

ORIGINAL RESEARCH ARTICLE: EXPERIMENTAL STUDY

RAJATA SINDOORA- EXPERIMENTAL STUDY ON SPERMATOGENIC ACTIVITY

YOUSUF SAHAD¹ SRINIVAS YADAV² SUREKHA MEDIKERI³

ABSTRACT

RajataSindoora (RS), a unique formulation mentioned in the various texts of Rasashastra is a *KupipakwaRasayana*(prepared using amber glass bottles). RS was prepared here as per the guidelines of the text RasendraSambhava with 4:4:1 proportions of *ShodhitaParada*(Purified Mercury), *ShodhitaGandhaka*(Purified Sulphur) and *ShodhitaRajata*(Purified Silver) respectively using modified method of Vertical Muffle Furnace. The author of the text Vaidya VishwanathDwivedi mentions that RS has rejuvenative, tissue boosting ,aphrodisiac, nootropic, beautifying properties and also possesses the potency to cure all the diseases on giving with appropriate *anupanas*(vehicles). *RajataSindoora* is widely claimed to have excellent *vajikarana*(spermatogenic) effect. Till date, no reported studies are available pertaining to spermatogenic effect of RS. Hence an attempt has been made to evaluate spermatogenic activity of RS experimentally on wistar strain male Albino rats and compared to Standard Fertisure-M under all necessary precautions. RS was taken in two different doses (250 mg/kgbw & 500 mg/kgbw) and compared with Control (distilled water & food), Induced (Oligospermic rats) and Standard (Tab Fertisure-M) groups. Study shows increase in sperm count (up to 95-99 million) which shows effect of RS on spermatogenesis. It showed statistically significant Spermatogenic activity compared to control group & standard group. Histopathological study also showed increase in size of seminiferous tubules suggestive of significant Spermatogenic activity.

Keywords: *RajataSindoora*, *Kupipakwa*, Tab Fertisure-M, Spermatogenic activity.

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INTRODUCTION

Rasashastra is a branch of Ayurvedic medicine, which deals with metals and minerals to produce the drugs with higher efficacy in lower doses and with good palatability. These days Rasashastra formulations have advanced rapidly and gained the wide acceptance of doctors and patients. At present Rasashastra formulations comprise the major share among prescriptions of Ayurvedic physicians.

In today's lifestyle because of stress and over exposure to synthetic and chemical drugs, there is increased incidence of male infertility. Male infertility refers to a man's inability to result pregnancy in a fertile female. In humans, it accounts for 40-50% of infertility and affects approximately 7% of all men. Male infertility may be due to one or a combination of low sperm concentration, poor sperm motility, or abnormal morphology.

The rejuvenation of genital tissue and to improve the quality and quantity of sperm, very few promising medications are available. The drugs which rejuvenate the genital tissue are rarely available in the contemporary medical science.

Ayurveda has explained till date many remedies, procedures in such condition along with many herbo-mineral formulations. *Swarnakalpas* are considered as standard formulations in improving the sperm count

and quality, but they are costly and not easily reachable for common people.

Rajata Sindoor^[1] is a novel and cost effective preparation explained in Rasendra sambhava as *Vrushya yoga*. *Rajata Sindoor* is the most popular preparation of Ayurvedic pharmaceuticals, prepared with purified *Rajata*, purified *Parada*, purified *Gandhaka* in the ratio of 1:4:4 by *Kupipakwa* method of preparation. The formulation was prepared by heating the above processed ingredients in an amber beer bottle using vertical muffle furnace with the heating pattern of 8 hours of *mridwagni* (125° C -250° C), 13 hours of *Madhyamgni* (250° C – 450° C) and 8 hours of *Teevragni* (450° C – 750° C). An XRD report of *Rajata Sindoor* showed the presence of vermilion in cubic crystal structure. EDX analysis showed significant percent of Hg, S and Ag.

The experimental study was carried out as per norms to assess the Spermatogenic activity thus providing the authenticated data and scientific explanation.

