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Electron microscope and cold plasma as new techniques for scanning weevil testes, *Rhynchophorus ferrugineus* (Oliver) (Coleopteran: Curculionidae).

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***Abstract**: Cold plasma is described as a new and safe method for pest control. This work aims to evaluate the effect of cold plasma on the fine structure of the male reproductive system in Rhynchophorus ferrugineus. One-day-old unmated males were exposed to cold plasma waves for 1 or 2 minutes at a distance of 5 cm. Normal and treated male testes were examined under SEM in the high vacuum mode after one day and seven days of treatment. The histological structure of normal testes showed that they consist of approximately 96 series of follicles. Each follicle has a large number of spermatogonia lying external to the zones of spermatocytes, which divide to form spermatids. Various stages of developmental sperm cells aggregate in the central region of the testis. Exposure of males to cold plasma for 1 or 2 minutes showed adverse effects on the testes. The external surface of the testes appeared highly irregular, with a number of small circular projections. Dividing sex cysts appeared abnormal, and the septate junctions were highly irregular, became ruptured in some parts and reduced in number in all examined testes. These alterations increased with exposure time and after exposure time. In conclusion, this study indicated that the exposure of males for 1or 2 minutes was sufficient to induce great damage to the testes. This study provides knowledge about the effect of cold plasma on insect spermatogenesis and opens a new scope of basic research into the suitability of cold plasma for a sterile insect technique.*

***Keywords:** cold plasma, red palm weevil, testes, ultrastructure, scanning electron*

I. Introduction

Red palm weevil, Rhynchophorus ferrugineus (Olivier) (RPW), is the most harmful pest of the date palm, Phoenix dactylifera, spreading rapidly in date palm growing countries across the globe (Abraham et al. 1998). RPW has been reported in over 50% of the date palm-growing countries in the Middle East (Faleiro 2006). In Egypt, the exclusion of infested trees has not been applied as soon as the pest was detected. The situation in Egypt is very worrying. It has been recorded in each of the Delta administrative districts, in some orchards along the road between Cairo and Alexandria, and even in the capital itself. Although the red palm weevil does not usually fly very much in the orchards where it is present, it probably flies to new orchards after killing all of the existing date palms and not finding enough food (Ferry and Gómez 2002).

In the physical sciences, "plasma" refers to the fourth state of matter (Fridman et al. 2008). This term refers to a partially ionized medium, usually gas. Importantly, plasma produces not only electrons and various ions but also neutral (uncharged) atoms and molecules, such as free radicals and electronically excited atoms that have a high chemical reactivity and the ability to emit UV radiation. In thermal plasma, gas temperatures can reach several thousand Kelvin. The effects of such thermal plasma on tissues are non-selective and difficult to control because they occur primarily through the transfer of intense heat (Vargo 2004). In contrast, in non-thermal plasma, gas can be maintained close to room temperature. Although it has been known for quite some time that electrical discharges generate non-thermal plasma, it has only recently been demonstrated that non-thermal atmospheric pressure plasma applied directly to living cells and tissues kills bacteria and induces blood coagulation without significant heating **(**Kieft et al. 2006). Non-thermal plasma treatment has also been shown to promote cell proliferation (Kalghatgi et al. 2011) and enhance cell transfection (Coulombe et al. 2006). A few researchers have studied the effect of plasma jet on insects (Mishenko et al. 2000; Keever et al. 2001; Donohue et al. 2006; Abd El-Aziz et al. 2014), but no research has been performed on the effects on RPW or insect tissues.

The advantage of cold plasma as a method for controlling insect pests is that plasma can be produced at ambient temperatures with reasonable electrical power requirements, the chemical reactive species produced by the plasma are not excessive, there is no ionizing radiation, and the plasma is effective against insects but safe to the end-user and the environment (Donohue et al. 2006).

During spermatogenesis, the spermatogonia pass through successive, synchronic divisions that result in a clump of primary spermatocytes. The number of cells per clump or cyst is species-specific but is usually 2^n, where n is the number of consecutive mitotic divisions that precedes meiosis **(**King and Büning 1985). Insect spermatozoa are generally filamentous and consist of a tiny triangular head connected to a very long tail (Jamieson et al. 1999). Irradiation of males kills the spermatogonial cells (Williamson et al. 1985) and induce dominant lethal mutation in spermatozoids. Consequently, mating between sterile males and wild females results in embryonic development arrest and mortality (Van Der Vloedt et al. 1978).

As previously stated, a few researchers have studied the effect of cold plasma on insects but none have described its effect on insect tissues, therefore this work aimed to illustrate its effect on RPW testes and spermatogenesis. We are hopeful that this step will help in understanding the effect of this safe, friendly technique on insects, as it may open new avenues of basic research into the use of this technique to control dangerous insect pests in the future.

