

Propranolol attenuates calorie restriction- and high calorie diet-induced bone marrow adiposity

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We investigated the effects of β -adrenergic activation on bone marrow adiposity and on adipogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). C57BL/6 mice were subjected to a control (CON), high calorie (HIGH) or low calorie (LOW) diet for 12 weeks. In each group, mice were treated with vehicle (VEH) or propranolol. The number of adipocytes per area bone marrow was increased in LOWVEH and HIGHVEH mice compared with CONVEH mice, which was attenuated by propranolol. Isoproterenol increased lipid droplet accumulation and adipogenic marker gene expression in 3T3-L1 preadipocytes and mouse BMSCs, which were blocked by propranolol. Conditioned medium obtained from MC3T3-E1 osteoblasts suppressed adipogenic differentiation of 3T3-L1 cells, which was significantly attenuated by treatment of MC3T3-E1 cells with isoproterenol. These data suggest that β -adrenergic activation enhances bone marrow adipogenesis via direct stimulation of BMSCs adipogenesis and indirect inhibition of osteoblast anti-adipogenic potential. [BMB Reports 2014; 47(10): 587-592]

INTRODUCTION

Bone marrow fat has been considered to be a metabolically inert filler for the void vacated by bone and/or hematopoietic cells. However, recent reports have suggested that marrow adipocytes may play a role as an energy source or as a modulator of adjacent tissue by the production of paracrine/autocrine factors (1). Osteoblasts and adipocytes are commonly derived from mesenchymal stem cells (MSCs) and it is generally accepted that there is reciprocal regulation between osteoblast

and adipocyte differentiation in bone (2). The abnormal regulation of osteogenesis and adipogenesis in the bone is involved in osteoporosis in aged people.

Mammalian bones are widely innervated by sympathetic and sensory nerves (3). Over the last decade, a line of research has emerged elucidating the role the sympathetic nervous system (SNS) plays in the regulation of bone. SNS activation drives bone loss mainly by reducing osteoblast differentiation and proliferation (4) and by stimulating osteoclast differentiation and activity (5). However, the regulatory mechanism of SNS on marrow adiposity and adipogenic differentiation of MSCs has not been fully elucidated. In the context of body fat metabolism, the regulatory mechanisms of SNS signaling has been demonstrated predominantly in the aspect of lipolysis and thermogenesis. β_3 -Adrenergic receptors are primarily expressed in adipose tissue and the activation of β_3 -adrenergic receptors stimulates not only lipolysis in white adipose tissues but also thermogenesis in brown adipose tissues (6-8).

Among the several factors inducing SNS activation, dietary calorie restriction and high calorie diet have been actively studied in association with bone loss and marrow adiposity. In the context of high calorie diet-induced obesity, potential factors including hyperleptinemia and hyperinsulinemia/insulin resistance have been proposed to contribute to the SNS activation (9). With fasting or weight reduction using calorie restriction, increased norepinephrine and/or epinephrine release have also been reported (10-12). Pharmacological blockade of β -adrenergic receptors (β -ARs) attenuates the loss of trabecular bone in the context of SNS activation associated with stressful conditions such as calorie restriction in adult rats (13) and ovariectomy in exercising adult rats (14). We previously demonstrated that both high and low calorie diets caused significant bone loss and that β -adrenergic blockade using propranolol attenuates trabecular bone loss as well as body fat mass increase in high calorie diet-fed mice (15). However, whether calorie alteration-induced SNS activation regulates bone marrow adiposity and whether β -adrenergic blockade prevents the changes in bone marrow adiposity have not been clearly proven.

In the present study, we investigated 1) the effect of low calorie and high calorie diet on bone marrow adiposity, 2) the effect of propranolol on calorie alteration-induced marrow

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adiposity change and 3) the effect of β -ARs activation on adipogenic differentiation of mouse bone marrow-derived MSCs (BMSCs) and 3T3-L1 cells.

