Effects of hyperbaric oxygen and inducible nitric oxide synthase inhibitor treatment on femoral head osteonecrosis in a rat model

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Abstract:

Introduction: Apoptosis is the final destiny of many cells in the body, though this process has been observed in some pathological processes. One of these pathological processes is femoral head non-traumatic osteonecrosis. Among many pro/anti-apoptotic factors, nitric oxide has recently been an area of further interest. Osteocyte apoptosis and its relation to pro-apoptotic action invite further research, and the inducible form of nitric oxide synthase (iNOS)—which produces a high concentration of nitric oxide—has been flagged. The aim of this study was to investigate the effect of hyperbaric oxygen (HBO) and inducible NOS suppressor (Aminoguanidine) in prevention of femoral head osteonecrosis in an experimental model of osteonecrosis in spontaneous hypertensive rats (SHRs).

Methods: After animal ethic approval 34 SHR rats were divided into four groups. Ten rats were allocated to the control group without any treatment, and eight rats were allocated to three treatment groups namely: HBO, Aminoguanidine (AMG), and the combination of HBO and AMG treatments (HBO+AMG). The HBO group received 250 kPa of oxygen via hyperbaric chamber for 30 days started at their 5th week of life; the AMG group received 1mg/ml of AMG in drinking water from the fifth week till the 17th week of life; and the last group received a combination of these treatments. Rats were sacrificed at the end of the 17th week of life and both femurs were analysed for evidence of osteonecrosis using Micro CT scan and H&E staining. Also, osteocyte apoptosis and the presence of two different forms of NOS (inducible (iNOS) and endothelial (eNOS)) were analysed by immunostaining and apoptosis staining (Hoechst and TUNEL).

Results: Bone morphology of metaphyseal and epiphyseal area of all rats were investigated and analysed. Micro CT findings revealed significantly higher mean fractional trabecular...
bone volume (FBV) of metaphyseal area in untreated SHRs compared with all other treatments (HBO, P<0.05, HBO+AMG, P<0.005, and AMG P<0.001). Bone surface to volume ratio also significantly increased with HBO+AMG and AMG treatments when compared with the control group (18.7 Vs 20.8, P<0.05, and 18.7 Vs 21.1, P<0.05). Epiphyseal mean FBV did not change significantly among groups.

In the metaphyseal area, trabecular thickness and numbers significantly decreased with AMG treatment, while trabecular separation significantly increased with both AMG and HBO+AMG treatment.

Histological ratio of no ossification and osteonecrosis was 37.5%, 43.7%, 18.7% and 6.2% of control, HBO, HBO+AMG and AMG groups respectively with only significant difference observed between HBO and AMG treatment (P<0.01).

High concentration of iNOS was observed in the region of osteonecrosis while there was no evidence of eNOS activity around that region.

In comparison with the control group, the ratio of osteocyte apoptosis significantly reduced in AMG treatment (P<0.005). We also observed significantly fewer apoptotic osteocytes in AMG group comparing with HBO treatment (P<0.05).

**Conclusion:** None of our treatments prevents osteonecrosis at the histological or micro CT scan level. High concentration of iNOS in the region of osteonecrosis and significant reduction of osteocyte apoptosis with AMG treatment were supportive of iNOS modulating osteocyte apoptosis in SHRs.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HBO</td>
<td>Hyperbaric oxygen</td>
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<tr>
<td>AMG</td>
<td>Aminoguanidine</td>
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<tr>
<td>ON</td>
<td>Osteonecrosis</td>
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<tr>
<td>FBV</td>
<td>Fractional trabecular bone volume</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ATA</td>
<td>Atmosphere absolute</td>
</tr>
<tr>
<td>MERF</td>
<td>Medical Engineering Research Facility</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>TNFR1</td>
<td>Tumour Necrosis Factor Receptor one</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR1–associated death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>FAS</td>
<td>Apoptosis Stimulating Fragment</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase-activated DNase</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
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<tr>
<td>ICAD</td>
<td>Inhibitor of CAD</td>
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*Effects of hyperbaric oxygen and inducible nitric oxide synthase inhibitor treatment on femoral head osteonecrosis in a rat model*
Effects of hyperbaric oxygen and inducible nitric oxide synthase inhibitor treatment on femoral head osteonecrosis in a rat model
Statement of Original Authorship

The work contained in this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously submitted or written by another person except where due reference is made.

I undertake to retain the original collated data on which this thesis is based for a minimum of five years, in accordance with University ethics guidelines.

Signed: ........................................

Date: 02/05/13

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Effects of hyperbaric oxygen and inducible nitric oxide synthase inhibitor treatment on femoral head osteonecrosis in a rat model
1.1 Background

Osteonecrosis (ON) – bone death – is one of the most challenging areas of orthopaedic surgery. The most common area of involvement is the femoral head, although other bones like the scaphoid and lunate (Kienbock’s disease), and talus can be affected. In broad terms, this disease is divided into two groups: traumatic and non-traumatic.

While traumatic ON has a well-established pathology related to disturbed blood supply, the non-traumatic ON pathology is not thoroughly understood. Femoral head ON is a devastating disease mostly affecting younger patients (average age of 38) (5) and due to inadequate pharmacological and surgical options, it usually leads to head replacement surgery of some sort like total hip arthroplasty (1). While many theories have been proposed, including marrow oedema and haemorrhage, lipocyte hyperplasia, thrombi or emboli in the microvasculature, cytotoxicity, osteoblast-osteoclast coupling, and recently osteocyte apoptosis (1,2), the exact pathogenesis of non-traumatic ON is not clearly understood. In this study, we sought to increase understanding of the disease by investigating the effects of inadequate femoral head oxygen supply and osteocyte apoptosis, and their interaction in an animal model.

Application of 100% oxygen at higher than atmospheric pressure through a chamber has been defined as Hyperbaric Oxygen treatment (HBO) (4). This concept of HBO in orthopaedics is relatively new and has focused primarily on other problems such as crush injuries, osteomyelitis, necrotising fasciitis, gas gangrene, and diabetic foot (8,9). HBO therapy has recently been investigated to address the inadequate blood supply to the femoral head (3-7). However, it also has potential for application in ON. Bone marrow oedema leading to ischemia is frequently associated with early-stage ON. Hypoxia in the oedematous zone around the necrotic zone (Fig. 1) has been demonstrated by detection of over-expression of
angiogenic proteins VEGF and CYR61 (12). Thus HBO treatment could be applied to relieve intramedullary ischemia by boosting arterial oxygen tension. Overcoming hypoxia may be crucial for survival of the bone marrow cells that are confined in non-yielding bony boundaries, and for acquiring the necessary metabolic conditions to initiate dead bone resorption and bone remodelling (5). Further, HBO has shown positive effects on collagen maturation, angiogenesis, and cell proliferation in vascular-deprived ON models and in vitro (5, 6, 11).

Figure 1: Schematic of the oedematous zone underneath a necrotic area of a femoral head with idiopathic ON, adapted from Arlet (12).
While HBO treatment of femoral head ON is based on a rational hypothesis and shows promising preliminary results, further research to determine the exact mechanisms of action is required, particularly its interaction with/effects on apoptosis of osteocytes. Thus, we chose to further investigate osteocyte apoptosis in an animal model with or without the application of HBO.

Apoptosis (programmed cell death) is the destiny of many cells in their natural history. However, stimulation or prevention of this natural process may become problematic in some situations like post-radiation cell death, stimulation by and withdrawal of glucocorticoids, and changes of growth hormones (10). The role of apoptosis in non-traumatic ON has been recently investigated (1, 20-22) and nitric oxide (NO)-mediated apoptosis introduced as the culprit agent behind steroid and alcohol induce human ON.

Nitric oxide plays a central role in the complex apoptosis signalling pathway, as well as in bone function (1), and is therefore a potential target for ON therapies. NO is a free-radical messenger molecule that is produced from the amino acid L-arginine by the enzymatic action of NO synthase (NOS) (19). Nitric oxide synthase (NOS) is available in both constitutive and inducible forms. The constitutive form is present in the vascular endothelium as endothelial NOS (eNOS), which produces low levels of NO and results in physiologic effects, including relaxation of vascular smooth muscle and neurotransmission (19). Inducible NOS (iNOS) is produced in response to microbes, cytokines, and other activating stimuli in many cell types (e.g., macrophages, bone marrow, osteoblasts, and osteoclasts), and plays an important role in inflammation (19). The potential for either pro- or anti-inflammatory effects of NO depends on the amount of NO produced, such that small amounts of NO produced from constitutive forms of NOS are thought to be physiologic and protective, whereas large amounts of NO produced by iNOS are likely to be pro-inflammatory and injurious (19) (Figure 2). High levels of iNOS have been found in femoral head ON, compared to osteoarthritis, and higher
amounts of iNOS are associated with osteoblast dysfunction and higher rates of apoptosis in osteoblasts, osteocytes and marrow cells (1). Thus, inhibition of iNOS could be a potential pathway to treat non-traumatic ON.

Figure 2: Different forms of nitric oxide synthase (NOS) and their effects on apoptosis. High levels of inducible NOS (iNOS) from macrophages and low levels of endothelial NOS (eNOS) from endothelial cells can lead to apoptosis and phagocytosis of non-apoptotic cells such as osteoblasts (24)

Aminoguanidine (AMG) is a potent inhibitor of iNOS, with little effect on constitutive NOS, eNOS (16, 17). Accordingly, AMG inhibits NO production in vivo, as measured by decreased plasma nitrate and nitrite levels (17). This agent has been used to treat experimental colitis in a rat model (16, 23) and significantly reduced iNOS-induced intestinal cell apoptosis (40). On the other hand, the combination of AMG with HBO treatment resulted in an even more distinct reduction of NO levels compared with HBO and AMG treatment alone (1616). To
the best of our knowledge, the role of iNOS inhibitors (e.g., AMG) has not been investigated for non-traumatic ON treatment.

Animal models of ON are the best platform to test the effects of HBO therapy and iNOS suppression on osteocyte apoptosis and the prevention of ON. *In vitro* testing of ON treatments is almost impossible, as the interaction between cells and vasculature is yet to be available *in vitro*. Animal models, on the other hand, have the major features necessary for studying ON, and may be the only possible pre-clinical method to investigate ON treatment modalities. Nonetheless, all animal models have limitations. None of the current models exactly follows the human osteonecrosis process, especially when ON is artificially induced. Housing large animals is difficult and expensive. Thus, a small animal model that spontaneously develops ON over a relatively short period of time would be ideal for studying potential ON therapies.

