EFFECT OF VITAMIN D ADMINISTRATION ON VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION AND ANGIOGENESIS NUMBER IN ORTHODONTIC TOOTH MOVEMENT OF PREGNANT WISTAR RAT

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Date Received:
February 06, 2019
Date Revised:
September 03, 2019
Date Accepted:
September 10, 2019

ABSTRACT

Objective: To analyze the effect of vitamin D administration on vascular endothelial growth factor (VGEF) expression and angiogenesis number during orthodontic tooth movement (OTM) in pregnant Wistar rats (Rattus norvegicus).

Methodology: This was an experimental study with post-test only control group design. Lameshow's sample size formula was used with random sampling method. Twenty eight female Wistar rats were divided into 2 control groups (C+7 and C+14) and 2 experimental groups (E+7 and E+14). Their pregnancy was synchronized, 30g/mm force closed coil spring was given between their incisors and 1st left maxillary molar. Vitamin D (0.2mg/kg) was given every 3 days to E+7 and E+14. Maxillary bone of all groups was extracted. Angiogenesis number was examined by means of Hematoxylin Eosin staining while VEGF expression was examined with Immunohistochemistry staining. Mann-Whitney and analysis of variance with Tukey HSD post-hoc test ($p < 0.05$) was performed based on Levene's test and Kolmogorov-Smirnov test result ($p > 0.05$).

Results: The lowest angiogenesis number was found in C+7 (11.11 ±5.95) and the highest was found in E+14 (23.83 ±7.10). Significant difference was obtained between C+7 and E+14 ($p = 0.003$, $p < 0.05$); E+7 and E+14 ($p = 0.013$, $p < 0.05$). The lowest VEGF expression was found in E+14 (3.09 ±1.14) while the highest was found in C+14 (6.26 ±2.39). There was no significant difference between each group ($p > 0.05$).

Conclusion: Vitamin D administration did not significantly increase VEGF expression and angiogenesis number in OTM of pregnant rats.

Key Words: Angiogenesis, Vascular endothelial growth factor, Orthodontic tooth movement, Pregnancy, Vitamin D

This article may be cited as: **Nareswari RAAR, Narmada IB, Djaharu'ddin I, Rahmawati D, Putranti NAR, Nugraha AP. Effect of vitamin D administration on vascular endothelial growth factor expression and angiogenesis number in orthodontic tooth movement of pregnant Wistar rats. J Postgrad Med Inst 2019; 33(3): 182-8.**

INTRODUCTION

There is an increasing orthodontic treatment demand in adult female population[1]. They have the possibility of pregnancy during orthodontic treatment. Pregnancy will cause significant physiological and anatomical changes in mother's body[2]. Hormonal changes, such as increasing level of sex hormones like estrogen and progesterone, also happen during pregnancy and might cause a decrease of remodeling activity which eventually will lead to increase in orthodontic treatment period[3]; whereas during pregnancy, the effectiveness of dental and oral health care management plays a very crucial role[4]. Alveolar bone and periodontal tissue vascularization plays an important role in OTM remodeling activity[5]. A good tissue innervation will ensure that an adequate nutrient, growth factors, hormones, cytokines and chemokines are achieved[6]. The success of orthodontic treatment is strongly influenced by periodontal tissue remodelling[7].

Vitamin D is a micronutrient that can be obtained by oral route or exposure of the skin to sun light[8-9]. There is high rate of vitamin D deficiency in South Asia and Southeast Asia with prevalence of 6-70% of the population. Vitamin D deficiency is defined as 25-hydroxyvitamin D [25 (OH) D] level below 50 nmol/l[10]. This may

be due to poor intake or disturbances in the absorption process of vitamin D[11]. Females in Asian countries have lower 25 (OH) D levels than males[10] as most of women in the Asian countries tend to limit their access to sunlight due to minimal outdoor activity or a heavy use of sunscreen. Some traditional clothing and skin pigmentation also effect the 25 (OH) D level in the Asian countries[12]. Pregnant women have been identified as a high-risk group of vitamin D deficiency, with incidence rates ranging from 20-80%[13].

Vitamin D can improve endothelial cell function and angiogenesis through the VEGF dependent pathway[14-15]. Angiogenesis will ensure that an adequate blood flow is maintained to provide sufficient supply of nutrition and oxygen[16]. The aim of this study was to determine the effect of vitamin D administration on VEGF and angiogenesis number in orthodontic tooth movement of pregnant Wistar rats model.

