

Basic Medical Research

## Effect of extracorporeal irradiation on segmental bone autograft incorporation in Sprague-Dawley rats

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### Abstrak

**Latar belakang:** Autograft tulang digunakan pada operasi rekonstruksi kasus tumor tulang. Extracorporeal irradiation (ECI) pada tulang digunakan untuk membunuh sel tumor. Namun radiasi juga menimbulkan efek negatif terhadap osteoinduksi dan osteogenesis untuk inkorporasi autograft tulang. Penelitian ini bertujuan mempelajari pengaruh iradiasi dengan dosis 50-300 Gy terhadap kemampuan inkorporasi graft tulang.

**Metode:** Pada 24 ekor tikus Sprague-Dawley dilakukan reseksi en bloc 7 mm pada diafisis tibia. Tikus dibagi menjadi 4 kelompok: kelompok 1 tidak mendapat radiasi; kelompok 2, 3 dan 4 berturut-turut mendapat iradiasi 50, 150 dan 300 Gy pada tulang yang direseksi, selanjutnya diimplantasi kembali. Inkorporasi tulang dievaluasi pada minggu ke-6 dan -8 secara radiologi dan pada minggu ke-8 dilakukan evaluasi histopatologis, hitung osteoblas dan pengukuran ekspresi BMP-2. Data dianalisis menggunakan uji Anova dan Kruskal-Wallis.

**Hasil:** Pada minggu ke-6, skor radiologis kelompok iradiasi 150 dan 300 Gy lebih rendah dibandingkan kontrol (4 vs 6 dan 4 vs 6;  $p = 0,01$ ). Demikian juga pada minggu ke-8 (5,40 vs 7,14;  $p = 0,009$  pada kelompok 150 Gy dan 5,60 vs 7,14;  $p = 0,018$  pada kelompok 300 Gy). Pemeriksaan histopatologis memperlihatkan bahwa median skor histologis kelompok iradiasi 50, 150 dan 300 Gy lebih rendah secara bermakna dibanding kontrol (6 vs 7,  $p = 0,017$ ; 4 vs 7,  $p = 0,005$ ; 6 vs 7,  $p = 0,013$ ). Hitung osteoblas dan ekspresi BMP-2 tidak menunjukkan perbedaan bermakna pada tiap kelompok.

**Kesimpulan:** ECI dengan dosis 50 sampai 300 Gy terhadap autograft tulang menyebabkan keterlambatan dalam inkorporasi autograft tulang, namun autograft tersebut masih memperlihatkan kemampuan osteoinduktif dan osteogenesis.

### Abstract

**Background:** Bone graft has been widely used in bone tumor reconstructive surgery. Extracorporeal irradiation (ECI) is commonly used to eliminate malignant cells before bone autograft. However, it may have negative effects on autograft incorporation. This study aimed to evaluate the ability of bone autograft incorporation after extra corporeal irradiation.

**Methods:** 24 Sprague-Dawley rats underwent 7-mm en bloc resection of tibial diaphysis, and were divided into 4 groups. The first group did not receive irradiation; the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> groups received 50, 150 and 300 Gy bone irradiation respectively, and then reimplanted. Radiologic score were evaluated at week-6 and -8, while histopathology, osteoblast count and BMP-2 expression were examined at week-8. Data were analyzed with ANOVA or Kruskal-Wallis tests.

**Results:** At week-6, radiologic scores in group 150 and 300 Gy were significantly lower compared to control group (4 vs 6 dan 4 vs 6;  $p = 0.011$ ;  $p = 0.01$ ). The same results were also obtained at week-8 (5.40 vs 7.14;  $p = 0.009$  in the group 150 Gy and 5.60 vs 7.14;  $p = 0.018$  in the group 300 Gy. Histopathological scores of the groups receiving 50, 150 and 300 Gy were significantly lower compared to the control group (6 vs 7,  $p = 0.017$ ; 4 vs 7,  $p = 0.005$ ; 6 vs 7,  $p = 0.013$ ). Osteoblast count and BMP-2 expression were not significantly different among all groups.

