STUDY OF THE PRENATAL EFFECTS OF SOME CHEMOTHERAPEUTIC AGENTS ON RATS AND THE MODULATIVE ROLE OF FOLIC ACID SUPPLEMENTATION

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ABBREVIATIONS

a	Atrium
Bl	Blood .clot
BPD	Biparietal diameter
C	Control
ca	Carotid sheath
cl	Clavicle
co	Concha
CP	Cyclophosphamide
CRL	Crown –rump length
d	Day
D	Doxorubicin
F	Fibula
FA	Folic acid
Fb	Frontal bone
Fe	Femur
fe	Fetus
FU	Fluorouracil
H	Heart
ha	Haematoma
hi	Hip bone
hu	Humerus
ia	Interatrial
ip	Interparietal
iv	Interventricular
k	Kidney
L	Lacrimal
li	Liver
lu	Lung
Lv	Lateral ventricle
M	Methotrexate

ma	Mandible
mc	Metacarpal
mt	Metatarsal
mx	Maxilla
Ns	Nasal septum
O	Ovary
oc	Occipital
oe	Oesophagus
p	Parietal
pa	Palate
Pet.Glu	Pteroylglutamic acid
R	Radius
ri	Ribs
S	Sternum
Sa	Subarachnoid space
Sc	Scapula
SL	Sternopericardial ligament
So	Supraocccipital
sp	Spinal cord
St	Stomach
T	Tibia
Th	Thyroid gland
thy	Thymus gland
To	Tongue
tr	Trachea
Tv	Third ventricle
U	Ulna
Uh	Uterine horns
V	Vagina
ve	Vertebrae
vn	Ventricles
vo	Vomeronasal cartilage
Wt	weight

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

"وَمَا مِن دَآبَّةٍ فِي الأَرْضِ وَلاَ طَائِرٍ يَطِيرُ
بِجَنَاحَيْهِ إِلاَّ أُمَمٌ أَمْثَالُكُمْ مَّا فَرَّطْنَا فِي الكِتَابِ
مِن شَيْءٍ ثُمَّ إِلَى رَبِّهِمْ يُحْشَرُونَ"

سورة: الأنعام - الأية:(٣٨)

CONTENTS

INTRODUCTION

All chemotherapeutic agents are potentially teratogenic and mutagenic because they act on rapidly dividing cells ***(Kerry et al., 1998).***

The prenatal exposure of rat embryos to doxorubicin results in variable anomalies affecting the skeletal system, alimentary tract, cardiovascular system and urogenital tract ***(Liu & Hutson, 2001 and Anderson et al., 2004).***

Cyclophosphamide has been found to be teratogenic in all animal species tested (rats, mice, rabbits and monkeys). The most common pattern of defects involves facial clefts and limb reduction defects ***(Chaube, 1967 and Neumann et al., 1985).***

5-Flurouracil is poorly eliminated by the exposed fetuses and can produce fetal anemia, defects of the nervous system, palate and skeleton ***(Shuey et al., 1994).***

Methotrexate demonstrates significant teratogenicity. Skull and limb abnormalities are the most frequent ***(Lioyd et al., 1999).***

Folic acid is now recommended to all women in preconception period and during the first trimester. A recent review has suggested that women exposed to methotrexate should continue supplementation of folic acid throughout pregnancy to reduce fetal abnormalities ***(Harten, 2005 and Tamura and Picciano, 2006).***

AIM OF THE WORK

The present work aimed at:

1-Study of the prenatal effects of the following four chemotherapeutic agents; (Doxorubicin, cyclophosphamide, 5-Fluorouracil and methotrexate), particularly on: morphology, skeleton and viscera of the rats.

2-Study of the modulative role of folic acid supplementation, on the prenatal effects of these drugs.

CHAPTER I

PRENATAL DEVELOPMENT OF THE RAT

Mice, rats and rabbits are the most frequently employed animals for teratogenic studies in view of their high fertility rate, easy handling and maintenance, ready provision in sufficiently large numbers, and the availability of breeding facilities in most laboratories. Moreover, it has been possible in these three animal species to demonstrate embryopathic activity by all chemical agents that were shown to be teratogenic in man ***(WHO, 1967).***

Table (1): Developmental stages of the rat embryo *(Edwards, 1968)*

Stage	Gestational age (days)	Main commencing features
1	10.5 – 10.75	Neural folds fuse at diencephalic-mesencephalic junction & Otic pits formed
2	10.75 – 11.5	Anterior neuropore and rhombencphalon close
3	11.5 – 12	Posterior neuropore and otic pit close
4	12 – 13	Endolymphatic sac appears "pinched off" from otic vesicle. Maxillary process reaches lateral nasal process.
5	13 - 14	Lens vesicle closes. Tubercles are visible on contiguous sides of mandibular and hyoid arches
6	14 – 14.5	First vibrissary papilla appears on maxillary process. First traces of digital condensation in fore-paws
7	14.5 – 15	Four raws of vibrissary papillae, with invagination starting.
8	15 – 15.5	Six raws of vibrissary papillae appear.
9	15.5 – 16.5	First trunk hair papilla appears.
10	16.5 – 17	Digits are fully separated on forepaws
11	17 – 18	Vibrissae appear from maxillary follicles, appearance of membraneous eyelids.
12	18 – 18.5	Umbilical hernia reduced

In 1925, Strong found that the stage of development and the time of appearance of ossification centers differed for the fetuses of the same gestational age of different litters. As a result, ***Burlingame & Long (1987)***, suggested that gestational age is not a definite time but is an approximate for the developmental stage. It is influenced by time of mating, strain of rats, methods of staining and visualizing the specimens, and sex of the fetus as females showed more advanced ossification than males.

Skeletal ossification in rat:

Skeletal ossification is generally considered to be an indicator of developmental maturity. In the rat, it normally begins during the seventeenth day after conception with ossification of the mandible and ribs and then proceeds very rapidly, adhering to a precise time schedule ***(Walker and Wirtschafter, 1957).***

In rat, as in other mammals, the skeletal system develops from mesoderm either in membrane (intramembranous osteogenesis) or in cartilage (endochondral osteogenesis). Several investigators have studied skeletal ossification during pre-and postnatal development in the rat ***(Fritz and Hiss, 1970 and Aliverti et al., 1979).***

Ossification of the skull:

The earliest centers of ossification appear in the mandible, maxillae and the frontal bones at 17^{th} day of gestation + 1 hour from insemination. Later at 17^{th} day of gestation + 8 hours, centers of ossification appear in premxiallae, parietals, squamosals, basioccipital, vomer, palatines, pterygoid process of basisphenoid and occipital bones. Ossification in lacrimals, nasals and jugal bones appear at 18^{th} day of gestation. A center of ossification in the interparietal, tympanic and basisphenoid bones,

appear at 18th day of gestation + 10 hours. Later at 19th day of gestation + 9 hours, a center of ossification appears in presphenoid and supraoccipital bones ***(Kettunen et al., 2006 and Rice, 2008).***

Ossification of the ribs, vertebrae, hyoid bone and sternum:

The ribs and Vertebrae:

The first ossification centers in the ribs appear at 17th day of gestation +1 hours. It is most extensive in the ribs associated with thoracic vertebrae VI to IX and it is lacking at this stage in the first and the last two ribs.

Last rib (13th rib) show ossification at 18th day of gestation. At 19th day of gestation +10 hours, the ossification occurs in nearly the whole extent of all ribs.

Ossification centers appear in the arches of the cervical vertebrae and the first two lumbar vertebrae at 17th day of gestation +8 hours. It is more developed in the cervical vertebrae. Ossification centers then appear in cranio-caudal pattern to first sacral at 18th day of gestation +10 hours.

Ossification centers in the vertebral bodies arise later than in the arches. They appear first at 18th day of gestation +10 hours, in a series beginning with the fourth and ending with the last lumbar vertebra. At 19th day of gestation +10 hours, ossification centers in the vertebral bodies extended to all thoracic and all four sacral vertebrae ***(Borisevich and Komarova, 1989).***

Hyoid bone and sternum:

It shows a median ossification center in the form of a short transverse bar at 19th day of gestation +10 hours.

The sternum consists of six sternebrae, ossification centers are present in all of these expect one or two at 19th day of gestation +10 hours ***(Aliverti et al., 1979).***

Ossification of limb bones:

Table (2): Time of first appearance of primary ossification centers within the forelimb skeleton of the rat per day *(Patton and Kaufman, 1995)*

Forelimb	Appearance of primary centres / days	
	First seen	Present in all specimens studied
Scapula	**NA**	**15**
Humerus	**NA**	**15**
Ulna	**NA**	**15**
Radius	**NA**	**15**
Carpus	**27**	**27**
Metacarpals		
1	**27**	**27**
2	**17**	**17**
3	**17**	**17**
4	**17**	**17**
5	**18**	**18**
Proximal phalanges		
1	**27**	**27**
2	**18**	**19**
3	**18**	**19**
4	**18**	**19**
5	**19**	**21**
Middle phalanges		
2	**19**	**21**
3	**19**	**21**
4	**19**	**21**
5	**21**	**21**
Distal phalanges		
1	**19**	**19**
2	**18**	**19**
3	**18**	**19**
4	**18**	**19**
5	**19**	**19**

NA, Information not available, since centres were already present in all samples of the earliest specimens studied, 27 day = 7th post natal day.

Table (3): Time of first appearance of primary ossification centers within the hindlimb skeleton of the rat per day *(Patton and Kaufman, 1995)*

Hindlimb	Appearance of primary centres/ days	
	First seen	Present in all specimens studied
Hiumerus	16	16
Ilium	17	17
Ischium	17	17
Pubis	17	17
Femur	15	16
Tibia	16	16
Fibula	16	16
Calcaneus	19	21
Talus	19	21
Tarsus	27	27
Metatarsals		
1	18	19
2	17	17
3	17	17
4	17	17
5	18	18
Proximal phalanges		
1	19	19
2	18	19
3	18	19
4	19	19
5	19	21
Middle phalanges		
2	19	21
3	19	21
4	19	21
5	21	21
Distal phalanges		
1	19	19
2	18	19
3	18	19
4	18	19
5	19	19
Patella	34	34
Fabellae	Not seen	Not seen

34^{th} day = 14^{th} post natal day

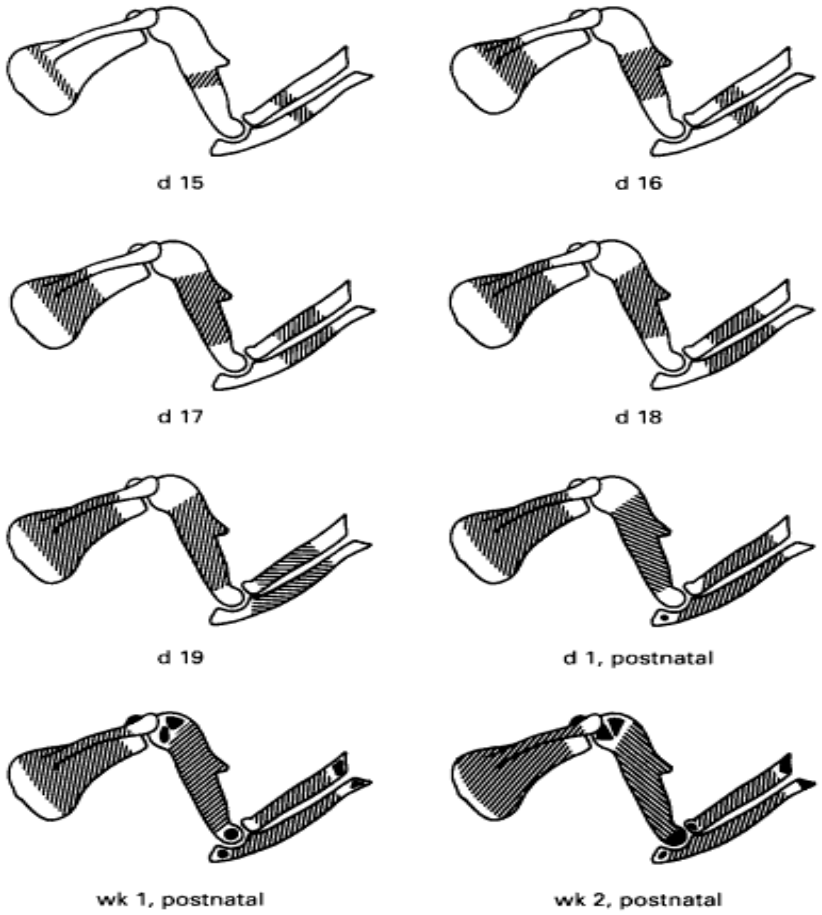


Fig. (1): Diagrammatic representation of the extent of ossification within the cartilage primordial of the scapula and forelimb long bones during the period between d 15 of pregnancy and d 14 postnatally. The shaded areas represent primary centers of ossification, while the small black areas located at the periphery of some of the cartilage primordial represent secondary centres of ossification ***(Patton and Kaufman, 1995)***

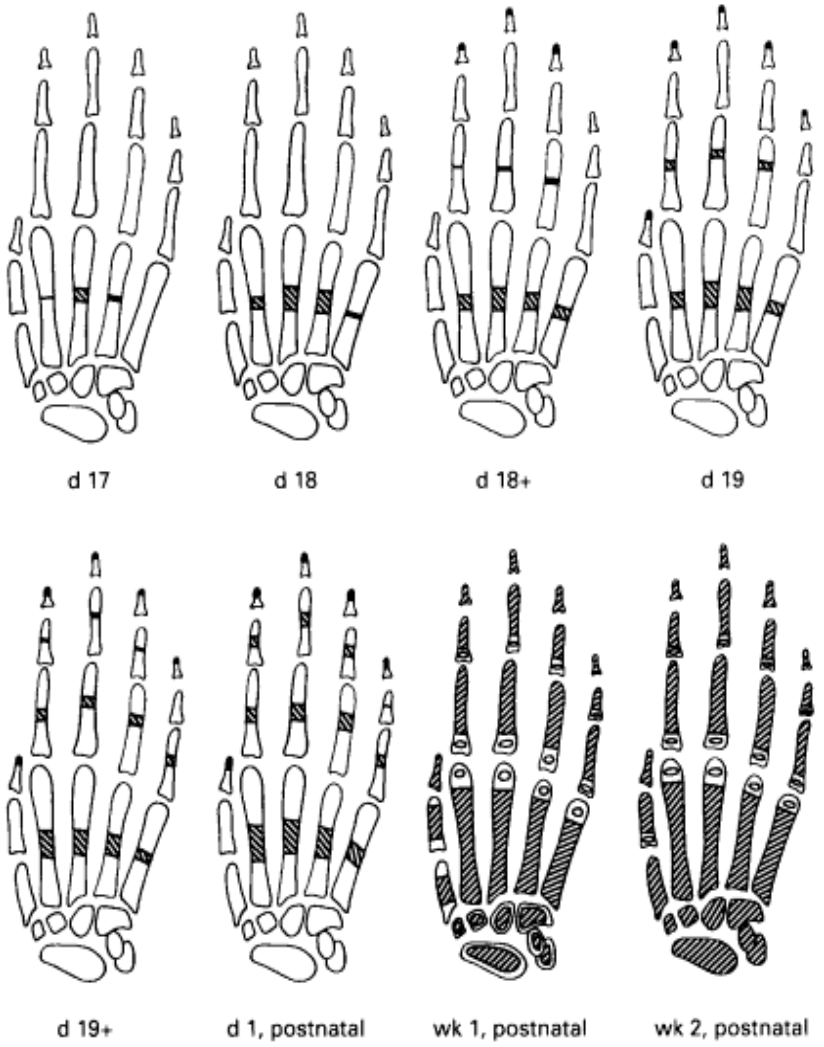


Fig.(2): Diagrammatic representation of the extent of ossification within the cartilage primordia of the carpal, metacarpal and phalangeal bones during the period between d17 of pregnancy and d 14 postnatally. Shaded areas represent primary centres of ossification, while the unfilled circles located at the periphery of some of the cartilage primordia represent secondary centres of ossification. ***(Patton and Kaufman, 1995)***

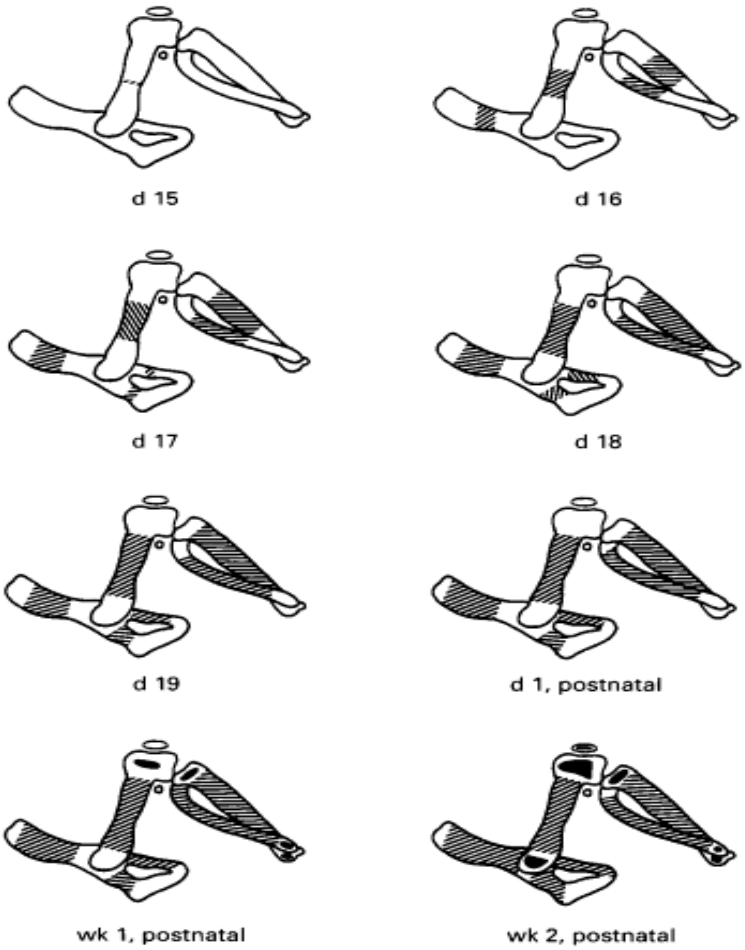


Fig (3): Diagrammatic representation of the extent of ossification within the cartilage primordial of the pelvic and hind limb long bones during the period between d15 of pregnancy and d 14 postnatally. . Shaded areas represent primary centres of ossification, while the unfilled circles located at the periphery of some of the cartilage primordia represent secondary centres of ossification ***(Patton and Kaufman, 1995)***

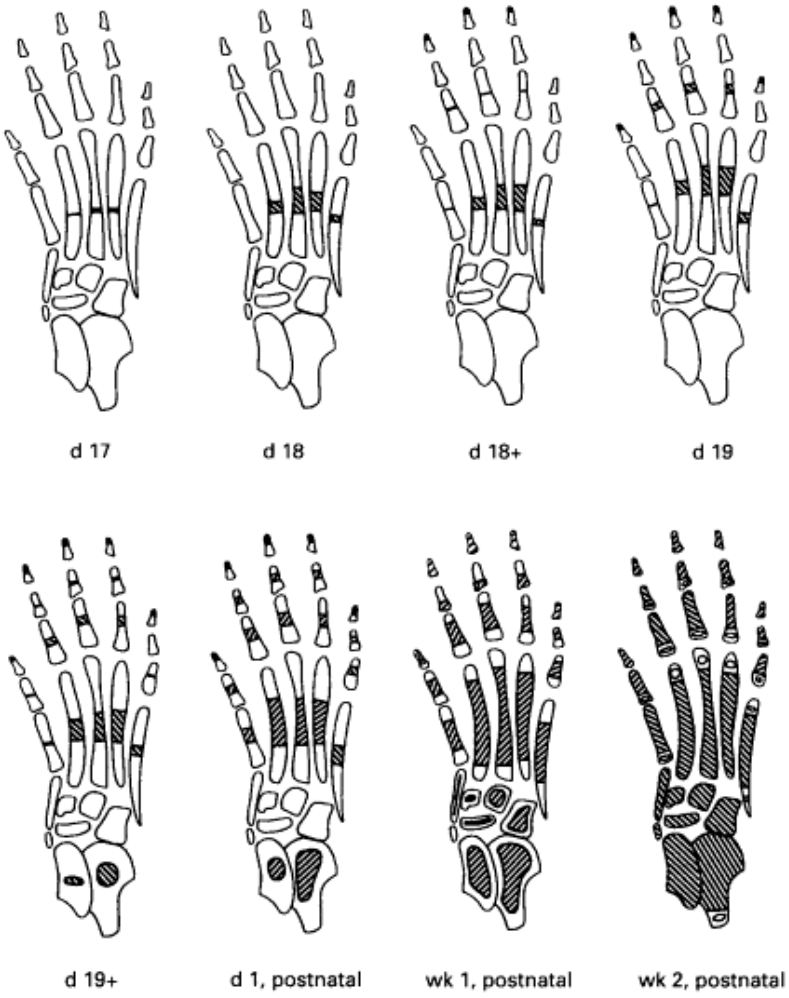


Fig. (4): Diagrammatic representation of the extent of ossification within the cartilage primordial of the tarsal, metatarsal and phalangeal bones during the period between d 17 of pregnancy and d 14 postnatally. The shaded areas represent primary centres of ossification, while the unfilled circles located at the periphery of some of the cartilage primordia represent secondary centres of ossification ***(Patton and Kaufman, 1995)***

Based on the previous literature, ***(Fadel et al., 1990),*** noticed that the following bones should show ossification by the 20th day of gestation in the rat fetus.

1- All of the bones of the skull.

2- A transverse anterior median center of the hyoid (body) bone.

3- The body of all ribs.

4- Three or four sternbrae.
5- All bones of the fore-and hind limbs except the small bones of the phalanges and some of the metacarpal and metatersal bones (especially 1^{st} and 5^{th}).
6- Ilium, ischium and pubis.
7- In the vertebral column, vertebral arch ossification centers appear earlier than in the centra. All cervical arches are ossified. Cervical arches 3 to 7 have an anterior center which appears before the posterior one. All the thoracic (13) vertebrae, lumbar (6), sacral (4) and 1 or 2 caudal arches are ossified. No ossification should be detected in all of the cervical and first thoracic centra. One to three caudal centra might be ossified.

Fritz & Hiss (1970), pointed out that the state of ossification of the axial skeleton at birth could be used as an indicator of the degree of developmental retardation. Delayed or retarded skeletal ossification at a given time near term is indicative of a non-specific retardation of fetal growth and development. Thus, evaluation of skeletal maturity is an important criterion in teratological studies.

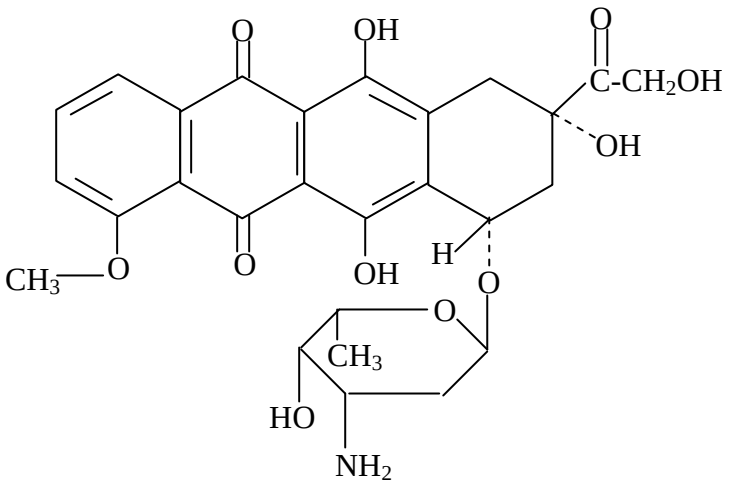
CHAPTER II

CHEMOTHERAPEUTIC AGENTS

Cancer is one of the major causes of death. Early micrometastasis is a characteristic feature of the neoplasm, indicating that a systemic approach such as chemotherapy will be required for effective cancer management. At present, about 50% of patients with cancer can be cured with chemotherapy. Cancer chemotherapy, as currently employed, can be curative in certain disseminated neoplasms that have undergone either gross or microscopic spread by the time of diagnosis. These include diffuse large cell lymphoma, Hodgkin's disease, choriocarcinoma and cancers of the breast, ovary and cervix ***(Bonadonna and Valagussa, 1985 and Hardman, 1996).***

1-DOXORUBICIN (D)

Doxorubicin is one of the most widely used anthracycline antibiotics, which are among the most useful cytotoxic anticancer drugs. It is produced by the fungus streptococcus peucetius var. caesius**.** The structure of doxorubicin is as follows ***(Bonadonna et al., 1995)***:



- **Mechanism of action:** The major actions of doxorubicin include:

(1)High – affinity binding to DNA through intercalation, with consequent blockage of the synthesis of DNA and RNA through effects on topoisomerase II.

