The Effect of Calcium Hydroxide and Chitosan Combination as A Pulp Capping Material on The Increased Expression of Osteopontin and Runt-Related Transcription Factor 2 in Dentine Reparative Formation in Osteoporosis

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Abstract

Direct pulp capping is performed to maintain the vitality of the exposed pulp by applying a dressing material to facilitate reparative dentinogenesis as a biological seal. Osteoporosis increases oxidative stress and inflammation therefore dentinogenesis was disrupted. Dentin bridge reparative produced by direct pulp capping material still inappropriate, there is a tunnel defect. Chitosan is a natural polysaccharide biopolymer, non-toxic, biocompatible, biodegradable, has broad spectrum antimicrobial properties, anti-inflammatory and antioxidant. OPN and RUNX2 secretion expressed by odontoblast-like cells can be a sign of reparative dentin formation in osteoporosis.

The aim of this research to analyse the effect of calcium hydroxide and chitosan combination on the increased expression of OPN and RUNX2 on day 1, day 3 and day 6 in reparative dentin formation in osteoporosis. This experimental study using a total of 42 wistar rat. Ovariohysterectomy was performed to induced osteoporosis and verified by tunel assay after 3 months. Rats divided into 6 groups, the control group on day 1, day 3 and day 6, the treatment group on day 1, day 3 and day 6. Necropsy was performed and teeth samples were obtained. After being decalcified, specimens underwent anatomical histopathology evaluation under light microscope to determine the direction of the perforation. Immunohistochemical examination was performed to determine the odontoblast like cells that express OPN and RUNX2.

The results were a significant increase in the expression of OPN and RUNX2 in the combination of calcium hydroxide with chitosan compared to the control group in osteoporosis.

The conclusion is in osteoporosis, the expression of OPN and RUNX2 in the combination of calcium hydroxide with chitosan on day 1, day 3 and day 6 was higher than calcium hydroxide.

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Introduction

According to Indonesia Basic Health Research 2018 (Riskesdas) reports, 88.8% of Indonesians have dental caries. The 55-64 age group had the greatest caries prevalence, at 96.8%[1]. Statistical data from International Osteoporosis Foundation in 2019 also reports that osteoporosis causes 8.9 million occurrences of bone fractures worldwide each year. Osteoporosis is a degenerative condition that characterized by reduced bone mass and degeneration of the bone microarchitecture[2,3].

Osteoporosis causes a reduction in calcium absorbtion[4]. Reduced calcium absorption

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damages the surface of tooth enamel, creating a space for bacteria and increase the risk of caries[5]. Direct pulp capping is performed to maintain the vitality of the exposed pulp by applying a dressing material to promotes reparative dentinogenesis as a biological seal[6].

Osteoporosis due to reduced estrogen level, decreased calcium level and aging can generate reduction of anti-oxidant enzyme and pulp cells[7]. The imbalance of Reactive Oxygen Species (ROS) production and anti-oxidant ability generate oxidative stress[8]. This condition increases pulp cell apoptosis and downregulate the differentiation of pulp stem cells and disrupt the mineralization process to dentinogenesis in osteoporosis condition[9,10,11].

The dentin bridge reparative produced by calcium hydroxide is not homogeneous and creates a tunnel defect. Bacteria recolonize there, causing the pulp inflammatory process to persist and the pulp capping treatment to fail[12,13]. Chitosan is simple to alter on various shapes[14]. Chitosan as a derivative compound has been shown to increase the release of inflammatory mediators such as PMNs and macrophages that play a role in the process of tissue regeneration[15]. Chitosan is non-toxic, biocompatible, biodegradable, has broad spectrum antimicrobial properties, anti-inflammatory and anti-oxidant[16]. As antimicrobial chitosan destroys bacterial cell membranes because of its cationic charge[17]. Due to its polyheterosaccharide structure, which resembles that of glycosaminoglycan, the primary component of the Extra Cellular Matrix (ECM), chitosan can also be employed as a template or scaffold in the proliferation and differentiation of cells. Chitosan's structure has the ability to increase the expression of anti-inflammatory cytokines while decreasing the expression of pro-inflammatory cytokines. Also, as an anti-oxidant chitosan has ability to bind the free radicals to create stable free radicals due to its hydroxyl and amino ion[18,19].

OPN and RUNX2 secretion began on day one and continued to rise, reaching a peak on day five. The ability of OPN and RUNX2 to induce odontoblast-like cells could be an indication that a direct pulp capping procedure was effective[20].

