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Effects on spermatogenesis in swiss mice of a protein isolated from the roots of *Ricinus communis* (Linn.) (Euphorbiaceae)

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ABSTRACT

This study was aimed to evaluate the effect on spermatogenesis of a 62 kDa protein (Rp) isolated from 50% ethanolic extract of the root of *Ricinus communis* in mice. A dose response study in mice revealed that 25 mg/kg body weight/day was the most effective dose. Swiss strain mature male mice of 30 days old were divided into two group namely control and Rp treated (25 mg/kg body weight/day). The study showed that sperm motility and count were decreased significantly in the treated group as compared to the control. The fertility index of the treated groups was reduced by 100%. The activity of HMG Co A reductase and cholesterol were increased significantly in the treated group. The testicular activities of 3βHSD, 17βHSD, glucose 6-phosphate dehydrogenase and malic enzyme and the level of serum testosterone were decreased significantly in the treated group. The expression of 3βHSD and 17βHSD were decreased and the expression of StAR increased significantly in the treated group as compared to the control. Proteolytic digestion of the native protein with trypsin and chymotrypsin showed that the proteolytic cleavage did not affect the spermicidal action of Rp. Hence this study can be concluded that Rp impaired spermatogenesis in vivo by suppressing the production of testosterone.

1. Introduction

Many plant and plant products regulate male and female fertility. One of the documented plants with antifertility effect is *Ricinus communis*. *R. communis* commonly known as castor bean belongs to the family of Euphorbiaceae. This is a plant grown widely throughout the world. Hence the plant and its products are freely available to humans. The root of the plant is an ingredient of various prescriptions in ayurveda for nervous diseases and rheumatic affections such as lumbago, pleurodynia and sciatica [1].

There were reports that the methanolic extract of *R. communis* seed has a reversible negative impact on male reproductive functions [2] and the antifertility and contraceptive efficacy of the seed was studied in women volunteers [3]. 50% ethanolic extract of the root of *R. communis* has an antidiabetic activity [4] and the methanolic extract has an anti-inflammatory activity in rats [5].

Previous studies conducted in our department showed that 50% ethanolic extract of the root of *R. communis* possess reversible antifertility effect [6] and preliminary studies showed that a protein present in the ethanolic extract was responsible for the antifertility effects.

The objective of this present study was to isolate the active protein, a 62 kDa protein (Rp), from 50% ethanolic extract of the root of *R. communis* and to study its effect on the spermatogenesis in swiss mice.

2. Experimental

2.1. Plant material

Roots of *R. communis* were collected from Thiruvananthapuram, India in 2009 and identified by Dr. Valsala Devi, Curator, Department of Botany, University of Kerala. A voucher specimen (KUBH 5786) is deposited at the Herbarium of Department of Botany, University of Kerala.

2.2. Separation of protein

The roots were dried in shade and 10gm of the dried material in 100 ml of 50% ethanol was refluxed at 65°C for 90 min and after that it was filtered and evaporated to dryness at 65 °C. The yield of the extract was 10 g/100 g dry root. About 1 g of the extract was dissolved in 25 ml saline, stirred, centrifuged at 2000 rpm for 10 min and the supernatant was dialyzed against double distilled water. Dialyzed sample was evaporated to dryness at 50 °C. The sample was then dissolved in potassium phosphate buffer (pH 7.0) and carried out diethyl amino ethyl cellulose (DEAE) ion exchange

