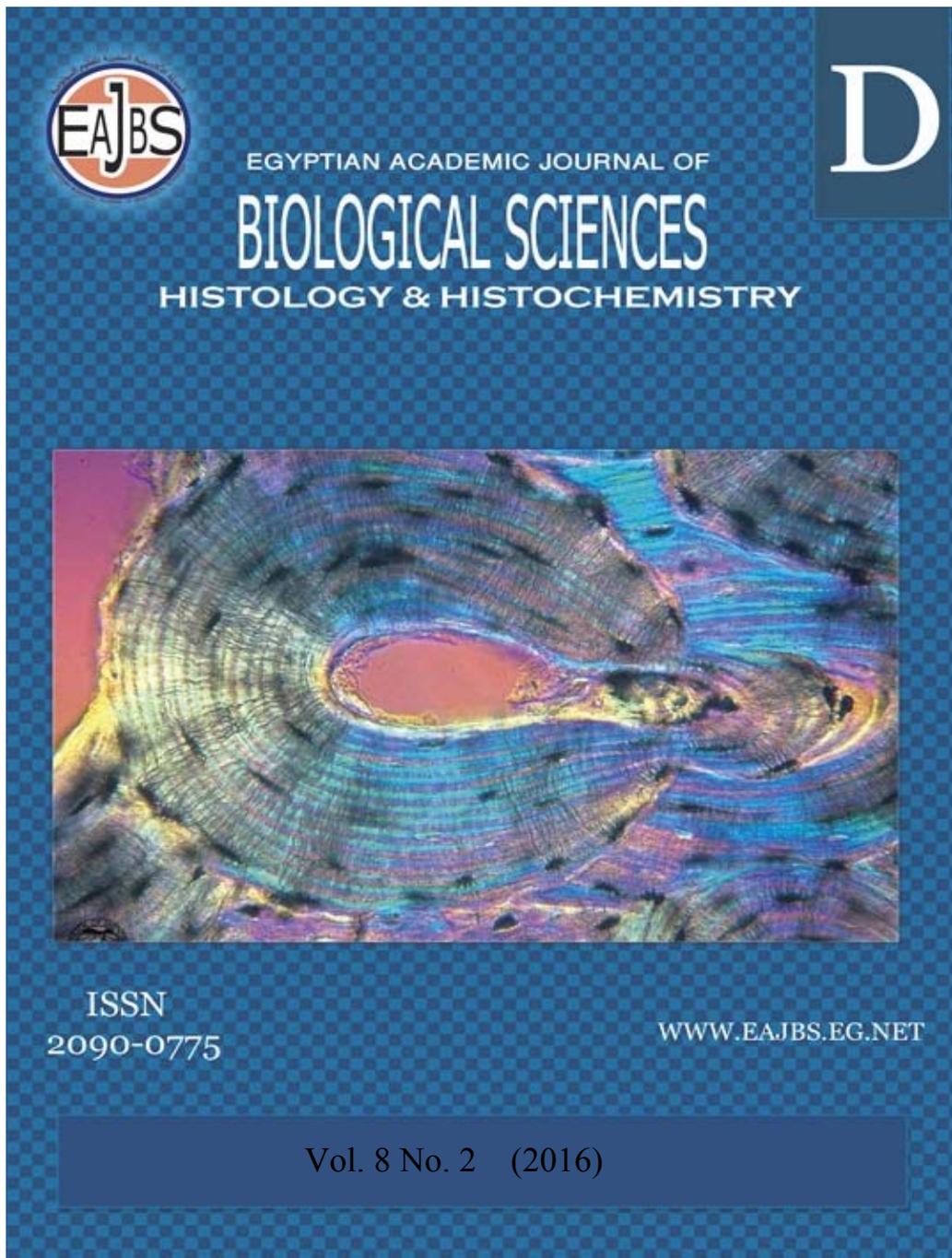


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Influence of Gamma Irradiation on Localization of Enzymatic Activity During Spermatogenesis of Green Vegetable Stink Bug *Nezara viridula* (Hemiptera: Pentatomidae)

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ABSTRACT

Nezara viridula which is serious pentatomid pest for many agricultural crops had irradiated with 40 Gray (Gy) dose of gamma radiation. The ultrastructure and cytochemical studies were carried out to evaluate the activities of acid phosphatase (ACP) and glucose-6-phosphatase (G-6-PDH) enzymes, during the spermatogenesis of non-irradiated and irradiated adult males. These studies were emphasis on the early and late spermatid stage and sperm flagellum, which involve the axoneme and mitochondria derivatives. The reaction products of (ACP) activity was associated with Golgi complex and nuclear membrane, which are relatively at the same level for both control and irradiated individual at early spermatid stage. The localization of (ACP) activity on late spermatid and spermatozoan flagellum showing strong reaction in the endoplasmic reticulum cisternae surrounding the axoneme and tail elements and was also observed on the mitochondria derivatives. The reaction products were approximately weak on irradiated male.