RESULTS

β -Adrenergic blockade attenuates bone marrow adiposity observed with both high and low calorie diets

To explore the effect of dietary calorie alteration in bone marrow adipogenesis, histological analysis was conducted on femoral sections obtained from each group. H&E staining showed that the number of adipocytes per mm^2 bone marrow was significantly higher in LOWVEH and HIGHVEH mice compared with CONVEH mice ($28.36 \pm 5.6/\text{mm}^2$, $15.64 \pm 3.1/\text{mm}^2$ vs $5.57 \pm 3.1/\text{mm}^2$, respectively, $P < 0.05$) (Fig. 1). The magnitude of marrow adiposity was more severe in LOWVEH than in HIGHVEH mice. Interestingly, β -adrenergic blockade using propranolol significantly mitigated the increased number of adipocytes per mm^2 bone marrow in both LOW and HIGH mice (Fig. 1).

Stimulation of β -adrenergic receptors increases adipogenic differentiation of BMSCs and 3T3-L1 cells

Because propranolol attenuated calorie alteration-induced increases in bone marrow adiposity, we hypothesized that β -ARs activation enhances adipogenic differentiation of MSCs in mouse bone marrow. We first evaluated the effect of β -adrenergic agonist on adipogenic differentiation in the 3T3-L1 preadipocyte cell line using isoproterenol. The cells were incubated in adipogenic medium for 24, 36 and 48 h in the presence or absence of $1 \mu\text{M}$ isoproterenol. Quantitative RT-PCR results showed that isoproterenol significantly increased expression levels of adipogenic marker genes (Fig. 2A). Oil Red O staining was also performed using 3T3-L1 cells incubated in adipogenic medium for 3 days. Oil Red O staining and the subsequent quantitation showed that the Oil Red O content of cells was at least 30% higher in the iso-

proterenol-treated group compared with control group (Oil Red O elution A_{490} : CON 0.12 ± 0.01 , ISO 0.16 ± 0.03 , $P < 0.001$), suggesting that isoproterenol significantly increased lipid droplet accumulation (Fig. 2B, right panels, 2C). To further confirm the effect of isoproterenol on adipogenic differentiation, mouse BMSCs were cultured for 21 days in the presence or absence of isoproterenol, and Oil Red O staining was performed. Similar to the results shown in 3T3-L1 cells, isoproterenol enhanced staining intensity in BMSCs (Fig. 2B, left panels). These results suggest that β -ARs activation enhances adipogenic differentiation of MSCs.

To verify that isoproterenol enhances adipogenic differentiation through activation of β -ARs, we examined the effect of propranolol, a global β -ARs blocker and inverse agonist (16, 17), on isoproterenol-induced stimulation of adipogenesis. BMSCs and 3T3-L1 cells were incubated in the presence or absence of $1 \mu\text{M}$ isoproterenol and $10 \mu\text{M}$ propranolol for 21 days and 3 days, respectively. Quantitative RT-PCR results showed that propranolol prevented isoproterenol-induced increases in adipogenic marker gene expression in both cell systems (Fig. 3A, 3B). Western blot analysis using 3T3-L1 cell lysates confirmed the stimulatory effect of isoproterenol and the inhibitory effect of propranolol on adipogenic marker gene expression (Fig. 3C). These results indicate that isoproterenol up-regulates adipogenic differentiation of BMSCs via β -ARs activation.

Stimulatory effect of isoproterenol on adipogenic differentiation is maintained in the presence of osteoblasts, and isoproterenol attenuates anti-adipogenic activity of osteoblasts

Given the suggested role of the SNS in the regulation of bone

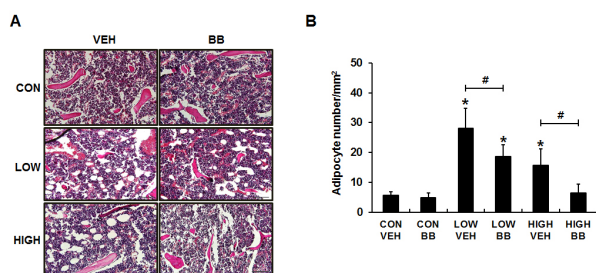


Fig. 1. β -Adrenergic blockade (BB) attenuated bone marrow adiposity observed with low calorie (LOW) and high calorie (HIGH) diet. H&E staining (A) and (B) histomorphometric analysis were performed with decalcified femoral sections. The number of bone marrow adipocytes is presented as the mean number adipocytes per mm^2 area (* $P < 0.05$ vs CONVEH, # $P < 0.05$).

