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# Impact of soft drink on skeletal formation indicated by RSPO2, HAO1, and RUNX2 gene expressions

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## ABSTRACT

The side effects of soft drinks are still an important challenge especially for the expressed genes of foetuses that regulate ossification. For that purpose, we investigate the expression of R-spending2 (RSPO2), Hydroxyacid Oxidase 1 (HAO1), and runt related transcription factor 2 (RUNX2) genes and the foetuses skeletal malformation due to maternal Pepsi consumption. Pregnant rats were divided into control group that was administrated orally with distilled water, group1 from day 1 to day 7 was orally administrated with 2.5 ml/day of Pepsi, group2 from day 1 to day 7 was administrated orally with 5ml/day of Pepsi, group 3 from day 8 to day 20 was orally administrated with 2.5 ml/day of Pepsi, and group 4 from day 8 to day 20 was orally administrated with 5 ml/day of Pepsi. Gene expression analysis revealed that the RSPO2 gene is significantly decreased with increasing the dosage of soft drinks during the 1$^{st}$ stage of pregnancy. Conversely, there is a significant increase in the HAO1 gene in 1$^{st}$ stage group relative to the control group. RUNX2 gene is significantly decreased in group1 and group 2 while it was significantly increased in groups 3 and 4 regarding the control group. Pepsi administration caused retarded body length and weight, ossification and lengths of some bones, and shortness of others. Different bones are seriously affected by Pepsi. Therefore, our findings reveal the effect of soft drink consumption on skeletal malformation and the RSPO2, HAO1, and RUNX2 genes that can be used as biomarkers for skeletal modulation.

**Key Words:**

Soft drinks; RSPO2; HAO1; RUNX1; Skeletal malformation.

## 1. INTRODUCTION

Diet is an important determinant of health of bone. The role of dairy products, calcium and vitamin (D) in health of bone has been widely reported [1,2]. Soft drinks called soda or carbonated beverages are one of the substantial components of the modern diet. Consumption of Soft drinks has increased in the entire world in the past two to three decades [3,4]. Pepsi typically contains predominantly water, caffeine, phosphoric acid, sucrose sugar and chemicals of colouring, preservatives and flavors [5]. Many health problems are associated with the consumption of soft drinks regularly. There were more than 25 separate harmful effects are associated with the consumption of soft drinks [3]. Studies of epidemiological pointed toward its associations with metabolic syndrome, diseases of liver, inflammation and bone diseases of adults and children [6,7]

Different studied reported the importance of pathways as wnt signaling pathway in skeletal system of the body [8].Wnt signaling pathway is upregulated as a result of binding of RSPO to LRP6 that cause obstruction of LRP6-DKK complex which boosts the canonical mechanism in Wnt signaling pathway [9]. The recently discovered secreted protein family, R-spondins have 4 members (RSPO1/2/3/and 4). R- spondins are cysteine rich that contain thrombospondin type 1 domain [10]. RSPO family members all have conserved around 60%

of its sequences [11]. It has been found that RSPO act as modulator for Wnt signaling pathway through G protein- coupled receptors that can do upregulation of the pathway due to stabilization of cytosolic β-catenin [12]. The G coupled- protein receptor consists of N terminal domain that can bind to the RSPO proteins [10]. Some studies reported the positive relationship between the RSPO family and the bone metabolism [13]. The mutations in RSPO4 were found to have critical association with finger and toe nails absence in humans [14]. It was found that RSPO2 was decreased during the early developmental stages of the chondrocyte differentiation [15]. RSPO2 plays critical role in the bone development. The knockout of RSPO2 of mice make it die just after birth and make different abnormalities including defects in limb differentiation [16].

HAO1 (Hydroxyacid Oxidase 1) is an enzyme from a family of enzymes that can transform the hydroxy acids to keto acids and reduce the oxygen to H2O2 [17]. Increasing Keto acids in body have a negative significant role in ossification. As described in previous studies, rats treated with keto acids as a part of ketone bodies reported bone loss in cancellous bones with some changes in L4 vertebral bone. In addition to decrease in strength of appendicular and axial bone [18]. Moreover, HAO1 considered as one of the antioxidant genes which have significantly changed expression that cause oxidative stress [19]. Bone remodeling can be differed due to the oxidative stress that causes unbalance between activities of osteoclast and osteoblast. The unbalance resulted in bone diseases including low bone mineral density and reduction in bone mass called osteoporosis which cause bone fracture [20].

RUNX is a family of transcription factors including RUNX1, RUNX2, and RUNX3. RUNX2 was reported to be an essential regulator for bone growth and is important for osteoblast differentiation [21]. In addition, mutations in RUNX2 results in skeletal malformation, multiple skeletal abnormalities and persistently open fontanelles, short stature, excessive teeth, hypoplastic clavicles, and other changes in skeletal development [22]. It was found that there is a complete loss of bone results from the inactivation of RUNX2 gene [23]. AS RUNX2 is necessary for differentiation of osteoblast, it was found that mice with RUNX2-/-exhibit dearth osteoblast differentiation and lack of osteoblast-specific gene expression that make the chondrocyte not developed [24].

