EFFECTS OF DI-N-BUTYLPHTHALATE ON THE LIVER, KIDNEY AND TESTES OF ADULT MALE ALBINO WISTAR RATS.

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SUPERVISOR: MR. J.C. ONYEANUSI

JUNE, 2010.

CERTIFICATION

This is to certify that this project titled EFFECTS OF DI-N-BUTYLPHTHALATE ON THE LIVER, KIDNEY AND TESTES OF ADULT MALE ALBINO WISTER rats was carried out by EZEOKEKE, EUNICE A., of Department of Medical Laboratory Sciences, Faculty of Health Sciences and Technology, University of Nigeria, Enugu Campus, under the supervision of:

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ABSTRACT

This study investigated the effects of di-n-butylphthalate on the liver, kidney and testes, after oral administration, to adult male albino Wistar rats. Twenty rats, weighing between 146.10g and 301.20g were arranged into groups A,B,C,D, of five rats each, and were fed with graded concentrations, 0 mg/kg, 2,000 mg/kg, 4,000 mg/kg and 6,000 mg/kg body weight of di-n-butylphthalate respectively for thirty days. Serum urea and creatinine levels were used as test of renal function, while bilirubin, alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase AST, served as indices of liver function. Estradiol and testosterone levels were also assayed as a test of reproductive function. In addition, the cell histology of liver, kidney, and testes of the rats were also examined. The results of all the liver parameters were significantly high ($P<0.05$), in groups B, C. and D. when compared to the level in the control group A. Conjugated bilirubin also recorded a significantly low level ($P<0.05$) in the treated groups B (2.14$\pm$0.04µmol/L), C (2.18$\pm$0.05µmol/L), and D (2.22$\pm$0.02µmol/L). There was also a significantly high level ($P<0.05$), of creatinine and urea in groups B (71.80±1.43µmol/L, 3.44±0.10Mmol/L), C (85.40±1.47µmol/L, 4.70±0.18Mmol/L), and D (108.00$\pm$0.95µmol/L, 6.30±0.13Mmol/L), when compared with the control group A. The toxicant, di-n-butylphthalate, also produced a significantly low levels ($P<0.05$) of estradiol and testosterone in the treated groups. The histological examination of the kidney in groups B, C, and D indicated glomerular expansion, generalized inflammation of some glomeruli and vascular congestion, while the liver cell histology revealed occasional portal inflammation mild fibrosis and moderate amount of nuclear

pyknosis. The testes histology showed normal seminiferous tubules with fewer cells when compared to the control group A. After thirty days of treatment, the control group showed a mean weight gain of 1.31%, whereas the treated groups B, C and D recorded a significant decrease in weight of 2.02%, 2.11% and 1.19% respectively. This study indicates that the chemical, di-n- butylphthalate is organotoxic, and may affect organ functions and sperm production in male rats at high concentrations.

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CHAPTER ONE

INTRODUCTION

1.1 Background of study

Di-n- butylphthalate, (DBP) belongs to a widely used group of chemicals called phthalates. It is a phthalic acid ester with the molecular formualr $C_{16}H_{22}O_4$. It is an inert, colourless, oily liquid with mild aromatic smell, low vapour pressure and is soluble in most organic solvents like benzene, ether and alcohol (Duty, 2005).

DBP is used mainly as a specialty plasticizer for nitrocellulose, polyvinyl acetate and polyvinyl chloride (PVC), where it may account for 20% W/W or more of the plastic materials. Its production has increased since 1950's when PVC was introduced (Centre for Disease Control and Prevention, CDC, 2005). When added to plastics, it allows the long polyvinyl molecules to slide against one another, hence softening and increasing their flexibility, transparency and durability. Its end applications include medical devices like plastic tubing for nasal or oral feeding, blood bags and catheters; pharmaceuticals, like enteric coatings of drugs; cosmetics and body care products like perfumes, nail polish, fingernail elongators, body lotions, hair spray, powder, liquid soap, eye

shadow, and moisturizer. Other products, like some building materials, aluminum foil used as food wraps, plastic tanks and containers, detergents, adhesives, printing ink, insect repellant, textile, paints, di-electric fluid in condensers, carpet backing, toys and rocket fuel contain phthalates (Brandt, 2005).

1.2 Statement of problem

Almost all the DBP present in the environment arose form anthropogenic sources rather than natural ones, and people are exposed to DBP daily through contact with everyday products and occupationally (National Occupational Exposure Survey, (1987). In Nigeria today, the use of products made from DBP and plastic materials for food packaging has been on the increase.

DBP and other phthalates are easily released into the environment because there is no covalent bond between the phthalates and plastics in which they are mixed. As the plastic ages and breaks down, the release of phthalates accelerates (Rudel and Perovich, 2008). DBP can easily leach out into the water or food contained in these packages or containers, like margarine, candy sweet, magi, fruit juice. It can also penetrate the skin and accumulate in the body through the use of some

drugs, cosmetics and other body care products made from DBP. Through poor waste management and incineration of waste plastic materials, it can find its way into the air and water sources like streams.

The persistent and ubiquitous distribution of this environmental contaminant has caused it to remain a potentially serious threat to human and animal health. The current research was therefore carried out to study some of the effects of DBP on male albino Wistar rats.

1.3 Aims and objectives

This research is aimed at studying the toxicological effects of orally administered di-n-butylphthalate on the liver, kidney and testes of adult male albino Wistar rats. The work therefore aims to:

i. Estimate the levels of serum bilirubin, ALP, ALT, and AST in the rats to assess the effects of DBP on the liver function.
ii. Estimate the levels of serum urea and creatinine in the rats to assess the effects of DBP on the renal functions.
iii. Estimate the levels of estradiol and testosterone in the rats to assess the effects of DBP on the reproductive functions.
iv. Examine the cell histology of their liver, kidney and testes for morphological changes due to the toxic effects of DBP.

v. Compare the levels of these biochemical parameters and the histological changes in the treated animals with those of the control animals.

1.4 Significance of study

The data generated from this study are assumed relevant to prediction of hazard to humans and will give some indications of the possible health implications of exposure to DBP. Further studies can be extended to humans. It will also help the policy makers to come up with a policy that will regulate its use in cosmetics, food, medical devices and everyday products to guarantee public safety.

1.5 Scope of study

This is a sub-acute study, and is limited to animal model - adult male albino Wistar rats of age 90 – 150 days old. This age was chosen based on the range of 70 -180 days stated as the peak reproductive age of male albino Wistar rats.

CHAPTER TWO

LITERATURE REVIEW

2.1.1 History of di-n-butylphthalate as a plasticizer

The development of cellulose nitrate in 1846 led to the official right approval patent of castor oil in 1856 for use as the first plasticizer. In 1870, camphor became the more favoured plasticizer for cellulose nitrate. Phthalates were first introduced in the 1920s and quickly replaced the volatile and odorous camphor (C.DC, 2005). In 1931, the commercial availability of polyvinyl chloride and the development of di-2-ethylhexylphthalate and di-n-butylphthalate began the boom of the plasticizer polyvinyl chloride industry.

As of 2004, manufactures produced about three hundred and sixty-three thousand tones of phythalates each year. They have been found to contribute 10-60% of plastic products by weight (Rudel and Perovich, 2008).

2.1.2 Properties of di-n-butylphthalate

Di-n-butylphthalate (DBP) is a manufactured chemical that does not occur naturally. It is a $-CH_3$ ester of phthalic acid. DBP has a clear

syrupy liquid consistency, and show low water solubility, high oil solubility and low volatility. It is an odourless depending on the degree of purity to mild aromatic liquid that is colourless to faint yellow in colour. The polar –COOH group contributes little to the physical properties (Environmental Protection Agency, EPA, 1998).

DBP has the molecular formular $C_{16}H_{22}O_4$ molecular weight of 278.35, minimum purity of 98%. Its weight per mililitre at 20^0C or specific gravity is 1.042 – 1.045g and maximum limits of impurities: - water – 0.1%, acidity - 0.1% and sulphated ash – 0.01%.

2.1.3 Sources of human exposure to di-n-butylphthalate

Diet is believed to be the main source of DBP and other phthalate exposure in the general population, although inhalational exposure through house dust, paints, insect repellants, new car smell in automobile is also significant (CDC, 2005). Infants breathe twice as much air as adults (per kilo body weight) and spend most of their time indoors, so they are suspected to be getting a particularly large doses of phthalates from house dust (Oie *et al;* 1997).

Baby care products containing DBP and other phthalates are a source of exposure for infants. Sathyanarayana (2008) observed that

reported use of infant lotion, powder, and shampoo, were associated with infant increased urine concentrations of phthalates. These findings suggest that dermal exposures may contribute significantly to phthalate body burden in this population. Some vendors of jelly rubber sex toys advise covering them in condom when used internally due to possible leaching of phthalates (Ethan *et al,* 2007).

Involuntary exposure to DBP do also occur through blood transfusion and other medical treatments using plastic devices (CDC, 2005). DBP is also found in medications, where they are used as an inactive ingredient in producing enteric coatings of some drugs. Some of those medications include omeprazole, didanosine, mesalamine and theophylline. DBP in medications raises concern about health risk due to the high level of exposure associated with taking these medications especially in vulnerable segment of the population, including pregnant women and children (Hernandez-Diaz *et al,* 2009).

2.1.4 Di-n-butylphthalate in the environment

Chemical substances enter the environment in different ways. Industrial chemicals like DBP are unintentionally released by volatilization, leaking or leaching, either during a products life time or

after ultimate disposal. Once a substance has passed through the environment, it can undergo different fates, such as further distribution between the environmental compartments - water, air and soil sediment as well as their sub-compartments. Degradation and transfer processes in the compartments and sub-compartments also occur (Lintelmann *et al.* 2003).

DBP, when released to the air as vapour, reacts with other chemicals in the air, and is usually broken down within a few days. It can also become attached to particles in the air and eventually settle to the land and water. Most of the DBP in water attaches to sediments and settles out of the water or is broken down by bacteria. Small amounts may evaporate to the air. When released to the soil, it attaches to soil particles and is broken down by bacteria. There is no evidence that it builds up in the food chain (Agency for Toxic substances and Disease Registry, ATSDR, 2001).

