

## THE EFFECTS OF GOSSYPOL ON SPERMATOGENESIS IN NMRI MICE\*

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**Abstract** – Infertility is a relatively common problem among couples, and Gossypol, a yellowish pigment detected in cottonseed, is one cause.

Adult NMRI mice (25-35 gr) were used in this study. The animals were kept in standard conditions with a dark and light cycle of 12:12, controlled humidity and temperature, food and water were available ad libitum.

Gossypol was dissolved in ethanol and diluted by sunflower oil as a vehicle (0.5: 4.5), and were used orally (13.425 mg/kg per day) for 15 days. The animals were sacrificed 24 hours after the last treatment.

The factors compared among these groups were testis and epididymis length and weight, sperm length and count and also histological differences. Statistical analysis by One Way ANOVA revealed that:

Gossypol causes a 69.33% & 67.22% decrease in the sperm number compared to control & sham groups, respectively. It also causes a 29.63% decrease in the epididymal weight, and changes in the histological structure of the testis.

In conclusion, although gossypol may be a good antifertility agent, it should be handled more cautiously. Furthermore, the usage of cottonseed oil in snacks should be limited and those field/factory-men who have contact with cotton should be more careful.

**Keywords** – Cottonseed, gossypol, antifertility, spermatogenesis

### 1. INTRODUCTION

In 1957 Liu reported that Wang village in Jiangsu province in China had not had a single childbirth for as long as 10 years during the 1930's and 1940's. While villagers before and after this period had been fecund [1].

The sensitivity of different species to gossypol is not equal. Among laboratory animals, hamsters seem to be the most sensitive at an effective dose of 5-15mg/kg per 6-13 weeks [2].

The decrease in sperm counts is attributed to the germinal epithelium damage. Only one layer of germ cells, including sertoli cells, was left intact after the treatment of male rats with a dose of 30 mg kg<sup>-1</sup> d<sup>-1</sup>GAA for 4-5 weeks [3].

Sterile men who had used crude cotton seed oil showed a reduced number of leydig cells with early signs of degeneration [4]. Rats treated with gossypol showed a decrease in cell size and a cytoplasmic volume of leydig cells [5].

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Infertility is a relatively common problem among young couples, which leads to considerable distress along with feelings such as anger, anguish, denial, depression, embarrassment, guilt, inadequacy, isolation and shock [6].

The male factor is incriminated in about 50% of infertility cases [7]. To the extent of our knowledge, the exogenous factors affecting fertility in the male is still limited, and the etiology remains unknown in 75% of cases [8].

In humans, various confounding factors (physical or chemical) affect sperm quality, fertility and pregnancy outcomes. These factors are related to socioeconomic state and lifestyle, which are not always taken into account [9]. One of the food derivatives, gossypol, is among them.

Cotton; a unique food and fiber plant, is produced perennially worldwide in tropical and subtropical regions. It is especially found in India, Southern Europe, and U.S. Cottonseed averages about 45% hull and linters, and 55% kernel [10]. The kernel contains innumerable pigment glands, the major component of which is gossypol [11]. Gossypol is a yellowish reactive sesquiterpene aldehyde, which was known as early as 1886 [12]. At least 14 other pigments are also present in the cotton plant, but their concentration is negligible. Gossypol is naturally found in the family Malvaceae and its genus species are *Gossypium Herbaceum* (Levant Cotton Root Bark) and *Gossypium Hirsutum* (Cotton Root Bark). Gossypol has a molecular weight of 518.54, and a structure of 2, 2'- binaphthalane -8, 8'- dicarboxyaldehyde - 1, 1'- 6,6'- 7, 7'-hexahydroxy- 5, 5'- diisopropyl - 3, 3'-dimethyl, which has been totally synthesized by Edwards [13]. If gossypol is recrystallized in different solvents, three crystalline substances with different melting points and different optical properties are obtained [14]. Its 3 tautomeric forms are: the aldehyde, the ketonoid and the hemiacetal form [15, 16].