MATERIALS AND METHODS

a) Ethics:

The experimental procedure was carried out in accordance with the ethical guidelines for animals proposed by Government of India. Ethical clearance obtained from Department of Pharmacology, PES College of Pharmacy, Bengaluru as per the protocol outlined in publication of the Committee for the Purpose of Control and Supervision of Experiments on Animals

standard guidelines (CPCSEA) and approval was obtained from Institutional Animal Ethics Committee (IAEC) with reference no:

IAEC/ABMRCP/2017-2018/23

Source of animals:

Male Albino wistar rats(180-210g) were purchased from Adita Biosys, Pvt Ltd. Plot No- SPL-Tumakuru-572106, and were maintained in the animals house of PES College of Pharmacy, Bengaluru

Drug and chemicals:

Cyclophosphamide^{[2][3]}, CMC, Fertisure, *Rajata Sindoor* and Distilled water.

Equipment:

Tuberculin syringe (1ml capacity), Gavage needle, Glouse, Digital plethysmometer, mortar and pestle.

b) Study design-

The Animals were divided, randomly into five groups of six each. The animals were kept for acclimatization for a period of two weeks. The study was conducted for 30 days.

Oligospermia was induced before the study in all the groups except group I. It was induced with the single dose administration of cyclophosphamide (100mg/kg bwpo) on 4th day.

Table No 1: Grouping of the animals

SL.No	Group Names	Type	Dose and route	Dose frequency
I	Normal Control	Normal rats	Vehicle	Daily for 30days
II	Disease Control	Oligospermic Rats	Vehicle	Daily for 30days
III	Test	Oligospermic Rats	RS (250 mg/kg bwpo) (Min)	Daily for 30days
IV	Test	Oligospermic rats	RS (500 mg/kg bwpo) (max)	Daily for 30days
V	Std	Oligospermic Rats	Std (35mg/kgbwpo)	Daily for 30days

Procedure-

- Animals was must be fully anesthetized.
- Topical ophthalmic anesthetic agent (one drop of tetracaine HCl) was applied to the eye to minimize the discomfort before bleeding.
- The animal was scuffed with thumb and forefinger and the skin around the eye is pulled taut.
- A capillary tube is inserted into the medial canthus under the nictitating membrane of the eye. Slight thumb pressure with rotation motion was applied to puncture the tissue and to enter the capillary tube tip into the plexus/sinus.
- As soon as the sinus is punctured, blood enters the tubing by capillary action.
- When the desired amount of blood was collected, the tube was withdrawn and a clean gauze pad was applied on the eye to ensure hemostasis.
- A small amount of triple antibiotic ophthalmic ointment is applied to the eye after the technique.

Serum parameter to be studied^[5]

The prepared serum was used for the estimation of serum testosterone by the enzyme-linked immunosorbent assay (ELISA) technique using the Fortress kit.

Semen collection by Microsurgical epididymal sperm aspiration (MESA)

Microsurgical epididymal sperm aspiration (MESA)- Once a surgical plane of anesthesia was reached, the caudal abdomen was massaged gently downward to ensure that the testes were in the scrotum . The mouse was laid on its back, and the skin was disinfected with 70% ethanol and rinsed with distilled water. . By using scissors, a sagittal cut of about 10 mm was made in the skin at the bottom of the scrotum. The 2 caudae epididymides were easily visible through the intact tunica vaginalis. One technician used curved forces to fix a cauda epididymis and applied gentle pressure; a second person used a cannula on an insulin syringe to puncture the tissue. After penetration of the cauda epididymis, negative pressure was applied on the syringe successful aspiration was confirmed by increased turbidity of the media in the syringe. The contents of the syringe were expelled into a culture dish and checked for the presence of motile sperm^[6].

Media and sperm survival

The motility characteristics of rat sperm over time were examined by using Dulbecco's Phosphate Buffered Saline with 10 mg/ml BSA supplemented with 1 mg/ml D-glucose.

Semen analysis^[7]

This was performed immediately after semen collection. Semen was squeezed from the caudal epididymis onto a pre-warmed microscope slide (27°C) and two drops of

warm 2.9% sodium citrate is added, the slide assessed on each field. The motility of 100 sperms was assessed randomly. Sperms was reported as motile, sluggish, or immotile. The percentage of motile sperms was counted as the number of motile sperms divided by the total number of counted sperms. It is then covered with a warm cover slip and examined under the microscope using x400 magnification. Ten fields of the microscope will be randomly selected and the sperm motility of 10 sperms will be assessed.

