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Effect of Deferoxamine on Rat Fetal Bone Formation and on it is Certain Regulating Genes

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Abstract

The aim of this study is to investigate the deferoxamine effect on rat fetal bone formation and on certain regulating genes expression that play a vital key role in bone formation. Sixty albino adult female rats three months old with average weight 170-200g were employed in this study. The rats were distributed randomly in to two groups. Control group (n=30) receiving 0.3ml ip physiological saline and deferoxamine methane intra peritoneal daily at a dose of 100 mg/kg throughout the pregnancy from the first day of pregnancy until last day of pregnancy. Skeleton samples and staining were done on sixty fetuses were chosen from each group at 20 days of pregnancy as well as liver samples from mothers and their fetuses for studying gene expression of Msx1 and Cx43. The results showed that many variations in certain bone formation and development markers were recorded as well as there was downregulation of studied genes. In conclusion, the study found that deferoxamine affect negatively on certain bone development markers and on gene expression of Msx1 and Cx43 represented as downregulation.

Keywords: **deferoxamine, fetal bone development, gene expression, Msx1, Cx43**

تأثير الديفيروكسامين على تكوين عظام الجنين وعلى بعض الجينات المنظمة

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الخلاصة

الهدف من هذه الدراسة هو التحري عن تأثير ديفيروكسامين على تكوين عظام الجنين لدى الجرذان وعلى بعض الجينات المنظمة التي تلعب دورًا رئيسيًا حيويًا في تكوين العظام. تم استخدام ستين جرذا بالغا بعمر ثلاثة أشهر ومتوسط وزن 170-200 غرام في هذه الدراسة. وزعت الجرذان عشوائياً إلى مجموعتين. مجموعة السيطرة (30) حقنت 0.3 مل من محلول الملح الفسيولوجي ومجموعة الديفيروكسامين ميثان حقنت داخل الخلب يوميًا بجرعة 100 مجم / كغم طول فترة الحمل من اليوم الأول للحمل حتى آخر يوم من الحمل. تم اختيار عينات الهيكل العظمي وصبغها بصبغة الأليزارين الحمراء والأليشيان الزرقاء على ستين جنينا من كل مجموعة في 20 يوما من الحمل فضلا عن جمع عينات الكبد من الأمهات وأجنتها لدراسة التعبير الجيني لـ Msx1 و Cx43. أظهرت النتائج أنه تم تسجيل العديد من الاختلافات في تكوين عظام معينة وعلامات التطور بالإضافة إلى انخفاض تنظيم الجينات المدروسة. في الختام، وجدت الدراسة أن الديفيروكسامين يؤثر سلبًا على بعض علامات نمو العظام وعلى التعبير الجيني لـ Msx1 و Cx43 الذي يمثله انخفاض التنظيم.

الكلمات المفتاحية: ديفيروكسامين ، نمو عظام الجنين ، التعبير الجيني ، Msx1، Cx43

Introduction

The importance of calcium in building the skeleton of the fetus increases day after day as pregnancy progresses, the placenta plays a vital role in transporting huge quantities of calcium to build fetal skeleton rapidly (1). At the time of organogenesis starting until end of gestation period, maternal bone resorption and development are both expanded (2). On other hand, evidence from studies conducted in both humans and rats suggests that iron is necessary for balanced bone metabolism, and moderate to severe dietary iron deficiency in rats results in altered bone morphology and microarchitecture، decreased density and strength in femurs and vertebrae, and increased urinary bone turnover markers (3,4). Deferoxamine (DFO) which is an iron chelator has been successfully used for the treatment of acute iron poisoning and chronic iron overload (5). The use of the DFO in pregnant thalassemia women with iron overload has been generally avoided due to fear of its potential teratogenicity (6). However, due to uncertainty of its safety for the developing fetus, it has generally been restrained during pregnancy and because of the concern over its teratogenic potential. Although there is an inadequacy of information regarding the maternal and embryo/fetal toxicity of DFO, only minor developmental effects represented as retardation of bone ossification, vertebral aplasia, bifurcation, and fusion of ribs were seen following DFO administration during pregnancy (7,8).

Daily DFO, more than the dose as long term exposure, could be related to bone abnormalities (9) and reduced bone formation and resorption maybe due to iron chelation (10). One of the developmental toxicity studies is the study of a substance-induced prenatal growth retardation as an outcome of a reduction of average fetal body weight (11). The relationship between body weight at term and extent of ossification of the fetal skeleton has not been extensively investigated and the spongy, incompletely, and poorly ossified bones as well as unossified ossification centers have also been taken as indicators of delayed ossification of the fetal skeleton. Therefore, the purpose of this study is to investigate the deferoxamine effect on rat fetal bone formation and on certain regulating genes expression that play a vital key role in bone formation.

Material and methods

Animals and experimental design

Sixty albino adult female rats three months old with average weight 170-200g were employed in this study. The rats were distributed randomly in to two groups. Control group (n=30) receiving 0.3ml ip physiological saline and (deferoxamine methane, NOVARTIS-Switzerland,) intra peritoneal daily at a dose of 100 mg / kg throughout the pregnancy from the first day of pregnancy until last day of pregnancy. Rats were placed in plastic cages under appropriate environmental conditions in terms of temperature (24 ± 1 ° C), humidity (60 ± 10%). Ventilation, adequate lighting, and water and feed were provided to them freely throughout the experiment period. At the end of the treatment for each group fetal samples for skeleton staining, and liver samples were collected for gene expression of Msx1 and Cx43 and kept at -80° C.

