Deanship of Graduate Studies

Al-Quds University

The Teratogenic Activity of Phthalates on Developing Chicks and Female Rats Fertility

By

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M.Sc. Thesis

June / 2010

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BY

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Medical Science in Biochemistry and Molecular Biology. From the Faculty of Graduate Studies, at Al-Quds University, Jerusalem Palestine

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June / 2010

DEDICATION:

This thesis is dedicated to …….

My parents: Abdul-Salam and Fawzia

My lovely sisters and Brother: Rula, Sana, Maisa & Iman

My best friend: Areen

And sweet lovely Aya

DECLARATION:

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or part of the same) has not been submitted for a higher degree to any other university or institution.

SIGNATURE: ----------

Name: **Safa Abdul-Salam Sami Abdul-Ghani**

Date: **May, 24, 2010**

ACKNOWLEDGMENTS

- First of all I would like to thank my Supervisor Dr. Ziad Abdeen for his help and support in this project.
- Second thanks to Dr. Munir Qazzaz from the Biology and Biochemistry Department at Birzeit University for his willingness to be the External Examiner.
- Thanks to Dr. Motaz Akkawi from the Biology Department for his agreement to be the Internal Examiner.
- Thanks to Prof. Joseph Yanai and Dr. Adi Penkis for allowing me to use the facilities of their lab in Hadassah Medical Center for measuring the teratogenic activity on Chicks Model, and for their scientific comments and advise.
- Thanks to Dr. Rula Abdul-Ghani for her help and advice on Molecular aspects
- Special thanks and appreciation to Mr. Rateb Hussein and Mr. Munther Metani for their technical support through the pregnancy period of my female rats, at the animal unit of Birzeit University.
- Many thanks to Dr. Tamer Essawi and Mr. Firas Hassan from the Faculty of Nursing and Allied Health Professions at Birzeit University for allowing me to use their Kits and Instruments for the Measurement of biochemical analysis.

CONTENT

ABSTRACT:

Phthalates are industrial chemicals widely used in consumer products including cosmetics, building material and medical equipment made with polyvinyl chloride (PVC) plastics and children toys, and the risk of exposure to phthalates is increasing continuously. In recent years many studies have been carried out on the possible health hazards of phthalates, including the effect on reproduction. However, there is still an inconsistency of teratological information on phthalates. **Therefore we used the Chick Model,** which provide a suitable model for the rapid evaluation of phthalates behavioral teratogenicity, and enable rapid screening for potential developmental disruptors by avoiding maternal toxicity, maternal-fetal unit and maternal-neonatal interactions.

Pre hatching exposure of chicks embryo to di(2-ethyl hexyl) phthalates DEHP in doses ranging from 20 – 100 mg / kg, have reduced percentage hatching from 80% in control eggs to 65%, and increased late hatching from 12.5 % in control eggs to 29.4 %. In addition it induced developmental defects characterized by a hole or weakening of abdominal muscles allowing internal organs to protrude externally with or without a sac (Omphalocele) or (Gastroschisis). The effect was dose dependent starting from 8% with DEHP (20 mg/kg) to 22 % with DEHP (100 mg/kg). Similar treatment with Di-butyl phthalates (DBP) 100 mg/kg has reduced percentage hatching to 57 % and increased late hatching to 37.5 %, with 14 % increase in developmental defects characterized as Gastroschesis.

Neurobehavioral measurements using imprinting test and locomotor activity on chicks, pretreated with DEHP 50-100 mg/kg, has shown a significant reduction of 21.6 % in imprinting performance which indicated neurobehavioral teratogenic activity.

DNA damage measurements using ELISA kit which measures the blood concentration of the metabolite 8-hydroxydeoxyguanosine (8-OH-dG), has shown a trend of increase by 39.7% following pre exposure to phthalates, which was significant with DEHP, indicating genetic toxicity of phthalates on embryonic development.

In Female Rats Model where the rats were injected twice weekly with DBP or DEHP (100 mg/kg) and cohabited with male rats for one month, we found a significant effects of DBP and DEHP on female fertility, by decreasing fertility rate from 87 % in control rats to 67 % and 50 % respectively and by increasing mortality rate in new born litters from 2.8 % to 52.3 % and 31.3 % respectively. Fecundity rate which express the average number of litters in each delivery was reduced from 8.2 in control treated rats to 7.3 in DBP treated and to 5.3 in DEHP treated rats.

No significant changes were observed in total body weight gain, or with the relative weight of the following organs, heart, spleen, liver, or brain. The only significant changes in relative weight were detected following treatment with DBP (100 mg/kg) , 27.5 % decrease in female sex organs ($P \leq 0.05$), and significant reduction of 7 % in kidneys. The change in female rats fertility following continuous treatment with DBP (100 mg/kg) were accompanied with a significant increase of 29.8% in blood serum 8-hydroxydeoxyguanosine (8-OH-dG), which is considered as a marker for DNA oxidative stress.

As biochemical changes in blood of female rats are concerned, phthalates induced a significant increase in GPT and GOT, and a significant reduction in alkaline phosphatase, uric acid and creatinine, which indicates a drug related injury to hepatic cells. No changes were observed in glucose, triglycerides, total protein, total cholesterol, HDL, and LDL.

In conclusion, our results provide evidence about the teratogenic activity of phthalates on chick embryonic development. Phthalates caused a significant decrease in egg hatching percentage and increasing late hatching and it also induced Gastroschisis and Omphalocele in 22% of the cases. The decrease in imprinting performance indicates neurobehavioral teratogenic activity. Part of the teratogenic activity is associated with oxidative stress and DNA damage. The elevated levels of alkaline phosphatase is due to a bony pathology or muscular dystrophy, which in turn might reduce muscle dry mass leading to decrease in creatinine and urea.

On female rat's fertility, Phthalates has decreased fertility rate, fecundity rate and increased mortality rate in new born litters, associated with significant reduction in relative weight of female sex organs, and increase DNA damage following treatment with DBP.

ملخص:

" تأثير مركبات الفثالات على الخصوبة و مراحل تطور الأجنة "

الفثالات هي مواد صناعية تستعمل بشكل كبير في منتجات المستهلك, بما في ذلك المستحضرات التجميلية

ومواد البناء والمعدات الطبية المصنوعة من PVC بلاستيك و أيضا ألعاب الأطفال, كما أن خطر التعرض للفثالات يزداد باستمرار.

في السنوات الأخيرة, أجريت دراسات عديدة للبحث في المخاطر الصحية للفثالات بما في ذلك التأثير على التناسل و تطور الجنين. لكن, ما زال هناك تناقض في المعلومات عن علاقة الفثالات بتشوه الأجنة. و لذلك استخدمنا نموذج الصيصان, الذي يقدم نموذج مناسب للتقييم السريع لتأثير الفثالات في تشوه الأجنة, و يقدم فحص سريع لإمكانية اختلال في التطور عن طريق تجنب تأثير السمية الناتجة عن الأم.

قبل الفقس تم حقن مادة DEHP (20-100ملغم \كغم). فانخفضت نسبة التفقيس من 80% إلى 65%, وازداد التفقيس المتأخر من 12.5% إلى 29.4%. بالإضافة إلى ذلك ظهرت عيوب في التطور الجنيني تميزت بوجود ثقب أو ضعف بعضلات البطن بحيث تبرز الأمعاء والأعضاء الداخلية للخارج مع أو بدون كيس تسمى الفتق الامنيوسي و الفتق المعوي على التوالي . كان هناك علاقة طردية بين التأثير و تركيز الفثالات من 8% مع استخدام DEHP بتركيز (20 مغم\كغم) إلى 22% مع استعمال DEHP (100 مغم\كغم). وعند حقن مادة DBP (100 مغم\كغم) انخفض نسبة التفقيس إلى 57% وزاد التفقيس المتأخر إلى 73.5%, كما أدى إلى زيادة بقيمة 14% في ظهور عيوب في التطور مثل الفتق المعدي.

باستخدام اختبارIMPRINTIG تم فحص السلوك العصبي و النشاط الحركي على الصيصان بعد العلاج بمادة DEHP (50-100مغم\كغم), فقد أدى إلى انخفاض واضح بقيمة 21.5% بالاختبار مما يدل على وجود تشوهات بنشاط السلوك العصبي.

تم قياس تلف الحمض النووي باستخدام ELISA ASSAY و التي تقيس تركيز (8-OH-dG) في الدم. أدى إلى ارتفاع تركيز مادة (8-OH-dG) بعد التعرض للفثالات والذي كان ملحوظا مع استعمال مادة DEHP , والتي تشير إلى سمية جينية للفثالات في مرحلة التطور الجنيني.

وعند دراسة تأثير الفثالات على إناث الفئران حيث تم حقنهم مرتين أسبوعيا بمادة DEHP او DBP (100مغم\كغم) و عاشوا مع الذكور لمدة شهر واحد, ظهر تأثير ملحوظ للمادتين DEHP, DBP على خصوبة الإناث عن طريق التقليل من معدل الخصوبة من 87% إلى 67% و50% على التوالي, وعن طريق زيادة معدل الوفيات عند حديثي الولادة من 2.8% إلى 52.3% و 31.3% على التوالي. معدل الإخصاب (و الذي يعبر عن متوسط عدد الفئران حديثي الولادة عند كل ولادة لهم), انخفض معدل الإخصاب من 8.2% إلى 7.3% عند استخدام DBP و 5.3% فى حالة استخدام مادة DEHP .

لم يلاحظ أي تغير بزيادة الوزن الكلي للجسم, أو بالنسبة للوزن النسبي للأعضاء التالية, القلب, البنكرياس, الكبد والدماغ . لقد حصلنا على تغيير ملحوظ بعد العلاج بمادة DBP (100مغم\كغم), وتجسد ذلك بانخفاض وزن الأعضاء التناسلية الأنثوية بنسبة 27.5%, وانخفاض بنسبة 7% بالوزن النسبي للكلية.

إن التغير بخصوبة إناث الفئران بعد العلاج المستمر ب DBP (100% مغم\كغم) رافق زيادة ملحوظة بنسبة 29.8% بتركيز (8-OHDG) بالدم. والذي يعتبر كعلامة لوجود إجهاد تأكسدي بالحمض النووي. وعند فحص كيميائيات الدم لدى إناث الفئران التي حقنت بالفثالات, لوحظ زيادة في GOT, GPT و انخفاض ملحوظ بالفوسفاتيز القلوي و حمض اليوريك و الكرياتينين. و لم يلاحظ أي تغير بالسكر, الدهون الثلاثية, إجمالي البروتين, إجمالي الكولسترول , الكولسترول الجيد و الكولسترول السيئ.

وفي الختام, قدمت نتائج هذا البحث أدلة حول قدرة الفثالات على التسبب بتشوهات خلقية في مراحل التطور الجنيني, و تقليل معدل الفقس وزيادة الفقس المتأخر. و عن طريق حدوث الفتق الامنيسوي و الفتق المعدي ب 22% من الحالات. الانخفاض بأداء اختبار IMPRINTING مما يدل على تشوهات و خلل بنشاط السلوك العصبي. جزء من هذه التشوهات مرتبطة بتلف الحمض النووي و الإجهاد التأكسدي, و بزيادة ضمور العضلات الهيكلية كما يتضح من ارتفاع الفوسفاتيز القلوي.

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ABBREVIATIONS

DEHP: Bis(2-ethylhexyl) Phthalate

DBP: Dibutyl Phthalate

PVC: Polyvinyl Chloride

I P: Intra-Peritoneal

min: Minutes

UDP-GT: Uridine 5'-Diphosphate-Glucuronosyl Transferase

NHANES: National Health and Nutrition Examination Survey

ADHD: Attention-Deficit/Hyperactivity Disorder

EPA: Environmental Protection Agency

IARC : International Agency for Research on Cancer

PPAR: peroxisome proliferator-activated receptors

8-OH-dG: 8-Hydroxy-2'-Deoxyguanosine

ALT / GPT: Alanine Aminotransferase

AST / GOT: Aspartate Aminotransferase

ALP: Alkaline Phosphatase

DNA : Deoxyribonucleic Acid

FDA: Food and Drug Administration

FSH : Follicle Stimulating Hormone

LDH : Lactic Dehydrogenase

LH : **Luteinizing Hormone**

NRC : **National Research Council**

WHO: **World Health Organization**

IMHV : **Intermedial Part of the Hyperstriatum Ventral**

HDL : **High Density lipoprotein**

LDL : **Low Density Lipoprotein**

ELISA: **Enzyme-linked immunosorbent assay**

CHAPTER ONE

1. INTRODUCTION

1.1 TERATOGENICITY:

Teratogenicity is the ability of producing fetal malformation. A wide range of different chemicals and environmental factors are suspected or are known to be teratogenic in humans and in animals. Classes of teratogens include radiation, maternal infections, chemicals, drugs, environmental chemicals, Tobacco, Alcohol, and Caffeine. Exposure to teratogens can result in a wide range of structural abnormalities such as cleft lip, cleft palate, dysmelia, anencephaly, ventricular septal defect (Shiotak et al, 1982). Exposure to a single agent can produce various abnormalities depending on the stage of development it occurs. Several factors affect the ability of a teratogen to contact a developing conceptus, such as the nature of the agent itself, route and degree of maternal exposure, rate of placental transfer and systemic absorption, and composition of the maternal and embryonic/fetal genotypes.(Heudorf et al, 2007).

The number of compounds which must be tested for teratogenicity has increased dramatically with the continuous development of therapeutic, cosmetic and food additive chemicals. It is unrealistic to attempt to perform complete in vivo teratogenicity tests on each and every one of these chemicals. Thus quicker, efficient and reliable tests must be developed.

In the last few years, a number of other systems have been proposed as possible screening tests for teratogenicity. The available in vitro systems are – mammalian organ culture, vertebrate embryos e.g., chick, fish, and amphibian embryos, invertebrate system like, drosophila, cricket and hydra, organ culture e.g. limb bud and cell culture system.(Kotwani et al 1994). In addition, pregnancy registries are large, prospective studies that monitor exposures women receive during their pregnancies and record the outcome of their births.

These studies provide information about possible risks of medications or other exposures in human pregnancies.

SCREENING FOR TERATOGENIC ACTIVITY

The methods for screening terarogenic activity include (a) studies in rodents and (b) surveillance of human epidemiology. Both these methods suffer from disadvantages. The former is too expensive for evaluation of a large number of substances. The second method i.e., surveillance system detects the teratogenic substances after defect has been produced.

1.1.1 Chick embryo Model

Chick embryo has been used for teratogenicity for many years but the predictive value of the chick model has been questioned. Newer techniques have been evolved and by standardization of test subjects, chick embryo is coming back as a screening method for teratogenicity. Moreover as the list of chemicals which must be tested has grown to an intolerable burden the chick embryo has received more favourable review. Several authors have described protocols in which the chick is utilized in a predictive test for teratogenicity. White leghorn fertilised eggs incubated in commercial apparatus at 30°C are usually used. To administer the test agent a hole is bored in the egg, which may be subsequently resealed with wax or paraffin. The test agent may be administered to the yolk sac, sub germinal cavity, allantois, amnion or air chambers depending upon the physicochemical properties of the compound and the individual preference of the investigators. Opinions on the most appropriate treatment time vary from 0 hour of incubation to 30 hours, 48 hours or 96 hours. The chick may be examined for abnormalities at any time during incubation, at hatching or may be allowed to mature to evaluate functional normality.

Chicks are a suitable model for the evaluation of neurobehavioral teratogenicity (Wormser et al 2005) because they provide a rapid and convenient model for screening; maternal variables that exist in rodents and other mammals and affect their offspring, such as changes in maternal stress, are completely devoid when using avian model (Sastry et al 1991). When examining prenatal exposure to different substances, the teratogen is administered directly into the media surrounding the embryo, without maternal mediation. Thus the

variables related to maternal physiology and behavior are being completely controlled. When examining neurobehavioral teratogenicity, the chick is an ideal model since it hatches in an advanced developmental stage that allows immediate physical and behavioral evaluation. A chick model provides an unlimited, cheap and rapid source for neural progenitors, since it is precisely timed and all the embryos are in a known and identical developmental stage (Kotwani et al 1995).

Several avian models for prenatal exposure to teratogens have been developed; it has been shown that nicotine, heroin and chlorpyrifos induce behavioral damage demonstrated in the imprinting behavioral test, that examines the chick's tendency to follow the first objects it encounter and is related with cholinergic muscarinic signaling in the left intermedial part of the hyperstriatum ventral (IMHV) (Izrael et al 2004).

