

Angiogenesis and hyperbaric oxygen in the chick embryo chorioallantoic membrane

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ABSTRACT Hyperbaric Oxygen Therapy (HBOT) is increasingly applied in different areas of medical practice. The oxy-hyperbarism effects are not well understood in cancer malignancy. One unique feature of cancer is the presence of hypoxic regions that are insensitive to conventional therapies. It is possible to alter the hypoxic state and produce reactive oxygen species for better treatment outcome by HBOT. In the present study, we determined the effects of HBOT on angiogenesis, a signature of cancer progression, by using the chick chorioallantoic membrane (CAM) *in vivo* assay. CAMs were exposed to 2.0 ATA (atmospheres absolute) for 30 min of hyperbaric oxygen on the 6th and 7th days of incubation (ED6, ED7). On the 10-11th day of incubation, CAMs were excised from eggs, fixed and analysed using APERIO ImageScope software. HBOT outcomes were evaluated quantifying the volumetric area occupied by blood vessels and calculating the number of blood vessel ramifications. Results indicated that CAMs treated at ED6 and ED7 had a significantly higher CAM vascularization and an increased number of blood vessel ramifications (+82% higher for ED6) compared to untreated CAMs (ED6=63.3±2.5 and ED7=57.7±5.5 vs. CTRL=34.7±2.5). Thus, HBOT induces an angiogenic response in treated CAMs through a classic sprouting mechanism.

KEY WORDS: *angiogenesis, chorioallantoic membrane, hyperbaric oxygen*

The variations of oxygen tissue tensions and the various conditions related to normoxia, hypoxia and hyperoxia are determining factors on the metabolic pathways and the formation of new blood vessels in several pathological conditions. The Hyperbaric Oxygen Therapy (HBOT) is a treatment used primarily to improve or cure disorders related to hypoxia and ischemia which, by increasing the amount and the partial tension of the oxygen (PpO₂) dissolved in the plasma, enhances its diffusion in the tissues.

The major international scientific societies defined several clinical indications for which the therapeutic use Oxygen Hyperbaric was approved including Gas Embolism Hypertension (EGA), Decompression Illness (DCI), Carbon Monoxide poisoning, acute traumatic ischemia and chronic wounds (Gesell, 2008; Mathieu, 2005).

Since its introduction in clinical practice, studies conducted on HBOT and cancer have been mainly focused on the stimulating role that pressurized oxygen may have on cancer growth. Systematic reviews of HBOT and cancer have described the use of HBOT in patients with malignancy (Daruwalla and Christophi, 2006; Hoggan and Cameron, 2014; Mayer *et al.*, 2005; Moen and Stuhr, 2012). However, several unsolved questions remain connected

with HBOT treatment, including severe DNA damage and genetic instability leading to reactivation or augmentation of existing cancer, development of fragile and porous capillaries and associated higher chance of metastasis, initiating aneuploidy causing delay in DNA repair of surrounding normal or stromal cells.

The effects of the mechanisms action of hypoxia are extensively investigated in clinical and experimental settings, but less known are the effects that the conditions of hyperoxia and mostly the one of oxy-hyperbarism may have in biological processes.

The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane which serves as a gas exchange surface and its function is supported by a dense capillary network. Because of its extensive vascularization and easy accessibility, the CAM has been broadly used to study the morphofunctional aspects of the angiogenesis process *in vivo* and to investigate the efficacy and mechanisms of action of pro-angiogenic and anti-angiogenic

Abbreviations used in this paper: CAM, chick embryo chorioallantoic membrane; HBOT, hyperbaric oxygen therapy.

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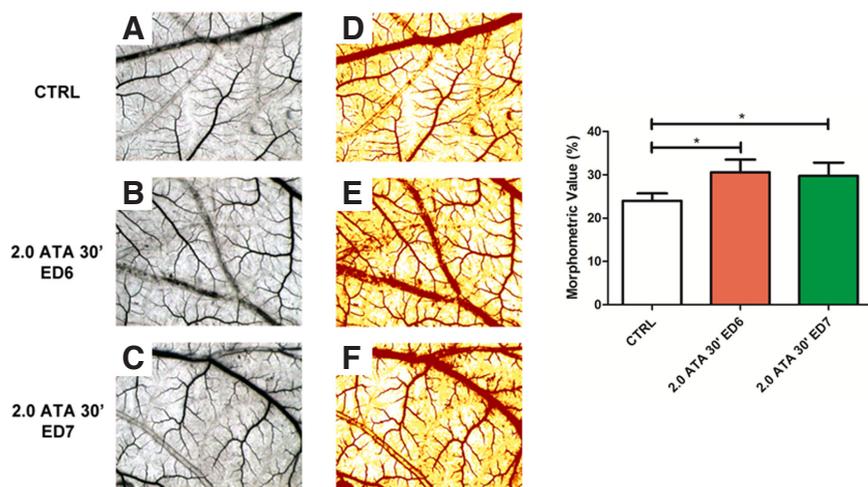


