Micron 77 (2015) 32–40

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

Managing of *Procambarus clarkii* by X-ray sterilisation of males: Cytological damage to gonads☆

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ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 26 May 2015
Accepted 26 May 2015
Available online 30 May 2015

Keywords:
Procambarus clarkii
Testicular tissue
Ultrastructure
SMRT
X-ray

ABSTRACT

Procambarus clarkii is an invasive alien species spreading worldwide. It is therefore mandatory to find new methods to manage this species since traditional techniques are not sufficient for this purpose. The present study investigates gonad damage induced by different doses of ionising irradiation: 20, 40 and 60 Gy. Testis were analysed after 10 and 30 days by means of light, scanning and transmission electron microscopy. Control unirradiated testes present an acinar structure with a well-defined germinative cells maturation from the distal proliferative zone to the proximal stalk of the lobes whilst, in irradiated testes, induced apoptosis of germinative and accessory cells and a high level of vacuolisation inside the acini were identified, progressively increasing in accordance to Gy dosage and time after exposure. We determined the dose of 40 Gy as the best compromise: it causes an extensive damage to germinative tissues without affecting crayfish vitality, differing from 60 Gy. From an applicative point of view, this dose reduces the efforts, in terms of cost and time, for the application of SMRT.

1. Introduction

The red swamp crayfish *Procambarus clarkii* (Girard; 1852), native to the Southern United States and now present worldwide (Penn, 1954; Barbaresi and Gherardi, 2000 Yue et al., 2008), with the exception of Australia and Antarctica, represents a highly invasive and dangerous alien species. In Europe, it was first imported into Spain in 1972 (Ackefors, 1999), while in Italy it has been present in several northern and central areas of the Country since '90s (Gherardi et al., 1999). The Sterile Male Release Technique (SMRT) has been chosen as part of a strategy to control the spread of the red swamp crayfish in Friuli Venezia Giulia (Italy). This technique is based on the release into the environment of sterile males which are sexually active and able to compete with untreated males for mating partners. The SMRT technique has been deemed reliable because it exclusively acts on target species without interacting with existing biomes, whilst alsobeing safe for human health (Holdich et al., 1999; Lodge et al., 2006). Sterilization by radiation has already been used in some commercial crustaceans such as *Penaeus japonicus* (Sellars and Preston, 2005), *Palaemonetes pugio* (Rees, 1962) and *Macrobrachium rosenbergii* (Lee, 2000). However, no extensive investigations have been carried out on the protocols used in radiation treatments and the methods for quantifying histological and behavioral damage. A previous study on the sterilization of *P. clarkii* male by X-rays defines the methodological protocol for the irradiation and the evaluation of the damage induced within crayfish, and demonstrates that the dose of 20 Gy induces a reduction of 43% in the number of offspring, while sexual behaviour is not altered (Aquiloni et al., 2009). Therefore, using the same protocols, we have decided to test different doses of X-rays – 20 Gy, 40 Gy and 60 Gy – in comparison with a control group, to assess the cytological effects that higher doses of radiation generate on the testes of males of *P. clarkii*.

The male reproductive system in crustaceans is composed of testis and *vasa deferentia* that lead to the paired gonophores. In the *vasa deferentia*, spermatozoa merge into spermatophores, which are structures specialized in transferring spermatozoa to females' body and placing them in internal or external storage receptacles (Krol et al., 1992). The male reproductive system of *P. clarkii* has not yet been fully investigated. In fact, just one study has been car-

☆ The study was supported by the European Project LIFE RARITY (LIFE 10 NAT/IT/000239).

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http://dx.doi.org/10.1016/j.micron.2015.05.016
0968-4328/

ried out on this issue, namely that of Moses (1961a,b). In detail, this analysis focuses on the development of spermatozoa. The literature shows other studies generally concerning the reproductive system of decapods, such as species of the Astacidae (Erkan et al., 2009; Rotllant et al., 2012) and Cambaridae (Word and Hobbs, 1958; Moses, 1961a,b; Wielgus-Serafinska, 1973). Our work aims to describe the male reproductive system in wild *P. clarkii* from the control group, quantifying the effects of different radiation doses on testisin order to improve the applicability of the SMRT.

2. Materials and methods

2.1. Animal collection and housing conditions

About 400 adult crayfish were collected in May 2013 from Cascine di Tavola and Ombrone Lake (Tuscany, Italy) before the reproductive season. Once in the laboratory, they were kept, sexes apart, at a density of 15 m^2 in plastic tanks (80 × 60 × 60 cm) containing 48 L of still tap water and halved terracotta pots as shelters. For the entire period of the study (May 2013–October 2013), experimental individuals were maintained under a 12:12 light/dark cycle at room temperature (20 °C) and fed ad libitum with live *Calliphora* sp. larvae. Water was changed every two days. Hard-shelled crayfish with both claws and all appendages intact were selected and periodically tested for their responsiveness to sexual partners.

2.2. Irradiation of males

A total of 120 males were randomly divided into four groups of 30 males each: a control group (hereafter, C), in which males were subjected to the same manipulation of the other groups but not irradiated, and 3 treatment groups at 20, 40 and 60 Gy (hereafter, 20, 40 or 60), respectively, in which males were exposed at different radiation doses. The minimum dose of 20 Gy was chosen in accordance with Aquiloni et al. (2009) who was the first one to describe the effects of such a dose in the same species. The irradiation was carried out at the beginning of July at the Careggi Hospital (Florence, Italy). Specimens belonging to the same experimental group were treated together. During the irradiation, crayfish were maintained in a plastic tank (17 × 29 × 36 cm) with 10 L of tap water and covered with a sheet of Plexiglas (thickness: 2 cm). A clinical linear accelerator (Philips S175) with a 6 MeV electron beam was used to generate X-rays yielding 2 Gy min^{-1} at 100 cm from the target (40 × 30 cm), so that the treatment doses were achieved with different time exposures (10 min for the 20 Gy treatment, 20 min for the 40 Gy treatment and 30 min for the 60 Gy treatment). After the treatment, crayfish were kept isolated for two weeks in individual aquaria (25 × 20 × 20 cm), each containing a shelter (a halved terracotta pot), and were observed daily to assess possible alterations in their general activity.