II. Materials and Methods

The experimental insects R. ferrugineus adults were obtained from cocoons and larvae collected from infested date palm trees in the Menasheet El-keram and Tal-Bani Tamim, Qalubia, Egypt. The insects were reared in the laboratory according to the method described by Mahmoud and Shoman (2009). The adult weevils were transferred into cylindrical glass jars (16 x 8 cm) and provided with pieces of sugarcane as food. *Food* was renewed every 2–3 days. The jars were covered with a network of metal wire. Newly laid eggs were collected in between the fibers of the sugarcane pieces using a 0.2 mm brush and were then inserted into Petri dishes (50 x 20 mm) containing tissue paper soaked in 3%benzoate solution to prevent bacterial and viral infection (Funke1983). The hatched larvae were maintained in plastic dishes (15 x 37 cm) with pieces of sugarcane (2larvae/dish to prevent cannibalisms).Third larval instars were collected in plastic containers provided with small pieces of sugarcane for feeding. The pupation occurred inside the cocoon of the moist tissue fibers of the sugarcane output from the adult and larvae feedings. The colony was maintained under controlled laboratory conditions (27°C and 85% RH),and a daily inspection was conducted until the adult emergence. All materials and chemicals were supplied from Gomhoria Trading Medicines Chemical, Cairo, Egypt.

Experimental conditions

A schematic of the pulsed atmospheric-pressure plasma jet (PAPPJ) discharge and the experimental set-up is shown in Fig. 1. The gas is fed through an annular region between the two metal electrodes that are 15 cm in length. The inner electrode is 5 mm in diameter and is powered with a pulsed high-voltage power supply, whereas the grounded outer electrode is separated from the inner electrode by a gap of a few millimeters. The PAPPJ device operates using a 10–20 kV power supply with a gap between two electrodes of 2–3mm under atmospheric pressure (El-Aragi 2009).

One-day-old unmated males were exposed to cold plasma waves at a distance of 5 cm from the nozzle of the PAPPJ apparatus. Six insects were exposed individually to the PAPPJ for 1 min. Three of the insects were dissected after one day of exposure, and the others were dissected after 7 days. Another group of three males was individually exposed to the PAPPJ for 2 min and dissected after one day only. Three normal 1-day-old unmated males were used as a control.

Histological study of normal testes: For the examination of normal testes with a normal microscope, five normal adult RPW males were dissected in a ringer lactate IV solution. The testes were isolated and fixed in Bouin's fluid, dehydrated and embedded in paraffin wax. Paraffin blocks were cut into sections 5.8µm thick, stained with Mayer's hematoxylin and eosin and finally mounted in Canada balsam. The sections were examined and photographed using alight microscope (Disbrey and Rack 1970).

Examination of testes with the scanning electron microscope (SEM)

For morphological examinations of normal and treated RPW testes with an SEM, males were dissected in a ringer lactate IV solution (from approximately 30 seconds to 1 min). The testes were isolated and frozen with liquid nitrogen and then put in the SEN chamber, with the SEM (Jeol-JSM-5600 LV in the SEM Unit) in the high vacuum mode. The micrographs were taken at approximately 5 min (to avoid dissolved ice).This technique resulted in the presence of a few small particles, which were white in color, on the micrographs that represented ice during the freeze drying of the specimens (Gasser et al. 2008).

III. Results

The reproductive organs in the RPW consist of paired testes located in the middle-posterior region of the abdomen. The normal histological structure of testes showed that each testis consists of a pair of yellow globular and kidney-shaped lobes opposed to one another. Each testis consists of a series of 96testis tubes or follicles. All of the follicles are covered by a common sheath, which hold them together (Figs.2a and b).There is a connection between the follicles in the peripheral and middle regions in each testis characterized by a thin layer junction (Fig.2a*). The SEM of normal testis showed that this thin layer consisted of two membranes that

were either regular or uneven with a cross striation or septa running between them, which produced a ladder-like appearance referred to as the septate junction (Figs.2c and 3a). Normally, the septate junctions appeared extended between the follicles, but in some sections, they did not appear to reach the basal region (Figs.2c and d).The terminal part of the septate junction ends in two branches (Fig.3a) and the membranes of these two branches have regular, flattened epithelial cells (Fig.3a*). A few parallel rows extended from both sides of the septate junction appear uneven across the membrane face (Fig.3a).

In the follicles, there is no distinct apical cell in the peripheral region of the germarium. Each follicle has a large number or groups of spermatogonia lying external to the zones of the spermatocytes, which have a large round nucleus located in the center of the cell (Figs.3c and d). The primitive spermatogonium moves backwards, repeatedly dividing and distending this sheath to form a cyst. At first, the cyst cells are rounded, but as they increase in size, they become small triangular cysts or multiple shapes (Figs.3b and c). Then, spermatocytes divide to form spermatids, which lie internally (Fig.3d). In the central region of the testes, the septate junction disappears and reveals zones of differentiation representing the various stages of development of aggregated sperm cells (Figs.3e and f).

To study the effect of cold plasma on male testes we chose to study reproductively mature 1-day-old males. The effect of 1min of exposure to the cold plasma on the testes was examined by a SEM and illustrated in Figs 4a–f. Figs 4a and c show small circular projections on the outer surface of the testes. The septate junction has winding extensions, some of which appear simple and the others wrap around each other such as the plexus (Figs.4c,c*, and d). The testes appear in a semi-stable condition and the spermatogonia are prevalent in the peripheral region, but their number is less than the control (Fig. 4b). The dividing sex cysts look abnormal; some of their contents appeared to be degenerating or decomposing and the content of others is heterogeneous (Figs.4a and b).The number of normally shaped sex cysts is few, whereas a high number showed various forms of changes (Figs.4a and e).Additionally, the sperm cells appeared less in number than the control (Fig.4f).

