ORIGINAL ARTICLE

Effect of *Mucuna pruriens* on oxidative stress mediated damage in aged rat sperm

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**Introduction**

There is a growing interest in the age-related changes that occur in the male reproductive system. Ageing process has been studied extensively for testicular steroidogenesis, prostatic function and intercourse frequency (Feldman *et al.*, 2000); there is apparent reduction in semen volume with an increase in age and it is more likely the reflection of impaired androgen action in the reproductive organs (Gooren, 1996). Characteristic signs of ageing are reduced serum levels of testosterone, diminished sperm production, degenerative changes in the seminiferous tubules, lipofuscin accumulation and thickening of the basement membrane. In addition, there are cell-specific vacuolization with an increase in the number and size of lysosomes (Zirkin *et al.*, 1993; Serre & Robaire, 1998). Different mechanisms play a role in the ageing of cells and tissues; understanding the mechanisms could help to delay the onset of age-related diseases (Pickett, 2007). Oxidative stress (OS) plays a significant role in the sperm quality and function. Pathobiology of duct system reflected in the sperm function and OS was known to play a major role in the aetiology of defective sperm function via mechanisms involving the induction of peroxidative

**Keywords:**
ageing, antioxidants, free radicals, *Mucuna pruriens*, sperm

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Received 3 October 2008; revised 12 November 2008; accepted 18 November 2008
doi:10.1111/j.1365-2605.2008.00949.x

**Summary**

*Mucuna pruriens* Linn., a leguminous plant, has been recognized as an aphrodisiac and spermatogenic agent. Protective efficacy of *M. pruriens* on reactive oxygen species (ROS)-induced pathophysiological alterations in structural and functional integrity of epididymal sperm in aged Wister albino rat was analysed. Animals were grouped as groups I, II, III and IV, i.e. young (control), aged, aged treated with ethanolic extract (200 mg/kg b.w.) of *M. pruriens* and young rats treated with *M. pruriens*, respectively. At the end of the experimental period, i.e. after 60 days animals were sacrificed, epididymal sperm were collected and subjected to count, viability, motility, morphology and morphometric analysis. Enzymatic and non-enzymatic antioxidants, ROS, lipid peroxidation (LPO), DNA damage, chromosomal integrity and mitochondrial membrane potential were estimated. Results obtained from the aged animals showed significant reduction in sperm count, viability and motility, increased morphological damage and an increase in the number of sperm with cytoplasmic remnant, and these alterations were significantly reversed in *M. pruriens* treated group. Significant increase in LPO, HO- and H₂O₂ production and significant decline in the levels of the enzymatic and non-enzymatic antioxidants were observed in the aged animals. Supplementation of *M. pruriens* significantly reduced ROS and LPO production and significant increase in both enzymatic and non-enzymatic antioxidant levels. There were significant DNA damage, loss of chromosomal integrity and increase in mitochondrial membrane permeability in aged rat sperm. This was significantly reduced in group III. Present observation indicates the antioxidant enhancing property, free radical quenching ability and spermatogenic efficacy of the *M. pruriens*. Collectively, sperm damage in ageing was significantly reduced by quenching ROS, improving antioxidant defence system and mitochondrial function.
damage to the plasma membrane (Griveau & Le Lannou, 1997).

*Mucuna pruriens* Linn. (Fabaceae), commonly known as ‘cowhage plant’ or ‘kapikacho’ or ‘kevach’ in Hindi, is the most popular drug in the Ayurvedic and Unani (Indian system of medicine). The plant is being cultivated in India, Sri Lanka, South East Asia and Malaysia (Kharel, 2004). *Mucuna pruriens* seeds extract was used to manage several free radical-mediated diseases, such as rheumatoid arthritis, diabetes, atherosclerosis, nervous disorders and male infertility (Rajeshwar *et al.* , 2005). Seed powder of *M. pruriens*, used in many conditions like controlling stress, increases the secretion of semen, acts as a restorative and an invigorating tonic or aphrodisiac in diseases characterized by weakness or loss of sexual power (Kumar *et al.* , 1994). It is also used in the management of Parkinsonism, as it is a good source of l-3,4 dihydroxyphenyl alanine (l-DOPA) (Molloy *et al.* , 2006). Seed of *M. pruriens* also possess antioxidant, hypoglycaemic, lipid lowering and neuroprotective activities (Sharma *et al.* , 1978). It contains alkaloids, mucunine, mucunadine, mucunadinine, prurienidine and nicotine, besides b-sitosterol, glutathione, lecithin, vernolic acid and gallic acid. Seed of *M. pruriens* reported to contain good number of other bioactive substances including tryptamine, alkalamines, steroids, flavonoids, coumarins, cardenolides and metals like magnesium, copper, zinc, manganese and iron (Misra & Wagner, 2007) and other compounds such as oleic acid, linoleic acid and palmitic acid (Adebowale *et al.* , 2005).