The Spontaneous Hypertensive Rat (SHR) has been used as a model of idiopathic femoral head ON and Legg-Calvé-Perthes disease because they frequently and spontaneously demonstrate femoral head necrosis and ossification disturbance (15). Table 1 shows the difference between SHRs and two other breeds of rats (Wistar and Wistar Kyoto) in terms of osteonecrosis and ossification disturbance (10).
Table 1: Ossification disturbance among different rat breeds, showing a higher rate of ossification disturbance and osteonecrosis (ON) in spontaneous hypertensive rats (SHRs) than Wistar Kyoto rats (WKY) and Wistar rats (WT). Ossification is classified as (A) No ossification, (B) ossification disturbance without ON, (C) ossification disturbance with ON, (D) abnormal ossification with ON, and (E) normal ossification without ON. Numbers represent femoral heads at 5, 10, 15, and 20 weeks. (10)

HBO has been implemented to prevent ON in SHRs, with 80% of rats showing normal femoral heads at the end of the experiment (7). It has been suggested that HBO either prevents or suppresses ON progression by increasing blood circulation as a result of decreased lateral epiphyseal vessel resistance and increased oxygen tension. In addition, the different ossification rates of HBO-treated SHRs suggest that HBO promotes ossification in the femoral head (7). However, its effect on osteocyte apoptosis has not been investigated.
1.2 Hypotheses:

Hyperbaric oxygen and/or Aminoguanidine reduce osteonecrosis in idiopathic osteonecrosis of rat femoral heads by reducing osteocyte apoptosis.

Research aims:

1. To determine if HBO and/or AMG treatment prevent idiopathic ON in SHRs

2. To elicit the potential mechanisms of HBO and AMG action in osteonecrosis by investigating:
   
   a). Expression of eNOS and iNOS in the SHR model of ON.

   b). Osteocyte apoptosis in the SHR model of ON.
In this chapter we reviewed the pathophysiology of apoptosis and then concentrated on available animal models of ON as well as the possible role of HBO and AMG on treatment of ON.

2.1 Apoptosis, nitric oxide, osteonecrosis

In this section, we reviewed apoptosis and role of nitric oxide in this process. Apoptosis, the beginning of the end, is the destiny of many cells through the natural aging process. This process is a well-regulated and controlled suicidal program that is part of normal physiological processes, but may also be involved in pathological processes. (62)

The physiological process of apoptosis attempts to control the cells’ population when they are not needed anymore simply, such as the uterine cells involved in women’s menstruation cycles, or polymorphonuclear cells (after they ingest the pathogen).

Pathological apoptosis can be initiated by:

- Post radiation; (59)
- Corticosteroid stimulation (27) and withdrawal; (60)
- Growth hormone changes and deprivation. (60, 61)

This process has been flagged as the aetiology of some diseases like: atherosclerosis, amyotrophic lateral sclerosis, and neurodegenerative disorders (Alzheimer, Huntington’s) (62), Ulcerative colitis and Crohn’s disease (65, 66), and non-traumatic osteonecrosis (1, 10).

Nitric oxide is one of the key regulatory factors of apoptosis, and can act as an inducer or inhibitor.
In their review article, Chung and associates (62) elaborated the dual role of nitric oxide in cell apoptosis. They mentioned that this complex action is mainly related to NO concentration. High concentration of NO activates the caspase protein family and ignites serial events and reactions leading to cell apoptosis. On the other hand, low/physiologic concentration of NO could be an anti-apoptotic agent, either by direct inhibition of caspase activity or by activating other protecting genes like heat shock protein. Nitric oxide plays a central mediator role in bone function, as well as a modulator role in bone cell apoptosis (1).

Brüne et al (63) also certified the pivotal action of NO, and mentioned the important fact that, although we can rationalize NO action, we cannot predict their action and it has to be investigated in individual circumstances.

The hypothesis that abnormal NO production in ON leads to osteoblastogenesis dysfunction and osteoblast apoptosis has been investigated by Calder et al (1). The femoral heads of twenty patients with ON undergoing total hip arthroplasty (Ficats stage III or IV, 112) (consisting of four idiopathic, seven steroid-induced, six sickle cell anaemia, and three alcohol-induced ON) were compared with 26 femoral heads of osteoarthritis patients. Standard immunohistochemistry examination and Western blotting were used to assess eNOS and iNOS. Presence of DNA laddering (hallmark of apoptosis) was evaluated by DNA extraction kit and confirmed by TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP neck end labelling) (Figure 3). They found out that eNOS was present in both groups, while iNOS was abundant in the osteonecrotic group and did not present in the control group. Also, the intensity of eNOS and iNOS bands was significantly stronger in ON group. In term of apoptosis, none of the control group revealed evidence of apoptosis,
whereas 15 patients of the ON group (except sickle cell-induced ON) had evidence of apoptotic osteoblasts, osteocytes and marrow cells (P<0.001).

![Figure 3: TUNEL assay of the apoptotic cells in ON. Adapted from Calder (1)](image)

Shibahara et al (10) evaluated the extent of apoptosis in SHR ON. Based on the previous investigation (44), they mentioned that SHRs develop ON due to one of these pathological processes: endocrine dysfunction, or blockage of vascular supply of the physis. Therefore, they hypothesized that these conditions could cause osteocyte apoptosis. Using TUNEL assay, they assessed osteocyte apoptosis in 5, 10, 15, and 20-week-old SHRs, compared to Wistar Kyoto and Wistar rats as the controls. They had five rats at each age group when they sacrificed them. At 5 and 10 weeks, the amount of apoptosis was not different among rats; however, at 15 and 20 weeks SHRs developed significantly higher apoptosis percentages comparing with controls. Their results suggested that apoptosis is an important pathologic process of osteonecrosis in SHRs (Figure 4).
Figure 4: This figure adapted from Shibahara study (10) with permission, reviewing apoptotic osteocytes. A: showing rate of apoptosis in 15 weeks old SHR, Wistar Kyoto (WKY) and Wistar (WT) rats. B: showing the changes took place at 20 weeks old rats with starts pointing significant differences.
**Glucocorticoid Induced ON**

“Glucocorticoids suppress bone formation and cause calcium loss in most species, but the mechanisms that cause severe and rapid changes in specific sites, particularly the femoral head and neck, are debated” (21). There are several proposed pathogeneses of glucocorticoid-induced ON like fat embolism (21); however, it does not appear to be the key mechanism. Other suggested mechanisms are microfractures, enhancement of osteoclast activity (RANK-Ligand expression), shift in osteoblastic differentiation, and finally osteoblast/osteocyte apoptosis (21).

Weinstein et al (14) hypothesised that glucocorticoid-induced bone disease is secondary to alteration of birth or death rate of bone cells. In their pilot study they realised that implantation of pellets releasing 0.5 mg/kg/d of prednisolone would not affect bone mineral density, whereas 1.4 mg/kg/d invariably led to loss of BMD. Thus, they used two doses, 0.7 (lower dose) and 2.1 mg/kg/d (higher dose) for 27 days in a murine model. Mice were sacrificed at the 27th day. Their serums and urines were taken to assess osteocalcin (marker of osteoblast activity) and deoxypyridinoline (as marker of osteoclast activity). Mice bone marrow was cultured to assess and quantify CFU-F- fibroblast and CFU-OB-osteoblast. Histomorphometric analysis was conducted on the left femur and lumbar vertebrae. Lastly, they used TUNEL assay to detect apoptotic cells.

Significant reduction of osteocalcin (34% and 50% in low and high dose groups respectively) indicated decreased osteoblast activity. A decreasing trend of osteoblast and osteoclast perimeters was found in the higher dose group. Mineral apposition, trabecular width and in general bone turnover decreased in a dose-dependent manner. CFU-F osteoclastogenesis response to 1,25(OH)2D3 reduced by 63% (P<0.05). They also detected 85% reduction in
CFU-OB colony formation in the high-dose group. It was noticed a threefold increase in vertebral osteoblasts’ apoptosis and a 28% increase of femoral osteocyte apoptosis. Therefore, they concluded that the inhibitory effect of glucocorticoids on early bone cell progenitors in the bone marrow is the culprit for decreased trabecular width.

In another study, Weinstein and associates (22) compared osteocyte apoptosis of a group of patients who underwent total hip arthroplasty. This group of patients comprised five patients with steroid-induced ON, another five with sickle cell-induced ON, three with alcohol-induced ON and one with trauma-induced ON. After the specimens were obtained, they were cut with a band saw in 5-mm slices and decalcified in formic acid for 2-3 weeks. Their control group was the iliac crest of healthy volunteers, and their positive control consisted of the trans-iliac biopsy of two patients with steroid-induced osteoporosis. Then all sectioned and samples systematically examined for presence of apoptosis under 400 magnification using TUNEL and Hoechst staining. Apoptosis rate was defined as predominant cell 3+, abundant cells 2+, rare 1+, and absence 0. Osteocyte apoptosis of the juxta-subchondral microfracture area were 3+ in corticosteroid-induced osteonecrosis, 1+ in alcohol-induced ON and zero in traumatic and sickle cell groups. They concluded that steroids provoked osteocyte apoptosis in the femoral head “a defect that could interrupt the mechanosensory network of the femoral head” and caused femoral head collapse. On the contrary, the rate of apoptosis in other groups did not support the role of osteocyte apoptosis in development of ON.
Figure 5: Cancellous bone of femoral head  
A: Apoptotic osteocytes after Hoechst staining display intense blue fluorescence, condense nuclei  
B: Showing positive control apoptotic osteocytes of steroid induced osteoporosis patients, arrows pointing apoptotic osteocytes  
(Hoechst staining) Adapted from (22),
Eberhardt and associates (21) evaluated the chronological sequence of events leading to ON in a rabbit model of steroid-induced ON. The rabbits were injected weekly with Methylprednisolone Acetate at 1.7 and 4 mmol/kg/day for four weeks. Assessment of the 1.7 mmol group showed minimal changes, and so did not warrant further evaluation. Their result showed no difference in cortical thickness and trabecular separation of the femoral shafts. Absence of bone formation in the subarticular trabecular bone (increased osteoclastic resorption and decreased formation) was confirmed by tetracycline metabolic labelling and electron microscopy scanning (50–80% resorption of the trabecular surface). Therefore, they realised that both enhanced resorption and diminished synthesis were involved, while none of the specimens showed inflammation, necrosis, or vascular changes. After performing TUNEL assays to confirm apoptosis, they stated, “The pattern of cell death involving cohorts of osteoblasts and osteocytes comprised up to half of the bone volume in affected regions and is consistent with an apoptotic mechanism” (21). Consequently, they concluded that apoptosis was an early event in the process of corticosteroid-induced ON, even before vascular, inflammation or microfracture occurrence.
2.2 Animal models of osteonecrosis

Animal models of osteonecrosis offer an invaluable model to study the osteonecrosis process, as it is impossible to mimic this process in vitro; however, none of the current models completely follow what is exactly happening in human atraumatic ON. Also, mimicking traumatic ON is another challenge in animals as only a few of them are not true quadrupeds. On the other hand, animals have a lifelong persistent physis that attempts to repair or regenerate the damage of the head; in this regard, it more or less follows the Legg-Calvé-Perthes disease in children.