(2) Binding to membranes to alter fluidity and ion transport.

(3) Generation of the semiquinone free radical and oxygen radicals through enzyme – mediated reductive process. This latter action may be responsible for cardiac toxicity through oxygen radical – mediated damage to membranes ***(Deffie et al., 1989 and Lubgan et al., 2006).***

- **Pharmacokinetics:** Doxorubicin is administered intravenously and cleared by hepatic metabolism and biliary excretion. Peak blood concentration decreases by 50% within the first 30 minutes after injection, but significant levels persist for up to 20 hours. Clearance is delayed in the presence of hepatic dysfunction, and at least a 50% initial reduction in dose should be considered in patients with abnormal serum bilirubin levels ***(Turchi and Villasis, 1988).***

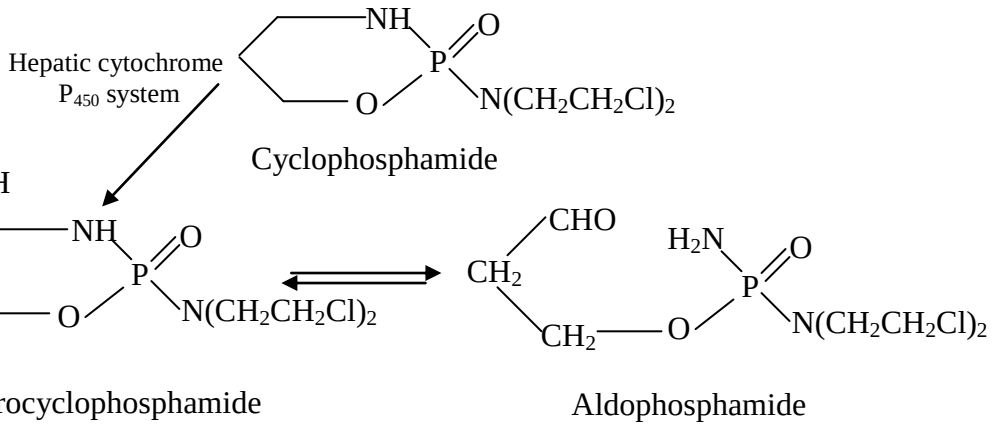
- **Therapeutic uses:** Doxorubicin is one of the most important anticancer drugs, with major clinical application in carcinomas of the breast, endometrium, ovary, testicle, thyroid, and lung and in treatment of many sarcomas, including neuroblastoma, Ewing's sarcoma, osteosarcoma, and rhabdomyosarcoma. It is also useful in hematologic cancers, including acute leukemia, multiple myeloma, Hodgkin's disease, and the diffuse non – Hodgkin's lymphomas ***(Reddy and Mandell, 1998).***

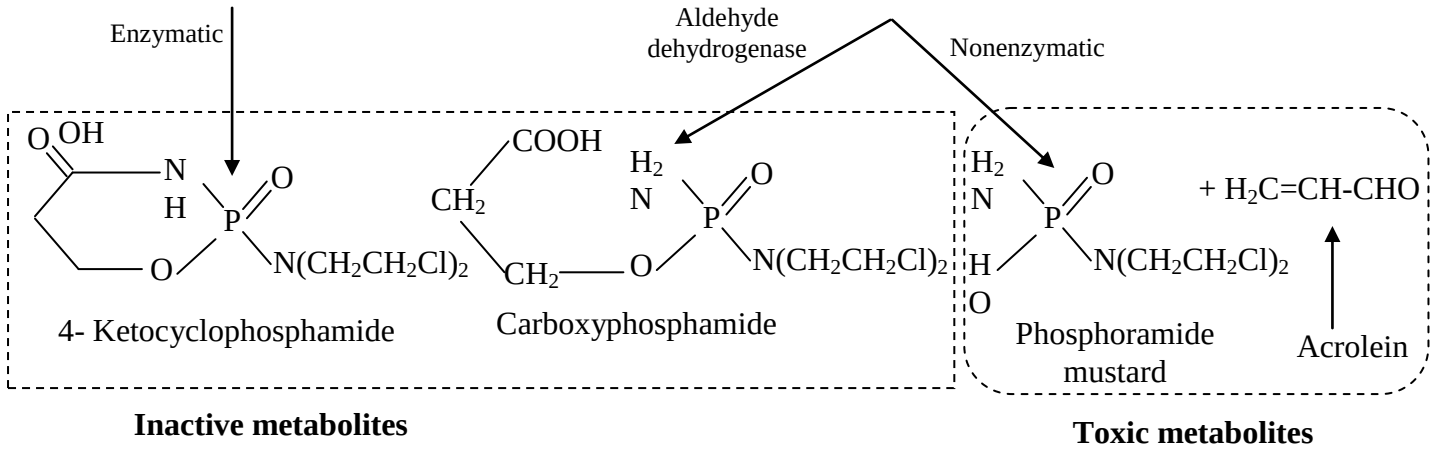
- **Adverse reactions:** In common with many other cytotoxic drugs, the doxorubicin causes bone marrow depression, which is of short duration with rapid recovery. Stomatitis, gastrointestinal disturbances,

and alopecia are common but reversible. Irreversible cumulative and dose – related cardiac toxicity, appears to involve excessive intracellar production of free radicals within the myocardium. Cardiac irradiation or administration of high doses of cyclophosphamide or another anthracycline may increase the risk of cardiotoxicity ***(Speyer et al., 1988 and Lipshultz et al., 1991).***

2-CYCLOPHOSPHAMIDE (CP)

CP is one of the most useful alkylating antineoplastic and immunosuppressant agent. It has a structure containing a bischloroethyl amine and metabolized to phosphamide mustard and acrolein, which are believed to be the ultimate teratogenic compounds. The structure of CP and the formation of its metabolites are as follows ***(Colvin, 1982)***:





- **Mechanism of action:** The alkylating agents exert cytotoxic effects via transfer of their alkyl groups to various cellular constituents. Alkylations of DNA within the nucleus represent the major interactions that lead to cell death. The major site of alkylation within DNA is N7 position of guanine, however, other bases are also alkylated to lesser degrees. This effect leads to DNA strand breakage ***(Hemminki and Ludlum, 1984).***

- **Pharmacokinetics:** Cyclophosphamide is a chemically stable solid drug that can be given orally. It is a prodrug that undergoes metabolic activation to produce two toxic metabolites : acrolein and phosphoramide mustard, which contains the N – $(CH_2\ CH_2Cl)_2$ group. Toxic metabolites of CP are excreted in the urine ***(Carmichael, 1994).***

- **Therapeutic uses:** The clinical spectrum of activity of CP is very broad. As a single agent, it is recommended for many neoplasms, such as lymphomas and chronic leukemias. CP is an essential component of many effective drug combinations for non – Hodgkin's lymphomas. Complete remissions and presumed cures have been reported when CP was given as a single agent for Burkitt's lymphoma. It is frequently used in combination with methotrexate (or doxorubicin) and fluorouracil as adjuvant therapy after surgery for carcinoma of the breast. Because of its potent immunosuppressive properties, CP has received considerable attention for the control of organ rejection after transplantation and in non neoplastic disorders associated with altered immune reactivity, including Wegener's granulomatosis, rheumatoid arthritis, and the nephrotic syndrome in children ***(Clements, 1991 and Hochster et al., 1994).***

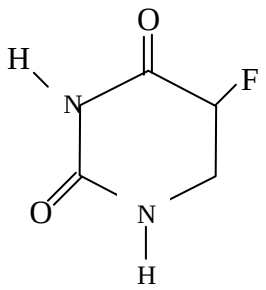
Adverse reactions: Nausea and vomiting, myelosuppression with platelet sparing, and alopecia are common to all regimes using CP

Mucosal ulcerations and less frequently, interstitial pulmonary fibrosis also may result from CP treatment. The occurrence of sterile hemorrhagic cystitis has been attributed to chemical irritation produced by acrolein. For routine clinical use, ample fluid intake is recommended. Administration of the drug should be interrupted at the first indication of dysuria or haematuria. It is important to be aware of the possibility of water intoxication, since these patients usually are vigorously hydrated ***(Defronze et al., 1973).***

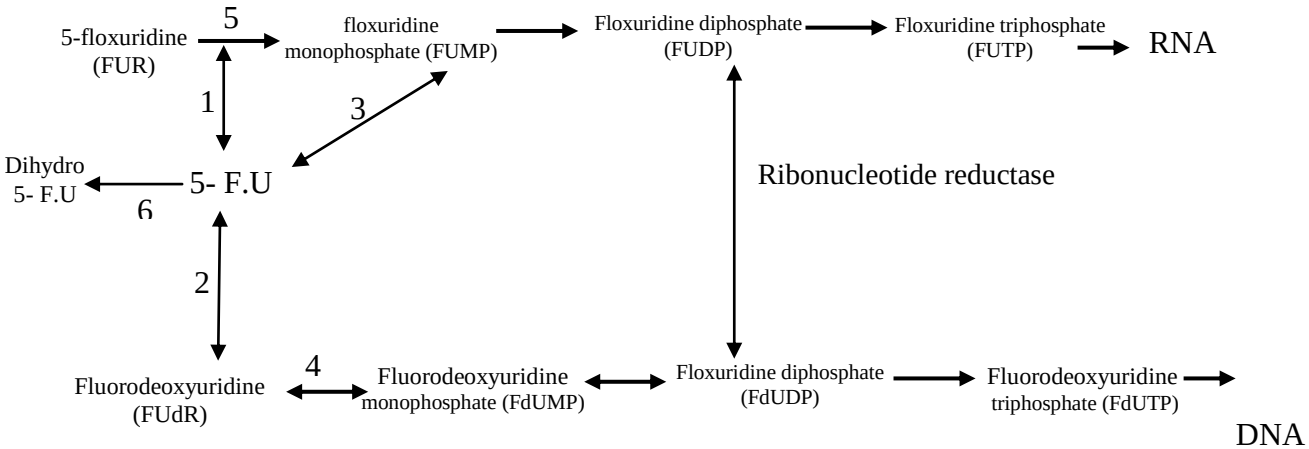
3- 5-FLUOROURACIL (5-FU)

5-FU is a pyridine analog antineoplastic agent that acts by interfering with the sythesis of DNA and RNA.

The structure of 5-FU is as follows:



- **Mechanism of action:** 5-FU requires enzymatic conversion to the nucleotide in order to exert its cytotoxic activity. The activation pathways for 5-F.U are as follows***(Canman et al., 1993)***:



1-Uridine phosphorylase . 4-Thymidine kinase

2-Thymidine phosphorylase 5-Uridine kinase

3-Phosphoribosyl transferase 6-Dihydropyrimidine dehydrogenase

The interaction between Fluorodeoxyuridine monophosphate and the enzyme thymidylate synthase leads to deletion of TTP, a necessary constituent of DNA. Fluorodeoxyuridine triphosphate incorporates into DNA in place of TTP. This may result in DNA breakage. 5-F.U incorporation into RNA also causes toxicity as the result of major effects on both the processing and functions of RNA ***(Canman et al., 1993).***

- **Pharmacokinetics:** 5-FU and floxuridine are administered parenterally, since absorption after ingestion of the drug is incomplete. Metabolic degradation occurs in many tissues, particularly the liver. 5-F.U is inactivated by reduction of the pyrimidine ring, this reaction is carried out by dihydropyrimidine dehydrogenase, which is found in liver, intestinal mucosa, tumor cells and other tissues. 5-FU has a short metabolic half-life and its metabolites are excreted in urine ***(Lu et al., 1993 and Milano et al., 1999).***

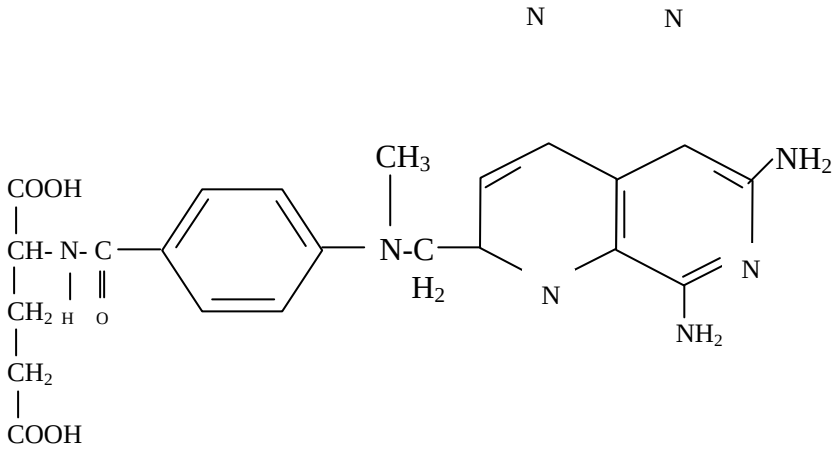
- **Therapeutic uses:** Fluorouracil is used systemically in the treatment of a variety of adenocarcinomas. Beneficial effects have been reported in carcinomas of the ovary, cervix, urinary bladder, prostate, pancreas, and oropharyngeal areas. A cream incorporating 5-F.U is used topically for treating skin cancers ***(De Gramont et al., 1998).***

- **Adverse reactions:** The earliest symptoms during a course of therapy are anorexia and nausea; these are followed by stomatitis and diarrhea. Mucosal ulcerations occur throughout the gastrointestinal tract and may lead to fulminant diarrhea, shock, and death. The major toxic effect is myelosuppression.

Loss of hair, nail changes, dermatitis, and increased pigmentaton and atrophy of the skin may be encountered. Cardiac toxicity, particularly acute chest pain may occur ***(Goodman and Gilman, 2001).***

4- METHOTREXATE (MTX)

MTX is a folic acid antagonist used in the treatment of autoimmune and neoplastic diseases. The structure of MTX is as follows ***(Bertram, 2001)***:



- **Mechanism of action:** Folic acid in its reduced form (tetrahydrofolic acid; THF) is an important biochemical intermediate. It is essetial for synthetic reactions that involve the addition of a single carbon atom during a biochemical reaction, such as the introduction of the methyl group into thymidylate and the synthesis of the purine ring system. During such reactions, THF is oxidized to dihydrofolic acid (DHF), which has to be reduced by dihydrofolate reductase back to THF before it can accept a further 1-carbon group. MTX has a very high affinity for and inhibits the active site of dihydrofolate reductase. This blocks purine and thymidylate synthesis and inhibits the synthesis of DNA, RNA and protein ***(Allegra et al., 1986).***

- **Pharmacokinetics:** MTX is routinely administered intravenously. It is also readily absorbed from the gastrointestinal tract at doses of less than 25mg/m^2 of body surface area, but larger doses are absorbed

incompletely. Distribution of MTX into body spaces, such as the pleural or peritoneal cavity, occurs slowly. However, if such spaces are expanded (e.g., by ascites or pleural effusion), they may act as a site of storage and release of the drug, with resultant prolonged elevation of plasma concentration and more severe toxicity. Approximately 50% of MTX is bound to plasma proteins and may be displaced from plasma albumin by a number of drugs, including sulfonamides, salicylates, tetracycline, chloramphenicol, and phenytion, so caution should be used if these are given concomitantly. Of these drug absorbed , about 90% is excreted unchanged in the urine within 48 hours, mostly within the first 8 to 12 hours. A small amount of MTX also is excreted in the stool, probably through the biliary tract ***(Stoller et al., 1977 and Sonneveld et al., 1986).***

- **Therapeutic uses:** MTX has been used in the treatment of severe, disabling psoriasis. It is also used in refractory rheumatoid arthritis. MTX is a useful drug in the management of acute lymphoblastic leukemia in children. The intrathecal administration of MTX has been employed for treatment or prophylaxis of meningeal leukemia or lymphoma and for treatment of meningeal carcinomatosis. MTX is of established value in choriocarcinoma and related trophoblastic tumors of women; cure is achieved in approximately 75% of advanced cases. Beneficial effects are also observed in patients with osteoasarcoma ***(Hoffmeister, 1983 and Borsi and Moe, 1987).***

- **Adverse reactions :** The primary toxicities of MTX affect the bone marrow and the intestinal epithelium. Such patients may be at risk for spontaneous hemorrhage or life-threatening infection, and they may require prophylactic transfusion of platelets and broad-spectrum antibiotics if febrile. Side effects usually disappear within 2 weeks, but

prolonged suppression of bone marrow may occur in patients with compromised renal function who have delayed excretion of the drug. The dosage of MTX must be reduced in proportion to any reduction in creatinine clearance. Additional toxicities of MTX include alopecia, dermatitis, interstitial pneumonitis, nephrotoxicity, defective oogenesis or spermatogenesis, abortion, and teratogensis. Hepatic dysfunction is usually reversible but sometimes leads to cirrhosis after long-term continuous treatment, as in patients with psoriasis. Intrathecal administration of MTX often causes meningismus and an inflammatory response in the CSF. Seizures, coma, and death may occur rarely ***(Jolivet, 1983).***

CHAPTER III

TERATOGENICITY OF CHEMOTHERAPEUTIC AGENTS

All chemotherapeutic agents are potentially teratogenic and mutagenic because they act on rapidly dividing cells. The potential exists for fetal malformations, intrauterine growth retardation, spontaneous abortion, stillbirth or premature delivery when a woman is exposed to chemotherapeutic agents prior to or during pregnancy ***(Schardein, 1993 and Meirow and Schiff, 2005).***

The greatest risk for birth defects occurs during first trimester exposure, second and third trimester exposures may result in transient bone marrow suppression, pancytopeni, intrauterine growth retardation, low birth weight and prematurity ***(Aviles, 1991 and Zemlickis, 1992).***

Rat fetuses exposed to doxorubicin (adriamycin) had a spectrum of cloacal and urogenital anomalies. Predominantly no urinary bladder and severe hydroureter/ hydronephrosis on one or both sides were observed. Male fetuses had a proximal blind- ending urethra communicating with dilated ureters. Female fetuses had a persistent urogenital sinus communicating with the ureters and cervix of the uterus. 57% of fetuses had an imperforate anus and some had recto- urethral fistulae (males) or recto-urogenital fistulae (females) ***(Liu and Hutson, 2000).***

The prenatal exposure of rat embryos to doxorubicin results in variable anomalies affecting the skeletal system, alimentary tract (e.g. imperforate anus, oesophageal atresia and tracheo-oesophageal fistula), cardiovascular system and urogenital tract particularly absence of the urinary bladder ***(Liu and Hutson, 2001 and Franca et al., 2004).***

Congenital uropathy is associated with significant morbidity and mortality in the human neonate. The prenatal exposure to doxorubicin leads to a spectrum of malformations including urinary tract anomalies. The most common anomaly was bilateral megaureters with a hypoplastic bladder. Other anomalies included unilateral or bilateral uretrohydronephrosis with a normal sized bladder, duplex kidney, and unilateral or bilateral renal agenesis ***(Mortell et al., 2004).***

Uretro-hydronephrosis was observed in 95% of fetuses exposed to doxorubicin. Also, oesophageal atresia in 92%, duodenal atresia in 92% and bladder hypoplasia in 90% were also observed ***(Franca et al., 2004 and Anderson et al., 2004).***

Cyclophosphamide is used in the treatment of Hodgkin's diseases, non-Hodgkin's lymphoma, neuroblastoma, Wilms' tumor, soft-tissue sarcomas, cancers of the breast, ovary, lung, and cervix, and connective tissue disorders such as systemic lupus erythematosus. It is commonly used in combination chermotherpy regimes ***(Hardman 1996 and Ostensen, 2006).***

Table (4): Teratogenic effects of cyclophosphamide (CP), in different species *(Zemlickis, 1996 and Heringova et al., 2003):*

	Species	Outcomes
Animal studies		This agent has been found to be teratogenic in all animal species tested. The most consistent pattern of defects involves facial clefts and limb reduction defects.
	Rats	Skeletal defects, cleft palate, exencephaly and encephalocele
	Mice	Central nervous system and limb and digit defects
	Rabbits	Cleft lip and / or palate and limb reduction defects
	Rhesus monkeys	Facial clefts meningoencephalocele and craniofacial dysmorphisms in late exposures
Human studies	Case reports	Most of the case reports involve the concomitant use of other chemotherapeutic agents or irradiation. The following malformed children were reported:
		1-Livebirth, 1,900g, absent big toes in both feet, flattening of nasal bridge, hypoplastic 5th finger and bilateral inguinal hernia
		2-Stillbirth (interrupted pregnancy) with absence of all toes and single left coronary artery
		3-Hemangioma and umbilical hernia
		4-Growth retardation, imperforate anus, rectovaginal fistula
		5-Dysmorphic facies, cleft palate, multiple eye defects.
		6-Abnormal shaped and low set ears and absent thumbs. Also, borderline microcephaly, hypotonia and developmental delay at 10months of age.
		7-Twins, one normal and the other with multiple malformation: lung deformity, oesophageal atresia, abnormal inferior vena cava, abnormal renal collecting system, radial anomaly with abnormal thumb and cryptorchidism with rudimental left testicle.
		There are many cases of 2nd or 3rd trimester exposure to cyclohosphamide without adverse effects to the fetus.
	Occupational exposure (case-control study)	Exposure to cyclophosphamide was significantly associated with early fetal loss.

Table (5): Teratogenic effects of 5-FU in different species *(Grafton et al., 1987 and Byrne 1998) :*

	Species studied	Outcomes
Animal studies	Rats	Fetal anemia, defects of the nervous system, palate and skeleton. 5-FU is poorly eliminated by exposed fetus, and can produce fetotoxicity at doses that are nontoxic to the dam
	Mice, rabbits, guinea pigs and hamsters	Hindfoot anomalies, cleft palate, microphtalmia and omphalocele
	Monkeys	Rib and vertebral anomalies intrauterine growth retardation and fetal death.
Hman studies	1st trimester exposure	There are only a few case reports of 5-FU systemic exposure during pregnancy.
	11-12wks of pregnancy; exposed also to irradiation	Therapeutic abortion at 16 weeks. The fetus had multiple defects including bilateral radial aplasia, absent thumbs, oligadactyly, hypoplasia of lungs, aorta, thymus and bile duct, and aplasia of the esophagus, duodenum and ureters.
	2nd and 3rd trimester	5-FU was administered in a patient in high doses over 5months during the 2nd and 3rd trimester. The baby was small but healthy.
	3rd trimester	A newborn exposed to 5-FU during the third timester was not malformed but had transient toxicity signs with cyanosis and jerking extremities during the neonatal period.