Sperm viability (life/dead ratio) ^[8]

This was performed by adding two drops of warm Eosin/ Nigrosin stain to the semen on a pre-warmed slide. A uniform smear was made and dried with air. The stained slide was immediately examined under microscope using x400 magnification. The live sperm cells unstained which dead sperm cells absorb the stain. The stained and unstained sperm was counted and the percentage was calculated.

Sperm morphology ^[10]

This was performed by adding two drops of Walls and Ewas or Eosin/ Nigrosin stain to the semen on a pre-warmed slide. A uniform smear was prepared and air dried. The stained slide is immediately examined under microscope using x 400 magnification. Five fields of the microscope will be selected randomly and the types and number of abnormal sperms was counted from the total

number of sperms in the five fields. The number of abnormal sperms was expressed as a percentage of the total number of sperms.

Sperm count^[9]

This was performed by removing the caudal epididymis from the right testes and blotted with filter paper. The caudal epididymis will be immersed in 5ml formal-saline in a graduated test-tube and the volume of fluid displaced was taken as the volume of the epididymis. The caudal epididymis and the 5ml formal-saline was poured into a mortar and homogenized into a suspension from which the sperm count was carried out using the improved Neubauer haemocytometer under the microscope.

Preparation of testes tissue homogenate ^[11]

After the animals were sacrificed at the end of the experimental protocols, the testes was surgically removed from the body and washed and reperused thoroughly with ice cold water to remove the traces of blood from the organs. They were gently blotted between the folds of filter paper and weighed on an analytical balance. 10% homogenate was prepared in 0.05 M phosphate buffer (pH 7) using a homogenizer at 4°C. The homogenate was centrifuged at 10,000 rpm for 20min for removing the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of

lipid peroxidation (MDA, Superoxide dismutase (SOD) and catalase.

Determination of tissue cholinesterase activity:

Procedure:

The supernatant liquid was collected for the estimation of acetylcholinesterase in the testis tissue using the ERBA kit method. R1 sample is equivalent to 3ml, in which R2=1 tablet was dissolved completely in R1. 1.5ml of R1 and 10µl of the test sample were added in one clear eppendroffs tube and waited for 30 min and was analysed using semi auto analyzer at 405nm and the conc was obtained.

Determination of tissue lipid peroxidation ^[12]

Requirements: Tissue homogenizer, UV spectrophotometer, phosphate buffer, thiobarbituric acid, acetic acid, butanol, pyridine, water bath.

Procedure: 4 ml of reaction mixture containing 0.4 ml of the tissue homogenate, 1.5 ml of 0.8 % TBA, 1.5 ml of acetic acid (20 %, pH 3.5) and distilled water was kept for 1 h in a boiling water bath at 95°C. After 1 hr., the reaction mixture was removed from the water bath, cooled and 1 ml of distilled water was added. 5 ml of butanol: pyridine mixture (15:1) was added to the reaction tube, mixed well and centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was measured at 532 nm against butanol: pyridine mixture.

Calculation: The level of lipid peroxides are expressed as nanomoles of MDA reactive substances per minute in mg protein using molar extinction co-efficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

LPO = Abs. X total volume X sample volume

$$1.56 \times 10^5 \times 10^{-9} \text{ mg of protein}$$

As an ex., if Abs. = 0.001 LPO = $\frac{0.001 \times 10 \times 0.4}{1.56 \times 10^5 \times 10^{-9}}$
= 25.64 nm/mg protein

Determination of tissue superoxide dismutase (SOD) activity ^[13]

Requirements: Tissue homogenizer, 0.1M EDTA, 1.5 mM NBT, 67 mM phosphate buffer, riboflavin, UV spectrophotometer.

Procedure: 0.01 ml of the homogenate was mixed with 0.2 ml of 0.1 M EDTA, 0.1 ml of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.6 ml. After adding 0.05 ml of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. All the tubes was illuminated uniformly for 15 min and absorbance of the blue color formed was measured again. Percent of inhibition was calculated after comparing the absorbance of sample with the absorbance of control (the tube containing no enzyme activity). The volume of the sample required to scavenge 50 % of the generated superoxide anion is considered as 1 unit of enzyme activity and expressed in U/mg protein.

Calculation:

% inhibition =

$$\frac{(\text{Abs. in tissue homogenate} - \text{Abs. in blank}) \times 100}{\text{Abs. in tissue homogenate}}$$

Abs. in tissue homogenate

As an ex.

