

The Effect of the Application of (*Garcinia mangostana* L.) towards PDGF-B Expression on Human Gingival Fibroblast Cell Culture After Wound Healing Scratch Test Assay (In-Vitro Study)

by Andra Rizqiawan

Submission date: 11-Apr-2022 11:13AM (UTC+0800)

Submission ID: 1807317750

File name: e_Application_of_Garcinia_mangostana_L._towards_PDGF-th.2021.pdf (558.53K)

Word count: 3238

Character count: 18224

The Effect of the Application of (*Garcinia mangostana* L.) towards PDGF-B Expression on Human Gingival Fibroblast Cell Culture After Wound Healing Scratch Test Assay (In-Vitro Study)

Felicia Laurens Lesmana¹, Andra Rizqiawan^{2*}, Indra Mulyawan², Ni Putu Mira Sumarta², David B. Kamadjaja², Coen Pramono D², Tobiumei Kei³, Gde Djodi Satria Rurus⁴, Naura Athiyyah Sativa⁴ and Rozhaline Apriliany Fanddhy⁴

1. Dept. of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

2. Department of Oral and Maxillofacial Surgery, Specialization of Cervico-Gnathostomology, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.

3. Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

Abstract

PDGF-B in fibroblast cell proliferation is critical during wound healing process. An increase in PDGF-B accelerates the proliferation and migration of fibroblast cells. Mangosteen peel comprises of numerous benefits, for example, having the ability to speed up the proliferation of fibroblast cells. Scratch tests also showed crucial cell migration and proliferation; thus, mangosteen peel extract is expected to speed up the wound healing process. This study investigated the effect of mangosteen peel extract on PDGF-B expression on scratched human gingival fibroblast cell cultures. Human gingival fibroblast cell cultures were divided into four categories: control for 24 hours, control for 48 hours, treatment for 24 hours, and treatment for 48 hours. All of the samples were initially scratched and then had mangosteen peel extracts applied to them. The concentration used in the experiment was 800µg/ml. This was followed by RNA extraction, which was processed by PCR assay. The 24-hour groups, both control and treatment, showed a brighter band than the 48-hour groups. The application of the mangosteen peel extract on scratched human gingival fibroblast cell culture increased the expression of PDGF-B during the first day (24 hours) and decreased it on the second day (48 hours).

Experimental article (J Int Dent Med Res 2021; 14(4): 1413-1418)

Keywords: PDGF-B, mangosteen peel, human gingival fibroblast cell, human and medicine.

Received date: 01 July 2021

Accept date: 26 October 2021

Introduction

Tooth extraction is the process of tooth removal, causing injury to the resulting socket.¹ The wounds can heal through a physical response which repairs damaged body parts, but this recuperative process is sometimes impeded due to infection and/or a dry socket. While the wounds are healing, fibroblasts proliferate, extracellular matrix is deposited, collagen is synthesized, and angiogenesis occurs. As a result, the damaged tissue can be restored to its normal condition.²

Fibroblasts represent the main cells found in

the wound healing process.³ When tissue is inflamed, fibroblasts migrate to the wound site, proliferate, and produce collagen matrix stimulated by platelet derived growth factor (PDGF) and fibroblast growth factor (FGF).⁴ In addition to that, the migration of fibroblasts to the location of the injury is also stimulated by transforming growth factor-β (TGF-β).⁵ Vascular endothelial growth factor (VEGF) is a potent stimulator in the process of angiogenesis and has an important overall role in inducing it.^{6,7}

Mangosteen peel contains several components useful for the medical field, namely xanthenes, tannins, flavonoids, and saponins, along with components which are antifungal, anti-inflammatory, antioxidant, antiviral, and antibacterial in function.^{8,9} This study aimed to identify differences in the expression of growth factors after the administration of mangosteen peel extract during the scratch assay in human gingival fibroblast cell cultures.

