Teratogenic Effects of Dietary Genistein and Daidzein are Mediated by Over regulation of *Oct-4* and Down Regulation of *Cdx2* Expression in Post Implantation Albino Rat Embryos

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***Abstract*—**Phytoestrogens are a class of endocrine disruptors that have been implicated in birth defects associated with hormone-dependent development. This study demonstrates the effects of dietary phytoestrogens at prenatal time on development of rat embryos. Thirty four female rats were divided into 2 groups; G1 (control) and G2 (High phytoestrogens). Implantation rate, expression of *Oct-4*, *Cdx2* in embryonic and uterine tissues, fetal teratology and serum alkaline phosphatase activity (ALP) were determined. Results revealed significantly decreased implantation rate, *Cdx2* expression and ALP while increased *Oct-4* expression at GD7 in treated group. Gross and skeletal malformations beside visceral abnormalities in foeti of treated group were observed. Serum ALP was significantly increased in foeti of dams treated with phytoestrogens than control. These effects could be attributed to the estrogenic action of phytoestrogens during embryonic development beside their down regulating effect on *Cdx2* that failed to down regulate *Oct-4* embryos around time of implantation.

Keywords*—Cdx2*, implantation, *Oct-4*, phytoestrogens, teratogenic effect.

I. Introduction

PHYTOESTROGENS are plant-derived, non-steroidal molecules that have structural and functional similarity to 17β-estradiol [1]. Phytoestrogens are present in high concentrations in soy and alfalfa products. Some well-known phytoestrogens are coumestrol from alfalfa and soy isoflavones; genistein, daidzein and daidzein's intestinal metabolite equol [2]. The increase in consumption of soy products raises concerns about the effects of increased prenatal exposure to phytoestrogens. Like 17β-estradiol, phytoestrogens have an affinity for estrogen receptors alpha and beta. However, most phytoestrogens have a greater affinity for the beta estrogen receptor than the alpha estrogen receptor [3]. The endocrine disruptor hypothesis proposes that exogenous compounds can interfere with endocrine function by altering the binding, release, or metabolism of endogenous hormones [4].

Caudal type homeobox-2 (*Cdx2*), a caudal-type homeodomain transcription factor (TF), has been reported to be specifically expressed in trophoectoderm (TE) at blastocyst stage, and expression is maintained within the proliferating extra-embryonic endoderm ExE. *Cdx2* is the earliest TF identified so far to be involved in specification of TE fate, formation of placenta [5]. Also it is required for repression of *Oct-4*/Nanog and normal blastocyst development [6].

The class V Pit-1, Octamer binding transcription factor (*Oct-4*), Unc-86 POU domain transcription factor (*Oct-4*) is a critical regulator of pluripotency in the mammalian embryo and is expressed in unfertilized oocytes, the inner cell mass ICM and epiblasts of pre-gastrulation embryos and in primordial germ cells [7], [8]. *Oct-4* is considered to be the important regulator, which is required throughout the embryonic development as well as for embryo survival [9].

Biological actions of isoflavones are manifold and include several physiological systems. Therefore, the impact of isoflavones on physiological processes in the organism seems to be very complex and may be related to large number of factors, which are not satisfactorily identified yet. Despite increasing number of studies, there is still a long way to a firm knowledge on the biological potency of isoflavones and their impact on human and animal health. Nevertheless, isoflavone phytoestrogens are very promising substances that may provide us with new ideas on the mechanisms of physiological regulations and therapeutic interventions [10]. Since phytoestrogens have the ability to mimic estradiol activity by binding to estrogen receptors, phytoestrogens could alter the physiologic and morphologic development of prenatal offspring [11], [12]. Therefore, this work aimed to

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investigate the effect of dietary phytoestrogens on implantation rate, *Cdx2* expression as crucial regulator for implantation and *Oct-4* regulation, Oct-4 expression as pluripotency determinator, teratogenic effects including (body weight & CVL, skeletal, visceral, histopathological studies) and serum alkaline phosphatase activity in GD20 foeti .

