World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086

Available online at: http://www.wjpsonline.org/
Original Article

Folic acid administration reduced selenite- induced abnormalities in the egg- cylinder of pregnant rat

Abdel- Fattah M. El- Shershaby[1] and Mai H. El- Dakdoky[2*]

[1]Zoology Department, Faculty of Science, Cairo University, Cairo, Egypt
[2]Zoology Department, Women's College for Arts, Science& Education, Ain Shams University, Cairo, Egypt

Received: 08-01-2015 / Revised: 04-02-2015 / Accepted: 04-02-2015

ABSTRACT

The present study was undertaken to evaluate the efficacy of folic acid in preventing selenium provoked alterations in early embryonic development. Pregnant rats were divided into four groups; control group, folic acid (FA) treated group (0.7 mg/kg b.wt.), sodium selenite (Se) treated group with 5 µg/ kg b.wt. and co-treated group with Se and FA at the same dosages. Dams were treated daily from gestational day zero to day 7 by gastric intubation. All animals were sacrificed on the eighth day of gestation at 10 a.m. Sodium selenite resulted in loss of maternal body weight, decrease in number of pregnant rats and relatively increase in number of pre-implantation loss. Also, sodium selenite caused retardation of the embryonic development as well as increase in the number of deformed, degenerated and aborted embryos. Folic acid supplementation reduced selenite-induced developmental abnormalities.

Key words: Egg cylinder; Folic acid; Implantation; Preimplantation loss; Sodium selenite.

INTRODUCTION

Selenium is an essential dietary element for health. It is important to reproduction due in part to its antioxidant activity via the selenoprotein glutathione peroxidase (GPx) [1]. Low concentrations of selenium are required for normal growth and development, moderate concentrations can be stored and maintain homeostatic functions, and elevated concentrations can result in toxic effects. Activity from industries and agriculture has accelerated the release of selenium from geologic sources, which could be absorbed by animals from contaminated food and water [2]. The toxic effect of selenium on wildlife can be increased by bioaccumulation and biomagnifications [3]. For example, Se tissue burden in the ovaries and liver of vitellogenic San Francisco Bay Delta white sturgeon were comparable with levels previously shown to cause reproductive toxicity in dietary Se experiments with captive white sturgeon [4].

Over dose exposure to selenium led to developmental toxicity in various species; selenate showed teratogenic potential in zebrafish (Danio rerio) embryos [2]. It was suggested that Se bioaccumulation was the cause of death and deformity of embryos of waterfowl nesting at a reservoir contaminated with Se [5]. Embryolethal effect and growth retardation were also observed by sodium selenite administered daily to pregnant rats from day 7 to day19 of gestation [6]. Most reproductive studies reported the effect of Se and other chemicals on mothers and fetuses during gestation period or after parturition [6- 10], but nothing was mentioned in the literatures concerning the effect of selenium on the structure of the cylindrical embryos of pregnant rats. Toxic chemical exposure during the preimplantation period, between conception and implantation, however, does not result in malformations or intrauterine growth retardation, but can cause the death of the blastocyst. It is assumed that since the cells of the blastocyst are totipotent, damaged cells can be replaced, up to a certain point, by undamaged ones [11,12]. By contrast, the postimplantation embryo does manifest adverse effects of chemical exposure resulting in resorption, varying types of malformation and delayed development seen as retarded or sub-optimal growth [12].

The mechanism of selenite toxicity may be derived from its sulfur-like chemical characteristics and its

***Corresponding Author Address: Mai H. El- Dakdoky,** *Zoology Department, Women's College for Arts, Science& Education, Ain Shams University, Cairo, Egypt;* **E-mail:** *mhgawad11@yahoo.com*

tendency to substitute for this element in amino acids during proteins assembly [13]. Also, it was suggested that selenium toxicity may be a result of oxidative damage [14].

Folic acid (FA) is a water-soluble B-complex vitamin. It is an essential methyl donor and is used for the generation of endogenous methionine [15]. It cannot be synthesized *in vivo* but instead it is absorbed in the body from green leafy vegetables and citrus fruits. Many studies have shown that 50-70% of neural tube defects can be prevented by folic acid supplementation before and during pregnancy [16, 17]. Additionally it has been reported that FA may prevent other birth defects such as heart defects, cleft lip and palate, limb deficiency and urinary tract anomalies [18-20]. The United State Department of Public Health, the Centers of Disease Control and Prevention recommended that women who are expected to be pregnant should take a FA supplement 400µg per day. Furthermore, FA has been defined as an effective free-radical scavenger [21, 22]; its supplementation can reduce the production of the superoxide radicals [23].

Therefore, the present investigation was undertaken to study the effect of selenium on early pregnancy in rat and to elucidate whether folic acid treatment is effective in preventing selenium provoked alterations in early embryonic development.

MATERIALS AND METHODS

Chemicals used: Sodium selenite is a colourless, water soluble salt. Its chemical formula is Na_2SeO_3, and its molecular weight is 172.94. The chemical purity of the compound is 99%. Sodium selenite was purchased from Sigma- Aldrich CAS: 19142-15-3, St Louis MO, USA. Sodium selenite was dissolved in distilled water, given orally at dosage of 5 µg/kg b.wt. according to Helal [6]. In addition, folic acid was obtained as a tablet from The Nile Co. for Pharmaceuticals and Chemical industries, Egypt. It was dissolved in distilled water and given orally at a dose of 0.7 mg/kg b.wt. according to Zhao et al. [24].