EVENT IN EMBRYONIC DEVELOPMENT

Fertilization before Egg Lying

Division and Growth of Living Cells
Segregation of Cells into Groups of Special Function (Tissues)

Between Laying and Incubation
No Growth; Stage of Inactive Embryonic Life

During Incubation:

First Day
16 hours - First Sign of Resemblance to a Chick Embryo
18 hours - Appearance of Alimentary Tract
20 hours - Appearance of Vertebral Column
21 hours - Beginning of Nervous System
22 hours - Beginning of Head
24 hours - Beginning of Eye

Second Day
25 hours - Beginning of Heart
35 hours - Beginning of Ear
42 hours - Heart Beats

Third Day
60 hours - Beginning of Nose
62 hours - Beginning of Legs
64 hours - Beginning of Wings

Fourth Day- Beginning of Tongue

Fifth Day - Formation of Reproductive Organs and Differentiation of Sex

Sixth Day - Beginning of Beak

Eighth Day - Beginning of Feathers

Tenth day - Beginning of Hardening of Beak

Thirteenth Day - Appearance of Scales and Claws

Fourteenth Day - Embryo gets into Position Suitable for Breaking Shell

Sixteenth Day - Scales, Claws and Beak becoming Firm and Horny

Seventeenth Day - Beak Turns toward Air Cell

Nineteenth Day - Yolk Sac Begins to Enter Body Cavity

Twentieth Day - Yolk Sac Completely Drawn into Body Cavity; Embryo Occupies Practically all the Space within the Egg except the Air Cell

Twenty-First Day - Hatching of Chick

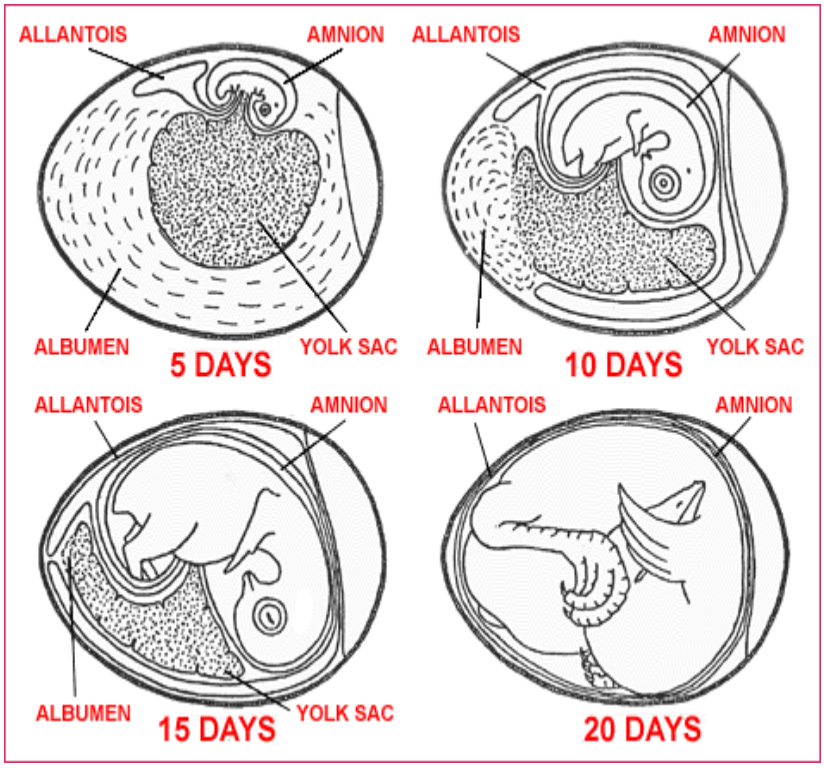


Fig (1.1): Successive changes in the position of the chick embryo and its embryonic membranes. (From A. L. Romanoff, Cornell Rural School Leaflet, September, 1939)

1.2 PHTHALATES.

Phthalates, or phthalate esters, are a class of industrial compounds widely used as softeners of plastics like polyvinyl chloride (PVC), solvents in perfumes, toys, food packaging and additives to hairsprays, lubricants, and insect repellents.

Concerns have been raised about some phthalates because studies on laboratory animals have shown that exposure can cause adverse health effects, including effects on development of the male reproductive system. Few data are available on the health effects of phthalates in humans, but studies show widespread human exposure to phthalates. The Environmental Working Group has focused on phthalates since 1998, when bis(2-ethylhexyl) phthalate was found in beauty product, found that dibutyl phthalate was present in the bodies of every single person tested for industrial pollutants.(Petersen et al.2000)

The Table (1.1) Below Represent the most widely used Phthalate Compounds and their Metabolites:

Parent Compound	Phthalate metabolite
Benzyl butyl phthalate (BzBP)	Mono-benzyl phthalate (MBzP) Mono-n-butyl phthalate (MBP)
Dibutyl phthalate (DBP)	Mono-n-butyl phthalate (MBP)
Di-isobutyl phthalate (DiBP)	Mono-isobutyl phthalate (MiBP)
Diethyl phthalate (DEP)	Mono-ethyl phthalate (MEP)
Di -2-ethylhexyl phthalate (DEHP)	Mono-2-ethylhexyl phthalate (MEHP) Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP) Mono-2-ethyl-5-oxohexyl phthalate (MEOHP)
Di-n-octyl phthalate	Mono-3-carboxypropyl phthalate (MCPP)
Dimethyl phthalate	Mono-methyl phthalate (MMP)

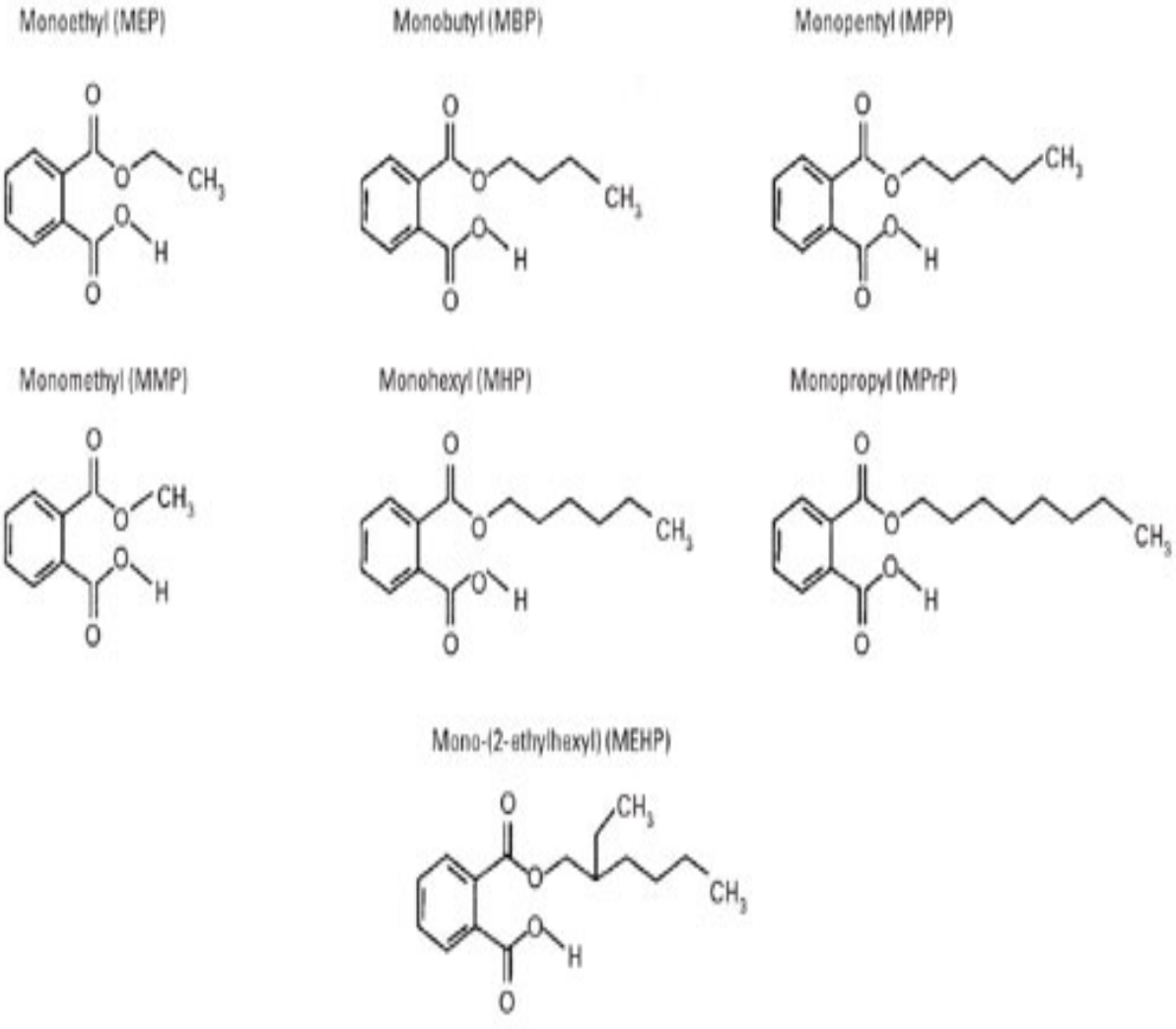


Fig (1.2): Phthalates Metabolites Chemical Structures

1.2.1 Di (2-Ethylhexyl) Phthalates:

Di (2-ethylhexyl) phthalate, commonly referred to as DEHP, one of the most widely used plasticizer; it is predominantly used as a plasticizer in the production of flexible polyvinyl chloride (PVC) products. At least 95% of DEHP produced is used as a plasticizer for PVC. PVC is made flexible by addition of plasticizers and is used in many common items such as wall coverings, tablecloths, floor tiles, furniture upholstery, shower curtains, garden

hoses, swimming pool liners, rainwear, baby pants, dolls, toys, shoes, automobile upholstery and tops, packaging film and sheet, sheathing for wire and cable, medical tubing, and blood storage bags. Numerous nonplasticizer uses of DEHP have been reported; however, it is not clear to what extent these uses are, or have ever been, important. Because of concerns regarding potential health effects from DEHP exposure, many toy manufacturers have discontinued use of DEHP in their products. The use of DEHP in domestically produced baby teethers and rattles has been discontinued, and DEHP is also no longer used as a plasticizer in plastic food wrap products.(1)

If you are exposed to DEHP, many factors determine whether you'll be harmed. These factors include the dose (how much), the duration (how long), and how you come in contact with it. You must also consider the other chemicals you're exposed to and your age, sex, diet, family traits, lifestyle, and state of health.

Occupational exposures may be significant, but the highest exposures to DEHP result from medical procedures such as blood transfusions (upper bound limit of 8.5 mg/kg/day) or hemodialysis (upper bound limit of 0.36 mg/kg/day), during which DEHP may leach from plastic equipment into biological fluids (Heudorf et al, 2007) .

1.2.1.1 Medical Tests for DEHP:

The most specific test that can be used to determine if you have been exposed to DEHP is the measurement of MEHP and other breakdown chemicals in your urine or blood. This test only provides a measure of recent exposure, since DEHP is rapidly broken down into other substances and excreted from your body. You also could be tested for another breakdown product (phthalic acid), but this test would not be specific for DEHP. One or 2 days after exposure, your feces could be tested for the presence of DEHP metabolites.

1.2.1.2 DEHP Health Effect:

One way to see if a chemical will hurt people is to learn how the chemical is absorbed, used, and released by the body. For some chemicals, animal testing might be necessary. Animal testing might also be used to identify health effects such as cancer or birth defects.

Without laboratory animals, scientists would lose a basic method to get information needed to make wise decisions to protect public health. Scientists have the responsibility to treat research animals with care and compassion. Laws today protect the welfare of research animals, and scientists must comply with strict animal care guidelines.

DEHP, at the levels found in the environment, is not expected to cause adverse health effects in humans. A man who voluntarily swallowed 10 g (approximately 0.4 ounces) of DEHP had stomach irritation and diarrhea. Most of what we know about the health effects of DEHP comes from studies of rats and mice that were given DEHP in their food, or the DEHP was placed in their stomach with the aid of a tube through their mouth (David et al, 2000). In most of these studies, the amounts of DEHP given to the animals were much higher than the amounts found in the environment. Rats and mice appear to be particularly sensitive to some of the effects of DEHP. Thus, because certain animal models may not apply to humans, it is more difficult to predict some of the health effects of DEHP in humans using information from these studies.

In recent years, concern has been raised that many industrial chemicals, DEHP among them, are endocrine-active compounds capable of having widespread effects on humans and wildlife (Crisp et al. 1998; Daston et al. 1997). Particular attention has been paid to the possibility of these compounds mimicking or antagonizing the action of estrogen, and more recently, their potential antiandrogenic properties. Estrogen influences the growth, differentiation, and functioning of many target tissues, including female and male reproductive systems, such as mammary gland, uterus, vagina, ovary, testes, epididymis, and prostate. Thus far, there is no evidence that DEHP is an endocrine disruptor in humans at the levels found in the environment.

The wealth of information in animals administered DEHP for periods ranging from a few days to lifetime studies indicate that DEHP is a developmental and reproductive toxicant by mechanisms not yet completely understood. As discussed below, the mechanisms do not appear to involve binding of DEHP to the estrogen or androgen receptors. DEHP administered perinatally to females is embryotoxic and teratogenic (reduced fetal body weight, increased rates of abortion and fetal resorptions, skeletal malformations and in males, it causes testicular toxicity.

1.2.2 Phthalates Physical and Chemical Properties:

Di-esters of ortho-phthalic acid (phthalates) consist of one aromatic ring and two usually aliphatic side chains. The phthalates are substances with low water solubility/high fat solubility and low volatility. The polar carboxyl group contributes less to the physical properties of the phthalates (unless the side chains are very short, as in di-methyl and diethyl phthalate).

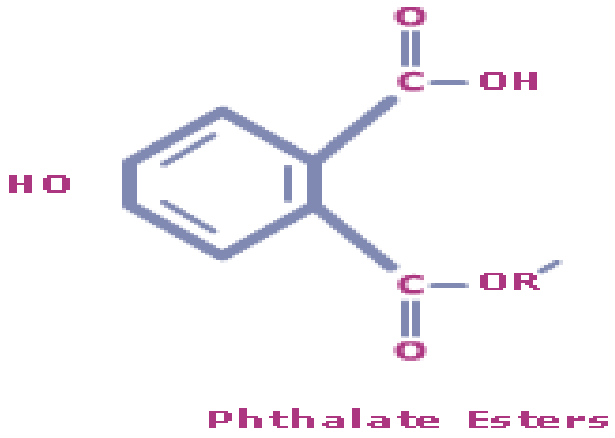


Fig (1.3): Phthalate Esters

They are fluid in wide temperature intervals, e.g. between –50ºC and 340ºC for DEHP. Due to their low water solubility, the phthalates hydrolyse relatively slowly, but the actual rate varies according to solubility and temperature. The properties of phthalates, such as solubility with different polymers, volatility, effect on the polymer at different temperatures and so, are modified with the side chains. These are most often similar but can also be different. They can be straight or branched aliphates but cycloaliphatic and aromatic side groups also occur. The physical and chemical properties of the phthalates have made them suitable as plasticisers in polymers such as plastic and also rubber.

1.2.3 Metabolism and Kinetics:

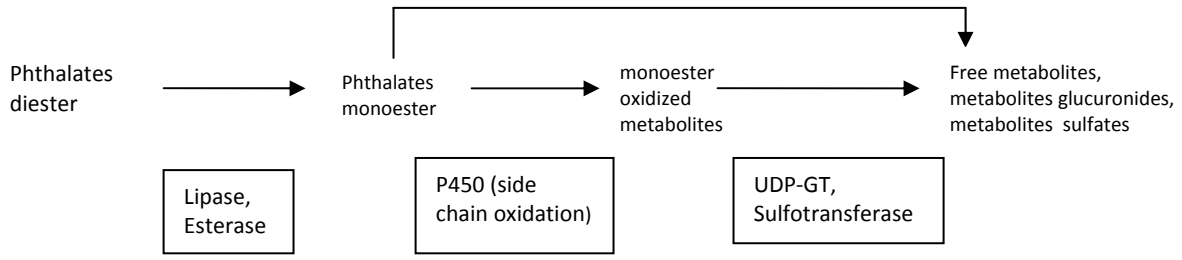


Fig (1.4) Phthalate Metabolism UDP-GT, Uridine 5'-Diphosphate-Glucuronosyl Transferase.