Boss et al (15) in their review of literature elaborated the current pathophysiologic understanding of different models of animal ON. Their study’s highlight breaks down into the following subjects:

Spontaneous Osteonecrosis in Laboratory Animals

Spontaneous hypertensive rats have been a well-established model of spontaneous ON. This process been studied in rats as young as 6 weeks old up to 8 months old. The main culprit (though not yet fully understood) seems to be a lack of ossification due to hindered mineralization controlling collagen X (42). It was suggested that this delay is related to underexpression of insulin-like growth factor 1. Hence the under-prepared cartilage does not serve as the base for mineralization process. This loose cartilaginous head and weight bearing add pressure to the fragile lateral epiphyseal arteries which supply the head and secondary ossification centres (43, 44, 45).

This process also takes place in small dogs, with the possibility of thrombotic venous occlusion inducing hypoxemia and the paucity of arteries being the main contributor to pathology (41).
Surgically Induced Osteonecrosis

The main challenge to surgically inducing ON is the dual blood supply of the physis by epiphyseal and metaphyseal vessels. Thus, simply disrupting the blood supply to the head does not exactly follow the sequence of events in either human ON or Legg-Calvé-Perthes disease. In these models after initial insult (by stripping the peristeme, dislocating the hip to disrupt ligamentum teres blood supply, or by damaging the metaphyseal blood supply), fibrous tissue invades the area and the battle between re-ossification and collapse starts. The battle ends in different stages of collapse or deformity, based on the animal breed and the weight-bearing status (6, 46, 47). Considering the dual blood supply, one at least needs to create an insult to drop the femoral head supply as low as 15% in pigs and 20% in canine model in order to achieve surgically-induced ON, which in turn demands the application of multiple operative interventions (48,49).

Traumatic Osteonecrosis

One needs to consider the fact that older animals have a mature dual blood supply, while younger ones still rely more or less on extraosseous blood supply on the ground of immature anastomosis of epiphyseal and metaphyseal vasculature. This is well established in older rabbits’ post-traumatic ON when ossification bridged the physis (50, 51)

Corticosteroid-Induced Osteonecrosis

Though the exact mechanism is unknown, evidence of lipid plug in subchondral capillaries and arterioles has been suggested as the primary pathophysiology of steroid-induced ON. In this regard, the femoral heads in steroid-induced osteonecrotic rabbits have shown bone marrow adipocyte hyperplasia. Also, their blood revealed a high ratio of low-density to high-
density lipoprotein, and lipid plug (especially in venous drainage) led to an increase in intraosseous pressure, further compromising blood flow (52, 53, 54). Thus treatment of steroid-induced ON with lipid-lowering agents grounds a logical place and has been tested in animal and human studies (2, 55).

**Physical Injury–induced Osteonecrosis**

Rabbits' femoral heads were treated with cryoprobe (liquid nitrogen) and the results revealed a necrotic area of the head three times bigger than the probe head and less active osteogenesis for the first 7 weeks after the insult, followed by complete healing 24 weeks after the event. Also, the role of hyperthermia has been evaluated in canine models; for instance, a temperature of 42.5°C for 60 minutes caused 5mm of necrosis around the probe tip with recovery after 12 weeks, while higher temperatures (e.g. 45°C) were poorly tolerated by osteocytes (56). On the other hand, physical insults like standing on the hind foot proved to cause ON in 40% of rats in high cages that required standing to reach the food and drinking apparatus (57).

To address the drawbacks of the current models, there have been some attempts to introduce new models to overcome the current dissimilarities. Manggold et al (37) introduced a new model of early-stage ON by injecting 8 ml of purified ethanol (rate of 1 ml/min) into the centre of femoral heads. They used 20 adult sheep to test their new model. After anaesthetizing the animal, using a k-wire and under guidance of an image intensifier, they located the femoral head and then withdrew the k-wire and replaced it with a cannulated needle to inject 8 ml of 99% ethanol at the rate of 1 ml/min. Animals were sacrificed at 3, 6 or 12 weeks to examine the femoral head. As depicted in figure 6, the empty lacunae present up to 12 weeks after the insult; this is the strength of their model as many other insults induce
ON repair earlier than 12 weeks in animals. In addition, all of these animals developed ON. Examining the other femur, they found only less than 5% empty lacuna.

![Figure 6: This bar graph represents percentage of empty lacunae in a sheep model of ON](image)

This model, along with other bipedal animal model like the emu, has been suggested as alternative animal model for ON and lately as a model of collapse as well. Fan et all (38) in their study managed to induce ON in femoral heads of emu using a probe to produce alternative freezing and heating insult by liquefied nitrogen and radiofrequency using their custom-made probe depicted in figure: 7. Then, the animals had MRI scans at 2, 4, 8, 12, 16 weeks; apart from three sacrificed at 12 weeks, the rest were sacrificed at 16 weeks. Their treatment led to a 75% rate of ON in the emu model and one complete head collapse. Emu is a valuable model as their hip biomechanics are quite similar to humans; however, producing ON in this animal and housekeeping of course is not free of challenges.
2.3 Hyperbaric oxygen and osteonecrosis in animal

In 1992, Kataoka et al (7) evaluated the effect of HBO on ischemic ON and ossification disturbance of spontaneously hypertensive rats. Their first group (A) consisted of 10 male SHRs aged 5 weeks and group B of 10 male SHRs aged 8 weeks. Both groups had HBO at 2.8 atmospheres absolute (ATA) for 6 weeks in a total of 30 hours. The control animals were 10 male SHRs (Group C) and 10 male Wistar Kyoto rats (WKY, Group D). Both group C and D did not have any treatment.

After sacrificing all rats at 17 weeks of life, group A did not reveal any evidence of ON with two femoral head ossification disturbances, while group B disclosed femoral head ON in two rats and ossification disturbance in one rat. In SHR control group they found 6 ON and 4 cases of ossification disturbance. Thus, they concluded that HBO prevented ON in spontaneously hypertensive rats.

Peskin and associates (5) examined the combination effect of HBO and non-weight-bearing in treatment of vascular deprivation-induced ON of rats’ femoral heads. By cutting the
ligamentum teres and disrupting the periosteal blood supply, they prepared their rats’ ON model. Using a strap (Figure 8) they sustained a non-weight bearing (NWB) situation, while the rat had free access to food and water.

![Figure 8](image)

**Figure 8: This figure showing the cage designed for NWB and free range of motion (5)**

From day 5 post-operation, 16 of the rats received 90 minutes of 100% O₂ in 250 KPa daily for 6 days a week. Twenty of rats had only NWB treatment and 18 rats were only kept in cage with no treatment. Half of each group were killed at day 30 and the rest at day 42. Contralateral non-operative hips also were used as an internal control in every other 5 cases to ensure no complication were induced by NWB.

They found out that continuing HBO for 12 days after day 30 would not achieve any benefit. Their results showed that NWB itself would not prevent femoral head collapse; nevertheless, the combination of HBO and NWB led to preserving femoral head architecture despite remodelling within the necrotic area. Also, higher degrees of hematopoietic tissue and new bone formation were observed in combination of HBO and NWB. While leaning toward a significant trend, none of their findings disclosed an actual significant difference among groups.
Levin and associates (6) found that HBO in vascular deprivation-induced rats’ femoral head ON did not affect the new bone formation, articular cartilage, and tissue viability at days 2, 7, and 21 post-operatively. However, they found out that osteogenesis, fibrogenesis and resorption of necrotic tissue was enhanced in HBO-treated rats but not to a significant degree. Extending their study to 42 days, they realised the amounts of osteogenesis (intramembranous and appositional) and necrotic bone resorption were significantly improved in HBO-treated rats.

Following the first case report on the healing effect of HBO on Crohn’s disease back in 1989 by Brady (65); Lavy et al (66) reported the effect of HBO on perianal Crohn’s disease in a series of ten patients receiving 250 KPa of HBO for 90 minutes, 6 times a week, 20 sessions in total. In their series if the patient did not improve with the first course, they received the same course for another 20 days. Five of patients’ perianal disease completely healed after one or two sessions, while three of them only improved to some extent and two did not improve at all.

After this report, many researchers attempted to explore this effect and understand the pathophysiology behind this healing effect. While the role of inflammatory cells like neutrophils, macrophages, and lymphocytes and their end product like eicosanoids, leukotrienes, and free radicals in the pathology of this disease is well established (39), some reported the effect of NO in this process as well.