5-Fluorouracil (5-FU) is commonly used to treat cancer of the breast, colon, stomach, pancreas, ovary, head, neck and bladder. It is used topically to treat human papilloma virus infections. The prenatal exposure to 5-FU results in one or more of the following anomalies, bilateral radial aplasia with absent thumbs and fingers, hypoplasia of the lungs, aorta, thymus and bile ducts, and aplasia of the oesophagus, duodenum and ureters ***(Grafton et al., 1987 and Kapetz et al., 2008).***

FU induces about 5% mortality with signicficant reduction in body weight and various dimensions of the developing brain. The brain shows microcephally, regression or absence of olfactory lobe and obliteration of the various fissures on the dorsal and ventral surfaces of the brain ***(Kumar et al., 2006)***

Methotrexate (MTX) has multiple therapeutic uses in women of reproductive age including treatment for ectopic pregnancy, neoplastic diseases, autoimmune disorders, and inflammatory conditions. Low-dose weekly of MTX is widely used in the treatment of inflammatory bowel disease, rheumatoid arthritis and is now increasingly used in other rheumatic conditions, including systemic lupus erythematosus and juvenile arthritis ***(Lioyd et al., 1999 and Ferrero and Ragni, 2004).*** More frequent use of methotrexate may result in an increased number of exposures in pregnant women and their fetuses. The minimal, low-dose and brief exposure to methotrexate in the first trimester results in multiple internal and external malformations including craniofacial, axial skeletal, cardiopulmonary and gastrointestinal abnormalities ***(Nguyen et al., 2002 and Affleck and Walker, 2007).***

MTX, a commonly administered chemotherapeutic agent, is a well-known human teratogen. Exposure of a fetus between 6 and 8 weeks of gestation is postulated to cause birth defects as cleft palate, agenesis and fusion of the toes ***(DeSesso and Goeringer, 1992 and Jay et al., 2003).***

Administration of low-dose MTX during pregnancy leads to an absent coronal and lambdoid sutures, wide posterior fontanelle, absent frontal bone, absent one toe, webbing between fingers, ear malformations, bifid uvula, hemivertebrae and multiple cardiac anomalies ***(Powell and Ekert, 1971 and Buckley et al., 1997).***

Table (6): Teratogenic effects of MTX in different species *(Rustin, 1984, Donnenfeld, 1994 and Bawle, 1995)*

			Outcomes
Animal studies	Mice, rats, rabbits and monkeys		Fetal death and doses required to produce this effect are higher in monkeys.
	Rats, mice and rabbits		Malformations are dose-dependent mostly cleft palate, limb and central nervous system malformation.
	Rhesus monkeys		No evidence of teratogenicity
Human studies	Case reports	1st trim exposure	A peculiar pattern of anomalies was described for at least seven children born to women treated with MTX during the first trimester of pregnancy. The main features of this syndrome are abnormal skull (clover- lead skull or oxycephaly) with a large head, large fontanelles, ocular hypertelorism with prominent eyes and wide nasal bridge. Skeletal, limb and CNS defects were also described. There are, also case reports and case series of successful outcomes after first trimester exposure. The risk are probably time and dose dependent, being greater with larger doses and smaller or even non-existent with lower doses.
		2nd and 3rd trimester exposures	1-A review of 5 cases of 2nd or 3rd trimester exposures without adverse effect. 2-A cases of severe myelosupression in an infant exposed to combined therapy during 1st, 2nd and 3rd trim. 3-A healthy girl born to a woman who received combination therapy including MTX for acute lymphoblastic leukemia. Karyotype revealed 46 chromosomes with the presence of gaps and a ring chromosome, which is similar to the observed in patients after cytotoxic chemotherapy. The clinical significance of this finding is still unknown but it can represent a risk of cancer, as well as a risk of genetic damage in the next generation.
		pregnancy loss:	MTX increases the rate of miscarriage in women exposed early in pregnancy and it is used as an abortifacient and in the treatment of ectopic pregnancy.

CHAPTER IV

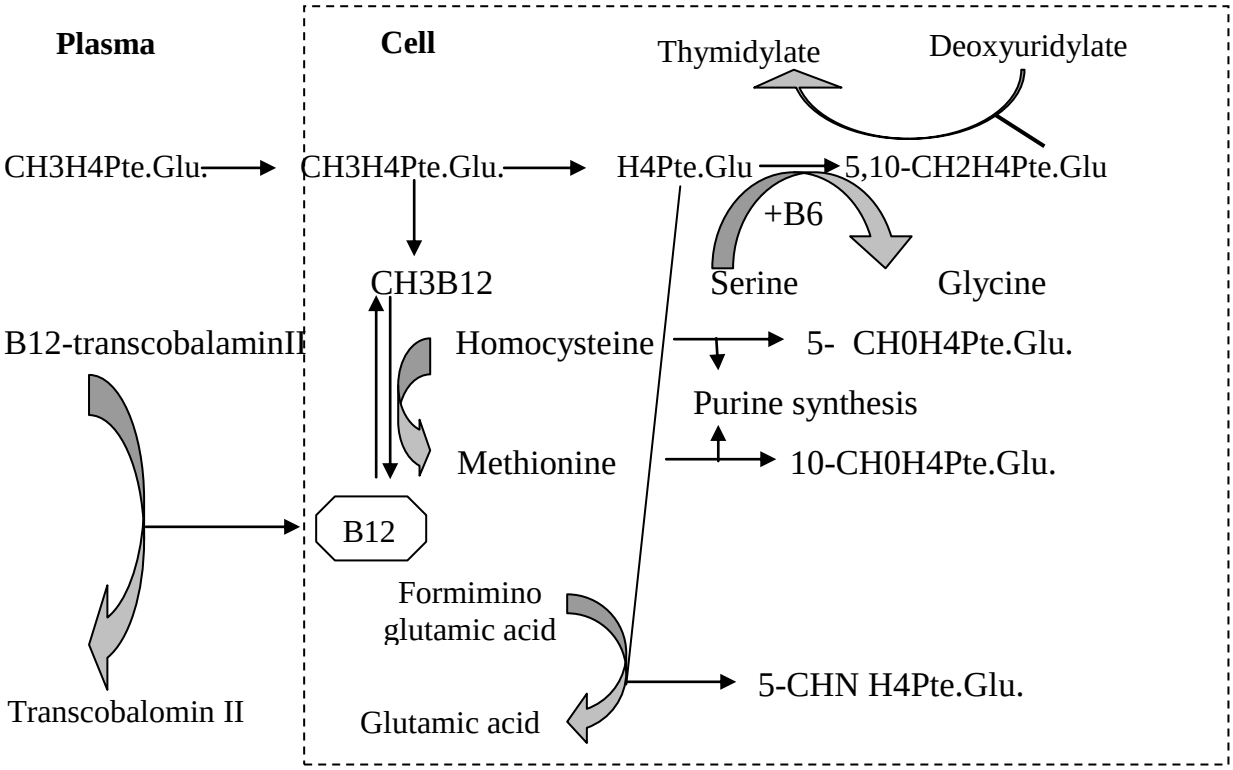
FOLATE AND FOLIC ACID

The folic acid was first described in 1937 and was called Wills' factor. Later on, this factor called vitamin M. the actual term folic acid, was coined in 1941. Folic acid is a dietary essential factor. Its deficiency results in defective synthesis of DNA in any cell in which chromosomal replication and division are taking place ***(Fenech et al., 1998).***

Chemistry and metabolic functions:

Pteroylglutamic acid (Pet.Glu.) is the common pharmaceutical form of folic acid following absorption. Pet.Glu. is rapidly reduced to tetrahydrofolic acid (H4Pte.Glu), which then acts as an acceptor of a number of one- carbon units as CH3 to form (CH3 H4 Pte.Glu.).

Each of the different forms of folic acid plays a specific role in intracellular metabolism, summarized as follows ***(Weir and Scott, 1983).***



The term folate includes all compounds that have the vitamin properties of folic acid including folic acid and naturally occurring compounds in food ***(Cornel and Erickson, 1997).***

Daily requirements:

Many food sources are rich in folates, especially fresh green vegetables, liver, yeast, and some fruits. However, lengthy cooking can destrory up to 90% of the folate content of such food. In normal adult, the recommended daily intake is 400μg, while pregnant or lactating women and patients with high rates of cell turnover such as patients with a hemolytic anemia may require 500 to 600μg or more per day ***(Suitor and Bailey, 2000).***

For the prevention of neural tube defects, a daily intake of at least 400μg of folate in food or in supplements beginning a month before pregnancy and continued for at least the first trimester is recommended ***(Crandall et al.,1998)***.

Table (7): Recommended daily intakes of folate *(Daly et al., 1997)*

Age in months	**Males and females (μg/day)**
0 to 6	65
7 to 12	80
Age in years	**Males and females (μg/day)**
1-3	150
4-8	200
9-3	300
≥14	400
Pregnancy	600
Lactation	500

Table (8): Selected food sources of folate *(Oakeley et al., 1996 and Shills et al., 1999):*

Food	Micrograms	% daily value
Beef liver, cooked, braised, 3 ounces	185	45
Cowpeas, cooked, boiled, ½ cup.	105	25
Spinach, frozen, cooked, boiled, ½ cup	100	25
Great northern beans, bolied, ½ cup	90	20
Asparagus, boiled, 4 spears	85	20
Vegetarian baked beans, canned, 1 cup	60	15
Spinach, raw, 1 cup	60	15
Green peas, frozed, boiled, ½ cup	50	15
Broccoli, chopped, frozen, cooked, ½ cup	50	15
Avocado, raw, all varieties, ½ cup	45	10
Peanuts, all types, dry roasted, 1 ounce	40	10
Wheat germ, crude, 2 tablespoons	40	10
Tomato juice, canned, 6 ounces	35	10
Orange juice, ¾ cup	35	10
Egg, raw, fresh, 1 large	25	6
Cantaloupe, raw, ¼ medium	25	6
Banana , raw, 1 medium	20	6

Absorption Distribution and Elimination:

Folates present in food are largely in the form of reduced polyglutamates, and absorption requires transport and the action of pteroyl- glutamyl carboxypeptidase associated with mucosal cell membranes. The mucosae of the duodenum and upper part of the jejumum are rich in dihydrofolate reductase and capable of methylating most or all of the reduced folate that is absorbed. Since most absorption occurs in the proximal protion of the small intestine, it is not unsual for folate deficiency to occur when the jejunum is diseased. Nontropical and

tropical sprue are common causes of folate deficiency and megaloblastic anemia. Once absorbed folate is transported rapidly to tissues as CH_3H_4Pte.Glu. a constant supply of CH_3H_4Pte.Glu is maintained by food and by an enterohepatic cycle of the vitamin. The liver actively reduces and methylates Pte.Glu and then transports the CH_3H_4Pte.Glu into bile for reabsorption by the gut and subsequent delivery to tissues. This pathway may provide 200μg or more of folate each day for recirculation to tissues. The importance of the enterohepatic cycle is suggested by studies in animals that show a rapid reduction of the concentration of folate in plasma following either drainage of bile or ingestion of alcohol, which blocks the release of CH_3H_4Pte.Glu. from hepatic parenchymal cells ***(Gloria et al., 1997).*** Following uptake into cells, CH_3H_4Pte.Glu. acts as a methyl donor for the formation of methylcobalamin and as a source of H_4PteGlu. Folate is stored within cells as polyglutamates ***(Lucock et al., 1989 and Lucock et al., 1995).***

Folate deficiency:

Folate deficiency is a common complication of diseases of the small intestine, which interfere with the absorption of folate from food and the recirculation of folate through the enterohepatic cycle. In acute or chronic alcoholism, daily intake of folate in food may be severely restricted, and the enterohepatic cycle of the vitamin may be impaired by toxic effects of alcohol on hepatic parenchymal cells, this is perhaps the most common cause of folate –deficient megaloblastic erythropoiesis. Disease states characterized by a high rate of cell turnover, such as hemolytic anemias, also may be complicated by folate deficiency. Additionally, drugs that inhibit dihydrofolate reductase (e.g., methotrexate, trimethoprim) or that interfere with the absorption and storage of folate in tissues (e.g., certain anticonvulsants and oral

contraceptive) are capable of lowering the concentration of folate in plasma and at times may cause a megaloblastic anemia. Folate deficiency has been implicated in the incidence of neural tube defects, including spina bifida, encephalocele, and anencephaly. This is true even in the absence of folate- deficient anemia or alcoholism ***(Paulozzi et al., 2001).***

Folate deficiency is recognized by its impact on the hematopoietic system. This fact reflects the increased requirement associated with high rates of cell turnover. Folate deficiency is best diagnosed from measurements of folate in plasma and in red cells by use of a microbiological assay. The concentration of folate in plasma is extremely sensitive to changes in dietary intake of the vitamin and the influence of inhibitors of folate metabolism or transport, such as alcohol. Normal folate concentrations in plasma range from 9 to 45nano-mole; below 9nano-mole is considered folate deficiency. The plasma folate concentration rapidly falls to values indicative of deficiency within 24 to 38 hours of steady ingestion of alcohol. The plasma folate concentration will revert quickly to normal once such ingestion is stopped. The amount of folate in red cells or the adequacy of stores in lymphocytes may be used to diagnose a long-standing deficiency of folic acid ***(Haslam and Probert, 1998).***

MATERIAL AND METHODS

Animals :

Fifty female albino rats and twenty male rats were used in this study. The female rats were isolated, about 120 days in age and ranging in weight from (160 to 200gms). The males were of the same range of weight and age.

The males and females were kept in a separate animal cages, under the prevailing atmospheric conditions. They were fed a balanced diet in the form of bread, barely, carrots, lettuce, milk and water. Each 3 females were placed over night with two males. If the vaginal smear in the following morning contained sperms, that day was considered as day zero of gestation.

The vaginal smear was done by the insertion of a vaginal pipette with attached rubber bulb into the vagina, and by gentle release of the bulb, sucking the secretion into the pipette, from the vaginal vault. The secretion is quickly poured across a glass slide and placed into a fixative (95% alcohol for about 4 minutes).

The pregnant female rats were classified into two groups

A-Control group: Ten pregnant female rats were used as a control.

B-Teated groups:

Forty pregnant rats, were subdivided into four subgroups each of which includes 10 rats. Each subgroup was given the corresponding drug daily from the 6^{th} to the 9^{th} days of gestation. The half of each subgroup (5 rats) was given also folic acid 100 microgram/ kg body weight daily orally by gastric intubation through a urethral catheter with flexible adapter ***(Bialostosky et al., 2002).***

1-Doxorubicin subgroup: includes two divisions:

- Division A1: Injected intravenously by doxorubicin (D) 2microgram /gram/day.
- Division B1 : As A1 plus folic acid 100 ug/kgm daily orally (D+FA).

2-Cyclophosphamide subgroup: includes two divisions:

Division A2 : Injected intravenously by cyclophosphamide (CP) 7microgram /gram/day.

Division B2: As A2 Plus folic acid 100 ug/kgm daily orally (CP+FA).

3-5-Fluorouracil subgroup includes two divisions:

Division A3: Injected intravenously by 5- fluorouracil (FU) 15microgram/ gram/day.

Division B3: As A3 plus folic acid100 ug/kgm daily orally (FU+FA)

4-Methotrexate subgroup: includes two divisions :

Division A4: Injected intravenously by methotrexate (M)5microgram/day.

Division B4 : As A4 plus folic acid 100 ug/kgm daily orally (M+FA).

Methods:

On the 20th day of gestation i.e. 12-24 hours before the expected day of delivery. All female rats, both the experimental and control were anaesthetized by inhalation of ether. The fetuses were immediately delivered by labarotomy to prevent the mothers from devouring any abnormal offspring. The anterior abdominal wall was incised and the uterus with the two ovaries were photographed, then removed after cutting of the mesometrium, mesovariun and vagina. The ovaries were examined using a magnifying lens in order to count the number of

corpora lutea of pregnancy in each ovary. They were large, with yellowish tinge to be differentiated from the corpora albicans which were small, with whitish colour.

The uterine horns were carefully inspected. The number of implantation sites, the resorption sites, live and dead fetuses were counted. The implantation sites indicate the original sites, live and dead fetuses were counted. These sites indicate the original sites of embryos irrespective of whether they have been survived or have undergone resorption. A resorption site was indicated by a dark brown blood spot and it refers to early post implantation death. However, late post implantation death appears as a large blood clot attached to the uterine wall at the site of implantation. The percentage of the pre-implantation loss was calculated from the difference between the number of corpora lutea and number of implantation sites. The sites of resorption and living fetuses in utero were counted and their exact distribution in each uterine horn was noted. The percentage of post implantation loss was calculated from the difference between the number of implantation sites and the total number of placentations of both living, dead or resorped fetuses.

The uterine horns were then cut longitudinally very gently with the tip of blunt scissors to deliver each fetus inside its amniotic sac. Each sac was incised at the dorsal aspect of the fetus and cautiously pealed from it. The umbilical cord was cut at its placental attachment and held by a forceps and each fetus was thoroughly washed with normal saline in a Petri-dish.

The number of fetuses whether alive or dead and their exact position in uterine horns was carefully noted. The crown rump length, head length and biparietal diameter were measured using a calibrated metallic gauge and the weight of the fetuses was recorded.

Using a magnifying lens each fetus was carefully examined for any apparent external gross abnormality in an ordinary manner from the head to the tail. The number of digits was counted in each limb. The results of this inspection were recorded in appropriately designed tables, as constructed by ***(Kalman, 1989).***

Two fetuses randomly were picked up from each litter delivered from each of the individual maternal rats. The fetuses were dehydrated and fixed by immersion in 95% ethyl alcohol for not less than seven days. This is for subsequent skeletal visualization by the method of ***Dawson (1926).*** Which will be described in detail later.

The remaining fetuses were placed for one week in Bouin's fluid consisting of a mixture of acetic aid and formalin along with picric acid in the respective proportions of 1: 15: 5 for efficient decalcification in order to allow easier performance of free hand serial sections in the soft fetal tissues ***(Mekota and Vermehren, 2005).***

The scheme of serial sectioning of the fetal tissues in the 20^{th} day rat fetuses as adopted by ***Kotb in (1973)*** was performed. The sections were of 1mm thickness and were subsequently examined under the magnification of the binocular dissecting microscope for the presence of any gross abnormalities in the internal organs.

Table (9): A list of serial sections done in this work *(Kotb, 1973):*

Serial number and level	Type of structure displayed by the section
A-Head and Neck 1-At the angle of the mouth.	In order to inspect the hard palate and nasal cavities.
2-Through eye balls.	Size of both eye balls and olfactory bulbs. Size and texture of lenses.
3-At the greatest transverse diameter of the head (between eyes and ears)	In order to inspect the third and fourth ventricles which are dilated in hydrocephalus states.
4-At the middle of the ear region (or just behind the ear)	In order to inspect the third and fourth ventricles which are dilated in hydrocephalus states.
5-Lower neck.	Examine trachea-esophagus and thymus gland.
B-Rest of the body: 6-Upper thorax	In order to inspect the arch of the aorta coursing diagonally to the left side of the trachea and esophagus. The apices of the lungs appear on either side.
7-Cardiac level (just below the axilla)	Right and left cardiac ventricles and the interventricular septum are shown.
8-Upper abdominal level (just above the umbilicus)	**In order to inspect:** - Lobes of the liver. -Stomach appearing on the left side. -Duodenum and pancreas appearing near the midline and ventrally.
9-Mid abdominal level (just below the umbilicus)	-Both kidneys have been cut through the renal pelvis. The size and configuration of the renal pelvis are important. The side-to- side and ectopia of kidney should be noted. -The intestines are removed from the caudal end of the trunk to achieve a good view of pelvic organs. The bladder is attached to the ventral body wall. -In female specimens, the two uterine horns are seen to emerge from the pelvic floor dorsal to the urinary bladder and coarse along the dorsal body wall towards the kidneys. -In male specimens, the testes are seen on either side of the bladder, if at more cephalic position, it would signify cryptorchidism.

Fig. (5): A scheme of cross sections of full term rate fetus (Razor section)

1-Section at the angle of the month.

2-Section at the region of the eyeballs.

3-Section at the greatest diameter of the head.

4-Section at the region of the ear.

5-Section in the upper part of the neck.

6-Section in the lower part of the thorax.

7-Section just below the axilla.

8-Section just above the umbilicus.

9-Section just below the umbilicus.

Skeletal system staining, *(Koji Yamakawa et al., 2003):*

1-After initial fixation in 95% alcohol for not less than one week period the fetuses were placed for 1-2 days in 1% KOH (Potassium hydroxide) solution to corrode the fetal soft tissues and expose the skeletal structures underneath.

2-Thorough washing of the fetal skeleton by tap water from all traces of the alkaline solution was then carried out before palcing it in reagent (solution B) (formed of a mixture of 150ml glycerin, 800ml distilled water and 10gm solid KOH) to which a few drops of 1% red alizarin (solution A) had been previously added. The bony parts of the skeleton were stained with a deep red violet colour within 3-5 days of exposure to this reagent.

3-Then the fetuses were transferred to fresh solution "B" for 7-14 days to remove the staining of soft tissues.

4-Then dehydration of the fetuses was done by passing them slowly through a mixtures of alcohol, glycerin and water in different percentages which were respectively (1: 2:7), (2:2:6), (4:4:2) and then the embryos were passed in solution of equal parts of alcohol and glycerin only.

5-The fetuses with the stained skeleton were preserved in pure glycerin with addition of 2 drops of formalin to prevent putrefaction.

6-The stained skeletons were studied under binocular dissecting microscope. The number of ribs and vertebrae were counted. Examination of the skull, limb bones and sternum was done for any skeletal abnormalities.

Skeletal system evaluation:

Each specimen was examined with the aid of a dissecting microscope. Ossification was scored as being complete, delayed or absent. Delayed centers can be identified when the center is either lightly stained (partial ossification) or asymmetrically developed (incomplete ossification). Abnormal ossification was also recorded.

Histolgocial techniques:

Ten fetuses of each group (control and experimental) were examined histologically, as follows:

Fixation:

The fetuses were directly fixed in Bouin's solution for 3 days to achieve proper degree of hardening of the soft tissues.

Bounin's solution *(Mekota and Vermehren, 2005):*

Saturated aqueous picric acid	25ml.
Formalin	75ml
Glacial acetic acid	5ml.

Dehydration and clearing:

The specimens were washed in several changes of 70% alcohol for 24 hours, and then dehydrated in ascending grades of alcohol (70%, 80%, 96% and 100%). After complete dehydration, the specimens were cleared in xylene.

Impregnation and Embedding:

The cleared specimens were impregnated in 3 changes of soft paraffin wax, (melting point 54-56C^{o}) each for one hour then impregnated in 3 changes of hard paraffin. Finally, the specimens were embedded in hard paraffin (melting point 56-58C^{o}).

Cutting into sections:

Serial sections 5-7μm thick were cut in the longitudinal and transverse planes.

Staining by Haematoxylin and Eosin Stain *(Joh et al., 1996 and Yang et al., 2007):*

Preparation of Ehrlich's haematoxylin solution:

-Haematoxylin 2.gm

-Absolute alcohol 100.0ml

-Dissolve in water bath and then cool and filter

-Distilled water 100.0ml

-Ammonium alum 2.0gm

-Dissolved by warming, mix the above two solutions together and then add:

-Glacial acetic acid 10.01ml

-Glycerol 100.0ml

-Mix thoroughly and leave exposed to sunlight to ripen for six weeks.

-Preparation of Eosin stock solution:

-Eosin 5.0gm

-Tap water 100.0ml.

Working solution 1% was prepared from the stock solution, few drops of formalin were added to prevent the growth of moulds in the solution. The alkalinity of tap water gave superior staining.

Methods of staining:

The sections were dewaxed in xylol and passed in descending grades of alcohol down to tap water. The sections were put in Ehrlich's haematoxylin for 4-8 minutes, washed in tap water and counter –stained in 0.5% eosin for 3minutes. The sections were then washed in tap water

for 15 minutes. Finally, the sections were passed through ascending grades of alcohol, cleared in xylol then mounted on slides using Canada balsam and cover slips. The nuclei stain from blue to black by the haematoxylin and eosin stain, while the cytoplasm stains pink. The red blood cells vary from orange to red.

STATISTICAL METHODS

The statistical methods used for data collection, presentation and analysis of the results in this study can be summarized as follow:

1-Frequency table and percentage:

Where the studied groups were presented according to the number of fetuses studied in each group and the different outcomes observed (live fetuses, pre-implantation loss and post implantation loss and resorption).

2-Arithmetic Mean ($\overline{X}$):

It equals the sum of all observation divided by their number. It can be calculated from the following equation:

$$\overline{X} = \frac{\sum X}{n}$$

Where:

$\overline{X}$ = Arithmetic mean.

$\sum X$ = Sum of values recorded.

n = Number of observations.

3-Standard deviation (S.D):

Calculated from the following equation:

$$S.D = \pm\sqrt{\frac{\sum (X - \overline{X})^2}{n-1}}$$

where:

$\sum (X - \overline{X})^2$ = Sum of square difference between each observation (x) and the mean value ($\overline{X}$) of all observation.

n= Number of observation

4-Range:

Range = Maximum – Minimum

5-Student's test (t-test):

It is a test of significance for the difference between two sample means (X1, X2). It can be calculated from the following equation:

Significant of Results:

The corresponding P value for each test was directly computed by the microprocessor, in which we used the one call test values:

P Values > 0.05 = Non –significant.

P values < 0.05 = significant.

P Values < 0.01 = Highly significant.

P values < 0.001 = Very Highly significant.

