The Egyptian Journal of Hospital Medicine (Oct. 2015) Vol. 61, Page 415-424

Protective Effect of Olive Oil Against Teratogenecity Induced by Mancozeb in Albino Rats

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ABSTRACT

Aim: this study aimed to investigate the protective effect of extra-virgin olive oil (EVOO) against teratogenicity of the fungicide mancozeb. **Methods**: after pregnancy confirmation, 32 pregnant rats were divided into 4-groups (n=8). The 1st group orally administered tap water (-ve control), the 2nd group (+ve control) was administered EVOO (0.5ml/dam) from the1st to 20th day of pregnancy. The 3rd and the 4th groups were administered 200 mg/kg mancozeb during the period of organogenesis, from the 6th to 15th day of pregnancy. The 4th group received the mentioned dose of EVOO prior to the pesticide administration. Cesarean section was performed on day 20 of pregnancy and the maternal and fetal parameters were recorded. **Results**: mancozeb induced maternal toxicity manifested as lower body weight gain of dams, increased number of late resorption sites/litter in comparison with the control group and mancozeb group pretreated with EVOO. Mancozeb evoked a decrease in fetal body weight, altered sex ratio (M/F) as well as increased incidence of fetal external, visceral and skeletal abnormalities. Treatment with virgin oil reduced the congenital malformations.

Conclusively, the present study elucidates the protective role of EVOO as a result of antioxidant activity which scavenges the reactive oxygen species which induced cytotoxicity and increased prenatal mortalities.

Keywords: Olive Oil, Teratogenecity, Mancozeb, Albino Rats.

INTRODUCTION

Mancozeb fungicide, a manganese-zinc complex of ethylene-bis-dithiocarbamates (EBDCs) is a commonly-used fungicide throughout the world, registered for use in many countries. Mancozeb is used to protect many fruits, vegetables, nuts and field crops against a wide spectrum of fungal diseases, including potato blight, leaf spot, scab and rust. It has been classified by EPA in the toxicity class IV [1]. Although the risk of intoxication by EBDCs mainly concerns industrial and agricultural workers [2]. Population can be chronically exposed to dietary residues present of such pesticides in food. Mancozeb, despite its low acute toxicity, has been shown to produce significant toxicological effects on thyroid [3] immune system [4] reproductive system [5] and nervous system [6], Moreover, Mancozeb was considered a multipotent carcinogen, inducing a variety of tumors of different origin in rats after chronic exposure [7], mutagenic [8] and possibly teratogenic effects [9]. Exposure to mancozeb causes normocytic type of anemia, significant decrease in blood glucose and globulin levels and a significant pathological changes were observed in liver, kidney, spleen while, heart showed congestion with slight enlargement and brain revealed few hemorrhage areas. [10,11].

Olive oil is an integral ingredient in the Mediterranean diet, full of nutrients and vitamins. Extra-virgin olive oil is derived from the first pressing of the olives. It has the most delicate flavor and antioxidant benefits in reduction of coronary heart disease risk, prevention of some cancers and modification of immune and inflammatory responses.[12] Virgin olive oil contains polyphenolic compounds [13] such as simple phenols (hydroxytyrosol, tyrosol); secoiridoids (oleuropein, the aglycone of ligstroside, and their respective decarboxylated dialdehyde derivatives and the lignans [(+)-1-acetoxypinoresinol and pinoresinol] which are of high antioxidant activity [14]and they are capable of scavenging free radicals produced, suggesting the chemoprotective effect of olive oil [15,16]

Received:12/9/2015
Accepted:25/9/2015

DOI: 10.12816/0017694

Therefore, the objective of the present study was to evaluate the possible protective effect of olive oil against maternal toxicity and teratogenicity induced by mancozeb fungicide

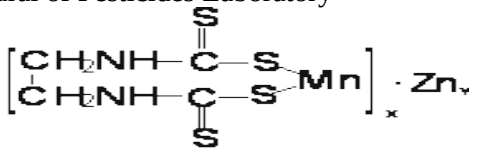
MATERIALS AND METHODS

1. Extra-virgin olive oil (EVOO):

A highly purified EVOO was obtained from agricultural research center (A.R.C.) Giza. Egypt

