4.0 MATERIALS AND METHODS

4.1 Reagents:

i) Spectroscopic grade glycerol. Marck Limited, Worli, Mumbai

ii) Potassium phosphate (K_2HPO_4). Marck Limited, Worli, Mumbai

iii) Ethylene diamine tetra acetic acid (EDTA). Marck Limited, Worli, Mumbai.

iv) Dehydroepiandrosterone (DHEA). Marck Limited, Worli, Mumbai

v) Nicotinamide adenine dinucleotide (NAD). Marck Limited, Worli, Mumbai

vi) Tetrasodium pyrophosphate (TNaPP). Marck Limited, Worli, Mumbai

vii) Testosterone. Marck Limited, Worli, Mumbai

viii) Ecoline ALAT (GPT) and ASAT (GOT) Kit. Marck Limited, Worli, Mumbai.

ix) Alkaline Phosphatase Kit. Marck Limited, Worli, Mumbai.

x) Urea Kit and Creatinine Kit. Coral Clinical System-Tulip Diagnostics, Goa, India.

xi) LDH (P-L) Kit. Coral Clinical System-Tulip Diagnostics, Goa, India.

xii) Estimation of FSH by Rat LH ELISA Kit (Wuhan Fine Biotech Co., Ltd.)

xiii) Estimation of LH by Rat LH ELISA Kit (Wuhan Fine Biotech Co., Ltd.).

xiv) Estimation of Estradiol by Rat E2 ELISA Kit (Wuhan Fine Biotech Co., Ltd.).

xv) Folic Acid pure, Riboflavin pure, Vitamin B_{12}. SRL (Sisco Research Laboratories Pvt. Ltd) South West Delhi, India.

xvi) Bovine serum albumin (BSA), thiobarbituric acid (TBA), reduced glutathione (GSH), 5-5´dithiobis-2-nitro benzoic acid (DTNB), and para nitro phenyl

phosphate (PNPP) were purchased from Sigma chemicals (St. Louis, MO). Sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), pyrogallol were supplied from SRL, India or MERCK, India.

xvii) Total protein kit purchase from Ranbaxy Diagnostic India Limited, Mumbai, India.

xviii) Lipid profiles done by standard kits from Ranbaxy Diagnostic Limited

xix) Sodium arsenite is purchased from HIMEDIA, Mumbai, India.

xx) The other chemicals and reagents were used as analytical grade as Merck, HIMEDIA, Mumbai, India or purchased from Sigma-Aldrich Diagnostics Ltd. USA.

4.2 Animals and their maintenance:

Wistar strain female rats aged 60 days, weighing about 120±10g were selected in this experiment. Rats were bred in the Central Animal Resource facility of Vidyasagar University, India. They were acclimatized under standard laboratory condition 12h L: 12h D and 25±2^{0}C temperature with free access to food (Standard Laboratory Diet) and water *ad libitum*. The Principle of Laboratory Animal Care (CPCSEA guide line) and the guideline of institutional ethical committee (ethical clearance no. 3.Vii.C) was followed throughout the all-experimental schedules.