The effect of cold plasma on the testes after 7 days of treatment for 1 min is illustrated in Figs5a–f. The external surface of the testes appeared highly irregular (Fig.5a). The septate junction is highly irregular; its cross striation disappears, and the extensions interfere with each other (Figs.5d and d*). The spermatogonia are prevalent in each testis in a small portion of the peripheral region (Fig.5b). Few of spermatogonia appeared completely degenerated (Fig.5b).The dividing sex cysts were very difficult to identify and the injury either appeared as a decomposition of all content or had an abnormal appearance (Figs. 5c and e). The necrosis can be observed in few numbers (Fig.5c).It is very difficult to quantify the number of sperm cells due to the irregularity and distortion of the testes organization or their small number (Fig.5f).

The effect of exposure of the 1-day-old adult male to cold plasma for 2 minat a distance of 5 cm on the testes is illustrated in Figs6a–f.The external surface of the testes was bulging in a few areas (Fig.6a). The structural organization of the testes became rampaged, erratic and clearly unstable (Fig.6b). The septate junctions became ruptured in some parts and were few in number in all testes (Figs.6b and c). Dividing sex cysts appeared abnormal, whether simple or oversized, and some cyst parts degenerated (Figs.6e and f). In the same figures, some sex cysts appeared huge and their internal content was empty, containing only one or approximately six abnormal spermatogonia. During examination by SEM, we observed that many testes contain few of these cysts, while few of the highly malformed testes are destroyed and contain large amounts of these sex cysts (Fig. 6e). The strong necrosis appeared on the external surface in large number of different sizes (Figs.6c and d).The aggregation of sperm cells in the central part of the testes cannot be detected, and their placement has different stages in abnormal cystic cells.

IV. Discussion

Most male reproductive organs consist of a pair of testes with paired seminal vesicles and an ejaculatory duct. Each testis consists of a series of follicles that vary in number from one in Coleoptera to over 100 in Acrididae (Chapman1998). In the present study, we found that each testis of the RPW consists of a series of 96 follicles. Mahmoud and Shoman (2009) found that the testes of the same insect, R. ferrugineus, consist of 16 follicles according to their section taken in the peripheral part. The number of follicles was studied in other coleopteran insects; Al-Taweel and Fox (1983) found that Dermestes ater have only sixfollicles per testis, whereas each testis in Dermestes frischii consists of 24 follicles (Fox 1969). De Almeida and Cruz- Landim (2000) found that Palembus dermestoides have six round follicles. Büning (1994) suggested that the number of follicles/testis is species specific, as are the number of ovarioles per ovary.

The ultrastructure of normal RPW testes showed that the terminal part of the septate junction ends in two branches, and a few parallel rows extended from both sides appear uneven across the membrane face. Each follicle has a large amount or groups of spermatogonia lying external to the zones of spermatocytes, which have a large round nucleus located in the center of the cell. Spermatocytes divide to form spermatids that lie

internally. In the central region of the testes the septate junction disappears and shows zones of differentiation representing the various stages of development of sperm cells aggregated in the central region.

The rapidly dividing germinal cells that are still in the process of differentiation are particularly radiosensitive, and because of their active division, they express radiation damage quickly (Hasan et al.1989).

The selection of the insect age that was irradiated was based on knowledge of the maturity time of insect reproductive organs. For many holometabolous species, a good time for irradiation is late in the pupal stage, or early in the adult stage, when germ tissues have formed (Anwar et al. 1971, Ohinata et al. 1978). A PAPPJ was directly applied to the 1-day-old male at a distance of 5 cm for 1 or 2 min. We examined morphological abnormalities in testes by using a SEM in the high vacuum mode. After one day of treatment for 1 min, we observed a decrease in the spermatogonia number in the peripheral region and the sex cysts appeared abnormal. The content of some cysts was degenerated, and in others, it was heterogeneous. In addition, the septate junction had winding extensions, some of which appeared simple and the others wrapped around each other, such as the plexus. After 7 days of treatment, the septate junction was highly irregular, the dividing sex cysts were very difficult to identify and their content either was degenerated or had an abnormal appearance. Moreover, the exposure of the male to the cold plasma for 2 min induced high alternation in the testes. The septate junction became ruptured in some parts and few in number. Dividing sex cysts appeared abnormal, some appeared huge and their internal content was empty, containing only one or approximately six abnormal spermatogonia. The sperm cells could no longer be detected. A small number of necrosis appeared on the surface of the testes after exposure to cold plasma for 1 min, whereas a large number of necrosis appeared with a different size after 2 min of exposure. Because of the high damage that was induced on the testes after the male's exposure to the cold plasma for 2min, there was no need to examine the testes after 7 days of treatment. It is obvious from the results that the cold plasma induced severe morphological abnormalities on the testes depending on the time of exposure and the time after treatment. The damages that were induced by the PAPPJ could be expected to have an effect on the insects' fertility and reproductive capacity.