Glucose-6-Phosphatase (G-6-PDH) was found labelling the nuclear envelope in irradiated early spermatid stage, however it was shown negative reaction in control individuals. The (G-6-PDH) was found mainly in the plasma membrane of sperm flagellum, endoplasmic cisternae surrounding axoneme and outer boarder of mitochondria derivatives. The activity of this enzyme was similar in both control and irradiated *N. viridula*.

INTRODUCTION

The polyphagous cosmopolitan, *Nezara viridula* is one of the most important pentatomid insect pests in the world. It infests many important vegetable crops in more than 30 families, with preference for legumes and brassicas (Panizzi, 1997). It feeds on all parts of the plant, including stems, leaf veins, growing shoots, immature fruits, seeds and even flowers. Efficient pest control of *N. viridula* is achieved mainly by the use of insecticides applied over wide areas and somewhat suppressed by biological control agents (Meglič *et al.*, 2001 and Knight and Gurr, 2007).

Autocidal methods and genetic manipulation can be effective against low-density populations dispersed across wide ranges and against high density pests over a limited range (Knipling, 1971). The Sterile Insect Technique (SIT) is one of various strategies to achieve minimally toxic control of insect pests (Klassen and Curtis, 2005).

Very little is known on the effects of radiation-induced sterilization in Hemiptera. Reported studies have dealt mainly with the determination of effective sterilizing doses (Ameresekere *et al.*, 1971). Partial sterility of adult hemipterans may be achieved after they are exposed to ionising radiation of 30-100 Gray (Gy) (Maudlin, 1976) and damage can be inherited (LaChance and Degrugillier, 1969). In *N. viridula*, radiation has been used to determine doses required for sterilization of eggs and adults (Mau *et al.*, 1967 and Dyby and Sailer, 1999). However, as well as inducing sterility, radiation is damaging to other organs (Banu *et al.*, 2006; Suckling *et al.*, 2011 and Paoli *et al.*, 2014).

The rationale for our study was in the context of the genetic methods of sterile insect technique for pest control. As radiation doses increase, the negative effect of radiation on different cells intensifies. It has been suggested that hemipteran insects display inherited sterility, whereby at low doses of radiation, irradiated insects can be fertile, yet their offspring sterile (Bloem *et al.*, 1999b and Soopaya *et al.*, 2011). A reduction in exposure to radiation improves the fitness of the irradiated insect making them a more viable option for a population management strategy (Bloem *et al.*, 1999a and Kean *et al.*, 2011). Drastic morphological changes of irradiated testes of *N. viridula* and spermatogenesis abnormalities had been observed recently (Ibrahim *et al.*, *in press*).

Spermiogenesis is the result of a complex process of cellular differentiation of genetic and energetic material required for fertilization involving biochemical and cytochemical changes (Andre, 1963; Anderson *et al.*, 1967; Phillips, 1970; Baccetti, 1972; Fawcett, 1975 and Fernandes and Bao, 1998). This phenomenon involves the

participation of several enzymes, including phosphatase enzymes (Fernandes and Bao, 1999).

Phosphatase enzymes have been known by their important functions in metabolism, including the phosphate cycle, tissue transformation, growth, nerve action and synthesis of fibrous protein (Mohmoud, 1988).

Acid phosphatase (ACP) is important in biological processes that need high level of energy, such as development, growth, maturation and histolysis (Ray *et al.*, 1984). Also, it plays an important role in the intermediary metabolism and the transportation of protein in insects. The (ACP) is a hydrolase which participates in the metabolism of phosphate which could be used for flagellum motility (Sridhara and Bhat, 1963 and Rousell, 1971).

Glucose-6-phosphatase (G-6-PDH) is an important enzyme in insects, mainly active in the fat body where it breaks down glucose as well as it appears to be a key enzyme in pentose metabolism (Horie, 1967). The activity of (G-6-PDH) has been shown in the endoplasmic reticulum and Golgi complex of insect spermatids (Bao and de Souza, 1994 and Furtado and Bao, 1996). Also in the axoneme and mitochondrial derivatives of the spermatozoa of some invertebrate and vertebrate species (Anderson and Personne, 1970 and Bigliardi, *et al.*, 1970). Evidence suggests that both enzymes are required for Spermiogenesis (Fernandes and Bao, 1999). The enzymatic activities of (ACP) and (G-6-PDH) for non-irradiated *N. viridula* had been described early by Fernandes and Bao (1999), but no information is available of the effect of gamma irradiation on the activities of such enzymes. The selection of these enzymes as an indicator of sterility, is based on the enzyme importance in the reproductive system of insects

(Lambremont, 1959 and Moore and Frazier, 1976).

