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Research Article

Adrenomedullin has no effect on segmental bone defect healing but increases bone mineral density in rat model

Mehmet Kaymakoglu[1], Eda Ciftci[2], Petek Korkusuz[3], Erdi Ozdemir[4], Mehmet Ege Erden[5], Egemen Turhan[6]

[1]Department of Orthopedics and Traumatology, Izmir University of Economics, Faculty of Medicine, Izmir, Turkey
[2]Department of Bioengineering, Hacettepe University Institute of Natural and Applied Science, Ankara, Turkey
[3]Department of Histology and Embryology, Hacettepe University Faculty of Medicine, Ankara, Turkey
[4]Department of Orthopedics and Traumatology, University of Health Sciences, Karabuk Training and Research Hospital, Karabuk, Turkey
[5]Hacettepe University Faculty of Medicine, Ankara, Turkey
[6]Department of Orthopedics and Traumatology, Hacettepe University Faculty of Medicine, Ankara, Turkey

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ORCID iDs of the authors:
M.K. 0000-0002-3548-5672;
E.C. 0000-0001-6900-4702;
P.K. 0000-0002-7553-3915;
E.O. 0000-0002-3147-9355;
M.E.E. 0000-0002-0148-2836;
E.T. 0000-0002-1971-704X.

ABSTRACT

Objective: This study aimed to investigate the effect of adrenomedullin on the healing of the segmental bone defect in a rat model.

Methods: Thirty-six Wistar rats were randomly divided into 6 groups based on follow-up periods and administered a dose of adrenomedullin hormone. In each group, bilaterally, a 2-mm bone defect was created at the diaphysis of the radius. Sodium chloride solution was administered to sham groups 3 times a week for 4 and 8 weeks intraperitoneally. Adrenomedullin was administered to the study groups 3 times a week: 15 μg—4 weeks, 15 μg—8 weeks, 30 μg—4 weeks, and 30 μg—8 weeks, respectively. After euthanasia, the segmental defects were evaluated by histomorphometric [new bone area (NBA)] and microtomographic [bone volume (BV), bone surface (BS), and bone mineral density (BMD)] analyses.

Results: Although the 4- and 8-week 15 μg administered study groups had higher NBA values than the other study and control groups, the histomorphometric analysis did not reveal any statistical difference between the control and study groups regarding NBA ($P > .05$). In microtomographic analysis, BV was higher in the 15 μg 4-week group than 30 μg 4-week group (296.9 vs. 208.5, $P = .003$), and BS was lower in the 30 μg 4-week group than in the 4-week control group (695.5 vs. 1334.7, $P = .005$), but overall, no significant difference was found between the control and study groups ($P > .05$). Despite these minor differences in histomorphometric and microtomographic criteria indicating new bone formation, the BMD values of the 15 μg 8-week study group showed a significant increase compared with the control group ($P = .001$, respectively).

Conclusion: Adrenomedullin positively affected BMD at 15 μg, but this study could not show healing in the segmental defect site at different dose regimens. Further studies are needed to assess its effects on bone tissue trauma.

Introduction

Adrenomedullin is a peptide hormone which consists of 52 amino acids and has a wide range of binding receptor distribution in many different tissues in our body presenting cardiovascular, endocrine, and neurological effects.[1-4] Adrenomedullin is now classified in the calcitonin gene family due to its very similar ring structure, and it has been shown in recent studies to have anabolic effects on bone tissue like another well-known member of the calcitonin gene family.[5-8] Despite the complex and ill-defined mechanism of action of adrenomedullin in various tissues, most of the studies on bone tissue showed an increase in bone tissue mass and proliferative effect on osteoblasts like other members of the calcitonin gene family.[6,7] Cornish et al[5, 9, 10] conducted extensive research about adrenomedullin's effect on bone tissue, reporting that adrenomedullin promoted increased proliferation and cell function in osteoblasts both in vivo and in vitro. There is no study in the literature investigating the effects of adrenomedullin on damaged or broken bone tissue. We hypothesized that adrenomedullin could increase bone formation at the segmental radial defect site via its anabolic effects shown in previous studies.[10,11].

The main study goal was to investigate whether adrenomedullin could enhance bone healing in a segmental bone defect model in rats to assess its potential in bone tissue trauma. ARRIVE Guideline 2.0 was used to improve the reporting of this animal study.[12]

Material and methods

Study design

After the ethics committee approval from Hacettepe University Ethics Boards and Commissions (protocol number: 2019/01-04), 36 male Wistar albino rats (>300 g) were randomly assigned to 1 of 6 groups, and each rat received a 2-mm segmental bone defect at radial shaft, bilaterally. All rats were healthy and did not have any genetic modification. There were 6 time- and dose-dependent groups (n = 12 radius per group, total n = 72); 2 groups were untreated placebo—C4 (control—4 weeks) and C8 (control—8 weeks)—and