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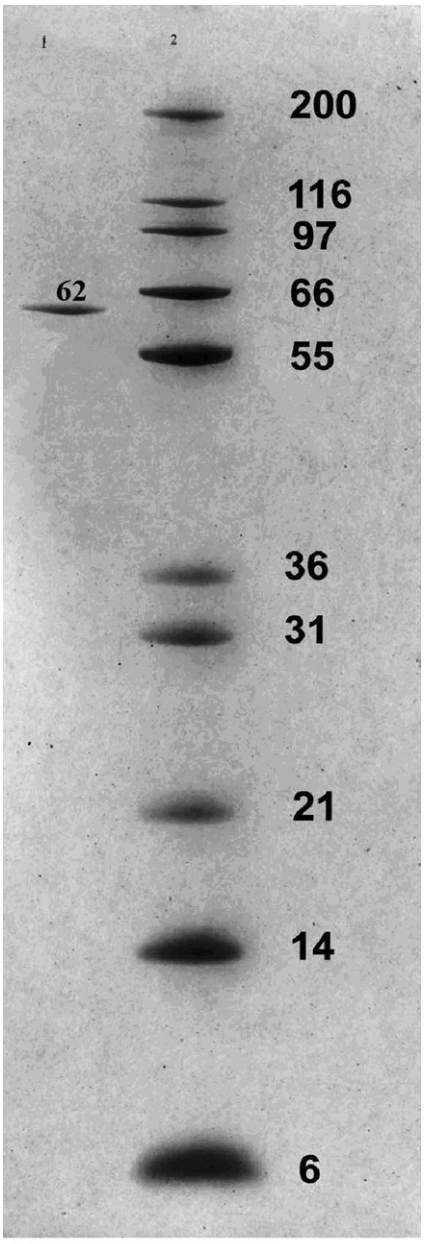

Fig. 1. Native polyacrylamide gel electrophoresis.

chromatography [7]. Gradient elution was carried out using potassium phosphate buffer (pH 7.0) containing KCl (50–200 mM) and each fraction was tested for sperm immobilization activity. The 200 mM fraction showed maximum sperm immobilization activity. This fraction was pooled, dialyzed against double distilled water to remove salt and concentrated. It was further subjected to gel filtration (Sephadex G100) using phosphate buffered saline. The fractions showing sperm immobilizing activity was pooled, concentrated, dialyzed against double distilled water and subjected to 20% native polyacrylamide gel electrophoresis(Native PAGE). A protein of molecular weight 62 kDa obtained from *R. communis* (Fig. 1) showed sperm immobilization activity. This protein has been named Rp. This native protein was electro eluted, dialyzed against double distilled water, concentrated and used for the studies. The molecular weight was quantified by Bio-Rad Gel Doc (California, USA) using Quantity One imaging software.

2.3. In vitro semen analysis

Semen was collected from cauda epididymis of healthy male mice (Swiss strain) and was diluted with normal saline and kept at 37 °C. Sperm count above 100 million/ml and viability above 60% with normal morphology, rapid and progressive motility were used for in vitro analysis. Sperm immobilization assay was done by treating the diluted sample with isolated protein fractions from each step of purification and the time taken for immobilization was recorded using a phase contrast microscope.

2.4. Experiment 1: Dose dependent studies

Swiss strain mature male mice of 30 days (body weight 25 ± 5 g) old bred in the University animal house and maintained on a normal laboratory diet (Amruth India Pvt. Ltd., Bangalore, India) were used for this study. Animal experiments were conducted in accordance with institutional ethics committee guidelines for the conduct of the experiments on laboratory animals as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Water and food were given ad libitum. The mice were housed in polypropylene cages in a room at 25 °C with 12:12 h light and dark cycle.

Mice were divided into 6 groups as follows:

Group 1: Control
Group 2: administered Rp at dose of 5 mg/kg body weight/day
Group3: administered Rp at dose of 10 mg/kg body weight/day
Group 4: administered Rp at dose of 25 mg/kg body weight/day
Group 5: administered Rp at dose of 50 mg/kg body weight/day
Group 6: administered Rp at dose of 100 mg/kg body weight/day

Rp was dissolved in saline and given orally by gastric intubation as detailed above. Duration of the experimental period was 35 days, since the time for the complete spermatogenic cycle in mice is 34.5 days [8]. At the end of the experimental period, all the animals were fasted overnight and sacrificed and semen was collected from cauda epididymis. Sperm motility, morphology and count were done according to WHO Manual [9].