The cytochemical approach is useful to determine the functional role of the different elements of spermatozoan and the role of enzymes in sperm movement and in fertilization process (Fernandes *et al.*, 2001).

The present study display the localization of acid phosphatase (ACP) and glucose-6-phosphatase (G-6-PDH) and their activities during spermatogenesis of non-irradiated and irradiated *N. viridula* insects with high dose of gamma radiation.

MATERIALS AND METHODS

Insects

Nezara viridula eggs were obtained from the wild and were placed in plastic containers with moistened filter paper and covered with screw-top lids. Nymphs and adults were fed on a diet of green beans and peanuts in an environmental chamber maintained at $25 \pm 2^\circ\text{C}$ and $50 \pm 5\%$ RH with photoperiod of 16:8 (L:D) hrs. (Panizzi and Mourão, 1999).

Irradiation

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). To ensure unambiguous radiation damage to bug gonads, the insects were contained in petri dishes (90mm diameter, 15 mm deep), and 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.

Enzyme cytochemistry TEM

Live control and irradiated male bugs were placed in a refrigerator at 4°C for 15 min prior to dissection to slow them down. Bugs were placed directly in chilled dissection buffer (0.1 M phosphate, 3% sucrose, pH 7). Dissection was rapidly performed using scalpel and small scissors to remove the bug's head, legs and dorsal terga, wings, and abdominal integument to expose the viscera, where upon the testes were located and carefully removed into the buffer and were then transferred directly to a light fixative (1% glutaraldehyde 0.1 M cacodylate buffer) for 15 minutes on a rotator. Samples were washed in buffer (0.1 M cacodylate, 5 mM CaCl_2 , pH 7.2) and cytochemical samples were transferred to either an acid phosphatase assay solution (7 mM cytidine-5'-monophosphate, 2 mM cerium chloride, 5% sucrose in a 0.1 M tris-maleate buffer at pH 5.0; following Pino *et al.*, 1981) or into a glucose-6-phosphatase assay solution (5 mM glucose-6-phosphate, 5 mM manganese chloride, 4 mM cerium chloride, 5% sucrose in 0.1 M tris-maleate buffer at pH 6.5; following Robinson and Karnovsky, 1983) for 1 hour at 37°C , then washed with buffer.

Transmission electron microscopy (TEM)

Cytochemically incubated and control samples were then processed for TEM by first placing in primary fixative (4% formaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate, 5 mM CaCl_2 , 3% sucrose, pH 7.2) for 30 minutes at room temperature $\approx 20^\circ\text{C}$ on a rotator and then overnight without fixation at 4°C . Subsequent steps before polymerisation were carried out at room temperature. Following primary fixation, samples were washed in buffer (0.1 M cacodylate, 5 mM CaCl_2 , pH 7.2), transferred to secondary fixative (1% osmium and 0.8% ferricyanide in 0.1 M cacodylate buffer) for 2 hours on the rotator, washed in ultrapure water, and dehydrated through an acetone series (70%, 80%,

90%, then 100% EM-grade dry acetone twice). Samples were infiltrated and embedded in procure 812-araldite 502 resin (50% resin/acetone, then thrice in 100% resin and polymerised for 22 hours at 60°C. Sections 80–100 nm thick were cut on a Leica Ultracut UCT fitted with a Diatome 45° diamond knife, post-stained briefly with 2% uranyl acetate then 0.02% lead citrate and viewed with a Morgagni (FEI) transmission electron microscope (TEM) operating at 80 kV. Efficacy of the cytochemical staining was assessed with respect to conventional TEM staining of the same structures in the control samples.