In this regard Rachmilewitz et al (39) investigated the effect of HBO treatment on two models of experimental colitis and in particular its interaction with NO synthase. NO caused
tissue injury by activating the superoxide family and its final product, peroxynitrite (67). Though NO and free radicals are generally accepted as final agents in the cascade of inflammation, there is no consensus about their exact role. This uncertain and pivotal role of NO is certified by studies indicating that the NO synthase inhibitor, NG-nitro-L-arginine methylester (L-NAME) (68), and nitroxide, a free radical scavenger (69), both ameliorated experimental colitis. Therefore, Rachmilewitz (39) set up his study to assess this uncertainty by evaluating NO synthase activities after HBO treatment. They induced inflammatory bowel disease in rats by implementing intraluminal acetic acid and for the second model by intracolonic administration of 0.25 ml 50% ethanol containing 30 mg TNB (trinitrobenzenesulphonic acid). Then, all experimental and control rats received 2.4 ATA of “HBO for one hour twice at six hour intervals on the day of colitis induction and once daily thereafter until they were sacrificed”. NOS activity was measured by conversion of L-[3H] arginine into citrulline in the mucosal scrapings of the colon. They also measured colonic weight, PGE2, and myeloperoxidase (MPO) activities in experimental and control rats’ colons. As depicted in figure 9, the lesion size and colon weight of the acetic acid-treated group (an indication of inflammation) was significantly reduced with HBO treatment. This was almost similar in the second experimental model induced with TNB. In the meantime, HBO treatment significantly reduced MPO and NOS activities in both experimental groups (Table 2). This study suggested that HBO could diminish NOS activity and therefore is an option in refractory bowel disease. We believe that HBO treatment is an option wherever NOS activity is troublesome, like ON.
Figure 9: The two bars on the left side illustrate size and weight the colonic lesions of acetic acid induced colitis group, and the two bars on the right side represents the same parameters of the other group, receiving acetic acid and HBO treatment. Lesion size is referenced on the left and weight on the right side of the figure. Stars indicating significant differences. (39)
Table 2: This table summarised Rachmilewitz’s findings in his two experimental colitis group with starts indicating significant reduction in NOS and MPO activities with HBO treatment (39)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetic acid</th>
<th>Acetic acid + HBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of rats</td>
<td>12–16</td>
<td>8–10</td>
</tr>
<tr>
<td>MPO (U/g)</td>
<td>5.6 (0.9)</td>
<td>3.2 (0.3)*</td>
</tr>
<tr>
<td>NOS (nmol/g/min)</td>
<td>7.1 (1.1)</td>
<td>4.0 (0.4)*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNB</th>
<th>TNB + HBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of rats</td>
<td>14–21</td>
<td>8</td>
</tr>
<tr>
<td>MPO (U/g)</td>
<td>6.9 (0.9)</td>
<td>4.0 (0.7)</td>
</tr>
<tr>
<td>NOS (nmol/g/min) + Ca^{2+}</td>
<td>11.8 (2.0)</td>
<td>4.2 (0.1)*</td>
</tr>
</tbody>
</table>

2.4 **Rationale behind our animal model**

Reviewing different animal models, we decided to choose spontaneous hypertensive rats, as the effect of HBO in treatment of SHRs’ ON has been established. Nevertheless, its role in preventing apoptosis of osteocytes has not yet been investigated. In addition, the high rate of osteocyte apoptosis (10) and high concentration of iNOS in these rats has been documented already (58). Apart from those reasons, SHRs were easy to obtain, and our facility, the Medical Engineering Research Facility (MERF), also had a hyperbaric oxygen chamber and could easily accommodate them (Figure 10).
Figure 10: A: The hyperbaric oxygen chamber used in our study, B: showing SHR rats inside the chamber at the beginning of their treatment
2.5 *Hyperbaric oxygen and osteonecrosis in human*

The ultimate goals of many animal experiments are to test a hypothesis and if it works, stretch it to the point to treat human diseases. HBO is not an exception in this regard.

Reis et al (4) evaluated the effect of HBO on the early stage of human femoral head ON. Out of 120 patients who have had HBO treatment for femoral head ON with various stages; they selected 12 patients with stage one of ON (symptomatic painful hip, normal radiograph, positive bone scan, positive MRI-Steinberg/Hayken classification) with subchondral lesions of 4 mm or thicker and 12.5 mm or longer. The aetiology of disease was idiopathic in 10 and corticosteroid-induced in 2. The patients had six daily sessions for 100 consecutive days with 2 to 2.4 atmospheres HBO\textsubscript{2} pressure for 90 minutes. All had MRI prior to treatment and the endpoint was either normal MRI or MRI at 2 years post-initiation of disease. Nine of them were reversed to normal, 2 remained unchanged at stage two and one progressed to collapse. This is the only article reporting effect of HBO in treatment of human ON.
Chapter 3: Material and methods

3.1 Treatment groups:
After obtaining animal ethic approval (0900000324) from Queensland University of Technology, we purchased thirty-four male SHRs from Animal Resource Centre (Perth, WA). The rats were divided into four groups:

I. Hyperbaric oxygen treatment group: HBO group

II. Aminoguanidine treatment group: AMG group

III. Combination of Hyperbaric oxygen and aminoguanidine: HBO+AMG group

IV. Control group

In order to address our facility’s limitation (mainly the HBO chamber, which would only accommodate 3 cages at the same time), we performed the study in two phases. In phase one, control and HBO-treated animals were purchased and then the HBO+AMG and AMG group were purchased and allocated into the groups. At the end, we allocated 8 SHRs to each treatment group and 10 SHRs to the control group.

SHRs at the time of experiment were in the fifth week of life, in some cases the younger ones had been looked after till they reached five weeks of age and then allocated into the groups.

Every two rats were looked after in one cage at MERF and followed the facilities’ standard procedure through the experiment period. The principal investigator and one of the MERF staff members monitored their well-being accordingly on a daily basis. All groups including control group were kept in the same laboratory room and under the same normal diet regimen.
At the 17th week of life, rats were captured by hand using the scruff technique and then sacrificed using an intraperitoneal injection of pentobarbitone sodium (Lethabarb) 325mg/ml at a dose of 0.5ml/kg. Then both femoral heads were extracted. After extraction, the samples labelled and kept in 4% paraformaldehyde until micro CT evaluation.

### 3.2 Implementing first aim:

Aim 1: To determine if HBO and/or AMG prevented idiopathic ON in a SHR model

In order to execute this aim, SHRs were commenced on HBO and AMG treatment. **Hyperbaric oxygen treatment:**

HBO group received 90 minutes of 100% O2 at 250 kPa daily, 6 days a week for 30 days (5) which made a total of 45 hours of HBO treatment using the hyperbaric oxygen chamber facility at MERF (Figure 10). The characteristics of the device allowed us to closely monitor rats’ behaviour through the device window during each HBO session. To overcome chamber size limitation, we had to re-allocate the cages at the time of HBO treatment. At that point, two rats of one cage were allocated to other cages; therefore, we had 1 cage with two rats and two more cages with three rats in the chamber. Thus, all the group members received their HBO at the same time and in the same fashion. At the end of HBO treatment, the rats were re-allocated again to their individual two-rats-per-cage basis.

To reach the desirable pressure, the oxygen input of the chamber was opened up, and usually the chamber pressure reached 250 kPa by 5 to 6 minutes. The whole decompression process took five minutes to be done safely. To decompress the chamber, the pressure was decreased at 85 minutes (after reaching the maximum pressure) to half of the pressure (125 kPa) over 30 seconds; after two minutes at this pressure, it was again decreased to half (62 kPa) over 30 seconds with a wait of 1.5 minutes, followed by free decompression which took another 30-60 seconds.
3.2.2 Aminoguanidine treatment:

AMG group received 1mg/ml of Aminoguanidine (34, 36) in their drinking water from the fifth to the 17th week of life (Figure 11), when they were sacrificed.

We decided to use Aminoguanidine bicarbonate (Sigma Aldrich), as this salt is easily soluble in water. Mixing was performed under a vacuum hood protective device at MERF and a concentration of 10 mg/ml of AMG was prepared and diluted with tap water to fill up the AMG and HBO+AMG groups’ drinking water apparatus.

3.2.3 Combination of HBO+AMG

The HBO+AMG group received both HBO and AMG treatments in exactly the same fashion as the individual HBO and AMG groups.

![Figure 11: This picture represents the drinking apparatus of the cages (yellow arrows)](image)

3.2.4 Control group

The control group did not receive any treatment. These rats were expected to develop ON themselves without any intervention.
3.3 **Micro CT scan assessment:**

Femoral heads were assessed for radiologic evidence of ON by micro-computed tomography at IHBI (Scanco µCT40, Brüttisellen, Switzerland). The femoral heads were kept in the best identical anatomical alignment in the micro CT scanner tube and fixed in place with foams and soaked in 70% ethanol while scanning. Calculations were made from 3D datasets of trabecular bone volume (BV), total volume (TV), fractional trabecular bone volume which is the ratio of BV to TV (FBV), trabecular thickness (µm), number (1/mm), and separation (µm).

The trabecular parameters were measured and tested using a threshold of 350 and the Micro CT setting described by Yamako (26): tube voltage 50 kV, tube current 36 µA, and sliced thickness of 19 µm.

Measurements were compared among groups. Such measurements have previously shown significant differences between normal and traumatic osteonecrotic rat femoral heads (26).

To perform these measurements in the epiphyseal area, we used the following strategy: In a pilot evaluation of the head, we realized that after around 20 voxel we reached the metaphyseal area (top yellow line, Figure 12); subsequently following the head down by another 100 voxel was enough to reach the head/neck junction (bottom yellow line, Figure 12). So area B is defined as the distance between metaphyseal plates to 100 voxel below that. However, the main volume of interest was the epiphyseal area (area C). We believe that to assess area C accurately, the best course of action was to measure all of area A (long arrow on the left side of figure 12) then to calculate area C as C=A-B.
Figure 12: Calculation method to assess epiphyseal bone formation. The area between two yellow horizontal lines depicts the metaphyseal area: B, blue arrow (A) shows total head and the difference between A and B is marked C as epiphyseal region

Following micro CT, femoral heads were prepared for histological examination by decalcification with 0.5 M ethylenediaminetetraacetate (EDTA), pH 7.6, for 3 weeks, and paraffin embedding. Samples were sliced, and the sections near the ligamentum teres insertion area were utilized and stained by standard hematoxylin and eosin staining to assess histological evidence of ON (12) in the epiphyseal area.

