Pertanika J. Sci. & Technol. 25 (1): 359 - 370 (2017)

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

# One-Time Prolonged Ultrasound Exposure during Early Pregnancy Affects Bone Strength in Young Aged *Oryctolagus Cuniculus*

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## ABSTRACT

The use of prenatal ultrasound has become controversial as it is increasingly being performed for business and social interests rather than for medical use. This nonmedical use of the modality has violated the US FDA guideline. Ultrasound scans have been proven to increase temperature in insonated tissue and their effects have been investigated via phantom and animal experiments. Absorption coefficient of the bone is the highest compared with any other structure. Thus, exposure to ultrasound, especially during osteogenesis, can cause significant damage to developing foetus. Twenty-two pregnant does of known gestation were enrolled in the control and experimental groups. No exposure was given to the control group while the experimental groups were exposed accordingly to the prenatal ultrasound in the 1st, 2nd and 3rd stage for 30, 60 and 90 minutes respectively. A total of 142 subjects aged between 1 and 5 months were analysed for bone strength. The Tb.Th of the experimental group was reduced significantly as compared to the control group. Po, TMD and empty lacunae were higher in the experimental group. It is thus concluded that one-time prenatal ultrasound can affect bone strength in young subjects.

*Keywords:* Bone histology, bone morphology, bone strength, prenatal ultrasound, tissue mineral density, young age

## INTRODUCTION

In recent times, it has become a trend for expectant ladies to go for ultrasound scans even during normal pregnancy to fullfil their social needs. Scans make them feel reassured about their pregnancy and knowing sex of their child-to-be gives them satisfaction (Lumley, 1990; Beck Black, 1992; Molander et al., 2010). The images and videos are kept as mementoes. Modern ultrasound imaging for diagnostic purposes has a wide range of

*Article history:*
Received: 16 June 2016
Accepted: 20 December 2016

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ISSN: 0128-7680 © 2017 Universiti Putra Malaysia Press.

applications. However, private doctors often recommend parents to perform scans to boost their healthcare business purpose. Hence, most pregnant women undergo up to 20 scans per pregnancy (Bashour et al., 2005; Gammeltoft & Nguyen, 2007).

According to The United States Food and Drug Administration (US FDA), diagnostic ultrasound for pregnancy is considered safe when it is performed for valid medical reasons whereby its benefits outweigh risks. The FDA prohibits any institutions to sell, promote or apply the ultrasound to make keepsake images or videos (US Food and Drug Administration, 2013). Several laboratory studies have proven that ultrasound exposure during pregnancy can have adverse effects on the tissue. Energy is deposited in the tissue by ultrasound absorption and thus diagnostic ultrasound cannot be considered completely safe. The effects of rising temperature during ultrasound scanning in animal model are well established, in particular, its effects on birth weight, organ weight, brain and bone. Vella et al. (2003) and Wu et al. (1995) have exposed phantoms that mimic biological tissue in diagnostic ultrasound. They found that ultrasound exposure can lead to temperature increase, particularly in the bone. Bone is sensitive to heat as it has high absorption coefficient (10 dB/cm/MHz) (Barnett et al., 1997) which is 30 times higher than any other structure.

According to Gent ( 1997), an adult bone is able to absorb 60% or more of ultrasound energy while the absorption is less in foetal bone. However, the absorption coefficient of the foetal bone differs depending on gestational age where there are changes in heat capacity, mineralisation and density (Drewniak et al., 1989; Shankar & Pagel, 2011). Ultrasound intensity and duration of exposure are two main parameters that lead to a linear rise in tissue temperature. In addition, beam angle to the bone, location of transducer, and the orientation of thermocouples in the tissue are able to indirectly increase the tissue temperature.

Our aim in this study was to investigate the effects of prolonged ultrasound exposure during pregnancy on bone status in young rabbits.

