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POTENTIAL APPLICATION OF CHITOSAN SYNTHESIZED FROM *PENAEUS MONODON* AS A DIRECT PULP CAPPING MATERIAL

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ABSTRACT : Chitosan could facilitate migration, proliferation and differentiation of pulp progenitor cells. The purpose of this study was to investigate the potential application of chitosan synthesized from *Penaeus monodon* shells, as a direct pulp capping material. Chitosan was synthesized by *Penaeus monodon* shells using deproteinase, demineralisation, depigmentation and deacetylation methods. The molar teeth of the male *Rattus norvegicus*-strain-Wistar had been prepared for class one in the occlusal part and were divided into two groups, namely Group one (G1) applied Ca $(OH)_2$ and Group two (G2) applied chitosan. Then, the histopathological evaluation was performed to observe odontoblast-like cells with hematoxylin eosin staining after seven and then 28 days following treatment. Measurement of the yield value, water content, molecular weight, deacetylation degree and cytotoxicity tests were carried out to determine the quality standards of chitosan. Statistics analysis used one-way analysis of variance (ANOVA) followed by an least significant different (LSD) post hoc test with multiple comparisons ($p<0.05$). This study showed that the yield value was 0.08%; water content was 8.73 %; molecular weight was 3012.83 g/mol; deacetylation degree was 86.22% and the cells viability was 97.83%. The application of chitosan was showed a significant difference in odontoblast-like cells on the seventh day of observation ($p=0.001$; $p<0.05$) and the 28th day of observation ($p=0.002$; $p<0.05$). Chitosan synthesized from *Penaeus monodon* shells from Indonesia has strong potentials as a direct pulp capping material.

Key words : Chitosan, *Penaeus monodon* shells, degree of deacetylation, direct pulp capping, odontoblast-like cells.

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INTRODUCTION

Direct pulp capping is a pulp perforation treatment in reversible pulpitis cases due to mechanical trauma or deep caries cleaning by giving topical material to perforated areas. This is undertaken to prevent bacteria entering soft tissue wounds and improving dentin tissue improving to opened areas, preventing the formation of irreversible pulpitis which leads to pulp dying (Mitsiadis, 2004). The gold standard material of a direct pulp capping treatment is calcium hydroxide (Ca $(OH)_2$). However, this material has a harmful side effect such as causing the rise of necrosis to the pulp's superficial layer. Ca $(OH)_2$ would form strong alkaline from its ionisation that produced Ca^{++} dan OH^-, hence it could increase the risk of pulp abnormality and apical periodontitis (Prasetyo *et al*, 2020; Prasetyo *et al*, 2021). Therefore, chitosan is a material developed as a candidate direct pulp capping.

Chitosan is a chitin derivative compound. Chitin is a structure composed from shrimp shells (Fadholly *et al*, 2020). Chitosan (poly-β-1,4-glucosamine) originates from the natural production of bio-polymer and N-deasetylase from chitin (Rezkita *et al*, 2020a). Generally, chitosan is biocompatible and biodegradable that is widely used in medicine to accelerate wound healing, adsorption and anti-infection material (Nugraha *et al*, 2020). Chitosan is a polycationic complex carbohydrate that can facilitate the migration and proliferation of progenitor cells (Rezkita *et al*, 2020b).

The effects of chitosan characteristic as a wound healing biomaterial, particularly in candidates needing direct pulpcapping, are moisture content, molecular weight and degree of deacetylation (Pang *et al*, 2005). One of the ideal biomaterials must be applied clinically and be non-toxic (Kuntjoro *et al*, 2020). Chitosan's mucoadhesive

characteristic is affected by its molecular weight in terms of wound healing. The higher the molecular wight, the slower dissolve. The higher deacetylation degree, the greater the possibility of impacting the chitosan cationic characteristics that are tied to protein, such as collagen (Rezkita *et al*, 2020a; Rahmitasari, 2016). This study aim is to investigate the *Penaeus monodon*'s shells synthesized production from Indonesia and its potential application as a direct pulp capping material.

MATERIALS AND METHODS

Chitosan synthesized preparation

Penaeus monodon shells were cleaned, dried and mashed. Then, it was proceeded to chitosan by deproteinization, demineralisation, depigmentation and deacetylation. The shells powder dissolved in the NaOH 3,5% at 65°C for two hours. Then, it was dissolved in the HCL 1 M. Subsequently, it was dissolved by acetone. The deacetylation of chitin to obtain chitosan is achieved by dissolving chitin powder with NaOH 50% and soaking at 65°C for three hours.

