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Influence of Calcium Chloride on Osteoblast Like Cells of Both Sexes in Rats in *In Vitro* Conditions

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ABSTRACT The aim of the study was to determine whether calcium chloride affects the proliferation of osteoblast like cells in a sex-dependent manner, as well as to determine the most effective concentration on proliferation of osteoblast like cells, in in vitro conditions. Bone marrow was used as biological material from young adult rats, both sexes, aged 90-95 days. Six different concentrations of calcium chloride were tested, determining the numerical representation of osteoblast like cells after 24 and 48 hours. Test results of mean values between males and females after 24 hours, indicate significant differences with a probability of $P<0.05$ at calcium chloride concentrations of: 0.25 mM and 1.8 mM. Results after 48 hours showed that there were no significant differences at most $CaCl_2$ concentrations. It was found that male osteoblast like cells show a higher affinity for different calcium chloride concentrations when compared to the female osteoblast like cells. The calcium chloride concentration of 0.25 mM affected the proliferation of osteoblast like cells most favorably, which is 26.6% higher than the control values.

Keywords: *Bone marrow, Calcium chloride, Cell proliferation, Osteoblast.*

ÖZ

In Vitro Ortamda Kalsiyum Klorürün Osteoblast Benzeri Hücreler Üzerine Etkisi

Bu çalışmada in vitro ortamda kalsiyum klorürün osteoblast benzeri hücrelerin proliferasyonu üzerine cinsiyet bağımlı olarak etkisi olup olmadığının belirlenmesi ve osteoblast benzeri hücrelerin proliferasyonlarını etkileyen en uygun konsantrasyonun belirlenmesi amaçlanmıştır. Her iki cinsiyetten 90-95 günlük ratların kemik ilikleri çalışmanın biyolojik materyalini oluşturmuştur. Altı farklı kalsiyum klorür konsantrasyonu test edildi ve 24 ve 48 saat sonra osteoblast benzeri hücrelerin sayısal değerleri tespit edildi. 24 saat sonunda erkekler ve dişiler arasındaki ortalama değerlerin test sonuçları, 0.25 mM ve 1.8 mM kalsiyum klorür konsantrasyonları istatistiki olarak anlamlı derecede farklılık göstermiştir($P<0.05$). 48 saat sonraki sonuçlar, çoğu $CaCl_2$ konsantrasyonunda önemli farklılıklar olmadığını gösterdi. Erkek osteoblast benzeri hücrelerin, dişi osteoblast benzeri hücrelere kıyasla farklı kalsiyum klorür konsantrasyonları için daha yüksek bir afinite gösterdiği belirlendi. 0.25 mM'lik kalsiyum klorür konsantrasyonu osteoblast benzeri hücrenin proliferasyonunu en olumlu şekilde etkileyen konsantrasyon olarak belirlenmiştir. Bu konsantrasyonda proliferasyon kontrol değerine oranla %26.6 daha fazla olduğu belirlenmiştir.

Anahtar Kelimeler: *Hücre proliferasyonu, Kemik iliği, Kalsiyum klorür, Osteoblast.*

INTRODUCTION

During one year in the human and animal skeleton, about 10% of bone tissue is replaced by new one, and this takes place by a balanced action of osteoblasts and osteoclasts through three phases – decomposition, construction and rest (Clarke 2008). Osteoblasts are defined as young types of bone cells that are responsible for building bone, by producing an organic extracellular matrix and partially controlling bone mineralization (Heino et al. 2004; Katica 2007; Çiçek and Tumer 2018). Osteoblast proliferation and differentiation are important events during bone processing and are controlled by both local growth factors and hormonal regulation. In their final phase of differentiation, osteoblasts become deeply embedded in the mineralized bone matrix during bone formation and become osteocytes (Heino et al. 2004).

A key stimulus for bone remodeling is the occurrence of focal bone micro-damage caused by mechanical stress, and

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in addition to its role in repairing such damage, this process plays an irreplaceable role in calcium homeostasis (Clarke 2008).