To facilitate comparison in the small group of samples, we divided the histology findings into three groups: ON and no ossification; anything less than 50% ossification of the epiphyseal area; and 50% or more ossification including normal ossification. This amount was calculated from the bisectional line to the line connecting the two epiphyseal-metaphyseal junctions together (Figure 13). So if the area of ossification did not reach this line, it was considered less than 50%; and if it reached or passed the bisectional line, then it was considered greater than 50% ossification.
Figure 13: This picture represents a bisectional line from epiphysial-metaphysial junction, which is a line perpendicular and exactly in the middle of the distance from the line connecting two epiphysial-metaphysial corners. The ossification calculated in the epiphysial area is relative to this line.
3.4 Implementing second aim:

Aim2: Elicit mechanisms of HBO and AMG action in ON

3.4.1 Osteocyte apoptosis in an established model of osteonecrosis

Figure 14: Diagram depicting apoptosis timeline in relation to cell function changes and available measurement methods. The two methods chosen in our study have been underlined.
The possible techniques to detect apoptosis have been illustrated in figure 14. Among them, two characteristics are more or less globally accepted as the hallmark of apoptosis: DNA fragmentation and DNA condensation (113). Therefore, Hoechst staining was employed to detect DNA condensation or fragmentation and to confirm with the DNA ladder detection method: TUNEL. TUNEL assessment was performed to confirm apoptosis of only two control samples, as this second method was quite expensive. In this part of study, Hoechst staining was used based on the described protocol by Weinstein (22) et al.

3.4.1.1 Hoechst staining protocol

1. Paraffin-embedded femoral head sections (those close to the ligamentum teres insertion) were deparaffinised in xylene bath, twice each time for 10 minutes each and washed in PBS bath after each session;

2. Samples were hydrated in distilled water;

3. Samples were placed in Tris-buffered saline, pH 7.6, for 5 minutes;

4. Excess buffer was wiped from the sections;

5. Area of interest was isolated with hydrophobic barrier pen;

6. Samples were placed in humidified dark container and 200–300 mL of a 50 ng/mL solution of Hoechst 33258 (Invitrogen) was added to these sections;

7. Samples were incubated in the dark humidifying chamber at room temperature for 2 minutes;

8. The sections were then washed in distilled running water for 6 minutes while container wall was covered with foil;

9. Samples were dried while protected against light;
10. Lastly, they were mounted with ProLong Gold and mounted.

In each staining session we used a combination of all the groups and right after staining, the samples’ labels were covered to blind them to the examiner. Then, the epiphyseal area was assessed for evidence of condensed or fragmented nuclei with a fluorescence microscope (Image Axio). As we reviewed the first sample, we noticed that minor adjustments brought different layers of osteocytes’ nuclei into the best visual focus (Figure 15); hence, this assessment was a real-time assessment.

To measure apoptotic osteocytes, we calculated dense or fragmented osteocyte nuclei in 10 random microscopic fields (*400 magnified) (Zeiss Axio Imager widefield fluorescence microscope). Previously, this technique was implemented by Shibahara (10). Each microscopic field had 1590×1170 µm dimensions, an area up to a total of 1.86 mm² per sample. This measurement was performed only on the samples with some degree of ossification, including osteonecrotic samples.
Figure 15: Figure A, B, C, and D showing the same area of Hoechst stained sample with minute focus adjustments bringing different layers of osteocytes into the best visual field. This picture clearly certifies that the nucleus condensation/fragmentation assessment was a real-time assessment. Arrows are pointing to apoptotic osteocytes; note how they fade with minute adjustments. The arrow heads (two normal osteocytes) come to the best focus on D.
3.4.1.2 **TUNEL assessment**

The presence of apoptosis in selected samples was confirmed by TUNEL assay (13, 27). This assay is based on labelling DNA strand breaks (Terminal deoxynucleotidyl transferase dUTP nick end labelling or TUNEL technology). We utilised the In Situ Cell Death Detection Kit (Roche, Germany) as described previously (64).

In situ apoptosis detection (TUNEL) protocol:

- Tissue sections were permeabilised for 30min at 37.0°C with Proteinase K solution (Roche, Germany)

  - Mid sagittal sections were incubated in Xylene bath twice for 10 minutes each;
  
  - Sections were hydrated with serial dilution of ethanol (70 to 90%);
  
  - Sections were immersed in PBS for 3 minutes;
  
  - Then, the area of interest was separated by hydrophobic barrier pen;
  
  - Samples were permeabilised for 30min at 37.0°C with Proteinase K solution (Roche, Germany);
  
  - Samples were rinsed twice in PBS;
  
  - 50µl of terminal deoxyribonucleotidyl transferase (TdT- Enzyme solution) was added to 450µl of deoxynucleotidyltransferase (fluorescein-dUTP- Label solution), mixed thoroughly and kept on ice;
  
  - Area around the sections was dried off;
  
  - 50 µl of TUNEL reaction mixture was added to each sample;
• Samples were incubated at 37°C in a dark container for 60 min;

• Samples were then rinsed 3x in PBS;

• Finally, samples were embedded with slow-fade embedding solution (incorporating DAPI stain)

4′,6-diamidino-2-phenylindole (DAPI) is a DNA-specific dye that can pass through intact cell membranes. An apoptotic cell membrane increases DAPI permeability, therefore leaving a stronger blue dye effect. For fluorescence microscopy, DAPI was excited with ultraviolet light and detected through a blue/cyan filter.

Fluorescein isothiocyanate (FITC) is a cell membrane phosphatidylserine (PS) stain. PS is a phospholipid exists in the inner leaflet of cell membrane in physiologic condition. Given that processes like apoptosis and necrosis expose this phospholipid, FITC binds to PS of apoptotic cells as well as necrotic cells, which one later indicates by stronger green fluorescence uptake (figure 41, 42)

A similar process was previously described by Gavrieli (13), though they did not use a fluorescence-labelled stain (FITC solution). Finally all samples were analysed under fluorescence microscope (Excitation wavelength 450-500 nm and detection in range of 515-565 nm).
3.4.2 Expression of eNOS and iNOS in an established model of osteonecrosis

The midline sections (again those close to or at insertion of the ligamentum teres) were evaluated by standard immunohistochemistry techniques to detect iNOS/eNOS protein expression using specific antibodies. We used following protocol for Immunohistochemistry.

3.4.2.1 Immunohistochemistry protocol

Indirect immunoperoxidase method:

- Paraffin embedded sections were deparaffinised in xylene bath twice for 10 minutes each time;

- Sections were dehydrated in a gradual dilution of ethanol (90% to 70%) bath and rinsed with PBS;

- Endogenous peroxidases were blocked by incubation in 0.3% peroxide for 30 min;

- Sections were washed in PBS;

- Sections were then incubated with Proteinase K (DAKO Multilink, CA, USA) for 20 min for antigen retrieval;

- Sections were treated with 0.1% bovine serum albumin (BSA) with 10% swine serum in PBS;

- Sections were incubated with optimal dilution of primary antibody (iNOS, or eNOS) overnight at 4.0°C in a dark humidified chamber;

- After 24 hours, sections were incubated with a biotinylated swine-anti-mouse, rabbit, goat antibody (DAKO Multilink, CA, USA) for 15 min;
Sections were then incubated with horseradish peroxidase-conjugated avidin-biotin complex for 15 min;

Antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 3 min;

The reaction was stopped by rinsing in PBS;

Sections were counterstained with Mayer’s haematoxylin for 10 sec each;

Sections were then rinsed with running tap water;

Sections were dehydrated with ascending concentrations of ethanol solution;

Sections were cleared with xylene twice;

Finally, they were mounted with coverslips using DePeX mounting medium.

Our pilot evaluation revealed that optimal eNOS antibody concentration was 1/10 in BSA (Anti-eNOS Polyclonal Antibody (PA1-037), Affinity BioReagents, Denver, CO, USA) and that of iNOS antibody (cat. Ab15323; Abcam) was 1/20 in BSA concentration. Then samples were evaluated on the same day with a light microscope (Axio Imager) for evidence of eNOS and iNOS activity around the growth plate and epiphyseal area.

3.5 Statistics:

Mean values reported ± standard deviation. Sample size was calculated to measure 50 percent reduction in final outcome: ON. The significance of relative apoptotic frequency was tested using student t-test analysis. Histological findings were analysed with Chi-square, and Fisher exact test when relevant. Micro CT findings were analysed with student t-test and post-hoc
tests (SPSS v14 statistic package). The level of significance for the mentioned tests were considered significant if the P value was <0.05.
Chapter 4: Results

4.1 Micro CT scan
All the samples were assessed with Micro CT scanner.

4.1.1 Metaphyseal findings:
The evaluation of the metaphyseal areas (referring to area B on figure 12) revealed the following results:

Fractional trabecular bone volume of control group was 0.65±0.01%. The difference between FBV of the control and HBO group was significant (0.60±0.00%, P<0.05), same as the difference between control and HBO+AMG group (0.59±0.01%, P<0.005) and AMG group (0.58±0.00%, P<0.001) (Figure 16, 17).

There was no significant difference between non-control groups.

![Figure 16: Picture representative of mid-sagittal section of femoral heads showing fractional trabecular bone volume ratio of AMG-treated (A) and control (B) SHRs. Note the dense trabecular area of the control epiphyseal region compared to the AMG-treated epiphyseal region.](image)
Figure 17: The bar graph represents mean fractional bone volume of control, HBO, HBO+AMG and AMG groups; the significant differences were highlighted in brackets. There was no significant difference among the three treatment groups.

Trabecular thickness, separation and numbers of the control group were 0.107±0.00 μm, 0.097±0.00 μm, and 7.007±0.09 per mm respectively. Those of the HBO group were 0.098±0.00 μm, 0.104±0.00 μm, 6.744±0.06 per mm; and those of the HBO+AMG group were 0.099±0.00 μm, 0.107±0.00 μm, and 6.668±0.15 per mm respectively. Evaluation of the AMG group revealed 0.097±0.00 μm of trabecular thickness, 0.110±0.00 μm of separation and finally 6.668±0.15 per mm of trabecular numbers.
Comparing trabecular thickness, separation and numbers of treatment groups to control group revealed the following results. Trabecular thickness and numbers significantly decreased with AMG treatment (P<0.05, P<0.005 respectively), while trabecular separation significantly increased with AMG and HBO+AMG treatment (P<0.005, and P<0.05 respectively) (Figures 18-21).

Figure 18: This bar graph represents mean trabecular thickness of control, HBO, HBO+AMG and AMG groups. The only significant difference was between control and AMG group, indicated with an orange bracket.
Figure 19: This picture demonstrates the mid-sagittal section of a 3D-reconstructed femoral head. Arrows indicate trabecular separation of AMG group (A) and control group (B).
Figure 20: This bar graph demonstrates mean trabecular separation of control, HBO, HBO+AMG and AMG groups. As indicated by solid and dashed lines, there was a significant difference between HBO+AMG and AMG versus the control group.