Chitosan characteristics

The chitosan that synthesized from Penaeus monodon shells was characterised to evaluate its quality which encircled yield value, water content, molecular weight, and deacetylation degree. The yield value was analysed by counting its powder mass to chitin powder mass that resulted from the chitosan-making process (Agustina *et al*, 2015). The water content was undertaken by the gravimetry method (Citrowati *et al*, 2019). Then, the molecule weight was undertaken by the Ostwald Viscosity method (Kasongo *et al*, 2020). Lastly, the deacetylate degree was done by testing chitosan powder through the infra-red spectrophotometry method (FTIR) (Swann *et al*, 2011).

Chitosan cytotoxicity test

The evaluation of cytotoxicity of chitosan material was conducted by MTT assay using fibroblast cell line (BHK-21). The MTT cytotoxicity test is based on the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoline bromide (known as MTT reagent) to form azure formazan in living cells enabling the evaluation of enzymatic activity of the succinate dehydrogenase, thereby determining the cellular vitality (Nugraha *et al*, 2020b; Kuntjoro *et al*, 2020).

In vivo experiment preparation

Thirty-two males of *Rattus norvegicus*-strain Wistar- (weight: 200-250 gr, age: 8-16 w) were used to follow the ethics committee for animal research in this study. All procedures were undertaken by using ketamine anesthetic. The molar teeth of the male Rattus norvegicus-strain Wistar - had been prepared for class one cavity in the occlusal part by a low-speed tapered round diamond bur and perforated by a dental sonde. Chitosan capping pulp material and $Ca(OH)_2$ was applied as 0.01 gr in the cavity. Then, the cavity was filled by Glass Ionomer Cement type IX, as dental restorative material.

Histology preparation

After post-healing for approximately seven and then 28 days, euthanasia was undertaken. The jaw and tooth were soaked in decalcification solution for 48 hours. The specimen was planted in the paraffin embedded block, then it was prepared by 4-5μ cutting and HE painting for observation of odontoblast-like cell activity. The calculation was done by 400x. The odontoblast-like cells were calculated by evaluating the number of cells on the left and right perforated sides.

Statistics analysis

Statistics analysis used one-way analysis of variance (ANOVA) followed by least significant different post hoc test multiple comparisons ($p<0.05$) by statistical package for social science (SPSS) 20.0 software (Illinois, Chicago, US).

RESULTS

This study used chitosan synthesized from *Penaeus monodon*'s shells. The chitosan was produced using the deproteinization, demineralisation, depigmentation and deacetylation method at 65°C for three hours. The characteristics analysis showed that this chitosan has a high-quality standard as be seen in Table 1.

The yield value showed 0.08%; water content ≤ 10%; molecular weight was 3012.83 g/mol and deacetylation degree ≥ 70%. The cytotoxicity test was done using the MTT assay method that represented the non-toxic chitosan to a CD50 parameter percentage of the viable cells above 50%. In this study, the cell viability was 97.83%. After seven and twenty-eight days *in vivo*, the odontoblast-like cells were observed in all the groups. There were significant differences between groups after seven days of healing ($p = 0.000$; $p<0.05$) and 28 days of healing ($p= 0.001$; $p<0.05$). At seven days, the number of the odontoblast-like cells in group two (17.75 ± 1.28) presented significantly higher than group one (13.62 ± 2.33) (Fig. 1a). Furthermore, at 28 days, there was a significantly higher number of odontoblast-like cells in group two (19.62 ± 1.68) than group one (15.75 ± 3.01) (Fig. 1b). The histopathology examination of odontoblast-like cell in each group can be seen in Fig. 2a and 2b.

