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RESEARCH ARTICLE

Expression Relative of RANK, RANKL and OPG Gene on Rat Femoral Fracture Healing Process in Delayed Union Model after Pulsed Electromagnetic Field Exposure

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Abstract

Pulsed electromagnetic field (PEMF) is application medical devices in healing bone fracture. Osteoblast and osteoclast cells have regulation in femoral fracture healing process. PEMF exposure has effect in cell nucleus activity. Genes that have critical role in bone remodelling are Receptor activator of nuclear factor kappa-B (RANK), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG).This research aimed to analysis effect PEMF exposure in improve bone fracture healing process with delay union model, which its expression level in RANK, RANKL and OPG gene. Our research used experimental design. Sample for this research was callus from rat femoral fracture with delay union. QRT-PCR techniques with Livak's measurement were used to detection expression relative. Statistic analysis used two way ANOVA with a significant differences value $p < 0.05$. The result showed that relative expression on RANK, RANKL and OPG have significance different in PEMF group and times (days) group. We found that the PEMF group can expedite the higher expression relative of RANK, RANKL and OPG than non PEMF group. The result has concluded using PEMF can improve the healing to normal union process on rat with delay union model.

Keywords: *Fracture healing, Delay Union, PEMF, OPG, RANK, RANKL.*

Introduction

Healing fractures in bones other than producing union can form delayed union and non-union. In this study focused on delayed union. Delayed union is fracture healing with ongoing clinical and radiological signs but fails to continue in the estimated time [1, 2]. The method of bone fracture therapy is in two ways; surgery or without surgery. In surgical method, there were closed reduction of the fracture with percutaneous skeletal fixation [3].

In a non-surgical method, one of was used pulsed electromagnetic field (PEMF) therapy. PEMF provides assistance effectively in helping to unite bone fractures. But the use of PEMF is rarely used at the beginning of bone healing. PEMF is used more after being diagnosed with a non-union or the final stage of a delayed union for more than 6 months [4] .Weak electromagnetic field could modulate electrochemical process at molecular interphase in the cell [5]. The combination of intracellular Ca2 + release from the intracellular composition (blocked by TMB-8) which leads to increased cytosolic Ca2 + in turn, leads to increased activation of calmodulin (blocked by W-7) and subsequent increase in bone cell proliferation.

The site of initial transduction with capacitive coupled stimulation (the calcium channel Ca2 + entry into cells) differs from combined electromagnetic field stimulation and by inductive coupling (intracellular Ca2 + release), all three stimulation methods have the same final pathway, namely cytosolic Ca2 + increase and activated

calmodulin increase [6]. The pulsed electromagnetic field create non-thermal fields with high-rates of amplitude changes. There are in-vitro effects of electromagnetic fields on gene expression, and focused to effects on Ca2+ transport across cell membranes and messenger RNA [7]. The duration of bone healing in mice after the fracture is about 28 days divided become the three main stages, namely inflammation, repair and remodelling [8].

The inflammatory stage occurs on days 0 to 4 but in the acute inflammatory response can continue until the day 7 [9, 10]. Repair stage occurs from days 3 to 14. The stages of bone remodelling begin simultaneously with repairs up to days 28 or more until day 35 [8]. Bone remodelling is a process of bone replacement by regulation of osteoblasts and osteoclasts [10].Osteoclasts remove old bones and then osteoblasts fill them with new bone[11, 12].

Communication between osteoblasts and osteoclasts is divided into three phases namely initiation, transition and termination [12,14]. Phase initiation consists of the recruitment of osteoclast precursors, osteoclast differentiation and activation, and maintenance of bone resorption. The transition phase is a period when back bone absorption is inhibited, osteoclasts undergo apoptosis, osteoblasts recruited and differentiated.

The termination phase consists of new bone formation, mineralization and inhibited osteoclast differentiation [14]. The system of RANK-RANKL-OPG is important for regulation of osteoclastogenesis [15]. RANK expression is detected in pre-osteoclasts, mature osteoclasts and dendritic cells [15, 16].

