

Investigating the Effect of Tumor Necrosis Factor Alpha on Placenta and Gene Related Bone Formation of Newborn Mice

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ABSTRACT

Background: The cytokine tumor necrosis factor α (TNF- α) is known to play an important role in modulation of bone cell function, regulation and differentiation. Its effects depend on dosage level and long of time exposure. Until now, there is little information about the effects of TNF- α on development of bone tissue and bone formation during pregnancy and early postnatal periods. Therefore, this study aimed to investigate the effects of TNF- α on bone formation in mice.

Methods: This study was experimentally conducted on 42 female NMARI pregnant mice. Pregnant mice were randomly divided into seven groups (6 mice in each groups) including: experimental 1 (TNF- α injection (100 ng) in 7th-9th pregnancy days), experimental 2 (TNF- α injection (10 ng) in 7th-9th pregnancy days), experimental 3 (TNF- α injection (10 ng) during pregnancy) and experimental 4 (TNF- α injection (100 ng) during pregnancy), control (without injection), vehicle 1 (PBS injection in 7th-9th pregnancy days), vehicle 2 (PBS injection during pregnancy). After breeding, the pups were collected, weighted and their number and length (crown-heel length, crown-rump length) were measured, then they were prepared for histological and gene expression study. The collected data were normalized, and analyzed through one-way ANOVA test, and presented as Mean+SEM. Herein the significance were considered as $p < 0.05$.

Results: The histologic study of the placenta showed a significant increase in inflammatory cells such as macrophages indicating the inflammation in placenta tissue of experimental groups $P < 0.05$. There was no significant difference in length, weight and numbers of newborn mice among groups ($P > 0.05$). The qReal time PCR results showed that the expression of osteoblast and bone forming related genes (RUNX2 and Osteocalcin) were significantly lower in experimental groups in comparison to control group (P value=0.02 for RUNX2 and (P value=0.02 for Osteocalcin).

Conclusions: TNF- α injection during pregnancy as long time or as single dose cause a dramatic alteration on bone formation of newborn mice.

Key words: Tumor necrosis factor alpha (TNF- α), Osteogenesis, Newborn

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BACKGROUND

Tumor necrosis factor alpha (TNF-alpha) is cytokine with molecular weight of 17 kDa and 157 amino acids [1,2]. TNF- α is mainly produced not only by

monocytes and macrophages, but also by other cellular sources including lymphocytes, mast cells, neutrophil, keratinocyte, astrocyte, microglia, Smooth muscle cells, paneth cells of intestine [3,4]. The biologic effects of TNF- α include cytotoxicity, antiseptic, modulator of cell growth and differentiation, effect on growth factor, and capacity to suppression and distortion of tumor cells [5,6].

Bone is a rigid and dynamic organ constituting the skeleton of body and is a main source of minerals such as calcium. A mature and developing bone includes three cells: Osteocyte, osteoblast and osteoclast. Osteoblasts are the special cells responsible for bone formation, developing and growth [7-10]. *Osteocalcin* and *RUNX2* as markers of osteoblast cells are main genes related to bone formation, developing and function. *Osteocalcin* is a non-collagenous protein found in different tissues but is known as the main marker of osteoblast. *RUNX2* is the main gene involved in osteoblast differentiation and affected by damages of osteoblast [11,12]. According to the previous reports, the inflammation has unsuitable effects on cellular components of skeleton and high level of TNF- α secretion increase the proliferation and differentiation of osteoclasts abnormally. In many bone inflammatory diseases including rheumatoid arthritis, activated osteoclasts decreases the mineral components of the bone [13]. Also, this cytokine has been reported to control the activity and differentiation of osteoblasts [14,15]. According to the high molecular weight of TNF- α (17 kDa), in the normal condition, it does not pass through placenta membrane, but in inflammatory condition due to weakness and increase of membrane permeability it pass from the membranes and reach to embryo [16,17]. Endogenous TNF- α is reported to be among the factors whose normal amount as a protector protects embryo from Teratogens stress [18-20]. But, by investigating and comparing the inflammatory models of laboratory such as LPS injection, it is expected that releasing of cytokines in inflammatory condition led to developmental disorders in organs [21]. It must be noted that releasing of TNF- α or other inflammatory cytokines can be harmful if they secrete continuously. Michalak *et al.* showed that TNF- α fatally affects nerve tissue through removing Heat-Shock protein (HSP) [22]. These studies show that the cytokine level specially TNF- α significantly increases in many autoimmunity diseases including rheumatoid arthritis and Periodontitis. All of above data replicates that TNF- α in pathologic condition can led to bone distraction, abnormal development and formation, but there aren't any reliable reports about teratogen effect of exogenous injection of TNF- α on bone formation in *in vivo* inflammatory condition. So, this study aimed to investigate the TNF- α effect on skeletal tissue formation and on the related cytokines involved in bone formation, differentiation and function during the embryonic development in *in vivo* inflammatory condition.