4.2.1 Composition of Standard Laboratory Diet:

i) Wheat meal: 65 %

ii) Casein: 15 %

iii) Dried skimmed milk: 5.0 %

iv) Yeast (dried): 1.0

v) Cod liver oil: 5.0 %

vi) Ground nut oil: 3.0 %

vii) Vitamin B complex (Pfizer Pharma): 1.0 %

viii) Dried grass meal: 2.0 %

ix) Salt mixture (**Hawk & Oser, 1976**): 3.0 %

4.3 Biochemical Assay of Ovarian Δ^5, 3β-HSD Activity:

The activity of this enzyme was measured by modified Talalay's method [**Talalay, 1962],** estimating the product, i.e., androstenedione from substrate dehydroepiandrosterone in presence of co-factor nicotinamide adenine dinucleotide (NAD). Homogenizing media contains a cocktail of 20 ml Spectroscopic grade glycerol, 10 ml K_2HPO_4 (0.05 M), 10 ml EDTA (0.01 M), 60 ml Redistilled water. The final pH of the medium was adjusted to pH 7.4 by addition of 0.1 (N) NaOH. Ovary was removed from the animal and immediately frozen. Excess fat body was removed from ovarian surface and then the whole ovary was transferred into ice cold homogenizing media making a tissue concentration of 10 mg / ml. It was then homogenized in chilling condition. The homogenate was centrifuged at 10,000g for 45 minutes at 4 ^{0}C. The supernatant solution was taken for assay of the enzyme activity. The assay was performed in a UV-spectrophotometer cuvette. The reaction medium contained 1.0 ml of 100 µmol TNaPP buffer (pH 8.9), 0.04 ml ethanol containing 30 mg of DHEA and 0.1 ml of redistill water containing 0.5 µmol of NAD. The reaction was started by the addition of 1.0 ml tissue supernatant. The rate of formation of reduced nucleotide was measured at 340 nm in a spectrophotometer

against a blank cuvette (without NAD). One unit of enzyme activity is defined as the change in absorbency of 0.001 per minute at the above wavelength and 1.0 cm light pathway at 25^0C.

4.4 Biochemical Assay of Ovarian 17β-HSD Activity:

This enzyme was assayed with little modification by spectrophotometric measurement of the rate of reduction of pyridine nucleotide at the step of conversion of testosterone to androstenedione. The rate of production of androstenedione was measured in this reaction. Above homogenizing media was used for preparation of ovarian tissue sample. Ovary was removed from the animal and immediately frozen. The reaction medium contained 1.0 ml of 440 μmol of TNaPP buffer (pH 10.2), 25.0 mg of BSA (added as 0.5 ml of a 5% solution), 0.04 ml of 95% ethanol containing 0.3 μmol of testosterone and 0.1 ml redistill water containing 1.1 μmol of NADP. The reaction was initiated by the addition of 1.0-ml tissue supernatant. The rate of formation of reduced nucleotide was measured at the time of conversion of testosterone to androstenedione at 340 nm in a spectrophotometer against a blank (without NADP). One unit of enzyme activity is defined as the change in absorbency of 0.001 per minute at the above wave length and 1cm light path at 25^0C **[Jaraback et al, 1962]**.

4.5 Estimation of FSH by Rat FSH (Folicle-stimulating hormone) ELISA Kit (Wuhan Fine Biotech Co., Ltd. Catalogue No.: ER0960)

This kit was based on Competitive-ELISA detection method. The provided microtiter plate in this kit has been pre-coated with FSH.

Before the testing, equilibrate the SABC working solution ,TMB substrate and wells were kept for minimum 30 min at room temperature (RT) (37 °C). After equilibrate

all reagents and samples as well as proper mixing of working reagents the testing was run. A plot a standard curve also created during each test.

1. Arranging standards, test samples and control (blank) wells on the pre-coated plate respectively, and recorded their locations. Before charging the standards, samples the precoated wells were rinsed for 2 times.
2. Then added 50µL of Standards, Samples in respective wells and only 50 µL diluents buffer was put into the blank well.
3. Then instantly 50µL working solution of Biotin-detection antibody was added in each well and covered with plate sealer and after mixing the plate is kept for 45 mins at 37°C.
4. After incubation the plate was washed for 3 times with provided wash buffer (approximately 350µl in each well) and then added 100µL of HRP-Streptavidin Conjugate (SABC) working solution.
5. Then the plate was sealed quickly and incubates at 37°C for 30 minutes.
6. After incubation the plate was again washed for 5 times as previously.
7. Then the TMB (Tetra methyl Benzedine) substrate is dispense at a quantity of 90µL by means of multi channel pipette and covered immediately and kept in dark place for 15 mins at 37°C.
8. After incubation add 50µl stop solution into each well and blue colour was turnd into yellow.
9. Then the OD (optical density) was measured of each well at once by ELISA reader at 450 nm weave length and value was taken by the standards curve.