Corresponding author:
Mehmet Kaymakoglu
kaymakoglumehmet@gmail.com

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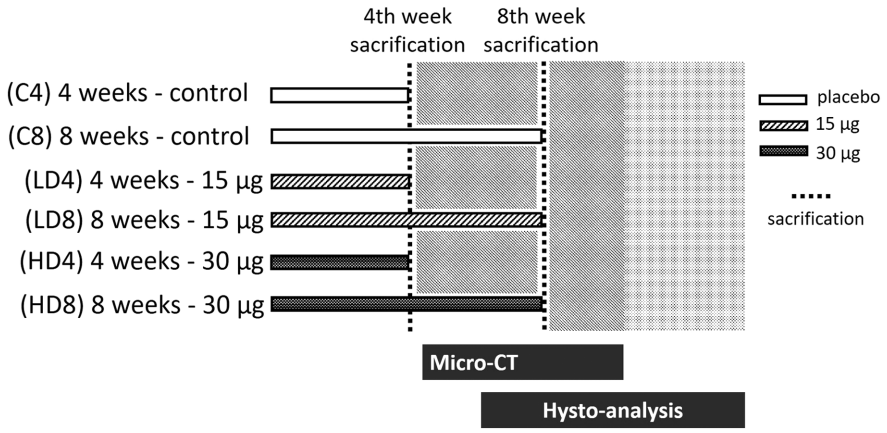

Figure 1. The schematic study plan that shows 6 dose- and time dependent groups. C4, control—4 weeks; C8, control—8 weeks; CT, computed tomography; HD4, high dose 30 µg—4 weeks; HD8, high dose 30 µg—8 weeks; LD4, low dose 15 µg—4 weeks; LD8, low dose 15 µg—8 weeks.

4 groups consisted of adrenomedullin-received study groups—LD4 (low dose 15 µg—4 weeks), LD8 (low dose 15 µg—8 weeks), HD4 (high dose 30 µg—4 weeks), and HD8 (high dose 30 µg—8 weeks) (Figure 1). Power analysis was performed to determine the minimum sample size for the study, and a total of 12 forearm samples per group were considered necessary to avoid type II error with a statistical power set to 80%. The bilateral forearms of every rat were used to reduce the number of animals.[13] Any statistically significant histological or radiological increase in new bone formation was determined as an outcome measure.

Surgical procedure

A preliminary surgery and microcomputed tomography (micro-CT) imaging were applied with a Wistar rat, which has been used in previous studies in our animal research center but was removed from the study due to the problems experienced during the experimental process. By this procedure, we were able to adjust the scaling of imaging in micro-CT and get used to the surgical procedure as described in other studies.[13-15]

The rats were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg body weight; Pharmacia, Erlangen, Germany) and xylazine (25 mg/kg body weight; Bayer, Leverkusen, Germany), and the surgical procedure was started after the cessation of response to the painful stimulus. After shaving the volar side of the forearm and asepsis with chlorhexidine solution, the volar approach was used to reach the shaft of the radius bilaterally, preserving the soft tissues. A 2-mm segmental defect was created without resecting the periosteum (Figure 2). The wound was closed after the incision line was irrigated with isotonic saline solution. Tramadol (15 mg/kg) was administered to rats for postoperative analgesia before they woke up from anesthesia.

HIGHLIGHTS

- Adrenomedullin is a relatively new discovered peptide hormone, which is classified in the calcitonin gene family, with possible anabolic potential for bone matrix.
- We investigated its effects on bone regeneration with a bone defect model in rats, but the results did not show a significant bone matrix regeneration in the defect area.
- Bone mineral density was found to be increased in the bone tissue adjacent to the bone defect area.
- More studies are needed to further investigate the effects of adrenomedullin in bony trauma.

Rats in the control group (C4 and C8) received 3 day/week intraperitoneal isotonic saline injections for 4 and 8 weeks, respectively, whereas study groups were treated with 3 days/week intraperitoneal injections of 15 µg (LD4 and LD8) and 30 µg (HD4 andHD8) Adrenomedullin 22-52 (Genscript Biotech, Piscataway, NJ, USA) for 4 and 8 weeks, respectively. Dosage was adjusted according to the effective molar dosage used in previous studies.[5,10] All animals were kept in a room with 21°C temperature and 12-hour cycles of darkness and light and fed ad libitum throughout the study. Treatment order was kept standard to minimize the risk of confounding and each step of experiment [surgical procedure (EO), treatment (MEE), radiological[16] and histological analysis (EC)] were made by different researchers to provide blinding. Any signs of infection in surgical site, loss of hair, lack of movement, and change in weight was noted for exclusion from the study. We lost

