used and hypoxia was achieved by placing the incubator in barochamber. The slaughter of the rats was performed on the 21th day, after sedation with ketamine 6mg/kgc (Oana *et al.* 2006). Eyeballs were enucleated for histopathological examinations. A 3 mm incision was made in the central area of the cornea (to facilitate penetration of the fixation solution), then eyeballs were fixed in

Stieve mixture for 24 hours. After, the eyeballs were sectioned at the limbus, under microscopic control, carefully removing the cornea, lens and vitreous. The pieces were then dehydrated with ethilic alcohol, clarified with butyl alcohol (n-butanol) and included in paraffin. Serial sections with 5 μ thickness were obtained and its were stained with Goldner's trichrome method. Examination of stained sections was made at a Olimpus BX41 microscope.

RESULTS AND DISCUSSION

In the control group, all histological sections made showed a normal aspect of the retinal vessels and retinal layers (Fig.1). In the experimental group of oxygen-induced retinopathy OHR, retinal histological sections revealed unusually large retinal vessels in the internal third of the retina, arranged at the level of the layer of optical fibers, parallel to the surface of the retina (Fig. 2). These vessels were present over the entire surface of the retina, from the posterior/center to periphery. In some areas, large-caliber vessels were distorted both surface and structure of the retina. From some vessels fine vascular branches started which entered initially perpendicular in the deep layers to the internal granular layer, and then obliquely, until the photoreceptor cell layer. These aspects have been observed all over the retina, both regarding the caliber of the superficial vessels and ramifications which penetrate in depth. In retinal periphery large vessels arranged parallel to the surface, in the thickness of the optical fiber layer were revealed (Fig. 3). In some cases, these vessels were extended also in ganglionic layer and were branched in depth (Fig. 4) and, at the level of internal granular layer its branched and penetrated obliquely in depth.

In some sections, at the retinal periphery (not only) numerous small-caliber vessels besides large-caliber vessels were revealed, which suggested that angiogenesis/neovascularization is in full progress. Îin very few cases, relatively large size vessels penetrate in depth until the external granular layer.

By increasing vascular branches in depth of the retinal layers, structural changes occurred whose magnitude was directly proportional with the number of vessels (Fig. 5). In situations when the vessels were less numerous and with smaller caliber, characteristic arrangement on layers was kept. By growth and branching in depth, the vessels anchored at photoreceptor cells layer exerted tractions on this layer leading to its zonal separation with forming of folds of different shapes and sizes. In the moment of folds forming by the separation of the photoreceptor cell layer from the pigmented cell layer, due to traction exerted by the blood vessels, a zonal microhemorrhage is produced (Fig. 6), followed by mobilization of macrophages, which come for phagocytosing red blood cells and debris resulting from their degradation (Fig. 7). Somewhat similar results were observed by Penn (1992) which showed on rat model, retinal folds in 50% of cases studied and retinal hemorrhages in 40% of cases. He asserted that the hemorrhages derived from new vessels and deep capillary vessels (red blood cells migrated to the retinal space).

First author which reported the susceptibility of the retinal vessels to the action of O2 beginning from clinical observations from neonatal-care units was Patz (1952). Conducting experiments on rats, he showed in his model of hyperoxia (80%), isolated cases of neovascularization on histopathological sections, but without an ulterior reproducing of them (Patz 1953, 1954). Postnatal exposure of the rats to hyperoxia caused severe vasoconstriction and vaso-obliteration followed by an abnormal proliferation of retinal vessels when, when its were returned to conditions of normoxia. (Smith 1990, Reynaud 1994, Madan 2003, Hardy 2005). Beauchamp (2004) said that continuous exposure to hyperoxia favors vasoconstriction while an alternating exposure to hyperoxia/hypoxia promotes vascular proliferation.

Similarly, Ventresca (1990) has conducted studies on animals exposed to hyperoxia and he highlighted retinal neovascularization in 16 of 20 animals. Reynaud (1994) also suggested that avascular retina initiates the events leading to neovascularization. Penn *et al.* (1994) compared the groups 80/40% (relative hypoxia) with groups 50/10% (hypoxia), cyclic exposure, in alternation, and he showed neovascularization 100% in 50/10% group, suggesting that, avoiding of hypoxic levels is more important in neonatal care, than difference of 40%. By exposing rats to hyperoxia in the first 14 days (80% O2) followed by normoxia, Cummingham (2000) obtained retinal vaso-obliteration at the border between vascular and avascular retina, morphologically similar to the human, but he not obtained also neovascularization, leading to conclusion that the hypoxia is an important element in promoting retinal neovascularization in newborn rats.

