QUANTIFICATION OF THE EFFECT OF HYPERBARIC OXYGEN ON ANGIOGENESIS IN DUCK *Anas luzonica* (Fraser 1839) EMBRYOS USING CAM ASSAY

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ABSTRACT

Wound healing researches revealed inductive effect of hyperbaric oxygen on angiogenesis. In this study, twenty *Anas luzonica* eggs implanted with gelfoam were divided into three groups: namely, unexposed (Control), normobaric (NB) and hyperbaric (HB). At 12 days post-laying, NB and HB were exposed to 100% oxygen at 1-atm and 2.5-atm respectively. Egg contents were later transferred and photographed for fractal analysis while gelfoams with some underlying chorioallantoic membrane were processed for histoanalysis via planimetric point counting method. The HB group featured the highest vessel count and mean fractal index, suggesting that hyperbaric oxygen does stimulate angiogenesis to a greater degree than normobaric oxygen. Thus, hyperoxia stimulates angiogenesis by increasing metabolic rate while increased ambient pressure effects still requires further study.

INTRODUCTION

Background of the study

Angiogenesis is of key importance in a wide array of physiologic and pathologic processes (Mignatti, *et al.*, 1989). Normal tissue growth as in embryonic development, wound healing, and the menstrual cycle is dependent upon new vessel formation for their supply of oxygen and nutrients as well as removal of waste products (Griffioen and Molema, 2000). Diseases such as tumor growth and metastasis (Folkman, 1985), rheumatoid arthritis (Koch *et al*, 1994), diabetic retinopathy (Murphy, 1995) also implicate angiogenesis as a critical factor to their progression. This role of angiogenesis propelled Dr. Judah Folkman, the pioneer of British angiogenesis research, to recognize the process as a possible target for therapy (Goldenberg, 1995).

The study of angiogenesis was limited due to lack of relevant in vitro and in vivo models. More and more models and assays for studying angiogenesis have sprouted as progress occurred in the study of the fundamental features of neovascularization (Goldenberg, 1995). Presently, many of the angiogenic stimulators and inhibitors have already been identified and these have led to angiogenic therapy now used worldwide. There are also various diseases that would benefit from the induction of angiogenesis called therapeutic angiogenesis. Studies in this area are fewer but most of the approaches used were successful in clinical trials (Pepper, 1997). One possible therapeutic angiogenic therapy is the hyperbaric oxygen therapy.

Hyperbaric oxygen has been described as "a therapy in search of diseases" (Tibbles and Edelsberg, 1996). Most of its traditional uses include removing gas or air emboli, overcoming ischemia and low hypoxia, reducing compartmental edema or neutralizing anaerobic necrotizing bacteria (Gregorevic, *et al.*, 2000). It has been employed by the medical community over the years in treating decompression sickness, arterial gas embolism, carbon monoxide poisoning and smoke inhalation. It is also a main step in cervical cancer chemotherapy since the 1970's and yields a greater percentage of cured patients although side effects were also noted (Dische, 1978). Studies related to wound healing (Tibbles and Edelsberg, 1996) and gastroduodenal ulcers (Leech, *et al.*, 1998) suggested its possible role in promoting angiogenesis. The particular effect of hyperbaric oxygen therapy could open new windows in the treatment of numerous medical conditions that involve angiogenesis. Although there are conventional therapies for accelerating angiogenesis, this otherwise novel therapy may be tapped in cases where a patient may manifest adverse reactions towards drugs used in conventional therapies.

Statement of the Problem

The main problem of study is: Will hyperbaric oxygen exposure influence the degree of angiogenesis in duck embryos?

Research Objectives

The general objective of this study is to investigate the effect of exposure to hyperbaric oxygen on duck embryonic angiogenesis. The specific objectives are as follows:

1. To examine the effect of varying concentration of oxygen on angiogenesis of the duck embryo.
2. To test whether an increased ambient pressure at twice that of sea level can cause a significant difference in the degree of angiogenesis in duck embryos.
3. To measure the quantitative effects of hyperbaric oxygen exposure on angiogenesis of the duck embryo on a microscopic and macroscopic level.

Significance of the Study

If it can be established that an increase in angiogenesis results from exposure to hyperbaric oxygen, then hyperbaric oxygen therapy can be a new and innovative method in treating medical conditions. This is particularly true for those conditions, like wound healing, in which angiogenic acceleration is greatly beneficial. This can also serve to support the angiogenic basis of hyperbaric oxygen therapy usage in the treatment of hypoxic cancer cells.

Scope and Limitations

The particular effects being investigated in the present study are morphological. Hence, no attempt is made to analyze any biochemical influences in the test specimens. A

single hyperbaric level was set only at two and a half times that of normal air pressure at sea level. Also, the hyperoxic condition was manipulated at a single concentration of 100% oxygen within exposure chambers. Thus, no multiple increments in ambient pressure and oxygen level were applied. The duration of each exposure session was limited to only one hour owing to the strict scheduling of the usage of the hyperbaric chamber. Therefore, the present study did not focus on the long-term exposure effects or time-dependent effects of hyperbaric oxygen.

REVIEW OF RELATED LITERATURE

The establishment and maintenance of a vascular supply is an absolute requirement for the growth of normal and neoplastic tissues, which is why the cardiovascular system is the first organ system to develop and become functional during embryogenesis (Pepper, 1997). Angiogenesis is the process by which new blood capillaries sprout from pre-existing blood vessels (Passaniti *et al*, 1992; Goldenberg, 1995; Pepper, 1997; Djonov *et al.*, 1999). It is distinguished from vasculogenesis, the formation of blood vessel from angioblasts. Vasculogenesis is of great importance in embryogenesis than in postnatal tissue growth (Allen and Wilson, 1993). Angiogenesis also occurs in a variety of normal and pathological conditions like wound healing, ovarian and menstrual cycling, rheumatoid arthritis, and tumor growth (Satterwhite *et al.*, 1999).

The process proceeds in a series of steps beginning with cell multiplication at the base of the sprouting vessel, followed by capillary cell migration into surrounding stroma, and finally differentiation of these migrated cells into mature microvessels (Mignatti *et al.*, 1989). Initiation of these steps, however, depends upon the production and release of certain regulators. Some factors that promote vessel formation are acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-a, tumor necrosis factor (TNF)-a, vascular permeability factor or vascular endothelium growth factor, monobutyrin, angiotropin, angiogenin, hyaluronic acid degradation products, and age-associated glycosylation end-products (Passaniti *et*

al., 1992). Among these, bFGF appears to be on of the more potent angiogenic inducers along with vascular endothelial growth factor whose actions might be synergistic (Mignatti *et al.*, 1989; De Castro and Oabel, 2002). The physiological reservoir for FGF may be the perivascular extracellular matrix or the basement membrane (Folkman *et al.*, 1988; Ribatti *et al.*, 1999). There, bFGF is found bound to heparan sulfate (heparin-like molecule) and it gains biologic activity upon binding with heparin (Flaumenhaft *et al.*, 1990). Capillary endothelial cells respond to bFGF by an increase in the rate of cell proliferation, by stimulation of endothelial cell migration along a gradient of angiogenic factor, and by increased production of proteolytic enzymes such as plasminogen activator and collagenase (Mignatti *et al.*, 1989). Production of such proteases is of pivotal importance for the degradation of basement membrane and for the invasion of extracellular matrix by endothelial cells (Ribatti *et al.*, 1999). Down regulation of some inhibitors is also required to facilitate the angiogenic process (De Castro and Oabel, 2002). Identified inhibitors include TIMP or a cartilage-derived inhibitor, identified as a tissue inhibitor of metalloproteinases, platelet factor-4, thrombospondin, laminin peptides, heparin/cortisone, monocycline, fumaqillin, difluoromethylornithine, and sulfated chitin derivatives (Passaniti *et al.*, 1992).