Contents of soft drinks have a harmful effect on the bone of humans. It causes bone demineralization, fracture to bone, and disruption in the formation of bone [25,26,27,28]. Increasing the intake of soft drinks reduce the intake of milk, magnesium and calcium, which can decrease the accumulation of minerals of bone and increase the osteoporosis and fracture [27,29,30]. Phosphoric acid in soft drinks reduced bone density, osteoporosis and fractures [31,32]. Sodium and sugar in soft drinks can also cause increased calcium loss [33]. Caffeine causes skeletal malformations to foetuses maternally treated with soft drinks [35]. Studies on soft drinks and skeletal malformations of foetuses are very limited. Therefore, this study has been done to show the effect of Pepsi on bone differentiation and the expression of genes that have been used as biomarker for loss of bone formation.

## 2. MATERIALS AND METHODS

### 2.1. Experimental animals

Pure inbred strain albino rat; Rattus norvegicus domesticus, provided from Theodor Bilharz research institute, Egypt, were used for this experimentation. Fifty virgin female and fertile male albino rats were acclimatized in the laboratory for ten to fourteen days under standard conditions of temperature and humidity. After 14 days of acclimation, the rats weighing approximately 280 gm [3] were used for experimentation. Mating was performed by housing one male with three females overnight. In the early morning, the presence of smear or vaginal plug was designated as day one of pregnancy [36]. The pregnant rats were divided into five groups; five groups, control group that was administrated orally with distilled water. The other groups were administered with Pepsi obtained from local markets in Egypt, with different doses. group1 (1st to 7th day of pregnancy) and group2 ($1^{st}$ to $7^{th}$ day of pregnancy) that were administrated with 2.5ml and 5ml of Pepsi, respectively, and group 3 ($8^{th}$ to $20^{th}$ day of pregnancy) group 4 (8th to 20th day of pregnancy) that were orally administrated with 2.5 ml and 5 ml of Pepsi [3], respectively as indicated in Figure1. At the $20^{th}$ day of pregnancy, the rats were anesthetized by inhaling light diethyl ether. Caesarean made to remove the uteri. For morphological studies, foetal length and weight measured and foetuses either living or dead were counted.

Bone tissues that were used in gene expression studies were kept in -80C. The experiment was approved by Zoology Department, Faculty of Science, Benha University IACUC with permit number ZD/FSc/BU-IACUC/2021-7.

**2.2. RNA extraction and cDNA preparation**

Total RNA has been isolated from each mice embryo (5 tissues /group) by using TRIzol reagent. The concentration of total RNA for each sample and their purity were measured by using Nanodrop (Nano Spectrostar, BMG, LABTECH). A verso cDNA synthesis kit and its random primer (Thermo Scientific, Hudson, NH, USA, cat# AB-1453/B) were usedto prepare cDNA in accordance with the manufacturer's instructions. Any DNA contamination was eliminated using buffer included in the cDNA synthesis kit.

**2.3. qPCR reaction**

Primer3 software (version0.2.0) was used for designing primers of each studied gene [37]. Table1 presents the NCBI accession number of each gene. qPCR was performed on PCR detection system, of Bio-Rad iCycler, using 80-fold diluted cDNA samples. qPCR reaction was carried out using a SYBR green master mix (Thermo Scientific, Hudson, NH, USA).The cDNA templates (0.006µg/µl)were added tomaster mix and the forward/reverse primers, 0.1nM/µl [38]. The initial denaturation step was carried out at 95°C for 7.00 min, that followed by number of 40 amplification cycles.The amplification cycle includedstep of denaturation at 95°C for 0.1 min, annealing step at 57−64°C, according to the melting temperature of each gene, and the final extension step at 60°C for 5 min. The mice GAPDH gene is a housekeeping genethat used for normalization [39]. The relative gene expressions were calculated using the $2^{-\Delta\Delta C}$ method[40].

**2.4. Skeletal examination**

For studying malformations of the skeletal system, Fresh foetuses were skinned and fixed in alcohol (95%) for 5 days, followed by acetone for two days. Then, the foetuses were stained for three days in 20 ml double staining freshly prepared solution at 4° C. The staining solution consists of:(1 ml Alcian blue (0.3%) in ethanol (70%), 1 ml Alizarin Red-S (0.1%) in ethanol (95%), 1 ml glacial acetic acid, 17 ml ethanol (70%).After the staining process was completed, the foetuses were washed with tap water, and then the specimens were immersed in ascending series of aqueous KOH solution (1%) and glycerol to complete digestion of soft tissues and then preserved in glycerin (100%) for investigation and photography [41,42]

**2.5. Statistical analysis:**

qPCR results were presented as (mean ± standard error). Data were analyzed using SPSS software.

T-test was performed and results at $p < 0.05$ indicated statistically significant difference.

## 3. RESULTS AND DISCUSSION

The present study focus on the effect of Pepsi on expression of some specific genes and malformations of the skeletal system produced in 20th day rat fetuses of pregnancy.

**3.1. Relative gene expression RSPO2, HAO1, and RUNX genes**

The abundance of gene encoding for RSPO2, HAO1, and RUNX2 was measured using qPCR. The expression of RSPO2 gene was decreased significantly by -1.62-fold in G1. In contrary, RSPO2 exhibited a significant reduction in G2 by -2.88-fold in relation to control group ($P \leq 0.05$). The expression of RSPO2 was significantly decreased in G4 by -0.99fold compared to the control group.

