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Viabilities of Odontoblast Cells Following Addition of Haruan Fish in Calcium Hydroxide

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Abstract

BACKGROUND: Haruan fish (*Channa striatus*) extract (HFE) contains all the essential amino acids and fatty acids that it is believed to have therapeutic value, accelerate wound healing, and anti-inflammation.

AIM: This study was aimed to examine the viability of odontoblast MDPC-23 cell lines following the addition of HFE in toxin of *Lactobacillus* sp. and/or $Ca(OH)_2$.

MATERIALS AND METHODS: First, to find antiproliferative effective doses, MDPC-23 cells were treated with HFE. The cell viability was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay on 24 h after the last treatment, while cell death was induced by addition toxin and/or $Ca(OH)_2$ following adding antiproliferative effective doses of HFE (25.0; 50.0; and 100 µg/mL). Untreated cells were used as control.

RESULTS: Adding of HFE at range 25.0 until 100 µg/mL increased the MDPC-23 cells viability. MDPC-23 on toxin and/or $Ca(OH)_2$ reported decrease the viability of cells, while supplemented with HFE significantly increase in cell viability compared to untreated cell ($p < 0.05$).

CONCLUSION: HFE effectively increased the viability of odontoblast MDPC-23 cells and has the potency to be used together to avoid the negative side effect of $(CaOH)_2$ as a capping agent.

Introduction

Inflammation of the pulp tissue may be initiated by various toxins, necrotic cells, or stimulation of odontoblasts. Damage of the odontoblasts induces the release of bioactive molecules and initiates an inflammatory response in the pulp [1]. On occasions, inflammation ceases rapidly and enables complete pulp healing with the formation of a barrier of reactionary dentin by the original surviving odontoblasts and/or reparative dentin by newly differentiated odontoblast-like cells in animal models [2], [3].

Odontoblast survival is sensitive to the remaining dentin thickness (RDT). A RDT of 1 mm has been suggested to protect the pulp from the harmful constituents of dental materials [4]. When the pulp-dentin complex of RDT is <0.5 mm, it should be protected from any injury with pulp capping materials [5]. The primary goal of capping the pulp is to preserve the underlying pulp and maintain pulp vitality through the formation of reparative dentin, which in turn increases the survival of teeth life expectancy and improves the overall oral health [6].

Calcium hydroxide ($Ca(OH)_2$) is the most widely used capping agent [7]. It has an antimicrobial effect and dissolves calcium ions to create an alkaline environment that can stimulate reparative dentin [8]. However, $Ca(OH)_2$ dressings have some drawbacks, tunnel defects were reported as incomplete dentin bridge formation that compromises the integrity of reparative dentin [9]. It is assumed that the high pH of $Ca(OH)_2$ is toxic to odontoblast cells, which may cause severe inflammation and incomplete cell regeneration.

Several measures were proposed in maximizing reparative dentin formation, such as to optimize direct capping techniques, improve the biocompatibility of the materials, and enhance the biological responses of the pulp tissues [10]. In a previous study, pulp inflammation was reduced by applying anti-inflammatory substances, but the results were not satisfactory [11].

Haruan fish (HF), *Channa striata*, is a natural ingredient rich in albumin that has been widely studied to have the ability to regenerate cells. Some studies reported that HF plays a role in increasing the viability of fibroblast cells [12], decreases macrophages [13], and

lymphocyte count in inflammation [14]. The chemical composition of HF content of amino acid glycine and proline was collagen sources potential [15]. HF can also increase the formation of new capillaries, which play an essential role in the healing process [16]. Palmitic acid which is isolated from *Gaultheria itoana* Hayata and *Sarcopyramis nepalensis* can suppress pro-inflammatory cytokines tumor necrosis factor-α, interleukin-6, cyclooxygenases-2, and nitric oxide in lipopolysaccharide-stimulated mouse peritoneal macrophages [17]. Therefore, we assumed that HF might be used in dental materials to improve the effectiveness of $Ca(OH)_2$ as a pulp capping agent since no studies have ever been performed in odontoblast cells.

Cell culture laboratory studies are beneficial for investigating some of the basic cell interaction/response requirements of the materials, although they have limitations because these cultures studies do not represent cell behavior in 3D structures [18]. This preliminary study is aimed to evaluate the viability of odontoblast MDPC-23 cells after the addition of Haruan fish (*Channa striatus*) extract (HFE) in toxin of *Lactobacillus* sp. and/or $Ca(OH)_2$.

