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RESEARCH ARTICLE

Anti-inflammatory loading of cinnamaldehyde on artificial bone scaffolds against the process of bone regeneration

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ABSTRACT

Cinnamaldehyde is an organic component in cinnamon and has anti-inflammatory, antibacterial, and osteogenic properties. Loading cinnamaldehyde with a concentration of 4% into an artificial scaffold from gypsum calcium carbonate hydrogel could reduce inflammation, accelerate healing, and promote new bone regeneration at bone implantation surgery. The aim of this study was to investigate the impact of cinnamaldehyde loaded on gypsum-hydrogel calcium carbonate on the process of wound healing and bone regeneration in rats. Implantation of bone scaffolding from gypsum hydrogel calcium carbonate with an addition of cinnamaldehyde was performed on 20 5-month-old Sprague-Dawley rats weighing 300-350 g in an artificial defect of condyle femoris bone dexter and sinister. Group A was a control with gypsum only, while groups B, C, and D were given gypsum and $CaCO_3$ with a combination of cinnamaldehyde or dehydrothermal treatment (DHT). Euthanasia was performed after implantation at 1, 4 and 8 weeks for 4 groups (n = 3). Femoris condyle bone was cut, made into histological preparations by hematoxylin eosin (HE) staining, and analyzed descriptively. The results showed that the loading of cinnamaldehyde on the scaffold was effective in weeks 1 and 4, but after 8 weeks of implantation, cinnamaldehyde was observed to inhibit defect closure. The cinnamaldehyde group combined with dehydrothermal (DHT) treatment was found to be better than those without DHT.

Keywords: bone scaffold; cinnamaldehyde; new bone regeneration

INTRODUCTION

Cinnamon oil or cinnamaldehyde, extracted from the bark of cinnamon tree, has been utilized in the nourishment industry as a flavouring and additive substance for centuries.[1] Cinnamon extract and essential oil offer advantages to human wellbeing and are recognized as an antimicrobial, antibacterial and anti-inflammatory dietary products.[2] Cinnamon essential oil (EO), cinnamon extract, and its main components show significant antimicrobial activity against oral pathogens and could be valuable for the prevention of caries, the treatment of periodontal and endodontic diseases, and candidiasis.[3] Cinnamaldehyde has also been shown to have antibacterial action,[4] anti-inflammatory,[5,6] anticancer[7] and antidiabetic properties.[8]

Several studies have found that antibacterial activity in cinnamon and its constituents, such as cinnamaldehyde and cinnamic acid, have the effect of reducing the number of pathogenic Gram-positive and Gram-negative bacteria.[2,4,9,10] As an aldehyde, cinnamaldehyde can also act as a crosslinker agent (cross binder); therefore, it can replace glutaraldehyde which tends to be toxic to tissues.[4] In the development of alloplastic bone scaffold modifications, these crosslinking agents largely determine the time of release of the active substance in bone defects. Surface properties, chemical properties, hydrophilicity, and topography are known to influence the interaction between cells and substrates in the environment surrounding the implanted material.[11] Cinnamaldehyde can act similarly to glutaraldehyde as a crosslinker for a type of polymer commonly used as a bone scaffolding material.[4]

Dewi et al in their research showed that cinnamaldehyde as an anti-inflammatory agent can be effectively loaded into $CaCO_3$ hydrogel-plaster of paris (POP) composite. The result showed that the addition of cinnamaldehyde to the hydrogel framework increased the contact angle, but still < 90° (hydrophilic).[11] Many studies have focused on modifying implant surfaces to improve implant success rates in patients. One modification strategy is to alter the surface topography of the implant.[1] The aldehyde chain of cinnamaldehyde as a crosslinking, serves as a plasticizer for the $CaCO_3$ hydrogel,[4] allowing the cinnamaldehyde to be loaded into the hydrogel- containing scaffold.