2.5. Experiment 2: Detailed studies

Selection of the animal and method of rearing the animal were the same as described in experiment 1.

Mice were divided into two groups of 12 each,

Group 1: Control
Group 2: Rp treated

Rp was dissolved in saline and given orally by gastric intubation at a dose of 25 mg/kg body weight/day. Duration of the experimental period was 35 days. At the end of the experimental period, six animals in each group were fasted overnight and sacrificed. Testis was removed to ice cold containers for various analyses. The rest of the animals in each group were mated with females (mature and swiss strain mice) in the ratio 1 male to 3 females for the evaluation of fertility index. The fertility index was calculated as the % of female mice that delivered healthy progenies from the total number of mice mated. (Number of mice delivered/number of female mice mated ×100.)

2.5.1. Biochemical analysis

Sperm motility, morphology and count were done according WHO Manual [9] Testicular lipids were extracted according to the method of Folch et al. [10] and cholesterol was estimated by the method of Abell et al. [11]. HMG Co A reductase activity

Table 1
Oligonucleotide primers used for RT-PCR.

Gene product	Oligonucleotide
3βHSD	Sense primer: 5′-TCTGAAAGGTACCCAGAACCTATT-3′ Anti-sense primer: 5′-TTGCTTGAACACAGGCCTCCA-3′
17βHSD	Sense primer: 5′-AGTGTGGGAGGCTTGATGGGA-3′ Anti-sense primer: 5′-CACTTCGTGGAATGGCAGTCC-3′
StAR	Sense primer: 5′-GAGCTCTCTGCTTGGTTCTCA-3′ Anti-sense primer: 5′-TTGAGTATGCCCAAGGCCTT-3′
GAPDH	Sense primer: 5′-GAAGGGCTCATGACCACAGT-3′ Anti-sense primer: 5′-GGATGCAGGGATGATGTTCT-3′

was assayed by the method described by Rao and Ramakrishnan [12]. The activities of 3β hydroxy steroid dehydrogenase (3βHSD) [13], 17β hydroxy steroid dehydrogenase (17βHSD [14], glucose 6-phosphate dehydrogenase [15] and malic enzyme [16] were analyzed. Serum testosterone level was assayed by RIA method using the kit for total testosterone purchased from Diagnostic Product Corporation, USA.

2.5.2. Quantification of 3β HSD, 17βHSD and StAR

Total RNA was isolated from testis using Trizol reagent (Sigma–Aldrich Pvt. Ltd.) and RNA with $A_{260/280}$ ratio 1.8 and above was used for reverse transcriptase-polymerase chain reaction (RT-PCR) to quantify the expression of 3βHSD, 17βHSD and StAR protein. The primer sequences of these are gene-specific and presented in Table 1. Total RNA was reverse transcribed and PCR was performed using Eppendorff RT-PCR kit with gene-specific primers [17]. PCR mixture was resolved on 2% agarose gel containing ethidium bromide. Molecular marker was simultaneously resolved in the first lane. Then the gels were subjected to densitometric scanning (Bio-Rad) to determine the OD of each and then normalized against internal control, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) by Bio-Rad Gel Doc (California, USA) using Quantity One imaging software.

2.5.3. Histopathology of testes

For histopathological studies, testes were fixed in Bauins' fixative and sections were taken in the microtome. Sections were stained using hematoxylin and eosin. Pathological changes were examined using a sensitive light microscope.

2.5.4. Proteolytic digestion of Rp

Proteolytic digestion of native protein (100 μg/ml) with the digestive enzymes trypsin (2 mg/ml) and chymotrypsin (1 mg/ml) were done separately at 37 °C for 1 h and then heated at 55 °C for 30 min to inactivate the proteolytic enzymes. The digested samples were applied on a Whatmann filter paper (No 1) and subjected to paper chromatography using butanol, glacial acetic acid and water in the ratio 12:3:5 as solvent. The chromatogram was developed using ninhydrin reagent (0.2% in acetone). These samples were eluted and sperm immobilization effect was evaluated in vitro.