Materials and methods

Research Samples

The samples used were female white rats (Rattus Novergicus) wistar strain with criteria of age 4 months, body weight 220-250 grams, fed Pap milk (PT Japfa comfeed) per rat 25 grams/day and the molars had grown perfectly.

Research Methods

42 rats were used as the subjects in this research. Ovariohysterectomy were performed to create osteoporosis condition. Rats were anesthetized using ketamine HCL 50 mg/Kg BW and xylazine HCl 10 mg/Kg BW intramuscularly. The 1 cm-long incision was made in the middle, between the pubis and the umbilicus. The right and left cornua uteri were identified and clipped using a haemostat and then ligated with catgut. The ovaries and uterus were excised using a blade. The musculature was sutured and injected intra-abdominal antibiotics ampicillin and intraperitoneal enrofloxacin. The sample was observed for 3 months.

The pulp capping procedure was performed on 6 groups of rats. Calcium hydroxide treated group used calcium hydroxide powder which was 0.1 mg weighed using an analytical balance to 2:1 ratio, 0.05 ml of distilled water was added which was taken using a micropipette. The treatment group used a combination of calcium hydroxide and chitosan which calcium hydroxide was weighed using an analytical balance in a ratio of 2:1, 0.1 mg of calcium hydroxide and 0.05 ml of chitosan was added using micropipette.

During procedure, all rats were treated antiseptic potassium iodide at the injection site, then anesthetized by peritoneal injection with ketamine HCl and xylazine HCl. A Class I cavity preparation (Black classification) was performed on the occlusal surface of the maxillary first molar using a low-speed handpiece with round diamond bur (0.8 mm diameter) until the perforation of the pulp chamber was confirmed using a probe with a tip diameter of 0.46 mm. After perforation, the cavity was dripped with sterile saline solution and dried with cotton pellets and paper points. In the control group, calcium hydroxide mixed with distilled water was applied. In the treatment group, calcium hydroxide combined with chitosan was applied. Application of the material on the pulp surface using a carrier and compacted with a cement

stopper. After the pulp capping material was applied, the cavity was restored with a glass ionomer cement filling material (Cention N, Ivoclar Vivadent). Rats were returned to cages and given the antibiotic Ampicillin (PT Meiji Indonesia, Vicillin®) a combination of Enrofloxacin (De Adelaar B.V., Interflox®) once a day for three days after treatment. Experimental animals from each group were necropsied according to the specified time, namely on the day one, three, and six after treatment.

Further examination was performed. Immunohistochemical examination (IHC) was carried out and the expression of OPN and RUNX2 were calculated. The IHC procedure uses enzyme-labelled antibodies so that protein and antibody binding can be visualized. The odontoblast-like cells that were counted were those expressing this bond with a brown colour in the cytoplasm and a purplish-blue colour (counterstaining) in the nucleus.

Statistical methods

Statistical analysis of the data calculated the mean and standard deviation of each group. Then the Saphiro-Wilk test was carried out to determine the normality of the data distribution, Saphiro-Wilk was chosen because the number of samples was less than 50 followed by the Leven homogeneity test. To differentiate the expression of OPN and RUNX2 at different observation times, the Tukey HSD test was used. To test the differences between groups at the same time, the independent t test was used.

Results

	Group	Mean ± SD	P value
Day one	Control group[a]	2.1429 ± 0.69007	0.001
	Treatment group	5.5714 ± 0.97590	0.001
Day three	Control group[b]	5.7143 ± 1.38013	0.001
	Treatment group	10.5714 ± 1.13389	0.688
Day six	Control group[c]	6.2857 ± 1.60367	0.001
	Treatment group	11.8571 ± 1.86445	0.217

Table 1. Mean, standard deviation (SD), and p value of OPN expression of treatment and control group. The different superscript letters are statistically different (ANOVA, P<0.05).

Table 1 showed the expression of OPN in the control group between day one and day three and between day one and day six shows a significant difference statistically (p<0.05), day three and day six shows no statistically significant difference (p>0.05). The expression of OPN in the treatment group between day one and day three as well as day one and day six shows a significant difference statistically (p<0.05) whereas day three and day six shows no statistically significant difference (p>0.05).

	Group	Mean ± SD	P value
Day one	Control group[d]	2.8571 ± 1.57359	0.001
	Treatment group	7 ± 1.41421	0.001
Day three	Control group[e]	6.2857 ± 1.49603	0.001
	Treatment group	11.4286 ± 1.39728	0.498
Day six	Control group[f]	7.2857 ± 1.79947	0.001
	Treatment group	12.2857 ± 2.42997	0.657

Table 2. Mean, standard deviation (SD), and p value of RUNX2 expression of treatment and control group. The different superscript letters are statistically different (ANOVA, P<0.05).