RESULTS

The spermatids of *N. viridula* undergo specific morphofunctional modification during Spermiogenesis. At early spermatid stage, the nucleus appears more or less round and has dispersed areas of chromatin (heterochromatic), nucleolus adhere the nucleus envelope. The cytoplasm rich with crescent-shape Golgi complex and parallel cisternae of endoplasmic reticulum (ER) (Fig. 1a). Cluster of mitochondrial aggregates which occurs in the cyst, adheres to the nucleus (Fig. 1b). At this stage, the (ACP) activity is located at the Golgi vesicles and nuclear membrane (Fig. 1a). In irradiated individual, the (ACP) reaction products is associated with nuclear membrane and endoplasmic reticulum at the same level as control insect. The sperm flagellum consists of an axoneme and two mitochondria derivatives (Fig. 2). The axoneme follows the 9+9+2 pattern of microtubules arrangement (9 accessories, 9 doublets and 2 central). The mitochondrial derivatives are symmetric in diameter and formed by one large Para crystalline region between two electron lucent areas and one mitochondria cristae region limited to the periphery of the derivatives (Fig. 2). Electron-dense

reaction product indicating the presence of acid phosphatase activity (ACP).

The reaction product was seen in endoplasmic reticulum cisternae surrounding axoneme and the tail elements of late spermatid for control *N. viridula*. It also seen in association with mitochondria derivatives (Fig. 3 a, b). It observed in the mitochondria cristae which are perpendicular along the axis of the mitochondria derivatives and spaced at regular intervals (Fig. 3 b). The (ACP) reaction product is scattered in the remnants of cytoplasm (Fig. 3 b). At the same stage of irradiated individual, (ACP) activity is detected but diffuse weak reaction (Fig. 4 a, b, c). Deposition of (ACP) reaction product was localized in spermatozoan axoneme, tow mitochondria derivatives and plasma membrane which enclosed these elements at non-irradiated *N. viridula* (Fig. 5).

The reaction of glucose-6-phosphatase (G-6-PDH) activity was not seen in the nucleus of early spermatid stage of control *N. viridula* (Fig. 6 a). However, a light labelling of (G-6-PDH) was observed on the nuclear envelope of early irradiated spermatid (Fig. 6 b).

Reaction product indicative of (G-6-PDH) activity was observed in the endoplasmic reticulum cisternae surrounding the axoneme and the tail elements. This reaction product was also detected on the mitochondrial derivatives of late spermatid for both non-irradiated and irradiated *N. viridula* (Fig. 7 a, b). The reaction exhibit the same level of activity.

DISCUSSION

The spermatogenesis process involves the structural and physiological transformation of organelles to more adapted forms at the fertilization process (Andre, 1963 and Anderson *et al.*, 1967). Several enzymes may be involved in the remodeling as well as in the chemical changes which occur during this process.

Hence the influence of gamma irradiation on localization of enzymatic activity during spermatogenesis are preserved, so the influence of radiation may attributed to the difference in sensitivity of enzyme loci that control the biosynthesis of the enzyme active protein (Abdel Megeed, 1987 and Protas and Charialo, 1991).

During the early spermatid stage in *N. viridula*, Our observations showed that (ACP) was detected cytochemically in the Golgi complex and nuclear membrane at the same level for both control and irradiated individuals. (ACP) reaction product was strongly observed in the endoplasmic reticulum cisternae surrounding the axoneme and the tail element. Also, it associated with the mitochondria derivatives of the late spermatid (presperm) and spermatozoan of non-irradiated insects. However, (ACP) showed weak reaction product in the irradiated males. The (ACP) activity has been associated with the Golgi complex, which is the site where proteins finally exit to respective cellular sites such as plasma membrane, secretory granules and lysosomes in germinal cells (Griffiths and Simons, 1986 and Grab *et al.*, 1997).

The presence of (ACP) has been mainly related to the axoneme which corresponds to those described for the spermatozoa of other insects (Anderson *et al.*, 1967; Bigliardi *et al.*, 1970; Baccetti *et al.*, 1971, 1973; Beaulaton and Perrin-Waldemar, 1973; Bao and Doler, 1990; Bao, 1991, Fernandes and Bao, 1998 and Bao and de Souza, 1994). Acid phosphatase is important in the metabolism of phosphate compounds which is essential for flagellar motility and activities of other enzymes in the axial filament (Bao and de Souza, 1994). The (ACP) is a hydrolase which participates in the metabolism of phosphate which could be used for the tail motility. This enzyme has been localized in proacrosomal vesicles (Anderson *et al.*, 1967; Anderson, 1968

and Souza *et al.*, 1988), on the axoneme (Anderson *et al.*, 1967 and Baccetti *et al.*, 1971) and plasma membrane (Anderson *et al.*, 1967 and Bao and de Souza, 1994).