Mammalian absorption and metabolism of phthalates (see figure 1.4) are rapid; initial de-esterification of one alkyl linkage occurs in the saliva or the gut after oral intake. The resulting monoesters have one or more carbons. Monoesters are the main detected metabolites of the low molecular weight phthalates, such as DEP and DBP (Silva et al.2007). However, phthalate monoesters with five or more carbons in the ester side chain (for example, MEHP, MOP, and MNP) are efficiently transformed further to oxidation metabolites. For esters with side chains of five or more carbons, the oxidized metabolites are the primary metabolite found in urine. Monoesters and oxidized metabolites are excreted free or conjugated as glucuronides- and to small extent sulfates- and mainly in urine (Silva et al 2003).

1.2.4 Source of Human and Environmental Exposure.

Wide spread exposure to phthalates has been recently documented among pregnant women in Jerusalem (Berman et al 2008). Previous studies in Israel have reported the presence of phthalate in soil surface, soil profiles and ground water (Muszkat et al 1993), and in the Jordan River. Studies around the world have shown that there is widespread exposure to phthalate in the general population. The largest of these studies, the NHANES study in 2.540 people in the US, found detectable levels for phthalate metabolite (MEP, MBP, MBzP and MEHP) in over 75% of the samples (Silva et al 2004).

Phthalates are easily released into the environment because there is no covalent bond between the phthalates and plastics in which they are mixed. As plastics age and break down

the release of phthalates accelerates. Phthalates in the environment are subject to biodegradation, photo degradation, and anaerobic degradation. Phthalate exposure can be through direct use or indirectly through leaching and general environmental contamination. Diet is believed to be the main source of DEHP and other phthalates in the general population. Fatty foods such as milk, butter, and meats are a major source. Low molecular weight phthalates such as DEP, DBP, and BBzP may be dermally absorbed. Inhalational exposure is also significant with the more volatile phthalates. Phthalates are also found in medications, where they are used as inactive ingredients in producing enteric coatings. It's not known how many medications are made using phthalates, but some include omeprazole, didanosinee, mesalamine, and theophylline. A recent study found that urinary concentrations of monobutyl phthalate, the DBP metabolite, of mesalamine users was 50 times higher than the mean of nonusers (some formulations of mesalamine do not contain phthalates).[(Hauser et al. 2004) another study evaluated whether such associations between use of phthalate-containing medications and urinary concentrations of phthalate metabolites may be present by using data from NHANES. (Harnandez Diaz et al.2009).

1.2.5 Children's Susceptibility:

Children sometimes differ from adults in their susceptibility to hazardous chemicals, but whether there is a difference depends on the chemical (Guzelian et al. 1992; NRC 1993). Children may be more or less susceptible than adults to health effects and the relationship may change with developmental age (Guzelian et al. 1992; NRC 1993).

There are often differences in pharmacokinetics and metabolism between children and adults. For example, absorption may be different in neonates because of the immaturity of their gastrointestinal tract and their larger skin surface area in proportion to body weight (Morselli et al. 1980; NRC 1993). The gastrointestinal absorption of lead is the greatest in infants and young children (Ziegler et al. 1978). Distribution of xenobiotics may be different. For example, infants have a larger proportion of their bodies as extracellular water and their brains and livers are proportionately larger (Fomon et al. 1982). The infant also has an immature blood-brain barrier and probably an immature blood-testis barrier (Adinolfi 1985). Many xenobiotic metabolizing enzymes have distinctive developmental patterns. At various stages

of growth and development, levels of particular enzymes may be higher or lower than those of adults, and sometimes unique enzymes may exist at particular developmental stages (Komori et al. 1990; Leeder and Kearns 1997; NRC 1993; Vieira et al. 1996). Whether differences in xenobiotic metabolism make the child more or less susceptible also depends on whether the relevant enzymes are involved in activation of the parent compound to its toxic form or in detoxification.

Children are mainly exposed to DEHP orally from mouthing toys and other soft PVC products and possibly food, and dermally from handling materials containing DEHP

1.3 PHTHALATES AND HUMAN HEALTH.

The effects of phthalates on human health are not yet fully known, but are being studied by several government agencies, including the Food and Drug Administration, the National Institute of Environmental Health Sciences, and the National Toxicology Program's Center for the Evaluation of Risks to Human Reproduction.(1)

Phthalates have been shown to cause a variety of effects in laboratory animals; however, their adverse effects on development of the reproductive system of male animals have led to particular concern. Those effects include infertility, decreased sperm count, cryptorchidism (undescended testes), hypospadias (malformation of the penis) and other reproductive tract defects and are referred to as the phthalate syndrome.

Epidemiology studies consistently linked multiple phthalates to a broad range of health effects, starting with birth defects in baby boys and reproductive problems in men, and extending to thyroid and immune disruption (Heudorf 2007).

1.3.1 Effects of Phthalates on Reproduction.

One of first studies from Harvard School of Public health to link phthalate exposure with harm to reproductive health among humans .The study recruited 168 men and found that those who had monobutyl phthalate (MBP) or monobenzyl phthalate in their urine tended to have lower sperm count. The study shows an inverse relationship between high concentration

of these chemicals and low sperm count. There are multiple studies in adult rats in which oral exposure to DEHP decreased the weights of the testes, prostate, seminal vesicles, and caused atrophy and degeneration of the somniferous tubules with consequent altered sperm measures and reduced fertility (Crisp et al, 1998).

Fertility studies with crossover mating have shown that active phthalates-like DEHP and DBP can decrease the fertility of rats and mice through male and female-mediated effects (Lamb et al.1987).Phthalates reduce concentrations of testosterone, an important androgen (or male sex hormone) that contributes to the development of male sex organs. This androgen deficiency causes the phthalate syndrome in laboratory animals if it occurs during time periods that are critical for male reproductive development.

Studies in rodents exposed to doses in excess of 100 mg/kg/day DEHP clearly indicate that the testes are a primary target tissue, resulting in decreased testicular weights and tubular atrophy (Gray and Butterworth 1980). Within the testis, Sertoli cells appear to be the target of DEHP toxicity (Li et al. 2000; Saitoh et al. 1997). Effects on spermatogenesis were also indicated by the appearance of damaged spermatogenic cells and abnormal sperm in rats exposed to 2,000 mg DEHP/kg/day in the diet for 15 days (Parmar et al. 1987). There are not enough data to draw conclusions concerning the role that hormones play in the testicular toxicity of DEHP; however, they do appear to have an effect. The co administration of testosterone with DEHP appeared to diminish but not abolish the testicular toxicity of DEHP in rats (Gray and Butterworth 1980). Luteinizing hormone aggravated the testicular toxicity of DEHP in rats (Oishi 1989).

Few studies have investigated the reproductive toxicity of DEHP in female animals. In contrast to males, it is generally thought that the female reproductive system is much less sensitive to phthalates. However, recent evidence suggests that phthalates can also induce adverse responses in females following pre and post exposure (Grande et al. 2006). Initial studies demonstrated that the ovary is a target site for DEHP. Davis et al 1994, reported that a high DEHP dose (2000mg/kg/day) results in prolonged estrous cycles, reduced serum estradiol levels and absence of ovulation in adult rats. These data indicate that oral exposure to DEHP can affect reproductive processes in female rodents.

Studies of long-term exposures in rats and mice have shown that high oral doses of DEHP caused health effects mainly in the liver and testes (David et al, 2000). These effects were induced by levels of DEHP that are much higher than those received by humans from environmental exposures. Toxicity of DEHP in other tissues is less well characterized, although effects in the thyroid, ovaries, kidneys, and blood have been reported in a few animal studies. The potential for kidney effects is a particular concern for humans because this organ is exposed to DEHP during dialysis and because structural and functional kidney changes have been observed in some exposed rats (Isenberg et al, 200).

1.3.2 Neurological Effects.

A new report by Korean scientists, published by Elsevier in the November 15th issue of *Biological Psychiatry*, adds to the potentially alarming findings about phthalates. They measured urine phthalate concentrations and evaluated symptoms of attention-deficit/hyperactivity disorder (ADHD) using teacher-reported symptoms and computerized tests that measured attention and impulsivity. They found a significant positive association between phthalate exposure and ADHD, meaning that the higher the concentration of phthalate metabolites in the urine, the worse the ADHD symptoms and/or test scores. The current findings do not prove that phthalate exposure caused ADHD symptoms. However, these initial findings provide a rationale for further research on this association. (Kim et al 2009).

1.3.3 Developmental Effects.

DEHP has been demonstrated to cause developmental toxicity including teratogenic effects in both rats and mice. Effects observed included decreased fetal/pup body weight, increased rates of abortion and fetal resorptions, or malformations.

In studies of pregnant mice and rats orally exposed to large doses of DEHP, effects on the development of the fetus, including birth defects and even fetal death, were observed.

Researchers observed alterations in the structure of bones and of parts of the brain, and in the liver, kidney, and testes of the young animals. These harmful effects suggested that DEHP or

some of its breakdown products passed across the placenta and reached the fetus. Therefore, humans exposed to sufficiently high levels of DEHP during pregnancy could possibly have babies with low birth weights and/or skeletal or nervous system developmental problems, but this is not certain (Sastry et al, 1991). Developmental effects of DEHP in rats exposed via maternal milk have been studied. Studies in animals also have shown that DEHP or some of its breakdown products can pass from mother to babies via the breast milk and alter the development of the young animals. This could also happen in humans because DEHP has been detected in human milk. (Main et al. 2006)

A variety of effects were observed in androgen-sensitive tissues of young male rats, including reduced (female-like) anogenital distance and permanent nipples, vaginal pouch, penile morphological abnormalities, hemorrhagic and undescended testes, testicular and epididymal atrophy or agenesis, and small to absent sex accessory glands. (Gray et al. 1999, 2000; Parks et al. 2000). These morphological effects, as well as reduced fetal and neonatal testosterone levels and adult sexual behavioral changes in male rats following gestational and lactational exposure, are consistent with an antiandrogenic action of DEHP. The changes in the development, structure, and function of the male reproductive tract observed in various studies indicate that effects of DEHP on reproduction and development are interrelated

.

Musculoskeletal Effects: No studies were located regarding the effect of phthalates on human musculoskeletal and no reports of musculo/skeletal effects of phthalates on animals were found in any of the studies reviewed.

1.3.4 Cancer.

The relationship between hepatic peroxisome proliferation, cell proliferation, and carcinogenicity has been evaluated in chronic studies of DEHP in rats and mice (David et al. 1999, 2000). It is well documented that long-term oral exposure to DEHP causes cancer of the liver in both rats and mice. There is no evidence that DEHP is genotoxic or a liver tumor initiator in rats and mice, although it does appear to have tumor promotion activity.

Based on the findings from one of the cancer studies, an NTP bioassay, EPA (Environmental Protection Agency) classified DEHP in Group B2 (probable human carcinogen) and derived a

cancer risk. Based largely on the same findings, the U.S. Department of Health and Human Services suggests that it is reasonable to consider DEHP as a human carcinogen. IARC (International Agency for Research on Cancer) recently (2001) updated its cancer classification of DEHP from Group 2B (possibly carcinogenic to humans) to Group 3 (not classifiable as to its carcinogenicity to humans). In making its overall evaluation of the carcinogenicity of DEHP to humans, IARC took into consideration that (1) DEHP produces liver tumors in rats and mice by a non-DNA-reactive mechanism involving peroxisome proliferation, (2) peroxisome proliferation and hepatocellular proliferation have been demonstrated under the conditions of the carcinogenicity studies of DEHP in rats and mice, and (3) peroxisome proliferation has not been documented either in human hepatocyte cultures exposed to DEHP or in the liver of non-human primates. Based on these three lines of evidence, IARC concluded that the mechanism by which DEHP increases the incidence of hepatocellular tumors in rats and mice is not relevant to humans. This conclusion is based on the assumption that peroxisome proliferation is the mechanism causing liver cancer.
Even though studies have shown that DEHP can cause liver cancer in rats and mice, the mechanism data suggests that these findings may not be relevant to the probability of DEHP causing cancer in humans.

1.3.5 Genotoxicity and Teratogenicity:

DEHP has been tested in a variety of short-term genotoxicity assays with predominantly negative or false-positive results. The observation that DEHP causes an early transient increase in liver DNA synthesis above a certain dose level is similar to Phenobarbital, a known rodent liver tumor promoter (Dalton et al. 2000), and strengthens the conclusion that DEHP is an epigenetic tumor promoting agent in rodents.

Most of the developmental toxicity evaluations of DEHP are traditionally designed studies in which physical development was evaluated just prior to birth in pups of rodents that were orally exposed during gestation only. These studies clearly show that gestational exposure to DEHP was embryotoxic and teratogenic in rats and mice. A range of effects were

observed including intrauterine deaths, skeletal and cardiovascular malformations, neural tube closure defects, increased perinatal mortality, and developmental delays.

1.4 MECHANISM OF ACTION.

1.4.1 Induction of Peroxisome Proliferation

There is strong evidence that hepatocarcinogenesis of DEHP and other peroxisome proliferators is due to their increased production of hydrogen peroxide by peroxisomes and enhanced cell proliferation; alteration of mitogenic/apoptotic balance might also contribute. A characteristic effect of exposure to DEHP in rodents, particularly rats and mice, is an increase in liver weight, associated with both morphological and biochemical changes. Liver enlargement is due to both hepatocyte hyperplasia and hypertrophy. Morphological examination reveals an increase in both the number and the size of peroxisomes in the liver. Peroxisomes are single membrane-limited cytoplasmic organelles found in the cells from animals, plants, fungi, and protozoa. Peroxisomes contain catalase, which destroys hydrogen peroxide, and a number of fatty-acid oxidizing enzymes, one of which, acyl CoA oxidase, generates hydrogen peroxide (Lazarow and deDuve 1976). The main biochemical alterations consist of induction of both peroxisomal and microsomal fatty acid-oxidizing enzyme activities. The activity of the peroxisomal fatty acid β-oxidation cycle is normally determined either by measuring the overall activity (e.g., as cyanide-insensitive palmitoyl CoA oxidation) or by determining the first rate-limiting enzyme of the cycle, acyl-CoA oxidase. An important observation is that while the β-oxidation cycle enzymes can be greatly induced by peroxisome proliferators, other peroxisome enzymes, such as D-amino acid oxidase and catalase, are increased to a much lesser extent. This induction imbalance has been postulated to play a major role in phthalate-induced liver carcinogenicity. In general, there is good correlation between enzyme activity and changes in peroxisome morphometry, allowing palmitoyl-CoA oxidation to be used as a specific biochemical marker of peroxisome proliferation. (Lake 1995)

Induction of peroxisome proliferation following treatment with DEHP is not due to the parent compound, but to DEHP metabolites. Studies with MEHP *in vitro* have demonstrated

that the proximate peroxisome proliferators are mono (2-ethyl-5-oxohexyl) phthalate (metabolite VI) and mono (2-ethyl-5-hydroxyhexyl) phthalate, (metabolite IX) and that for 2-ethylhexanol, the proximate proliferators' is 2-ethylhexanoic acid (Elcombe and Mitchell 1986). Similar findings were observed by Maloney and Waxman (1999), who showed that MEHP (but not DEHP) activated mouse and human PPARα and PPARγ, while 2-ethylhexanoic acid activated mouse and human PPARα only, and at much higher concentrations. Based on its potency to induce enzyme activities, such as the peroxisomal fatty acid β-oxidation cycle and carnitineacetyltransferase, DEHP might be considered a relatively weak proliferators'.

The role of PPAR in peroxisome proliferators-induced toxicity has been examined in several studies. In a study by Ward et al. (1998), treatment of PPARα wild-type mice with DEHP for up to 24 weeks resulted in typical up regulation of mRNA for peroxisomal and CYP4A enzymes in the liver and kidney, while treated null mice were no different from control wild-type or null mice. Whereas treated wild-mice had liver, kidney, and testicular toxicity, treated PPARα-deficient mice did not exhibit liver toxicity, but showed delayed moderate kidney and testicular toxicity. This suggested that while DEHP-induced liver toxicity is mediated solely by PPARα activation, both renal and testicular toxicities have both a receptor- and nonreceptor-mediated response.