G3 showed a significant increase in RSPO2 gene by 1.7-fold relative to the control group

($p \geq 0.05$, Figure2). The gradual down-regulation of RSPO2 in the 1st trimester resulted in the reduction in the bone development. It was reported the critical role of wnt signaling pathway in the bone activity and its great importance in osteoclastogenesis and bone remodeling [43]. RSPO2 is from secreted factors that cooperate in Wnts pathway to enhance the β-catenin stability[44] and during the osteoclastogenesis, the B-catenin is showed to be expressed. Previous studies reported the necessity of RSPO gene in the development of hind limb and fibular development [45]. The four R-spondins share the samemechanism of action [46]. It was declared that RSPO1 can protect the mice fromcartilage damage and bone fraction[47]. Recently, studies on human proved the therapeutic use of RSPO1 in specific bone or cartilage diseases [43].

HAO1 gene showed a significant increase by 2.88-fold in the G1 over the control group, the expression of HAO1 gene is significantly increased G2, G3, and G4 relative to the control group. It has been reported that

the HAO1 have reported its highest expression value in G2 by 2.89-fold compared to the control group ($p \geq 0.05$, Figure2). The oxidative stress is known as the the changes between antioxident level and prooxidant [48]. The gradual significant increase of HAO1 resulted in the increasing of oxidative stress. Previous studies demonstrated that the soft drinks stimulate the oxidative stress which causes changes in certain genes [49]. Production of reactive oxygen species contribute in mineral tissue homeostasis that lead to enhancement of bone resorption [50]. Studies have been done to confirm that oxidative stress participate in bone pathogenesis including bone complications and inflammation in joints [51].

The expression of RUNX2 gene showed a significant decrease in G1 and G2 by -0.41-fold and -2.32-fold, respectively, relative to the control group. While RUNX2 gene showed a significant increase in abundance in both starved G3 and G4 vs. the control group by 3.23 and 0.99- folds, respectively (Figure 2). Consistent with our investigation, RUNX2 gene was suggested to have great effect on craniofacial variability [52]. RUNX2 is considered as the master transcription factor of bone formation and cell transition that is the essential steps of osteogenesis [53,54]. All RUNX family (RUNX1, RUNX2, and RUNX3) share the same motif that appear to play a critical role in craniofacial development [55].

### 3.2. External Morphology:

The examination of external morphology of foetuses at 20$^{th}$ day of pregnancy showed that pepsi administration caused retardation of the growth of foetuses assessed by the minimization of foetal body length reaching to 5.1 ± 0.11 at control , 4.5 ± 0.1 at G1, 3.3± 0.12 at G2, 4.8± 0.08 at G3 and 3.9± 0.12cm at G4 (Figure 3). The mean of foetal weight of maternally administered with Pepsi was also significantly reduced. It was 5.6 ± 0.08 at control, 4.8± 0.1 at G1, 3.6± 0.12 at G2, 5.1± 0.09 at G3and 4.2 ± 0.07 gm at G4 (Figure 4). G2 showed the highest minimization of foetal body length and weight compared to the other administrated groups (Figures 3 &4). Large amount consumption of soft drinks minimised foetal growth and caused spontaneous abortion [56, 57]. Maternal administrated with caffeine caused low foetal birth length and weight in rat [58,59]. Pepsi - administration minimized the number of alive foetuses and increased the rate of dead fetuses. The percent of change of the total foetuses of the administrated groups was 84.5% at G1, 60.3% at G2, 93.1% at G3 and 75.9% at G4 in comparison with control. The percentage of change of a live foetus was 15.5% at G1, 39.6 % at G2, 6.9% at G3, and 24.1 % at G4 in compare with live foetuses of the control. This result indicated that the more effective foetuses are in G2 that foetuses maternally administered orally with high dose of Pepsi at (1$^{st}$ -7$^{th}$) day of pregnancy.Maternal administration of caffeine in rat reduced the number of implantation sites, miscarriage and number of live births [59, 60].

#### 3.2.1. Skeletal examination

Osteological malformation of 20$^{th}$ day foetuses indicated that administration of Pepsi in all groups to the pregnant rat affected on the skeletal system development of foetuses at the 20$^{th}$ day of gestations compared with the control. This effect included the reduction in decrement and lengths of cartilages and bones (Figure 5; G1-G4). Pregnant female administrated with Caffeine showed osteological malformation in rat fetuses [34,61]. Ossification of both dermal and cartilaginous skull bones was reduced. It was recorded incomplete ossification of the skull bones of foetuses maternally administered with Pepsi. In addition, a clear shortage in volume and length of the skull were recorded (Figure 6; G1-G4). There is a slight ossification of dentary and moderate ossification of lower jaw bones in all administrated groups (Figure 6; G1-G4). Previous studies on pregnant rats administrated with Caffeine administration was reported delay in the skull ossification of feutuses [62,63]. Examination of the vertebral column showed that the axis and atlas vertebrae not well ossified in all Pepsi administrated groups. The centra of the first six cervical vertebrae have lost their ossification and the vertebral elements in the caudal region are less ossified in G3 and G4. The cervical and lumbar vertebrae were moderate ossified in G1 and G2. Moreover, caudal and sacral vertebrae were completely non-ossified (Figure 7; G1-G4). Caffeine administration to pregnant rats showed cellular disorganization of chondrocytes of vertebral bodies, delayed ossification in between deteriorated chondrocytes, and decreased developed secondary centers of ossification in feotuses [63].