Skeleton sampling and staining

Sixty fetuses were chosen from each group at 20 days of pregnancy from mothers. Fetuses were removed by cesarean section after euthanized the animals. Fetuses were fixed with ethanol 95% up to 7 days in preparation for the skeletal staining process (12,13) according to following protocol.

Fixation process; the skin of the rat fetuses was carefully removed and eviscerated completely after fixation with 95% ethanol for 7 days at room temperature, and then left in ethanol for an additional two weeks. Then the fetuses were transferred to one-liter plastic containers containing pure acetone to be rid of the adipose tissue from the fetuses and kept for 24 hours at room temperature.

Staining process; in this step, the stain was prepared as follows: Prepared 0.1% of Alizarin red-S in 95% ethanol by dissolving 250 mg of stain in 250 ml 95% ethanol. Prepared 0.3% of Alcian blue in 70% ethanol by dissolving 750 mg of stain in 250 ml (70%

ethanol) plus 250 ml ice acetic acid, after which 0.1% Alizarin red-S stain was added to 0.3% Alcian blue and the final volume was completed to 5 liters of 70% ethanol. For the staining of the fetuses, the fetuses were transferred from the acetone container to other containers containing the mixture of the two stains and placed in the incubator at 40° C for one week. After which the fetuses were washed from the stain with tape water until all the stain was removed from the body. The fetuses and the process took 3-5 hours according to the amount of saturated pigment in the body of the embryo. Then fetuses were transferred to the final stage of the staining process, which is the process of digestion and visible the skeleton.

Digestion and clearing process; in this step, the fetuses were transferred to a container containing 2% KOH solution for 48 h. Then, the fetuses were placed in a 20% aqueous solution of 1% KOH glycerol and left until the skeleton was clearly visible. The skeletal samples were transferred to other plastic containers containing 1: 1 glycerol: 95% ethanol solution for 24 hours at room temperature. The skeletons were passed through two concentrations of a glycerol solution - ethanol, 50% and 80%, for one week and for each concentration. The skeletons were then transferred to other container containing 100% pure glycerin. The skeletons were examined under a stereo-microscope and images of the various parts of the skeleton were taken by the camera attached to the microscope.

Detection of Msx1 and Cx43 mRNA expression using semi-quantitative PCR

RT-qPCR protocol

Liver samples that kept at -80° C were used to extract RNA using an RNA extraction kit (RNA General Extraction Kit, Genome, USA). According to the kit instructions. Transcription of RNA to cDNA was done by using a special kit to transcribe the RNA into DNA (wizScript RT FDmix (dT20), south korea) where, the Rt FDmix tube was placed on the PCR tube rack of the PCR polymerase chain, and 5µg of the extracted RNA was added to each tube and added to it. 20µl of RNase-free water. The tubes were placed in the Thermal cycler according to the instructions for the kit used and as follows: 25°c /10 min, 42°c /30 min, 85°c /5 min, and 4°c /hold. The cDNA samples are ready for the last stage of gene expression measurement and the samples were stored at -20 ° C for qPCR.

Gene expression analysis for Msx1 and Cx43 was performed according to the following sequence Msx1; F: 5'-GCCTGCACCCTACGCAAGCA-3' R:5'-AGCAGGCGGCAACATTGGCT-3'.Cx43; F: 5'-TCCTTTGACTTCAGCCTCCAAGGAG-3'

R: 5'-GCAGACGTTTTCGCAGCCAGG-3'. (14). Real-time PCR was performed to analyze the gene expression using a BIO-RAD CFX96 system and SYBR green method using FastStart Universal SYBR Green Master (Rox) kit (Roche's diagnostics GmbH, Germany). Relative gene expression was determined by relative qPCR by the use of the comparative CT method. RT-qPCR reaction in all samples was conducted in the total reaction mixture volume of 25 µl as follows: 12.5 µl FastStart Universal SYBR Green Master (Rox), 1 µl forward primer (20 pmol/µl), 1 µl Reverse primer (20 pmol/µl), 2 µl cDNA and 8.5 µl sterile water. The thermal cycling conditions for qPCR were an initial denaturation for three minutes at 95 oC, followed by 35 cycles of annealing 95 oC for one minute, and 60 oC for one minute, and extension at 72 oC for one minute. The Bio-Rad CFX Manager 3.1.1517.823 software was used to analyze the relative quantitative PCR, and values obtained from each sample were normalized to glucose-6-phosphate dehydrogenase expression F: 5'-GGCTCTCTGCTCCTCCCTGTTCTA-3' R: 5'-TGCCGTTGAACTTGCCGTGG-3'. (Integrated DNA technologies, Singapore).

Statistical analysis

All data were expressed as mean ± S.E, independent t-test was employed for determining the significant variation between studied groups. Whereas, the Chi-square test was employed for determining the significant variation among studied variations by using spss software v. 23. and *P*-value 0.05 was considered significant.