Materials and Methods

Materials

Materials that were used in this study include Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin 10000 U/mL, trypsin-EDTA 0.25%, sodium dodecyl sulfate, and amphotericin B were purchased from Gibco, Grand Island, NY, while 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich, St. Louis, MO.

Research design

This was an experimental laboratory study with "post-test only control group design," conducted in the Laboratory of Hasanuddin University Hospital under the approval of the Ethics Commission of the Dental and Oral Hospital, Faculty of Dentistry, Hasanuddin University (0015/PL.09/KEPK FKG-RSGM/UNHAS/2018). The experiment design can be seen in Figure 1.

Sampling and extraction

The selected HF that is still alive came from the ponds of Tanjung Redeb, Berau, East Kalimantan. The fish used was firstly cleaned from the heads, guts, and scales. The flesh was extracted by steaming in a closed pan for ±30 min until the pale yellowish liquid was obtained and separated. The liquid extract was further processed in powder using the freeze dryer (Büchi, German) and stored at the freezer, called HFE. Gently weighed 10 mg of HFE and dissolved in 10 mL DMEM medium (1000 µg/mL) as a stock solution, the diluted until reach 25.0; 50.0; and 100 µg/mL.

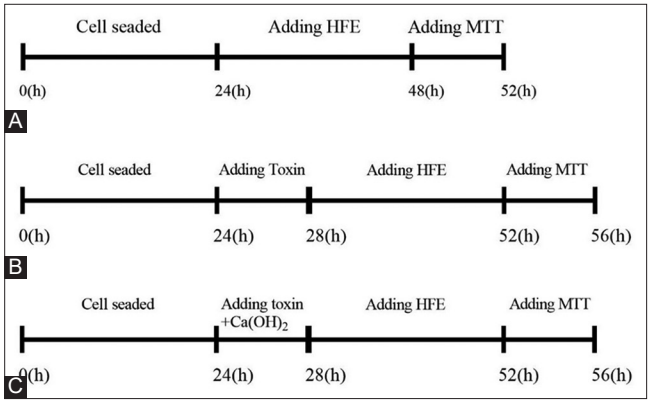

Figure 1: Scheme of the experiment protocol in the present study. Cell viability following application of HFE (A), induced by toxin (B), induced by toxin and $Ca(OH)_2$

Cell lines culture

The following established *in vitro* cell lines were applied in odontoblast MDPC-23 cells (ATCC-CRL 2537). The MDPC-23 cells were cultured in DMEM medium supplemented 10% FBS, penicillin (100U/mL), streptomycin (100µg/mL), and 0.5% fungizone in plastic flasks (Iwaki, Japan) at 37°C in a 5% CO_2 incubator. After reaching confluence, cells were harvested by 0.25% trypsin-EDTA.

Determination the effect of HEF on cell viability

The harvesting cells were seeded into well plate 96 (10^5 cell/mL). All the cells were treated with HFE and incubated for 24 h. The medium discarded and washed with PBS. The cell viability was measured by MTT assay. About 100 µL MTT (0.5 mg/mL in DMEME) was added to each well, and the plate was incubated for 4 h at 37°C in a CO_2 incubator. Formed formazan crystals were solubilized by adding 100 µL of dimethyl sulfoxide per well. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the microplate reader at 540 nm. The viability of the cell was calculated using the formula: Cell viability (%) = Mean OD/Control OD × 100%.

Experimental models

Spread the harvested cells into well plate 96 (10^5 cell/mL). To induce injury and death, the cells were contacted with 0.04 µL toxin of *Lactobacillus* sp.

alone or with $Ca(OH)_2$ for 4 h then added with HFE. The number of cells was determined using the MTT method as described before (determination the effect of HEF on cell viability). The viability of the cell was calculated using the formula: Cell viability (%) = Mean OD/Control OD × 100%.

Statistical analysis

Data were collected in an Excel worksheet and analyzed by ANOVA. Data are presented as the mean ± standard deviation after deductible with baseline (untreated cell as 100%). Differences with $p < 0.05$ were considered significant. All of the experiments were evaluated in three replicates (n = 3).