2.3. Histological procedure

On the 10th day after the treatment, we dissected 5 males of the control group and 5 of the 20 Gy group, after being anesthetized with ice for one hour. The same operation was also repeated on the thirtieth day but we dissected 5 males of each group (C, 20 Gy, 40 Gy, 60 Gy). Dissections were used for gonad analyses. Testes were subsequently fixed in modified SPAFG solution (Ermak and Eakin, 1976) (0.8% paraformaldehyde, 2.5% glutaraldehyde and 7.5% saturated aqueous solution of picric acid in 0.1 M phosphate buffer saline, pH 7.4, with 1.5% sucrose). Samples for light microscopy and transmission electron microscopy (TEM) were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol (50%, 70%, 95% and absolute) and propylene oxide, and finally embedded in Epon 812-Araldite mixture (Electron Microscopy Sciences, Fort Washington, PA). For optical microscopy, Pabisch TOP Ultra 150 was used to cut resin semi-thin sections (1 μm) which were stained with toluidine blue and examined with Olympus BX50; images were acquired with a digital Olympus E-P1 camera. The analysis of the images was performed with the open-source program ImageJ. For transmission electron microscopy, ultra-thin sections (120 nm) were cut with a Leica Ultracut UTC Ultratome, stained with uranyl acetate and lead citrate, and examined with a Philips EM201 electron microscope at 100 kV; images were acquired with a Veleta – 2k × 2k side-mounted TEM CCD Camera (Olympus, Germany) provided with an iTEM imaging platform and saved in TIF format. For scanning electon microscopy (SEM) analysis, the samples were dehydrated in a graded 50–100% ethanol series, sputter coated with gold in a Edwards S150A apparatus (Edwards High Vacuum, Crawley, West Sussex, United Kingdom), and examined with a Leica Stereoscan 430i scanning electron microscope (Leica Cambridge Ltd., Cambridge, United Kingdom). For the terminology of testis histology we used the one proposed by Hobbs et al. (2007).

2.4. Morphometric data and statistical analysis

For each group (control, 20 Gy at 10 days after irradiation, 20 Gy, 40 Gy and 60 Gy at 30 days after irradiation) major and minor diameters of 250 acini from sections at 5 different testes were measured. Subsequently, the average of two diameters was calculated. All statistical analysis were performed using R version 2.3.1 software (R Development Core Team 2011). The diameters of the acini of control and irradiated animals were checked for normality with the Shapiro–Wilk test and the homogeneity of variance among groups was checked with the Bartlett test. The differences were assessed by non-parametric statistics, Kruskal–Wallis test and pairwise comparisons using Wilcoxon test with Bonferroni correction, since the null hypothesis of the Shapiro–Wilk and/or the Bartlett test could not be rejected. The boxplot were drawn with the boxplot command. Measurements are expressed as mean ± standard error.

2.5. Ethical note

The experiments comply with the current laws of Italy, the Country in which they were done. No specific permits were required for the studies that did not involve endangered or protected species. Individuals were maintained in appropriate laboratory conditions to guarantee their welfare and responsiveness. After the experiments were completed, crayfish were sacrificed by hypothermia.

3. Results

P. clarkii males present a tri-lobed testis which is located in the cephalothorax, placed ventral to the pericardium. The mature testis has a pair of cephalic lobules, extending in the lateral cephalic region above the posteroventral lobes of the hepatopancreas, and a median caudal lobule located dorsal to the midgut. Lobes are connected through the fusion of their stalks from which emerge the paired *vasa deferentia* which extend caudolaterally over the dorsal surface of the caudal lobes of the hepatopancreas, then descending to the gonopores. In dorsal view, the right *vas deferens* is more developed than the left one, which appears thinner and atrophic. Each lobule has an acinar structure and resembles a bunch of grapes, where berries are its acini and the stalk corresponds to its protubules (distal) and collecting tubules (proximal) (for an exhaustive description of the testicular acinar structure of Cambaridae see Hobbs et al., 2007).

The SEM analysis shows that the testes of animals irradiated by 20 Gy, 40 Gy, 60 Gy are smaller than those of the control group. In addition, the medial-sagittal fractures of testicular lobes show a

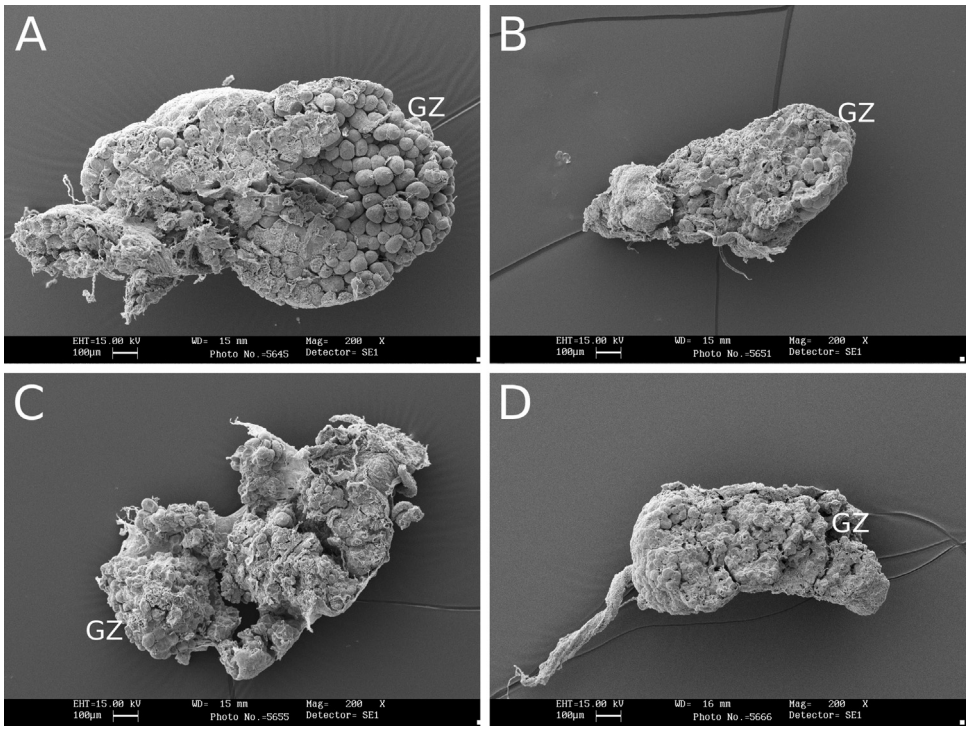

Fig. 1. SEM general view of the medial-sagittal fractures of the testicular lobes of *Procambarus clarkii*: A – control; B – animal irradiated with 20 Gy; C – 40 Gy; D: – 60 Gy.