Results

Effect of DFO injection during pregnancy on fetal bone formation

Intrauterine exposure to DFO up to 20 days during pregnancy revealed a significant variation in fetal bone formation characterized by clear variations in the degree of mineralization, ossification, bone development, and bone fragmentation as well as other markers.

Loss of mineralization, the percentage of loss of mineralization in head and thorax region showed significant increase in bones of DFO fetuses group compare to control fetuses' bones at *P*-values 0.018 and 0.013, respectively. Despite the elevation of loss of mineralization % in pelvic and limbs' bones of DFO fetuses' bones with respect to control fetuses' bones, but still non-significant and *P*-values 0.091 and 0.268 respectively. (Figure 1)

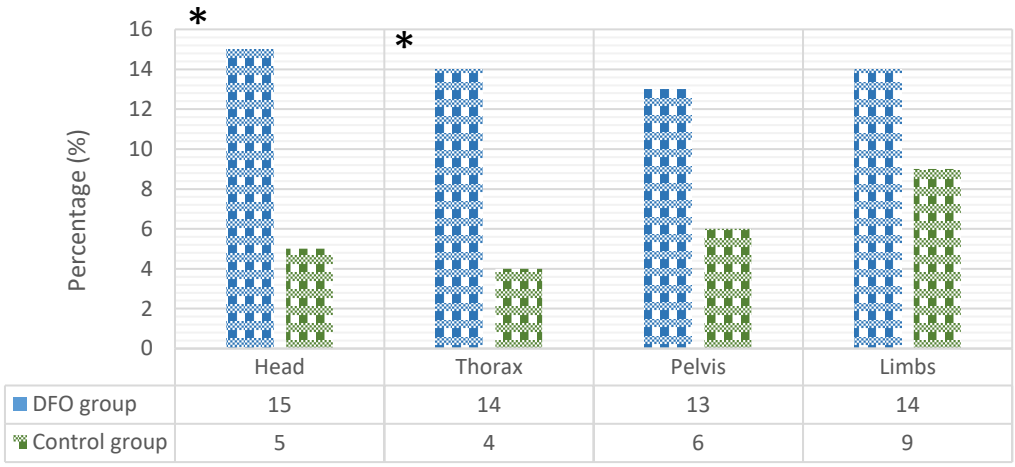

Figure 1: Effect of DFO on mineralization degree in fetal bones

Development delayed, the percentage of delayed bone development exhibited significant elevation of head bones in DFO fetuses' group compare to control group (*P*-value 0.027). Whereas, the elevation of the rate of the development delay in limbs bones was non-significant (*P*-value 0.093). Figure 2

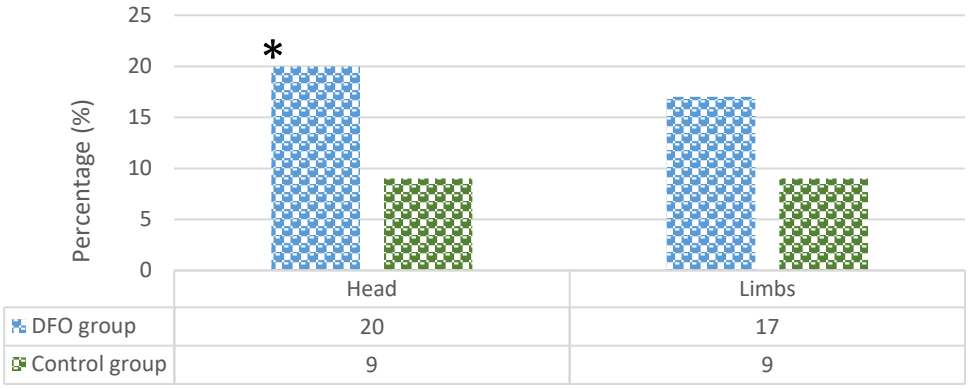

Figure 2: Effect of DFO on the rate of bone development delay of fetuses' bones

Bone fragmentation was recorded in the bones of the pelvis and limbs and significantly higher in the bones of DFO fetuses' group compared to control fetuses (*P*-value 0.000). Whilst, the head bone s did not show any variation in the rate of bone fragmentation between studied groups (*P*-value 0.651). Figure 3

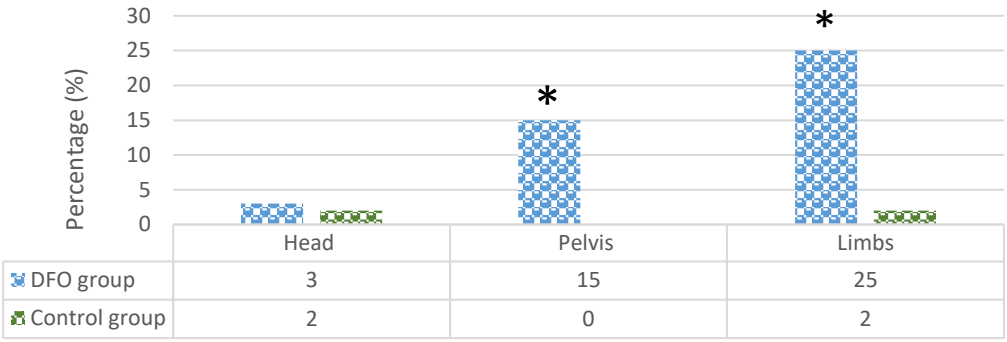

Figure 3: Effect of DFO on bone fragmentation rate in fetal bones

Soft plate development data revealed significant increase in soft plate development of fetuses' head bones belongs to DFO fetuses group compared to control fetuses' head bones (*P*-value 0.017). Figure 4