**Conclusion:** ECI with the dose of 50 to 300 Gy is associated with delayed bone autograft incorporation. However, the osteoinductive and osteogenesis capacity for autograft incorporation were maintained.

**Keywords:** autograft incorporation, BMP-2, extracorporeal irradiation (ECI), osteoblast, osteoinductive, osteogenesis

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Nowadays, bone graft is widely used in orthopedic surgery, either for fracture healing or bone tumor reconstructive surgery. Reconstruction of the bone tumor needs a massive bone graft, either by autograft or allograft as a substitute of prostheses, depends on the case and graft availability. Bone allograft, which are common in the countries that have tissue banks, has some disadvantages including the risk of viral transmission and the possibility of rejection due to recipient's immune system. In the absence of tissue bank facility, bone autograft become an option. This autograft brings additional advantages such as a much lower risk of immune reaction, as well as a lower cost for the patients. However, the possibility of residual bone tumor in the autografted segment represents a significant drawback. An attempt to overcome this problem is by extracorporeal irradiation (ECI) of bone segment to be implanted. In this method, the bone undergoes en bloc resection, irradiation and re-implantation. Irradiation dose of 50-300 Gy is usually used and it has been known to be quite effective to kill tumor cells. But, radiation may also have negative effects on cells of the bone graft.<sup>1,2</sup> The bone components, such as the osteogenic and bone marrow cells are radiosensitive, while the inorganic matrix, the major components of the bone is relatively radio resistant.

Osteogenic cells are precursor cells which play roles in the formation of the new bone. A lot of studies have been conducted with the aim to identify the effect of irradiation on the bones, bone healing and bone graft incorporation.<sup>3,4</sup> Besides osteogenic cells, the bone matrix also contains growth factors which are essential for bone healing and bone graft incorporation. One of the growth factors is bone morphogenic protein-2 (BMP-2), a derivate of transforming growth factor- $\beta$  (TGF- $\beta$ ). Study that has been conducted by Tarmo et al, showed that a irradiation up to 25 kGy on BMP-2 extracted in gelatin capsules did not affect BMP-2 function which acts as osteoinductive factor.<sup>5</sup>

The effect of different doses of ECI on bone autograft incorporation and BMP-2 expression in the osteotomy healing area has not been clearly identified. This study was aimed to evaluate the influence of extracorporeal irradiation on the incorporation ability of bone autograft. In addition, its influence on histological features and expression of BMP-2 were also evaluated.

## METHODS

This was an experimental study using post-test only control group design conducted at the Laboratory of Experimental Animal and Toxicology, Research and Development Unit, Ministry of Health, Indonesia. Extra corporeal irradiation was performed at National Nuclear Energy Agency of Indonesia (BATAN), Jakarta; while histopathological and immunohistochemical studies were conducted at Department of Pathological Anatomy, Faculty of Medicine, Universitas Indonesia.

The study was conducted on 24 white Sprague-Dawley rats, 8-12 weeks, 250-350 g BW, which were bred at the Laboratory of Experimental Animal and Toxicology, Research and Development Unit, Ministry of Health, Indonesia, between December 2010 and February 2011. The rats were divided into four groups, the control group without irradiation and three groups receiving 50 Gy, 150 Gy and 300 Gy irradiation, respectively. The protocol of this study has been approved by Ethical Committee of the Faculty of Medicine, Universitas Indonesia (472/PT02.FK/ETIK/2010).

## Surgical procedure

The rat was placed in supine position, under aseptic condition an anterior incision (1.5 cm) was made on the left tibia. En bloc resection of the left tibial diaphysis was made about 7 mm in size. The bone was temporarily fixed with 0.8 mm Kirschner wire, soft tissues and skin was stitched using 4.0 vycril filament. The resected bone segments were put into transportation device equipped with dry ice. The bone for irradiation was taken to BATAN and received gamma irradiation of 50 Gy, 150 Gy and 300 Gy emitted by a calibrated radiotherapy Cobalt-60 source. After irradiation, the bone segments were reimplanted, fixated with a 0.8 mm K-wire and put into the medullary canal in a retrograde fashion toward proximal tibia and subsequently inserted to distal. After reimplantation, the experimental animal was kept at animal laboratory.