2.Pesticide:

Mancozeb of a Chemical Class, ethylene, bis, dithiocarbamate (EBDC) (tridex 80 % 80% WP (wettable powder as prepared by Mebedco Company) was provided from Central Agricultural of Pesticides Laboratory



3.Experimental protocol:

Male and female adult albino rats of Wister strain weighing 130-150g were purchased from the National Research Center. Rats were housed in polypropylene cages with wood shavings as bedding and given access food and tap water. All animals were fed a commercial standard diet during the experiment. Animals were acclimatized two weeks to the laboratory conditions and then kept for mating. Each two virgin female rats in estrus phase were kept with one adult male. Pregnancy was confirmed at the next morning by presence of sperms in the vaginal swab and this day was considered as zero day of gestation. After that, 32 pregnant rats were divided into 4 groups (8dams/group). Negative (–ve) and positive (+ve) control groups were orally administered tap water and extra-virgin olive oil (EVOO) (0.5 ml/dam) respectively all over the gestation period. The 3rd and 4th groups received mancozeb 200 mg/kg b.wt alone or in combination with EVOO respectively. All pregnant females were examined daily throughout the gestation period for mortality, morbidity (a clinically irreversible condition leading inevitably to death), general appearance, and behavior. The maternal body weights and weight gain were recorded on 7th, 14th and 20th days of gestation.

4. Teratological study:

Dams were sacrificed by cervical dislocation and subjected to cesarean section on the 20th day of gestation. The ovaries and uterus of each dam were removed and examined for the number of the corpora lutea in each ovary as well as the number of implantation sites in each horn per litter. The uteri with no evidence of implantation were stained with a 2% ammonium sulphide solution to identity the presence of early and late resorption sites [17]. All live fetuses were removed, dried on absorbent paper, weighed and examined for sex ration, crown- rump and gross malformations. About one- half of live fetuses from each litter were fixed in 70% ethanol for subsequent maceration with 2% aqueous KOH until clearance of skeleton and stained with Alizarin red S and investigated [18]. The other half of fetuses was preserved in Bouin's solution sectioning technique for freehand razor sectioning technique. All sectioned tissues were examined under dissecting microscope for visceral examinations [19].

5. Statistical analysis

Data were expressed as the mean ± SE. of eight replicates. The data were subjected to statistical analysis using one-way Analysis of Variance (ANOVA) and complemented with Duncan's Multiple Range Test. Statistical difference was set at $p<0.05$ by using [20] program. The data obtained from skeletal and visceral malformations were statistically evaluated using non parometric Mann-Whitney U by using SPSS program. [20].

RESULTS

Table 1 revealed that maternal toxicity assessed by a significant reduction ($p<0.05$) in the final body weight gains of dams administered repeated dose of mancozeb at a dose level of 200mg/Kg b.wt. during the organogenesis period as compared to the control groups. The significant decline in body weight gain started from the 14th day of pregnancy. However, no mortality occurred at this dose level

Maternal and fetal parameters recorded in table 2 showed insignificant difference in the number of corpora lutea, number of implantation sites, fetal length and number of live fetuses in all the treated groups. Mancozeb treatment showed a significant increase in number of late resorption sites/litter ($p<0.05$) in comparison with the control groups. Fetal and placental weights were significantly decreased ($p<0.05$) in fetuses obtained from mancozeb-treated dams

compared to the control groups. Meanwhile, fetuses obtained from dams formerly supplemented with olive oil exhibited an improvement in placenta weight, but not the fetal weight. In addition, a significant increase in number of male fetuses manifested by an increase in sex ratio was observed in +ve control compared to -ve control group and in mancozeb group pretreated with olive oil compared to +ve control group

Table 3 revealed limited external abnormalities induced by mancozeb, such as external hydrocephaly, scoliosis (Figs.1 C,D), haematoma (at the temporal and lumber regions), growth retardation, short tail, associated with malformations in skeleton especially vertebral column, as well as urogenital malformations in fetuses compared to the control groups. These malformations almost prevented by olive oil supplementation.