We employed dehydrothermal (DHT) treatment to generate the formation of intermolecular crosslinks through a condensation reaction in a functional group of gelatin to decrease the crosslink effect capability of cinnamaldehyde.[12] Crosslinking gelatin can be achieved by physical treatments, such as heat (DHT), UV radiation, and gamma radiation, and chemical treatments, for example aldehydes (such as formaldehyde, glutaraldehyde (GA) or glyceraldehyde), poly-epoxides, and isocyanates.[13] GA is currently the most used crosslinking molecule because it is inexpensive, and it works well with collagen-based materials. However, GA is cytotoxic.[14] Therefore, DHT treatment is used to improve the properties of collagen because this does not involve the use of any chemical reagent.

In the process of wound healing in implantation surgery, it has been reported that either mild or severe inflammation always happens. Apart from having an anti-inflammatory and anti-bacterial activity, cinnamaldehyde has been shown to have osteogenic properties[15] that can trigger osteogenesis and initiate bone formation by promoting osteoblastic differentiation and inhibiting osteoclast activity in ovariectomy mice. This suggests that cinnamaldehyde can be a dynamic substance to promote osteogenesis and possibly play role in clinical administration of osteoporosis care.[15,16,17] Modification to the implant surface by adding cinnamaldehyde can reduce inflammatory reaction after surgery. The objective of this study was to determine whether there was an effect of cinnamaldehyde loaded on gypsum-hydrogel calcium carbonate on wound healing and bone regeneration in in vivo study of Sprague-Dawley rats after implantation for 1, 4 and 8 weeks.

MATERIALS AND METHODS

Animal experimentation in this research was approved by the Ethics Committee of the Integrated Research and Testing Laboratory of Universitas Gadjah Mada, Yogyakarta (project number 00003/04/LPPT/II/2017). Twenty 5-month-old male Sprague-Dawley rats weighing 300-350 g were put in an animal care facility for 7 days in

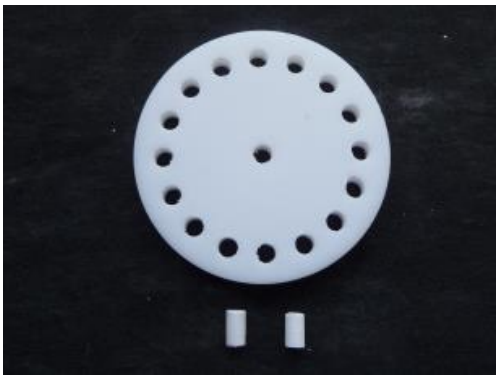

Figure 1. The cylindrical sample with 2.5 milimeters in diameter and 3 milimeters in thickness

Table 1. Sample Distribution

Groups	Gypsum	Hidrogel $CaCO_3$	Ratio gypsum: Hidrogel $CaCO_3$	Sinamaldehid 4%	DHT
A	+				
B	+	+	1:1		+
C	+	+	1:1	+	
D	+	+	1:1	+	+

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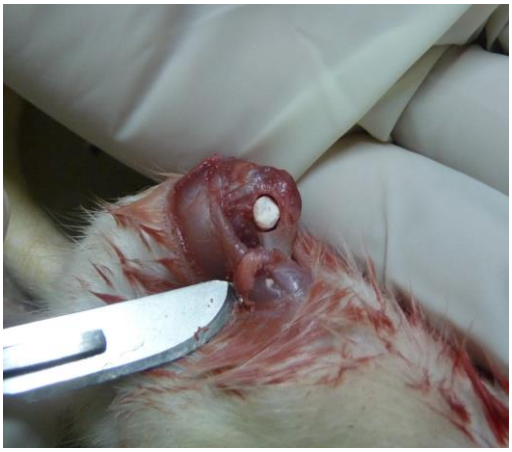


Figure 2. Articial defect in rat's femure condyle dexter and sinister

a standard cage with a 12-hour light/12-hour dark cycle. They were fed everyday and given water *ad libitum*. National guidelines for the care and use of research facility animals were followed during the study. These experimental animals were put in cages in the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada.

