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6th IBC

PROCEEDINGS

THE 6th INDONESIAN BIOTECHNOLOGY CONFERENCE

"ENHANCING INDUSTRIAL COMPETITIVENESS THROUGH BIOTECHNOLOGY INNOVATION"

Surakarta, 6-7 September 2016

Editors:

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Prof. Dr. Ir. Ahmad Yunus, M.S
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THE EXPRESSION ANALYSIS OF TGF-B1. IGF. AND FGF ON SUPERFICIAL AND DEEP OSTEOCHONDRAL DEFECTS OF KNEE JOINT IN SPRAGUE DAWLEY RATS (PRELIMINARY STUDY)

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Abstract

The growth factor is one of the elements that contribute to healing and regeneration of osteochondral defects in knee joint. Stem cell work together with growth factors to produce tissue regeneration. The timing of growth factor expression is very important to determine the timing of observation, during stem cell intervention in osteochondral defect. Here, we showed that TGF-β1, IGF and FGF expressed on chondrocyte cells in osteochondral defect of Sprague Dawley rats temporally. Early expression was observed after 7 days post defect, and disappeared on day 28th . Macroscopic examination exhibit improvement on day 7. The morphogical improvement stayed until day 28th post defect. Taken together, the osteochondral defect induced the expression of TGF-β1, IGF, and FGF temporarily.

***Keywords**: Growth factors. TGF-β1. IGF. FGF. chondrocyte cells*

1. Introduction

Signal molecules were one of the factors that triggered the cell response and the production of extra-cellular matrices hyaline cartilage. The signal molecules can be growth factors like TGF-β1, IGF-1, FGF, BMP [1-6] or mechanical stimulation[2]. TGF-β1repaired cartilage, and triggered chondrogenesis [4]. TGF-β1 receptor was large quantities found in chondrocyte and cartilage [7]. IGF-1 played a role in the proliferation and cartilage formation. Together, IGF-1 and TGF-β1 triggered chondrogenesis sinergically [1]. FGF induced cell regeneration, chondrocytes proliferation and cartilage formation [7].

Research showed that mechanical stimulation triggered the differentiation of progenitor cells, albeit without the help of growth factors external addition [8-9].This is because the mechanical stimulation triggered the release of growth factors internally, that served to regenerate cells. In the intervention stem cell therapy, the release time of growth factors on the mechanical stimulation like osteochondral defect, was very important. The stem cell intervention that accompanied by the release of growth factors on appropriate time, initiated regeneration of cells. The objective of our study was to observe the timing of growth factor expression during cell regeneration in osteochondral defect of the knee joint.

2. Methods

Rats and study design

Using an animal model, we examined the expression of growth factors in knee osteochondral defect. Two male and two female non-engineered SD rats were purchased from Indonesian Food and Drug Administration, Jakarta. Rats at 11 weeks of age, 292 15 gram body weight were housed in Animal Laboratory at Research and Health Care Development Center, Indonesian Ministry of Health, Jakarta and undergone adaptation with

twelve-hour lighting cycle and free access to food and tap water. Each rat was manipulated with two defects including superficial defect in proximal trochlear and deep defect in distal trochlear region in the right knee joint at one time (Figure. 1). Establishment two defects in one knee has been done to minimize the number of rats being used and to minimize the operating procedures which are painful to the experimental animals.These were consistent with the ethical principle for animal experiment[10]. The preliminary study of defect modeling showed that osteochondral defect model with two types of defect (superficial and deep defect) can be made on one trochlear of rat without damaging the bone, deformity formation, pain and death (data not shown). Superficial defect was produced by drilling right trochlear with 1 mm stopper end which had 1.1 mm diameter (30% trochlear width). The depth of superficial defect was created according to the reference [11]. Deep defect was produced by drilling superficial defect with 0.8 mm K wire until it reached subchondral bone. Then, the knee joints were irrigated with saline solution. After the surgery, rats were returned to their cages and could move freely. The rats were fed as needed and received paracetamol 300 mg/kg bodyweight and amoxicillin at 150 mg/kg body weight subcutaneously for 5 days.Rats were randomexecuted on day 1, 7 and 28. Treatment with superficial defect and deep defect were given in all rats on day 0. Macroscopic and microscopic evaluations performed.