Results obtained in this model of alternating exposure to hyperoxia/hypoxia, are similar to those reported by the authors mentioned. Particular is the fact that number of vessels that proliferated in retinal thickness was great and the caliber of some of them impressive, the biggest being present at the level of optical fibers layer.

The apparition of a more pronounced neovascularization than that reported by most authors who have similar experiences, is probably due to the fact that alternation hyperoxia (80%)/hypoxia (10%) used in this experiment had a more brutal effect on the developing of the retinal vascularization than alternation 50/10% or 80/40% (Penn *et al.,* 1992). This effect is due to a larger difference of variation of O2 (80% -10%), and also due to hypobaric conditions. As asserted Reynaud (1994), both incidence and severity of neovascularization intensity grow with intensity of periods of hypoxia. Although some authors observed in their studies that retinal neovascularization is reversible, with restoration of normal retinal vascularization, in case of newborn rats, cytoarchitectural retinal changes persist (Dorfmann, 2008) In this study, the exaggerated neovascularization obtained is accompanied by cytoarchitectural severe and irreversible abnormalities, bilateral, the loss of visual function being an inevitable consequence. The aspects obtained in this experimental study, allow us to say that, the variation of O_2 concentration has a negative effect on retinal neurovascular development, with apparition of neovascularization and developmental abnormalities of neural retina.

CONCLUSIONS

In this experimental model of exposure of newborn rats to alternating variations of oxygen concentration 80%-10%, an exaggerated retinal neovascularization on surface of the retina was obtained, both regarding the number of vessels, as well as their caliber.

Ramifications of these new vessels in depth retina, cause both zonal decolation of photoreceptor cells layer by pigment cell layer and breaking of some small vessels, with the development of microhemorrhages in the layer of cones and rods.

Excessive proliferation of blood vessels causes severe bilateral and irreversible histoarchitectural changes, with serious damage of retinal function, the degree of damage being in some cases different from one animal to another.

The apparition of more severe vascular and histoarchitectural changes than in most cases reported in the literature, suggests that these changes increase in intensity depending on the severity of hypoxia.

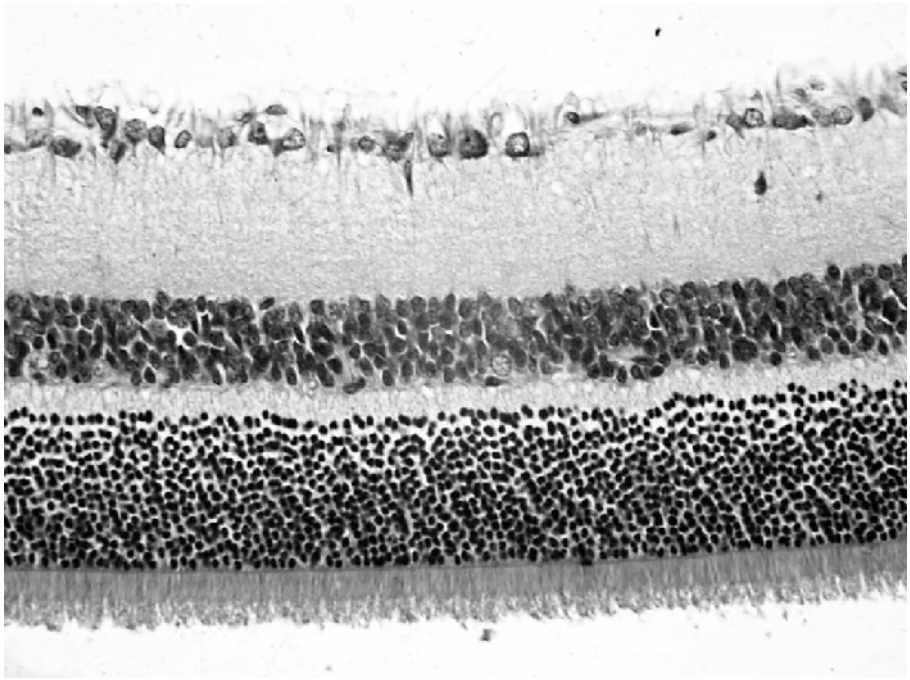

Fig. 1 Control group – normal structure (Goldner's Trichrome ob. 40X)