Animal Treatment and Biological Sample Preparation: Thirty two adult female Wister rats, weighing 170- 180 g, and fifteen sexually mature male of the same strain (180- 200 g) were obtained from the central animal house of the farm animal of the Egyptian Organization for Vaccine and Biological Preparation at Helwan- Egypt and maintained on a 12:12 light/dark cycle with a temperature of 25 ºC. Standard chow and tap water were available ad libitum. Experiment was carried out according to the internationally valid guidelines and the institutional animal ethics committee. After two weeks of acclimatization two females rats were housed overnight with a mature male rat. The day of sperm detection in the vaginal smear was considered to be day 0 of gestation. Pregnant rats were weighed and randomly divided into four groups, eight rats each. Group (1) control group received 0.5 ml of distilled water by gavages, group (2) administered orally folic acid (FA) at 0.7 mg/kg b.wt., group (3) received 5 µg/kg b.wt. of Sodium selenite (Se) by gavages and group (4) administered both Se and FA together in the same manner and dosages. Doses were given daily, in the morning at 10 a.m., on gestational days (GD) 0 to GD 7. The dosing volume was 0.5 ml/rat. All females were observed daily for clinical signs of toxicity. Maternal body weights were recorded on GD 0 and GD8. All animals were sacrificed by cervical dislocation on the eighth day of gestation at 10 a.m. The ovaries and uteri were dissected out; the number of implantation sites (uterine swellings) was counted along with corpora lutea in both ovaries. The percent of pre-implantation loss was calculated as ((number of corpora minus number of implants)/number of corpora) x 100. The implantation sites, with early embryos, were fixed in 10% neutral formalin. Specimens were then dehydrated in alcohol, cleared in xylol and embedded in paraffin wax. Then sectioned at 5µm thickness and stained with haematoxylin and eosin (H&E) [25].

Statistical analysis: Statistical analysis was performed using the statistical package for social science (SPSS, Chicago, IL) version 17 statistical software. Data were analyzed by one-way analysis of variance followed by post hoc-least significant difference analysis, the level of significance was set at $p < 0.05$. Data were expressed as the mean ± standard error (SE).

RESULTS

Clinical signs including decreased activity, emaciation, soft stool and body weight loss were observed in Se as well as Se and FA co-treated rats. The results also indicated that all the mated control and folic acid treated females became pregnant and showed normal number of corpora lutea and implantation sites. Treatment with 5µg of sodium selenite from GD 0 to GD7 caused partial inhibition of pregnancy wherein 3 out of 8 rats were not pregnant. The uteri of 2 out of 5 pregnant rats showed internal bleeding and signs of abortion. There was a non-significant decrease in the number of implantation sites in Se treated group when compared with that of the control or FA treated group. The pre-implantation loss was approximately 10 % in the control or FA- treated

group, but it was 18 % in the group treated with sodium selenite. Co-treatment with FA ameliorated the increase of pre-implantation loss (approximately 11 %) and reduced the internal uterine bleeding (one case out of 7 pregnant rats) mediated by selenite (Table1 and Figs. 1&2).

Microscopical examination of the embryo obtained from control or FA treated dam at its maximum diameter on day eight of pregnancy at 10 a.m. showed normal cylindrical embryo embedded in a mass of spongy decidual tissue and is consisted of three main regions; ectoplacental cone, extraembryonic region and embryonic region (Figs. 3-7). The latter two regions consist of an inner ectoderm and an outer proximal endoderm. The inner ectoderm is divided into an extraembryonic ectoderm and an embryonic ectoderm the former consisting of spherical or ovoid cells located at the mesometrial half of the egg-cylinder (Figs. 5& 6). The embryonic ectoderm consists of pseudostratified layer of low columnar cells with regular ovate nuclei, located at the antimesometrial half of the embryo (Fig. 7). In the center of the ectoderm, the proamniotic cavity is present. The proximal endoderm at the lower half of the embryonic region consists of a single layer of cells which are flat in section, except when they became rounded during division (Fig. 7). The extraembryonic endoderm differs from the lower embryonic endoderm in that the cells are columnar in shape, with an orientation along the radial axes of the egg cylinder (Figs. 5& 6). The distal endoderm and the trophectoderm are in- close contact with each other and are located around the circumference of the whole embryo. Cells of these two layers are flattened or cylindrical in shape having eosinophilic cytoplasm and large ovoidal basophilic nuclei. They grow distally and became hardly distinguishable from each other and from the decidual cells. Some trophectodermal cells showed dense basophilic bodies in their cytoplasm probably representing dead uterine epithelial cells at this stage of development. The ectoplacental cone is formed mostly from cells of the trophectoderm and extraembryonic ectoderm. Cells of the ectoplacental cone exhibited round or irregular outlines. They showed less basophilic cytoplasm, markedly basophilic nuclei and prominent nucleoli (Fig. 4). The yolk sac cavity is found around the egg -cylinder and lined with the proximal and distal endoderm. In all embryonic layers, few cells are seen containing large vacuoles with collapsed cytoplasm and dense basophilic bodies, probably abnormal chromosomes. Also dense basophilic dead cells with pyknotic nuclei are frequently recognized. In the embryonic ectoderm, the dead cells are seen pushing their way to the proamniotic cavity. Mitotic cells are commonly observed in all layers. Also, few disintegrated decidual cells are observed very near to the developing embryo.