Bone tissue is a kind of calcium depot, in order to balance, if necessary, the concentrations of calcium in the muscles and serum (Brini et al. 2013; Modi et al. 2019). Calcium is, therefore, the most abundant mineral in the body with different physiological roles. Extracellular calcium is extremely important for the formation of skeletons and teeth, as well as for the normal course of hemostasis, and the regulation of signal transduction pathways, neurotransmission, etc. (Živančević-Simonović 2006; Brini et al. 2014; Modi et al. 2019).

Calcium deficiency in the body can be regulated by replacement with calcium salts. The most commonly used salts are: calcium carbonate, calcium citrate malate, calcium chloride, calcium gluconate, calcium gluceptate, calcium glucoheptonate and calcium acetate. They differ in calcium content, solubility, bioavailability and taste (Trailokya et al. 2017; Modi et al. 2019).

The effect of some calcium salts and/or calcium ions on the proliferation and/or expression of osteoblast like cells salts has already been described (Lei et al. 2018; Modi et al. 2019; Nakamura et al. 2020).

However, the effect of calcium chloride salt on osteoblast like cells in in vitro conditions is not completely clear. For this reason, we conducted an in vitro experimental study using rat bone tissue, to establish the beneficial or possibly undesirable effect of different concentrations of calcium chloride salt on osteoblast like cells proliferation.

MATERIAL AND METHODS

Ethical Declaration

This research was approved by the Ethics Committee of the University of Sarajevo, Faculty of Veterinary Medicine, under registration number 01-02-421-2/21, Sarajevo, Bosnia and Herzegovina.

Rats

Fourteen young adult Wistar rats, aged 90 to 95 days, weighing 170-210 grams, both sexes were used in the study.

Rats were housed in cages and acclimatized to standard laboratory conditions (temperature 20°C to 24°C; 12 hours light, 12 hours dark), with humidity 60% ± 70%. All experimental animals consumed pelleted food that contained a mixture of different dietary components such as protein, fiber and minerals (Katica and Gradascevic 2017).

Femur preparation

The proximal parts of the femur were dissected for bone marrow extraction. The procedure was performed in – α minimal essential medium (α MEM), which contained the usual antibiotic (Katica and Tepeköy 2020).

Isolation and primary cell culture of bone marrow cells

Bone marrow suspension was dispersed by pipetting, filtered through 70-μm mesh nylon filter, and centrifuged at 200 × *g* for 10 min. After centrifugation, the supernatant containing erythrocytes was decanted and the bone marrow cells were resuspended with 10 ml α- MEM, where the common antibiotics were previously placed: 100 μg/ml Penicillin G, 50 μg/ml Gentamicin sulphate. The cells in the medium supplemented with 10% FBS were transferred to a plastic culture flask. The prepared vials with a half-closed stopper were immediately transferred to a special incubator with a temperature ambient of 37^0C, with a presence of 95% air and 5% CO_2. The cells were approximately seeded in the density of $1X10^3$ cell/vial, after the first preliminary microscopic examination. We constantly monitored the maturation of OB-like cells under a light microscope, with the magnification of 10X or 40 X. On the third day the medium was changed by adding 0,7 ml 10% of FBS. On day six, the medium was enriched with 10 mM Na-β-glycerophosphate (β-GP), 10^{-8} M Dexamethasone (D_x) and 50 μg/mL ascorbic acid (Maniatopoulos et al. 1988; Katica and Tepeköy 2020). After the sixth day, the medium was being changed every second day.

Subculture of bone marrow cells

We approached day 12 by subculturing the bone marrow cells of the examined rats (I passage), applying the conventional methods of porcine trypsin use obtained from the exocrine portion of the pancreas (Maniatopoulos et al. 1988; Katica and Tepeköy 2020). We emptied the medium from small plastic vials and injected 1ml of pork trypsin, which was previously heated in a water bath at 37^0C, for 3-5 minutes. 18 ml α MEM was added into the same plastic culture flasks and then using a sterile pipette, after one minute of "shaking" the cells in suspension, they were transferred to a prepared larger pot in which the medium was previously placed. Finally, 1.8 ml FBS was added. The subcultured cells were placed back into an incubator at the temperature of 37^0C, 100% relative humidity, and 5% of CO_2 and 95% of pure air presence. The medium was being changed every other day, and the cultured cells constantly monitored using a light microscope. Over the next six days, the cells gradually progressed and the preconditions were made for the passage II, that is, for their preparation in suspension for the test.

