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Ultrastructure and Morphological Changes in the Testes of Southern Green Stink Bug Nezara viridula (Hemiptera: Pentatomidae) Irradiated by Gamma Radiation

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INTRODUCTION

The southern green stink bug, Nezara viridula, is an important pentatomid insect pest. It is cosmopolitan and highly polyphagous on many crops (Mau et al., 1967; DeWitt and Godfrey, 1972; Todd, 1989; Panizzi and Mourão, 1999 and Esquivel and Ward, 2014). Control of Nezara viridula relies heavily upon insecticides, most of which are disruptive to beneficial insects. Consequently there is momentum toward the development of targeted methods for control (Žunič et al., 2002 and Knight and Gurr, 2007).

The Sterile Insect Technique (SIT) is one of various strategies to achieve minimally toxic control of insect pests (Knipling, 1955 and Klassen and Curtis, 2005). The SIT programme is a species specific with no negative off-target effects. Gatenby et al. (1929) had early reported that the spermatogonia of insects are radiosensitive and they destroyed by high doses but any that remain show a relatively low mutagenicity. Sterility has to be genetic in origin and based on aspermia, infecundity, physiologically compromised gametes or inability to mate.

ABSTRACT

The effect of gamma radiation on Nezara viridula male gonad cells was studied using transmission electron microscopy. When fourth instar nymphs were irradiated with a dose of 40 Grays by a 60Co gamma unit, the gonads from the resulting adult males were abnormal in shape and colour compared with those of non-irradiated males. The ultrastructure of the developing spermatids and sperms from irradiated bugs was variable compared with non-irradiated bugs, which all appeared uniform in structure. In particular, there were structural abnormalities in the mitochondrial derivatives and axonemes. Further, supernumerary mitochondrial derivatives and axonemes were observed in the flagella of spermatids and sperms from irradiated males. In spite of these differences, some appear to be normal in structure. The results from high dose radiation-induced damage can be used to aid the development of the sterile insect technique (SIT) for control of N. viridula and other hemipteran pests.
These radiation-induced abnormalities might be expected to have an effect on the insect fertility and reproductive capacity (Robinson, 2002). For a sterile insect release programme to be successful irradiated insects need remain sexually competitive (Knipling, 1955). A reduction in fitness would require a larger number of insects to be released to overcome reductions in sexual fitness (Kean et al., 2011).

Previous radiation work on hemipteran insects had demonstrated mainly the effective of sterilizing doses, and with the biotic effects of such sterilization (Ameresekere et al., 1971). Partial sterility of adult *Rhodnius prolixus* (Hemiptera, Reduviidae) was achieved after exposure to ionizing radiation, with evidence of fertility rates returning to normal over subsequent generations (Maudlin, 1976). In *N. viridula*, studies had investigated the dose required to sterilize eggs and adults (Mau et al., 1967 and Sales, 1977) and eggs were more radio-resistant than the adults. As little as 20 Grays (Gy) appeared to be enough to render adult *N. viridula* sterile, and at lower doses irradiated adults produced a higher proportion of nonviable eggs and had significantly lower fecundity than controls (Dyby and Sailer, 1999 and Žunić et al., 2002).

The researches on spermatogenesis ultrastructure in Hemiptera are not much and concerned to some species (Dallai and Afzelius, 1980; Danilova et al., 1983; Afzelius et al., 1985; Bão and DeSouza, 1994; Carcupino et al., 1998; Fernandes and Bão, 1998, 1999; Fernandes et al., 2001; Chawanji et al., 2005, 2006, 2007 and Araújo et al., 2011 and Özyurt et al., 2013). Moreover, up to the authors’ knowledge, studies on the effect of radiation-induced damage on the spermatogenesis ultrastructure of *N. viridula* has not reported yet. The aim of this study was to investigate the morphogenetic and spermatogenesis ultrastructural changes of male *N. viridula* gonads caused by exposure to gamma radiation.

**MATERIALS AND METHODS**

**Insects**

*Nezara viridula* eggs were field collected from Auckland, New Zealand and maintained in a laboratory. After hatching the nymphs were fed on fresh green beans (*Phaseolus vulgaris*) and raw peanuts (*Arachis hypogaea*) in an environmental chamber maintained at 25 ± 2°C, 50 ± 5% RH and 16:8 h (L:D) photoperiod following the method of Panizzi and Mourão (1999). Fourth instar nymphs were collected from the colony after two generations, and were used for the subsequent trials.