With these richest natural sources of bioavailable and bioactive antioxidant compounds in the seed of *M. pruriens*, this prospective study was aimed to evaluate the antioxidant property of the ethanolic seed extract on the levels of reactive oxygen species (ROS) and sperm damage in aged rat epididymal sperm.

**Materials and methods**

**Animals**

Animals were maintained as per the national guidelines and protocols, approved by our institutional ethical committee (IAEC No. 01/031/05). Healthy male Wistar albino rats (*Rattus norvegicus*) is divided into four groups: group I – control (young weighing 175–200 g 3–6 month); group II – aged (weighing 425–450 g 26–28 month old rats); group III – aged and treated with *M. pruriens* seed extract and group IV – young rats treated with *M. pruriens* seed extract (extract treated control). Extract was administrated orally (200 mg/kg body weight), once daily for 60 days. The colony was maintained under controlled conditions and in room temperature (23 ± 2 °C), humidity (50 ± 5%) and a 12 h light–dark cycle. The animals were housed in sanitized polypropylene cages. The animals were fed with standard rat pellet diet and drinking water ad libitum. The animals were sacrificed by cervical decapitation.

**Chemicals**

Ethidium bromide, acridine orange, 2’,7’-dichlorofluorescin diacetate (DCFH-DA), 4’,6-diamidino-2-phenylindole (DAPI) and 2-(6-amino-3mino-3h-xanthene-9-yl) benzoic acid methyl ester (rhodamine 123) were purchased from Sigma (St. Louis, MO, USA). Other chemicals were purchased from SISCO Research Laboratory (SRL, Mumbai, India).

**Plant material and extract preparations**

The seeds of *M. pruriens* were procured locally after authentication, and the voucher specimen (Herbarium Voucher No. 6907) was deposited in the Department of Plant Biology and Plant Biotechnology (The Presidency College, Chennai, India). Seeds were washed twice using tap water and then washed again in distilled water to remove the dust. The seeds were dried in the shade for 7–12 days, and then crushed into coarse powder. Later, it was transferred into a container and ethanol was added as a solvent until the coarse particles of the seed were completely soaked. The container was gently shaken for few minutes at 1-h interval for 72 h (until the colour of the solvent becomes colourless), and the filtrate was vacuum concentrated to remove the moisture content (Harborne, 1973). Percentage of yield was around 20%.

**Sperm count, viability and motility**

Sperm count was carried out according to the procedure described by Atessahin *et al.* (2006). Briefly, spermatozoa were collected from caudal portion of the epididymis, by mincing caudal epididymis with anatomical scissors in 5 mL of pre-warmed (35 °C) physiological saline, placed in a rocker for 10 min and incubated at room temperature for 2 min. The supernatant fluid was diluted 1 : 100 with a solution containing 5 g sodium bicarbonate, 1 mL formalin (35%) and 25 mg eosin per 100 mL H2O. Total sperm number was determined with a haemocytometer. Approximately, 10 μL of diluted sperm suspension was transferred to each counting chamber and was allowed to stand for 5 min and counting under a light microscope at 400x magnification.

About 20 μL of sperm suspension was mixed with an equal volume of 0.05% eosin-Y and nigrosin. After 2-min incubation at room temperature, slides were viewed by bright-field microscope under 400x magnification (Nikon...
Corporation, Tokyo, Japan). Dead sperm appeared pink and live sperm were not stained (Wyrobek et al., 1983). Two hundred sperms were counted for each sample and viability percentages were calculated. Percentage of motile sperm was assessed using graded semi-quantitative scale of 0–5, and the spermatozoa were evaluated for the rate of forward movement and graded accordingly, i.e. 0 = no movement; 1 = sluggish or tail movement alone; 2 = intermittent sluggish movement; 3–4 = fair & good movement; 5 = maximum movement in forward direction.