The sternebrae of the control group were taller than all Pepsi administrated groups. The sternebrae of G1 and G2 are the most affected ones (Figure 8; G1-G4). The ribs were shorter than the control one. The chondrification of ribs were reduced as they exhibited less blue coloration in their cartilaginous portion than the normal feotuses. No alterations in the number of the ribs in all administrated groups (Figure 8; G1-G4). Rib alterations were noted in pregnant mice in early developmental stages of foetuses maternally administrated with caffeine [64]. The components of the fore limb and pectoral girdle showed that reduction in the degree of ossification and size. Fore limbs and Pectoral girdle of all examined foetuses of G1 and G2 showed serious shortage of ossification as well as the highest reduction in their lengths (Figures 9&10; G1-G4). There was a positive association between soft drink consumption and fracture risk in wrist and forearm in children [65]. The hind limb and pelvic girdle of the foetuses showed that the ossification degree and the length of fibula, tibia, femur, pubis, ischium and ilium were affected. Moreover, a series of phalanges was affected. The chondrification degree of the pubic symphysis, metatarsals and tarsals was also affected especially at G1 and G2 (Figures 11&12; G1-G4). Malformed phalanges of the developing limbs were seen in mice foetuses maternally administered with caffeine [66]. Young rats fed food rich in fat and soft drinks suffered from injuries in the growth plates of tibia and the parameters of the bone structure degenerate. Analysis of RNA sequence of the growth plates showed deterioration of proliferation, differentiation and mineralization processes [4]. Many studies among children and adolescents also found a direct association between soft drinks consumption regularly and fracture [27, 65]. A Study on among postmenopausal women showed that soft drinks consumption every day was associated with a fourteen percent increased risk of fracture of hip [67, 68].

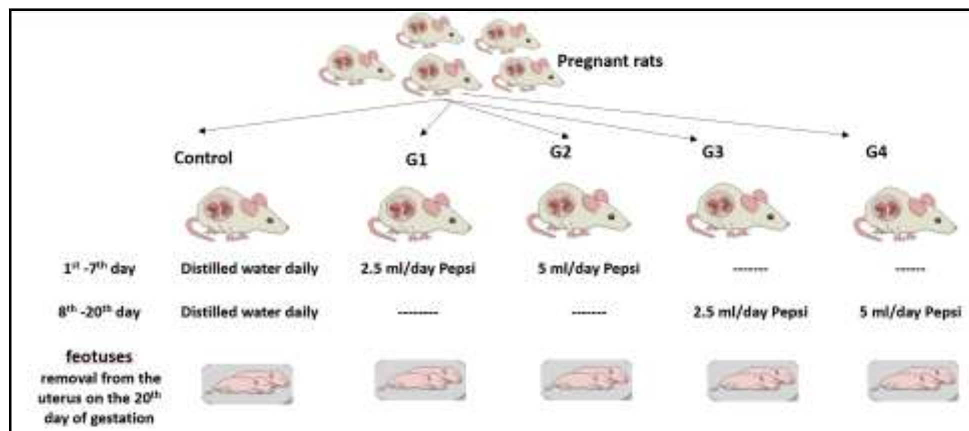
Many mechanisms may explain the association between consumption of Pepsi and skeletal malformations. One mechanism may be due to low intake of calcium, but high intake of phosphorus may stimulate hormone of parathyroid and cause resorption of bone [69]. The high intake of phosphoric acid changes the imbalance of phosphorus and calcium ratio and the acid-base ratio in the body, resulting in reduce the renal activation of 25-hydroxyvitamin D and affects calcium homeostasis and reduced density of bone, osteoporosis and fractures [31,32,70]. Certain ingredients in Pepsi can also affect bones as sodium and sugar that can increase the loss of calcium [33]. Caffeine in soft drinks crosses the placental barrier easily, where it can directly affect the foetus in several different aspects; spontaneous abortion and skeletal malformations [35,71]. The half-life of caffeine is increased more in pregnancy, as it cannot be metabolized by the foetus or the placenta [72]. Caffeine consumption regularly also increase the risk of fracture [61, 73].