2.1.5 Levels of DPB and other phthalates in some foods

A major human exposure to phthalates is believed to be from foods which have absorbed the chemical from their packaging or from manufacturing processes. The United Kingdom Ministry of Agriculture,

Fisheries and Food (MAFF) has done a range of studies on foods. In their study on formula baby milk published in 1996, all fifteen brands of baby milk formula tested contained phthalates. The highest total phthalate concentration found was 10.2mg/kg. using the manufacturers' feeding guides, it was estimated that a new born infant would receive on average 0.13mg/kg body weight/day of total phthalate, falling to 0.10mg/kg/day at six months (MAFF, 1996).

MAFF studied also found that phthalate levels in foods like crisps, chocolate bars and cheese, are in tens of mg per kg range. The highest level found in daily produce was 114mg/kg in soft cheese.

Phthalates, particularly DBP and di-ethylhexylphthalate (DEHP), were also found in products packaged in paper and board, such as cakes, fats and confectionery. The highest concentrations of DBP found were in gravy granules (62mg/kg), vegetable burger mix (10mg/kg), vegetable fat (8.4mg/kg), chocolate coated cakes (5.8mg/kg), and sausages (4.3mg/kg). The highest concentrations of DEHP were 25mg/kg in cookies and 11 mg/kg in vegetable fat.

A survey of phthalate levels in fatty food samples taken as part of a "Total Diet Survey" by MAFF found phthalates to be present in every sample, including meat, fish, egg, milk and milk products. These

analyses led to a prediction that the daily intake of phthalates by an adult averaged 0.8mg/person/day (0.013mg/kg body weight/day), up to 1.6mg/person/day (0.027mg/kg body weight/day), for someone with a diet high in phthalate containing products (MAFF, 1996).

2.1.6 Metabolism of di-n-butylphthalate

Studies on laboratory animals have shown that DBP is rapidly absorbed from the gastrointestinal tract after oral administration, distributed primarily to the liver and kidneys of rats and excreted in urine as metabolites (Foster *et al;* 1982). Available data indicate that in rats, following ingestion, DBP is metabolized by non specific esterases mainly in the skin and plasma, to yield monobutylphthalate (MBP), with limited subsequent biochemical oxidation of the alkyl side chain of MBP. MBP is stable and resistant to hydrolysis of the second ester group. The MBP and other metabolites are excreted in the urine mainly as glucuronide conjugates and unchanged DBP at a low level. Accumulation has not been observed in any organ. However about 35% of the dose recovered in urine was primarily excreted in bile mainly as MBP-Glucuronide and underwent hepatobiliary recycling (Foster *et al,* 1982).

Williams and Blanchfield, (1975), had reported that after oral administration of ^{14}C labeled DBP to rats, 90 to 96% of the administered dose was excreted in urine within forty-eight hours. Phthalic acid, MBP, MBP-Glucuronide and mono-3-hydroxybutyl phthalate have been identified as metabolites of DBP in urine (Tanaka *et al,* 1978).

Assuming that oral exposure is prevented by personal hygienic measures, the risk characterization for workers is limited to the dermal and respiratory exposure paths. From in vivo study with rats, it has been determined that approximately 10% of the applied dose of neat DBP is absorbed per day, leading to a total absorption of 72% within seven days (Elsisi *et al,* 1989). The absorption flux was calculated to be 20 to 40mg/cm^2/hr. From in vitro studies, the absorption flux in rats determined in meaning or unoccluded conditions was very similar (39 to 42mg/cm^2/hr (Mint and Hotchkiss, 1994). A non-negligible amount of DBP remained within the skin at the end of the experiment, which suggested that the skin might be a reservoir for DBP. In human skin, the absorption flux was found to be about 20 to 50 fold lower than that in rats (Scott *et al* 1987).

2.1.7 Potential health effects of DPB

Di-n-butylphthalate (DBP) appears to have relatively low acute toxicity and reported LD_{50} value, following oral administration was 8,000mg/kg body weight in rats (Brandt, 1985).

DBP has been found to be one of the endocrine disruptors (Dweailly *et al* 1994). These are exogenous chemical substances or mixture that alter the function(s) of the endocrine system, and thereby cause adverse effects at the level of the organism, its progeny, the population or sub-population (Endocrine Distruptor Screen Testing Advisory Committee, EDSTAC, 1998). Studies on rodents exposed to certain phthalate in high doses have been shown to change hormone levels and cause birth defects (CDC, 2005).

A seminal study by Swan *et al* (2005), showed that human phthalate exposure during pregnancy resulted in decreased anogenital distance among baby boys later born-a change that in rodents exposed to DBP is associated with genital abnormalities. In their study, DBP metabolites were measured in urine samples collected from pregnant women who gave birth to the infants. After birth, the genital features and anogenital distance of these women's babies were measured, and

correlated with the residue in the mothers' urine. Boys born to mothers with the highest levels of phthalates were seven times more likely to have shortened anogenital distance. The Swan study is thought by some to suggest that male reproductive development in humans could be affected by prenatal exposure to environmentally relevant levels of phthalates (Tilson, 2008).

A more resent study on body with undescended testes suggested that exposure to a combination of phthalates and anti-androgenic pesticides may have contributed to that condition (Toppari *et al,* 2006).

In 2004, a joint Swedish – Danish research team found a very strong link between allergies in children and the phthalates, DBP and DEHP (Bornehag *et al,* 2004).

A recent British study by Hallmark *et al,* (2007), showed that the phthalate, DBP, its metabolite, MBP, suppresses steroidogenesis by fetal type Leydig cells in primates as in rodents. Also in 2007, a cross-sectional study of males in the United States of America concluded that urine concentrations of four phthalate metabolites, correlate positively with waist size, and three phthalate metabolites correlate with cellular resistance to insulin, a precursor to type II diabetes (Stahlhut *et al,* 2007).

Children exposed intravenously to phthalate diesters experienced relatively little metabolic conversion of the intravenous phthalate diester to its toxic monoester metabolite (Huber *et al;* 1996).

Occupational exposure to high levels of phthalates has been reported to lead to miscarriages and other complications of pregnancy (Institute for Environment and Health, IEH (1995). Both DBP and DEHP caused irregularities in male sexual differentiation, when given to pregnant rats (Gray *et al*, 1999).

Another research suggests that there is an association between PVC flooring and the development of bronchial obstruction in children (Jaakkola *et al*, 1999). This research suggests that the increasing incidence of asthma could be partially due to the increasing household use of plastics containing phthalates over the last few decades.

2.2.8 Mechanism of endocrine disruption by DBP

Phthalates are fat soluble, so they tend to concentrate in materials such as butter, margarine and cheese. In addition, they are likely to accumulate in the body fat. Steroids are often in association with fat. Several phthalates particularly DBP are testicular toxicants. Part of this toxicity is believed to involve depletion of testicular zinc, and may

include the death and disintegration of the testicular germ cells (Peters *et al*, 1997).

Research at the Chemical Industry Institute of Toxicology, confirmed that DBP does damage to the reproductive system of male rats at low exposures by disrupting the androgen system, rather than imitating estrogen hence demonstrating that DBP is an endocrine disruptor, though may be not an estrogen. (Foster, 1997). Further research has established that DBP cannot bind to androgen receptor but does disrupt androgen regulated male sexual differentiation (Mylchreest *et al*, 1999).

2.2.1 Liver detoxification

The liver is the principal organ in the body when it comes to detoxifying or getting rid of foreign substances or toxins, especially from the gut. It plays a key role in most metabolic processes especially detoxification of environmental chemicals and endogenous biochemicals. Most of the toxic chemicals that enter the body are fat soluble and have a high affinity for fat tissues and cell membranes, which are composed of fatty acids and proteins. In these fatty tissues of the body, toxins may be stored, being released during times of exercise, stress or fasting.

To avoid the accumulation of these toxins, the liver detoxifies and disposes them by filtering the blood to remove large toxins, synthesizes and secretes bile full of cholesterol and other fat soluble toxins and enzymatically disassembles unwanted chemicals. The enzymatic process usually occurs in two steps, referred to as phase I and phase II. (Keit, *et al,* 2001).

Filtration of toxins is absolutely critical as the blood from the intestines contains high levels of bacteria, bacteria endotoxins, antigen-antibody complexes, and various other toxic substances. When working properly, the liver clears 99% of bacteria and other toxins during the first pass (Sturgill and Lambert, 1997).

The liver's second detoxification process involves the synthesis and secretion of bile which serves as carrier in which many toxic substances are dumped into the intestines. In the intestines, the bile and its toxic load are absorbed by fibre and excreted. However, when the excretion of bile is inhibited, that is cholestasis, toxins stay in the liver longer, causing hepatocellular damage.

The liver's third role in detoxification involves a two-step enzymatic process for the neutralization of unwanted chemical compounds. Phase I either directly neutralizes a toxin or modifies the toxic chemical to form

activated intermediates by oxidation, reduction or hydroxylation. During this process, free radicals are produced, which if excessive can damage the liver cells. Phase II detoxification is called the conjugation pathway, where by the liver cells add another substances like cysteine, glycine or a sulphur molecule to a chemical to render it less harmful. This makes the toxin water soluble, so that it can then be excreted from the body via watery, fluids, such as bile or urine.

There are essentially six phase II detoxification pathway, namely glutathione conjugation, amino acid conjugation, methylation, sulphation, acetylation and glucuronidation. These reactions take place in the microsomal fraction of the hepatocytes, where many environmental chemical and endogenous biochemicals are also processed, and by the same mechanisms. Synthesis and secretion of enzymes of the hepatic microsomal system are increased by enzyme induction. The production of hepatic enzymes is increased in response to an environmental signal such as toxin or drug. Specifically, the enzymes of the cytochrome p450 system are responsible for metabolism of drugs or toxins and are induced by the presence of drugs or toxin in the liver (Murray *et at,* 2006).

