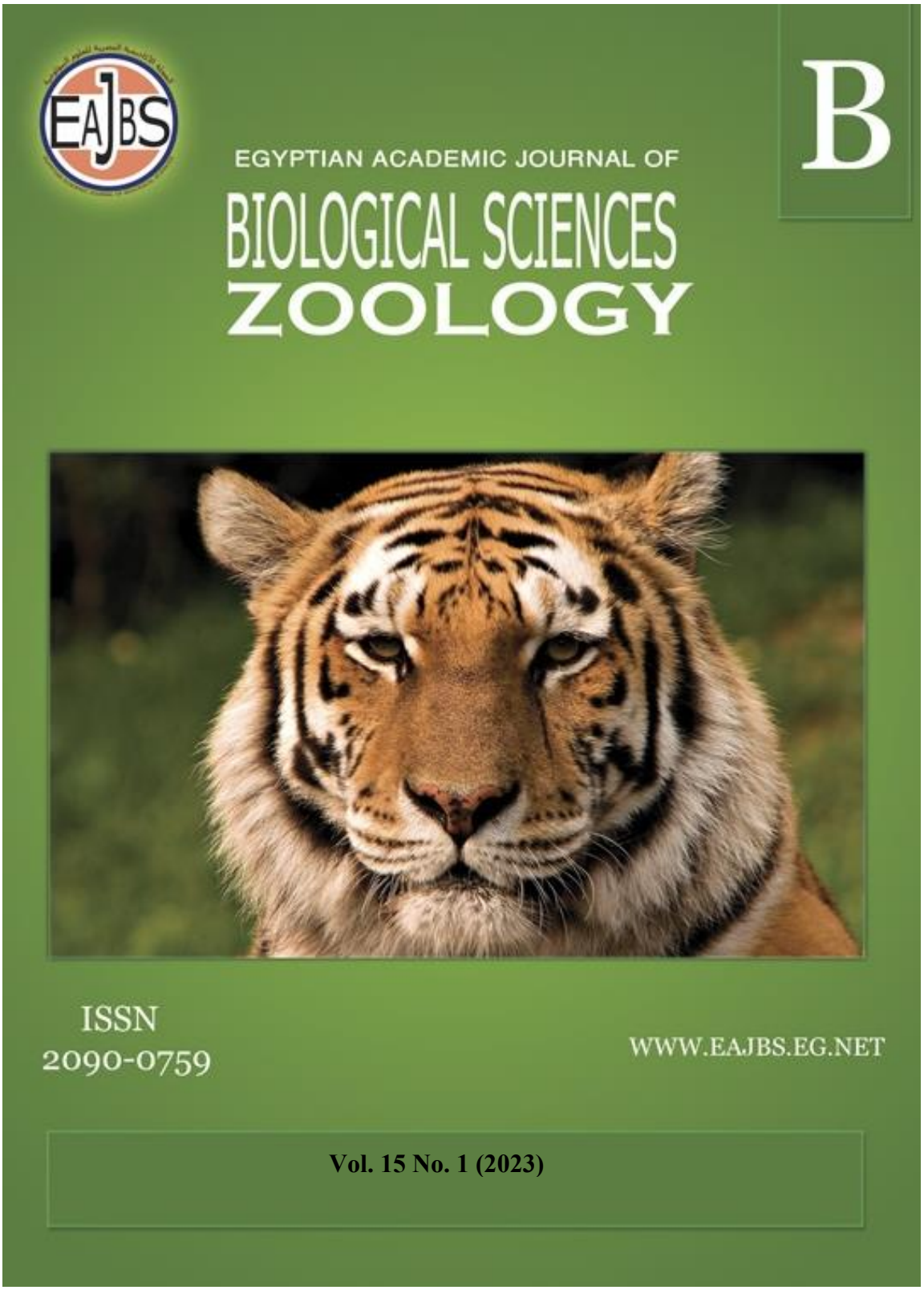

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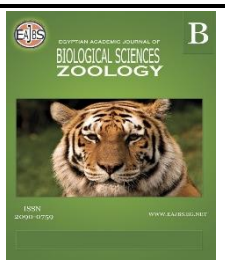

Reproductive Toxicity of Sodium Nitrite and Its Modulation by Ascorbic Acid as An Antioxidant in Pregnant Female Mice

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ABSTRACT

Sodium nitrite ($NaNO_2$) is a water-soluble compound, well-known as a principal food preservative and colorant in the food industry. Besides the variety of industrial and medicinal applications, toxicity to humans and animals is well documented after nitrite overexposure. The present work was carried out to investigate the maternal and developmental toxicity of sodium nitrite and its modulation by ascorbic acid as an antioxidant in pregnant female mice. Forty-eight pregnant female mice were divided into equal sex groups (8 per group). Group I was used as control. Group II received 100 mg/kg of ascorbic acid. Groups III& IV received 0.016 and 0.032 mg sodium nitrite/g body weight. Group V and VI received 0.016 and 0.032 mg sodium nitrite /g body with 100 mg ascorbic acid/kg body weight. Pregnant female mice were orally administered doses at days from 8 to 17 of gestation and sacrificed on day 18 of gestation. Sodium nitrite treatment during late pregnancy induced maternal toxicity as indicated by a reduction in the maternal body weight and incidence of both partial and complete resorption of implants and miscarriage of fetuses. Examination of life fetuses from $NaNO_2$-treated dams showed fetal growth retardation and a significant increase in the percentage of malformed fetuses per dam and the % of dams with malformed fetuses. These malformations were clearly recorded in both gross morphology and skeleton of the fetuses (sternebrae). The assessment of skeletal ossification of life fetuses showed marked retardation in the major parts of the skeleton. The results could be concluded that ascorbic acid administration may ameliorate the maternal toxic effects of $NaNO_2$.

INTRODUCTION

Nitrite most commonly applied as curing agent in the meat industry to preserve the red-pinkish colour of the meat, aroma and flavor; impart a better taste and prevent the risk of contamination by *Clostridium botulinum* bacteria of the cured meat. Unluckily, recent research has demonstrated some negative effects of this technique (Ferysiuk & Wójciak, 2020). Acute exposure to high nitrite levels has been associated with death, primarily owing to methemoglobinemia (Chui *et al.*, 2005). Chronic exposure to low amounts of nitrite causes birth abnormalities, respiratory tract diseases,

nervous system damage, and paralysis, among other things. Long-term nitrite exposure can also be carcinogenic and mutagenic (Ansari *et al.*, 2017). One of the main mechanisms through which nitrite exert its toxicity is oxidative damage. Vitamin C, that also known as ascorbic acid, is a powerful antioxidant found in biological systems (Duarte *et al*., 2005). It was found to be an antioxidant in the reproductive environment (Nazirolu, 2003). Data about its influence on the reproductive system are controversial. So, our study aimed to assess the maternal and developmental toxic effects of two (16 & 32mg/kg/d) $NaNO_2$ doses on pregnant female mice and its fetuses and to evaluate the possible reversibility these effects by using ascorbic acid.

MATERIALS AND METHODS

Experimental Animals and Determination of Doses:

Determination of Acute Oral Toxicity (LD_{50}) of Sodium Nitrite in Female Mice:

To estimate the LD_{50} of sodium nitrite, forty virgin female mice were used. The graphical method of Miller and Tainter, (1944) was used. Virgin females weighing 25-30 grams were randomly divided into five groups. Each one consists of 8 animals. An oral dose was administered to each animal based on its body weight. Mortality rates were recorded throughout 24 hours. The percent of mortality was plotted against the logarithm of the applied dose after the application of the correction formula as follows :

Zero% dead animals = 100 × 0.25/n

100% dead animals = 100 × [(n- 0.25) /n]

Where, (n) is the number of animals used in each group. The dose corresponding to 50% mortality was found to be an acute oral median lethal dose, which was expressed as mg/kg body weight (b.w) .

Forty-eight pregnant female *Mus musculus* mice weighting 28±2 grams were purchased from VACSERA at Helwan, Egypt. Animals were housed and acclimatized to the animal house in accordance with normal laboratory conditions with access to food & water ad libitum, with 12 h dark and light cycles. This study has been approved by Fayoum University Institutional Animal Care and use Committee (FU-IACUC) Code No. of proposal: AEC 2225, Jan.16, 2023.

The pregnant female animals were divided into six groups of 8 mice per group. Female mice of the different studied groups were exposed to two doses of $NaNO_2$ and/or ascorbic acid from day 1 till day 7 of gestation, and sacrificed on18^{th} day under the same conditions. Animal in control (G1) administered distilled water. The ascorbic acid group (G2) was administered 100 mg/kg/day of ascorbic acid. Sodium nitrite low dose group (G3) administered 0.016 mg $NaNo_2$ /g body weight as 1/8 of LD_0. Sodium nitrite high dose group (G4) administered 0.032 mg $NaNo_2$/g body weight as 1/4 of LD_0. Group 5: administered a low dose of 0.016 mg $NaNo_2$ /g body weight in combination with 100 mg/kg/day of ascorbic acid. Group 6: pregnant female administered 0.032 mg $NaNo_2$ /g body weight as a high dose in combination with 100 mg/kg/day of ascorbic acid.

Maternal Evaluation:

Animals were weighed on the morning of gestational day one (GD1) and o evaluation each morning until the 18^{th} day of gestation (GD_{18}, sacrificing day). For clinical toxicity signs, dams were daily observed during treatment. The pregnant females were killed by cervical dislocation on the 18^{th} day of gestation; the maternal body weight at sacrifice, maternal carcass weight and gravid uterine weight were recorded. A further evaluation for maternal toxicity is the maternal weight change throughout gestation corrected for gravid uterine weight. The uterus was opened along the antimesometrial side and the uterine contents such as implantation and resorption sites, dead and live

fetuses were recorded as the mean number and percentage. The percentage of postimplantation loss was also estimated according to **Manson & Kang, (1994)**. Also, live fetuses were dissected away from the uterus for the further examination.

Fetal Evaluation:

Assessment of Fetal Growth, External and Visceral Malformations:

Live fetuses, weighed and their length crown rump length (CRL) were recorded. Also, they examined under the dissecting microscope for the presence of genital papillae and sexed by measuring anogenital distance. External examinations of live fetuses for general body curvature, presence of anasarca (edema) and any hemorrhagic spots as well as external malformation were recorded in different body regions. The head was examined in different views, to record the size, shape, and presence of anomalies in the jaws, ears, eyes, lips and snouts. The limbs were examined also for size, shape and position, also, the digits for the number and depth of digital furrow. The tail was also checked for presence, size and shape. Fetuses were dissected and examined for visceral malformation. Fetuses with external and visceral malformation were recorded, counted and photographed.

Assessment of Skeletal Ossification and Malformation:

Skeletal Staining:

Fetuses scheduled for skeletal analyses were skinned and eviscerated, post fixed in 10% buffered formalin for two days, then washed in tap water and distilled water for 2 hours each (Wassersug, 1976). Blot of excess water and place in cartilage staining solution, which is made of (9 mg alcian blue 8 GX, 60 ml absolute ethanol and 40 ml glacial acetic acid). Staining for 48 hours then transfer to absolute ethanol. Blot specimens and macerate in 2% potassium hydroxide and stained with alizarin red S (0.25% in 2% KOH). Transfer specimens to pure glycerol via a graded series of glycerol-KOH and stored them in fresh pure glycerol. The cartilaginous elements appear blue and the bones appear red.

Skeletal Examination:

The stained skull was examined from lateral, ventral and dorsal views. The degree of ossification of each individual bone of the skull was recorded. Skulls with malformed bones and distended fontanella were also recorded and photographed.

Stained vertebral columns were examined for the number and ossification degree of cervical, thoracic; lumber, sacral and caudal as well as vertebral arches and centra. Any abnormalities in the center were also recorded and photographed. Ribs and bones of sterna are counted and examined for number, size, shape and degree of ossification. Both pectoral and pelvic girdles together with fore and hind limbs were examined for normal development and degree of ossification of long bones and the number of ossified phalanges. During the examination of the skeleton, the extent of ossification of individual bone was noted as ossified, incompletely ossified, and cartilaginous or unossified. Only the data of the ossified condition were tabulated. Any deviations from the normal development of bones and cartilage were recorded and photographed.

Statistical Analysis:

ANOVA, the analysis of variance and Duncan's Multiple Range tests were performed for the statistical analysis to determine differences among different treatment means at $p < 0.05$ (significant level) and at $p < 0.01$ (highly significant). Standard errors were also estimated, Dytham (1999). All statistics were run on the computer using the SPSS program. The curves were fitted with office (2007) computer program.

RESULTS

Graphical calculation of LD_{50} according to Miller and Tainter (1944):

The mortality percent was graphically applied in relation to the log dose (Fig1). From this figure, the LD_{50} and the lowest maternal toxic dose LD_{10} or LD_0 of sodium nitrite are deduced from the doses corresponding to 50%, 10%, or 0% of mortalities respectively (Table 1).

Median lethal dose:

LD_{50} = log 2.414 = 259.25 mg/kg

The lowest maternal toxic dose:

LD_{10} = log 2.161 =144.877 mg/kg

LD_0 = log 2.107 = 128 mg/k

Table 1: Graphical calculation of LD_{50} of sodium nitrite according to Miller and Tainter (1944).

Group	Dose mg/kg	Log dose	Dead	Survived	Dead %	Correction%
1	128	2.107	0	8	0	3.125
2	182	2.26	2	6	25	25
3	259.25	2.414	4	4	50	50
4	366.583	2.564	6	2	75	75
5	518.5	2.747	8	0	100	96.9

- 0.0 % of dead animals = 100 × 0.25/n - 100% of dead animals = 100 (n – 0.25/n)

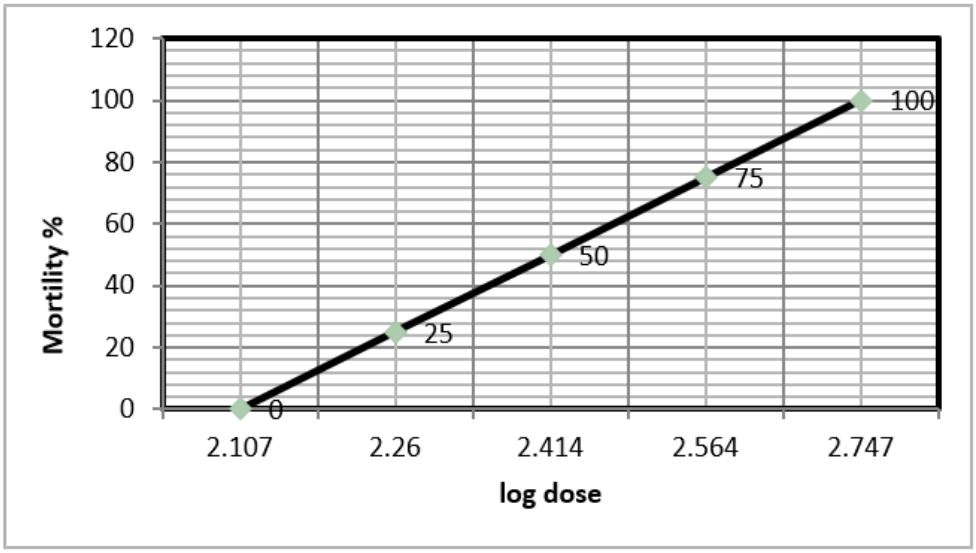

Fig. 1: Graphical curve of LD50 of Sodium nitrite.