Table 4 indicated higher incidence of skeletal anomalies in fetuses obtained from dams exposed to mancozeb only compared to the control groups. Skeletal anomalies recorded in fetuses of mancozeb treated dams were manifested by delayed/incomplete ossification of skull bones (Figs.1 G,H), missing and incomplete ossification of hyoid bone, bipertite sternbrae, incomplete ossification and misaligned sternbrae (Figs.1 A,B), missing one or more sternebrae and xiphoid process (Fig. C), incomplete ossification, misaligned and/or missing of caudal vertebrae and wavy ribs, , misaligned and/or incomplete ossification of centrum and arch of lumbar and sacral vertebrae, missing of coccygeal vertebrae, malformations of the spine, scoliosis (Fig1s C,D), were also apparent. Pretreatment with olive oil almost prevent these anomalies.

Table 5 demonstrated several visceral anomalies in fetuses obtained from dams received mancozeb attested by anophthalmia and/or microphthalmia, external hydromicrocephaly and internal hydrocephaly (Figs.1 E,F), dilated ventricles, cardiomegaly and small and/or collapsed lung, hepatomegaly, large stomach, unilateral renal agenesis and/or hypoplasia, malpositioned pelvic kidney due to insufficient ascent of metanephros during embryonic development and/or enlarged adrenals and abnormalities of genital system revealed by unilateral testicular agenesis and/or hypotrophy, hermaphroditism (male pseudohermaphrdoite as well as unilateral true hermaphrodite fetuses. Pretreated females with olive oil showed amelioration in certain malformations

DISCUSSION

Pesticides, including their presumed inert ingredients, are among a growing list of chemicals that have been shown to interfere with sexual development, reproduction, and fertility when exposure occurs during vulnerable life stages [21]. There is increasing evidence to suggest an association between environmental exposure to certain agricultural pesticides and adverse reproductive outcomes in men and women working on/or living near farms [22]. The environmental agents interfere with the developmental processes thus derailing them from giving their proper end results [23].

The principal factor leading to this reduction in weight gain of mothers during gestation exemplified higher incidence of embryocidal effects in mancozeb experimental group. The maternal weight loss may be associated with the marked reduction in the fetal weights. Similar results have been reported by **Narotsky and Kavlock** [24].

Post-implantation loss may be attributed to fetal death and resorption where, *in utero* exposure of the pups to the chemical may led to alteration of the maternal hormonal levels as a result the effect of chemical on the CNS, suppressing the brain release of gonadotropins apparently due to acetyl cholinesterase activity. This alteration may result in change of the intrauterine environment leading to fetal death and resorption [25].

The decreased fetal weight is classified as an indicator of intrauterine growth retardation and occurrence of a delay in ossification in several skeletal districts coincided with it. The reduction in fetal weight observed in this study may be accompanied by reduction in maternal weight gain, suggesting an association between the two maternal and fetal weights. The probable reason for such association might be attributed to maternal stress, which in turn retards the fetal growth. Mancozeb may act as prooxidants inside the cell that induce oxidative stress due to the mitochondrial-rich placenta. [26].So, oxidative stress may alter the uterine environment through

direct effect on the uterus and indirectly through its activity on the brain [27].

The results of the present investigation have shown skeletal defects with special reference to non-ossified skeletons, this might also be one of the reasons for the decrease in fetal weight. This relationship between low body weight of fetuses and retarded ossification are similar with the results that has also been reported by **Akhtar *et al.*** [28].The skeletal defects are linked with acetyl-cholinesterase inhibition, disruption of cholinergic system and influx of calcium across the cell membranes. [29] Acetylcholine inhibition affects proliferation, differentiation and migration of target cells and any hindrance to the functioning of acetyl cholinesterase (AchE) during early development would cause harmful effects. [30].The induction of retarded ossification in the skull may be as a result of the sensitivity of cells to chromosomal damage which delays the cell division [31].