**Irradiation**

To ensure unambiguous radiation damage to bug gonads, the fourth instar nymphs were irradiated to a dose of 40 Gy using a Theratron T-80 Co-60 teletherapy external beam treatment unit (ESR, Christchurch). The insects, contained in petri dishes (90mm diameter, 15 mm deep), were placed at a distance of 50 cm from the radioactive point source. This point source geometry limited the dose gradient through the sample to 6%. A four millimetre thick piece of Perspex was added to the beam entrance side of the containers to ensure that full dose deposition to the insects occurred. The irradiated nymphs were returned to the aforementioned rearing conditions and were maintained until they moulted into the adult stage. All insects were dissected 24-48 h post final moult.

**Transmission electron microscopy (TEM)**

Control and irradiated alive male *N. viridula* were placed in a refrigerator at 4°C for 15 min to reduce their activity prior to dissection. After placing them in chilled dissection buffer (0.1 M phosphate, 3% sucrose, pH 7), dissection
was immediately performed using a scalpel and small scissors to remove their head, legs, thorax, wings, and abdominal integument to expose the viscera. The testes were carefully isolated and transferred directly to the primary fixative (4% formaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate, 5 mM CaCl₂, 3% sucrose, pH 7.2) where they were fixed for 4 h on a rotator. Primary fixation and subsequent steps before polymerisation were carried out at room temperature. Samples were then washed in buffer (0.1 M cacodylate, 5 mM CaCl₂, pH 7.2) and transferred to the secondary fixative (1% osmium tetroxide and 0.8% ferricyanide in 0.1 M cacodylate buffer) for 2 h on the rotator, washed in ultrapure water, and dehydrated through an acetone series (70%, 80%, 90%, 15 min each, then 100% EM-grade dry acetone twice for 20 min). Samples were then infiltrated and embedded in procure 812-araldite 502 resin (50% resin/acetone, then thrice in 100% resin and polymerised for 22 h at 60°C. Sections 80–100 nm thick were cut on a Leica Ultracut UCT fitted with a Diatome 45° diamond knife onto 100 mesh formvar coated copper grids, poststained briefly with 2% uranyl acetate then 0.02% lead citrate and viewed with a Morgagni (FEI; fei.com) transmission electron microscope (TEM) operating at 80 kV.

RESULTS
The internal reproductive organs of male *N. viridula* comprised paired testes, vasa deferentia, vesiculae seminalis, as well as accessory glands. The testes of the control reproductive organs were elongate ovoid in form, lay across the body cavity and covered by an orange peritoneal sheath (Fig. 1 A, B). The irradiated gonads had the same position in the body cavity but were smaller in size, and variable in colour. In three of the four irradiated samples, the colour was similar, but the size of the testes significantly reduced (Fig. 2 A, B). In one case, the testes were grey in colour, small in size and appeared deflated in shape, vas deferent and the accessory glands also appear grey (Fig. 3 A, B). Irradiated testes investigated with TEM were small in size, but not grey in colour (similar to Fig. 2 A, B).

In testes of non-irradiated insects, germinal cells in different stages of development are distinguished in the testicular follicles. These include spermatogonia, spermatocytes, spermatids and spermatozoa. The cysts are formed by the grouping of germ cells of the same stage. In non-irradiated *N. viridula*, the spermatocytes are generally large with heterochromatic nuclei and the nuclear membrane is double layered and containing distinct pores. The nuclei are surrounded by a cytoplasm containing different cell organelles. Parallel cisternae of rough endoplasmic reticulum lie round the nucleus. Dense mass of mitochondria aggregated close to the nucleus and Golgi elements are scattered throughout the cytoplasm (Fig. 4). In early stage of spermatid, the mitochondria (M) become spherical and are clustered together (Fig. 5). They undergo a complex series of rearrangements and fusions that lead to their integration into a large spherical mass, the nebenkern (NK). It located behind the nucleus and is composed of outer membranous array of vesicles enclosing coiled spireme (Fig. 6). In later stages of spermatid morphological transformation process, the nebenkern divided into two tortuously interwoven mitochondria which are closely associated with the developing axial filament. Most gonadal cells of irradiated male showed morphological changes than normal. The early spermatid of irradiated *N. viridula* exhibit a variety of abnormalities. Deteriorated mitochondria were observed within the cyst cell, the mitochondrial cristae were loose. The nucleus appear to have no regular
outlines and chromatin is very poor (Fig. 7). In non-irradiated *N. viridula*, the spermatid showed the development of the interwoven mitochondria into unequal two mitochondria derivatives which showed electron lucent areas peculiar to derivatives of Hemiptera, surrounded by cristae and flanked the axoneme (Ax) (Fig. 8). In irradiated *N. viridula*, the interwoven mitochondria was not obvious as normal. Irregular mitochondria distributed in lysed cyst cell in an unusual manner and not flanked the axoneme (Fig. 9). In non-irradiated *N. viridula*, presperm the later stage of the spermatid morphological transformation process, two mitochondrial derivatives which exhibit irregular cristae are closely associated with developing axial filament (Fig. 10). In Irradiated late spermatid (presperm) exhibit a variety of abnormalities as appearance of supernumerary axonemes, typically two to four were common and sometimes multiple mitochondria were observed (Fig. 11).