Cotton plant seed naturally contains 0.6% of the polyphenol gossypol [17, 18]. A mean gossypol content of 1.32% (0.59-2.35%) n=46 was reported from *G.Hirsutum* [19], also 0.39-1.7% gossypol was found in 8 varieties in 13 different locations [20, 21]. Furthermore, the gossypol content of the seed positively correlates with rainfall, but negatively with temperature.

## 2. MATERIALS AND METHODS

In this research the antifertility effects of gossypol have been studied.

In the present research, 36 adult male Albino-NMRI mice (25-35gr) were used. The animals were kept under standard conditions of animal house and reweighed every five days and at the end of the treatment. (i.e. Commercial mice chow and water were available adlibitum. They were kept in controlled room humidity of 20% and temperature ( $23 \pm 2^\circ \text{C}$ ) on a 12L: 12 D light schedule.)

Gossypol M.W=518.6 (Cat. No. G-8761) was purchased from Sigma. Each 20mg GAA contains 17.9 mg gossypol [22], so instead of using 15 mg GAA, we administered 13.425 mg gossypol. Each 13.425 mg of gossypol was dissolved in 0.5ml absolute ethanol and 4.5 ml sunflower seed oil was added. Solutions were made up weekly.

The solution was administered with an oral dosing needle at a dose of 13.425mg kg<sup>-1</sup> body weight for a 15 day duration. The amount of solution administered varied between 0.1 and 0.2 ml according to body weight. The sham group was dosed with the same amounts of vehicle only. The dose was repeated everyday at the same time, with the last dose being given 24 hrs before the mice were killed. They were sacrificed by a lethal dose of ether. Testes and epididymes were removed and isolated from the surrounding tissues. All other body organs were also dissected in a search for toxicity signs in the mice's body.

#### **a) Sperm Count**

The epididymides were minced for sperm count. They were first macerated, completely homogenized, and then diluted in PBS (diluted 5 times). After thorough mixing, the number of spermatozoa was counted on an Improved Neubauer Hemacytometer. The method applied to count the sperm was based on the method described by Searle and Beechey [23], which was also used by Suzan Hunt and Ursula Mittwoch in 1984 [24].

#### **b) Organ Weight and Length**

All organs were weighed to the nearest 0.01mg and their wet weight recorded. In order to measure the length of epididymides, they were isolated from the testes, then their length was measured by a scale lens (on  $\times 10$ PF lens), which was placed on a stereomicroscope (magnification  $\times 7$  and  $\times 14$ ).

#### **c) Sperm Length**

After sperm count, we used the same solution to measure their mean length, the sperm was collected randomly and the measurement repeated 3 times. Sperm length was measured by a scaled lens ( $\times 40$ ).

#### **d) Histological Process**

In each mouse one testis was fixed in formalin for tissue preparation and the other was decapsulated and macerated in PBS to prepare smears. After tissue preparations sections were cut at 6-8  $\mu$  by a Rotary Microtome, sections were stained with Harris' Hematoxylin and Eosin. Smears were stained either by Fuchsin or Harris' Hematoxylin and Eosin.

Microscopic slides were then studied. All measurements were accomplished using a scaled lens ( $\times 40$ ), and the results repeated 3 times. Also, other comparisons are presented on the basis of studying 3 different regions of each slide.

#### **e) Statistical Analysis**

By using Microsoft excel software the results were analyzed and the graphs designed. Analytical tests were performed for the variables: (1) Testis length and weight, (2) Epididymis length and weight, (3) Sperm count and length. These variables were compared among the control, sham and treatment groups by One-Way ANOVA. The standard error was defined by Mean  $\pm$  SEM and the significant difference among groups has been demonstrated by stars.

### **3. RESULTS**

Treated mice showed manifestations as languid, solid heartbeats, rale, inappetence and lower body weight.

#### **a) Sperm Count**

The gossypol group showed a significant 66.33% and 67.22% decrease in sperm count compared to the control and sham groups, respectively. Also, the sham group showed a significant 6.44% decrease compared to the control group. Mean  $\pm$  S.E =  $260.8 \times 10^4 \pm 36410.3$  (Fig.1).