*Corresponding author:

Andra Rizqiawan,
Department of Oral and Maxillofacial Surgery, Faculty of
Dental Medicine, Universitas Airlangga. Prof. Dr. Moestopo
No. 47 St., Surabaya, Indonesia.
E-mail: andra-r@fkg.unair.ac.id.

Materials and methods

Samples and cell culture

In January-April 2017, human gingival tissue was carefully extracted from the permanent third molar region of healthy subjects (aged 18-32 years old) at the Dental Hospital of Universitas Airlangga. Ethical clearance (117/HRECC.FODM/VIII/2017) was approved, and informed consent was obtained from all individual participants. Human gingival fibroblasts (HGFs) were isolated and cultured in a humidified atmosphere of 5% CO₂ at 37°C. The cells were cultured in 75 cm² cell culture flasks containing Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) and added with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY) and antibiotic (100 µg/mL streptomycin and 100U/mL penicillin G, Sigma Chemical Co., St. Louis, MO, USA).

The cells were sub-cultured using 0.25% trypsin and 0.05% EDTA (Invitrogen, Carlsbad, CA) after reaching 90% confluency. Cells between the third and fifth passages were used for experiments.

Powder extract preparation

Garcinia mangostana Linn. was collected from suppliers in Blitar, East Java, Indonesia. The mangosteen peels were collected, chopped, and placed in a drier tunnel at the Phytochemistry Laboratory, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. Extraction was performed by means of a maceration method using distilled ethanol with a concentration of 70% as the solvent to collect mangosteen peel extract (MPE).¹⁰

RT-PCR

An RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate the total RNA according to the protocols stipulated by the manufacturer. Meanwhile, ReverTra Ace (TOYOBO, Osaka, Japan) was used to synthesize cDNA. Semiquantitative RT-PCR was performed involving thirty 30-second cycles of denaturation at 95°C. It was then annealed for 30 seconds and extended for 60 seconds at 72°C. PCR primers were as follows:

- PDGF-B *forward* 5'-
GATCCGCTCCTTTGATGATC-3' (385bp)
- PDGF-B *reverse* 5'-
GTCTCACACTTGCATGCCAG-3' (385bp)
- TGF-β1 *forward* 5'-

CGAAATCTATGACAAGTTCAAGCA-
3'(192bp)

- TGF-β1 *reverse* 5'-
GAGGTATCGCCAGGAATTGTT-3' (192bp)
- FGF-2 *forward* 5'-
CTTCTTCTGCGCATGCACC-3' (262bp)
- FGF-2 *reverse* 5'-
CACATACCAACTGGTGTATTT-3' (262bp)
- VEGF *forward* 5'-
CTGCTGTCTTGGGTGCATTG-3' (499bp)
- VEGF *reverse* 5'-
CTCGGCTTGTACACATACGC-3' (499bp)
- GAPDH *forward* 5'-
ACCACAGTCCATGCCATCAC-3' (353bp)
- GAPDH *reverse* 5'-
CAGCCCCAGCGTCAAAGGTG-3' (353bp).

PCR Products in 2% agarose gel were separated by electrophoresis and visualized by means of staining with ethidium bromide, the results of which were observed by means of a gel doc machine (Bio-Rad, Mississauga, Canada).

Wound Healing Assay

Cells were seeded at a density of 1.5 x 10⁵ cells/well in 24-well culture plates. A wound was created by scratching the confluent cells in each well with a 200 µl pipette tip¹². As much as 800 µg/ml mangosteen peel liquid extract was applied to members of the treatment group. After incubation periods of 24 hours and 48 hours, extraction of RNA was performed, at which point its levels and purity were measured. Comparison of the control and treatment groups involved the calibration of results by means of ImageJ application.

Results

Human gingival fibroblast cells were divided into four categories: 24-hour control, 24-hour treatment, 48-hour control, and 48-hour treatment. Figure 1 describes the differences in the PDGF-B, TGF-β1, FGF, and VEGF expressions between the control group and treatment group at 24 hours and 48 hours.