From figure (1):

- 50% mortality (LD_{50}) corresponding to log dose 2.414
- 10% mortality (LD_{10}) corresponding to log dose 2.161
- 0.0% mortality (LD_0) corresponding to log dose 2.107

So that:

Median lethal dose:

LD_{50} = log 2.414 = 259.25 mg/kg

The Lowest Maternal Toxic Dose:

LD_{10} = log 2.161 =144.877 mg/kg

LD_0 = log 2.107 = 128 mg/kg

Maternal Toxicity:

The data of maternal toxicity was recorded as clinical symptoms of toxicity and maternal body weight gain from GD1 to GD7 and till GD18 (gestation day) at the end of the experiment.

Clinical Symptoms:

In this study, all the treated dams survived to the termination of the experiment. The treated dams showed some clinical symptoms such as loss of appetite, ataxia, dizziness, diarrhea, dyspnea, nausea, abdominal cramp and general weakness. The symptoms were more pronounced on the last days of the experiment, especially with the high dose (32 mg/kg/d).

Maternal Body Weight and Weight Change:

A significant reduction in the maternal body weight at the 18^{th} day of gestation was observed at 32 mg/kg (high dose) and 16 mg/kg (low dose) as compared with the control group. The mean value was 48.12±1.10 grams for control dams, 48.15±1.13 for the ascorbic acid group, 37.48±1.95 grams for dams treated with the high dose, 43.48±1.13grams for dams treated with 16mg/kg and 37.48±1.95 grams for dams treated with the 32mg/kg $NaNO_2$. On the other hand, the mean value was 47.49±1.16 grams for dams treated with a low dose and co-administered with ascorbic acid and 45.66±1.14 grams for dams treated with a high dose and co-administered with 100mg/kg of ascorbic acid.

At different doses of sodium nitrite treatment, the changes in the maternal body weight during 1st to 7th days of gestation (treatment period) showed a significant reduction. This reduction becomes evident at the high dose of 32 mg/kg (mean value 1.62±0.19, and the mean percent of change 6.37±0.83), compared with the control group (mean value 2.75±0.13and the mean percent of change 10.71±0.06). Also, a dose-dependent significant reduction was observed in sodium nitrite-treated groups for maternal weight change during the gestational period from day zero till the 18th day of gestation (Table 2).

Moreover, a significant drop in the weight of the gravid uterus was recorded in low and high doses. This clear drop in weight was strongly influenced by females' entirely resorbed fetuses and by those who showed reduced fetal body weight. However, dams treated with ascorbic acid (100mg/kg) concurrent with both sodium nitrite doses showed normal or less similar gravid uterus weights values comparing to the control group (Table2).

Table 2: Maternal toxicity of sodium nitrite individually or co-administered with ascorbic acid as antioxidant in SWR mice following administration of oral doses on gestational days 1 through 7-

	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	$NaNo_2$ low dose +ascorbic acid	$NaNo_2$ high dos +ascorbic acid
Maternal pregnancy status:						
Number (%) of dead dams [(a)]	0.0	0.0	0.0	0.0	0.0	0.0
Number of pregnant dams at sacrifice [(b)]	8	8	8	8	8	8
Number (%) of dams with totally non-live implants [(c)]	0.0	0.0	2 (25) *	3 (37.5) *	0.0	0.0
Number (%) of dams with live implants[(d)]	8 (100)	8 (100)	6 (75) *	5 (62.5) *	8(100)	8(100)
Maternal body weight:						
Gestational day GD_0	25.37±0.95	25.40±0.93	25.336±0.96	25.176±0.95	25.12±0.94	25.52±0.98
First treatment day GD_1	25.63±0.94	25.66±0.93	25.61±0.92	25.43±0.91	25.43±0.95	25.85±0.93
Mid-treatment day GD_4	26.91±0.97	26.94±0.95	26.73±0.97	26.18±0.96	26.72±0.98	26.95±0.94
Last treatment day GD_7	28.38±0.98	28.41±0.96	27.95±1.14	27.04±0.89*	28.22±0.97	28.43±0.98
Sacrifice day GD_{18}	48.12±1.10	48.15±1.13	43.48±1.13**	37.48±1.95**	47.49±1.16	45.66±1.14**
Corrected (absolute) body weight at sacrifice. [(e)]	32.62±1.1	32.55±1.3	31.59±1.22	26.76±2.12*	32.52±1.15	31.24±1.13
Maternal body weight change:						
Throughout the treatment period GD_{1-7}	2.75±0.13	2.75±0.15	2.33±0.19**	1.62±0.19**	2.79±0.14	2.57±0.16
% Change throughout treatment period GD_{1-7}	10.71±0.06	10.71±0.09	9.12±0.78**	6.37±0.83**	10.96±0.05*	9.98±0.12*
Throughout the post-treatment period GD_{7-18}	19.74±0.43	19.74±0.46	15.05±0.82**	10.45±0.67**	18.78±0.47*	16.57±0.38**
% Change throughout post-treatment period GD_{7-18}	69.57±0.02	69.48±0.07	53.84±0.16**	38.63±0.60**	66.54±0.07*	58.28±0.09**
Throughout the gestation period GD_{0-18}	22.75±0.48	22.75±0.46	17.66±0.50**	12.32±0.55**	21.88±0.44*	19.48±0.47**
% Change throughout gestation period GD_{0-18}	89.69±0.02	89.56±0.06	69.71±1.6**	48.95±1.5**	87.10±0.08*	78.95±0.06*
Corrected (absolute) body weight change [(f)]	7.25±0.18	7.16±0.11	6.25±0.24**	1.58±0.28**	7.4±0.16	5.72±0.1**
Gravid uterine weight	15.50±0.43	15.59±0.41	11.41±0.50**	10.74±0.53**	14.48±0.49	13.76±0.50*

- (a): Number of pregnant females' dead/total number of pregnant females. - (b): Includes all dams with implants.
- (c): Dams with all implantation sites present as resorption or dead fetuses at a sacrifice
- (d): Dams with one or more live fetuses at sacrifice. - (e): Body weight at a sacrifice – gravid uterine weight.
- (f): Body weight change through gestation period GD_{0-18} – gravid uterine weight.

*P<0.05 Significant. **P<0.01 Highly Significant.

$$\%\ Body\ weight\ change = \frac{\text{body weight}_2 - \text{body weight}_1}{\text{body weight}_1} x\ 100$$

Fetal Study (assessment of developmental toxicity of sodium nitrite in Mice) For All Dams:

Maternal indicators were the numbers of corpora lutea, implantation sites and percentage of preimplantation loss per dam (Table 3). These parameters were comparable between control and sodium nitrite treated groups indicating that the treated dams were different in their reproductive status from the control one.

Assessment of other gestational parameters indicated that sodium nitrite exerts strong embryotoxic and/or fetotoxic as well as embryolethal or fetolethal effects (Table3). A dose-dependent reduction was seen in the number of live fetuses per dam with a concurrent increase in the number and percent of resorbed fetuses per dam. The percentage of resorbed fetuses per dam was increased significantly from 0% (mean number per dam 0) for control to 17.54% (mean number per dam 1.57) for low dose, and to 25.25% (mean number per dam 2.21) for high dose. On the other hand, mice treated with ascorbic acid (100 mg/kg) concurrent with a low and high dose of sodium nitrite, showed a percentage of resorbed fetuses with values (of 2.47% and 2.72%, respectively) Furthermore, the number and percentage of pregnant females with one or more resorption sites, showed marked variations among treated groups depending on the used doses. These values were 50% (4 out of 8) in low dose and 62.5% (5 out of 8) in high dose, compared with 0% for the control one. Moreover, mice treated with ascorbic acid (100 mg/kg) concurrent with a low and high dose of sodium nitrite showed 12.5% (1 out of 8) (Table 3 & Fig2).

The number of late fetal death per dam was increased significantly at low-dose and high-dose only from mean value 0 represented (0%) for control to 0.42±0.15

representing (4.69%) for low-dose and 0.66±0.18represent (7.54%) for high-dose. Moreover, mice treated with ascorbic acid (100 mg/kg) concurrent with a low and high dose of sodium nitrite showed 0 represented (0%). The incidence of resorption or late fetal death was expressed as the mean number and % of non-live implants (dead and resorbed) per dam. The number and percentage of non-live implants showed a significant increase in low and high doses. The mean value was 1.99±0.82 represents (22.23%) for low dose and 2.87±0.69 (32.8%) for high dose, compared with the control value of 0 (0%).On the other hand, mice treated with 100mg/kg of ascorbic acid concurrent with a low and high dose of sodium nitrite showed 0.25±0.23represents (2.47%) for low dose and 0.27±0.21 represents (2.72%). These findings were recorded in 4 dams (50%) and 5 dams (62.5%) for low and high doses respectively, compared with 0 dams (0%) in the control group, mice treated with ascorbic acid (100 mg/kg) concurrent with a low (16) and high (32) doses of sodium nitrite were recorded in 1 dam (12.5). Similar findings were obtained when non-live implants were expressed as a percent of post-implantation loss (Table 3 & Fig2).

Significant increases in dams with complete resorption of all implants at 2 dams (25%) in dams treated with a low dose and 3 dams (37.5%) in dams treated with a high dose. Meanwhile, the aborted dams appear at a low dose in 2 dams (50%) and it appears at a high dose in 3 dams (37.5%) (Table 3).

Table 3: Developmental toxicity in SWR mice after oral administration of sodium nitrite individually or co-administered with ascorbic acid as an antioxidant during gestational days 1through7.

	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	$NaNo_2$ LD + ascorbic acid	$NaNo_2$ HD + ascorbic acid
All dams [(a)]:	**8**	**8**	**8**	**8**	**8**	**8**
Number of corpora lutea / dam	10.6±0.27	10.7±0.29	9.8±1.3	10.1±0.25	10.5±0.22	10.3±0.28
Number of implantation sites/dam	10.4±0.22	10.5±0.23	8.95±0.4	8.75±0.25	10.1±0.21	9.9±0.26
% Implantation sites/dam	98.11	98.13	91.13	86.63	96.19	96.11
% Preimplantation loss / dam (b)	1.88±0.49	1.86±0.45	8.67±0.25*	13.36±0.47**	3.80±0.51	3.88±0.44
Number (%) of dams with preimplantation loss	1 (12.5)	1(12.5)	6 (75)	8(100)	1(12.5)	1(12.5)
Number of resorption sites/dam (c)	0	0	1.57±0.7*	2.21±0.72*	0.25±0.65*	0.27±0.70*
% Resorption site/dam	0	0	17.54**	25.25**	2.47*	2.72*
Number (%) of dams with one or more resorption sites	0	0	4 (50) **	5 (62.5) **	1 (12.5)	1 (12.5)
Number of late fetal death/dam	0	0	0.42±0.15*	0.66±0.18*	0	0
% of late fetal death/dam	0	0	4.69*	7.54*	0	0
Number (%) of dams with one or more late fetal death	0	0	2 (25) *	3 (37.5) *	0	0
Number of non-live implants/dam (d)	0	0	1.99±0.82*	2.87±0.69*	0.25±0.23*	0.27±0.21*
% Non-live implants/dam	0	0	22.23**	32.8**	2.47*	2.72*
Number (%) of dams with non-live implants	0	0	4 (50) **	5 (62.5) **	1 (12.5) *	1 (12.5) *
% Postimplantation loss/dam (e)	0	0	22.23**	32.8**	2.47*	2.72*
Number (%) of dams with postimplantation loss (f)	0	0	4 (50) **	5 (62.5) **	1 (12.5) *	1 (12.5) *
Number (%) of dams with complete resorption of all implants	0	0	2 (25) *	3 (37.5) *	0	0
Number (%) of dams with live implants (g)	8 (100)	8 (100)	6 (75) *	5 (62.5) *	8 (100)	8 (100)
Number (%) of aborted dams	**0**	**0**	**2 (25) ***	**3 (37.5) ***	**0**	**0**

(a): Includes all dams pregnant at sacrifice. Litter size = implantation site / dam

(b): % Pre implantation loss = $\frac{\text{Number of corpora lutea – Number of implantation sites}}{\text{Number of corpora lutea}}$ **x 100**

(c) : Early, mid and late resorption. % resorption = $\frac{\text{Sites resorption of Number}}{\text{Number of implantation sites}}$ **x 100**

(d): Dead fetuses plus resorbed fetuses

(e): % Post implantation loss = $\frac{\text{Number of implantation sites – Number of live fetuses}}{\text{Number of implantation sites}}$ **x100**

(f): Dams with post implantation loss at one or more sites.

(g): Dams with at least one live fetus.

***P<0.05 Significant. **P<0.01 Highly Significant**

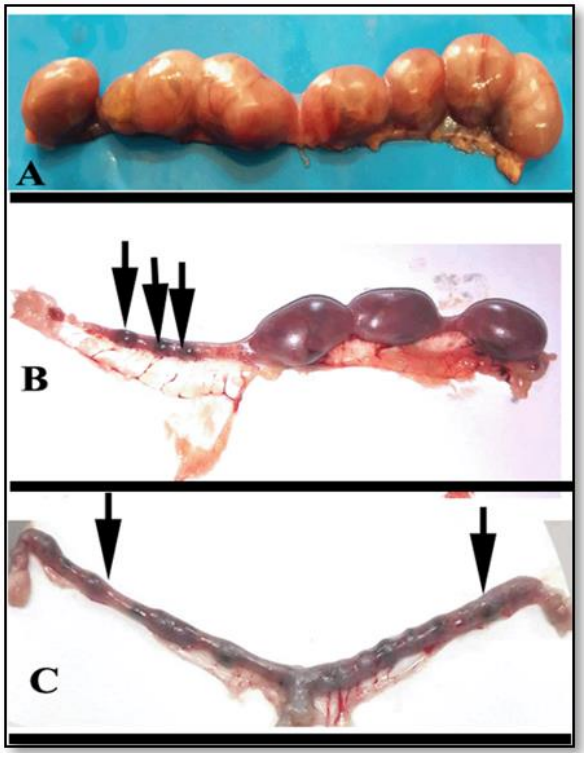

Fig. 2: Showed (A) control uterus, (B) site of resorption in sodium nitrite treated group and (C) complete resorption of all implantation sites in Sodium nitrite treated group (high dose. Note the resorption site (arrow).

For Dams with Live Fetuses:

A dose-dependent reduction in the number and percentage of dams with live fetuses examined on day 18th of gestation were induced after the daily oral administration of $NaNO_2$ to pregnant female mice from the 1st day of gestation to the 7th day. These findings were 6 out of 8 (75%), and 5 out of 8 (62.5%) for low and high doses respectively, in comparison with the control group 8 out of 8 (100%). While treatment of ascorbic acid concurrently with both low and high doses of nitrite showed marked improvement 8 out of 8 (100%), and 8 out of 8 (100%) respectively.

The number of live fetuses per dam was highly significantly reduced in low and high doses of sodium nitrite. The mean number of live fetuses per dam was 6.76±0.69for low dose, and 5.47±0.64 for high dose, compared with 10.33 in the control group. The number of dams with live fetuses was strongly reduced and influenced by females with complete resorption of all implants, others with one or more resorption sites and aborted dams. However, dams treated with ascorbic acid (100mg/kg) concurrent with low and high doses of sodium nitrite, revealed betterment of 8.58±0.45and 8.32±0.39 respectively (Table 4).