Four percent of cinnamaldehyde was loaded into a blend of gypsum and calcium carbonate hydrogel in a predetermined proportion. The diameter of the cylindrical sample was 2.5 millimeters and the thickness was 3 millimeters (Figure 1).

Sample was sterilized using ethylene oxide gas (EOG) at Bethesda Hospital. The research group was separated into 4 (Table 1), the control group with gypsum (group A) and the treatment groups (groups B, C, and D), with a combination of cinnamaldehyde, gypsum hydrogel calcium carbonate, and with or without dehydrothermal treatment (DHT).

Experimental animals had been examined for general health before being used for research. All equipment and research materials were accessible on the operating table under sterile conditions. All fundamental drugs were also accessible, weighed according to the dosage per individual rat, labelled, and recorded for each rat. The surgery was performed under general anaesthesia with intramuscular (IM) injection of xylazine 0.1-0.2 mg/kg body weight after an IM ketamine injection of 10-15 mg/kg body weight. The lateral fur of the left and right legs of the rats was shaved, washed with PBS, and applied with povidone iodine disinfectant. Longitudinal incisions were performed to the depth of the bone alternately. After the bone area was visible, bone opening was carried out using a round shape bone bur to create defects and proceeded with an implant bur with a diameter of 2.5 mm to a depth of 3 mm at a speed of 800-1000 rpm.

The surgical implant motor used Implant cube from Saeshin®, Daegu, South Korea, which had high speed low torque specifications to guarantee the artist's lege in implantation management. All through the opening and creation of the defect, a stream of physiological solution was applied to suppress the excess of temperature rise within the operating range. After reaching the desired defect size, implantation was carried out according to the randomization table. The scar was cleaned, closed, and sutured using paint gut and vicryl suture 2.0. The operating area was cleaned as much as possible and disinfected once more. A topical application of gentamicin 0.1% was applied to the scar surface.[15]

To avoid postoperative infection, antibiotics were administered through subcutaneous (SC) injection with interflox-100 10 mL/20-40 kg body weight on the day of surgery to the third day postoperatively. Pain was anticipated by giving xylazine for pain relief of 0.01-0.02 mg/kg body weight through intravenous (IV) injection after IV injection of butorfanol 0.01-0.02 mg/kg body weight from the first day to the third day postoperatively. Examination of the condition of the scar area and the general condition of the experimental animal was carried out the first 2 weeks after surgery. Medical records for each experimental animal were made.[18]

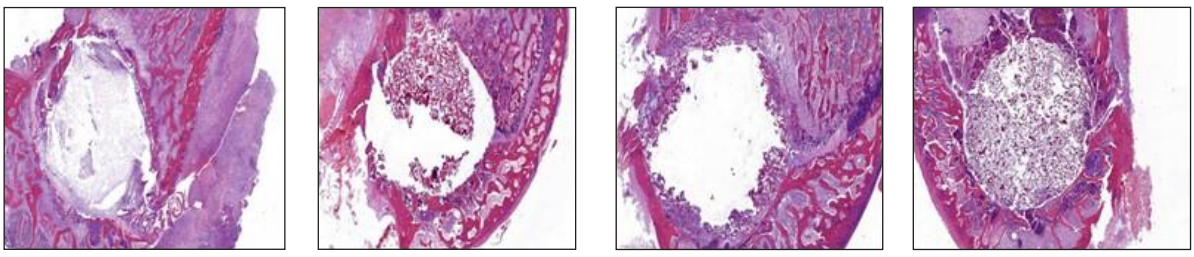
The experimental animals were killed according to the required period (1, 4, and 8 weeks post-implantation) by an overdose of ketamine injection. Vital signs were examined to confirm that the experimental animals were dead. Area of the surgery was dissected by cutting the femoral condyle. The specimen was fixed using 10%