On comparing the activities of the (ACP) in the non-irradiated and irradiated *N. viridula*, it was found that control males had high level of activity than that in the irradiated males. These is might attributed to their direct or indirect role in energy production. The enzyme (ACP) acts as mean by which phosphates can be added to the phosphate pool which is necessary for production of energy needed to flight activity and another physiological processes (Gilbert and Huddleston, 1965; Baker and Lloyds, 1973; Bogitsh, 1974 and Moore and Frazier, 1976). Kumar (2012) studied the impact of cell phone radiation on various biochemical and physiological aspects of semen of drone honey bee *Apis mellifera* (L.). It was observed that the activities of seminal enzymes such as (ACP), (G-6-PDH), Alkaline phosphatase and Hexokinase had decreased leading to reduced utilization of the biomolecules and hence increase in their concentration. Chilton (1974) investigated the effects of gamma radiation on the amount of acid phosphatase (ACP) in the mid-gut of adult beet army-worms, *Spodoptera exigua* (Hubner). There was no difference between control moths (0Gy) and irradiated moths (200, 400, and 1200 Gy).

In the present study, early spermatid displayed glucose-6-phosphatase (G-6-PDH) activity at nuclear membrane of irradiated *N. viridula* and were not detected in the non-irradiated insects. In contrast to the control, the irradiated male *N. viridula* was associated with increasing activity of enzyme assayed in the present study. The observed increase might reflect an accumulation of the unused enzyme which may result from the impairment of the normal growth, development and

maturation of gonads. These finding was in agreement with (Mohammed, 2006), which detected accumulation of assayed enzymes in sterile female of cowpea beetle, *Callosobruchus maculatus*.

The resistance to high doses of gamma irradiation is due to the higher capacity for DNA repair in damaged cells, possibly through significantly high levels of relevant enzymes (Cavalloro *et al.*, 1985). Cytochemical studies have demonstrated the presence of sugar residues in intracellular compartment mainly in nucleus associated with dense chromatin (Vannier-Santos *et al.*, 1991; Bao and de Souza, 1992; Craveiro and Bao, 1995 and Bao *et al.*, 1997). The (G-6-PDH) was located in the endoplasmic reticulum in germinal cells of *N. viridula* (Fernandes and Bao, 1999). This enzyme is also detected on the axoneme and mitochondria derivatives of late spermatid in both non-irradiated and irradiated males. The presence of (G-6-PDH) in spermatid and spermatozoan indicates the presence of glycogenolytic pathways (Fernandes and Bao, 1999). The activity of (G-6-PDH) has been shown in the endoplasmic reticulum and Golgi complex of insect spermatids (Bao and de Souza, 1994; Furtado and Bao, 1996). Also in the axoneme and mitochondrial derivatives of the spermatozoa of some invertebrate and vertebrate species (Anderson and Personne, 1970 and Bigliardi, *et al.*, 1970).

Generally, inhibition of the enzyme activities after irradiation may attributed to the disturbance in some physiological and biochemical metabolism. This suggestion could be confirmed by the conclusion of La Brecque and Smith (1963) and Abdel Megeed *et al.* (1987) that the effect of gamma irradiation may be attributed to the interruption of any of the complicated steps of metabolic rate by hormonal, biochemical or genetic factors. The marked sensitivity of enzymes is suggestive of the potential

gamma irradiation to interfere with various energy requiring processes. Thourburn (1972) had theorised that irradiation might interrupt energy supplies blocking of key enzymes which could stop the normal metabolism.

Finally, it is recommended to carry out the quantitative and qualitative studies in the near future to estimate the activity of these two enzymes, (ACP) and (G-6-PDH) present in the germ cells of male *N. viridula* insects before and after irradiation.

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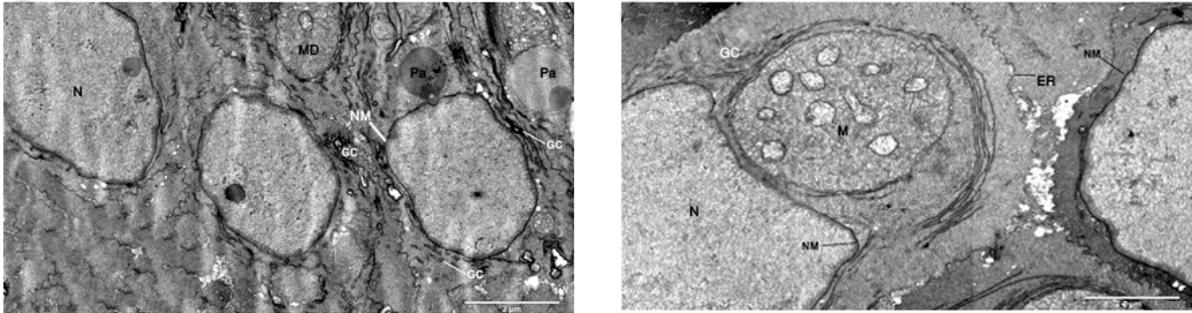


Fig. 1 (a): Electron- micrograph showing acid phosphatase (ACP) localization in early spermatid stage of non-irradiated *N. viridula*. The reaction products labelling the Golgi complex (GC) and nuclear membrane (NM). Key: Proacrosome (Pa), Mitochondria (M). Bars: 2µm.