The fetal weight and length were also recorded as sensitive indicators for intrauterine fetal growth. The mean values of fetal weight following maternal treatment with a high dose of sodium nitrite reflected significant embryotoxic effects. The mean fetal weight was 0.97±0.04for for high doses, and 1.18±0.10for low doses compared with 1.24±0.04 in the control group. However, mice administered ascorbic acid (100 mg/kg/day) in combination with both low (16mg/kg) and high (32mg/kg) doses of sodium nitrite showed increase significant in the fetal weight 1.22±0.05 and 1.21±0.06, respectively. Also, a reduction in the relative weight of fetuses (fetal weight/maternal weight) was recorded for low and high doses treated groups (Table 4).

The mean fetal length (Crown Rum Length CRL) was significantly reduced in

low and high doses (Table 4). These values were 2.12±0.10 for low dose and 1.94±0.12 for high dose, compared with 2.26±0.02for control fetuses. On the other hand, mice treated with ascorbic acid (100 mg/kg) concurrent with a low and high dose of sodium nitrite, showed a significant increase in the fetal length of 2.26±0.03 and 2.25±0.05, respectively. Also, the ratio of fetal weight to fetal length showed no treatment-related effect, except that for the highest dose level (Table 4.).

The placental weight shows a highly significant reduction in high-dose treated dams, the mean placental weight was 0.093±0.005 for low dose, and 0.064±0.008 for high dose compared with 0.096±0.004 in the control group. Moreover, female mice administered low and high doses of sodium nitrite in combination with 100mg/kg ascorbic acid revealed significant improvement in placental weight 0.094±0.004 and 0.094±0.002, respectively (Table 4).

Table 4: The status of the live fetuses from control and pregnant female mice after exposure to NaNO2 individually or in combination with ascorbic acid on gestational days 1 to day 7.

	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	$NaNo_2$ low dose + Ascorbic acid	$NaNo_2$ high dose + Ascorbic acid
Dams with live fetuses [a]:						
Number (%) of dams with live fetuses	8 (100)	8(100)	6(75)	5(62.5)	8(100)	8(100)
Number of live fetuses /dam	10.33±0.25	10.51±0.21	6.76±0.69**	5.47±0.64**	8.58±0.45*	8.32±0.39*
Absolute fetal weight (g)	1.24±0.04	1.24±0.05	1.18±0.10	0.97±0.04**	1.22±0.05	1.21±0.06
Relative fetal weight [b]	0.038±0.001	0.039±0.001	0.037±0.004	0.036±0.001	0.041±0.003	0.041±0.002
Fetal length (CRL) [c]	2.26±0.02	2.25±0.03	2.12±0.10*	1.94±0.12**	2.26±0.03	2.25±0.05
Mean fetal weight / length (cm / g)	0.548±0.02	0.551±0.02	0.556±0.05	0.500±0.04*	0.539±0.03	0.537±0.05
Placental weight (g)	0.096±0.004	0.096±0.005	0.093±0.005	0.064±0.008**	0.094±0.004	0.094±0.002
All malformations:						
Number (%) malformed fetuses [d]	11(13.75)	10(12.5)	16(38.0)*	21(60.0)*	10(13.3)	11(14.6)
Number (%) dams with malformed fetuses [e]	2(25)	2(25)	3(50) *	4(80)**	2(25)	2(25)
Percent malformed fetuses / dam	13.75	12.5	38.0*	60.0**	13.3	14.6

Data expressed as Mean ± SD.
(a): Includes only dams with live fetuses. Litter size is the number of live fetuses per dam.
(b): Fetal weight /maternal weight.
(c): Crown-Rump length (cm).
(d): Total number of fetuses with malformations (external & skeletal)
(e): Dams with at least one malformed fetus .
*P <0.05 Significant.
**P <0.01 Highly Significant.

All Malformations:

The percentage of dams with one or more malformed fetuses and the percentage of malformed fetuses, as well as the percentage of malformed fetuses per dam, were significantly increased in a dose-dependent manner, maybe, as a result of a significant increase in both external and skeletal malformations. The malformed fetuses were obtained from a significantly large number of dams in sodium nitrite-treated groups. In control group, 25% of the examined dams showed at least one malformed fetus, in comparison with 50% at a low dose, and 80% at a high dose. In the ascorbic acid group, 25% of the examined dams showed at least one malformed fetus and in mice treated with low and high doses of $NaNO_2$ in combination with 100mg/kg ascorbic acid, 25% of the examined dams showed at least one malformed fetus. Also, a significant increase in the percentage of malformed fetuses was observed. In this respect, only 13.75% of the examined fetuses in the control group were malformed, compared with 38% in low doses and 60% in high doses. In the ascorbic acid group, 12.5% of the examined fetuses were malformed. 13.3% and 14.6% of the examined fetuses were malformed respectively in

female mice treated with 16mg/kg (low) and 32mg/kg (high) doses of sodium nitrite in combination with the studied ascorbic acid dose. The percent of malformed fetuses per dam was also increased significantly in a dose-dependent manner as shown in Figure 3.

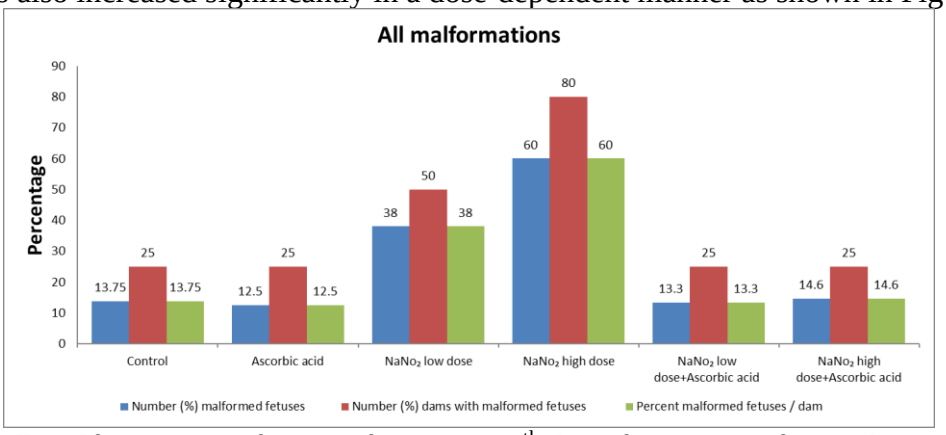

Fig. 3: All malformations of mouse fetuses at 18th day of gestation after oral treatment of sodium nitrite doses individually or co-administered with 100mg/kg ascorbic acid to pregnant dams from gestational days 1 through 7.

External Malformations:

Here, limited numbers of fetuses were available for examination in the most studied groups probably due to prenatal loss. The incidences of external malformations were significantly increased in fetuses from sodium nitrite treated groups than the control one. On the other side, sodium nitrite treatment induced significant increase in the occurrence of malformed fetuses. Approximately, 15.9% of fetuses from dams treated with a low dose and 50% of fetuses from dams administered 32mg/kg $NaNO_2$ (high dose) were grossly malformed, compared with 3.75% in the control group. A dose-dependent increase in the number and percent of dams with externally malformed fetuses were recorded. The percent of dams with one or more malformed fetuses was 37.5% with the low dose and 100% with the high dose, compared with 12.5% in concomitant control (Table 5).

Table 5: Summary of external malformations recorded in mouse fetuses at the day eighteen of gestation after maternal administration of oral doses (16 & 32mg/kg/d) of $NaNO_2$ individually or co-administered with 100mg/kg ascorbic acid on gestational days 1 through 7.

Groups / Parameters	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	$NaNo_2$ low dose + ascorbic acid	$NaNo_2$ high dose +ascorbic acid
Number of dams examined	**8**	**8**	**6**	**5**	**8**	**8**
Number (%) dams with malformed fetuses [(a)]	**1 (12.5)**	**0.0**	**3(50)****	**3(60)****	**1(12.5)**	**1(12.5)**
Number of fetuses examined	**80**	**80**	**42**	**35**	**75**	**71**
Number (%) malformed fetuses [(b)]	**3(3.75)**	**0.0**	**10(23.8)****	**9(25.71)****	**3(4)**	**3 (4.2)**
Number (%) malformed fetuses/dam	**0.375 (3.75)**	**0.0**	**1.66(15.9)****	**1.80(34.88)****	**0.375(3.75)**	**0.375(3.75)**

Data represented as the number and that between parentheses represent incidence percent.

(a): Dams with one or more malformed fetuses. (b): Fetuses with at least one malformation.

*P <0.05 Significant. **P <0.01 Highly Significant

$$\text{Incidence percent} = \frac{number\ of\ malformed\ fetuses}{total\ number\ examined} X100$$

The most frequent anomalies observed were edema, hematoma (Table 6). Most of these malformations occurred in high doses of sodium nitrite and some of them were observed in the low-dose treated group.

The incidence of gross malformations observed in the fetuses of treated groups was found to be dose-dependent. The present incidences and types of malformations observed by individual fetuses indicated an increase in the incidence of external malformations. The incidence of these malformations was higher in the high-dose group than in other groups. In the control animals, only 3anomalized fetuses (3.75%) were recorded in one dam (12.5%). this dam has a fetus with edema and two with hematoma in different regions of the body (Table 6).

Hematoma was found in different locations of the body. The incidence percent of hematoma was 21.42% and 22.85% in fetuses from dams treated with low and high doses respectively, compared with 2.5% for the control group. But, the percentage was 1.33% and 2.66% in mice treated with both doses (16mg/kg and 32mg/kg) of sodium nitrite in combination with ascorbic acid respectively, (Table 6& Fig. 4.).

Table 6: Incidence (%) of external malformations in mouse fetuses at 18th day of gestation after maternal administration of oral doses of sodium nitrite individually or co-administered with ascorbic acid as antioxidant on gestational days 1 through 7.

Groups / Malformations	Control	Ascorbic acid	NaNo2 low dose	NaNo2 high dose	NaNo2 low dose + ascorbic acid	NaNo2 high dose + ascorbic acid
Edema	1 (1.25)	0.0	1 (2.3)	1(2.85)	0.0	0.0
Hematoma	1 (1.25)	0.0	9 (21.42)**	8 (22.85)**	1 (1.33)	2 (2.8)
Micrognathia	0.0	0.0	0.0	0.0	0.0	0.0
Kyphosis	0.0	0.0	0.0	0.0	0.0	0.0
Microdactly	0.0	0.0	0.0	0.0	0.0	0.0
Club foot	0.0	0.0	0.0	0.0	0.0	0.0
Club hand	0.0	0.0	0.0	0.0	0.0	0.0
Kinky tail	0.0	0.0	0.0	0.0	0.0	0.0

Data represented as the number and that between parentheses represent incidence percent.

(a): Dams with one or more malformed fetuses. (b): Fetuses with at least one malformation.

*P<0.05 Significant. **P<0.01 Highly Significant

$$\text{Incidence percent} = \frac{number\ of\ malformed\ fetuses}{total\ number\ examined} X100$$

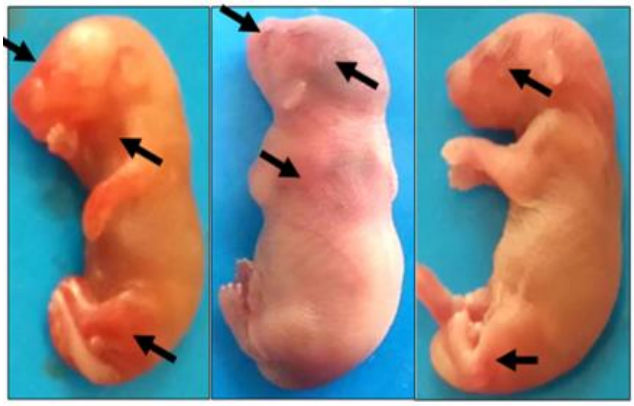

Fig. 4: Gross morphology of mouse fetuses at 18th day of gestation showing external Malformations which represented as hematoma (arrows)

Skeletal Malformations:

The type and incidence of skeletal malformations recorded by the individual fetus of dams that orally intoxicated with sodium nitrite are illustrated. The number and percentage of malformed fetuses and the percentage of dams with one or more malformed fetuses were increased significantly in the nitrite treated groups. About 23.80% of the examined fetuses at a low dose and 34.28% at a high dose were malformed, compared with 10% in the control one and 7.5% in the ascorbic acid group. 9.3% and 10.6% of the examined fetuses were malformed respectively in mice treated with 16 and 32mg/kg of sodium nitrite doses in combination with ascorbic acid. Also, fetuses with skeletal malformations occurred in a significantly large number of dams (50% at low dose and 80% at high dose) compared with 12.5% in the control group and 12.5% in the ascorbic acid group. The number and percentage of malformed fetuses per dam were significantly increased in sodium nitrite treated groups. These data were 1.6 (23.80%) at low and 2.4(34.28%) at high doses, compared with 1.0 (10%) in the control group and 0.75(7.5%) in the ascorbic acid group. On the other side, in female mice treated with ascorbic acid concurrent with both low (16mg/kg) and high (32mg/kg) doses of sodium nitrite, the number and percentage of malformed fetuses per dam were found in the normal level of the control 0.87(9.3%) and 1(10.6%) respectively (Fig5.).

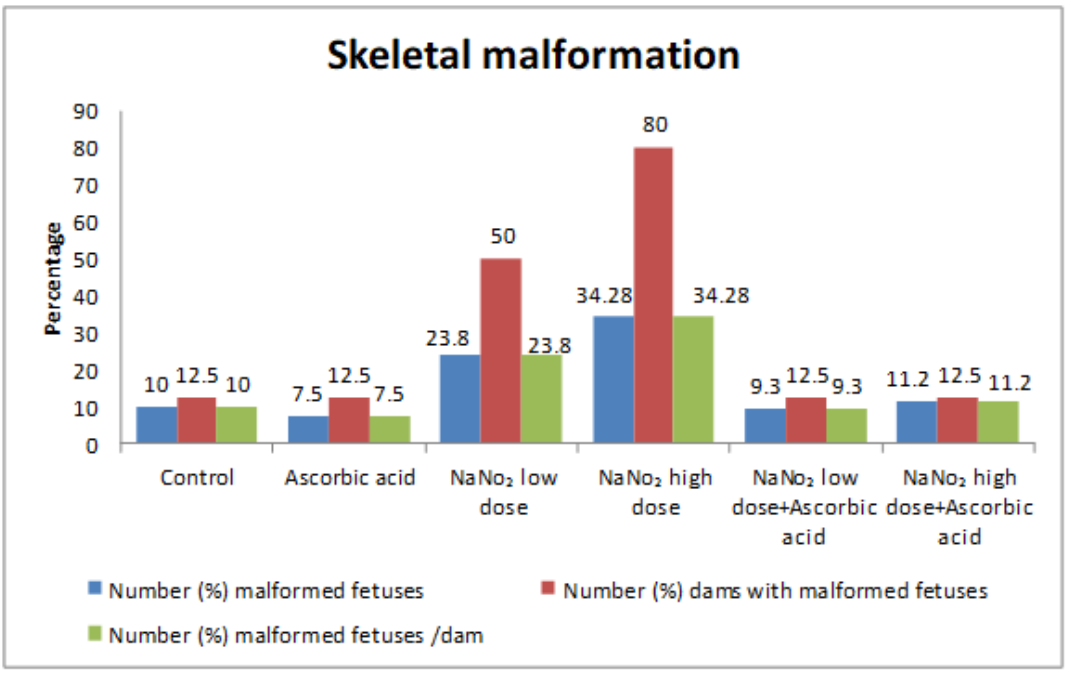

Fig. 5: Skeletal malformations of mouse fetuses at the day eighteen of gestation after oral sodium nitrite administration (16 & 32mg/kg/d) individually or co-administered with 100mg/kg/d of ascorbic acid to pregnant dams from gestational days 1 through 7.