Other studies have been performed on the influence of the different radiation types on the RPW and other coleopteran male reproductive systems, andon the mating behavior and efficacy of the sterile insect technique (SIT). Rahalkar et al. (1973) determined the effectiveness of exposure to X-rays in sterilizing males and the mating behavior of the RPW. Hasan (1995) reported on the effect of gamma irradiation in Tribolium spp., noting that high doses of gamma radiation can inactivate sperm, or at least have a lethal effect in cells, and lower doses can have significant effects on sperm production. Banu et al. (2006) found a reduction in the growth of the Tribolium castaneum testis after gamma radiation. This reduction is assessed by a decrease in or the absence of spermatogenic activity as a possible consequence of radiation damage to the germ cells that lead to sterility, as characterized by in fecundity. Mahmoud and Shoman (2009) observed that radiation induced the retardation of spermatogenesis, resulting in abnormal spermatocyte shapes, damaged spermatids, and sperm bundles, some of which were completely absent. The results were reinforced by studying the fecundity and fertility of the adult male that appeared to be significantly reduced by increasing the dose level when compared with the untreated control. Al-Waneen et al. (2009) observed that the major damage induced by gamma radiation in the zone of growth in the testes of the RPW male was a rupture of the spermatic tubules with scattered lysed cyst cells. Al-Ayedh and Rasool (2010) found no adverse effects of gamma radiation on the mating behavior parameters of the RDPW, such as mate recognition time, mating duration, mating frequency within a 30-min period and the duration between consecutive mating. Llácer et al. (2013) studied the testicular deterioration of gamma irradiated 1-day old RPW males; the deterioration was rated according to their appearance (turgidity and sparkling).The researchers found that the higher the dose, the sooner the testes lost turgidity, and irradiation did not affect male sexual competiveness but it did affect sperm quality.

Oxidative stress (OS) has harmful effects on sperm number, motility, quality, and function, including damage to the sperm's nuclear DNA (Cocuzza et al. 2007). The major free radicals produced by the plasma jet are reactive oxygen species (ROS) and nitric oxide (NO), which are derived from oxygen and nitrogen under reducing conditions (Yang et al. 2008).UV irradiation, ions and electrons are also emitted in the plasma discharges, which also interact with biological material or tissues (Laroussi and Leipold 2004; Vleugels et al. 2005). Free radicals play an important role in a number of biological processes. However, cells exposed to large amounts of ROS respond by either ending their proliferation or by cell death (apoptosis or necrosis), depending on the phase of the cell cycle they undergo at the moment of cell lysis. Nitrogen monoxide (NO) the one hand, ROS play a central role for sperm physiology, such as sperm maturation and capacity. On the other hand, abnormal ROS production is associated with defective sperm function. The delicate balance between ROS production and recycling is essential for spermatogenesis. The excessive generation of seminal ROS can cause male infertility (Hsieh et al. 2006). Excessive free radical generation often involves errors in spermiogenesis and, as a result, the release of spermatozoa from the germinal epithelium with abnormally high levels of cytoplasmic retention (Sanocka and Kurpisz 2004). Lipid peroxidation can profoundly affect sperm quality, including the percentage of motility and specific motility parameters **(**Bansal and Bilaspuri 2011). In addition,

oxidative stress impedes spermatogenesis, resulting in the generation of spermatozoa with poorly remodeled chromatin. These defective cells have a tendency to default to an apoptotic pathway associated with motility loss and the activation of free radical generation by mitochondria. The latter induces lipid peroxidation and oxidative DNA damage, which leads to DNA fragmentation and cell death. At the same time, spermatozoa produce small amounts of ROS that play a significant role in many of the sperm's physiological processes such as capacitation, hyper activation, and sperm-oocyte fusion (Aitken et al. 2004). Excessive production of free radicals or reactive oxygen species (ROS) can damage sperm (Agarwal et al.2005).

The formations of septate junctions in polarized epithelia are necessary to maintain the integrity of epithelial barriers in insects (Tepass and Hartenstein 1994). The septate junction of the living tissue is composed of an extremely immobile multi-protein complex (Oshima and Fehon 2011). Proteins can undergo direct and indirect damage following their interaction with ROS. The consequences of protein damage are altered cellular functions, such as energy production, interference with the creation of membrane potentials, and changes in the type and level of cellular proteins (Kohen and Nyska 2002).

In conclusion, the present study could provide key information about the damage effects of cold plasma on insect spermatogenesis. There are a number of clear and understandable questions that may be asked as a result of this study, such as does cold plasma induce permanent sterility and does it affect the mating behavior of these males. Such questions must be investigated to detect the suitability of cold plasma for a sterile insect technique. However, additional studies are needed to evaluate the cost and technique of the treatment for a large number of dangerous pests.