Fig. 1 (b): Electron- micrograph showing acid phosphatase (ACP) localization in early spermatid stage of irradiated *N. viridula*. The reaction products is associated with nuclear membrane (NM) and endoplasmic reticulum (ER) at the same activity level as control. Bars: 2 µm.

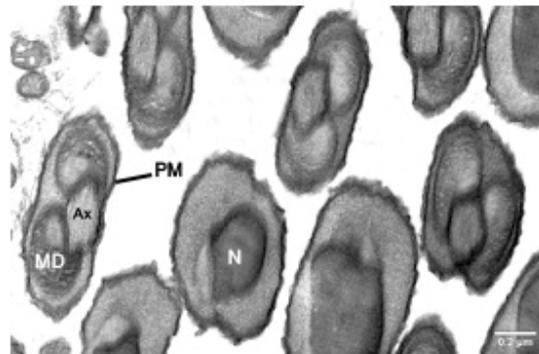


Fig. 2: Transverse section of sperm flagellum of non- irradiated *N. viridula* showing axoneme (Ax) and two mitochondria derivatives (MD) enclosed by plasma membrane of cell (PM). Bars: 0.2 µm.

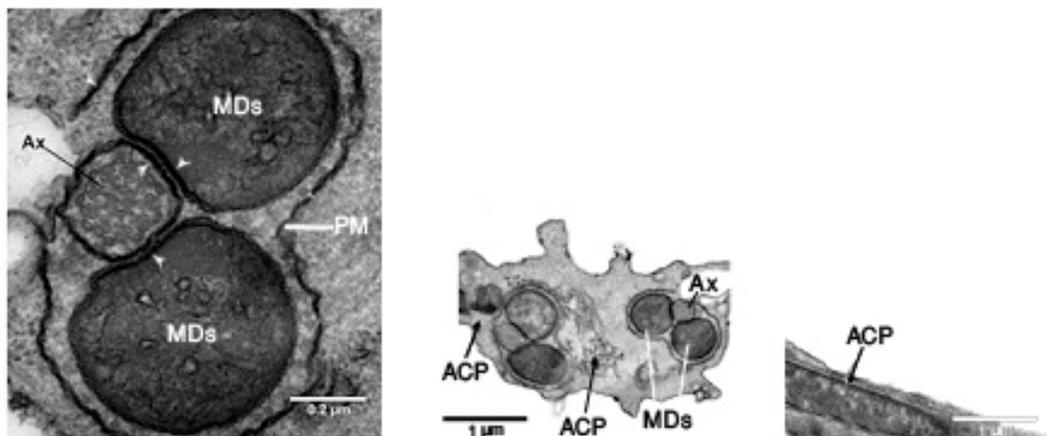


Fig. 3 (a& b): Transverse section of late spermatid (presperm) stage of non- irradiated *N. viridula* showing reaction product of acid phosphatase (ACP) on the endoplasmic reticulum cisternae (ER) surrounding axoneme (Ax) and tail elements and mitochondria (MD) (arrow head). Bars: 0.2 µm.

Fig 3 (c): Longitudinal section through late spermatid (presperm) stage showing the reaction product of (ACP) in the mitochondria cristae which are perpendicular along the axis of mitochondria derivatives (MD) and spaced at regular intervals (arrow head). Dark granules in cytoplasm (G) (arrow). Bars: 1 µm.