Skeletal malformations were recorded in the vertebrae and sternum of fetuses obtained from dams treated with sodium nitrite-treated groups, compared with the control group. A significant increase in the incidence of fetuses with malformed sternebrae was recorded. The percentage of fetuses with malformed sternebrae was 35.8% with the low dose and 54.2% with the high dose, in comparison with 10% in the control group and 8.7% in the ascorbic acid group. The percentages were 10.6% and 10.6% respectively for mice treated with ascorbic acid concurrent with a low and high dose of sodium nitrite. The fetuses with malformed sternebrae occurred in a significantly

large number of dams 80.5% for low and 100% (all dams) for high doses, compared with 12.5% in the control one and 12.5% in the ascorbic acid group. Moreover, the percentages were 12.5% and 12.5% respectively for mice treated with ascorbic acid concurrent with a low and high dose of sodium nitrite. Concerning the type of malformations observed in the sternebrae, a significant increase in the occurrence of the maligned sternebrae was recorded. This type of malformation occurred at an incidence percent 14.2% in low dose and 20% in high dose, compared with 5% in the control group and 3.75% in the ascorbic acid group. The percent was 5.3% and 4% respectively for mice treated with ascorbic acid concurrent with both low and high dose of sodium nitrite. Dumb-bell sternebrae were also observed at incidences of 7.1% at a low dose and 14.2% at a high dose, compared with 5% in the control one and 3.75% in the ascorbic acid group. The percent was 2.6% and 4% respectively for mice treated with 100mg ascorbic acid concurrent with both the low and high doses of sodium nitrite (Table 7 & Fig6.).

Asymmetric sternebrae were also observed at incidences of 11.9% at a low dose and 14.2% at a high dose, compared with 1.25% in the control group and 1.25% in the ascorbic acid group. The percent was 2.6% and 2.6% respectively for mice treated with ascorbic acid concurrent with a low and high dose of sodium nitrite. While some sternbrae are absent at low and high doses in percent (2.3%) and (5.7%) respectively (Table 7 & Fig. 6.).

Table 7: Incidence of skeletal malformations in mouse fetuses at day eighteen of gestation after maternal treatment orally by 16 and 32mg/kg/d of $NaNO_2$ doses individually or co-administered with 100mg/kg/d of ascorbic acid on gestational days 1 through 7.

Groups / Parameters	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	$NaNo_2$ low dose + ascorbic acid	$NaNo_2$ high dose + ascorbic acid
Number of fetuses examined (1), (2)	80	80	42	35	75	71
Number of dams examined (3)	8	8	6	5	8	8
Sternum						
% Fetuses with malformed sternebrae	10	8.7	35.8**	54.2**	10.6	11.2
% Dams with fetuses with malformed sternebrae	12.5	12.5	80.5**	100**	12.5	12.5
Dumb-bell sternebrae	3 (3.75)	3 (3.75)	3(7.1)*	5(14.2)*	2(2.6)	3(4.2)
Malalignedsternebrae	4 (5)	3 (3.75)	6(14.2)*	7(20)**	4(5.3)	3(4.2)
Asymmetric sternebrae	1 (1.25)	1 (1.25)	5(11.9)**	5(14.2)**	2(2.6)	2(2.8)
Hemi sternebrae	0.0	0.0	0.0	0.0	0.0	0.0
Forked sternebrae	0.0	0.0	0.0	0.0	0.0	0.0
Absent sternbrae	0.0	0.0	1(2.3) *	2(5.7)*	0.0	0.0

Data expressed as number of fetuses with alterations, ( ) represent incidence percent.
(1): A single fetus may be represented more than one; an alteration may appear at one or more site in a single fetus
(2): Only live fetuses were examined for skeletal alterations.
(3): Dams with live fetuses only include.
*P < 0.05 Significant. ** P < 0.01 highly significant.

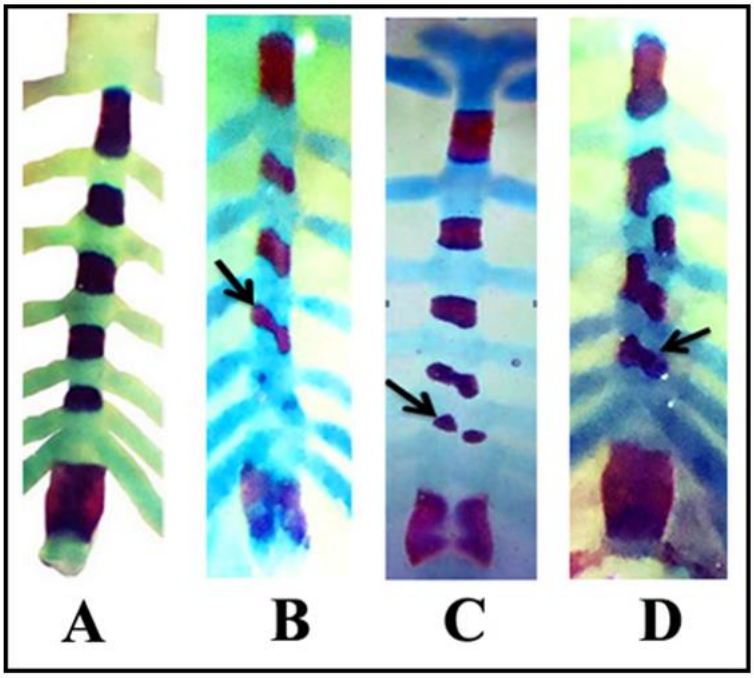

Fig. 6: Sternum alterations of the of mouse fetuses on the 18th day of gestation after maternal $NaNO_2$ treatment.
(A): Fetus from control dam showing normal well six ossified sternebrae.
(B-D): Sternum of fetuses from sodium nitrite treated dams showing:
(B): Malaligned sternebrae (ML).
(C): Dumb-bell (DB).
(D): Asymmetric (AS).

Ossification of the Skeleton:

Total Ossification of the Skeleton

The occurrences of several skeletal variants in the examined foetuses of the control and sodium nitrite-treated groups on the 18th day of gestation were significantly increased. Among these variations, a marked reduction in the degree and extent of ossification of the skeleton was recorded. Many fetuses failed to display a similar pattern of ossification as that shown in the control one. In general, about 57.1% of the examined fetuses at a low dose and 85.7% at a high dose showed reduced ossification in one or more bones, compared with 30.0% in the control group. Also, the present findings showed that fetuses with delayed skeletal ossification obtained from a significantly large number of dams in sodium nitrite-treated groups. This delay was represented by 66.6% in the low nitrite dose and 80% in the high dose, comparing with 25% in the control group. Both the number and percentages of fetuses with delayed ossification per dam increased significantly from 3 (30%) in the control group to 4 (57.1) in the low dose and 6 (85.7) in the high dose. The increased incidence of reduced or delayed ossification in some skeletal elements (or rudiments) was also seen in both axial and appendicular skeletons.

On expressing the data as the mean percent of total ossification of the skeleton, a significant reduction was recorded in sodium nitrite-treated groups. The mean percent was 87.2% at a low dose and 82.8% at a high dose, compared with 92.4% for the control one and 92.8% for the ascorbic acid group. Moreover, females treated with low and high doses of nitrite (0.016 and 0.032 mg/g) in combination with ascorbic acid (100 mg/kg) showed 92% and 91.2% respectively. The percent of total ossified bones of the skull was significantly reduced in low and high doses. It was 90.7 at a low dose and 82.9% at a high dose, compared with 98.5% in the control one.

Great reduction in the vertebral column ossification was showed only at a high nitrite dose (73.5%), compared with 84.1% in control group. The ribs were not affected displaying 99.8%, and 99.7% for the used two doses respectively, compared with 100% in the control group. Also, the mean percent of ossified sternebrae was significantly reduced in treated groups. The mean percent was 89.6% with a low dose and 84.8% with a high dose, compared with 98.2% in the control group.

The fore and hind limbs were also greatly affected in sodium nitrite-treated groups. The mean percent of total ossification of the fore limb and pectoral girdle was 88.1% at a low dose, and 82.2% at a high dose, compared with 91.4% in the control group. On the other hand, the hind limb and pelvic girdle displayed a mean percentage of 79.4% at a low dose and 74.2% at a high dose, compared with 82.3% in the control group (Table 8 Fig.7).

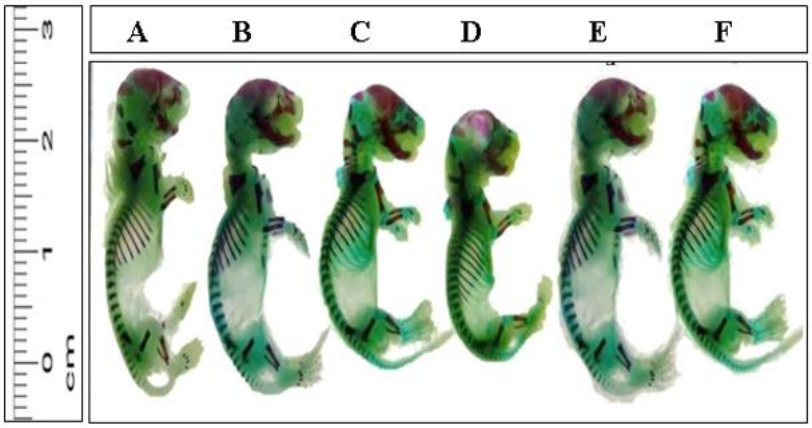

Fig. 7: Lateral view of the whole-mount skeleton of mice fetuses on the 18th day of gestation showing skeletal ossification retardation.
(A): Fetus from control dam.
(B): Fetus from ascorbic acid (100 mg/kg) treated dam
(C): Fetus from low dose (16 mg/kg) treated dam.
(D): Fetus from high dose (32 mg /kg) treated dam.
(E): Fetus from low dose + ascorbic acid treated dam.
(F): Fetus from high dose + ascorbic acid treated dam.

Table (8): Summary of skeletal ossification in mouse fetuses at 18th day of gestation after maternal oral treatment with sodium nitrite doses (16&32mg/kg/d) individually or co-administered with 100mg/kg/d of ascorbic acid from gestational day 1 to day 7.

	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	NaNo2 low dose + ascorbic acid	$NaNo_2$ high dose + ascorbic acid
Number of fetuses examined (1)	80	80	42	35	75	71
Number of dams examined (2)	8	8	6	5	8	8
Number (%) of fetuses with delayed ossification (3)	24 (30.0)	22(27.5)	24(57.1)*	30(85.7)*	24(32.0)	25(35.2)
Number (%) of dams with fetuses with delayed ossification (4)	2 (25)	2 (25)	4(66.6)*	4(80)*	2(25)	2(25)
Number (%) of fetuses with delayed ossification / dam	3 (30)	3(27.5)	4(57.1)*	6(85.7)*	3(32.0)	3.1(33.3)

- (1): Only live fetuses examined.
- (2): Includes only dams with live fetuses.
- (3): Fetuses with delayed ossification in one or more bone.
- (4): Dams with one or more fetuses with delayed ossification.

* P < 0.05 Significant. ** P < 0.01 Highly Significant.

The Skull:

The skull elements are well ossified in control group at the 18th day of gestation. The premaxilla articulates with the nasal and maxillary bones. The nasal bones are quadrangular in shape and articulate with the maxilla and with two large frontal bones with clearly visible internasal and frontonasal sutures. The frontal bones are formed of two halves limited anteriorly with the anterior fontanella. The anterior portion of the brain covers by the nasal and frontal bones. The squamosal bones, zygomatic arch and the posterior fontanella are well distinct. The posterior portion of the cranial roof is represented by interparietal bones. The parietal bone represents by two large halves forming part of the roof and sides of the cranium. The squamosal bone is found to intervene between the auditory capsule and the orbit. The zygomatic arch consists of squamosal bone, zygomatic bone and zygomatic process of maxilla. Elements of the face and upper jaw include the premaxilla, maxilla, squamosal and zygomatic arch. Both the exoccipital and supraoccipital bones are separated by a gap called the occipital ring. The occipital ring surrounds the foramen magnum and forms the posterior wall of the cranial cavity.

From the ventral aspect, the ethmoid, presphenoid and basisphenoid bones are found in one plane while the basioccipital is found at a slight angle with the basisphenoid. The tympanic ring is frequently ossified and sometimes ear ossicles are also ossified (Fig. 8). The lower jaw consists of two well-ossified mandibular bones. Each mandible consists of a single dentary bone. In the front, two dentary bones are connected with each other by a cartilaginous symphysis.

In sodium nitrite-treated groups, the skull of the examined fetuses was grossly reduced in both the anteroposterior and transverse lengths, with a clear reduction in the extent and degree of ossification. In addition, a significantly large number of fetuses failed to show a similar pattern of ossification of the skull bones as observed in the control fetuses. Only 47.6% of the examined fetuses at a low dose and 22.8% at a high dose showed normal ossification of the skull bones, compared with 86.25% in the control one. On the other side, pregnant females treated with ascorbic acid (100 mg/kg) concurrent with a low and high dose of sodium nitrite, showed normal ossification of the skull bones with percent 81.3, and 76.0% respectively. Also, the percentage of dams giving fetuses with normally ossified skull bones in the mid and high doses respectively was significantly reduced. Only 20 % of dams in the high dose, showed normal ossified skull bones compared with 75.0% in the control group. Meanwhile, 50% of dams at low doses showed a non-significant reduction in ossified skull bones. Moreover, mice treated with low and high doses of sodium nitrite (0.016 & 0.032 mg/g) in combination with ascorbic acid (100 mg/kg) showed a non-significant reduction in ossified skull bones with percent 75% and 75% respectively (Fig. 8).

In a large number of specimens, the pattern of ossification of some skull bones was less compared to that observed in the control fetuses. In the low-dose, bones of the premaxilla, maxilla, nasal, parietal, exoccipital, squamosal and presphenoid showed a non-significant incidence, compared with the control incidences and it ranged from 94% to 98%. In addition, the frontal, interparietal, supraoccipital, zygomatic arch, basioccipital, basisphenoid, mandibles and tympanic ring displayed a significant reduction in the incidence of ossification and it ranged from 79.3% to 93.5%.