METHODOLOGY

All experimental protocols were approved by the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Airlangga University (approval number: 087/HRECC.FODM/VII/2018). This was an experimental study with post-test only control group design. Sample size was determined using Lameshow's formula. All samples were selected through simple random sampling. The sample consisted of 28 female Wistar rats between the ages of 16-20 weeks and weight of 200-250 gram. Experimental animals were in good health without any systemic disorder characterized by soft fur and normal mobility.

The subjects were divided into four groups: two control groups, (n=7) pregnant female Rattus norvegicus with OTM, without vitamin D; and two experimental groups, (n=7) pregnant female Rattus norvegicus with OTM and vitamin D, depending on their examination time (day 7 and day 14 post appliance insertion and activation). Pregnant mare serum gonadotropin (PMSG) (Sigma Aldrich, US) and human chorionic gonadotropin (HCG), (Sigma Aldrich, US) injection were given to all experimental animals. One male Wistar rat was given to each female to synchronize pregnancy. Determination of pregnancy was done with clinical examination, abdomen expansion confirmed by dissection and observation of the fetuses in womb after being sacrificed[3].

To place the mechanical device, an intra-muscular injection of rat cocktail (Sigma Aldrich, US) which consisted of xylazin hydrochloride 1.25 ml, ketamine hydrochloride 2 ml, acepromazine 0.33 ml and saline 6.41 ml were done with the ratio of 0.3 ml/100 g body weight. Round bur was used to make an outline on the distocervical part of rat's 1st left maxillary molar. Ni-Ti coil spring was placed between maxillary central incisor and 1st left maxillary molar and were fixed by 0.07 stainless steel ligature wire. In addition, dental composite resin was given and was activated with light cure. The force was measured with a tension gauge to reach 30g/mm force. The springs were not reactivated during the experiment. Vitamin D was given at a the rate of 0.2 mg/kg every 3 days after appliance insertion to the experimental group (E+7 and E+14).

After the specified time (7 and 14 days after coil spring activation), experimental animals were euthanized and their maxillary bones were extracted up to the first molar area. The extracted tissue specimen was placed in 10% formalin for four days. It was transferred to ethylene diamine tetra acetate (EDTA), (Sigma Aldrich, US) 10% for 21-30 days to decalcify the bone structure. EDTA were changed every day until the extracted tissue specimen was soft. Then, the specimen was washed with phosphate buffer saline (PBS), (Sigma Aldrich, US) for 3-5 times to remove the remaining contaminants. After that, the specimen was fixed with 10% formalin and dehydrated with multilevel alcohol (starting from 30% alcohol, 50% alcohol, 70% alcohol, 80% alcohol, 96% alcohol and ended with an absolute alcohol) for 60 minutes each. Xylol (Sigma Aldrich, US) was used as clearing agent. This step was done twice, 60 minutes each, at 48°C temperature. Hard paraffin block was made in the mould for 24 hours and then it was placed on holder. Rotary microtome was used to cut the paraffin into 4-6 µm thickness and gelatin 5% was used to mount the paraffin to the object glass. Object glass was placed in xylol for 5 minutes, twice. Then, rehydration was performed using multilevel alcohol (One Med, Indonesia) starting from absolute alcohol, 96% alcohol, 80% alcohol, 70% alcohol, 50% alcohol and ended with 30% alcohol for 5 minutes each. After that, the object glass was washed with H_20 (One Med, Indonesia) for 5 minutes.

Hematoxylin and Eosin (H&E), (HE staining kit, Sigma Aldrich, US) staining method and histopathology examination was used to study angiogenesis in all groups. Angiogenesis was quantified under 400x magnifications, with Nikon Eclipse Ci electron microscope, on five different fields of view and taken in average. Angiogenesis was examined by histopathologist. Immunohistochemistry (IHC) staining using monoclonal antibody anti-VEGF (VEGF (C-1) Antibody, Cat No. sc-7269, Santa Cruz Biotechnology, Inc, US) with 3,3'-Diaminobenzidine (DAB) brown chromogen was used in all groups to analyze the VEGF expression. VEGF was quantified under 1000x magnifications, with Nikon H600L, on five different field of view and taken in average. Immuno-Reactive Score (IRS) was used to do semi-quantitative count on VEGF. VEGF was observed by 2 experts.