If, Abs. in test (liver tissue homogenate) =

0.290, Abs. in blank = 0.005, then

$$\% \text{ inhibition} = \frac{(0.290 - 0.005) \times 100}{0.290}$$

0.290

= 98.27%

We know, 50% inhibition = 1 unit of SOD

So for 98.27%, $98.27 \times 1/50 = 1.96$ units of

SOD

1.96 units is in 10 μ l (0.01 ml) of enzyme,

total volume 1ml = 1000 μ l

$$= (1.96/10) \times 1000$$

$$= 196.54 \text{ units SOD}$$

SOD activity = 196.54 units/ 100 mg liver tissue proteins

= 1.96 units/mg liver tissue protein.

Reference values (rats):

Liver = 2.32 units/mg tissue protein Brain = 0.6 unit/mg tissue protein

Determination of tissue catalase (CAT) activity ^[14]

Requirements- Tissue homogenizer, colorimeter/UV spectrophotometer, phosphate buffer (0.5M, pH7), 11mM H₂O₂.

Procedure- 0.1ml of the tissue homogenate (approximately 0.1mg protein) was combined with 1.9ml of the phosphate buffer (0.5M, pH 7). The decrease in extinction was measured at

240nm, 1 min interval for 3min immediately after adding a ml of 11mMH₂O₂ solution in buffer. A sample control was placed in the reference cuvette containing 0.1ml of tissue homogenate and 2.9 ml of buffer. Activity of catalase was calculated using the mmoles (mM) extinction coefficient $40\text{cm}^{-1} \cdot \mu\text{moles}$ of H₂O₂ decomposed/ min/ mg protein.

Catalase (U/mg protein) =

$$\frac{\Delta A / \text{min} \times 1000 \times 3}{40 \times 0.1 \text{mg protein in sample}}$$

40 x 0.1mg protein in sample

Where, $\Delta A = (\text{abs. of blank} - \text{abs. of test})$

As an ex., In liver tissue

If Abs. in 1st, 2nd and 3rd min are 0.14, 0.13 and 0.11 respectively and Abs. of blank = 0.16

So, catalase activity in 1st min =

$$\frac{(0.16 - 0.14) \times 1000 \times 3}{40 \times 0.1} = 15 \text{ U/mg protein}$$

2nd min =

$$\frac{(0.16 - 0.13) \times 1000 \times 3}{40 \times 0.1} = 22.5 \text{ U/mg protein}$$

3rd min =

$$\frac{(0.16 - 0.11) \times 1000 \times 3}{40 \times 0.1} = 37.5 \text{ U/mg protein}$$

Histological examination of testes ^[15]

Histopathology study was carried out at reputed lab in Bangalore

Procedure:

- A portin of the Spermatogenic activity in the male rat testis was dissected out and

fixed in 5% buffered neutral formalin solution for histological observations.

- Epididymis of all the rats were taken into 5ml of 1% of Sodium Citrate Solution and squashed thoroughly with the help

of needle and forceps until a milky suspension was obtained.

The sections were examined with the help of compound microscope and photomicrographs were taken

RESULTS

Table no 2: Body weight of various groups

Sl.no	Group name	Initial weight	Final weight	%change
1	Normal	182.5±12.34	175.6±12.03	3.78%
2	Disease control	231.3±8.63***	226.6±11.9***	2.03%
3	Oligospermic rats (RS-250mg/kg .po)	227.6±14.36	228±16.28	-0.175%
4	Oligospermic rats(RS-500mg/kg.po)	234.3±12.80	235.5±13.56	-0.42%
5	Standard control(6mg/kg.po)	208±5.74###	205±3.83###	1.44%

The values were expressed as Mean±SEM in each group. ***P<0.0001, was considered as significant. The statistical analysis was done by

using Graph pad prism- Dunnet's test comparison of selected pair of coloums with disease control.