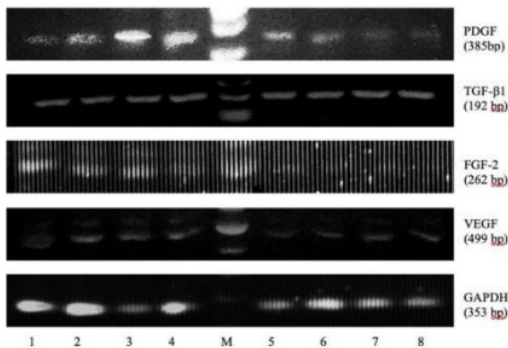


Figure 1. PCR result visualization. 1,2: 24-hour control group; 3,4: 24-hour treatment group; 5,6: 48-hour control group; 7,8: 48-hour treatment group; M: Marker.

In Figure 2, PDGF-B expression in the 24-hour treatment group increased compared to that in the 24-hour control group. This was also the case for the 48-hour treatment group compared to its 48-hour control counterpart.

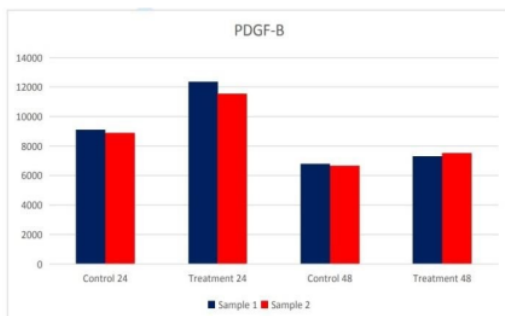


Figure 2. PDGF-B expression graphic from calibration with ImageJ software.

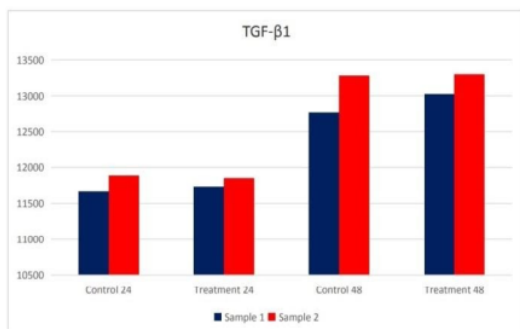


Figure 3. TGF-β1 expression graphic from calibration with ImageJ software.

In Figure 3, the TGF-β1 expression in each control group produced bands which were less vivid than that of the treatment groups. Based on the imageJ calibration of band luminance, an increase was observed in the control and treatment groups at both 24 hours and 48 hours.

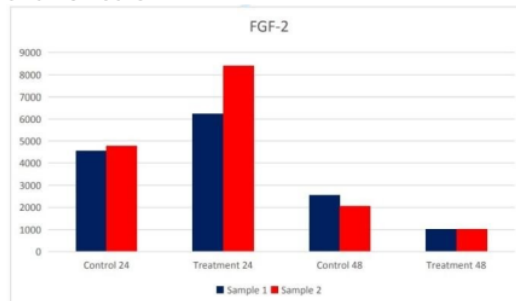


Figure 4. FGF-2 expression graphic from calibration with ImageJ software.

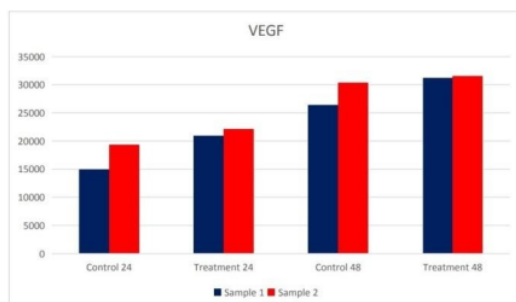


Figure 5. VEGF expression graphic from calibration with ImageJ software.

In Figure 4, the expression of FGF-2 in the 24-hour treatment group was at a higher level than that in the 24-hour control group, while in the 48-hour group, the treatment group exhibited a lower level of FGF-2 than the control group.