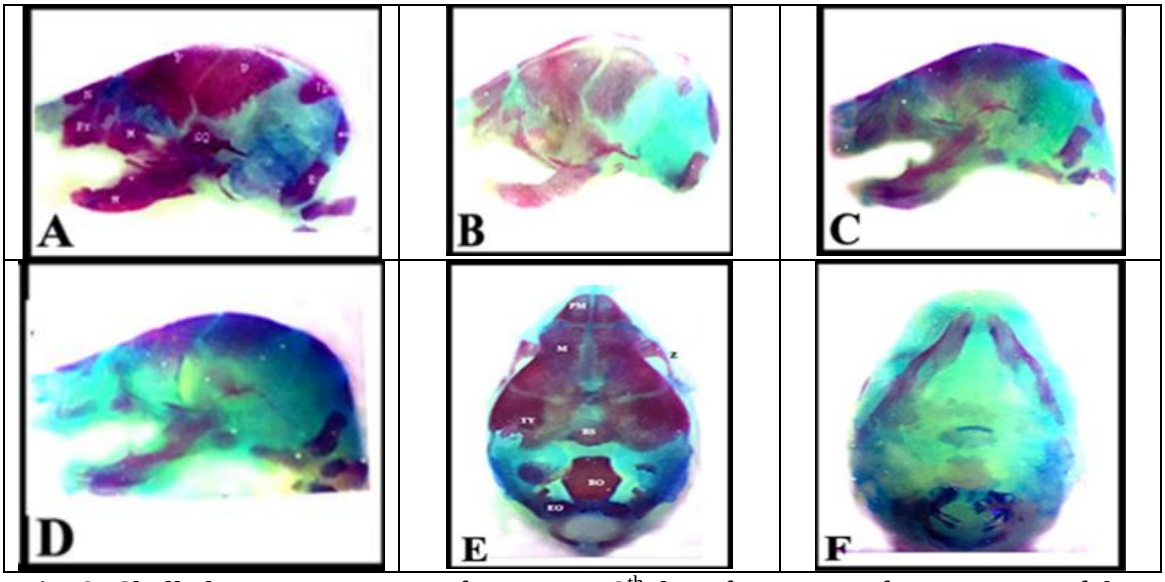

Fig. 8: Skull alterations in mouse fetuses on 18th day of gestation after treatment of dams with sodium nitrite during organogenesis.
(A): Dorsal aspects of the skull of control fetus showing the ossified elements: basioccipital (BO), basisphenoid (BS), exoccipital (EO), interparietal (IP), maxilla (M), premaxilla (Pm), nasal (N), parietal (P), supraoccipital (SO), tympanic (Ty) and zygomatic arch (Z). **(B, C& D)**: Dorsal aspect of the skull of fetus from nitrite treated dam showing incomplete ossification of the skull (arrow). Note, large number of skull bones is still in cartilaginous stage (N) nasal and (P) parietal. **(E)**: Ventral aspect of the skull of control mouse fetuses showing normal ossified skull bones (F): Skull of fetuses from sodium nitrite treated dams showing weak ossification. Alizarin, Alcian blue, X 5.

While in the high dose, the effect of sodium nitrite was clearly obvious where all the skull bones were either poorly ossified or still in mesenchymal or cartilaginous states. Pre-maxilla, nasal, frontal, parietal, exoccipital, squamosal zygomatic, basioccipital and mandibles. interparietal, supraoccipital, basisphenoid, presphenoid bones and tympanic ring and ear ossicles showed reduced ossification and occurred at incidences ranging from 70.2% to 93% in treated groups, compared with 95% to 100% incidences in the control group. Moreover, in mice treated with ascorbic acid (100 mg/kg) concurrent with a low and high dose of sodium nitrite, the incidence percent of the ossified skull bones was comparable to the control incidences and it ranged from 92.9% to 100%. Also, the mean percent of total ossified skull bones was greatly influenced by sodium nitrite treatment. It displayed a significant reduction in the centers of ossification of the skull bones in all treated groups. The mean percent was 90.78%, and 82.92% at both low and high doses respectively, compared with 98.5% in the control group. On the other hand, the mean percent was 97.8%, and 96.79% respectively at low and high doses in combination with ascorbic acid, compared with 98.5% in the control one (Fig 9).

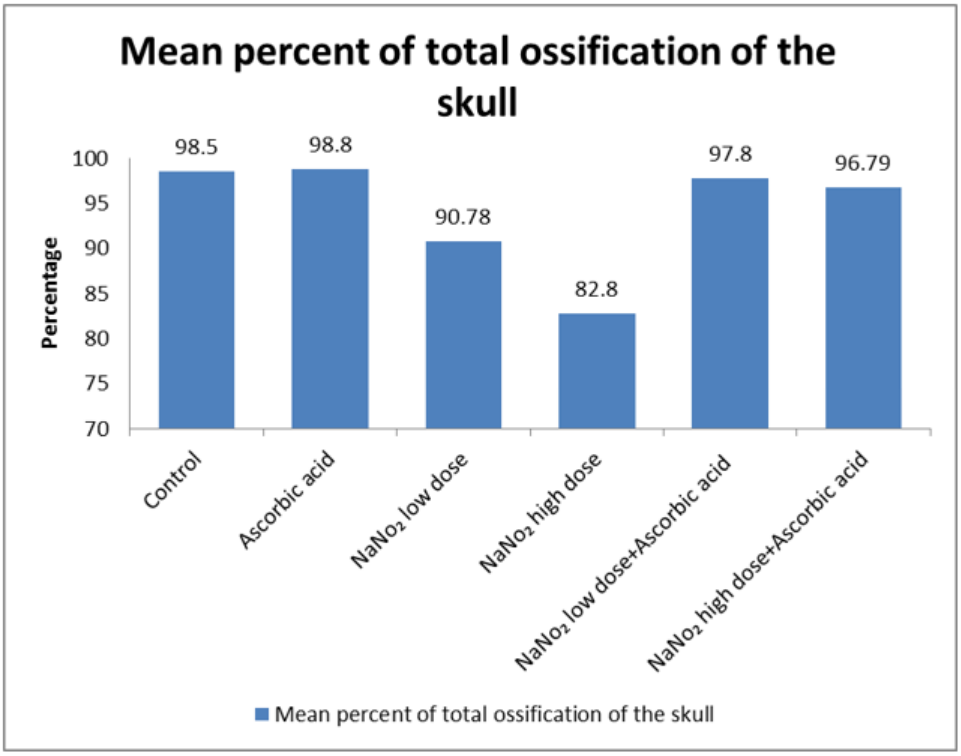

Fig. 9: Showing ossification of skull of mouse fetuses at 18th day of gestation after administration of oral doses (16 &32mg/kg/d) of sodium nitrite individually or co-treated with 100mg/kg of the ascorbic acid to pregnant females from gestational days 1 through 7.

The Vertebral Column:

The vertebra is the basic structural unit of the vertebral column. In the control mice at the 18th day of gestation, each vertebra consists of a ventral centrum and a pair of lateral neural arches. The vertebral column articulates anteriorly with the occipital condyles of the skull. The vertebral column is subdivided into five regions, as in all mammals, cervical, thoracic, lumbar, sacral and caudal regions. The vertebral column of the normal mouse fetuses consists of 7 cervical, 13 thoracic, 6 lumber, 4 sacral and a variable number of caudal vertebrae (28-30) depending on the tail length. Generally, all the vertebral centra are fully ossified but still surrounded by cartilaginous rings. In caudal vertebrae, only 10 centra are ossified and the remaining centra are still cartilaginous. The neural arches are almost completely ossified except for a cartilage tip at the fusion of the two arches.

In the cervical region, the first vertebra (atlas) and the second (axis) are specialized for articulation with the skull and rotation on the vertebral column. The remaining five vertebrae are normal in appearance. Each of the thoracic vertebrae articulates laterally with a pair of ribs. Each of the lumber vertebrae has a centrum, neural arch, neural spine and a pair of transverse processes. In the sacral region, cartilages of the transverse processes of the sacral vertebrae I, II and III are normally fused together on each side providing extra support for the pelvis. The caudal vertebrae showed a progressive reduction in the elements from the base of the tail towards the posterior end. The posterior vertebrae formed only from the cartilaginous centra. About 8-10 centra of the anterior vertebrae are ossified.

In $NaNO_2$-treated groups, the percentage of ossified vertebrae was greatly influenced by the use of nitrite. In this concern, the percent of ossified centra of thoracic,

lumber and sacral vertebrae showed non-significant incidences at both low and high doses. When the data were expressed as the mean percent of ossified centra, low and high doses showed a significant response. About 64.2% of the examined vertebrae at a low dose and 60.4% at a high dose showed normal ossified centra, compared with 79.8% in the control group and 81.2% in the ascorbic acid group. The percent was 78.4% and 77.1 respectively at low and high doses in combination with ascorbic acid.

On the other hand, the percent of ossified neural arches at cervical, thoracic, lumbar and sacral vertebrae was comparable between control and both low and high doses. A similar response was obtained when data were expressed as the mean percent of ossified arches. Low and high doses showed a non-significant response. When data were expressed as mean percent of centra and arches, low and high doses showed a significant response with incidences of 75.7% and 73.5%, compared with 84.1% in the control and 86 % in the ascorbic acid group. The percent showed improvement in mice treated with low and high doses in combination with ascorbic acid 84.1 % and 82.6 % respectively (Table 9).

The incidence of ossification of the cervical and caudal vertebrae is illustrated in table (9). An increased percentage of individuals with a reduced number of ossified cervical centra were observed. At both low and high doses, all 7 cervical centra showed a highly significant reduction in ossification. It ranged from 7% to 35% at mid-dose, and 7% to 22% at high doses respectively, compared with 68% to 90.0% in control group and 69% to 91% in ascorbic acid group. The mean percent of ossified cervical centra was also significantly reduced in all treated groups. It was 20.42%and 13.4% in low and high doses respectively, compared with 70.6% respectively, in control group and 71.42 in the ascorbic acid group. The percentages were 61.14% and 57% in mice treated with low and high doses in combination with ascorbic acid.

In all treated groups, the number and incidence of ossified caudal centra were significantly reduced. Only seven caudal centra were normally ossified at a low dose, and six centra were observed for high doses. It occurred at reduced incidences and reached 75.0%, 67.0%, 57.0%, 55.0%,48.0%, 39.0% and 26.0% for the first seven centra at a low dose, and 63.0%, 53.0%, 47.0%, 50.0%, 38.0% and 30.0% for the first six centra at high dose, compared with 10 ossified centra in control ones (90.0% for 1^{st}, 2^{nd} and 3^{rd}, 67.0%,65.0%,60.0% and 50.0% for 4^{th}, 5^{th},$6^{th}$and,7^{th}one). A significant reduction in the mean percent of ossified centra of caudal vertebrae was also observed among treated groups. It was 36.7%and 28.1% for low and high doses respectively, compared with 61.2% in the control group and 62.9% in ascorbic acid group **(**Fig10).

In sodium nitrite-treated groups, the percentage of fetuses with normal ossified vertebrae was significantly reduced. It was 23.8%and 20% in both low and high doses respectively, compared with 80% in the control and 82.5% in the ascorbic acid group. The percentages were 80% and 78.6% for mice treated with low and high doses in combination with ascorbic acid. Also, the percentage of dams giving fetuses with normal ossified vertebrae was reduced significantly in the low and high doses. About 33.3% of dams at a low dose and 20.0% of dams at a high dose give fetuses with normal ossified vertebrae, compared with 75% in the control and 75% in the ascorbic acid groups. The percentages were 75% and 62.5% for females administered the low and high doses in combination with the ascorbic acid (Table 9).

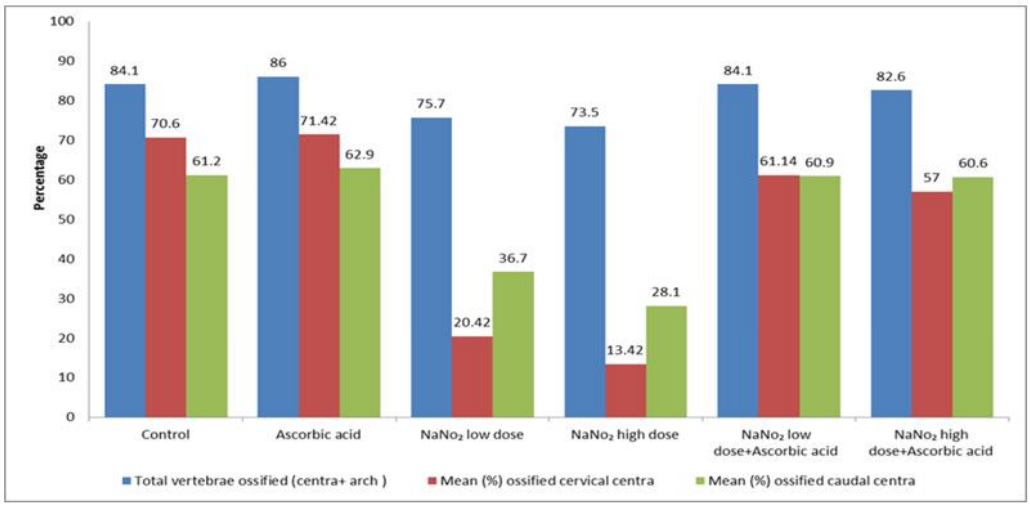

Fig. 10: Percent of total vertebrae ossified percent of ossified cervical and caudal centra of mouse skeletal fetuses at 18th day of gestation after maternal oral administration of sodium nitrite doses individually or co-administered with 100 mg/kg/d of ascorbic acid from gestational days 1 through**.**

Table 9: Incidence (%) of ossification of the centra of cervical & caudal vertebrae of mouse fetuses at 18th day of gestation after maternal administration of oral doses of nitrite individually or co-administered with ascorbic acid on gestational days 1 through 7.

	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	$NaNo_2$ low dose + ascorbic acid	$NaNo_2$ high dose +ascorbic acid
No. fetuses examined	80	80	42	35	75	75
No. dams examined	8	8	6	5	8	8
No. (%) fetuses with normal ossified vertebrae	64 (80)	66(82.5)	10(23.8)**	7(20)**	60(80.0)	59(78.6)*
No. (%) dams with normal fetuses	6 (75)	6(75)	2(33.3)**	1(20)**	6(75)	5(62.5)*
Cervical vertebrae						
% Ossified cervical centrum (1)	90	91	35	22	70	65
% Ossified cervical centrum (2)	82	85	30	19	66	61
% Ossified cervical centrum (3)	69	67	30	18	62	60
% Ossified cervical centrum (4)	59	61	21	13	51	48
% Ossified cervical centrum (5)	61	64	11	9	59	55
% Ossified cervical centrum (6)	65	63	9	6	61	56
% Ossified cervical centrum (7)	68	69	7	7	59	54
Mean (%) ossified cervical centra	70.6	71.42	20.42**	13.42**	61.14	57*
Caudal vertebrae						
% Ossified caudal centrum (1)	90	95	75**	63**	90	91
% Ossified caudal centrum (2)	90	93	67**	53**	91	90
% Ossified caudal centrum (3)	90	91	57**	47**	88	89
% Ossified caudal centrum (4)	67	65	55**	50**	65	63
% Ossified caudal centrum (5)	65	66	48**	38**	65	64
% Ossified caudal centrum (6)	60	61	39**	30**	58	59
% Ossified caudal centrum (7)	50	52	26**	0	51	50
% Ossified caudal centrum (8)	40	43	0	0	39	41
% Ossified caudal centrum (9)	40	40	0	0	41	40
% Ossified caudal centrum (10)	20	23	0	0	21	19
Mean (%) ossified caudal centra	61.2	62.9	36.7**	28.1**	60.9	60.6

$$Percent\ of\ ossified\ centrum = \frac{\text{Number } ossified}{\text{Number examined}}\ x\ 100$$

The Ribs:

All fetuses obtained from normal pregnant mice have 13 pairs of well-ossified ribs. Each one consists of three rudiments: a cartilaginous tip proximally lying to the arch, ossified middle portion & distal cartilaginous portion. The distal cartilages of the first seven pairs articulate directly with the sternum, while those of the 8th and 9th pairs

are curved upward and fused to those of the 7$^{th.}$ The last four pairs are free distally, have no connection with the sternum and are described as floating ribs.