2.2.2 The cytochrome P450 enzyme system

Cytochrome p450 enzyme system involves a group of enzymes that are involved in drug metabolism, and are known to be found in high levels in the liver. The enzymes of the cytochrome p450 enzyme system alter many drugs including the anticancer drugs, into less toxic forms that are easier for the excretion from the body. Cytochrome p450 enzyme system is a super family of various related haemeproteins that are found throughout the phylogenetic spectrum, ranging from animals, plants, bacteria and fungi, and include numerous complex oxygenases, called mixed function oxygenases. These enzymes serve two major functions in animals, such as the biosynthesis of fatty acids, steroids, bile acids, and the metabolism of endogenous and a large variety of exogenous substrates including drugs and toxins.

Cytochrome p450 enzyme is classified with relation to the similarities of the sequences rather than its functions, into the CYP gene families, with more than its functions, into the CYP gene families, with more than 40% homology, and subfamilies with more than 59% homology (Keith *et al*, 2001). Drug metabolism is caused by many enzymes of the CYP_1, CYP_2 and CYP_3 gene families. The cytochrome P450 enzyme system substrates include the metabolic intermediates, like

the lipids, the steroid hormones, toxic chemicals and drugs. The commonest reaction that is catalysed by the cytochrome p450 enzyme system is a mono-oxgenase reaction that involves the insertion of an oxygen atom into an organic substrate, while the other oxygen atoms are reduced to water thus.

$RH + O_2 + 2H^+ + 2^{e-} \rightarrow ROH + H_2O$. where RH represents drug, carcinogen, pesticide, pollutant, steroids or eicosanoid in their reduced form.

The haeme iron centre is the active site of the cytochrome p450 enzyme system wherein the iron is tethered to the P450 protein through a thiolate ligand that is derived from the cysteine residue. The cysteine residue and the residues that are flanking to it are much conserved (Keith *et al*, 2001). There are about 60 genes of humans that code for the various enzymes of the cytochrome p450 enzyme system. Certain specific functions of cytchrome p450 enzymes include vital role in the synthesis of steroid hormones by the gonads, adrenal and peripheral tissues (Murray *et al,* 2006).

2.2.3 Toxic effects of xenobiotics

The central role of the liver in drug and toxin metabolism predisposes it to toxic injury, not only because of the bio-accumulation of toxins in the liver, but also because drug or toxin metabolism may go awry, leading to the formation of electrophilic toxic metabolites and immunogenic protein adducts. An imbalance between phase I and Phase II detoxification reactions can occur when a subject is exposed to large amounts of toxins, or exposed to toxins for a long period of time. In these situations, the critical nutrients needed for phase II detoxification are depleted, which allows the highly toxic activated intermediates to build up (Tredger and Sherwood, 1997). The toxic effects of xenobiotics include cell injury or cytotoxicity, which may be severe enough to result in cell death.

The mechanism by which xenobiotics injure cells include covalent binding to cell macromolecules, of reactive species of xenobiotics produced by metabolism. These macromolecular targets include DNA, RNA, and proteins. If the macromolecule to which the reactive xenobiotic binds is essential for short term cell survival, example, a protein or enzyme involved in some critical cellular function, such as oxidative phosphorylation or regulation of the permeability of the plasma

membrane, the severe effects on cellular function could become evident quite rapidly.

Also, the reactive species of a xenobiotic may bind to a protein, altering its antigencity. The xenobioitc is said to act as a hapten, ie a small molecule that by itself does not stimulate antibody synthesis but will combine with antibody once formed. The resulting antibodies can damage the cell by several immunologic mechanisms that grossly perturb normal cellular biochemical processes.

Furthermore, reactions of activated species of chemical carcinogen with DNA are thought to be of great importance in chemical carcinogenesis. Some chemical require activation by monooxygenases in the endoplasmic reticulum to become carcinogenic. Other chemical like various alkylating agents, can react directly with DNA without undergoing intracellular chemical activation (Murray *et al* 2006).

2.2.4 Enzyme markers of liver cell damage

Serum levels of numerous cytosolic, mitochondrial and membrane associated enzymes are increased in individuals with various forms of liver damage. They include aspartate transaminase (AST), Alanine transaminase (ALP), and gamma glutamyl transferase (GGT). The

detailed mechanism by which enzymes are released from the cytosol and mitochondria of hepatocytes into the blood stream are unknown. Clinical observations and experimental studies have shown that subtle membrane changes are sufficient to allow passage of intracellular enzymes to the extracellular space.

A very large concentration gradient between the hepatocyte and the sinusoidal space usually exists for enzymes. Cell damage increases membrane permeability causing cytosolic enzymes to spill into the sinusoids, and from there, into peripheral blood. Permeability of mitochondria membrane also is increased (Murray *et al,* 2006).

2.3 Excretion of toxins

Excretion of drugs and chemicals from the body occurs through intestinal, pulmonary or renal routes. Although each route represents a possible mechanism of toxin elimination, renal excretion is a major pathway for the elimination of most water soluble toxins metabolites.

Alteration in renal function may have a profound effect on the clearance and apparent half life of the parent compound or its active metabolites. Thus, decreased renal function causes elevated serum toxin concentration and increases the toxic response. The composition

of blood and urine reflects not only functional disorders of the nephron but also various systemic disorders (Burtis *et al,* 2001).

2.4 Preventing exposure to DPB

Di-n-butylphthalate is considered hazardous waste, and should be regulated as pollutant in air and water. Presently, phthalates, including DBP are unregulated in food, cosmetics, and medical products. Because they are found in everyday products, some exposures may be unavoidable. One way to reduce exposure is to switch to phthalate free cosmetics and body care products. Choose body care and cleaning products that do not list "fragrance" as an ingredient, eat less food packages in plastic and not microwave food in plastic containers.

Polytetheylene terephthalate ethylene (PETE) is the main substance used to package bottled water. Products containing PETE are labeled "Type 1": while some PVC plastics labeled "Type 3" contain phthalate esters. Although the world phthalate appear in the name, PETE does not contain phthalate plasticizers. The terephthalate polymer, PETE, and the phthalate ester plasticizers are chemically different substances (Rudel and Perovich, 2008). Plastic industries are hereby

advised to replace phthalate plasticizers with non-phthalate plasticizers, such as 1,2, cyclohexane dicarboxylic acid, diisononyl ester and citrates.

CHAPTER THREE

MATERIALS AND METHODS

3.1 SUBJECTS – ARTS

Twenty adult male albino Wistar rats, weighing between 146.10g and 301.20g were used for this study. They were obtained from the Animal House of University of Nigeria Teaching Hospital (UNTH), Enugu. The rats were arranged into four groups (A, B, C, D) of five rats each, and were kept for three days to acclimatize before initiating the study. They were allowed free access to clean drinking water, and were fed on standard rat feed (top feed), throughout the period of the study.

The rats in group A received no treatment and served as control, while those in groups B, C and D were treated orally with graded concentrations of DBP 2,000mg/kg, 4,000mg/kg and 6,000mg/kg body weight respectively for thirty days. Doses were chosen based on the LD50 value of 8,000mg/kg body weight. Two rats from each of the groups were sacrificed at the end of the treatment period, and their liver, kidney and testes were harvested.

3.2 Materials

The chemical, DBP was purchased from Analar grade Laboratory regents and chemical dealer at bridge Head market, Onitsha, Anambra State.

3.3 Specimen collection and preparation

The specimens were blood samples collected through the retro-bulbar plexus of the nasal canthus, at the end of thirty days, for the estimation of the chemical parameters. Serum samples were prepared from the whole blood as follows:

Three millitres of whole blood was collected into clean plain test tube.

This was allowed to clot and retract, and then centrifuged at 3,000 rpm for five minutes. The supernatant (serum) was separated from the sediment (red cells) and transferred into another clean plain test tube, for the chemical analyses.

Two rats from each group were killed by euthanasia, with chloroform, their liver, kidney and testes dissected out and fixed in 10% formol saline solution, for histological studies.

3.4 Analytical methods, principles and procedures

Histopathological examination

The liver, kidney, and testes tissues were processed in an automatic tissue processor and embedded in paraffin wax. Thin sections (about 4-5 microns thick) were cut using rotary microtome, and stained by heamatoxylin and eosin (H & E) method, and examined using a light microscope.(magnification X100)

Staining principle: Haematoxylin was used as a nuclear stain preceding staining of cytoplasm and connective tissues with eosin. Sections were stained with haematoxylin with a stain of sufficient power and for long enough to ensure some overstraining of nuclei, the superfluous and obscurative coloration of structures being removed by treatment with acid alcohol. Eosin stains connective tissues and cytoplasm in varying intensity and shades of primary colour giving a most useful differential stain with haematoxylin.

BIOCHEMICAL ANALYSIS OF SERUM SAMPLES

TESTOSTERONE AND ESTRADIOL ESTIMATION IN SAMPLES

Testosterone was analysed using Microwell Testosterone EIA Kit from Syntron Bioresearch, Inc,. Carlsbad, USA.

Estradiol was analyzed using Microwell Estradiol (E_2) EIA Kit from Syntron Bioresearch Inc., Carlsbad, USA.

The EIA measurement is highly reproducible with the co-efficient of less than 10%.

Principle of testosterone assay: The microwell testosterone enzyme immunoassay is a solid phase enzyme immunoassay utilizing the competitive binding principle. Testosterone present in the test sample competes with enzyme labeled testosterone for binding with anti testosterone antibodies immobilized on the microwell surface. The amount of conjugate that binds to the microwel surface decreases in proportion to the concentration of testosterone in test sample. The unbound sample and conjugate are then removed by washing and the colour development reagent (enzyme substrates) were added. Upon exposure to the bound enzyme, a colour change takes place. The intensity of the colour reflects the amount of bound enzyme-

testosterone conjugate and is inversely proportional to the concentration of testosterone in the test sample measured within dynamic range of the assay, using a spectrophotometer at 450nm. The testosterone concentration in the sample and concurrently run controls were determined from the standard curve.

Procedure for testosterone assay:

Incubation:

1. Bring the unopened zip-lock microwell bag test components and sample specimens to room temperature prior to testing.
2. Remove the desired number of microwells from the zip-lock bag and place in a well holder.
3. Dispense 25µL of each reference standard, control and test sample into the appropriate well. Complete pipetting within 5minutes.
4. Dispense 50µL of enzyme conjugate into each well. Gently rock the wells for 20 seconds, then seal by covering with parafilm.
5. Incubate at 37^{0C} for 60 minutes.
6. Remove and discard cover seal, decant the incubation mixture thoroughly by flicking into a sink with disinfectant.