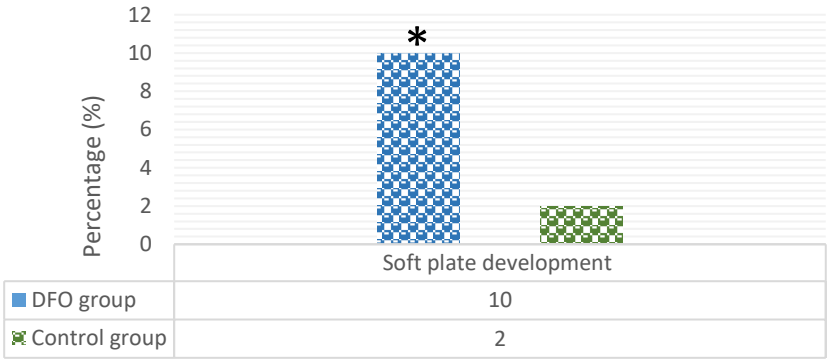

Figure 4: Effect of DFO on soft plate development in fetal head bones

Distinct proximal epiphyseal plate non-differentiation was recorded in 18% of the fetuses of the DFO group compared to that of the 0% undifferentiated distinct proximal epiphyseal plate of the fetuses of the control group. This percentage is considered significantly high at the *P*-value 0.000. Figure 5

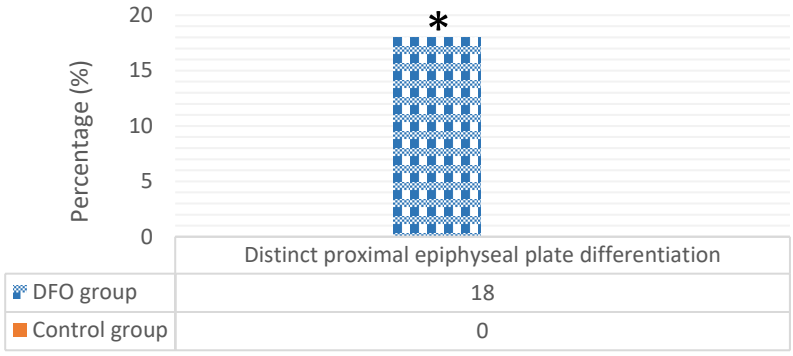

Figure 5: Effect of DFO on distinct proximal epiphyseal plate differentiation in fetal bones

Conformation of long bones showed well conformation of fetal long bones and exhibited no significant variations between studied groups regarding the percentage of non-conforming of the long bones in the limbs despite their height in DFO group (*P*-value 0.088). Figure 6

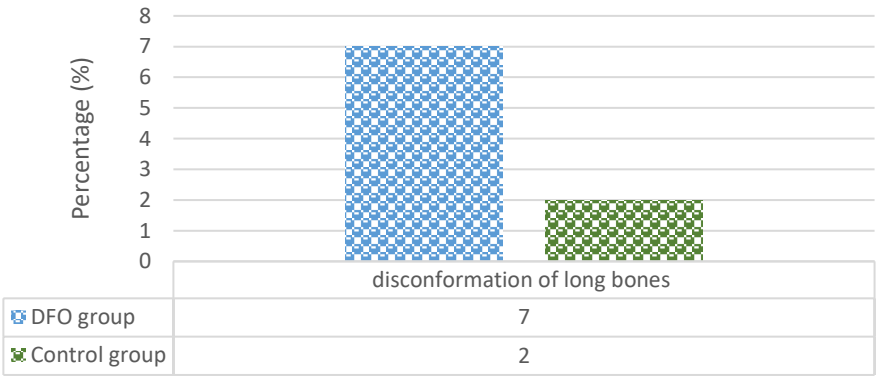

Figure 6: Effect of DFO on conformation of long bones in fetal bones

Osteochondral lines development in fetal bones, the statistical analysis of the percentage of non-development of the osteochondral lines of the thorax bones resulted in a significant increase in the fetuses' bones of the DFO stressed group compared to control fetuses bones (*P*-value 0.002). While, no differences were recorded for this criterion of the bones of the pelvis and limbs between the fetuses of all groups (*P*-value 0.165, 0.579 respectively). Figure 7