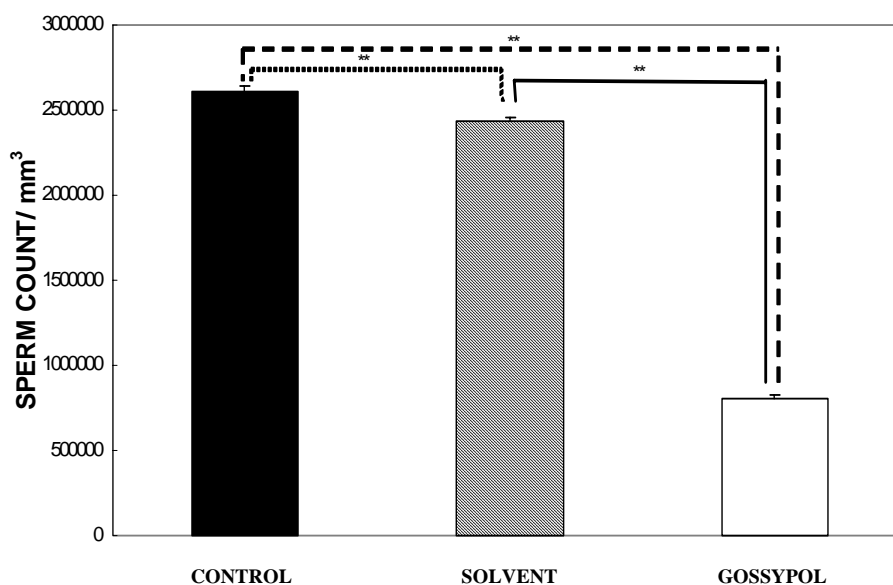


Fig. 1. Comparing Sperm count among the control, sham (solvent) & gossypol groups, Significant differences were detected at  $P < 0.001$  in the gossypol group in contrast to the control and sham groups. \*\*\* =  $P < 0.001$

#### b) Organ Weight & Length

The gossypol group differed significantly in epididymal weight, but the control and sham group did not show a significant difference. The gossypol group showed a 29.63% decrease compared to the control groups. Mean  $\pm$  S.E =  $4.2 \pm 0.16$  (Fig. 2).

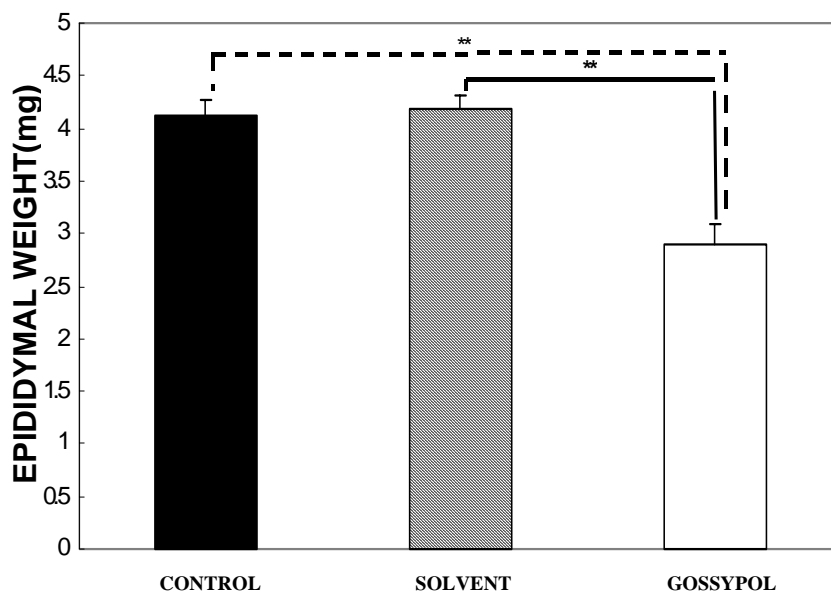


Fig. 2. Comparing Epididymal weight among the control, sham (solvent) & gossypol groups, Significant differences were detected at  $P < 0.001$  in the gossypol group in contrast to the control and sham groups. \*\*\* =  $P < 0.001$

### c) Sperm Length

The length of sperm, length of epididymidis and the weight and length of the testes did not differ significantly.