Angiogenesis is fundamental to reproduction, development, and repair. However, inappropriate expression of normal functions can occur, such the unregulated growth of blood vessels. This is a case when angiogenesis becomes pathologic and sustains progression of neoplastic and non-neoplastic diseases (Folkman, 1995). In neoplastic tumors, the rate of proliferation of cells is equilibrium with cell apoptosis when there is

no growth of new vessels. However, when a subgroup of cells switches to an angiogenic phenotype, one or more of the positive regulators of angiogenesis is over-expressed. The tumors become vascularized via proliferation of host microvasculature and penetration of new capillaries into the tumor implant (Ausprunk *et al.*, 1975). Only after such penetration will tumors enter a phase of rapid growth and metastases leading to symptom onset. On the other hand, in non-neoplastic tissues, otherwise known as angiogenic diseases, an abnormal growth of microvessels or angiogenesis which may be excessive or deficient develops. Examples of diseases due to excessive angiogenesis are ocular-neovascularization (which causes blindness), artherosclerosis plaques, hemangiomas of infancy, rheumatoid arthritis, and psoriasis (Pepper, 1997). Diseases due to deficient angiogenesis include ischemia, wounds, fractures, peptic ulcers in animals, and duodenal and gastric ulcers in humans (Passaniti *et al*, 1992; Schaper and Ito, 1996; Pepper, 1997).

One type of a therapy that was believed to induce angiogenesis is the hyperbaric oxygen therapy. This therapy is done using a hyperbaric chamber which as defined by the international group UK Divers as an equipment used to recompress divers with decompression illnesses by slowly bringing them back to normal pressure allowing nitrogen, or other gases, to be exhaled harmlessly. Hyperbaric oxygen is 100% oxygen at roughly two to three times the atmospheric pressure (29.4 psi, 1520 mmHg, 2 kg/cm^2) at sea level leading to arterial oxygen tension in excess of 2000 mmHg and oxygen tension in tissue of almost 400 mmHg (Tibbles and Edelsberg, 1996). At sea level, the plasma oxygen concentration is 3ml/L and tissues of normal perfusion rate at rest require about 60 ml of oxygen per liter of blood flow to maintain normal cellular metabolism but at 3

atm, plasma oxygen concentration rises to 60 ml/L. This oxygen concentration is almost sufficient to supply the resting total oxygen requirement of many tissues without contribution of oxygen that are bound to hemoglobin (Leach *et al.*, 1998).

Hyperoxia improves angiogenesis since hypoxia inhibits collagen matrix formation, an essential in angiogenesis (Leach *et al.*, 1998). It is also found to be effective in promoting angiogenesis and wound healing in irradiated tissues (Leach *et al.*, 1998). Not only does it promote wound healing, it also protects wounds from infection. Hypoxia predisposes wounds to infection since the amount of neutrophil-mediated free radicals are decreased but this is restored by hyperbaric oxygen and even increases the rate of killing of some common bacteria by phagocytes (Tibbles and Edelsberg, 1996). It was reported to be effective in deep sternal wound infection in a heart transplant wherein there is clean granulation and closing of wound in nine weeks (Petzold *et al.*, 1999).

Hyperbaric oxygen was also found to be effective in treating muscle injuries. It was found to increase the maximum force-producing capacity of regenerating muscles and the size of the regenerating muscle fibers after myotoxic injury (Gregorevic *et al.*, 2000).

Hyperbaric oxygen has also been used as a supplemental treatment to tumors. Hypoxia is a problem in tumors as shown by the presence of hypoxic cancer cells. These are cells adjacent to necrotic areas in tumors or areas that are 100 to 150 μm away from the tumor's blood supply (Brown, 1999). The lowering of oxygenation of these cells made them more resistant to damage by ionizing radiation and may also lead to metastasis by induction of proangiogenic proteins (Brown, 1999). The trials done by the

British Medical Research Council on hyperbaric oxygen effect on radiotherapy left no doubt that hyperbaric oxygen increased the response observed from radiotherapy of certain malignancies even though there was also increased effect on normal tissue observed (Dische, 1978).

Other therapeutic uses of hyperbaric oxygen include carbon monoxide poisoning, decompression sickness, arterial gas embolism, necrotizing infection and osteomyelitis (Leach *et al.*, 1998), and hemorrhagic shocks (Tibbles and Perrotta, 1994; Faglia *et al.*, 1996; Tibbles and Edelsberg, 1996; Leach *et al.*, 1998). In decompression sickness and arterial gas embolism, hyperbaric oxygen reduces bubble volume, replaces inert gas bubbles with oxygen thus hastening their dissolution via cell metabolism, and prevents formation of new bubbles (Tibbles and Edelsberg, 1996).

However, patients undergoing hyperbaric oxygen therapy have been reported to experience side effects. The most common are reversible myopia, mild-to-severe pain from rupture of the middle ear or the cranial sinuses, and reversible tracheo-bronchial symptoms (Tibbles and Edelsberg, 1996). These risks may be avoided as long as the pressure does not exceed 300 kPa and the length of treatment is less than 120 minutes or two hours and patients should be allowed to adequately drain pneumothoraces before treatment (Leach, *et al.*, 1998). Another disadvantage of using hyperbaric oxygen therapy is the cost of the treatment, which ranges from $300 to $400 at the United States for one session alone (Tibbles and Edelsberg, 1996).

Due to the previously mentioned pathological conditions related to angiogenesis, quantification of angiogenesis is very important and beneficial to clinical diagnosis and

prognosis. Many models used to quantify angiogenesis have already been developed. The most common among these is the chorioallantoic membrane assay (CAM) used for testing angiogenic substances (Ribatti *et al.*, 1997; De Castro and Oabel, 2002). It provides for a more rapid assay than utilizing rabbit cornea (Goldenberg, 1995).

The avian chorioallantoic membrane is an extraembryonic membrane formed on day 4 of incubation. It is a very thick capillary network that is in direct contact with the shell to mediate gas exchanges with the extraembryonic environment before hatching (Ribatti *et al.*, 2000). The CAM is formed by fusion of the splanchnic mesoderm of the allantois and the somatic mesoderm of the chorion (Leeson and Leeson, 1963; Lusimbo, 2000). It is a superficial and readily accessible organ rich in endothelial, epithelial and mesenchymal cells and serves to support extra-embryonic respiratory capillaries. It also actively transport sodium and chloride from the allantoic sac and calcium from the eggshell into the embryonic vasculature (Coleman and Terepka, 1972; Lusimbo, 2000). The most common model used for assays is the 8 to 10 day old CAM (Allen and Wilson, 1993) owed to the fact that cells of it undergo a predictable sequence of differentiation with clear morphological end points. It is also easily exposed to injurious agents and is easily harvested and examined.

The CAM assay may be done in ovo or in vitro. The ovo method involves placing the eggs in an incubator as soon as embryogenesis starts and making a square window in the shell after removal of 2 to 3 ml of albumen to detach the CAM from the shell. This is then sealed with a glass and incubation goes on until the day of experiment (Ribatti *et al.*, 2000). The in vitro method is performed by transferring the embryo to a

petri dish on either the third or fourth day of incubation. The CAM develops at the top of the transferred embryo as a flat membrane. It reaches the edge of the dish to provide a two-dimensional monolayer onto which multiple grafts can be placed so the entire membrane can be seen (Ribatti *et al.*, 2000).

Substances to be tested maybe laid upon inert synthetic polymers allowing its sustained release at constant rates or inoculated directly into the cavity of the allantoic vessel (Ribatti *et al.*, 2000). One example of synthetic polymers is Gelfoam. It is a ready-to-use sponge for treatment of alveolus based on the ability of the sponge to take up a great deal of blood and thereby aid in forming a fibrin plug. Gelfoam is composed of solidified gelatin foam (95% DAB 8) and colloid silver (5% DAB 6). The gelatin evokes no reaction from the body since it is completely nontoxic and can confer anti-microbial effect. It could remain in the wound to stabilize tissue defect and is completely resorbed in four to six weeks. An additional in vivo system has recently been developed wherein Matrigel, supplemented with heparin and fibroblast growth factor (FGF), is injected subcutaneously (Goldenberg, 1995). Matrigel contains basement membrane components, such as collagens, laminin, and proteoglycans, and matrix degrading enzymes or their inhibitors and growth factors (Iida, 1996).