loss of the acinar organization. In the control group acini are more distinguishable and bigger than those present in the testicular lobes of irradiated animals; in fact, the latter are compact and confluent due to the necrosis induced by radiation (Fig. 1).

The projections of testicular lobes' maximum areas of control animals and of 20 Gy, 40 Gy and 60 Gy irradiated animals after 30 days were measured and a highly significant difference is recorded among the groups (Kruskal–Wallis test: chi-squared = 22.2556, df = 3, $p = 5.771e\text{-}05$). The pairwise comparison using the Bonferroni correction shows that the control group's testicular lobes areas ($3.92 \pm 0.18\,mm^2$) are significantly larger than those of the irradiated groups ($p < 0.0001$; 20 Gy $1.40 \pm 0.13\,mm^2$; 40 Gy $1.24 \pm 0.10\,mm^2$; 60 Gy $1.26 \pm 0.11\,mm^2$), while the lobes of the irradiated animals do not differ between them. The measurements of acini diameters reveal the same trend. The statistical analysis shows highly significant differences between control and irradiated groups (Kruskal–Wallis chi-squared = 395.0381, df = 4, $p < 2.2e\text{-}16$). In particular, pairwise comparisons with Bonferroni correction show that testicular acini of animals irradiated with 20 Gy after 10 days (diameter of $100.9 \pm 2.6\,\mu m$) are highly significantly smaller than those of the control animals ($p < 0.001$ – diameter of $135.9 \pm 4.6\,\mu m$) and after 30 days, at all doses (diameters: 20 Gy $53.2 \pm 1.5\,\mu m$, 40 Gy $49.9 \pm 1.4\,\mu m$, 60 Gy $52.0 \pm 1.3\,\mu m$), the acini are highly significantly smaller than those of animals irradiated by 10 days ($p < 0.001$). There is, however, no significant difference among groups of animals irradiated after 30 days (Fig. 2).

In order to further investigate damage at the spermatogenesis level through light and electron microscopy, semithin sections of testicular lobes have been prepared. A sagittal section of a lobe of unirradiated animal presents a gradual maturation of acini from distal germinative zone (GZ), with a progressive increase in the diameter moving towards the proximal region. This area reveal a testicular pedicle formed by groups of collecting tubules (CT), into which mature acini release the newly formed sperm (Fig. 3).

A higher magnification allowed for visualisation of all stages of spermatogenesis. Acini are delimited by a basal lamina and present a layer of accessory cells (Sertoli-like cells) and the germinative cells inside. The peripheral distal layer of a testicular lobe house buds from collecting tubules forming the primordial acini containing spermatogonia with a mean diameter of $17.31 \pm 0.52\,\mu m$ and a nucleus of $11.36 \pm 0.20\,\mu m$ ($n = 52$, Fig. 4A), then a layer of acini containing primary spermatocytes with a mean diameter of $20.78 \pm 0.58\,\mu m$ and a nucleus of $13.39 \pm 0.30\,\mu m$ ($n = 32$, Fig. 4B) and secondary spermatocytes II (Fig. 4C), whereas the third layer contains acini with early and mature spermatids (Fig. 4D) and the last layer present acini with free spermatozoa with a mean diame-

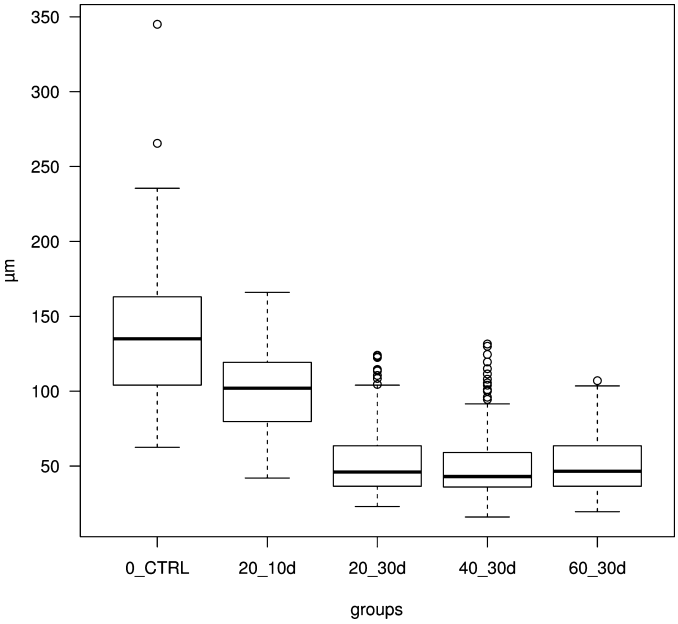

Fig. 2. Boxplot of the diameters of testicular acini in semithin sections of control animals (0_CTRL), of animals irradiated with 20 Gy after 10 days from the treatment (20_10d), of animals irradiated with 20 Gy (20_30d), 40 Gy (40_30d) and 60 Gy (60_30d) after 30 days of treatment.