![Bar graph showing mean trabecular separation](image)

Figure 21: This bar graph represents mean trabecular numbers of control, HBO, HBO+AMG, and AMG groups. Solid bracket indicate significant difference

Bone surface to volume ratio of the control group was 1.87±0.67; those of the HBO, HBO+AMG, and AMG groups were 2.07±0.37, 2.08±0.66, 2.11±0.37, respectively. Although we did not find significant difference among treatment groups per se, HBO+AMG and AMG groups showed significant changes of bone surface to volume ratio when compared with the control group (Figure 22).
4.1.2  Epiphyseal calculation

Epiphyseal area, area C, (referring to figure 12) was assessed via micro CT scanner. Due to the fact that some of the femoral heads did not have trabecular bone in the epiphyseal area, we could not assess trabecular parameters and bone surface to volume ratio; thereby, only fractional bone volume was measured. Analysis of data did not show any significant difference of epiphyseal FBV among the groups (Figure 23-A). Sub-analysis of FBV in less than 50% ossification SHRs also did not reveal any significant difference among groups (Figure 23-B).
Figure 23: Mean FBV of epiphyseal area. A: depicts FBV of epiphyseal area among all samples. B: sub-analysis of FBV among the samples with less than 50% ossification.
4.2 **Histology:**

One of the control femoral heads was lost during preparation for histology; thus, we evaluated the histology of only 19 control femoral heads, even though we still had all 16 samples per treatment group.

In the control group, we detected six with no ossification and ON (three ON- Figure 24), 11 with less than 50 percent and 2 with more than 50 percent ossification (Figure 25-A). The breakdown in the HBO group was: 7 with no ossification and ON (4 ON), 6 with less than 50 percent ossification and 3 with more than 50 percent ossification (Figure 25-B). Those of the HBO+ AMG comprised: 3 with ON and no ossification (two ON, Figure 26), 9 with less than 50 percent ossification, and 4 with more than 50 percent ossification. The AMG group contained only one with no ossification and ON (Figure 27-A), 14 with less than 50 percent ossification and one with more than 50 percent ossification (Figure 27-B).

Comparing the rate of no ossification/ON to other histological findings, we found significant differences between AMG and HBO group (P<0.01) and no other significant difference between other groups or between the control and treatment groups.

Also, a rate of less than 50% ossification was significantly different only between HBO (37.5%) and AMG group (87.5%) (p <0.001) (Figures 28, 29).
Figure 24: H&E-stained control SHR femoral heads. A: shows the area of ON in the epiphyseal region (arrow); note the disorganised bone stroma with empty lacunae. B: also represents another osteonecrotic control femoral head (arrow), shown by the lack of hematopoietic cells in the bone cavity and empty lacunae. Scale bar represents 250 µm.
Figure 25: A: This picture represents 50% or more ossification of one of the control SHRs. B: more than 50% ossification of one of the HBO-treated femoral heads. Scale bar represents 1000 µm.

Figure 26: This picture represents ON of HBO+AMG treated SHR. The picture at the top right shows 100x magnification of the osteonecrotic area.
Figure 27: Histological findings of AMG-treated SHRs. A: The only ON in this group. B: more than 50 percent ossification (two arrows showing the boundaries of ossification region). Scale bar represents 1000 µm.

Figure 28: Histological findings among the groups. Numbers represent the fraction of each histological finding in each group. Brackets indicate significant differences.
Figure 29: This bar graph demonstrates the ossification rates among different groups; brackets indicate significant differences.

4.2.1 Growth plate changes

By examining the growth plate we also noticed changes in the growth plate, which was consistent with coagulative and liquefactive necrosis of the germinal layer, as well as chondromucinous (CM) changes. In attempt to probe this finding, we divided the area of necrosis into two groups: one group when the changes were limited to the zone of proliferation and maturation, and another group with more severe damages extending into
the zone of calcification and ossification (Figure 30 and 31). The breakdowns of this
classification among treatment groups are summarized in figures 32 and 33. The differences
between the control and AMG group as well as the HBO and AMG group were statistically
significant (P<0.001).

Figure 30: process of normal growth plate proliferation and maturation (adapted from
http://www.ouhsc.edu/histology/text%20sections/bone.html)

Figure 31: A: This picture represents chondromucinous changes of the growth plate of a
control SHR. Downward arrows indicate changes limited to growth plate, and upward
arrow indicates extension of these changes into the epiphyseal area. B: another example in control SHRs. Scale bar represents 250 µm.

Figure 32: This bar graph represents the breakdown of growth plate CM changes in each group. Brackets indicate statistically significant differences. Note that CM changes in AMG and HBO+AMG group were significantly higher than in the HBO and control groups.
Figure 33: Chart represents grade of growth plate CM changes among groups. Note that all AMG treated group had highest rate of severe growth plate changes following by HBO+AMG also the least damaged growth plate belong to HBO treated SHRs.

4.2.2 Ossification disruption of AMG group

Another coincidental finding of our study was the correlation between growth plate changes and epiphyseal ossification progress in AMG-treated rats. Of 14 AMG-treated SHRs with less than 50% ossification, half of them demonstrated the following observation. Ossification was impeded at an area of grade four growth plate damage (Figure 34).
Figure 34: Growth plate CM changes A: Ossification halts at the area of grade 4 growth plate CM changes in AMG-treated SHR (downward arrow). B: another AMG-treated SHR with ossification disruption at the region of grade 4 growth plate CM changes (right arrow).

4.3 Immunohistochemistry

NOS antibody immunostaining of the selected samples from each group clearly disclosed the relation between inducible nitric oxide synthase enzyme and the zone of ON as well as the zone of growth plate changes, while endothelial nitric oxide synthase enzyme distribution failed to represent this portrait (Figures 35-37).
Figure 35: Figure A represents iNOS activities in the area of ON in HBO-treated SHR (arrow shows iNOS, and triangles indicate iNOS activity at region of CM changes of growth plate). Figure B represents minimal eNOS activities in the same HBO-treated SHR, (arrow indicates nil eNOS activity in the area of ON and triangle also indicates minimal eNOS activities in growth plate CM changes area).
Figure 36: Figure A indicates high iNOS activity in the area of ON in AMG-treated SHR (arrow). B: clearly depicts minimal, if any, activity of eNOS in the same area of ON (arrow).

Figure 37: This iNOS stained section illustrates high iNOS concentration in area of chondromucinous changes of the growth plate in one of the HBO-treated rats (arrows).
4.4 Apoptosis:

To perform epiphyseal apoptosis assessment, we had to exclude the samples with no ossification in this area. Therefore this analysis performed on ten controls, nine AMG, nine HBO and ten HBO+AMG femoral heads meeting the inclusion criteria.

The Hoechst stained sections (Figures 38, 39) were analysed under fluorescence microscope. Epiphyseal osteocyte apoptosis in the control group was 19.1± 4.9 per 1.86 mm$^2$ and that of AMG was 9.6±6.0 per 1.86 mm$^2$ (P<0.005). Also, the difference between AMG and HBO (19.7±12.1 per 1.86 mm$^2$) was statistically significant (P<0.05). There was no difference among the rest of the groups (HBO+AMG: 15.1±12.2 per 1.86 mm$^2$) (Figure 40).

Figure 38: Figure A represents Hoechst staining of osteonecrotic area of a control sample, with arrows indicating some of the dense nucleus. Figure B represents another area of the same sample with arrows indicating some of the dense nucleus.
Figure 39: This Hoechst stained section reveals the difference between normal osteocytes (triangles) and apoptotic osteocyte (arrow) nucleus in one of the HBO-treated SHRs.
Figure 40: This figure depicts the 95% confidence interval of mean apoptosis among groups. Brackets indicate significant differences.
The presence of apoptosis was also confirmed with TUNEL assessment of selected samples (Figures 41, 42), as these two techniques have been used previously in conjunction.

Figure 41: This picture represents a TUNEL stained section of the control group; figure A represents DAPI and figure B represents FITC staining. Arrows indicate some of the apoptotic osteocytes. Note the higher level of green fluorescence of necrotic cells (arrow heads) in figure B in comparison with apoptotic osteocytes (arrows).

Figure 42: Another TUNEL stained section of the control group, with figure A representing DAPI and figure B FITC staining. Arrows indicate some of the apoptotic osteocytes.
Chapter 5: Discussion

5.1 Apoptosis

Our results supported the role of iNOS in the process of osteocyte apoptosis of SHRs. Although the high presence of iNOS and its association with osteocyte apoptosis in ON process has been an area of attention recently (1, 15), to the best of our knowledge this is the first study to explore the role of AMG (iNOS suppressor) in the prevention of ON.

Our data clearly document the concentration of inducible nitric oxide synthase enzyme in the area of ON; however, our immunostaining technique was not a quantitative measurement, so we are unable to quantify the correlation of iNOS suppression and rate of ON or apoptosis. However, AMG treatment decreased the rate of apoptosis significantly; thus, we contemplate that it is related to the suppression of iNOS based on the presence of iNOS in the area of ON.