## 4. CONCLUSION

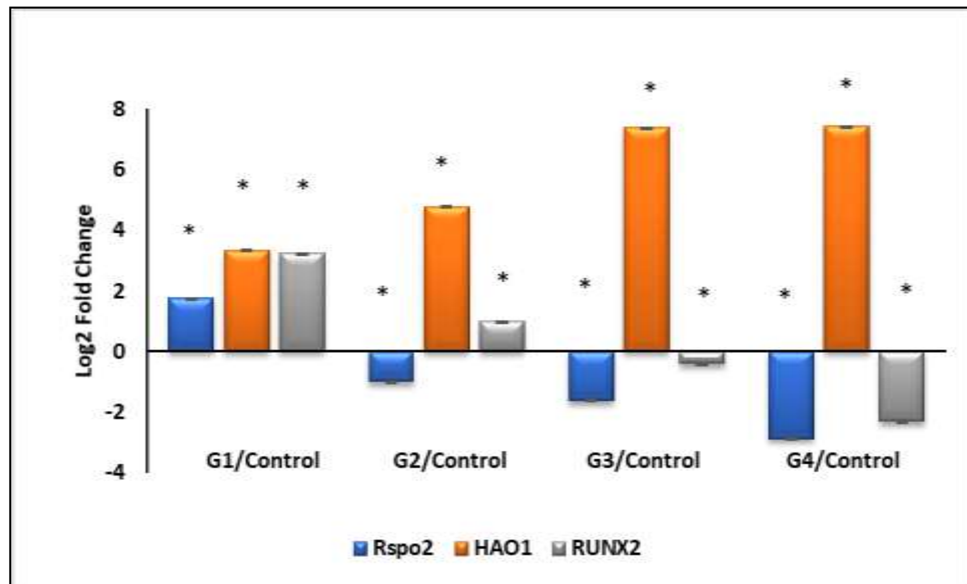
The results reported herein concluded some genes and pathways that involved in bone resorption or delay ossification process. RSPO2, HAO1, and RUNX2 gene expressions are changed and their effect on the skeletal system in response to maternal exposure to Pepsi consumption was reported. Pregnant rats administrated with low and high doses of Pepsi in 1st to 7$^{th}$ days, showed significant down-regulation of RSPO2 and RUNX1 genes of their feotuses relative to the control group. while the HAO1 gene of feotuses exhibited a significant increase in the same groups relative to the control group. Administration of high dose of Pepsi to the pregnant rats in days 8$^{th}$ to 20$^{th}$ showed significant decrease in RSPO2 and RUNX1 genes compared to the control group. Consistent with the gene expression results, osteological malformation of 20$^{th}$ day foetuses indicated that administration of Pepsi in all groups to the pregnant rat affected the foetuses skeletal system development at the 20$^{th}$ day of pregnancy assessed with the control one of the similar age. The impact of Pepsi consumption included the decrement in lengths of cartilages and bones of skull, vertebrae, sternum, limbs and girdles. Therefore, our findings supported the RSPO2, HAO1, and RUNX2 genes to be used as biomarkers for ossification. In some years, the dissolved oxygen is below the permissible limit.

**Table1**: Designed primers and their accession numbers

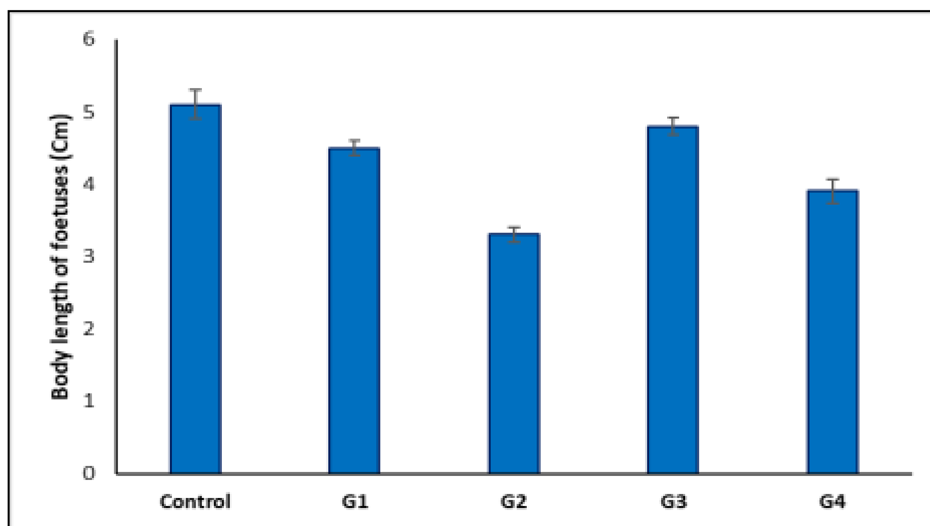
| | **Primer** | **Accession number** |
|---|---|---|
| **RSPO2_L** | ATGGGGAACGTGTAGCAGAA | XP_038935678.1 |
| **RSPO2_R** | AAGACGCTGTGCTGTTCTTG | |
| **HAO1_L** | CTCAGACGGTTGACCTCACT | NP_001101250.1 |
| **HRO1_R** | TTCCACAGCCTCAACGATCT | |
| **RUNX2_L** | TTCCCAGGCATTTCATCCCT | NP_001265412.1 |
| **RUNX2_R** | GGGAACTGATAGGACGCTGA | |
| **GAPDH_L** | AACGACCCCTTCATTGACCT | NP_058704.1 |
| **GAPDH_R** | CCCCATTTGATGTTAGCGGG | |