METHODS

Laboratory animal

In this study, 42 female NMARI mice of 20 g weight (6-8 week old) were used and all experiment was performed in accordance with guidelines of ethical committee of Kerman University of Medical Sciences, Kerman, Iran (IR.KMU.REC.1394.680). They were housed in controlled temperature (21°C-23°C) and 12/12 h light/dark cycle. They freely accessed to water and food. Two female mice with one male mouse were kept in a cage. After observing vaginal plug, the zero day of pregnancy was considered. If the vaginal plug was negative, non-pregnant mice were separated from male ones for 24 hours, and then they were returned to cage of male mice. If it was negative, mice were not involved in the experiment for a week.

Drugs

In the study, TNF- α drug with following specifications was used: Recombinant mouse TNF- α (mTNF- α , aa.80-235) (R&D-USA.410-MT) 10 μ g. The vial weight was 10 μ g which was reconstituted with 90 μ l of PBS containing 1% of BSA on the basis of related protocol. The first concentration of vial was 100 μ g/ml which was poured in 10 vials of 1.5 litre. By diluting each new vial, this drug was supplied with 10 mg and 100 mg concentration for intraperitoneal injection into experimented groups and was kept in -20°C. Reserving condition of the drug is -70°C. After dilution and aliquot, these vials were kept in -20°C for 1-3 months and in -70°C for over these times.

Groups and methodology

Animals were randomly and equally grouped as follows. Control group: newborn of pregnant mice who did not receive any injection (n=6) Vehicle group 1: Newborns of pregnant mice receiving PBS in 7th-9th pregnancy days (n=6). Vehicle group 2: Newborns of pregnant mice daily receiving PBS during pregnancy (n=6). Experimental group 1: Newborns of pregnant mice receiving TNF- α (100 ng/kg) in 7th-9th pregnancy days (n=6). Experimental group 2: Newborns of pregnant mice receiving TNF- α (10 ng/kg) in 7th-9th pregnancy days (n=6). Experimental group 3: Newborns of pregnant mice injected TNF- α (10 ng/kg) during pregnancy (n=6). Experimental group 4: Newborns of pregnant mice receiving TNF- α (100 ng/kg) during pregnancy (n=6). In each group, six pregnant mice were experimented and their three mice underwent the surgery and a placenta of each mouse was conveyed to Formalin (10%). Three segments of each placenta were experimented and measured, after performing all tissue passage steps. In each group, number of embryo was counted. At the end, other three mice bred their newborns in 21st day. Newborns of each mouse were counted and their length was measured by digital coulisse from crown to heel (CHL) and from crown to the ramp (CRL) and their weight was measured by digital scale. 6 newborns of each mouse were selected to measure the gene expression. Left side of Lower limb of newborns were conveyed to cryovial to evaluate the

expression of *osteocalcin* and *RUNX2* genes and were kept in nitrogen tank for an hour, then were kept in -70°C until the RNA purification. Also, right lower limbs were removed for histological studies.

Histological sample preparation

For assessed inflammation in placenta, from each group six sample of placenta fixed in formalin(10%) and then histological passage were done with ethanol (70%-100%) and embedded with paraffin [23]. 5 μm thick section were prepared with microtome and stained with H&E [24,25] and mounted on slide and light microscope image were taken.

RNA purification from tissue sample

The left lower limb of new born mice fetuses were dissected and frozen immediately in liquid nitrogen and were stored at -70°C until RNA extraction. Total RNA from all six samples of each experimental group were extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, Cat.No. 74804).

Real Time PCR for *RUNX2* and *Osteocalcin* mRNA

First Strand cDNA from total RNA was prepared using Prime Script™ Reagent Kit (Takara-Japan-Cat. No. RR037Q). Quantitative real time PCR for *RUNX2* and *Osteocalcin* was performed with a Step One plus Real Time PCR instrument (Applied Biosystems) with SYBR premix EX Tag11 (Tli Plus) (Takara-Japan-Cat. No.RR820L) [26]. The primers that used in this study are presented in Table 1.

Table 1: The list of primer sequences used for qRT-PCR

Gene	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)
<i>RUNX2 (F)</i>	CCTGACTCTGCACCAAGTC	55	234
<i>RUNX2 (R)</i>	GAGGTGGCAGTGTATCATC	55	234
<i>Osteocalcin (F)</i>	TCTGACAAAGCCTTCATGTCC	57	198
<i>Osteocalcin (R)</i>	AAATAGTGATACCGTAGATGCG	57	198
<i>18S (F)</i>	GTAACCCGTTGAACCCATT	60	151
<i>18S (R)</i>	CCATCCAATCGGTAGTAGCG	60	151

Analysis

All data were analyzed using SPSS version 21. The differences between groups were normalized, and analyzed through one-way ANOVA and post hoc Tukey test. Real time PCR results were analyzed with $2^{-\Delta\Delta\text{ct}}$ method. Data presented as Mean+SEM, herein the significance were considered as $p < 0.05$.