7. Wash the microwell three times with diluted washing buffer and dry the wells by firmly tapping the plate on a clean paper towel to remove excess washing solution.

Colour development

1. Dispense one drop each of buffer hydrogen peroxide solution and buffered 3,3',5,5'-tetramethylbenzidine solution into each well. Gently rock the wells for twenty seconds, incubate at room temperature for 15 minutes.
2. Stop the reaction by adding one drop of 1N hydrochloric acid to each well and gently rock the wells
3. Read the absorbance at 450nm of each well no longer than 30 minutes after stopping the reaction.

Principle of estradiol assay: The microwell estradiol enzyme immunoassay is also a solid phase enzyme immunoassay utilizing the competitive binding principle. Estradiol present in the patient sample will compete with enzyme labeled estradiol for binding with rabbit anti-estradiol antibody linked with goat anti-rabbit immobilized on the microwell surface. The amount of conjugate that binds to the anti-

estradiol antibody is inversely proportional to the concentration of estradiol in the patient sample.

The unbound sample and conjugate are then removed by washing and colour development reagent (substrates) are added. Upon exposure to the bound enzyme, a colour change will take place. The intensity of the colour reflects the amount of bound enzyme-estradiol conjugate and is inversely proportional to the concentration of unlabeled estradiol in the patient sample within the dynamic range of assay. After stopping the reaction, the resulting colour is measured at 450nm using a spectrophotometer. The estradiol concentration in the concurrently run samples and controls can be determined from the standard curve.

Procedure for estradiol assay

Incubation

1. Bring the unopened zip-lock microwell bag test components and sample specimens to room temperature prior to testing.
2. Remove the desired number of microwells from the zip-lock bag and place in a well holder.

3. Dispense 50µL each of reference standard, control and sample into the appropriate well. Complete pipetting within five minutes.
4. Dispense 50µL of enzyme conjugate into each well. Gently rock the wells for twenty seconds, then seal by covering with parafilm. Incubate at 37^{0C} for 60 minutes.
5. Remove and discard the cover seal, decant the incubation mixture thoroughly by flicking into a container containing disinfectant.
6. Wash the microwells three times with diluted washing buffer and dry the wells by firmly tapping the plate on a clean paper towel to remove excess washing solution.

Colour development.

1. Dispense 50µL each of substrate and buffered chromogen solution into each well . Gently rock the wells for twenty seconds and incubate at room temperature for 15 minutes.
2. Stop the reaction by adding one drop of 1N H_2SO_4 to each well and gently rock the wells.
3. Read the absorbance at 450nm in a spectrophotometer within 30 minutes after stopping the reaction.

ALKALINE PHOSPHATASE ESTIMATION

Alkaline phosphatase activity was analyzed by the King and King method of 1954.

Principles of alkaline phosphatase assay: Alkaline phosphatases are a group of phosphomonoester enzyme which has optimal activity when the P^H is in neighborhood of 9.8. ALP hydrolyses disodiumphenylphosphate, librating phenol and phosphoric acid. The phenol is estimated by its reaction with 4-aminoantipyrene to produce a red complex in the presence of alkaline oxidizing agent. The coloured complex is measured photometrically at 500nm.

Procedure for alkaline phosphatase assay.

1. Set up four test tubes labeled test, test blank, standard, and standard blank respectively.
2. To the tube labeled test, and test blank, add 2ml of buffered substrate and warm for 5 minutes at 37^{0C} in a water bath.
3. Add 0.1ml serum to the test and incubate both tubes for 15 minutes at 37^{0C}.
4. To the tube labeled standard test add 1.1ml of buffer and 1ml of working phenol standard and mix.

5. To the tube labeled standard blank, add 1.1ml of buffer and 1ml of water and mix.
6. To all the tubes, add 0.8ml of 0.5N NaOH and 1.2ml 0.5N $NaHCO_3$ and mix.
7. Add 0.1ml serum sample to only the tube labeled test blank.
8. Add 1.0ml each of 4-aminoantipyrene and potassium ferricyanide to all the tubes and mix.
9. Read the absorbance immediately in a spectrophotometer at 520nm against the blank.

ESTIMATION OF TRANSMINASE ACTIVITIES

Aspartate and alanine transaminase actgivites where by Reitman and Frankel method (1959).

Principle of transaminase assay: Aspartate transaminase catalyses the reaction:- oxogluterate + L – aspartate $\xrightarrow{AST}$ Oxaloacetate + glutamate;

whereas alanine transaminase catalyses the reaction:-

oxogluterate + L – alanine $\xrightarrow{ALT}$ L – glutamate + pyruvate. The oxoacids produced couple with 2,4- dinitrophenylhydrazine to form

oxoacidhydrazone, (oxaloacetate hydrazone and pyruvate hydrazone) which in alkaline medium is reddish brown. The intensity of the colour read at 540nm is directly proportional to the enzyme concentration present in the sample. The ALT and AST concentration in each sample and concurrently run controls were determined by interpolation using the absorbance intensity obtained from AST and ALT standard curves respectively.

Procedure for ALT and AST assay.

1. Set up three test tubes labeled test, serum blank, and reagent blank.
2. Pipette 0.5mlof substrate into the three test tubes and warm at 37^{0C} in a water bath for 5 minutes.
3. Add 0.1ml serum to the tube labeled test and mix.
4. Incubate the three test tubes at 37^{0C} for 60 minutes for AST and 30 minutes for ALT.
5. At the end of incubation period add 0.5ml of 2,4-dinitrophenylhydrazone reagent to the three tubes, remove tubes from water bath and mix.
6. Allow to stand at room temperature for 20 minutes.

7. Add 5ml of 0.4N NaOH, mix and read the absorbance at 546nm against the blanks after 5 minutes.

For serum blank: Mix 0.5ml substrate and 0.5ml 2,4-dinitrophenylhydrizone and add 0.1ml serum then continue from stage 6.

For reagent blank: Mix 0.5ml substrate, 0.1ml water, and 0.5ml 2,4-dinitrophenylhydrazine and proceed as from stage 6.

BILIRUBIN ESTIMATION

Total and conjugated bilirubin were analysed by Groff and Gendrassic method.

Principle of bilirubin assay: Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total billirubin was determined in the presence of caffeine which releases albumin bound bilirubin. The intensity of the colour measured photometrically at 546nm is directly proportional to the

bilirubin concentration present in the sample and the result accepted when the value of the control serum is acceptable.

Procedure for bilirubin assay.

1. Set up four test tubes labeled total blank, total test, conjugated blank, and conjugated test for total and conjugated bilirubin respectively.
2. Pipette 0.2ml sulphanilic acid into the four tubes.
3. Add one drop of sodium nitrite to the tubes labeled total test and conjugated test.
4. Add 1ml of caffeine each to the tubes labeled total blank and total test, then 2ml of normal saline to tubes labeled conjugated test and blank.
5. Add 2ml of sample to the four tubes, mix and allow to stand at room temperature in the dark.
6. For conjugated bilirubin, read the absorbance of test sample against sample blank after 5 minutes at 546nm.
7. For total bilirubin, add 1ml of tartrate, mix and allow to stand for further 20 minutes at room temperature then read the absorbance against blank at 578nm.

SERUM UREA AND CREATININE ESTIMATION

Serum urea concentration was estimated by the modified diacetylmonoxime method of Wybenga et al. 1971; while serum creatinine, concentration was determined using alkaline picrate method of Fabiny and Ertingshaysen, (1971).

Principle of urea assay: In acid solution, diacetyl monoxime is hydrolysed to diacetyl, which reacts with urea to form a pink condensation product, which is measured at 480nm. The concentration of urea in each sample was determined by interpolation from urea standard curve.

Principle of creatinine assay: Creatinine in the sample react with picrate in alkaline medium, forming a red-orange coloured complex. The intensity of the colour read at 500nm is directly proportional to the concentration of creatinine present in the sample. The complex formation rate is measured in a short period to avoid interference from non-creatinine chromogens and creatinine present in the samples were interpolated from the crteatinine standard curve.

Procedure for urea assay.

1. Set up three test tubes labeled blank, standard, and test respectively.
2. Into each test tube add 2ml of mixed colour reagent and 2ml of mixed acid reagent.
3. Add 20µL of water, urea standard, and serum sample to the respectively labeled tubes.
4. Mix, plug with cotton wool and place in a boiling water bath for ten minutes.
5. Cool quickly in cold water and read the absorbance of the pink coloured product at 520nm against the blank.

Procedure for creatinine assay.

1. Set up three test tubes labeled blank, standard, and test.
2. Add 1.5ml 32mmol/L picric acid to the three test tubes.
3. Add 0.2ml of distilled water, creatinine standard, and serum sample to the respectively labeled tubes.
4. Mix and centrifuge for 5 minutes at 3,000rpm

5. To 1ml each of the supernatant fluid in another correspondingly labeled tubes, add 50µl of 0.2N NaOH, mix and stand at room temperature for 30 minutes.
6. Read the absorbance against reagent blank at 540nm.

5.3 ANALYSIS OF RESULTS

Results were expressed as mean $\pm$ standard error of mean. Significant difference between means were determined by one way analysis of variance (ANOVA) using statistical package for social sciences (SPSS).

5.4 STEPS TAKEN TO AVOID CONTAMINATION

Since phthalates frequently occur as plasticizers in analytical equipment and as contaminants in laboratory solvents, a great deal of care is needed to prevent contamination during the experiment. These precautionary measures include:

1. Glass and metal containers and feeders were used in feeding the rats during the treatment period.
2. Apparati used were washed scrupulously clean, free from detergents, with tap water before use.

CHAPTER FOUR

RESULTS

The results showed that the rats in the control group A, which received no treatment with the chemical, DBP, recorded 1.31% weight gain but the rats in groups B,C and D which were treated with graded concentrations of DBP recorded 2.02%, 2.11% and 1.19% weight loss respectively as detailed in table 1.

