

## Original Article

# Proliferation and osteogenic differentiation of bone marrow-derived mesenchymal stem cell after exposure to red flesh dragon fruit extract

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## ABSTRACT

**Background:** Induction of the proliferation and differentiation of stem cells could represent a viable alternative therapeutic method for treating bone diseases. Stem cells are essential to bone tissue regeneration; although, their availability is limited. One possible method of increasing the number of stem cells and promote osteogenic differentiation is the application of red flesh dragon fruit extract supplement. The present study was performed to identify and analyze proliferation and osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMMSCs) after exposure to red flesh dragon fruit extract.

**Materials and Methods:** This *in vitro* study was posttest only control group design. Red flesh dragon fruit extract was produced by means of water extraction method and subsequent dilution with different amounts of water to produce a range of concentrations. BMMSCs were obtained from the femurs of three White New Zealand rabbits. BMMSCs were then treated with 50, 100, 200, 300, and 400 µg/ml red flesh dragon fruit extract concentrations. The *in vitro* proliferation assay was determined by means of an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Osteogenic differentiation was determined by means of the degree of nodule mineralization. There were two groups as follows: group I with the addition of 50 µg/ml of red flesh dragon fruit extract and Group II without the addition of red flesh dragon fruit. Data were analyzed using analysis of variance and the Student's *t*-test ( $P = 0.05$ ).

**Results:** 50, 100, 200, 300, and 400 µg/ml of red flesh dragon fruit extract demonstrated the capacity to significantly increase the proliferation of BMMSCs ( $P \leq 0.05$ ). Red flesh dragon fruit extract could significantly increase osteogenic differentiation ( $P \leq 0.05$ ).

**Conclusion:** Red flesh dragon fruit extract enhances proliferation and osteogenic differentiation of BMMSCs.

**Key Words:** Cell differentiation, cell proliferation, fruit, mesenchymal stem cells

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## INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can be isolated from various

tissues, including bone marrow, adipose tissue, skin, umbilical cord, placenta, and dental tissue. MSCs are

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very promising for regenerative medicine since they are differentiated into a variety of cell types, including osteoblast, chondrocytes, and adipocytes.<sup>[1]</sup> Bone marrow-derived MSCs (BMMSCs) are cells that can be used for bone regeneration because they possess the ability to self-renew with a high proliferation capacity and osteogenic differentiation potential.<sup>[2]</sup>

Signaling molecules such as growth factors, cytokines, and chemokines are important factors involved in tissue regeneration and can be employed to modulate cellular functions including cell proliferation and differentiation.<sup>[3]</sup> Several cytokines and growth factors such as bone morphogenetic protein (BMP), transforming growth factor beta and fibroblast growth factor-2 are involved in the regulation of mesenchymal cell proliferation and differentiation.<sup>[4]</sup> BMP is the gold standard factor for increasing proliferation and differentiation of stem cells. BMP induces the mitogenesis of MSCs and other osteoprogenitors and their differentiation toward osteoblasts.<sup>[5,6]</sup> However, there are several problems regarding the use of growth factors, such as safety, the potential for ectopic bone formation and expense.<sup>[7]</sup>

Pitaya or dragon fruit is popular in numerous countries around the world.<sup>[8]</sup> There are three species that have been commercialized, namely *Hylocereus polyrhizus* (*H. polyrhizus*), which has red-skinned fruit with red flesh; *Hylocereus undatus*, which has red-skinned fruit with white flesh; and *Hylocereus megalanthus*, which has yellow-skinned fruit with white flesh.<sup>[9]</sup> Dragon fruit is usually consumed fresh and can be processed in fruit juice and puree. Processed red flesh dragon fruit is the most popular processed dragon fruit product because of its attractive color.<sup>[9]</sup> Red flesh dragon fruit (*H. polyrhizus*) contains Vitamin B1, B2, B3, and C; protein, fat, carbohydrates, crude fiber; flavonoids, thiamine, niacin, pyridoxine, cobalamin, phenolic, betacyanin, polyphenols, and carotene.<sup>[10]</sup> At present, red flesh dragon fruit extract has been used as a medicinal properties such as antioxidant, anticancer, hypocholesterolemic, antimicrobial, and prebiotic effects.<sup>[9]</sup>

Natural compounds produced from natural ingredients have a significant impact on the treatment and prevention of human diseases. In many developing countries, traditional medicine is an important alternative treatment.<sup>[11]</sup> A number of studies has reported that some plants or herbals could induce proliferation and differentiation of BMMSC.