PBS-formalin by transcardiac perfusion. Once the femoral bone had been fixed and cleared of all soft tissues, the condyle portion of the femoris was cut into smaller pieces with diamond bur and fixed with 10% formalin buffer for 1 day. The sample was then dehydrated using a graded series of ethanol from 50% to 100%, and the implant was cut in cross-sectional, perpendicular direction on longitudinal axis. Staining used hematoxylin eosin and examined under a light microscope (Leica Microsystem AG, Wetzlar, Germany). The parameters for evaluating the success of bone regeneration included looking at inflammatory cells (leukocytes, PMNs) and the ratio of recurrence with descriptive analysis.[18]

RESULTS

Observation of the results of implantation of artificial scaffolds with cinnamaldehyde in vivo in 5-month-old experimental animals of Sprague-Dawley rats for 1, 4 and 8 weeks showed new bone regeneration in the defect area (Figures 3, 4 and 5).

The implant material was still visible in all groups (Figures 3 A-D), marked with more purple painted parts. Inflammatory cells, especially PMN cells such as neutrophils, and monocytes were found around the rest of the implant material inside the artificial defect. A small amount of residual gypsum was observed after 1 week of implantation. The distribution of PMN cells was still visible around the rest of the gypsum. This is in accordance with research by Dewi et al[18] which showed that gypsum degrades faster when exposed to body fluids in the defect area, so the implant material disappears quickly before the bone regeneration process is fully formed (Figure 3 A).[18] Group B with gypsum and calcium carbonat inside, showed esidual implant material was observed in the defect area with an uneven location (Figure 3 B). The calcium carbonate hydrogel scaffold was shown to withstand the degradation rates of gypsum. The distribution of PMN cells followed the location where the remaining implant material was still present. In group C, artificial scaffolding was charged with cinnamaldehyde as an anti-inflammatory and antibacterial agent. Cinnamaldehyde can also act as a crosslinker agent for calcium carbonate hydrogel due to its aldehyde content. The remaining implant material in group C (Figure 3 C), which was without treatment with DHT, was less than in preparation B (Figure 3 B). The rest of the implant material was still visible on the edge of the inner bone defect. PMN cells were scattered around the rest of the implant material. The center of the defect area appeared empty. This empty part was prone to being infiltrated by fibrous tissue, thus disrupting the process of forming new bone. In group D, implantation in the defect area used samples of artificial bone scaffolding with cinnamaldehyde loading combined with DHT treatment. A large



(A) (B) (C) (D)

Figure 3. The results of implantation for 1 week. Artificial defect in femoral condyle bone of 5-month-old Sprague-Dawley rats. Paraffin embedding was followed by the staining process using hematoxylin eosin. The implant was cut in cross-sectional, perpendicular direction on longitudinal axis. Observation used a light microscope (Leica Microsystem AG, Wetzlar, Germany) with a magnification of 10 x 10. (3 A) Bone defects in the control group with gypsum implantation. The treatment groups (B, C, and D) were implanted with gypsum and calcium carbonate hydrogel bone scaffold. (3 B) bone scaffold with DHT treatment, (3 C) bone scaffold with cinnamaldehyde as crosslinker and (3 D) bone scaffold with cinnamaldehyde as crosslinker and DHT treatment.