To better understand the pivotal role of NO concentration in process of apoptosis, it is crucial to review the apoptosis cascade. Apoptosis, or programmed cell death, is defined as “the orchestrated collapse of a cell, staging membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of corpses by neighbouring cells” by Thompson et al (75). This process is initiated in cells through the tumour necrosis factor family receptors like TNFR1, the receptor for TRADD, and the CD95/Fas/Apo-1 complex. This ligand binding trimerized intracellular molecules (FADD, TRADD) to form the death-inducing signalling complex, which is the key to activate the first reaction of the apoptosis cascade (caspase 8). Afterward, caspase 8 initiates the chain reaction that causes cell apoptosis (Figure 43) through the CAD complex (caspase-activated DNase) (62). First indications of NO-induced apoptosis were reported back in 1993 (107, 108).
Figure 43: This figure summarises the apoptosis cascade and the possible anti-apoptotic effect of NO in this process (marked with red arrows). Note the DISC complex at the top of the cascade and then its activating role on caspase 8 the initiator of apoptosis cascade. CAD, caspase-activated DNase; DISC, death inducing signaling complex; ICAD, inhibitor of CAD; HSP70, heat shock protein 70; TNFR, tumor necrosis factor receptor. Adapted from Chung (62).
NO is synthetised by an enzyme called Nitric Oxide Synthase (NOS). Among three types of NOS, two types are constitutive: endothelial (eNOS) and neuronal (nNOS), which are generally activated through intra-cytosolic calcium release and inflict a transient effect. In contrast, the inducible form (iNOS) is expressed after an immunological or inflammatory reaction by many cells (like macrophages) and lingers in the area for several days. (70, 71)

NOS’s final product, NO, is a diffusible and multifunctional messenger with numerous physiologic and pathologic implications with a couple of other reactive end products like NOx, peroxynitrite (ONOO⁻), and metal–NO. NO’s biological effect can be divided into two broad categories: cGMP-dependent and cGMP-independent pathways (7273) with cGMP and concomitant intracellular phosphorylation being the main pathway (74). The pro-apoptotic effect of NO is cGMP-independent (62)

This pro-apoptotic effect came to researchers’ attention only after the following observations: the high concentration of NO-induced apoptosis in macrophages (76), some neurons (7776), tumour cells (78), and pancreatic islands (79). Also, it causes cytotoxicity in tumour cells and surrounding tissues (80). Subsequently, NO-mediated apoptosis was investigated further and it was found that it is associated with redox state and transition metal complex activities (81) as well as its interaction with the survival gene (82). Thus, it is obvious that the apoptosis threshold of NO would be cell-dependent and varies from one to the other.

In general, pro-apoptosis action of NO can be divided into four categories (figure 44):

- Direct Activation of Mitochondrial Apoptotic Pathway
- Activation of Caspase Signaling Pathway by NO-Induced p53 Expression
- Activation of JNK/SAPK and p38 Kinase
- Apoptosis by NO-Mediated Ceramide Generation
In the first path (direct mitochondrial), NO directly activates cytochrome c and subsequently the apoptosis cascade. In this path, NO interacts with the electron-transferring chain and produces peroxynitrite, which causes mitochondrial dysfunction (83). This is the same agent responsible for neural and tumour cell apoptosis (84, 85, respectively). An interesting point about peroxynitrite-induced apoptosis is that it does not induce apoptosis in normal healthy human endothelial and mononuclear cells (85). Therefore, its effect depends on the level of intracellular redox like iron, glutathione, and superoxide (62).

Messmer et al (86) studied the cytotoxic effect of NO on tumour cells and found that the damaged tumour cell had an accumulation of p53 in the DNA. This observation led to discovery of the second pathway. In this path, p53 accumulation increases the Bax/BCL-xL ratio (pro- and anti-apoptotic respectively), inducing Cyt c release and caspase activation.
Figure 44: This diagram summarises the four NO pro-apoptotic pathways. Adapted from Chung (62). Cyt c, cytochrome c; GSNO, S-nitrosoglutathione; PKG, protein kinase G; SNP, sodium nitroprusside.

“NO activates the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) group of mitogen-activated protein kinases (MAPKs)” (62) which is the third pathway of NO-induced apoptosis. Interestingly, blockage of the MAPK complex in RAW264.7 cells
prevented SNP-mediated apoptosis, indicating the crucial role of JNK/SAPK and p38 MAPK on NO-mediated apoptosis and caspase 3 activation (87, 88). It appears that caspase 3 is the key modulator of this pathway.

The final NO-mediated apoptosis pathway is through ceramide activity. It has been observed that NO donor increases ceramide activity of HL-60 and mesangial cells (89, 90). Ceramide itself is a potent initiator of several apoptosis signalling like: Cyc c activation, caspase 9 and 3 activation, JNK/SAPK activation (88), suppression of Bcl-2 (92), and inhibition of protein kinase B/Akt (93).

As mentioned before, NO also is an anti-apoptotic agent; it can prevent oxidative stress (94) and glucose deprivation-induced (95) apoptosis. This effect mainly depends on the particular cell behaviour and also the concentration of NO. This anti-apoptotic effect can be divided into three categories:

- Inhibition of Apoptotic Signalling by NO/cGMP Pathway
- Inhibition of Caspase Activity by S-Nitrosylation
- Regulation of Antiapoptosis-Related Gene Expression by NO

It was shown that NO prevented apoptosis of hepatocytes (96), some neuronal cells (95), B lymphocytes (97), and eosinophils (98); nevertheless; another study revealed that inhibiting soluble guanylate cyclase halted this effect of NO (99). Thus the cGMP-dependent and cGMP-independent anti-apoptotic effects of NO became clear to researchers. The dependent action of NO is mainly due to cytochrome c release (94), ceramide generation (100), and caspase activation (96), while the independent effect of NO is prominently on expression
levels of Bcl-2 (97) and the activation of Akt/PKB (101) causing Bad phosphorylation which is an anti-apoptotic protein (62).

NO can directly inhibit the caspase protein family, a family of proteins consisting of 14 isoforms with a cysteine at the catalytic site. This site is susceptible to redox modification; therefore, it can be inactivated S-nitrosylation by NO and its side products (102). In vivo studies support this inactivating effect of NO on caspase 3 and caspase 8 (81, 103). This effect of NO is dependent on electron-accepting molecules like oxygen, iron, and copper. NO is a weak chemical agent and only after reacting with this redox agent can it become a potent agent, NO⁺ (104).

The final pathway is related to the inductive role of NO on heme-oxygenase proteins like HSP 70 and HSP 32. After induction by NO, these two proteins protect hepatocytes from TNFα-induced apoptosis (82, 94). The exact mechanism behind this pathway is still unclear, though there are two possibilities. HSP 70 may hinder Apa-f-1 oligomerisation causing aptosome formation suppression (105), or it may import a precursor protein into mitochondria to hinder Cyt c action (106).

Apoptosis of osteocytes and osteoblasts has been reported in the ON process of human and different animals, namely SHRs (1, 4, 10, 15). Our study clearly confirmed a high degree of osteocyte apoptosis in the control SHRs, in keeping with Shibahara’s findings (10); meanwhile, AMG treatment significantly decreased this process in SHRs. Surprisingly, we did not observe apoptosis reduction with HBO and HBO+AMG treatment. Given that Rachmilewitz (39) et al. found that HBO reduced NOS activities in their experimental colitis model, we expected to observe some preventive role of HBO per se and in combination with AMG treatment through suppressing NOS and subsequently apoptosis. However, our experiment failed to prove it. Maybe HBO activated some pro-apoptotic pathways or even
prevented the anti-apoptotic effect of NO through one of the mentioned pathways. Also, given that HBO increases dissolved oxygen tension in blood (at 2.4 ATA dissolved oxygen increase from 0.32 volume % to 4.8-5.7 volume %) (4), it is possible that this newly available oxygen acts like a booster for the redox status of the epiphyseal cells and increases apoptosis. However, our HBO treated rats did not show significant enhancement of apoptosis rates compared with the control group, but they were significantly higher than the AMG-suppressed group.

It has been established that the weak area of hypertrophic cartilage on the grounds of insufficient ossification prepared a recipe to compress and obstruct the vulnerable vessels entering the physis area of SHRs (15). In this regard, the protective effect of non-weight bearing and caloric restriction (and consequently lower body weight) certifies this theory (5, 15). Following obstruction/stenosis of the vessels, oxygen deprivation will modify expression of some of the stress proteins, including metalloenzymes like nitric oxide synthase (NOS). Of the three forms of NOS, inducible NOS is expressed by macrophages, neutrophils, endothelial cells, hepatocytes, cardiac myocytes, chondrocytes, and many other cell types under the influence of cytokines.

Given that the antiapoptotic action of AMG (iNOS suppressor) in the study of Bcl2/Bax expression has been well-documented (31) and that another study supports the pro-apoptotic action of iNOS (34), we hypothesised that suppressing iNOS in ON could be also a potential innovative treatment for SHRs’ femoral head ON. From those studies we believed that iNOS action in the pathology of SHRs is through the second pro-apoptotic pathway: “Activation of Caspase Signaling Pathway by NO-Induced p53 Expression,” which is the only mechanism involving Bcl-2/Bax expression alterations. Having said that, the fourth pathway, “Apoptosis by NO-Mediated Ceramide Generation,” also indirectly interacts with Bcl-2 family, so it might be another possible involved mechanism.
5.2 Osteonecrosis of SHRs

In our study we used only male SHRs to make sure we would not have any issue with breeding of the study’s animals. This is a well-known strategy implemented in many animal-based studies. Our findings failed to prove that AMG could prevent ON at the histological level when compared with the control group (P<0.06); however, it did significantly improve ossification compared with HBO treatment.

Shibahara (10) in his experiment with ten SHRs reported no ossification in the epiphyseal area at the fifth week of life; then, at the 15th week, two SHRs had normal ossification, five had abnormal ossification with ON, two ossification disturbance with ON, and one ossification disturbance without ON. This picture significantly changed at the 20th week; five of 10 had normal ossification and other five had ON either with ossification disturbance or abnormal ossification (50% with ON). In another study by Kataoka (7), the rate of ON was reported as high as 6 of 16 femoral heads (37%) at the 7th week of life, with another 4 having severe ossification disturbance. However, our control SHRs only developed three ON (19%) and another three showed severe ossification disturbance with no ossification at all. These findings may contribute to the fact that we failed to prove that AMG and HBO+AMG treatment could prevent ON at the histology level.

Another justification for our findings could be the fact that osteocytes’ apoptosis is part of ON process in SHRs; nevertheless, it is not the only process involved. So to prevent ON in this model, something more substantial is required than merely suppressing osteocytes’ apoptosis.
Given the low rate of ON in our control group, we will be cautious to declare that this treatment is not definitely effective on ON. A more reliable and predictable animal model of ON would be useful to make a strong conclusion in this regard.

We did not start any treatment for the first 5 weeks of life as previous studies indicate that SHRs do not develop any ossification by that age; only after they start to step into the realm of ossification issues (10). However, that statement is based on histological findings and none of the previous studies looked into the exact commencement of osteocyte apoptosis in SHRs. Hence, there is a possibility that apoptosis was already started at the fifth week of life.