Animal care and use statement

All procedures had been approved by Animal Care and Use Committee (ACUC) Bimana the Ethical Committee Centre for Non-human Primate, Bogor Agricultural University, Number R.03-12-IR. The procedures involving animal model SD rats in this study were conducted strictly based on the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health[10]. All surgery was performed concordance with surgery in human which is sterile and aseptic. Anesthetic has been done using ketamine and xylazine in anesthetic dose. The euthanasia was performed at the end of the study, using pentobarbital lethal dose. All procedures involving animal model were conducted under supervision of a veterinarian and were made to minimize suffering from surgery.

Macroscopic evaluation

Each defect was examined macroscopically in day 1, 7 and 28. Rats were sacrificed by euthanized with pentobarbital intraperitoneally. We performed morphological evaluation to observe the repair tissue of the defect, then documented using Olympus SLR E-620 The macroscopic evaluation used a macroscopic score according to Nishimori's modification [12].

Microscopic evaluation, histology,and immunohistochemistry (TGF-β1, IGF,and FGF) examination

To confirm regeneration tissue of the defect and expression of growth factors we performed histology and immunohistochemistry examination. Specimens at day 1, 7 and 28, were fixed with 4 % paraformaldehyde and 70 % alcohol alternatively for 24 hours at 4 ○C, decalcified with Plank &Rychlo for 10 days, dehydrated with alcohol then encased in paraffin and sectioned in 5 □m thick slices and stained with Hematoxylin and Eosin (HE), TGF-β1, IGF, and FGF staining [13]. The miscroscopic evaluation score was performed according to Pineda's modification [14]..

The specimens used for immunohistochemistry TGF-β1, IGF, and FGF examinations were deparaffinized, rehydrated with running water, and washed in aqua. Then each slides incubated in a sequence with protein blocker (BIOCARE's Background Sniper solution from BIOCARE Medical, combined with normal horse serum), and only one antibody, anti rat TGF-β1 antibody (Abcam), anti rat IGF antibody (Abcam), anti rat FGF antibody (Abcam)), BIOCARE's Trekkie Universal Link solution (Starr Trek Universal HRP Detection System from BIOCARE Medical), and BIOCARE's TrekAvidin-HRP (Starr Trek Universal HRP Detection System from BIOCARE Medical). After incubations, the specimens were washed with PBS and diamino benzidine (DAB) solution, then counterstained with hematoxylin. For the last procedures, the specimens were washed again with aqua, dehydrated, cleared and mounted. Qualitative and quantitative examinations were performed using a light microscope and microphotography tools.

3. Results and Discussion

General condition of the animals

There were no ill, deformed or dead rats, until the end of study. The rats walked, climbed, eat, drink and moved as usual. The rat's weight decreased non significantly on day 7, and regained on day 21 (Figure 1).

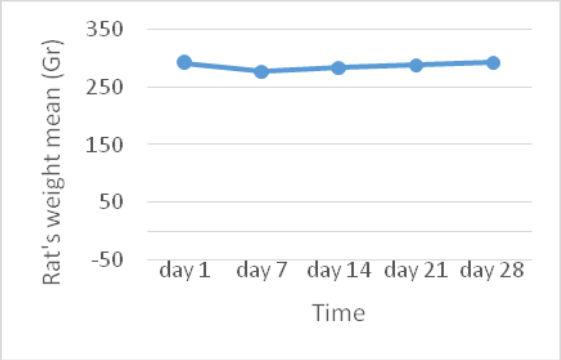

Figure 1. Rat's weight mean.

Macroscopic evaluation

Scoring and macroscopic examination successfully performed on all defects on days 1, 7, 28. There were no signs of infection and allergies in all defect. Surface and boundary defects were changed. Superficial defects surface was changed until day 28, while the deep defects surface unchanged on day 28. Superficial defect surface covered with a transparent layer. Superficial and deep defect margin changed on day 7 and 28 (Figure 2).

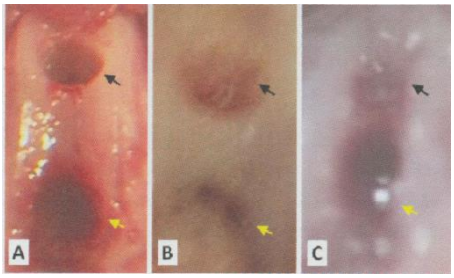

Figure 2. Deep defect and superficial defect model of Sprague Dawley Rats.