Numerous angiogenic factors, both stimulatory and inhibitory, have been described using the CAM assay. These factors vary in nature from peptide growth factors to fungal antibiotics (Allen and Wilson, 1993). The CAM assay has also been used to study neovascularization of heterologous tumor implants (Ausprunk *et al.*, 1975)

and to measure proliferative response of blood vessels to tumor angiogenesis factor (Cavallo, *et al.*, 1973; Ausprunk *et al.*, 1975).

The main advantages of the CAM assay are its low cost, simplicity, reliability, and lend itself to large-scale screening. However, it is nonspecific to inflammatory reactions and the presence of pre-existing vessels makes it difficult to determine the extent of anti-angiogenic agents (Ribatti *et al.*, 2000).

Quantification of angiogenesis can be used for clinical diagnosis and prognosis, as well as prediction of metastasis and recurrence. More importantly, it is useful for the development of therapy that may be either be angiogenic or anti-angiogenic.

However, quantifying angiogenesis is a relatively difficult undertaking. This is generally performed by measuring microvessel density using different types of morphometric analysis methods. Most researchers in this field favored the planimetric method of point counting (Ribatti *et al.*, 1997) for a microscopic level of examination. Another method that is useful at macroscopic level would be fractal analysis. Fractal analysis is used in the biological field as a method for quantifying structural complexity most often observed in tumor microvasculature (Sabo *et al.*, 2001). Fractal geometry has a number of advantages like it remains constant over a wide range of scales making variations in the magnification or processing artifacts discountable (Cross *et al.*, 1994).

MATERIALS AND METHODS

Test Specimens

Twenty, 7-days post-laying duck eggs (Plate 1) were obtained from a local supplier in Pateros, Metro Manila. The eggs were wiped with a slightly damp towel to remove dirt and then incubated under conditions of 50 to 55% humidity and a maintained temperature of 37°C at a laboratory in the College of Arts and Sciences Building in University of the Philippines Manila.

Implantation of Gelatin Sponges

Gelfoam (Roescheisen GmbH + Co., Germany) materials were cut to a size of 1.0 mm^3. Twenty pieces of 1.0 mm^3 Gelfoam were prepared for the twenty eggs. On the fifth day of incubation, in which the eggs were 11-days post-laying, the rounder end of each egg was wiped with alcohol using cotton balls. A small square window was opened by tapping the shell with a metal spoon. The prepared Gelfoam was then placed on top of the exposed CAM under a hood with a membrane filtration unit and a flame to ensure sterile conditions (one piece of 1.0 mm^3 Gelfoam per egg). The window was sealed using parafilm and paraffin wax prior to being returned in the incubator.

The eggs were divided into three groups: the unexposed group (Control), the normobaric oxygen group (NB) and the hyperbaric oxygen group (HB). Seven eggs had been allotted to each of the exposure groups NB and HB. Six eggs were allotted for the Control group.

Exposure Proper

On the sixth day of incubation, in which the eggs were 12-days post-laying, the eggs were transported to the Armed Forces of the Philippines (AFP) Medical Center at V. Luna St., Quezon City. This is where the Haux Multiplace Chamber (Haux Life Support, D-76307 Karlsbad, Fabrik.-NR.9501, manufactured in 1995) is housed (Plate 2). Incubator temperature (37°C) was maintained for the eggs for the duration of the travel. The NB and HB groups were separately placed in transparent plastic containers with one hole large enough to insert oxygen tubes (Plate 3). Each sealed container was placed inside the chamber and the oxygen tubes were inserted into the holes. The chamber was closed and the pressure for both the chamber and the oxygen was adjusted to 2.5 atm for the HB group, while a level of 1.0 atm was later adjusted for the NB group. The duration of the exposure was for one hour for each group. The duration excluded the 20-minute period for the sealing, pressurization and depressurization of the chamber before and after exposure proper. All eggs were then transported back to the laboratory in University of the Philippines Manila.

Processing for Quantitative Analysis

On the ninth day of incubation, in which the eggs were 15-days post-laying, the parafilm of each egg was peeled off. The contents were transferred to a Petri dish (Plates 5 and 6), taking extra care for the CAM to remain intact. The Gelfoam (Plate 4), the underlying and immediately adjacent CAM portions were then removed and transferred to a vial filled with 10% formalin. The remaining embryo and its chorioallanatoic

membrane were photographed using a digital camera for later analysis. Each CAM was then detached from the embryo, laid flat onto a white surface and again photographed. One vial each was provided for the Gelfoam of each egg.

The fixed Gelfoams with CAM were submitted to the Philippine General Hospital (PGH) Dermatopathology Laboratory. Each was cut parallel to the surface of the CAM into 8-mm serial sections. Staining was done with 0.5% aqueous solution of toluidine blue.

Quantification of the Angiogenic Response

Histological analysis employed the planimetric method of point counting as described by Ribatti *et al.* in 1997 (Appendix A). A mesh consisting of twelve lines per side, with a total of 144 intersection points, was inserted in the eyepiece of a double-headed photomicroscope. Six randomly chosen microscopic fields of each section were viewed. This method involved simultaneous identification and counting of intersection points with transversely cut microvessels. The total number of intersection points per field of view was averaged for each group and expressed as mean values ± standard deviation. The microvessel area was designated as a percentage of the final mean number of intersection points over the total number of intersection points. Statistically significant differences between the mean values of the intersection points were analyzed using one-way analysis of variance (ANOVA).

In addition, fractal analysis at the macroscopic level was also performed using the box counting method (Appendix B). A photograph of the image of each CAM specimen

was divided into grid of boxes whose size, designated as "s", was initially at four square inches. The boxes that are occupied by the vessels were counted. The size "s" was progressively reduced into smaller sizes by more or less a factor of ½ from one grid to the next and their corresponding occupied boxes were counted. This count is designated as N(s). The logarithm of N(s) versus the logarithm of 1/s gives a line whose slope corresponds to the box dimension. The fractal index is determined from the slope by subtracting its value from 1. Fractal dimension values range from 1 and 2. Fractal dimensions of classical geometrical figures are identical to the classic Euclidean dimension being 1 for a line, 2 for a square and 3 for a cube. Geometrical structures with complex contours on the other hand are best represented by rational numbers (Cross *et al.*, 1994). The more branched and tortuous the contours of the blood vessels the larger the fractal dimension, thus, the higher the vessel complexity, the closer to 2 is the fractal dimension value. Statistically significant differences between the mean fractal indices were also analyzed using one-way analysis of variance (ANOVA).

RESULTS

Out of the twenty eggs at 15-days post-laying, five had been spoiled by contaminating agents such as molds. Fifteen viable eggs remained, four from the Control group, six from the normobaric oxygen group, and four from the hyperbaric oxygen group. These remaining specimens were subjected to histological analysis (Plates 7, 8, and 9) and fractal analysis (Plates 10, 11, and 12) of this study.

From the fifteen remaining eggs, one of the gelfoams of the normobaric oxygen group disintegrated during slide sectioning preparation. Thus, only five gelfoams were therefore sectioned and analyzed histologically as part of the normobaric oxygen group.

Histological analysis of the fourteen gelfoams with immediate CAM surroundings revealed that the hyperbaric oxygen group had the highest vessel count of 10.09 (± 2.01) and a microvessel density of 6.88%, while the normobaric oxygen group had a vessel count and a microvessel density of 7.13 (± 0.87) and 4.95% respectively. The lowest vessel count of 2.81 (± 0.77) and microvessel density of 1.85% was observed from the control group (Table 1).

Intersection points per field of view of each egg range from 1 to 29 intersection points for the hyperbaric group, 1 to 19 intersection points for the normobaric group, and 0 to 9 intersection points for the control group (Appendix C). The statistical analysis (Appendices E and F) revealed that the values for the NB and HB groups were significantly different from the Control group, but not from each another (Figure 1).

The average fractal dimension values of each of the three groups ranged from 1.70 to 1.74 (Table 2). The mean fractal indices computed for each egg (Appendix D) ranged from 1.73 to 1.75 for the HB group, 1.65 to 1.75 for the NB group, while the Control group had a range of 1.68 to 1.73. Statistical analysis (Appendix G) showed, however, that the differences among the three mean fractal indices of the three groups were not significant (Figure 2).