(Daniel,1991)

RESULTS

The pre-implantation loss and post- implantation loss as well as resorption are explained in table (10). It is observed that the highest percentage of preimplantation loss in the treated groups is evident in the cyclophosphamide (CP) treated group (27.27%). While the lowest percentage is seen in the cyclophosphamide with folic acid (CP+FA) treated group (6.97%). Also, it is observed that the highest percentage of post- implantation loss and resorption is evident in the fluorouracil (FU) treated group (26.08%). While the lowest percentage is seen in the CP+FA treated group (0%).

With respect to the weight, it was found that the weight is decreased in all treated groups. It is observed that the highest decrease in weight is evident in the fluorouracil (FU) treated group. While the lowest decrease is seen in the doxorubicin with folic acid (D+FA) treated group (Table 11).

The crown- rump length (CRL) is decreased in all treated groups. It is observed that the highest decrease in (CRL) is evident in the (FU) treated group. While the lowest decrease in seen in the (D+FA) treated group (Table 12).

The biparietal diameter (BP) is decreased in all treated groups. It is observed that the highest decrease in (BP) is evident in the (FU) treated group. While the lowest decrease is seen in the (D+ FA) group (Table13).

The head length is decreased in all treated groups. It is observed that the highest decrease is evident in (CP), (FU) and (M) treated groups. While the lowest decrease is seen in the (D+FA) treated group (Table 14).

In table (15) the comparison between (D) and (D+FA) treated groups, showed that the variable parameters are improved with the use of folic acid. The best improvement is seen in (CRL) parameter while the worest improvement is seen in head length parameter.

In table (16) the comparison between (CP) and (CP+FA) treated group showed the variable parameters are improved with the use of folic acid. The best improvement is seen in weight parameter. While the worest improvement is seen in (BP)parameter.

In table (17) the comparison between (FU) and (FU +FA) treated groups showed that the variable parameters are improved with the use of folic acid. The best improvement is seen in weight parameter. Wile the worest improvement is seen in head length parameter.

In table (18) the comparison between (M) and (M+FA) treated groups, showed that the variable parameters are improved with the use of folic acid. The best improvement is seen in (CRL) parameter. While the worest improvement is seen in head length and weight parameters.

Table (10): Live fetuses, pre implantation loss and post implantation loss and resorption.

Group	Live fetuses		Preimplantation loss		Post implantation loss and resorption		Total number of corpus lutea of pregnancy
	No.	%	No.	%	No.	%	
Control	80	88.88	10	11.12	0	0	90
Doxorubicin	32	69.56	12	26.09	2	4.35	46
Doxorubicin+folic acid	35	74.46	11	23.40	1	2.14	47
Cyclophosphamide	25	56.81	12	27.29	7	15.90	44
Cyclophosphamide+folic acid	40	93.02	3	6.98	0	0	43
Fluorouracil	30	65.22	4	8.69	12	26.09	46
Fluorouracil + folic acid	33	73.33	4	8.88	8	17.79	45
Methotrexate	28	63.63	6	13.63	10	22.74	44
Methotrexate+ folic acid	30	62.50	8	16.66	10	20.84	48

Preimplanation losses = total number of corpus lutea of pregnancy minus the number of implantation sites.

Post implantation loss and resorption = Total number of implantation sites minus all fetuses either live or died

Histogram (1): Shows live fetuses, pre implantation loss and post implantation loss and resorption.

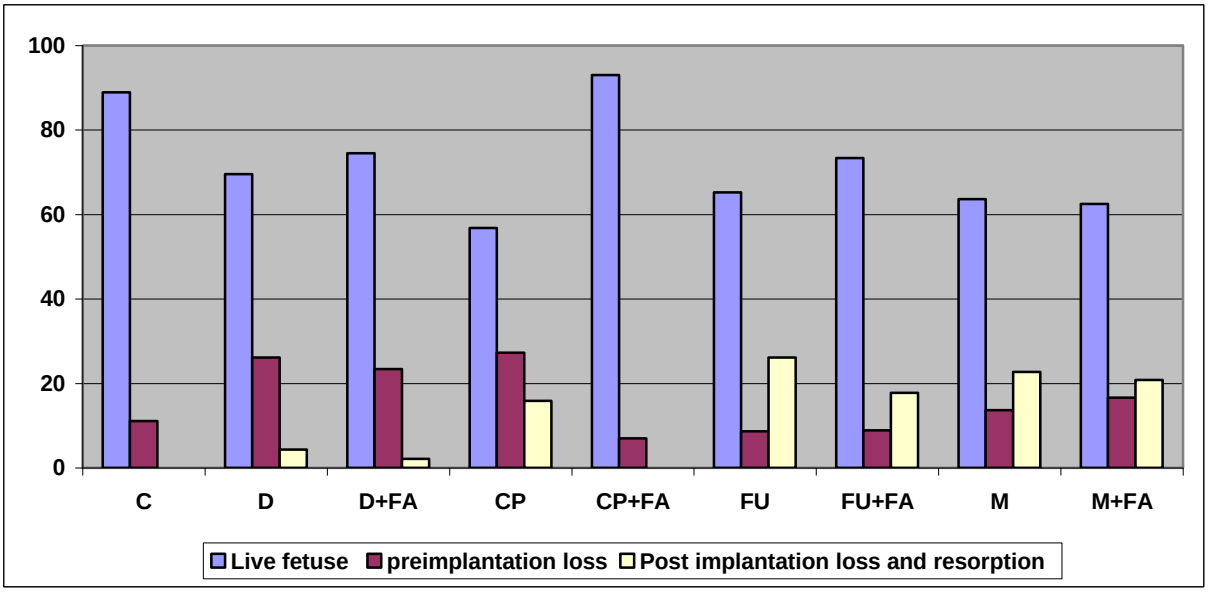


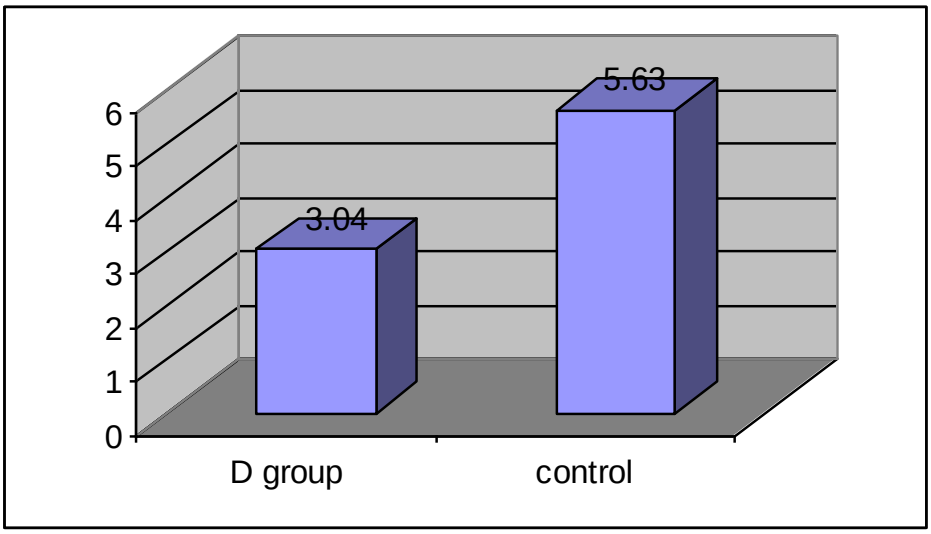
Table (11): Mean ± SD of the weight (Wt) (gm) in the control and different treated groups .

Group	Mean ± SD	Control Mean ± SD	T	P	Significance
Doxorubicin (D)	3.04 ± 0.0565	5.63 ± 0.02	64.75	0.00	**
Doxorubicin+folic acid (D+FA)	4.26 ± 0.0100[a]		88	0.00	**
Cyclophosphamide (CP)	1.73 ± 0.0200[a]		246	0.00	**
Cyclophosphamide + folic acid (CP+FA)	2.90 ± 0.0919		41	0.001	**
Fluorouracil (FU)	1.43 ± 0.0919		60	0.00	**
Fluorouracil + folic acid (FU+FA)	1.99 ± 0.0282		182	0.00	**
Methotrexate (M)	1.60 ± 0.4879		201	0.00	**
Methotrexate+ folic acid (M+FA)	1.65 ± 0.0200[a]		251	0.00	**

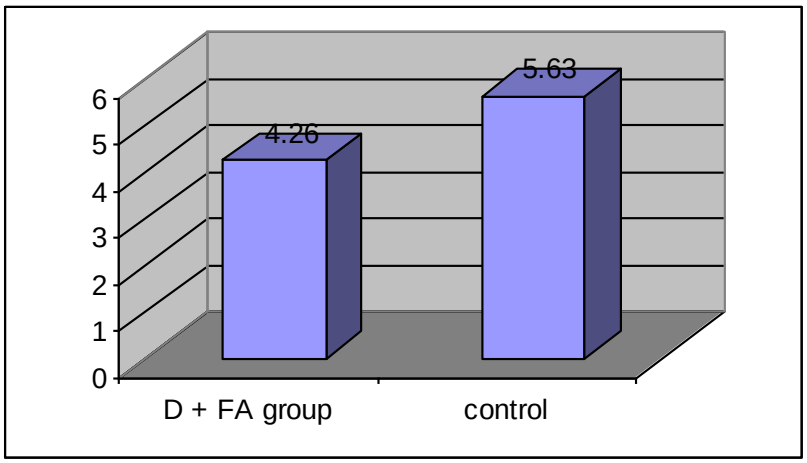
****** Highly Significant

Histogram (2): Shows the mean ± SD of the weight (Wt) (gm) in the control and different treated groups .

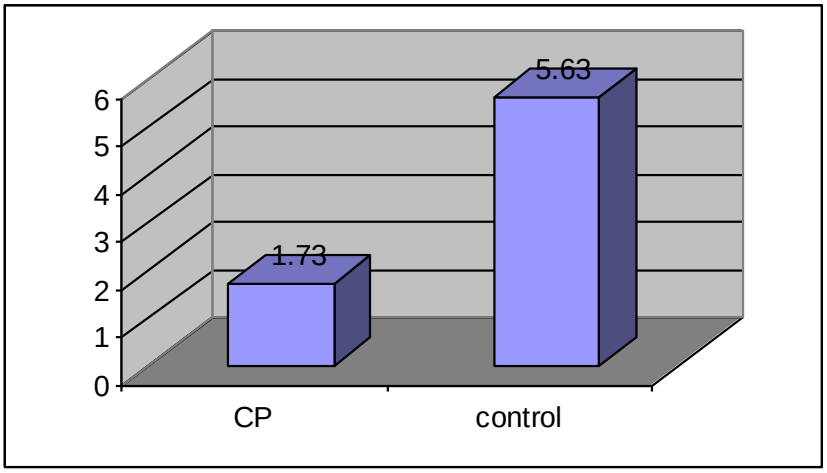
A



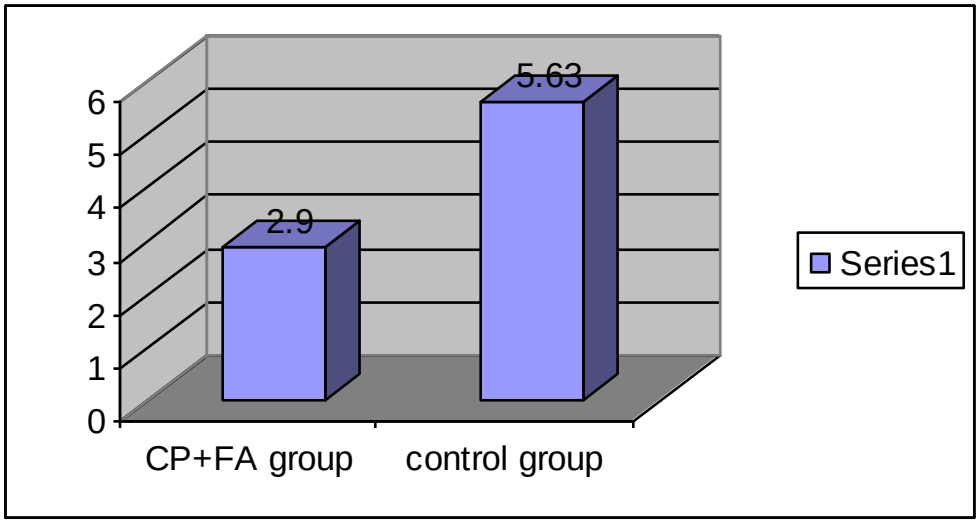
B


6
5
4
3
2
1
0
4.26
5.63
D + FA group
control

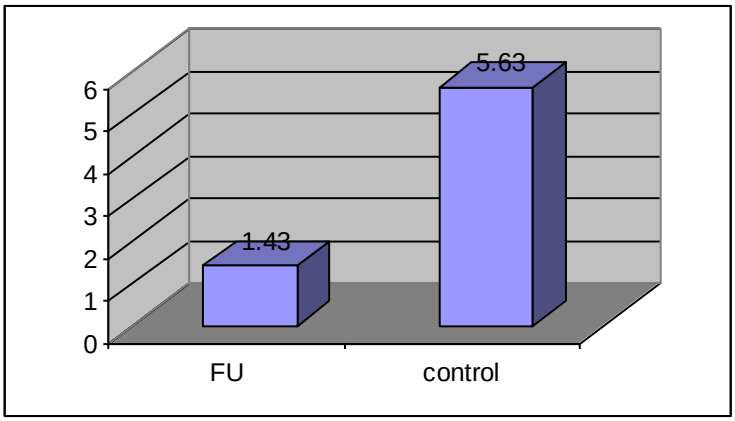
C


6
5
4
3
2
1
0
1.73
5.63
CP
control

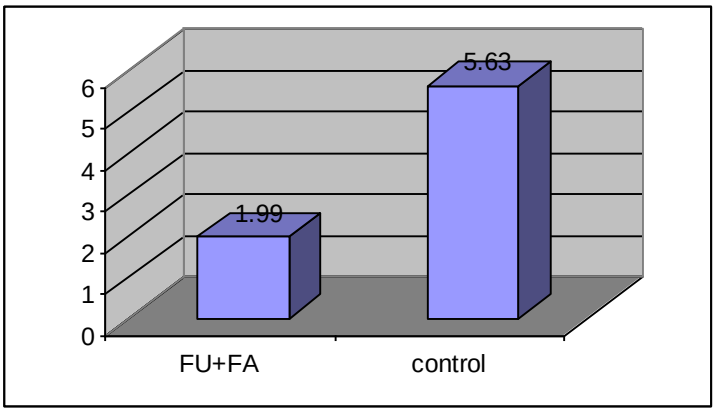
D


6
5
4
3
2
1
0
2.9
5.63
CP+FA group
control group
Series1

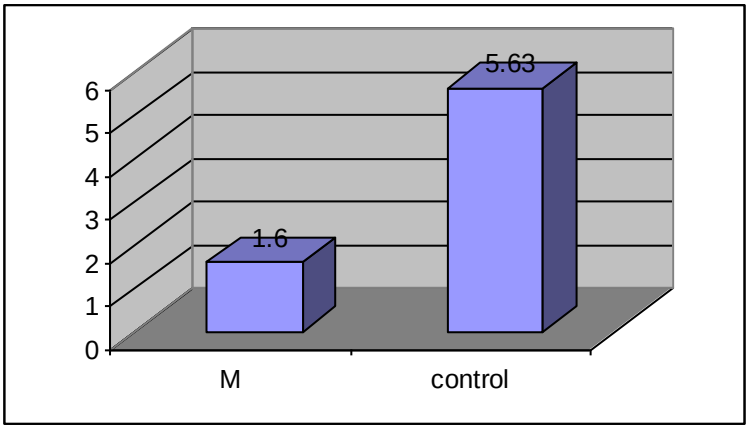
E


6
5
4
3
2
1
0
5.63
1.43
FU
control

F


6
5
4
3
2
1
0
5.63
1.99
FU+FA
control

G


6
5
4
3
2
1
0
5.63
1.6
M
control

H

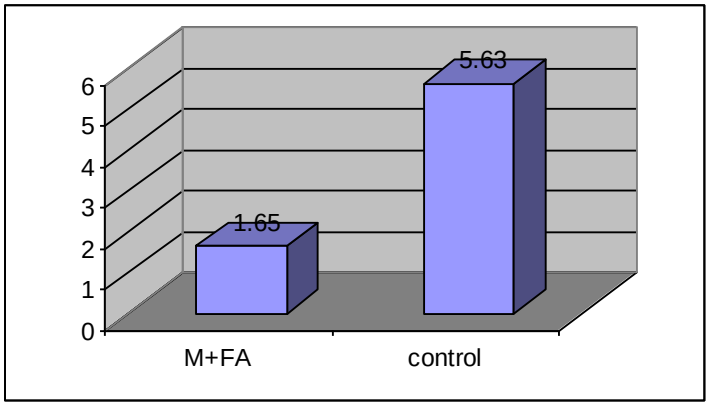

6
5
4
3
2
1
0
5.63
1.65
M+FA
control

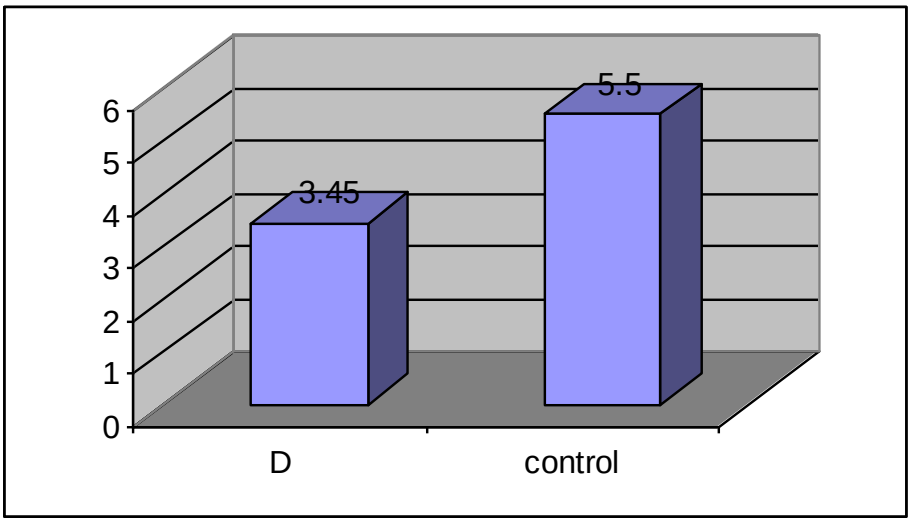
Table (3): Mean ± SD of the crown- rump length (CRL) (cm) in the control and different treated groups.

Group	Mean ± SD	Control Mean ± SD	T	P	Significance
D	3.45 ± 0.0707	5.5 ± 0.00	41	0.001	**
D+FA	3.95 ± 0.0707		31	0.001	**
CP	2.60 ± 0.2828		14.5	0.005	*
CP+FA	3.30 ± 0.1414		22	0.002	**
FU	2.40 ± 0.1414		31	0.001	**
FU+FA	2.90 ± 0.0200[a]		130	0.00	**
M	2.45 ± 0.3535		12.03	0.008	**
M+FA	2.60 ± 0.0200[a]		13.6	0.005	**

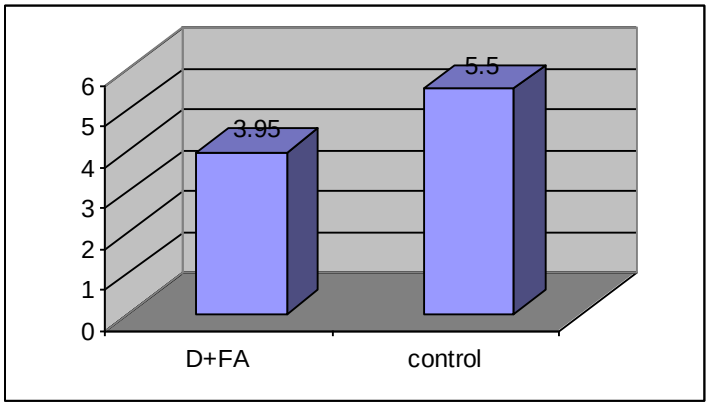
*Significant ** Highly significant

Histogram (3): Shows mean ± SD of the crown- rump length (CRL) (cm) in the control and different treated groups.

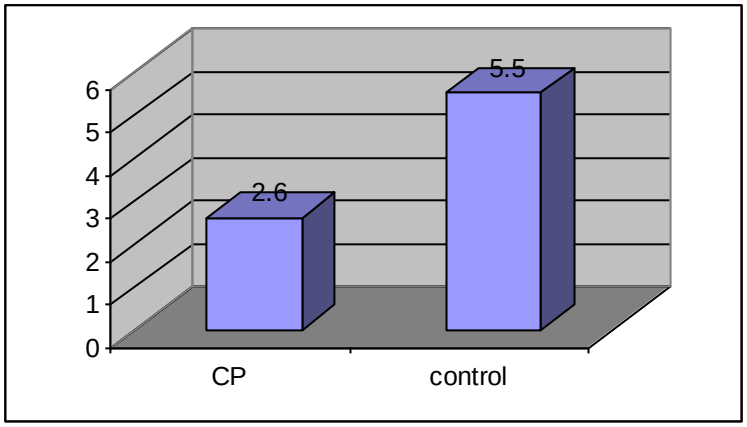
A



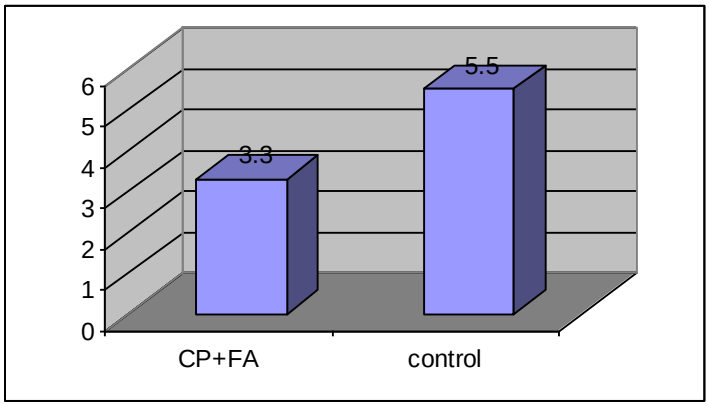
B


6
5
4
3
2
1
0
3.95
5.5
D+FA
control

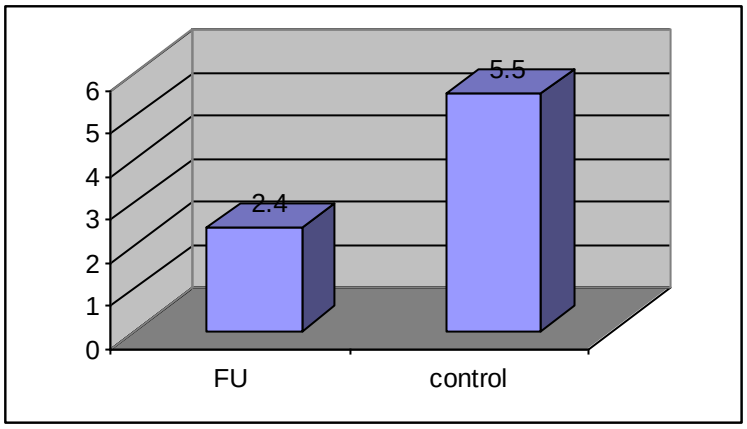
C


6
5
4
3
2
1
0
2.6
5.5
CP
control

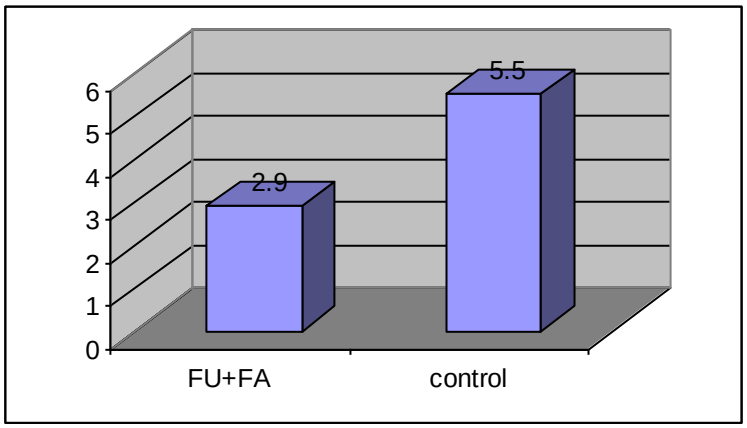
D


6
5
4
3
2
1
0
3.3
5.5
CP+FA
control

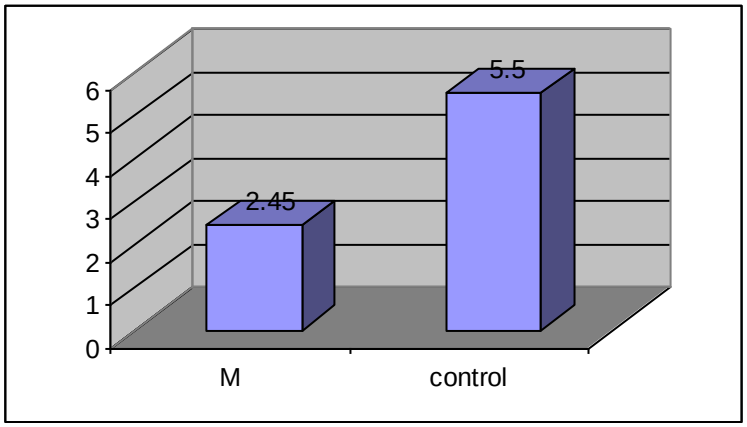
E


6
5
4
3
2
1
0
2.4
5.5
FU
control

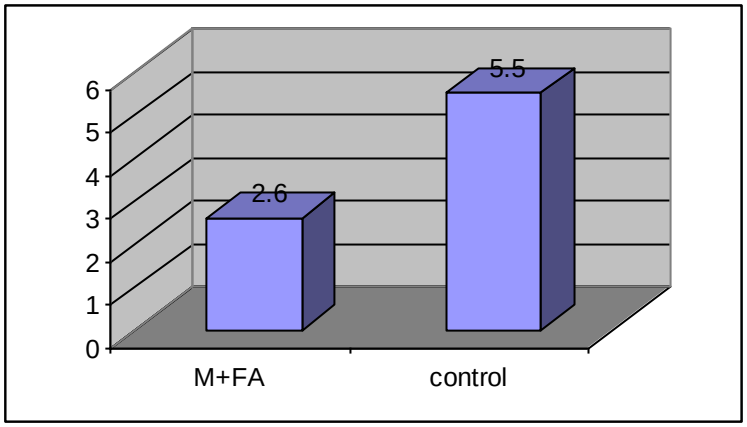
F


6
5
4
3
2
1
0
2.9
5.5
FU+FA
control

G


6
5
4
3
2
1
0
2.45
5.5
M
control

H


6
5
4
3
2
1
0
2.6
5.5
M+FA
control

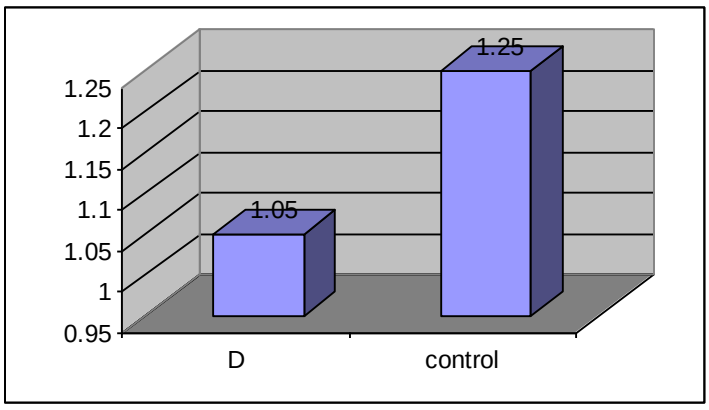
Table(13): Mean ± SD of the biparietal diameter (BP) (cm) in the control and different treated groups.