Microscopical examination of the rat egg- cylinder obtained from Se treated dams on day eight of pregnancy at 10 a.m., showed various degrees of degeneration varying from minor to severe conditions. Some of the examined embryos exhibited more or less normal forms which were histoarchitecturally similar to those obtained from normal pregnancy at 10 a.m. Other embryos showed developmental retardation with numerous disintegrated cells, ghost cells and cell debris in the proximal endoderm and extraembryonic ectoderm (Fig 8). Abnormal dissociated embryonic ectodermal cells with large intercellular spaces were also observed. In addition, massive haemorrhage and many inflammatory cells were observed within the deciduoma at both mesometrial and antimesometrial poles of the embryo. In some other retarded embryos the embryonic ectoderm was in continuous around the proamniotic cavity of this layer, representing a stage of development just before the studied stage (Fig. 9). Some other examined embryos showed deformed histoarchitecture with no proamniotic cavity and instead some cells containing large irregular vacuoles filled all the cytoplasmic area were found along the dorsoventral axis of the egg-cylinder (Fig. 10). These embryos showed in the ectoplacental cone a considerable number of dead cells with highly vacuolated and degenerated cytoplasm and pyknotic nuclei (Fig. 11). Many cells of the extraembryonic and embryonic ectoderm as well as the proximal endoderm were vacuolated with pyknotic nuclei (Figs. 12-14) and other cells showed mass of densely stained dead or dying cells (Fig. 13). Haemorrhagic batches were clearly observed within the deciduoma around the circumference of the deformed embryos. Other specimens showed completely deteriorated embryos with indistinguishable embryonic layers and detached ectoplacental cone from the uterine wall (Fig. 15). In such case, the degenerated egg-cylinder was represented by very dense dead basophilic cells; many of them are disintegrated in the yolk- sac cavity. Some decidual cells showed vacuolar degeneration and some others showed pyknotic nuclei. In some specimens, uterine lumen was seen filled with destructed decidual tissue that invaded with masses of maternal blood, numerous leucocytes and macrophages denoting cases of abortion (Fig.2).

Co-treatment with selenium and FA revealed more or less normally developed embryos. In such case, no deformed or degenerated embryos were observed at this stage of development. The egg-

cylinder observed under this treatment showed normal extraembryonic and embryonic regions and well formed ecto-placental cone (Figs. 16-19). Most of the embryonic cells in all layers were viable cells. Low columnar cells of the proximal endoderm with brush borders were observed in the extraembryonic region of the embryo and flattened cells were found at the embryonic region of the egg- cylinder (Figs. 18&19). The distal endoderm and trophectoderm were observed in close contact to each other. Some trophectodermal cells at the antimesometrial pole of the embryo showed basophilic bodies in their cytoplasm (Fig.16). These bodies represent phagocytosed dead uterine epithelial cells. The ectoplacental cone and the decidual tissue were well developed and were similar to that found in normal pregnancy.

DISCUSSION

The results obtained in the present study suggested that treatment with 5 µg of sodium selenite for 8 days of early pregnancy seems to be an effective dose as 37.5% of the mated rats failed to be pregnant. Also, the results indicating that when sodium selenite is administered early after fertilization results in histoarchitectural abnormalities of successfully implanted embryos and caused partial abortion in some cases. Toxicity of Se has been previously reported in some animal species [4, 6, 26, 27]. Selenite increased the incidence of embryonic malformation and inhibited the embryonic growth of rat embryos culture at day 9.5 of gestation at dosage 20 µg M [28]. Exposure of zebrafish embryos to higher concentrations of selenite (>10 µM) caused delayed development, decreased hatching rate, mortality, and obvious malformation, including a bent trunk and tail [2].

In the current study, it was found that selenite administration at dose level 5 µg increased the number of retarded and deformed embryos with dissociated cells in some embryonic regions. It was suggested that cells of the developing embryos interact with each other by cell to cell contact and by production of biochemical messengers. This type of interaction provides a mean of coordinating the differentiation and the development of various cell types within the embryo [29]. Therefore, the death of cells as a result of toxicants exposure may affect normal development by disrupting the cell to cell communication. Thus, the loose and dissociated embryonic cells and little increase in the number of dead cells may cause retardation in growth and deformation of the embryo [30]. The current result also found that selenite exposure increased the number of egg- cylinders with numerous disintegrated embryonic cells, massive haemorrhage with leucocytic infiltration very near to and around the embryo and degenerated decidual cells. These embryos are probably subjected to resorptions as gestation proceeds. Helal [6] found that daily administration of sodium selenite at dose level 5 µg to pregnant rats from 7th to 19th day of gestation increased significantly the number of resorbed fetuses and post-implantation loss.

In the present study, dead cells were described more or less in all embryonic layers and these dead cells increased in Se treated mothers. This indicated that Se may cause a harmful effect on the embryonic layers and this may lead to loss of synchrony in the developmental process. The source of dead cells in the developing embryos might be the cells with minor chromosomal abnormalities and aberration that result from differentiation of other embryonic cells. This result was in accordance with that of Kent [29] who reported that cells are not only preprogrammed to replicate and differentiate, but also some cells are programmed to die at specific time. Also, this author revealed that exposure of mouse embryo to methyl- methane sulphate, which breaks DNA strands, spontaneously abort or produce small offspring. The current results also found that selenite treatment increased the number of completely destructed embryos and the number of hemorrhagic deciduas that separated from the rest of uterine wall denoting cases of abortion. In this context, Yonemoto et al. [7] reported that sodium selenite at 58.8 µg mol/kg caused abortion to pregnant mice treated at day 12 of gestation. It has long been recognized that the balance between cell proliferation and apoptosis is a key factor in embryogenesis; Ma et al. [2] observed abnormal proliferation and apoptosis in selenite-treated zebrafish embryos that may have contributed to the ultimate failure of embryonic development. Selenium has been reported to induce cell growth and cell proliferation, but also cell death by necrosis or apoptosis. The biological action of selenium is dependent on both its specific chemical form and concentration. At high levels, selenium induces oxidation and cross-linking of protein thiol groups and generation of reactive oxygen species, ultimately leading to cell death [26]. Spallholz [14] confirmed that Se toxicity induced oxidative damage.