Sperm is differentiated from presperm by great elongation of the tail. An electron micrograph of non-irradiated *N. viridula*, individual sperm in cross section enclosed by membranous sheath and has pair of equal mitochondria derivatives which has paracrystalline area, has no distinct cristae and they are flanked the axoneme. The axoneme is formed by 20 fine microtubules which are arranged as wheel like pattern of (9+9+2) with 2 central microtubules, encircled by 9doublets which in their turn are surrounded by 9 accessories microtubules (Fig. 12). Irradiated sperm or presperm which male may fail to develop mature sperm exhibit a variety of abnormalities as appearance of two axonemes and malformed mitochondria derivatives (Fig. 13).

**DISCUSSION**

In spite of a detailed irradiated ultrastructure comparison for hemipteran insects is not possible due to the lack of literature reports, the dose of 40 Gy was selected because it ensures negative impact on the testes and would evaluate the possible ultrastructural changes on spermatogenesis in the male gonads of *N. viridula*.

There were morphologically significant variations in the samples had investigated between irradiated and non-irradiated insects. However, not all the irradiated samples had the extreme differences in morphological features of the testes. A significant reduction in the size and functionality of male *N. viridula* reproductive organs would probably lead to reduction in overall fecundity and fertility. Degeneration and necrosis in reproductive organs of the house fly, *Musca domestica* (L.); the secondary screw worm fly, *Cochliomyia macellaria* (Fab.) and in the black blow fly, *Phormia regina* (Meigen) which resulted in empty cysts were observed by using of X-ray dosages of 2.5, 5.5 and 8.5 kR (≈ 22, 48 and 74 Gy) (Riemann, 1967; Riemann and Thorson, 1969). The morphological and histological changes resulting from exposure of nymphs and adults of the hemipteran leaf hopper *Circulifer tenellus* to 60Cobalt radiation were demonstrated by Ameresekere et al., (1971). Treatment of those males resulted in pronounced cell necrosis and sperm depletion, as well as reduction in various stages of spermatogenesis and vacuolation of the testicular sacs, with slight thickenings of the walls. Cell necrosis and nuclear fragmentation were recorded after the desert locust, *Schistocerca gregaria* had irradiated with 300 rad X-ray dosage, the fertility and the reproductive capacity were reduced (Coggins, 1973). Gamma radiation had also affected the gonads of red flour beetle, *Tribolium castaneum* treated with 15 Gy resulted in significant size reduction of testes and ovaries, leading to sterility in males and in females (Banu et al., 2006). Irradiated *Rhynchophorus*
male beetles with 15 Gy of gamma radiation were damaged spermatic tubules, lysed spermatic cysts and the breakage at the junction of spermatic tubes and vas efferent that disconnected the normal passage of sperm (Al-Waneen et al., 2009). The quick degeneration of testes in gamma irradiated males of *R. ferrugineus* could be related to injury in testicular tissues (Llácer et al., 2012). Solar radiation had used also as abiotic factor and demonstrated severe effect and reduced the size of testes and the accessory glands of the male beetles *Callosobruchus maculatus* (Khaled et al., 2015).