RESULTS

The inflammation assessments of placenta

For assessment of inflammation in pregnant mice, the placenta were removed and prepared to inflammatory markers analysis. The results showed

that the inflammatory cells such as macrophages, were significantly increased in treated groups in compared to control (untreated) groups ($p < 0.5$) (Figure 1).

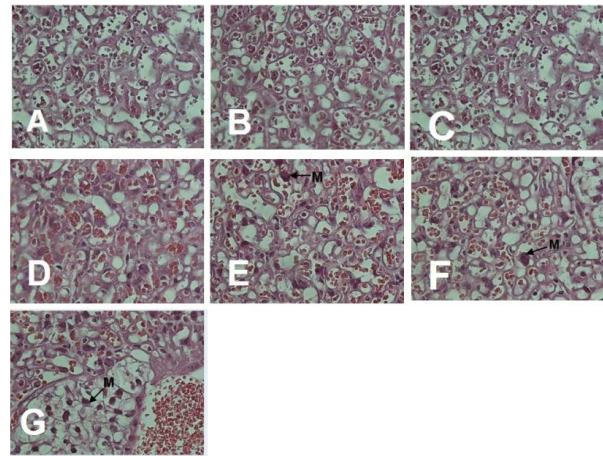


Figure 1: Results from placental histopathology (40X) (A) Control group, (B) Vehicle group 1, (C) Vehicle group 2, (D) Experimental 1, (E) Experimental 2, (F) Experimental 3 and (G) Experimental 4. Inflammation has been observed in placenta in TNF- α receiving group. This inflammation shown by arrow is qualitatively assessed in the form of increasing the inflammatory cells including macrophages in placenta

Macroscopic finding

In this section, the parameters like, crown-rump length, crown-heel length and the number of newborns between groups were analyzed and presented in Figures 2 and 3.

As shown in the Figures 2 and 3, there was no significant difference in the mean of length (crown-rump length and crown-heel length) among experimental groups in compared to control group ($P > 0.05$) (Figures 2 and 3). The number of newborns and their weights between experimental groups was significantly altered in compared to control group ($P > 0.05$) (Figure 4).

Expression of genes related to bone formation (*RUNX2*, *Osteocalcin*)

Real Time PCR resulted showed that *RUNX2* mRNA expression was significant decreased among Experimental 3 in compared to control group (P value=0.02), there weren't any significant alteration in *RUNX2* between experimental groups 1, 2 and 4 in compared to control group ($P > 0.05$). The gene expression level of *osteocalcin* has been measured by qRT-PCR also. Results show that there is significant decrease among experimental groups in compared with control group (P value=0.02) (Figure 4).

DISCUSSION

Based on our results, TNF- α injection to the pregnant mice led to placenta inflammation and a dramatically abnormal alteration in bone structures, genes related to bone formation, differentiation and remodeling. The rate

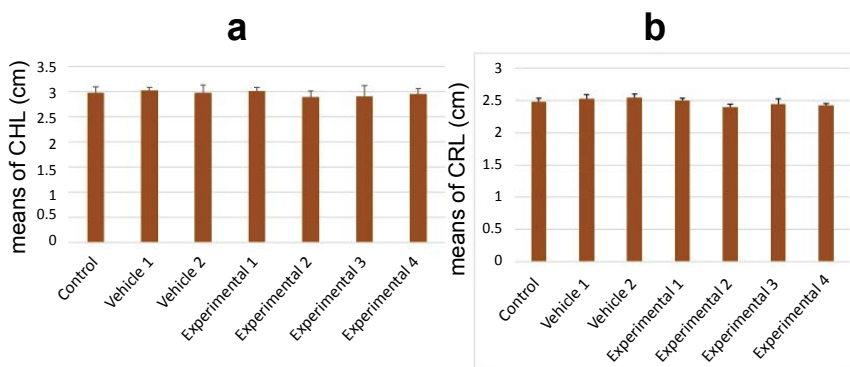


Figure 2: Results from the quantitative comparison of data related to the mean of CHL (a) and CRL (b) in these studied groups including the control group, vehicle group and groups receiving TNF- α show that there is no significant difference among these studied groups ($P>0.05$). This quantitative comparison was done through one-way ANOVA test