II. Materials And Methods

A. Animals and experimental design:

A total of 51 Albino rats, 34 regular cyclic females and 17 males were purchased from Lab. Animal House, National Research Center, Dokki, Cairo. They were kept in metallic cages, the females were housed separately five per cage and males were housed separately in one large cage. Animals were kept at room temperature under natural day light rhythm. Food and water were allowed *ad libitum*. Animals were kept for 2 weeks for acclimatization before starting the experiment and fed casein based diet. The females will be divided into two groups: Group I, control group, n= 10, They were fed on a casein based diet and Group II, receive high soy (26%) phytoestrogens diet, n= 10. Soybean was autoclaved at 110^0c for 30 minutes according to Westfall and Hauge [13] to inactivate trypsin inhibitor, tannins, saponins, phytate, protease inhibitors, lectins and goitrogens. Both diets were formulated to fulfill all the nutritional requirements of adult rat according to NRC, 1995 [14] and were offered for 30 days. Males were offered control diet.

B. Isoflavones extraction:

Isoflavones, genistein and Daidzein, were extracted from the diet according to the method described by Thiagarajan et al., [15]. Concentrations of both isoflavones were analyzed in the extract by HPLC [15] using Genistein, HPLC standard, (Cas No. 446-72-0, Applichem GmbH Co., Germany) and Daidzein, HPLC standard (Code No. 308-05871, Fullcco Co., Japan).

C. Breeding procedures , feed intake and body weight:

Mature males were introduced with proestrous virgin females in a ratio (one male / two females / cage) at the afternoon for overnight. Mating was confirmed by the presence of sperm in the vaginal smears or the presence of vaginal plug and this was designated as the zero day (D0) of pregnancy [16]. The pregnant females were removed from the mating cages and rehoused in separate cages, each contained 5 animals. Feed intake was recorded from zero day of pregnancy till gestation day 20 (GD20). Female rats were kept under daily observation along the gestation period. At the end of 20th day of pregnancy the females were weighed then sacrificed to obtain foeti and their placenta from gravid uteri.

D. Implantation rate at GD7:

Fetomaternal junctions and post implantation embryos were dissected from 7 females from each group (control and high phytoestrogens) at gestation day 7 (GD7). Before immersion in 10% neutral buffer formalin saline; Number of corpora lutae (CL) was count under stereo microscope. Implantation sites were also count to determine implantation rate.

Implantation rate (%)= number of implantation sites / total number of CL x 100.

E. Immunohistochemistry:

Sections were dewaxed in xylene, rehydrated and pretreated with 3% hydrogen peroxide for blocking endogenous peroxidise activity. Microwave –assisted antigen retrieval was then performed for 20 minutes. Sections were incubated overnight at 4^oC with the corresponding antibody (Primary antibody for *Cdx2* (Cat. No. 3977 New England BioLabs GmbGH Co., Frankfurt- Germany) and *Oct-4* (C52g3) Rabbit mAb (Cat. No. 2890, New England BioLabs GmbGH co., Frankfurt- Germany).

Cdx2 and *Oct-4* antibodies were added after dilution by PBS (1:300 and 1:600 respectively). Biotinylated polyvalent secondary antibody (Cat. No. 32230, Thermo Scientific Co., UK) was applied to tissue sections and co-incubated for 30 minutes after washing. The reaction was visualized by adding Metal Enhanced DAB Substrate according to Bancroft & Cook [17]. For quantitative analysis, the intensity of immunoreactive parts was used as a criterion of cellular activity after substracting background noise. Measurement was done using an image analyzer (Image J program). From each slide of both experimental groups, 9 fields were randomly selected. The integrated density (IntDen) of nine random parts within each field were analyzed and the mean for them was expressed as field IntDen.