RANKL is stimulates pre-differentiation osteoclasts by binding to RANK and activating bone absorption[17]. Osteoprotegerin (OPG) acts as a competitor of receptors from RANKL [12, 18].RANK bind with RANKL will activate osteoclastogenesis, while OPG inhibits RANKL to bind to RANK so that osteoclastogenesis does not occur [12, 16]. Fracture healing is a complex physiological process. In knowing the effectiveness of PEMF by applying advances in molecular biology and genetics, we began to examine how the molecular pathways affected by PEMF.

Research by Ross et al (2015).Showed the effect of PEMF on bone marrow cell differentiation [19]. The effect of PEMF on osteoclasts is reported by Wang (2017) which shows an effect on the ability to absorb murine macrophages or monocytes through the expression of the RANK gene [20]. The balance between osteoblasts and osteoclasts is necessary for healing bone fractures. In this study we observed effects of PEMF in transcription process on RANK, RANKL and OPG gene.

Material and Methods

Subjects

This design study was experimental in vivo with used 24 Sprague Dawley rats with femur bone fracture delay union. Delayed union model of bone fracture was referring to Kasman D study used stripping periosteum circularly with surgical knife around 5 mm from fracture line to proximal and then distal [21].

Subjects were divided into 8 groups. Groups were based from 5, 10, 18 and 28 day group for PEMF exposure and non PEMF exposure. PEMF machine from Umiatin study with maximal exposure 1,6 mT and frequency 60 Hz in 4 hour per day [22].Fractures of the femur bone were cut transverse as much as one piece at the middle of the callus, placed in RNA later and stored at -80^{o}C. Received permission from Research Ethics Committee of Faculty of Medicine University Indonesia (Approval letter No. 195/UN2.F1/ETIK/2018).

RNA Isolation

For RNA isolation we used 30 mg of callus. It was mashed using mortar & pestle. Total RNA isolation used Trizol and Qiagen RNA easy kit. The procedure of RNA isolation was performed in accordance with the procedure of the kit. Total RNA was stored at -80oC.

CDNA Synthesis

Total RNA from RNA isolation was denaturated at 65oC for 5 minutes used thermal block and then stored in ice immediately. cDNA synthesis was done using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit. Measuring the concentration and purity of cDNA used spectrophotometer.

QRT-PCR Analysis

CDNA that already establish was used to qRT-PCR Analysis. We used Sensi FAST SYBR Lo-Rox kit for master mix qPCR.

The primers used were designed by IDT software (http://sg.idtdna.com/PrimerQuest) with primary sequences in Table 1.Samples were amplified for 40 cycle with denaturation on 95°C for 12.5 seconds, annealing on 55°C for 10 seconds and elongation on 72°C for 20 seconds and then the Ct value was obtained. Analysis was carried out by the Livak method as a quantification method to show the level of relative expression [23, 24].

Table 1: Primer mRNA

No	Gene	Primer sequence (5' – 3')	PCR Product (bp)
1	RANKL	Forward : CATCGCTCTGTTCCTGTACTT Reverse : CGAGTCCTGCAAACCTGTAT	118
2	RANK	Forward : GCTCTTCCCTGACACTCATAAA Reverse : CACCACTACCACAGAGATGAAG	95
3	OPG	Forward : ACTTGGCCTCCTGCTAATTC Reverse : CGCACAGGGTGACATCTATT	104
4	β actin	Forward : GATCTGGCACCACACCTTCT Reverse : GGGGTGTTGAAGGTCTCAAA	106

Data Analysis

Statistical analysis was performed using SPSS 25. Data was tested for normality using Shapiro-Wilk with results p> 0.05 indicating that the data had a normal distribution. Homogeneity test using Lavene test produced data p> 0.05, which showed homogeneous data.

Both of these results are requirements for the Two Way ANOVA parametric test which aims to determine the significant differences on days 5, 10, 18 and 28 with the PEMF exposure and non PEMF exposure group. Differences were then tested using Post Hoc Turkey.

Results

Differences in the relative expression of RANK were seen in treatments non exposure to PEMF exposure. The highest relative expression RANK in exposure group was 5 days after exposure and then decrease at day 10. While in non-exposure group, the highest relative expression RANK on day 10 and then decreased on day 18.