Morphology and morphometry
The fixed sperm were smeared on a glass slide and stained with phosphate buffered saline solution of Giemsa (Merck, Darmstadt, Germany) (Hafez, 1977). Sperm analysis for head and tail defects was performed as per WHO Laboratory Manual (WHO, 1999) for semen analysis. The length of the head and flagellum was evaluated in 100 spermatozoa (intact sperm) per animal under the light microscope by using ocular micrometer scale at 400× magnification.

Cytoplasmic droplet
The presence of cytoplasmic droplet was determined using the method described by Syntin & Robaire (2001). A minimum of 100 spermatozoa/animal were evaluated.

Estimation of protein and enzymatic antioxidant
Protein content was determined by the method of Lowry et al. (1951). The activity of superoxide dismutase (EC 1.15.1.1, SOD) was assayed according to the method of Marklund & Marklund (1974). The enzyme activity was expressed as units/mg protein. The activity of catalase (EC 1.11.1.6, CAT) was assayed by the method of Sinha (1972). The activity of CAT was expressed as units/mg protein (one unit is the amount of enzyme that utilizes μmole of hydrogen peroxide/min). The activity of glutathione peroxidase (EC 1.11.1.9, GPx) was determined by the method of Rotruck et al. (1973). The enzyme activity was expressed as units/mg protein (one unit is the amount of enzyme that converts μmole reduced glutathione (GSH) to oxidized glutathione (GSSG) in the presence of hydrogen peroxide/min). The activity of glutathione reductase (EC 1.6.4.2, GR) was determined by the method of Staal & Vegel (1969). Activity of GR was expressed as micromoles of NADPH (nicotinamide adenine dinucleotide phosphate) oxidized/min/mg protein. This glutathione-S-transferase (EC 2.5.1.18, GST) enzyme was assayed by the method of Habig et al. (1973). Activity of GST was expressed as units/mg protein (one unit is the amount of enzyme that conjugate 1 nmol of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH/min).

Estimation of non-enzymatic antioxidants
The level of vitamin E was estimated by the method of Desai (1984). The level of vitamin E was expressed as μg/mg protein. The level of ascorbic acid was estimated by the method of Omaye et al. (1979). The level of ascorbic acid was expressed as μg/mg protein. The level of reduced glutathione was measured by the method of Moron et al. (1979). The level of glutathione was expressed as μg/mg protein.

Estimation of ROS and LPO
Hydrogen peroxide production was estimated by the method of Pick & Keisari (1981) and the production was expressed in μmole/mg protein. Hydroxyl radical production was estimated by the method of Pantarulo & Cedebaum (1991) and the production was expressed in μmole/min/mg protein. The level of lipid peroxidation (LPO) was measured by the method of Ohkawa et al. (1979). The thiobarbituric acid reactive substance was expressed in nmole of malondialdehyde (MDA) formed/min/mg protein.

Confocal microscopic study for ROS production
Generation of ROS was determined by using DCFH-DA as a probe, according to the method of Wang et al. (2007) with little modification. A total of 500 μL of sperm were mixed with equal amount of 5 μM/mL of DCFH-DA and incubated at 37 °C for 30 min. After incubation, the tubes were centrifuged at 5600 g for 5 min and the supernatant was discarded to remove excess dye. Sperm pellet was re-suspended in 1 mL PBS. A drop of suspension was placed on a micro slide and then a cover slip was placed without air bubble formation. Slides were observed under confocal microscope (Leica Microsystem, Wetzlar, Germany) at an excitation and emission wavelength of 488 and 525 nm respectively.

Sperm chromosomal integrity study (Acridine orange staining)
Sperm chromosomal integrity study was performed by the method of Erenpreiss et al. (2001). Sperm heads with good DNA integrity had green fluorescence, and those with diminished DNA integrity had orange-red staining. Samples were observed within 48 h of staining. A minimum of 100 spermatozoa/animal were evaluated.