Mann-Whitney and ANOVA with Tukey's honest significant difference (HSD) post-hoc test were per-

formed ($p<0.05$) based on Levene's test and Kolmogorov-Smirnov test result ($p>0.05$) using SPSS version 20.0 (SPSS™, Chicago, United States).

RESULTS

Regarding the number of angiogenesis, the lowest angiogenesis number was found in control group on day 7 (11.11 ±5.95), while highest angiogenesis number was found in experimental group on day 14 (23.83 ±7.10) as shown in Figure 1 and Figure 2. Data were normally distributed ($p>0.05$) but were not homogeneous ($p<0.05$) as shown in Table 1. An overall significant difference was found ($p=0.028$). Significant difference was observed between control group on day 7 compared to experimental group on day 14 ($p=0.003$); and experimental group on day 7 compared to experimental group on day 14 ($p=0.013$) as shown in Table 2.

Regarding VEGF expression, the lowest amount of VEGF level was found in experimental group on day 7 (3.09 ±1.14); while highest amount of VEGF level was found in control group on day 14 (6.26 ±2.39) as shown in Figure 3 and Figure 4. The data were normally distributed ($p>0.05$) and homogeneous ($p>0.05$) as shown in Table 1. There was no significant difference between each group ($p>0.05$) as shown in Table 2.

Table 1: Kolmogorov-Smirnov normality test and Levene's homogeneity test of angiogenesis

Variable	Kolmogorov- Smirnov Test		Levene's Test	
	Group	P Value	A	P Value
Angiogenesis	C+7	0.841*	0.05	0.002
	C+14	0.453*		
	E+7	0.973*		
	E+14	0.956*		
VEGF	C+7	0.906*	0.05	0.212*
	C+14	0.995*		
	E+7	0.918*		
	E+14	0.497*		

*Information: Normal distribution and homogeneous variant ($p>0.05$)

Table 2: Multiple comparison test result of angiogenesis using Mann-Whitney Test

Variable		C+7	C+14	E+7	E+14
Angiogenesis	C+7	-	-	-	-
	C+14	0.653	-	-	-
	E+7	0.337	0.565	-	-
	E+14	0.003*	0.159	0.013*	-
VEGF	C+7	-	-	-	-
	C+14	0.260	-	-	-
	E+7	0.885	0.058	-	-
	E+14	0.997	0.188	0.930	-

* Significant difference ($p<0.05$).

Figure 1: Angiogenesis histological comparison of all groups. A. Angiogenesis result in control group day 7 (C+7). B. Angiogenesis result in control group day 14 (C+14). C. Angiogenesis result in experiment group day 7 (E+7). D. Angiogenesis result in experiment group day 14 (E+14). Examination was done at the tension area. Angiogenesis numbers (yellow arrow) were quantified under 400x magnification