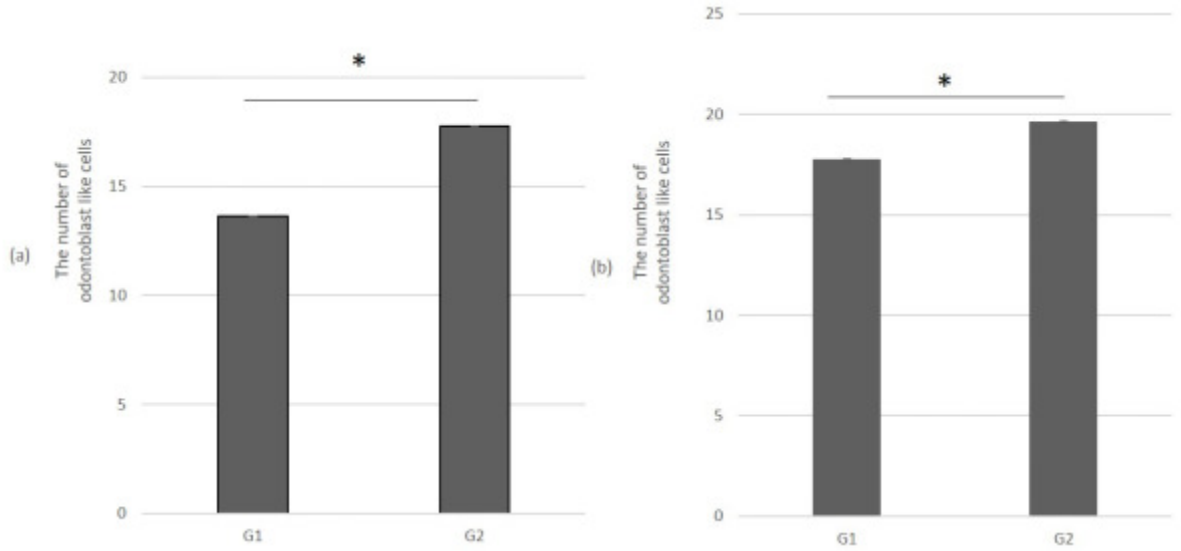

Fig. 1 : (a) The averagenumber of odontoblast like cells after seven day in G1 and G2. (b) The average number of odontoblast like cells after 28 days in G1 and G2. *information: significant at $p < 0.05$.

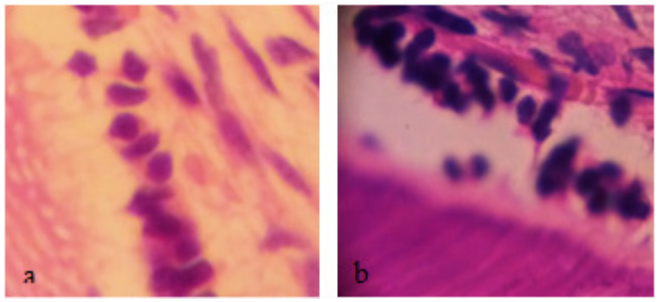

Fig. 2 : The histopathology examination of (a) Odontoblast-like cell at Group one applied $Ca(OH)_2$, (b) Odontoblast-like cell at Group one applied Chitosan with 400x magnification.

Table 1 : The chitosan characteristics.

Parameter	Value
Yield value	0.08%
Water content	8.73%
Molecular weight	3012.83 g/mol
Deacetylation degree	86.22%
Cell viability	97.83%

DISCUSSION

In this study, chitosan synthesized from *Penaeus monodon's* shells using deproteinization, demineralization, depigmentation and deacetylation method at 65°C for three hours produced high quality chitosan as a biomaterial. The yield value and water content analysis showed that synthesized chitosan has economic value by using those methods. The MTT assay proved that chitosan is one of the material prerequisites to be applied in vivo in this study, due to the non toxic properties.

This chitosan has a high degree of deacetylation at 86.22 %, which has good solubility in water (Agustina *et al*, 2015). The source of chitin influenced the deacetylation degree and molecular weight of chitosan. This study used chitosan synthesized from *Penaeus monodon* shells that obtained from Indonesia. They are cultivated traditionally and have a high quality for international trading. The molecular weight of chitosan is influenced by the synthesis method, deacetylation temperature, deacetylation degree, particle size and the source of the chitin. By increasing the deacetylation degree of chitosan, more acetyl units will be removed. Futhermore, it will increase the number of amide groups (NH_2) and extend the length of the polymer chain. Finally, the molecular weight will be higher (Citrowati *et al*, 2019; Kasongo *et al*, 2020).