Extra virgin olive oil (EVOO) pre-treatment could successfully prevented occurrence of these skeletal anomalies induced by mancozeb. The positive effects of olive oil may be due to its higher contents of Mono Unsaturated Fatty Acids (MUSFAs), which have a positive association with bone mineral density [32]. Olive oil prevents the bone loss and improves bone mineral density in rats [33]. Olive oil enhances intestinal absorption of calcium [34]. Olive oil has been reported to favor the mineralization and development of bones [35]. It is a complex compound made of fatty acids, vitamins, volatile components and water soluble components. Olive oil is rich in monounsaturated fatty acids (mainly oleic acid). In addition, it contains adequate amounts of linoleic acid. It contains a group of related natural products with potent antioxidant properties, which are esters of tyrosol and hydroxytyrosol, including oleocanthal and oleuropein as well as vitamin E. [36].

As observed in this study, the fetal organs primarily affected by mancozeb are the brain, the kidney and the skeletal system [37]. The major visceral anomaly noticed in the head region was the hydrocephaly and enlarged cerebral ventricles (ventriculomegaly) appeared as an enlargement of the head caused by an abnormal accumulation of cerebrospinal fluid (CSF) in the cranium due to an imbalance between the production and absorption of CSF. This forces the ventricles to enlarge (ventricular dilatation), which in turn exerts pressure on the surrounding brain tissue, causing the brain tissue to shrink and the head to enlarge. The risk of these anomalies is that the majority of congenital hydrocephaly cases have other additional birth defects such as congenital heart disease [38]. Teratogenic effects of EBDCs in young animals may be due to disruptions in the development of the neural systems where, Ethylene thiourea (ETU) cross the placenta and caused birth defects in laboratory mammals and interfere with normal thyroid function[39] They added that the fetal brain development requires adequate thyroid hormone secretion. **Axelstad *et al*.** [39] also reported that the decrease in thyroxine levels in dams dosed with mancozeb by gavage indicated that mancozeb acts mainly via disruption of the thyroid hormones and are mainly suspected to disrupt brain development.

The pseudohermaphroite fetuses observed in this study may be due to chromosomal or hormonal abnormalities. Male reproductive diseases or disorders like malformed reproductive organs reflect various stages of Testicular Dysgenesis Syndrome (TDS), arising during gestation. These disorders included androgen insensitivity syndrome, various types of pseudohermaphroditism and malformed testes result from androgen resistance syndrome and defective biosynthesis of testicular androgen which are associated to one or more of the chemicals through the chemical-gene associations [40]. Anti-androgens are chemicals that interfere with the normal function of testosterone and similar male hormones. Exposure to such chemicals, particularly during periods of vulnerability prenatally, can result in feminization of males. The developing fetuses are extremely sensitive to the hormonal environment in the uterus and natural differences in hormone levels surrounding rats fetuses has been shown to influence the timing of sexual development and the animal behavior in adult life [41]. This explains the decrease in sex ratio observed in mancozeb treated group in comparison with EVOO +ve control group observed in this study.

The mechanism of the teratogenic effect of EBDCs may involve the chelating agent, trapping zinc required for many important enzyme systems. Furthermore, because of their chelating properties, EBDC may inhibit enzymes activity by complexing with metal-containing enzymes; these metals, such as Cu, Zn and Fe may lead to intraneuronal accumulations and implicated in promoting lipid peroxidation, oxidative stress and enzyme inhibitions causing neurotoxic effects [42]. The teratogenic effect of mancozeb may be attributed to oxidative stress whereas; mancozeb may act as prooxidants inside the cell. This mechanism is based on the presence of coordinated transitional metals, like manganese and zinc present in the chemical structure of this pesticide that will catalyze the formation of ROS through the Fenton reaction, where metals have a strong catalytic power to generate highly reactive radicals. [26, 42] Association existed between mancozeb and neural tube defects, as it can damage DNA and initiate tumors in fetal cells. [8]