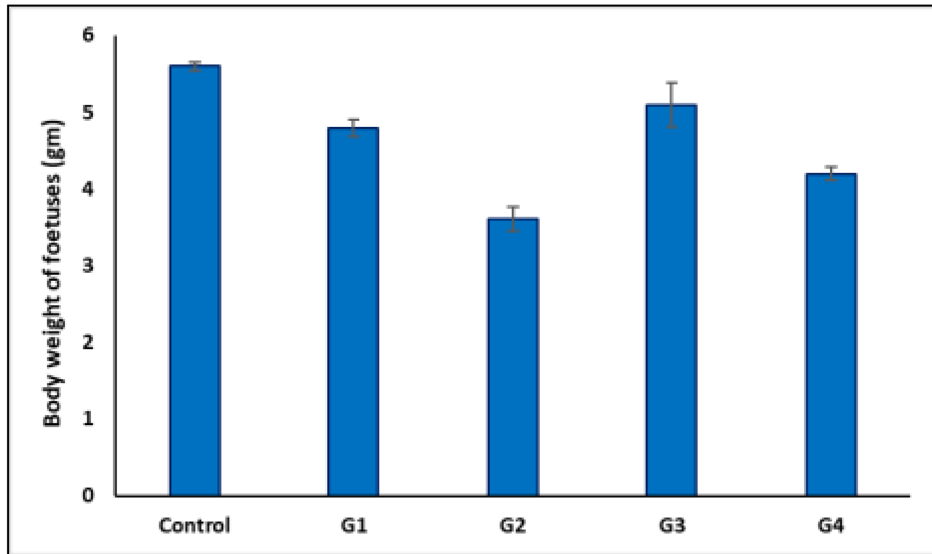

**Figure(1):** Schematic diagram showing the experimental design of the study



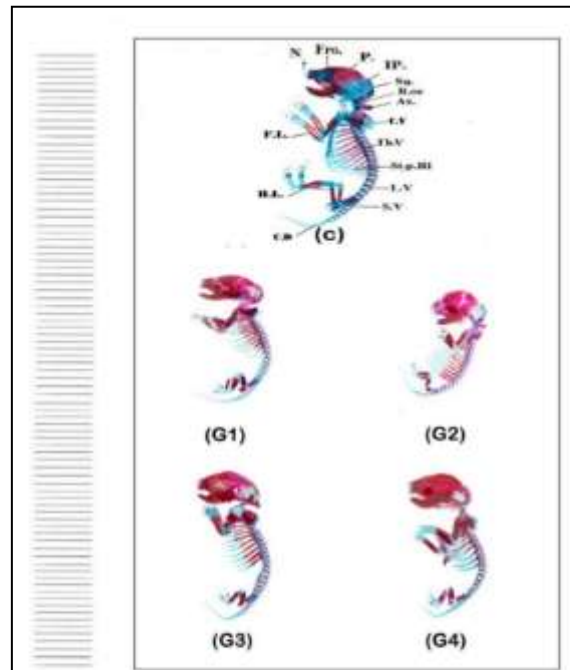

**Figure(2):** RSPO2, HAO2, and RUNX2 differential gene expressions of feotuses in the four Pepsi treated groups (G1, G2, G3, and G4) vs control groups. The data normalization was done using the GAPDH gene. Fold change represented the expressions of genes between groups ± standard error. * : indicates data statistical significance at $p \leq 0.05$ and n=5.



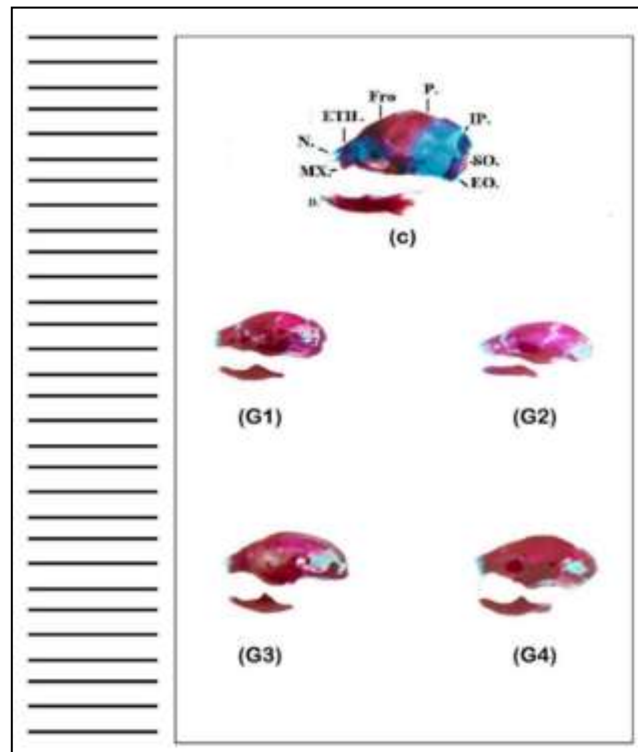

**Figure(3):** Histogram showing the mean change of body length (Cm) of foetuses of maternally administrated groups with Pepsi compare with control on day 20$^{th}$ of pregnancy.



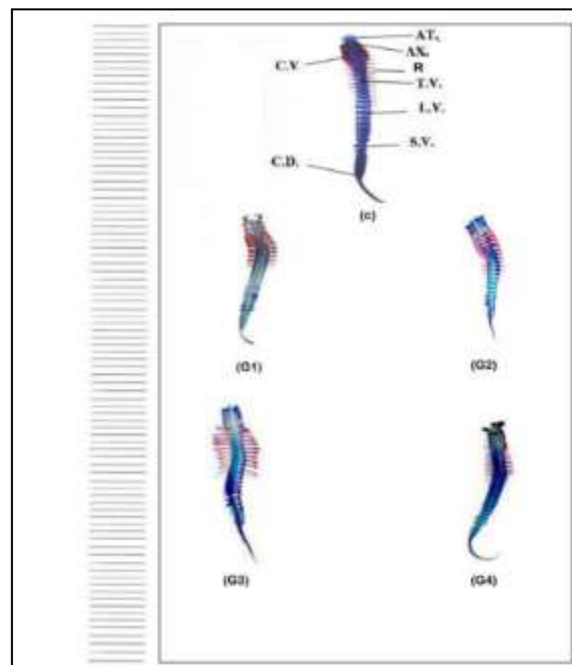

**Figure(4):** Histogram showing the mean change of body weight (gm) of foetuses of maternally administrated groups with Pepsi compare with control on day 20th of pregnancy.