The high molecular weight and high deacetylation degree of this chitosan may increase the biocompatibility for the healing process particularly the number of the odontoblast-like cells. The high molecular weight and high deacetylation degree at 86.22% impacted chitosan characteristics to odontoblast-like cell. Chitosan with high molecular weight, has more monomer N-acetylate compared to the lower molecular weight chitosan. The high molecular weight would increase the viscosity. The high viscocity may increase the mucoadhesive characteristic, making it stronger and easier to apply to viscous tissues. The average of odotoblast-like cells is higher to a chitosan group than a $Ca(OH)_2$ group. The comparison of chitosan and $Ca(OH)_2$ towards the number of the odontoblast-like cells has a significant difference on the seven and the twenty-eight days following treatment. This may be caused by high deacetylation degree used as a direct pulp capping chitosan material. The high deacetylation degree of chitosan is followed by a high percentage of the acetyl units. The high deacetylation degree of chitosan is a source of active *N*-

acetyl-D-glucosamine dimer. If it is applied to wound areas, the inflamed cells would release a lysozim enzyme (Rezkita *et al*, 2020a,b). The neutrophil produced would migrate to wound areas by inflamed cells after several hours of injury and would maximum concentrations in 24 hours. *N-acetyl-D-glucosamine* monomer is tied to the main receptor–the mannose receptor – by chitosan macrophages. It also triggers macrophage migration and proliferation. Macrophage, which is the predominant cell, would migrate to wound areas after 24 hours and for approximately five days, and subsequently there was a significant decrease in inflamed cells decreasing after 7 days (Matsunaga *et al*, 2005). The deacetylation degree impacts chitosan biologically included chitosan biodegradation by lysozyme, chitosan produced by neutrophil and macrophage inflammation cells. Chitosan consisted of active dimer *N-acetyl-D-glucosamine*, and if experiencing biodegradation would be forming cross-links with glycosaminoglycan and glycoprotein that play a part in the biological process. Thus, it encircles cell interaction, cell matrix and also factors involved in growth activation (Ikeda *et al*, 2005). Growth factors and bone morphogenetic protein-2 (BMP-2), that is still related to transforming the growth factor superfamily, would stimulate osteoblastic cell differentiation (Prahasanti *et al*, 2020; Saskianti *et al*, 2020). Chitosan is able to accelerate wound healing through a fibrinogenic mediator, due to the growth factor. This is caused by chitosan's ability to form a polyelectrolyte complex with polyanion heparin, that could increase and prolong the half-life growth factors to cell differentiation stimuli. Chitosan is also suspected to be able to stimulate osteoprogenitor cells and bone swaging (Rezkita *et al*, 2020a,b). Chitosan has played a role in the electrostatic interaction by anionic glycosaminoglycan and proteoglycan that would effectively increase growth. The osteoblast cell culture that was stimulated by chitosan showed increasing Alkaline Phosphatase (ALP) mRNA expression, after three days and bone morphogenetic protein-2 (BMP-2) mRNA after seven days. Chitosan is able to directly stimulate multipotent mesenchymal progenitor and osteogenic cells. Chitosan stimulate the differentiation of osteogenic cells and therefore has a real potential to accelerate bone regeneration (Lahiji *et al*, 2000).

CONCLUSION

Although, there are many studies on chitosan, this study has suggested that chitosan synthesized from *Penaeus monodon's* shells from Indonesia as a direct pulpcapping material, may increase the number of the odontoblast-like cells on seven and twenty-eight days.

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REFERENCES

Agustina S, Swantara I M D and Suartha I N (2015) Isolasi kitin, karakterisasi, dan sintesis kitosan dari kulit udang. *J Kimia* **9**, 271-278.

Citrowati A N, Satyantini W H and Mahasri G (2019) Pengaruh kombinasi NaOH dan suhu berbeda terhadap nilai derajat deasetilasi kitosan dari cangkang kerang kampak (*Atrina pectinata*). *J Aquac Fish Health* **6**, 48.

Fadholly A, Ansori A N M, Proboningrat A, Nugraha A P, Iskandar R P D, Rantam F A and Sudjarwo S A (2020) Apoptosis of HeLa cells via caspase-3 expression induced by chitosan-based nanoparticles of *Annona squamosa* leaf extract: *In vitro* study. *Indian J. Pharmaceutical Education and Res.* **54** (2), 416-421.

Ikeda T, Yanagiguchi K, Matsunaga T, Yamada S, Ohara N and Ganno T (2005) Immunohistochemical and electron microscopic study of the biodegradation processes of chitin and chitosan implanted in rat alveolar bone. *Oral Med. Pathol. J.* **2**, 131-138.

Kasongo K J, Tubaidi D J, Bampole L D, Kaniki T A, Kanda N J M and Lukumu M E (2020) Extraction and characterization of chitin and chitosan from *Termitomyces titanicus*. *SN App. Sci.* **2**, 406.

