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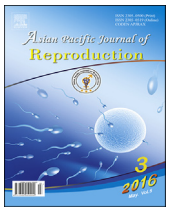

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An overview of the current methodologies used for evaluation of anti-fertility agents

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ABSTRACT

Discoveries in the past two decades have continued to improve our understanding of the mechanism of fertilization and animal models have played a significant role to define the basic mechanism of anti-fertility agents. *In vivo* models have been developed in the past years to study the anti-fertility agents. Methods that are used in anti-fertility study can be categorized into method including estimation of sex hormones, assessment of sperm motility and count, assessment of sperm viability and morphology, mating trial test body, sex organ weights, abortifacient activity, post-coital anti-fertility activity, effect on estrous cycle, anti-estrogenic activity, anti-gonadotrophic effect and quantification of fructose in seminal vesicle, histopathology, and biochemical methods. This review aims to highlight some of the new and currently, used experimental models that are used for the evaluation of anti-fertility agents.

1. Introduction

The population explosion is a leading cause of poverty and pollution in developing countries [1]. Exponentially growing population has been adversely affecting the social, economical and technological development of human race [2]. Therefore to reduce/control our number has to be the first on a priority list. A good number of synthetic contraceptives are available in market, each one with either a limited success or side effects [2]. It created a population control programme, which includes studies of traditional medical practices [1]. Since ancient times, plants have been a source of drugs, but scientific medicines tend to ignore the importance of herbal medicine [3]. The World Health Organization suggested that effective, locally available plants can be used as substitutes for drugs [1].

Medicinal plants in India have been screened for contraceptive potential and anti-fertility effects, since the country has always been concerned about population explosion [1]. Exploration of drugs having anti-fertility activity is the need of current time, and many time plant extracts have been investigated for their anti-fertility effect in animals [4]. Since herbal drugs are easily available and with no side effects, the current study was undertaken [2].

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2. Evaluation of antifertility agents

The therapeutic value, efficacy and toxicity of drug may be evaluated in animals experimentally, followed by clinical trials. *In-vivo* animal models are employed to assess anti-fertility activity in experimental animals like rat and mice.

2.1. Parameters used to evaluate anti-fertility agents

For the study of anti-fertility activity many *in-vivo* models have been used.

2.2. Estimation of sex hormones

Blood samples were collected from rats for estimations of serum levels of sex hormones. Sera were separated into clean bottles, stored frozen and used within 12 h of preparation for the estimation of testosterone, estrogen level, prolactin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [5].

2.3. Assessment of sperm motility and count

Progressive motility was tested immediately. The right cauda epididymis was incised and semen was squeezed on a pre-warmed slide. Two drops of warm 2.9% sodium citrate was added to semen and mixed by a cover-slip. The percentage of progressive sperm motility was evaluated visually at 400× magnification. Motility estimates were performed from three

different fields in each sample. The mean of the three successive estimations was used as the final motility score. For sperm count, the left cauda epididymis was incised and semen that oozed was quickly sucked into a red blood pipette to the 0.5 mark, and then diluted with warm normal saline up to the 101 mark. A drop of the semen mixture was placed on the Neubauer counting chamber and viewed under the magnification of ×40. The total numbers of sperm cells were counted and expressed as 10^6/mL [5].

2.4. Assessment of sperm viability and morphology

A viability study (percentage of live spermatozoa) was done using eosin/nigrosin stain. A drop of semen was squeezed onto a microscope slide and two drops of the stain were added. Thin smears were then prepared and observed under a light microscope at ×400 magnification. Viable sperm remained colorless while non-viable sperm stained red. The stained and the unstained sperm cells were counted using ×40 microscope objectives and an average value for each was recorded from which percentage viability was calculated. To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin–nigrosin (5 slides/rat) viewed under a light microscope at 400× magnifications. A total of 300 sperm cells was examined on each slide (1 500 cells for each rat), and the head, tail and total abnormality rates of spermatozoa were expressed as a percent [5].