Fig. 1. Differences in vascularization between control, ED6 and ED7. Chicken eggs were treated with 2.0 ATA for a 30-min exposure of Hyperbaric Oxygen on the 6th (ED6) and 7th (ED7) day of incubation respectively, while untreated eggs were used as controls (CTRL). On the 10-11th day of incubation, CAMs were excised from eggs, fixed and examined. Stereomicroscope images were converted in gray-scale (A-C) and analyzed using the Aperio Positive Pixel Count algorithm; see mark-up images (D-F). CAMs treated at ED6 and ED7 show a significant higher percentage of strong positive pixels than control ones (ED6=29.76±3.05 and ED7=30.58±2.94 vs CTRL=23.98±1.75). Mark-up image color code: Red, strong positive pixels; Orange, medium positive pixels; Yellow, weak positive pixels. * $p < 0.05$.

natural and synthetic molecules (Ribatti, 2008).

The aim of this study is to evaluate the possible effects that exposure to Hyperbaric Oxygen (HBO) on the process of angiogenesis by using as experimental *in vivo* model the chick embryo CAM.

Results and Discussion

Image analysis shows significant differences between control CAM's and treated CAMs. The percentage of strong positive pixels which identifies blood vessels in the stereomicroscopic images shows a significant difference between treated and control CAMs. Specifically, CAMs treated at ED7 showed a higher percentage of strong positive pixels (mean = 30.58; SD = 2.939) when compared with control CAMs (mean = 23.98, SD = 1.750). A higher percentage of strong positive pixels was found in CAMs treated at ED6 (mean = 29.76; SD = 3.051) when compared with control CAMs (mean = 23.98, SD = 1.750), as well. No statistical significance was found between the two treated groups (Fig. 1).

Counting of primary, secondary and tertiary blood vessel ramifications in a magnified particular stereomicroscopic of all the images revealed significant differences between the experimental groups. In particular, the number of blood vessel ramifications were higher in CAMs treated at ED7 (mean = 57.67, SD = 5.508) when

compared with control CAMs (mean = 34.67, SD = 2.517). CAMs treated at ED6 also showed a higher number of blood vessel ramifications (mean = 63.33, SD = 2.517) when compared with control CAMs (mean = 34.67, SD = 2.517). No statistical significance was found between the two treated groups (Fig. 2). The presence of an higher number of blood vessel ramifications in CAMs treated at ED7 when compared with control CAMs was confirmed also by histological analysis (Fig. 3).

Vascular remodeling is a fundamental physiological process during development and acclimatization to adverse environments, and a multitude of factors are involved in the angiogenic response during vascular remodeling. Oxygen tension is positively correlated with collagen production, bacterial killing, epithelialization, and angiogenesis (Knighton, 1990).

The results of this study have demonstrated that HBO is able to induce an angiogenic response in the treated CAMs as compared to control ones, and are in accord with a general effect of high tension of oxygen of enhancement of growth of chick embryo, as previously demonstrated by Stock and co-workers (Stock *et al.*, 1983).