DISCUSSION

The results of the histological analysis of the gelfoam sections revealed that the hyperbaric-exposed group showed greatest mean vessel density, and therefore the greatest extent of angiogenesis. The significant difference between the NB and HB groups histologically suggests that 100% oxygen with an increased level of pressure stimulated angiogenesis.

It has been established that hyperbaric oxygen application delivers a greater than typical oxygen concentration in tissues (Petzold, 1999). Tibbles and Edelsberg in 1996 reported that from roughly 20% oxygen in normal ambient air as that of the Control group to 100% oxygen at a normobaric pressure level of the normobaric oxygen group, blood (plasma) oxygen concentration rises from 0.3 ml per deciliter to 1.5 ml per deciliter. Increasing the environmental pressure level to 3-atm raises the blood oxygen concentration to an even higher 6 ml per deciliter which is more or less what can be inferred for the blood oxygen concentration effects on the hyperbaric oxygen group exposed at 2.5 atm (Tibbles and Edelsberg, 1996). With such doses of oxygen, numerous biochemical, cellular and physiological effects are triggered (Petzold, 1999).

Hyperoxic environments, initially decrease the rate of capillary growth by decreasing the release of certain growth factors that stimulate angiogenesis. However, hyperoxic animals provide all substrates necessary for cellular metabolism. This leads to an eventual increase in vessel formation due to the faster growth of the organism (Knighton, 1981).

It should also be noted that in previous studies, such as those involving tumor progression and metastasis (Brown, 1999), hypoxia was found to be inductive to angiogenesis. The hypoxic system involved normally result to structural adaptations to improve the oxygenation of its immediate surroundings. This is achieved by the release of known angiogenic factors such as ADP and lactic acid in cases of fetal hypoxia (Karimu, 1994). Other growth promoting factors such as vascular endothelial growth factors are also released in these conditions (Brown, 1999). The low capillary oxygen level, however, impairs cellular metabolism and lead to slowing down of the growth rate (Knighton, 1981). Once the hypoxic gradient is removed, capillary growth ceases. This suggests that extremes of oxygen concentration, hyperoxia and hypoxia, both promote angiogenesis.

The significant difference between the histological results of the normobaric oxygen group and the hyperbaric oxygen group suggests that ambient pressure did affect angiogenesis. Increase in ambient pressure is associated with increased oxygen tension in body fluids. Hence, this results in blood oxygen concentration which is more elevated than when the body has been exposed to 100% oxygen at normobaric levels as what Tibbles and Edelsberg reported in 1996. One study on the direct effect of pressure on angiogenesis that resulted to angiogenic stimulation involved changes to fetal perfusion pressure (Karimu, 1994). This pressure, however, is intravascular in nature. Whether or not ambient pressure has a direct mechanical effect on angiogenesis has yet to be verified.

Results of the fractal analysis did not show significant differences among the macroscopic vessel density of three groups. Nonetheless, the high value of the mean fractal index for the HB group among the three test groups concur with the data of the planimetric procedure. The latter method was found to be more accurate in studying angiogenesis since the vessels to be measured are ensured to be newly formed blood vessels by using gelfoam implants.

With the given results, hyperbaric oxygen could be of therapeutic use to medical conditions, such as ischemia, wound healing, reconstructive surgery and implantation islets of Langerhans, that are likely to benefit from an induction of the angiogenic process. It may even be found to be beneficial to the treatment of infarcted brain tissues associated with thromboembolic events since promotion of angiogenesis in such case prevents neuronal degeneration and quite possibly results to longer survival (Pepper, 1997).

Hyperbaric oxygen therapy may be used to supplement tumor treatment in that it could remove the hypoxic gradient in tumor cells that promote angiogenesis specifically hypoxic cells. Hypoxia confers tumor cell resistance against ionizing radiation (Brown, 1999). Hence, removing the hypoxic state renders the tumor cells more vulnerable to radiotherapy.

CONCLUSION

Various stimuli have been implicated in angiogenesis. Based on microscopic and macroscopic observations in the present study, exposure to hyperbaric oxygen was found to have stimulated angiogenesis to a greater extent than exposure to normobaric oxygen. Hyperbaric oxygen induces a state of hyperoxia, which by increasing metabolic rates also increases angiogenic capabilities.

RECOMMENDATIONS

Contamination of the opened eggs that resulted to spoilage was the greatest problem encountered through the course of the experiment. Hence, it is suggested that a consistent practice of sterilization techniques be maintained. The working area and equipment should be decontaminated at frequent intervals. It is advised that future researches resterilize the gelfoam using a hot air sterilizer for one or two hours at 150°C.

The use of computerized image analysis systems (Cross, 1994) program and clearer photographic images could improve the precision of fractal analysis. The line dimension of the grids manually drawn over the image could contribute to instrumental bias in the box-counting method.

Lastly, multiple exposures of test specimens may amplify the effect of hyperbaric oxygen. Most promising results on human patients are often manifested following multiple exposures.

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TABLES

TABLE 1. Vessel counts (± standard deviation) and microvessel density of gelfoam with underlying chorioallantoic membrane of duck embryos in the three treatment groups.

TREATMENT GROUPS	n	VESSEL COUNT (mean intersection points per field of view ± s.d.)	MICROVESSEL DENSITY (%)
Control group (1.0 atm at 20% O_2)	4	2.81 ± 0.77 a	1.95
Normobaric (NB) group (1.0 atm at 100% O_2)	5	7.13 ± 0.87 b	4.95
Hyperbaric (HB) group (2.5 atm at 100% O_2)	4	10.09 ± 2.01 c	6.88

* n = no. of samples

*Values in a column with a common letter notation are not significantly different from each other at α=0.05.

TABLE 2. Mean fractal indices (± standard deviation) of vascular complexity of duck embryonic chorioallantoic membrane in the three treatment groups.

TREATMENT GROUPS	n	MEAN FRACTAL INDEX ± s.d.
Control group (1.0 atm at 20% O_2)	4	1.70 ± 0.03
Normobaric (NB) group (1.0 atm at 100% O_2)	6	1.72 ± 0.04
Hyperbaric (HB) group (2.5 atm at 100% O_2)	4	1.74 ± 0.01

*n = no. of samples

FIGURES

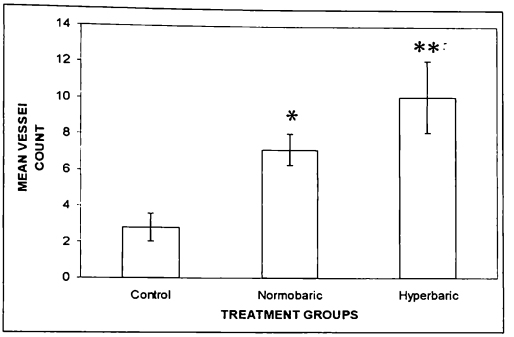


FIGURE 1. Mean vessel counts of serial sections of gelfoam with the chorioallantoic membrane of 15-days old postlaying duck eggs. Range bar indicates the standard deviation of the mean. The * and ** indicate significant difference from the control.

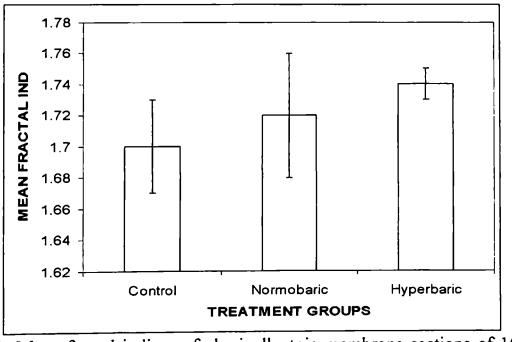


FIGURE 2. Mean fractal indices of chorioallantoic membrane sections of 15-days old postlaying duck eggs. Range bar indicates the standard deviation of the mean.

PLATES

PLATE 1. Twenty seven-day old duck eggs from Pateros, Metro Manila.

PLATE 2. Haux Multiplace Chamber of the AFP Medical Center.