All the liver parameters of the treated rats were significantly high ($P<0.05$), except conjugated bilirubin which was significantly low ($P<0.05$) when compared with the untreated control group A, as shown in table 2.

There were significantly higher values, ($P<0.05$) of urea and creatinine in groups C (4.70$\pm$0.18Mmol/L, 85.40$\pm$1.47µmol/L) and D (6.30+0.13Mmol/L, 108.00$\pm$0.95µmol/L), which were fed with higher doses (4,00mg/kg and 6,000mg/kg respectively) of the toxicant, but their levels in group B (3.44$\pm$0.10Mmol/L, 71.80$\pm$1.43µmol/L) were not as highly significant ($P<0.05$) when compared with the control group A (3.28$\pm$0.08Mmol/L, 60.00$\pm$1.14µmol/L) as can be seen in table 3.

The mean levels of testosterone and estradiol in the treated groups B (3.22±0.14ng/ml, 113.80±1.28pg/ml), C (2.38±0.12ng/ml, 103.40±1.91pg/ml) and D (1.60±0.09ng/ml, 84.20±2.65pg/ml) were significantly low ($P<0.05$) when compared to the control group A (7.12±0.14ng/ml).

In summary, there was a progressive increase in variation in the levels of the biochemical parameters, with increase in doses of DBP given to the rats. The renal and liver parameters showed increases while reproductive parameters showed decreases.

The liver cell histology showed portal inflammation, mild fibrosis and nuclear pyknosis, while the kidney cell histology revealed vascular congestion, glomerular expansion and inflammation of some glomeruli. Finally, the testes cell histology showed normal seminiferous tubules but with reduced number of cells when compared with the control.

TABLE 1

WEIGHTS OF THE RATS AT THE ON-SET AND END OF THE EXPERIMENT (Mean ± Standard Error of Mean).

MEAN WEIGHT(g)	GROUP A	GROUP B	GROUP C	GROUP D
On– set of exp:	228.22±11.87	157.38±11.87	175.20±11.87	293.68±11.87
End of exp:	231.20±11.97	154.20±11.97	171.50±11.97	290.18±11.97
Diff in wt:	2.98±0.6	3.30±0.06	3.70±0.06	3.50±0.06
Percentage diff. in wt :	1.31%	2.02%	2.11%	1.19%

TABLE 2:

THE LEVELS OF INDICES OF LIVER FUNCTION

(Mean ± Standard Error of Mean)

PARAMETER	GROUP A	GROUP B	GROUP C	GROUP D
Total Bilirubin (µmol/L)	4.26±0.06	5.68±0.12	6.00±0.13	6.30±0.15
Test of significance: F (4,16) =58.229; P < 0.05				
Conji. Bilirubin (µmol/L)	3.40±0.06	2.14±0.04	2.18±0.05	2.22±0.02
Test of significance: F (4,16) =183.937; P < 0.05				
ALP (U/L)	32.40 ±2.20	50.60±1.29	76.80±0.92	100.00±1.78
Test of significance: F (4,16) =337.454; P < 0.05				
ALT (U/L)	15.00±1.10	19.00±0.55	26.60±1.08	27.20±1.40
Test of significance: F (4,16) = 30.780; P < 0.05				
AST (U/L)	23.00±0.84	31.60±1.70	44.40±1.40	64.20±1.50
Test of significance: F (4,16) =165.292 P< 0.05				

TABLE 3:

THE LEVELS OF INDICES OF RENAL FUNCTION

(Mean ± Standard Error of Mean)

PARAMETER	GROUP A	GROUP B	GROUP C	GROUP D
Urea (Mmol/L)	3.28±0.08	3.44±0.10	4.70±0.18	6.30±0.13
Test of significance: F (4,16) =115.145; P < 0.05				
Creatinine (µmol/L)	60.00±1.14	71.80±1.43	85.40±1.47	108.00±0.95
Test of significance: F (4,16) = 265.342; P < 0.05				

TABLE 4:

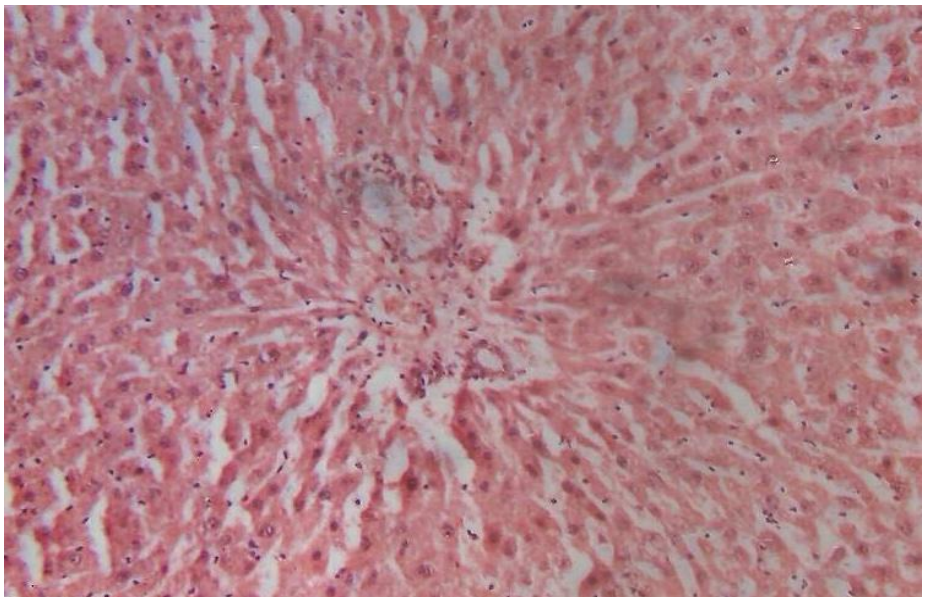
THE LEVELS OF INDICES OF PRODUCTIVE FUNCTION

(Mean ± Standard Error of Mean)

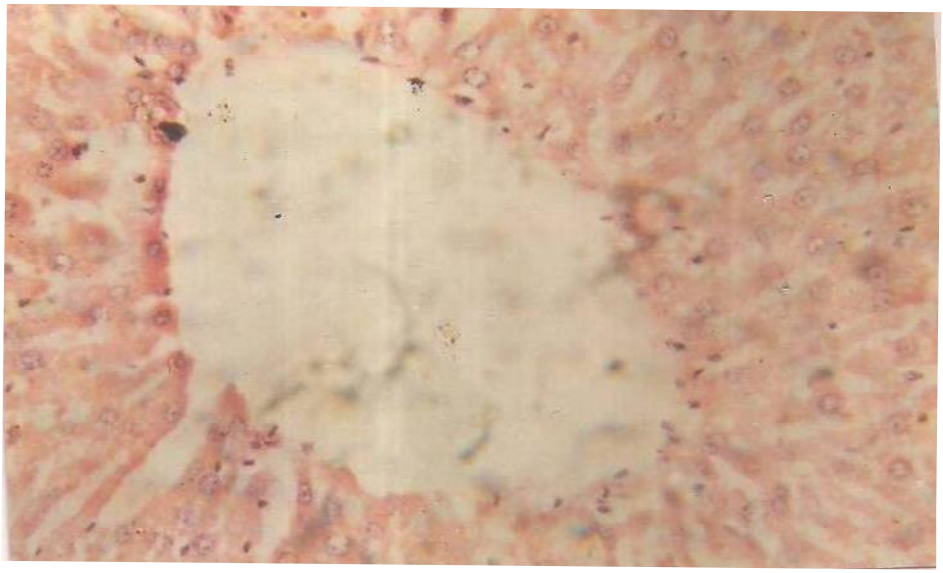
PARAMETER	GROUP A	GROUP B	GROUP C	GROUP D
Testosterone (ng/ml)	7.12±0.14	3.22±0.14	2.38±0.12	1.28±0.12
Test of significance: F (4,16) =381.964; P < 0.05				
Estradiol (pg/ml)	278.00±1.050	113.80±1.28	103.40±1.91	84.20±2.65
Test of significance: F (4,16) =126.732; P < 0.05				

Figure 1

Photomicrograph of liver cell histology



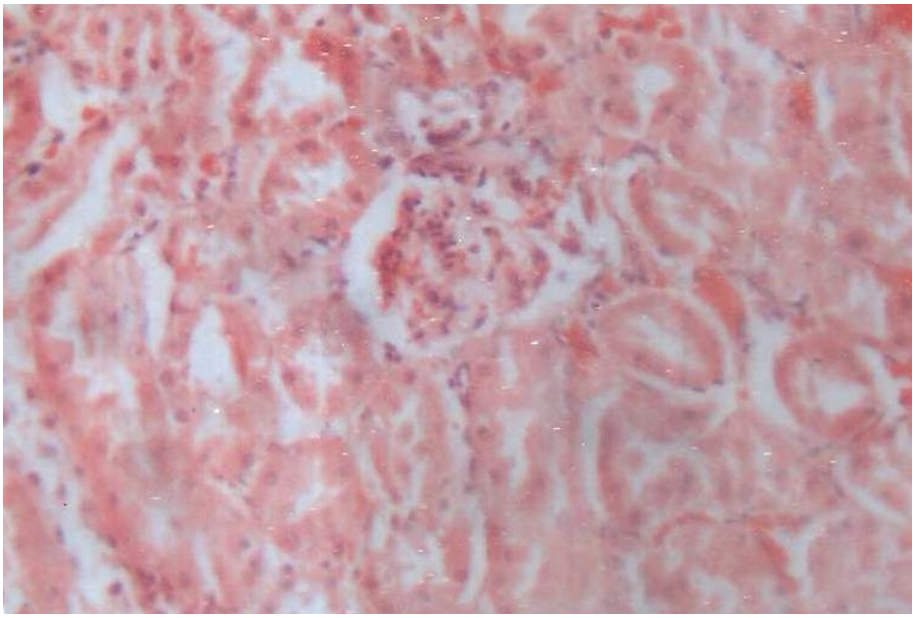
Control (No treatment with DBP)