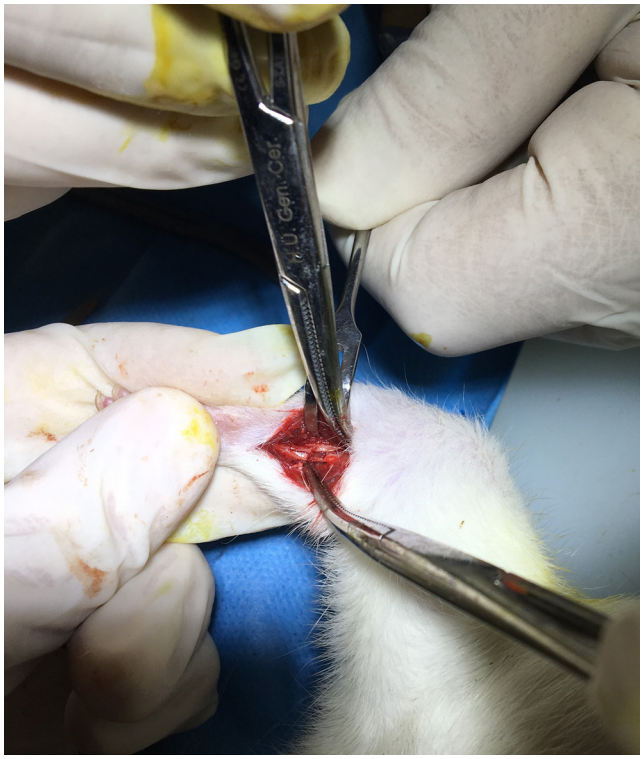

Figure 2. Image of the 2-mm segmental defect created without resecting the periosteum.

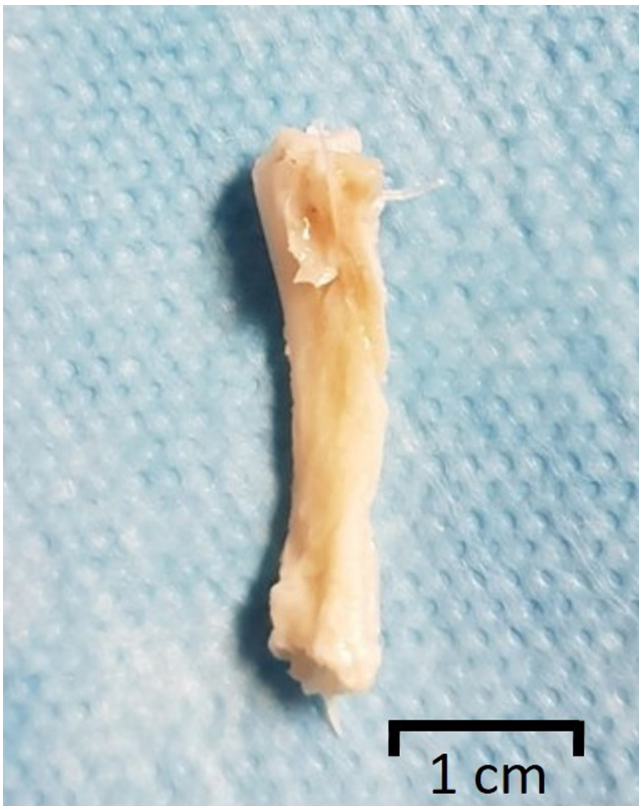

Figure 3. Image of the dissected forearm of a rat without damaging the osteotomy site.

only 1 rat due to inadequate dosage of anesthetics at the beginning of our study, and we replaced it with a newer one. All rats were euthanized with overdose anesthesia at the end of the treatment period determined for each group, and all forearms were dissected immediately after the sacrification for radiological and histological analysis (Figure 3).

Plain radiographic and micro-CT analysis

Following the dissection of the forearms after fourth and eighth weeks, samples were immediately fixed with formalin solution, and micro-CT (Bruker – Skyscan 1272; Billerica, Mass., USA) analyses were performed under 70 kV voltage and 114 μA current, with an integration time of 204 ms and an average of 1200 images for a sample. Then, the raw images obtained were reconstructed as 2-dimensional in a 2052 × 2052 image matrix using a voxel size of 7 μm. The region of interest area was accurately calculated in osteotomy gap, and using the Bruker computed tomography analysis (CTAn ver. 1.16.1.0) software, bone volume (BV), bone surface (BS), and trabecular thickness (Tb.Th) were evaluated. Bone mineral density (BMD) in the intact bony structure adjacent to the defect area was calculated. Three-dimensional reconstruction from 2-dimensional raw images was also obtained for every sample to better visualize the defect area (Figure 4). We also received high-resolution plain radiographs of the segmental defect area with the same micro-CT device, and the defect area was evaluated by 2 independent observers (E.C. and M.K.) with modified Lane and Sandhu defect scoring system, and the mean value was accepted as the main value for each sample (Table 1).[17-19]

Histomorphometric analysis

Tissue samples were fixed in 10% formaldehyde solution in phosphate buffer (pH 7.0) at room temperature and gradually decalcified in de Castro solution for 4 weeks at room temperature.[20] The samples were embedded in paraffin following dehydration and clearing in graded alcohols and xylenol in a fixed vacuum tissue processor. Serial sections of 3-4 μm thickness were taken from paraffin blocks on a sliding microtome (Leica, Weztlar, Germany). Consecutive hematoxylin eosin and Masson's trichrome-stained sections revealed general morphology and quantification of new bone formation, respectively, under bright-field microscope (DM-6B, Leica).[20] Micrographs were transferred to a computer, and the new bone area (NBA) [21] and total defect area (TDA) in the segmentary defect area were quantitatively measured in square millimeters with the image analysis program (LAS v3, Leica, Germany).[20] The sections were evaluated based on the zone where new bone tissue was separated from the damaged bone ends and by measuring the length of the defect created (Figure 5). The ratio of NBA/TDA was calculated as percentage.[20]