F. Teratogenic investigation:

The foeti were pulled out from uterus and counted. The number of implantation and resorption sites was examined by magnifying lens. The implantation sites appear as black spot after being immersed in 10% ammonium sulphide for 20 minutes [18]. Foetal body weight and placental weight were recorded. Fetal crown-vertebral length (CVL) was determined, the foeti were examined for any gross external malformations.

The foeti obtained from each dam were classified into three groups, the first group was eviscerated and put in absolute ethyl alcohol to be stained alizarin red 2% (Cas No. 130-22-3, Sigma- Aldrich Co., Egypt) and examined according to Hayes [19]. The second group was injected intraperitoneally with 0.2 ml Bouin's solution, and then kept in glass jar containing Bouin's solution to examine gross and visceral abnormalities. The third group was injected subcutaneously by 0.2 ml of 10% neutral buffer formalin and kept in glass jar containing 10% neutral buffer formalin with placentae after recording their weight for histopathological examination [19].

G. *Histopathological examination:*

Formalin fixed foeti and their placenta were dehydrated in gradiance of alcohol and finally embedded in paraffin wax. Serial sections of 5 µm were stained with hematoxylin and eosin then examined by light microscopy [20].

H. *Serum alkaline phosphatase activity:*

Fetal blood was obtained by an axillary incision and serum samples from each offspring were pooled within the same litter and stored at -20°c. Alkaline phosphatase activity was determined using commercial kit (Cat. No. 92214, Biolabo reagents Co., Maizy, France) by colorimetric end point method, according to Tietz, [21].

I. *Statistical analysis:*

All data in the present study were expressed as mean ± SE. they were subjected to student T test using SPSS® software (Statistical Package for Social science, version 17.01, IIIinois, USA). The probability criterion for significance was P> 0.05 and P<0.01 for high significance.

III. Results

HPLC analysis to the experimental diet reveals that; control diet contains 45 µg/g genistein and 28 µg/g daidzein respectively, while the high phytoestrogens diet contains 1320 µg/g genistein and 704.7 µg/g daidzein, respectively.

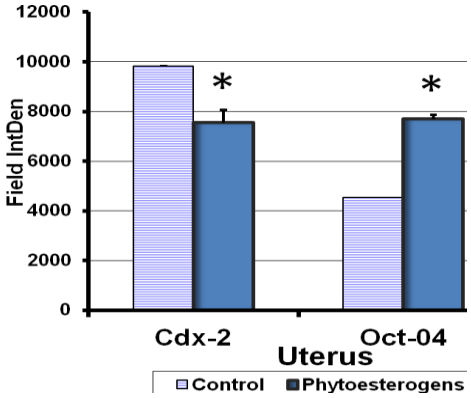

Fig.1 Effect of high dietary phytoestrogens on expression of *Cdx2* and *Oct-4* in gravid uterus at GD7.

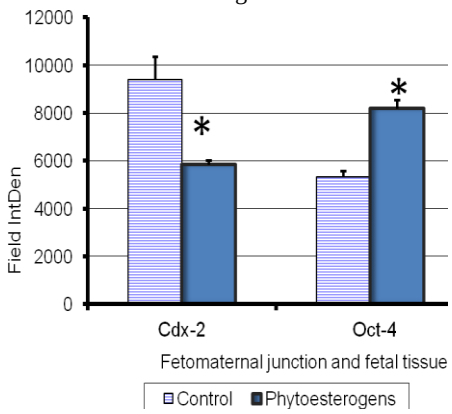

Fig.2 Effect of high dietary phytoestrogens on expression of *Cdx2* and *Oct-4* in fetomaternal junction and fetal tissue at GD7.

Body weight and feed intake of high phytoestrogens-fed dams at GD20 were significantly (P<0.05) lower than control. Implantation rate of maternally treated foeti with high phytoestrogens showed significant (p<0.01) reduction than control (Table I). Uterine and fetomaternal junction *Cdx2* expression was significantly (P<0.01) decreased in high phytoestrogens-fed group when compared to control. On the other hand, *Oct-4* expression (in uterus, fetomaternal junction and fetal tissues) was significantly (P<0.01) higher in high phytoestrogens fed group than control at GD 7 (Fig. 1, 2 &5).