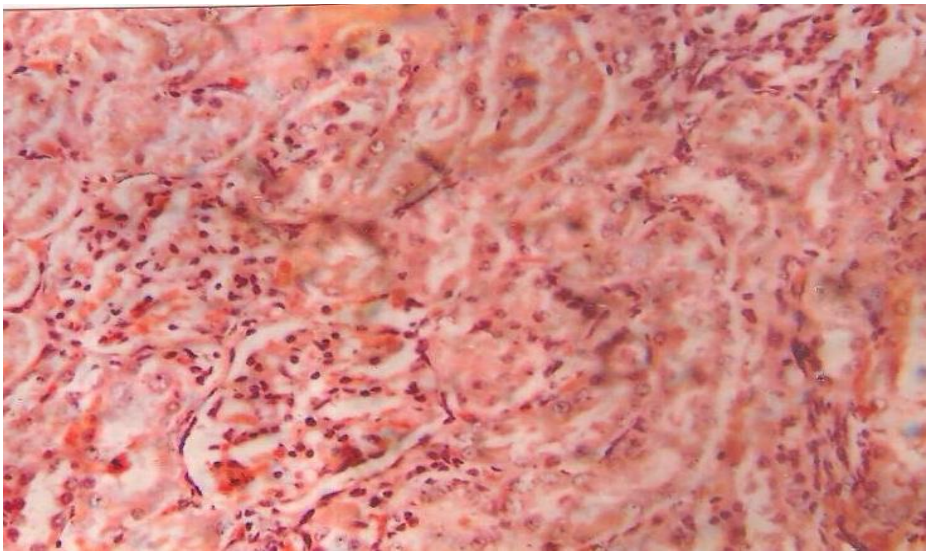
Test (treated with DBP)

Figure 2

Photomicrograph of kidney cell histology



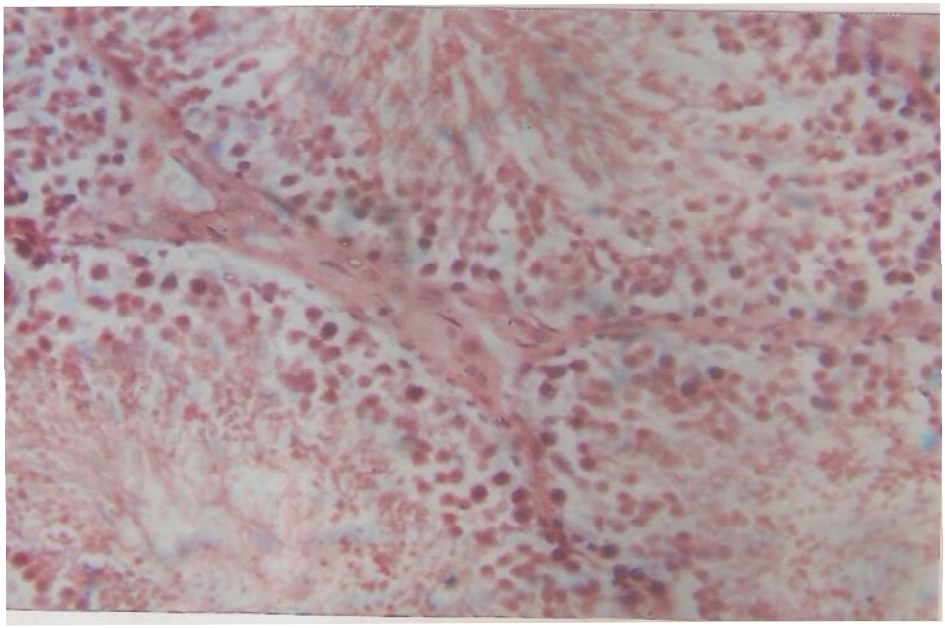
Control (No treatment with DBP)



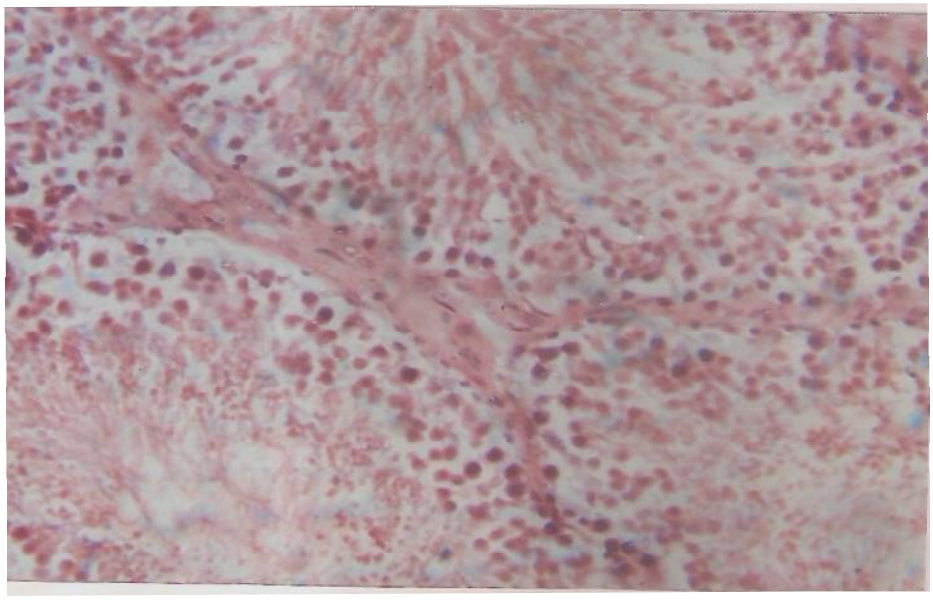
Test (Treated with DBP)

Figure 3

Photomicrograph of testes cell histology



Control (No treatment with DBP)



Test (Treated with DBP)

CHAPTER FIVE

DISCUSSIONS

The results of the sub-acute study on male albino Wistar rats, showed that di-n-butylphthalate (DBP) given orally to rats on daily doses of 2,000mg, 4,000mg and 6,000mg per kilogram body weight, produced some biochemical and pathological changes in the rats.

The results revealed a significantly higher total bilirubin in the treated rats and a significantly lower ($P < 0.05$) conjugated bilirubin level when compared with the control group. Foster *et al,* (1982) investigated the metabolism of DBP following oral and intravenous doses in rats and hamsters, and reported that monobutylphthalate (MBP) undergo glucuronidation in the liver forming monobutylphthalate glucuronide (MBP-GLUC), which is the major metabolite in urine. The decrease in the level of conjugated bilirubin may be attributed to the depletion or diversion of the available glucuronic acid to conjugation of MBP, hence elimination of the toxicant from the system.

The levels of serum ALP, ALT, and AST from the study were signifigantly higher ($P<0.05$) in the treated rats in comparison with the control rats. Elevated activities of these enzymes are a common sign of

toxic injury to the liver. It has also been shown by Litterest *et al,* (1972) that polychlorinated biphenyls, compounds structurally similar to DBP, can induce hepatic microsomal enzymes in rats in an attempt to eliminate the poison quickly. Similar process of enzyme induction and hepatocellular damage may also apply in this study and implicated as the cause of the increased liver enzyme activities.

It was further observed that there was significantly higher ($P < 0.05$) serum urea and creatinine levels in the treated rats when compared with the rats in the control group. This can be attributed to the impairment of the kidney, muscle wasting (weight loss), dehydration (diarrhea) caused by the toxicant. In addition, the reference interval for the ratio of serum urea nitrogen to serum creatinine for a normal subject on normal diet is 12 - 20:1 (Burtis *et al,* 2001). But from the results, all the rats in the treated groups had higher ratios (Group A 18:1, Group B 22:1, Group C 26:1 and Group D 27:1) with elevated creatinine, which is usually seen in post renal obstruction, pre-renal uremia superimposed on renal disease.

During this investigation, it was also noted that testosterone and estradiol were significantly lower ($P < 0.05$) in the treated rats compared with the control group. This is in agreement with the findings

of Samir *et al*, (2000), that one phthalate can cause reduction in level of estradiol in female rats. His cell studies showed that phthalates can replace estradiol on estrogen receptor sites. Main, and Skakkeback, (2006), reported that baby boys consuming breast milk high in certain phthalates tend to have altered body levels of both testosterone and ratio of leutenizing hormone to testosterone. Also Thompson et al, (2003) reported that in-utero exposure to di-n-butylphthalate leads to a variety of male reproductive abnormalities similar to those caused by androgen receptor antagonist. However, DBP demonstrates no affinity for the androgen receptor but rather leads to diminished testosterone production by the fetal testes, by diminishing cholesterol transport across the mitochondrial membrane as well as diminishing function at each point in the testosterone biosynthesis pathway except 17β-hydroxysteroid dehydrogenase. Therefore high dose DBP exposure led to the low levels of testosterone through diminution of the expression of several proteins required for cholesterol transport and steroidogensis.

Dosage effect was also observed among the treated rats, suggesting a hepatobilliary saturation occurring at the higher doses, hence clearance of DBP and its metabolites from the plasma being

higher at lower dose than at higher doses; implying more severe damage to the rats on higher doses.

It was also noted that after the first seven days of treatment, the treated groups began to feed less readily, showed dullness and had watery stool, in comparison with the control group. At the end of thirty days, the body weights of the control rats had significantly increased, unlike the treated rats that lost weight. All these are the symptoms of the damage done by the toxicant on the internal organs like intestines, liver and the kidney.

The kidney morphology showed some mild changes in the glomerular structure of the treated rats in comparison with the control group as indicated by glomerular expansion, inflammation and vascular congestion while the pathological changes in the liver morphology of the treated groups manifested as portal inflammation, mild fibrosis and nuclear pyknosis. The testes morphology of the treated rats showed normal seminiferous tubules with fewer cells when campared with the control rats, where the cells were more numerous. On the contrary, several development studies have shown testicular atrophy, being evident within a few days of DBP administration, suggesting that younger rats seem to be more susceptible than older ones. These

alternations in the structures of the liver, kidney and testes of the treated rats can be implicated in the corresponding alteration from normal, of the biochemical parameters assayed for, in the rats.