A study using human hepatoma cells expressing PPARα, β/δ, or γ showed that the DEHP MEHP, activated all three isoforms of PPAR in a dose-related fashion, but DEHP did not (Lapinskas andCorton 1997). In addition, the metabolite 2-ethylhexanoic acid was isoform-specific since it activated PPARα but not β/δ or γ. These data are consistent with observations *in vivo* and *in vitro* indicating that the toxicity of DEHP is due mainly to MEHP.
The exact mechanism(s) by which peroxisomal proliferating agents such as DEHP induce hepatic cancer in rodents are not precisely known, but might be related to the modulation of peroxisomal β oxidation, the PPAR α receptor, gap junctional intercellular communication, and replicative DNA synthesis (Isenberg et al. 2000, 2001). Two major mechanisms have been proposed to account for peroxisome proliferators-induced hepatocarcinogenicity in rodents: induction of sustained oxidative stress and enhanced cell proliferation and promotion. Suppression of hepatocellular apoptosis has also been suggested to play a role.

In Summary, there is strong evidence that hepatocarcinogenesis of DEHP and other peroxisome proliferators is due to their increased production of hydrogen peroxide by peroxisomes and enhanced cell proliferation; alteration of mitogenic/apoptotic balance might also contribute. These events are triggered by the activation of gene expression via a nuclear receptor, PPARα. It should be noted that if liver cancer in humans can be promoted by DEHP via a mechanism not involving peroxisome proliferation (i.e., inhibition of gap junctional intercellular communication), the fact that this was not measured in human liver and that promotion must occur on initiated liver cells for long periods of time at a concentration that exceeds a potential threshold level (a characteristic of chemical tumor promoters) might still implicate DEHP as a potential human liver tumor promoter. However, because the model chemical, Phenobarbital, is also a rodent tumor promoter and has not been shown to be a human liver tumor promoter, it is reasonable to conclude that normal exposures to DEHP will not be a significant risk factor for human liver cancers.

1.4.2 Oxidative Stress:

Several investigators have hypothesized that liver tumor formation arises from an imbalance between hydrogen peroxide generation and degradation within the peroxisome (Rao and Reddy 1987; Reddy and Lalwani 1983; Reddy and Rao 1989). This imbalance is the result of a much greater induction by peroxisome proliferators of hydrogen peroxide-generating enzymes than induction of catalase. This might be compounded by a reduction in enzyme activities that detoxify active forms of oxygen and organic hydroperoxides. Hydrogen peroxide that escapes the peroxisome might damage intracellular membranes and/or DNA (Reddy and Rao 1989). Lipid peroxidation and lipofuscin deposition have been observed in hepatocytes from rats treated with DEHP and other peroxisome proliferators (Cattley et al. 1987; Conway et al. 1989). Tagaki and coworkers have examined the possibility of DNA damage by DEHP by measuring the induction of 8-hydroxydeoxyguanosine (8-OH-dG), a marker of DNA oxidation, in the liver and kidney from male rats administered DEHP for various periods of time (Sai-Kato et al 1995). Increased levels of 8-OH-dG were seen in the liver after 1 or 2 weeks or 12 months of treatment, but no increases were seen in the kidney. In general, the increases were small (2-fold) and in some cases, were not sustained with prolonged DEHP treatment (Cattley and Glover 1993). Moreover, the increased levels of 8-

OH-dG do not correlate with carcinogenic potency, as similar levels of induction have been associated with divergent carcinogenic activities (Marsman et al. 1988, 1992). Furthermore, DEHP and other peroxisome proliferators have consistently lacked initiation activity unlike other DNA-damaging agents. The overall evidence suggests that increased production of hydrogen peroxide and DNA oxidation are not solely responsible for peroxisome proliferators-induced liver tumor formation.

1.4.3 Effects on Reproductive System.

The role of zinc in DEHP-induced testicular atrophy has been examined in several studies since a reduction in testicular zinc is a primary event following administration of DEHP. A decrease in testicular zinc, but not in serum or liver zinc, was reported in rats given DEHP (Oishi 1985). After a 45-day recovery period, when there was morphological evidence of seminiferous tubule regeneration, testicular zinc was still lower than in controls (Oishi 1985). Simultaneous oral administration of DEHP and oral or intraperitoneal administration of zinc did not prevent testicular atrophy in rats, and zinc supplementation did not increase the concentration of zinc in the testis despite increases in liver and serum (Oishi and Hiraga 1983). This suggested that DEHP-induced testicular effects do not result from interference with gastrointestinal absorption of zinc, but that atrophy might be related to endogenous testicular zinc, thus, cannot be prevented by zinc supplementation (Oishi 1985).

The effects of DEHP on hormones that influence testicular maturation and function have also been explored. As was the case with zinc. DEHP administered to mice significantly reduced the concentration of testosterone in the testis, but no testicular atrophy was observed (Oishi1980). Increases in testicular concentration of testosterone along with decreases in testicular content of testosterone seen after DEHP treatment suggested that testosterone-producing Leydig cells are normal, but that the total number of cells is less than in controls or that the cells are less active in testosterone production (Oishi et al, 1985).

In a later study, Oishi (1989) reported that co-administration of DEHP and testosterone apparently aggravated the testicular damage caused by DEHP, an effect that seemed to be due to testosterone prolonging the biological life and the mean residence time of MEHP in the testis. A mechanism for such an effect was not discussed. In a similar study, luteinizing

hormone-releasing hormone significantly enhanced the testicular toxicity of DEHP when given together with DEHP (Oishi et al. 1989).

Results from both *in vivo* and *in vitro* studies have indicated that the Sertoli cell is the main target for DEHP-induced testicular toxicity and that MEHP is the ultimately active testicular toxicant (Chapin et al. 1988; Gray and Beamand 1984). However, effects on Leydig cells have also been reported (Jones et al. 1993). The Sertoli cell is a somatic cell type whose integrity and functionality is required for the growth and maintenance of the germ cells as they divide and differentiate from spermatogonia to spermatocytes and ultimately to spermatids. The latter are released by the Sertoli cell into the lumen as sperms. Gray and Butterworth (1980) had suggested that Sertoli cell and not the germ cell was the direct target of DEHP toxicity since the germinal cells affected were those inside the Sertoli cell barrier. Alterations in Sertoli cell cytoskeleton after exposure to phthalates also have been reported (chapin et al, 1988).

1.5 ANIMAL TO HUMAN EXTRAPOLATIONS:

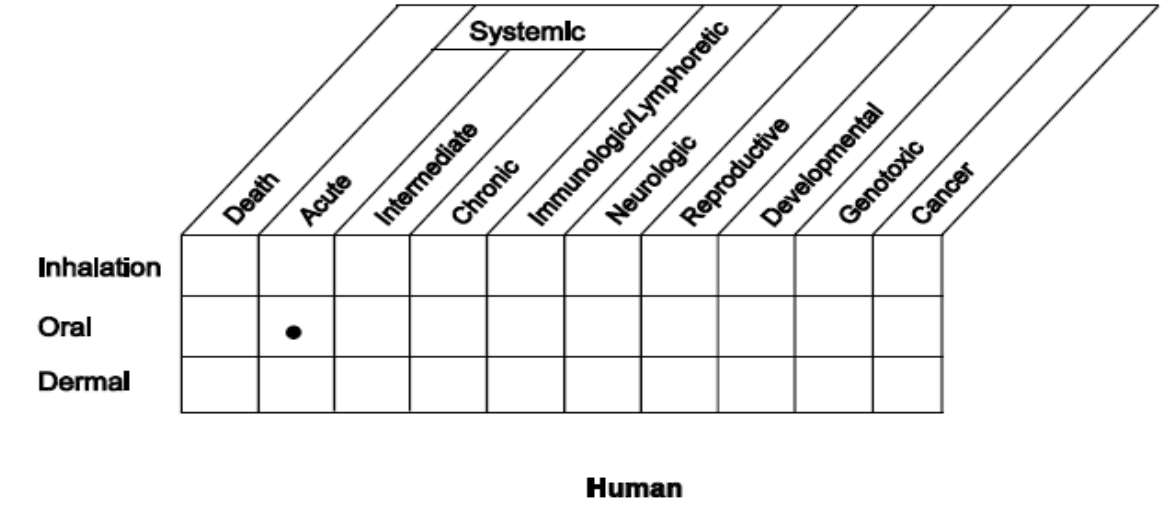
There is ample evidence suggesting that there are species differences in both the pharmacokinetics and toxicity of DEHP; strain differences have also been described. In some cases, the differences in toxicity can be explained by differences in pharmacokinetics. The issue of greatest importance to be considered is whether DEHP can induce liver cancer and reproductive toxicity in humans, as seen in rodents. As previously mentioned the hepatocarcinogenic response to DEHP in rats and mice is associated with peroxisome proliferation and increased hepatocyte replication. Studies in animals have shown that after exposure to peroxisome proliferators, rats and mice exhibit the greatest response, hamsters exhibit an intermediate response, whereas primates, guinea pigs, and dogs are either unresponsive or refractory (Cattley et al. 1998).

Elcombe and Mitchell (1986) isolated MEHP metabolites from rat urine and tested them in cultures from rat, guinea pig, marmoset, and human liver. Metabolite VI, biochemically and morphologically identified as the proximate proliferators in the rat, had little or no effect in marmoset, guinea pig, or human hepatocytes. These findings suggested the existence of intrinsic species differences of liver cells to peroxisome proliferators.

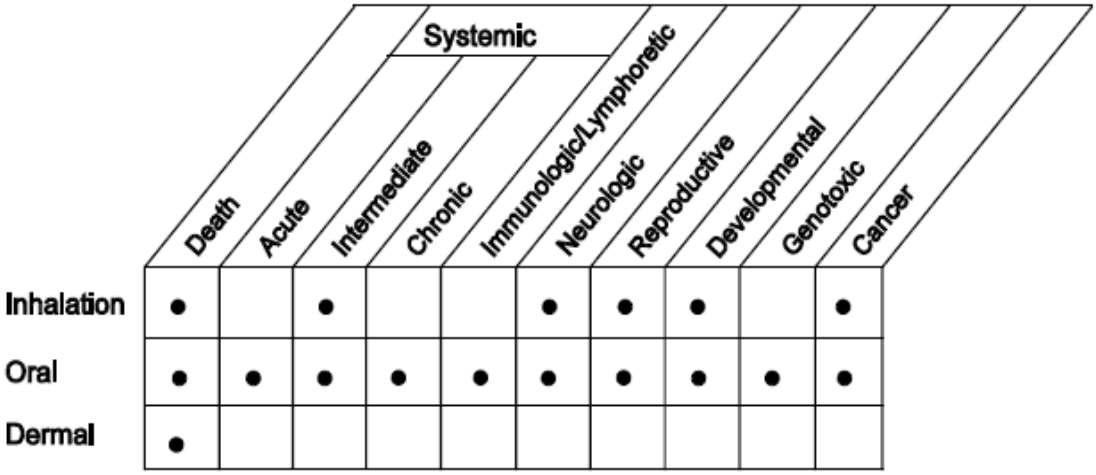
If peroxisome proliferation and liver carcinogenicity is mediated by PPARα, the species differences could reflect either variation in PPARα itself or in the gene networks regulated by PPARα (Green 1995). However, MEHP activated both human and mouse PPARα and both preparations were equally sensitive. This suggested that differential sensitivity of human PPARα cannot alone account for the lack of peroxisome proliferation response seen in humans, but other factors, such as the much lower level, as found by Palmer et al. (1998), are also likely to be important. Another important factor might be species differences in responsiveness of genes to PPARα-mediated transcription.

As for testicular toxicity, which does not seem to be related to peroxisomal proliferation to the extent that liver cancer is, differential sensitivity among animal species has been found. Studies *in vivo* have shown that rats and guinea pigs are highly sensitive while mice are fairly sensitive, and hamsters and monkeys are highly resistant (Gray et al. 1982; Kurata et al. 1998).

A lack of information precludes ranking humans relative to other species. Differences in pharmacokinetics might play a role in the differential sensitivity between species, but differences in tissue sensitivity might play a role as well mixed cultures of Sertoli cells and germ cells from rat testes were more sensitive to MEHP toxicity than cultures from hamster testes (Gray and Beamand 1984). Also, cultures from older rats were less sensitive than cultures from young animals, suggesting that intrinsic cell factors might account for different susceptibility. Studies with the knockout mice for PPARα have suggested that other receptor subtypes (PPARδ or γ) might play a role in the delayed testicular toxicity observed in these mice or that the high dose of DEHP might modify the pharmacokinetics of DEHP in the (-/-) mice (Ward et al. 1998).



Human



Animal

● Existing Studies

Fig (1.5): Existing information about the Effect of DEHP on Human Health

1.6 LEGAL STATUS:

In Europe, any substance known or suspected to be carcinogenic, mutagenic or to cause reproductive effects in laboratory animals at any dose cannot be used in cosmetics this Includes phthalates such as dibutyl phthalate. The restriction states that the amount of phthalates may not be greater than 0.1% mass percent of the plasticized part of the toy. Some phthalates are allowed at any concentration in other products and other phthalates are not restricted. (2)

1.7 AIMS OF THIS STUDY:

- This study is designed to establish a chick model for the evaluation of the developmental effects of phthalates using pre- and posthatch developmental markers.

- To see whether pre-hatch exposure to phthalates can effect behavioral development and whether the behavioral defects may be related to DNA damage.

- To study the biochemical changes in blood serum of animals pre-treated with phthalates compared to untreated controls

- To provide public health officials, physicians, toxicologists, and other interested individuals and groups with an overall perspective on the toxicology of DEHP and DBP.

- To understand the reasons for differences in susceptibility to phthalates based on species differences.

CHAPTER TWO

MATERIALS AND METHODS

2.1 CHEMICAL AND REAGENTS:

Dibutylphthalate (DBP) and Bis(2-ethylhexyl)phthalates (DEHP) were purchased from Sigma Aldrich. DNA Damage ELISA Kit from Assay Designs, Inc. 5777 Hines Drive, Ann Arbor, MI48108USA. Kits for biochemical assays from SEPPIM S.A.S. –Zone Industrielle - 61500 SEES France. All chemicals and drugs were of analytical grade and were purchased from Sigma Chemicals Co P.O.Box. 14508, St. LouisMO.63178USA. Dextrostix strips were purchased from Ames, (Miles, Paris)

2.2 CHICKS MODEL FOR TERATOGENIC ACTIVITY:

Chick Model Teratogenic Activity: As developed by Yani et al (2008).

2.2.1 Teratogen Treatment:

Fertile chicken eggs (*Gallus gallusdomesticus*) of the Lohmann line of the leghorn breed were obtained from a local breeder and placed in an incubator. To administer the DEHP, a hole was drilled in the chorioallantois end (pointed end) of the shell and was sealed with medical silicon (type A, Dow Corning). (DEHP), dissolved in corn oil was then administered on incubation day (ID) 0 with at doses of 5, 20, 50, and 100 mg/kg egg. Control eggs received equivalent volumes (60µl/kg of egg) of corn oil vehicle solution. Eggs were placed in a commercial incubator at 37.5 ^{0}C with 50-60% humidity. Embryonic survivals were monitored via candling and hatch rate and physical attributes at hatching were noted. The chicks were trained to follow an imprinting object and were tested for imprinting performance.

2.2.2 BEHAVIORAL TESTS:

2.2.2.1 Testing of Imprinting:

As modified from earlier descriptions by McCabe et al 1981, the chicks were transferred to the imprinting apparatus contained three 20 cm diameter running wheels with the sides covered with black, permitting the chicks to see only forward or backward. The

imprinting objects were an illuminated red box or a bule cylinder (both 15x10x18 cm high), located 50 cm from the front open side of the running wheel, lit from within by a 40W bulb with holes covered with red or blue filters. Imprinting training and testing will be both assessed with this apparatus.

The chicks were hatched in total darkness and handling was done in the dark, aided by a dim green light, which has a minimal effect on imprinting (Kovach et al 1971). Each chick was tagged and then transferred to an individual dark, enclosed wooden chamber warmed to 30 ^{0}C where they were physically and visually isolated from each other. Fourteen to twenty-four hours post-hatch, the chicks underwent 45 min of "priming" 30 min exposure to a light followed by 15 min of darkness. Immediately after, they were placed individually on the running wheel for training. The chicks were divided into group trained for 60 min with either blue or red imprinting objects. The numbers of wheel rotations made by the chick towards or away from the imprinting object were recorded by a self-made computerized system. After training, the chicks were returned to the enclosed chambers for 60 min, after which testing took place. Recorded maternal calls were played continually throughout training but not during testing.