On the other side, folic acid (FA) is one of the most commonly deficient vitamins in women. In the early 1990s, landmark studies provided strong evidence that 400 µg of daily FA consumption at least 1 month before conception and through the first trimester of pregnancy is effective in preventing both the occurrence and reoccurrence of neural tube defects (NTDs) [31]. Zhao et al. [24] found that FA in combination with soybean isoflavone reduced the malformation incidence rate

and increased fetus' development in pregnant rats exposed to cyclophosphamide. In the present result, folic acid treatment reduced the number of embryonic abnormalities and the number of degenerated embryos which were increased under the effect of Se administration. Such result was in agreement with Ma et al. [2] who demonstrated that FA protected zebrafish embryos from selenite-induced neural and cardiac defects. Several data indicated that selenite toxicity may be derived from its pro-oxidant ability to catalyze the oxidation of thiols and to produce superoxide simultaneously [14, 32, 33] also, Se toxicity may lead to imbalance of intracellular glutathione (GSH) redox [27]. It was reported that exogenous antioxidants as ascorbic acid reduced the embryotoxicity of selenium in culture rat embryos [34]. In *in vitro* and *in vivo* studies, Zhao et al. [24] found that FA in combination with soybean isoflavone improved parameters related to antioxidative stress in rat embryos exposed to cyclophosphamide. Joshi et al. [21] and Sahin et al. [22] reported that folic acid has been defined as an effective free-radical scavenger. Its supplementation can reduce the production of the superoxide radicals [23]. Moreover, folic acid are involved in the synthesis, repair, and functioning of DNA and are required for the production and maintenance of cells [35]. Therefore, folate plays an important role for cells undergoing rapid turnover, such as tissues in the developing fetus [36]. Thus, folic acid may be involved in the protective effect on selenite-induced embryonic toxicity by its antioxidant properties and by its highly preserving capacity on DNA.

In conclusion, selenite administration at early time after fertilization resulted in loss of preimplanted embryos and caused retardation of embryonic development as well as an increase in the number of deformed, degenerated and aborted embryos. Folic acid supplementation may reduce selenite-induced developmental abnormalities.

Table (1): **Initial and final body weight (g) and pregnancy outcome of pregnant rats on the 8th day of gestation of control (Cont), folic acid (FA), selenium (Se) as well as selenium and folic acid (SFA) treated groups (mean ± S.E.).**

	Cont	**FA**	**Se**	**SFA**
Initial body weight (g)	180.3 ± 7.23	178.0 ± 10.7	171.4 ± 6.6	171.5 ± 6.2
Final body weight (g)	193.2 ± 7.81	191.5 ± 10.78	159.0 ± 5.44^{a2b1}	162.87 ±7.72 ^{a2b1}
No. females with +ve sperm	8	8	8	8
No. pregnant rat	8	8	5	7
No. corpora lutea	10.25 ± 0.7	9.37 ± 0.60	9.80 ± 0.73	9.86± 0.86
No. implantation sites	9.13 ±0.48	8.38 ± 0.42	8.00 ± 0.84	8.71 ± 0.64
Preimplantation loss index	10.01 ± 3.29	9.72 ± 3.84	18.00 ± 7.54	10.80 ± 3.40

a2: significant different from the control at level $p<0.001$ & b1 significant different from folic acid treated group at level $p<0.05$.

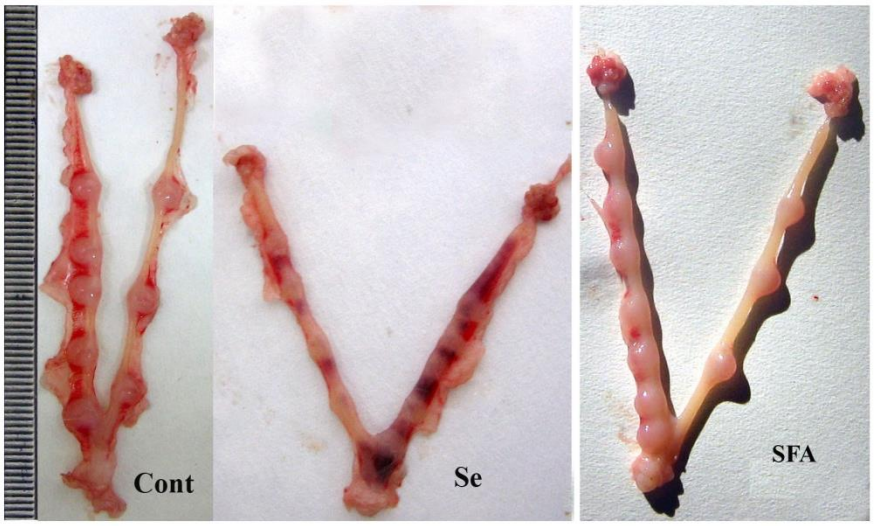


Fig. (1)