TABLE I
EFFECT OF DIETARY PHYTOESTROGENS ON FEED INTAKE FROM ZERO DAY TILL GD20, BODY WEIGHT (AT GD20), NUMBER OF CORPORA LUTAE, IMPLANTATION RATE (%) AT GD7 IN PREGNANT FEMALE ALBINO RATS.

Parameters /group	Control	High phytoestrogens
Feed intake from 0 day till GD 20 (g)	232.80±13.50	211.65±12.71*
Body weight of pregnant rats (g)	235.50±10.76	225.25±2.99*
Number of C. L/ dam at GD7	9.5±0.58	10.8±0.42
Implantation rate % at GD7	95.60±2.10	79.14±5.71**

* Significant at (P<0.05).
** Highly significant at (P<0.01).

Foetal weight and CVL were decreased with higher significance (P<0.01) in high phytoestrogens treated foeti through placenta than control. Placental weight showed non-significant difference between both high phytoestrogens treated dams and control (Table II).

Alizarin red stained foeti showed wide opened fontanella, incomplete ossification of cranial bone(Fig. 3D), absence of sternebrae (Fig. 3F), phalanges of fore and hind limbs (Fig. 3D) and absence of coccygeal vertebrae (Fig. 3E) in foeti treated with dietary phytoestrogens through placenta compared with control foeti who showed normal features (Fig. 3A-C). Gross examination of Bouin's fixed foeti revealed dome shaped head ill marked ear pinna was evident and the eyes were pin like shaped (absent eye placode) (Fig. 4B). The visceral examination of maternally treated foeti on GD 20 revealed the following abnormalities; enophthalmia and microphthalmia (Fig. 4F). Unilateral or bilateral renal agenesis (Fig. 4H) and hypoplasia of foetal lungs (Fig. 4D) were evident.

TABLE II
EFFECT OF MATERNAL DIETARY PHYTOESTROGENS ON NUMBER OF CL/ DAM AT GD20, FOETAL WEIGHT (G), PLACENTAL WEIGHT (G), CVL (CM) AND FETAL ALKALINE PHOSPHATASE (IU/ L).

Parameters /group	Control	High phytoestrogens
Number of CL/ dam at GD20	10.10±0.40	11.20±0.23
Foetal weight / g	3.92±0.04	2.45±0.10**
placental weight / g	2.16±1.58	0.49±0.03
CVL / cm	4.46±0.03	2.74±0.17**
Fetal alkaline phosphatase IU/ L	129.65±4.71	367.31±20.74**

** highly significant at (P<0.01)

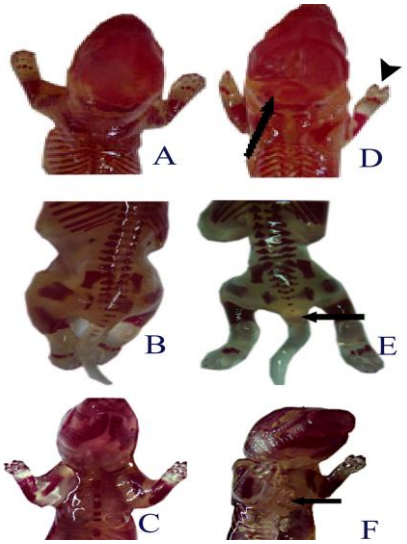

Fig. 3 Skeletal system of control rat foeti at GD20 stained with alizarin red (A-C). (A) normal head and phalanges; (B)coccygeal vertebrae; (C) normal sternebrae. Skeletal system of maternally treated rat foetus with high dietary phytoestrogens at GD20 stained with alizarin red (D-F). (D) showing wide open fontanella (arrow) and absence of phalanges (arrow head); (E) showing absence of coccygeal vertebrae (arrow); (F) showing absence of sternebrae (arrow).