In Figure 5, the 24-hour treatment group showed a higher level of VEGF expression than the 24-hour control group. The 48-hour treatment group also showed a higher VEGF expression level than the 48-hour control group.

Discussion

Fibroblasts are vital to the wound repair process since they are responsible for producing collagen, the protein structure essential for tissue reconstruction. Collagen links the wound margins and influences the re-epithelizing process in

wound healing.^{3,11} The activity of fibroblast division is hardly observed under normal circumstances. However, these cells become more active when an injury occurs by producing extracellular matrices. During the wound healing process, various growth factors naturally stimulate fibroblast proliferation. For example, the polypeptides initiate the acceleration of differentiation and cell metabolism, while also regulating the tissue repair process.¹¹⁻¹⁴

Scratch test assays in HGF cultures affect the increase in growth factor expression as indicated by increased fibroblast proliferation. Cells migrate and proliferate more rapidly, and those at the margin of the scratch tend to migrate more quickly to the open wound to close it until contact between cells is re-established.¹⁵

The contents of mangosteen peel extract have an effect in increasing the expression of the growth factors: xanthenes, flavonoids, tannins, and saponins. The first two factors act as anti-inflammatory agents by inhibiting the action of cyclooxygenase enzymes more rapidly. They are characterized by increased fibroblast cell proliferation, which indicates the involvement of PDGFR- β , a PDGF-B receptor.¹⁶ PDGFR- β plays a significant role in the proliferation, transformation, and secretion of collagen by fibroblasts. Several studies on vascular repair and angiogenesis have revealed that PDGFR- β functions via the PI3K/Akt signaling pathway. PDGFR- β activation is involved in the regulation of proliferation, migration, differentiation, and angiogenesis of cells. PDGF- β induces tyrosine phosphorylation in PDGFR- β and increases the production of inositol triphosphate. PDGFR- β subsequently engages PI3K, phospholipase C γ 1, Src family kinase, and phosphotyrosine phosphatase SHP-2. PI3K activates Akt, which functions as a multifunctional regulator of cell growth and resistance and stimulates proliferation, migration, and angiogenesis.¹⁷

The content of xanthone derived from mangosteen peel extract can accelerate the process of angiogenesis through VEGF expression. Antioxidants contained in mangosteen peel extract or in xanthenes can bind to an element of radical superoxide (O₂⁻) and subsequently release free nitric oxide (NO). NO can increase VEGF expression via PI3K (Phosphoinositide 3-kinase)-Akt, which transduces intracellular signals. As a result, the HIF-1 α gene activates and involves a

transcription regulator, Hypoxia-inducible factor (HIF-1) as a key for several angiogenic factors. The signal affects the nucleus which contains a VEGF gene to express VEGF into the cells will later be excreted in the form of a VEGF protein affecting the increase in angiogenesis and potentially accelerate wound healing.¹⁸

Flavonoids in mangosteen peel extract can affect the expression of FGF-2 which was higher in the 24-hour treatment group. This is related to an increase in nitric oxide (NO), which is influenced by flavonoids. This mechanism begins with the direct signaling process of FGF-2, constituting the transfer of molecules from one cell to another across cell membranes. The binding of extracellular FGF-2 to its receptor (FGFR-2), located on the surface of fibroblast cell membranes, produces the complex formation of FGF-FGFR-HSPG with heparan sulfate proteoglycan (HSPG) as a cofactor. This complex then activates the intracellular tyrosine kinase FGFR-2 domain through phosphorylation of tyrosine residues. The active receptors will send signals via the Ras-MAPK pathway (mitogen-activated protein kinase) since their activation increases the cytosolic concentration of Ca²⁺ through the production of inositol-1,4,5-triphosphate by phospholipase C γ and Ca²⁺. This is liberated from the endoplasmic reticulum, mediated by the activation of Ca²⁺ channels. Increasing Ca²⁺ will form a Ca²⁺-calmodulin (Ca²⁺/CaM) complex, which results in the activation of eNOS, an enzyme that will synthesize NO. Synthesized NO subsequently activates the cytosolic guanylyl cyclase, which will induce cGMP that activates protein kinase G (PKG) in the form of RAF kinase. RAF kinase will phosphorylate and activate the MEK, which will also phosphorylate and activate MAPK. MAPK will activate Ets4 and Ets5, members of the family of DNA transcription factors, which will affect the transcription process of FGF-2 expression in the nucleus.^{19,20}