Extra virgin olive oil (EVOO) contains significantly more antioxidants than refined virgin olive oil. Olive oil contains a group of antioxidants that are esters of tyrosol and hydroxytyrosols, including oleuropein and vitamin E. The main phenols in olive oil, only the hydroxytyrosol and oleuropein are catechols compounds with a catechol group which exhibit antioxidant activity is able to scavenge and stabilize a variety of endogenous and exogenous free radicals through the formation of intramolecular hydrogen bonds**.** The phenolic compound hydroxytyrosol is capable of protecting cells from hydrogen peroxide damage blocking cell cycle progress. One mechanism associated with consumption of antioxidants hydroxytyrosol and oleuropein is prevention of oxidative damage to DNA and RNA and reduce the risk of mutagenesis and inducing apoptosis. [43, 44,45] Olive oil, enriched in double bonded, monounsaturated, chemoprotective compounds, antioxidant, oleic acid and/or linoleic acid was able to reduce resorption and malformation rates, possibly by acting through pathways that regulate prostaglandins (PGs), PGI2 and PGE2 generation [46] Linoleic acid; a polyunsaturated omega-3 fatty acid that makes up 1.5% of extra-virgin olive oil. [47]They added that supplementation with oils rich in linoleic acid, can prevent malformations**.** Linoleic acid is the precursor of arachidonic acid, an essential fatty acid highly required throughout gestation [48]**.** Impairments in arachidonic acid metabolic pathways were involved in malformations. The oil-supplemented diet is responsible for providing essential fatty acids such as arachidonic acid, which the embryo requires for development and may help to prevent maternal malformations**.** Oleic acid, present in high concentrations in olive oil, is able to activate one of the three Peroxisome Proliferator-Activated Receptors (PPARs) isoforms, PPARd that regulate several metabolic, developmental pathways [48]**.** Oleic acid is important in neuronal differentiation in neuroblastoma through the activation of PPARd, which is essential for placental development; its activation has been clearly involved in embryo implantation, and it promotes embryonic stem cell proliferation and is expressed during rat embryo early organogenesis [49]**.**

5-Conclusion and Recommendations

In conclusion: fetal and maternal developmental toxicity of mancozeb included increase in post-implantation loss, decreased dams and fetal body weight, increased fetal external, visceral and skeletal abnormalities. This study clarified the important role of antioxidants and bioactive components of olive oil against congenital malformations induced by mancozeb. Olive oil exhibited chemopreventive effect mainly against the external and the skeletal anomalies, while provoked ameliorative effect on the visceral malformation resulting from exposure of pregnant dams to mancozeb during the critical period of organogenesis. Therefore, the incorporation of olive oil in the food products is recommended especially for pregnant dams exposed to xenobiotics and pesticides such as manozeb. Also, inexpensive oil rich olive cake is recommended for field animal's diet.

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Table 1. Maternal body weight gain of dams orally administered mancozeb (200mg/kg.b.wt.) with or without extra virgin (0.5 ml olive oil (EVOO)) compared to the controls.

Days of treatment	Control (1ml/Kg b.wt)	Olive oil (0.5ml/dam)	Mancozeb (200 mg/Kg b.wt)	Mancozeb(200mg/Kg b.wt) &olive oil(0.5ml/dam)
7-days	**11.50** ±0.82	**9.04** ±0.48	**11.33** ±1.20	**11.58** ±0.99
14-days	**26.96** ±0.03	**23.81** ±1.28	**18.43** ±1.06[a,b]	**22.03** ±1.67[a]
20-days	**51.48** ±2.59	**46.20** ±1.4	**38.31** ±1.61[a,b]	**37.47** ±1.65[a,b]

Data expressed as mean ± S.E. (a) Significant different from corresponding (-ve) control group by one-way ANOVA at $P \leq 0.05$. (b) Significant different from corresponding (+ve)control group by one-way ANOVA at $P \leq 0.05$. (c) Significant different from corresponding mancozeb group by one-way ANOVA at $P \leq 0.05$.

Table 2. Assessment of teratogenicity of fetuses maternally treated with mancozeb (200mg/kg.b.wt.) with or without extra virgin (0.5 ml) olive oil (EVOO) compared to the controls.