Results

To select the optimal HFE concentrations for the cell culture experiments, we investigated the effect of HFE on cell viability after incubation for 24h by MTT reduction assay. Viability of MDPC-23 cells increased significantly following the addition of each concentration of HFE, as seen in Figure 2. It is meaning a safe dosage range to be used for further biological.

The effect of HFE on the viability of MDPC-23 cells induced *Lactobacillus* sp. toxin was illustrated in Figure 3. In general, despite the presence of the toxin, the viability of MDPC-23 cells increased significantly on each concentration of HFE. The toxin group indicates the decreasing of cell viability (−5.51 ± 7.00%) without the influence of the HFE. For the HFE, the treated cells with the lowest concentration (25.0 µg/mL) showed a significant increase in viability with a value of 9.54 ± 7.00%. When moderate and high concentration (50.0 and 100.0 µg/mL), they showed a relatively similar effect on the percentage of viability cells with a value of 26.38 ± 14.00 and 28.04 ± 10.07%.

The viability of MDPC-23 cells increased significantly, albeit $Ca(OH)_2$ and toxin of *Lactobacillus* sp. were added on each concentration of HFE as seen in Figure 4 at a dose-dependent manner. On the other hand, HFE 100 µg/mL showed the highest effect on the viability of the cell.

Discussion

The dental pulp is a connective tissue that responds to stimuli and insults. Maintenance of healthy pulp tissue is essential for the function and survival of

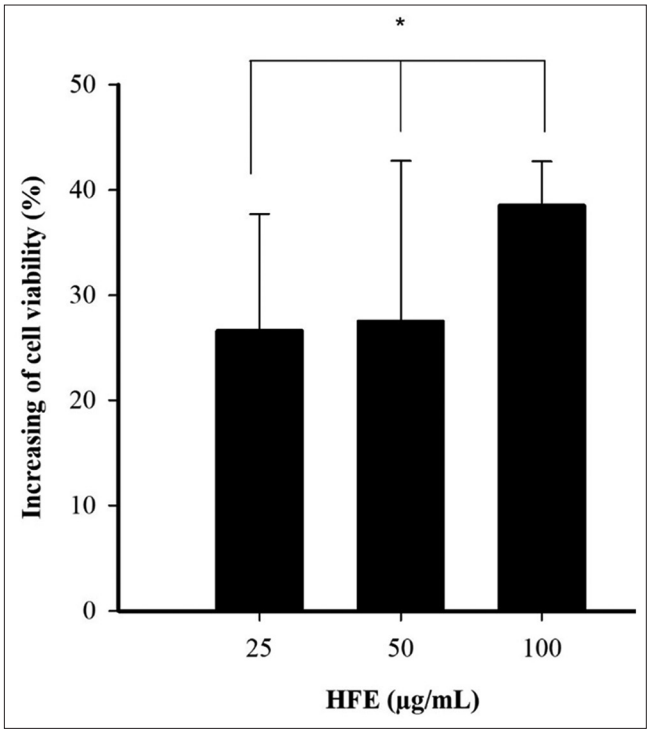

*Figure 2: Effect of Haruan fish (Channa striatus) extract (HFE) on the viability of MDPC-23 cells. Cell viability following incubation with indicated concentrations of HFE for 24h was determined using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability is expressed as a percentage of the increasing cell compared to the untreated cells. *p < 0.05 (HFE 100 µg/mL compared to 25 and 50 µg/mL)*