### d) Microscopic Results

It seems that the thickness of tunica albuginea had roughly increased. A fewer number of vessels was detected either in the area of interstitial space or the capsule. The most significant change detected in this group was the detachment of spermatogonia from the basement membrane and the forming clusters of spermatocytes. Sertoli cells were not detected easily. A fewer number of spermatogenic cells were detected in contrast to control and sham groups. As a result, fewer spermatozooids and more residual bodies were detected. Other differences included a paler staining in contrast to the two other groups. In relation to the interstitial space, the area was increased. Also, the area of the Leydig cells was increased. We came across a higher density of residual bodies and weaker staining in the gossypol group. The area of seminiferous tubules and spermatogenic cells fluctuated widely. Therefore they were less reliable as indicators of a lower fertilizing capacity.

## 4. DISCUSSION

We administered a dose of  $13.425 \text{ mg kg}^{-1}$  gossypol orally for a 15-day duration and came across effects in macroscopic studies, such as spoiled gastrointestinal tract, gas in the stomach and intestine, spoiled respiratory system, especially the trachea region, larger livers, gallbladders and kidneys in the treatment group, edema, and distorted hearts. Other symptoms were breathing with difficulty, sharp heartbeats, dark yellow urine and also a yellowish color spreading on all the organs. Similar damage and symptoms were reported from gossypol treated animals [25, 26, 27]. All these symptoms confirm that gossypol is a toxic agent.

We came across a 69.33% and 67.22 % decrease in the number of spermatozoa compared to the control and sham groups, respectively, which was significant in both cases. Also, the sperm count showed a 6.44% decrease in the sham group compared to the control group. This result confirms previous results [28]. Due to the sharp decrease in sperm number (gossypol group), we can conclude that gossypol may be a useful contraceptive. By the way, regarding the slight decrease in sperm number (sham group), it is assumed that sunflower oil may also have decreasing effects, although more studies are required before this can be concluded. We came across a 29.63% decrease in the epididymal weight, which was significantly different from controls at  $p < 0.001$ . There was not a great difference in the length of the epididymis, and also the weight and the length of the testes have to be taken into consideration as factors affecting fertility. Different records are not in complete agreement. The reports of Shi [28] and Coulson [29] include reduced weights of accessory glands, whereas testicular weights remained the same or were increased. Hunt also came across a slight decrease in the weight of caput epididymis and Wang [30] reported no effect on accessory glands. No specific report on testis length and weight was found to compare our results with. The decrease in sperm number can explain the reduced weight of the epididymis, although endocrine studies are also necessary.

In microscopic studies, we came across a thicker tunica albuginea (Fig. 3), clusters of spermatocytes and other spermatogenic cells, and a reduction in the number of spermatozoa, which is consistent with the results of epididymal ligation indicating that the target organ of gossypol is the testis and not the epididymis [31]. The decrease in sperm count is attributed to the germinal epithelium damage, which is due to detachment of spermatogonia from the basement membrane,

higher density of residual bodies and weaker staining, an increase in the area of interstitial space and an increase in the number and area of Leydig cells. Hikim [32] reported no significant deviation in leydig cell morphology number and volume, Wang reported a reduced number of leydig cells (Fig. 4), and a reduction in the number of Sertoli cells, which seems to be dose dependent; low doses have no effect, while higher doses show a decrease in number [32, 33] (Fig. 5). Dilation of vessels either in the membrane or in the interstitial space, in the gossypol group and ion- channel of sperm and Gossypol also causes a 29.63% decrease in the epididymal weight, whereas other organs show no significant difference [34, 35, 36, 37].