Statistical analysis

Independent variables were defined as groups and time; dependent variables are histomorphometric parameters, plain-radiographic and micro-tomographic measurements. Shapiro–Wilk test was used for the distribution of normality. Comparison between groups of nonparametric data was determined with the Kruskal–Wallis test and, 2-group comparisons were examined by Mann–Whitney *U* test. Correlations of 2 different parameters were analyzed with the Spearman test. Statistical analyses were performed by Statistical Package for the Social Sciences (SPSS) software version 23.0 (IBM SPSS Corp.; Armonk, NY, USA) with 95% CI range. Definitional statistics were presented with minimum, maximum, and median.

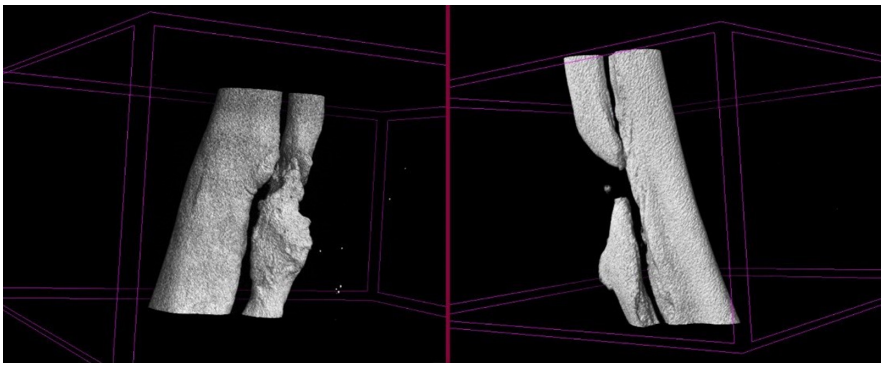

Figure 4. Three-dimensional micro-computed tomography images of 2 different samples showing bony union and non-union in the defect area.

Table 1. Modified Lane and Sandhu defect scoring system

Modified Lane and Sandhu defect scoring system		
Healing status	*Radiologic finding*	*Score*
Bone formation	No evidence of bone formation	0
	Bone formation occupying ≥ 25% defect area	1
	Bone formation occupying ≥ 50% defect area	2
	Bone formation occupying ≥ 75% defect area	3
	Bone formation occupying = 100% defect area	4
Union (proximal and distal evaluated separately)	Non-union	0
	Possible union	2
	Radiographic union	4
Remodeling	No evidence of remodeling	0
	Remodeling of medullary canal	2
	Full remodeling of cortex	4

Results

Radiological findings

There was no statistical difference between 6 groups in terms of union on plain radiographs using modified Lane and Sandhu defect scoring system (P = .608). The results are given in Table 2. Mean BV values were 234.5 and 298.5 mm^3 (C4 and C8, respectively) for the control groups. The BV values for study groups were 296.9, 221.2, 208.5, and 295.7 mm^3 (LD4, LD8, HD4, and HD8, respectively). Microtomography revealed a significantly higher BV value in the LD4 group comparedto HD4 (P=.003, Figure 6A). Mean BS values were 1233.7 and 459.4 mm^2 (C4 and C8, respectively). Bone surface values for study groups were 1058.8, 598.9, 695.5, and 502.1 mm^2 (LD4, LD8, HD4, and HD8, respectively). There was a significant decrease in the