2.6. Statistical analysis

The results were analyzed using a statistical programme SPSS/PC+, version 11.5 (SPSS Inc., Chicago, IL, USA) using student's *t* test for the comparison of two groups. A one-way ANOVA was employed for comparison among six groups. Duncan's post-hoc multiple comparison tests of significant tests of significant differences among groups were determined. $P \leq 0.05$ was considered to be significant [18].

3. Results

3.1. Isolation of the active protein (Rp)

The result of native PAGE analysis revealed the presence of a 62 kDa protein (Fig. 1). This protein is named as Rp. This Rp was electro eluted, dialyzed against double distilled water, concentrated and used for further studies. Rp caused the complete immobilization of sperm within seconds. So dose dependent studies were conducted with Rp.

3.2. Dose dependent studies

Result of the dose dependent study showed that as the concentration of Rp was increased the spermatotoxicity was also increased. 25 mg/kg body weight/day was the minimum effective dose which caused maximum spermatotoxicity (Table 2). So this dose was selected for detailed *in vivo* studies.

3.3. Detailed studies

The effect of Rp on sperm motility, morphology, fertility index and sperm count

Sperms belonging to the treated group were motile for shorter periods than the sperms of the control group. Maximum morphological abnormalities were seen in the Rp treated group when compared to the control. The fertility index of the treated group was reduced by 100% and sperm count was also decreased significantly in the treated group as compared to the control (Table 3).

3.3.1. The effect of Rp on testicular parameters

The activity of HMG CoA reductase is measured as the ratio of HMG CoA to mevalonic acid. Hence lower the ratio indicates higher the activity. The testicular activity of HMG CoA reductase and cholesterol were increased significantly in the treated group. The activities of 3βHSD and 17βHSD were decreased significantly in the treated group. There was also a significant decrease in the levels of serum testosterone in the treated group in comparison to the control. The activities of glucose 6-phosphate dehydrogenase and malic enzyme in the testis were decreased significantly in the treated group as compared to the control (Table 4).

Table 2
Experiment 1: Dose dependent studies of Rp.

Group	Sperm motility (time taken for complete immobilization of sperm in minutes)	Sperm morphology (%)	Sperm count (million/ml)
Group 1 (Control)	30.75 ± 2.81	8	51.25 ± 4.46
Group 2 (5)	20.81 ± 1.91[a]	15	48.10 ± 4.12
Group 3 (10)	12.50 ± 1.14[a]	30[a]	41.46 ± 3.78[a]
Group 4 (25)	4.23 ± 0.46[a]	75[a]	25.75 ± 2.81[a]
Group 5 (50)	2.20 ± 0.20[a]	85[a]	22.72 ± 3.64[a]
Group 6 (100)	Within seconds[a]	95[a]	15.17 ± 1.38[a]

Values are mean ±SEM.
[a] $p < 0.05$ between control and treated group.

Table 3
The effect of Rp on sperm motility (%), morphology (%), fertility index and sperm count (millions/ml).

Groups	Sperm motility at 0′ and mode of movement	Time taken for complete immobilization of sperms in minutes	Total abnormal sperms (%)	Fertility index (%)	Sperm count (millions/ mL)
Control	Rapid progressive, 100% motility	30.75 ± 2.81	8	100%	51.25 ± 4.46
Rp	Slight nonprogressive, 40% motility	4.23 ± 0.46[a]	75[a]	0	25.75 ± 2.81[a]

Values are mean ±SEM.
[a] $p < 0.05$ between control and treated group.

3.3.2. RT-PCR analysis of 3βHSD, 17βHSD and StAR

The expression of 3βHSD and 17βHSD were decreased significantly in the Rp treated group as compared to the control (Figs. 2 and 3). The expression of StAR increased significantly in the Rp treated group as compared to the control (Fig. 4).