2.5. Mating trial test

Mating trial test of male rats was done, 5 d before the termination of the experiment. Each male rat was cohabitated overnight with proestrous females in a ratio of 1:2 and housed in a single cage. Positive mating was confirmed by presence of sperm and vaginal plug in the vaginal smear the following morning. Each sperm positive female was kept under observation and the resultant pregnancies were noted, when dam gave birth. The following reproductive parameters were then computed:

Mating success % = number mated/number paired × 10;

Fertility success % = number pregnant/number paired × 100;

Fertility index = number pregnant/number mated × 100 [5].

2.6. Body and sex organ weights

The initial and final body weights of the animals were recorded. The testes, epididymides, seminal vesicle and ventral prostate were dissected out, freed from adherent tissues and blood, and weighed to the nearest milligram. Organ weights were reported as relative weights (organ weight/body weight × 100) [5].

2.7. Quantification of fructose in seminal vesicle

For fructose quantification, seminal vesicular homogenate was prepared at a tissue concentration of 50 mg/mL. The supernatant (seminal plasma) was deproteinized by adding 50 μL of zinc sulfate and sodium hydroxide to make a total dilution of seminal plasma 1:16, followed by centrifugation at 2 500 r/min for 15 min. For fructose measurement, 200 μL of clear seminal plasma was used and the optical density of standard and samples were measured against blank at 470 nm. The concentration of fructose was obtained by plotting the value in standard curve and the value expressed in the unit of μmol/mL of seminal plasma [5].

2.8. Abortifacient activity (Anti-implantation activity)

The plant extracts were tested in female albino rats for abortifacient activity. The vaginal smears of caged female rats of known fertility were monitored daily. Unstained material was observed under a light microscope. The proportion among the cells observed was used for the determination of the estrous cycle phases. Female rats were caged with males of proven fertility in the ratio of 2:1, in the evening of proestrous and examined the following day for the evidence of copulation. Rats exhibiting thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day one of pregnancy. These rats were randomly distributed into four groups, a control group and three experimental groups of six animals each. Group I received vehicle only and served as control. Groups II, III, and IV received different extracts. On the 10th day of pregnancy the animals were laparotomized under light ether anesthesia using sterile conditions. The two horns of uteri were examined to determine the implantation sites. Thereafter the abdominal wound was sutured in layers. Post operational care was taken to avoid any infection. The extract to be tested were then fed to operated pregnant rats, specified by an intragastric soft rubber catheter from day 11 up to the 15th day of pregnancy. The animals were allowed to go to full term. After delivery the pups were counted and the antifertility activity of extract was evaluated. Litters were examined for any malformation [1,6,7].

Percentage abortifacient activity = number of resorptions/number of corpus luteum × 100 [8].

2.9. Post-coital antifertility activity (Pre-implantation activity)

The anti-implantation activity is expressed as the percentage of animals showing absence of implantations in uteri when laparotomised on day 10 of pregnancy. Vaginal smears from each rat were monitored daily and the rats with normal estrous cycle were selected. Rats found in proestrus phase of cycle were caged with males of proven fertility, in the ratio 2:1 and examined the following morning for evidence of copulation. Rats exhibiting thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day 1 of pregnancy and those rats were divided into five groups containing six rats in each group. The extract was administered from day 1–7 of pregnancy. The powdered drug was also administered from day 1–7 of pregnancy. Control rats received the vehicle (distilled water). On day 25, laparotomy was performed under light ether anesthesia and semisterile conditions. The uteri were examined to determine the number of implantation sites and no of corpora luteagraviditis [9–12].

Frequency of pre-implantation losses = missing no. of implants (corpora lutea implants)/no.of corpora lutea × 100

2.10. Effect on estrous cycle

The female animals were artificially brought into estrus phase (heat) by administering either suspension of ethinyl estradiol orally at the dose of 100 μg/animal 48 h prior to the pairing and subcutaneous administration of progesterone at the dose of 1 mg/animal 6 h before the experiment or alternatively by the sequential administration of estradiol benzoate (10 μg/100 g body weight) and progesterone (0.5 mg/100 g body weight) through subcutaneous injections, 48 and 4 h respectively. Estrous cycle was determined between 8 am and 10 am using vaginal smear method. Vaginal secretion was collected with a plastic pipette with 10 μL of normal saline. The vagina was flushed three times with the pipette and the vaginal fluid was placed on a glass slide. Different slides were used for each animal. The unstained secretion was observed under a light microscope. After confirmation of regular four day cycle for 2 weeks the animals were selected for study and divided into six groups and treated with test drugs. The effect of test drugs on the estrous cycle was monitored for 28 d [9,13].