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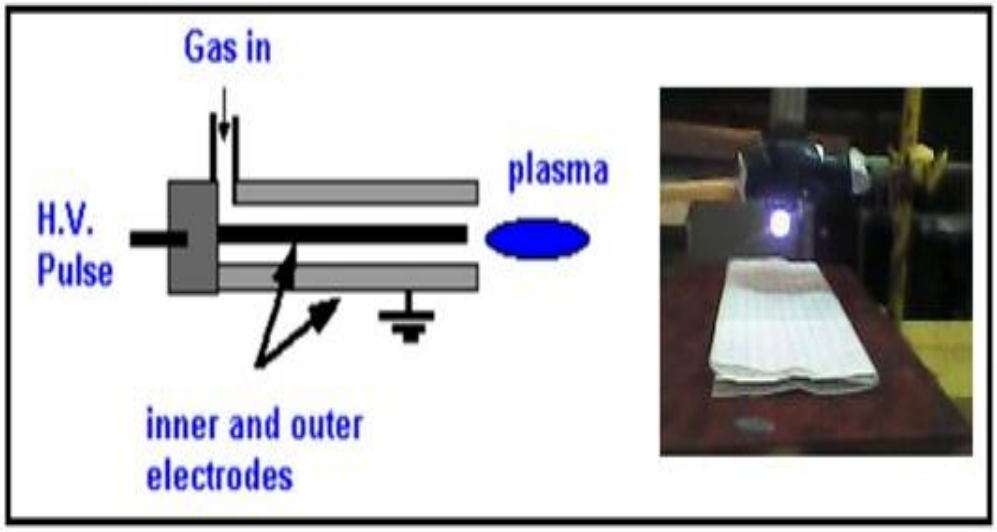


Fig. 1. A schematic of the PAPPJ discharge and the experimental set-up. 148x57mm (96 x 96 DPI)

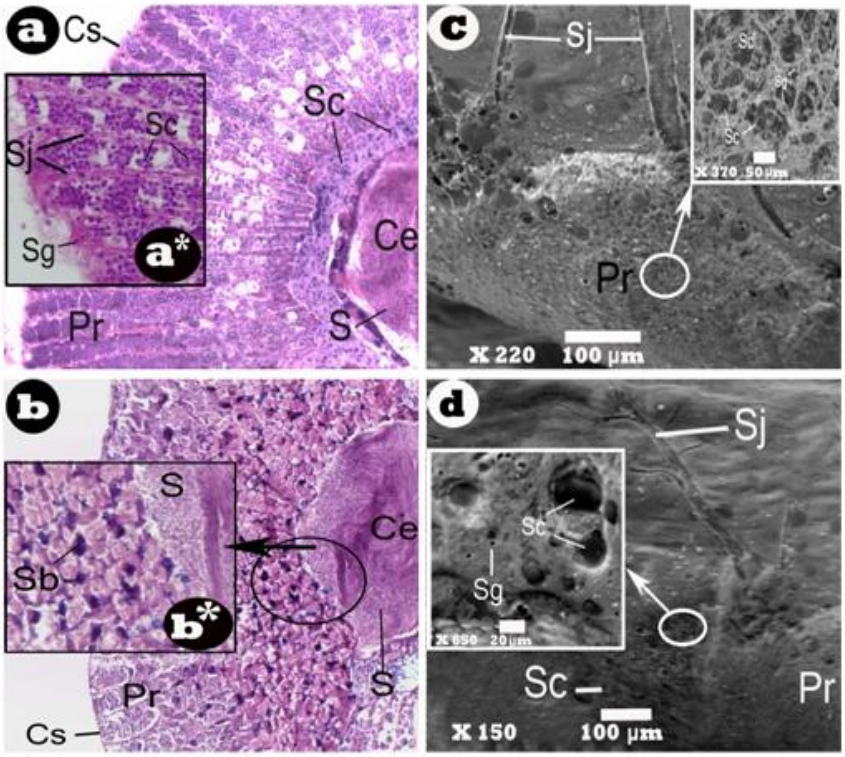
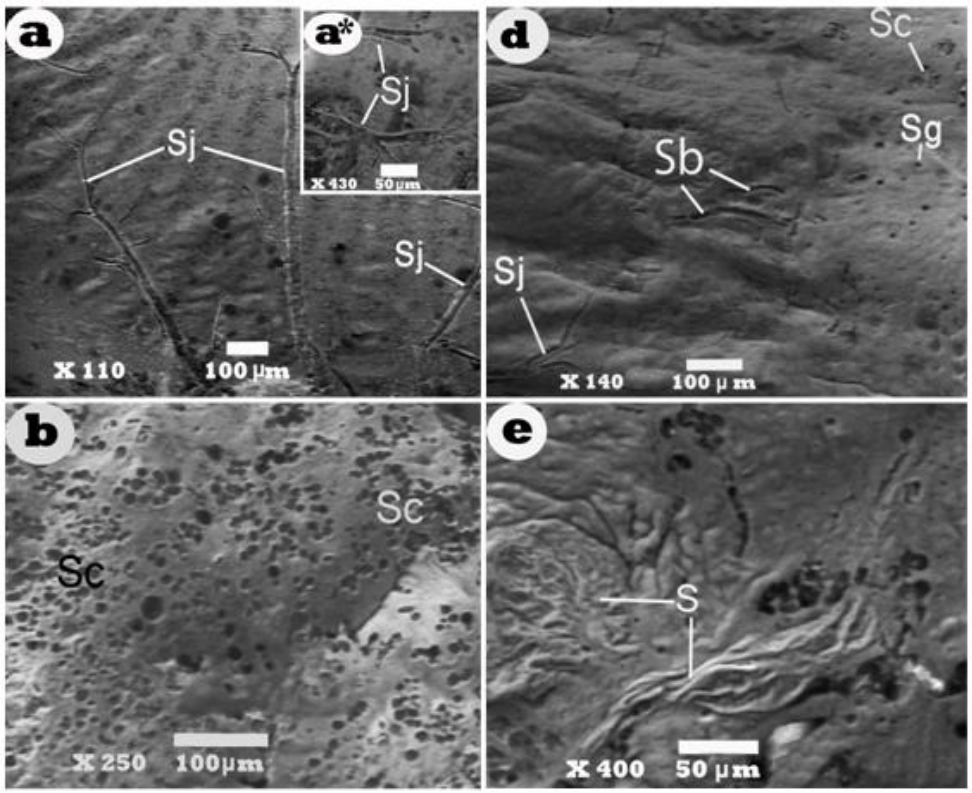