In conclusion, the toxicant, DBP altered the biochemical parameters in the treated animals by significant elevations and decreases in some parameters. Its toxicity on the liver, kidney and testes is also evident from the cell histology of these organs. By its ubiquitous and persistent distribution in the environment, reasonable quantities can easily be ingested.

DBP, being organotoxic, may affect sperm production in adult male albino Wistar rats and possibly lead to secondary infertility.

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APPENDIX I

INSTRUMENTS/EQUIPMENT USED

1. **Centrifuge:** 80 – 1 Electronic centrifuge. B Brain scientific and instrument company England.
2. **Refrigerator:** Westpoint refrigerator:
3. **Weighing balance:** Hangping YP601N: Mettler Toledo AB104 – S England.
4. **Incubator:** Galenkamp water bath, England.
5. **Spectorphotometer:** Spectronic 20 and Syntron MSR – 1000 ELISA Machine.
6. **Computer Software:** Statistical Package for Social Sciences (SPSS)

APPENDIX II

GLOSSARY

TABLE OF ABBREVIATIONS

Wt.	-	Weight
Conj. bil.	-	Conjugated bilirubin
Total bil.	-	Total bilirubin
ALP	-	Alkaline phosphatase
ALT	-	Alanine transaminase
AST	-	Aspartate transaminase
Ur	-	Urea
Cr	-	Creatinine
Testo.	-	Testosterone
Est.	-	Estradiol
Grp.	-	Group