4.6 Estimation of LH by Rat LH (Luteinizing Hormone) ELISA Kit (Wuhan Fine Biotech Co., Ltd).

This kit was based on Competitive-ELISA detection method. The provided microtiter plate in this kit has been pre-coated with LH.

Before the testing, equilibrate the SABC working solution ,TMB substrate and wells were kept for minimum 30 min at room temperature (RT) (37 °C). After equilibrate all reagents and samples as well as proper mixing of working reagents the testing was run. A plot a standard curve also created during each test.

1. Arranging standards, test samples and control (blank) wells on the pre-coated plate respectively, and recorded their locations. Before charging the standards, samples the precoated wells were rinsed for 2 times.
2. Then added 50µL of Standards, Samples in respective wells and only diluents buffer was put into the blank well.
3. Then instantly 50µL working solution of Biotin-detection antibody was added in each well and covered with plate sealer and after mixing the plate is kept for 45 mins at 37°C.
4. After incubation the plate was washed for 3 times with provided wash buffer (approximately 350µl in each well) and then added 100µL of HRP-Streptavidin Conjugate (SABC) working solution.
5. Then the plate was sealed quickly and incubate at 37°C for 30 minutes.
6. After incubation the plate was again washed for 5 times as previously.

7. Then the TMB (Tetra methyl Benzedine) substrate is dispense at a quantity of 90µL by means of multi channel pipette and covered immediately and kept in dark place for 15 mins at 37°C.
8. After incubation add 50µl stop solution into each well and blue colour was turned into yellow.
9. Then the OD (optical density) was measured of each well at once by ELISA reader at 450 nm weave length and value was taken by the standards curve.

4.7 Estimation of ESTRADIOL by Rat E2 (Estradiol) ELISA Kit (Wuhan Fine Biological Technology Co).

This kit was based on Competitive-ELISA detection method. The provided micro titer plate in this kit has been pre-coated with E_2.

Before the testing, equilibrate the SABC working solution ,TMB substrate and wells were kept for minimum 30 min at room temperature (RT) (37 °C). After equilibrate all reagents and samples as well as proper mixing of working reagents the testing was run. A plot a standard curve also created during each test.

1. Arranging standards, test samples and control (blank) wells on the pre-coated plate respectively, and recorded their locations. Before charging the standards, samples the precoated wells were rinsed for 2 times.
2. Then added 50µL of Standards, Samples in respective wells and only diluents buffer was put into the blank well.
3. Then instantly 50µL working solution of Biotin-detection antibody was added in each well and covered with plate sealer and after mixing the plate is kept for 45 mins at 37°C.

4. After incubation the plate was washed for 3 times with provided wash buffer (approximately 350µl in each well) and then added 100µL of HRP-Streptavidin Conjugate (SABC) working solution.
5. Then the plate was sealed quickly and incubates at 37°C for 30 minutes.
6. After incubation the plate was again washed for 5 times as previously.
7. Then the TMB (Tetra Methyl Benzedine) substrate is dispense at a quantity of 90µL by means of multi channel pipette and covered immediately and kept in dark place for 15 mins at 37°C.
8. After incubation add 50µl stop solution into each well and blue colour was turned into yellow.
9. Then the OD (optical density) was measured of each well at once by ELISA reader at 450 nm weave length and value was taken by the standards curve.

4.8 Biochemical assays of transaminase, phosphatase, and total protein:

In this investigation serum glutamate pyruvate transeaminase (SGPT) or alanine aminotransferase (ALT) and serum glutamate oxaloactate transaminase (SGOT) or aspartate aminotransferase (AST) , alkaline phosphatase (ALP), total protein (TP), were measured by using standard kit from Marck Limited, Worli, Mumbai. Serum containing SGPT converted L-Alanine and α-Ketoglutarate into pyruvate and glutamate. This pyruvate produced hydrazone by reacting with 2, 4 dinitrophenol in alkaline media forming brown colour complex whose intensity was measured at 340 nm weave length (WV). On the otherhand SGOT reacts with L-aspertate and α-ketoglutarate which generates oxaloacitate and glutamate. Then oxaloacetate reacts with NADH in presence of malate dehydrogenase to form NAD by oxidation in