This may indicate that highest recruitment of osteoclast precursors in exposure group faster than non-exposure group. Decrease of relative expression RANK indicated that osteoclast *differentiation* and osteoclast activation at day 10 for exposure group and 18 day for non-exposure group (see Figure 1).

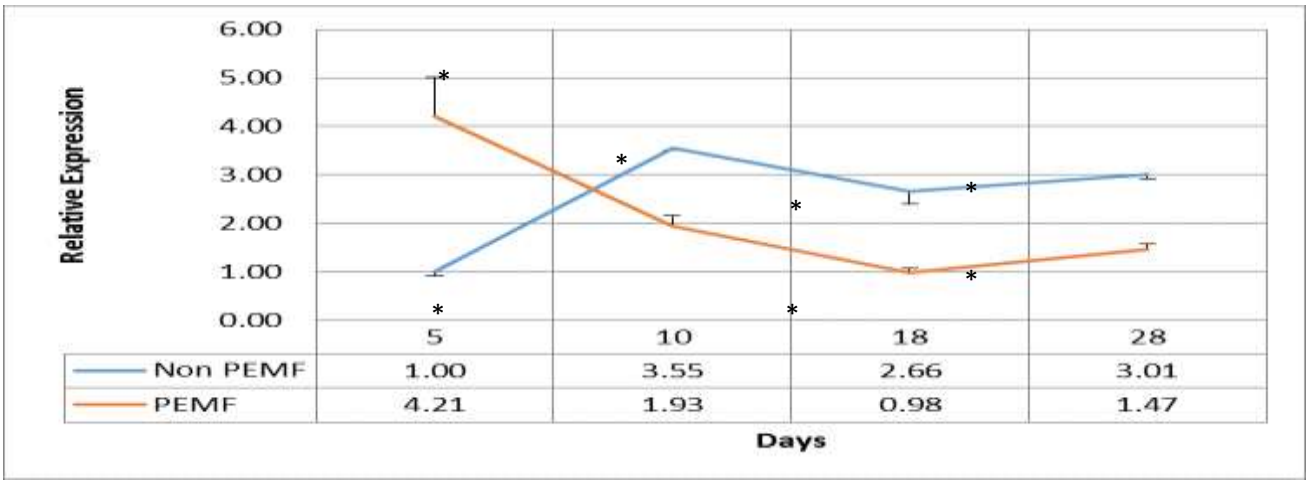

Figure 1: Relative expression RANK in PEMF exposure group and non PEMF group. The graph showed that relative expression in PEMF group has highest at 5 days, while group of non PEMF at 10 days. After fifth day in PEMF group, relative expression RANK has decrease and then increase at 28 days. In group non PEMF, after increase highest at 10 days, the relative expression RANK has decrease at 18 day and then increase at 28 day. *= (p<0, 05)

The relative expression RANKL in the PEMF exposure group showed a two-fold increase, the first increase occurred on the 10th day. In the group non-exposure showed one an increase and occurred on the 18th day. During exposure, PEMF showed an increase in graph acceleration compared to non exposure. On day 5 the relative expression of

RANKL is higher than non exposure, then rises on day 10, decreases on day 18, and rises again on day 28. This may show a pattern of RANKL gene that rises after active osteoclasts then drop when osteoclasts stop active and increase again when osteoblasts are active (see Figure 2).

