Experimental Model:

Swiss Albino Male Mice, 6-8 weeks old and of same weight were selected randomly from a colony which is maintained under controlled condition of temperature light and humidity. The selected animals were kept in separate cages. Females of same age as that of male mice were introduced in the cages 1 week prior to autopsy date.

The animals were divided in the following groups based on the quantity and type of food:

**Group I:** Control

**Group II:** Sham

**Group III:** Experimental

The animals of experimental group were given food pellets exposed to a microwave radiation in microwave oven at 320 watt for 10 minutes. The sham group was given the same food pellets but not microwaved and in low quantity whereas control was given normal food in sufficient amount. The experimental group was administered with fixed amount of microwave exposed mice pellets daily for 2 weeks (Experiment 1), 3 weeks (Experiment 2) and 4 weeks (Experiment 3). The recovery group (Experiment 4) was given microwave pellets for 4 weeks, thereafter given normal mice fed for 4 weeks. After the termination of each experiment the animals of each group, i.e. treated, sham and control, were sacrificed by cervical dislocation blood was collected and liver was perfused carefully with cold saline whereas the testis, seminal vesicle and prostate were collected in 0.1% NaCl. The body weight and organ weight (testis) was recorded at each intervals.
Dose
The daily mice feed consists of pellets manufactured at Hindustan Lever Pvt. Ltd. The feed which is prepared from whole wheat, corn, jawar etc. is rich in proteins, amino acids, carbohydrates, fats, fiber, vitamins, minerals and other essential nutrients. The other cereal grains used in the feed are soya, maize, ground nut cake, cannula seed cake, roasted black gram, wheat flakes, and rice polish/bran. Besides this, ingredients like skimmed milk powder, casein, calcium, edible oil (refined) and molasses are also present. Mineral mixture in the diet ensures the presence of vitamin A, D, E, K, C and B complex along with choline which are of prime importance for proper growth of laboratory animals. The mineral mixture constituting all the above referred essential vitamins along with calcium carbonate, calcium pantothenate, niacin, vitamin B₁₂, choline chloride, manganese, iodine, iron, zinc, copper and cobalt is added to the feed (as per the information provided by manufacturer).

On the basis of preliminary study and careful observation on the normal diet of an individual animal it was determined that 15g of microwave exposed mice pellets should be fed orally to each mice daily.

Experimental Design

Parameters

a) Nutritional assay

Male organism for its normal reproductive function has certain nutrient requirements. The greatest difficulty encountered in the appraisal of reproductive disorders due to ‘malnutrition’ is to distinguish between primary effects due to the lack of a specific
dietary component such as a vitamin, a trace element, or an essential amino acid, and secondary effects arising from diminished appetite, inadequate food intake and, finally, inanition, all of which are sequel of deficient diets. Therefore, a nutritional assay was performed, wherein essential parameters were taken into consideration such as

- Carbohydrate
- Total Protein
- Magnesium
- Zinc
- Vitamins: Vitamin A, Vitamin C, Vitamin D, Vitamin E

b) Physical Test

**Sperm Count**

A small aliquot of sperm suspension is diluted with sperm diluting fluid and counted on a hemocytometer. This count is used to calculate the total number of sperms per epididymis (cauda region) and calculated by the equation:

\[ \text{Concentration/ml} = (\text{Dilution Factor}) \times (\text{Count in 5 squares})(0.05 \times 10) \]

By convention, sperm concentration is usually expressed in terms of sperm \(X10^6/\text{ml}\).

**Sperm Morphology**

Sperm abnormality was tested according to the method of Wyrobek et al (1975). The epididymes were excised and minced with fine scissors in physiological saline in a petri dish. Smears were made on clean, greasefree slides with a mixture of normal saline (9:1) for 45 min. The slides were air-dried and stained with Papanicolaou stain
for subsequent examination under microscope and different abnormalities were recorded. Cytological evaluation for sperm abnormalities was carried out using a binocular microscope. The following categories of defects were recorded and observed: Head shape/size defects or double heads, or any combination of these. Neck and midpiece defects, including absence of tail, non inserted or bent tail, abnormally thin midpiece or any combination of these. Tail defects, including short, multiple, broken, irregular width, coiled tails, tails with terminal droplets, or any combination of these and cytoplasmic droplets greater than one-third of the area of a normal sperm head.