decreasing manner which was measured by 340 nm WV. In each assay 0.1 ml serum was used [**Bergmeyer et al., 1978; Schumann et al., 2002**]. To measure the activity of serum ALP, 0.1 mL of serum was mixed with a mixture of Tris-HCl (pH 8.0) and p-nitrophenyl phosphate and incubated at 37ºC. The rate of generation of p-nitrophenol in increasing manner was measured at 405 nm WV [**Copeland et al., 1985**]. Total protein was measured at 540nm WV following Biuret method [**Henry & Winkelman, 1974**].

4.9 Detection of urea and creatinine levels:

The urea and creatine were measured by standard kit was measured by modified method where 10 μL of serum was mixed with urease at 37ºC and finally chromogen and hypochlorite induced green color was measured at 570 nm [**Fawcett and Scott, 1960**]. Following the modified Jaffe's kinetic method, creatinine was determine using alkaline picrate with 0.1 mL plasma at 37ºC and reading was taken at a of 520 nm WV [**Pardue et al., 1987**].

4.10 Estimation of Malondialdehyde and Conjugated Dienes Levels:

Tissues were homogenized (20% w/v) in ice-cold phosphate buffer (0.1 mol/L, pH 7.4). The homogenates were centrifuged at 15,000×g at 4°C for 3 min. The supernatants were collected for the assessment of malondialdehyde (MDA) and conjugated dienes (CD).

MDA was determined from the reaction of thiobarbituric acid with MDA. The amount of MDA formation was measured by taking the OD at 530 nm (ε=1.56×105 mol^{-1} cm^{-1}) [**Buege & Aust, 1978; Jendryczko and Drozdz, 1988**].

CD was determined by a standard protocol where lipids were eliminated with the mixture of chloroform and methanol (2:1), and by the centrifugation at 1,000×g for 5 min. The residual lipid was dissolved in 1.5 ml of cyclohexane, and at last, the formation of hydroperoxide amaunt was measured at 233 nm [**Okhawa et al., 1989**].

4.11 Colorimetric Assay of Super oxide Dismutase (SOD) and Catalase Activities:

Tissues were homogenized in 100 mmol/L ice-cold Tris-HCl buffer having 0.16 mol / L KCl (pH 7.4) with tissue concentration of 10% (w/v) and centrifuged it at 10000 g at 4°C for 20 min. The activity SOD was estimated from the supernatant following standard protocol [**Steinman, 1978**]. The reaction mixture was prepared by mixing 800 µl of TDB (Merck), 40 µl of 7.5mmol / L NADPH (Sigma), 25 µl of EDTA-$MnCl_2$ and 100 µl of the tissue supernatant. The SOD activity in this mixture was measured at 340 nm by the rate of NADPH oxidation.

The activity of Catalase was estimated using calorimeter [**Sinha, 1972**] by the rate of conversion of dichromate in acetic acid into perchromic acid followed by chromic acetate during heating in the presence of H_2O_2. Then the chromic acetate was evaluated at 620 nm. The catalase preparation was allowed to split H_2O_2 in time manner. The reaction was stopped at different time intermissions by the adding of a dichromate-acetic acid mixture and the residual H_2O_2 was determinant as chromic acetate. The expression of one unit of activity was as a mole of H_2O_2 consumed/ min/mg protein.

4.12 Assessment of SOD and Catalase by native gel electrophoresis:

For evaluation of SOD and CAT activity by native gel electrophoresis tissue was homogenized (20%; w/v) in chiliad PBS (0.1M pH 7.4) and rotted it at 10,000 × *g centrifugal* force for 20 min at 4°C. The SOD activity gel (12%) assay system was based on the inhibition of the reduction of nitroblue tetrazolium (NBT) by SOD and based on the capacity of $O2^{\bullet-}$ to interact with NBT reducing the yellow NBT within the gel to generate a blue precipitation and SOD active site developed a clear area of achromatic bands competing with NBT for the $O2^{\bullet-}$. The gel became purple except at the position containing SOD. The gel was scanned when the maximum contrast between the band and background has been achieved.