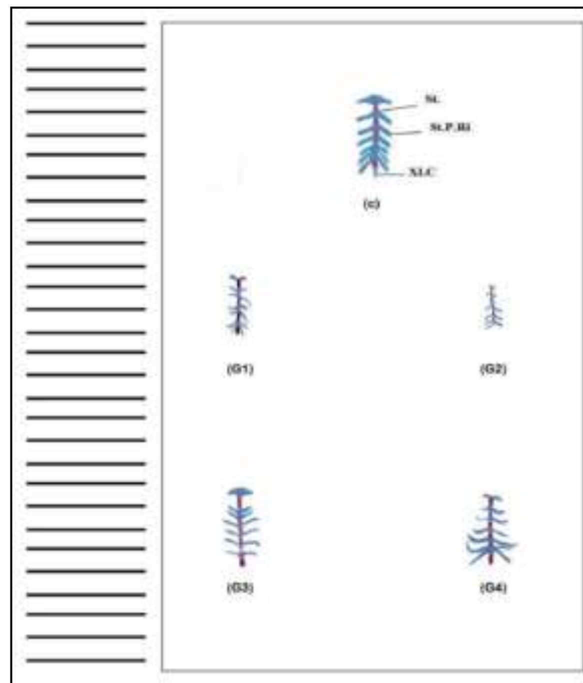

**Figure(5)**: A lateral view of the skeletal system of 20th day of pregnancy of foetuses of rat showing: Control (C) and G1, G2, G3, & G4 are the 4 Pepsi-treated groups.Ax.: Axis; C. D.:Caudal vertebrae; C. V.: Cervical vertebrae; F. L.: Fore limb; Fro.:Frontal; H. L.: Hind limb; IP.:Interparietal; L.V.:Lumbar vertebrae; N.:Nasal; P.:Parietal; R.:Ribs; S.V.:Sacral vertebrae; Sq.:Squamosal; St. p. Ri.:Sternal portion of ribs; T.V.:Thoracic vertebrae.



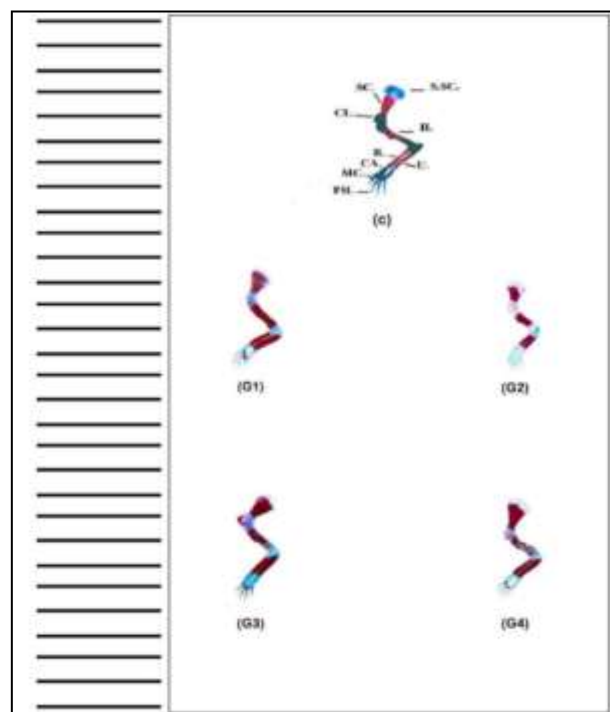

**Figure(6):** A lateral view of the skull of foetuses of rat at 20$^{th}$ day of pregnancy of foetuses of rat showing: Control (C) and G1, G2, G3, & G4 are the 4 Pepsi-treated groups. D.: Dentery; ETH.: Ethmoid; EO.: Exooccipital; Fro.: Frontal; P.: Parietal; MX.: Maxilla ; IP.: Interparietal; N.: Nasal; SO.: Supraoccipital.



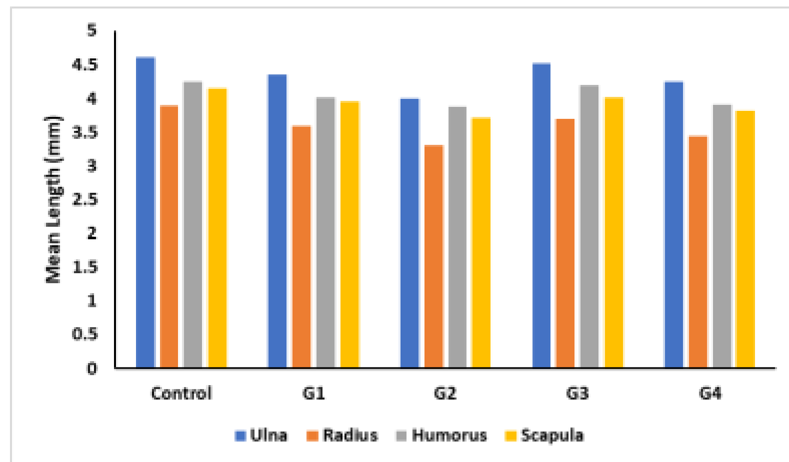

**Figure(7):** A ventral view of the vertebral column of foetuses of rat at 20$^{th}$ day of pregnancy of foetuses of rat showing: Control (C) and G1, G2, G3, & G4 are the 4 Pepsi-treated groups. AT.: Atlas; AX. : Axis; C.D.: Caudal vertebrate; C.V.: Cervical vertebrae; L.V.: Lumbar vertebrae; R.:Ribs; S.V.: Sacral vertebrae; T.V.: Thoracic vertebrae.