## MATERIALS AND METHODS

Prior to the investigation, all procedures performed on the animals were approved by Universiti Teknologi MARA Animal Research and Ethics (UiTM CARE) Committee. Female (doe) and male (buck) Malaysian breed New Zealand white rabbits (*Oryctolagus cuniculus)* aged between five to eight months were enrolled in this study. In order to allow the use of 22 pregnant does of known gestation, the rabbits were time-mated. Four does were used as control and 18 were assigned in experimental groups. The control group was allowed to have full term delivery without exposure to the ultrasound while the experimental group was given a one-time exposure to ultrasound. The full term gestational period for a doe is between 30 and 33 days (Kaplan & Timmons, 1979) and divided into three stages namely as 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ gestational periods. The rabbits were given ultrasound exposure of 30, 60 and 90 minutes respectively using a 2-D B-mode Philips HD3 ultrasound system with a 5-9 MHz broadband high-resolution linear array transducer (L9-5, Philips Electronics E.V., Germany). The transducer had a focal depth of approximately 5.5 cm, and directed to where the foetuses were located. During the exposure, the recorded mechanical index (MI) and thermal index (TI) were 1.0 and 0.2 respectively. The output power and spatial peak temporal average ($I_{SPTA}$) were varied from 0.4 W to 0.7

W and 0.13 to 0.19 W/cm$^2$ respectively as calculated based on the previous characterisation of the transducer (Ahmad Zaiki et al., 2013). The exposure factors remained constant for all exposures. 'My Rabbit Burrow,' a rabbit restrainer designed by Dom (Md. Dom et al., 2012) was utilised to keep the does calm and cooperative during the scanning.

The offsprings were taken as a subject when they reached 1 and 5 month old. All rabbits (bucks, does and offsprings) were kept under the same laboratory environment. They were given ad lib water supply, and pelleted feed which was measured at 5 % of their body weight. The animal house was set for 16 hours: 8-hour light and dark cycle with temperature between 14ºC – 28°C (Matics et al., 2013). BioGS air purifier was utilised to provide proper ventilation from the harmful gases released by the rabbits such as carbon dioxide and ammonia (Lebas, 1997). A total of 142 subjects were analysed for bone strength.

The subjects were euthanised by administering a dose of ketamine hydrochloride (50 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight). Femoral bone was dissected using a scalpel. The Skyscan 1176 (Skyscan 1176, SkyScan bvba, Aartselaar, Belgium) was used to provide data on bone morphology in terms of trabecular thickness (Tb. Th), trabecular separation (Tb.Sp), tissue mineral density (TMD) and porosity (Po) (Che Isa et al., 2015). After micro-CT scanning, the femurs were processed for histological examination and subsequently decalcified using hydrochloric acid- (HCL) based decalcifying agent. The paraffin-embedded specimens were sectioned 5 µm (Smith et al., 2001) in a longitudinal orientation and stained with hematoxylin and eosin (H&E). The slide was observed for empty lacunae using a light microscope (Primo Star iLED™, Carl Zeiss Microimaging, Oberkochen, Germany). An illumination system of a commercial video-microscope (AM413T5 Dino-Lite Pro, AnMo Electronics Corporation, Hsinchu, Taiwan) was utilised to obtain microscopic pictures. The percentage of empty lacunae was calculated from the number of empty lacunae compared with the total number of lacunae counted in each slide.

Data from both the experimental group and control group were compared. Analysis of variance (ANOVA) was used to analyse the data using Statistical Package for the Social Sciences (SPSS) version 21.0. All differences were assumed statistically significant at $p \leq 0.05$.