Fifty microgram (50µg) protein per sample was loaded in 8% (vol/vol) native gels for separation of catalase. Catalase eliminated the peroxides from the region of the gel it occupied. Exclusion of peroxide did not allow for the potassium ferricyanide (a yellow substance) to be reduction into potassium ferrocyanide that reacted with ferric chloride to form a Prussian blue precipitate. A green-blue background dominated by white broad bands indicating the presence of enzyme has been achieved in catalase activity gels (8%) [**Wedert & Cullen, 2010**].

4.13 Assessment of Uterine Peroxidase activity and electrozymogram of Peroxidase:

Uterine tissues were homogenized in 0.1 M of phosphate buffer solution (pH 7.0) and centrifuged at 18,000 *g* for 15 min at 5°C [**Sadasivan & Manicckam, 1996**] for enzymatic assay of peroxidase. A cocktail of 20 mM of guiacol and 0.1mL of homogenate supernatant was read in the presence of 0.3 mL of H_2O_2 (12.3 mM) as substrate. The time was noted when the absorbance increased (436 nm) by 0.1 unit.

For electrozymogram study of uterine peroxidase uterine horns were homogenized (20%;w/v) in ice cold PBS (0.1M pH 7.4) and centrifuged at 10,000 × *g* for 20 minutes at 4°C and extracted protein was loaded in 8 % native gel. Peroxidase zymogram was developed by benzidine in 30% H_2O_2 solution till the appearance of brown colour [**Hasan & AbURAHMA**, **2014**].

4.14 Assessment of serum total Lactate Dehydrogenase (LDH):

Using Tulip Assay kit from Tulip India Group total serum LDH activity was assayed by decreasing absorbance of the oxidation of NADH to NAD. This decrease in absorbance was proportional to the LDH activity in the sample.

For electrozymographic study of the enzyme agarose gel of 1.2% in 50 mM Tris-HCl buffer pH 8.2 was used and 20 µl serum was uploaded into different holes of gel. The gel was electrophoresed at 170 V until the bromophenol blue has traveled to within 1 mm of the positive electrode end of the gel. Agarose gel was developed with a few modifications in presence of H_2O, 1 M Tris, tetrazolium-blue, phenazine-methosulphate, Na-lactate and NAD and then incubated at 37^{o}C to develop color reaction for 30 minutes following the **r**insing of the gels with water and observed under light exposure [**Brandt et al., 1987**].

4.15 Estimation of lipid profile:

Lipid components, such as total cholesterol (TC) [**Allain et al., 1974**], HDL-C [**Warnick et al., 1985**] and triglyceride (TG) [**Werner et al., 1981**] were estimated in serum by using standard kits supplied by Ranbaxy Diagnostic Limited (Mumbai, India). For total cholesterol estimation, 1.0 mL of working reagent containing cholesterol esterase, cholesterol oxidase, and peroxidase were mixed with 10 µL of serum sample and incubated at 37°C for 5 minutes. The OD was taken against a

blank at 505 nm. For HDL-C estimation, serum is mixed with precipitating reagents containing polyethylene glycol to precipitate the VLDL and LDL. Then 50 µL of the supernatant is mixed with the reagent containing cholesterol oxidase, phenol and peroxidase and incubate for 5 min at 37°C Then the OD is measured at 505 nm [**Warnick et al., 1985**]. For estimation of triglyceride, 10 µL of serum is added with 1.0 mL of working reagent containing lipoprotein lipase, glycerol kinase, glycerol-3-phosphate, and peroxidase, followed by incubation for 5 minutes, and absorbance was taken at 570 nm [**Werner et al., 1981**]. LDL-C was calculated from the value of TC (CHL) and HDL-C, using the formula of Friedwald and Fredickson's [**Friedewald et al., 1972**].