**Gonadotropic Ratio**

The body weight and organ weight (testis) was recorded at each interval. Gonadotropic ratio is expressed as ratio of organ weight to body weight.

c) Hormonal Assay

**Follicle Stimulating Hormone (FSH)**

FSH is secreted by the anterior lobe of pituitary under the influence of hypothalamic gonadotrophic releasing hormone (GnRH). FSH facilitates the development and maintenance of gonads in both males and females. Gonadal tissues synthesize and secrete steroidal hormones particular to sex, which regulates FSH concentrations by negative feedback mechanism. High level of FSH are reported in males when the testes fail to attain functional maturity, or, in cases of infertility due to primary testicular failure, due to any reason.
Total FSH levels were analyzed in serum via Radioimmunoassay (RIA) by outsourcing.

**Testosterone**

Testosterone is the principal hormone among the androgens secreted by male. This steroid hormone primarily is secreted in the testes of males, and also by the ovaries of females. The adrenal gland also secretes this hormone, although in small amounts. It plays important role in health and well being of both males and females. Measurement of the free or unbound fraction of serum testosterone has been proposed as a mean of estimating the physiologically bioactive hormone, therefore, testosterone levels were analyzed in serum via Radioimmunoassay (RIA) by outsourcing.

**Luteinizing hormone (LH)**

The anterior lobe of pituitary under the influence of gonadotropic releasing hormone (GnRH) secreted by hypothalamus in both males and females produces LH. In females this hormone is accountable for cyclic changes that occur during menstrual cycle, including maturation of graafian follicle, ovulation and steroid production. In males LH along with FSH bring about maturation of spermatozoa in the seminiferous tubules. Main function of LH is to produce testosterone by the stimulation of interstitial cells .Any change in the level of LH leads to dysfunctioning of reproductive organs, and disturbance of reproductive cycles. Therefore, total LH levels were analyzed in serum via radioimmunoassay (RIA) by outsourcing.
d) Biochemical Studies

**Fructose**

Fructose is the primary source of energy for sperm. The level of fructose is androgen-dependent and it is produced in the seminal vesicles. It was estimated by Seliwanoff’s Method (1887) using 5% Perchloric acid and Resorcinol reagent as the essential chemicals in its estimation.

**Principle:**

Seliwanoff's test is based on the principle in which ketoses are dehydrated on heating and forms 5-hydroxy methyl furfural which reacts with resorcinol to form a red coloured complex. The intensity of color is directly proportional to the amount of fructose in the sample.

**Calculation:**

\[
\text{Reading of unknown} \times \text{conc. of standard} = \text{Conc. in mg/gm}
\]

Reading of standard

Readings were taken using spectrophotometer at 540nm wavelength.

**Reduced glutathione (GSH)**

GSH level in the hepatocytes was determined by the method as described by Moron *et al.* method (1979).
Principle

DTNB (5,5’-Dithiobis(2-nitrobenzoic acid)), known as Ellman’s Reagent, was developed for the detection of thiol compounds. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in a sample solution can be determined by the measurement at 412 nm. GSH is generated from GSSG by glutathione reductase, and reacts with DTNB again to produce 2-nitro-5-thiobenzoic acid. Therefore, this recycling reaction improves the sensitivity of total glutathione detection.

Calculation

\[
\text{Reading of unknown} \times \text{conc. of standard} = \text{Conc. in mg/gm}
\]

Reading of standard

Readings were taken using spectrophotometer at 412 nm wavelength.

**Cholesterol**

The concentration of cholesterol was estimated by Lieberman and Buchard’s reaction method (1952).

Principle:

The method is based on Lieberman and Buchard’s reaction. The reaction involves 3-hydroxyl-5-ene part of cholesterol 3, 5-diene and then oxidize by H\textsubscript{2}SO\textsubscript{4} to link two molecules together as bis-cholesterol-3,5-diene. This compound is further sulphonated by H\textsubscript{2}SO\textsubscript{4} to produce mono or di sulphonic acid which is highly coloured
(green). In the presence of ferric ions, disulphonic acids are red coloured complex. The intensity of color is proportional to the concentration of cholesterol.

Calculations:

\[
\text{Reading of unknown} \times \text{Conc. of standard} \times 1000 = \text{Conc. in mg/gm}
\]

\[
\text{Reading of standard} \times \text{weight of tissue taken}
\]

Readings were taken using spectrophotometer at 540nm wavelength.

**Glycogen**

Glycogen was estimated quantitatively by Montogomery’s (1957) method.

Principle:

The procedure is based on the phenolic sulfuric acid method for determination of alkali soluble polysaccharides present in a tissue. Carbohydrates in presence of sulfuric acid and phenol undergo dehydration within the formation of hydroxyl baldheads which are subsequently converted to furfural derivatives. The reaction gives rise to a pink color. The intensity of which is proportional to the amount of glycogen present.