## RESULT AND DISCUSSION

Morphological and histological data of 1 and 5 months subjects is presented in Table 1. Ultrasound exposure has the potential to raise temperatures in insonated tissues. As osteogenesis is very sensitive to heat, a temperature rise can lead to denaturation of the enzymatic and membrane proteins (Augustin et al., 2012), microcirculation blockage, bone tissue necrosis and activation of bone marrow macrophages (Yoshida et al., 2009). Prenatal ultrasound exposure in the 1$^{st}$ stage has caused Tb.Th to reduce significantly from the control ($p<0.05$) among 1 month subjects (Figure 1). However, in 5 months subjects, Tb.Th was reduced in the 2$^{nd}$ stage (Figure 2). In terms of Tb.Sp, no difference was noted in 1 month subjects (Figure 3) but in 5 months subjects, Tb.Sp increased in the 2$^{nd}$ stage after 90 minutes of exposure (Figure 4). Cortical bone region was likely affected by the ultrasound exposure. A significant increase in porosity was found in both 1 month (Figure 5) and 5 month subjects (Figure 6) especially in 1$^{st}$ and 2$^{nd}$ stage. In addition, 1 (Figure 7) and 5 month old subjects

(Figure 8) in the experimental group showed higher TMD compared with the control group. Dom et al. (2012) observed the heat-induced changes in rabbit foetal mineral density. The bone strength can be altered by poor status of the cortical bone and likely lead to impaired resistance to fractures.

Table 1
*In-house normal range for metaphyseal bone analysis based on control group data*

| Group | Parameters | ($\bar{x}$) | SD |
|---|---|---|---|
| 1 month old | Tb.Th (μm) | 1.44 | 0.2 |
| | Tb.Sp (μm) | 3.42 | 0.49 |
| | Po (%) | 0.83 | 0.16 |
| | TMD ($g.cm^{-3}$) | 16.42 | 3.01 |
| | EL (%) | 6.09 | 1.01 |
| 5 months old | Tb.Th (μm) | 2.00 | 0.15 |
| | Tb.Sp (μm) | 4.23 | 0.72 |
| | Po (%) | 0.06 | 0.02 |
| | TMD ($g.cm^{-3}$) | 40.16 | 7.84 |
| | EL (%) | 6.17 | 1.04 |

*Abbreviations:* $\bar{x}$, mean; SD, standard deviation; S.E.M, standard error mean; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Po, porosity; TMD, tissue mineral density; EL, empty lacuna

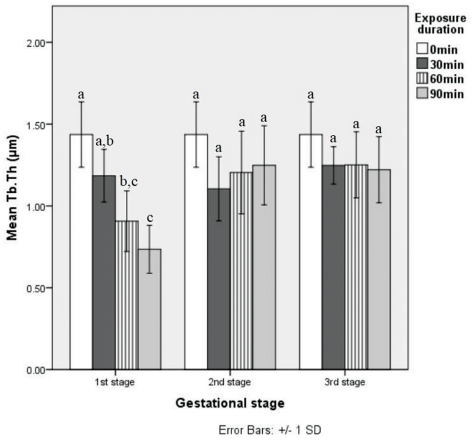


*Figure 1*. Graph of mean trabecular thickness (Tb.Th) of 1 month old subjects. Data in each stage sharing same alphabets is not significant ($p>0.05$)

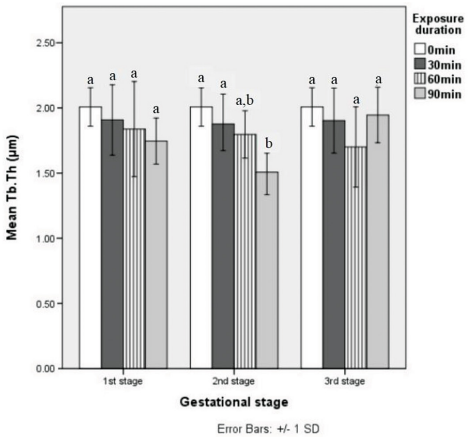


*Figure 2*. Graph of mean trabecular thickness (Tb.Th) of 5 month old subjects. Data in each stage sharing same alphabets is not significant (p>0.05)