Group	Mean ± SD	Control Mean ± SD	T	P	Significance
D	1.05 ± 0.0717	1.25 ± 0.00	4	0.057	•
D+FA	1.20 ± 0.1414		0.5	0.667	•
CP	0.90 ± 0.0717		6.8	0.021	*
CP+FA	0.95 ± 0.0717		6	0.027	*
FU	0.80 ± 0.0300[a]		17.6	0.003	**
FU+FA	0.90 ± 0.0400[a]		11.1	0.008	**
M	1.00 ± 0.0500[a]		6.6	0.022	*
M+FA	1.10 ± 0.0200[a]		7.5	0.017	*

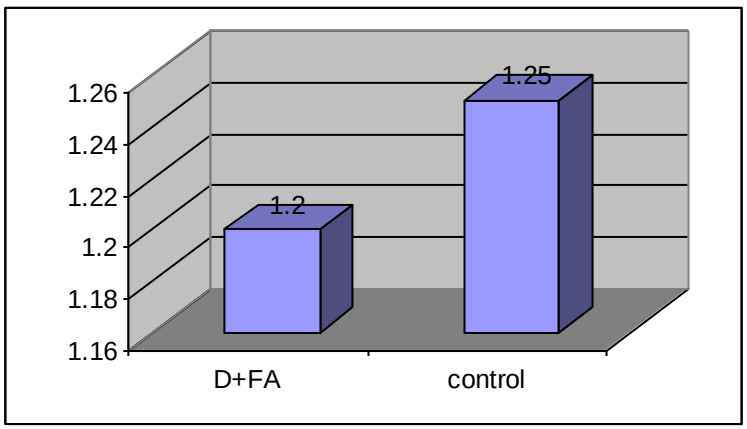
• Non significant * significant ** Highly significant

Histogram (4) : Shows mean ± SD of the biparietal diameter (BP) (cm) in the control and different treated groups.

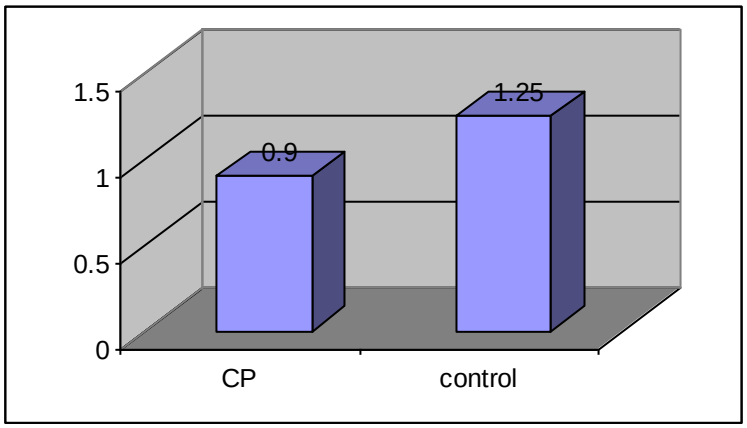
A



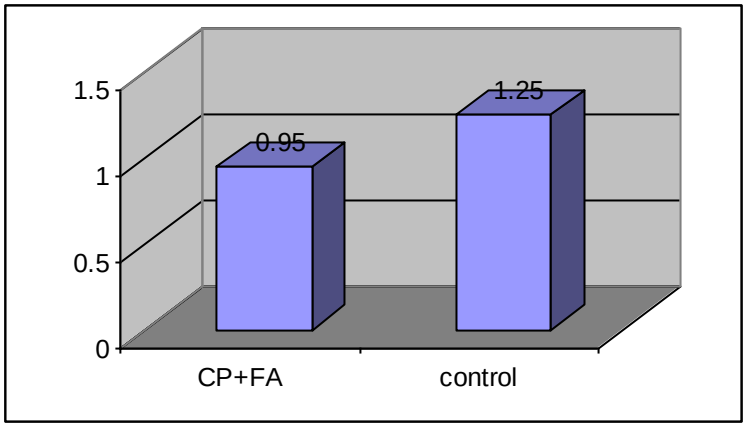
B



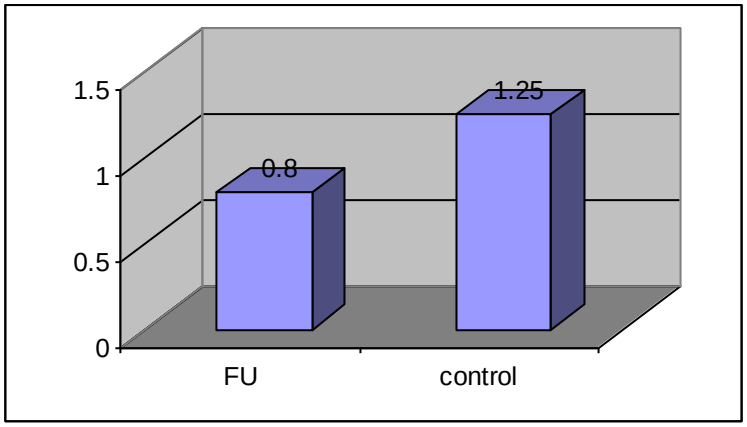
C


1.5
1
0.5
0
0.9
1.25
CP
control

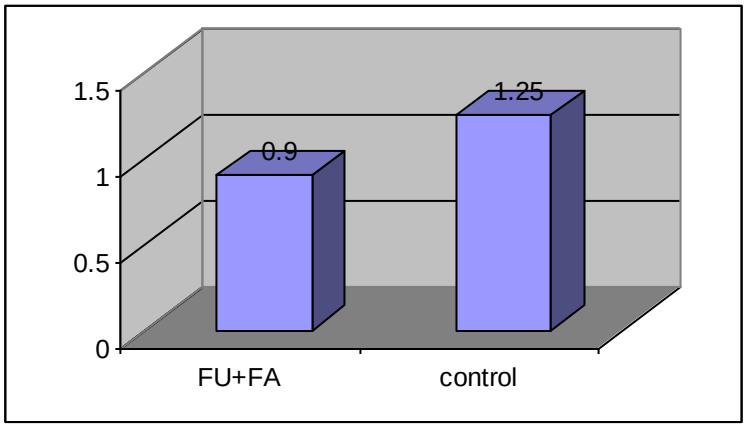
D


1.5
1
0.5
0
0.95
1.25
CP+FA
control

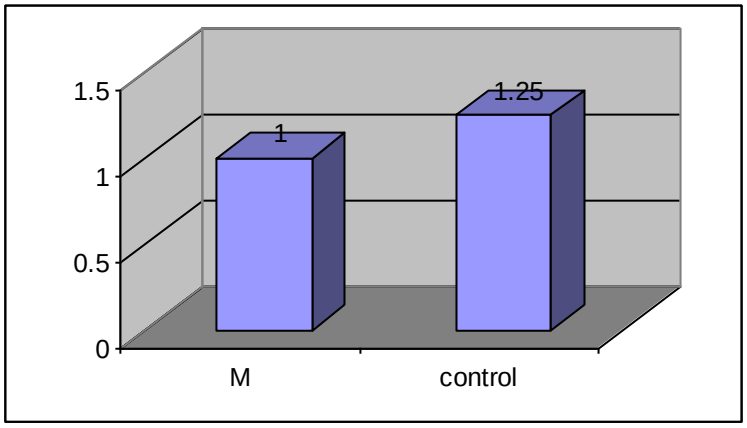
E


1.5
1
0.5
0
0.8
1.25
FU
control

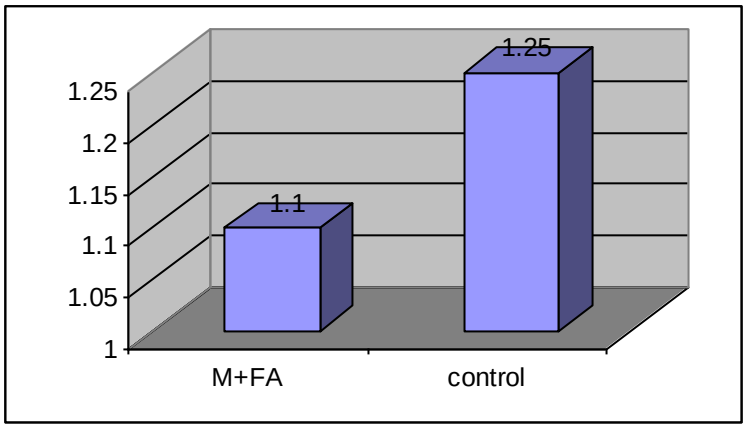
F


1.5
1
0.5
0
0.9
1.25
FU+FA
control

G


1.5
1
0.5
0
1
1.25
M
control

H


1.25
1.2
1.15
1.1
1.05
1
1.1
1.25
M+FA
control

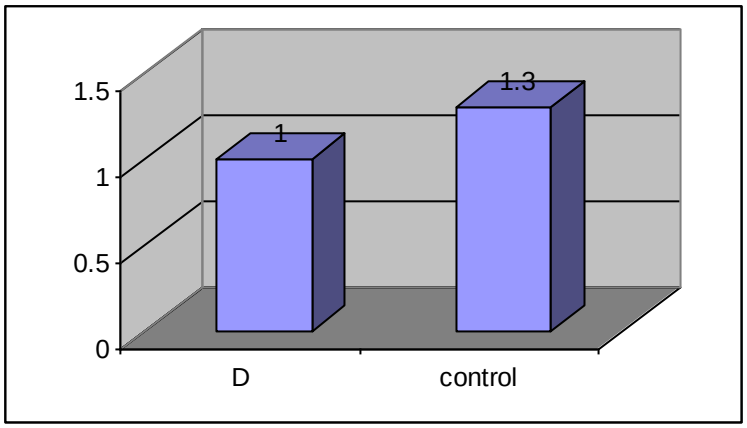
Table(13): Mean ± SD of the head length (cm) in the control and treated groups:

Group	Mean ± SD	Control Mean ± SD	T	P	Significance
D	1.00 ± 0.0707	1.30 ± 0.00	5.8	0.028	*
D+FA	1.10 ± 0.0500		5.25	0.034	*
CP	0.95 ± 0.0717		7	0.020	*
CP+FA	1.00 ± 0.0200		15	0.004	**
FU	0.95 ± 0.0717		31	0.001	**
FU+FA	1.00 ± 0.0400		9.5	0.011	*
M	0.95 ± 0.0717		11	0.008	**
M+FA	1.00 ± 0.0300		11.7	0.007	**

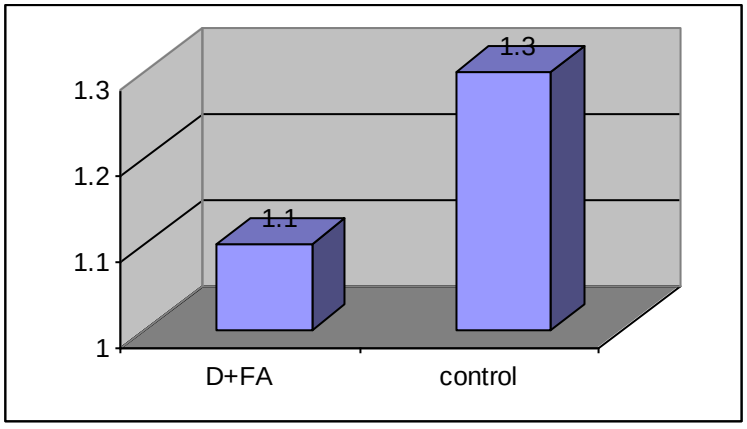
* Significant ** Highly significant

Histogram (5): Shows mean ± SD of the head length (cm) in the control and treated groups

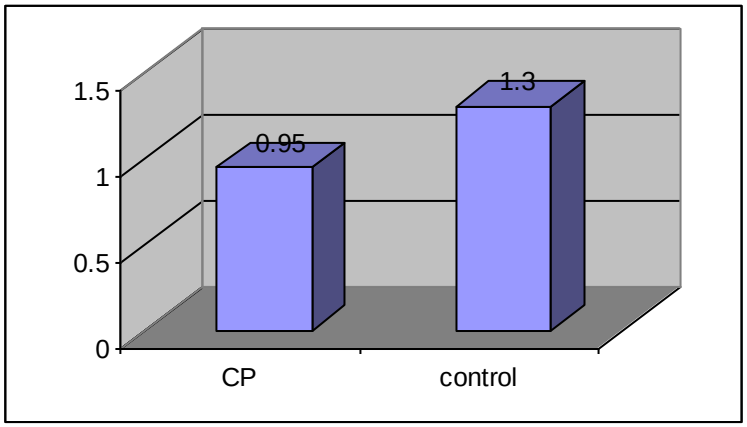
A



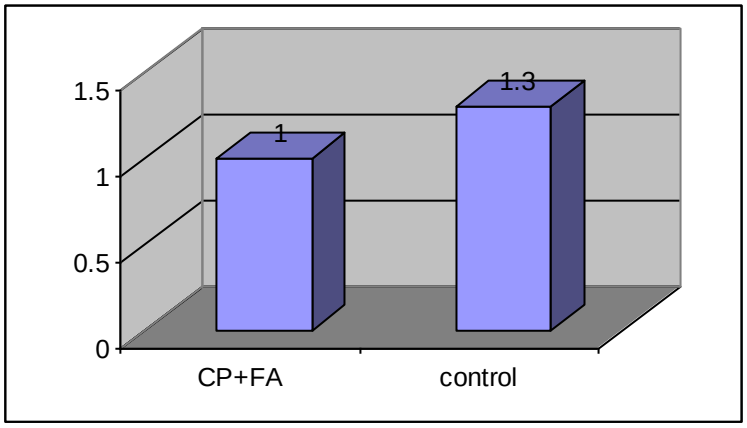
B



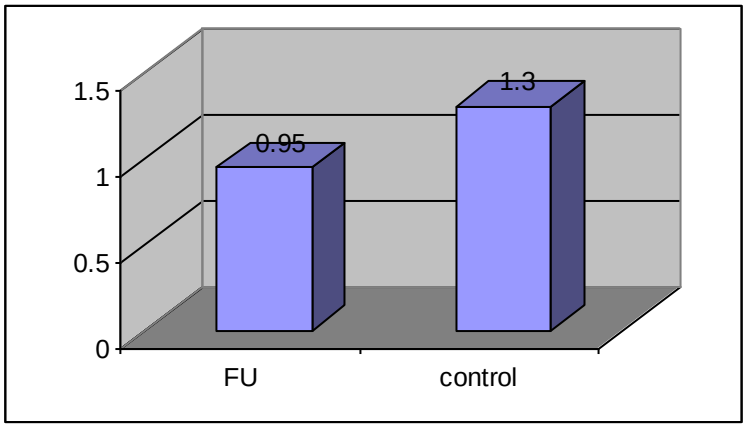
C


1.5
1
0.5
0
0.95
1.3
CP
control

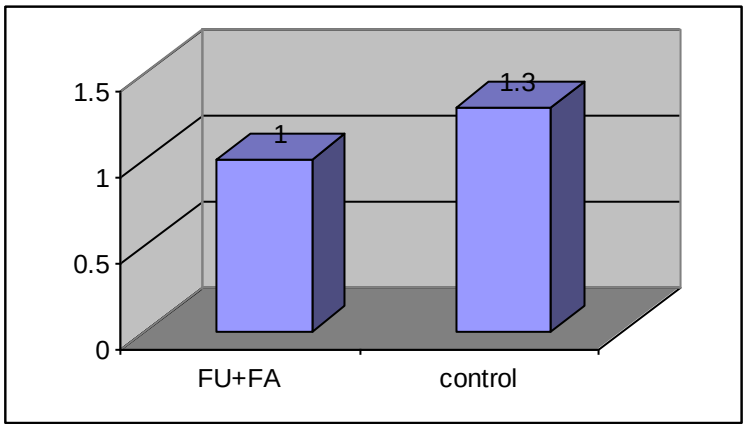
D


1.5
1
0.5
0
1
1.3
CP+FA
control

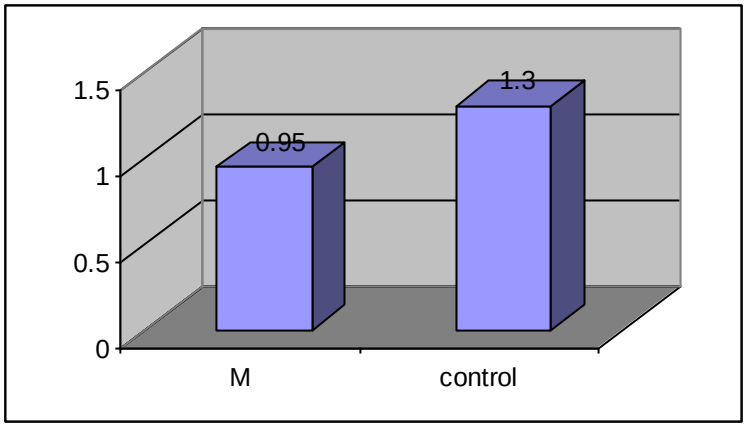
E


1.5
1
0.5
0
0.95
1.3
FU
control

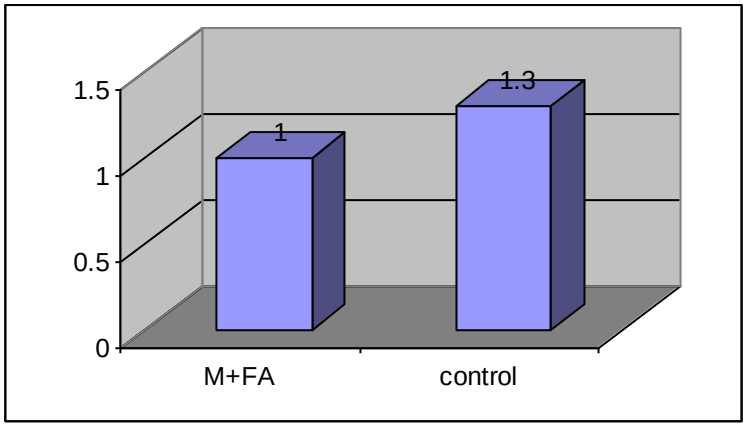
F


1.5
1
0.5
0
1
1.3
FU+FA
control

G


1.5
1
0.5
0
0.95
1.3
M
control

H


1.5
1
0.5
0
1
1.3
M+FA
control

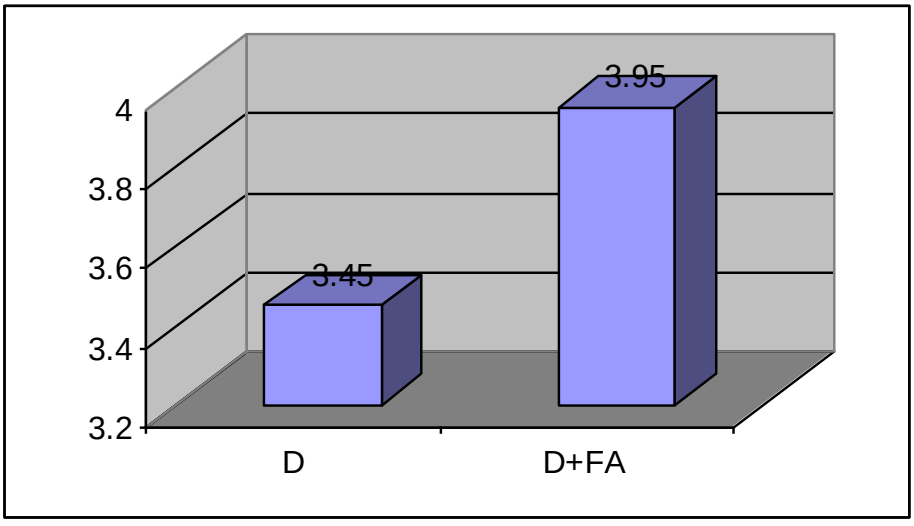
Table(14): Mean ± SD of the crown-rump length (cm), head length (cm), biparietal diameter (cm) and weight (gm) of D and D+FA treated groups.