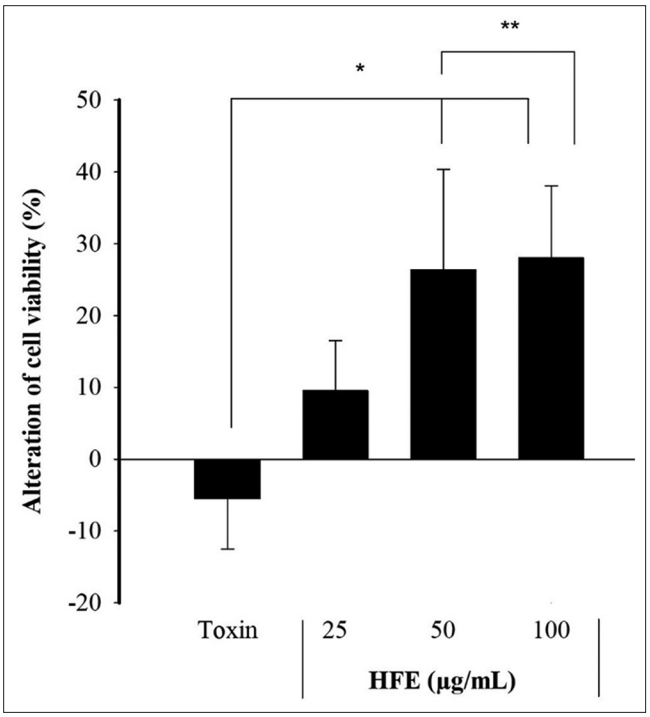

*Figure 3: Effect of Haruan fish (Channa striatus) extract on the viability of MDPC-23 cells was induced by 0.04 µL toxin of Lactobacillus sp. The viability of cells was determined after 24h of incubation by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability is expressed as a percentage of the increasing cell compared to the untreated cells. *p < 0.05 (All treatment group compared toxin); **non-significant effect between the group*

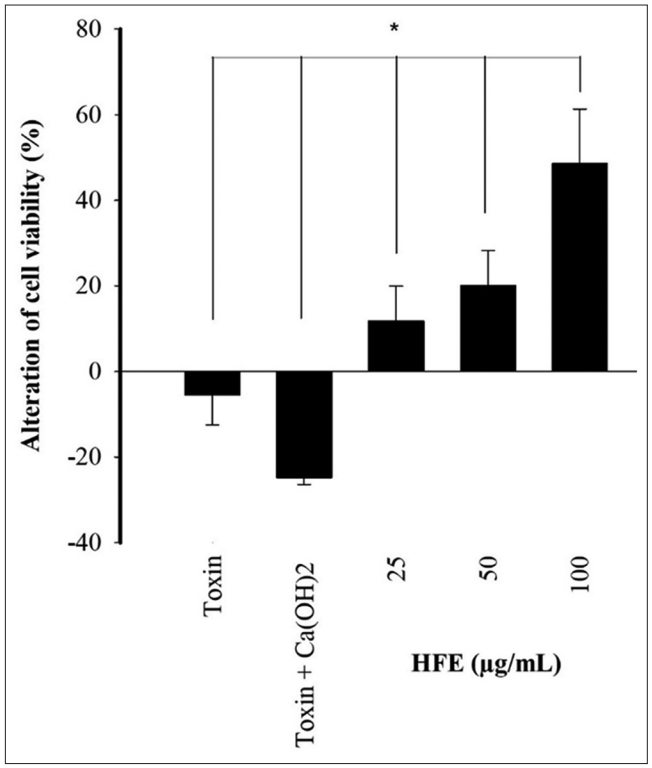

*Figure 4: Effect of Haruan fish (Channa striatus) extract on the viability of MDPC-23 cells was induced by 0.04 µL toxin of Lactobacillus sp. and $Ca(OH)_2$. The viability of cells was determined after 24h of incubation by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability is expressed as a percentage of the increasing cell compared to the untreated cells. *p < 0.05 (All treatment group compared toxin and $Ca(OH)_2$)*

tooth [2]. Formation of secondary and tertiary dentin serves to protect the tooth from caries, infections, and traumatic dentin exposure. On the other hand, trauma, wear, or disease may impair the barrier and lead to pulpal necrosis by many insults during the lifetime of the tooth [19], [20]. Various toxins, necrotic cells, or stimulation of odontoblasts may all cause harmful effects on the pulp tissue with both acute and chronic responses depending on the magnitude and duration of the insult [21].

Cavity base materials are designed for the purpose of protecting the pulp from damage by toxins or material components. The potential ability of cavity base materials in inducing tertiary dentin formation was believed to be necessary for their function. This ability is mediated through the release of growth factors and other bioactive molecules [22]. $Ca(OH)_2$ is a cavity base material that induces tertiary dentin formation through the activation of blast cells in the pulp. These cells are recruited as new hard tissue-forming cells replacing the destroyed odontoblasts. $Ca(OH)_2$ produces a zone of superficial necrosis, which serves as a scaffold for an underlying hard tissue repair, forming a dentin bridge. This bridge is seldom continuous and complete, which compromises the barrier [9]. Several bioactive molecules have been experimentally tested for intended use as pulp capping and reparative dentin-forming materials and some products have shown some potential.