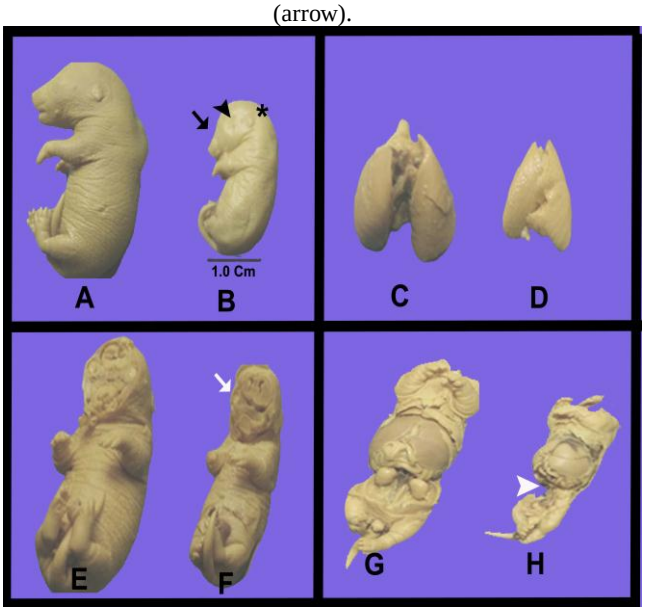

Fig. 4 photographs of control rat foeti (A,C, E, G) and maternally treated rat foeti with high dietary phytoestrogens diet (B, D, F,H) at GD20. (A) normal foetus; (B) showing dome shaped head (arrow), absence of eye placode (arrow head) and ill marked ear pinna (astrick); (C) normal lung; (D) hypoplastic and collapsed lung; (E) normal eye cut section; (F) microphthalmia and enophthalmia (arrow); (G) normal kidney, (H) bilateral agenesis of kidneys (arrow head).

Histopathological examination of maternally treated foeti and placenta revealed the following abnormalities: Foetal liver showed congestion, dilatation of sinusoids, necrosis in hepatocytes and karyolysis (Fig. 6B). Foetal lungs showed variable degrees of degeneration, hemorrhage exudates in the lumina of alveoli and thickening of the alveoli and inter alveolar septa with persistent fetal epithelium lining the alveoli (Fig. 6D). Foetal kidney showed hemorrhagic areas especially in the cortical region, the glomeruli were dilated and renal tubules exhibited necrosis and cloudy swelling (Fig. 6F). Foetal testes showed degeneration in some spermatogonia as well as karyolysis in some spermatogonia (Fig. 7B). Foetal ovary showed marked reduction in size, hemorrhagic areas and degeneration in most oocytes (Fig. 7D). Foetal cartilage showed degeneration in chondrocytes besides hemorrhage (Fig.7F). Placenta showed degeneration of the basal layer, necrotic trophoblasts within the labyrinthine layer and hemolysed blood cells (Fig. not shown). Alkaline phosphatase level activity was significantly ($P<0.01$) higher in foeti maternally treated with soy phytoestrogens than those of control.