3.3.3. Histopathology of testes

The testicular section of control group showed normal seminiferous tubule and the lumen was filled with spermatozoa (Fig. 5(a)). Rp did not cause any change in the histology of testes, but the number of spermatozoa were reduced significantly (Fig. 5(b)).

3.3.4. Proteolytic digestion of Rp

Proteolytic digestion of Rp with trypsin and chymotrypsin showed that chymotrypsin digestion gave 4 peptides and trypsin digestion gave 2 peptides (Fig. 6). The sperm immobilization effect of these peptides was evaluated in vitro. It showed that 4th fraction of chymotrypsin and 2nd fraction of trypsin digestion had maximum sperm immobilization effect (Table 5).

4. Discussion

Dose dependent study showed that the minimum dose for the maximum spermatotoxicity of Rp was 25 mg/kg body weight/day. So this dose was used for detailed in vivo studies. The duration of the experiment was 35 days so as to cover the complete spermatogenic cycle in mice which is 34.5 days [8].

The detailed in vivo studies revealed that the native protein Rp from 50% ethanolic root extract of *R. communis* impaired spermatogenesis. A significant reduction in sperm motility and count were observed in Rp treated group as compared to the control. Histopathological studies also support this. Maximum morphological abnormalities of sperm were found in the Rp treated group as compared to control. This is in agreement to our earlier studies where in we had reported the spermatotoxicity of the ethanolic extract of *R. communis* [6]. Similar spermatotoxicity was also observed in rats followed by the administration of areca nut extracts [19]. Only few studies have documented the spermatotoxicity of proteins. Reddy et al, 2004 [20] observed that Nisin, a 34 amino acid cationic peptide produced by *Lactococcus lactis* has sperm immobilization effect. A 52 kDa protein isolated from 50% ethanolic extract of the root bark of *Cananga odorata* [21] and a 58 kDa protein from *Achyranthes aspera* [22] were also reported to have similar spermatotoxicity in male animals.

The detailed in vivo studies in mice showed that in Rp treated group, there was an increase in the level of cholesterol. This may be due to increased biosynthesis of cholesterol as evidenced by the enhanced activity of the rate limiting enzyme HMG CoA reductase.

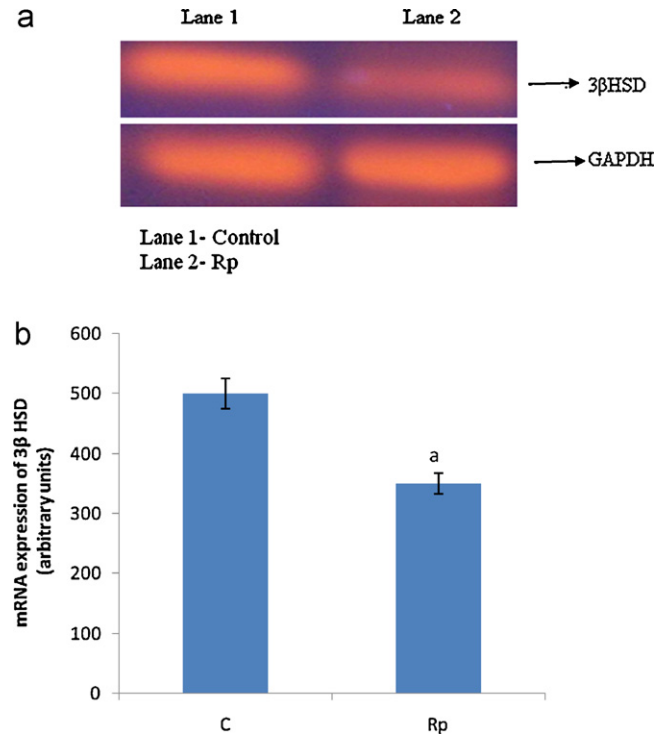

Fig. 2. Expression of 3βHSD. Parts (a) and (b) represents the effect of mRNA expression of 3βHSD relative to GAPDH. Each bar represents mean ± SEM. [a] $p < 0.05$ between control and treated group.