In addition, our experience with HBO treatment was not as successful as previously described (5-7). Despite some changes at the micro CT level, our histological and osteocyte apoptosis findings fail to confirm its preventing effect on the treatment of SHRs. Kataoka (7) used 2.8 ATA (equal to 280 kPa, each 1 Atm=101325 pascal) HBO on their rats for 60 minutes, 5 days a week and total of 30 hours. Thus, SHRs received HBO 5 days a week for total of 6 weeks. Their decompression process took 30 minutes as they gradually decreased the pressure. Taking this into account may increase the time animals were exposed to HBO to even more than 30 hours. We did not use their protocol as our chamber safety gauge was set as 270 kPa. Instead, we implemented Peskin’s (5) methods by using 250 kPa, for 90 minutes, 6 days a week for total of 30 days. In his study on surgically-induced ON, 16 HBO-treated SHRs were divided into two groups: one group of 8 received 32 sessions and another eight received 22 sessions. Therefore, 8 of their samples were exposed to a total of 48 hours and another eight to 33 hours. They realised that adding another 10 sessions did not help with the prevention of ON as at the end of 22 sessions they had 50% of grade 2 or more residual necrotic area of femoral head as opposed to 66% of control group which amended to 37.5% by adding another 10 sessions. They mentioned that HBO treatment brought a favourable outcome by preserving femoral head geometry; however, looking into their data showed that
the statement was on a statistically non-significant trend (P<0.08). Interestingly, they mentioned the possible detrimental effect of high dose of HBO on the overactivity of osteoclasts, which can lead to a collagen-rich repair process that is not strong enough to carry rats’ weight and leads to femoral head collapse. In our study, SHRs were exposed to a total of 45 hours. Also in our practice, the decompression process only took 5 to 6 minutes, which meant that rats were exposed to 250 kPa for a total of almost 90 minutes. Considering that Kataoka’s method 7) of measuring ossification rate and trabecular density was quite different from our advanced micro CT method and it was only based on a single mid-sagittal histological section, it is difficult to blame our HBO findings on the amount and interval of exposure to HBO. However, it is impossible to rule it out at the same time. We believe a comparison study of these two HBO regimens can draw a stronger conclusion in this regard.

5.3 Controversial micro CT findings

Our micro CT scan findings showed that AMG and HBO+AMG treatments could have a detrimental effect on bone morphology of metaphyseal area. However, analysis of epiphyseal region, as the main area of interest of our study, did not reveal any significant changes in terms of bone morphology and even revealed an improvement trend of FBV of the epiphyseal area with HBO, HBO+AMG and AMG treatment which was our area of interest with micro CT evaluation (Figure 23-B). Kataoka (7) reported a significant difference between SHRs’ and Wistar Kyoto rats’ trabecular density as well as a significant difference of this density after HBO treatment from age 5 to 11 weeks compared to the control group. Their experiment did not show any meaningful changes of trabecular density when SHRs received HBO at 8 to 14 weeks (which was another reason we used HBO from 5 to 10 weeks of age). Figure 45
depicts Kataoka’s method to calculate trabecular density based on H&E and toluidine blue-stained histological sections (as briefly mentioned in section 5.2). Though it was state of the art in 1992, obviously it is not as accurate as the current micro CT methods. The same statement applies to the method they used to assess the ossification rate.

Yamako et al (26) evaluated the effect of surgically-induced traumatic ON on Wistar rats’ metaphyseal bone morphology using micro CT scan. At day 7 of post-ON induction when ON was quite established, micro CT measurement revealed a significant reduction of FBV of the ON group compared to the control group. In addition, trabecular thickness and number also significantly decreased at day 7, and trabecular separation increased (P<0.05). All of those figures returned to almost normal by day 42 when the reparative process was accomplished. The mechanism of traumatic-induced osteonecrosis (as elaborated before, 15) is different from the idiopathic process of SHRs; therefore, we believe our metaphyseal findings are not entirely comparable to their findings.

Han et al (109) used a P-glycoprotein inducer, Rifampicin, to prevent ON in a steroid-induced rat model of ON. The rationale behind this treatment was that P-glycoprotein could neutralize the adipocytogenic effect of steroid on bone marrow cells and Rifampicin as a potent enhancer of P-glycoprotein could boost this effect. They proved that these rats significantly developed less ON comparing to their control group. In their study, they implemented Parfitt’s plate model (110) to reconstruct 3D structural parameters of the epiphyseal area based on two-dimensional histology pictures. Their epiphyseal findings revealed an increase in trabecular thickness and number and a reduction of trabecular separation. In addition, Rifampicin treatment also decreased epiphyseal cell apoptosis, though they did not comment which cells were included in the TUNEL assessment.
As mentioned earlier, one of the main pathologies of steroid-induced ON is osteocyte apoptosis. Therefore, comparison of our findings with Hans’s findings concerns us with regard to the detrimental effect of AMG alone and in combination with HBO on metaphyseal trabecular morphology. However, their measurement technique (plate model opposed to direct calculation model) has been an area of controversy (111) as it caused volume-dependent bias in the measurement of trabecular thickness and separation. Moreover, our trabecular findings were on the metaphyseal region; we did not calculate epiphyseal trabecular bone morphology, as we believed the samples with no ossification would cause bias on such findings.

Figure 45: This diagram depicts the implemented method to calculate ossification rate and trabecular density based on H&E and toluidine blue stained mid-sagittal section by Kataoka-7

5.3.1 Micro CT, Metaphyseal changes
After reviewing the data and noticing no ossification of some of samples, we had to make a plan to measure epiphyseal bone formation. We decided to calculate total bone formation of the femoral head, and then calculated metaphyseal bone formation to be able to calculate the epiphyseal area. In order to perform that, we used the method described earlier to calculate area C. Murata et al (11), using monoclonal antibody (anti-4HNE and anti-8OHdG), confirmed oxidative stress changes of epiphysis and metaphysis microcirculation of corticosteroid-induced rat’s femoral head ON, though the ON was not detectable at the histology level in the metaphyseal area. Calder et al (20) also reported the extent of ON of the femoral head beyond femoral head up to 4 cm below the lesser trochanteric area in patients with ON undergoing total hip arthroplasty. This is endorsed with our micro CT findings in SHRs. Thus, we were not astonished to detect bone morphology changes in the metaphyseal area with our treatment.

5.4 Growth plate changes

The CM changes of the growth plate (Figures 31, 37) had been reported in different breeds of rats with as high as 50% incidence (28). Although the process is closely related to aging of the rats, the exact pathology is uncertain (28). These changes were observed in SHR rats but their exact correlation to ON is yet to be defined.

Some authors have suggested that these changes are age-related and reparative, which are normal in the aging process. Also, the role of blood supply to the growth plate and possibility of thrombosis-induced CM changes have also been postulated (30). However, to the best of our knowledge, the high concentration of inducible nitric oxide synthase in this area has not been reported before. Whether this growth change has a role in the process of ON remains unclear, though it seems that they have a more or less reparative-protective effect, as the
iNOS treated group showed the highest degree of growth plate changes on the ground of better ossification and less ON.

5.5 Ossification disruption of AMG-treated rats

We also noticed that in AMG-treated rats, the area of normal ossification stops at a decent-sized growth plate CM spot (Figures 31, 34) with evidence of iNOS activity in those regions (Figures 37). It is possible to justify this finding, as AMG treatment improved the ossification ratio of the SHRs, but this ossification process reached the critical point of blood supply challenge as described before. Then, the adjacency to the maximal ossification region—inhernently a weak spot to keep up with blood flow—could not cope with the rate of ossification and collapsed (CM changes), halting further ossification. During this interaction, inflammatory cells were activated; despite AMG suppression, iNOS still acted in the vicinity of CM change.

Another way to look at this picture is to suggest that AMG aggravated CM changes. However, the fact that all of the AMG-treated group developed at least less than 50% ossification (as opposed to three cases of no ossification in each control and HBO group) makes it harder to believe the current justification.

5.6 Pivotal effect of AMG

In our study, we decided to use aminoguanidin to suppress iNOS. Previously (16), 100 mg/kg of this agent was applied intraperitoneally twice a day in a rat model of experimental colitis, which successfully suppressed iNOS in this animal model of inflammatory bowel disease. Various doses of AMG have been used for different treatment strategies in the past. Dosages ranging from 1.5 µmol/kg each day in the drinking water of Wistar rats (26) to
1mg/ml (35) and as high as 4mg/ml have all been used (31). Mohamad et al (31) found that the dosages of 2mg/ml and 4mg/ml were significantly more effective in prolonging tumour doubling time as opposed to the control and 1-mg/ml groups. However, van't Hof findings (32) indicated an essential role of iNOS in IL-1-induced bone resorption; this made us cautious about using a high dose of AMG, which could potentially cause a deleterious effect on the reparative action of osteoclasts. It probably did to some extent in our series, owing to the fact that it deteriorated metaphyseal bone morphology. Whether using a different dose of AMG would result in improved ossification, fewer growth plate CM changes, better metaphyseal trabecular bone morphology and more ossification is a question that needs further investigation.
5.7 Conclusion

Our histological and epiphyseal micro CT findings failed to prove that any of our treatments could prevent ON process of SHRs. We confirmed that AMG suppression of iNOS significantly reduced osteocyte apoptosis. The presence of high iNOS concentrations at the region of ON supports our hypothesis that iNOS plays a pro-apoptotic role in the process of osteocyte apoptosis in the SHR model of ON.

Also, the observed reproving trabecular morphology changes with AMG and HBO+AMG treatments raise some concerns about the possible detrimental effect of AMG on metaphyseal trabecular bone morphology. Despite an improving trend of FBV in the epiphyseal area with all of our treatments, none of those were statistically significant.
5.8 *Future study*

Weinstein (22) et al looked into the deleterious effect of steroid-induced apoptosis in ON. They were so convinced about the process that they stated this term (steroid-induced ON) was a misnomer, and the disease should be named glucocorticoid-induced osteocyte apoptosis. Given those findings and our results, we believe the iNOS suppressor (AMG) may show an even more outstanding protective measure against corticosteroid-induced ON. This will definitely be another area of potential future study.

As our micro CT and apoptosis findings did not keep up together and they were controversial, we believe it is worth assessing the exact action of different doses of AMG on osteocyte apoptosis, and the exact pathway they implement to affect apoptosis. In the meantime, measuring osteoclast activity can also shed some light about the possible detrimental effect of AMG or even HBO on resorption capability of the bone, and it may explain AMG-induced metaphyseal trabecular bone morphology changes.
Chapter 6: References


Animal monitoring sheath

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*Any animals showing altered behaviours, self harm or significant weight loss should be isolated & details completed in the welfare sheet.*