*Herba Epimedii* has been found to enhance the osteogenic differentiation of BMMSC through BMP and Wnt/ $\beta$ -catenin signaling pathway.<sup>[12]</sup> Panax notoginseng saponins enhanced the differentiation of the osteoblastic lineage of bone marrow stromal cells through the mitogen-activated protein kinase (MAPK) signaling pathways.<sup>[13]</sup> Red flesh dragon may possibly induce proliferation and differentiation of stem cell due to its powerful compounds. Several studies have suggested that certain vitamins and minerals, such as Vitamins B3, C, and D; folic acid, selenium, and retinoic acid, play a role in proliferation and differentiation of stem cells.<sup>[14-17]</sup> To the best of our knowledge, there have been no studies to date evaluating the effect of red flesh dragon fruit extract on proliferation and osteogenic differentiation of BMMSCs. Thus, the purpose of this study was to identify and analyze the proliferation and osteogenic differentiation of BMMSC after exposure to red flesh dragon fruit extract.

## MATERIALS AND METHODS

This *in vitro* study was designed as a posttest only control group. Two evaluations were made: proliferation and osteogenic differentiation of BMMSCs after exposure to red flesh dragon fruit extract.

### Preparation of red dragon fruit extract

The identification of phytochemical analysis of red flesh dragon fruit conducted at Badan Penelitian dan Konsultasi Industri, Surabaya, Indonesia, confirmed the following ingredients: alkaloids (5.12%), saponin (4.06%), tannins (3.08%), flavonoids (1.05%), terpenoid (2.15%), polyphenol (4.18%), and Vitamin C (29.5 mg/100 g).

Red dragon fruits used in this study, approximately 50-day-old, were obtained locally from Purwodadi, East Java, Indonesia, which were washed and stored at  $-20^{\circ}\text{C}$  before the use. The fruit was peeled, and 850 g of flesh were cut into pieces and extracted using fruit extractor. Water extract of red flesh dragon fruit was filtered and frozen at  $-40^{\circ}\text{C}$ . The completely frozen extract was freeze-dried using a freeze dryer (CHRIST LMC-2, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) under a pressure of 4.6 Pa and at a temperature of  $-54^{\circ}\text{C}$  for 72 h. The freeze-dried was ground to obtain homogeneous powder. The freeze-dried powder was storage at  $-40^{\circ}\text{C}$ .<sup>[18]</sup> The powders were diluted with phosphate buffer saline (PBS) (Sigma) at a

stock concentration of 200 mg/ml before the use for analysis.

### Isolation of bone marrow-derived mesenchymal stem cell

Ethical clearance for the research was obtained from the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (approval number 13/KKEPK.FKG/I/2016). BMMSCs were obtained from the femurs of three White New Zealand rabbits, 4-month-old, 1 kg in weight. The bone marrow was flushed out by Dulbecco's modified Eagle medium (DMEM) (Sigma), and the collected cell suspension was centrifuged at 500 g for 5 min and suspended with culture medium. Bone marrow cells were seeded at a density of 0.1 ml aspiration/35-mm tissue culture dish (Corning) and cultured in 2 ml DMEM with 10% fetal bovine serum (FBS) (Sigma) and antibiotics (100 units/ml penicillin G and 100 ug/ml of streptomycin) before being incubated in a 37°C humidified tissue culture incubator at 5% CO<sub>2</sub>. Three days after seeding, floating cells were removed, and new medium added to the cells attached to the base of the culture dish. The medium was subsequently changed once every 3 days. Passage was undertaken when the cells were 80%–90% confluent.<sup>[19]</sup> To prove that the cells obtained were MSC, CD105, and CD45 were examined.<sup>[20]</sup>