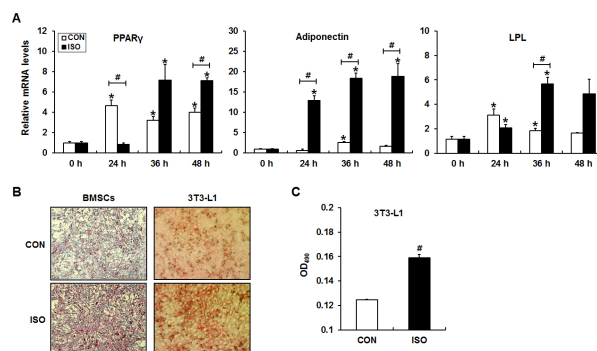


Fig. 2. Isoproterenol increased adipogenic differentiation of BMSCs and 3T3-L1 cells. (A) 3T3-L1 cells were incubated in adipogenic medium for the indicated periods in the presence or absence of $1 \mu\text{M}$ isoproterenol (ISO). The mRNA levels of adipogenic marker genes were then examined by quantitative real time-PCR. Data are presented as the mean \pm SD of triplicates (* $P < 0.05$ vs CON 0 h, # $P < 0.05$). (B, C) Adipogenic differentiation was induced in BMSCs and 3T3-L1 cells for 21 days and 3 days, respectively, and Oil Red O staining was performed (B). Oil Red O content in 3T3-L1 cells was quantitated (C).

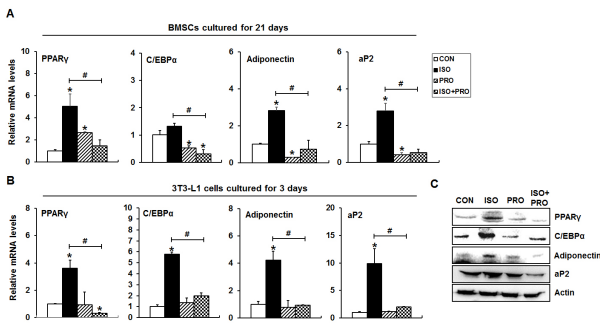


Fig. 3. Propranolol, a β -adrenergic antagonist, blocked isoproterenol-induced adipogenic differentiation. BMSCs (A) and 3T3-L1 cells (B, C) were incubated in adipogenic medium for 21 days and 3 days, respectively. Adipogenic marker gene expression was analyzed with real time-PCR (A, B) and Western blot analyses (C). * $P < 0.05$ vs control (CON), # $P < 0.05$ for the indicated comparison. ISO, 1 μ M isoproterenol; PRO, 10 μ M propranolol.

marrow adipogenesis via the osteoblasts (18), we next examined the regulatory role of osteoblasts on adipogenic differentiation. Osteogenic differentiation of MC3T3-E1 cells was induced by incubation with ascorbic acid and β -glycerophosphate, and conditioned medium (CM) was obtained from the day 3 and day 7 cells incubated for 24 h after medium change (Fig. 4A, upper panel). Confluent 3T3-L1 cells were then incubated for 2 days in a 1 : 1 mixture of adipogenic medium and osteoblast CM (or fresh osteogenic medium) (Fig. 4A, lower panel). The levels of adipogenic stimuli (dexamethasone, insulin, methylisobutylxanthine, indomethacin) in the medium mixture were adjusted to be the same as those in adipogenic medium. Interestingly, addition of fresh osteogenic medium significantly increased the expression levels of adipogenic marker genes compared to the adipogenic medium-only group. However, osteoblast CM significantly suppressed adipogenic gene expression compared to the fresh osteogenic medium group. Additionally, the inhibitory effect of osteoblast CM was much higher in day 7 CM compared to that in day 3 CM (Fig. 4B). These results suggest that secreted factors from osteoblasts exert inhibitory effects on adipogenic differentiation.