There were four testing sessions in counterbalanced randomized order, each lasting 5 min; in two of the tests, the chick was allowed to run toward the imprinting rotating object and in the other two, toward the control rotating object. The red-light box was used as the imprinting object and the blue-light box served as the control object for chicks trained to follow a red object and vice versa for the chicks trained to follow the blue-light box. The number of wheel rotations completed by the chick toward the imprinting or the novel (control) object, and the running away (backward) from the objects was recorded by the apparatus.

The imprinting is expressed as preference scores where: Preference score = Running toward the training light / (Running toward the training light + Running toward a novel light). The preference score is a measure of the strength of learning; assessing the selective preference that arises from the experience of the training object (Sluckin et al 1972). The expected range of the preference score is 0.0-1.0, where 0.5 indicates no imprinting.

2.2.2.2 Locomotor Activity:

Locomotors activity influences the number of wheel rotations, it's the number of rotations of the wheel made by the chicks during **training** (Forward and backward). The locomotor activity during imprinting **testing** (both training and novel lights) is expressed as the total number of rotations of wheel (forward and backward) made by the chicks.

2.3 RATS MODEL OF FERTILITY:

2.3.1 Experimental Design for Fertility Studies:

Healthy White Albino female rats weighing 120-200 g each were used in these experiments. Animals were divided into three groups, 6 animals in different cages. Group B was treated with DBP 100 mg / kg, group C was treated with the same dosage of DEHP, and group A was treated with vehicle solution of Corn Oil. All the experimental animals in groups A, B, C, were maintained under normal conditions of humidity, circadian cycle and temperature and with free access to food and water unless required otherwise. A standard rat pellet diet was used for all the experiments.

The female rats were injected intraperitoneally (IP) twice a week with DBP or DEHP 100 mg/kg body mass and control group was injected (IP) with the same volume of Corn Oil. Each week rats were fasted for 15 hours for the measurement of body weight and blood glucose. After one week females were cohabited for one month with two male rats in each cage, and to avoid aspects of male fertility the male rats were distributed equally between the cages. Thus the females in group A, B and C were exposed equally to the same male rats. All the male rats were not treated with the drugs. After one month of exposure the male rats were removed and we continued to measure the body weight, blood glucose, and to follow up carefully the cases of pregnancy, until delivery. During this period we recorded the number of pregnant females and the number of litters in each delivery for the measurement of mortality rate, fertility rate and fecundity rate. In addition we observed carefully the appearance of any behavioral changes including motor disorders on female rats or the new born litters.

PROTOCOL OF EXPERIMENTS

1ST DAY: Measurements of total body weight and glucose in fasted female rats divided into three groups

Group A: Control Treated with Corn oil 0.2 ml

Group B Treated with DBP (100 mg/kg)

Group C: Treated with DEHP (100 mg/kg)

Injections of 0.2 ml of each drug were made i.p twice a week for 67 days

7th Day: Female rats were Cohabited with two male rats for mating.

37th Day: Male rats were removed.

67th Day: Female fasted rats were anaesthetized with ether for:

a) Removal of blood samples from the femoral artery for:

- Biochemical Measurements
- DNA Damage Measurements

b) Removal of Internal organs for the measurement of Relative weight of: heart, kidneys, spleen, liver, brain and Sex organs.

** Pregnant female rats were separated and on delivery the number of new born litters was counted.

2.3.2 Total Body Weight and Relative Weight of internal Organs

At the end of experiment rats were anaesthetized with ether and the following organs were removed for the measurement of absolute and relative weight. Heart, kidneys, spleen, liver, brain. sex organs including the ovaries, oviducts, uterus and vagina.

Blood samples were collected from the femoral artery for separation of blood serum and measurement of blood biochemistry and DNA damage.

2.4. BIOCHEMICAL MEASUREMENTS:

During examination rats were anaesthetized with ether and at the stage of light anesthesia characterized by loss of pain sensation and somatic motor activity with positive corneal reflex, 1 ml blood was drawn from the femoral artery into a test tube. Blood sample was centrifuged at 2000 g for 10 min; serum was isolated and stored at – 80 °C for Biochemical analysis and DNA damage test.

2.4.1 Measurements of Blood Glucose:

Blood samples for glucose analysis were taken from the tail tip, and plasma glucose was measured using Glucometer instrument and Dextrostix strips (Washka& Rice 1961).

2.4.2 Measurements of Basic Biochemical Compounds:

Cholesterol Total, Cholesterol HDL and LDL, Triglycerides, Total protein, Urea, Uric acid Creatinine were all determined by the colorimetric assay of (Eli-Tech diagnostics) Following the Kits instructions.

Enzymatic colorimetric determination of total cholesterol was measured according to the method described by Tietz (1995), and Vassault et al (1999). Cholesterol HDL Direct was measured as described by Rifai et al 2001. Cholesterol LDL Direct was measured as described by Rifai et al 2001 and Naito 2003. With total protein we used the Biuret reaction as described by Christensen 1983 and Scherwin 2003 and for the enzymatic colorimetric measurement of triglycerides we used the method as described by Naito 2003 and Fossati&Prencipe 1982. Urea

was measured as described in details by Newman and Price 2001, Uric acid acid determination as described by Tietz 1995. While Creatinine according to Allston 1993.

Following the Kits instructions, total protein is expressed in g/dl while all the other compounds as glucose, urea, uric acid, creatinine, triglycerides, cholesterol total, cholesterol-HDL and cholesterol-LDL are expressed as mg/dl.

2.4.3 Enzymatic Measurements:

In these experiments the activity of the following enzymes were measured. Kinetic determination of Alkaline phosphatase (AlP) was measured as reported by the Scandinavian Society of Clinical Chemistry (1972) and the German Society for clinical chemistry (1972). Alanine aminotransferase (ALT/ GPT) activity was measured as reported by (Schiele 1982) and Aspartate aminotransferase (AST / GOT) as reported by expert panel of the IFCC (1976). In all cases enzyme activity was expressed as U/L,

2.5. MEASUREMENT OF DNA DAMAGE:

Assay Designs' DNA Damage ELISA (enzyme-linked immunosorbent assay) is a fast and sensitive competitive immunoassay for the detection and quantitation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in serum as well as urine samples. 8-OHdG has become a biomarker of oxidative DNA damage and oxidative stress, the method uses an 8-OHdG monoclonal antibody to bind in a competitive manner. Details of the procedure are described in details in catalog number: EKS-350, and as published previously (Chiou et al 2003; Lezza et al 1999 ; Alam et al 1997). DNA Damage ELISA Kit was used for detection and quantitation of 8-hydroxy-2'-deoxyguanosine in serum samples of controls and treated animals.

ASSAY PROCEDURE SUMMARY:

1. 8-OHdG Immunoassay Plate, 20X Wash Buffer, Sample Diluent, Antibody Diluent, HRP Conjugate Diluent, TMB Substrate and Stop Solution 2 were brought to room temperature.

2. 8-OHdG Standard and samples were prepared in Sample Diluent.

3. 50µL prepared standards and samples in duplicate were added to wells of 8-OHdG Immunoassay Plate.

4. 50µL diluted Anti-8-OHdG was added to each well, except the blank. Immunoassay plate was then covered.

5. Plate was incubated at room temperature for 1 hour.

6. Wells 6X were washed using 300µL/well of 1X Wash Buffer.

7. 100µL diluted Anti-Mouse IgG: HRP Conjugate was added to each well, except the blank. Immunoassay plate was then covered.

8. Plate was incubated at room temperature for 1 hour.

9. Wells were washed 6X using 300µL/well of 1X Wash Buffer.

10. 100µL TMB Substrate was added to each well.

11. Incubation took place at room temperature for 15 minutes (in the dark).

12. 100µL Stop Solution 2 was added to each well.

13. Absorbance was measured at 450nm.

14. The 8-OHdG standard curve was plot and 8-OHdG sample concentrations were calculated.

2.6. STATISTICAL ANALYSIS:

All values are presented as Mean ± SEM for the number of experiments indicated in brackets and the data were analyzed using Students t-test.

CHAPTER THREE

RESULTS

3.1 TERATOGENIC ACTIVITY OF PHTHALATES ON CHICKS DEVELOPMENT:

3.1.1 EFFECT OF PHTHALATES ON HATCHING AND DEFECT PRODUCTION

Teratogenic activity of DEHP and DBP was tested on chicks embryonic development. Eggs were injected before incubation with 60 µl DBP (100 mg/kg egg) or DEHP 5 to 100 mg/kg. Control eggs were treated with the same volume of corn oil.

Table 3.1 and Fig 3.1 show that that eggs which were injected with the vehicle solution, produced 80 % hatching, and all the new born chicks were normal without any defects. Eggs which were exposed to DEHP 5 mg/kg, percentage hatching was reduced to 64 % without having any defects. Higher dose of 20 mg/kg has decreased percentage hatching to 62 %, and produced 8 % defects. The chicks were born with a hole in abdominal muscles, allowing the intestinal track to protrude externally without a sac, a pathological case called (Gastroschisis) Fig 3.1.B. Higher doses of DEHP (50 mg/kg), and (100mg/kg) produced similar percentage of hatching but increased percentage defects by 11 % and 22 % respectively. The types of defects were mixed, Gastroschisis and Omphalocelewhere the internal organs are protruded externally in a translucent sac, (Fig 3.1- C + D).

Eggs treated with DBP 100 mg/kg reduced percentage hatching from 80% to 57 % and increased percentage of defective chicks from 0 % in control eggs to 14 %. All the defective chicks were Gastroschisis and animals were not able to move or stand on their legs as shown in (Fig. 3.1 – E).

Late hatching in control eggs was 15.6 %, in DEHP treated eggs 29.4 %, and DBP 37.5 %

TABLE – 3.1

TERATOGENIC ACTIVITY OF BIS(2-ETHYLHEXYL) PHTHALATES (DEHP) &DIBUTYLPHTHALATES (DBP) ON CHICKS DEVELOPMENT

	CONTROL	DEHP				DBP
		5 mg/kg	20mg/kg	50 mg/kg	100 mg	100 mg/kg
NUMBER OF EGGS:	40	11	13	19	9	14
(CANDLING EGGS)						
HATCHING:	32	7	8	13	6	8
% HATCHING:	80 %	64 %	62 %	68 %	67 %	57 %
NORMAL HATCHING:	22	6	7	6	5	5
LATE HATCHING:	5	1	1	7	1	3
DEFECTS:	0	0	1	2	2	2
% DEFECTS:	0 %	0 %	8 %	11 %	22 %	14 %
OBSERVATIONS:	Normal	Normal	*(1)	* (1)	** (2)	* (1)
				** (1)		
						*** (1)

*Gastroschisis: Hole in or weakening in abdominal muscles, allowing internal organs to protrude externally. Without a Sac.

**Omphalocele: Hole in or weakening in abdominal muscles, allowing internal organs to protrude externally in a Translucent Sac.

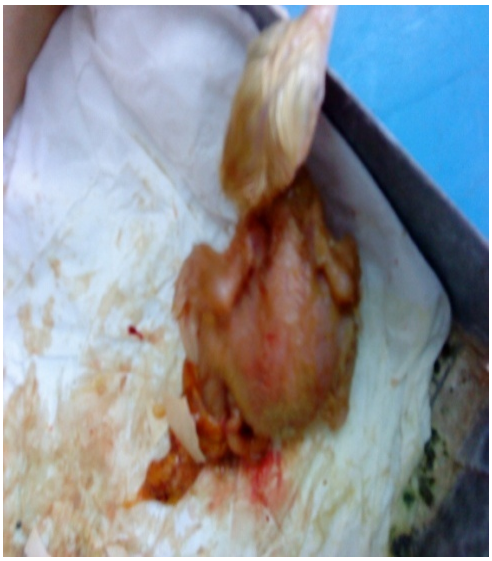
***Motor Disorders: Not able to move or stand on his legs

FIG 3.1 THE EFFECT OF PHTHALATES ON DEVELOPING CHICKS

A)Normal Chicks (Control)

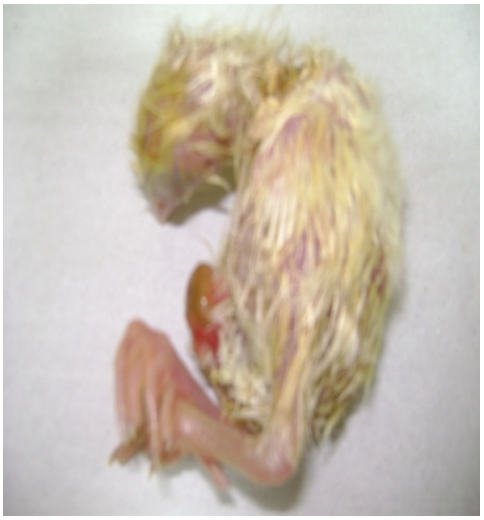
(Normal)

B) **20 mg/kg DEHP**



(Gastoschisis**)**

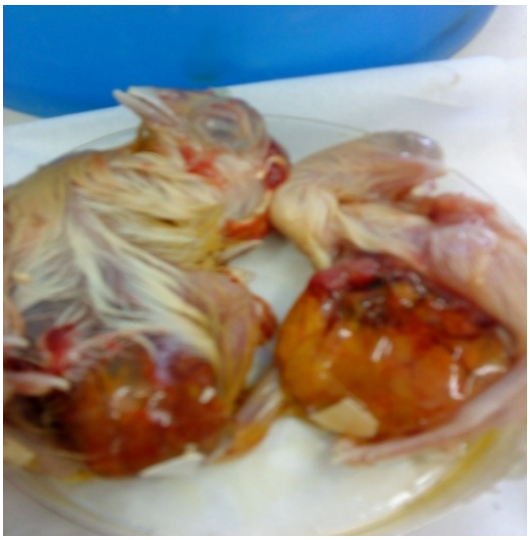
C) **50 mg/kg DEHP**



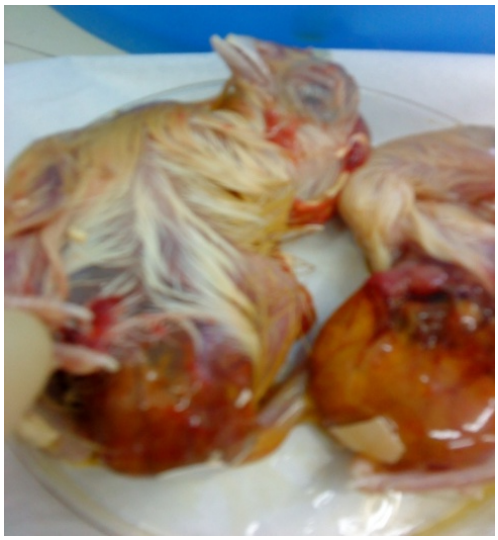
(Omphalocele)

(Gastroschisis)

D) **100 mg/kg DEHP**

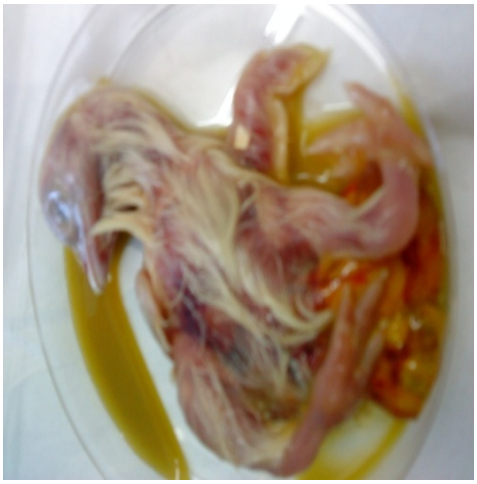


(Omphalocele)



(Omphalocele)

E)<u>100 mg/kg DBP</u>



(Gastroschisis)

(Motor Disorder)

3.1.2 BEHAVIORAL TEST:

3.1.2.1 IMPRINTING TEST:

Preference ratio in control chicks and chicks with prehatch exposure to DEHP. Data represent mean ± SEM obtained from 6 control and 9 DEHP-exposed chicks. * $p < 0.05$ for the difference between the groups.