Table 2 showed the expression of RUNX2 in the control group shows a significant difference statistically (p<0.05) in all day. The expression of RUNX2 in the treatment group between day one and day three as well as day one and day six shows a significant difference statistically (p<0.05) whereas day three and day six shows no statistically significant difference (p>0.05).

	Group	Mean ± SD OPN Expression	Mean ± SD RUNX2 Expression	P value OPN Expression	P value RUNX2 Expression
Day one	Control group[g]	2.1429 ± 0.69007	2.8571 ± 1.57359	0.001	0.001
	Treatment group[h]	5.5714 ± 0.97590	7 ± 1.41421	0.001	0.001
Day three	Control group[i]	5.7143 ± 1.38013	6.2857 ± 1.49603	0.001	0.001
	Treatment group[j]	10.5714 ± 1.13389	11.4286 ± 1.39728	0.001	0.001
Day six	Control group[k]	6.2857 ± 1.60367	7.2857 ± 1.79947	0.001	0.001
	Treatment group[l]	11.8571 ± 1.86445	12.2857 ± 2.42997	0.001	0.001

Table 3. Mean, standard deviation (SD), and p value of OPN and RUNX2 expression of treatment and control group. The different superscript letters are statistically different (t-test, P<0.05)

Table 3 showed that there is a significant difference statistically between control group and treatment group on day one, three, and six with p values = 0.001 (p < 0.05), respectively. The table shows a higher expression of both OPN and RUNX2 in the treatment group as contrasted to only control group.

Discussion

The combination of calcium hydroxide with chitosan as a direct pulp capping agent in

osteoporosis is considered due to the antioxidant and anti-inflammatory properties of calcium hydroxide and chitosan.

Ovariohysterectomy procedure was performed to induce osteoporosis in rats. Bone loss following ovariohysterectomy reaches more than 60% at 13-14 weeks post operatively[21]. Therefore, the pulp capping procedure was performed three months after the ovariohysterectomy.

Rats that have undergone ovariohysterectomy experience estrogen deficiency. Estrogen deficiency causes ROS accumulation which triggers oxidative stress and decreases antioxidant enzymes (AE). An increased level of ROS will activate the p53 genes and the oxidative stress pathway resulting in ROS-induced apoptosis of osteocytes which increases bone loss and bone fragility. Hence, the morphology and vitality of osteocytes act as indicators of bone quality. Tunnel assay used in this study shows that the osteoporosis groups' osteocyte cells were discovered to be smaller in size and flatter in shape, demonstrating the alteration in bone mass and structure[22].

The Tukey HSD statistical tests showed differences between OPN and RUNX2 expression on the control group on day one and three, as well as day one and six. There was no difference between the third and sixth days. On day one and three, as well as day one and six, there were difference in the expression of OPN and RUNX2 in the treatment group whereas no difference showed on day three and day six. This demonstrates that the expression of OPN and RUNX2 differs significantly between day one and daythree, as well as between day 1 and day 6 whereas no significant differences between day three and day six. There is a significant increase in the expression of OPN and RUNX2 with increasing days. In reparative dentinogenesis, OPN and RUNX2 secretion began on day one and continued to rise, reaching a peak on day five[23].

OPN in the dentin-pulp complex is found at the dentin and tertiary dentinal junctions. Immunocompetent cells such as macrophages and dendritic cells secrete OPN, which are deposited at the dentin-predentine junction before differentiation into odontoblast-like cells. OPN deposition in the calcification process is important for the secretion of type I collagen by odontoblast like cells to form reparative dentin during pulp healing[22]. Therefore, the process of reparative dentin mineralization is induced by an increase in OPN expression from day one to day six.

Runt-related Transcription Factor 2 (RUNX2) is a transcription factor in differentiation of odontoblast-like cells in the early stages of reparative dentinogenesis calcification. RUNX2 immunoreactivity was detected in odontoblast-like cells on day 1 and peaked on day 5[23]. Thus, increasing RUNX2 expression from day one to day six induces the mineralization of reparative dentin.

Conclusions

The expression of osteopontin (OPN) and Runt-related Transcription Factor (RUNX2) on the application of calcium hydroxide combine with chitosan on day one, three and six was higher than the application of calcium hydroxide in osteoporosis.

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Ethical policy and institutional review board statement

Ethical clearance had been obtained from the Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya (284/HRECC.FODM/VI/2020).

Declaration of Interest

The authors report no conflict of interest.

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