	Group	Mean ± SD	T	P	Significance
CRL	D	3.45 ± 0.0717	7.07	0.019	*
	D+FA	3.95 ± 0.0717			
Head length	D	1.15 ± 0.0717	1.64	0.24	•
	D+FA	1.00 ± 0.0500			
BP diameter	D	1.05 ± 0.0717	1.34	0.312	•
	D+FA	1.20 ± 0.1414			
Wt	D	3.04 ± 0.0565	30.5	0.001	**
	D+FA	4.26 ± 0.0100			

• Non significant * Significant ** Highly significant

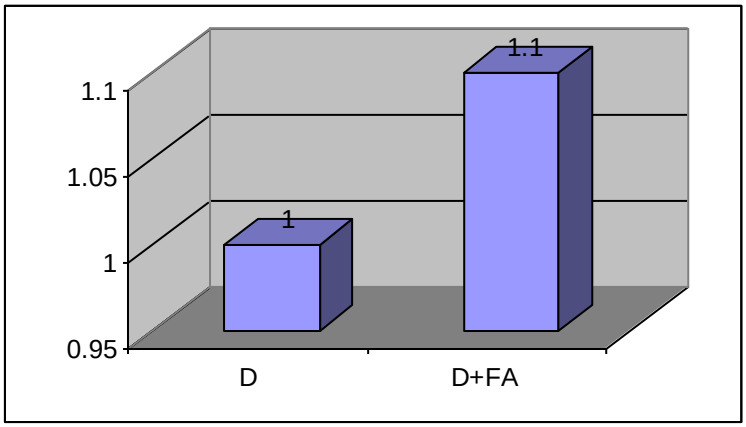
Histogram (6): Shows mean ± SD of the crown-rump length (cm), head length (cm), biparietal diameter (cm) and weight (gm) of D and D D+FA treated groups.

A



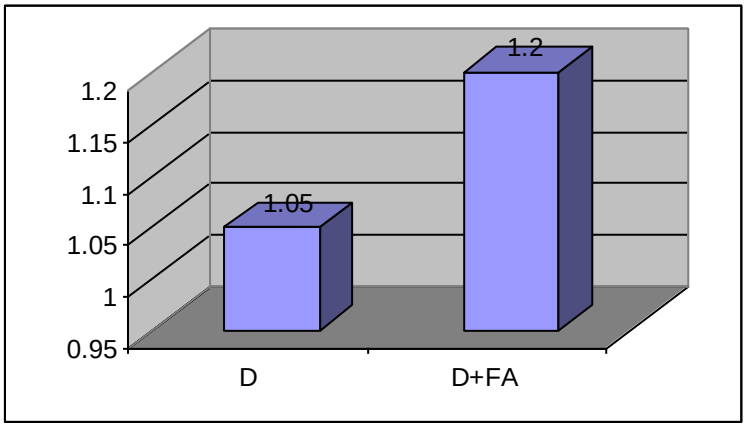
CR length

B



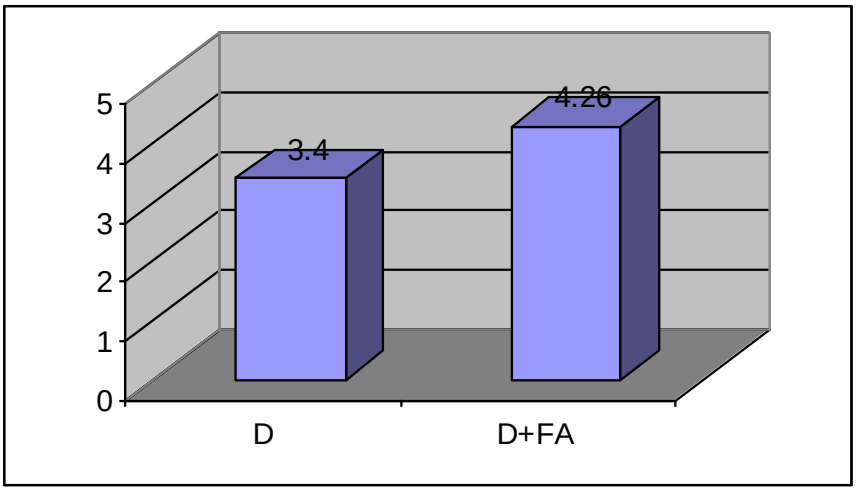
Head length

C



Biparietal diameter

D



Weight

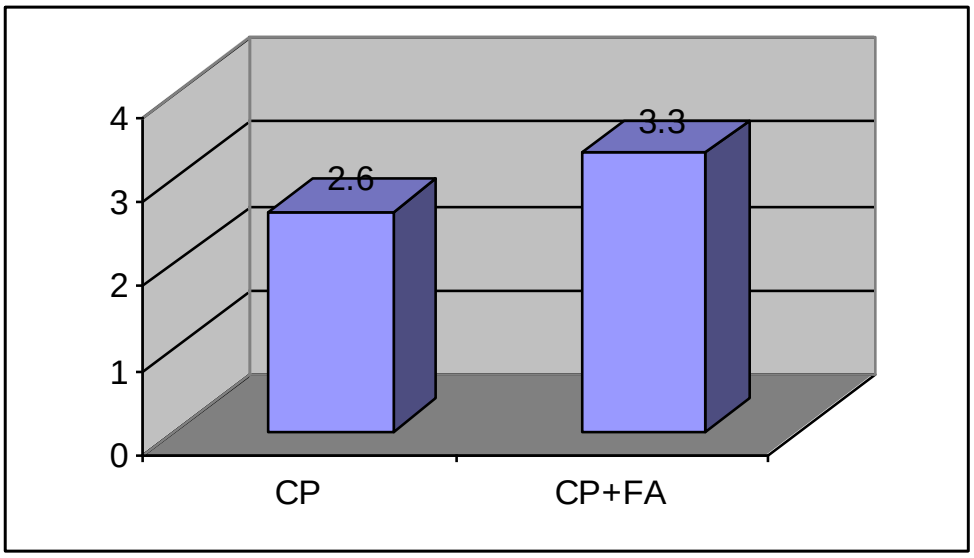
Table(15): Mean ± SD of the Crown – rump length (cm), head length (cm), biparietal diameter (cm) and weight (gm) of CP and CP + FA treated groups.

	Group	Mean ± SD	T	P	Significance
CRL	CP	2.60 ± 0.2828	3.13	0.089	•
	CP+FA	3.30 ± 0.1414			
Head length	CP	0.95 ± 0.0717	1	0.423	•
	CP+FA	1.00 ± 0.0200			
BP diameter	CP	0.90 ± 0.0717	0.7	0.55	•
	CP+FA	0.95 ± 0.0717			
Wt	CP	1.73 ± 0.0200	18.07	0.003	**
	CP+FA	2.90 ± 0.0919			

•Non significant ** Highly significant

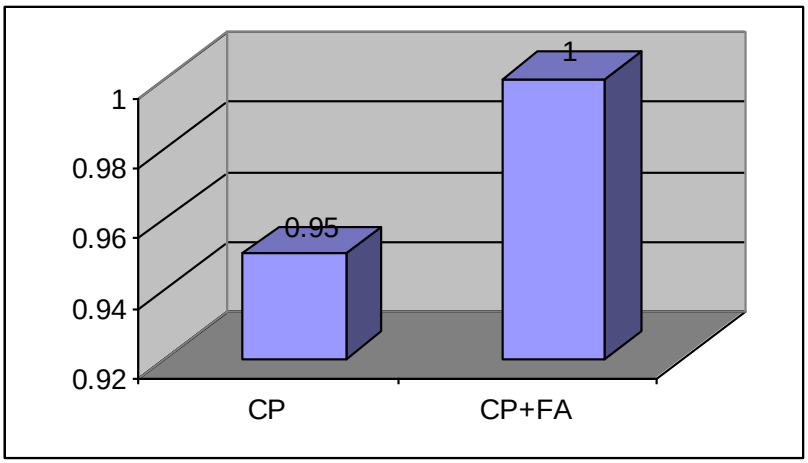
Histogram (7): Shows Mean ± SD of the Crown – rump length (cm), head length (cm), biparietal diameter (cm) and weight (gm) of CP and CP + FA treated groups.

A



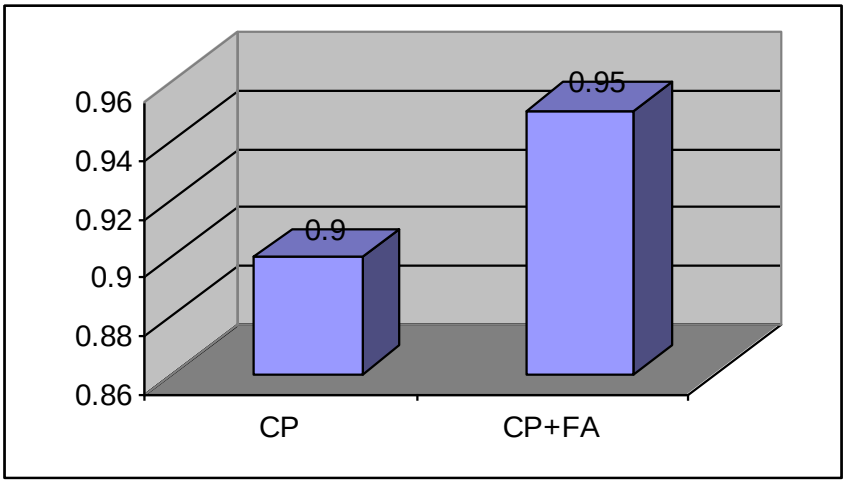
CR- Length

B



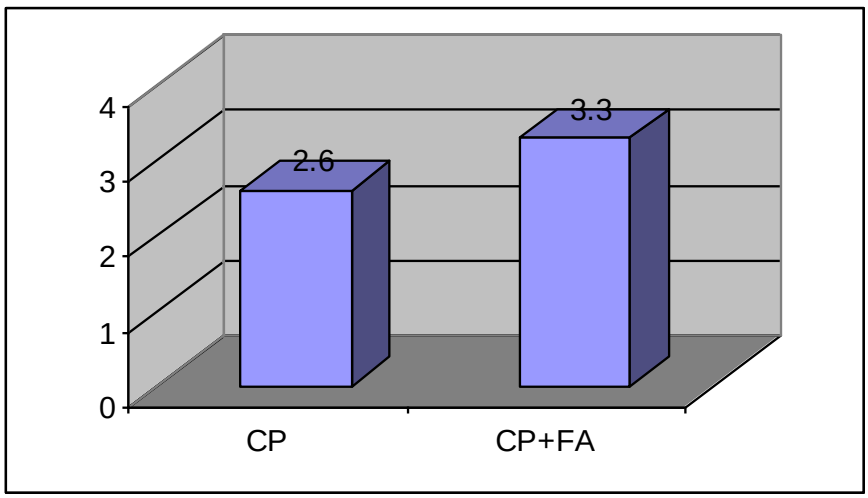
Head length

C



Biparietal diameter

D



Weight

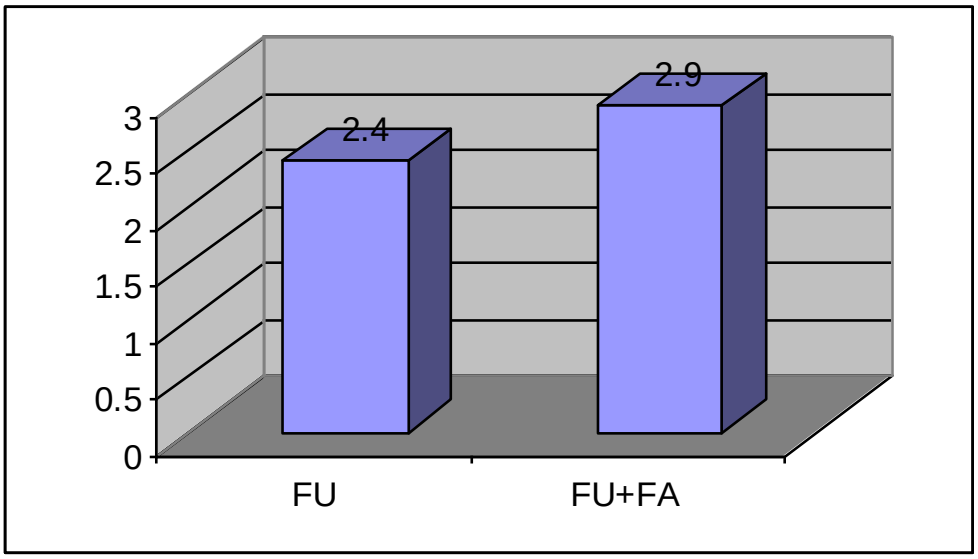
Table(16) : Mean ± SD of the crown - rump length (cm), head length (cm) biprietal diameter (cm) and weight (gm) of FU and FU +FA treated groups.

	Group	Mean ± SD	T	P	Significance
CRL	FU	2.40 ± 0.1414	5	0.038	*
	FU+FA	2.90 ± 0.0000			
Head length	FU	0.95 ± 0.0717	1	0.423	•
	FU+FA	1.00 ± 0.0400			
BP diameter	FU	0.80 ± 0.0300[a]	2.83	0.11	•
	FU+FA	0.90 ± 0.0400[a]			
Wt	FU	1.43 ± 0.9899	7.69	0.016	*
	FU+FA	1.99 ± 0.0282			

• Non significant * Significant

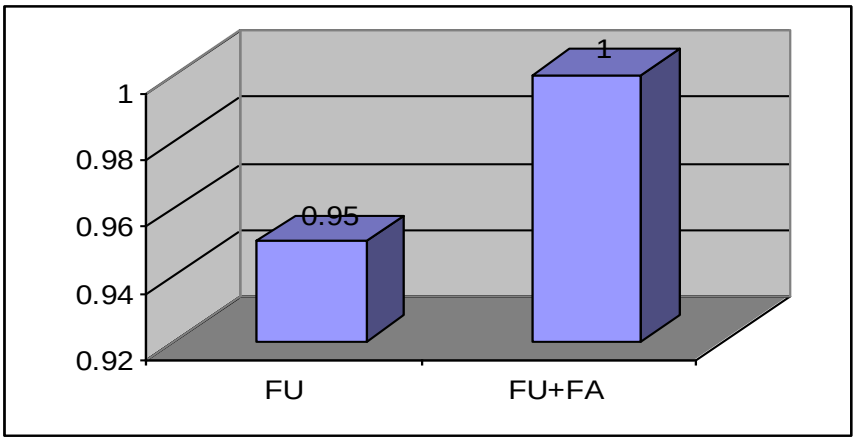
Histogram (8): Shows mean ± SD of the crown - rump length (cm), head length (cm) biprietal diameter (cm) and weight (gm) of FU and FU +FA treated groups.

A



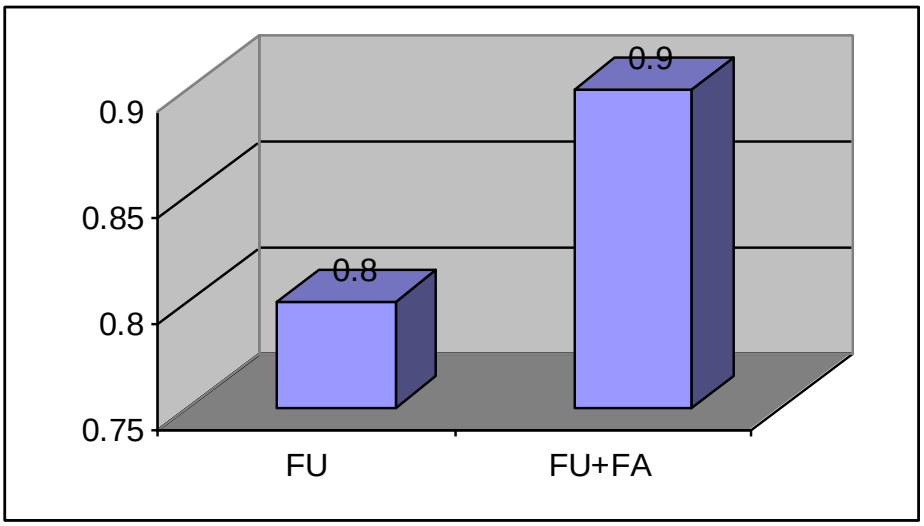
CR Length

B



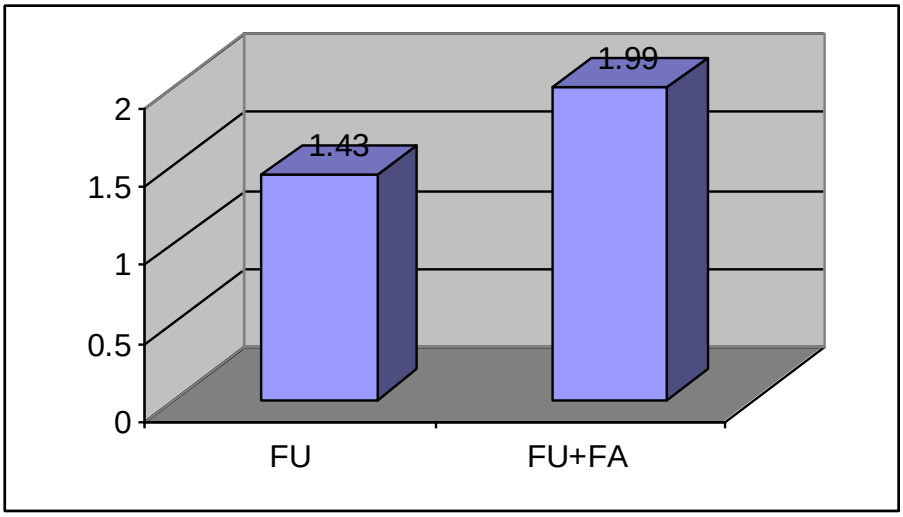
Head length

C



Biparietal diameter

D



Weight

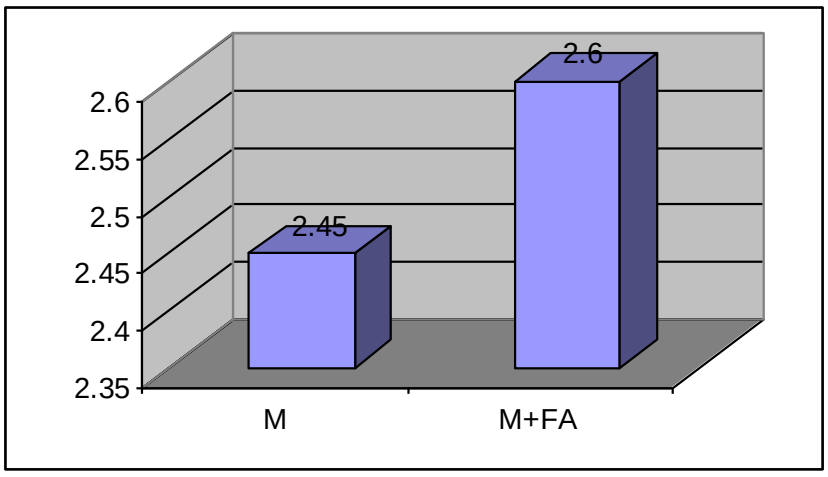
Table(17): Mean ± SD of the crown –rump length, head length (cm), biparietal diameter (cm) and weight (gm) of the M and M+ FA treated groups.

	Group	Mean ± SD	T	P	Significance
CRL	M	2.45 ± 0.3535	0.6	0.607	•
	M+FA	2.60 ± 0.0200			
Head length	M	0.95 ± 0.0717	1	0.423	•
	M+FA	1.00 ± 0.0300			
BP diameter	M	1.00 ± 0.0500[a]	2.6	0.12	•
	M+FA	1.10 ± 0.0300[a]			
Wt	M	1.60 ± 0.4879	0.15	0.255	•
	M+FA	1.65 ± 0.0000			

• Non significant

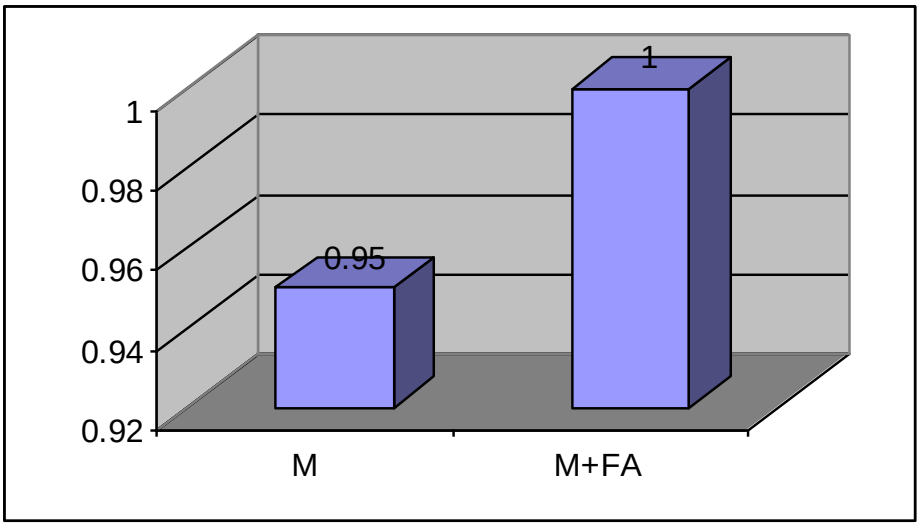
Histogram (9): Shows mean ± SD of the crown –rump length, head length (cm), biparietal diameter (cm) and weight (gm) of the M and M+ FA treated groups.

A



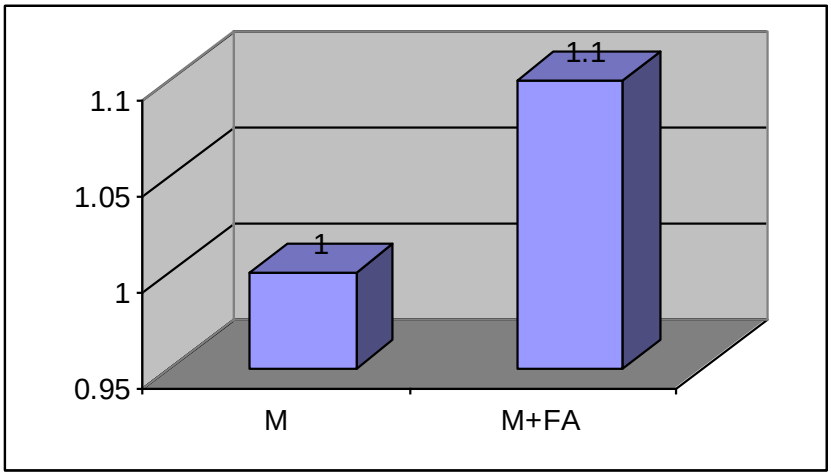
CR length

B



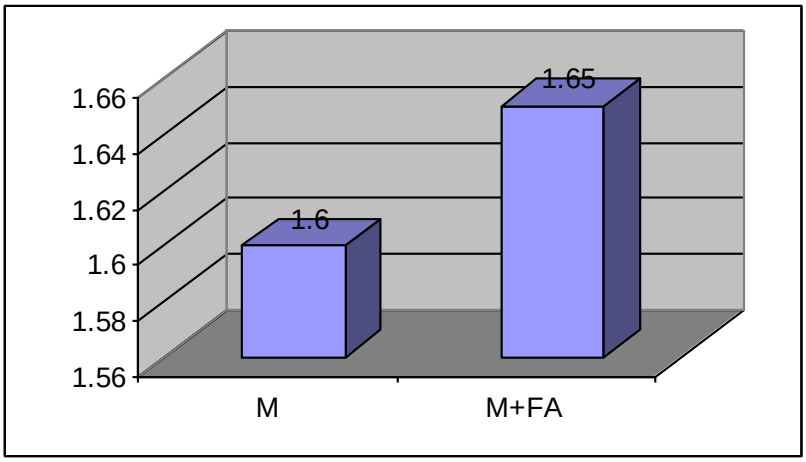
Head length

C



Biparietal diameter

D



Weight

I-EXTERNAL EXAMINATION

For studying the normal implantation, preimplantation loss and postimplantation loss as well as resorption a number of figures (6-11) were performed. Fig. (6) shows the normal control non pregnant female uterus, vagina and ovary which contains corpus albicans (whitish in color).

Normal corpus lutea of pregnancy (appearing yellowish tinge in color) is seen in (Fig. 7). In (Fig. 8), the two uterine horns are seen empty, indicating complete preimplantation loss of all embryos. Notice normal corpus lutea of pregnancy in the ovary. Fig. (9) shows dork brown spots indicating early postimplantation loss. Normal corpus lutea of pregnancy can be seen.

In fig. (10) normal pregnant uterus with live fetuses can be observed. In the treated female rat, large blood clots indicating a site of late postimplantation resorption is seen in the uterus figs. (11&12). Fig. (13) shows maceratd fetuses indicating prenatal fetal death.

In fig. (14) a large abdominal haematoma is seen in a [CP] treated rat fetus. Also, haematoma in the back of a (M) treated rat fetus is seen.