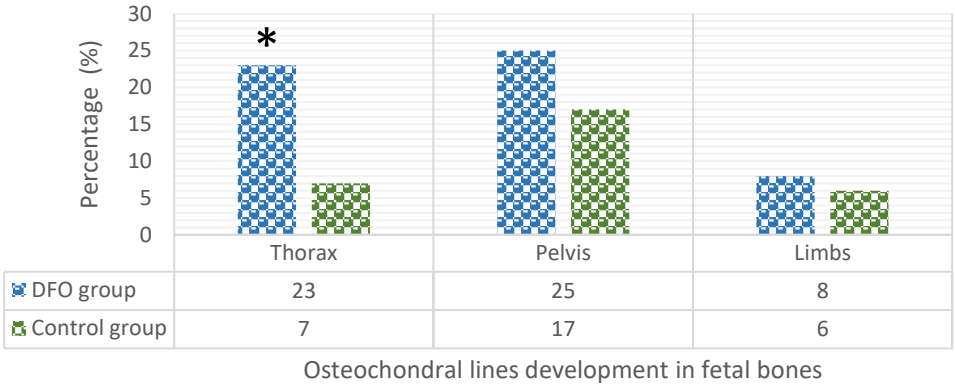

Figure 7: Effect of DFO on osteochondral lines development in fetal bones

Crookedness/Malformation/Tortuous, the percentage of crookedness/malformation/tortuous were significantly higher in some thorax bones reaching 18% in the thorax bones of the DFO stressed group than in the fetuses of the control group 3% at *P*-value 0.001. No significant difference was recorded for the same criterion in the limbs' bones between the fetuses' bones of studied groups *P*-value 0.708. Figure 8

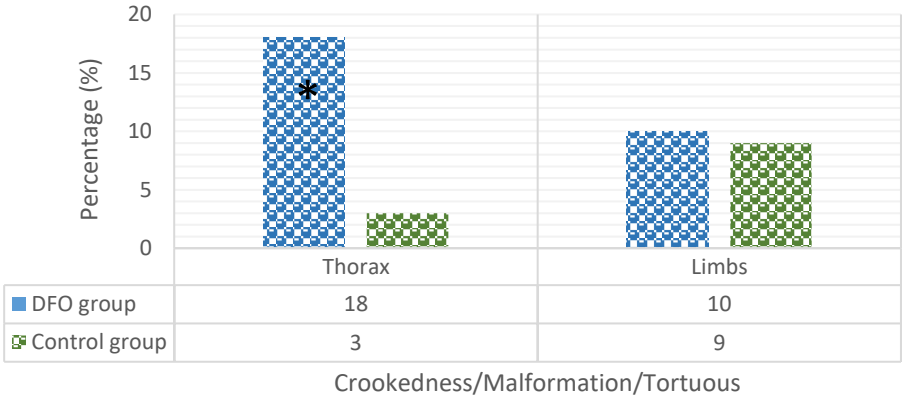

Figure 8: Effect of DFO on crookedness/malformation/tortuous in fetal bones

Absence of bones, examination of the fetuses of the DFO stressed group resulted in a significant increase in the percentage of bone loss compared to the fetuses of the control group of the head bones (*P*-value 0.043). Whereas no significant differences were recorded for the thorax bones between the fetuses' bones of all groups (*P*-value 0.651). Figure 9

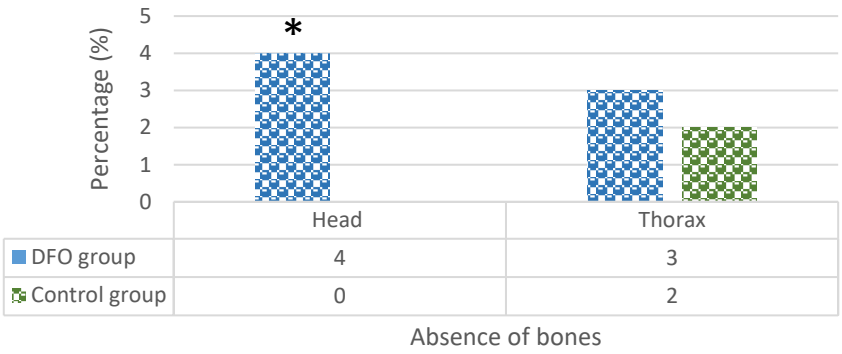

Figure 9: Effect of DFO on absence of bones in fetal bones

Curving of the bones, statistical analysis using chi-square test did not result in any significant differences in the percentage of curving bones of the thorax and limbs between the fetuses' bones of the studied groups (*P*-value 0.297 and 0.249) respectively. Figure 10