The principal mechanisms of action of HBO are based on the intracellular production of Reactive Oxygen Species (ROS) including superoxide anions (O_2^-), hydroxyl radicals (OH^\cdot), and hydrogen peroxide (H_2O_2). Increased release of ROS generated

by cyclooxygenase-1 in endothelial cells might produce vasoconstriction by inhibiting the synthesis or the action of vasodilatory components such as prostacyclin or nitric oxide (Stuart *et al.*, 1984). Moreover, this latter is a strong modulator of the angiogenesis (Aicher *et al.*, 2003). At micromolar concentrations, H_2O_2 induces vascular endothelial

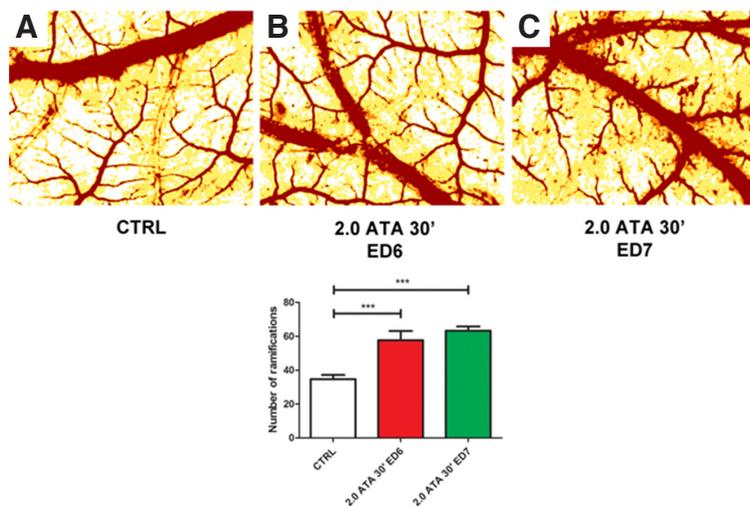


Fig. 2. Blood vessel ramifications counting between control, ED6 and ED7. Chicken eggs were treated with 2.0 ATA for a 30-min exposure to hyperbaric oxygen on the 6th (ED6) and 7th (ED7) day of incubation respectively, while untreated eggs were used as controls (CTRL). On the 10-11th day of incubation, CAMs were excised from eggs, fixed and examined. Mark-up images (A-C) analyzed using the Aperio Positive Pixel Count algorithm were used to count primary, secondary and tertiary blood vessels ramifications. CAMs treated at ED6 and ED7 showed a significant increase in the number of blood vessel ramifications compared to control ones (ED6=63.33±2.52 and ED7=57.67±5.51 vs CTRL=34.67±2.52). *** $p < 0.001$.

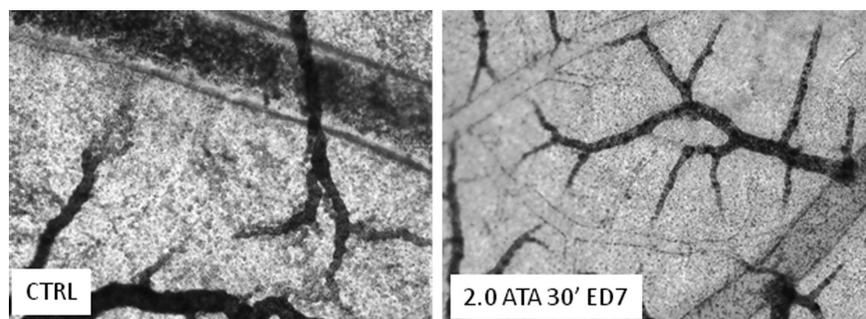


Fig. 3. Microscopic features of vascular tree in control CAM (CTRL) and CAM treated at ED 7 (2.0 ATA 30' ED7). Note an higher number of vascular ramifications in the experimental condition. Original magnification, 5 x.

cell growth factor (VEGF) expression (Sen, 2002).

On the contrary, oxidative stress caused by high oxygen exposure suppress expression of angiogenic factors such as VEGF (Thébaud and Abman, 2007). Moreover, hyperoxia reduces the number of circulating endothelial cell precursors in blood and lung of newborn mice (Balasubramaniam *et al.*, 2007), induces apoptosis of endothelial cells (Gu *et al.*, 2003), and inhibits endothelial cell proliferation (D'Amore and Sweet, 1987).