Imprinting preference score in the control group was 0.649 (Table 3.2) and (Fig.3.2), well above the "no preference" score of 0.5 ($p<0.001$). Prehatch exposure to DEHP decreased the imprinting score to 0.509 ($p<0.05$). The percentage decrease in neurobehavioral test is 21.6 % ($p<0.05$)

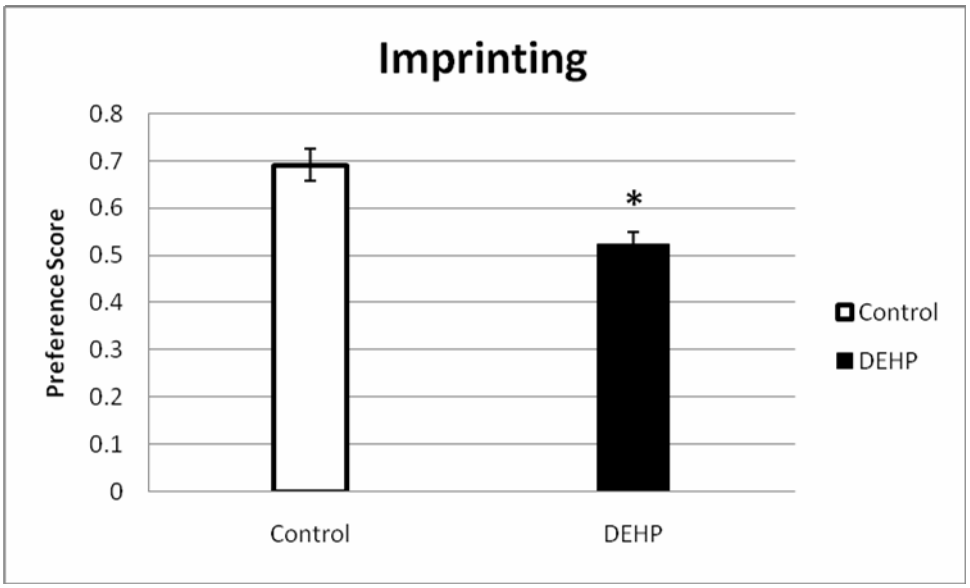
TABLE – 3.2

IMPRINTING TEST ON CHICKS TREATED WITH BIS(2-ETHYLHEXYL) PHTHALATES (DEHP)

CONTROL:	**0.649 ± 0.041 (6)**
Eggs injected with 60µl Corn Oil	
TREATED:	**0.509 ± 0.023 (9)**
Eggs Injected with DEHP	
(50 – 100 mg/ kg)	
% CHANGE:	**↓21.6 %**
	P≤0.05

DEHP was injected ip in doses of 50 and 100 mg/kg and in a volume of 60µl into fertilized eggs before incubation. Control eggs were injected with the same volume of corn oil. Candling eggs were kept in complete darkness before hatching for imprinting test.

Fig 3.2 Preference Score of Imprinting Test



* $p<0.05$

3.1.2.2 Locomotor Activity:

We assessed locomotor activity (The number of rotations of the wheel made by the chick during training). Since locomotion can be itself influence imprinting.

The locomotor activity during imprinting testing (both training and control light) was expressed as the total number of rotations of the wheel made by the chicks (Table 3.3).

We evaluated general activity to exclude potential confounding effects on activity in the imprinting evaluations. Whereas DEHP has an effect of 28 % increase on activity during training although it was not significant. Activity during testing was not changed significantly.

Table 3.3

The Effect of Prehatch Exposure to Phthalates on the Activity Level (Wheel Rotation during Training (left) and during Testing (right)

TREATMENT	ACTIVITY DURING TRAINING	ACTIVITY DURING TESTS

CONTROL	**158.6 ± 45.1 (7)**	**50.0 ± 8.6 (8)**
DEHP	**203.0 ± 55.4 (10)**	**42.2 ± 11.2 (11)**
% CHANGE	**↑ 28 %**	**↓ 15.6 %**
P	**NS**	**NS**

There were no statistical significant differences between control and DEHP exposed group between activity during imprinting training and imprinting testing.

3.1.3 BIOCHEMICAL MEASUREMENTS IN CHICKS SERUM:

Pre-hatch exposure of eggs to Phthalates has produced the following biochemical changes in blood serum of non-fasted chicks (Table 3.4).

Alkaline phosphatase activity was increased significantly by 296 % from 90.00 ± 25.50 (4) to 357.00 ± 73.00 (8) (U/L) ($P \leq 0.05$), and GPT activity was decreased by 76 % from 32.00 ± 8.20 (6) to 7.82 ± 2.06 (11) (U/L) ($P \leq 0.05$) , while urea was reduced by 41 % from 64.15 ± 4.10 (4) to 37.72 ± 2.83 (12) (mg/dl) ($P \leq 0.05$), and creatinine by 69 % from 1.67 ± 0.38 to 0.51 ± 0.07 (10) (mg/dl) ($P \leq 0.05$).

No significant changes were observed in cholesterol total, cholesterol-LDL, cholesterol-HDL, total protein, triglycerides, and glucose or on the activity of GOT enzyme.

TABLE – 3.4

BIOCHEMICAL CHANGES IN CHICKS TREATED WITH BIS(2-ETHYLHEXYL) PHTHALATES (DEHP)

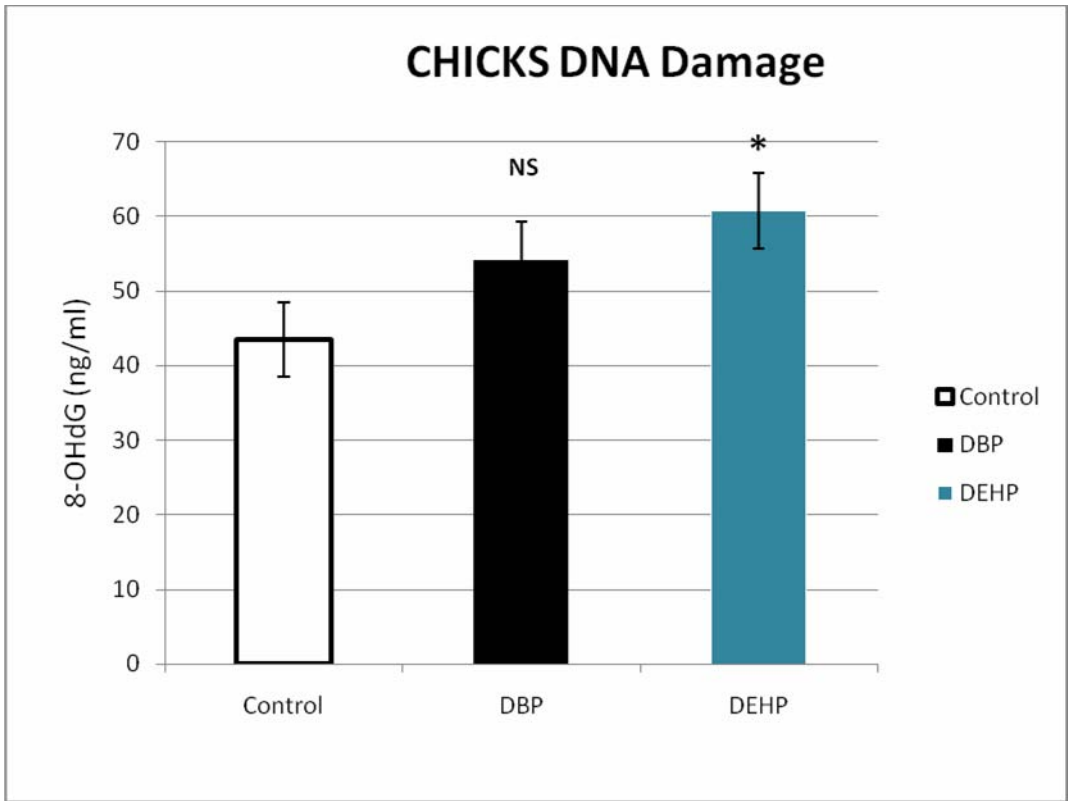
	CONTROL	PHTHALATES
GLUCOSE: (mg/dl)	**232.60 ± 11.4 (6)**	**207.04 ± 10.19 (10)**
ALKALINE PHOSPHATASE: (U/L)	**90.00± 25.50 (4)**	**357.00 ± 73.00 (8)**
GPT: (U/L)	**32.00 ± 8.20 (6)**	**7.82 ± 2.06 (11)***
GOT: (U/L)	**148.80 ± 11.7 (5)**	**164.33 ± 15.44 (12)**
UREA: (mg/dl)	**64.15 ± 4.10 (4)**	**37.72 ± 2.83 (12) ****
CREATININE(mg/dl)	**1.67 ± 0.38 (4)**	**0.51 ± 0.07 (10)***
URIC ACID: (mg/dl)	**7.37 ± 1.90 (6)**	**5.22 ± 0.58 (12)**
TOTAL PROTEIN: (g/dl)	**3.13± 0.56 (6)**	**2.28 ± 0.11 (11)**
CHOLESTEROL – TOTAL:(mg/dl)	**396.00 ± 36.06 (6)**	**378.91 ± 16.89 (11)**
CHOLESTEROL- HDL (mg/dl)	**155.60 ± 11.8 (5)**	**149.55 ± 7.81 (12)**
CHOLESTEROL – LDL (mg/dl)	**261.00 ± 35.00 (6)**	**207.04 ± 10.19 (10)**
TRIGLYCERIDES: (mg/dl)	**103.50 ± 16.00 (6)**	**68.15 ± 3.50 (12)**

Values shown are Mean ± SEM for the number of experiments indicated in brackets. At the end of each experiment, blood was collected from the common carroted artery of chicks, centrifuged and blood serum was separated for the measurement of biochemical compounds. Analysis was performed using Bio-analyzer for measuring all the chemical compounds.* $P \leq 0.05$ ** $P \leq 0.02$

3.1.4 CHICKS DNA DAMAGE:

DNA damage was estimated by measuring the concentration of 8-OHdG in blood serum of new born chicks. Fig. 3.3 shows that pre exposure to DEHP has increased 8-OHdG significantly by 39.7 % ($P \leq 0.05$), while the 25 % increase induced by DBP was not significant.

FIG. 3.3 EFFECT OF DEHP & DBP ON CHICKS DNA DAMAGE



Values are Mean ± SEM and measured in ng 8-OHdG in ml blood for 6 control chicks, 3 chicks treated with DBP and 6 chicks treated with DEHP. * $P \leq 0.05$, NS = not significant.

3.2 EFFECT OF PHTHALATES ON RATS FEMALE FERTILITY:

3.2.1 EFFECT OF PHTHALATES ON FERTILITY RATE, FECUNDITY & MORTALITY RATE

In control samples, female rats were injected (I P) with 200 µl corn oil twice a week for 3 months and cohabited with adult male rats for a period of one month. Female rats produced pregnancy in 87% of the cases (13 / 15). Each pregnant female rat delivered between 6 – 12 litters (an average of 8.2 litters per delivery), and average weight of each litter is 7 to 8 grams. All the new born litters developed normally. Mortality rate was 2.8 %, since 3 litters died from a total number of 106 litters.

Female rats which were injected (I P) with DBP 100 mg / kg twice a week and were cohabited for one month with male rats. DBP has reduced fertility rate from 87 % in control group to 67 % (6 female rats got pregnant from a total number of 9 rats (Table 3.5). Fecundity rate was reduced to 7.3 litters per delivery and mortality rate was increased to 52.3 %. Litters weight round 6 gram each. Observation studies have shown 2 cases with motor disorders, one rotating (10 rotations per min.) and one rearing (5 times per min.).

Other group of female rats was injected (IP) with DEHP 100 mg / kg for the same periods and cohabited with the same male rats for a period of one month. Treatment with DEHP decreased fertility rate to 50 % and reduced fecundity rate to 5.3 litters per delivery. Mortality rate among the new born litters was elevated from 2.8 % in control animals to 31.3 % in DEHP treated animals. From observations studies only one rat suffered from tremor. (Table 3.5).

TABLE - 3.5

EFFECT OF DIBUTYLPHTHALATES (DBP) &BIS(2-ETHYLHEXYL) PHTHALATES (DEHP) ON RATS FEMALE FERTILITY

	CONTROL	DBP (100 MG/KG)	DEHP (100 MG/KG)
FEMALES NUMBER:	**15**	**9**	**6**
FEMALES COHABITED:	**15**	**9**	**6**
PREGNANT FEMALES:	**13 / 15**	**6 / 9**	**3 / 6**
LITTER: (NEW BORN):	**106**	**44**	**16**
DIED LITTERS:	**3**	**23**	**5**
(percentage lethal cases)			
FERTILITY RATE:	**87 %**	**67 %**	**50 %**
(% Pregnant Females / Females Cohabited):			
FECUNDITY RATE:	**8.2** per delivery	**7.3** per delivery	**5.3** per delivery
(Average number of litters per delivery):			
OTHER OBSERVATIONS:	**- Normal**	**- Rotating** (1) (10 /min) **Head bending to the right** **Rearing (1) (5 / min)**	**- Tremor** (1)

Female rats weighing 120-200gm received twice a week ip injection of DBP and DEHP (100mg/kg). Control animals were injected with the same volume with corn oil. Female rats were cohabited with 2 male rats for one month. Mortality rate: is the percentage of died litters compared to new born litters. Fertility rate: is the percentage of pregnant females per the number of females cohabited. Fecundity rate: is the average number of litters per delivery

3.2. 2 EFFECT OF PHTHALATES ON TOTAL AND RELATIVE BODY WEIGHT:

As total body weight gain is concerned no significant changes were obtained in female rats treated with DBP or DEHP compared to control animals (Table 3.6). Similar pattern of change in total body weight gain was observed during one month matting or in pre-matting or post-matting stage.

TABLE – 3.6

CHANGES IN BODY WEIGHT GAIN IN NORMAL RATS COMPARED TO RATS TREATED WITH DBP & DEHP

	CONTROL	DBP(100 mg/kg)	DEHP(100 mg/kg)
BODY WEIGHT: (gram)			
- INITIAL	**143.5 ± 5.6 (15)**	**138.0 ± 7.7 (10)**	**120.8 ± 3.8 (6)**
(8 days before mating)			
- MATING	**168.6 ± 5.8 (15)**	**162.3 ± 8.3 (10)**	**137.3 ± 4.4 (6)**
(1st day)	**↑ 17.5 %**	**↑ 17.6 %**	**↑ 13.7 %**
- MATING	**208.8 ± 8.7 (15)**	**186.6 ± 5.8 (10)**	**179.7 ± 5.7 (6)**
(1 months):	**↑ 45.5 %**	**↑ 35.2 %**	**↑ 48.8 %**
- POST MATING	**217.1 ± 7.5 (11)**	**203.0 ± 6.3 (6)**	**188.3 ± 8.7 (6)**
(After 1 month):	**↑ 51.3 %**	**↑ 47.1 %**	**↑ 55.9 %**

Values shown are Mean ± SEM for the number of experiments indicated in brackets. Body weight was measured one week before mating, the first day of mating, the last day of mating and one month after removal of the male rats. Significance of differences between treated and control were assessed using Student's t-test. For experimental details see legend of table (3-1) DBP, dibutylphthalates. DEHP, bis (2-ethylhexyl) phthalates.

At the end of the experiments after one month matting and one month after matting rats were anaesthetized with ether and the internal organs were removed and weight for the measurement of relative weight of the following organs: Heart, kidneys, spleen, liver, brain and sex organs. Female sex organs includes: Ovaries, Oviducts, Uterus and Vagina.

Relative weight of the kidneys was reduced significantly in DBP treated rats from 0.685 ± 0.013 (10) gram to 0.638 ± 0.012 (9) gr. ($P \leq 0.05$), and the relative weight of sex organs was reduced by 27.5 % from 1.787 ± 0.129 (9) to 1.296 ± 0.126 (9) gr. (P 0.05).

With DEHP treatment no significant changes were observed and the clear reduction of 25.5 % in relative weight of sex organs was not significant.