Table no 3: Serum parameters

Group	Group name	Serum Testosterone(ng/ml)
I	Normal	6.29
II	Disease control	10.5
III	Oligospermic rats (RS-250mg/kg .po)	13.8
IV	Oligospermic rats(RS-500mg/kg.po)	16.2
V	Standard control(6mg/kg.po)	3.78

Table no 4: Tissue parameters

Group n=6	Group names	SOD activity (unit/mg protein)	Catalase activity (unit/mg protein)	MDA level (nM/mg protein)	Tissue cholinesterase (U/L)
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I	Normal control	1.73±0.02	12.16±1.18	525.64±1.37	130.03
II	Disease control	1.92±0.07**	57.5±1.87**	602.56±0.40**	1249.74
III	RS(low dose)	1.88±0.02## #	31.5±1.11	205.12±0.67###	228.64
IV	RS(high dose)	1.90±0.01## #	28.75±1.54	243.58±1.12	232.71
V	Std	1.89±0.11##	13.67±1.21##	358.97±0.81##	176.68

The values were expressed as Mean± SEM in each group. ***P<0.0001, was considered as significant. The statistical analysis was done by using Graph pad prism-Dunnet's test comparison of selected pair of columns with disease control.

Statistical analysis

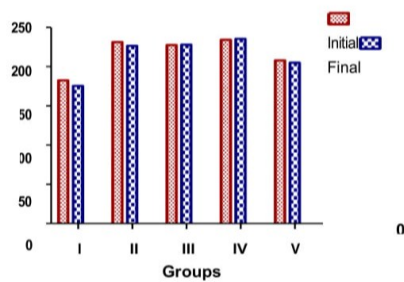


Fig 1. Effect of RS on body weight

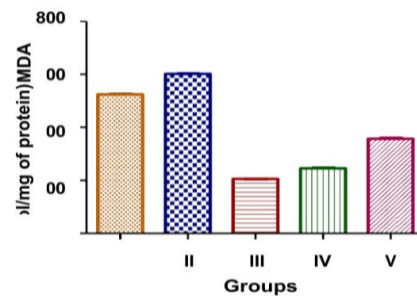


Fig 2. Effect of RS on MDA

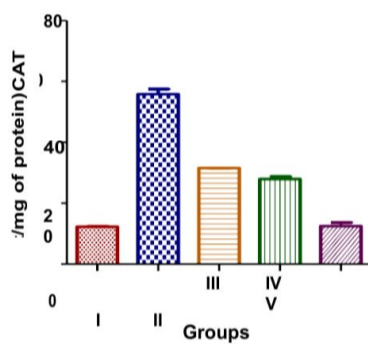


Fig 3. Effect of RS on CAT

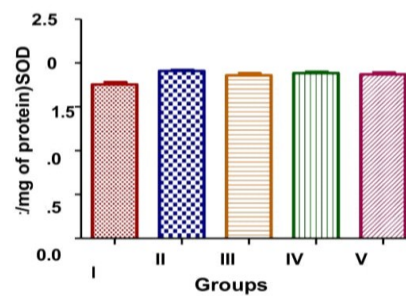


Fig 4. Effect of RS on SOD

Histopathology studies

Source of Specimen: Rat Testis

- The section of the testis of the control group when compared with treated group albino male rats showed observable differences in various stages of Spermatogenesis observed in different treated groups.
- In control group's albino male rats all stages of Spermatogenesis were clearly observed viz. Spermatogonia, primary spermatocytes, secondary spermatocytes, spermatid and spermatozoa, beside connective tissue, blood vessels, lymph ducts and Leydig's cell were observable and distinct.
- The proliferation was evidently more perceptible in case of treated group animals as compared to control.
- *Rajata Sindoor* 2.25 mg/kg.b.wt and 2.7/mg/kg.b.wt treated groups showed

increase in size of seminiferous tubules. Basement membrane was tightly bound with germinal epithelium. The lumen of seminiferous tubule was filled with bundles of spermatozoa.

- There was also increase in number of Leydig's cell as cytoplasm was highly stained with eosin.
- Under normal condition the Sertoli cells lie down near the basement membrane and are spaced at quite regular intervals where by they perform their functions of supporting the developing Spermatogenic cells, in general the nucleus is at right angles to the wall and the cell is pyramidal in shape.
- In case of *Rajata Sindoor* treated groups, the primary Spermatogenic cells, the spermatogonia are the first state of repetitive cell division.