Note:
(A) Day 1: The superficial defect demonstrated no bleeding. The deep defect demonstrates bleeding (yellowarrow). No bone destruction. (B) Day 7: Surface defects and defect area boundary were the same as day 1. (C) Day 28: Surface defects covered by transparent layer boundary defects began to faint.

There was no different between margin defect score of superficial defect and margin defect score of deep defect. Macroscopis score from male rat and female rat were the same on day 7 and 28. (Tabel 1).

Table 1. Macroscopic Scores of Superficial Defect and Deep Defect on Day 1. 7 and 28

Time	Day 1		Day 7		Day 28	
	M	F	M	F	M	F
Superficial defect						
Sign of infection	0	0	0	0	0	0
Sign of allergy	0	0	0	0	0	0
Defect surface	3	3	3	3	2	2
Defect margin	3	3	2	2	2	2
Total	6	6	5	5	4	4
Deep defect						
Sign of infection	0	0	0	0	0	0
Sign of allergy	0	0	0	0	0	0
Defect surface	3	3	3	3	3	3
Defect margin	3	3	2	2	2	2
Total	6	6	5	5	5	5

Note : infection (no=0, yes=1), allergy (no=0, yes=1), surface (normal=0, whittish layer=1, transparent=2, not covered=3), margin (cannot differentiated=0, difficult to differentiated=1, easy to differentiated=2, clearly differentiated=3). M = Male, F = Female.

Microscopic evaluation

HE staining was successfully performed days 1, 7 and 28. Staining was successfully illustrate the shape and regenerated tissue tissue of the defects(Figure 3).

Superficial defect **Deep defect**

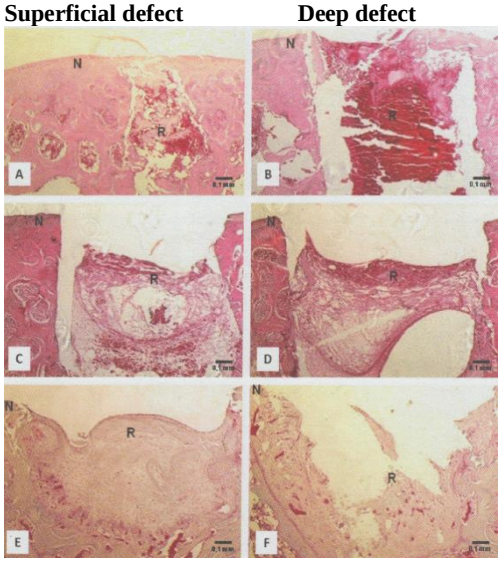

Figure 3. Microscopic HE staining images of rat osteochondral defect on day 1, 7 and 28.

Note:
(A-B) Day 1: Neither superficial defect or deep defect were filled with regenerated tissue. (C-D) Day 7 : Superficial defect and deep defect were begin to fill with regerenared tissue. (E-F) Day 28 : Superficial defect and deep defect were filled with fibrous tissue. N = Normal tissue. R = Regenerated. TissueBar = 0.1 mm.

Expression of growth factor evaluation

Immunohistochemical staining to analysis the growth factor TGF-β1, IGF and FGF expression, successfully carried out day 1, 7, and 28. Growth factor expression was observed in chondrocyte cells on cartilage defects, the expression began appeared on the day 7 and disappeared on from to day28 (Figure 4-6).

Superficial defect **Deep defect**

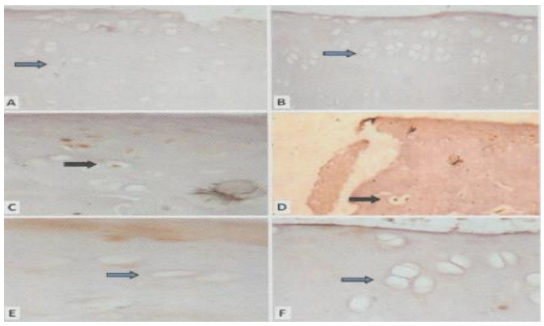

Figure 4. TGF-β1 immunohistochemistry staining of rat osteochondral defect on day 1, 7 and 28.

Note:
(A-B) Day 1 : There is no sign of TGF-β1. Chondrocyte cell did not absorbed brown staining (blue arrow) on superficial defect and deep defect.
(C-D) Day 7 :. Chondrocyte cell absorbed brown staining (black arrow) on superficial defect and deep defect.
(E-F) Day 28 : The expression of TGF-β1 dissapeared. Chondrocyte cell did not absorbed brown staining (blue arrow) on superficial defect and deep defect. Magnification = 100 x.