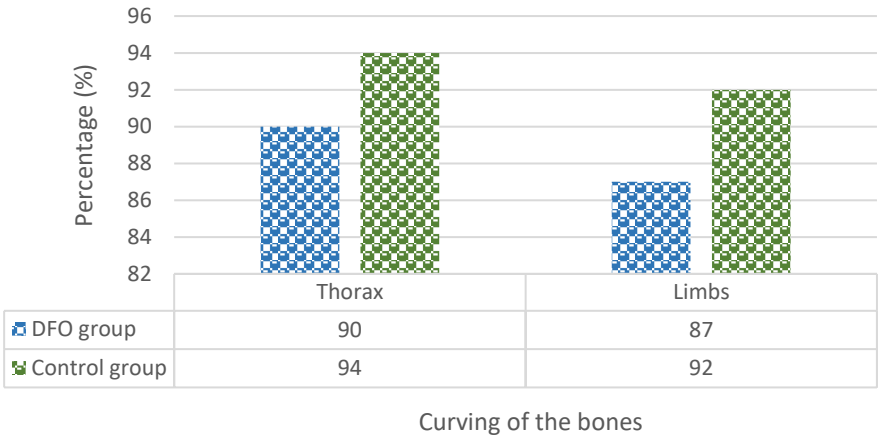

Figure 10: Effect of DFO on curving of the bones in fetal bones

Effect of DFO injection during pregnancy on Cx43 and Msx1 gene expression

Effect of DFO on Cx43 gene expression in mothers and their fetuses

The statistical analysis of the ΔCT mean value of the Cx43 gene expression in both fetuses and mothers revealed no significant differences despite the decrease of the ΔCT mean value in cDNA samples for DFO groups (*P*-values 0.110 and 0.297) respectively (Figure 11). Figure 12 illustrated a plot showing the gene expression of the Cx43 gene for the pregnant rats and their fetuses.

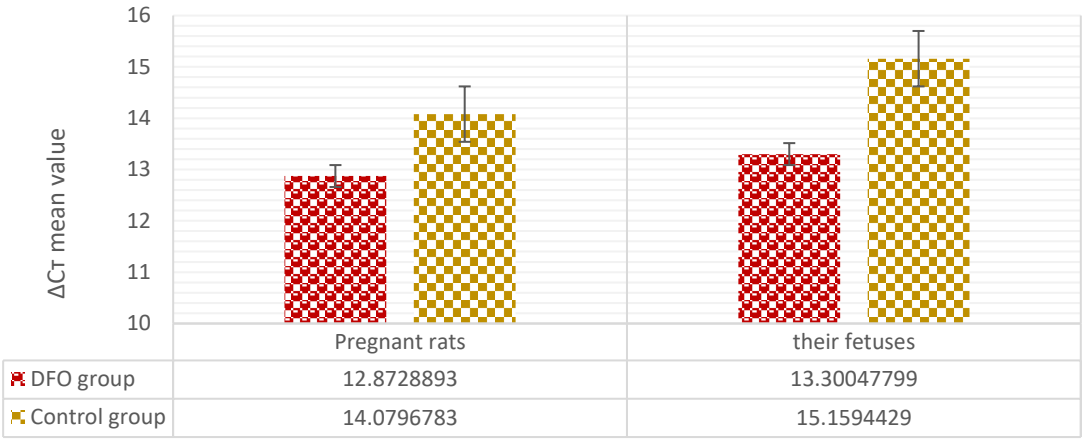

Figure 11: Effect of DFO on Cx43 gene expression in both pregnant rats and fetuses

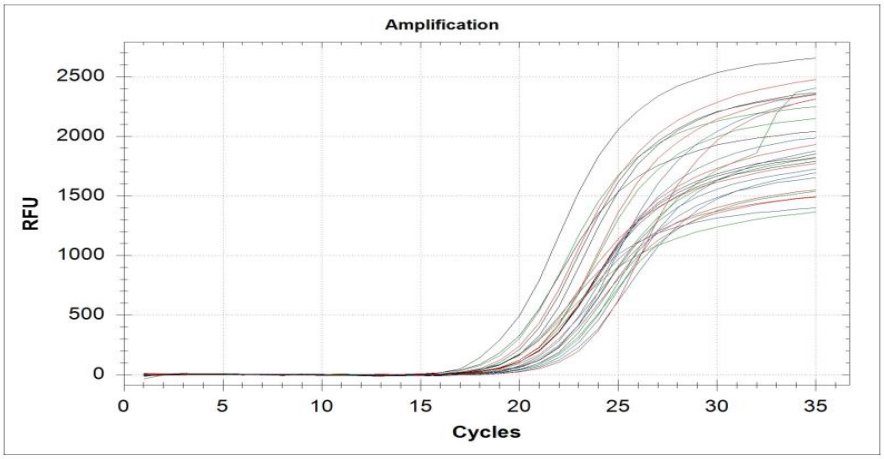

Figure 12: Scheme showing gene expression of Cx43 from cDNA isolated from fetuses and mothers of experimental rats. The green line represents the gene expression of the mothers of the control group. The black line represents the gene expression of the mothers of the DFO group. The blue line represents gene expression of control group fetuses. The red line represents the gene expression of the fetuses of the DFO group

Effect of DFO on Msx1 gene expression in mothers and their fetuses

The statistical analysis of the ΔCT Mean value of the Msx1 gene did not result in significant differences despite the decrease of the ΔCT Mean value in cDNA samples for DFO groups for both fetuses and mothers (*P*-value 0.163 and 0.168) respectively (Figure 13). Figure 14 illustrated a plot showing the gene expression of the Msx1 gene for the pregnant rats and their fetuses.