Fig. no. 1 Normal Control

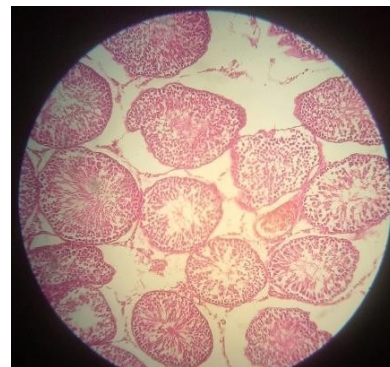


Fig. no. 2 Disease Control (CYP)

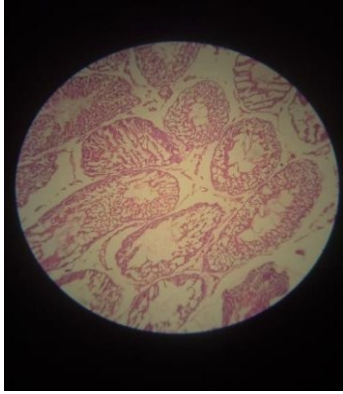


Fig. no. 3 Test-RS (lowdose-250mg/kg)

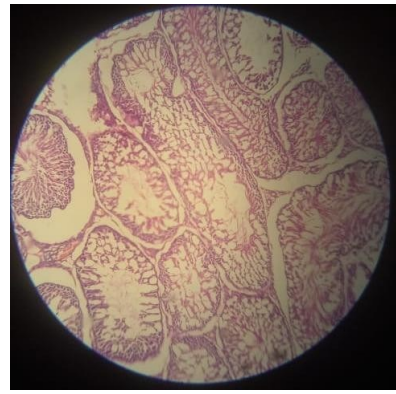


Fig. no. 4 Test-RS (Max dose-500mg/kg)



Fig. no. 5 Standard control (Fertisure)

DISCUSSION: Administration of *Rajata Sindoor* extracted in weight gain in treated albino male rats. Testis and epididymis weight were also increased significantly. The increase in body and organ weights was comparable with control group. A significant anabolic effect upon administration of *Rajata Sindoor* was observable as compared to the control group.

The experiment clearly suggests enhancement of sexual activity has been directly co related to the enhancement of sexual pleasure. Penile erection and increase in mobility, viability, morphology and count of sperm are important

for evaluating the effect of administration of *Rajata Sindoor* on erectile function. The aqueous extract of *Rajata Sindoor* increase the penile erection, mobility and count of semen of male rats. An increase in penile erection was observed in treated groups, indicating the involvement of nitrous oxide based intervention.

The effect in *Rajata Sindoor* treated group was much more pronounced when compared to the administration of Tab. Fertisure-M. Administration of *Rajata Sindoor* (22.5mg/kgbw and 45 mg/kgbw) modified both the orientation as well as sexual

behavior, conclusively suggesting a better sexual performance after administration of Rajata Sindoor. This activity was also validated by the histopathological on testis section.

The result thus confirms that, the drug extract can be useful in the enhancement of overall sexual performance of male rats. Drug also shows prolonged and sustained effect in overall sexual performance conducted during this study also increase spermatogenic activity. This may be due to the change in neuro-transmitter level or their action in the cell could also change sexual behaviour.

Spermatogenesis involves a complex interplay between the structural element of testis and the endocrine system. FSH (Follicular stimulating hormone) stimulates Spermatogenesis, also Testosterone cause direct stimulation of spermatogenesis. Our results also show that there is increase in Spermatogenesis and increase in weight of sexual organ in extracted *Rajata Sindoor* treated group as comparison to control group. The improvement of sperm count suggests an improved spermatogenic activity of the test extract. Similarly, the sperm count improvement also indicates that the *Rajata Sindoor* the quality of sperm count which eventually assist in better reproductive potential.

CONCLUSION

Rajata Sindoor is a *Sagandha*, *Sagni*, *Murchana Kalpana* which is *Kanthastha* and *Bahirdhooma Kupipakwa Rasayana*. Study shows increase in sperm count (upto 95-99 million) which shows effect of *Rajata Sindoor* in spermatogenesis. It showed statistically significant ($p < 0.05$) spermatogenic activity compared to control group ($p < 0.05$) and standard group ($p < 0.05$). *Rajata Sindoor* is competent with Standard drug Fertisure in Spermatogenic activity. Histopathology report of Testis of Albino rats shows normal Spermatogenesis. This shows Spermatogenic effect of *Rajata Sindoor*. With the factual evidence obtained by the above experimental data, it has been concluded that *Rajata Sindoor* is having significant spermatogenic activity.

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