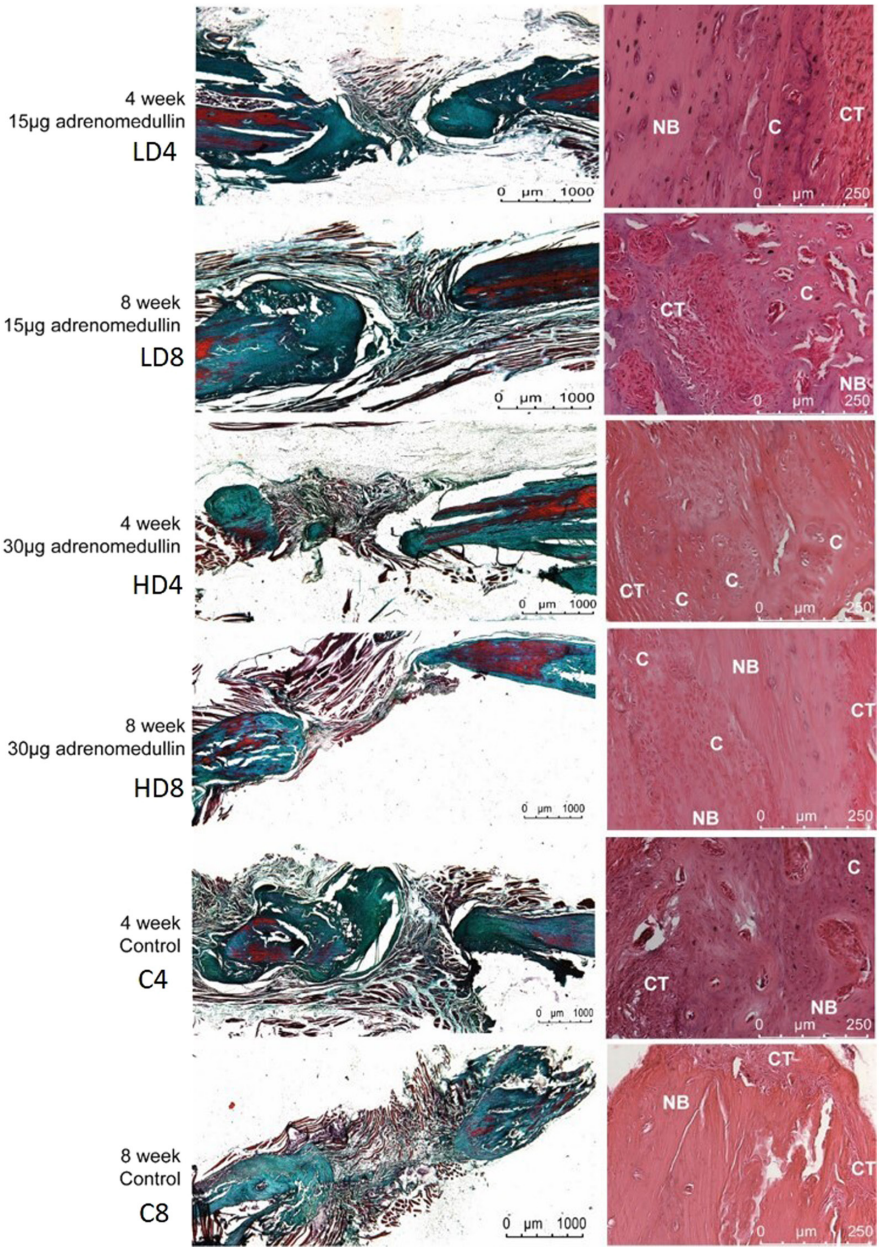

Figure 5. The bone formation in a critical-sized defect model after adrenomedullin injection at different doses and different time points. The left column presents low and the right column high power magnifications of the control and experimental groups' micrographs. The defect area is shown in the left column. The new bone at the edge of the defect with fibrous connective tissue (16) and cartilage islands at the right. Left column: Masson's trichrome, 40×; right column: hematoxylin eosin, 200×. NB, New Bone; C, Cartilage; CT, Connective Tissue.

Table 2. Results of the plain radiographic findings of the study groups according to the Lane and Sandhu defect scoring system

Groups	Number of samples (n)	Lane and Sandhu defect score	*P*
C4	12	1.09 ± 0.72	.608
C8	12	1.11 ± 0.96	
LD4	12	1.18 ± 0.64	
LD8	12	1.08 ± 0.74	
HD4	12	0.98 ± 0.82	
HD8	12	1.25 ± 0.77	

C4, control—4 weeks; C8, control—8 weeks; HD4, high dose 30 μg—4 weeks; HD8, high dose 30 μg—8 weeks; LD4, low dose 15 μg—4 weeks; LD8, low dose 15 μg—8 weeks.

BS value of HD4 group when compared with the C4 group ($P = .005$, Figure 6B). Full bony union was observed in only 2 samples of the LD4 group. Since this number was very small, the union rates could not be compared statistically between the groups.

Mean BMD values were 1.15 and 1.25 BMD-g/cm^2 (C4 and C8, respectively) for the control groups. The BV values for study groups were 1.67, 1.70, 1.37, and 1.57 BMD-g/cm^2 (LD4, LD8, HD4, and HD8, respectively). Although BMD values showed an increase in adren omedullin-received groups overall, only BMD value of LD8 group was significantly higher when compared with the C8 group ($P = .001$) (Figure 6-C)

Pearson correlation tests showed a significant positive correlation between Tb.Th and BV ($R^2 = 0.254$, $P = .012$) (Figure 7A) and also between BV and BS/BV ratio ($R^2 = 0.625$, $P < .001$), Figure 7B).

Histomorphometric findings

Various amounts of new bone formation were observed among control and study groups. Despite full bony union in 2 samples in the LD4 group, we found diffuse fibrous callus and cartilage islands at the damaged bone ends in most of the samples (Figure 4). There was no statistical difference between NBA/TDA ratio of the control and study groups; however, we found higher NBA values in 15 μg adren omedullin-received study groups (LD4 and LD8) compared with control and 30 μg study groups (C4, C8, HD4, and HD8) (Figure 8).