### Examination of bone marrow-derived mesenchymal stem cell proliferation with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.  $5 \times 10^4$  cells were subcultured in 96-well tissue culture. After 24 h of incubation, the medium was change into red flesh dragon fruit extracts containing media at a concentrations of 50, 100, 200, 300, 400, 500, 600, and 700 µg/ml. Cells were incubated for 20 h at a temperature of 37°C in 5% CO<sub>2</sub>. After treatment with red dragon fruit extract, the cell was washed with ×1 PBS. A new medium-containing 0.5 mg/ml MTT was added to each well and incubated at 37°C for 4 h in a humidified tissue culture incubator at 5% CO<sub>2</sub>. After incubation, dimethyl sulfoxide was added to each well. Optical density examination was performed using enzyme-linked immunosorbent assay reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 595 nm.<sup>[19]</sup>

### Examination of bone marrow-derived mesenchymal stem cell osteogenic differentiation

BMMSC were cultured at a density of  $1 \times 10^5$  in 24-well tissue culture with osteogenic medium. DMEM was used as osteogenic medium which added 10% FBS, 10 mM-glycerophosphate, 100 nM dexamethasone, and 50 µg/ml ascorbic acid (AA)-2-phosphate.<sup>[21]</sup> There were two groups as follows: group I featuring the addition of 50 µg/ml of red flesh dragon fruit extract and Group II, the control group to which red flesh dragon fruit extract was not added. In this study, 50 ug/ml red dragon fruit extract was used since this concentration represented the highest increase in the proliferation of BMMSCs. The cells were incubated in a 37°C humidified tissue culture incubator at 5% CO<sub>2</sub> and maintained for 21 days. After 21 days, the culture medium was aspirated, and the cell layer washed twice with PBS and fixed with 95% ethanol for 10 min at room temperature. They were then washed twice with distilled water and stained for 30 min with 1% alizarin red S solution (Sigma-Aldrich; Merck KGaA) at room temperature. The cell layers were washed with water and the red positions were recognized as calcium deposits. The staining results were observed and visualized under using phase contrast microscope (CKX41, Olympus, Japan) at ×40. The mineralized nodules or calcium deposits were counted.<sup>[19]</sup>

### Data analysis

Data are expressed as the mean ± standard deviation. The significance of difference was evaluated by analysis of variance, with Student's *t*-test when comparing the two groups.  $P < 0.05$  was considered as statistically significant.

## RESULTS

### Bone marrow-derived mesenchymal stem cell proliferation after exposure to red dragon fruit extract

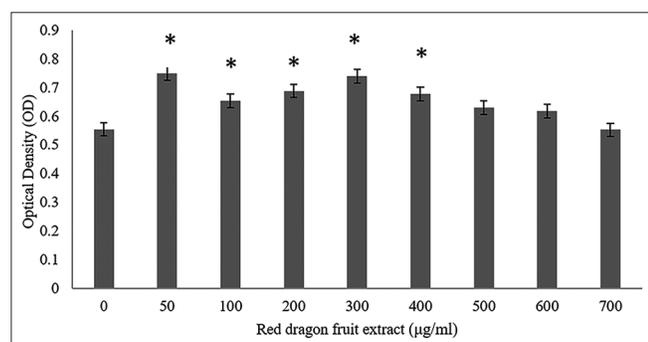
The effect of adding red flesh dragon fruit extract to BMMSC proliferation was examined by MTT assay. The addition of red flesh dragon fruit extract at concentrations of 50, 100, 200, 300, and 400 µg/ml can significantly increase the BMMSC proliferation ( $P < 0.05$ ), while concentrations of 500, 600, and 700 µg/ml cannot. The highest increase in the proliferation of BMMSC was obtained by adding 50 µg/ml of red flesh dragon fruit extract [Table 1 and Figure 1].