APPENDIX III

RAW DATA

CONCENTRATIONS OF CHEMICAL PARAMETERS

S/NO	Wt. (g)	Tot. Bill (µMmol/L)	Conj. Bil (µMmol/L)	ALP (U/L)	ALT (U/L)	AST (U/L)	Ur. (Mmol/L)	Cr. (µmol/L)	Testo (ng/ml)	Est. (Pg/ml)	Grp.
1.	146.10	4.20	3.40	39.00	17.00	25.00	3.30	58.00	7.00	280.00	A
2	196.20	4.10	3.40	26.00	11.00	22.00	3.30	57.00	6.70	280.00	
3.	253.20	4.40	3.60	35.00	17.00	25.00	3.50	63.00	7.50	279.00	
4.	260.50	4.40	3.40	32.00	15.00	22.00	3.30	60.00	7.30	275.00	
5.	285.10	4.20	3.20	30.00	15.00	21.00	3.00	62.00	7.10	276.00	
6.	149.70	5.40	2.20	49.00	20.00	31.00	3.50	72.00	2.80	117.00	B
7.	152.20	5.40	2.20	49.00	19.00	28.00	3.30	72.00	3.30	112.00	
8.	157.40	5.80	2.10	52.00	19.00	36.00	3.30	72.00	350	114.00	
9.	163.70	6.00	2.20	55.00	20.00	35.00	3.30	67.00	3.50	110.00	
10.	163.90	5.80	2.00	48.00	17.00	28.00	3.80	76.00	3.00	116.00	
11	170.20	6.20	2.20	77.00	28.00	45.00	5.00	88.00	2.60	100.00	C
12.	170.20	5.80	2.00	75.00	26.00	42.00	5.00	85.00	2.60	101.00	
13.	175.20	5.80	2.20	75.00	24.00	45.00	4.70	88.00	2.20	109.00	
14.	180.20	5.80	2.20	77.00	25.00	41.00	4.50	80.00	2.00	107.00	
15.	180.20	6.40	2.30	80.00	30.00	49.00	4.80	86.00	2.50	100.00	
16.	286.20	6.60	2.30	98.00	24.00	60.00	6.00	105.00	1.50	82.00	D
17.	288.80	6.20	2.20	103.00	26.00	62.00	6.60	110.00	1.80	78.00	
18.	295.50	6.30	2.20	101.00	25.00	64.00	6.30	107.00	1.60	80.00	
19.	296.70	6.60	2.20	105.00	31.00	67.00	6.60	108.00	1.30	91.00	
20	301.20	5.80	2.20	95.00	30.00	68.00	6.00	110.00	1.80	90.00	

```
ONEWAY VAR00001 BY VAR00002
 /STATISTICS DESCRIPTIVES
 /MISSING ANALYSIS
 /POSTHOC=TUKEY BTUKEY GH ALPHA(0.05).
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Oneway

[DataSet0]

Descriptives

TOTAL BILIRUBIN

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Group A	5	4.2600	.13416	.06000	4.0934	4.4266	4.10	4.40
Group B	5	5.6800	.26833	.12000	5.3468	6.0132	5.40	6.00
Group C	5	6.0000	.28284	.12649	5.6488	6.3512	5.80	6.40
Group D	5	6.3000	.33166	.14832	5.8882	6.7118	5.80	6.60
Total	20	5.5600	.83817	.18742	5.1677	5.9523	4.10	6.60

ANOVA

TOTAL BILIRUBIN

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	12.228	3	4.076	58.229	.000
Within Groups	1.120	16	.070		
Total	13.348	19			

Post Hoc Tests

Multiple Comparisons

Dependent Variable :TOTAL BILIRUBIN

	(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Group A	Group B	-1.42000*	.16733	.000	-1.8987	-.9413
		Group C	-1.74000*	.16733	.000	-2.2187	-1.2613
		Group D	-2.04000*	.16733	.000	-2.5187	-1.5613
	Group B	Group A	1.42000*	.16733	.000	.9413	1.8987
		Group C	-.32000	.16733	.262	-.7987	.1587
		Group D	-.62000*	.16733	.009	-1.0987	-.1413
	Group C	Group A	1.74000*	.16733	.000	1.2613	2.2187
		Group B	.32000	.16733	.262	-.1587	.7987
		Group D	-.30000	.16733	.312	-.7787	.1787
	Group D	Group A	2.04000*	.16733	.000	1.5613	2.5187
		Group B	.62000*	.16733	.009	.1413	1.0987
		Group C	.30000	.16733	.312	-.1787	.7787
Games-Howell	Group A	Group B	-1.42000*	.13416	.000	-1.8874	-.9526
		Group C	-1.74000*	.14000	.000	-2.2324	-1.2476
		Group D	-2.04000*	.16000	.000	-2.6186	-1.4614
	Group B	Group A	1.42000*	.13416	.000	.9526	1.8874
		Group C	-.32000	.17436	.325	-.8787	.2387

		Group D	-.62000*	.19079	.049	-1.2371	-.0029
	Group C	Group A	1.74000*	.14000	.000	1.2476	2.2324
		Group B	.32000	.17436	.325	-.2387	.8787
		Group D	-.30000	.19494	.461	-.9279	.3279
	Group D	Group A	2.04000*	.16000	.000	1.4614	2.6186
		Group B	.62000*	.19079	.049	.0029	1.2371
		Group C	.30000	.19494	.461	-.3279	.9279

*. The mean difference is significant at the 0.05 level.

[DataSet0]

Descriptives

CONJUGATED BILIRUBIN

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Group A	5	3.4000	.14142	.06325	3.2244	3.5756	3.20	3.60
Group B	5	2.1400	.08944	.04000	2.0289	2.2511	2.00	2.20
Group C	5	2.1200	.10954	.04899	1.9840	2.2560	2.00	2.20
Group D	5	2.2200	.04472	.02000	2.1645	2.2755	2.20	2.30
Total	20	2.4700	.56017	.12526	2.2078	2.7322	2.00	3.60

ANOVA

CONJUGATED BILIRUBIN

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	5.794	3	1.931	183.937	.000
Within Groups	.168	16	.011		
Total	5.962	19			

Post Hoc Tests

Multiple Comparisons

CONJUGATED BILIRUBIN

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	1.26000*	.07483	.000	1.0099	1.5101
	Group C	1.28000*	.08000	.000	1.0201	1.5399
	Group D	1.18000*	.06633	.000	.9311	1.4289
Group B	Group A	-1.26000*	.07483	.000	-1.5101	-1.0099
	Group C	.02000	.06325	.988	-.1844	.2244
	Group D	-.08000	.04472	.364	-.2358	.0758
Group C	Group A	-1.28000*	.08000	.000	-1.5399	-1.0201
	Group B	-.02000	.06325	.988	-.2244	.1844
	Group D	-.10000	.05292	.333	-.2911	.0911
Group D	Group A	-1.18000*	.06633	.000	-1.4289	-.9311
	Group B	.08000	.04472	.364	-.0758	.2358
	Group C	.10000	.05292	.333	-.0911	.2911

*. The mean difference is significant at the 0.05 level.

[DataSet0]

Descriptives

ALP								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Group A	5	32.4000	4.92950	2.20454	26.2792	38.5208	26.00	39.00
Group B	5	50.6000	2.88097	1.28841	47.0228	54.1772	48.00	55.00
Group C	5	76.8000	2.04939	.91652	74.2553	79.3447	75.00	80.00
Group D	5	1.0040E2	3.97492	1.77764	95.4645	105.3355	95.00	105.00
Total	20	65.0500	26.67835	5.96546	52.5641	77.5359	26.00	105.00

ANOVA

ALP					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	13312.550	3	4437.517	337.454	.000
Within Groups	210.400	16	13.150		
Total	13522.950	19			

Post Hoc Tests

Multiple Comparisons

ALP

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	-18.20000*	2.55343	.001	-26.8485	-9.5515
	Group C	-44.40000*	2.38747	.000	-52.9944	-35.8056
	Group D	-68.00000*	2.83196	.000	-77.1635	-58.8365
Group B	Group A	18.20000*	2.55343	.001	9.5515	26.8485
	Group C	-26.20000*	1.58114	.000	-31.3910	-21.0090
	Group D	-49.80000*	2.19545	.000	-56.9899	-42.6101
Group C	Group A	44.40000*	2.38747	.000	35.8056	52.9944
	Group B	26.20000*	1.58114	.000	21.0090	31.3910
	Group D	-23.60000*	2.00000	.000	-30.5285	-16.6715
Group D	Group A	68.00000*	2.83196	.000	58.8365	77.1635
	Group B	49.80000*	2.19545	.000	42.6101	56.9899
	Group C	23.60000*	2.00000	.000	16.6715	30.5285

*. The mean difference is significant at the 0.05 level.

[DataSet0]

Descriptives

ALT								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Group A	5	15.0000	2.44949	1.09545	11.9586	18.0414	11.00	17.00
Group B	5	19.0000	1.22474	.54772	17.4793	20.5207	17.00	20.00
Group C	5	26.6000	2.40832	1.07703	23.6097	29.5903	24.00	30.00
Group D	5	27.2000	3.11448	1.39284	23.3329	31.0671	24.00	31.00
Total	20	21.9500	5.72598	1.28037	19.2702	24.6298	11.00	31.00

ANOVA

ALT					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	530.950	3	176.983	30.780	.000
Within Groups	92.000	16	5.750		
Total	622.950	19			

Post Hoc Tests

Multiple Comparisons

ALT

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	-4.00000	1.22474	.064	-8.2666	.2666
	Group C	-11.60000*	1.53623	.000	-16.5199	-6.6801
	Group D	-12.20000*	1.77200	.001	-17.9478	-6.4522
Group B	Group A	4.00000	1.22474	.064	-.2666	8.2666
	Group C	-7.60000*	1.20830	.003	-11.7963	-3.4037
	Group D	-8.20000*	1.49666	.009	-13.6380	-2.7620
Group C	Group A	11.60000*	1.53623	.000	6.6801	16.5199
	Group B	7.60000*	1.20830	.003	3.4037	11.7963
	Group D	-.60000	1.76068	.985	-6.3214	5.1214
Group D	Group A	12.20000*	1.77200	.001	6.4522	17.9478
	Group B	8.20000*	1.49666	.009	2.7620	13.6380
	Group C	.60000	1.76068	.985	-5.1214	6.3214

*. The mean difference is significant at the 0.05 level.

DataSet0]

Descriptives

AST								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Group A	5	23.0000	1.87083	.83666	20.6771	25.3229	21.00	25.00
Group B	5	31.6000	3.78153	1.69115	26.9046	36.2954	28.00	36.00
Group C	5	44.4000	3.13050	1.40000	40.5130	48.2870	41.00	49.00
Group D	5	64.2000	3.34664	1.49666	60.0446	68.3554	60.00	68.00
Total	20	40.8000	16.16559	3.61474	33.2343	48.3657	21.00	68.00

ANOVA

AST					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	4810.000	3	1603.333	165.292	.000
Within Groups	155.200	16	9.700		
Total	4965.200	19			

post Hoc Tests

Multiple Comparisons

AST

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	-8.60000*	1.88680	.016	-15.1857	-2.0143
	Group C	-21.40000*	1.63095	.000	-26.9027	-15.8973
	Group D	-41.20000*	1.71464	.000	-47.0535	-35.3465
Group B	Group A	8.60000*	1.88680	.016	2.0143	15.1857
	Group C	-12.80000*	2.19545	.002	-19.8874	-5.7126
	Group D	-32.60000*	2.25832	.000	-39.8566	-25.3434
Group C	Group A	21.40000*	1.63095	.000	15.8973	26.9027
	Group B	12.80000*	2.19545	.002	5.7126	19.8874
	Group D	-19.80000*	2.04939	.000	-26.3696	-13.2304
Group D	Group A	41.20000*	1.71464	.000	35.3465	47.0535
	Group B	32.60000*	2.25832	.000	25.3434	39.8566
	Group C	19.80000*	2.04939	.000	13.2304	26.3696

*. The mean difference is significant at the 0.05 level.

[DataSet0]

Descriptives

UREA								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Group A	5	3.2800	.17889	.08000	3.0579	3.5021	3.00	3.50
Group B	5	3.4400	.21909	.09798	3.1680	3.7120	3.30	3.80
Group C	5	4.7000	.41231	.18439	4.1880	5.2120	4.00	5.00
Group D	5	6.3000	.30000	.13416	5.9275	6.6725	6.00	6.60
Total	20	4.4300	1.27159	.28434	3.8349	5.0251	3.00	6.60

ANOVA

UREA					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	29.362	3	9.787	115.145	.000
Within Groups	1.360	16	.085		
Total	30.722	19			

Post Hoc Tests

Multiple Comparisons

UREA

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	-.16000	.12649	.608	-.5688	.2488
	Group C	-1.42000*	.20100	.002	-2.1383	-.7017
	Group D	-3.02000*	.15620	.000	-3.5472	-2.4928
Group B	Group A	.16000	.12649	.608	-.2488	.5688
	Group C	-1.26000*	.20881	.004	-1.9794	-.5406
	Group D	-2.86000*	.16613	.000	-3.4035	-2.3165
Group C	Group A	1.42000*	.20100	.002	.7017	2.1383
	Group B	1.26000*	.20881	.004	.5406	1.9794
	Group D	-1.60000*	.22804	.001	-2.3464	-.8536
Group D	Group A	3.02000*	.15620	.000	2.4928	3.5472
	Group B	2.86000*	.16613	.000	2.3165	3.4035
	Group C	1.60000*	.22804	.001	.8536	2.3464

*. The mean difference is significant at the 0.05 level.

[DataSet0]

Descriptives

CREATININE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Group A	5	60.0000	2.54951	1.14018	56.8344	63.1656	57.00	63.00
Group B	5	71.8000	3.19374	1.42829	67.8344	75.7656	67.00	76.00
Group C	5	85.4000	3.28634	1.46969	81.3195	89.4805	80.00	88.00
Group D	5	1.0800E2	2.12132	.94868	105.3660	110.6340	105.00	110.00
Total	20	81.3000	18.49068	4.13464	72.6461	89.9539	57.00	110.00

ANOVA

CREATININE

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	6368.200	3	2122.733	265.342	.000
Within Groups	128.000	16	8.000		
Total	6496.200	19			

Post Hoc Tests

Multiple Comparisons

CREATININE

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	-11.80000*	1.82757	.001	-17.7192	-5.8808
	Group C	-25.40000*	1.86011	.000	-31.4423	-19.3577
	Group D	-48.00000*	1.48324	.000	-52.7862	-43.2138
Group B	Group A	11.80000*	1.82757	.001	5.8808	17.7192
	Group C	-13.60000*	2.04939	.001	-20.1641	-7.0359
	Group D	-36.20000*	1.71464	.000	-41.8857	-30.5143
Group C	Group A	25.40000*	1.86011	.000	19.3577	31.4423
	Group B	13.60000*	2.04939	.001	7.0359	20.1641
	Group D	-22.60000*	1.74929	.000	-28.4266	-16.7734
Group D	Group A	48.00000*	1.48324	.000	43.2138	52.7862
	Group B	36.20000*	1.71464	.000	30.5143	41.8857
	Group C	22.60000*	1.74929	.000	16.7734	28.4266

*. The mean difference is significant at the 0.05 level.

[DataSet0]

Descriptives

TESTOSTERONE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Group A	5	7.1200	.30332	.13565	6.7434	7.4966	6.70	7.50
Group B	5	3.2200	.31145	.13928	2.8333	3.6067	2.80	3.50
Group C	5	2.3800	.26833	.12000	2.0468	2.7132	2.00	2.60
Group D	5	1.2800	.27749	.12410	.9355	1.6245	1.00	1.60
Total	20	3.5000	2.27318	.50830	2.4361	4.5639	1.00	7.50

ANOVA

TESTOSTERONE

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	96.828	3	32.276	381.964	.000
Within Groups	1.352	16	.085		
Total	98.180	19			

Post Hoc Tests

Multiple Comparisons

TESTOSTERONE

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	3.90000*	.19442	.000	3.2773	4.5227
	Group C	4.74000*	.18111	.000	4.1580	5.3220
	Group D	5.84000*	.18385	.000	5.2502	6.4298
Group B	Group A	-3.90000*	.19442	.000	-4.5227	-3.2773
	Group C	.84000*	.18385	.008	.2483	1.4317
	Group D	1.94000*	.18655	.000	1.3408	2.5392
Group C	Group A	-4.74000*	.18111	.000	-5.3220	-4.1580
	Group B	-.84000*	.18385	.008	-1.4317	-.2483
	Group D	1.10000*	.17263	.001	.5470	1.6530
Group D	Group A	-5.84000*	.18385	.000	-6.4298	-5.2502
	Group B	-1.94000*	.18655	.000	-2.5392	-1.3408
	Group C	-1.10000*	.17263	.001	-1.6530	-.5470

*. The mean difference is significant at the 0.05 level.

[DataSet0]

Descriptives

ESTRADIOL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Group A	5	2.7800E2	2.34521	1.04881	275.0880	280.9120	275.00	280.00
Group B	5	1.1380E2	2.86356	1.28062	110.2444	117.3556	110.00	117.00
Group C	5	1.0340E2	4.27785	1.91311	98.0883	108.7117	100.00	109.00
Group D	5	66.4000	36.89580	16.50030	20.5878	112.2122	1.00	91.00
Total	20	1.4040E2	85.22564	19.05703	100.5132	180.2868	1.00	280.00

ANOVA

TRADIOL

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	132431.600	3	44143.867	126.732	.000
Within Groups	5573.200	16	348.325		
Total	138004.800	19			

Post Hoc Tests

Multiple Comparisons

ESTRADIOL

Games-Howell

(I) CATEGORY	(J) CATEGORY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	164.20000*	1.65529	.000	158.8514	169.5486
	Group C	174.60000*	2.18174	.000	167.1258	182.0742
	Group D	211.60000*	16.53360	.001	144.5624	278.6376
Group B	Group A	-164.20000*	1.65529	.000	-169.5486	-158.8514
	Group C	10.40000*	2.30217	.011	2.7752	18.0248
	Group D	47.40000	16.54992	.140	-19.5741	114.3741
Group C	Group A	-174.60000*	2.18174	.000	-182.0742	-167.1258
	Group B	-10.40000*	2.30217	.011	-18.0248	-2.7752
	Group D	37.00000	16.61084	.255	-29.7452	103.7452
Group D	Group A	-211.60000*	16.53360	.001	-278.6376	-144.5624
	Group B	-47.40000	16.54992	.140	-114.3741	19.5741
	Group C	-37.00000	16.61084	.255	-103.7452	29.7452

*. The mean difference is significant at the 0.05 level.