The transformation process of spermatids to sperm involves several morphological reorganization of the cells. The changes are presaged by eccentric displacements of the nucleus and the caudal displacement of the cytoplasm, the fusion of the mitochondria forming the nebenkern, which is common in insect sperm (Nicotra and Murd, 1985). In presperms the nucleus elongates, the tail develops and the nebenkern divides forming the mitochondrial derivatives, which extend caudally along the developing tail, in front of the axial filament, the axoneme (Mohammad et al., 2009). All mitochondrial developmental stages are very sensitive to radiation (Coggins, 1973; Hodges, 1983 and Mahmoud and Shoman, 2009). Gatenby (1941), had indicated that the nebenkern develops under the influence of the neck body (centriole and centriole adjunct). Coggins (1973), suggested that the failure of the mitochondria to fuse into a nebenkern may due to temporary interference with either the genes controlling this formation process and/or the breakdown or the formation of the bounding membrane. We observed non-symmetrical mitochondrial derivatives in the irradiated insects. Mitochondrial derivatives appeared symmetrical in shape in non-irradiated Pentatomidae (Araújo et al., 2011) and in normal *N. viridula* males. Abnormalities in irradiated insects would reduce the ability of the sperm to swim via whipping of the flagellum. Abnormalities in the mitochondrial derivatives could reduce the overall energy available to the sperm and decrease its ability to fertilize any ova (Gatenby 1941; Coggins, 1973 and Paoli et al., 2014).

The axoneme, which is believed to be the motile element of the sperm, arises behind the nucleus in the sperm head and extends longitudinally flanked by the accessory bodies along the sperm tail (Mohammad et al., 2009). Production of supernumerary axonemes in the spermatids and sperms were observed in irradiated samples of *N. viridula*. The supernumerary abnormal structure of the axonemes in many insects were reviewed by Hodges (1983). He mentioned four explanations were postulated by different researchers. Creighton and Evans (1941) were speculated that these abnormalities had resulted from the suppression of either the first or second meiotic divisions. Pollister and Pollister (1943) had observed many centrioles and development supernumerary tail filament in the snail *Vivipera sp*. They also noted that the number of chromosomes lost were corresponded to the number of centrioles gained. They theorized that each kinetochore of the lost chromosome had taken on the role of a centriole and might responsible for the formation of the axoneme. Tahmisian and Devine (1961) suggested that radiation caused an intracellular induction of these organelles of irradiated *Melanoplus differentialis* grasshopper. Coggins (1973) was suggested that the effect of radiation on unstable genes resulted in centriole multiplication in the irradiated desert locust *Schistocerca gregaria*. Hodges (1983) had suggested that the appearance of many axonemes in irradiated *Dermamestes frischii* beetle with X- ray radiation were resulted from suppression
of either the first or second meiotic division. Paoli et al. (2014) observed many axonemes in irradiated red palm weevils, *Rhynchophorus ferrugineus*. The axoneme gives structure to the flagellum, yet is flexible. An excess of axonemes may increase the rigidity of the flagellum, reducing the amount of thrust available to the sperm or produce multiple-tailed sperm (du Plessis and Soley, 2011).

In conclusion, the 40 Gy gamma radiations have demonstrated severe effect on the shape and size of the testes and it influenced the spermatogenesis processes in *N. viridula*. Further studies over a variety of doses coupled with biological studies would help to quantify the variation in spermatogenesis abnormalities between individuals exposed to radiation as well as variation sterility rates.

**ACKNOWLEDGMENTS**

This work was funded by Plant & Food Research through a Blue sky Project started at April 2015up to the end of August 2015 at the Biosecurity Dept. We wish to thank Dr. H. Duane for his technical support and scientific advice. Also, would like to extent great appreciation to Prof. M. Ibrahim, Entomology Department, Faculty of Science, Ain Shams University for the scientific advice and for reviewing the manuscript.

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Fig. 1: A Photograph of the internal reproductive system of non-irradiated male *Nezara viridula* testes (Te), accessory gland (EG) and salivary gland (SG). (B) Higher magnification of (A) showing the testis. Bars: 1mm.

Fig. 2: A Photograph of the internal reproductive system of 40 Gy of irradiated testes of male *N. viridula* reproductive organs. (B) Higher magnification of (A) showing reduces size of the testes (Te). Bars: 1mm.