Maternal and Feto-indses	Control (1ml/Kg b.wt)	Olive oil (0.5ml/dam)	Mancozeb (200 mg/Kg b.wt)	Mancozeb(200mg/Kg b.wt) &olive oil(0.5ml/dam)
No of corpora lutea	**8.75** ±0.59	**9.25** ±0.49	**8.625** ±0.53	**8.25** ±0.59
corpora lutea/litter	**1.28** ±0.06	**1.19** ±0.04	**1.48** ±0.14[a,b,d]	**1.30** ±0.08
No of implantation	**8.13** ±0.72	9.25 ±0.53	7.63 ±0.38	**7.88** ±0.64
Early resorption	**0**	0	**0**	**0**
Late resorption	**0.11** ±0.04	**0.05** ±0.02	**0.27** ±0.07[a,b,d]	**0.11** **0.**05
Live fetus	**7.00** ±0.42	7.25 ±0.31	**6.88** ±0.67	**6.88** ±0.52
Fetal length (cm)	**3.69** ±0.06	3.70 ± 0.06	**3.60** ±0.05	**3.74** ±0.12
Fetal weight (g)	**3.60** ±0.10	**3.36** ±0.1	**3.37** ±0.08[a]	**3.26** ±0.08[a]
Placenta weight (g)	**4.06** ±0.19	**3.57** ±0.27	**3.46** ±0.0.16[a,b,d]	**3.71** ±0.23[c]
Sex ratio	**0.61** ±0.08	**2.45** ±0.24[a,c,d]	**0.64** ±0.11[b,d]	**1.39** ±0.08[a,b,c]

Data expressed as mean ± S.E. (a) Significant different from corresponding (-ve) control group by one-way ANOVA at $P \leq 0.05$. (b) Significant different from corresponding (+ve)control group by one-way ANOVA at $P \leq 0.05$. (c) Significant different from corresponding mancozeb group by one-way ANOVA at $P \leq 0.05$. (d) Significant different from corresponding mancozeb and olive oil group by one-way ANOVA at $P \leq 0.05$.

Table 3. External malformations of fetuses obtained from dams orally administered mancozeb (200mg/kg.b.wt.) with or without extra virgin (0.5 ml) olive oil (EVOO) compared to the controls.

Days of treatment	Control (1ml/Kg)	Olive oil (0.5ml/dam)	Mancozeb (200 mg/Kg b.wt)	Mancozeb(200mg/Kg b.wt) &olive oil(0.5ml/dam)
No. of examined fetuses	60	60	56	58
Haematoma	-	-	3 (5.36)	-
Scaliosis	-	-	2 (3.57)	-
Short tail	-	-	-	1 (1.72)

Table 4. Skeletal anomalies in rat fetuses obtained from dams orally administered mancozeb (200mg/kg.b.wt.) with or without extra virgin (0.5 ml) olive oil (EVOO) compared to the controls.

Parameters	Control (1ml/Kg b.wt)	Olive oil (0.5ml/dam)	Mancozeb (200 mg/Kg b.wt.)	Mancozeb(200mg/Kg b.wt) &olive oil(0.5ml/dam)
No of detected fetus	**30**	**30**	**28**	**29**
Abnormalities of vertebral column	-	-	2(7.14)	-
Sternbrae	-	-	7(25)*	-
Absence of hyoid bone	-	-	1(3.57)	-
Incomplete ossification of hyoid bone	-		2(7.14)	
Incomplete ossified skull	-		10(35.71)*	
Bipertite sternbrae	-		2(7.14)	
Absence of tail vertebral	-		1(3.57)	1(3.44)
Wavy ribs	-		2(7.14)	

*Significance comparing with controls using Mann-Whitney U.

Table 5. Visceral anomalies in rat fetuses obtained from dams orally administered mancozeb (200mg/kg.b.wt.) with or without extra virgin (0.5 ml) olive oil (EVOO) compared to the controls.

Parameters	Control (1ml/Kg b.wt)	Olive oil (0.5ml/dam)	Mancozeb (200 mg/Kg b.wt.)	Mancozeb (200mg/Kg b.wt) &olive oil(0.5ml/dam)
No of detected fetus	**30**	**30**	**28**	**29**
Internal Hydrocephaly	-	2(6.67)	7(25)*	10(34.48)*
Microcephaly	-	-	5(17.86)*	2(6.90)
Hydrocephaly &Microcephaly	-	-	9(32.1)*	2(6.90)
Anaphthalmia	-	-	-	1(3.44)
Microphthalmia	-	-	-	1(3.44)
Pelvic Kideny	-	-	2(7.14)	2(6.90)
Unilateral Hermaphrodite	-		3(10.71)*	2(6.90)
Hepatomegaly	-	-	-	1(3.44)
Small lung			1(3.44)	1(3.44)
Cardiomegaly				1(3.44)

*Significance comparing with controls using Mann-Whitney U.