## Evaluation procedure

At the 6<sup>th</sup> and 8<sup>th</sup> week after bone autograft, implantation antero-posterior (AP) and lateral projections of tibial radiograph were performed. At the 8<sup>th</sup> all experimental animals were sacrificed, the tibial bone was resected and sent for histologic evaluation. Paraffin-embedded block was made for each rat's bone and histological slides

were stained with hematoxylin eosin (HE) and some were left unstained for immunohistochemical analysis. The HE stained slides were evaluated for radiological score using modified Salkeld histological score and for osteoblast count. Immunohistochemical analysis was performed using antibody antiBMP-2 reagent to evaluate BMP-2 expression. These parts of study were conducted at the Department of Pathological Anatomy, Faculty of Medicine, Universitas Indonesia.

### Radiographic assessment

The radiographic evaluation of bone graft incorporation was made based on radiographic grading score as described by Lane and Sandhu.<sup>6</sup> Score are shown in table 1. The assessment was performed by observing the proximal and distal osteotomy line on AP and lateral projection of radiograph at sixth and eighth week. The assessment was carried out using X-ray instrument by Philips bulky diagnosis, 46 kV 5.00 mAs. The distance between the light source and the film was 90 cm. The total score of complete radiographic assessment between proximal and distal distance was 8. The assessment was carried out by an expert in musculoskeletal radiology.

### Osteoblast count

On microscopic examination (40X magnification, HE stain), osteoblast count was evaluated by examining the proximal to distal osteotomy area and autograft cortex. The evaluation was performed by an expert in pathological anatomy. The evaluation criteria are shown in table 1.

### BMP-2 assessment

Immunohistochemical analysis was performed using the BMP-2 (P275) pAb reagent kit (Bioworld Technology, USA) with product category number of BS3473 to detect BMP-2 expression in rat bones. The evaluation was performed by using a microscope at 40x magnification and analyzing three fields of view in intramedullary area of the bone autograft. The number of cells expressing BMP-2 was counted.<sup>7,8</sup> The semi-quantitative assessments of BMP-2 expression are shown in table 1.

### Histopathological assessment

The assessment of autograft incorporation was evaluated histologically after performing the hematoxilyn eosin (HE) staining based on modified

**Table 1.** Evaluation criteria of osteoblast count, BMP-2 expression, histological and radiographic score

	Score	Criteria
Osteoblast count	(-)	No osteoblast was found
	(1+)	Osteoblast was found in several field
	(2+)	Osteoblast was found in every field
BMP-2 expression	(-)	No cell with positive staining
	(1+)	< 1/3 cells with positive staining
	(2+)	1/3-2/3 cells with positive staining
	(3+)	> 2/3 cells with positive staining
Histological scoring	0	No healing
	1	Healing with fibrous union
	2	Healing with fibrocartilaginous or cartilage union
	3	Bone healing with mineralized cartilage union
	4	Mature bone healing
Radiographic scoring	0	No healing
	1	No callus formation
	2	Initial bone formation
	3	Nearly disappeared osteotomy line
	4	Complete bone healing

Salkeld fracture healing scoring. The proximal and distal area of osteotomy was evaluated. The histology scoring was the total score of proximal and distal osteotomy area. The range of score was between 0 and 8.<sup>9</sup>

### Data analysis

Data are presented descriptively to indicate the characteristics of the tested bones and their similarity to control group. ANOVA test and Kruskal-Wallis test were used to analyze the correlation between independent and dependent variables.

## RESULTS

### Radiographic assessment of autograft incorporation

At the 6<sup>th</sup> week, the post-hoc analysis revealed p = 0.005 for 150 Gy compared to the control group. The median score of bone graft incorporation rate at 6<sup>th</sup> week after 150 Gy irradiation was 4.0 (2.0-4.0) and were significantly lower compared to control

group 6.0 (5.0-7.0). Following 300 Gy irradiation, the median score of bone graft incorporation rate at 6<sup>th</sup> week was 5.0 (4.0-6.0) which was significantly lower compared control group, which is 6.0 (5.0; 7.0). There was no significant difference among the irradiation groups.