Pearson correlation tests presented a significant positive correlation between micro-CT Tb.Th and NBA/TDA ratio ($R^2 = 0.14$, $P = .072$) (Figure 9).

Discussion

Adrenomedullin hormone is a member of the calcitonin gene family, and its effects on different tissues have been investigated with increasing number of studies in recent years.[22-24] Additionally, certain fragments like –(22-52) and –(27-52) were found to be potent in the

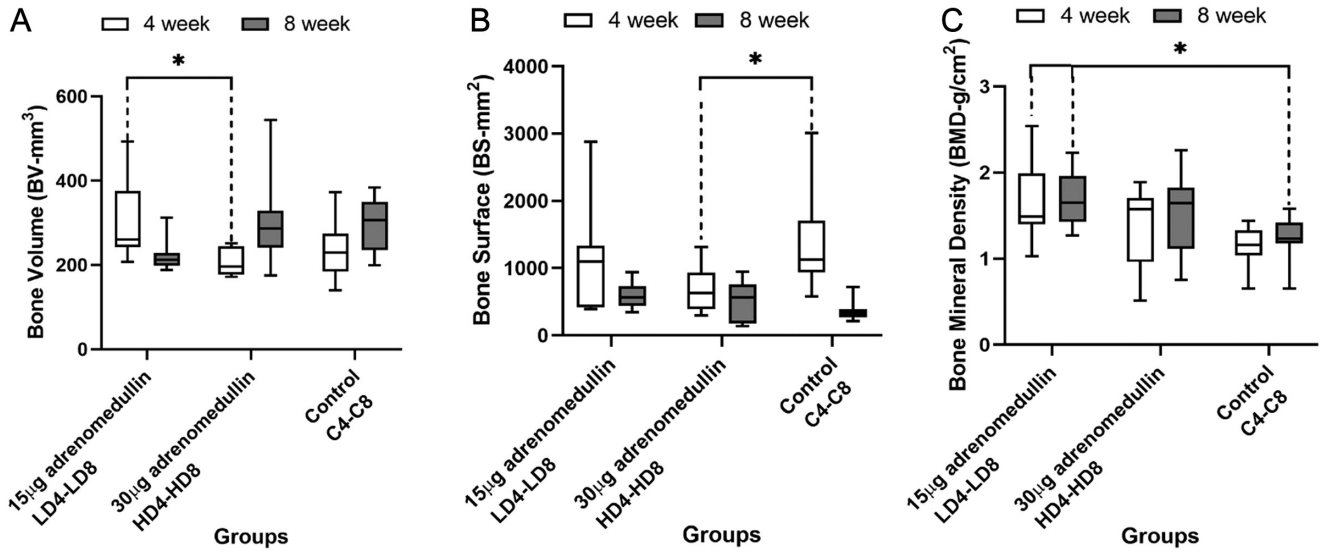

Figure 6. Box plot graphic presents the descriptive data of microtomographic analyses at weeks 4 and 8. The bone volume (BV), bone surface (BS), and bone mineral density are shown on the vertical axis,; and the groups on the horizontal axis. C4, control— - 4 weeks; C8, control— - 8 weeks; HD4, high dose 30 μg— - 4 weeks; HD8, high dose 30 μg— - 8 weeks; LD4, low dose 15 μg— - 4 weeks; LD8, low dose 15 μg— - 8 weeks. (*P < .05).

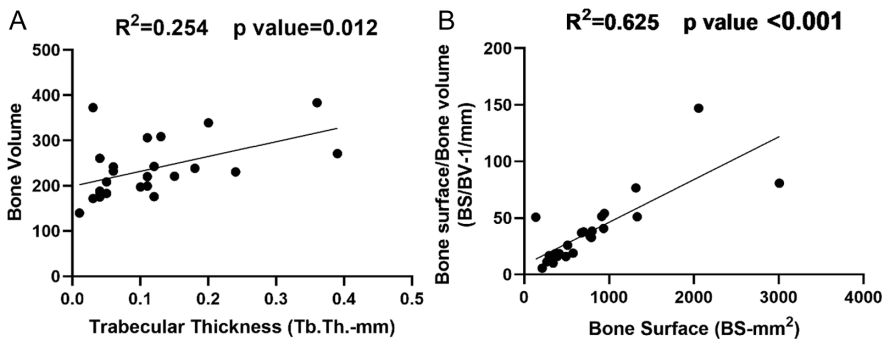

Figure 7. In the correlation graph showed (A) the bone volume (BV) and trabecular thickness, (B) bone surface/bone volume ratio, and bone surface correlate positively with each other. C4, control— - 4 weeks; C8, control— - 8 weeks; HD4, high dose 30 μg— - 4 weeks; HD8, high dose 30 μg— - 8 weeks; LD4, low dose 15 μg— - 4 weeks; LD8, low dose 15 μg— - 8 weeks.