Quantitative assessment of DNA damage (comet assay)
Neutral single cell electrophoresis (Hughes et al., 1996) was performed with some modifications. The slides were
observed under fluorescent microscope in green light at 590 nm. A minimum of 100 spermatozoa/animal were evaluated. The length of the comet tail was measured by using ocular micrometer scale.

Mitochondrial membrane potential

The changes in the sperm mitochondrial membrane potential were estimated using the fluorescent cationic dye rhodamine 123 (Rh123) by the modified method of Windsor (1997). Rh123 dissolved in 0.01 M PBS was added to sperm samples at a final concentration of 40 μg/mL and incubated at 37 °C for 30 min. After incubation, the tubes were centrifuged at 5600 g for 5 min and the supernatant was discarded to remove excess dye. The sperm pellet was re-suspended in 1 mL PBS then added 50 μL of DAPI and mixed well. A drop of suspension was placed on a micro slide and cover slip was placed without air bubble and observed under fluorescent microscope, at an excitation and emission wavelength of 490 and 515 nm, respectively.

Statistical analysis

The data were statistically analysed using ‘ANOVA’. When the ‘F’ ratio was statistically significant, the data were subjected to the ‘one-way ANOVA’ test (Zar, 1974). Values were considered significant at p < 0.05.

Results

Sperm analysis

Significant reduction in the percentage of sperm viability was observed in the group II when compared with group I. Supplementation of M. pruriens significantly reversed this in group III (Table 1). Figure 1 shows the sperm motility in control and experimental groups; significant decline in sperm motility was observed in group II when compared with group I; this was significantly reversed in group III. No alterations in motility were observed in the M. pruriens alone treated animals (group IV).

The sperm concentrations and motility were markedly decreased in the aged rats (Fig. 2); these alterations were significantly reversed in group III. No significant changes were observed in the morphometrical study in all the experimental groups (results not shown). Morphological analysis shows wide degree of abnormality in aged rat sperm, such as defects in the head (microcephalic, bicephalous, amorphous and acephalic), neck and tail. These morphological defects were significantly reduced in group III (Table 1 & Plate 1).

There was an increase in sperm with cytoplasmic droplets in group II rats. These cytoplasmic droplets were located at various regions of the sperm; however, tail remnants were more in number. Morphological defects appeared to be reduced in group III, and the group IV was similar to the group I (Table 1).

Estimation of enzymatic antioxidant

The activity of enzymatic antioxidants, such as SOD, CAT, GPx, GR and GST in young and experimental groups was represented in Fig. 3. Significant reduction in the activity of enzymatic antioxidants was observed in group II animals when compared with the group I rats. The activity of SOD, CAT, GPx, GR and GST was normal in group III when compared with group II. These activities were slightly increased in group IV than in group I.

Table 1 Showing sperm parameters studied in various experimental groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability (%)</td>
<td>96.5 ± 3.11</td>
<td>72.5 ± 2.2</td>
<td>93.98 ± 2.17</td>
<td>98.78 ± 2.98</td>
</tr>
<tr>
<td>Dead</td>
<td>3.5 ± 1.23</td>
<td>28 ± 3.01</td>
<td>6.02 ± 3.04</td>
<td>1.22 ± 1.78</td>
</tr>
<tr>
<td>Motility (semi-quantitative)</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>98.5 ± 8.5</td>
<td>53.5 ± 3.12</td>
<td>90.76 ± 3.53</td>
<td>98.87 ± 3.12</td>
</tr>
<tr>
<td>Normal</td>
<td>1.5 ± 0.89</td>
<td>46.5 ± 4.02</td>
<td>9.29 ± 3.53</td>
<td>1.13 ± 0.89</td>
</tr>
<tr>
<td>Abnormal</td>
<td>–</td>
<td>11.83 ± 0.75</td>
<td>5 ± 1.09</td>
<td>–</td>
</tr>
<tr>
<td>Head</td>
<td>–</td>
<td>12.33 ± 1.21</td>
<td>3 ± 1.10</td>
<td>–</td>
</tr>
<tr>
<td>Microcephalic</td>
<td>–</td>
<td>14.5 ± 1.05</td>
<td>3.83 ± 1.98</td>
<td>–</td>
</tr>
<tr>
<td>Bicephalic</td>
<td>–</td>
<td>10.5 ± 1.64</td>
<td>4.67 ± 1.86</td>
<td>–</td>
</tr>
<tr>
<td>Acephalic</td>
<td>–</td>
<td>12.5 ± 1.05</td>
<td>4.33 ± 1.51</td>
<td>–</td>
</tr>
<tr>
<td>Amorphous</td>
<td>–</td>
<td>21 ± 1.26</td>
<td>6 ± 1.90</td>
<td>–</td>
</tr>
<tr>
<td>Neck</td>
<td>4 ± 1.01</td>
<td>32 ± 2.41</td>
<td>7.5 ± 1.01</td>
<td>4.5 ± 0.89</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SD.
Estimation of non-enzymatic antioxidant