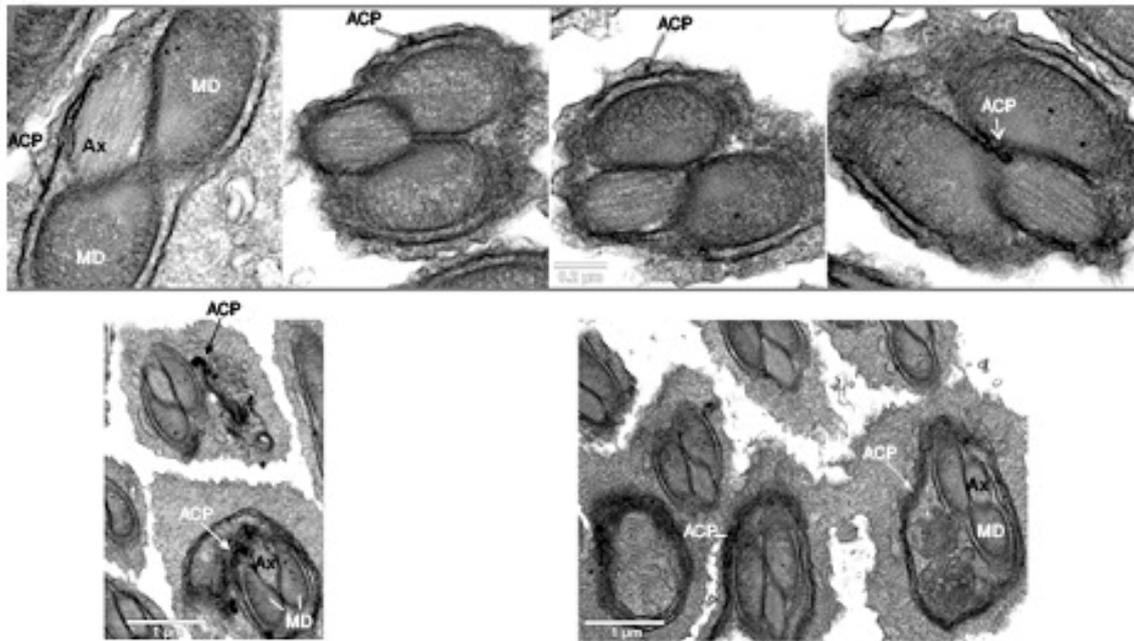


Fig. 4 (a & b & c): Transverse section of late spermatid (presperm) stage of irradiated *N. viridula* showing weak reaction product of acid phosphatase (ACP) on the endoplasmic reticulum cisternae (ER) surrounding axoneme (Ax) and tail elements. Bars: 0.2 and 1 μm .

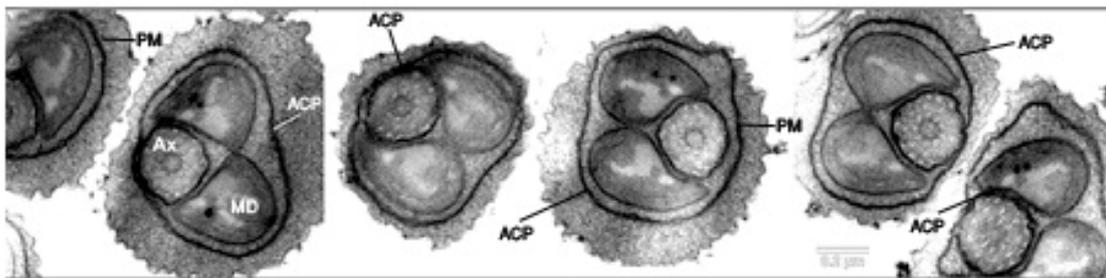


Fig. 5: Transverse section of sperm flagellum of non-irradiated *N. viridula* showing localization of acid phosphatase (ACP) on the axoneme cistern (Ax). Plasma membrane enclosed cell elements (PM) and mitochondria derivatives (MD). Bars: 0.2 μm .

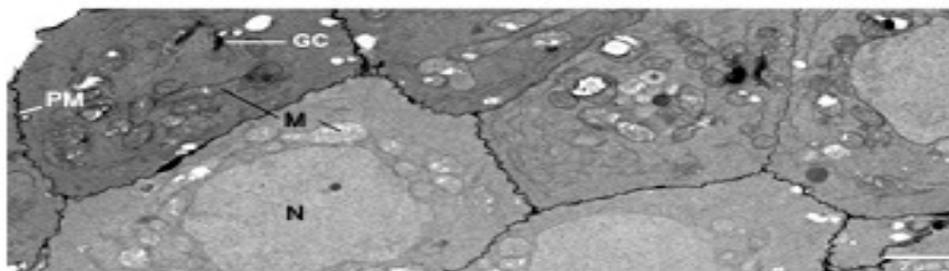


Fig. 6 (a): Electron micrograph in the early spermatid stage of non-irradiated *N. viridula* showing negative reaction of glucose-6-phosphatase (G-6-pDH). Nucleus (N), mitochondria (M), cytoplasm (Cy). Bars: 2 μm .