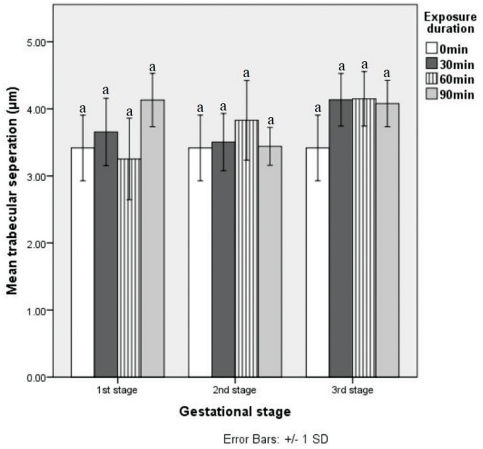


*Figure 3.* Graph of mean trabecular separation (Tb.Sp) of 1 month old subjects. Data in each stage sharing same alphabets is not significant (p>0.05)

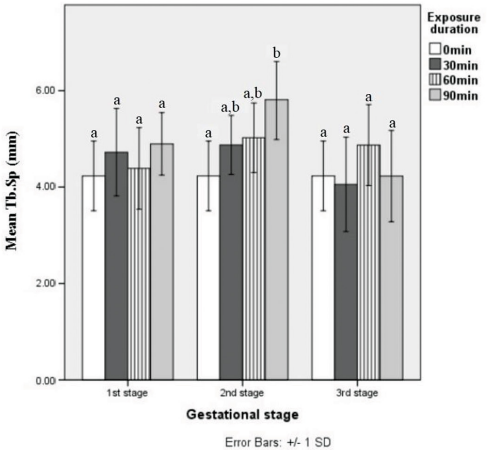


*Figure 4.* Graph of mean trabecular separation (Tb.Sp) of 5 month old subjects. Data in each stage sharing same alphabets is not significant (p>0.05)

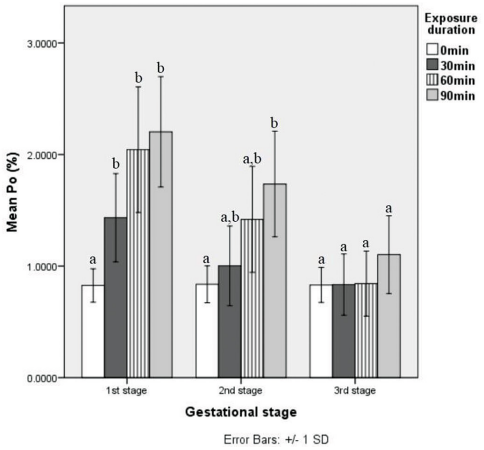


*Figure 5.* Graph of mean total porosity (Po) of 1 month old subjects. Data in each stage sharing same alphabets is not significant (p>0.05)

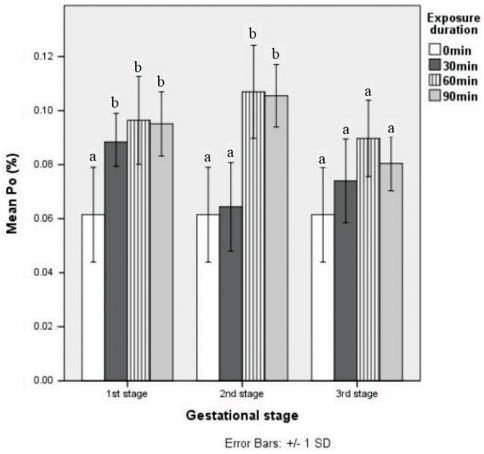


*Figure 6.* Graph of mean total porosity (Po) of 5 month old subjects. Data in each stage sharing same alphabets is not significant (p>0.05)