Application of Calcium Chloride ($CaCl_2$)

Prepared cells in suspension after treatment with trypsin were transferred to a sterile plastic plate with appropriate dents (24 pieces). 1 ml of cells in suspension were placed into every dent using an automatic pipette and two plates were filled, 48 dents. A third, separate plate with 24 dents was a control one. $CaCl_2$ was applied, fresh magistral preparation, prepared immediately before the test at six (6) different concentrations (mM): 0.25, 1.0, 1.3, 1.8, 2.3 and 2.5. Each concentration was tested four times, counting the cells after 24 and 48 hours.

Counting cells

The cells were counted manually, using Thoma's hemocytometer, under the binocular light microscope Motic Type 102 M, with magnification 40X, according to the below-mentioned formula: Counted cell number X 1000 X dilution factor, to obtain the number of cells in $1mm^3$ in the suspension (1×10^{-3}/ml) (Davis 1996; Katica and Tepeköy 2020). Most representative fields were electronically recorded using Motic Images Plus 2.0 software.

Statistical Analysis

All data obtained in the study were processed and analyzed using IBM SPSS Statistics for Windows, Version 24 for statistical data processing: Standard Deviation, Statistical Significance (T-Test), T-Test (difference) for small dependent samples, where a value of $P<0.05$ was considered statistically significant.

RESULTS

In Table 1, significant differences from control osteoblast like cells were observed when $CaCl_2$ was administered to males after 24 hours, with a probability of P<0.01 at $CaCl_2$ concentrations of 0.25 mM, 1,3 mM, 2.3 mM and 2.5 mM. Significance of differences with a probability of P<0.001 was recorded at a concentration of 1mM (Table 1). It was noticed that osteoblast like cells treated with a concentration of 1 mM were more numerous than osteoblast like cells from the control group by 5.4%.

Results after 48 hours in males after administration with calcium chloride (Table 2) indicate a tendency of a slight decrease of about 5% of the mean values of the number of treated osteoblast like cells compared to the mean values in males of 24 hours (Table 1).

In Table 2, significant differences were found in mean values compared to control values after $CaCl_2$ treatment after 48 hours and differ from the results of those of 24 hours. Significant differences with a probability of P<0.001 were found at concentrations of: 1.8 mM, 2.3 mM and 2.5 mM. Significant differences with a probability of P<0.01 were found at concentrations of: 0.25 mM and 1.3 mM, and at concentrations of 1.0 mM there were no significant differences (Table 2).

When examining the effect of $CaCl_2$ on the proliferation of female osteoblast like cells after 24 hours (Table 1), significant differences were recorded compared to control osteoblast like cells at the following concentrations: 0.25 mM, 1.3 mM, 1.3 mM and 2.3 mM with a probability of P<0.01. At a concentration of 2.5 mM, no cells were found (mean value was 0) (Figure 1C) and the significance of the differences in this case was expected, P<0.001. No significant difference was recorded at a concentration of 1.0 mM, and these osteoblast-like cells were more represented than the controls by 2.9%.

Mean values after application of $CaCl_2$ of 2.3 mM after 48 hours decreased when compared to the same 24 hours by 66.6%, and the mentioned mean values ($CaCl_2$ of 2.3 mM) of 24 and 48 hours were lower than the control values by 63% or 87%. Only $CaCl_2$ concentration of 0.25 mM had a favorable effect on proliferation and the mean value was 26.6% higher than 24 and 48 hours compared to the control values.

The test results of mean values between males and females by t-test, and after 24 hours, indicate significant differences with a probability of P<0.05 at $CaCl_2$ concentrations of: 0.25 mM and 1.8 mM (Table 3). Testing of the same parameters, but after 48 hours showed that there were no significant differences at most $CaCl_2$ concentrations. Exceptions were concentrations of 1.0 mM and 2.3 mM, where significant differences were found (Table 3).

The results of the study of the significance of the differences between the findings after 48 and 24 hours by t-test of differentiation in males for $CaCl_2$ concentration of 0.25 mM, the average difference was -0-3 which corresponds to a significant difference with probability P<0.05 (Table 4). Other t-test results showed no significant differences. The same analysis in females found the presence of a significant difference in $CaCl_2$ concentration of 2.3 mM. It had an average difference value of -0.8, and the t-test was 6.92, which is a significant difference with a probability of P<0.01 (Table 4).