In group II, the levels of ascorbic acid, α-tocopherol and reduced glutathione were found to be significantly reduced when compared with the group I. These were significantly increased in group III. Activities of non-enzymatic antioxidants in young and experimental groups were represented in Fig. 4.

HO₂⁻, H₂O₂, LPO

Levels of HO₂⁻, H₂O₂ and LPO in young and experimental animals were analysed. Data showed significantly high levels of HO₂⁻, H₂O₂, LPO in group II, when compared with group I. *M. pruriens* treated animals (group III) showed a significant reduction in HO₂⁻, H₂O₂, LPO levels (Fig. 5).

Confocal study for ROS production

In situ ROS detection of epididymal sperm in young and experimental groups was interpreted based on their staining intensity. Analysis showed increased ROS production in the group II when compared with group I. In group III, the ROS production was reduced. Group IV showed low level of the ROS production when compared with group II (Plate 2).

Chromosomal integrity

Chromosomal integrity test showed significant loss of chromosomal integrity in group II (43 ± 1.23%, orange to red fluorescent and 57 ± 0.98%, of green fluorescent) rat sperm when compared with group I (5 ± 1.01% and 95 ± 2.01%). The chromosomes were protected in the group III (8.23 ± 1.05% and 91.77 ± 1.68) when compared with group II. Group IV showed good chromosomal integrity as in group I (6.89 ± 0.99 and 93.11 ± 2.02) (Plate 2).

DNA damage (comet assay)

Comet assay revealed significant DNA damage in group II sperm (comet tail length 223.83 ± 2.34 μm), when compared with group I. Administrations of *M. pruriens* significantly reduced the DNA damage in group III (comet tail length 75.94 ± 3.01 μm). Group IV analysis showed normal DNA migration. DNA fragmentation analyses by comet assay of young and experimental animals were shown in Plate 2. No tail formation was observed in groups I and IV, indicating no DNA damage.

Mitochondrial membrane permeability assay

The mitochondrial membrane permeability assay (MMP) of young and experimental groups was represented in the Plate 3. Data indicate that MMP was increased in group II, when compared with group I. Supplementation of *M. pruriens* in group III was reduced MMP.

Discussion

Directly or indirectly, the OS plays a major role in pathophysiology of reproductive dysfunction and infertility in ageing male animals and human (Levy et al., 1999; Feldman et al., 2000). In this study, age-related decline in the sperm count may be caused by the increased OS or defects in the protective function of the epididymis (Serre & Robaire, 1998); this could have caused sperm cell DNA damage or apoptosis. Sperm counts were significantly increased in aged animals treated with *M. pruriens*; this might be as a result of reduced sperm cell apoptosis in testis and improved epididymal sperm maturation through increased antioxidant system (Rajeshwar et al., 2005; Ahmad et al., 2007; Shukla et al., 2007). Further, spermatogenesis might be improved by its primary
component, i.e. L-DOPA (Siddhuraju et al., 1996) by enhancing the activity of testicular somatic cells through hypothalamo-pituitary and testicular axis viz., dopaminergic system (Herberg & Rose, 1990; Amin et al., 1996; Sriram et al., 2003).