HFE is a natural ingredient that has been studied to have the ability to regenerate fibroblast cells [12]. This is in line with the results of this study that the viabilities of the odontoblast cell line significantly increase at each concentration of HFE added to the cells (Figure 2). The viability of these cells slightly decreased when a toxin was incorporated (Figure 3). This may be explained that the toxin used was an inactive toxin of *Lactobacillus* sp., with the purpose of injuring the cells that simulate the conditions of the oral cavity where bacterial toxin triggers injury and inflammation of the pulp cells [23]. In addition, bacterial toxins do not directly contribute to the cell's viability but act as an inflammatory agent. Lipoteichoic acid, the endotoxin of *Lactobacillus* sp. can provoke the immune response and activate odontoblast cells through TLR2, phosphorylate the intracellular pathways, activate the transcription factor nuclear factor-κB, and mitogen-activated protein kinase, which in turn synthesize various pro-inflammatory mediators and disrupt the normal functioning of the body [6], [24]. Therefore, the addition of HFE could increase the viability of cells in the presence of toxin (Figure 3).

$Ca(OH)_2$ has long been recommended for capping the exposed pulp. The main goal of a pulp capping treatment is to maintain pulp vitality of the affected tooth. A cascade of physiologic, biochemical events occurs, starting from a slight inflammatory reaction to generating chemical signals that will lead dental pulp progenitor cells to migrate to the affected area. These cells have been shown to proliferate and differentiate to form a mineralized bridge that will separate the pulp tissue from the noxious stimuli to avoid further damage and eventually to enable the pulp to heal [18], [25]. Contrastly higher dose, $Ca(OH)_2$ is toxic to normal cells, which may cause severe inflammation and incomplete cell regeneration [26].

In this study, $Ca(OH)_2$ and toxin significantly decreased the viabilities of cells to 24.7% (Figure 4). $Ca(OH)_2$ is known to have a high pH that has a degenerative effect on cells, although it is generally accepted as the material of choice for capping the exposed pulp. It was reported that in some cases, the pulp remains chronically inflamed, and the tissue degenerates. The hydroxyl ions released are free radical and highly reactive. It causes protein denaturation, proliferation inhibition, and ATP depletion resulting in necrosis and cell lysis [11], [27]. This may partly explain the decreased cells viability. However, the addition of HFE significantly reincreased the viability of the cells and was in accordance with a dose-dependent manner (Figure 4). These results are similar to the previous study, who reported the highest percentage increase in the number of fibroblast cells at a concentration of 100%. This might be attributed to the high albumin content in HFE which is a crucial nutrient for recovery of damaged cells [12], [14], [16], [28], [29]. Albumin also plays an important role in maintaining the capillary

osmotic pressure that can prevent excessive edema and avoid cell death [30].

Albumin of bovine serum has ever been studied and developed as a biodegradable cavity liner, a hydrogel with the potency as a capping agent. Hydrogel constitutes injectable scaffolds, cells, and bioactive molecules carriers for tissue engineering. Using the rat model, hydrogel showed homogenous dentinal bridge formation compared to Dycal capping with tunnel disturbances after 3 weeks. The inflammation was weak or barely detectable after hydrogel capping. In contrast, Dycal capping has a continuously high pH which causes superficial burn covering a scar at the pulp surface with the consequence of inflammation in a limited area. This inflammation is still observed after 4 weeks and a mild necrotic area was noticed between the reparative dentinal bridge and vital pulp [31]. Investigations of HFE are still in their infancy, and whether it has the potency as albumin of bovine serum needs further studies.

Under the limitations of this study, HFE has shown its effectiveness to increase the viability of odontoblast MDPC-23 cells despite the presence of toxin and $Ca(OH)_2$. Further studies should be carried out using an animal model to evaluate pulp tissue reactions when exposed to these bioactive substances that might be utilized in dentistry.

Conclusion

The HFE enhanced the proliferation of odontoblast cells on MDPC-23 cells induced toxin and $Ca(OH)_2$. It suggests that HFE has the potency to be used together with $Ca(OH)_2$ as a capping agent to induce the formation of reparative dentin.

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