Table 1. Results of application of $CaCl_2$ to osteoblasts like cells in males and females after 24 hours.

Ca Cl_2 (mM)	Males (n=7)		Females (n=7)		t -test Significance difference compared to the control group	
after 24 hours	Mean value (10^3)	SD	Mean value (10^3)	SD	Males	Females
2.5	0.2	0.14	0.0	0.0	t=23.33 P<0.01	t=33.00 P<0.001
2.3	1.6	0.21	1.2	0.15	t=11.70 P<0.01	t=16.47 P<0.01
1.8	1.4	0.12	1.2	0.17	t=34.29 P<0.001	t=15.87 P<0.01
1.3	1.9	0.31	2.2	0.15	t=7.69 P<0.01	t=13.91 P<0.01
1.0	3.7	0.24	3.4	0.30	t=1.15 NS	t=0.55 NS
0.25	4.8	0.07	4.5	0.12	t=10.17 P<0.01	t=10.23 P<0.01
Control	3.5	0.24	3.3	0.20	-	-

NS: Not significant, SD: Standard deviation, n: Number of rats used in the experiment.

Table 2. Results of application of $CaCl_2$ to osteoblasts like cells in males and females after 48 hours.

Ca Cl_2 (mM)	Males (n=7)		Females (n=7)		t -test Significance difference compared to the control group	
after 48 hours	Mean value (10^3)	SD	Mean value (10^3)	SD	Males	Females
2.5	0.1	0.00	0.0	0.0	t=33.00 P<0.001	t=31.50 P<0.001
2.3	1.4	1.10	0.4	0.07	t=18.85 P<0.001	t=58.00 P<0.001
1.8	1.3	0.20	1.0	0.12	t=15.33 P<0.001	t=37.55 P<0.001
1.3	2.0	0.21	2.0	0.28	t=9.89 P<0.01	t=9.19 p <0.01
1.0	3.7	0.18	3.1	0.18	t=2.26 P<0.001	t=2.00 P<0.001
0.25	4.5	0.18	4.4	0.07	t=8.31 P<0.01	t=22.00 P<0.001
Control	3.4	0.28	3.3	0.43	-	-

SD: Standard deviation, n: Number of rats used in the experiment.

Table 3. Significance of differences in mean values between males and females after $CaCl_2$ application after 24 and 48 hours.

$CaCl_2$ (mM)	Testing significance of differences in average values between male and female by t-test	
	After 24 hours	After 48 hours
2.5	t=2.82 NS	t=2.82 NS
2.3	t=3.02 NS	t=20.00 P<0.001
1.8	t=3.25 P<0.05	t=3.00 NS
1.3	t=1.44 NS	t=0.00 NS
1.0	t=0.75 NS	t=4.53 P<0.05
0.25	t=4.24 P<0.05	t=1.06 NS
Control	t=1.26 NS	t=1.06 NS

NS: Not significant.

Table 4. Results of testing the significance of differences after application of $CaCl_2$ 48 hours and 24 hours by t-test of differences between females and males.

$CaCl_2$ (mM)	Average difference (d)		t-test of difference	
	Males	Females	Males	Females
2.5	-0.1	0.0	t=0.471 NS	t=0.462
2.3	-0.2	-0.8	t=1.188 NS	t=6.92 P<0.01
1.8	-0.1	-0.2	t=0.926 NS	t=2.19 NS
1.3	0.1	-0.2	t=0.451 NS	t=1.18 NS
1.0	0.0	-0.3	t=1.18 NS	t=1.12 NS
0.25	-0.3	-0.1	t=3.0 P<0.05	t=1.73 NS
Control	-0.1	0.0	t=0.594 NS	t=0.597 NS

NS: Not significant.