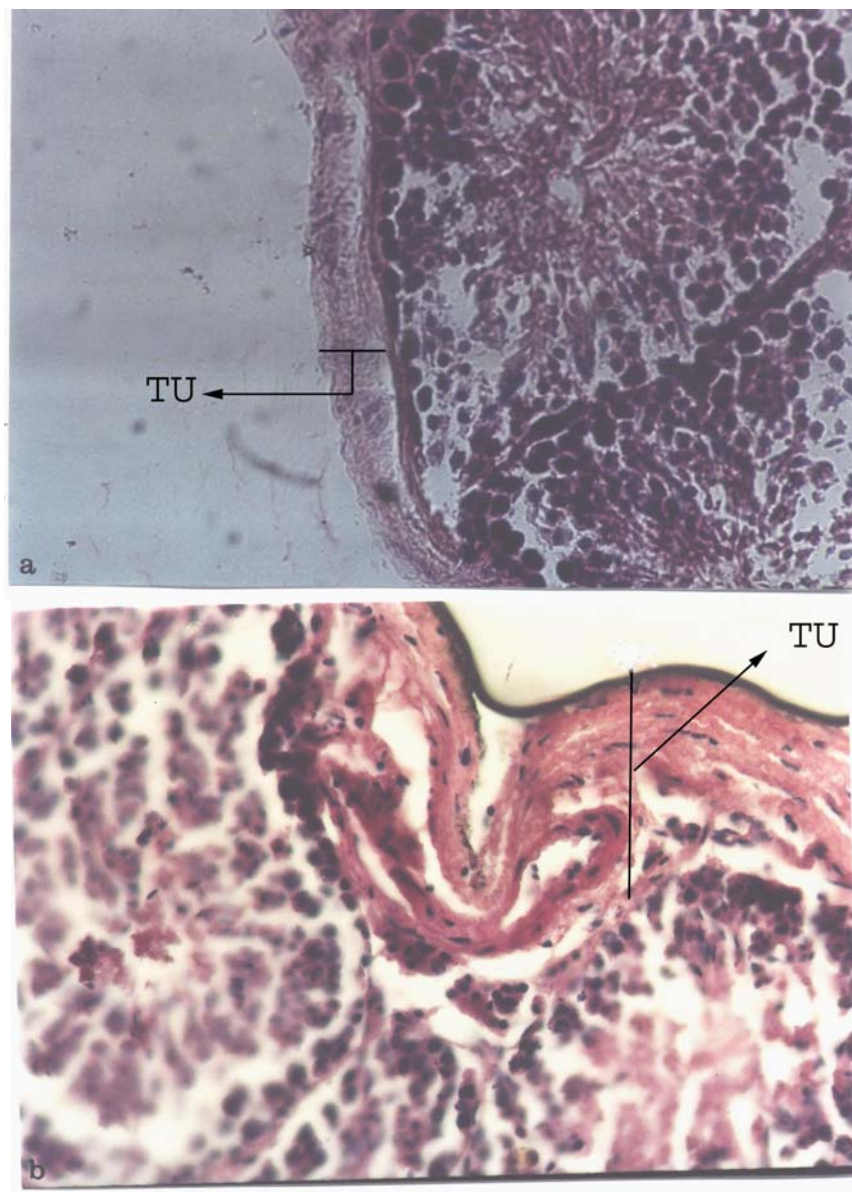


Fig. 3. Tunica albuginea(tu) after staining by H&E method, (a) control (b) gossypol treated mouse. Note the thickness in gossypol treated mouse. Micrograph by WILD microscope (mag.  $\times 40$ )