Oxidative stress affects motility by altering axoneme structure that leads to tail abnormality in sperm (Syntin & Robaire, 2001) and decrease in sperm motility (Giorgio et al., 2007). This study showed high levels of ROS in aged rat sperm; this would increase mitochondrial membrane permeability, interrupting respiratory chain and ATP production and decreased phosphorylation of axonemal proteins (Flaherty et al., 2006), thus affecting sperm quality. Significant mean improvement in baseline sperm morphology and motility following 60 days of *M. pruriens* treatment was observed in this study.

In aged rats, there was increase in the number cytoplasm containing spermatozoa; as a result of spermatogenesis impairment ensuing OS in ageing (Levy et al., 1999). With depleted secretion and absorption or phagocytic activities of the epididymis in ageing make it unable to manage these spermatozoa (Syntin & Robaire, 2001). The presence of more number of cytoplasm containing spermatozoa elevates the levels of cytoplasmic enzymes, especially glucose-6-phosphate dehydrogenase. Thus, super physiological level of ROS produced by the fuelling activity of putative NADPH oxidase located in the plasma membrane of sperm (Gomez et al., 1996; Aitken et al., 1997). Cytoplasm containing spermatozoa were considerably reduced in aged rats treated with *M. pruriens*, this might be the influence of spermatogenenic (Shukla et al., 2007) and antioxidants and androgenic potential (Amin et al., 1996) of *M. pruriens*.

**Figure 3** Activity of SOD, CAT, GR, GPx and GST in epididymal sperm of young and experimental groups. Each bar represents mean ± SEM of six animals. Significance at $p < 0.05$, a – group I; $*$ – group II; $**0.001$.  

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There was a significant increase in the production of \( \text{HO} \cdot \), \( \text{H}_2\text{O}_2 \) in spermatozoa of aged rats, because of the oxidant and antioxidant imbalance (De Lamirande & Gagnon, 1992). Sperm are more vulnerable to excess ROS because of high amount of polyunsaturated fatty acid present in their plasma membrane (Jones et al., 1979). Increased LPO and altered membrane function can render defects in head (amorphous, microcephalic and acephalic), neck and tail of sperm. Further, ageing brings DNA denaturation influenced by excessive ROS (Manicardi et al., 1995); DNA damage was significantly reduced in \( M. \text{pruriens} \) treated aged rats. Suppression of ROS production and increase in free radicals scavenging before they interact with the plasma membrane might be influenced by the hydrogen sharing ability of the \( M. \text{pruriens} \) (Rajeshwar et al., 2005; Shukla et al., 2007), thereby preventing membrane damage by impeding self-propagating LPO reaction.

Superoxide dismutase, which is involved in conversion of the \( \text{O}_2 \cdot^- \) to \( \text{H}_2\text{O}_2 \) and oxygen, was significantly decreased in aged rats. Decreased SOD activity indicates either reduced synthesis or elevated degradation or inactivation of the enzyme. Increased concentration of \( \text{H}_2\text{O}_2 \) and \( \text{HO} \cdot \) in aged rat sperm observed in this would inhibit SOD activity (Weir & Robaire, 2007). SOD activity in \( M. \text{pruriens} \) treated aged rat sperm was good as those of young animals. \( M. \text{pruriens} \) ability to increase the synthesis or decrease the degradation, increase the bioavailability and scavenging the excess amount of \( \text{O}_2 \cdot^- \) (Tripathi & Upadhyay, 2001, 2002; Rajeshwar et al., 2005; Shukla et al., 2007) would have played major role in improving SOD level.

Catalase is well-known to scavenge \( \text{H}_2\text{O}_2 \) and neutralize into \( \text{H}_2\text{O} \) and \( \text{O}_2 \). There was a significant reduction in the activity of CAT in aged rats, and this may be because...
of the reduced conversion of \( \text{O}_2^{\cdot-} \) to \( \text{H}_2\text{O}_2 \) by SOD that leads to the accumulation of \( \text{O}_2^{\cdot-} \); this inhibits the activity of CAT (Freeman Bruce & Crapo James, 1982; Kono & Fridovich, 1982). Decline in CAT activity in ageing was reversed by \textit{M. pruriens} treatment; this may be effected by the conversion of inactive CAT to active form or increase in bioavailability of the CAT through removal of the excess amount \( \text{O}_2^{\cdot-} \) and \( \text{H}_2\text{O}_2 \) (Rajeshwar et al., 2005; Shukla et al., 2007).