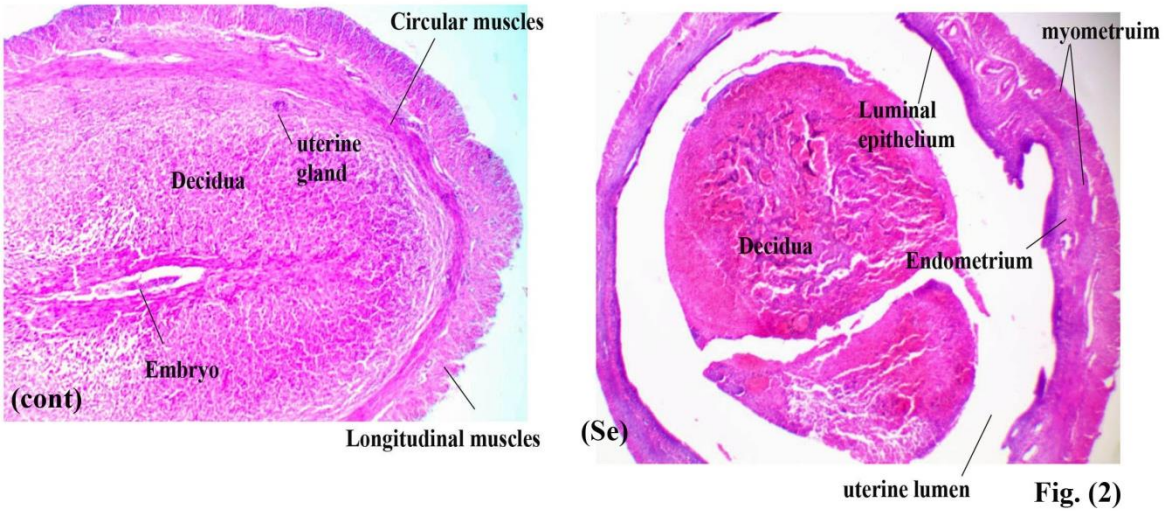


Fig. (2)

Fig. (1) :Uteri of pregnant rats on the 8th day of gestation showing normal implantations of control (cont), internal bleeding and placental scares of selenium treated rats (Se) and normal implantation sites of a rat co-treated with selenium and folic acid (SFA).

Fig. (2): Transverse sections of 8-day pregnant uteri of rats showing normal decidual tissue around the implanting embryo (cont) and destructed deciduoma with massive hemorrhage separated from the uterine tissue from selenium treated rat mother. [Stained H & E x 40]

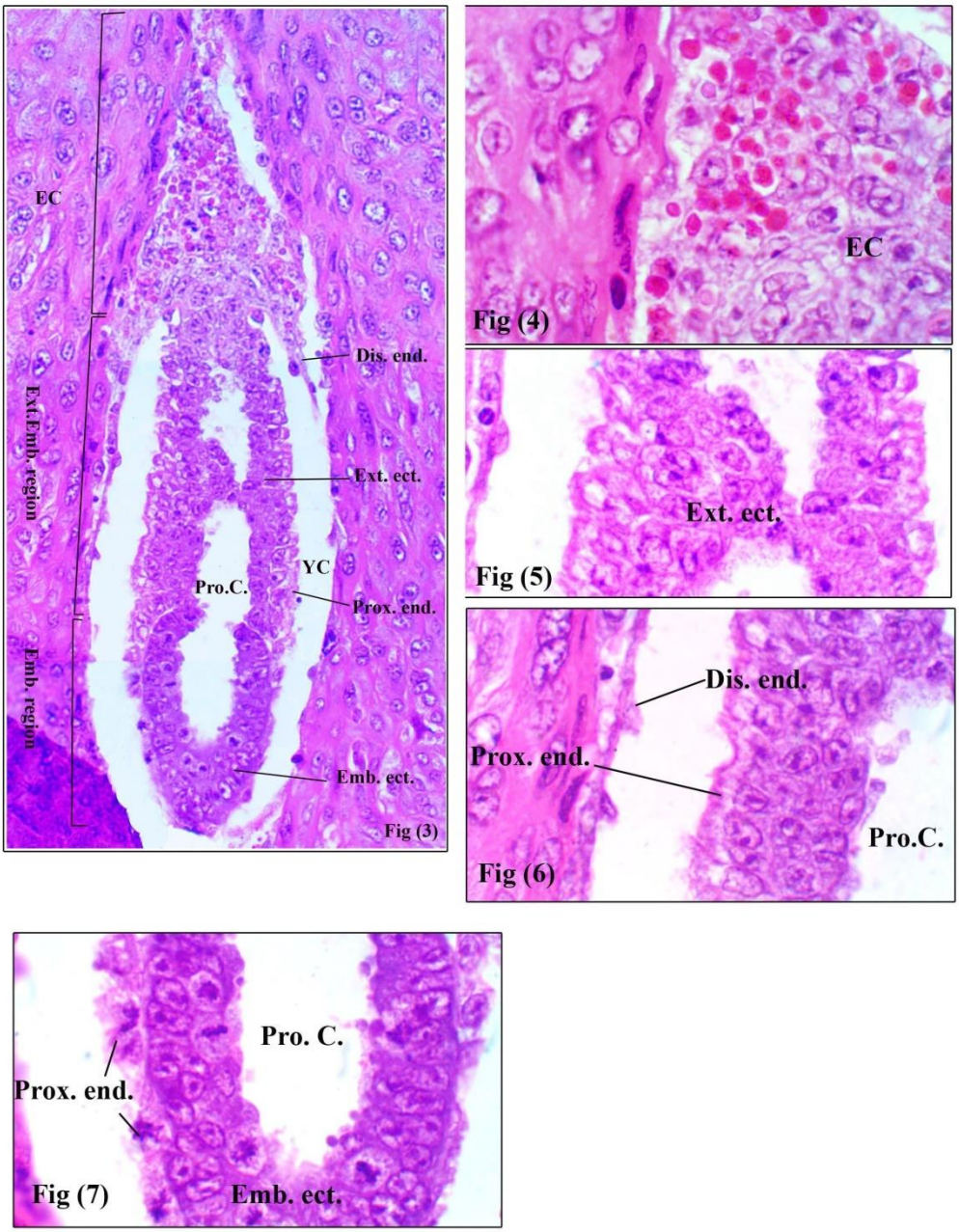
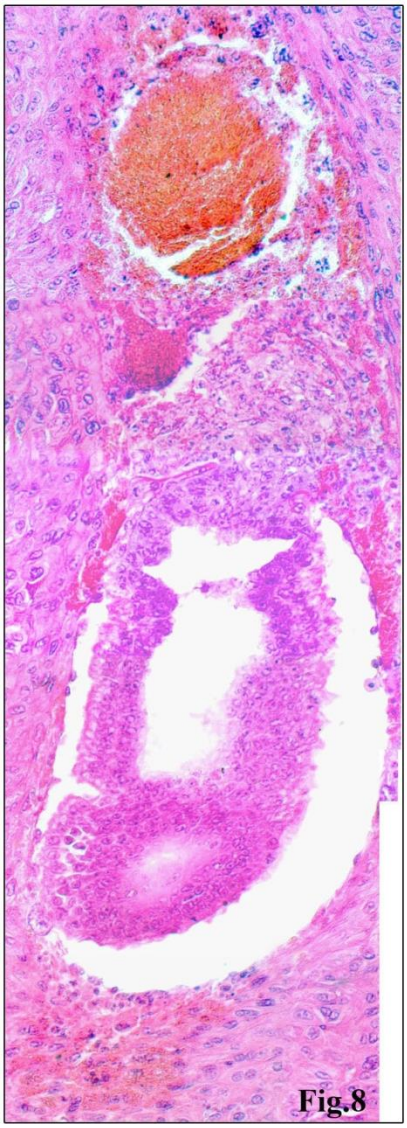
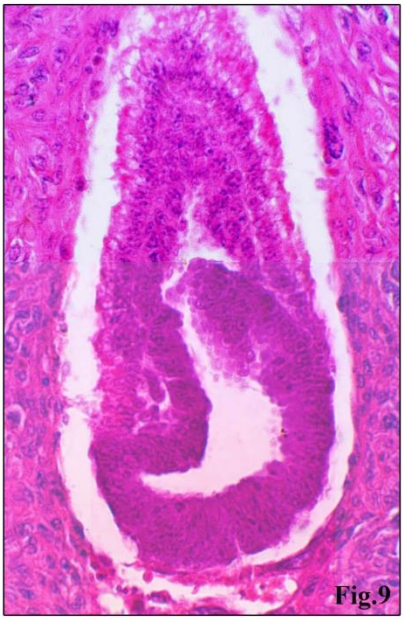