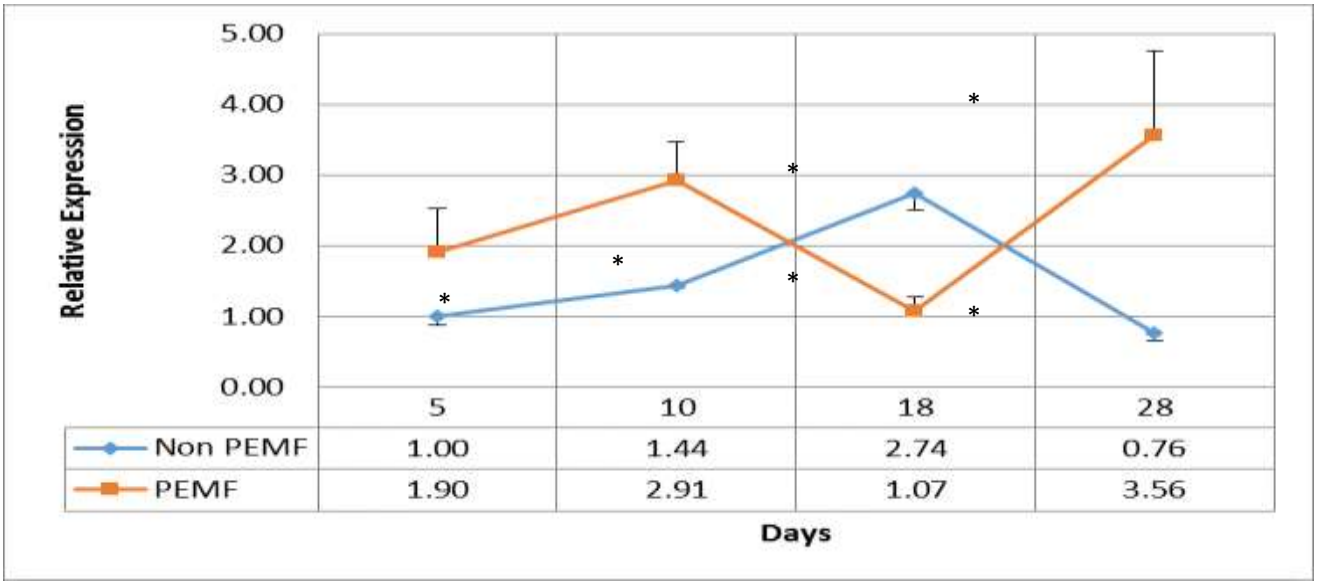

Figure 2: Relative expression RANKL in PEMF exposure group and non PEMF group. The graph showed that relative expression in PEMF group has highest at 28 days, while group of non PEMF at 18 days. Relative expression RANK in PEMF group has increase until tenth days, and then decrease at 18 days then increase until 28 days. In group non PEMF, increase until 18 days, then decrease again until 28 days.*= (p<0, 05)

In this study, the relative expression of OPG on day 5 with exposure to PEMF was higher than non-exposure (see Figure 3). In the non-exposure, the relative expression of OPG shows a pattern of increase from days 5 to 10, and then decreases on days 18 to 28. This may indicate OPG comes from the secretion of cytokines in the inflammatory stage. From the two ways ANOVA test showed a significant difference on the 10th and 28th days non exposure where the 10th day was the highest relative expression of OPG while the 28th day was the lowest, indicating that on the 28th day the osteoblasts were not active due to the decline.