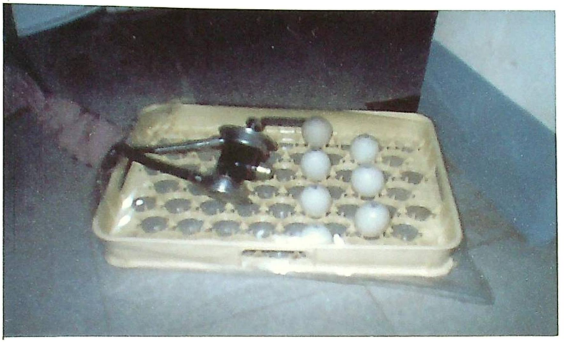


PLATE 3. The exposure setup done for the hyperbaric oxygen group that was placed inside the hyperbaric chamber and supplied with 100% oxygen at 2.5-atm. (The same setup was also done for the normobaric oxygen group but the supply of 100% oxygen was only at 1-atm)

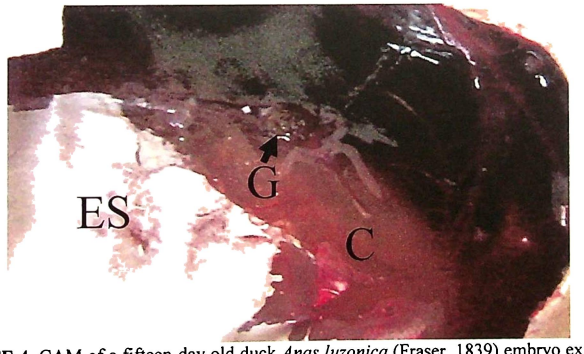


PLATE 4. CAM of a fifteen-day old duck *Anas luzonica* (Fraser, 1839) embryo exposed to 100% oxygen at 1-atm for one hour with gelfoam (G). Eggshell, ES; CAM, C.

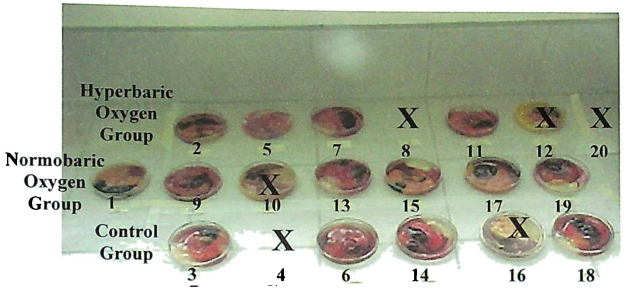


PLATE 5. The experimental setup – the control group and the experimental groups (normobaric oxygen group and hyperbaric oxygen group) of fifteen-day old duck embryos. X = spoiled eggs.

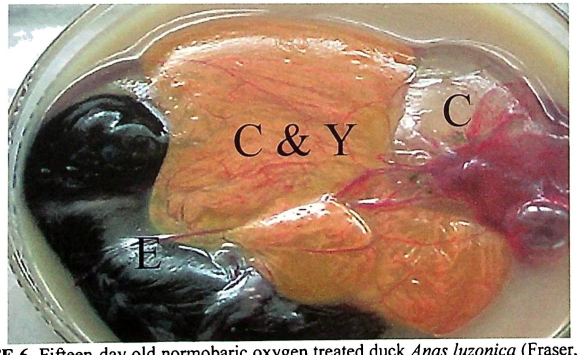


PLATE 6. Fifteen-day old normobaric oxygen treated duck *Anas luzonica* (Fraser, 1839) egg in a petri dish. Embryo, E; Chorioallantoic membrane (CAM), C; Yolk, Y.

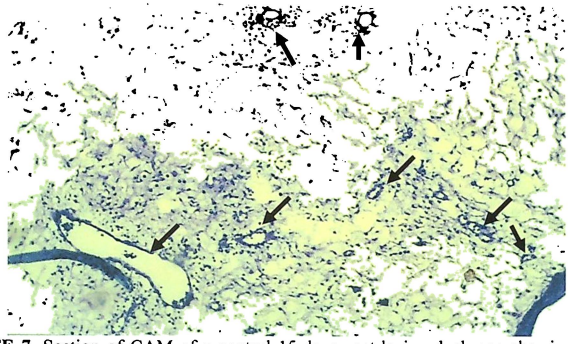


PLATE 7. Section of CAM of a control 15-days post-laying duck egg showing lesser vessel quantity than the experimental groups. Magnification 100X. Blood vessels are indicated by arrows.

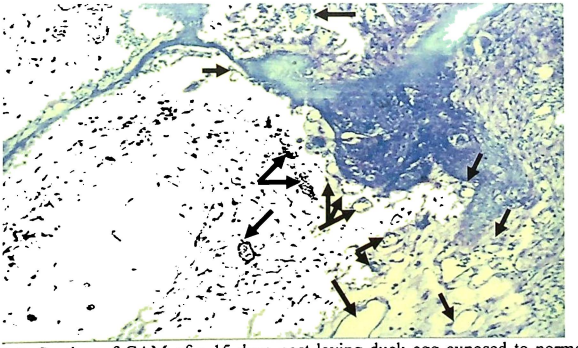


PLATE 8. Section of CAM of a 15-days post-laying duck egg exposed to normobaric oxygen (100% oxygen at 1-atm) for one hour showing greater vessel quantity than the control group. Magnification 100X. Blood vessels are indicated by arrows.

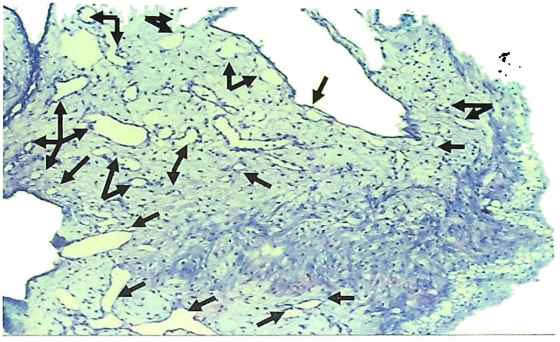


PLATE 9. Section of CAM of a 15-days post-laying duck egg exposed to hyperbaric oxygen (100% oxygen at 2.5-atm) for one hour showing greater vessel quantity than the control group. Magnification 100X. Blood vessels are indicated by arrows.

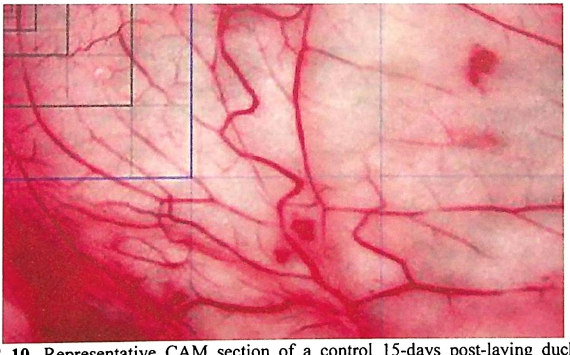


PLATE 10. Representative CAM section of a control 15-days post-laying duck egg showing lesser vascular complexity than the experimental groups. Drawn grids represent zones for fractal analysis. Magnification 3X+.

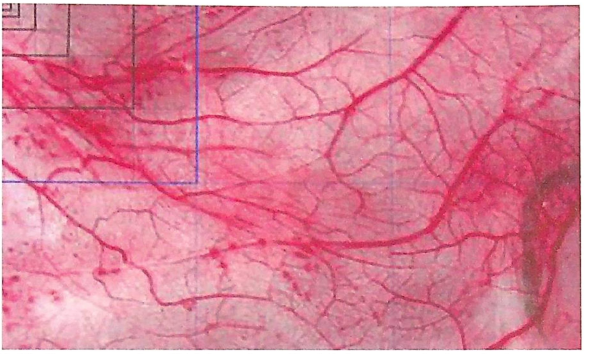


PLATE 11. Representative CAM section of a 15-days post-laying duck egg exposed to normobaric oxygen (100% oxygen at 1-atm) for one hour showing greater vascular complexity than the control group. Drawn grids represent zones for fractal analysis. Magnification 3X.