Fig.**2**. **a,b**. Transverse sections of normal R. ferrugineus testes showing the follicles with different developmental stages of sex cysts. **c,d**. SEM of normal R. ferrugineu. Shown are the Central region (Ce), Common sheath (Cs), Peripheral region (Pr), Septate junction (Sj), Sperm bundle (Sb), Sperm cell (S),Spermatogonia (Sg), Sex cyste (Sc). a,b X=160 ,a*b* X=400. Scale bars in c 100, 50 microns and d 100, 20 microns



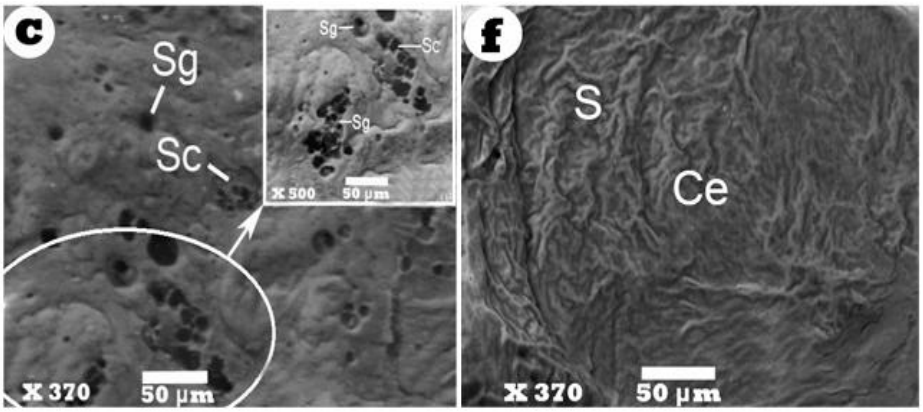


Fig.**3**. SEM of normal 1-day-old *R. ferrugineu.* **a, a***. Showing the septate junction (Sj). **b**. several sex cysts (Sc) in different developmental stages. **c**. High magnification of sex cyst (Sc) with amount of spermatogonia (Sg). **d**. Amount of sperm bundle (Sb) beside to the terminal part of septate junction. **e**. Move of sperm cell (S) in direction to the central region (Ce). **f**. The various stages of development of sperm cells in the central region.