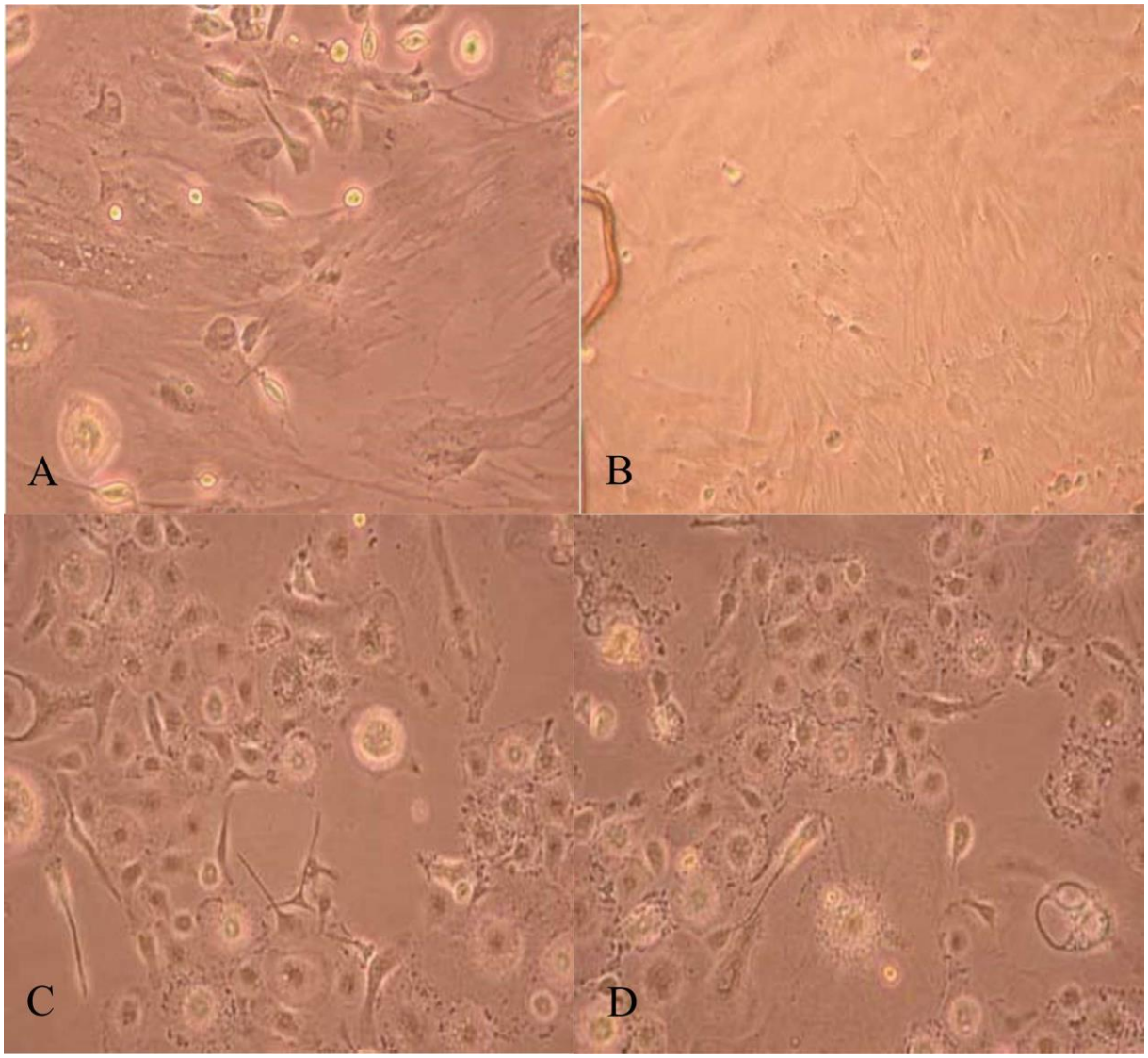

Figure 1. Osteoblast like cells after application of different concentration of $CaCl_2$.

DISCUSSION AND CONCLUSION

In cell culture, osteoblasts are difficult to distinguish from fibroblasts. They most commonly take on spindle shape (Figure 1A, 1B, 1C, 1D), although there are not all spindle cells in in vitro fibroblast cell culture (Davis 1996). All genes found in fibroblasts are also expressed in osteoblasts and vice versa, thus osteoblasts can be viewed as a sophisticated version of fibroblasts, and as such are called osteoblast like cells (Wlodarski 1990; Katica 2007). The exceptions are two gene products identified in osteoblasts, one is Cbfa1 transcription factor and the other is osteocalcin, as well as the presence of a mineralized extracellular matrix (Katica 2007).