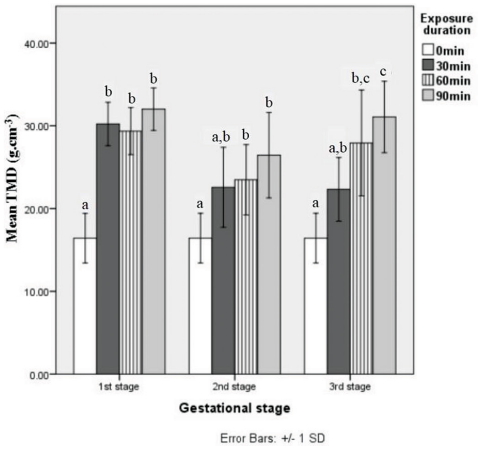


*Figure 7.* Graph of tissue mineral density (TMD) of 1 month old subjects. Data in each stage sharing same alphabets is not significant (p>0.05)

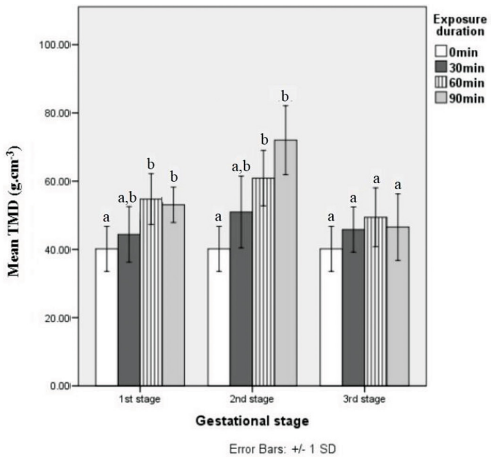


*Figure 8.* Graph of tissue mineral density (TMD) of 5 month old subjects. Data in each stage sharing same alphabets is not significant ($p>0.05$)

In order to determine the temperature rise expected in an ultrasound examination during pregnancy, it is important to consider bone density in the foetus. In this study, the formation of a major structure, including the bone, was disrupted as the exposures were given during organogenesis period. Bones in the early stage of pregnancy (1st stage of the gestational period) has a high ability to absorb heat even though it is in osteoid form and not yet ossified. This is mainly because the osteoid tissue is rich in collagen (National Council on Radiation Protection Measurements, 1992). On the other hand, ultrasound-induced heating has a direct effect on bone density during 2nd and 3rd stage of the gestational period as it is thicker and denser.

The anatomic and amount distribution of cortical and trabecular bone in the femoral region might be a major indication in determining resistance to fracture. This anatomic distribution is crucial for the mechanical performance of the bone as a whole. As indicated by decreased Tb.Th and, increased Tb.Sp, Po and TMD in 1 and 5 month subjects, the ultrasound exposure has led to poor bone strength.

The empty lacunae was found higher in 2nd and 3rd stage after a 90-minute exposure in 1 month (Figure 9) and 5 month old subjects (Figure 10). No histological damage attributable to ultrasound was noted in a previous investigation based on thermal damage caused by low-intensity pulsed ultrasound (Shimazaki et al., 2010). However, another study demonstrated osteocyte damage and necrosis characterised by empty lacunae and pyknotic cells following focused ultrasound energy (Smith et al., 2001). The authors observed that acoustic power levels of 39, 52 and 65 W had caused osteocytes loss to be pronounced and had suggested that longer ultrasound exposure could possibly cause more severe and irreparable damage.