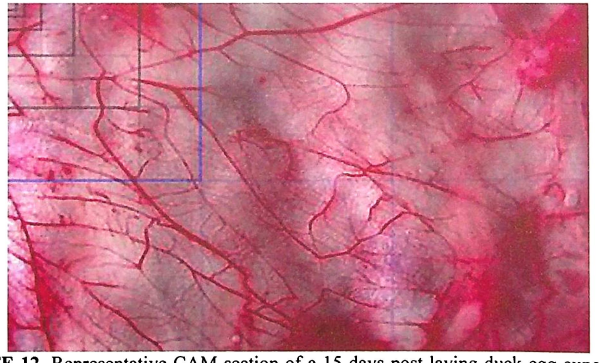


PLATE 12. Representative CAM section of a 15-days post-laying duck egg exposed to hyperbaric oxygen (100% oxygen at 2.5-atm) for one hour showing greater vascular complexity than the control group. Drawn grids represent zones for fractal analysis. Magnification 3X.

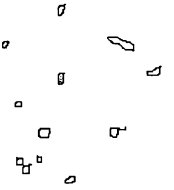
APPENDICES

APPENDIX A

Histological Analysis using the Planimetric Method of Point Counting (Ribatti *et al.*, 1997)

A mesh consisting of 12 lines per side, having a total of 144 intersection points, is inserted in the eyepiece. Under the microscope, it should look like the figure below.

Next, a section of a serial slide is viewed under LPO. Six fields of view per section are chosen randomly by the researcher. A chosen field of view with the 144-mesh inserted in the eyepiece will look like the figure below under the microscope.



Assuming that all objects within the field of view other than the 144-mesh in the previous figure were blood vessels, the number of blood vessels that intersected with the grids of the 144-mesh are then counted. From the previous figure, 7 blood vessels intersected with the 144-mesh.

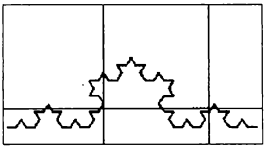
The same process of counting the number of blood vessels that will intersect with the grids of the mesh is repeated per field of view. The number of section to be viewed is ideally ten sections, which is every third section in a series of 30 sections.

The mean number of intersection points per field of view will then be obtained for each treatment group which will be regarded as the vessel count of that treatment group. Microvessel density is then obtained by dividing the vessel count by 144 and is represented in percentage.

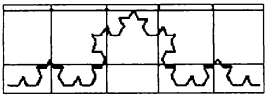
APPENDIX B

Fractal Analysis using the Box-counting Method (Richardson and Gillespy III, 2000)

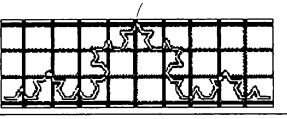
First, an image of the structure to be measured is obtained. An arbitrary grid is then drawn over the structure. Boxes in the grid that are occupied by the structure were then counted. Like the structure below, it occupied six boxes.



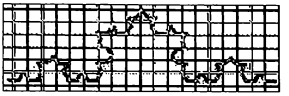
Next, a grid half the size of the previous one is drawn over the structure. The boxes occupied by the structure are then counted. With the grid below, the structure filled 9 boxes.



The grid size will again be halved and the boxes occupied by the structure are counted. With the grid below, the structure filled 18 boxes.



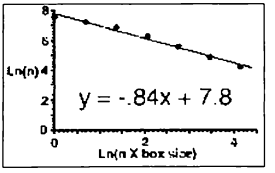
The size of the grids can be reduced indefinitely on the choice of the analyst and the process of counting the boxes occupied by the structure is repeated on each grid. For the structure analyzed here, box counting will be done one last time. With the final grid below, the structure filled 59 boxes.



Box-counting data of the structure analyzed is summarized in the table below:

Box Size (1/s)	N(s)
1	6
¼	9
1/16	18
1/256	59

The data from the table above are plotted on a log-log plot as shown below. A linear regression is done to find the best fit and the slope of the line is obtained. The best fit equation is y = -.84x + 7.8 and the slope is -.84.



This fractal index is calculated as 1 minus the slope of the line. Thus, for this fractal object, the fractal index is D = 1 - (-.84) = 1.84.

APPENDIX C

Fractal Analysis: Box-counting Method

APPENDIX C.1. Fractal analysis on vascular complexity of 15-day old duck (*Anas* luzonica, Fraser 1839) embryonic CAM subjected to 100% oxygen at 1-atm and 2-atm pressures.

TREATMENT	LOG [N(s)] (s in inches)					SLOPE	FRACTAL INDEX	MEAN FRACTAL INDEX
	LOG 4	LOG 1	LOG ¼	LOG 1/16	LOG 1/256			
1 (NB)	0.6021	1.0792	1.6232	2.2227	2.8075	-0.7514	1.7514	1.7500
	0.6021	1.0792	1.6232	2.2253	2.8082	-0.7518	1.7518	
	0.6021	1.0792	1.6232	2.2201	2.8082	-0.7513	1.7513	
	0.6021	1.0792	1.6232	2.2068	2.8075	-0.7511	1.7511	
	0.6021	1.0792	1.6232	2.2253	2.8082	-0.7518	1.7518	
	0.6021	1.0792	1.6232	2.1987	2.7868	-0.7427	1.7427	
2 (HB)	0.6021	1.0792	1.6232	2.2201	2.7966	-0.7477	1.7477	1.7403
	0.6021	1.0792	1.6232	2.2201	2.7875	-0.7448	1.7448	
	0.6021	1.0792	1.6232	2.1903	2.7634	-0.7348	1.7348	
	0.6021	1.0792	1.6232	2.2201	2.7931	-0.7466	1.7466	
	0.6021	1.0792	1.6232	2.2148	2.7275	-0.7412	1.7412	
	0.6021	1.0792	1.5798	2.1614	2.4082	-0.7268	1.7268	

APPENDIX C.1. (continued from previous page.)

TREATMENT	LOG [N(s)] (s in inches)					SLOPE	FRACTAL INDEX	MEAN FRACTAL INDEX
	LOG 4	LOG 1	LOG ¼	LOG 1/16	LOG 1/256			
3 (Control)	0.6021	1.0792	1.6128	2.2014	2.7774	-0.7402	1.7402	1.7288
	0.6021	1.0792	1.6128	2.1959	2.7701	-0.7374	1.7374	
	0.6021	1.0792	1.6232	2.2175	2.7825	-0.7430	1.7430	
	0.6021	1.0792	1.6232	2.2253	2.7910	-0.7464	1.7464	
	0.6021	1.0792	1.5911	2.1303	2.6812	-0.7041	1.7041	
	0.6021	1.0792	1.5911	2.1173	2.6776	-0.7018	1.7018	
4 (Control)	-	-	-	-	-	-	-	-
5 (H)	0.6021	1.0792	1.6021	2.1987	2.7193	-0.7220	1.7220	1.7338
	0.6021	1.0792	1.6232	2.2201	2.7832	-0.7435	1.7435	
	0.6021	1.0792	1.6128	2.2041	2.7597	-0.7349	1.7349	
	0.6021	1.0792	1.6232	2.2014	2.7536	-0.7325	1.7325	
	0.6021	1.0792	1.6232	2.2068	2.7701	-0.7382	1.7382	
	0.6021	1.0792	1.6232	2.2122	2.7482	-0.7318	1.7318	
6 (Control)	0.6021	1.0792	1.6128	2.1761	2.7474	-0.7285	1.7285	1.6766
	0.6021	1.0792	1.5441	2.1072	2.7135	-0.7132	1.7132	
	0.6021	1.0792	1.6021	2.0170	2.6128	-0.6722	1.6722	

APPENDIX C.1. (continued from previous page.)