Fig. 3: Mid- sagittal section of day 8 embryo of normal pregnancy of rat showing the main germinal layers of the extraembryonic [Ext. Emb.] and the embryonic regions [Emb.] together with the ectoplacental cone [EC], proamniotic cavity [Pro.C.] and yolk-sac [YC] cavity. [Stained H & E x 400].

Figs. (4-7): Magnified portions of figure 3 showing the ectoplacental cone [EC], the proximal endoderm [Prox. end.], the distal endoderm [Dis. end.], the extraembryonic ectoderm [Ext. ect.] and the embryonic ectoderm [Emb. ect.] [Stained H & E x 1000].





Figs. (8&9): Mid- sagittal sections of day 8 embryos maternally treated with selenium showing different forms of retarded rat egg- cylinder. [Stained H & E x 400]

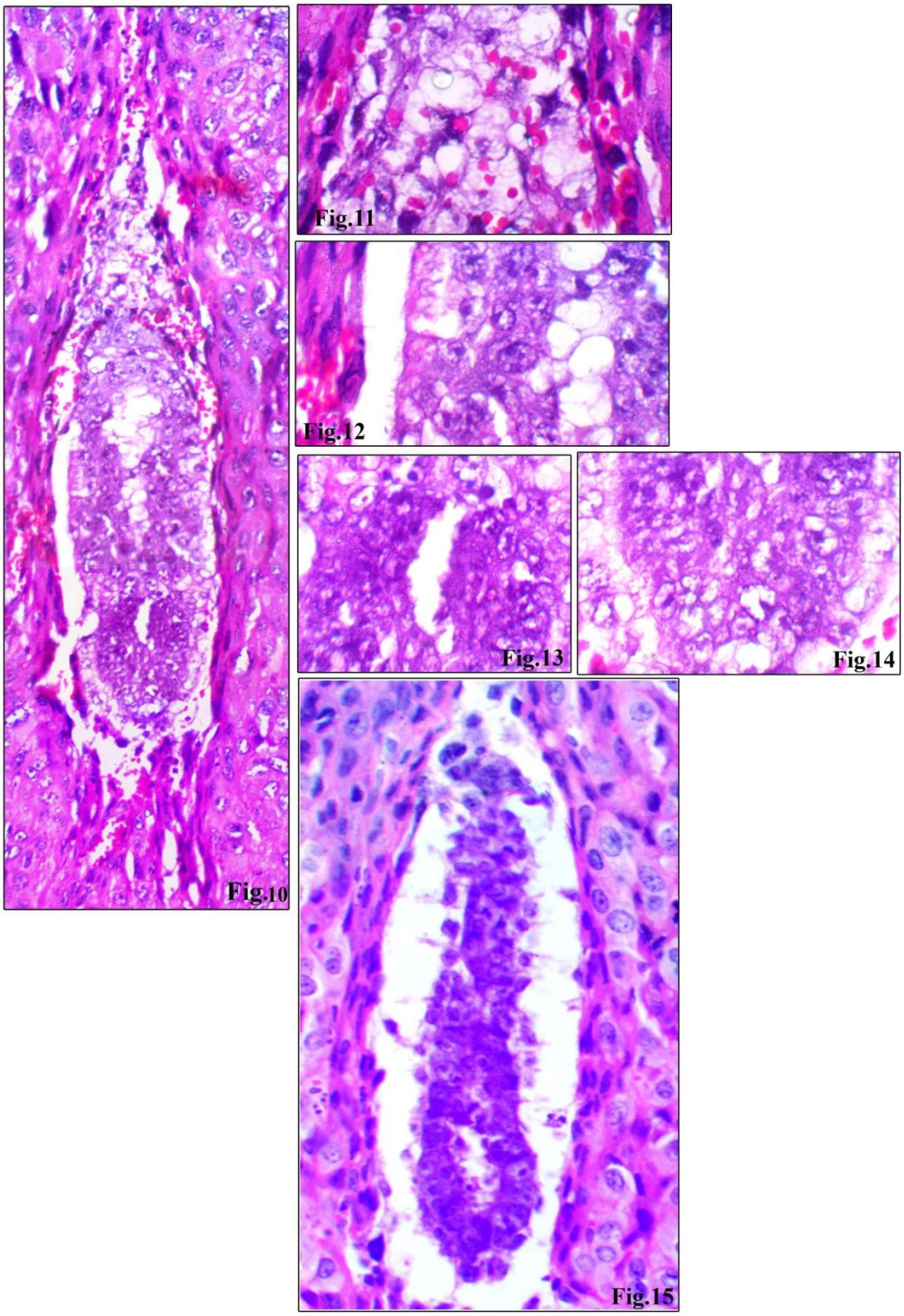
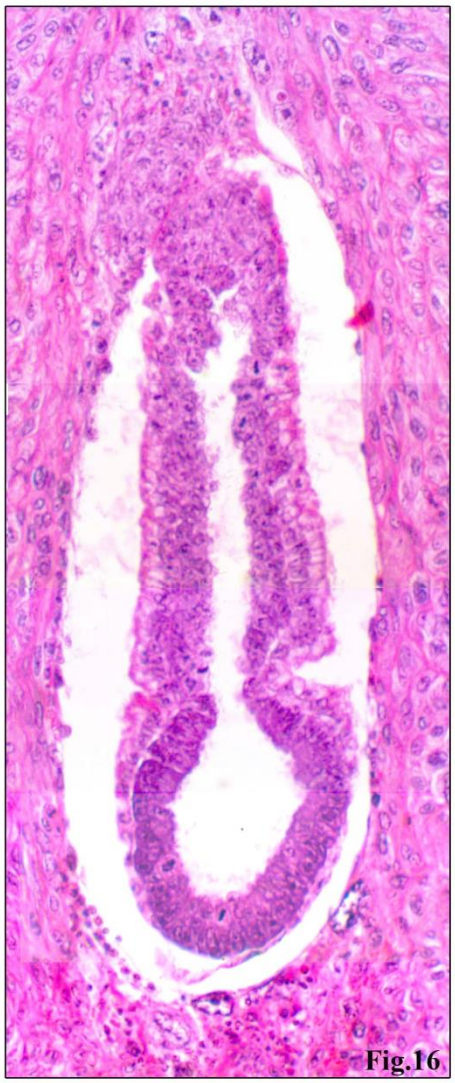
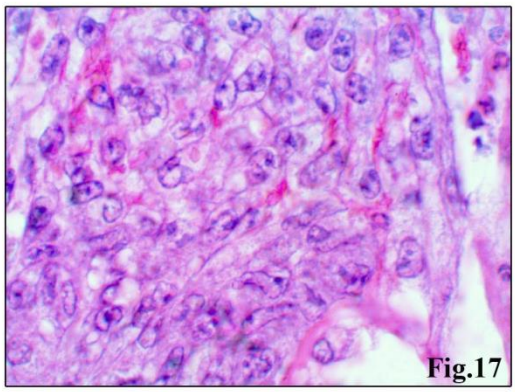


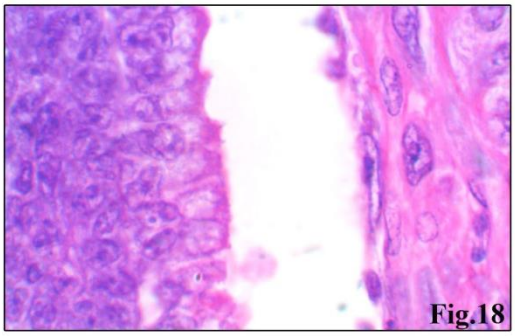