At the 8<sup>th</sup> week, the mean score of bone autograft incorporation rate following 150 Gy irradiation is  $5.40 \pm 1.14$  which was significantly lower compared to the control group ( $7.14 \pm 0.69$ ,  $p < 0.05$ ). In the group receiving 300 Gy irradiation, the score was  $5.60 \pm 1.14$  ( $p < 0.05$  vs control group). No significant difference among the irradiation groups (Table 2).

### Comparison of histopathological score

Table 3 shows comparison of histopathological, osteoblast count and BMP-2 expression. The median value of bone histopathological score following 50, 150, and 300 Gy irradiation are 6.0 (5.0-6.0), 4.0 (4.0-6.0), and 6.0 (4.0-6.0). All of these values were significantly different from control group [7.0 (6.0-8.0),  $p < 0.05$ ]. There was also significant different between the group of 150 Gy and 50 Gy ( $p = 0.028$ ).

### DISCUSSION

This study used radiographic grading score as described by Lane and Sandhu<sup>6</sup> to assess fracture union. By using this score, the quality of healing in proximal and distal osteotomy can be evaluated. The timing of X-ray assessment at the 6<sup>th</sup> week was based on the fact that the X-ray assessment for tibial fracture union showed no significant changes after



Figure 1. Tibial bone X-Ray at 8<sup>th</sup> week

6 weeks.<sup>10</sup> In other word, on the sixth week, normal tibial bone fracture had undergone radiographical healing. We expected to see delayed bone healing by performing the X-ray at the 8<sup>th</sup> week.

At the 6<sup>th</sup> week, animals in the control group and those receiving 50 Gy irradiation showed radiographic healing, or at least had undergone bone formation. In fact, there were segments which showed complete bone healing according to Lane and Sandhu score. This finding is consistent with the results of the study conducted by Voggenreiter, et al<sup>6</sup> which showed that

Table 2. Comparison of radiographic scoring for bone graft incorporation at 6<sup>th</sup> and 8<sup>th</sup> week after extracorporeal irradiation

Score of incorporation	Control (n = 7)	50 Gy irradiation (n = 5)	150 Gy irradiation (n = 5)	300 Gy irradiation (n = 5)	Statistical test	p
Median at 6 <sup>th</sup> week	6.0 (5.0 ; 7.0)	6.0 (4.0 ; 7.0)	4.0 (3.0 ; 5.0)	4.0 (4.0 ; 6.0)	<i>Kruskal-Wallis</i>	0.012
Mean at 8 <sup>th</sup> week	7.14 ± 0.69	6.60 ± 1.14	5.40 ± 1.14	5.60 ± 1.14	<i>One-Way ANOVA</i>	0.027

Table 3. Comparison of histopathological, osteoblast count and BMP-2 expression

Variable	Control (n = 7)	50 Gy irradiation (n = 5)	150 Gy irradiation (n = 5)	300 Gy irradiation (n = 5)	Statistical test	p
Median of histopathological score	7.0 (6.0 ; 8.0)	6.0 (5.0 ; 6.0)	4.0 (4.0 ; 6.0)	6.0 (2.0 ; 6.0)	<i>Kruskal-Wallis</i>	0.004
Median of osteoblast count	constant	2.0 (1.0 ; 2.0)	1.0 (1.0 ; 2.0)	2.0 (1.0 ; 2.0)	<i>Kruskal-Wallis</i>	0.16
Median of BMP-2 expression	2.0 (2.0;3.0)	2.0 (1.0 ; 3.0)	1.0 (1.0 ; 3.0)	2.0 (1.0 ; 3.0)	<i>Kruskal-Wallis</i>	0.77

on the 6<sup>th</sup> week, the bone autograft, which was not exposed to irradiation had been healed with bone formation, and immature bone (woven bone) formation can be shown histologically. Meanwhile, most of bones which are exposed to 150 Gy and 300 Gy irradiations, were still in callus form and just began to have bone formation; although, there were 2 cases with complete bone healing. Thus, it can be assumed that 150-300 Gy irradiations may cause delayed bone autograft incorporation; while 50 Gy irradiation group showed similar incorporation rate with control group at 6<sup>th</sup> week observation.