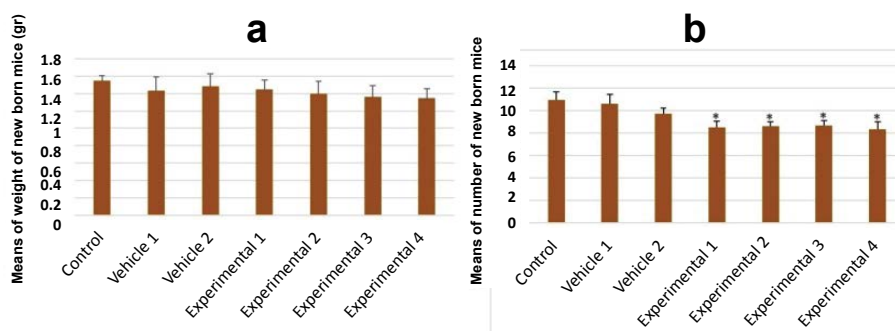


Figure 3: Results from the quantitative comparison of data (done by one-way ANOVA test) related to the mean of weight (a) and the mean of number of these newborn mice (b) in these studied groups including control group, vehicle group and groups receiving TNF- α show that there is a significant difference in number of newborn mice but not weight among these studied groups ($P<0.05$)

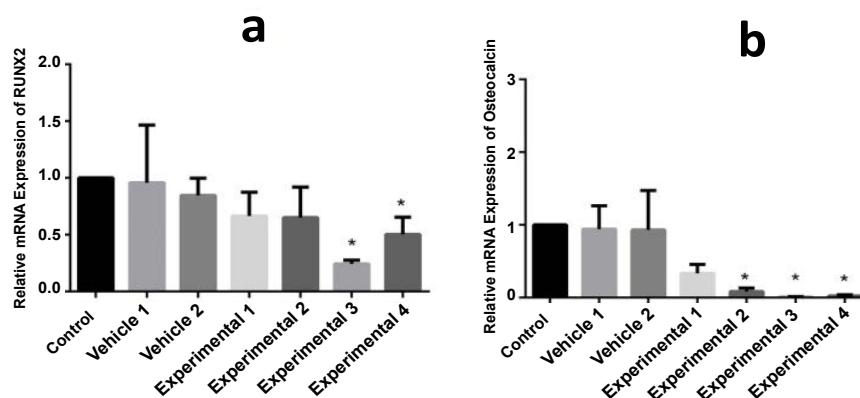


Figure 4: (A) Results from the quantitative comparison of data related to relative mRNA expression of *RUNX2* of newborn mice in studied groups show that there is significant decrease among experimental 3 with control group (P value=0.02) Also, (B) relative mRNA Expression of *Osteocalcin* of newborn mice show that there is significant decrease among Experimental 3 and 4 with control group (P value=0.02)

of these effects relates to dose level, time and length of TNF- α exposure.

Based on many studies, TNF- α injection lead to placenta inflammation. Zaretsky showed that TNF- α transfer across the placenta membrane, and thereby led to increase in concentrations of proinflammatory cytokines in amniotic fluid. These reply to the presence of intra-amniotic inflammation and increase the risk of preterm birth, cerebral palsy, and bronchopulmonary dysplasia [17].

Chronic inflammation is known to mediate bone loss in a variety of conditions including rheumatoid arthritis and aging. In the study on Mesenchyme Stem Cell of human by Ding et al., TNF- α has been shown to inhibit gene expression of *RUNX2* [27,28]. Hess et al., had showed that effect of TNF- α on osteogenesis is dose dependent [29,30]. Similarly, the results of this study showed that there is significant decrease in *RUNX2* expression in the pregnancy mice that exposed to TNF- α and these changes are more depends on dose and time exposure.

Osteocalcin is another gene related to bone formation. In the study, the gene expression level of *osteocalcin* has been measured by qRT-PCR. Results show that there is significant decrease among pregnancy mice treated by TNF- α . In a research on mesenchyme stem cells extracted from bone marrow, Lacey *et al.* indicated that TNF- α has inhibiting effect on gene expression of *osteocalcin* [31]. The difference between this study and previous ones is that this study has been carried out *in vivo* and the TNF- α effect has been evaluated in pregnant mice. Also, it is found if the drug is injected in subsequent days in pregnancy time, the gene expression level of this gene will be mostly decreased. In these studies, the mean of the number of newborns, their weight and length (CHL and CRL) were measured and there was no significant difference in length, numbers and but weight of newborn mice among groups. Fraker *et al.* had showed that intra peritoneal injection of TNF- α (25 and 100 $\mu\text{g}/\text{kg}$) twice daily were decreased daily food intake and weight in rat [32]. It has been found that doses (10,100 ng/kg) have slight effect on this gross parameter of new born mouse fetus that is not observable and measurable.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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