4.16 Estimation of Non-protein soluble thiol (NPSH): The NPSH in 10,000 × g supernatant from liver-tissue homogenate (prepared in 0.1 M of phosphate buffer, pH 7.4) was considered by standard DTNB (5,5'-dithiobis2-nitrobenzoic acid) protocol, with a few modification [**Mieyal et al., 2008; Forman et al., 2009**]. In concise, the protein was precipitated by sulfosalisialic acid, and clear cytosol was mixed with 0.1 M of sodium phosphate buffer containing 5 µM of DTNB. The level of NPSH was estimated against a reduced glutathione (GSH) standard curve.

4.17 DNA analysis:

Tissues were used for DNA preparation and cell pellet was treated with 500 µL lysis buffer (50mMTris pH 8.0, 20 mM EDTA, 10 mMNaCl, 1% SDS, 0.5 mg/ml proteinase K) for 15 min at 4°C and centrifuged in chilling environment at 12,000 rpm for 20 min. The collected supernatant was treated with 1:1 mixture of phenol: chloroform with gentle agitation for 5 min and precipitated in two parts of cold ethanol and one tenth part of sodium acetate. After spinning down and decantation,

the precipitate was resuspended in 30 µl of deionized water–RNase solution (0.4 ml water+5 µl of RNase) at 37°C. The 8.0 % agarose gel with ethidium bromide was run at 65 V and documented in gel documentation system [**Garcia-Martinez et al., 1993**].

4.18 Comet assay:

According to Singh et al. (**1988**) with slight modified comet assay was performed. To a 25 ml of cell suspension was added in low melting point agarose (0.6%) in PBS at 37°C and fixed onto a glass slide precoated with 1% agarose. Following the solidification of agarose the coverslips were removed and the slides were soaked in ice cold lysis buffer (2.5 mM NaCl, 85 mM EDTA, 10 MmTrizma base, 1% Triton X-100, 10% DMSO and 1% sodium lauryl sarcosinate, adjusted to pH 10) for 1hr at 4°C. The slides were washed 3 times in PBS at RT after lysis. The slides were then incubated at 37°C for 45 min. The coverslips were then removed and the slides were washed in water 2 times more to remove excess salt and placed in a submarine gel electrophoresis chamber (Bio-Rad, USA) filled with alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) for 25 min. Following the completion of electrophoresis for 30 min at 25 V and 300 mA slides were then neutralized with PBS and stained with a solution of 10 mg/ml ethidium bromide for 5 min. Washing with water excess stain was removed. Slides were analyzed using a fluorescence microscope (Nikon, Eclipse LV100 POL), with the Vis Comet [**Impuls Bildanalyse]** software.

4.19 Histological study:

Whole ovaries, pieces of uterine horns and parts of liver and kidney were fixed in 10% formalin solution for overnight, dehydrated in series of graded ethanol and

embedded in paraffin wax. Paraffin sections (5.0 μm) were done by a semiautomated microtome [Leica Biosystems] and the intact ovaries, pieces of uterine horn, liver, kidney were stained with haematoxylin and eosin. Histological analyses were done under microscope (Nikon, Eclispse LV100) to evaluate the histological changes [**Fischer et al., 2008**]. Fractalyse 2.4 software was used to analyze the surface morphology and fractal dimension of uterus from its histological image to assess possible fibrotic status.

For scanning electron microscopy cutting tissues are kept in 2.5% gluteraldehyde (in Phosphate buffer). Further wash the tissues with phosphate buffer for several times. Then the tissues are dehydrates with alcohol at a critical point dying (CPD). Then the tissues are coated with gold and viewed in scanning electron microscope [**Jones, 2012**].

4.20 Densitometry and Statistical Analysis:

UN-SCAN-IT gel 7.1software was used to measure the band intensity with respect to the control band (100 %).

4.21 Statistical Analysis of Data:

For statistical analysis of our data, ANOVA followed by a multiple two-tailed t test was used. Analysis of variance (ANOVA) tests the difference between the variances of two or more groups. We analyzed of our data by one- way ANOVA, which is used to investigate the effects of single independent variable on dependent variable. Multiple two-tailed t test with Bonferroni modification used to find out whether the difference of mean value in each parameter between different group combination is significant [**Das, 1998**].