After the 8<sup>th</sup> week, radiographic assessment in the control group revealed that there were 2 rats experiencing complete bone healing on both segments; while the other still demonstrated osteotomy line though the line has almost been disappeared. Some rats in the groups receiving irradiation also demonstrated complete bone healing at 8<sup>th</sup> week, which showed diminished osteotomy line. After post-hoc analysis was performed, a significant difference was found regarding the average rate of bone graft incorporation between control group and those with 150 Gy and 300 Gy irradiations.

A study conducted by Voggenreiter, *et al.*<sup>6</sup> demonstrated that at the 9<sup>th</sup> week, the bone autograft without irradiation had experience bone healing by bone formation; moreover, in some cases the osteotomy line had disappeared and the osteotomy area was hardly recognized. It can be said that the radiographic results of the control group at 8<sup>th</sup> week in our study are similar with the results of Voggenreiter at 9<sup>th</sup> week. Higher dose of irradiation (1kGy) has shown significant difference between the irradiated non irradiated autograft.

It can be concluded from the radiographic grading score that 150-300 Gy irradiation causes delayed incorporation of bone autograft. However, the irradiated bone still has the capacity for incorporation, as indicated by the increased score between the 6<sup>th</sup> and 8<sup>th</sup> week. Radiographically, the irradiation with 50 Gy does not give any signs of delayed incorporation.

The union process between autograft and recipient bones is similar to fracture healing process. However, the autograft transplantation of cortex bone may be accompanied by a process of creeping substitution, which includes revascularization at the beginning

of healing process, and subsequently followed by resorption of bone graft and formation of the new bones.<sup>11</sup>

In this study, the union process of proximal and distal segment of bone autograft against recipient bone was evaluated histologically using the modified Salkeld score, which assess the quality of the union.<sup>10</sup> We used K-wire during implantation for fixation and after implantation the rats directly went through weight bearing on their operated extremities; therefore, the healing model would be through the process of endochondral ossification.

Endochondral ossification involves recruitment, proliferation and differentiation of undifferentiated mesenchymal cells into calcified cartilage that eventually will be replaced by bone. The characteristic of such healing process includes some stages: hematoma formation, inflammatory phase, cartilage formation, calcification of the cartilage, cartilage resorption, bone formation and eventually bone remodeling.<sup>12</sup>

It has been known that irradiation may damage cells, both directly and indirectly. By direct mechanism, the irradiation ionizes cell DNA or other essential components of cell causing chemical changes in the cells or DNA. Bone graft irradiation may affect osteogenesis and osteoinductive components that play role in bone autograft incorporation.

The effect of irradiation on bone at initial stage includes vascular dilatation accompanied with injured myeloid cells and subsequently followed by bone resorption and cell lost in bone marrow. It can be due to macrophage disintegration in the bone marrow, which releases enzymes and causes myeloid aplasia. Occurrence of bone resorption is indicated by increased osteoclast lining of resorbed trabecula.<sup>13</sup> Damage in bone marrow may affect autograft incorporation since the stroma of bone marrow is a major contributing factor in osteogenesis. However, it should be noted that after each damage, the repairing process will follow.<sup>14</sup>

Maeda, *et al.*<sup>15</sup> revealed that at initial stage of radiation, there were decreases of bone cells and osteoid, resulting in lower osteoblastic and osteoclastic activity. After this period, there will be regeneration of the dead cells; however, osteoblastic activity requires longer time to be reactivated than the bone resorption.

Osteoblast is one of cell component of the bone which has essential function in collagen synthesis to develop osteoid and calcification process of the osteoid. Osteoblast is derived from osteoprogenitor cells of bone marrow stroma or from the adjacent soft tissues, and act as component of osteogenesis. Active osteoblast can change into bone lining cell and osteocyte.<sup>11,12,16</sup>

It has been known that the higher the levels of cell proliferation and differentiation, the more resistant the cells against radiation are. A study conducted by Jacobson et al on rat bones with irradiation dose of 40 Gy indicated that the osteocytes was still viable.<sup>17</sup>

A study conducted by Dare, et al<sup>18</sup> demonstrated that following 40 Gy irradiation in rats, there was reduced capacity of osteoblast differentiation. Moreover, it was also found that there was reduced alkaline phosphatase activity.