Fig. 3: A Photograph of the internal reproductive system of the 40 Gy of male non-irradiated male *N. viridula* (B) Higher magnification of (A) showing atrophied testes (Te) with greyish appearance. Bars: 1mm.
Ultrastructure and morphological changes in the testes of southern green stink bug *Nezara viridula*

Fig. 4: Electron micrograph of the spermatocytes of non-irradiated *N. viridula* showing: Nucleus (N), Nucleolus (Nu), Chromatin (Ch), Nuclear membrane (Nm), Cytoplasm (Cy) including aggregateso-fmitochondria (M), Rough endoplasmic reticulum (RER), Golgi vesicles (Gv), Ribosomes (R). Bars: 5mm

Fig. 5: Electron micrograph of the early spermatid of non-irradiated *N. viridula* showing cluster of mitochondria (M). Bars: 2um

Fig. 6: Electron micrograph through spermatid of non-irradiated *N. viridula* showing the nebenkern body (NK). Bars: 2um

Fig. 7: Electron micrograph of spermatid of irradiated *N. viridula* showing: Nucleus (N) with irregular nuclear membrane (Nm), less dense chromatin (Ch), disappearance of cytoplasmic organelles (Cy) except few mitochondria (M) with collapse cistrena. Bars: 2um

Fig. 8: Electron micrograph through spermatid of non-irradiated *N. viridula* showing interwoven mitochondria (IM), lucent area (L) and axoneme (Ax). Bars: 2um.

Fig. 9: Electron micrograph through spermatid of irradiated *N. viridula* showing malformed interwoven mitochondria (IM) and axoneme (Ax). Bars: 2um.
Fig. 10: Electron micrograph of late spermatid (presperm) of non-irradiated *N. viridula* showing formation of the axial filament (Ax) flanked with mitochondria derivatives (MDs). Bars: 2um.

Fig. 11: Electron micrograph of late spermatid (presperm) of irradiated *N. viridula* showing supernumerary axonemes (Ax) and loose mitochondria derivatives (MDs). Bars: 2um.

Fig. 12: Electron micrograph of the sperm of non-irradiated *N. viridula* showing the axoneme (Ax) is formed by microtubules which are arranged as wheel like pattern of (9+9+2) and two mitochondria derivatives (MDs). Bars: 0.2 um

Fig. 13: Electron micrograph of presperm or the sperm of irradiated *N. viridula* showing abnormalities as appearance of two axoneme (Ax) and malformed mitochondria derivatives (MDs). Bars: 2 um.
Ultrastructure and morphological changes in the testes of southern green stink bug Nezara viridula

ARABIC SUMMERY

دراسة التغيرات المورفولوجية والترابيب الدقيقة فخصية البقة الخضراء المشععة بأشعة جاما (فصيلة Nezaravirid (Hemiptera: Pentatomidae) الأجنحة: بنتانوميدي)

حاتم عبد الفتاح إبراهيم 1- لويدي ستيوينجر 2- ماكس دي سكلينج 2

1- قسم علم الحشرات، كلية العلوم، جامعة عين شمس
2- قسم الأدوية الحيوي، معهد النبات والغذاء، نيو زيلاندا

إن تأثيرات اشعاعية جاما على خلايا المناسل الذكري في البقة الخضراء قد تم دراسته باستخدام الميكروسكوب الإلكتروني النافذ. عندما تم تعرض الطور النخسي المتبوع لجرعة عالية من أشعة جاما، وجد أن خلايا المناسل أو الخصى للذكور البالغة قد تغيرت حجمها وأشكالها وأصبحت مشابهة للأشكال الطبيعية للذكور غير المعرضة للإشعاع.

لقد أوضحت صور الميكروسكوب الإلكتروني النافذ أن الخلايا المنوية الأولية والخلايا المنوية قد كانت لها تراكيب مختلفة عن خلايا الطبيعية. وقد وجد تقريرا أن الميتوكوندريا ومحاور النيل للحيوان المنوي كان بهم تغيرا كبيرا. كما لوحظ أكثر من محور في بعض المحاور (4 على الأكثر) في الذكور المعرضة للإشعاع.

إن استخدام الجرعات العالية من اشعة جاما لحداث تغير وتدمير الخلايا المنوية لابد من استخدامه تحت أساليب التعقيم الذكري لمكافحة هذه الافة المنتشرة حول العالم وأيضا في مكافحة أمراض البعيد الأخرى.