As regards the physical growth of the offsprings of the mothers treated with chemotherapeutic agents, it was noticed that, the lowest size is observed in the fluorouracil (FU) treated group, followed by methotrexate (M), cyclophosphamide (CP) and then doxorubicin (D) treated groups in comparison to that of the control (C) (Fig. 15). After folic acid supplementation the lowest size is observed in (M +FA) treated groups in comparison to that of the control (C) (Fig. 16).

Severe malformation with rudimentary limbs in the methotrexate (M) treated group in comparison to the control (C) is seen in (Fig. 17).

Fig. 6: A photograph of the control non pregnant female genital system showing corpus albicans (arrow) appears whitish in color in the ovary (O). The two uterine horns (Uh) and vagina (V) can be seen.

Fig. 7: A photograph showing a control pregnant rat with the ovary (O) containing corpus lutea of pregnancy (arrow) which appear large, with yellowish tinge color.

Fig.8: A photograph of (FU) treated female rat genital system showing corpus lutea of pregnancy (arrow) in the ovary (O). The uterine horn (Uh) and vagina (V) can be seen. The two horns are empty, indicating complete preimplantation loss of all embryos.

Fig. 9: Photograh of the (M) treated female rat genital system showing the uterine horn (Uh) with dark brown spots (black arrow) indicating early post implantation loss. Also, corpus lutea of pregnancy (blue arrow) in the ovary (O) and the vagina (V) can be seen.

Fig. 10: A photograph of the control female pregnant rat showing four fetuses (arrows) in right uterine horn (Uh) and five fetuses (arrows) in the left horn.

Fig. 11: A photograph of the (CP) treated female rat with pregnant uterus showing large blood clot (Bl) in one of the uterine horns (Uh) indicating a site of late post implantation resorption. Also corpus lutea of pregnancy (blue arrow) in the ovary (O) can be seen.

Fig. 12: A photograph of the (FU) treated female rat with pregnant uterus showing large blood clot (Bl) in one of the uterine horns (Uh) indicating a site of late post implantation resorption. Also corpus lutea of pregnancy (blue arrow) in the ovary (O) can be seen.

Fig.13 : A photograph of the (M) treated female rat with opened uterine horns showing maceratd malformed fetuses (fe).

Fig. 14: A photograph showing cyclophosphamide treated rat fetus (CP) showing a large abdominopelvic haematoma (ha) and rudimentary hindlimbs (arrow). Also, a methotrexate treated rat fetus (M) with haematoma (ha) in the back.

Fig. 15: A photograph showing the control rat fetus (C), doxorubicin treated rat fetus (D), cyclophosphamide treated rat fetus (CP), methotrexate treated rat fetus (M) and fluorouracil treated rat fetus (FU). The lowest size is noticed in the fluorouracil treated rat fetus, followed by methotrexate, cyclophosphamide and doxorubicin treated rat fetuses in comparison to the control.

Fig. 16: A photograph showing the control rat fetus (C), doxorubicin with folic acid treated rat fetus (D+FA), cyclophosphamide with folic acid treated rat fetus (CP+FA), fluorouracil with folic acid treated rat fetus (FU+ FA), and methotrexate with folic acid treated rat fetus (M+FA). The lowest size is noticed in M+FA followed by FU+ FA, CP+FA and D +FA treated rat fetuses in comparison to the control.

Fig. 17: A photograph showing the control rat fetus (C) and methotrexate treated rat fetus (M) showing severe malformation with rudimentary limbs (arrows).

II-ALIZARINE RED STAIN

To detect skeletal deformities, alizarine red stain was used. The completely ossified bones take the stain completely and appear red in colour, incompletely ossified bones partially take the stain and appear lightly stained. However, non ossified bones don't take the stain completely.

In the control rat group, most of the bones are ossified (Figs. 18,19 and 20), including skullbones (mandible, maxilla, frontal, lacrimal, parietal, interparital, supraoccipital and occipital bones), trunk bones (vertebrae, ribs and sternum) and limb bones (clavicle, scapula, metacarpal, hip, femur, tribia, fibula and metatarsal bones).

In the doxorubicin (D) treated group there is evident failure of ossification of the caudal vertebrae and incomplete ossification of skull bones (parietal, interparietal, supraoccipital and occipital bones), sternum, vertebrae, clavicle, metacarpal, scapula, hip, femur and metatarsal bones. Irregular base of the mandible, maxilla and ribs are seen. Also, extraossification centers for the vertebrae are seen (Figs. 21,22 and 23).

The supplementation with folic acid improved most of the skeletal changes. However, incomplete ossification of some bones [sternum, vertebrae, clavicle, metacarpal, metatarsal and occipital bones] and irregular ribs are seen (Figs.24, 25 and 26).

In the cyclophosphamide (CP) treated group there is evident failure of ossification of the clavicle, sternum, metacarpal, metatarsal and caudal vertebrae. Incomplete ossification of skull bones (parietal, interparietal, supraoccipital and occipilal bones), scapula, hip, femur, vertebrae, metacarpal and metatarsal bones are seen. Also, irregular mandible,

scapula and ribs which fuse together, short 13th ribs and extraossification centers in the vertebrae are seen (Figs. 27,29 and 29).

The supplementation with folic acid improved most of the skeletal changes. However, incomplete ossification of some bones skull bones; (parietal, interparietal, supraoccipital and occipital bones), sternum, hip, metacarpal and metatarsal bones). Irregular ribs, short 13th rib and extra ossification centers in the vertebrae are seen (Figs. 30, 31 and 32).

In fluorouracil (FU) treated group there is failure of ossification of limb bones and incomplete ossification of skull bones, vertebrae and ribs as seen (Fig. 33).

The supplementation with folic acid improved some of the skeletal changes. However, failure of ossification of the posterior part of the skull, metacarpal, metatarsal ad caudal vertebrae are seen. Also, incomplete ossification of vertebrae, femur, (tibia and fibula) which are fused together and irregular ribs are seen (Fig. 34).

In methotrexate (M) treated group there is evident failure of ossification of sternum, metacarpal, metatarsal, parietal, interparietal bones and caudal vertebrae. Incomplete ossification of clavicle, tibia, fibula and suprooccipital bones are seen. Also, irregular scapula and wavy ribs are seen (Figs. 35, 36 and 37).

The supplementation with folic acid improved some of the skeletal changes. However, failure of ossification of metacarpal, metatarsal and sternum. Incomplete ossification of skull bones (parietal, interparietal, supraoccipital, , occipital and mandible), clavicle and hip bones are seen. Also, irregular ribs, mandible and scapula are seen (Figs. 38,39 and 40).

Fig. 18: A photograph of the control rat fetus (anterior view), showing completely ossified maxilla (mx), mandible (ma), clavicle (cl), sternum (S), metacarpal (mc) and metatarsal (mt) bones. (Alizarine red slain)

Fig. 19: A photograph of the control rat fetus [posterior view], showing completely ossified skull bones (parietal (p), interparietal (ip), supraocipital (So) and occipital (oc). Also, completely ossified scapula (Sc), ribs (ri), vertebrae (ve) hip bone (hi) and femur (Fe) can be noticed (Alizarine red stain).

Fig. 20: A photograph of the control rat fetus (lateral view), showing completely ossified skull bones (maxilla (mx), mandible (ma), lacrimal (L) and frontal (Fb) bones). Also, completely ossified clavicle (cl), scapula (Sc) humerus (hu) radius (R), ulna (Ul), metacarpal (mc) ribs (ri), vertebrae (ve), femur (Fe), Tibia (T), fibula (F) and metatarsal (mt) bones (Alizarine red stain).

Fig. 21: A photograph showing the control rat fetus (C) and doxorubicin treated rat fetus (D) (anterior view), showing incomplete ossification of clavicle (cl), sternum (S), metacarpal (mc), metatarsal (mt) and maxilla (mx) bones. Also, irregular base of the mandible (ma) can be seen (Alizarine red stain).

Fig. 22: A photgraph showing the control rat fetus (C) and doxorubicin treated rat fetus (D) (Posterior view), showing incomplete ossification of skull bones; (parietal (p), interparietal (ip), supraoccipital (So) and occipital (oc) bones). Also, incomplete ossification of scapula (Sc), ribs (ri), vertebrae (ve), hip (hi) and femur (Fe) bones can be seen. Also, vertebral extra ossification centers (black arrow) can be seen. (Alizarine red stain).

Fig. 23: a photograph showing the control rat fetus (C) and doxorubicin treated rat fetus (D) (lateral view) showing brachygnathia, irregular maxilla (mx), mandible (ma) and ribs (ri). Also, incomplete ossification of vertebrae (ve) metacarpal [mc] and metatarsal (mt) bones. Also, failure of ossification of caudal vertebrae (blue arrow). Completely ossified (hu), radius (R), ulna (U), femur (Fe), tibia (T) and fibula (F) are seen (Alizarine red stain).

Fig. 24: A photograph showing the control rat fetus (C) and doxorubicin with folic acid treated rat fetus (D+FA) (anterior view), showing incompletely ossified clavicle (cl), sternum (S), metacarpal (mc) metatarsal (mt) and maxilla (mx) bones. Normally ossified skull bones and mandible (ma) can be seen. (Alizarine red stain).

Fig. 25: A photograph showing the control rat fetus (C) and doxorubicin with folic acid treated rat fetus (D +FA) (posterior view) showing incomplete ossification of occipital bones (oc), vertebrae (ve) and hip bone (hi). All other bones are normally ossified. (parietal (p) , interparietal (ip), supraoccipital (So), scapula (Sc), ribs (ri), and femur (Fe)). (Alizarine red stain).

Fig. 26: A photograph showing the control rat fetus (C) and doxorubicin with folic acid treated rat fetus (D +FA) [lateral view], showing irregular ribs (ri). All other bones are normally ossified (frontal bone (Fb), lacrimal bone (L) , maxilla (mx), mandible (ma), clavicle (cl), scapula (Sc), humerus (hu), radius (Ra), ulna (U), metacardpal bones (mc), ribs (ri), vertebrae (ve), femur (Fe), tibia (T), fibula (F), and metatarsal bones (mt)).(Alizarine red stain).

Fig. 27: A photograph showing the control rat fetus (C) and cyclophosphamide treated rat fetus (CP) (anterior view) showing absence of the clavicle (cl), sternum (S), metacarpal (mc) metatarsal (mt) and maxilla (mx). Normal skull bones and mandible (ma) can be seen. (Alizarine red stain).

Fig. 28: A photograph showing the control rat fetus (C) and cyclophosphamide treated rat fetus (CP) (posterior view), showing incomplete ossification of all skull bones (parietal (p), interparietal (ip), supraoccipital (So) and occipoital (oc) bones) , scapula (Sc), hip bone (hi), femur (Fe) and vertebrane (ve). Also, irregular fused upper ribs (ri), short 13^{th} ribs (green arrow) and extraossification centers (black arrow) (Alizorine red stain).

Fig. 29: A photograph showing the control rat fetus (C) and cyclophosphamide treated rat fetus (CP) (lateral view), showing brachygnathia, irregular mandible (ma), scapula (Sc), and ribs (ri). Incomplete ossification of metacarpal (mc), tibia (T), fibula (F) and metatarsal (mt) bones can be seen. Also, there is absence of caudal vertebrae (blue arrow) Normally ossified humerus (hu), radius (R), ulna (U) and femur (Fe) can be seen (Alizarine red stain).

Fig. 30: A photograph showing the control rat fetus (C) and cyclophosphamide with folic acid treated rat fetus (CP + FA) (anterior view), showing incomplete ossification of sternum (S), metacarpal (mc), metatarsal (mt) bones and maxilla (mx). Normally ossified mandible (ma) and clavicle (cl) can be seen. (Alizarine red stain)

Fig. 31: A photograph showing the control rat fetus (C) and cyclophosphamide with folic acid treated rat fetus (CP+FA) (posterior view), showing incomplete ossification of all skull bones (parietal (p), interparietal (ip), supraoccipital (So) and occipital (oc) bones], hip bone (hi) and femur (Fe). Also, irregular ribs (ri) and short 13^{th} rib (green arrow) can be seen. Extra ossification centers are seen in lumbar and sacral regions (black arrow). (Alizarine red stain).

Fig. 32: A photograph showing the control rat fetus (C) and cyclophosphamide with folic acid treated rat fetus (CP + FA) (lateral view), showing brachygnathia and incomplete ossification of metacarpal (mc) and metatarsal (mt) bones. Irregular humerus (hu) and ribs (ri) can be seen. Also, there is absence of caudal vertebrae (blue arrow). Normally ossified skull bones (frontal (Fb), lacrimal (L), maxilla (mx) and mandible (ma)), clavicle (cl), scapula (Sc), radius (R), ulna (U), femur (Fe), tibia (T) and fibula (Fi) can be seen (Alizarine red stain).

Fig. 33: A photograph showing fluorouracil treated rat fetus (FU) showing failure of assification of all limb bones and incomplete ossification of vertebrae (ve) and ribs (ri) (Alizarine red stain).

Fig. 34: A photograph showing the control rat fetus (C) and fluorouracil with folic acid treated rat fetus (FU+ FA) (lateral view), showing brachygnathia, failure of ossification of the posterior part of the skull (black arrow), metacarpal (mc), metatarsal (mt) and caudal vertebrae (blue arrow). Also, incomplete ossification of vertebrae (ve), femur (Fe) and (tibia (T) and fibula (F)) which are fused together. Fused radius (R) and ulna (U) can be seen. The ribs (ri) are irregular. frontal bone (Fb) lacrimal (L), maxilla (mx), mandible (ma), clavicle (cl), scapula (Sc) and humerus (hu). (Alizarine red stain).

Fig. 35: A photograph showing the control rat fetus (C) and methotrexate treated rat fetus (M) (anterior view), showing failure of ossification of sternum (S), metacarpal (mc) and metatarsal (mt) bones. (Alizarine red stain).

Fig. 36: A photograph showing the control rat fetus (C) and methotrexate treated rat fetus (M) (posterior view), showing failure of ossification of parietal (p) and interparietal (ip) bones. Incomplete ossification of supraoccipital (So) bone and irregular occipital (oc) bone. Also, wavy ribs (ri) and irregular scapula (Sc) are seen (Alizarine red stain).

Fig. 37: A photograph showing the control rat fetus (C) and methotrexate treated rat fetus (M) (lateral view), showing failure of ossification of metacarpal (mc), metatarsal (mt) bones and caudal vertebrae (blue arrow). Irregular incompletely ossified limb bones, frontal bone (Fb), lacrimal (L), maxilla (mx), mandible (ma), clavicle (cl), scapula (Sc), humerus (hu), radius (R), ulna (U), femur (Fe), tibia (T) and fibula (F) can be seen (Alizarine red stain).

Fig. 38 : A photograph showing the control rat fetus (C) and methotrexate with folic acid treated rat fetus (M +FA) (anterior view), showing failure of ossification of metacarpal (mc), metatarsal (mt) bones and sternum (S). Also, incomplete ossification of the clavicle (cl), mandible (ma) and maxilla (mx) are seen. (Alizarine red stain).

Fig. 39: A photograph showing the control rat fetus (C) and methotrexate with folic acid treated rat fetus (M+ FA) (posterior view), showing incomplete ossification of skull bones (parietal (p), interparietal (ip), supraoccipital (So) and occipital (oc) bones), scapula (Sc), vertebrae (ve), hip (hi) bones and femur (Fe). Also, irregular ribs (ri) are seen (Alizarine red stain).

Fig. 40: A photograph of the control rat fetus (c) and methotrexate with folic acid treated rat fetus (M+FA) (lateral view), showing brachygnathia, irregular mandible (ma), scapula (Sc) and ribs (ri). Also, incomplete ossification of clavicle (cl), humerus (hu), radius (R), ulna (U), metacarpl (mc), femur (Fe), tibia (T), fibula (F) and metatarsal (mt) bone are seen (Alizarine red stain).

III-RAZOR SECTIONS

For studying any gross abnormalities in the internal organs in different groups, razor sections were made at different levels. At the level of the angle of the mouth there is normal conchae, nasal septum, palate, tongue and mandible in the control group (fig. 41). At the level of the angle of the mouth there is a buccal haematoma in doxorubicin (D) treated group (fig. 38). Malformed nasal conchae appear in all other groups which are also large in fluorouracil (FU) and methotrexate (M) treated groups. Thick nasal septum is observed in cyclophosphamide (CP) and (FU) treated groups, which is also deviated in (M) treated group. Malformed mandible is seen in (FU) and (M) treated groups. Also, relatively large tongue obliterating the oral cavity in (M) treated group is observed figs. (43, 44 and 45).

At the level of the largest transverse diameter of the head, malformed brain and bilaterally dilated lateral ventricles were observed in (CP) and (M) treated groups. Also, the third ventricle is dilated in (FU) treated group in comparison to the control beside dilated subarachnoid space (Figs. 46, 47 and 48).

At the level of the root of the neck small thymus gland and haematomas are noticed in doxorubicin (D) treated group (fig. 50). While in fluorouracil (FU) treated group there are malformed spinal cord, thymus and thyroid glands in comparison to the control (fig. 51).

At the level of the thoracic region just below the axilla malformed spinal cord is noticed in all treated groups except fluorouracil (FU) treated group. Small lungs appear in all treated groups which are also malformed in doxorubicin (D) and methotrexate (M) treated groups. Malformed hearts in all treated groups which also contain and surrounded

by haematomas in (D) treated group and with thick walled atria in (FU) treated group. Also, long sternopericardial ligaments are noticed in (D) and (FU) treated groups in comparison to the control (figs. 52 to 56).

At the level of the abdominal region just above the umbilicus malformed kidneys are noticed in all treated groups. Also, small thick walled stomach is observed in fluorouracil (FU) and methotrexate (M) treated groups in comprison to the control (Figs. 57 to 60).

Fig.41: A photograph of section of the control rat fetus at the region of the angle of the mouth showing normal nasal conchae (co), nasal septum (Ns) palate (pa), tongue (To) and mandible (ma). (Razor section).

Fig. 42:A photograph of section of doxorubicin treated rat fetus (D) at the region of the angle of the mouth showing buccal haematoma (ha). Normal nasal conchae (co), nasal septum (Ns), palate (ha), tongue (To) and mandible (ma) are seen (Razor section).

Fig. 43: A photograph of section of cyclophosphamide treated rat fetus (CP) at the region of the angle of the mouth showing malformed nasal conchae (co) and thick nasal septum (Ns). Normal palate (pa), tongue (To) and mandible (ma) are seen. (Razor section)

Fig.44: A photograph of section of fluorouracil treated rat fetus (FU) at the region of the angle of the mouth showing large irregular nasal conchae (co), thick nasal septum (Ns) and malformed mandible (ma). Normal palate (pa) and tongue (To) are seen. (razor section)

Fig. 45: A photograph of section of methotrexate treated rat fetus (M) at the region of the angle of the mouth showing large irregular nasal conchae (co), deviated nasal septum (Ns), large tonge (To) obliterating the oral cavity and malformed mandible [ma]. Normal palate (pa) is seen (Razor section).

Fig.46: A photograph of section of the control rat fetus at the level of the greatest transverse diameter of the head showing normal lateral ventricle (Lv), third ventricle (Tv) and regular subarachnoid space (sa). (Razor section).

Fig. 47: A photograph of section of the cyclophosphamide treated rat fetus (CP) at the level of the greatest diameter of the head showing malformed brain, bilaterally dilated lateral ventricles (Lv) and and subarachnoid space (sa). Normal third ventricle (Tv) is seen. (Razor section).

Fig. 48: A photograph of section of the fluorouracil treated rat fetus (FU) at the level of the greatest diameter of the head showing malformed brain, dilated lateral (Lv), third (Tv) ventricles and subarachnoid space (sa). (Razor Section).

Fig. 49: A photograph of a section of the control rat fetus at the level of the root of the neck showing normal spinal cord (sp), oesophagus (oe), trachea (tr), thyroid (Th) and thymus (thy) glands. (Razor section).

Fig. 50: A photograph of a section of doxorubicin rat fetus (D) at the level of the root of the neck showing small thymus gland (thy) and haematomas (arrow). Normal spinal cord (sp), oesophagus (oe), trachea (Tr) and thyroid gland (Th) are seen. (Razor section).

Fig. 51: A photograph of a section of fluorouracil treated rat fetus (FU) at the level of the root of the neck showing malformed spinal cord (sp), thymus (thy) and thyroid glands (Th). Normal oesophagus (oe) and trachea (tr) are seen. (Razor section).

Fig. 52: A photograph of a section of the control rat fetus at the level of the thoracic region just below the axilla showing normal spinal cord (sp) and lungs (lu). A normal heart with two ventricles (vn), atria (a), interventricular septum (iv), interatrial septum (ia) and sternopericardial ligaments (SL) can be seen. (Razor section).

Fig. 53: A photograph of a section of doxorubicin treated rat fetus (D) at the level of the thoracic region just below the axilla showing malformed spinal cord (sp), small lung (lu) and malformed heart (atria (a), interatrial septum (ia), ventricles (vn) and interventricular septum (iv)) with large haematoma (blue arrow) (Razor section).

Fig. 54: A photograph of a section of doxorubicin treated rat fetus (D) at the level of the thoracic region just below the axilla showing malformed spinal cord (sp), small malformed lungs (lu), malformed heart with large haematoma (H) and long sternopericardial ligaments (SL) (Razor section).

Fig. 55: A photograph of a section of fluorouracil treated rat fetus (FU) at the level of the thoracic region just below the axilla showing small lungs (lu), malformed heart with thick walled atria (a) and mildly elolngated sternopericardial ligaments (SL). Normal spinal cord (sp), ventricles (vn) and interventricular septum (iv) are seen. (Razor section).

Fig. 56: A photograph of a section of methotrexate treated rat fetus (M) at the level of the thoracic region just below the axilla showing small malformed spinal cord (sp), malformed lungs (lu) and small malformed heart (H). (Razor section).

Fig. 57: A photograph of a section of the control rat fetus at the level of the abdominal region just above the umbilicus showing normal spinal cord (sp), kidney (k), stomach (St) and liver (li). (Razor section).

Fig. 58: A photograph of a section of cyclophosphamide treated rat fetus (CP) at the level of the abdominal region just above the umbilicus showing large malformed kidneys (k). Normal spinal cord (sp), stomach (St) and liver [li] are seen. (Razor section).

Fig. 59: A photograph of a section of fluorouracil treated rat fetus (FU) at the level of the abdominal region just above the umbilicus showing small malformed kidneys (k), and small thick walled stomach (St). Normal spinal cord (sp) and liver (li) are seen. (Razor section).

Fig. 60: A photograph of a section of methotrexate treated rat fetus (M) at the level of the abdominal region just above the umbilicus showing small thick walled stomach (St) and abnormal kidney (k). Normal spinal cord (sp) and liver (li) are seen. (Razor section).

IV-HISTOLOGICAL SECTIONS

For studying any gross abnormalities in the internal organs in different groups at different levels histological sections were made. At the level of the angle of the mouth, malformed mandible and rudimentary maxillae are noticed in all treated groups. Also, malformed vomeronasal cartilage, nasal septum and nasal conchae which are rudimentary are noticed in doxorubicin (D) treated group. Also, hypertrophied nasal septum is noticed in cyclophosphamide (CP) treated group in comparison to the control (figs. 61 to 64).

At the level of the largest transverse diameter of the head, bilaterally dilated lateral and third ventricles are seen in cyclophosphamide (CP) and fluorouracil (FU) treated groups in comparison to the control (figs. 65 to 67).

At the level of the root of the neck, malformed spinal cord and vertebrae are observed in all treated groups. Rudimentary thymus in all treated group except fluorouracil (FU) treated group is seen. The oesophagus is deviated to the right in cyclophosphamide (CP) and doxorubicin (D) treated groups while it is central in (FU) treated group. The oesophagus is divided into two compartements by mucosal folds in (D) treated group. The thyroid gland and internal jugular vein are large in methotrexate (M) treated group in comparison to the control (Figs. 68 to 72).

Fig. 61: A photograph of a section of the head of the control rat fetus at the level of the angle of the mouth showing normal nasal septum (Ns), vomeronasal cartilage (vo), conchae (co), maxilla (mx), palate (pa), tongue (To) and mandible (ma). (Hx & E X 10).