In nitrite-treated groups, the mean percent of an ossified segment of the rib was not significantly reduced at low and high doses revealing mean incidences of 99.8, 99.7 respectively compared with 100% in the control group.

The percentage of fetuses with normal ossified ribs was significantly reduced in high doses. It was 88.5% in high doses, compared with 100% in the control group. These fetuses are obtained from a relatively small number of dams. The percentage of dams giving fetuses with normal ossified ribs was 83.3%and 60% for low and high doses respectively, compared with 100% for the control group.

Table 10: Incidence (%) of ribs ossification in mouse fetuses at day eighteen of gestation after maternal oral administration of 16 &32mg/kg/d of sodium nitrite individually or co-treated with 100mg/kg of ascorbic acid on gestational days 1 through 7.

	Control	Ascorbic acid	$NaNo_2$ low dose	$NaNo_2$ high dose	$NaNo_2$ low dose + ascorbic acid	$NaNo_2$ high dose +ascorbic acid
No. fetuses examined	80	80	42	35	75	71
No. dams examined	8	8	6	5	8	8
No. (%) fetuses with normal ossified ribs	80 (100)	80 (100)	40(95.2)	31(88.5)*	75(100)	71(100)
No. (%) of dams with fetuses with normal ossified ribs	8 (100)	8 (100)	5(83.3)*	3(60)**	8(100)	8(100)
% Ossified rib (1)	100	100	100	100	100	100
% Ossified rib (2)	100	100	100	100	100	100
% Ossified rib (3)	100	100	100	100	100	100
% Ossified rib (4)	100	100	100	100	100	100
% Ossified rib (5)	100	100	100	100	100	100
% Ossified rib (6)	100	100	100	100	100	100
% Ossified rib (7)	100	100	100	100	100	100
% Ossified rib (8)	100	100	100	100	100	100
% Ossified rib (9)	100	100	100	99.9	100	100
% Ossified rib (10)	100	100	100	99.9	100	100
% Ossified rib (11)	100	100	100	99.6	100	100
% Ossified rib (12)	100	100	100	99.5	100	100
% Ossified rib (13)	100	100	98.2	98	100	100
Mean (%) of ossified ribs [1]	100	100	99.8	99.7	100	100

(*): Significantly different from control at $p < 0.05$
(**): Highly Significant different from control at $p < 0.01$

$$\text{Percent of ossified rib bone} = \frac{Number\ ossified}{Number\ examined}\ x\ 100$$

The Sternum

The sternum of the control fetuses at the 18th day of gestation consists of six well-ossified pieces called sternebrae, of which the first one is large and called manubrium and the last one is also large and called xiphisternum. The last sternebra (xiphisternum) is terminated with an expanded cartilaginous plate called xiphoid cartilage. Each sternebrae is formed from two symmetrical bars. They are fused together in the ventral mid-line. The cartilaginous junctions of the sternebrae are articulated with the sternal ribs or costal cartilages of the first seven pairs of ribs. In all treated groups, the number of ossified sternebrae was greatly reduced especially the 5th and the 6th ones, indicating incomplete ossification of the sternum (Fig.11).

The number and percent of fetuses with normally ossified sternebrae were greatly influenced by sodium nitrite treatment and showed a significant reduction. In the low dose, only 54.7% of the examined fetuses showed normal ossified sternebrae and 40% at the high dose, compared with 95.0% in the control group. While the mean percent was 94.6%, and 92.9 % respectively at low and high doses in combination with ascorbic acid. Also, the number and percent of dams with fetuses showing normal ossified sternebrae were significantly reduced and reached about 16.6% at a low dose and non at a high dose, compared with 87.5% for the control group. While the percent were 87.5%, and

75% respectively at low and high doses in combination with ascorbic acid. In the control group, the percent of ossified sternebrae was 100% for the 1st, 2nd, 3rd and 4thsternebrae. Meanwhile, the 5th and 6thsternebrae displayed 95.0% and 94.0 % respectively. In the low dose, the percent of ossified sternebrae (4th, 5th and 6th) showed a significant reduction to percent 85.7%, 63.6% and 89.9% respectively. At the high dose, the percent of ossified sternebrae was reduced to 94.3% and 91.8% for 2nd t and 3rdsternebrae and 81.6%, 58.7%and86.9% for 4th, 5th, and 6th respectively. At low and high doses in combination with ascorbic acid, the percent of ossified sternebrae was less compared to that observed in the control fetuses. The mean percent of the total sternebrae ossified was significantly reduced from 98.2% in the control group to 89.6 and 84.8% for low and high doses respectively. While the percentage was 98%, and 97.9% respectively at low and high doses in combination with ascorbic acid.

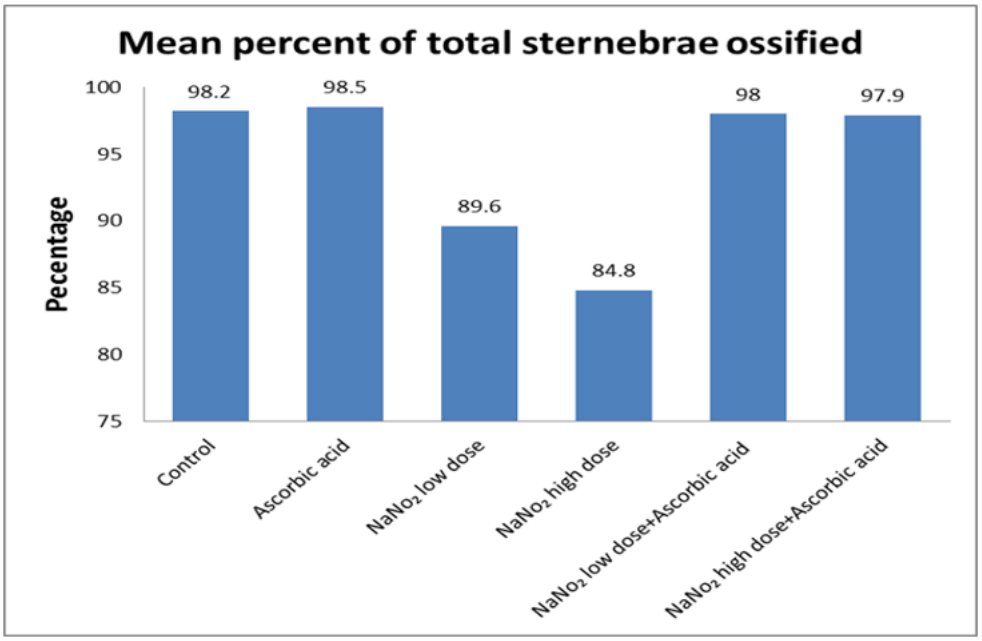

Fig.11: Showing ossification of sternebrae of mouse fetuses at 18th day of gestation after oral maternal administration of two sodium nitrite doses individually or co-administered with ascorbic acid from gestational days 1 through 7.

The Pectoral Girdle and Fore Limb:

The pectoral girdle has two elements, dorsal scapulae and ventral clavicles. The scapula is a flat trapezoidal bone from which various processes are developed. The clavicle is elongated curving slender connected at each lateral side by a ligament with the manubrium of the sternum and with the acromion process of the scapula. The skeleton of the fore limb of control fetuses at the 18th day of gestation is built up of well-ossified bones; the humerus supports the upper arm (brachium); the ulna and radius support the lower forearm (ant brachium) and the carpals, metacarpals and phalanges in the forefoot (manus). Each foot has five digits and has phalangeal formula (2: 3: 3: 3: 3). The metacarpals are partially ossified, while the carpals are still cartilaginous. Each of the metacarpals has five metacarpalia located between carpals and phalangeal rows. In sodium nitrite-treated groups, the number and percent of fetuses with normal ossified fore limb bones were reduced significantly in both the low and high doses. Only 81% of the examined fetuses at low dose showed normal ossification of the fore limb bones, and 42.8% at the high dose, compared with 86.3% in the control and 87.5 in an ascorbic acid group. The percent was 86.6% and 81.6% respectively for mice treated with ascorbic acid concurrent with a low and high dose of sodium nitrite. The fetuses with normally

ossified bones of the fore limb were found in a small number of dams, about 33.3% of the examined dams at a low dose of 20% the high dose, compared with 75.0% at the control group and ascorbic acid group. The percent was 75% and 75% respectively for mice treated with ascorbic acid concurrent with a low and high dose of sodium nitrite. The low dose and high dose showed comparable incidences as recorded in the control group in (scapula &clavicle) pectoral girdle, humerus, radius and ulna. Also, a significant reduction in the percent of ossified metacarpals and phalangeal rows was frequently observed in sodium nitrite-treated groups. The percent of ossified metacarpals was 63%and 33% at the level of low and high doses respectively, compared with 78.8% in the control and 79.1% in the ascorbic acid group. The percent was 77.5% and 73% respectively for mice treated with ascorbic acid concurrent with a low and high dose of sodium nitrite. The mean percent of ossified phalanges at the level of the first; second and third rows were significantly reduced in sodium nitrite-treated groups. In the first row (proximal), the mean percent was 82%and 79% in low and high doses respectively, compared with 95.7% in the control group and 95.2% in the ascorbic acid group. The percent was 94.3% and 92.8% respectively for mice treated with ascorbic acid concurrent with the results in both16 and 32mg/kg/d doses of sodium nitrite groups. At the level of the second row (middle), the mean percent of ossified phalanges was 86.9%and 63.8% at low and high doses respectively, compared with 96.1% in the control group and 96.9% in the ascorbic acid group. In the third row (distal), the mean percent was 81.1%and 47.7% for low and high doses respectively, compared with 93.0% in the control group and 94.2% in ascorbic acid group. The percent was 91.5% and 86.7% respectively for mice treated with ascorbic acid concurrent with the low and high dose of nitrite The mean percent of total ossification of the fore limb was greatly influenced by sodium nitrite treatment (Figs. 12&13). The mean percent was significantly reduced from 82.9% in the control group and 83.17 in ascorbic acid group to 76.6% at a low dose and 65.4% at a high dose. The percent showed improvement in mice treated with ascorbic acid concurrent with the low and high doses of nitrite 82.1% and 80.4%, respectively.

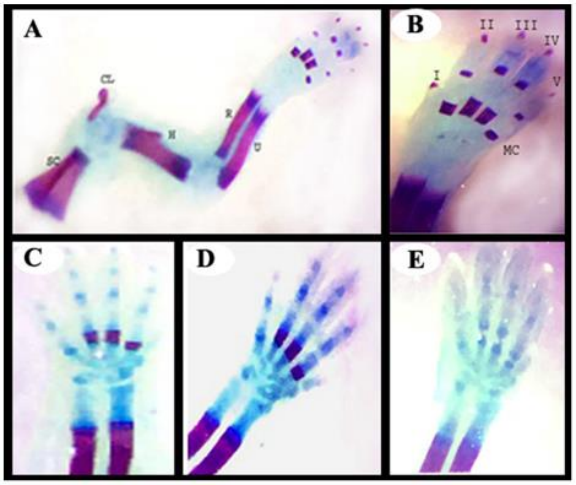

Fig.12: Showing alterations of the pectoral girdle and fore limb of mouse fetuses on day eighteen of gestation after maternal sodium nitrite treatment.

A&B: Skeleton of the fore limb, clavicle and scapula of control fetus showing well ossified scapula (Sc), clavicle (Cl), humerus (Hu), radius (R) and ulna (U). The metacarpals (MC) of 2nd, 3rd, 4th and 5th fingers are well ossified, meanwhile the metacarpals of the 1st finger is unossified. Each of the 2nd, 3rd, $_{4th}$ and 5th fingers has three ossified centers; meanwhile, the 1st finger has one center only. All the epiphyses and carpalia are still cartilaginous.

C&D&E: Fetuses from sodium nitrite treated dams showing ossified metacarpalia of 2nd, 3rd and 4th fingers. The metacarpalia of the 5th is faintly ossified, meanwhile the metacarpalia of the 1st and 5th digit

and the phalanges are un-ossified.

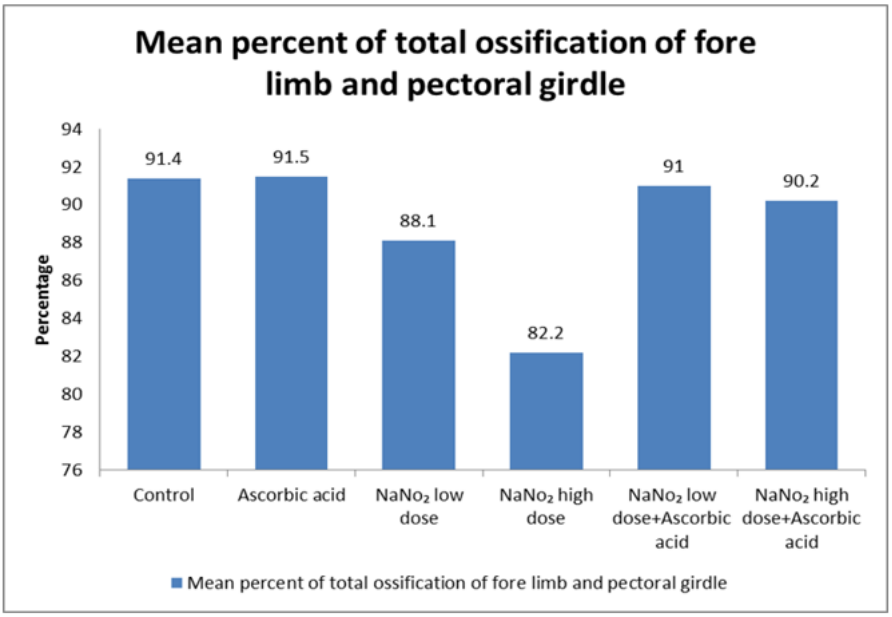

Fig.13: Ossification of fore limb and pectoral girdle of mouse fetuses at 18th day of gestation after administration of oral sublethal doses of sodium nitrite individually or co-administered with ascorbic acid as antioxidant to pregnant dams from gestational days 1 through 7.