Tannins and saponins in mangosteen peel extract can affect the increasing expression of TGF- β 1. The tannins themselves can stimulate TGF- β , leading to fibroblast proliferation. Tannins constitute antioxidants that bond to proteins and polysaccharides and are capable of forming strong complexes that can increase expression of TGF- β 1.²¹ Saponins will activate the function of TGF- β . They activate the TGF- β receptor, TGF- β R1, to stimulate an increase in TGF- β 1 in

fibroblast receptors. Increased TGF- β 1 can stimulate cell migration towards injury sites and increase fibroblast proliferation.²² Saponin stimulates synthesis, secretion, and activation in fibroblast cells, while also altering TGF- β receptor expression and modifying signal transduction at TGF- β post-receptors.^{8,16}

In this study, the expression of PDGF-B and FGF-2 in the control and treatment 48-hour sample groups experienced decreases compared to the 24-hour sample groups. This contrast is most likely related to the completion of proliferation of HGF cell cultures in the in vitro study reported here. Fibroblast cells cease growing after forming a single, flat layer (confluent monolayer) at the base of the culture medium due to contact inhibition. Cells will no longer proliferate once they have come into contact with others.^{23,24} PDGF-B increases during the early stages of wound healing before decreasing once the neovascularization process has been completed, and perfusion in the wound area has returned to normal.^{4,22,25} Another factor contributing to low FGF-2 expression in the 48-hour group is the transient effect of a concentration of 800 μ g/ml of mangosteen peel extract. Given the continuous expression of FGF-2, overexpression will ensue. This can eventually lead to the development of various forms of cancer such as prostate cancer, melanoma, and hepatocellular carcinoma.¹⁹

Conclusions

The application of the mangosteen peel extract on scratched human gingival fibroblast cell culture increased the expression of PDGF-B during the first day (24 hours) and decreased it on the second day (48 hours).

Acknowledgements

The author would like to thank the Faculty of Dental Medicine, Universitas Airlangga for supporting this study.

Declaration of Interest

The authors report no conflict of interest.