TREATMENT	LOG [N(s)] (s in inches)					SLOPE	FRACTAL INDEX	MEAN FRACTAL INDEX
	LOG 4	LOG 1	LOG ¼	LOG 1/16	LOG 1/256			
6 (Control)	0.6021	1.0414	1.5315	2.0043	2.5514	-0.6587	1.6587	1.6766
	0.6021	1.0792	1.5798	2.1004	2.3444	-0.5958	1.5958	
	0.6021	1.0792	1.5798	2.0719	2.6551	-0.6909	1.6909	
7 (HB)	0.6021	1.0792	1.6232	2.2175	2.7731	-0.7401	1.7401	1.7364
	0.6021	1.0792	1.6232	2.2175	2.7810	-0.7426	1.7426	
	0.6021	1.0792	1.6232	2.1818	2.7505	-0.7298	1.7298	
	0.6021	1.0792	1.6232	2.2041	2.7235	-0.7233	1.7233	
	0.6021	1.0792	1.6232	2.1987	2.7612	-0.7346	1.7346	
	0.6021	1.0792	1.6232	2.2227	2.7973	-0.7481	1.7481	
8 (HB)	-	-	-	-	-	-	-	-
9 (NB)	0.6021	1.0414	1.5563	2.1206	2.7497	-0.7306	1.7306	1.7383
	0.6021	1.0792	1.5911	2.1703	2.7973	-0.7442	1.7442	
	0.6021	1.0792	1.5798	2.1335	2.7649	-0.7309	1.7309	
	0.6021	1.0792	1.6128	2.1303	2.7796	-0.7345	1.7345	
	0.6021	1.0792	1.6128	2.1987	2.7987	-0.7467	1.7467	
	0.6021	1.0792	1.6128	2.1959	2.7875	-0.7429	1.7429	

APPENDIX C.1. (continued from previous page.)

TREATMENT	LOG [N(s)] (s in inches)					SLOPE	FRACTAL INDEX	MEAN FRACTAL INDEX
	LOG 4	LOG 1	LOG ¼	LOG 1/16	LOG 1/256			
10 (NB)	-	-	-	-	-	-	-	-
11 (HB)	0.6021	1.0792	1.6232	2.2068	2.7839	-0.7425	1.7425	1.7507
	0.6021	1.0792	1.6232	2.2253	2.8116	-0.7529	1.7529	
	0.6021	1.0792	1.6232	2.2227	2.8041	-0.7503	1.7503	
	0.6021	1.0792	1.6232	2.2253	2.8116	-0.7529	1.7529	
	0.6021	1.0792	1.6232	2.2253	2.8109	-0.7527	1.7527	
	0.6021	1.0792	1.6232	2.2253	2.8109	-0.7527	1.7527	
12 (HB)	-	-	-	-	-	-	-	-
13 (NB)	0.6021	1.0792	1.6232	2.1761	2.7868	-0.7407	1.7407	1.7217
	0.6021	1.0792	1.5911	2.1584	2.7642	-0.7327	1.7327	
	0.6021	1.0414	1.5441	1.9777	2.7126	-0.7064	1.7064	
	0.6021	1.0792	1.6021	2.1430	2.7767	-0.7350	1.7350	
	0.6021	1.0792	1.6232	2.1584	2.7118	-0.7155	1.7155	
	0.6021	1.0792	1.5441	2.0864	2.6767	-0.6998	1.6998	
14 (Control)	0.6021	1.0792	1.5911	2.1367	2.6972	-0.7097	1.7097	1.7042
	0.6021	1.0792	1.6021	2.1673	2.7235	-0.7205	1.7205	

APPENDIX C.1. (continued from previous page.)

TREATMENT	LOG [N(s)] (s in inches)					SLOPE	FRACTAL INDEX	MEAN FRACTAL INDEX
	LOG 4	LOG 1	LOG ¼	LOG 1/16	LOG 1/256			
14 (Control)	0.6021	1.0414	1.5315	2.0828	2.5132	-0.6535	1.6535	1.7042
	0.6021	1.0792	1.6232	2.2041	2.7574	-0.7339	1.7339	
	0.6021	1.0792	1.5911	2.1303	2.6730	-0.7015	1.7015	
	0.6021	1.0792	1.6021	2.1367	2.6866	-0.7061	1.7061	
15 (NB)	0.6021	1.0414	1.5185	1.9868	2.3096	-0.5812	1.5812	1.6458
	0.6021	1.0414	1.4624	1.9542	2.2504	-0.5609	1.5609	
	0.6021	1.0792	1.5911	2.0969	2.6721	-0.6982	1.6982	
	0.6021	1.0792	1.6021	2.1072	2.7324	-0.7179	1.7179	
	0.6021	1.0792	1.5798	2.1492	2.7459	-0.7264	1.7264	
	0.6021	1.0792	1.5798	2.0864	2.3304	-0.5902	1.5902	
16 (Control)	-	-	-	-	-	-	-	-
17 (NB)	0.6021	1.0792	1.6232	2.2068	2.7152	-0.7209	1.7209	1.7152
	0.6021	1.0792	1.6128	2.1818	2.7076	-0.7165	1.7165	
	0.6021	1.0792	1.6128	2.1673	2.7059	-0.7147	1.7147	
	0.6021	1.0792	1.6128	2.1818	2.7059	-0.7160	1.7160	
	0.6021	1.0792	1.6128	2.1987	2.6972	-0.7148	1.7148	

APPENDIX C.1. (continued from previous page.)

TREATMENT	LOG [N(s)] (s in inches)					SLOPE	FRACTAL INDEX	MEAN FRACTAL INDEX
	LOG 4	LOG 1	LOG ¼	LOG 1/16	LOG 1/256			
17 (NB)	0.6021	1.0792	1.6232	2.1903	2.6803	-0.7085	1.7085	1.7152
18 (Control)	0.6021	1.0792	1.6021	2.0086	2.6085	-0.6701	1.6701	1.6769
	0.6021	1.0792	1.5798	2.1072	2.4843	-06404	1.6404	
	0.6021	1.0792	1.6021	2.0374	2.6149	-0.6747	1.6747	
	0.6021	1.0414	1.5441	2.0170	2.5705	-0.6653	1.6653	
	0.6021	1.0792	1.5441	2.1004	2.6702	-0.6990	1.6990	
	0.6021	1.0792	1.6021	2.1492	2.7007	-0.7117	1.7117	
19 (NB)	0.6021	1.0792	1.6232	2.2014	2.7686	-0.7372	1.7372	1.7340
	0.6021	1.0792	1.6232	2.2148	2.7945	-0.7466	1.7466	
	0.6021	1.0792	1.6232	2.2068	2.7520	-0.7325	1.7325	
	0.6021	1.0792	1.6232	2.2068	2.7627	-0.7358	1.7358	
	0.6021	1.0792	1.6232	2.1987	2.7168	-0.7207	1.7207	
	0.6021	1.0792	1.6232	2.2148	2.7451	-0.7310	1.7310	
20 (HB)	-	-	-	-	-	-	-	-

* HB – Hyperbaric oxygen treatment group; NB – Normobaric oxygen treatment group; Control – Control group;

*- – Spoiled eggs

APPENDIX D

Histological Analysis: Planimetric Method of Point Counting

APPENDIX D.1. Histological analysis on gelfoam with underlying CAM obtained from 15-day old duck (*Anas* luzonica, Fraser 1839) eggs subjected to 100% oxygen at 1-atm and 2-atm pressures.

TREATMENT	SECTION	NO. OF INTERSECTION POINT PER FIELD OF VIEW						MEAN NO. OF INTERSECTION POINT PER FIELD OF VIEW
		1	2	3	4	5	6	
1 (NB)	-	-	-	-	-	-	-	-
2 (HB)	A	13	13	14	13	8	6	13.07
	B	24	15	18	9	3	8	
	C	20	8	15	16	18	13	
	D	2	9	18	18	20	9	
	E	15	17	9	18	14	9	
3 (Control)	A	1	5	5	3	2	3	3.40
	B	3	4	3	3	4	3	
	C	4	2	1	5	5	1	
	D	8	9	2	5	4	5	
	E	4	6	0	2	2	1	
4 (Control)	-	-	-	-	-	-	-	-
5 (HB)	A	4	5	15	7	8	9	8.97
	B	4	4	5	5	10	7	
	C	5	3	7	13	25	9	
	D	7	6	5	5	26	9	
	E	10	9	26	9	5	7	
6 (Control)	A	4	3	4	5	6	4	2.77

APPENDIX D.1. (continued from previous page.)