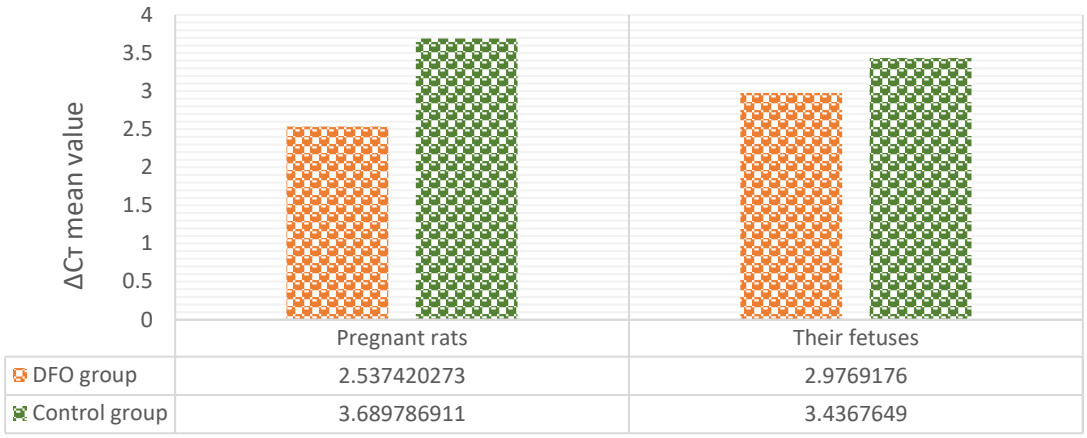

Figure 13: Effect of DFO on Msx1 gene expression in both pregnant rats and fetuses

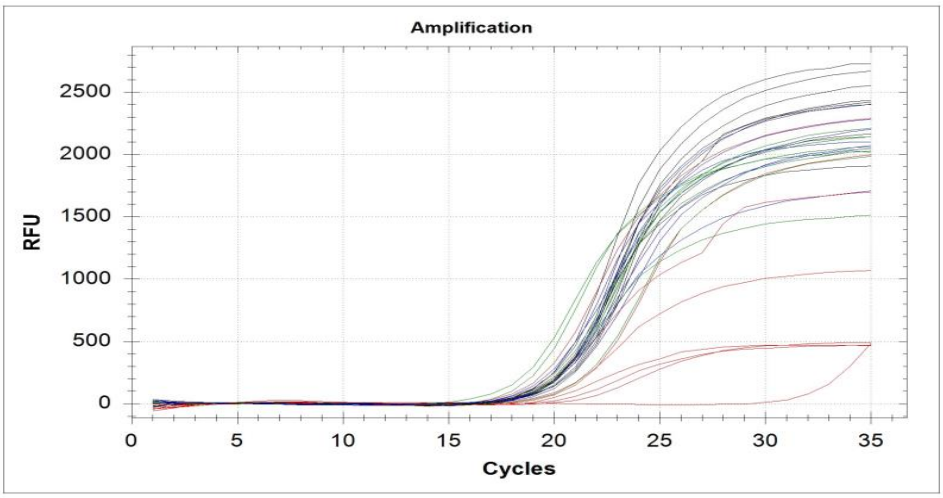

Figure 14: Scheme showing gene expression of Msx1from cDNA isolated from fetuses and mothers of experimental rats. The green line represents the gene expression of the mothers of the control group. The black line represents the gene expression of the mothers of the DFO group. The blue line represents gene expression of control group fetuses. The red line represents the gene expression of the fetuses of the DFO group

Discussion

Pregnancy is a challenge that faces the mothers and the developing fetuses under all natural and abnormal conditions. The use of some medicines during pregnancy may be critical under certain circumstances that may cause harmful negative effects for mothers and/or their fetuses. The aim of current study is to investigate the deferoxamine effect on rat fetal bone formation and on certain regulating genes expression that play a vital key role in bone formation. Ossification begins in the bones of the skeleton of rats on the 16-17th day of pregnancy, specifically the bones of the lower jaw and ribs (15,16). This period is very critical in the formation of the skeleton as well as the organogenesis that are exposed to many internal and external factors that affect negatively or positively on their formation and development (11).

The current study has demonstrated clear changes in the skeleton formation of the fetuses, represented by varying degrees of mineralization and ossification, as well as anatomical changes in some different bones of the body. These changes might be due to the

indirect effect of desferal on the induction of oxidative stress because of its interference with the level of oxygen in the fetal tissues as a result of desferal interaction on iron metabolism (17). The reason for these changes may be attributed to the enzymatic activity associated with the growth and development of the embryos and its increased influence on the metabolic processes in the placenta. This is due to the imbalance of oxygen levels in the placental tissue, which results in an excessive increase in the synthesis and production of free radicals (18). It is known that placental hypoxia is closely related to the conversion of the enzyme xanthine dehydrogenase into xanthine oxidase, the main source and important in the generation of cytotoxic free superoxide radicals (19). This supports our hypothesis that the lack of oxygen resulting from the oxidation of iron in cytosol and its conversion from the Fe^{+2} to the Fe^{+3} that negatively affects the transport of oxygen to the placental cells (20). As a result of the increase in free hydroxyl radicals by an amount greater than the capacity of cell defenses from antioxidants transforming the placental environment into an oxidizing environment not conducive to the growth and development of embryos (21). Other reason for the changes in the growth and development of bones may be attributed to the defect in the level of IGF-1, which is important in converting cartilage cells into osteons and contributing to the mineralization process (22). Where (23) pointed to the vital and critical role of IGF-1 in regulating the growth of embryos and the formation of different tissues during the embryonic stage and the postpartum stage through the ability of this factor to promote division, migration and survival of cells in addition to protein synthesis. A study done by (24) showed that postnatal growth and survival rates in newborns with mutations in IGF-1 suffer from delayed bone growth and development. This support our hypothesis that oxidative stress caused inhibition of IGF-1, which in turn led to a defect in the processes of ossification and the transformation of cartilage into bone. This may be due to its interference with the genes responsible for building and developing bone and cartilage naturally, for example Cx43 and Msx1 who have an active role in the growth and development of bones.