References

1. Horowitz R, Holtzclaw D, Rosen PS. A review on alveolar ridge preservation following tooth extraction. *J Evid Based Dent Pract* 2012;12(3):149–60.
2. Brunello G, De Biagi M, Crepaldi G, Rodrigues FI, Sivoletta S. An Observational Cohort Study on Delayed-Onset Infections after Mandibular Third-Molar Extractions. *Int J Dent* 2017;2017:1–5.
3. Baxter LC, Frauchiger V, Textor M, ap Gwynn I, Richards RG. Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces. *Eur Cell Mater* 2002;4(1):1–17.
4. Velnar T, Bailey T, Smrkolj V. The Wound Healing Process: An Overview of the Cellular and Molecular Mechanisms. *J Int Med Res* 2009;37(5):1528–42.
5. Kanzaki T, Tamura K, Takahashi K, Saito Y, Akikusa B, Ohashi H, et al. In vivo effect of TGF- β 1 in rabbit arteries injured with a balloon catheter. *Arterioscler Thromb Vasc Biol* 1995;15(11):1951–57.
6. MacKay D, Miller AL. Nutritional Support for Wound Healing. *Altern Med Rev* 2003;8(4):359–77.
7. Polverini PJ. Angiogenesis and wound healing: basic discoveries, clinical implications, and therapeutic opportunities. *Endod Top* 2011;24(1):130–45.
8. Pedraza-Chaverri J, Cárdenas-Rodríguez N, Orozco-Ibarra M, Pérez-Rojas JM. Medicinal properties of mangosteen (*Garcinia mangostana*). *Food Chem Toxicol* 2008;46(10):3227–39.
9. Kresnadi U, Hadioesanto Y, Prabowo H. Effect of mangosteen peel extract combined with demineralized freeze-dried bovine bone xenograft on osteoblast and osteoclast formation in post tooth extraction socket. *Dent J (Majalah Kedokt Gigi)* 2016;49(1):43.
10. Novilla A, Djamhuri DS, Fauziah N, Maesaroh M, Balqis B, Widowati W. Cytotoxic Activity of Mangosteen (*Garcinia mangostana* L.) Peel Extract and α -mangostin toward Leukemia Cell Lines (HL-60 and K-562). *J Nat Remedies* 2016;16(2):52.
11. Tanaka F, Rizqjawan A, Higashikawa K, Tobiume K, Okui G, Shigeishi H, et al. Snail promotes Cyr61 secretion to prime collective cell migration and form invasive tumor nests in squamous cell carcinoma. *Cancer Lett* 2013;329(2):243–52.
12. Zhu R, Liu C, Gundersen GG. Nuclear Positioning in Migrating Fibroblasts. In *Seminar in Cell Dev Biol*. Academic Press. 2018;82:41–50.
13. Yarrow JC, Perlman ZE, Westwood NJ, Mitchison TJ. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC Biotechnol* 2004;4(1):21.
14. Huang C, Rajfur Z, Borchers C, Schaller MD, Jacobson K. JNK phosphorylates paxillin and regulates cell migration. *Nature* 2003;424(6845):219–23.
15. Guo S, Di Pietro LA. Factors affecting wound healing. *J Dent Res* 2010;89(3):219–29.
16. Liang C-C, Park AY, Guan J-L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2007;2(2):329–33.
17. Fan H, Ma L, Fan B, Wu J, Yang Z, Wang L. Role of PDGFR- β /PI3K/AKT signaling pathway in PDGF-BB induced myocardial fibrosis in rats. *Am J Transl Res* 2014;6(6):714–23.
18. Kimura H, Esumi H. Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis. *Acta Biochim Pol* 2003;50(1):49–59.
19. Ornitz DM, Itoh N. The Fibroblast Growth Factor signaling pathway. *Wiley Interdiscip Rev Dev Biol* 2015;4(3):215–66.
20. Villalobo A. Enhanced Cell Proliferation Induced by Nitric Oxide. *Dyn Cell Biol* 2007;1:60–4.
21. Sakagami H, Kushida T, Makino T, Hatano T, Shirataki Y, Matsuta T, et al. Functional Analysis of Natural Polyphenols and Saponins as Alternative Medicines. In: Kushida T, editor. *A Compend. Essays Altern. Ther.*, Rijeka: IntechOpen; 2012:269–302.

22. Souders CA, Bowers SLK, Baudino TA. Cardiac Fibroblast. *Circ Res* 2009;105(12):1164–76.
23. Gonzalez AC de O, Costa TF, Andrade Z de A, Medrado ARAP. Wound healing - A literature review. *An Bras Dermatol* 2016;91:614–20.
24. Hendiani I, Carolina DN, Arnov ST, Rusminah N, Amaliya A, Susanto A, Komara I. Effectiveness of Mangosteen (*Garcinia mangostana* L.) Peel Gel on the MMP-8 Levels in Chronic Periodontitis Patients after Scaling and Root Planing. *Journal of International Dental and Medical Research* 2021;14(2):654-9.
25. Rizqiawan A, Aprilia O, Pakpahan RG, Rodherika E, Setyowati A, Kei T. Application of Mangosteen Peel Extract (*Garcinia mangostana* Linn.) to TGF-1, PDGF-B, FGF-2 and VEGF-A Expression on Human Gingival Fibroblast Cell Culture (In Vitro Study). *Journal of International Dental and Medical Research* 2021;14(1):119-24.