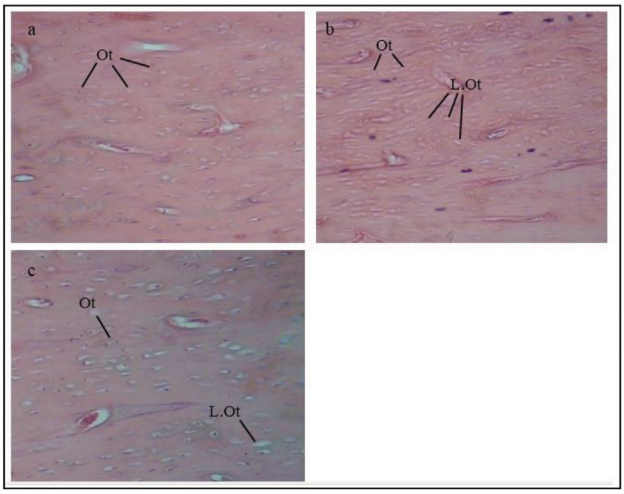


*Figure 9.* Histological results in 1 month old subjects. (a) 90 min of exposure in the 1st gestational stage, (b) 90 min of exposure in the 2nd gestational stage, (c) 90 min of exposure in the 3rd stage. (Ot=lacunae filled with osteocytes, L.Ot=empty lacunae)

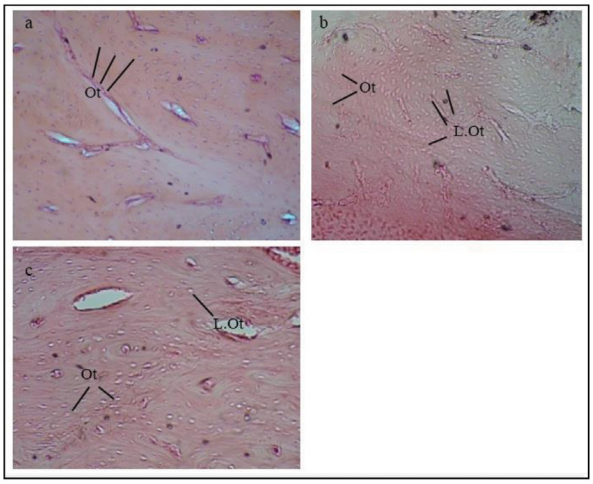


*Figure 10.* Bone histological images of 5 month old subjects. (a) 90 min of exposure in the 1st gestational stage, (b) 90 min of exposure in the 2nd gestational stage, (c) 90 min of exposure in the 3rd stage. (Ot=lacunae filled with osteocytes, L.Ot=empty lacunae)

As osteocytes are potential regulators of bone modelling and remodelling (Lanyon, 1993), bone fragility may be increased due to reduction in osteocyte cells viability. As a result of minimal short-term effects of radiation-induced cell death, loss of osteocytes is likely to affect bone strength in short term, and could cause fractures in the long term (Power et al., 2001). Loss of osteocytes in the femoral neck might include local vasculature disruption thus affecting the nutrient supply to the adjacent bone cell population (Power et al., 2001). Low rate of remodelling could also be one of the effects of osteocytes loss in bone tissue (Wand et al., 1992).

Bone strength is dependent on quality, quantity, and balanced remodelling of the bone tissue. In a normal condition, mammalian bone undergoes continuous remodelling process where formation of new bones takes place replace older ones. This remodeling process is balanced in young adults and becomes imbalanced as a result of ageing. While it is known that bone architecture changes according to aging, there are other factors, such as social activities, nutrition, and co-morbidities, that affect bone metabolism, independent of direct mechanical stimuli (Parkinson & Fazzalari, 2013). In this study, disruption during osteogenesis caused by ultrasound exposure has affected bone metabolism of the subjects. Even though bone can repair the damage, it was not observed in this study. The damage in the subjects' bone was at a faster rate than can be repaired by normal bone remodelling. As a result, bone becomes more fragile and loses its mass.

## CONCLUSION

This study has proven that prolonged exposure to ultrasound during early intrauterine life can cause disturbance in osteogenesis. The effects last until after birth leading to poor bone strength and increasing bone fragility.

## ACKNOWLEDGEMENTS

We wish to thank product specialists from Crest NanoSolutions for their dedicated work, and the staff of Medical Lab Technology Department, Universiti Teknologi MARA (UiTM), Puncak Alam Campus, in providing the and facilities for this study.

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