Sugimoto, et al<sup>13</sup> found that during irradiation up to 50 Gy, the osteocytes stayed intact and only several empty lacuna were detected. Moreover, after a few weeks of irradiation there was significant lower capacity of developing new bone and reduced number of cells in bone marrow. However, when followed for a longer period, increased new bone formation, bone marrow cells and reduced porous bone could be observed.<sup>13</sup> Since new bone formation is the function of osteoblast, it can be concluded that immediately after irradiation, osteoblast damage occurs. However, since the bone marrow has a capacity for regeneration and it is one of osteoprogenitor cells sources, therefore our study did not find any significant different between the control group and irradiated groups. It is assumed that at 8<sup>th</sup> weeks following the irradiation, the bone marrow cells have regenerated and produced osteoblast cells.

BMP-2 is part of transforming growth factor- $\beta$  superfamily and it is one of bone growth factor components and has chemotactic characteristic for osteoprogenitor cells in vitro.<sup>19</sup> A study conducted by Bostrom, et al<sup>20</sup> showed the expression of BMP-2 in fracture callus. Thus, they concluded that BMP-2 has essential role in fracture healing.

Bone autograft has osteoinductive properties. It has a capacity to recruit mesenchymal cells in the host and its surrounding to differentiate into osteoblast and chondroblast. The recruitment is induced by

growth factor inside the autograft, such as BMP-2.<sup>11</sup> Anderson, et al<sup>21</sup> has conducted a study to identify BMP-2 expression in bone. By performing immunohistochemical analysis, the study found BMP-2 expression in osteoblast, chondrocytes, osteoprogenitor cells of medullary bone stroma and cells that have role in vascular formation.

In our study, there was no significant difference of BMP-2 expression at least in two groups. It is similar to the study conducted by Anderson, et al.<sup>21</sup> There was no significant difference between control and irradiated groups, because after 8 weeks, cell regeneration has occurred in bone marrow; therefore, during the assessment, it has been able to express BMP-2. Another rational for this includes BMP-2 denaturation has not been proven to be present following irradiation in a dose of 50-300 Gy. It is in accordance with the study conducted by Wintroub et al,<sup>22</sup> which demonstrated that BMP still has osteoinductive function at dose range of 50-70 kGy on demineralized allograft. Osteogenesis was evaluated by osteoblast count and osteoinductive factor was assessed by BMP-2 expression. Both factors have important role in autograft incorporation. The osteoblast count and BMP-2 expression at 8<sup>th</sup> week did not show significant difference between control group and autograft that had received irradiation.

If we consider the histological and radiographical score, it can be said that at 8<sup>th</sup> week, there has been regeneration of bone marrow cells. Thus, osteoblast count and BMP-2 expression show non-significant difference. This is consistent with a study conducted by Sugimoto, et al<sup>13</sup> which indicated that following irradiation, the bone marrow has a capacity for regeneration. The larger the dose of irradiation, the longer the time needed for regeneration.

A study by Tarmo, et al<sup>5</sup> showed that BMP-2 extracts which received 4 kGy irradiation still has osteoinductive function. It can be also considered that bone autograft irradiation up to 300Gy does not cause denaturation of BMP-2.

The onset of regeneration and the increase of osteoblast activity following 150-300 Gy irradiation is assumed to occur between 6-8 weeks. However, following 50 Gy irradiation, bone marrow regeneration seems to begin earlier, as indicated by non-difference of radiographical score with control group at the 6<sup>th</sup> week.

It can be concluded from our study, that extracorporeal irradiation at the dose of 50-300 Gy is associated with delayed incorporation without impairing the capacity of bone autograft incorporation since it still retain osteoinduction and osteoconduction capabilities.

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### Conflic of interest

All authors have nothing to disclose.

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