The Effect of the Application of (Garcinia mangostana L.) towards PDGF-B Expression on Human Gingival Fibroblast Cell Culture After Wound Healing Scratch Test Assay (In-Vitro Study)

ORIGINALITY REPORT

13%

SIMILARITY INDEX

7%

INTERNET SOURCES

10%

PUBLICATIONS

%

STUDENT PAPERS

PRIMARY SOURCES

- 1 C O'Brien. "Measurement of movement is an objective method to assist in assessment of opiate withdrawal in newborns", Archives of Disease in Childhood - Fetal and Neonatal Edition, 2004
Publication 1%
- 2 ajtr.org
Internet Source 1%
- 3 Hang Wang, Yangguang Yin, Wei Li, Xiaohui Zhao et al. "Over-Expression of PDGFR- β Promotes PDGF-Induced Proliferation, Migration, and Angiogenesis of EPCs through PI3K/Akt Signaling Pathway", PLoS ONE, 2012
Publication 1%
- 4 V. C. de Vries. "Mast Cell Degranulation Breaks Peripheral Tolerance", American Journal of Transplantation, 10/2009
Publication 1%

5	www.freepatentsonline.com Internet Source	1 %
6	J. G. Kingma, D. M. Yellon. "Limitation of myocardial necrosis with verapamil during sustained coronary occlusion in the closed-chest dog", <i>Cardiovascular Drugs and Therapy</i> , 1988 Publication	1 %
7	mafiadoc.com Internet Source	1 %
8	spotidoc.com Internet Source	1 %
9	Indra Mulyawan, Coen Pramono Danudiningrat, Pratiwi Soesilawati, Aulanni'am Aulanni'am et al. "The Characteristics of Demineralized Dentin Material Sponge as Guided Bone Regeneration Based on the FTIR and SEM-EDX Tests", <i>European Journal of Dentistry</i> , 2022 Publication	1 %
10	Kresnoadi Utari, Ruslianda Diva Amelia, Laksono Harry. "The potential for induction of the expression of bone morphogenetic protein-7 and alkaline phosphatase in postextraction tooth sockets using a combination of mangosteen peel extract and	1 %

11

Higinio Arzate. "Cyclosporin A promotes mineralization by human cementoblastoma-derived cells in culture", Journal of Periodontal Research, 6/2005

Publication

1 %

12

Alexandra C. Maki, Paul J. Matheson, Jessica A. Shepherd, R. Neal Garrison, Cynthia D. Downard. "Intestinal Microcirculatory Flow Alterations in Necrotizing Enterocolitis are Improved by Direct Peritoneal Resuscitation", The American Surgeon, 2012

Publication

<1 %

13

H Yamabe, H Osawa, M Kaizuka, N Tamura, S Tsunoda, K Shirato, F Tateyama, K Okumura. "Angiotensin II further enhances type IV collagen production stimulated by platelet - derived growth factor and fibroblast growth factor - 2 in cultured human mesangial cells", Nephrology, 2001

Publication

<1 %

14

contempclindent.org

Internet Source

<1 %

15

ijbms.mums.ac.ir

Internet Source

<1 %

16

dergipark.org.tr

Internet Source

<1 %

17

T. Imatani. "Histatin 5 inhibits inflammatory cytokine induction from human gingival fibroblasts by Porphyromonas gingivalis", Oral Microbiology and Immunology, 12/2000

Publication

<1 %

Exclude quotes Off

Exclude matches < 10 words

Exclude bibliography On

The Effect of the Application of (Garcinia mangostana L.) towards PDGF-B Expression on Human Gingival Fibroblast Cell Culture After Wound Healing Scratch Test Assay (In-Vitro Study)

GRADEMARK REPORT

FINAL GRADE

/0

GENERAL COMMENTS

Instructor

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6