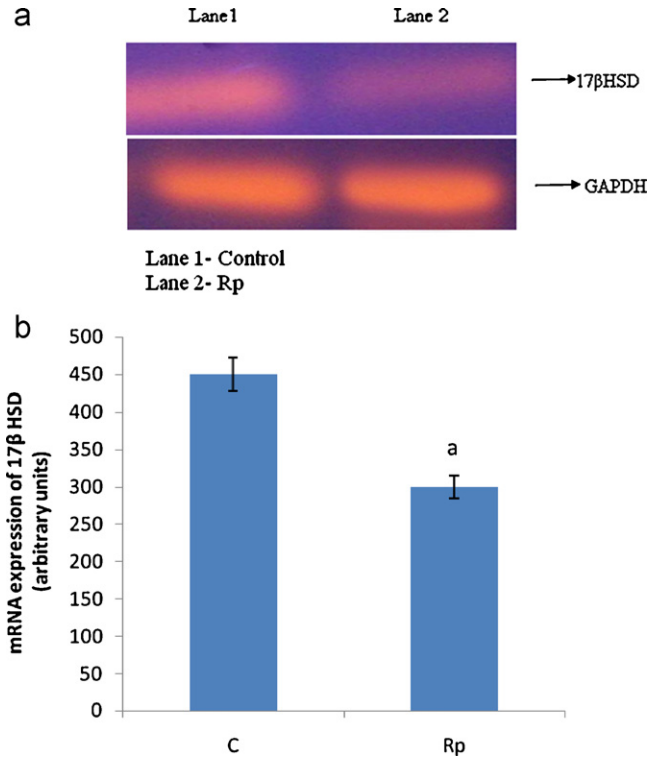

Fig. 3. Expression of 17βHSD. Parts (a) and (b) represents the effect of mRNA expression of 17βHSD relative to GAPDH. Each bar represents mean ± SEM. [a] $p < 0.05$ between control and treated group.

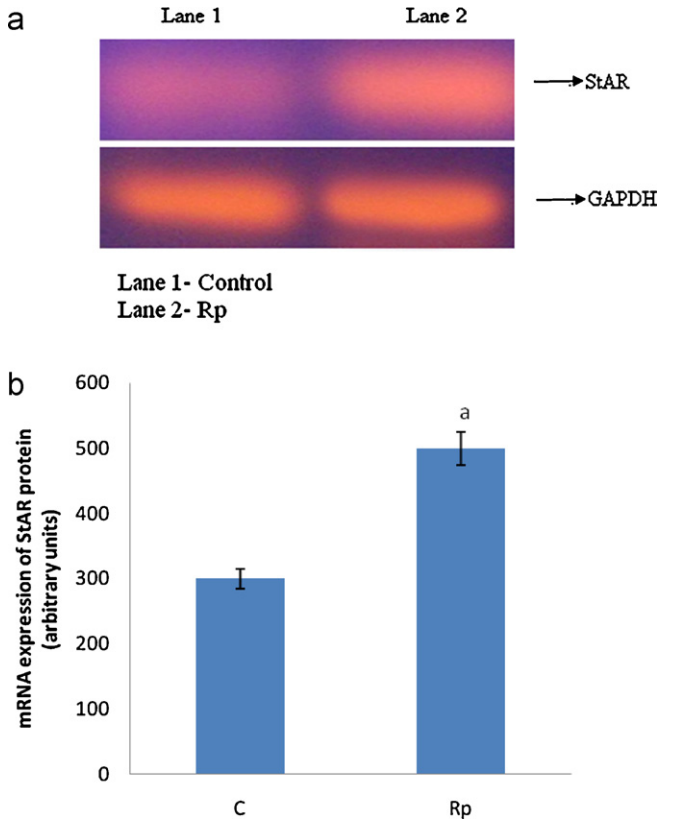

Fig. 4. Expression of StAR. Parts (a) and (b) represents the effect of mRNA expression of StAR protein relative to GAPDH. Each bar represents mean ± SEM. $^{a}p < 0.05$ between control and treated group.