The Pelvic Girdle and Hind Limb:

The pelvic girdle of mouse fetuses at the 18th day of gestation is formed from 2 halves that articulate ventrally at the pubic symphysis. Each half is made up of three elements; an antero-dorsal bone called ilium, a postero-dorsal one called ischium and a ventral flattened slender bone called *os*pubis. The last two bones are separated by obturator foramen. All three bones share in the formation of a cup-shaped depression called acetabulum. Also, the skeleton of the hind limb of fetuses obtained from pregnant dams at the 18th day of gestation is built up of the femur supporting the thigh, tibia and fibula supporting the shank, and the tarsal bones supporting the ankle. The metatarsals and phalanges are the bones of the hind paw. Each foot has five digits and has a digital formula (2:3:3:3:3). All five metatarsalia as well as the phalangeal rows and one of the ankle bones are found in a good ossified condition. In sodium nitrite-treated groups, there was a marked reduction in the percentage of fetuses with normal ossified bones of the pelvic girdle and hind limb. At the low dose, only 60% of the examined fetuses showed normal ossified hind limb bones and reached a dramatic diminution of 20% at the high dose, in comparison with 83.75% in the control group. While the percentage was 80%, and 77.3% respectively at low and high doses in combination with ascorbic acid. Also, a significant decrease was recorded in the percentage of dams giving fetuses with normally ossified pelvic girdles and hind limb bones. Only 33.3% of the examined dams were at a low dose and 20% at a high dose giving fetuses with normal ossified bones of the hind limb and girdle, compared with 75.0% of dams in the control group. But the percent was 62.5%, and 50 % respectively at low and high doses in combination with ascorbic acid, compared with 75.0% of dams in the control group. A non-significant reduction in ossification of bones of the pelvic girdle was recorded in sodium nitrite-treated groups. It was 100% and 98% for the femur; 100 %and 97% for tibia and 100% and 97% for fibula at both the low and high doses respectively, compared with 100% incidences in the control group for each bone. The percentage of ossified metatarsals was

significantly reduced in treated groups and revealed 55.5% at a low dose and 25% at a high dose, compared with 82.7% in control group. On the other hand, the percent was 79.2%, and 65.1% at low and high doses in combination with ascorbic acid, respectively. The mean percent was 15% and 15%, for astragalus and calaneum high doses respectively compared with 25 % in control group for each bone indicating a non-significant change at low, meanwhile, a significant reduction was observed at high doses. The mean percent of ossified phalanges was highly reduced in sodium nitrite-treated groups. It was 71% at a low dose of 68.4 % at a high dose for the first row (proximal), compared with 72.8% in control group. The percent was 72%, and 71.2% at low and high doses in combination with ascorbic acid respectively. The percent was 66.8% at a low dose and 47.73% at a high dose for the second row (middle), compared with 78.8% in the control group and 21.1%. The percent was 73.5%, and 69.6% at low and high doses in combination with 100mg/k/d of ascorbic acid respectively. The percent was 50.9% at the low dose and 21.1% at the high dose for the third row compared with 63.2% in the control group. The percent was 60.2%, and 54.3% at low and high doses in combination with ascorbic acid respectively. The mean percent of total ossification of the hind limb was significantly reduced and reached about 58.8% at low dose and 48.4% at a high dose, compared with 63.8% in the control group. The percent was 63.2%, and 60.18% at low and high doses in combination with ascorbic acid respectively (Figs. 14&15).

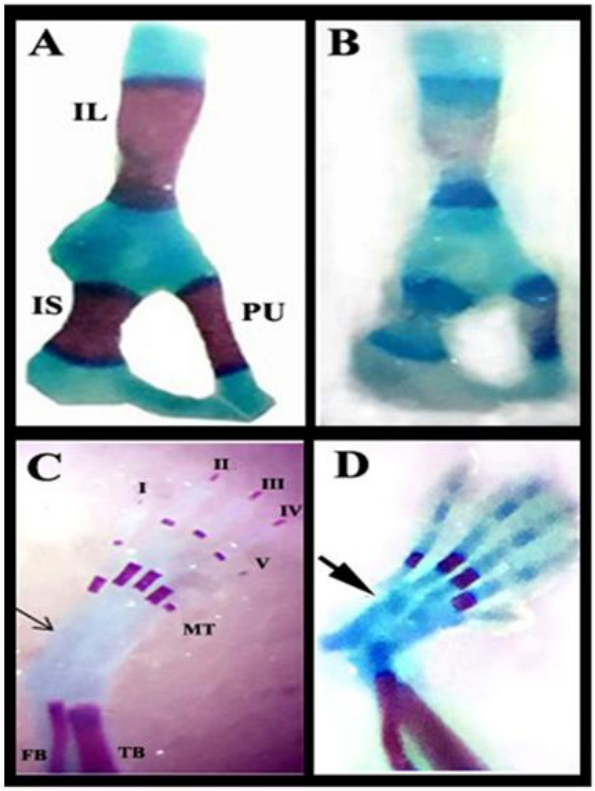

Fig.14: Alterations of the pelvic girdle and hind limb of mouse fetuse on the 18th day of gestation after sodium nitrite treatment.

(A): Skeleton of the pelvic girdle of the control fetus showing well-ossified ilium (IL), ischium (IS) and pubis (Pu).

(B): Pelvic girdle of fetuses from sodium nitrite treated dams showing weakly ossified ilium, ischium and pubic bone.**(C & D):** Skeleton of the hind limb:**(C):** Skeleton of the hind limb of control fetus showing well-ossified tibia (TB), fibula (FB), astragalus and calcaneum (arrow). The metatarsals (Mt) of all five digits are well-ossified. Each of the 2nd, 3rd, 4th and 5th digits has 3 ossification centers meanwhile; the 1st digit has 2

ossified centers. All tarsalia and epiphysis are still cartilaginous. **(D):** Hind limb of fetuses from sodium nitrite treated dams showing cartilaginous astragalus and calcaneum (arrow) metatarsalia (Mt) and phalanges (Ph).

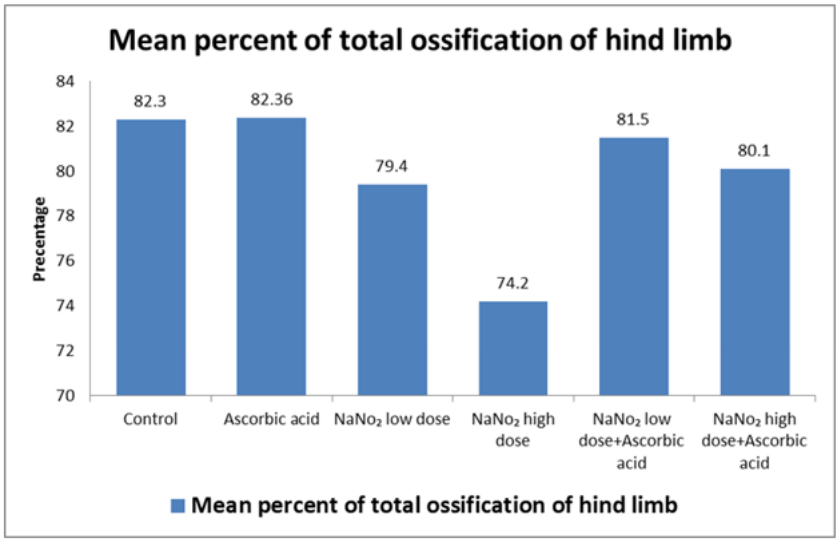

Fig.15: Ossification of hind limb and pelvic girdle of mouse fetuses at the day eighteen of gestation after oral maternal administration of two sodium nitrite doses individually or co-administered with ascorbic acid from gestational days 1 through 7.

DISCUSSION

Maternal Toxicity:

Due to the urgency of the symptoms of acute poisoning, nitrite toxicities mainly focused on the mechanism of acute nitrite poisoning and its clinical treatment. Other researchers focused on the chronic nitrite poisoning, given its ability to affect human health harshly. Nitrite can retard the development of the central nervous system, particularly dendritic development and may cause mental retardation (Chen, *et al*., 2016). Mammalian maternal toxicity disrupt prenatal development, with general systemic maternal toxicity, including decreased weight gain to morbidity, causative for decreased fetal weights/litter and increased skeletal fetal variations/litter, but not, in the author's opinion, for increased fetal malformations, decreased litter sizes or full litter losses (Tyl, 2012). Although sodium nitrite ($NaNO_2$) is frequently used as a food additive, its extensive intake has toxic effects on females fertility and fetal development but its mechanism remains unknown (Ge *et al*., 2019). In the stomach, sodium nitrite may react with amines in food to form free radicals and nitrosamines. Different organs may be harmed by such products (Choi *et al*., 2002). Also, in the mouth cavity, bacteria convert nitrate to nitrite, while in the gastrointestinal system, intestinal microflora converts nitrate to nitrite (Kross *et al*., 1992). Wu, *et al*. (2022) reported that, exposure to 120 mg/kg nitrite in saline as high dose + one pregnancy cycle gavage (from G_0 - pup birth) lead to sterility in a time-dependent manner ($p<0.05$), and 2-round exposure increase sterility than that one-round exposure in adult female mice ($p<0.01$). Also, mouse sterility can be induced by oxidative stress response and cell apoptosis following the exposure to nitrite suggesting that nitrite exposure in a time-dependent manner. Cell apoptosis and oxidative stress response are involved in infertility caused by nitrite (Wu, *et al*., 2022). In the current study, prior to initiation of treatment, no differences were observed among the control and $NaNO_2$-treated groups for indices, indicating that animals were similar in their health condition and reproductive status. Here, the oral

treatment of nitrite to pregnant female mice during the fetal periods' induced obvious signs of maternal and developmental toxicity. The observed maternal toxicity symptoms associated with sodium nitrite during the treatment and gestation periods were; reduced maternal body weight gain, corrected body weight gain, and reduced weight of gravid uterus compared with the control, ascorbic acid treated groups in a dose-dependent manner indicating that sodium nitrite exerts a toxic impact on the maternal body of the experimental animals. Also, the $NaNO_2$-treated dams showed some clinical symptoms such as loss of appetite, ataxia, dizziness, nausea, dyspnea, diarrhea, abdominal cramp, and general weakness. Similarly, Fouad *et al*. (2017) revealed that adult female albino rats weighing 150±10g received orally sodium nitrite at 1.5 mg/rat equivalent 1/20 from LD_{50} (200 mg/kg b.wt.) showed obvious clinical signs including a decrease in body weight, decrease water and food intake when compared with control. Also, Isidori *et al*. (2005) stated that, the reduction in maternal weight gain can be due to; a loss of appetite, diarrhoea as mentioned previously, metabolic disorders in the maternal body as showed by reduced corrected maternal weight gain or a decrease in the sex hormones in pregnant women that leads to significantly reverse in the gain acquired in the body weight during pregnancy, way by decreasing the mass of the muscles and bones. The reduced maternal weight gains appeared to be a secondary effect to developmental toxicity as displayed by the reduced weight of gravid uterus due to the decreased number of live fetuses and mean fetal weight and increased incidence of early and late resorptions and abortions. Also, female rats were treated orally and daily with 1/20 from LD_{50} of sodium nitrite (200 mg/kg b.wt.) or 1.5 mg/rat plus 10 mg/kg b.wt of Vit. C for 6 months showed normal behaviour with normal feed and water consumption (Fouad *et al*., 2017).

Developmental Toxicity: Reproductive outcome

Embryo/fetal development, adversely affect by a number of sicknesses and adverse conditions during pregnancy, so, many scientists suppose that any major disturbance of maternal homeostasis that produced by chemical exposure may ultimately produce a teratogenic effect. Although there is little doubt that developmental toxicity may be maternally mediated, the idea that, in principle, any maternal toxicity leads to birth defects is contested. if embryotoxicity was noted only during the range of maternal toxic dose, it is not possible to ensure whether it is in fact maternally mediated or not (i.e., the development of embryo may have been impaired by the direct effect of the chemical at doses that also adversely affect the mother; in these conditions, it will still be an eclectic developmental toxicant) (Paumgartten, 2010). Nitrite toxicity can be said affect other functions, such as, reproduction and infertility because nitrite can pass the placenta and abortion of fetal result (Fan & Steinberg, 1996; Aly, *et al*., 2010).

Nitrates can penetrate through the placental blood barrier, exposing the fetus while still in the womb (Bruning-Fann *et al*., 1993). There is some evidence to suggest that nitrous oxide exposure causes spontaneous abortion (Olfert, 2006). In experimental animals, nitrous oxide has been proven to cause prenatal deformities, increase fetal mortality, and reduce litter size (Rowland *et al*., 1995). Pathological analysis of the placentas detect that when sodium nitrite enters the animal body, structural changes in the placental show that there are placental and maternal circulatory disorders concurrent with destructive and dystrophic processes (Ivanova *et al*., 2014).

In our study sodium nitrite treatment during fetal life was associated with many developmental toxic impacts affecting pregnancy outcome including a significant increase in resorption rate and % of postimplantation loss per dam. Significant increase in the percentage of dams with postimplantation loss at one or more sites, complete resorption of all implants, complete miscarriage of all fetuses and a significant decrease in females producing live fetuses (decrease in the number of live fetuses / dam). This

reduction in the number of live fetuses may be attributed to a reduced number of implantation sites and postimplantation loss. In addition, pregnant female mice treated with the studied doses of sodium nitrite and thereafter with 100 mg/kg of ascorbic acid showed significant improvement in all maternal parameters.

Nitrate, nitrite, and *N*-nitroso compounds may navigate the placenta and can harm the fetus *in uteroas* as indicated in animal studies (Gruener *et al*., 1973; Fan *et al*., 1987; Bruning-Fann and Kaneene 1993). L'hirondel (2002) suggested that the placental membranes are effective in separating blood circulation between mother and fetus from the 4th month of pregnancy, thus preventing the crossing of methemoglobin molecules. Also, Hartman (1982) suggested that the nitrate or the reduced form (nitrite) may be able to pass to the fetus through active transport system that similar to that of iodide, and fetal plasma levels of nitrate may exceed that of the mother.

A previous study of Vlachou *et al*. (2020) has found that excessive $NaNO_2$ exposure via intraperitoneal injection or drinking water could impair the reproductive function and causes congenital fetal disabilities. In addition, growth restriction, spontaneous abortion, in utero, and many congenital disabilities have been associated with $NaNO_2$ exposure in drinking water (Manassaram *et al*., 2006). In addition, results of Ge, *et al*. (2019) showed that increased levels of ROS, decreased oocytes in the early stages of apoptosis, decreased numbers of the offspring and survival rates in female mice treated with 60 mg/kg/day or 120mg/kg/day of $NaNO_2$ by intra-gastric gavage for 3 weeks indicating the adverse effects of $NaNO_2$ on female reproductive capabilities in female mice.

Fetal Growth Retardation:

The result of the present study showed that fetuses obtained from sodium nitrite-treated dams on the 18th day of gestation exhibited reduced live litter size and this reduction was related to decreased number of the sites of implantation and postimplantation loss. With regard to living fetuses, fetal growth retardation was reflected by the significant decrease in the fetal body weight (only at high doses), fetal length (at high and low doses) and weight per length ratio (only at high doses) and a decrease in placental weight (only at high dose).In the present study, fetal weight and length were considered sensitive indicators for intrauterine fetal growth. The fetal growth retardation observed in this study may arise from the direct action of the used sodium nitrite on fetal tissues and may be secondary to maternal toxicity. The fetal growth retardation was attributed to impaired protein synthesis. The thyroid hormone plays an important role in intrauterine fetal growth Kilby (2005). On the 16th day of incubation, sodium nitrite caused numerous teratogenic consequences in developing embryos, according to HA and AM (1994). In comparison to the control group, resorption, mortality, and growth retardation were all significantly higher in the experimental group. Furthermore, the total length of treated chick embryos was lowered, a statistically significant reduction. Sun & Zhang (2022) revealed that, brain damage in fetal rats of pregnant female that exposed to water containing 0.05, 0.15, and 0.25% of $NaNO_2$. Also, the number of live foetuses, the number of fetuses per litter, fetuses' weight, and brain weight were significantly decreased in the model group than in the control one. Mainly, nitrate is inactive and becomes active biologically after its reduction to nitrite. Subsequently nitrite can react with amides and amines from diet forming *N* nitroso compounds (NOCs). Nitrate and NOCs are soluble in water, so can exposing the fetus in the uteruses (Bruning-Fann & Kaneene, 1993).