### Osteogenic Differentiation of bone marrow-derived mesenchymal stem cell

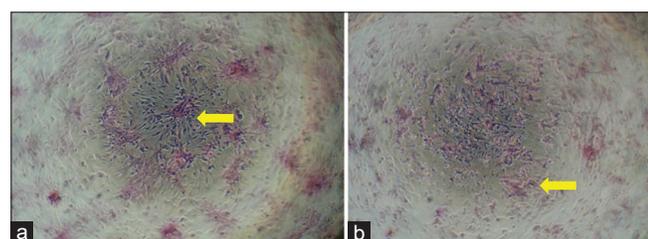
To evaluate the effect of red flesh dragon fruit extract on osteogenic differentiation, culture staining was performed with alizarin red on day 21. Both groups underwent osteogenic differentiation into osteoblasts characterized by the mineralization of nodules [Figure 2]. The red flesh dragon fruit extract group showed a significantly high number of mineralized nodules ( $P < 0.05$ ) than the control group [Table 2 and Figure 3].

## DISCUSSION

This study analyzed the effect of adding red flesh dragon fruit extract to the proliferation and osteogenic



**Figure 1:** Red flesh dragon fruit extract enhanced the proliferation of bone marrow-derived mesenchymal stem cell, which were cultured with the addition of 50, 100, 200, 300, 400, 500, 600, and 700 µg/ml red flesh dragon fruit extract for 20 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to measure cell proliferation. Red flesh dragon fruit extract at concentrations of 50, 100, 200, 300, and 400 µg/ml significantly enhanced the proliferation of bone marrow-derived mesenchymal stem cell. The mean ± standard deviation of four cultures is shown in the figure. \*Significantly different ( $P < 0.05$ ) as compared to the control.



**Figure 2:** Osteogenic differentiation of bone marrow-derived mesenchymal stem cell. Bone marrow-derived mesenchymal stem cell cultures maintained in the osteogenic medium (a) or in osteogenic medium with 50 µg/ml red flesh dragon fruit extracts (b). Calcification levels were estimated by the mineralization of nodules with Alizarin Red S Staining (yellow arrow) using phase contrast microscope at a ×40.

differentiation of BMMSC. The addition of red flesh dragon fruit extract at concentration of 50, 100, 200, 300, and 400 µg/ml can enhance BMMSCs proliferation, the highest level of which was found at 50 µg/ml. This study also indicated that the addition of red flesh dragon fruit extract increased the osteogenic differentiation of BMMSC.

High proliferation of BMMSCs through the addition of red flesh dragon fruit extract is probably due to the influence of AA, the active component of red flesh dragon fruit. AA has been known to stimulate the proliferation of various mesenchymal cell including osteoblasts, adipocytes, chondrocytes, and odontoblasts.<sup>[22-24]</sup> The addition of AA during culture can modulate the proliferation and differentiation of MSC, while at certain concentrations is a potent modulator of MSC proliferation without causing loss of differentiation capacity of the MSC.<sup>[25]</sup>

The active components of red flesh dragon fruit such as AA, saponins, tannins, flavonoids, polyphenols, terpenoids, and alkaloids are antioxidant ingredients. Antioxidants have an influence on stem cell proliferation.<sup>[26]</sup> Antioxidants are resistant to reactive oxygen species (ROS) and stimulate stem cells to enter the S phase of the cell cycle by inhibiting cyclin-dependent kinase (CDK) and CDK4 inhibitors (p21 and p27), as well as increasing the

**Table 1: Mean values of the proliferation of bone marrow-derived mesenchymal stem cell after addition of red flesh dragon fruit extract for 20 h (mean±standard deviation)**

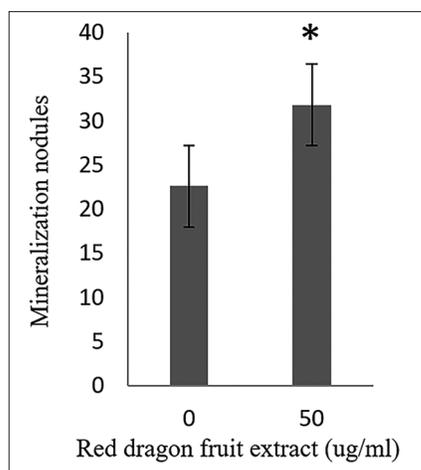
Red flesh dragon fruits (ug/ml)	Mean±SD
0	0.555±0.024
50	0.75±0.0218
100	0.655±0.014
200	0.689±0.02
300	0.741±0.035
400	0.679±0.045
500	0.631±0.039
600	0.619±0.024
700	0.553±0.018