As concerns the CAM vascularization, primitive vessels proliferate and differentiate into an arterio-venous system until day 8, thus originating a network of capillaries that migrate to occupy an area beneath the chorion and mediate gas exchanges with the outer environment. Rapid capillary proliferation goes until ED11; thereafter, their mitotic index declines just as rapidly, and the vascular system attains its final arrangement on ED18, just before hatching (Ausprunk *et al.*, 1974). A morphometric investigation by DeFouw *et al.*, has shown rapid extension of the CAM surface between ED6 and ED14, and during this period, the number of feed vessels increased predominantly due to growth and remodeling after ED10 (DeFouw *et al.*, 1989). According to Schlatter *et al.*, CAM vascularization undergoes three phases of development with both sprouting and intussusceptive microvascular growth: in the early phase (days 5-7) multiple capillary sprouts invade the mesenchyme, fuse and form the primary capillary plexus. During the second (intermediate phase) (days 8-12), sprouts are no longer present since they have been replaced by tissue pillars expression of intussusceptive microvascular growth, with a maximal frequency at day 11 (Schlatter *et al.*, 1997). During the late phase (day 13 and older), the growing pillars increase in size to form inter-capillary meshes more than 2.5 μm in diameter.

Development of the response to hypoxia is better described than that to hyperoxia. Dusseau and Hutchins studied the effects of hypoxia on the number of pre and post-capillary vessels in the CAM and demonstrated that the low oxygen regimen stimulated a preferential increase in the number of arterioles (Dusseau and Hutchins, 1989). In our experimental condition, HBO induces an angiogenic response through a classic sprouting mechanism, because the time of treatment correspond to early phase of CAM vascular growth, in which angiogenic occurs. This is also confirmed by an increase in the number of blood vessel ramifications in experimental group, as compared to control ones. However, also in this case, in contrast with our study, other Authors have previously studied the effects of hyperoxia on the angiogenesis process in the CAM. Chick eggs were incubated in various oxygen atmospheres beginning on the 7th day of development, and vascularity was measured on the 14th day. Measurements included vessel end-point density, length density, fractional image area, and a vascular

density index, using a computerized image analysis system, and the results demonstrated that hyperoxia inhibits angiogenesis (Strick *et al.*, 1991).

Materials and Methods

Two hundred fertilized White Leghorn chicken eggs were incubated at 37°C at constant humidity (60%). On day 3 of incubation, a square window was opened in the egg shell, and 2 to 3 ml of albumen was removed to allow detachment of the developing CAM from the shell. The window was sealed with a glass, and the eggs returned to the incubator. Embryo manipulation did not alter its development. The possibility to look inside the embryo through the window allows to verify the vitality of the embryo. For each batch of chicken eggs supplied, three randomized groups were set up: treatment with Hyperbaric Oxygen on the 6th day of incubation (ED6), treatment with HBO at 7th day of incubation (ED7) and a control group (CTRL).

For security reasons the system embryo/bag saturated in oxygen was then placed in a small recompression chamber for experimental use (GlobalMed Pontenure srl-Italy) and pressurized air at a pressure of 2.0 ATA (atmospheres absolute) for a 30-min exposure of HBO. After treatment, the embryos were removed from the bag and placed back in an incubator. After four days of post-treatment incubation, CAMs were excised and then fixed with Karnovsky's fixative for 2 h at room temperature. CAMs were then examined and photographed with a stereomicroscope equipped with a camera (Olympus Italia, Rozzano, Italy) at 10 X magnification. Finally, CAMs were processed for light microscopy. CAM portions were removed and embedded in paraffin. Fifteen μm sections were cut according to a plane parallel to the surface of the CAM, stained with 0.5% aqueous solution of toluidine blue (Merck Biochemica), and observed under a Leitz-Dialux 20 light microscope (Leitz, Wetzlar, Germany).

Image analysis

Microscopic images obtained from the stereomicroscope were converted into gray-scale and analyzed using the Aperio Positive Pixel Count algorithm embedded in the ImageScope v.11.2.0.780 (Leica Biosystems Nussloch, Germany) as previously described. Briefly, the algorithm input parameters were initially set to obtain the identification of pixels related to the blood vessels as strong and positive as to the background medium and weak positive and tuned to minimize non-specific pixel recognition as strong positive. The ratio between the number of strong positive pixels and the sum of weak, medium and strong positive pixels is the morphometric value used to quantitate CAM vascularization across the experimental groups.

In additions, random areas of the images were magnified and the primary, secondary and tertiary blood vessel ramifications were counted in all the experimental groups. All the analysis were performed on images with equal area.

Statistical analysis

Fold change data are reported as means \pm SD. The Graph Pad Prim 5.0 statistical package (GraphPad Software, San Diego, CA, USA) was

used for the analysis and $P < 0.05$ was considered as the limit for statistical significance.

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