Animal studies have suggested higher rates of fetal death, cycle irregularities, and decreased numbers of offspring and longer days to litter among female cattle and mice that treated with nitrate (Wigle, *et al*., 2008). Also, as shown in observational

studies in humans, neonatal death has been associated with drinking water nitrate (Aschengrau *et al*., 1993), intrauterine growth retardation (Migeot, *et al*., 2013; Coffman *et al*., 2021), prematurity (Sherris, *et al*., 2021), very low birth weight (Bukowski, *et al*., 2001; Stayner, *et al*., 2017) and congenital malformation (Dorsch, *et al*., 1984; Croen, *et al*., 2001) at exposure levels under the drinking water standard. In addition, Vuong, *et al*. (2016) stated that the preterm birth has been associated with prenatal exposure to secondary or tertiary amines (nitrosatable drugs), in conjunction with higher levels of dietary nitrite intake may due to of the increased formation of NOCs.

Birth defects may be causes infant mortality and associated with long-term disability and substantial morbidity. Endogenous and exogenous nitrite and nitrate may react with Nitrosatable drugs to form *N*-nitroso compounds, causing teratogenicity effect in experimental animals. *N*-nitroso compounds, that formed endogenously after the ingestion of nitrite and nitrate increase central nervous system birth defect in mice (Platzek, *et al*., 1983).

On the other side, the present work showed an increase in the fetal body weight, fetal length and placental weight after maternal treatment of ascorbic acid. The shortage of information on the dietary source of nitrate and nitrite, antioxidants intake that might modify the effects of nitrate exposure on the risk of birth defects, and the use of nitrosatable drugs that may promote the formation of N nitroso compounds (Blaisdell, et al., 2019). Also, Ward, *et al*. (2018) showed that the endogenous formation of NOCs depends on the presence of inhibitors (e.g. vitamins E and C) and nitrosation precursors (e.g. nitrosatable drugs & red meat), the concentration of components involved, and acidity in the stomach.

Malformations

Exposure to some chemicals that may alter special maternal functions at the critical point during pregnancy resulted in increased fetal malformations. Also, genetic/epigenetic alterations cause malformations, specifically altered molecula,r proteins pathways, etc. Information to inform maternal and developmental toxicities includes uterine implantation profile, degree of litter reduction, ovarian corpora lutea counts, extent and timing of maternal toxicity relative to those of opposing fetal - embryo effects, etc. (Tyl, 2012).

The results of the present study indicated that maternal exposure to nitrite might carry a selective degree of risk to developing fetuses that is involved in a significant increase in the number of malformed fetuses and the percentage of dams with malformed fetuses. $NaNO_2$ treatment associated with skeletal and external malformations. External malformations affecting the gross morphology of fetuses including hemorrhage (Hematoma). Skeletal malformations were clearly observed in the sternum. Among these abnormalities hemisternebrae, dumb-bell sternebrae, malaligned sternebrae, asymmetric sternebrae, and absent sternebrae were seen.

Ossification:

One of the standard components of developmental toxicology testing in experimental animals is the evaluation of the skeleton. Perturbations of skeletal development include delays of the ossification, alterations in the shape, number, and size of ossification centres, alterations in the number of vertebrae and ribs. Transient delays in development can result in apparent results of the defective skeletal structure because the skeleton is undergoing developmental changes when foetuses are investigated in most study designs. It is crucial to determine whether it indicates a long-term alteration in embryo development that will have negative effects on the organism (DeSesso & Scialli, 2018).

As a consequence of sodium nitrite-impaired fetal growth, skeletal ossification

was also retarded. A large number of dams produced fetuses with delayed ossification of the skeleton. The impaired ossification was clearly observed by reduced ossification centers in the skull. Also, reduced ossification of cervical centra and caudal vertebrae, and sternebrae was observed. The fore and hind limbs displayed a marked degree of reduced ossification, mainly in the distal phalanges, metacarpals, and metatarsals. Also, bones of the ilium, ischium, pubis, and clavicle displayed different degrees of ossification retardation. Among sodium nitrite-treated groups a significantly large number of dams produced a large number of individuals with retarded ossification of the skeleton. HA, & AM, (1994) mentioned that skeletal abnormalities were caused by sodium nitrite, as evidenced by a decrease in ossification of the vertebrae, skull, ribs, and limbs.

REFERENCES

Aly, H. A., Mansour, A. M., Abo-Salem, O. M., Abd-Ellah, H. F., & Abdel-Naim, A. B. (2010). Potential testicular toxicity of sodium nitrate in adult rats. *Food and chemical toxicology*, 48(2), 572-578.

Ansari, F. A., Ali, S. N., Arif, H., Khan, A. A., & Mahmood, R. (2017). Acute oral dose of sodium nitrite induces redox imbalance, DNA damage, metabolic and histological changes in rat intestine. *PLoS One*, 12(4), e0175196.

Aschengrau, A., Zierler, S., & Cohen, A. (1993). Quality of community drinking water and the occurrence of late adverse pregnancy outcomes. *Archives of Environmental Health: An International Journal*, 48(2), 105-113.

Blaisdell, J., Turyk, M. E., Almberg, K. S., Jones, R. M., & Stayner, L. T. (2019). Prenatal exposure to nitrate in drinking water and the risk of congenital anomalies. *Environmental research*, 176, 108553.

Bruning-Fann, C. S., & Kaneene, J. B. (1993). The effects of nitrate, nitrite and N-nitroso compounds on human health: a review. *Veterinary and human toxicology*, 35(6), 521-538.

Bukowski, J., Somers, G., & Bryanton, J. (2001). Agricultural contamination of groundwater as a possible risk factor for growth restriction or prematurity. *Journal of occupational and environmental medicine*, 377-383.

Celis, J. E. AND Celis, A. (1985). Cell cycle dependent variations in the distribution of the proliferating cell nuclear antigen in cultured cells: subdivision of S phase. Proceedings of the National Academy of Sciences of the United States of America, 82: 3262-3266.

Chen, Y., Cui, Z., Wang, L., Liu, H., Fan, W., Deng, J., & Deng, J. (2016). The impairment of learning and memory and synaptic loss in mouse after chronic nitrite exposure. *Environmental toxicology*, *31*(12), 1720-1730.

Choi, S. Y., Chung, M. J., & Sung, N. J. (2002). Volatile N-nitrosamine inhibition after intake Korean green tea and Maesil (Prunus mume SIEB. et ZACC.) extracts with an amine-rich diet in subjects ingesting nitrate. *Food and Chemical Toxicology,* 40(7), 949-957

Chui, J. S. W., Poon, W. T., Chan, K. C., Chan, A. Y. W., & Buckley, T. A. (2005). Nitrite induced methaemoglobinaemia aetiology, diagnosis and treatment. *Anaesthesia,* 60(5), 496-500.

Coffman, V. R., Jensen, A. S., Trabjerg, B. B., Pedersen, C. B., Hansen, B., Sigsgaard, T., ... & Stayner, L. T. (2021). Prenatal exposure to nitrate from drinking water and markers of fetal growth restriction: a population-based study of nearly one million Danish-born children. *Environmental health perspectives,*

129(2), 027002.

Croen, L. A., Todoroff, K., & Shaw, G. M. (2001). Maternal exposure to nitrate from drinking water and diet and risk for neural tube defects. *American Journal of Epidemiology*, 153(4), 325-331.

DeSesso, J. M., & Scialli, A. R. (2018). Bone development in laboratory mammals used in developmental toxicity studies. *Birth defects research*, 110(15), 1157-1187.

Dorsch, M. M., Scragg, R. K., McMichael, A. J., Baghurst, P. A., & Dyer, K. F. (1984). Congenital malformations and maternal drinking water supply in rural South Australia: a case-control study. *American Journal of Epidemiology*, 119(4), 473-486.

Duarte, T. L., & Lunec, J. (2005). Review part of the series: from dietary antioxidants to regulators in cellular signalling and gene expression review: when is an antioxidant not an antioxidant? *A review of novel actions and reactions of vitamin C. Free radical research*, 39(7), 671-686.

Dytham, C. (1999). Choosing and Using Statistics: A Biologist's Guide. Blackwell Science Ltd. London, UK OpenURL, pp 147.

Ebdrup, N. H., Schullehner, J., Knudsen, U. B., Liew, Z., Thomsen, A. M. L., Lyngsø, J., ... & Ramlau-Hansen, C. H. (2022). Drinking water nitrate and risk of pregnancy loss: a nationwide cohort study. *Environmental Health*, 21(1), 1-13.

Fan, A. M., & Steinberg, V. E. (1996). Health implications of nitrate and nitrite in drinking water: an update on methemoglobinemia occurrence and reproductive and developmental toxicity. *Regulatory toxicology and pharmacology*, 23(1), 35-43.

Fan, A. M., Willhite, C. C., & Book, S. A. (1987). Evaluation of the nitrate drinking water standard with reference to infant methemoglobinemia and potential reproductive toxicity. *Regulatory Toxicology and pharmacology*, *7*(2), 135-148.

Ferysiuk, K., & Wójciak, K. M. (2020). Reduction of nitrite in meat products through the application of various plant-based ingredients. Antioxidants, 9(8), 711.

Fouad, S. S., Mohi-Eldin, M. M., Haridy, M. A., & Khalil, A. M. (2017). Ameliorative effects of ascorbic acid (vit C.) against sodium nitrite toxicity in albino rats: hematological, biochemical and histopathological studies. *American-Eurasian Journal of Toxicological Sciences* 9(1), 01-06.

Ge, L., Han, Z., Gao, Y. Q., Zhou, C. J., Wang, D. H., Ma, Y. Z., & Liang, C. G. (2019). Sodium nitrite negatively affects reproductive ability and offspring survival in female mice. *Toxicology*, 427, 152284.

Gruener, N., Shuval, H. I., Behroozi, K., Cohen, S., & Shechter, H. (1973). Methemoglobinemia induced by transplacental passage of nitrites in rats. *Bulletin of environmental contamination and toxicology*, *9*(1), 44-48.

HA, M., & AM, E. Z. (1994). Hisopathological, biochemical and teratogenic effects of sodium nitrite [antimicrobial agent] on chicken.

Hammoud, G. (2014). Protective effect of grape seeds extract against sodium nitrite-induced toxicity and oxidative stress in albino rats. *Al-Azhar Journal of Pharmaceutical Sciences*, 49(1), 1-34.,

Hartman, P. E. (1982). Nitrates and Nitrites: Ingestion, pharmacodynamics, and toxicology. *Chemical mutagens*, 211-294.

Ivanova, A. S., Peretiatko, L. P., Demidov, V. I., & Nazarov, S. B. (2014). Effect of nitric oxide on the morphology of the placenta and the activity of placental

macrophages during uncomplicated pregnancy in the experiment. *Arkhiv Patologii*, 76(4), 35-38.

Kilby, M. D., Barber, K., Hobbs, E., & Franklyn, J. A. (2005). Thyroid hormone action in the placenta. *Placenta*, *26*(2-3), 105-113.

Kross, B. C., Ayebo, A. D., & Fuortes, L. J. (1992). Methemoglobinemia: nitrate toxicity in rural America. *American family physician*, *46*(1), 183-188.

L'hirondel, J. (2002). *Nitrate and man: toxic, harmless or beneficial?*. CABI.

Manassaram, D. M., Backer, L. C., & Moll, D. M. (2006). A review of nitrates in drinking water: maternal exposure and adverse reproductive and developmental outcomes. *Environmental Health Perspectives*, *114*(3), 320-327.

Manson, J.M., Kang, Y.J., (1994). Test methods for assessing female reproductive and developmental toxicology. In: Hayes, A.W.(Ed.), Principles and Methods of Toxicology, second ed. Raven

Migeot, V., Albouy-Llaty, M., Carles, C., Limousi, F., Strezlec, S., Dupuis, A., & Rabouan, S. (2013). Drinking-water exposure to a mixture of nitrate and low-dose atrazine metabolites and small-for-gestational age (SGA) babies: a historic cohort study. *Environmental research*, 122, 58-64.

Olfert, S. M. (2006). Reproductive outcomes among dental personnel: a review of selected exposures. *Journal of the Canadian Dental Association*, *72*(9), 821–825.

Patel, V. P., & Chu, C. T. (2011). Nuclear transport, oxidative stress, and neurodegeneration. *International journal of clinical and experimental pathology*, *4*(3), 215.

Paumgartten, F. J. (2010). Influence of maternal toxicity on the outcome of developmental toxicity studies. *Journal of Toxicology and Environmental Health, Part A*, 73(13-14), 944-951.

Platzek, T., Bochert, G., & Rahm, U. (1983). Embryotoxicity induced by alkylating agents. *Archives of Toxicology*, 52(1), 45-69.

Rowland, A. S., Baird, D. D., Shore, D. L., Weinberg, C. R., Savitz, D. A., & Wilcox, A. J. (1995). Nitrous oxide and spontaneous abortion in female dental assistants. *American journal of epidemiology*, *141*(6), 531-538.

Sherris, A. R., Baiocchi, M., Fendorf, S., Luby, S. P., Yang, W., & Shaw, G. M. (2021). Nitrate in drinking water during pregnancy and spontaneous preterm birth: A retrospective within-mother analysis in California. *Environmental Health Perspectives*, 129(5), 057001.

Stayner, L. T., Almberg, K., Jones, R., Graber, J., Pedersen, M., & Turyk, M. (2017). Atrazine and nitrate in drinking water and the risk of preterm delivery and low birth weight in four Midwestern states. *Environmental research*, 152, 294-303.

Sun, J., & Zhang, W. (2022). Supplementation with dietary omega-3 PUFA mitigates fetal brain inflammation and mitochondrial damage caused by high doses of sodium nitrite in maternal rats. *Plos one*, 17(3), e0266084.

Tyl*, R. W. (2012). Commentary on the role of maternal toxicity on developmental toxicity. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, 95(3), 262-266.

Vlachou, C., Hofstädter, D., Rauscher-Gabernig, E., Griesbacher, A., Fuchs, K., & König, J. (2020). Risk assessment of nitrites for the Austrian adult population with probabilistic modelling of the dietary exposure. *Food and Chemical Toxicology*, *143*, 111480.

Vuong, A. M., Shinde, M. U., Brender, J. D., Shipp, E. M., Huber Jr, J. C., Sharkey, J. R., ... & Canfield, M. A. (2016). Prenatal exposure to nitrosatable drugs, dietary intake of nitrites, and preterm birth. American *Journal of Epidemiology*, 183(7), 634-642.

Ward, M. H., Jones, R. R., Brender, J. D., De Kok, T. M., Weyer, P. J., Nolan, B. T., ... & Van Breda, S. G. (2018). Drinking water nitrate and human health: an updated review. *International journal of environmental research and public health*, 15(7), 1557.

Wassersug, R. J. (1976). A procedure for differential staining of cartilage and bone in whole formalin-fixed vertebrates. *Biotechnic & Histochemistry*, 51(2), 131-134.

Wigle, D. T., Arbuckle, T. E., Turner, M. C., Bérubé, A., Yang, Q., Liu, S., & Krewski, D. (2008). Epidemiologic evidence of relationships between reproductive and child health outcomes and environmental chemical contaminants. *Journal of Toxicology and Environmental Health, Part B*, 11(5-6), 373-517.

Wu, S., Hu, S., Fan, W., Zhang, X., Wang, H., Li, C., & Deng, J. (2022). Nitrite exposure may induce infertility in mice. *Journal Of Toxicologic Pathology*, 35(1), 75-82.