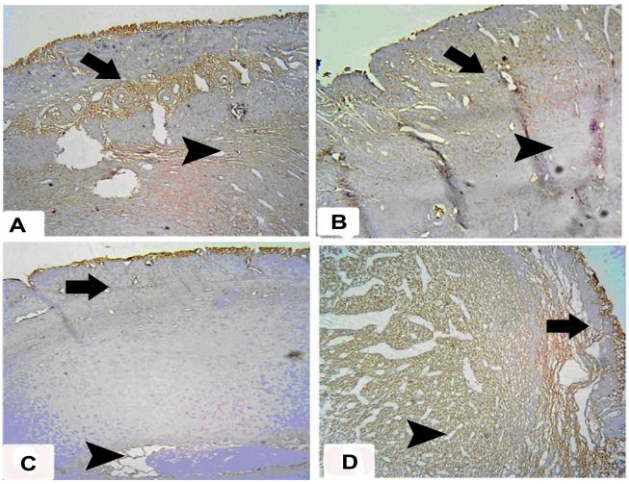

Fig. 5: Immunohistochemical sections in uterus, fetomaternal junction and fetal tissue of albino rat at GD7 showing expression of *Cdx2* and *Oct-4* (A-D). The intensity of *Cdx2* immunostaining in control rats (A) is higher at both fetomaternal junction (arrow head) and uterus (arrow) than high dietry supplemented phytoestrogens group (B). On the other hand immunostaining of *Oct-4* is more deeper in uterine (arrow), fetomaternal and embryonic cells (head arrow) of high dietry supplemented phytoestrogens group (D) while control is more lighter. X100

IV. Discussion

Administration of high phytoestrogens diet containing 1320 µg/g genistein and 704.7 µg/g daidzein, respectively leads to variable changes in dams and offsprings. The maternal mortality, which is also a sign of toxicity, didn't occur in the present study. However, other variables can be indicative of subtler adverse effects, such as alterations during the treatment in the body mass and the pregnancy parameters. Ingestion of dietary phytoestrogens in this study caused significant ($P<0.05$) reduction in feed intake associated with significant reduction in body weights of pregnant dams at GD20. These results are in agreement with those of Casanova et al.[22], Flynn et al. [23] and Delclos et al. [24] who found a significant reduction in body weight gain at high concentration of genistein administration. While they disagree with Odum et al.[25] and Soucy et al. [26] who found that repeated administration of genistein and ingestion of phytoestrogens in diet by pregnant female rats had no

effect on maternal body weight nor their feed intake. Reduction in feed intake may be due to the appetite repressing action or anorectic effect of estrogen [27], [28] as high dietary phytostrogens decreased feed intake and hence decreased body weight. The decrease implies that the estrogenic hormone action of phytoestrogens is beneficial to body fat regulation and the decreased level of leptin that is produced in adipose tissue that influences hypothalamic neuropeptide Y (NPY) levels which regulates feeding behaviour [29].

Ingestion of high phytoestrogens diet in this study significantly decreased implantation rate and expression level of *Cdx2* associated with higher expression of *Oct-4* in both uterus and fetomateal junction at GD7. *Cdx2* is required for correct cell fate specification and differentiation of TE [6]. These results suggested that the embryonic losses occurred prior the implantation period could be attributed to the lower rate of *Cdx2* expression that failed to down regulate *Oct-4* expression which is required for proper implantation and placentation. Both *Cdx2* and *Oct-4* show reciprocal pattern of expression in early embryonic life [30]. Down regulation of *Oct-4* in outer cells leading to TE lineage and maintenance of ICM appears to be one of the crucial events enabling proper preimplantation and embryo development [7].

The teratogenic effects of dietary phytoestrogens were detected in the present study via body weight and CVL, skeletal, visceral and histopathological studies. The results revealed That prenatal exposure to dietary phytoestrogens produced decrease in both CVL and foetal weight with higher significance ($P<0.01$) than control that may be due to crossing of dietary phytoestrogens through placenta to cause its effects on embryos [26]. The reduced fetal weight and CVL creates a wider level of contacts with teratogens and interfere with the normal development of the embryos inducing anomalies [31].

At skeletal level the study shows clear teratogenic effect of dietary phytoestrogens which clarified by incomplete ossification of cranial bones, absence of sternaebrae, phalanges and coccygeal vertebrae. Delaying in ossification of foetal skeleton in high phytoestrogens-fed group can be attributed to blockage of gene expression in osteoblast [32], [33]. Also high dietary phytoestrogens caused increase in fetal serum ALP activity (increased to about 2 folds) which is indicative for bone degenerative changes. In the presence of high levels of endogenous estrogenic activity, as in case of the present study, genistein and daidzein may interrupt ER-derived increase in ALP activity. Further studies are necessary to define the nature of these interactions that may help to develop a prognostic indicator and treatment for these abnormalities. It may also to work out new screening tests that would allow us to identify women or female animals that are particularly predisposed for bearing a handicapped offspring.