The numerical representation of the cultured osteoblast like cells was not large in our study (Figures 1A, 1B, 1C, 1D and Table 1, 2). The reason for the low numerical representation can be related to the age of rats of 90-95 days. The experimental rats were in the young adult stage, which is equivalent to a 20-year-old man (Ćupić et al. 1999). In animals of this age, the presence of osteoblasts in bone tissue is not pronounced, as is the case in young animals in the period of early puerperium. Therefore, osteoblasts in young adult rats, in our case, are in a certain physiological balance in opposition to antagonists, osteoclasts, and their number is not evident for these reasons.

In vivo studies have found that mineral supplements, primarily calcium, in the diet, support osteoblast proliferation, which helps during bone remodeling in osteoporosis (Marie and Kassem 2011).

Results of study, Modi et al. (2019), indicate that calcium glucoheptonate has an increased effect on the cell proliferation of osteoblasts of similar MG-63 cells. Our study unequivocally found that calcium chloride induced osteoblast like cells proliferation in a dose-dependent manner. Lower concentrations of $CaCl_2$ (0.25 mM and 1.0 mM) in males and females, after 24 and 48 hours, favorably affect the proliferative processes of osteoblast like cells (Figure 1A). Obtained results correspond to a similar study by Modi et al. (2019), where identical calcium glucoheptonate concentrations of 0.25 and 1.0 mM, significantly increased alkaline phosphatase activity, suggesting increased osteoblast like cells activity. Higher concentrations of $CaCl_2$: 1.3, 1.8, 2.4 mM, adversely affect the population of osteoblast like cells in cell culture (Figures 1C, 1D), where the destructive influence of $CaCl_2$ is visible with the appearance of degenerated osteoblast like cells. A concentration of 2.5 mM $CaCl_2$ proved to be very toxic (Figure 1B).

The answer to why calcium salts in sufficient concentration yield good results in terms of supporting proliferation and maturation of osteoblast like cells, lies in the accumulation of calcium in the extracellular space. It is inevitable binding to hydroxyapatite crystals, in fact is a key factor in supporting and maturing osteoblast like cells (Bermudez-Reyes et al. 2018).

The results of our study show that the proliferation of osteoblast like cells in males, after treatment with different concentrations of $CaCl_2$ is more pronounced when compared to females. Our results are consistent with a similar study by Katica and Tepeköy (2020), where it was found that different concentrations of calcitriol also have a more favorable effect on the proliferation of the male population of osteoblast like cells compared to the population of females. It is indicative that the control results of male osteoblast like cells, within our study, are slightly higher than the control results in females. Osteoporosis, various forms of osteoarthritis and pathophysiological disorders of the spine are generally more prevalent in the female population than in males (Tosi et al. 2005). The female population has higher levels of estrogen, compared to testosterone in males (Jochems et al. 2010). So, it is to be assumed that this hormonal factor, as well as a higher susceptibility to osteopathies, may to some extent affect lower osteoblast like cells in female population. According to D'Amelio et al. (2008) evident differences in bone properties, including morphological and genetic aspects in male and female cells contribute to sexual dimorphism between men and women.

However, we can conclude that the results of this study favor a 0.25 mM concentration of calcium chloride, which most effectively acts on cell proliferation similar to osteoblasts. Also, male osteoblast like cells were found to show higher affinity for different $CaCl_2$ concentrations when compared to the osteoblast like cells of the female population. This finding provides guidance that calcium chloride treatment could also affect the proliferation of osteoblast like cells in a sex-dependent manner. A better understanding of osteoblast biology, as the most representative bone tissue cell, is necessary as a prerequisite for more effective curative treatment of various osteopathies.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest for this study.

AUTHOR CONTRIBUTIONS

Idea / Concept: MK
Supervision / Consultancy: NG, NHA
Data Collection and / or Processing: NHA, NG
Analysis and / or Interpretation: MK
Writing the Article: NG, NHA
Critical Review: MK

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