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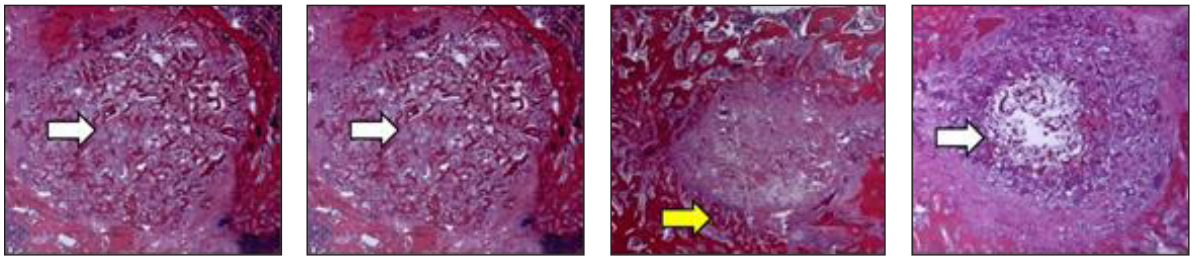
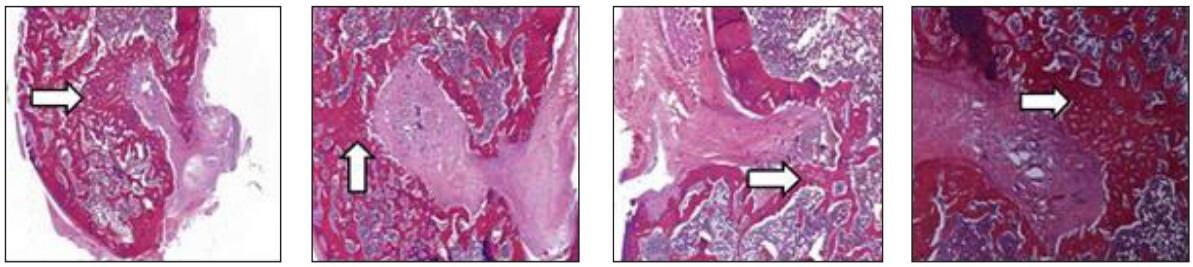


(A) (B) (C) (D)

Figure 4. Implantation results for 4 weeks. Artificial defect in femoral condyle bone of 5-month-old Sprague-Dawley rats. Paraffin embedding was followed by the staining process using hematoxylin eosin. The implant was cut in cross-sectional, perpendicular direction on longitudinal axis. Observation used a light microscope (Leica Microsystem AG, Wetzlar, Germany) with a magnification of 10 x 10. (4 A) Bone defects in the control group with gypsum implantation. The treatment groups (B, C, and D) were implanted with gypsum and calcium carbonate hydrogel bone scaffold. (4 B) bone scaffold with DHT treatment, (4 C) bone scaffold with cinnamaldehyde crosslinker and (4 D) bone scaffold with cinnamaldehyde crosslinker and DHT treatment. White arrows indicates residual implant material (4 B and 4 D). Yellow arrows indicate the regeneration of new bone at the beginning of the peripheral defect area.



(A) (B) (C) (D)

Figure 5. Implantation results for 8 weeks. Artificial defect in femoral condyle bone of 5-month-old Sprague-Dawley rats. Paraffin embedding was followed by the staining process using hematoxylin eosin. The implant was cut in cross-sectional, perpendicular direction on longitudinal axis. Observation used a light microscope (Leica Microsystem AG, Wetzlar, Germany) with a magnification of 10x10. (5A) Bone defects in the control group with gypsum implantation. The treatment groups (B, C, and D) were implanted with gypsum and calcium carbonate hydrogel bone scaffold. (5B) Bone scaffold with DHT treatment, (5C) bone scaffold with cinnamaldehyde crosslinker and (5D) bone scaffold with cinnamaldehyde crosslinker and DHT treatment. White arrow indicates new bone regeneration after 8 weeks of implantation.

amount of implant material in group D (Figure 3 D) remained and filled the entire defect area. This showed that cinnamaldehyde loading combined with DHT treatment gave better results at implantation for 1 week. Results showed that the degradation of artificial bone scaffolding continued during the first 1 week. The resulting distribution of PMN cells also appeared more uniform and was present around the rest of the remaining implant material (Figure 3 D). PMN cells appeared in response to the scaffolding material, indicating acute inflammation due to implantation wounds. The addition of cinnamaldehyde could relieve inflammation and accelerate healing.