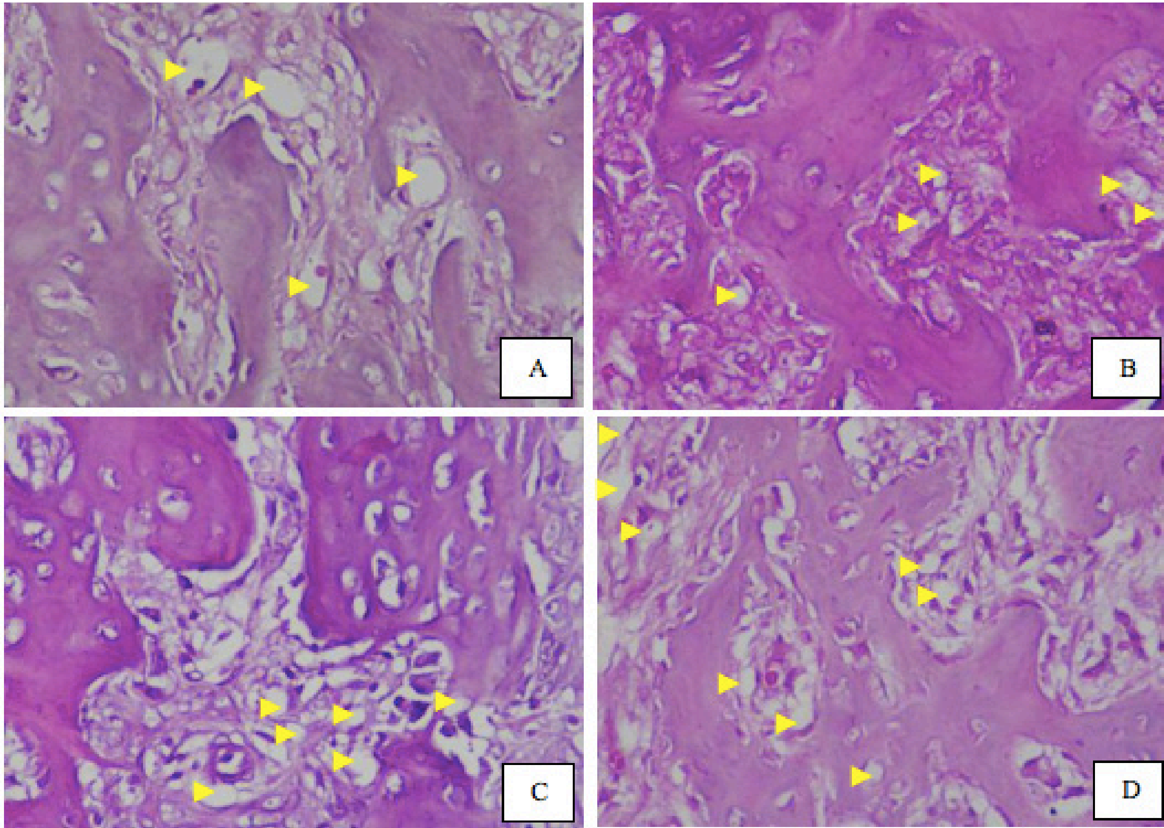


Figure 2: Mean and standard deviation (SD) of angiogenesis number in each group

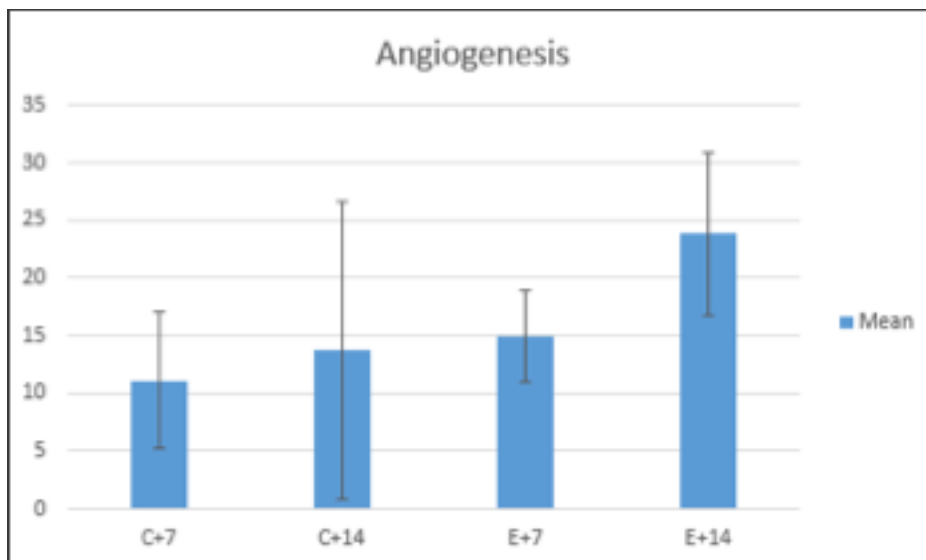


Figure 3: VEGF histological comparison of all groups: VEGF result in control group day 7 (C+7). VEGF result in control group day 14 (C+14). VEGF result in experiment group day 7 (E+7). VEGF result in experiment group day 14 (E+14). Examination was done at the tension area. VEGF expression (red arrow) were quantified under 1000x magnification