TREATMENT	SECTION	NO. OF INTERSECTION POINT PER FIELD OF VIEW						MEAN NO. OF INTERSECTION POINT PER FIELD OF VIEW
		1	2	3	4	5	6	
6 (Control)	B	2	4	1	3	2	3	2.77
	C	4	1	1	1	2	2	
	D	2	2	3	2	2	3	
7 (HB)	E	4	5	2	3	2	1	9.50
	A	9	5	4	5	3	8	
	B	16	9	13	17	9	9	
	C	18	20	17	5	6	8	
	D	7	29	9	3	8	8	
	E	8	5	8	9	5	5	
8 (HB)	-	-	-	-	-	-	-	-
9 (NB)	A	2	4	7	10	7	2	6.50
	B	10	14	8	6	12	16	
	C	10	13	6	5	3	8	
	D	4	3	3	4	5	1	
	E	4	5	8	6	6	3	
10 (NB)	-	-	-	-	-	-	-	-
11 (HB)	A	6	7	4	16	8	11	8.80
	B	4	8	13	8	13	7	
	C	2	7	3	7	13	14	
	D	10	18	7	7	8	11	
	E	6	8	8	12	9	9	
12 (HB)	-	-	-	-	-	-	-	-
13 (NB)	A	3	8	7	6	8	8	8.20
	B	7	6	8	13	12	3	

APPENDIX D.1. (continued from previous page.)

TREATMENT	SECTION	NO. OF INTERSECTION POINT PER FIELD OF VIEW						MEAN NO. OF INTERSECTION POINT PER FIELD OF VIEW
		1	2	3	4	5	6	
13 (NB)	C	10	18	12	9	8	8	8.20
	D	15	6	7	13	12	8	
	E	11	4	4	5	4	3	
14 (Control)	A	1	1	2	0	0	1	1.73
	B	0	2	1	2	1	2	
	C	1	3	3	1	0	7	
	D	5	3	4	1	3	3	
	E	0	1	0	2	2	1	
15 (NB)	A	8	8	8	2	2	3	6.03
	B	7	5	4	8	6	3	
	C	8	7	6	4	6	1	
	D	5	13	5	7	13	11	
	E	8	7	3	4	6	3	
16 (Control)	-	-	-	-	-	-	-	-
17 (NB)	A	2	8	8	6	2	12	7.33
	B	6	11	6	5	6	6	
	C	4	4	3	6	19	10	
	D	9	11	11	9	8	10	
	E	8	6	3	5	6	10	
18 (Control)	A	4	3	2	3	7	1	3.33
	B	2	2	6	6	1	1	
	C	3	0	2	1	7	1	
	D	1	4	4	7	3	4	

APPENDIX D.1. (continued from previous page.)

TREATMENT	SECTION	NO. OF INTERSECTION POINT PER FIELD OF VIEW						MEAN NO. OF INTERSECTION POINT PER FIELD OF VIEW
		1	2	3	4	5	6	
18 (Control)	E	5	3	4	2	3	8	3.33
19 (NB)	A	3	3	5	11	7	7	7.60
	B	11	5	14	19	8	3	
	C	11	16	13	2	4	4	
	D	4	2	7	11	8	7	
	E	8	5	9	10	7	4	
20 (HB)	-	-	-	-	-	-	-	-

*HB – Hyperbaric oxygen treatment group; NB – Normobaric oxygen treatment group; Control – Control group

*- – No histological analysis

APPENDIX E

Statistical Analysis of the Mean Vessel Count of the Three Treatment Groups Based on One-way ANOVA

APPENDIX E.1. Mean number of intersection points of vessels per field of view from histological analysis of gelfoam with underlying CAM sections of each duck egg in their respective treatment groups (based on Appendix C.1).

TREATMENT GROUPS		
CONTROL	NORMOBARIC OXYGEN TREATMENT	HYPERBARIC OXYGEN TREATMENT
3.40	6.50	13.07
2.77	8.20	8.97
1.73	6.03	9.50
3.33	7.33	8.80
	7.60	

$\sum x = 87.23$
$\sum x^2 = 709.6303$
$\sum C = 11.23$
$\sum N = 35.66$
$\sum H = 40.34$
$CF = 585.3133$
$n = 13$
$k = 3$

x – mean number of intersection points per field of view
C – mean fractal indices of the control group
N – mean fractal indices of the normobaric treatment group
H – mean fractal indices of the hyperbaric treatment group
n – total number of samples
k – number of treatments

APPENDIX E.2. One-way ANOVA of Table E.1 with α set at 5%, degrees of freedom of 2 and 10, and a critical F-value of 4.10.

SOURCES OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	COMPUTED F
TREATMENTS	107.37	2	53.69	31.58*
ERROR	16.95	10	1.70	
TOTAL	124.32	12		

*$F_{computed}$ (31.58) > $F_{critical}$ (4.10), therefore at least one of the treatments has a significantly different vessel count as compared to the other two treatments.

APPENDIX E.3. One-way ANOVA of Table E.1 with α set at 1%, degrees of freedom of 2 and 10, and a critical F-value of 7.56.

SOURCES OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	COMPUTED F
TREATMENTS	107.37	2	53.69	31.58*
ERROR	16.95	10	1.70	
TOTAL	124.32	12		

*$F_{computed}$ (38.21) > $F_{critical}$ (7.56), therefore at least one of the treatments has a significantly different vessel count as compared to the other two treatments.

APPENDIX F

Pair Comparison of Mean Vessel Count of the Three Treatment Groups Based on Least Significant Difference Test

A. Control vs Normobaric Treatment Group

$(\text{Vessel Count})_{\text{Control}} \pm$ standard error $= 2.81 \pm 0.77 = 2.04$ and 3.58

$(\text{Vessel Count})_{\text{NB}} \pm$ standard error $= 7.13 \pm 0.87 = 6.26$ and 8.00

*The vessel counts of the two groups are significantly different since no overlapping occurred.

B. Control vs Hyperbaric Treatment Group

$(\text{Vessel Count})_{\text{Control}} \pm$ standard error $= 2.81 \pm 0.77 = 2.04$ and 3.58

$(\text{Vessel Count})_{\text{HB}} \pm$ standard error $= 10.09 \pm 2.01 = 8.08$ and 12.10

*The vessel counts of the two groups are significantly different since no overlapping occurred.

C. Normobaric Treatment Group vs Hyperbaric Treatment Group

$(\text{Vessel Count})_{\text{NB}} \pm$ standard error $= 7.13 \pm 0.87 = 6.26$ and 8.00

$(\text{Vessel Count})_{\text{HB}} \pm$ standard error $= 10.09 \pm 2.01 = 8.08$ and 12.10

*The vessel counts of the two groups are significantly different since no overlapping occurred.

APPENDIX G

Statistical Analysis of the Mean Fractal Indices of the Three Treatment Groups Based on One-way ANOVA

APPENDIX G.1. Mean fractal indices of CAM of each duck egg in their respective treatment groups (based on Appendix B.1).

TREATMENT GROUPS		
CONTROL	NORMOBARIC OXYGEN TREATMENT	HYPERBARIC OXYGEN TREATMENT
1.7288	1.7500	1.7403
1.6766	1.7383	1.7338
1.7042	1.7217	1.7364
1.6769	1.6458	1.7507
	1.7152	
	1.7340	

$\sum x = 24.0527$
$\sum x^2 = 41.3365$
$\sum C = 6.7865$
$\sum N = 10.3050$
$\sum H = 6.9612$
Correction Factor (CF) = 41.3237
$n = 14$
$k = 3$

x – mean fractal indices
C – mean fractal indices of the control group
N – mean fractal indices of the normobaric treatment group
H – mean fractal indices of the hyperbaric treatment group
n – total number of samples
k – number of treatments

APPENDIX G.2. One-way Anova of Appendix D.1 with α set at 5%, degrees of freedom of 2 and 11, and a critical F-value of 3.98.

SOURCES OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	COMPUTED F
TREATMENTS	3.82×10^{-3}	2	1.91×10^{-3}	2.34*
ERROR	8.98×10^{-3}	11	8.16×10^{-4}	
TOTAL	1.28×10^{-2}	13		

*$F_{computed}$ (2.34) < $F_{critical}$ (3.98), therefore, no significant difference between the mean fractal indices of the three treatments.