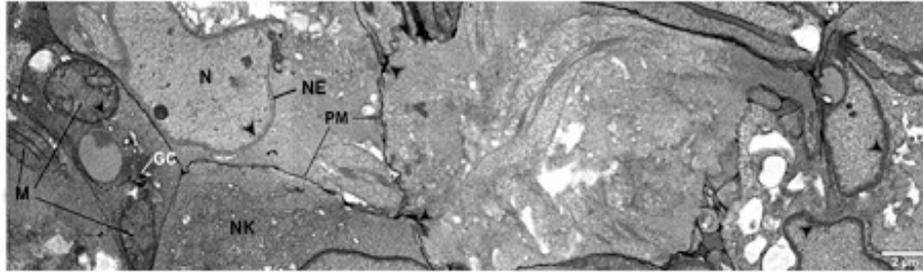


Fig. 6 (b): Electron micrograph in the early spermatid stage of irradiated *N. viridula* showing the reaction product of glucose-6-phosphatase (G-6-PDH) on the nuclear envelope (NE) (arrow head). Bars: 2 μ m.

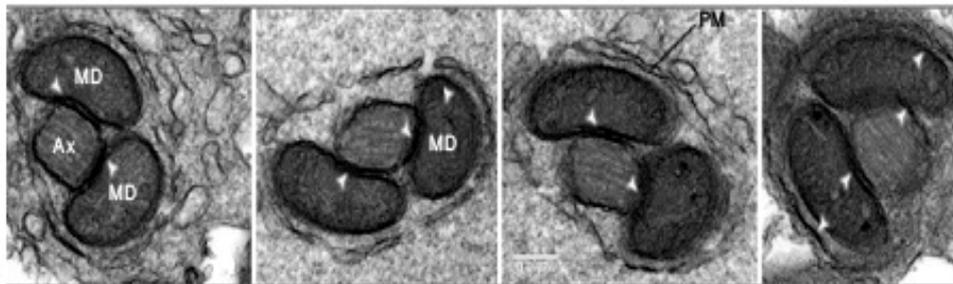


Fig. 7 (a): Transverse section through late spermatid (presperm) in non-irradiated *N. viridula* showing the reaction product of (G-6-PDH) in the endoplasmic reticulum cisternae (ER) surrounding axoneme (Ax) and tail elements. Outer border of mitochondria derivatives (MD) which have distinct cristae (arrow head). Bars: 0.2 μ m.

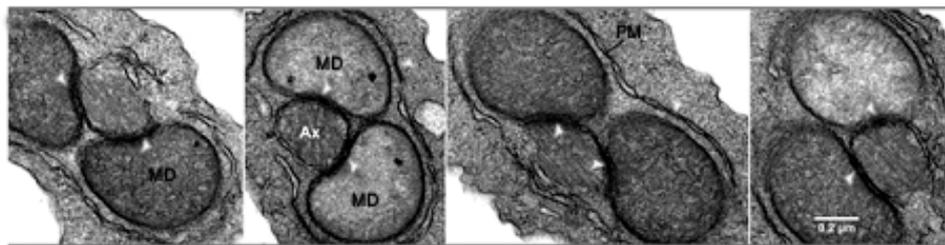


Fig. 7 (b): Transverse section through late spermatid (presperm) in irradiated *N. viridula* showing the reaction product of (G-6-PDH) in the endoplasmic reticulum cisternae (ER) surrounding axoneme (Ax) and tail elements. Outer border of mitochondria derivatives (MD) which have distinct cristae (arrow head). Bars: 0.2 μ m.

ARABIC SUMMARY

تأثير الاشعاع بأشعة جاما علي اماكن نشاط الانزيمات خلال عملية تكوين الحيوانات المنوية للبقعة الخضراء
(رتبة نصفية الاجنحة: بنتاتوميدي) (*Nezara viridula* (Hemiptera: Pentatomidae))

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إن البقعة الخضراء تعتبر آفة خطيرة للعديد من المحاصيل الزراعية المهمة ، وقد تم تعريفها لاشعة جاما ب (٤٠ ج) من أشعة جاما. وقد تمت دراسة تأثير الاشعاع على نشاط انزيم الاسيد فوسفاتيز وانزيم ٦ جلوجوز فوسفاتيز اثناء تكوين الحيوانات المنوية. ووجد إن هناك تغير في نشاط انزيم الاسيد فوسفاتيز في الحشرات المشععة قد أنخفض عن الحشرات التي لم يتم تشعيها. أما بالنسبة لانزيم ٦ جلوجوز فوسفاتيز لم يلاحظ تغير كبير في نشاطة للحشرات المشععة والغير مشععة من ذكور البقعة الخضراء.