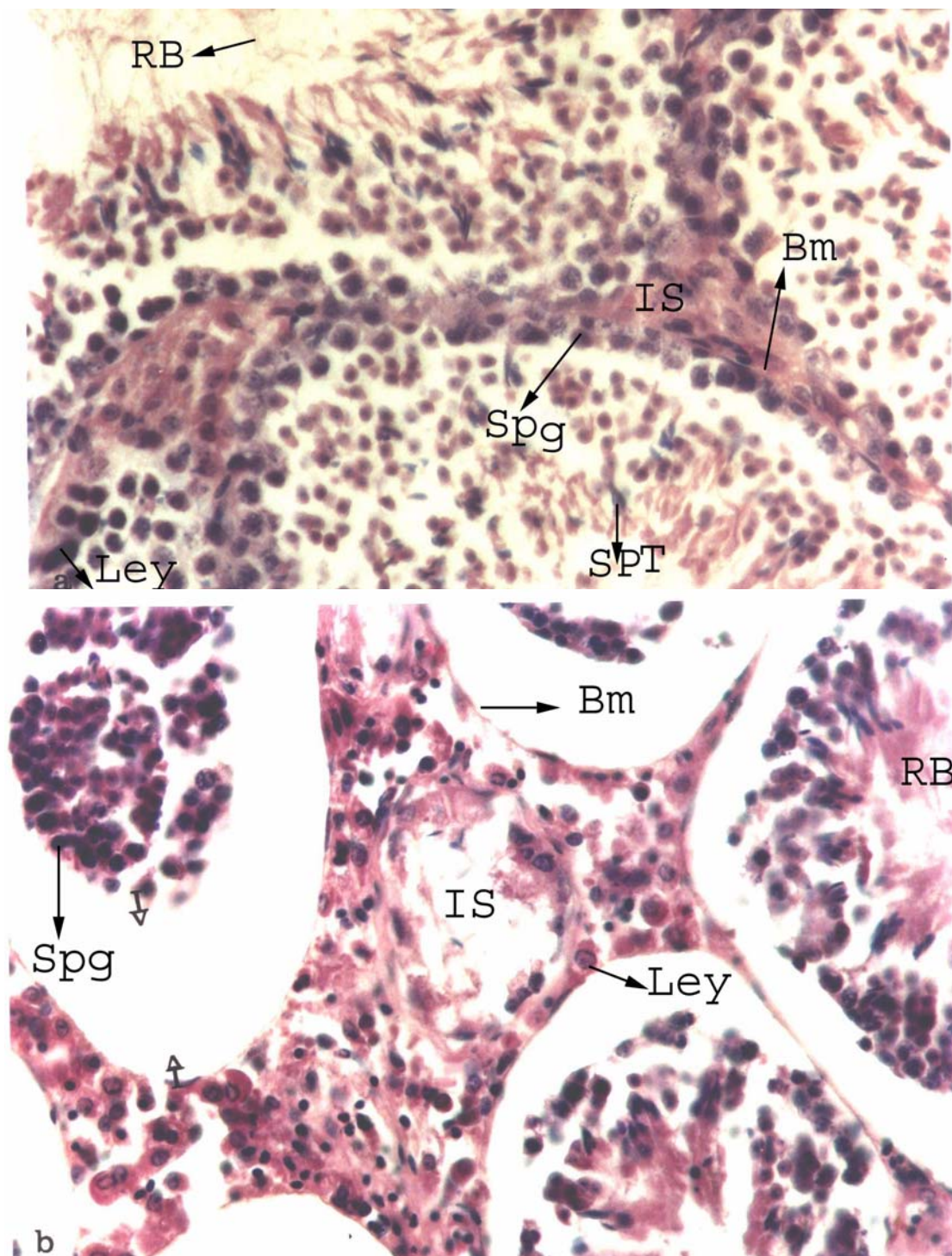


Fig. 4. Spermatogenic cells (*spg*), residual bodies (*rb*), spermatids (*spt*), Interstitial space (*is*) and Leydig cells (*ley*) after staining by H&E method, (a) control (b) gossypol treated mouse. Note the lower density, clusters of spermatogenic cells and the detachment of spermatogonia from basement membrane, also note the higher density of spermatids and residual bodies respectively, in control and gossypol treated mouse. Micrograph by WILD microscope (mag.  $\times 40$ )