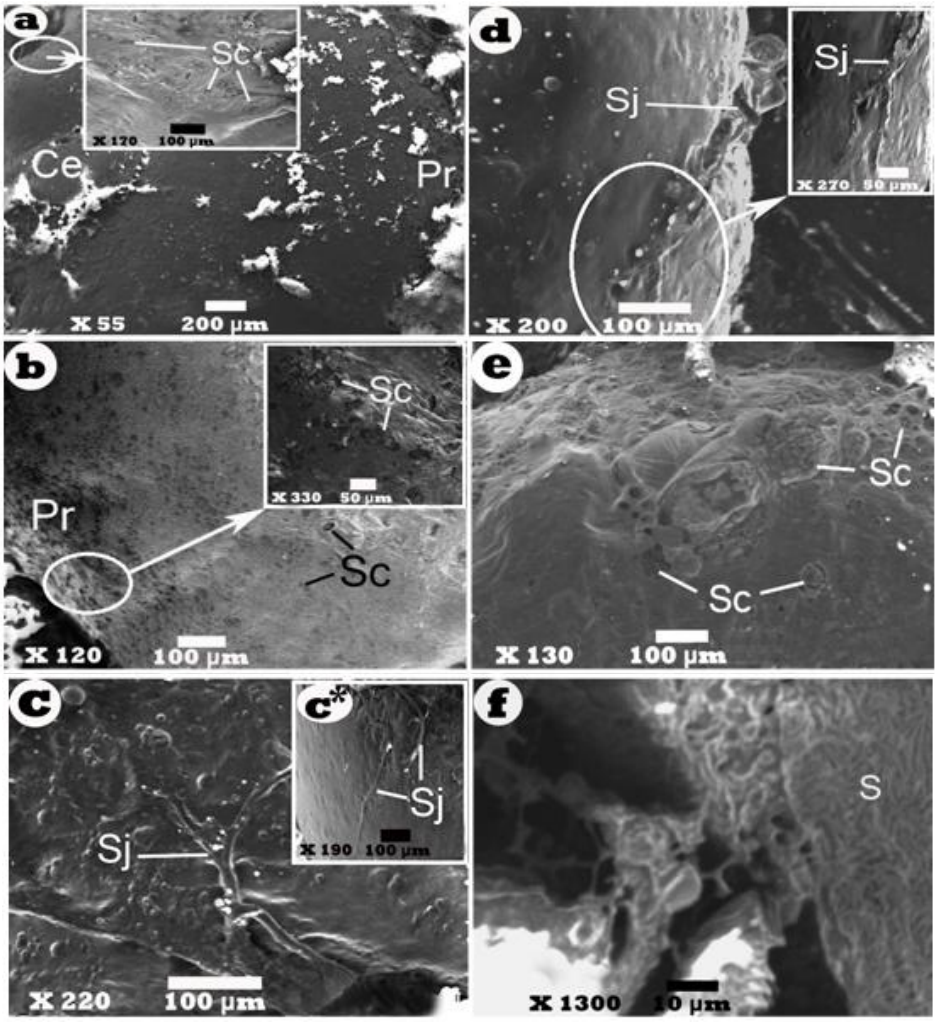


Fig.**4**. SEM of 1 day old *R. ferrugineu* post exposure to 1 minutes of cold plasma. **a**. Small circular projections appear on the outer surface of testes. **b**. Spermatogonia are prevalent in the peripheral region but their number is decreased , the high magnification showed various forms of degenetaring. **c**.The abnormality of septate junction. **c***. The irregularity of terminal ends of septate junction. **d**. The septate junction wrap around each other. **e**. High number of sex cysts showed various forms of changes. **f**. Less number of sperm cells. Central region (Ce), Peripheral region (Pr), Septate junction (Sj), Sex cyste (Sc) and Sperm cell (S).