Fig. (10): Mid- sagittal section of deformed day 8 embryo maternally treated with selenium showing unidentifiable layers. [Stained H & E x 400]

Figs. (11- 14): Magnified portions of figure 9 showing different parts of the deformed embryo. [Stained H & E x 1000].

Fig. (15): Mid- sagittal section of complete degenerated rat egg-cylinder on day 8 of pregnancy maternally treated with selenium showing very dense unrecognizable layers and many separated dead embryonic cells are shown in the yolk sac cavity. [Stained H & E x 400].







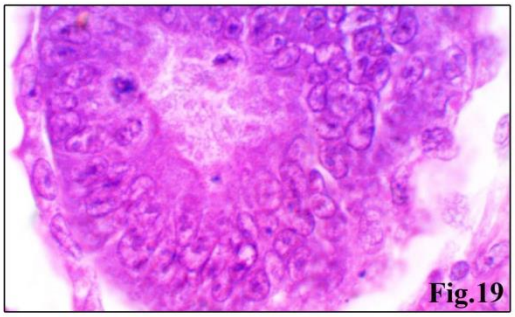


Fig. (16): Mid- sagittal section of day 8 embryo maternally co-treated with selenium and folic acid showing more or less normal egg-cylinder with viable cells in all the embryonic layers [Stained H & E x 400].
Figs. (17-19): magnified portions of rat egg cylinder maternally co-treated with selenium and folic acid showing normal embryonic layers and normal ectoplacental cone.