We next examined whether isoproterenol treatment on MC3T3-E1 cells modulates anti-adipogenic potential of these cells. MC3T3-E1 cells were incubated in osteogenic medium for 7 days in the presence or absence of isoproterenol. Osteoblast CM was obtained from day 7 cells, followed by incubation of 3T3-L1 cells for 2 days in a 1 : 1 mixture of adipogenic medium and osteoblast CM. Quantitative RT-PCR results showed that expression levels of adipogenic marker genes were much higher in 3T3-L1 cells incubated with CM from isoproterenol-treated osteoblasts compared to those treated with CM from control osteoblasts (Fig. 4C). Oil red O staining demonstrated the same results as RT-PCR results (Fig. 4D). These results suggest that β -ARs activation in osteoblasts down-

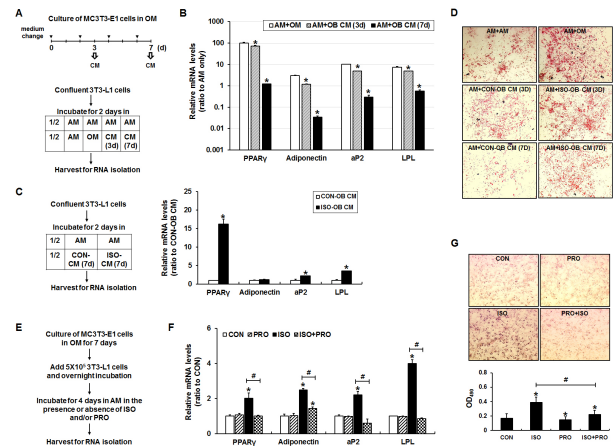


Fig. 4. Stimulatory effect of isoproterenol on adipogenic differentiation was maintained in the presence of osteoblasts, and isoproterenol downregulated anti-adipogenic potential of osteoblasts. (A, B) MC3T3-E1 osteoblasts were cultured in osteogenic medium (OM) and conditioned medium (CM) was obtained from the day 3 and day 7 cells. Confluent 3T3-L1 cells were then incubated for 2 days in a 1 : 1 mixture of adipogenic medium (AM) and osteoblast CM (or fresh OM). Real time-PCR data are presented as the relative fold changes normalized to those in the cells incubated in AM only (* $P < 0.05$ vs AM + OM). (C, D) MC3T3-E1 osteoblasts were incubated in osteogenic medium for 3 or 7 days in the presence or absence of 1 μ M ISO and CM was prepared from day 3 and day 7 cells. 3T3-L1 cells were incubated for 2 days (C) or 4 days (D) in a 1 : 1 mixture of AM and CM, and real time-PCR (C, * $P < 0.05$ vs CON-OB CM) and Oil red O staining (D) was performed. (E-G) MC3T3-E1 and 3T3-L1 cells were co-cultured as illustrated in (E) and real time-PCR (F, * $P < 0.05$ vs CON, # $P < 0.05$ for indicated comparison) and Oil red O staining (G) was performed.

regulates the anti-adipogenic potential of osteoblasts.

We next performed co-culture of 3T3-L1 and MC3T3-E1 cells to observe the effect of isoproterenol on adipogenic differentiation in conditions that mimic the bone microenvironment in which osteoblasts are in close proximity to MSCs. Osteogenic differentiation of MC3T3-E1 cells was induced for 7 days and 3T3-L1 cells were seeded on top of MC3T3-E1 cells and incubated in adipogenic medium for an additional 4 days, in the presence or absence of isoproterenol and propranolol (Fig. 4E). Even in co-culture conditions, isoproterenol significantly increased expression levels of adipogenic marker genes, which were blocked by propranolol (Fig. 4F). Stimulatory effect of isoproterenol on lipid droplet accumulation was also confirmed by Oil red O staining (Fig. 4G). These results suggest that the stimulatory effect of β -adrenergic activity on adipogenic differentiation is maintained in the presence of osteoblasts.

DISCUSSION

These experiments extend previous work demonstrating that blockade of β -adrenergic signaling attenuates the decrease in

trabecular bone mass in high calorie diet-fed obese mice (15). To our knowledge, this is the first study providing a side-by-side comparison of the effects of low calorie or high calorie diet on bone marrow adiposity and demonstrating the impact of β -adrenergic blockade on alterations in marrow adiposity in the context of prolonged calorie restriction or high calorie diet. Our data showed that 1) both low and high calorie diets upregulate bone marrow adipogenesis, 2) blockade of β -adrenergic signaling using propranolol significantly attenuates the increase of marrow adiposity observed in low and high calorie diet-fed mice and 3) the β -ARs agonist isoproterenol increases adipogenic differentiation of BMSCs and preadipocytes, which was blocked by a β -ARs antagonist propranolol.