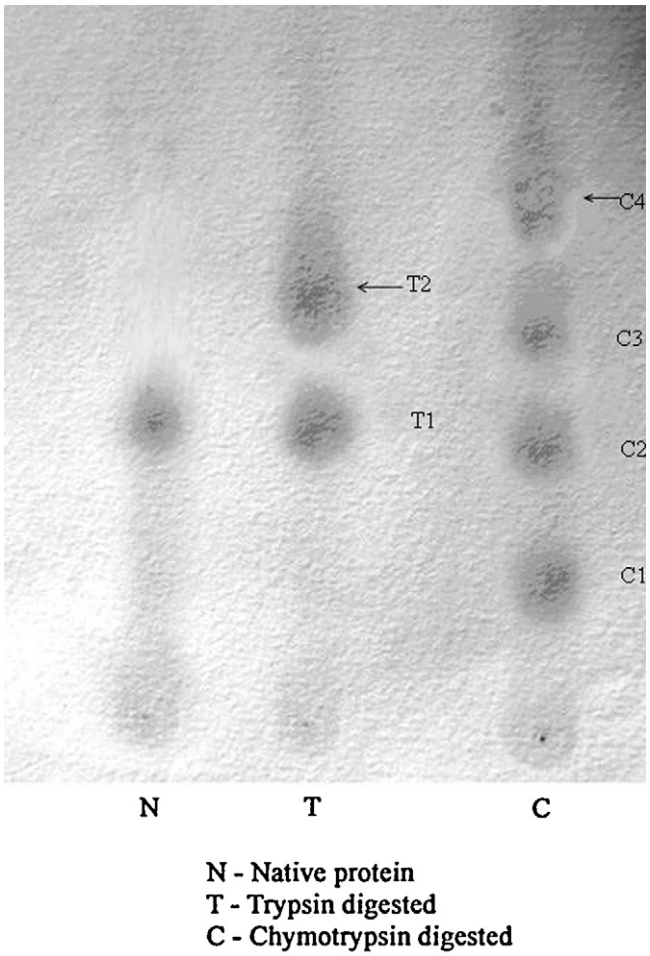

Fig. 6. Proteolytic digestion of Rp.

The increased level of cholesterol may be also due to the decreased androgen production, since there was decrease in the serum testosterone level.

Cholesterol is metabolized to testosterone. The key enzymes involved in this are 3βHSD and 17βHSD. The activities of 3βHSD and 17βHSD were decreased significantly in the treated groups as compared to the control. This might have caused decreased biosynthesis of testosterone. There was also decrease in the activities of glucose 6-phosphate dehydrogenase and malic enzyme in the testis in the treated group. As a result, there may be a lower supply of NADPH which is essential for the conversion of cholesterol to testosterone. This may be also a factor for the lower level of testosterone.

To further strengthen our above findings, mRNA expression of 3βHSD, 17βHSD and StAR protein were also assayed. In agreement with enzymatic studies in Rp treated group, the expression of 3βHSD and 17βHSD were decreased in comparison with the control. But the expression of StAR protein was increased significantly in the treated group as compared to the control. StAR is a transport protein that regulates cholesterol transfer within the mitochondria. Its expression is usually increased in steroidogenic tissues in response to agents that stimulate steroid production [23]. But contrary to these reports testosterone was reduced in the treated