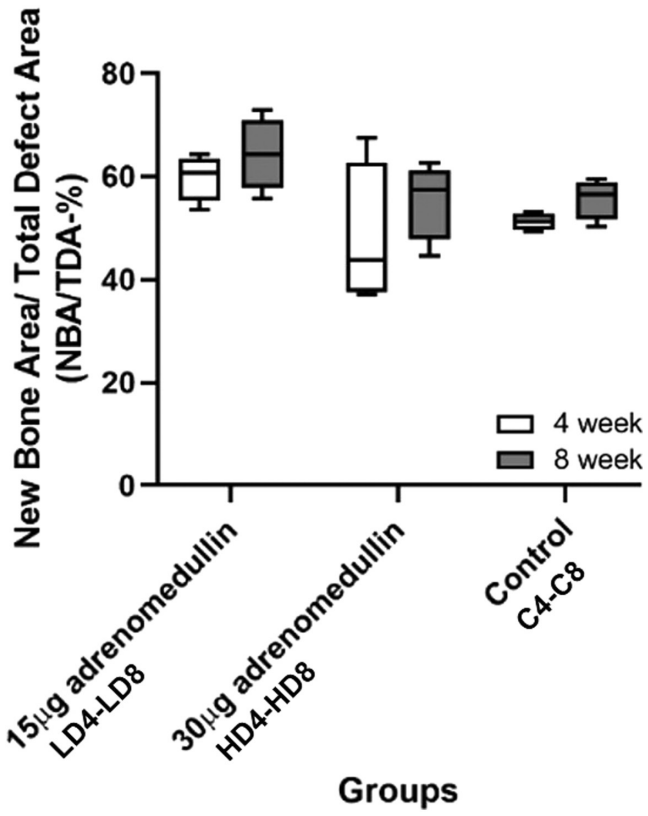

Figure 8. Box plot graphic presents the descriptive data of histomorphometric analysis at weeks 4 and 8. The new bone area to total defect area percentage is shown on the vertical and the groups on the horizontal axis.

bone tissue without showing a vasoactive effect, and animal studies showed its potential as an anti-osteoporotic molecule.[6,7,25] In the light of this information, we decided to use –(22-52) instead of –(27-52) fragment because of its easy accessibility to exclude adrenomedullin's vasoactive effect and obtain a pure bony active fragment to assess its effect on segmental radius defect. No significant effect on callus formation and bony union was observed via –(22-52) fragment

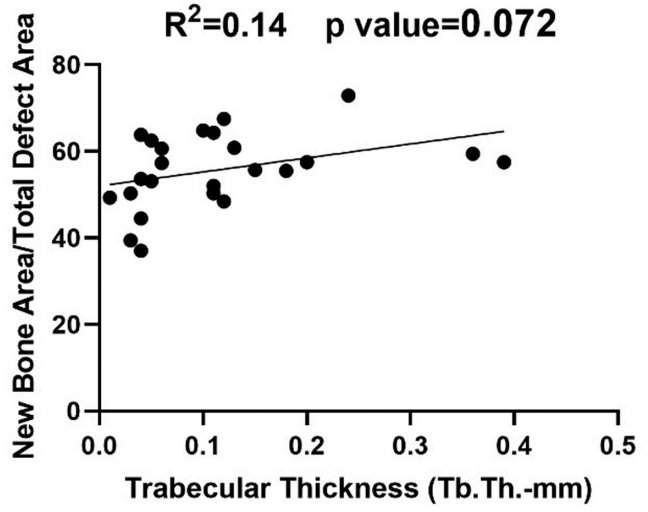

Figure 9. In the correlation graph showed, the new bone area to the total defect area percentage and trabecular thickness correlate positively with each other. C4, control– - 4 weeks; C8, control– - 8 weeks; HD4, high dose 30 μg– - 4 weeks; HD8, high dose 30 μg– - 8 weeks; LD4, low dose 15 μg– - 4 weeks; LD8, low dose 15 μg– - 8 weeks.

of adrenomedullin in this study, but increased BMD was observed in the study groups.

Bone defects are often used to investigate union capability of bone matrix biomaterials or for new systemically applied agents that may enhance bone metabolism. Segmental bone defects, which are smaller than the critical bone defects (up to 3 mm in rats), are used to obtain a nonunion model as well as larger defects in the form of critical bone defects are used to achieve defect union stimulation with biomaterials. Demineralized bone matrix or scaffolds were used in some studies to demonstrate the increased union capability with stem cells, osteoprogenitor cells, or platelet-rich plasma in critical long bone defect models.[13,14,26] In this current study, a shorter segmental defect model (up to 2 mm) was used instead of critical bone defect as critical bone defects need a scaffold or biomaterial for union.[27,28] It was possible to simulate a non-union model with a shorter segmental bone defect and assess systemically administered adrenomedullin's contribution to this non-union in terms of new bone formation. Holstein et al[29] demonstrated increased union in the segmental bone defect model in the rat femur with erythropoietin administration, but because femoral or tibial segmental defects do need an intramedullary or extramedullary fixation, a simpler defect model in the rat forearm was chosen in this study. Various studies reported that radial defect model in rat is easy to apply and inexpensive, as the forearm of rodents does not require fixation because of the synostotic relation of radius and ulna with limited pronation and supination.[13,26,27,30,31] The current study also did not use a fixation material for the radial segmental defect model in rat.