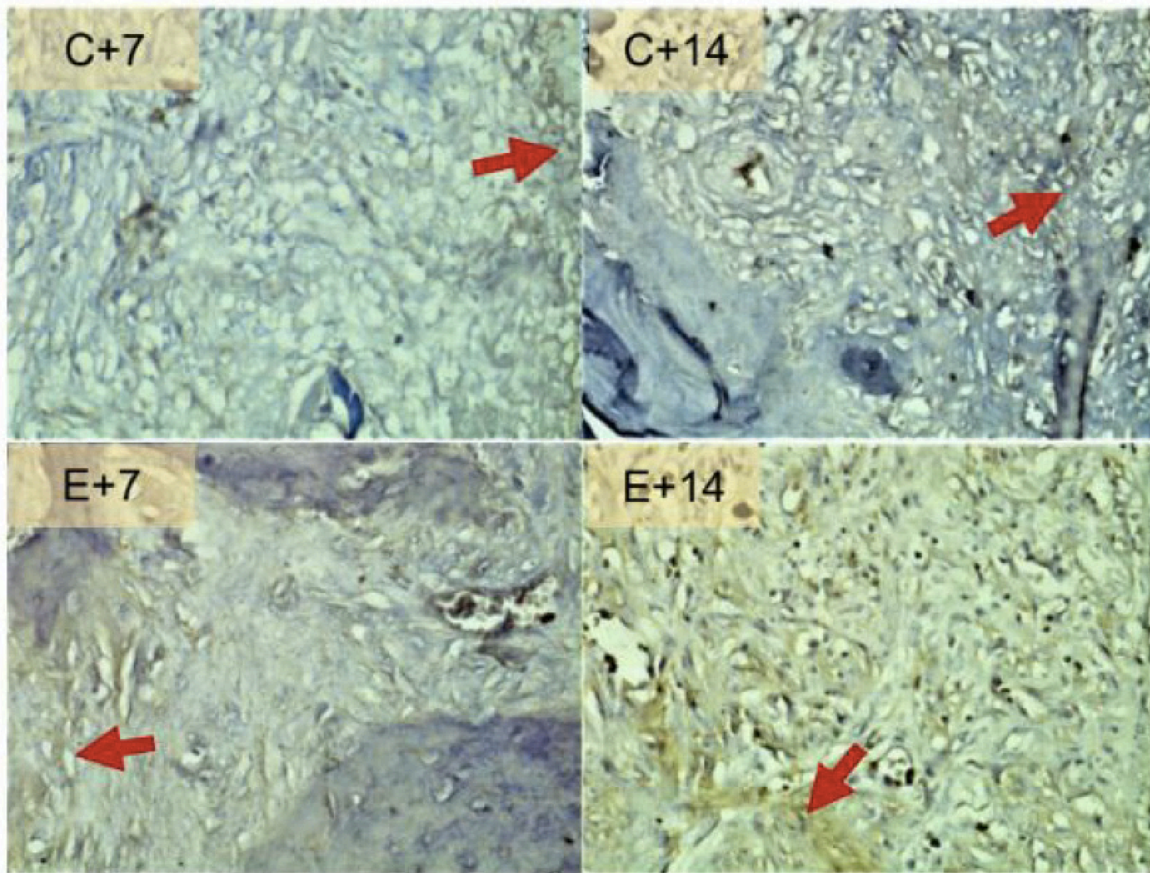
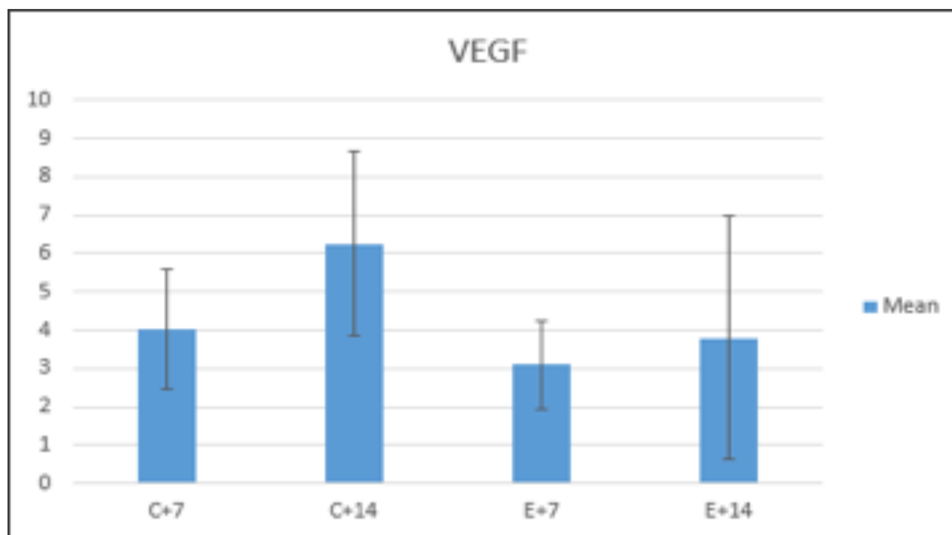