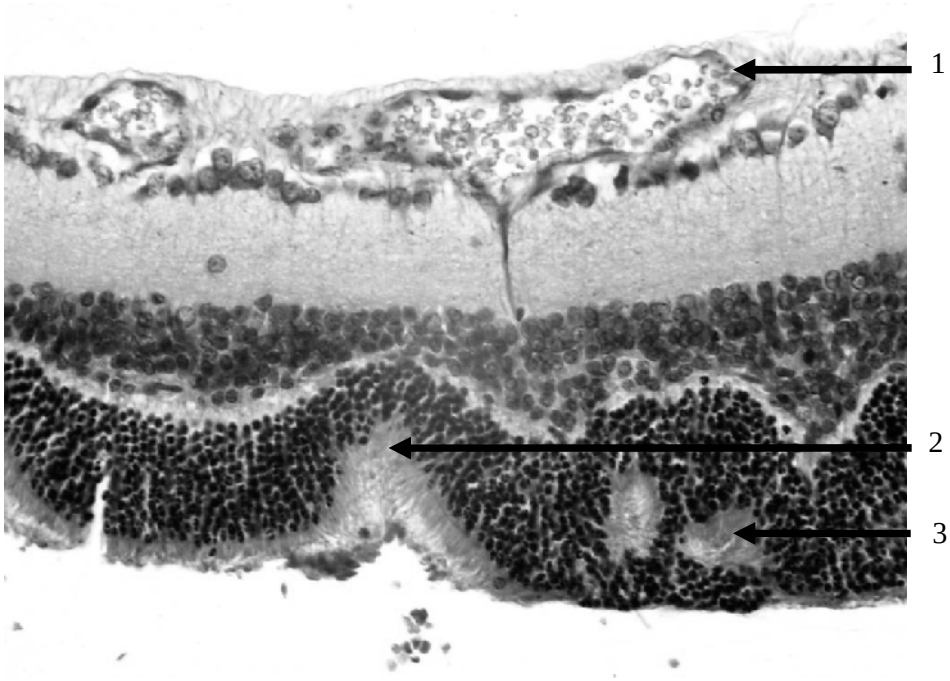

Fig. 2. Hyperoxia/hypoxia group (Goldner's Trichrome ob. 40X)
1- large vessels in the layer of optical fibers 2- retinal folds; 3- rosettes

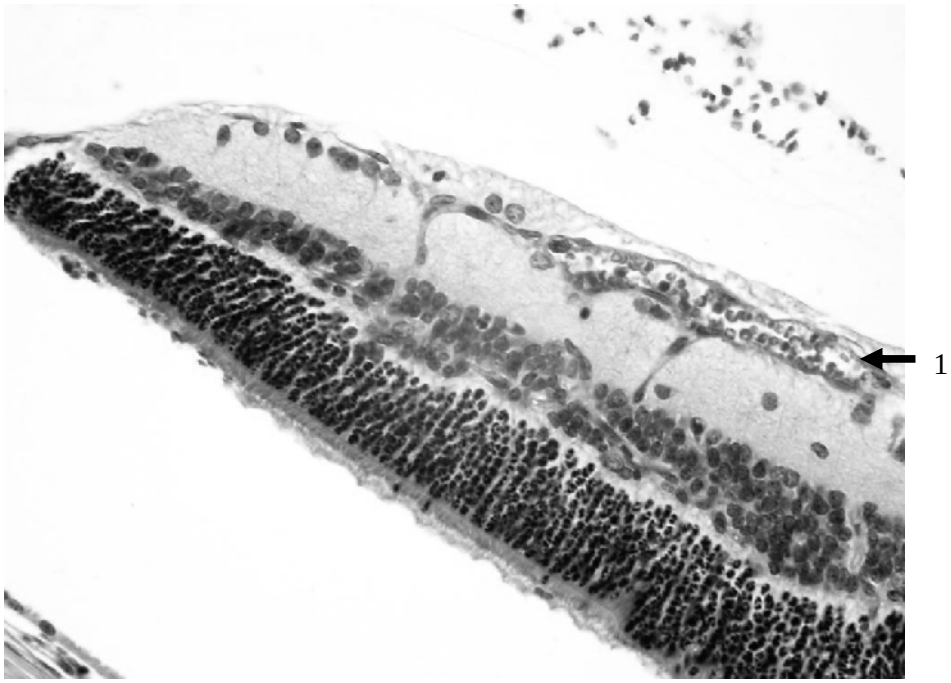

Fig. 3. Hyperoxia/hypoxia group (Goldner's Trichrome ob. 40X)
1-large vessels in peripheral region of the retina