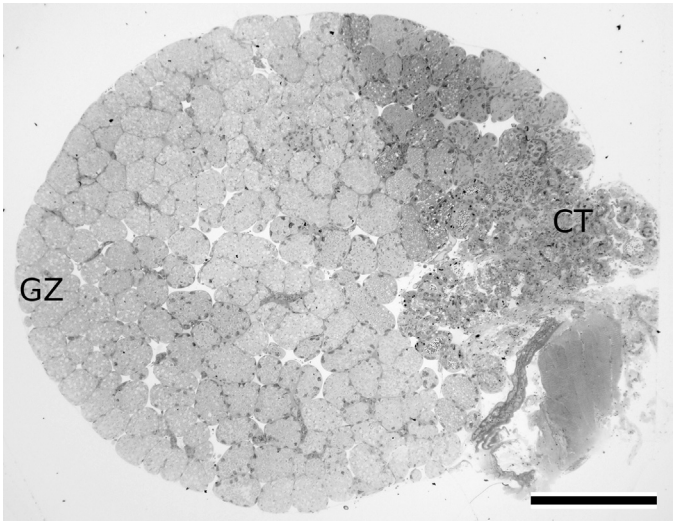

Fig. 3. Median sagittal semi-thin section (1 μm – toluidine blue) of a cephalic testicular lobe of an unirradiated male of *Procambarus clarkii*. The lobe presents a gradual maturation of acini from the distal germinative zone (GZ) to proximal region where groups of collecting tubules (CT) form the lobe stalk. Calibration bar = 0.5 mm.

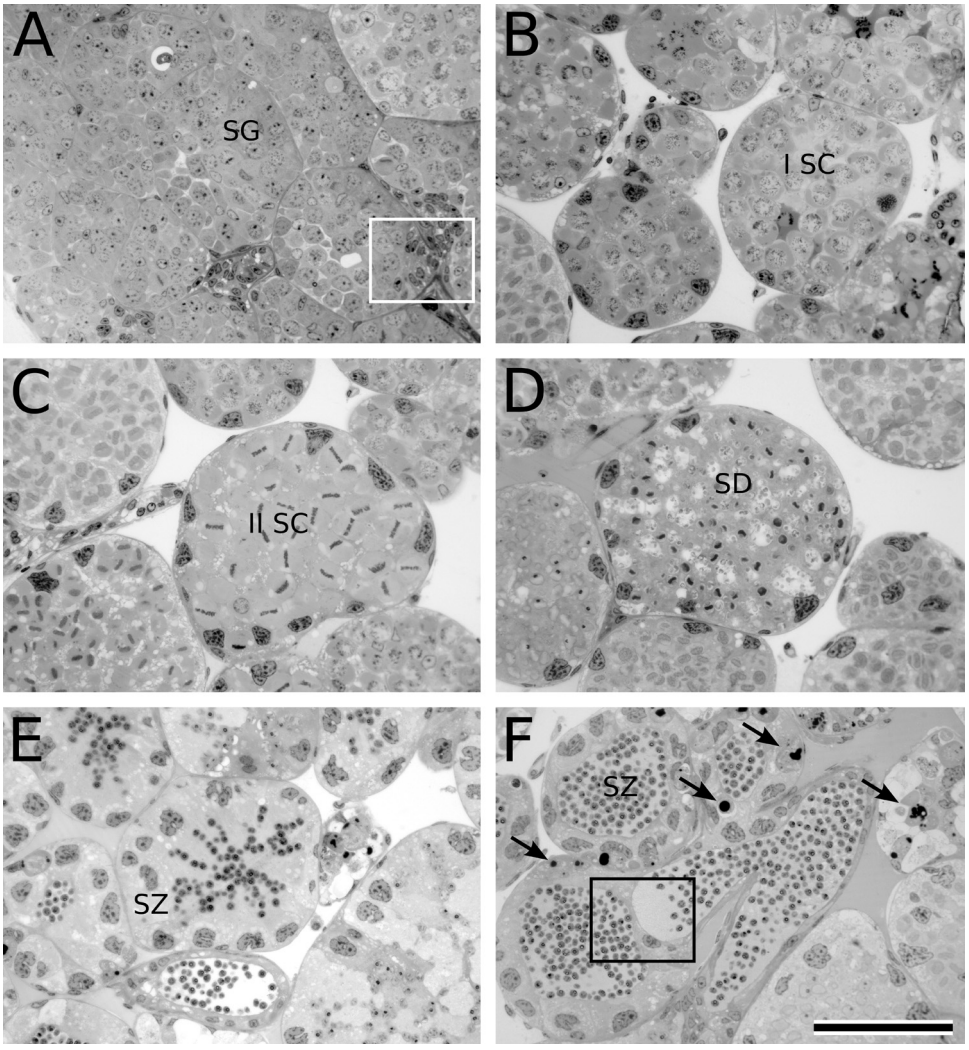

Fig. 4. Semi-thin section (1 μm – toluidine blue) of testicular acini of unirradiated males of *Procambarus clarkii*. The acini lumen shows spermatogonia (A), primary spermatocytes (B), secondary spermatocytes (C), mature spermatid (D), almost mature spermatozoa being moved to the central area (E) and matures acini, containing free spermatozoa, the accessory cells started to show apoptotic degenerative signs such as cytoplasm condensation, nuclear indentation/buds (rectangles) with chromatin condensation and peripheralization, pyknosis, karyorrhexis and apoptotic bodies (arrows) (F). SG – spermatogonia; I SC – primary spermatocytes; II SC – secondary spermatocytes; SD – spermatid; SZ – spermatozoa. Calibration bar = 100 μm.