REFERENCES

1- Rayman MP. The importance of selenium to human health. The Lancet 2000; 356: 233– 41.
2- Ma Y et al. Embryonic developmental toxicity of selenite in zebrafish (Danio rerio) and prevention with folic acid. Food Chem Toxicol 2012; 50: 2854-63.
3- Lenz M, Lens PN. The essential toxin: the changing perception of selenium in environmental sciences. Sci Total Environ 2009; 407: 3620–33.
4- Linares-Casenave J et al. Selenium tissue burden compartmentalization in resident white sturgeon (Acipenser transmontanus) of the San Francisco Bay Delta estuary. Environ Toxicol Chem 2015; 34(1):152-60.
5- Wu L. Review of 15 years of research on ecotoxicology and remediation of land contaminated by agricultural drainage sediment rich in selenium. Ecotoxicol Environ Saf 2004; 57:257–69.
6- Helal MA. Toxicological study of sodium selenite on fetal development and DNA fragmentation in liver cells of pregnant rats. Biol Trace Elem Res 2011; 140:114-26.
7- Yonemoto J et al. Toxic effects of sodium selenite on pregnant mice and modification of the effects by vitamin E or reduced glutathione. Teratology 1983; 28: 333–40.
8- Hegazy AAK, El-Shershaby AM. Effect of the herbicide Dual on pregnancy in rats. Bull Fac Sci Assiut Univ 1996; 25(3-E): 43-59.
9- Favero AM et al. Teratogenic effects of diphenyl diselenide in Wistar rats. Reprod Toxicol 2005; 20:561–68.
10- Weisa SN et al. Repeated administration of diphenyl diselenide to pregnant rats induces adverse effects on embryonic/ fetal development. Reprod Toxicol 2007; 23:175–81.
11- El-Shershaby AM, Hinchliffe JR. Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate. J Embryol Exp Morphol 1974; 31(3):643-54.

12- Behnke M, Eyler FD. The consequences of prenatal substance use for the developing fetus, newborn, and young child. Int J Addict 1993; 28:1341–91.
13- Maier KJ, Knight AW. Ecotoxicology of selenium in freshwater systems. Rev Environ Contam Toxicol 1994; 134: 31–48.
14- Spallholz JE. Free radical generation by selenium compounds and their prooxidant toxicity. Biomed Environ Sci 1997; 10(2-3):260–70.
15- Henning SM et al. Male rats fed methyl- and folate-deficient diets with or without niacin develop hepatic carcinomas associated with decreased tissue NAD concentrations and altered poly (ADPribose) polymerase activity. J Nutr 1997;127: 30–6.
16- Czeizel AE et al. Population-based case control study of folic acid supplementation during pregnancy. Teratology 1996; 53(6):345-51.
17- Mills JL, Signore C. Neural tube defect rates before and after food fortification with folic acid. Birth Defects Res A Clin Mol Teratol 2004; 70(11):844-5.
18- Tolarova M, Harris J. Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and multivitamins. Teratology 1995; 51(2):71-8.
19- Yang Q et al. Does periconceptional multivitamin use reduce the risk for limb deficiency in offspring? Epidemiology 1997; 8(2):157-61.
20- Serrano M et al. Fetal alcohol syndrome: cardiac birth defects in mice and prevention with folate. Am J Obstet Gynecol 2010; 203: 75e7 – 15.
21- Joshi R et al. Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. Free Radic Biol Med 2001; 30(12): 1390-9.
22- Sahin K et al. Dietary Vitamin C and Folic Acid Supplementation Ameliorates the Detrimental Effects of Heat Stress in Japanese Quail. J Nutr 2003; 133:1882-6.
23- Hwang SY et al. Folic acid supplementation inhibits NADPH oxidase-mediated superoxide anion production in the kidney. Am J Physiol Renal Physiol 2011; 300:F189–98.
24- Zaho H et al. Folic acid and soybean isoflavone combined supplementation protects the postneural tube closure defects of rodents induced by cyclophosphamide *in vivo* and *in vitro*. Neuro Toxicol 2010; 31:180–7.
25- Bancroft CF, Gamble M. Theory and practice of Histological techniques 5th ed; Churchill Livingstone,Edinburgh, London, 2002.
26- Kim IY, Kim TS, Chung YW, Jeong D. Selenium-induced apoptosis. Selenium. 2nd ed; Springer US, 2007 Part III pp 379– 85.
27- Misra S, Niyogi S. Selenite causes cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes by inducing oxidative stress. Toxicol In Vitro 2009; 23: 1249-58.
28- Usami M et al. Effects of methionine on selenium embryotoxicity in cultured rat embryos. Teratog Carcinog Mutagen 2002; 22: 301–8.
29- Kent C. Teratogens. In: Basics of Toxicology. John Wiley and Sons, London, 1998.
30- El-Shershaby AFM et al. Teratogenic and embryolethal effects of Edifenphos during early pregnancy in mice. J Union Arab Biol 2006; 25 A Zoology: 67-87.
31- Wu DY et al. Knowledge and use of folic acid for prevention of birth defects amongst Honduran women. Reprod Toxicol 2007; 23:600–6.
32- Stewart MS et al. Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis. Free Radic Biol Med 1999; 26:42.
33- Raideep K et al. Effect of sub-chronic selenium toxicosis on lipid peroxidation, glutathione redox cucle and antioxidant enzymes in calves. Vet Hum Toxicol 2003; 45(4):190–2.
34- Usami M et al. Effects of ascorbic acid on selenium teratogenicity in cultured rat embryos. Toxicol Lett 1999; 105: 123–8.
35- Kamen B. Folate and antifolate pharmacology. Semin Oncol 1997; 24:S18-30 -9.
36- Nieuwenhuijsen, J. et al. Chlorination Disinfection By-Products in Drinking Water and Congenital Anomalies: Review and Meta-Analyses Mark. Environ Health Perspect 2009; 117:1486–93.