According to the plain radiographic findings in our study, no statistically significant difference was found between the study and control groups according to the Lane and Sandhu defect healing criteria.[17,18] According to Tawonsawatruk et al,[18] Lane and Sandhu scoring system is more appropriate in defect models in terms of calculating new bone formation than other scoring systems in the literature, but they also implied that the sensitivity and specificity of the classification systems that score the defect area according to plain radiographs decreases with the lack of callus formation.[19] This expected result was got, since there was no big difference in the callus formation of control and study groups on plain radiography in the current study.

Bone volume and BS are both correlated with the new bone formation in the defect area. There was a statistically different increase in BV in the LD4 group compared with the HD4 group, and HD4 group also had lower BS value than the C4 group. These results alone are not fully consistent with our hypothesis that adrenomedullin could trigger new bone formation via osteoblastic activity. The fact that the improvement was not greater in the high dosage groups and that there was no obvious increase in the 8-week groups compared to the 4-week groups suggested 2 possibilities: adrenomedullin does not have a positive effect like parathormone above a certain dose, or the result we found may be misleading due to the small number of samples in the experiment.

Looking at the results in terms of BMD values, despite the fact that only statistical difference was observed in the LD8 group compared with the C8 group, the increase in BMD values in all adrenomedu llin-received groups was remarkable. We are of the opinion that although the positive effect of adrenomedullin could not be shown in the defect area, increased BMD values in adjacent intact bony structures were consistent with the anabolic potential of adrenomedullin.[5,9,11] There was no study measuring the BMD after adrenomedullin

–(22-52) administration in vivo in the literature, but members of the calcitonin gene family have been shown to increase BMD.[32] Similarly, Cornish et al found that systemic administration of a similar fragment of adrenomedullin –(27-52) increased the trabecular BV and cortical width in a mice model.

Masson trichrome stain was used to evaluate the histomorphometry of new endochondral bone formation at the defect region.[33] We could not find a statistical difference between groups, but the groups that received low dose (15 μg) had more NBA on histomorphometric analysis. There could be a correlation between this finding and increased BMD, but nevertheless, it is impossible to draw a certain conclusion with these results as there were no statistically significant correlations in micro-CT results and hystomorphometric analysis.

There are also opposing views on adrenomedullin's effect on bone tissue in the literature. In a study by Martinez-Herrero et al,[34] contrary to what was expected, less osteoporosis was reported in the adrenomedullin inhibitor given group in an osteoporosis model in ovariectomized mice. Ah Kioon et al[25] also reported that the –(22-52) fragment of adrenomedullin could decrease the osteoporosis via inhibiting the inflammation, but they attribute this to the fact that the –(22-52) fragment of adrenomedullin could actually act as an inhibitor of adrenomedullin. In another study, serum adrenomedullin levels were found to be high in patients with idiopathic osteoporosis, but the authors could not state whether high adrenomedullin levels lead to osteoporosis or adrenomedullin increases secondary to osteoporosis.[35] Yet, there are many unknowns about adrenomedullin, and we are just at the very beginning of obtaining the exact mechanisms and effects of adrenomedullin. There are even studies showing that polymorphisms in human adrenomedullin gene are very common and that there is less cancer in people with lower serum adrenomedullin levels due to this polymorphism.[36,37] Another interesting finding was the increase of serum adrenomedullin hormone levels in brain during the brain injury.[38-40] This raises the question of whether the unknown mechanism of the increase in callus in head trauma patients is related with adrenomedullin levels.

Our most important limitation was the small number of subjects and the fact that the experiment was an animal experiment.[31,41] Additionally, despite the advantages of being economical and easy to apply, fixation might be required to better enhance bone regeneration in forearm defect models.[13,42] Other long bone defect models like femur or tibia with proper fixation could give different results, as forearm is not a weight-bearing bone segment. But, on the other hand, fixation with implants includes problems such as cost increase and risk of infection.

To sum up, adrenomedullin increased the NBA and BMD in some study groups in this segmental defect model, but more comprehensive studies are needed to obtain stronger evidences and support our findings. Studies on adrenomedullin may be illuminating to find new treatments on bone tissue trauma.

Ethics Committee Approval: This study was approved by Ethics committee of Hacettepe University Ethics Boards and Commissions (Protocol No: 2019/01-04)

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – M.K., E.T.; Design – M.K., P.K.; Supervision – E.T., P.K.; Resources – E.C. M.E.E.; Materials – E.C., E.O.; Data Collection or Processing – M.K., E.C.; Analysis or Interpretation – E.C., M.K.; Literature Search – M.K., E.C.; Writing – M.K., E.C.; Critical Review – M.E.E., P.K.

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Declaration of Interests: The authors have no conflict of interest to declare.

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