Figure 4: Mean and standard deviation (SD) of of VEGF expression in each group



DISCUSSION

In this study, histopathological examination showed increased angiogenesis in both control and experimental groups which lead to remodeling process on the tension side as reported in previous studies[17]. OTM is a biological response from an orthodontic force, a remodeling process of alveolar bone and periodontal ligament in both tension and compression side[18]. Alveolar bone and periodontal tissue angiogenesis plays an important role in the remodeling process during OTM[5]. Angiogenesis is a process of new vessels formation from pre-existing ones[16,19]. Angiogenesis will provide blood flow in order to supply sufficient nutrition and oxygen to the area[16]. In this process, differentiation of osteoblasts also starts from stem cells in the bone marrow which eventually moves into the blood vessels[20].

There was an increasing level of angiogenesis in control group on day 7 and 14, however, the difference was not significant. It might be attributed to pregnancy. Some recent studies have shown that the presence of VEGF like receptors, known as soluble Fms-like Tyrosine Kinase (sFLT1), are found during pregnancy. They are absent in non-pregnant female or males[21]. These act as VEGFA and placental growth factor (PIGF) antagonist by preventing their interaction with their receptors, VEGF R1 and VEGF R2. The sFLT1 neutralize the angiogenic activities of VEGF and PlGF during pregnancy, resulting in decreased angiogenesis and VEGF in circulation[22]. This condition might explain the insignificant difference between day 7 and day 14 in both control and experimental groups of VEGF (C+7 compared to C+14; E+7 compared to E+14).

Significant difference was found in angiogenesis on day 7 (both in control and experimental groups) compared to day 14 in experimental group. This probably happened due to the longer time between coil activation and the time of observation on E+14 (14 days) compared to C+7 and E+7 (7 days). Study by Narimiya et al[17], has also shown that increased angiogenesis in the tension side during OTM is time-dependent. Vitamin D can increase angiogenesis through VEGF-dependent pathway[14,15]. Vitamin D Receptor Element (VDRE) activation stimulates VEGF synthesis and leads to the increasing level of VEGF in the circulatory system[23]. Endothelial cells produce angiopoietin growth factors and matrix metalloproteinase (MMP) enzymes to start the new blood vessels production[20].

Study by Garcia et al[24], showed that vitamin D also increase other mediators of angiogenesis such as fibroblast growth factor (FGF), induces the secretion of endothelial proteinases and plasminogen activators that cause breakdown of the vessel basement membrane, allowing the branching process of blood vessels. Study by Grundmann et al[25] showed that vitamin D also increase pro-MMP-2 activity which along with MMP-2 play an important role in angiogenesis and remodeling process as these are able to degrade extracellular matrix proteins in the process of angiogenesis. Furthermore, study by Kogawa et al[26] showed that vitamin D increases ephrin B2 which along with Ephrin B4 have the ability to regulate VEGF with resultant prevention of excessive angiogenesis[27]. Such regulation of VEGF might explain the decrease in VEGF level in experimental group compared to the control group. Contrarily, there was still increased angiogenesis in experimental group compared to the control group.

CONCLUSION

Vitamin D administration does not significantly increase VEGF expression and angiogenesis number in OTM of pregnant rat model.

ACKNOWLEDGEMENT

The authors would like to thank Orthodontic Department, Faculty of Dental Medicine and Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya for the support and help. This study was funded and supported by Penelitian Dosen Pemula, Faculty of Dental Medicine, Universitas Airlangga, Surabaya-Indonesia.

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CONTRIBUTORS

RAARN conceived the idea, designed the study, wrote the initial manuscript and remained involved in all stages of the study. IBN refined the initial script, helped in preparing the subjects, executed the plan and collected data. ID, DR and remained involved in all stages of the project including literature search and critical appraisal, analysis of data and referencing. NARP and APN helped in correction of the manuscript, taking care of ethical aspects and kept liaison among the investigators while executing the plan according to the protocol. All authors contributed significantly to the submitted manuscript.