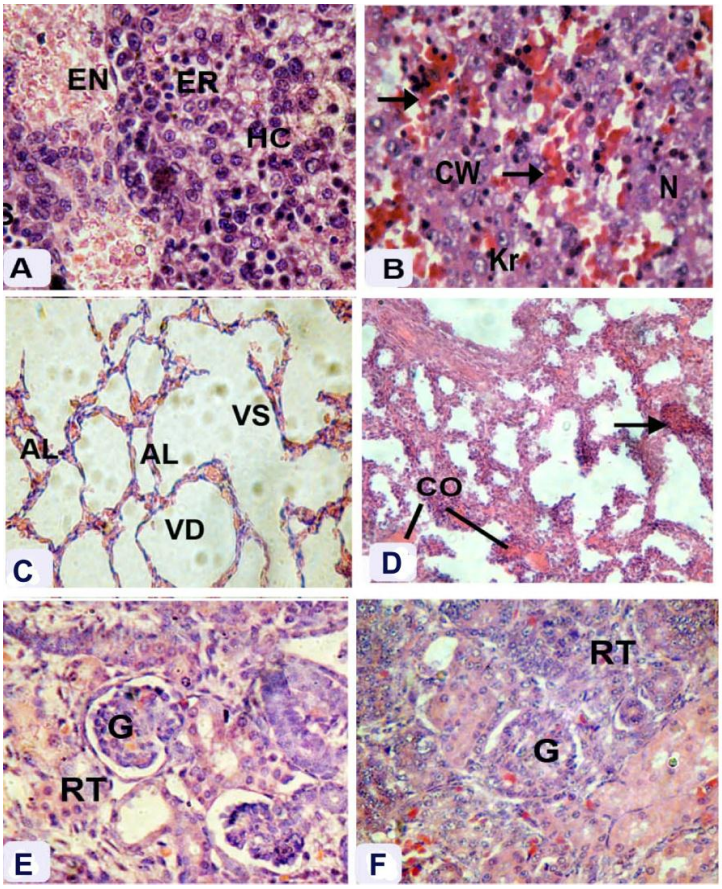

Fig 6 . Histopathology of rat featus organs at GD 20. (A) section of control rat foetus liver, showing blood sinusoids (S) containing erythroblast (ER) and hepatic vein lined with endothelial cell (EN). (X400). (B) liver section of maternally phytoesterogen-treated rat foetus showing congestion sinusoids with hardly distinct endothelial lining (arrow), beside karyolysis(KR) of most hepatocytes nuclei. (X400). (C) lung section of control rat foetus (GD19), showing alveolar duct (VD), alveolar sac (VS), alveoli (AL) and blood vessels. (X200). (D) lung section of maternally phytoesterogen-treated rat foetus showing congested vessels (CO), thickened alveolar walls and interalveolar septa and hemorrhage (arrow) in pulmonary tissue. (X200). (E) kidney section of control albino rat foetus, showing glomerulus (G) and normal renal tubules (RT). (X400). (F) kidney section of maternally phytoesterogen -treated rat foetus showing diltation of glomerular capillary (G) and necrotic renal tubules (RT). (X400)

At visceral level dome enophthalmia and microphthalmia were observed in embryos. Also hypoplasia of foetal lungs and unilateral or bilateral absence of kidney as well as absence of ureter were evident. They are consistent with a possible disruption of physiological functions. In any case, interpretation of these changes could be attributed to the estrogenic action of phytoestrogens during embryonic development or may be due to increased *Oct-4* expression. Where a critical amount of *Oct-4* is required to sustain stem cells self-renewal and up or down regulation induces divergent developmental programme [34]. Also transcriptional activation of *Oct-4* could alter a lot of genes or

interacts with another transcription factors regulating self renewal and pluripotency negatively or positively [8], [35].
Concerning the surprising effect of dietary phytoestrogens on urinary system including aplasia of kidney and ureter that could be explained by the vital role played by ERs in urinary system [36]. This point requires further investigations to clarify the mechanism by which phytoestrogens as SERM can play a role in development of urinary system.