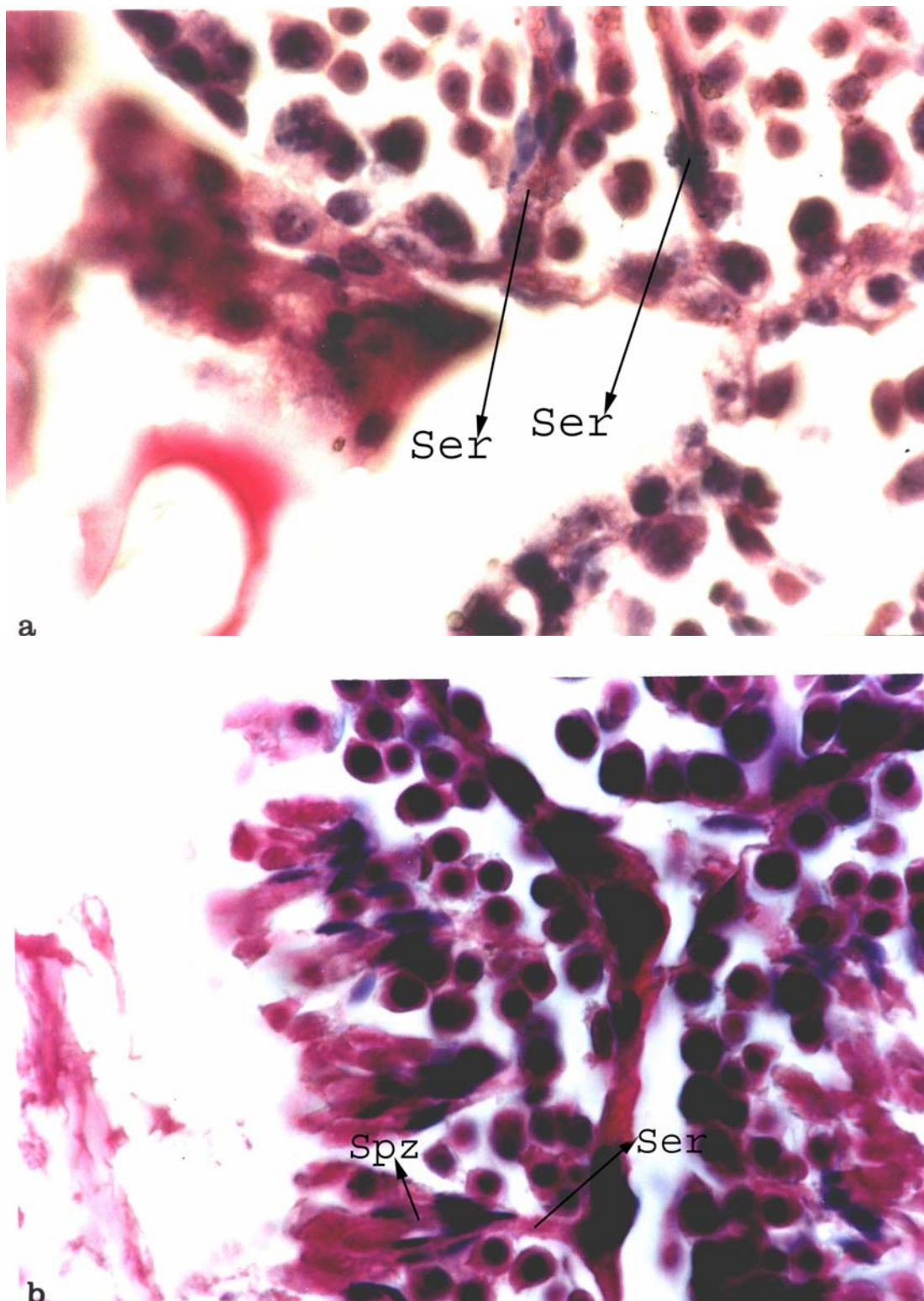


Fig. 5. Sertoli cells (*ser*) after staining by H&E method, (a) control (b) gossypol treated mouse. Note the reduced number of Sertoli cells in gossypol treated mouse. Micrograph by WILD microscope (mag.  $\times 100$ )