At 4 weeks of implantation (figure 4), it indicated that degradation scaffold in all groups. New bone grew from the edge towards the center of the defect. The gypsum in the control group was shown to degrade quickly, leaving an empty room in the defect area. Inflammatory cells were still visible at 4 weeks of implantation (Figure 4 A). Group B showed the distribution of inflammatory cells (Figure 4 B) among the remaining implant material that was still visible in some places. Similar to that in groups A and B, the defect in groups C and D appeared to have not closed. The defect area in group C tended to be filled with fibrous tissue (Figure 4 C) although bone formation

progressed from the periphery to the middle. The defect area in group D was still predominated by the remaining implant material, which persisted until 4 weeks after implantation. Inflammatory cells at week 4 were dominated by lymphocytes and macrophages (Figure 4 D).

At 8 weeks of implantation (Figure 5), it appeared that the defect had not closed in all groups. In group A (gypsum), it appeared that the defect had closed about 75% (Figure 5 A), while in groups B, C, and D, the closure only ranged from 25% to 50% (Figures 5 B, 5 C, and 5 D). The rest of the implant material in all groups was almost completely absent. The defect was dominated by fibrous tissue, mainly in groups B and C (Figures 5 B and 5 C).

DISCUSSION

Cinnamaldehyde or cinnamic aldehyde (3-phenyl-2-propenal) is the main component in cassia oil and cinnamon bark oil.[19] Cinnamaldehyde is a chemical ingredient of cinnamon that has anti-inflammatory,[5,6] anti-bacterial,[4] and osteogenic properties.[15] Gypsum-based artificial bone scaffolding and calcium carbonate hydrogel have been shown to increase osteogenesis.[4,18] Cinnamaldehyde loaded in gypsum calcium carbonate hydrogel scaffolding has been shown to reduce swelling when immersed in phosphate buffered saline (PBS) and reduce its degradability rate. In addition, a 4% concentration of cinnamaldehyde loaded in artificial scaffolding was shown to have no toxic effect on human gingival fibroblast cells.[4] In previous research, Dewi et al have shown that the construction of calcium carbonate gypsum hydrogel scaffolding increases its mechanical strength after being mixed with cinnamaldehyde.[19] The speed of degradability of the scaffold in the artificial defect bone can be seen from the description of the remaining implant material up to 4 weeks after implantation.[19] In control group (group A) with gypsum, the rest of the implant material only lasted up to 1 week compared to other groups. The treatment groups (B-D) were implanted with gypsum and calcium carbonate hydrogel bone scaffold. Group B (bone scaffold with DHT) and group C (bone scaffold with cinnamaldehyde) showed similar patterns in the defect area, consisting mainly of fibrous tissue. Regeneration of new bone in groups B and C was slower than that in group D (bone scaffold with combination of cinnamaldehyde loading and DHT treatment). This may indicate that the effect of cinnamaldehyde as a crosslinker is mutually reinforcing with DHT treatment of crosslinking in hydrogel systems. If this crosslinking was detached, gypsum and calcium carbonate would be released into the extracellular environment, triggering osteogenesis.

Research by Dewi et al shows that the addition of cinnamaldehyde affects the average degradation of bone scaffold.[4] The higher the concentration of cinnamaldehyde, the lower the average degradation.[4] The ratio of the amount of hydrogel also affects the release of the active substance of the scaffold because the process of hydrogel swelling when exposed to body fluids initiates its degradation. The hydrophilicity of the hydrogel results in increased swelling of the polymer network and creates a larger pore size.[19] The speed of release of the active substance depends on the selected crosslinker agent and the type of treatment. Babu et al showed that cinnamaldehyde can add crosslink effects to chitosan nanoparticles as baicalin carriers.[20] The effect of cinnamaldehyde crosslinks is replaced by physical treatment using heat, namely dehydrothermal (DHT), which creates intermolecular crosslinks through condensation reactions in the gelatin functional group.[20]