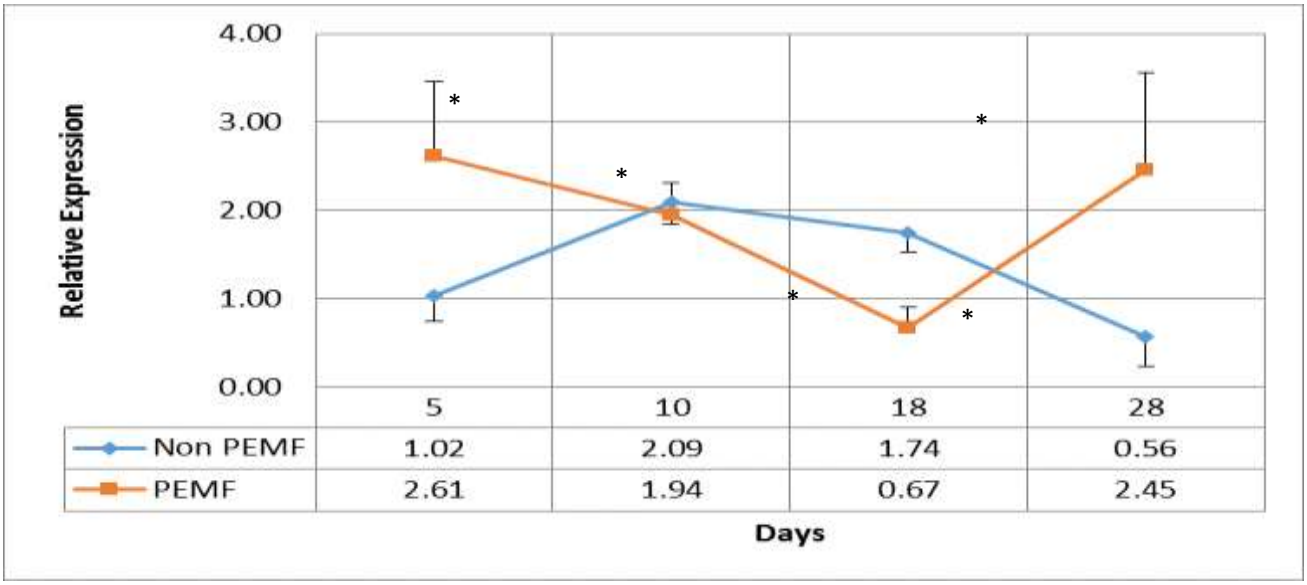

Figure 3: Relative expression OPG in PEMF exposure group and non PEMF group. The graph showed that relative expression in PEMF group has highest at 5 days, while group of non PEMF at 10 days. Relative expression RANK in PEMF group has decrease until 18 days, and then increase until 28 days. In group non PEMF, increase until 10 days, then decrease again until 28 days.

Discussion

In the PEMF exposure group, the fifth day of the highest expression of relative RANK decreased after the 18th day and increased to 28th day. This indicates that on the 5th day osteoclasts have been actively resorbing the

bone so that the next day they return to the osteoclast active seen with a decrease in the relative expression of RANK. The results of two ways ANOVA exposure to PEMF differed significantly on days 5, 18 and 28. Based on the graph non exposure PEMF showed recruitment and activation of osteoclasts from days 10 to 28 while exposure to PEMF had the effect of accelerating osteoclast activation in bone absorption on day 5.

RANK genes are expressed by osteoclast precursors and mature osteoclast [25]. Our result showed that RANK gene had a highest expression in 5th day exposure PEMF. This indicated that resorption of necrotic cell after fracture had been occurred in 5th day after PEMF exposure. In normal healing process, inflammatory stage occurred in 5th day.

In the inflammatory stage there is a process of bone resorption that experiences necrosis by osteoclast cells [24].On the 10th day with a 10-day exposure period, the relative expression of RANK showed a decrease in expression compared to non exposure. On the 10th day, the stages of healing bone fractures naturally enter the repair stage where there are osteoblasts, osteoclasts and chondrocytes [25].

On day 18 naturally enter the stage of repair with coarse callus formation containing myelopoietic and hematopoietic cells [25]. Because osteoclasts not active at this stage, a decrease in RANK gene expression occurs. On day 28, the relative expression of RANK from PEMF exposure appears to increase slightly.

According to Ralston, the preparation of bone remodelling begins with the withdrawal of osteoclast precursors in the peripheral region to the remodelling region [26]. This shows the duration of exposure of PEMF to affect the acceleration of activation of the RANK gene compared to non exposure. From the two ways ANOVA test showed a significant difference in the length of exposure compared to the use of PEMF exposure.

In the group non exposure the difference was significant on days 5.10, 18 and 28. The PEMF exposure group was significant on days 18 and 28. On days 5 and 10 exposure High RANKL expression was thought to be due to osteoclast activation. According to Hofbauer&Schoppett that RANKL stimulates RANK as a receptor to activate osteoclasts. RANKL is also known to stimulate maturation and osteoclast activation in absorbing bone [27]. On day 10, it naturally enters the repair stage with osteoblasts, osteoclasts and chondrocytes in it. The possibility of relative expression of RANKL increases because it is expressed by osteoblasts and their interaction with the RANK gene to activate osteoclasts [28]. This is thought to be an effect of the duration of exposure to PEMF by increasing RANKL expression.