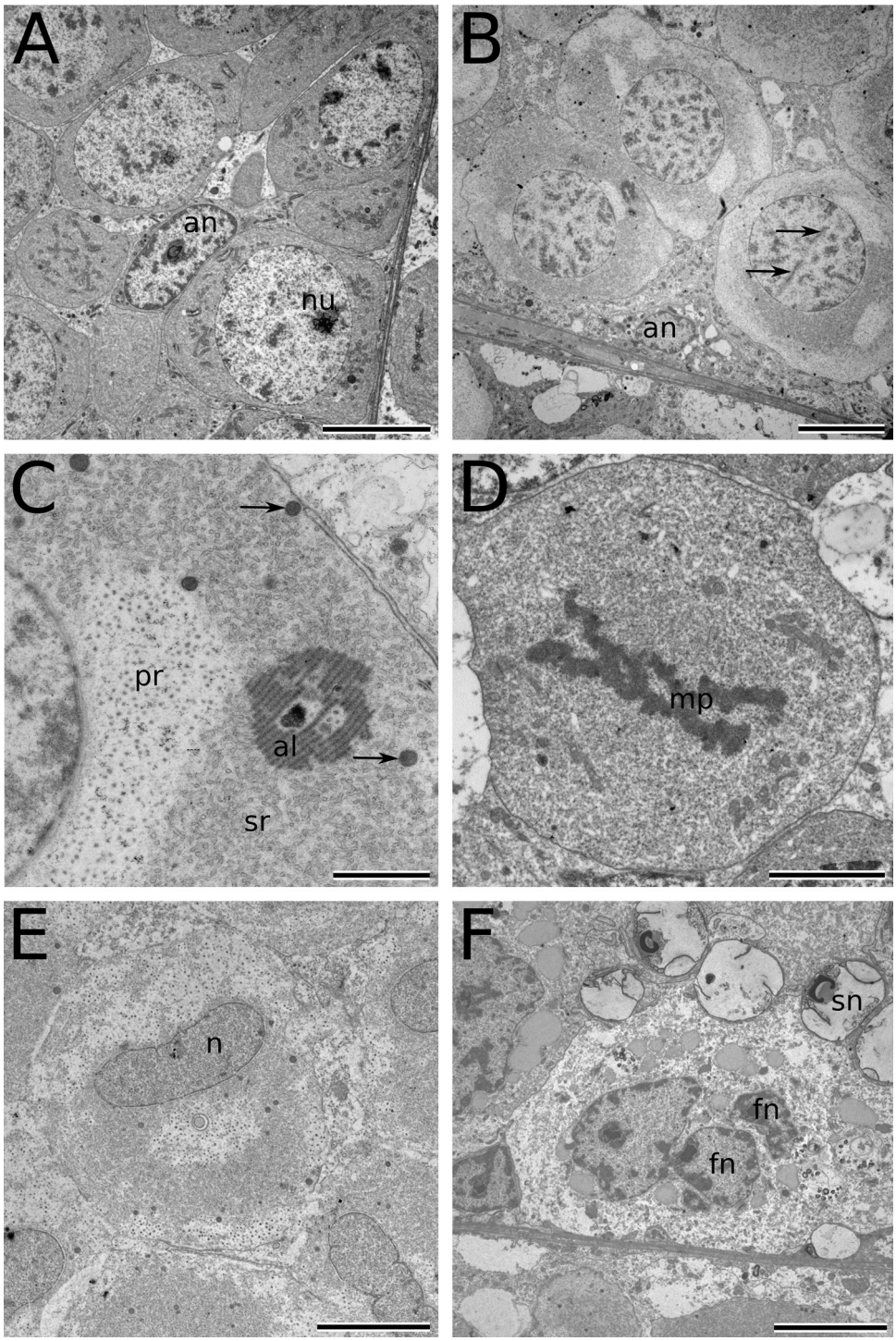

Fig. 5. Ultrastructure of testicular acini of unirradiated males of *Procambarus clarkii*. A – spermatogonia: an – accessory cells nucleus, nu – nucleolus; B – primary spermatocytes: arrows for synaptonemal complex; C – primary spermatocytes: pr – area rich in polyribosomes, sr – area rich in endoplasmic reticulum; al – *annulate lamellae;* arrows – electrondense vesicles; D – secondary spermatocytes: mp-metaphase plate; E – spermatids at an early stage: *n*-nucleus; F – acinus at an almost mature stage: fn – fragmented nuclei of apoptotic accessory cell, sn – spermatozoon nucleus. Calibration bars: A, B and F = 10 μm; C = 2 μm; D and E = 5 μm.

ter of 5.37 ± 0.15 μm being moved to the central area (Fig. 4E) and, then, in the newly formed acinar lumen (Fig. 4F) with a continuous gradient of maturation from the periphery to the proximity of testicular pedicle. Germinative cell stages are synchronized within a single acinus and in a particular region of the testicular lobe. In fully matured acini, containing free spermatozoa, the accessory cells start to show apoptotic degenerative signs such as cytoplasm condensation, nuclear indentation/buds with chromatin condensation and peripheralization, pyknosis (condensed nuclei), karyorrhexis (fragmented nuclei separation) and apoptotic bodies (Fig. 4F). The acinus life ends with the appearance of a lumen in the epithelium of a developing collecting tubule and the release of spermatozoa into this lumen. Empty, collapsed acini are characterised by highly vacuolated accessory cells showing advanced apoptotic features.

The analysis of the ultrastructure of the testis of control animals allows the identification of the different developmental stages of spermatogenesis inside the acini, such as spermatogonia (Fig. 5A), spermatocyte I (Fig. 5B and C) characterised by the presence of