In this study, GPx activity was significantly decreased in the aged rats. Decrease in the activity of GPx may be because of the lower level of GSH or decreased synthesis or elevated degradation or inactivation of the enzyme (Miller, 1987). Aged rats treated with \textit{M. pruriens} extract GPx activity was significantly restored to the level of young rats. This may be because of normalization in the level of GSH and reduced ROS in the presence of \textit{M. pruriens} (Tripathi & Upadhyay, 2002; Rajeshwar et al., 2005).

The GSH serves as a sensitive marker of OS, and it also plays an important role in maintaining integrity of the cell system. GSH can scavenge peroxynitrite and HO• as well as convert \( \text{H}_2\text{O}_2 \) to water with the help of GPx (Maher, 2005). Our observations showed that GSH content was significantly decreased in aged rat sperm and reduced glutathione in \( \text{H}_2\text{O}_2 \) detoxification. However, \textit{M. pruriens} treatment elevated the level of GSH in aged rat sperm. This might be the influence of increased bioavailability and enzyme activity or reduce the degradation and increase the synthesis through scavenging the excess amount of free radicals (Rajeshwar et al., 2005; Shukla et al., 2007).

![Plate 1](image1.png)

Plate 1 Light micrograph of normal and abnormal morphology seen in epididymal sperm of aged animals. Giemsa stain.

![Plate 2](image2.png)

Plate 2 Confocal micrograph of epididymal sperm from young and experimental groups. Detection of ROS production in sperm using 2',7'-dichlorofluorescin diacetate (DCFH-DA), which is converted into dichlorofluorescin (DCF), scale bar = 12 µm. Chromatin integrity of sperm various experimental groups using Acridin orange (AO) stain. Good integrity – green fluorescent and loss of integrity – orange to red fluorescent (arrow head), scale bar = 6 µm. Comet assay of sperm cells from various experimental groups, scale bar = 1.5 µm. \textit{Mucuna pruriens} (MP).
Glutathione radical, formed during scavenging of ROS, is readily neutralized by combining with another Glutathione radical to produce GSSG; this can be reversed by NADPH-dependent GR. GR activity was significantly decreased in this study suggesting an inadequate level of reducing equivalents (NADPH) and a failure to maintain GSH levels in aged rat (Maher, 2005). The decreased enzyme activity can cause free radical mediated OS and consequent damage to sperm. The activity of GR was increased in aged rat treated with *M. pruriens*. It may indirectly restore GR by increasing the bioavailability and activity of GSH (Tripathi & Upadhyay, 2001, 2002).

Vitamins C and E are well-known for their antioxidant properties in animal tissues (Frei *et al.*, 1989; Kujo, 2004). α-Tocopherol and ascorbic acid levels were significantly reduced in aged rats; this might be because of their participation in scavenging that increased ROS production or increased degradation in aged rats. Whereas, *M. pruriens* treated rats showed increase in the levels of these vitamins viz. increased free radical scavenging or increased the biosynthesis of these vitamins (Rajeshwar *et al.*, 2005).

In conclusion, our results demonstrated that the oxidative damages in aged rat sperm were reversed by *M. pruriens* treatment. To author’s knowledge, this is the first report on the action of ethanolic seed extract *M. pruriens* on intracellular ROS and structural and functional integrity of epididymal spermatozoa in aged rat. Present observation suggests that *M. pruriens* may serve as a good therapeutic agent for male infertility related with andropause or ageing.

**References**


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**Plate 3** Fluorescence analysis of mitochondrial membrane potential in epididymal sperm using with rhodamine stain (Green – bright in aged) and DAPI stain (Blue – nucleus-bright in young, arrow indicates a spermatozoon with nuclear damage and head deformity). Superimposed images of sperm from young and aged rat-rhodamine and DAPI staining. Fluorescence micrograph showing increased membrane permeability in aged. Scale bar = 12 μm. *Mucuna pruriens* (MP).
biochemical parameters in seminal plasma of infertile men. *Fertility and Sterility* 90, 627–635.