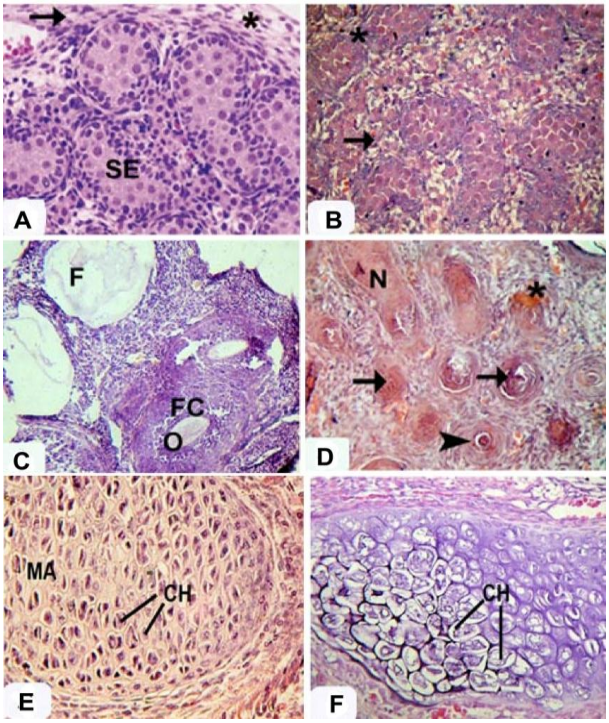

Fig . 7 Histopathology of rat featus organs at GD 20. (A) testes section of control albino rat foetus (GD20) showing a thick tunica albugenea (astrick), tunica vasculosa (arrow) and seminiferous tubules (SE). (X200). (B) testes section of maternally phytoesterogen-treated rat foetus showing alteration of the general architecture of seminiferous tubules and disorganization of the germinal epithelium (arrow head). (X200). (C) ovary section of control albino rat foetu showing ovarian follicles (F) contain oocyte (O) surrounded by follicular cells (FC). (X200). (D) ovary section of maternally phytoesterogen-treated rat foetus showing hemorrhagic areas (astrick), necrosis of follicular cells (N), severely damaged oocytes (arrows) and attretic oocytes (arrow head). (X200). (E) sternum cartilage section of control albino rat foetus (GD20) showing chondrocytes (CH) and matrix (MA). (X200). (F) sternum cartilage section of maternally phytoesterogen-treated rat foetus showing chondrocyte degeneration (CH). (X200).

Effects of phytoestrogens on visceral level were noted and confirmed by the histopathological findings that revealed congestion, and variable degrees of degeneration in liver, lungs, kidney and cartilage of maternally treated foeti. Moreover foetal testes and ovaries showed germ cells degeneration. These results provide an additional evidence that dietary exposure to oestrogenic compounds as phytoestrogens could adversely affect reproductive organs development and the future fertility of the offsprings. As phytoestrogens, as endocrine disruptors, could reduce androgen [37], [38], [39] and estrogen production by altering aromatase activity [39] during fetal life. where during development of reproductive system androgenic and estrogenic hormones were needed to exert their organizational effects that permanently shape the reproductive system and its function. Moreover, phytoestrogens produced degenerative and necrotic changes in placenta.

V. Conclusion

Prenatal exposure to soy phytoestrogens produced a reduction in implantation rate and teratogenic effect in offsprings. These effects could be attributed to the estrogenic action of phytoestrogens during embryonic development beside their down regulating effect on *Cdx2* that failed to down-regulate *Oct-4* embryos around time of implantation.

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