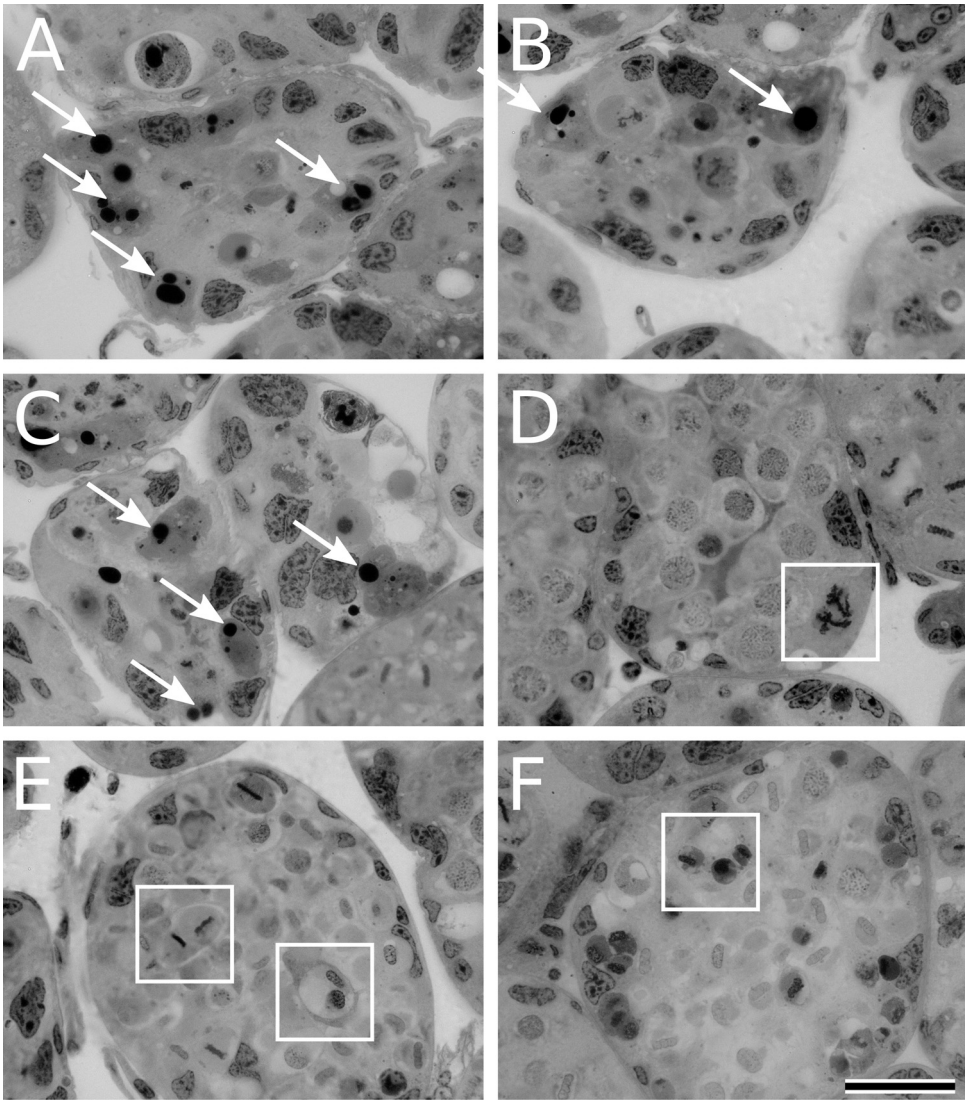

Fig. 6. Semi-thin section (1 μm – toluidine blue) of testicular acini of males of *Procambarus clarkii* irradiated with 20 Gy, 10 days after the treatment. Accessory cells with micronuclei (arrows) and cells with abnormal meiotic division with incomplete chromosomes segregation and germinative cells with condensed chromatin (white squares) are shown. Calibration bar = 20 μm.

well-developed smooth endoplasmic reticulum, *annulate lamellae* and clusters of free ribosomes (Fig. 5C), spermatocyte II during metaphase II (Fig. 5D), and developing spermatozoa (Fig. 5F). The apoptosis of accessory cells starts when almost mature spermatozoa are transported centripetally (Fig. 5F).

Testicular lobes of animals irradiated by 20 Gy, after 10 days, do not show the gradient of maturation – as control animals do – and present acini containing apoptotic cells showing degenerative features throughout all testicular area. The apoptotic accessory cells are not limited to fully matured acini but they are present at all acinar stages. They show up to 3 deeply stained fragmented nuclei due to karyorrhexis (Fig. 6A–C). In addition, there are: germinative cells with condensed chromatin (Fig. 6A and B), binucleated cells (Fig. 6E), cells showing anomalous meiotic division with incomplete chromosomes segregation (Fig. 6D–F). Moreover, germinative cell stages are not synchronized within a single acinus (Fig. 6F).

Acini of animals irradiated by 20 Gy, after 30 days, appear further deteriorated, thus making impossible to see the gradient of development stages of spermatogenesis. No cells with meiotic division are visible and a high number of deeply stained fragmented nuclei are present in almost all acini. Cell apoptosis regards all the lobes' surface and vacuolisation expands even further (Fig. 7A). Deterioration gradually increases in the sections of animals irradiated with 40 Gy (Fig. 7B) and 60 Gy, where the apoptosis/necrosis of germ and accessory cells appear widespread along all testicular lobes. In these sections, the different stages of spermatogenesis are not visible because acini only have cells with a high level of vacuolisation, condensed chromatin and areas with reassembling of membranes.

Ultrastructure of acini of irradiated animals after 10 days reveal in spermatogonia typical of early autophagic structures, *i.e.* cytoplasmic autophagic vacuoles and autophagosomes (Fig. 8A). Later spermatogenesis stages are characterised by cellular shrinkage and chromatin condensation and fragmentation (Fig. 8B and C). Spermatocytes exhibiting cellular swelling and karyolysis are also present (Fig. 8B), this degradative processes usually occur after cell death in necrosis. In spermatids a remarkable development of irregularly shaped membranes and solid electron dense – circular in cross section – material is observed (Fig. 8D). Cells with anomalous meiotic chromosome segregation are also common (Fig. 8B). Interestingly, no signs of degeneration are recorded in fully developed spermatozoa of irradiated crayfish both at light and electron microscopy levels and the ultrastructure reveals the decondensed