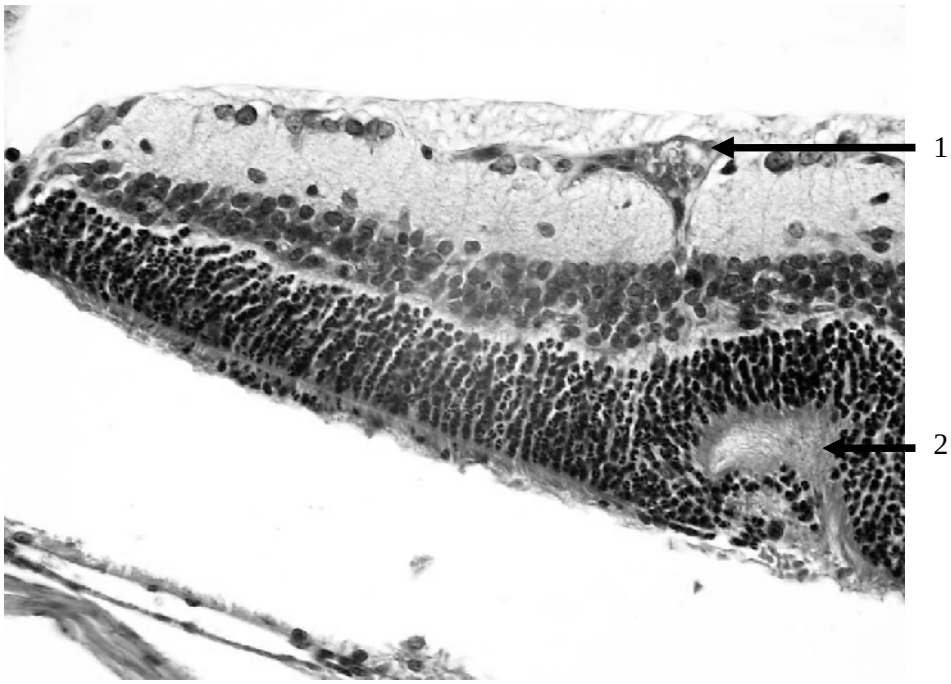

Fig. 4. Hyperoxia/hypoxia group (Goldner's Trichrome ob. 40X)
1-branched vessels ; 2- rosettes

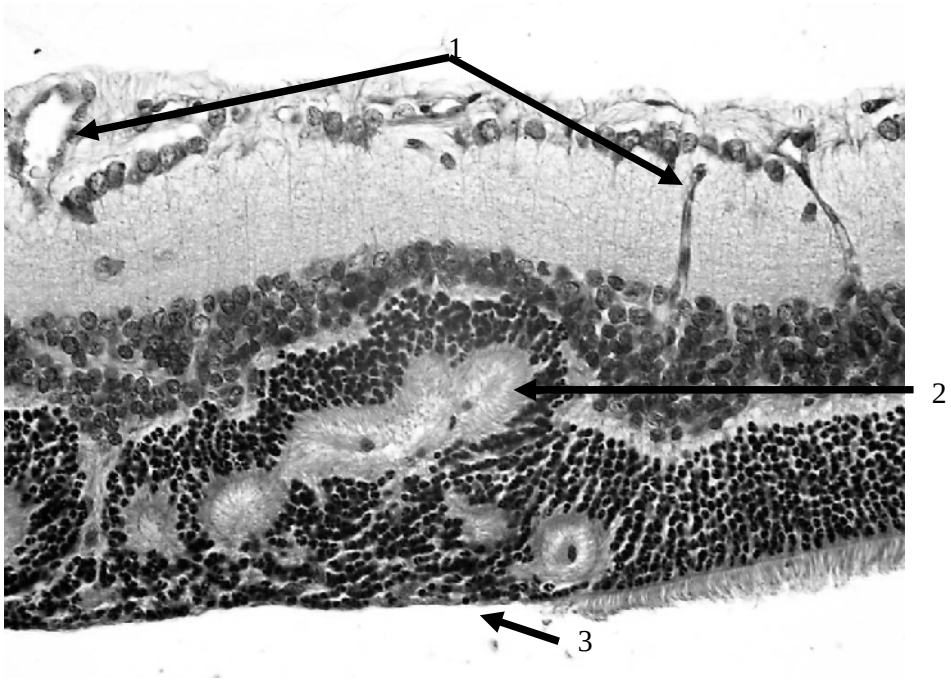

Fig. 5. Hyperoxia/hypoxia group (Goldner's Trichrome ob. 40X)
1. vessels of different calibers ; 2- polymorphous rosettes;
3- regional absence of cons and rods