Increased levels of catecholamines are observed in the context of obesity and profoundly affect lipid metabolism (19, 20). Classically, it has been suggested that catecholamines are strong activators of lipolysis that lead to the breakdown of triglyceride into free fatty acids. It was demonstrated that isoproterenol stimulates, and propranolol inhibits, lipolysis in 3T3-L1 adipocytes (21). In addition, there are reports in the literature that adipogenic marker gene expression is inhibited by β -adrenergic stimulation in adipocytes (22). Recently, Li H *et al.* demonstrated for the first time that MSC is also a target for β -adrenergic regulation and that β -adrenergic signaling activation (major β_3 -signaling) negatively affects MSC adipogenesis. Cyclic-AMP/PKA-mediated β -ARs signaling on MSC adipogenesis was also demonstrated (23). Inconsistent with these classical views, however, our results showed that isoproterenol increases adipogenic differentiation of adipocyte lineage cells and mouse BMSCs, which was prevented by propranolol. These data are consistent with *in vivo* data showing that propranolol attenuates diet calorie alteration-induced bone marrow adipogenesis. Recently, a growing body of evidence has demonstrated that tissue-specific ablation of β -ARs or use of β -ARs antagonists in rodents and humans is associated with increased bone mass and that bone marrow adipogenesis is correlated with the bone loss observed with aging and metabolic diseases. While information regarding the underlying mechanisms is only beginning to emerge, it is likely that SNS stimulation upregulates adipogenesis, at least in bone marrow.

The crosstalk with osteoblastogenic programming of BMSCs in adipocyte differentiation is being explored (24). In light of the reports showing that the molecular regulators such as PPAR γ , Runx2, Wnts and TAZ inversely regulate osteogenesis and adipogenesis, it could be proposed that the SNS-regulated osteoblast signaling is a contributing factor in the predisposition of BMSCs to the adipocyte versus osteoblast lineage. In the present study, the data provide evidence that the stimulatory effect of β -adrenergic activation on bone marrow adipogenesis is dependent on direct stimulation of adipocyte precursor cells as well as on the effect mediated partially via osteoblasts. In the context of β -adrenergic signaling in osteoblasts, it is widely accepted that SNS activation of β -ARs sup-

presses osteoblast differentiation (4). Our results demonstrated that osteoblast CM inhibits adipogenic differentiation of 3T3-L1 cells. Additionally, more differentiated cells exhibit a stronger suppressive effect, suggesting that differentiated osteoblasts secrete anti-adipogenic factors. In addition, CM obtained from isoproterenol-treated osteoblasts showed less of a suppressive effect than CM from control osteoblasts. Therefore, it is suggested that in addition to direct stimulation of adipogenic differentiation, SNS activation indirectly contributes to the generation of a favorable bone marrow microenvironment for adipogenic differentiation of BMSCs via inhibition of osteoblast differentiation.

In summary, both the high calorie and low calorie dietary intake over 12 weeks in mice caused significant marrow adiposity in limb bones, and the magnitude of marrow adiposity induced by the low calorie diet was greater than that of the high calorie diet. β -Adrenergic blockade using propranolol attenuated marrow adiposity observed in high or low calorie diet-fed mice. The β -adrenergic agonist isoproterenol increased adipogenic differentiation in 3T3-L1 cells and mouse BMSCs, which was blocked by propranolol. Isoproterenol treatment of MC3T3-E1 osteoblasts attenuated anti-adipogenic activity of osteoblasts on 3T3-L1 cells. Taken together, these data suggest an important role for β -adrenergic signaling in bone marrow adipogenic responses to alterations in dietary calorie intake. Mounting evidence supporting a causative role of bone marrow adipogenesis in the bone loss associated with aging, chronic drug treatment, and metabolic disease such as diabetes and osteoporosis has emerged. Even with limitations and a disagreement of classical opinion regarding SNS regulation on fat metabolism, our results could be an attractive prospect for understanding the role of SNS signaling that influences or determines the phenotypic fate of BMSCs toward osteoblasts or adipocytes in the bone marrow microenvironment.