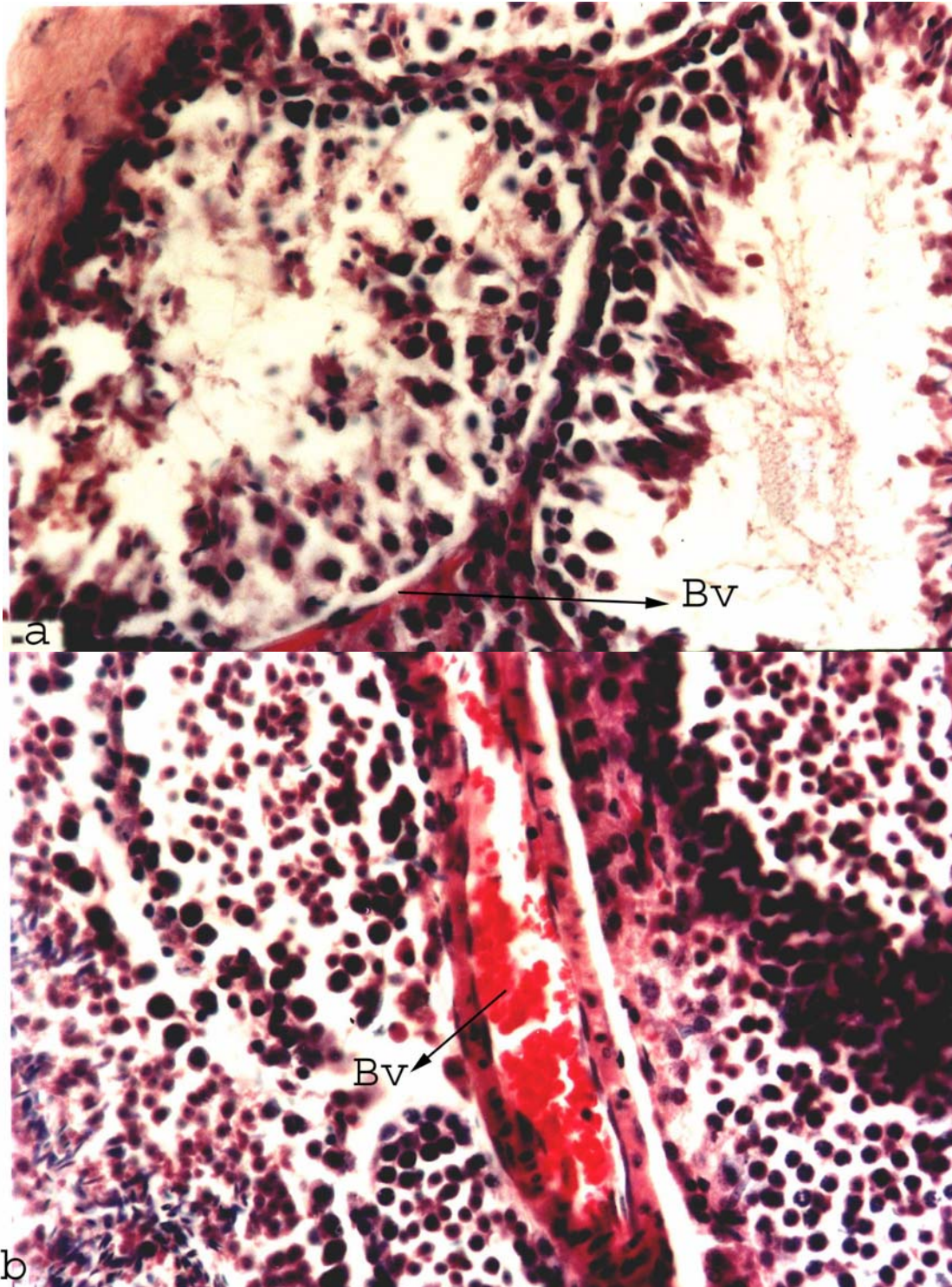


Fig. 6. Blood vessels (*Bv*) after staining by H&E method, (a) control (b) gossypol treated mouse. Note the dilation of blood vessels in gossypol treated mouse. Micrograph by WILD microscope (mag.  $\times 40$ )

In conclusion, the major effect of gossypol is on sperm. Gossypol causes a 69.33% & 67.22% decrease in the sperm number compared to control & sham groups, respectively.

These results are important in light of great concerns about using gossypol as a male antifertilizing agent, suggesting more studies be conducted on its toxic and side effects. Besides, heedless use of gossypol, with its great effects on spermatogenesis in snacks and working carelessly in fields and factories may lead to undesired results. It may be useful to study the effects of gossypol on other body organs and its effects on embryos at different developmental stages both in vitro and in vivo.

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