Superficial defect **Deep defect**

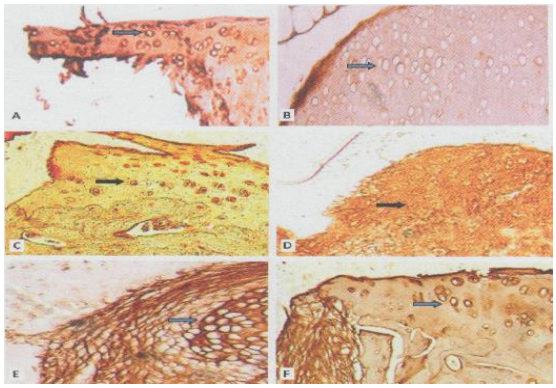

Figure 5. IGF immunohistochemistry staining of rat osteochondral defect on day 1, 7 and 28

Note:
(A-B) Day 1 : There is no sign of IGF. Chondrocyte cell did not absorbed brown staining (blue arrow) on superficial defect and deep defect.
(C-D) Day 7 :. Chondrocyte cell absorbed brown staining (black arrow) on superficial defect and deep defect.
(E-F) Day 28 : The expression of IGF dissapeared. Chondrocyte cell did not absorbed brown staining (blue arrow) on superficial defect and deep defect. Magnification = 100 x.

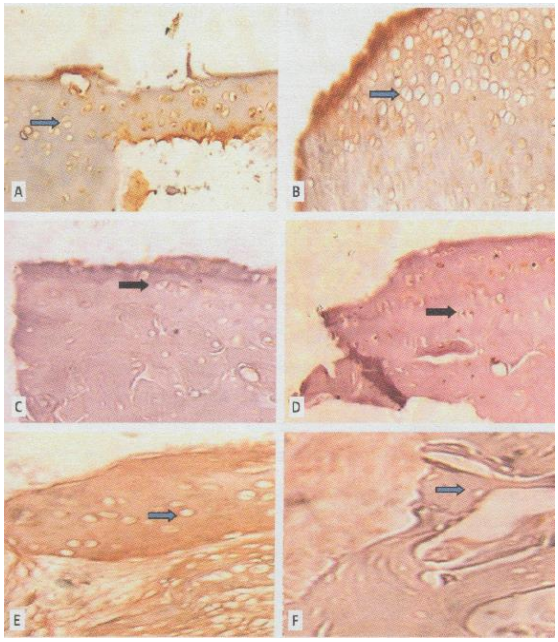

Figure 6. FGF immunohistochemistry staining of rat osteochondral defect on day 1. 7 and 28

Note:
(A-B) Day 1 : There is no sign of FGF. Chondrocyte cell did not absorbed brown staining (blue arrow) on superficial defect and deep defect. (C-D) Day 7 :. Chondrocyte cell absorbed brown staining (black arrow) on superficial defect and deep defect. (E-F) Day 28 : The expression of FGF dissapeared. Chondrocyte cell did not absorbed brown staining (blue arrow) on superficial defect and deep defect. Magnification = 100 x.

Macroscopic scores began to decline on the 7th day and decreased on day 28. The macroscopic score changes showed morphological changes from time to time. The macroscopic score showed that tissue repairment or regeneration process, started from the 7th day. This is in line with the expression of TGF-β1, IGF, and FGF which occurred on day 7, and ends on day 28. This is also consistent with the microscopic data, which showed fibrous tissue formation in the defect. This fact occurred in male and female rats. This showed that rat's knee had internal ability repairment, when knee's tissue defect was occurred.

These results indicate that the growth factor has a temporary expression. Temporary expression of growth factor occured because cartilage tissue were damage . When cartilage damage, a lot of chondrocyte cell died. Cell proliferation and regeneration occured 7 after the the tissue damage [15]. The growth factors induced the chondrocyte proliferation [1,3-5,7]. After weeks later, chondrocyte proliferation decreased and at the end dissapeared [15]. Chondrocyte proliferation reduction, in line with the need of extracellular matrix proteoglycans formation [16].

4. Conclusions

The study proved the expression of growth factors TGF-β1, IGF and FGF in line with regeneration tissue of the osteokondral defect of knee joints in SD rat.

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