MATERIALS AND METHODS

Animals and experimental design

Sixty C57BL/6 mice, aged 5 weeks at purchase (Orient Bio Inc., Seoul, Korea), were assigned into six groups of ten animals and fed for 12 weeks as follows: ad-lib fed controls treated with vehicle (CONVEH) or β -blocker (CONBB), 30% calorie restricted mice treated with vehicle (LOWVEH) or β -blocker (LOWBB), and high fat diet (60% calories from fat) mice treated with vehicle (HIGHVEH) or β -blocker (HIGHBB). β -Blocker groups were administered propranolol ((\pm)-propranolol hydrochloride, Sigma-Aldrich, St. Louis, MO) via drinking water (0.5 g/L) (25). Experimental procedures and diet information were described previously (15). All procedures in this study were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-110531-2).

Bone marrow adipocyte quantification

To examine bone marrow adiposity, femurs were fixed in 4%

phosphate-buffered formaldehyde and routinely processed for decalcification, paraffin embedding and H&E stain. The histological images were obtained with a SPOT system (Diagnostic Instruments, Sterling Heights, MI) interfaced with a color video camera (DXC-390P, Sony, Tokyo, Japan). For assessing the marrow adiposity between the endocortical edges, a defined region of interest was established approximately 0.2 mm from the growth plate and encompassing 2.5-3 mm². Subsequently, the number of marrow adipocytes was counted.

Cell culture

MC3T3-E1 preosteoblasts and mouse BMSCs were maintained in alpha minimum essential medium (α MEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA). Murine preadipocytic 3T3-L1 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM; Hyclone) supplemented with 10% FBS. Mouse BMSCs were kindly provided by Prof. T. Yi (Inha University, Incheon, Korea) (26).

Osteogenic differentiation of MC3T3-E1 cells was induced by addition of 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid to growth medium. To induce adipogenic differentiation, 3T3-L1 cells and BMSCs were cultured for 3 and 21 days, respectively, in adipogenic media (consisting of regular growth media plus 1 μ M dexamethasone, 50 μ M indomethacin, 10 μ g/ml insulin and 0.5 mM methylisobutylxanthine). Adipogenic differentiation was confirmed by examining adipogenic marker gene expression levels and Oil Red O staining of lipid droplets in cells (27).

When indicated, co-culture of MC3T3-E1 and 3T3-L1 cells was performed. MC3T3-E1 cells of ~80% confluency in 60 mm tissue culture dishes were incubated in osteogenic medium for 7 days and then 3T3-L1 cells (5×10^7 /dish) were seeded over the MC3T3-E1 cells and incubated overnight in DMEM plus 10% FBS. On the next day, medium was changed to adipogenic medium and cells were incubated for an additional 4 days. At the end of the culture, cells were harvested for RNA isolation.

Reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate mRNA expression, total RNA was isolated and quantitative RT-PCR was performed using the kits described previously (25, 27). PCR primer sequences for murine peroxisome proliferator-activated receptor gamma (PPAR γ), CCAAT/enhancer binding protein alpha (C/EBP α), the fatty acid binding protein adipocyte protein 2 (aP2), lipoprotein lipase (LPL) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were previously described (25). Primer sequences for adiponectin were as follows: forward 5'-CCCAAGGGAAGTTGGCAGGTTGGATG-3', reverse 5'-GTTGGTATCATGGTAGAGAAGAAAGCC-3'. For quantification, GAPDH was used as the reference for normalization of each sample.

Western blot analysis

Protein levels of adipogenic marker genes were examined by Western blot analysis as described previously (25). Adiponectin antibody was purchased from Abcam (Cambridge, UK).

Statistical analysis

To analyze the numbers of adipocytes in bone marrow, two-way ANOVA was performed with post hoc testing as appropriate using the SAS (v.9.3) statistical package. *In vitro* experiments were analysed with Student's t-test. A P value < 0.05 was considered statistically significant. The data are presented as the mean \pm SD.

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