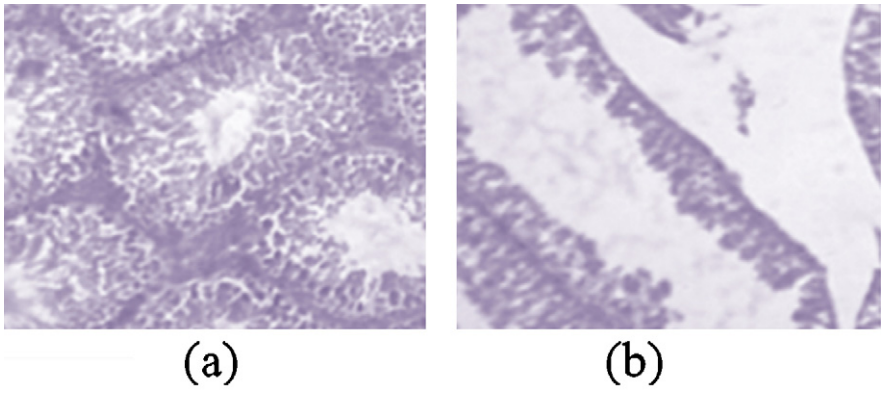

Fig. 5. Histopathology of testes. (a) Testicular section of control group which show normal seminiferous tubule. Germinal cells show varying degree of maturation and lumen filled with spermatozoa. (b) Testicular section of mice administered with Rp which show normal seminiferous tubule. Germinal cells proliferated, lumen show reduced spermatozoa.

Table 4
The effect of Rp on testicular parameters.

Groups	HMG CoAreductase (HMG/mevalonate)	3βHydroxy steroid dehydro genase (absorbance*/mg protein)	17βHydroxy Steroid Dehydro genase (absorbance*/mg protein)	Glucose6-phosphate dehydro genase (molesof NADPH formed/min/mg protein)	Malic enzyme (units#/mg protein)	Cholesterol (mg/100g tissue)	Serum testosterone (ng/mL)
Control	3.74 ± 0.34	7.18 ± 0.65	9.23 ± 0.84	3.63 ± 0.33	8.82 ± 0.80	205.0 ± 18.70	1.54 ± 0.14
Rp	1.65 ± 0.15[a]	4.10 ± .37[a]	5.13 ± 0.47[a]	2.16 ± 0.20[a]	5.13 ± 0.47[a]	256.25 ± 23.38[a]	0.18 ± 0.02[a]

Values are mean ± SEM.
* Change in absorbance of 0.001/min at 340 nm.
# Enzyme that cause increase in optical density (OD) of 0.01/min.
[a] $p < 0.05$ between control and treated group.

Table 5
Proteolytic digestion of Rp.

Sample	Time taken for complete immobilization of sperm (s)
Rp	63.04 ± 5.76
Trypsin digested	
T1	59.97 ± 5.48
T2	43.57 ± 3.98[a]
Chymotrypsin digested	
C1	53.63 ± 5.14
C2	48.75 ± 4.68[a]
C3	43.88 ± 4.20a
C4	30.75 ± 2.81a

Values are mean ±SD.
[a] $p < 0.05$ between Rp and treated groups.

groups, although there was increase in cholesterol level. It has been observed that StAR could increase the expression of cholesterol transporters [24]. So the enhanced cholesterol level observed might have induced StAR protein. But since the activities and expression of the key enzymes of testosterone production were reduced, there was reduction in testosterone level.

Since the active principle was a protein, doubts were raised whether the protein will be digested when given orally. So the present studies were extended up to determine whether the proteolytic digestion of Rp affects the spermatotoxic activity. Proteolytic digestion of the protein with trypsin and chymotrypsin showed that the proteolytic cleavage did not affect the spermicidal action of Rp. This was evidenced by the fact that even after proteolytic cleavage, the peptides in the protein preparation caused the complete immobilization of sperm and the mechanism of action of Rp may be mediated through the small peptides of Rp.

5. Conclusion

It can be concluded that the protein Rp impaired spermatogenesis in vivo. Rp exerts its action by suppressing the production of testosterone by inhibiting the production of the key enzymes 3βHSD and 17βHSD and the effect may be due to the action of some peptides of the native protein.

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