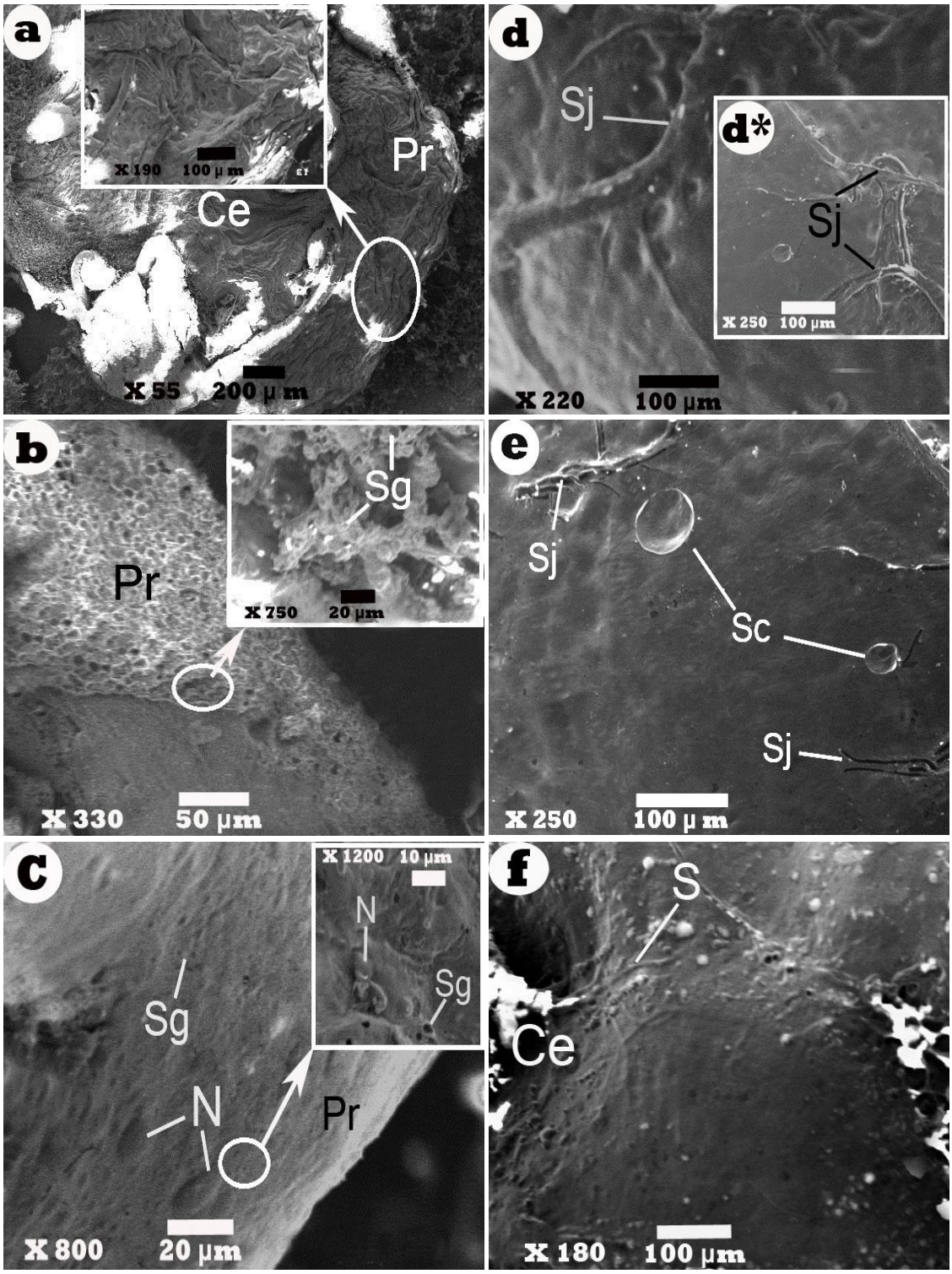


Fig. **5**. SEM of 7 day old *R. ferrugineu* post exposure to 1 minutes of cold plasma. **a**. Highly irregular of the outer surface of testes. **b**. The peripheral region with high magnification of spermatogonia where few of them completely degenerated. **c**. The peripheral region with few number of weak necroses. **d**. Highly irregular of septate junction and their extensions interfere with each other. **e**. Highly irregular of terminal end of junction and two sex cysts completely degenerated. **f**. The central region with less number of the sperm cells. Central region (Ce),Necroses (N),Peripheral region (Pr),Septate junction (Sj), Sex cyste (Sc), Sperm cell (S) and Spermatogonia (Sg).

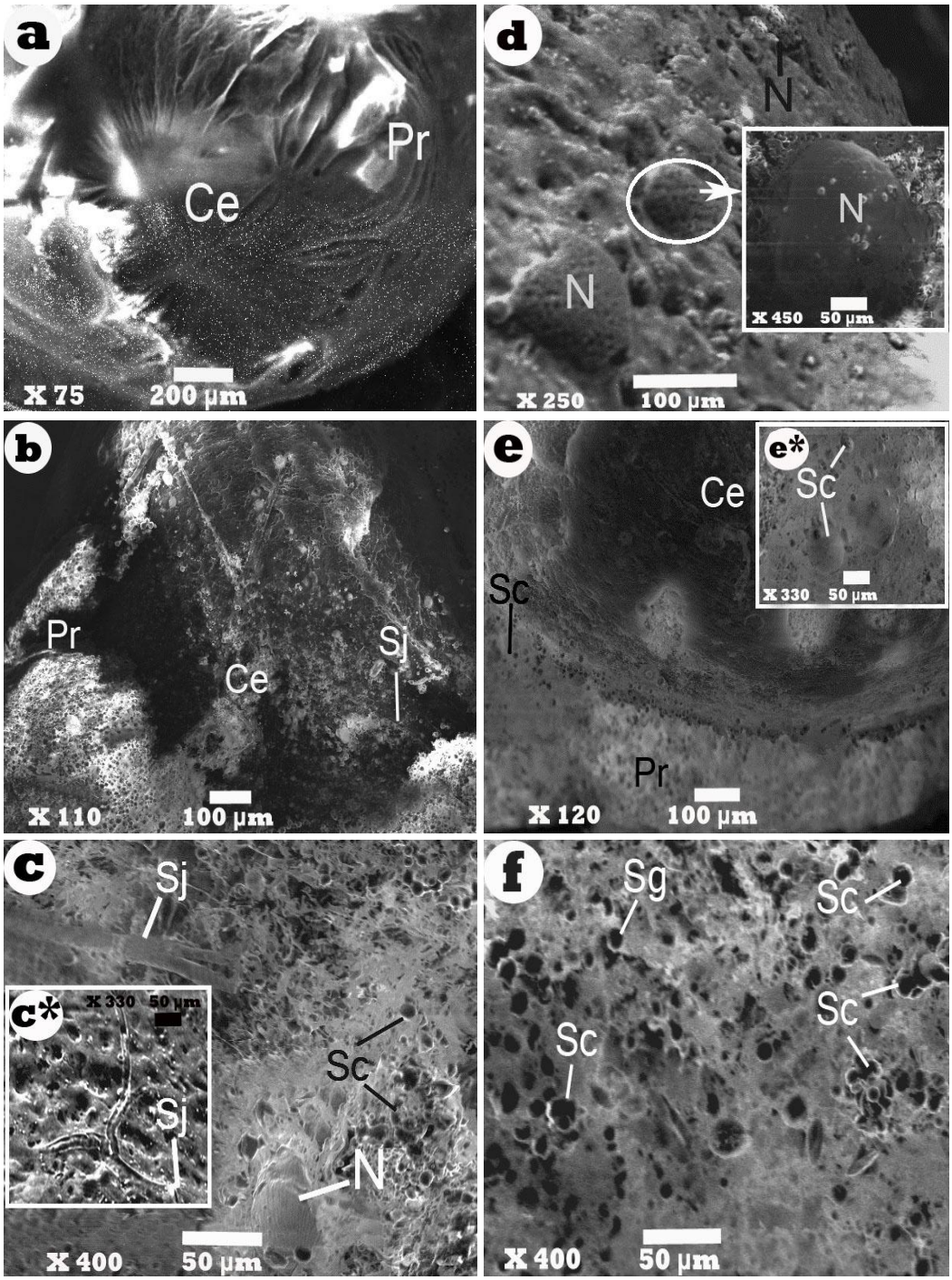


Fig.**6**. SEM of 1 day old *R. ferrugineu* post exposure to 2 minutes of cold plasma. **a**. The external surface of testes appears bulging in few areas .**b**. The testes appeared erratic and unstable. **c, d**. The strong necroses appeared on external surface in large number. **e, f**. Appeared abnormal of sex cysts. Central region (Ce), Necroses (N), Peripheral region (Pr), Septate junction (Sj),Sex cyste (Sc),Sperm cell (S) and Spermatogonia (Sg).