Kuntjoro M, Prasetyo E P, Cahyani F, Kamadjaja M J K, Hendrijantini N and Laksono H (2020) Lipopolysaccharide's cytotoxicity on human umbilical cord mesenchymal stem cells. *Pesqui Bras Odontopediatria Clín. Integr*. **20**, e0048.

Lahiji A, Sohrabi A, Hungerford D and Frondoza C (2000) Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. *Biomed Mater. Res. J*. **51**, 586-595.

Matsunaga S, Yanagiguchi K, Yamada S and Ohara H (2005) Chitosan monomer promotes tissue regeneration on dental pulp wounds. *J. Biomed Mater. Res.* **76A**, 711-720.

Mitsiadis T (2004) Pararells between tooth development and repair: conserved molecular mechanism following carious and dental injury. *Dent. Res. J.* **83**, 896-902.

Muzzarelli R A, Mattioli-Belmonte M, Pugnaloni A and Biagini G (1999) Biochemistry, histology and clinical uses of chitins and chitosans in wound healing. *EXS* **87**, 251-264.

Nugraha A P, Prasetyo E P, Kuntjoro M, Ihsan I S, Dinaryanti A, Susilowati H, Hendrianto E, Narmada I B, Ernawati D S, Nugraha A P and Rantam F A (2020b) The effect of cobalt (II) chloride in the viability percentage and the induced hypoxia inducible factor-1α of human adipose mesenchymal stem cells (HAMSCs): An *in vitro* study. *Sys. Rev. Pharm.* **11**(6), 308-314.

Nugraha A P, Rezkita F, Puspitaningrum M S, Luthfimaidah M S, Narmada I B, Prahasanti C, Ernawati D S and Rantam F A (2020a) Gingival mesenchymal stem cells and chitosan Scaffold to accelerate alveolar bone remodelling in periodontitis: A narrative review. *Res. J. Pharm. Tech*. **13**(5), 2502–2506.

Prahasanti C, Nugraha A P, Saskianti T, Suardita K, Riawan W and Ernawati D S (2020) Exfoliated human deciduous tooth stem cells incorporating carbonate apatite Scaffold enhance BMP-2, BMP-7 and attenuate MMP-8 expression during initial alveolar bone remodeling in Wistar rats (*Rattus norvegicus*). *Clinical,*

Cosmetic and Investigational Dentistry **12**, 79–85.

Prasetyo E P, Widjiastuti I, Cahyani F, Kuntjoro M, Hendrijantini N and Hariyani N (2020) Cytotoxicity of calcium hydroxide on human umbilical cord mesenchymal stem cells. *Pesqui Bras Odontopediatria Clín. Integr*. **20**, e0044.

Prasetyo E P, Kuntjoro M, Goenharto S, Juniarti D E, Cahyani F, Hendrijantini N, Nugraha A P, Hariyani N and Rantam F A (2021) Calcium hydroxide increases human umbilical cord mesenchymal stem cells expressions of apoptotic protease-activating factor-1, caspase-3 and caspase-9. *Clin. Cosmet Investig. Dent.* **13**, 59-65.

Rahmitasari F (2016) Scaffold 3D kitosan dan kolagen sebagai graft pada kasus kerusakan tulang (studi pustaka). *J PDGI* **150**, 1-7.

Rezkita F, Sarasati A, Wijaya F N, Nugraha A P, Hendrijantini N, Ridwan R D, Ramadhani N F, Fadholly, Ansori A N M and Susilo R J K (2020a) Chitosan Scaffold, concentrated growth factor and gingival mesenchymal stem cells as th osteoporotic jaw therapy: A review. *Biochem. Cell. Arch*. **20**(Supplement 1), 2913-2919.

Rezkita F, Wibawa K G P and Nugraha A P (2020b) Curcumin loaded chitosan nanoparticle for accelerating the post extraction wound healing in diabetes mellitus patient: A review. *Res. J. Pharm. Tech.* **13**(2), 1039–1042.

Saskianti T, Nugraha A P, Prahasanti C, Ernawati D S, Suardita K and Riawan W (2020) Immunohistochemical analysis of stem cells from human exfoliated deciduous teeth seeded in carbonate apatite scaffold for the alveolar bone defect in wistar rats (*Rattus novergicus*). *F1000Res.* **22**(9), 1164.

Swann G E A and Patwardhan S V (2011) Application of fourier transform infrared spectroscopy (FTIR) for assessing biogenic silica sample purity in geochemical analyses and palaeoenvironmental research. *Clim Past*. **10**, 65-74.