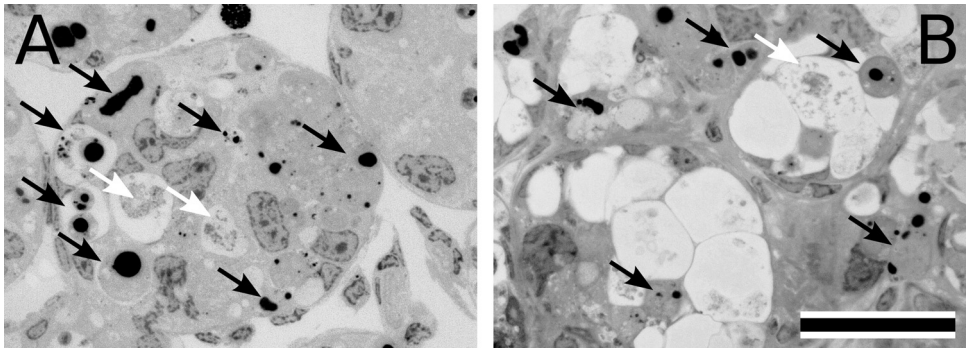

Fig. 7. Semi-thin section (1 μm – toluidine blue) of testicular acini of males of *Procambarus clarkii* irradiated with 20 Gy (A) and 40 Gy (B), 30 days after the treatment. Apoptotic accessory cells (black arrows) and germinative cells exhibiting cellular swelling (white arrows) are shown. No viable germinative cells are present. Calibration bar = 50 μm.

chromatin, typical of decapods, with nucleosomes of about 12 nm in diameter (Fig. 8E). The testicular tissues of irradiated animals after 30 days, at all doses, corroborate the LM observations, revealing an almost totally advanced stage of acini necrosis and no visible germinative viable cells; few viable accessory cells show deeply complex phagosomes containing onion-like bodies and membranaceous material derived from autophagosomes of apoptotic germinative cells (Fig. 8F). The degenerated acini present a highly convoluted

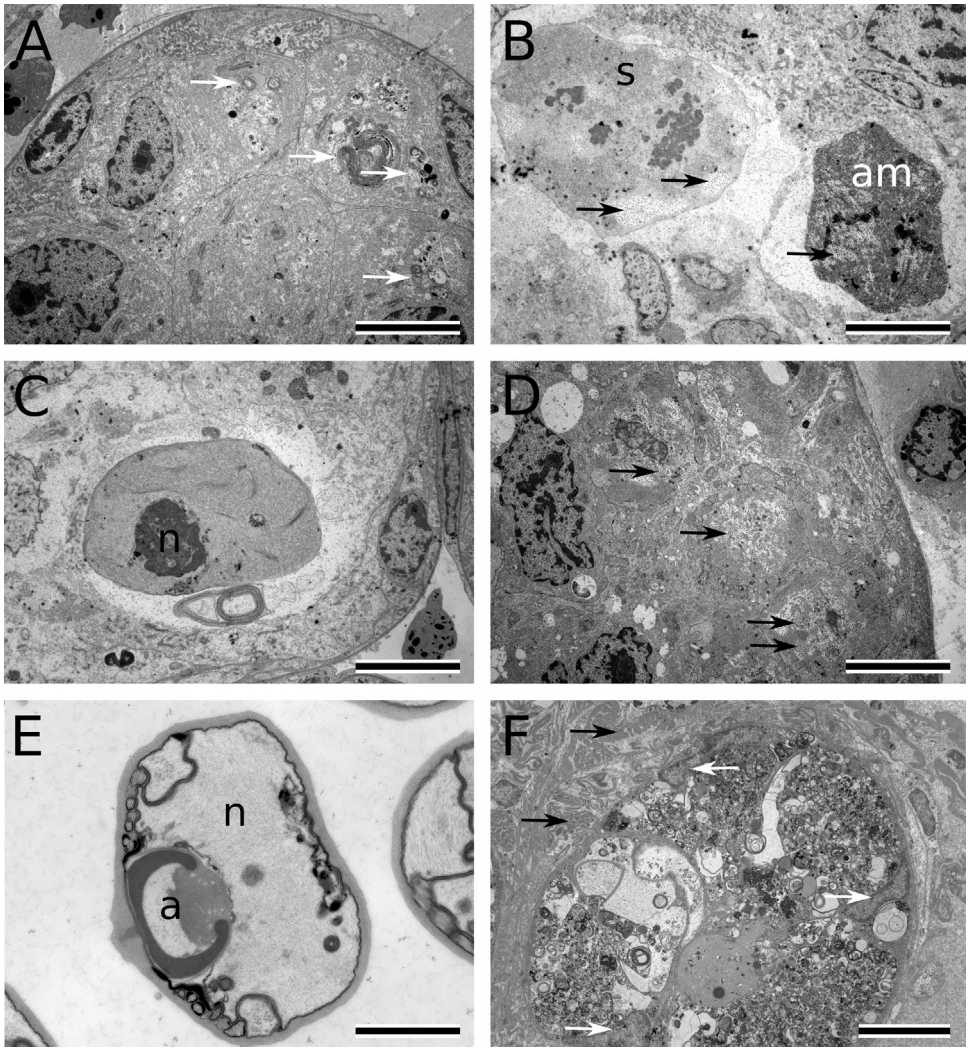

Fig. 8. Ultrastructure of testicular acini of males of *Procambarus clarkii* irradiated with 20 Gy, 10 days after the treatment (A–E) or with 40 Gy, 30 days after the treatment (F). ; A – spermatogonia presenting autophagic structures (arrows). B – spermatocytes exhibiting cellular swelling (s) or anomalous chromosomes segregations (am). Area rich in solid electron dense material circular in section are present (arrows). C – spermatocyte showing typical apoptotic features as cell shrinkage, pyknotic nucleus (n), dense cytoplasm with the integrity of the plasma membrane. D – spermatids with a remarkable development of irregularly shaped membranes (arrows). E – spermatozoon with decondensed chromatin in the nucleus. a – acrosome. F – an almost totally advanced stage of acinus necrosis with a highly convoluted basal lamina around and viable accessory cells containing complex phagosomes characterised by various concentric membranes. The nuclei of viable accessory cells are shown by arrows. fn – fragmented nuclei of apoptotic accessory cell, sn – spermatozoon nucleus. Calibration bars: A–D = 10 μm; E = 2 μm; F = 10 μm.

basal lamina around them, indicating the shrinkage of these functional units of the spermatogenesis as a result of cell death. Only few nuclei of viable supporting cells are present. The latters show a highly vacuolated cytoplasm with complex phagosomes containing apoptotic bodies and autophagosomes derived from germinative cells and from other dead accessory cells as a result of irradiations (Fig. 8F).