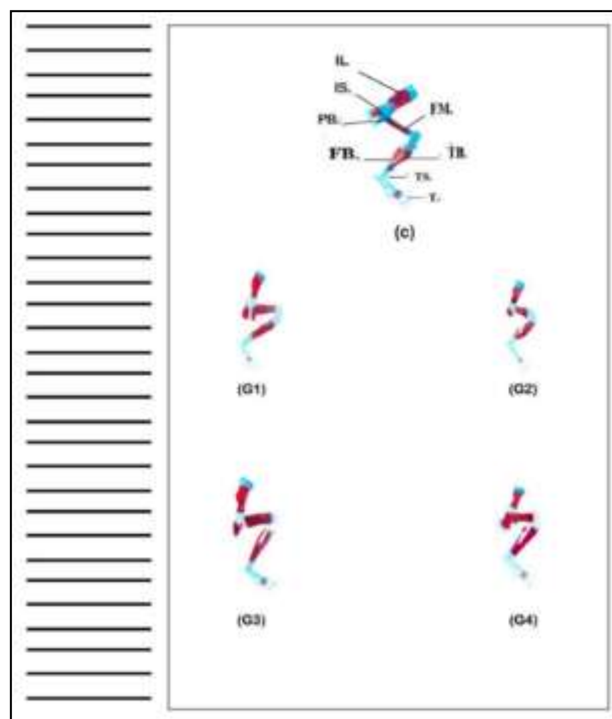
**Figure(8):** A ventral view of the sternum and sternebrae of foetuses of rat of 20th day of pregnancy of foetuses of rat showing: Control (C) and G1, G2, G3, & G4 are the 4 Pepsi-treated groups. ST.P.Ri.: Sternal portion of ribs; ST.: Sternebrae; XI.C.: Xiphoid cartilage.



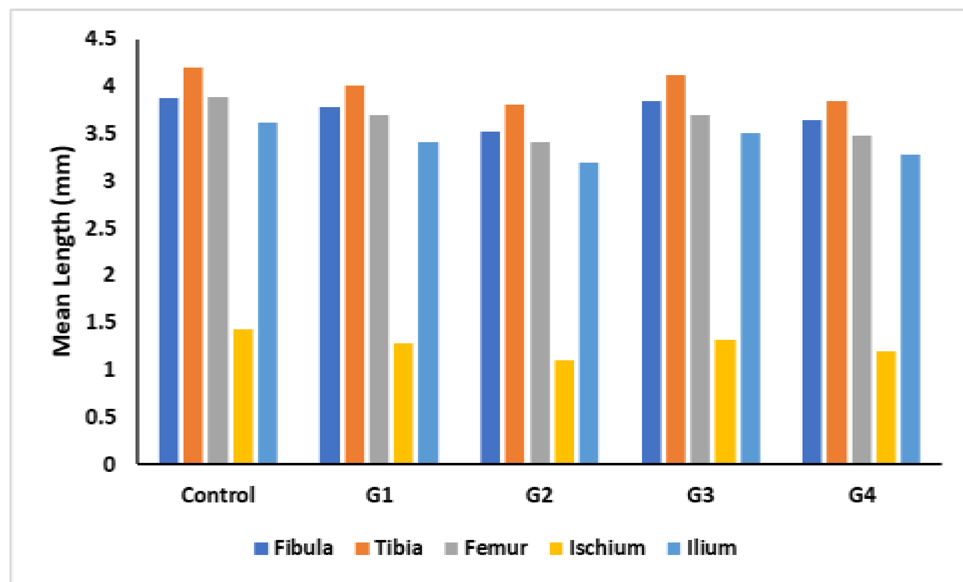
**Figure(9):** A lateral view of the fore limb and pectoral girdle of foetuses of rat of 20th day of pregnancy of foetuses of rat showing: Control (C) and G1, G2, G3, & G4 are the 4 Pepsi-treated groups. CA.: Carpales; CL.: Clavicle; H.: Humerus; MC.: Metacarpalia; PH.: Phalanges; S.SC.: Supra-scapula; R.: Radius; SC.: Scapula; U.: Ulna.




**Figure(10):** Mean ossified length (mm) of ulna, radius, humerus and scapula of foetuses of maternally administrated groups with Pepsi compare with control on day 20$^{th}$ of pregnancy.




**Figure(11):** A lateral view of the hind limb and pelvic girdle of foetuses of rat of 20$^{th}$ day of pregnancy of foetuses of rat showing: Control (C) and G1, G2, G3, & G4 are the 4 Pepsi-treated groups.FB.:Fibula; FM.: Femur; T.:Toes; TS.: Tarsalia; TB.: Tibia;




**Figure(12):** Mean ossified length (mm) of fibula, tibia, femur, ischium and ilium of maternally administrated groups with Pepsi compare with control on day 20th of pregnancy.

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