In normal bone growth, calcification of the cartilage matrix is associated with a specific stage in the development of chondrocytes, and with the progression of the primitive bone marrow that is involved in removing the chondrocytes and replacing them with bone (25). In this stage the chondrocytes enlarge and the cytoplasm disintegrates, and the enlargement of the chondrocytes and the enlargement of their gaps in the cartilage matrix gradually reduces the thin, irregular and permeable septum, after which the remaining hyaline matrix is calcified (26). These parts will be colored red and blue by the Alizarin red (bone stain) and Alcian blue (cartilage stain) according to the degree of mineralization and ossification of the skeleton (27). The lack of oxygen plays an important role in the differentiation and normal growth of bone cells, as the lack of oxygen negatively affects the growth and differentiation of bone cells by activating the extracellular signal-regulated kinases and mitogen-activated protein (ERK1/2 MAPK) pathway. This pathway transports the MAPK into the nucleus, causing the reduction of the gene expression responsible for the ossification process. The reason for the variation in the mineralization and ossification is attributed to the increased phosphorylation of ERK1/2 MAPK that contribute to the differentiation of bone cells under hypoxic conditions (28). This supports our hypothesis that DFO caused a lack of oxygen in the bone tissue of the fetuses and that the lack of oxygen inhibited the ossification process through the aforementioned pathway. The other reason for the low ossification rate may also be attributed to the direct interaction of the excessive formation of free radicals, and the low level and efficiency of antioxidants. This in turn increases the inhibition of bone deposition when primary ossification centers form during embryonic development through the interaction with the processes of bone mineralization and the intensity of the bone turnover processes, thus directly affecting the formation and development of the fetus' skeleton.

There are many genes that have a distinct role in the process of regulating bone formation through the process of regulating mineral deposition in bone tissue during the period of skeletal formation, for example the Cx43 and Msx1 genes. Msx1 is known as a homeodomain transcription factors that encode genes is a much needed for the development of the cranial, limb and nervous system. The genes of the Msx family have been classified as regulatory proteins, and their responsibility is *in-vitro, in-vivo,* and transcription inhibitors. This is evident in the phenotypic abnormalities that appear in rats, mice and humans because this gene is vital and essential in the normal formation of the cranial organs, and the limbs (29). In the development of vertebrate embryos, the gene expression of Msx1 and Msx2 is strongly observed in the developing craniofacial areas in an overlapping manner. This indicates the role of Msx genes in craniofacial development (30). The downregulation of the Msx1 gene during the growth of mouse embryos resulted in decreased hyoid bone and cranial neural-crest derivatives that were identified by the lack of calcified tissue in different parts of the fetal skull and this is consistent with what was reported (31). (32), reported that the gene expression of Msx1 in both osteoblast and osteoclast cells and that differentiation of periosteoclasts is associated with downregulation of Msx1, while in the endo-skeletal, it is associated with both differentiated osteoblasts and osteoclasts via expression Genetic for homeoprotein. In previous studies that support the current results, they were proved that the main factor in the formation of the cranial skeleton is Msx1 and that any changes in gene expression or mutations lead to defects in the growth of teeth and delayed growth. In addition to that abnormalities in the teeth and cranial bones and cleft palate (33-35). This is demonstrated by our current study of high cleft palate, as well as delayed bone growth in fetuses.

Connexin 43 (Cx43) is the most abundant gene from the connexin family of proteins in bone and is expressed in osteoclasts, osteoblasts, and osteocytes (36, 37). The Cx43 gene is called a gap junction protein, forming a channel called a gap junction channel that facilitates communication between adjacent cells in bone as well as in other tissues. gap junction protein channel is formed by the fusion or coupling of two channels of this protein (Cx43) on the membranes of the opposite cells to allow direct exchange (38). Cx43 contributes to peak bone mass in addition to the cortical modeling of long bones and the maintenance of bone quality. Cx43 also contributes in a variety of ways to the bone's response to hormonal and mechanical signals (39). (40), noted that the Cx43 protects the bone cells from oxidative stress damage and that the decrease in its gene expression causes the overproduction of ROS, which in turn increases the rate of programmed death of these cells. The other reason is osteocytes are affected by oxidative stress may be attributed to the stress of endoplasmic reticulum, which plays an important role in the gene expression of Cx43 (41). These evidences also support our findings about role of Cx43 in regulating bone formation and development.

Conclusion

The study concludes that pregnant rats' model exposed to DFO during pregnancy were affected negatively on fetal bone formation and developments through clear variations in the degree of mineralization, ossification, bone fragmentation, cleft palate development and other studied bone development markers. Furthermore, the DFO affect in direct and clear manner on gene expression, although it is insignificant Msx1 and Cx43 genes showed downregulation expression in both pregnant rats and their fetuses.

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Conflict of interest

The authors have no conflicts of interest.

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