2.11. Antiestrogenic activity

All the rats were ovariectomized by the same methods described in previous procedure and the weight of the ovaries were recorded. The ovariectomized rats were randomly taken and divided in thirteen groups. Except control, other groups were administered with different doses of estradiol (0.1 μg/rat and 1.0 μg/rat) and followed by test compounds respectively for 4 consecutive days. On eleventh day, the rats were anaesthetized using ketamine (60 mg/kg, i.p.) and the remaining right sided ovaries were dissected out from all the animals. Properly cleaned, dries and their respective weights were recorded. The ovaries weight variations prior to and after treatment with extracts were calculated. Percentage inhibition of ovarian weight was calculated using the following equation:

$$\text{Percentage inhibition in ovarian weight} = [1 - (XE - C)]/E - C \times 100.$$

Where, C indicates mean ovarian weight from rats treated with vehicle, E for estradiol and XE indicates the mean ovarian weight of rats treated with extract and estradiol [9].

2.12. Antigonadotrophic effect

Female rats were studied for 5 consecutive normal estrus cycles by vaginal smear method. The rats were anaesthetized using ketamine (60 mg/kg) pretreated with atropine (1 mg/mL) and left side ovariectomy was performed. Left ovary was dissected out carefully from surrounding fatty tissue and dried by soaking on filter paper and weighed. The ovariectomized rats were divided into six groups and treated. On 12th day after treatment, the remaining right ovaries of all rats properly dissected out using same anesthetic condition. Cleaned, dried and their respective weights were recorded and percentage increase in ovarian weight compared with weight of the left ovaries were calculated. Percentage increase in the weights of ovary was calculated using the formula [9].

$$\text{Percentage increase in ovarian weight} = (\text{weight of right ovary} - \text{weight of left ovary})/\text{weight of left ovary} \times 100.$$

2.13. Histological analysis

Testes and uteri were carefully dissected out following abdominal incision from male and female rats respectively and fixed in 10% normal-saline and processed routinely for paraffin embedding. Sections of 5 μm from both were obtained with rotary microtome, stained with Hematoxylin and Eosin Stalin (H/E) respectively and observed under a light microscope [5,14].

2.14. Measurement of some biochemical and blood parameters

Blood samples were collected from the heart of each rat at the time of scarification into non-heparinized and heparinized tubes. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphates, creatinine and urea in addition to red blood cell (RBC) count, total leucocytic count (TLC), hemoglobin (Hb) concentration, packed cell volume (PCV),cholesterol and total proteins were determined by standard methods [5,15].

2.15. Determination of testicular and serum cholesterol (Chod PAP method)

Cholesterol is the precursor in the synthesis of many physiologically important steroids such as bile acids, steroid hormones and vitamin D and its requirement for normal sexual activity has been well established. Testicular and serum cholesterol concentrations may be determined by the Chod–PAP method as briefly, 0.02 cm^3 of the working reagent and the absorbance of the resulting mixture is read after 5 min at 546 nm [13].

2.16. Determination of total protein

A timed rate biuret method was used to measure the concentration of total protein in serum or plasma. Proteins in the sample combined with the reagent producing alkaline copper-protein chelate. The rate change in absorbance was monitored by a detector at 545 nm. The observed rate of chelate formation is directly proportional to the total protein concentration in the sample [16].

3. Conclusion

Drug discovery and development consists of a series of processes starting with the demonstration of pharmacological effects in experimental animal models and ending with drug safety and efficacy studies in patients/animals. The main limitation is often the unacceptable level of toxicity with herbal drugs. The study of antifertility agents has been an important field of research. The article explains various types of animal models which are employed in the study of antifertility agents.

Conflict of interest statement

We declare that we have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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