In groups B, C, and D (Figure 4), the healing process appeared to still be underway after 4 weeks of implantation. In addition, remnants of implant material were still present, and the distribution of inflammatory cell infiltrated around it. It showed that cinnamaldehyde has positive effect as crosslink on preventing the rate of scaffold degradation. However, at 8 weeks implantation (Figure 5), almost all defects have not closed. The gypsum control group showed better bone regeneration than groups B, C, and D, especially

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8 weeks post-implantation. A possible explanation for this might be because the remaining gypsum implant material ran out the fastest, causing the inflammatory process to subside faster, and the bone regeneration to occur faster. In actual clinical conditions, sometimes bone defects can involve large wounds and irregular defects. In large wounds and extensive defects, the healing process must be assisted by external treatment with bone scaffold implantation because it cannot heal on its own. Sometimes anti-inflammatory and antibacterial agents are needed and added into the scaffold to speed up healing. Cinnamaldehyde has been recognized as an antimicrobial, antibacterial and anti-inflammatory agent that can be added to bone scaffold.

At week 8 of implantation (Figure 5), bone defects in experimental animals still did not close as expected (groups B, C, and D). It might be because the concentration of cinnamaldehyde was too high, and as a result, the content of gypsum and calcium carbonate hydrogel as a trigger for osteogenesis was lower than the expected result. It also seemed possible that cinnamaldehyde was not resorbed as quickly in the defect area, thus interfering with the process of osteogenesis. At 1 to 4 weeks, the process of wound healing and regeneration of new bone appeared to show good results but was different at 8 weeks after implantation. Supposedly cinnamaldehyde should not interfere with the process of new recurrence in the area. The concentration of cinnamaldehyde of 4% in this study referred to the research of Dewi et al, which was tested *in vitro* against fibroblast cells in cell culture.[4] Cinnamaldehyde of 4, 6, 8, 10% with dilutions of 25, 37.5, and 50 mg/mL was shown to be safe for fibroblast cells. This suggests that the concentration of cinnamaldehyde for cell testing in *in vitro* culture should be redetermined if it is to be tested in vivo in experimental animals because the extracellular environment in living tissue may be different from the environment in cell culture. Inflammatory reactions in soft tissue are usually more severe than bone tissue, so the selection of in vivo testing sites also determines the dose. Further research can be done to test the concentration of cinnamaldehyde on artificial bone scaffolds to achieve better results without interfering with the bone regeneration process.

In daily clinical examination of experimental animals during the implantation process, it was found that healing of soft tissue wounds, including skin and musculus around the implantation defect, occurred between 4 to 5 days after surgery and hair grew normally covering the surgical area. The experimental animals showed active behaviors immediately 1 day after surgery, and nutritional intake was considered normal. This may indicate that cinnamaldehyde loading with a concentration of 4% did not interfere with the healing process of open wounds in the soft tissues around the implant. The healing process in the area of bone defects showed good results up to 4 weeks. After 8 weeks of implantation, it appeared that there was a delay in the bone regeneration process.

CONCLUSION

The loading of cinnamaldehyde as an anti-inflammatory agent on gypsum and calcium carbonate hydrogel bone scaffolding proved effective at 1 week and 4 weeks of implantation. At 8 weeks of implantation, the bone regeneration process of the group with cinnamaldehyde loading was impaired, as evidenced by the suboptimal closure of the artificial defects compared to the group without cinnamaldehyde. The combination of cinnamaldehyde loading with DHT treatment showed better results than a single use of either cinnamaldehyde or DHT alone. The loading of 4% concentration of cinnamaldehyde on the scaffolding should be readjusted to the bone tissue in experimental animals. The addition of cinnamaldehyde to the gypsum-CaCO3 scaffold was proven to act as an anti-inflammatory agent and a crosslinking, and it was effective in weeks 1 to 4 of implantation. This study did not provide data on how long it takes for cinnamon to be fully released since a certain number of inflammatory cells also determine wound healing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with the data contained in the manuscript.

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