Calculations:

\[
\text{Reading of unknown} \times \text{Conc. of standard} \times 1000 \times 4 = \text{mg/gm tissue taken}
\]

\[
\text{Reading of standard} \times \text{tissue taken} \times 2
\]

Where,

4 = Amount of precipitate dissolved in 4 ml of distilled water.

2 = Volume taken from the above in a tube for estimation.
**Materials and Methods**

Readings were taken using colorimeter at 570 nm wavelength.

**pH(Seminal vesicle, Prostrate)**

pH has pivotal role in providing motility to sperm. The pH is determined by acidic secretions of the prostate and alkaline secretions of the seminal vesicles. It should normally be in the range of 7.2-8.0.

To test pH, pH paper range 6.1 to 10.0 is used. If the pH exceeds 8.0, infection should be suspected with decreased secretion of acidic products by the prostate, such as citric acid. Abnormal pH may also be recorded in cases of incomplete ejaculation. Extremely acidic pH (<6.5) is found in cases of agenesis (or occlusion) of the seminal vesicles.

**Sialic Acid**

This was estimated by Svennerholm’s (1956) method as given by Glick (1960).

Principle:

Sialic acid, a class of important ketoses that contain nine carbon atoms, are acetylated derivatives of neuraminic acid (2-keto-5-amino-3, 5-dideoxy-D-nonulosonic acid. The principle of this method depends on the formation of chromogen from the addition of resorcinol reagent in to the test tube. The chromogen formed was extracted by butyl acetate/methanol reagent and measured at 580 nm. Sialic acid exhibits purple colour in an acidic medium with resorcinol.

Calculations:

\[
\text{Reading of unknown} \times \text{concentration of standard} \times 1000 = \text{mg/gm of sialic acid}
\]

\[
\text{Reading of known} \times \text{tissue taken}
\]
Colorimetric readings were taken using green filter of 580 nm.

**Alkaline Phosphatase (ALP)**

Quantitative biochemical estimation of Alkaline Phosphatase (ALP) marker for bone destruction enzyme activity was estimated by outsourcing (by using ALP kit (Accurex). Alkaline Phosphatase is based on kinetic method using p-nitrophenyl phosphate (p- NPP). In this method, ALP catalyzes the hydrolysis of ρ-Nitrophenyl phosphate (pNPP) to ρ-Nitrophenol. pNPP is colorless but ρ-Nitrophenol has a strong absorbance at 405 nm. The rate of increased absorbance at 405 nm is proportional to the enzyme activity. The procedure is standardized by means of the millimolar absorptivity of ρ-Nitrophenol (18.75 at 405 nm) under the specified conditions. The results are based on the change in absorbance per unit of time. Accurex Autozyme Acid Phosphatase is based on kinetic method using α- naphthylphosphate. Blood samples were outsourced to veterinary diagnostic centre.

**Acid Phosphatase (ACP)**

Quantitative biochemical estimation of Acid Phosphatase (ACP) marker for lysosomal activity was estimated by outsourcing (by using ALP kit (Accurex). Naphthol acid phosphate is hydrolyzed by acid phosphatases present in the tissue, and napthol derivatives are produced. The napthol derivatives couple with the unstable diazonium salt, hexazonium pararosanilin, to produce a red azo dye to mark the site of enzyme activity. Blood samples were outsourced to veterinary diagnostic centre.
d) Histological study of Testis

Collected tissues (tissue) after being washed in saline were fixed in Bouin’s fluid. The tissues were dehydrated by passing through alcohol and processed in paraffin wax. The tissue is cut in 5 microns thick sections with help of microtome. Staining was done with Haematoxylin and Eosin and then it was permanently mounted. These slides were examined and histology and histological deformities were observed.

STATISTICAL ANALYSIS

The data obtained by present set of experiments was subjected to statistical analysis. The statistical calculations are based on biological statistics. The values are expressed as mean ± standard error (SE). The data were analysed statistically using student’s “t” test. The possibility for obtaining “t” value for a given Degree of Freedom (df) was determined by comparing the “t” values. The “p” values were signified according to the following convention:

<table>
<thead>
<tr>
<th>$P$</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&gt; 0.05$</td>
<td>Non significant</td>
</tr>
<tr>
<td>$&lt;0.05$</td>
<td>Significant</td>
</tr>
<tr>
<td>$&lt;0.01$</td>
<td>Highly Significant</td>
</tr>
<tr>
<td>$&lt;0.001$</td>
<td>Very Highly Significant</td>
</tr>
</tbody>
</table>