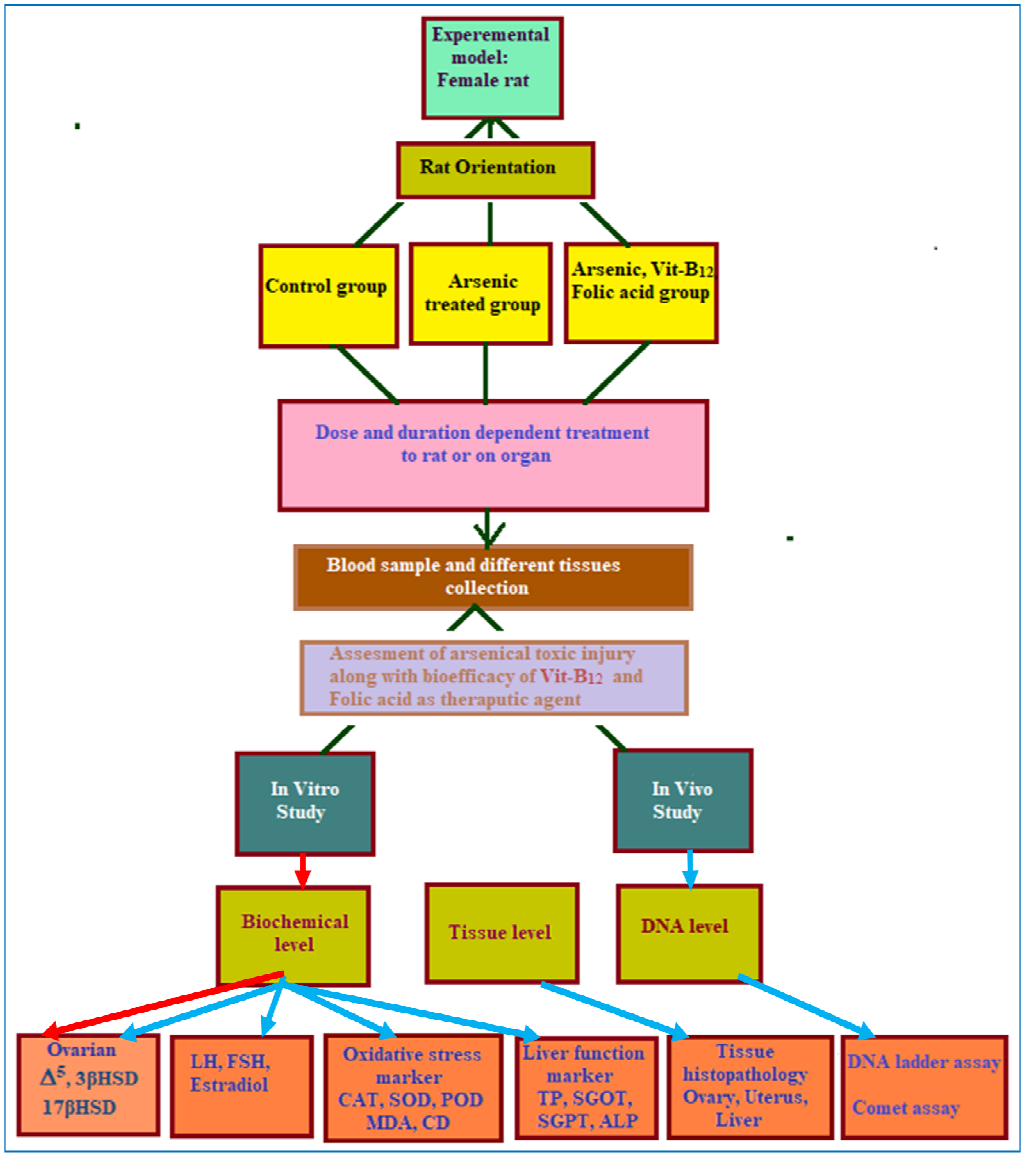

Figure 3: A schematic diagram of the present experimental deigned.