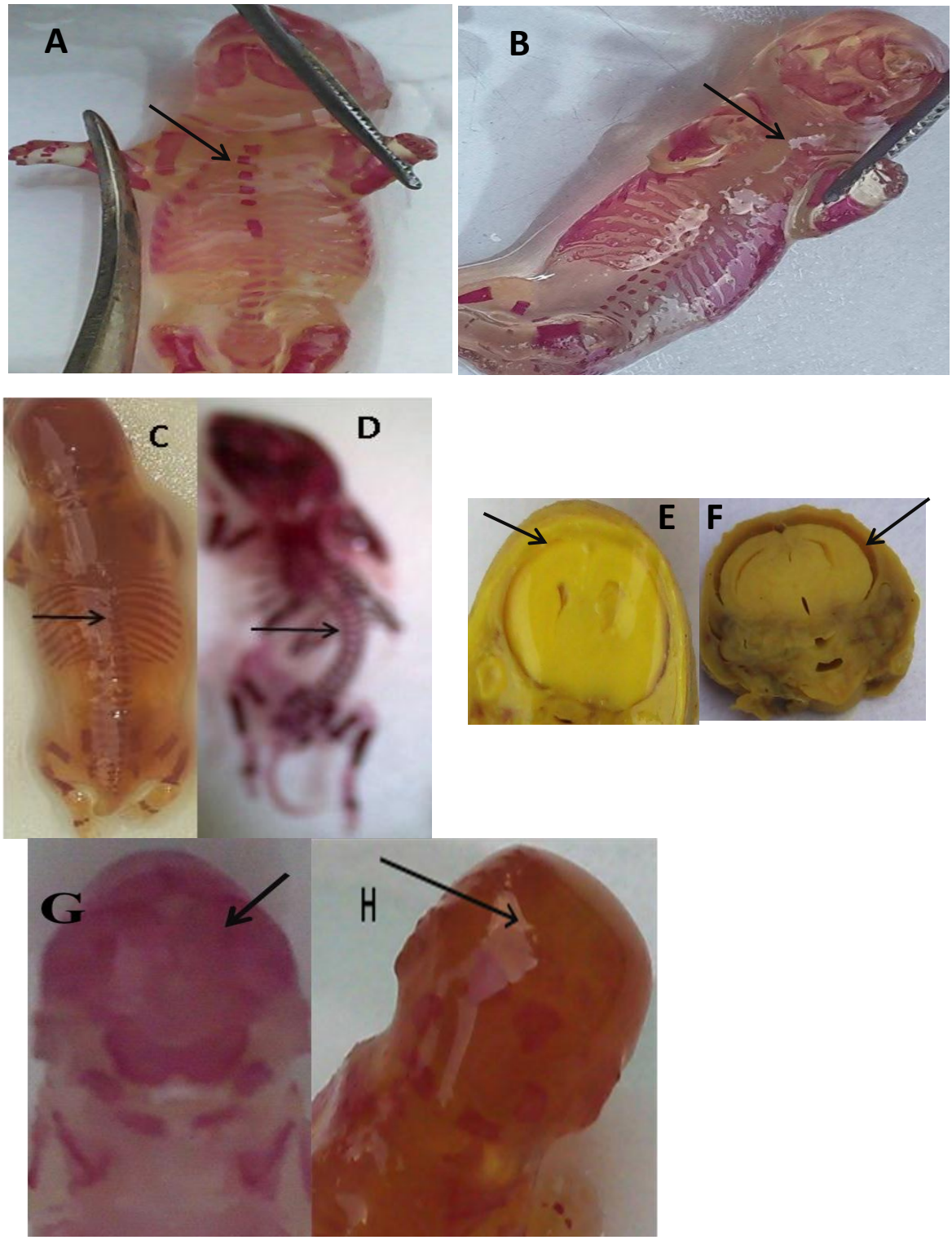

Fig.1. (A): normal sternbrae (B): absence of all sternbrae (C): normal vertebral column (D): scoliosis (curved vertebral column) (E): normal brain (F): hydrocephaly (G): normal skull (H): decreased ossification of skull.