TABLE – 3.7

EFFECT OF DIBUTYLPHTHALATES (DBP) & BIS(2-ETHYLHEXYL) PHTHALATES (DEHP) ON RELATIVE WEIGHT OF BODY ORGANS IN FEMALE RATS

	CONTROL	DBP (100 mg/kg)	DEHP (100 mg/kg)
HEART:	**0.404 ± 0.013 (10)**	**0.358 ± 0.012 (9)**	**0.373 ± 0.018 (6)**
KIDNEYS:	**0.685 ± 0.013 (10)**	**0.638 ± 0.012 (9)*** **↓ 7 %**	**0.690 ± 0.038 (6)**
SPLEEN:	**0.281 ± 0.016 (9)**	**0.276 ± 0.010 (9)**	**0.286 ± 0.023 (6)**
LIVER:	**3.247 ± 0.171 (10)**	**3.118 ± 0.173 (9)**	**3.490 ± 0.218 (6)**
BRAIN:	**0.767 ± 0.023 (10)**	**0.769 ± 0.026 (9)**	**0.732 ± 0.036 (6)**
SEX ORGANS:	**1.787 ± 0.129 (9)**	**1.295 ± 0.126 (9)*** **↓ 27.5 %**	**1.329 ± 0.160 (6)** **↓25.6 % NS**

Values shown are Mean ± SEM for the number of experiments indicated in brackets. The relative weight shows the percentage weight of different organs compared to total body weight in each animal. Three groups of female rats treated twice a week with DBP or DEHP 100mg/kg or with the same volume of vehicle solution. Female rats were cohabited with male rats for one month as mentioned in table-1 . At the end of experiments rats were anaesthetized with ether and the selected organs: heart, kidneys, spleen, liver, brain and sex organs were removed. Sex organs included ovaries, oviducts, uterus and vagina. * $P \leq 0.05$

3.2. 3 BIOCHEMICAL CHANGES IN BLOOD SERUM FOLLOWING TREATMENT WITH PHTHALATES:

The level of biochemical compounds in blood serum of overnight fasted rats was measured in control animals and in animals treated with DBP or DEHP.

Fig 3.8 shows that Alkaline phosphatase activity was reduced significantly by DBP from a control values of 33.85 ± 5.86 (12) to 17.33 ± 3.22 (12) U/L ($P \leq 0.05$) and by DEHP to 18.02 ± 3.24 (6) U/L ($P \leq 0.05$).

GPT values were increased with DBP by 42 %, from 50.33 ± 5.73 (9) to 71.25 ± 2.10 (12) U/L ($P \leq 0.02$). While GOT values were increased by DBP from 98.38 ± 6.52 (8) to 142.00 ± 11.38 (10) U / L and with DEHP they were elevated to 181.25 ± 30.80 (6) U/L ($P \leq 0.05$).

Uric acid was reduced with DBP treatment from its normal values of 1.20 ± 0.12 (12) to 0.86 ± 0.06 (12) mg/dl ($P \leq 0.05$) and by DEHP treatment to 0.77 ± 0.05 (5) mg/dl ($P \leq 0.05$). Creatinine levels were reduced significantly by DBP from 0.41 ± 0.09 (10) to 0.18 ± 0.02 (12) mg/dl ($P \leq 0.05$).

Both phthalates were not able to produce any significant changes with the serum levels of glucose, total proteins, triglycerides, cholesterol HDL or cholesterol LDL.

TABLE – 3.8

BIOCHEMICAL CHANGES IN RATS BLOOD SERUM TREATED WITH DIBUTYLPHTHALATES (DBP)

&BIS(2-ETHYLHEXYL) PHTHALATES (DEHP)

	CONTROL	DBP (100 mg/kg)	DEHP(100 mg/kg)
GLUCOSE: (mg/dl)	**83.71 ± 8.35 (8)**	**87.7 ± 4.3 (7)**	**68.0 ± 5.0 (6)**
ALKALINE PHOSPHATASE: (U/L)	**33.85 ± 5.86 (12)**	**17.33 ± 3.22 (12)***	**18.02 ± 3.24 (6)***
GPT: (U/L)	**50.33 ± 5.73 (9)**	**71.25 ± 2.10 (12)****	**60.50 ± 12.90 (6)**
GOT: (U/L)	**98.38 ± 6.52 (8)**	**142.00 ± 11.38 (10)***	**181.25 ± 30.80 (6)***
UREA: (mg/dl)	**61.81 ± 5.62 (13)**	**48.67 ± 4.30 (12)**	**72.00 ± 6.90 (6)**
CREATININE(mg/dl)	**0.41 ± 0.09 (10)**	**0.18 ± 0.02 (12)***	**0.47 ± 0.13 (6)**
URIC ACID: (mg/dl)	**1.20 ± 0.12 (12)**	**0.86 ± 0.06 (12)***	**0.77 ± 0.05 (5)***
TOTAL PROTEIN: (g/dl)	**7.19 ± 0.31 (13)**	**6.54 ± 0.19 (12)**	**7.74 ± 0.21 (6)**
CHOLESTEROL – TOTAL: (mg/dl)	**115.97 ± 7.68 (12)**	**106.70 ± 5.69 (12)**	**127.60 ± 16.90 (6)**
CHOLESTEROL- HDL (mg/dl)	**74.05 ± 5.7 (12)**	**72.23 ± 5.68 (12)**	**97.10 ± 11.20 (6)**
CHOLESTEROL – LDL (mg/dl)	**22.70 ± 2.73 (12)**	**18.55 ± 1.72 (12)**	**19.35 ± 1.30 (6)**
TRIGLYCERIDES: (mg/dl)	**62.29 ± 4.70 (8)**	**58.32 ± 5.68 (10)**	**68.90 ± 11.00 (6)**

Values shown are Mean ± SEM for the number of experiments indicated in brackets. At the end of experiments blood was collected from the femoral artery of anaesthetized rats, centrifuged and blood serum was separated for the measurement of biochemical compounds. Analysis was performed using glucometer for the measurement of blood glucose and Bio-analyzer for measuring all the rest of the chemical compounds.* $P \leq 0.05$ ** $P \leq 0.02$

3.2.4 FEMALE RATS DNA DAMAGE:

Female rats treated with DBP 100 mg / kg has caused oxidative stress and DNA damage by increasing 8-OHdG concentration significantly by 29.8 % from 30.63 ± 1.33 (9) to 39.77 ± 3.30 (6), while DEHP has no effect whatsoever.

FIG. 3.4

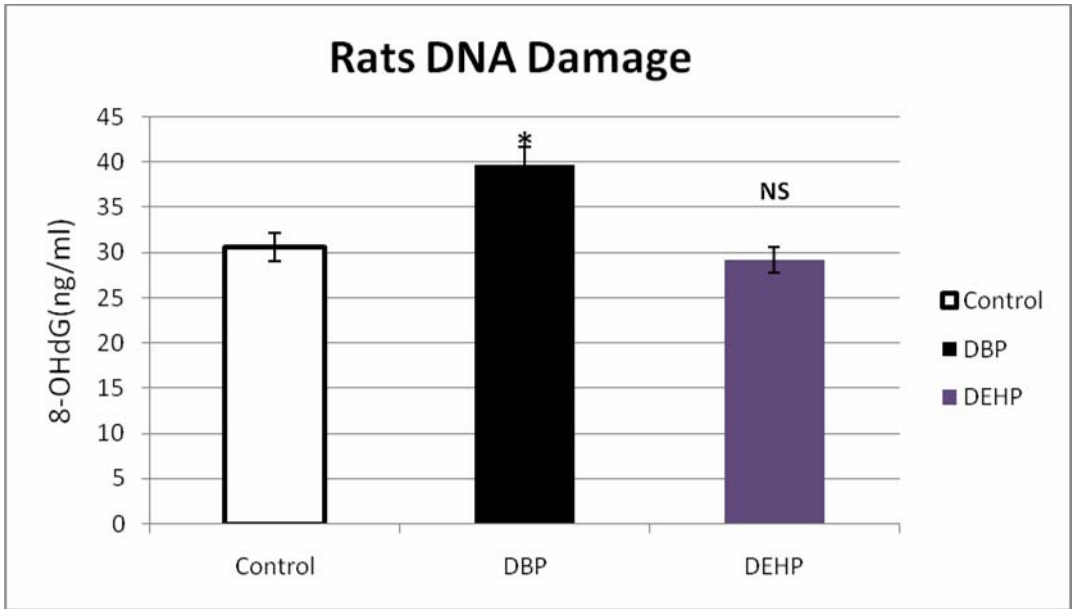


Table 3.9 shows that DBP is more effective in causing oxidative stress in female rats more than in chicks embryonic development, while DEHP was more effective with the chicks model.

TABLE 3.9

EFFECT OF DIBUTYLPHTHALATES (DBP) &BIS(2-ETHYLHEXYL) PHTHALATES

(DEHP) ON DNA DAMAGE IN FEMALE RATS AND DEVELOPING CHICKS

	CONTROL	DEHP	DBP
FEMALE RATS:	**30.63±1.33 (9)**	**39.77±3.30 (6)**	**29.20 ±1.43 (6)**
		↑ 29.8%	**↓ 4.7 %**
		P ≤ 0.05	**NS**
NEW BORN CHICKS:	**43.47 ± 6.50 (5)**	**54.22±2.87 (3)**	**60.74±2.02(6)**
		↑ 14.3%	**↑ 39.7%**
		NS	**P ≤ 0.05**

Values are Mean ± SEM for the number of experiments indicated in brackets, the concentration of 8-hydroxyguanosine (8-OHdG) in blood serum in (ng/ml). DBP and DEHP 100 mg/ kg were injected intraperitoneally into female rats, and the same concentrations were injected in a volume of 60 µl to eggs before incubation. Control samples were treated with the same methods of injection and with the same volume of corn oil.

CHAPTER FOUR

DISCUSSION

Plastics have become an integral part of our everyday lives. Phthalates are plasticizers widely used in the manufacture of polyvinylchloride and other plastics, including products, clothing, food packing, children's products and media devices. Thus, the general population can be exposed to phthalates in food, water, and air via ingestion or inhalation. Phthalates, in general, are colorless, high-boiling liquids, soluble in organic solvents but immiscible in water, and they are degraded very slowly in the ambient environment. Diethylhexyl phthalates (DEHP), for example, closely resembles organochlorine pesticides (DDT, PCB) in rate of uptake and storage. In recent human studies residues of phthalates have been found in milk, human tissues and blood plasma. Recent reports on the level of phthalates monoesters metabolites in urine samples collected from the third U.S. National Health and Nutrition Examination Survey (NHANES ШI) (Bount et al 2000) showed that four metabolites , MEP. MEHP, MBP and MBzP were present in more than 75% of subject sampled. The first report on human exposure to phthalates in the region, suggests that pregnant women in Jerusalem are exposed to a range of phthalates, seven phthalate metabolites were detected in 100% of the samples and nine phthalate metabolites were detected in at least 95% of the samples, MEP and MBP were found at the highest concentrations, in agreement with findings among pregnant women in the United States (Adibi et al 2003; Swan et al 2005) and Taiwan (Huang et al 2007).

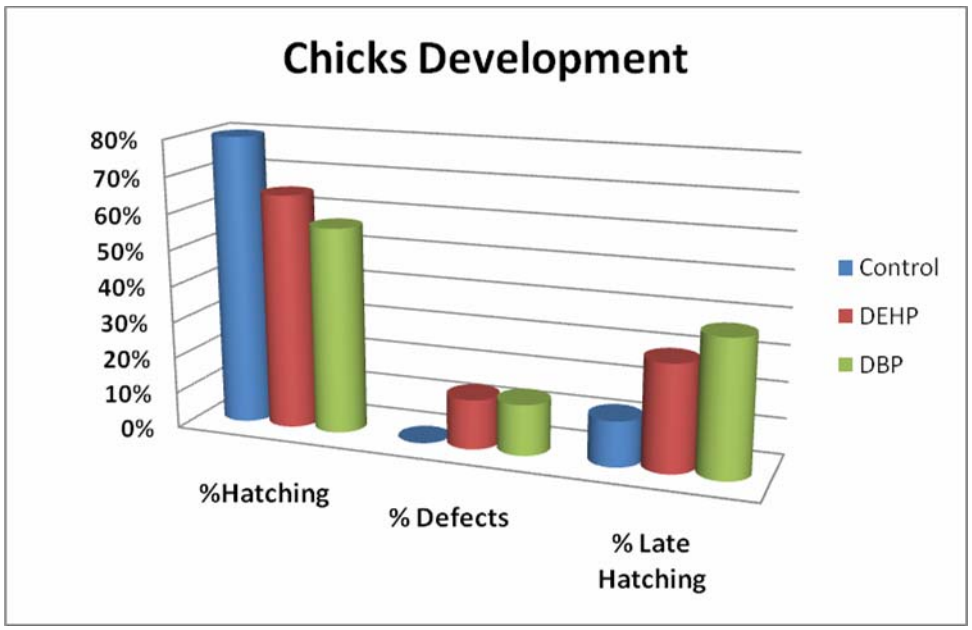
Therefore, there is scientific and public concern about potential human health risks from exposure to phthalates. The Food and Drug Administration and the National Institute of Environmental Health Science have a continuing interest and concern regarding the safety of phthalates. These concerns stem from studies showing that large populations are exposed to phthalates, as well as from animal studies consistently showing that some phthalates are developmental and reproductive toxicants. However there is still inconsistency of teratological information of phthalates. Therefore, the present study was designed to develop an avian model for the neurobehavioral teratogenicity of phthalates.

4.1. TERATOGENIC ACTIVITY OF PHTHALATES ON EMBRYONIC DEVELOPMENT:

As our results (Fig 4.1) showed that embryonic exposure to DEHP 5mg/kg reduced hatching rate by 20 % without inducing any defects. Embryonic exposure to DEHP at higher doses of 20-50-100 mg/kg reduced hatching rate by 20 % and induced post-hatch defects in dose depending manner. Chicks treated with 100 mg/ kg of DBP has reduced hatching percentage to 57% and increased defect chicks percentage by 14 %. Not all phthalates are equivalent in the severity of their effect; some phthalates exhibit less or more sever effects.

The most effective dose of Phthalates in inducing defects characterized as Omphalocele, was DEHP 100 mg/kg. Where a single dose injected into the egg before incubation has induced birth defects in 22% of the cases. All the defects were characterized as Omphalocele with a hole in abdominal muscle, allowing internal organs to protrude externally in a translucent sac, while similar dose of DBP has induced birth defects in 14% of the cases, and the types of defect was characterized as Gastroschisis where the internal organs protruded externally without a sac.

FIG. 4.1 Percentage of Hatching, Late Hatching and Defects on chicks exposed to Phthalates



Our results demonstrate the usefulness of the chick model for teratogenic evaluation of phthalates and provide initial information about two important phthalates members DEHP and DBP. In a model that exclude any potential contribution of maternal effects. Three main defects; Omphalocele, Gastroschisis and motor disorder, were observed at doses equal to or below those found to produce defects in mammalian models, and were never seen in control chicks but occurred in doses above 20 mg/kg.

An omphalocele is caused by an opening (defect) in the middle of the abdominal wall at the bellybutton (umbilicus). The skin, muscle, and fibrous tissue are absent. The intestines protrude through the opening and are covered by fine membranes. Some cases of omphalocele are believed to be due to an underlying genetic disorder, such as Edward's syndrome or Patau Syndrome. Gastroschisis a similar birth defect, but the umbilical cord is not involved, the lesion is not usually midline, and not enclosed in a membranous sac.(3)

Gastroschisis is an opening in the abdominal wall (muscles and skin of the abdomen) that appears during fetal development. The opening occurs almost always to the right of the umbilical cord. As a result, the stomach, small and large intestines are not enclosed in the abdominal wall and appear outside of the fetus's body, leaving the intestine exposed to amniotic fluid, which contains the baby's urine. This contact may irritate the bowel, causing it to swell and shorten. Normally, during early development, the intestines, stomach and liver protrude to the outside of the body. As the fetus grows, these organs are "pulled in" and the abdominal wall forms around them. This does not occur in gastroschisis. As with many birth defects, the reason behind it is unknown. Some scientists think that genetics or an environmental exposure during pregnancy can cause gastroschisis, but the exact cause is still unknown. Unlike other abdominal wall defects such as omphalocele, gastroschisis is typically not associated with chromosomal anomalies or other structural malformations. (4)

Because the chick model unlike mammalian models, dose not involve maternal effects, our studies indicate that phthalates evoke neurobehavioral teratogenesis through direct effect on developing brain, rather than through indirect compromise of maternal function or maternal-neonatal interactions, Furthermore, chicks are more mature at hatching than are newborn rats or mice. Cognitive behavior, in the form of imprinting performance, can be evaluated immediately. Prior to any potential impact of prenatal treatment on newborn chick tends to follow the first object it sees after hatching and can thus be imprinted upon an artificial object. In this way it becomes a suitable subject for studying the effect of prenatal treatment on imprinting behavior (Sluckin W et al. 1972). In the present study we evaluated whether DEHP has an adverse effect on imprinting performance in the chick model. Preference scores reflect the strength of learning during imprinting (Bateson et al 1966). The expected range of the preference score is 0-1. Where 0.5 indicates no imprinting, 1 represent maximal imprinting, and 0 represents avoidance (running away from the imprinting object). Control chicks showed typical-high imprinting score of about 0.649, chicks exposed to DEHP lost their imprinting ability to 0.509, reflecting adverse impact on the development of the hippocampus and the cognitive centers in the brain (McGrath et al. 2004; Yanai et al. 2009). It could also be due to its effect in reducing the concentration of PKC isoforms in the left IMHV as reported by (Izrael et al. 2004; Slotkin et al. 2005; Slotkin et al. 2008). The neurobehavioral

defects could happen with the sub toxic doses of phthalates. The effects on imprinting were not secondary to loss of motor function (locomotor activity), since overall activity levels in the imprinting apparatus were not significantly affected.