SD: Standard deviation

**Table 2: Mean values of the osteogenic differentiation of bone marrow-derived mesenchymal stem cell after addition of red flesh dragon fruit extract for 21 days (mean±standard deviation)**

Groups	Mean±SD
Control	22.583±0.768
Red flesh dragon fruits	31.8±2.853

SD: Standard deviation



**Figure 3:** Red flesh dragon fruit extract enhanced the osteogenic differentiation of bone marrow-derived mesenchymal stem cell. Bone marrow-derived mesenchymal stem cell cultured in osteogenic medium with the addition of 50 µg/ml red dragon fruit extract for 21 days. Red flesh dragon fruit extract significantly increased the number of mineralization nodules. Mean ± standard deviation of four cultures is shown in the figure. \* $P < 0.05$  as compared with control.

expression of CDK2, CDK4, and CDC2. Previous reports show that polyphenols can block ROS production, reduce apoptotic cell, and maintain the viability of BMMSC cells.<sup>[27]</sup> Saponins may also affect the proliferation of BMMSC. Previous studies have shown that saponins from *Panax notoginseng* can increase the proliferation and osteogenesis of bone marrow stromal cells dose-dependent.<sup>[28]</sup> It was also reported that saponins can increase the proliferation of BMMSC through the ERK and p38-MAPK mechanism.<sup>[12]</sup>

The addition of 500, 600, and 700 µg/ml of red flesh dragon fruit extract does not enhance the BMMSC proliferation, which is probably due to the antioxidants contained in red flesh dragon fruit at these concentrations having a slightly toxic effect on BMMSC. Previous research has shown that antioxidants in large doses produce toxic and harmful effects on neural stem cells.<sup>[29]</sup>

Osteogenic differentiation of BMMSC examination demonstrated that the red flesh dragon fruit extract group contained a greater number of mineralization nodules compared to the control group. The active ingredients in red flesh dragon fruit, such as flavonoids, polyphenols, saponins, and AA may possibly help the osteogenic differentiation process of BMMSC.<sup>[10,29,30]</sup>

Flavonoids are a group of chemicals with a diphenylpyran structure which have been demonstrated

as having antioxidant properties. High levels of ROS caused by oxidative stress block and reduce osteoblast activity and differentiation.<sup>[34]</sup> Flavonoids can regulate the expression of core-binding factor alpha 1 (Cbfa1) which is an important regulator in the process of osteoblast differentiation.<sup>[31]</sup> Flavonoids through increased BMP and Wnt β-catenin activity promote the osteoblastic differentiation.<sup>[13]</sup>

Polyphenol was reported to be successful in inducing bone formation *in vivo*.<sup>[32]</sup> Previous studies reported that polyphenols block ROS production, reduces apoptotic cell death, and maintain the BMMSC cell viability.<sup>[27]</sup> Polyphenol has been reported as capable of increasing Cbfa1/runt-related transcription factor 2 (Runx2), osterix, osteocalcin, type 1 collagen, and alkaline phosphatase (ALP) in MSC, which shows its potential to induce osteogenic differentiation.<sup>[33]</sup> Polyphenols can also stimulate osteoprotegerin production.<sup>[34]</sup>

In this study, the increasing number of mineralization nodules due to the addition of red flesh dragon fruit extract may also be due to the influence of AA, which is an important regulator of osteoblasts. AA influences the process of differentiating bone marrow stromal cells into mature osteoblasts and is important for increasing the osterix expression that occurs during osteoblast differentiation.<sup>[35]</sup> Several studies have shown that the addition of AA to osteoblast culture can stimulate the formation of collagenous extracellular matrix followed by the induction of ALP, osteocalcin, osteopontin, osteonectin, and Runx2.<sup>[36-38]</sup> It is not clear in this study which molecular mechanism is related to the effect of red flesh dragon fruit extract on proliferation and differentiation of BMMSC.

## CONCLUSION

Red flesh dragon fruit extract can enhance the proliferation and osteogenic differentiation of BMMSC. Further investigation is necessary to understand the molecular biological mechanism underlying the effects of red flesh dragon fruit extract on proliferation and differentiation of BMMSC.

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## Conflicts of interest text should be

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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