On day 18 the relative expression of RANKL at PEMF exposure was lower. On day 18, coarse callus formation occurs so that RANKL expression decreases because the one active at this stage is myelopoietic and hematopoietic cells [29]. This is also thought to be an effect of the duration of exposure to PEMF by accelerating the decrease in RANKL expression. On day 28 the expression of relative RANKL increased higher than non exposure.

The possibility of high relative expression of RANKL is due to the differentiation of osteoblasts. In bone, osteoblasts, osteocytes and stromal cells express RANKL.[28] On the 28th day they have entered the bone remodelling stage where osteocytes are being formed.

This is thought to be an effect of the duration of exposure to PEMF by increasing RANKL expression. Based on the two way ANOVA test, we found significant difference between PEMF exposure on days 5, 18 and 28. The relative expression of the highest OPG on day 5 was probably derived from an increased cytokine in the inflammatory stage as an effect of PEMF exposure. Then it decreases until day 18 and increases again on day 28 which is likely to originate from osteoblasts.

The possibility of high expression of OPG is derived from cytokines expressed after inflammation. In accordance with Schopett et al., it was stated that cytokines derived from inflammation induce an increase in OPG [30]. This is thought to also originate from the effect of PEMF on increasing OPG expression.

On the 10th day there was a decrease in OPG expression at PEMF exposure. It is likely that on the 10th day the decrease in OPG expression comes from inhibitors of osteoclast activity.

Schopett et al. reported that bone absorption suppresses OPG expression.[30] On the 18th day OPG relative expression on PEMF exposure decreases. It is possible that on the 18th day there was a rough callus formation and OPG has not been needed that day. This is thought to be an effect in reducing OPG expression. On day 28 OPG expression at PEMF exposure increased higher than exposure and was significantly different. It is possible because OPG originates from osteoblasts which differentiate into osteocytes and are aided by the effect of PEMF in increasing OPG expression [29].

Based on the graph of non-exposure, there is a visible increase in the relative expression of RANK, RANKL and OPG from days 5 to 10, indicating recruitment of osteoclast precursors while decreases occur after day 18. Differentiation and activation osteoclasts were seen on day 18. On the relative expression of RANK and OPG while RANKL has increased until the 28th day, it is seen making preparations for the replacement of osteoblasts with osteoclasts. At exposure to PEMF, the relative expression of RANK on the highest 5th day compared to RANKL with OPG, it is seen that active osteoclasts and OPG are produced at the inflammatory stage.

On the 10th day the relative expression of RANKL increased, while the RANK and OPG decreased. Day 18, relative expression of RANK, RANKL and OPG decreased then an increase on day 28. On day 28 it had entered osteoblast activation. Based on the comparison between non-exposure and exposure, the expression of RANK, RANKL and OPG in the non-exposure had delayed expression, indicating that delayed union models were occurring. Exposure accelerates the increase and decrease in expression, making the effect of PEMF change the delayed union process to normal. PEMF shows an effect on osteoclast activation. Based on Funk that mentions cell differentiation is aided by progressive hyperpolarization. PEMF targets the plasma membrane and has the ability to bind ligands with its receptors. In signal transduction where the resting membrane potential turns into action. The addition of Ca ++ enters the cell so that making a positive feedback loop between Ca ++ entering the Ca ++ channel affects the G Channel voltage by activating several paths[31].

On day 5, high RANK is active in osteoclasts. On days 10, 15 and 28 RANK expression decreased steadily in the repair stage after inflammation and increased again in the remodelling stage. RANKL and OPG reach their highest expression at day 28. According to Wang, PEMF with an intensity of 0.5 mT decreases bone absorption by inhibiting osteoclast maturation and formation, whereas 3 mT increases bone absorption ability [20]. In this study showed that PEMF with 1.6 mT increases osteoclast activation in the inflammatory stage.

Conclusions

PEMF Exposure to Rat Femoral Fracture Healing Process in Delayed Union Model gave affect in relative expression of RANK, RANKL and OPG by indicated showing the normal healing of union process.

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Ethics

This article is original and contains unpublished material. This research has received permission from Research Ethics Committee of Faculty of Medicine University Indonesia with Approval letter No. 159/H2.F1/ETIK/2013

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