When measuring the biochemical markers, there was a significant increase in alkaline phosphatase by 296% and decrease of urea and creatinine and GOT activity. The elevation of alkaline phosphatase is due to a bony pathology or muscular dystrophy this in turn might reduce muscle dry mass leading to decrease in creatinine and urea.

The primary importance of measuring alkaline phosphatase was to check the possibility of bone disease or liver disease. Since the mucosal cells that line the bile system of the liver are the source of alkaline phosphatase. When the liver, bile ducts or gallbladder system are not functioning properly or blocked, this enzyme is not excreted through the bile and alkaline phosphatase is released into the blood stream. Thus the serum alkaline phosphatase is a measure of the integrity of the hepatobiliary system and the flow of bile into the small intestine. In addition to liver, bile duct, or gallbladder dysfunction, an elevated serum alkaline phosphatase can be due to rapid growth of bone since it is produced by bone-forming cells called osteoblasts (5). The decrease in GPT activity obtained in blood of chicks treated with DEHP roles out the possibility of liver damage. The weakness in abdominal muscles and the weakness of skeletal muscles and motor disorders indicate muscular dystrophy.

Previous results using other types of phthalate esters like dibutoxyethyl phthalates, di-2-methoxyethyl phthalates and octylisodecyl phthalates on developing chicks, has induced other congenital malformation such as crania bifida and anophthalmia resulting from an absence of bone tissue forming the orbit of the eye and blindness. (Bower et al.1970)

To study the genotoxicity induced by DEHP and DBP at the molecular level we employed DNA Damage ELISA (enzyme-linked immunosorbent assay) it is a fast and sensitive competitive immunoassay for the detection and quantitation (8-OHdG), a marker of DNA oxidation. Several studies have demonstrated that the 8-OHdG in bodily fluids can act as a biomarker of oxidative stress (Takane M et al. 2005), and potentially involved in carcenogenesis in various experimental models.

Oxidative stress can result in DNA damage, including oxidation of nucleosides. Therefore, oxidative DNA damage was assessed in rats and chicks in vivo from serum levels of the DNA repair product (8-OHdG).

Our results indicate that in new born chicks pre exposure to DEHP significantly increased levels of 8-OH-dG by 39.7% ($P \leq 0.05$), while the 25 % increase induced by DBP was not significant. On the other side using female rat fertility model, showed a significant increase of 29.8 % ($P \leq 0.05$) was achieved on rats treated with DBP (100 mg/kg) while the changes induced by DEHP were not significant compared with control group. (8-OHdG) is a product of oxidative DNA damage by reactive oxygen species and serves as an established marker of oxidative stress. It has been shown to cause G→T and A→C transversions (Cheng et al. 1992). Hydroxylation of guanosine occurs in response to both normal metabolic processes and a variety of environmental factor. Elevated level of urinary 8-OHdG has been detected in patient with various cancers. In human atherosclerotic plaques there were increased amounts of urinary 8-OHdG in diabetes correlated with the severity of diabetic nephropathy and retinopathy (Wu LL et al. 2004). In south Indian population, patients with essential hypertension a significant increase of urinary 8-OHdG was observed correlated with decrease serum TAS levels, which reflect increased oxidative damage (Subash et al.2010). The increased levels of 8-OH-dG do not correlate with carcinogenic potency, as similar levels of induction have been associated with divergent carcinogenic activities (Marsman et al. 1988, 1992). Furthermore, DEHP and other peroxisome proliferators have consistently lacked initiation activity unlike other DNA-damaging agents. The overall evidence suggests that increased production of hydrogen peroxide and DNA oxidation are not solely responsible for peroxisome proliferators'-induced liver tumor formation.

Recent studies reported that DEHP can induce DNA damage in nerve system of mice and oxidative damage in internal organs of mice with apparent dose-dependent manner, they employed comet assay to measure the DNA damage induced by DEHP in cells of brain (Martino-Andrade et al. 2009). Tagaki and coworkers (1992) have examined the possibility of DNA damage by DEHP by measuring the induction of (8-OH-dG), in the liver and kidney from male rats administered DEHP for various periods of time. Increased levels of 8-OH-dG were seen in the liver after 1 or 2 weeks or 12 months of treatment, but no increases were seen

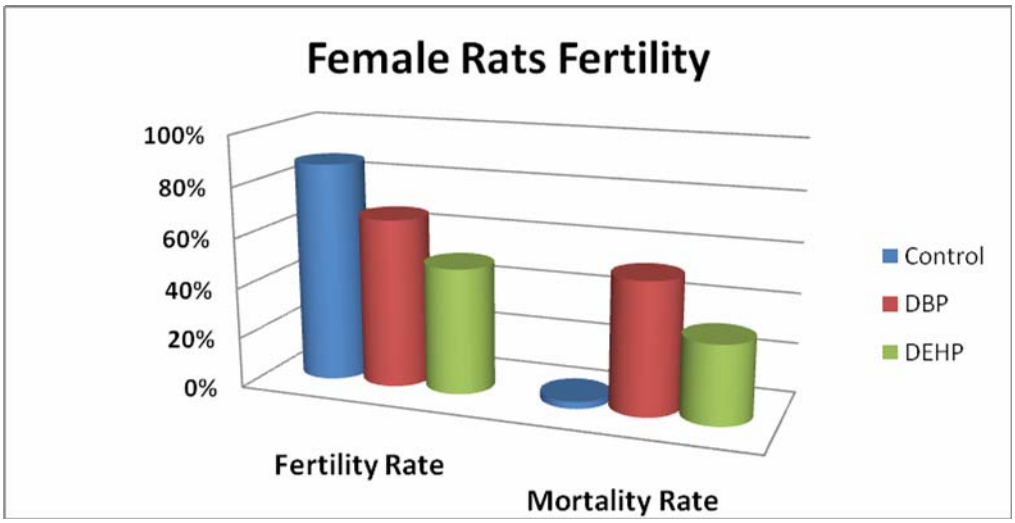
in the kidney. In general, the increases were small (2-fold) and in some cases, were not sustained with prolonged DEHP treatment (Cattley and Glover 1993). A study by Duty SM et al 2003, represent the first human data to demonstrate that urinary MEP, at environmental levels, is associated with increased DNA damage in sperm.

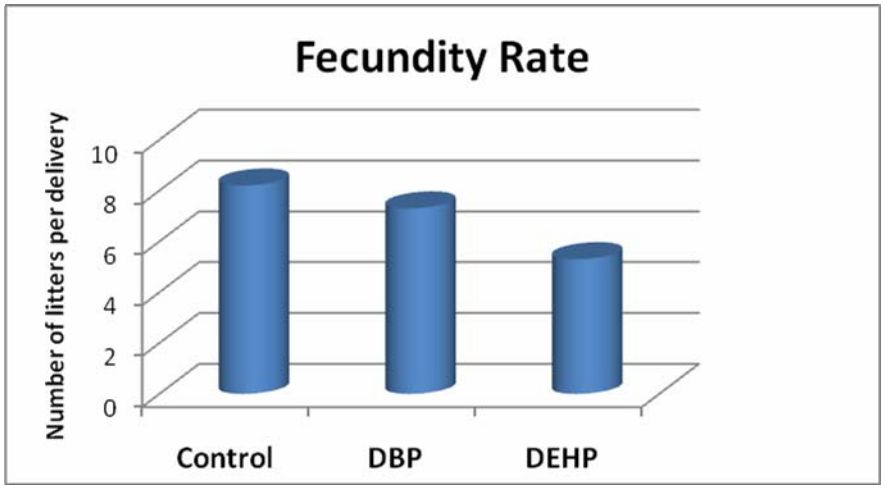
The reason that serum 8-OHdG was selected as a marker of oxidative DNA damage in this study was because of its convenient use in clinical practice and also they allow the assay to be non-invasive and it is well known that the study of oxidative DNA damage is clinically important. However, no data on serum 8-OHdG in rats and chicks treated with both DEHP and DBP are available.

4.2. EFFECT OF PHTHALATES ON FEMALE FERTILITY:

The present experiments were undertaken to further investigate the action of DEHP and DBP upon female rat reproduction. The effects of DBP and DEHP on female rats fertility are summarized in Fig 4.2.

FIG. 4.2: Effect of DEHP and DBP on Female Rats Fertility Rate, Fecundity and Mortality Rate





The Fertility rate expressed as percentage of pregnant females per females cohabited. was reduced by DBP from 87 % in control rats to 67 % and by DEHP to 50 %. Fecundity rate expressed as average number of new born litters per delivery, was reduced from 8.2 per delivery in control rats to 7.3 in DBP treated rats and to 5.3 per delivery in DEHP treated female rats. Mortality rate was increased significantly by DBP and DEHP from 2.8 % to 52.3 % and 31.3 % respectively.

Few studies have investigated the reproductive toxicity of DEHP in female animals. The present study coupled with other recently published studies serve to confirm some of the

previously reported effects of phthalates on the female reproductive system and the effect of DEHP on decreasing fertility rate. Fertility rate was decreased to 50% and 67% by DEHP and DBP respectively. In contrast to males, it is generally thought that female reproductive system is much less sensitive to phthalates. However, recent evidence suggests that phthalates can also induce adverse responses in females following pre and postnatal exposure (Gray et al. 2006). Initial studies demonstrated that ovary is a target site for DEHP. Davis et al 1994 reported that high doses of DEHP results in prolonged estrous cycles, reduced serum estradiol levels and absence of ovulation in adult rats, which can explain the reduction in pregnancy in female rats treated with DBP and DEHP. Fertility studies with crossover mating have also shown that active phthalates can decrease the fertility of rats and mice through male and female-mediated effects (Lamb et al.1987), which support our results. Shiota et al 1982, has reported that mice ingestion of high doses of phthalates caused intrauterine growth retardation and delayed ossification with an apparently dose related manner and caused neural tube closure in developing embryo.

In males the first finding of phthalates induced testicular injury in experimental animals was reported by Shaffer et al. in 1945. The testicular effects are characterized by decreased testis weight and atrophy of seminiferous tubules. The alterations manifested in male offspring include cryptorchidism, hypospadias (ectopic opening of the urethra), atrophy or agenesis of sex accessory organs, testicular injury, reduced daily sperm production, permanent retention of nipple and decreased (feminized) anogenital distance. (Martino-Andrade et al. 2009). In addition, phthalates induced testicular dysgenesis by affecting sertoli cells and leydig cells (Liu et al 2005).

However, the alteration in spermatogenesis observed after exposure to high doses of DEHP could be due to dysfunction in sertoli cells (Kavlock et al. 2002), or through the effect of follicle stimulating hormone action on sertoli cells (Lloyd et al. 1988; Heindel and Powell. 1992), or by targeting leydig cells which induce testosterone (Jones et al. 2004). In general it was found that phthalates with medium side chain like DBP or branched long side chain like DEHP are more toxic and more effective than those with linear long side chains (Heindel and Powell. 1992; Lamb et al. 1987). Furthermore DEHP was found to reduce sperm production (Andrade et al. 2006; EFSA 2005).

As total body weight gain is concerned no significant changes were obtained in female rats treated with DBP or DEHP compared to control animals, these results were consistent before mating during one month mating and one month after pregnancy.

The relative weight of the following internal organs: Heart, kidneys, spleen, liver, and brain were not affected following treatment with DBP or DEHP, while the relative weight of the female sex organs which includes: Ovaries, Oviducts, Uterus and Vagina, was reduced significantly by 27.5 % in DBP treated rats. The 25% reduction achieved with DEHP was not significant. Similar decrease in male sex organs following treatment with Phthalate esters was reported by Martino-Andrade et al 2009.

Our results had shown no changes in rat blood glucose level. Previous studies were performed to investigate phthalates exposure and its association with abdominal obesity and insulin resistance, suggest that exposure to phthalates may contribute to the population burden of obesity, insulin resistance and related clinical disorders. (Stahlhut RW et al 2007). In this study, when female rats were exposed to DEHP, they were found to have increased serum glucose and decreased insulin, as well as thyroid and adreno-cortical dysfunction (Goyathri et al 2004). These studies does not support our results, since we have not seen any change in glucose levels following treatment with DBP or DEHP in any of the models used, fasted rats or non fasted chicks. From biochemical measurements obtained in this study no significant changes were observed in serum levels of glucose, total proteins, triglycerides, cholesterol HDL or cholesterol LDL.

In rats model a significant increase of GOT and GPT activity accompanied with decrease in creatinine and uric acid was obtained. This is due to drug related injury to liver cells, which increase GOT and GPT and leads to decrease in dry mass which results in low creatinine and uric acid. While results obtained from experiments on chicks model there was an increase in alkaline phosphatase and a reduction in GPT which is more classical to bony muscular dystrophy.

Our recent results with DNA damage and all other reports suggests that competitive ELISA for 8-OHdG appears to be simple method for quantifying the extent of oxidative stress. Several evidence show that oxidative damage may be an important mechanism underlying several pathophsiolgical states, for example, atherosclerosis caused by oxidative modification of low-density lipoprotein (Ross et al. 1999); diabetic complications caused by oxidative damage of lipids, protein (Baynes et al. 1999) and DNA (Dandona et al. 1995); aging caused by oxidative damage of proteins and myocardial damage\loss through oxidative injury. These results supported with our results that showed oxidative stress is increased upon exposure to DEHP and DBP in animal models and could be the mechanism underlying phthalates toxicity.

5. CONCLUSIONS:

Nowadays it is well known that phthalates are the most commercially important plasticizer for PVC plastics, and one of the serious contaminations in the whole world. DEHP is the most distributed phthalate and the greatest potential risk to human health.
It has reproductive toxicity, developmental toxicity, embryonic toxicity and potential carcinogenicity.

Our results show that the medium side chain phthalate (DBP) and the long branched side chain (DEHP) are very effective both on chick model and on female rats fertility model. It was most effective in inducing teratogenic activity in developing chicks following pre-exposure to single dose of phthalate (20-100mg\kg), and for the first time it was reported to induce Gastroschisis and Omphalocele in new born chicks and in 8-20% of the cases with DEHP, these were associated with oxidative stress and DNA damage. Oxidative damage induced by DEHP and DBP is an experimental evidence for molecular mechanism of

phthalates toxicity. In addition, it has reduced hatchability and caused neurobehavioral deficits as shown in preference scores which reflect the strength of learning during imprinting test.

These results indicate that in addition to the effect of phthalates in reducing male fertility as reported before, it has reduced female fertility and fecundity and increased mortality rate in new born litters. It also showed that phthalates are more toxic when injected to early stages of embryonic development, while the effect on adult males or females rats needs continues injection of the drug or continuous gestational exposure to very high doses.

The mechanism of action of Phthalates could be due to induction of peroxisome proliferation, oxidative stress, or through zinc deficiency. Therefore further experiments are needed to elucidate its mechanism of action. We recommend more epidemiological studies using larger human population including (follow-up) studies of infants exposed to phthalates. More teratogenic screening experiments should be performed to find the risk of exposure to small doses. To avoid even neurobehavioral changes, which affects cognition, learning and memory.

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