Notice:

1-Nasal cartilage	2-Ethmoid	3-Temporoparietalis muscle
4-Angle of the mouth	5-Meckel's cartilage	6-Depressor angluli muscle
7-Hypoglossal nerve	8-Genioglossus muscle	9-Mylohyoid muscle
10-Submental artery	11-Sublingual duct	12-Submandibular duct
14-Levator anguli	13-Maxillary sinus	14-Levator anguli
15-Buccinator muscle	16-Facial artery	17-Platysma muscle

Fig. 62: A photograph of a section of the head of doxorubicin treated rat fetus (D) at the level of the angle of the mouth showing malformed nasal septum (Ns) vomeronasal cartilage (vo) and mandible (ma). Rudimentary conchae (co) and maxilla (mx) are seen. Also, normal tongue (To) and palate (pa) are seen. (Hx & E X 10).

Notice:

1-Nasal cartilage	2-Ethmoid	3-Temporoparietalis muscle
4-Angle of the mouth	5-Meckel's cartilage	6-Depressor angluli muscle
7-Hypoglossal nerve	8-Genioglossus muscle	9-Mylohyoid muscle
10-Submental artery	14-Levator anguli	13-Maxillary sinus
14-Levator anguli	15-Buccinator muscle	16-Facial artery
17-Platysma muscle		

Fig. 63: A photograph of a section of the head of cyclophosphamide treated rat fetus (CP) at level of the angle of the mouth showing malformed mandible (ma). Hypertrophied nasal septum (Ns) and narrow nasal cavity are seen. Also, rudimentary maxilla (mx) is seen. Normal conchae (co), vomeronasal cartilage (vo) and palate (pa) are seen. (Tongue was lost during processing). (Hx & E x10).

Notice:

1-Nasal cartilage	2-Ethmoid	3-Temporoparietalis muscle
4-Angle of the mouth	5-Meckel's cartilage	6-Depressor angluli muscle
7-Hypoglossal nerve	8-Genioglossus muscle	9-Mylohyoid muscle
10-Submental artery	11-Sublingual duct	12-Submandibular duct
14-Levator anguli	13-Maxillary sinus	14-Levator anguli
15-Buccinator muscle	16-Facial artery	17-Platysma muscle

Fig. 64: A photograph of a section of the head of fluorouracil treated rat fetus (FU) at the level of the angle of the mouth showing malformed mandible (ma) and rudimentary maxilla (mx). Also, normal nasal septum (Ns), vomeronasal cartilage (vo), conchae (co), palate (pa) and tongue (To) are seen. (Hx & E X 10).

Notice:

1-Nasal cartilage	2-Ethmoid	3-Temporoparietalis muscle
4-Angle of the mouth	5-Meckel's cartilage	6-Depressor angluli muscle
7-Hypoglossal nerve	8-Genioglossus muscle	9-Mylohyoid muscle
10-Submental artery	11-Sublingual duct	12-Submandibular duct
14-Levator anguli	13-Maxillary sinus	14-Levator anguli
15-Buccinator muscle	16-Facial artery	17-Platysma muscle

Fig. 65: A photograph of a section of the head of the control rat fetus at the level of the largest transverse diameter showing normal lateral ventricle (Lv), third ventricle (Tv), subarachnoid space (sa) and tongue (To). (H x & E X 10).

Notice:

18 – Pia mater	19-Lateral pterygoid muscle
20-Tooth bud	21-Masseter muscle
22-Mandibular branch of facial nerve	23-Anterior belly of digastric
24-Nasopharynx.	-Mandible (ma)

Fig. 66: A photograph of a section of the head of cyclophosphamide treated rat fetus (CP) at the level of the largest transverse diameter showing dilated lateral (Lv), third (Tv) ventricles and subarachnoid space (sa). (Hx & E X 10).

Notice:

18-Pia mater	25-Telachoroidae of 3rd ventricle.
26-Cerebellum	27-Temporomandibular joint
28-Inner ear.	29-Sternomastoid muscle
30-Splenius capitis	31-Longissimus capitis
32-Pharyngeal constrictors	33-Larynx

Fig. 67: A photograph of a section of the head of fluorouracil treated rat fetus (FU) at the level of the largest transverse diameter showing dilated (Lv) and third (Tv) ventricles. (Hx & E X 10).

Notice:

18-Pia mater	25-Telachoroidae of 3rd ventricle.
27-Temporomandibular joint	28-Inner ear.
29-Sternomastoid muscle	30-Splenius capitis
31-Longissimus capitis	32-Pharyngeal constrictors
33-Larynx	34-choroid plexus
35-Septum pellucidum	36-Thalamus
37-Caudate nucleus	

Fig. 68: A photograph of a section at the root of the neck of the control rat fetus showing normal spinal cord (sp), vertebra (ve), oesophagus (oe), trachea (tr), carotid sheath (ca), thyroid (Th) and thymus (thy) glands. (H x & E X 10).

Notice

38-Trapezius	39-Rhomboideus cervicis
40-Splenius capitis	41-Semispinalis capitis
42-Longissimus muscles	43-Dorsal root ganglion
44-Semispinalis cervicis	45-Multifidus
46-Shoulder joint	47-Pectoralis muscle

Fig. 65: A photograph of a section at the root of the neck of doxorubicin treated rat fetus (D) showing malformed spinal cord (sp) and vertebra (ve). Rudimentary thymus gland (thy) is seen. Also, the oesophagus (oe) is divided by mucosal folds into two compartments which is deviated to the right. Normal trachea (tr), carotid sheath (ca), thyroid gland (Th) and scapula (Sc) are seen. (Hx & E X 10).

Notice

38-Trapezius
39-Rhomboideus cervicis
40-Splenius capitis
41-Semispinalis capitis
42-Longissimus muscles
44-Semispinalis cervicis
45-Multifidus
46-Shoulder joint
47-Pectoralis muscle
48-Vertebral lamina
49-Manubrium sterni

Fig. 70: A photograph of a section at the root of the neck of cyclophosphamide treated rat fetus (CP) showing malformed spinal cord (sp) and vertebra (ve). The oesophagus (oe) is deviated to the right. Also the thymus gland (thy) is rudimentary. Normal trachea (tr), carotid sheath (ca) and thyroid gland (Th) are seen. (Hx& E X 10).

Notice

38-Trapezius
39-Rhomboideus cervicis
40-Splenius capitis
41-Semispinalis capitis
*(44& 45)-Semispinalis cervicis and multifidus
46-Shoulder joint
47-Pectoralis muscle
48-Vertebral lamina
49-Manubrium sterni

Fig. 71: A photograph of a section at the root of the neck of fluorouracil treated rat fetus (Fu) showing malformed spinal cord (sp) and vertebrae (ve). Central oesophagus (oe) and small thyroid gland (Th) are seen. Normal trachea (Tc), carotid sheath (Ca) thymus (thy), scapula (Sc) and clavicle (Cl) are seen. (Hx & Ex 10).

Notice

38-Trapezius	39-Rhomboideus cervicis
40-Splenius capitis	*(44& 45)-Semispinalis cervicis and multifidus
46-Shoulder joint	47-Pectoralis muscle
48-Vertebral lamina	

Fig. 72: A photograph of a section at the root of the neck of methotrexate treated rat fetus (M) showing malformed spinal cord (sp) and vertebrae (ve). Also, rudimentary thymus gland (thy) is seen. Normal trachea (tr) and oesophagus (oe) thyroid gland (Th), carotid sheath (ca) and scapula (Sc). (Hx & E X 10).

Notice

38-Trapezius
39-Rhomboideus cervicis
40-Splenius capitis
41-Semispinalis capitis
42-Longissimus muscles
*(44& 45)-Semispinalis cervicis and multifidus
46-Shoulder joint
47-Pectoralis muscle
48-Vertebral lamina
49-Manubrium sterni

DISCUSSION

Approximately 20-25% of people in the western world die form cancer. Surgery and radiotherapy are valuable for treating localized cancers but are less effective in prolonging the patient's life once the tumour has spread to produce metastases. The introduction of cytotoxic chemotherapy to kill rapidly proliferating neoplastic cells has a major impact on the successful treatment of malignant disease, especially diffuse tumours ***(Carmichael, 1994)***

The results of the present study revealed that the pre-and post implantation losses and resorption were high among all of the treated groups. This was in line with the study of ***Kerry et al. (1998)***, who stated that there was a significant increase in the incidence of fetal malformations, intrauterine growth restriction, spontaneous abortion, still birth or premature delivery after exposure to chemotherapeutic agents prior to, or during pregnancy.

The variable parameters (weight, crown-rump length, biparietal diameter and head length) were used via statistical methods for the comparison between the different treated groups and that of the control. The statistical methods revealed that all the chemotherapeutic agents used have a significant difference in comparison to the control. With comparing different treated groups with each other, it was clear that methotrexate and fluorouracil treated groups were more teratogenic than cyclophosphamide and doxorubicin treated groups. The modulative

effects of folic acid were evident in all of the treated groups. These results were in accordance with ***Kumar et al. (2006),*** who found that the use of fluorouracil during pregnancy induces fetal mortality in about 5% of cases with significant reduction in body weight and various dimentions of the developing brain. The brain shows microcephally, regression or absence of olfactory lobe and obliteration of the various fissures on the dorsal and ventral surfaces of the brain.

Donnenfeld (1994) and Bawle (1995), found that the use of methotrexate during pregnancy induces fetal death, abortion, abnormal skull especially cleft palate, limb and central nervous system defects.

Heringova et al. (2003), found that the use of cyclophosphamide during pregnancy leads to skeletal defects, growth retardation, early fetal loss and still birth. The variable parameters were improved with use of folic acid. These data was in agreement with ***Paula Kurtzweil (1999),*** who stated that, folate is needed both before and in the first weeks of pregnancy and can help to reduce the risk of certain serious and common birth defects. The major neural tube birth defects as anencephaly and spina bifida are reduced by adequate folate intake. Babies with anencephaly do not develop a brain and are stillborn or die shortly after birth. Those with spina bifida have a defect of the spinal column that can result in varying degrees of handicap.

Miller et al. (1989), found that folic acid deficiency causes embryonic deformities, the most common being cleft palate, limb

abnormailities, poor brain development and neural tube defects. The deficiency also causes major fetal abnormalities leading to abortion. ***MRC vitamin study research group (1991),*** found that folic acid deficiency causes partial deletion of chromosome 18, Downs syndrome, bilateral talipes, pes equinovarus, hydropic fetus, complex cardiac malformation, Keinfelter's syndrome, cervical hygroma and adrenal haematoma. ***Phenkoo (1997)***, stated that supplementation of 4mg folic acid daily until the 12^{th} week of pregnancy reuduces the risk of a mother having a child with neural tube defect by 72%. So, the folic acid supplementation is now routinely advised in the first trimester of pregnancy.

Jordan et al. (1977) found that administration of methotrexate which is folic acid antagonist during pregnancy leads to hydrocephalus, microophthalmia, cleft lip and palate, abnormal ossification of long bones especially in the distal parts of the limbs and malformation of caudal vertebrae in up to 75% of fetuses.

The complications of exposure to folic acid antagonists during pregnancy are summarized in the following:

Emerson, (1962), found that administration of folic acid antagonist during pregnancy leads to absent parietal bones, rudimentary temporal and frontal bones, hydrocephalus, cerebral hypoplasia and talipes eqinovarus.

Powell and Ekert, (1971) and Buckley et al., (1997), found that administration of folic acid antagonist during pregnancy leads to oxycephaly, large anterior fontanelle, fusion of coronal suture and webbing between fingers. brachycephaly, retrognathia, ear malformation, bifid uvula, dorsal kyphosis, two hemivertebrae, syndactly and multiple cardiac abnormalities.

In the present work haematomas were detected in all of the treated groups except the fluorouracil treated group. These results go in hand with ***Lioyd et al. (1999),*** who stated that folic acid deficiency during pregnancy leads to fetuses with adrenal haematoma. Also , low- dose of methotrexate during pregnancy causes ileal perforation in the fetuses leading to abdominal haematomas. ***Bawle (1995),*** found that the administration of methotrexate during pregnancy leads to severe myelosupression in the infants with increased risk of haemorrhage. ***Heringova et al. (2003),*** mentioned that the exposure to cyclophosphamide during pregnancy leads to haemangioma and umbilical hernia.

With staining the skeletons of the rat fetuses by alizarine red stain, it revealed that all the used chemotherapeutic agents have a teratogenic effects on the fetal bones. These effects were more extensive in fluorouracil and methotrexate treated groups. The use of folic acid partially improves theses skeletal changes. These results coincided with the results of ***Morrell (2002),*** who stated that folic acid supplementation

may protect against birth defects by overcoming an abnormality in homocystine metabolism.

Liu and Hutson (2001), found that doxorubicin induces a variable anomalies affecting the skeletal system especially radial limb dysplasia and other limb abnormalities and vertebral defects.

Heringova et al. (2003) and Zemlickis (1996), found that cyclophosphamide induces skeletal defects, craniofacial dysmorphisms, limb reduction, absent big toes in both feet, absent thumbs, flattening of nasal bridge and hypoplastic 5^{th} fingers. ***Grafton et al., (1987) and Byrne (1998)*** stated that fluorouracil during pregnancy causes skeletal defects, cleft palate, rib and vertebral anomalies, hind foot anomalies, bilateral radial aplasia, absent thumb and oligodactly.

Donnenfeld (1994) and Bawle (1995), found that methotrexate during pregnancy induce skeletal defects especially limb defects, cleft palate and abnormal skull with a large head and large fontanelles.

The study of the organs gross anomalies in different groups by razor sections at different levels, it was clear that all the used chemotherapeutic agents have a teratogenic effect on a particular system or organ. The anomalies at the level of the angle of the mouth include malformed nasal conchae, thick nasal septum and large tongue obliterating the oral cavity. Malformed brain, dilated lateral and third ventricles were observed at the level of the largest transverse diameter of the head.

Malformed spinal cord, thymus and thyroid glands were observed at the level of root of the neck. Malformed lungs, heart and long sternopericardial ligaments were observed at the level of the thoracic region. Malformed kidneys and stomach were observed at the level of the abdominal region above the umbilicus.

These results were in line with a significant number of clinical and research reports which prove that the intrauterine exposure to chemotherapeutic agents are associate with increased incidence of fetal malformations.

Kerry et al. (1998) and Meirow and Schiff (2005), mentioned that all chemotherapeutic agents are potentially teratogenic and mutagenic because they act on rapidly dividing cells. The potential exists of fetal malformations, intrauterine growth restriction, spontaneous abortion, stillbirth or premature delivery when a woman is exposed to chemotherapeutic agents prior to, or during, pregnancy.

In the present study, the histological sections reveald that there was malformed nasal septum, nasal conchae and oesophagus in (D) treated group. Hypertrophied nasal septum was detected in (CP) treated group. Dilated ventricles were detected in (CP) and (FU) treated groups. Malformed spinal cord was detected in all of the treated groups. These results went in hand with ***Liu and Hutson (2001)***, who stated that the prenatal exposure of rat embryos to doxorubicin result in viarable anomalies affecting the skeletal system, the alimentary tract (e.g. imperforate anus, oesophageal atresia and tracheo-oesophageal fistula),

the cardiovascular system and urogenital tract. ***Heringova et al. (2003),*** mentioned that the prenatal exposure to cyclophosphamide produces skeletal and central nervous system defects. ***Byrne (1998)***, mentioned that the prenatal exposure to fluorouracil produces fetal anemia, defects of the nervous system, palate and skeleton**.** ***Bawle (1995)***, mentioned that prenatal exposure to methotrexate leads to cleft palate, limb and central nervous cystem malformations.

SUMMARY AND CONCLUSION

Chemotherapeutic agents have multiple therapeutic uses in women of reproductive age including treatment of neoplastic diseases, inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythromatosus, juvenile arthritis and human papilloma virus infections.

In the present study fifty female albino rats and twenty male albino rats were used. The females were virgin, about 120 days in age and ranging in weight from (160 to 200gms). The males were of the same range of age and weight. The animals were fed a balanced diet. Each 3 females were placed over night with 2 males. If the vaginal smear in the following morning contained sperms, that day was considered as day zero of gestation. The pregnant rats were classified into two main groups; control group (10 rats) and treated group (40 rats).

The treated group was subdivided into 4 subgroups (10 for each). Each subgroup was given the corresponding drug daily from 6^{th} - 9^{th} day of gestation. Half of each subgroup was also given folic acid 100 microgram/kg body weight daily orally by gastric intubation.

The drugs given were; doxorubicin in a dose of 2microgram/gram body weight/ day for the first subgroup, cyclophosphamide in a dose of 7 microgram/ gram body weight / day for the second one, fluorouracil in a dose of 15 microgram /

gram body weight / day for the third one and methotrexate in a dose of 5 microgram/ day for the fourth one. All drugs were given via an intravenous injection.

The fetuses of the pregnant mothers were obtained by labarotomy at the 20^{th} day of gestation. Two fetuses picked up from each pregnant female, dehydrated, fixed by immersion in 95% ethyl alcohol and were used for skeletal visualization by alizarine red stain. The rest of fetuses were placed in Bouin's solution and were used for razor sections as well as histological section after external examination.

The present study revealed that the preimplantation loss and postimplantation loss and resorption increased in all the treated groups. The addition of folic acid has improved this percentage. The CRL, head length, BPD and weight parameters showed statistical significance between the treated groups and the control group. Also, the modulative effect of folic acid was evident. The sizes of the fetuses of the treated groups were smaller than those of the control group especially in the methotrexate and fluorouracil treated groups.

With alizarine red stain, many skeletal deformities were present in all the treated groups. The supplementation with folic acid improves some of these skeletal changes. Using razor techniques, gross anomalies of organs were detected, these were in the form of malformed nasal conchae, thick nasal septum, large tongue obliterating the oral cavity, dilated brain ventricles, malformed spinal cord, thymus and thyroid glands, lungs, heart, kidneys and stomach.

The histological study revealed malformed nasal septum, nasal conchae and oesophagus in the (D) treated group. Hypertrophied nasal septum was detected in the (CP) treated group. Dilated brain ventricles and subarachnoid space were detected in the (CP) and (FU) treated groups. Malformed spinal cord was detected in all of the treated groups.

Based on this study it can be concluded that all chemotherapeutic agents included in this study are teratogenic in a variable degrees. In this study, the doxorubicin and cyclophosphamide appears to be less teratogenic than the other groups. The use of folic acid reduces the teratogenic effects of the chemotherapeutic agents. Based on this study the use of doxorubicin or cyclophosphamide at the lowest effective dose and supplementation with folic acid can markedly reduce the teratogenic effects of chemotherapy and increase the chance of mothers with neoplastic diseases to get a healthy child.

The use of methotreaxate and fluorouracil should be avoided during pregnancy.

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الملخص العربى

أدوية العلاج الكيميائى لها استخدامات علاجية عديدة للمرأة خلال سن الإنجاب مثل علاج الأورام الخبيثة ومرض الأمعاء الملتهبة والتهاب المفاصل الروماتزمى والذئبة الحمراء العامة والتهاب المفاصل الأحداثى والتهابات فيروس الأورام الحليمية البشرى٠

فى الدراسة الحالية تم استخدام خمسين فأرة بيضاء من الإناث المعزولة وعشرين فأراً أبيض من الذكور ٠ وكانت الإناث المعزولة تبلغ من العمر حوالى مائة وعشرون يوماً وتتراوح أوزانها بين ١٦٠-٢٠٠جم وكانت الذكور من نفس العمر والوزن، وتمت تغذية الفئران بوجبة متكاملة٠ وتم وضع كل ثلاث إناث مع ذكرين فى قفص واحد لمدة ليلة واحدة ويتم فحص مسحة مهبلية للإناث فى الصباح التالى فإذا كان يحتوى على حيوانات منوية يعتبر هذا اليوم هو بداية الحمل٠ تم تقسيم الفئران الحوامل إلى مجموعتين رئيستين، مجموعة ضابطة (١٠فئران) ومجموعة معالجة (٤٠ فأراً)٠

تم تقسيم المجموعة المعالجة إلى أربع مجموعات فرعية (١٠ فئران لكل مجموعة) وقد أعطيت كل مجموعة فرعية الدواء المتماثل يومياً من اليوم السادس إلى اليوم التاسع من الحمل٠ وقد تم إعطاء حمض الفوليك لنصف كل مجموعة فرعية بجرعة يومية مقدارها ١٠٠ ميكروجرام لكل كجم من وزن الجسم عن طريق أنبوبة معدية ٠

الأدوية المعطاة هى الدكسوروبيسين بجرعة يومية مقدارها ٢ ميكروجرام لكل جرام للمجموعة الفرعية الأولى، والسيكلوفوسفاميد بجرعة يومية مقدارها ٧ ميكروجرام لكل جرام للمجموعة الثانية، والفلورويواسيل بجرعة يومية مقدارها ١٥ ميكروجرام لكل جرام للمجموعة الثالثة، والميثوتركسيت بجرعة يومية مقدارها ٥ ميكروجرام للمجمموعة الرابعة وتم اعطاء كل الأدوية عن طريق الحقن الوريدى٠

وتم أخذ أجنة الفئران الحوامل بفتح بطنها فى اليوم العشرين من الحمل٠ وتم أخذ جنينين من كل أم حامل وتم تجفيفهم وتثبيتهم بوضعهم فى ٩٥% كحول وتم استخدامهم لايضاح الهيكل العظمى مستخدماً صبغة الأليزارين٠ وباقى الفئران تم وضعهم فى محلول بوان لاستخدامهم فى مقاطع الموس والمقاطع الهستولوجية بعد إجراء الفحص الخارجى لهم٠

وقد أظهرت النتائج أن فقد الأجنة في الفئران زاد في كل المجموعات المعالجة وأن إضافة حمض الفوليك قد قلل هذه النسبة٠ وأظهرت الدراسة أيضاً أن أطوال الأجنة وأوزانها وطول وعرض الرأس فيها تأثرت تأثراً له دلالة إحصائية في المجموعات المعالجة مقارنة بالمجموعة الضابطة وأيضاً ظهر بوضوح التحسن الناتج عن استعمال حمض الفوليك٠

وقد كانت أحجام الأجنة في المجموعتين المعالجتين بالميثو تركسيت والفلوروبوراسيل اصغر في احجامها من اللتين عولجتا بالدكسوروبيسين والسيكلوفوسفاميد٠

ومع استخدام صبغة الأليزارين الحمراء ظهرت تشوهات في عظام الأجنة في المجموعات المعالجة مقارنة بالمجموعة الضابطة وقد خفض حمض الفوليك بعض هذه التشوهات ومع استخدام طريقة الفحص على شكل مقاطع ظهرت تشوهات ضخمة فى الأعضاء مثل تشوهات بالقرائن الأنفية وتضخم بالحاجز الأنفى واللسان الذى أصبح يملاً تجويف الفم وتشوهات بالحبل الشوكى واتساع في بطينات الدماغ وتشوهات بالغدة التيموسية والدرقية والرئتين والقلب والكليتين والمعدة٠

وأظهرت مقاطع الهستولجى تشوهات بالحاجز الأنفى والقرائن الأنفية والمرئ في مجموعة الدكسوروبيسين٠ وقد ظهر أيضاً تضخم في الحاجز الأنفى في مجموعة السيكلوفوسفاميد وإتساع

فـي بطينـات الـدماغ فـي مجمـوعتى السيكلوفوسـفاميد والفلورويوراسـيل٠ وظهـر تشـوهاً فـي الحبـل الشوكى في كل المجموعات٠

وقد أكدت هذه الدراسة على أن كل أدوية العلاج الكيميائى للسرطان التى تمت الدراسة عليها لها أثاراً تشويهية بدرجة متفاوتة٠

مما سبق نستخلص أن استخدام الدكسوروبيسين والسيكلووفوسفاميد بأقل جرعة علاجية ممكنة مع إضافة حمض الفوليك يقلل التشوهات المصاحبة للعلاج الكيميائى للسرطان ويزيد من فرصة الأم المصابة بالسرطان في أن تلد طفلاً صحيحاً٠

وإن استخدام الميثوتركسيت والفلورويوراسيل يجب أن يُتجنب أثناء الحمل٠

دراسة تأثير بعض أدوية العلاج الكيميائى على الفئران قبل الولادة والدور المعدل لأضافة حمض الفوليك

رسالة دكتوراه مقدمــة مـــن

الطبيب/ محمود السيد محمد

حاصل على ماجستير التشريح والأجنة – كية طب بنها

توطئة للحصول على درجة الدكتوراه في التشريح والأجنة

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بكلية طب بنها

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