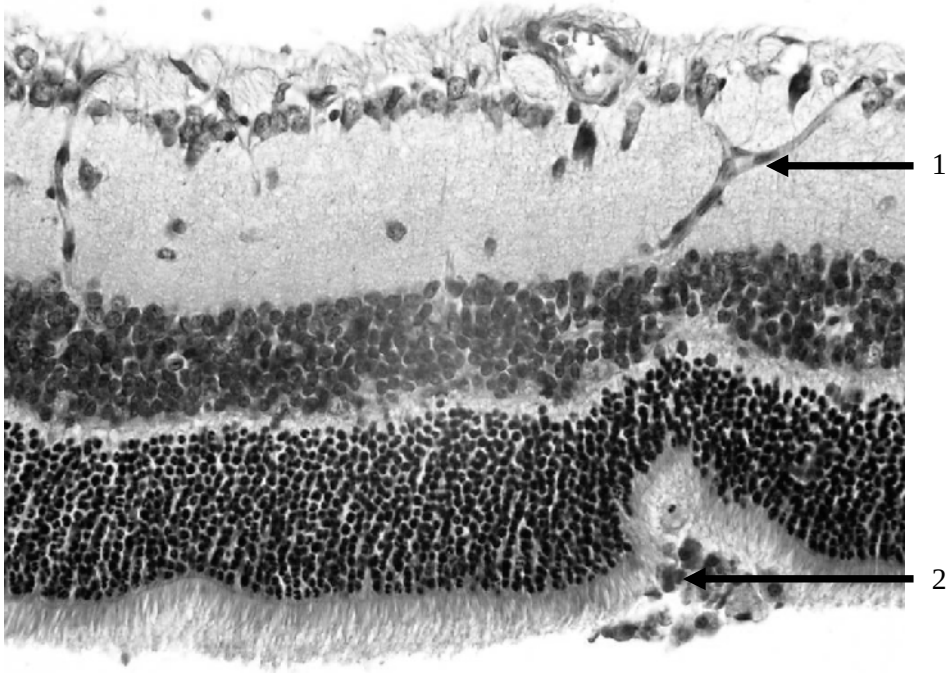

Fig 6. Hyperoxia/hypoxia group (Goldner's Trichrome ob. 40X)353
1- branched vessels; 2- microhemorrhage in the layer of cones and rods

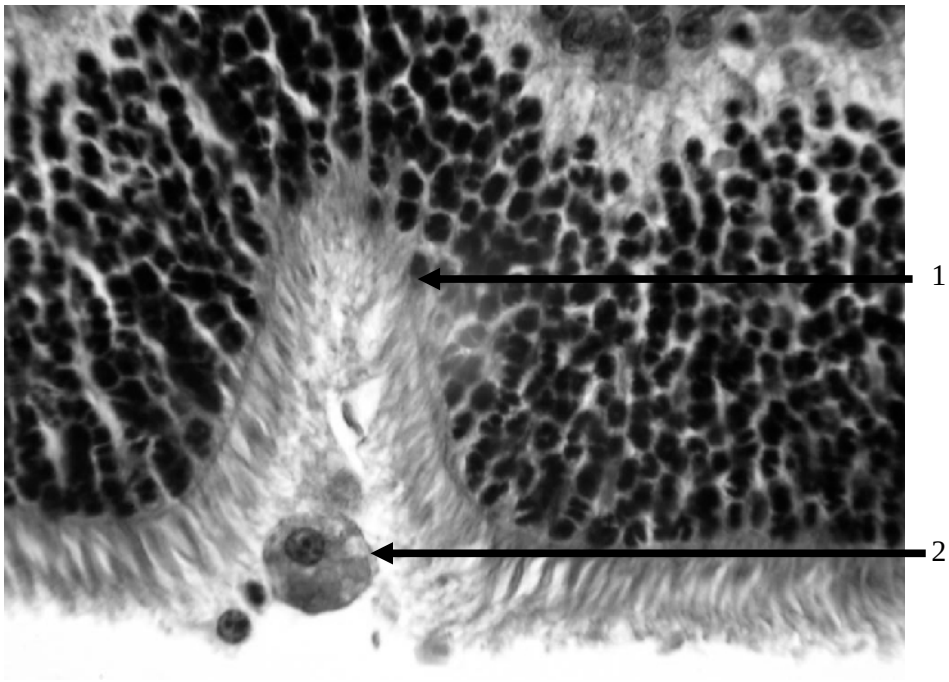

Fig7. Hyperoxia/hypoxia group (Goldner's Trichrome ob. 100X)488
1- retinal fold; 2- macrophage with phagocitated erythrocytes

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