4. Discussion

Exposure to ionizing radiation is currently the preferred method to make insects sterile for integrated pest management (Bakri et al., 2005). The main parameter is the irradiating dose that must be tightly tailored to ensure a high degree of sterility and low damage to somatic cells in order not to affect the reproductive behaviour of animals and assuring that they can compete for mates with wild males. The dose needed for sterilization in arthropods varies from less than 5 Gy to more than 300 Gy (Bakri et al., 2005) but data currently are scant for crustaceans in the present literature. Untreated females of *P. japonicus* crossed with males treated with 10 or 20 Gy showed a significantly lower hatch rate (% of nauplii hatching from the eggs) than those crossed with control males (11.9% *vs* 40.7%: a reduction of 70.8%; Sellars and Preston, 2005). Interestingly the authors demonstrated that the position in the box in which the shrimp were irradiated is relevant to define the actual dose received by animals: for the proposed dose of 20 Gy the dose received by shrimps varies ±5.5 Gy (Sellars and Preston, 2005). In the present study, the testicular damage induced by 20, 40 and 60 Gy are presented. The gross morphology analysed by means of SEM clearly demonstrate that the overall acinar testicular structure is deeply damaged after 30 days of radiation. The measurements of the mean diameter in section of the acini further corroborate this evidence. Moreover, we clearly demonstrate an effect of time-dependent accumulation of damages: the acini after 10 days after the irradiation of 20 Gy are significantly smaller than the control ones whilst the acini after 30 days are significantly smaller than those after 10 days of radiation. The cytological analysis in control animals shows that the accessory cells maintain the functional integrity of the acinus by mediating phagocytosis: -1 of germ cells died *via* apoptosis caused by abnormal meiotic division and, later on, -2 of cytoplasm derived from developing spermatids during spermiogenesis. When the mature spermatozoa are transported into the central lumen of the acinus the accessory cells begin to show degenerative signs that end with their apoptosis in the emptied acini. In animals irradiated with 20 Gy, after 10 days, the DNA/chromosome damage induced by radiation result in anomalous mitotic and meiotic segregations followed by apoptosis of germinative cells that, in turn, induce phagocytosis in accessory cells by stimulating their scavenger role. Subsequently, the accessory cells undergo apoptosis induced by phagocytosis of the germ cells as they normally do in the mature acini. After 30 days of irradiation, at the 3 doses, no germinative cells are distinguishable and only degenerated acini with highly vacuolated accessory cells are present. Our data are in agreement with those present in literature for insects. In a review of the control of insects by induced sterilization Proverbs (1969) highlighted that “the various developmental stages of germ cells show differential susceptibility to killing and sterilization by radiation” and that “radiation is most injurious to the early stage of spermatogenesis”. In the dipteran *Cochliomya hominivorax*, the testis of flies emerged from pupae irradiated with the dose of 6200 roentgen (equivalent approximately to 54 Gy) of gamma rays showed after 3 weeks that all spermatogonia and many primary spermatocytes were killed but no morphological alterations were recorded in spermatids and in immature sperms that developed into normal appearing mature sperm (Riemann, 1967). The apparent lack of ultrastructural damages in spermatozoa is surprising because X-rays break the double helix and in mammalian cells a dose of 1 Gy results in 20–40 DNA double strand breaks (Ward, 1988). Moreover Wang et al. (2014) postulated that decondensed, open chromatin would make the DNA helix more vulnerable to ionizing radiation than condensed one. Actually recent findings confirmed that in decapods, from early spermatid to mature spermatozoon, contrary to spermiogenesis in most taxa, the developing nucleus undergoes chromatin decondensation (Kurtz et al., 2009). This characteristic is confirmed in *P. clarkii* in the present paper by spermatozoa ultrastructure analysis, where decondensed chromatin is evident in the nucleus (Fig. 8E). It seems reasonable that a high number of double strand breaks are present in the spermatozoa, that do not show signs of degeneration due to their nature of fully differentiated cell. The reduction of the reproductive success by (only) 43% of males, crossed with untreated females, treated with 20 Gy in a previous study on *P. clarkii* (Aquiloni et al., 2009) could be explained with the use of non-virgin females who had spermatophores with viable spermatozoa in the seminal receptacle, *annulus ventralis*.

5. Conclusions

Our findings confirm that all doses of X-radiation after 30 days damage the early stages of spermatogenesis in *P. clarkii* and prevent the regular development of germinative cells, thus proving to be an excellent technique to sterilize males of this species. In particular, we observe that different radiation doses have similar effects in damaging testes, although they determined a different reduction in the hatchlings: 50% fewer at 20 Gy, 57% fewer at 40 Gy, 67% fewer at 60 Gy (L. Aquiloni 2014, pers. comm.). Taking into account that the position in the box in which the crayfish are irradiated is relevant in the actual dose received (Sellars and Preston, 2005), we consider a radiation with 40 Gy the best solution as even animals in a peripheral position with respect to the emitted beam should receive a sufficient dose of X-rays, to reduce their fertility over the 50%. It represents a major compromise between the damage that higher doses of X-rays can cause to testes and crayfish vitality (L. Aquiloni 2014, pers. comm.). Accordingly, our study confirms the capacity of X-rays to sterilize the males of *P. clarkii* to be used in SMRT technique to fight, prevent and reduce the spread of this invasive crayfish species in integrated pest management. Further long term studies are needed to assess if the cytological damage in gonadal tissues could be repaired in the subsequent breeding season, or if they are permanent.

Acknowledgements

This study was financed by the RARITY Project (LIFE 10 NAT/IT/000239). The authors are indebted with Enrico A. Ferrero for his critical suggestions. We are extremely grateful to Massimo Zanetti, Giorgio Tonizzo and Sandro Zoccolan (Ente Tutela Pesca), Manuela Balzi e Paola Faraoni (University of Florence) for their precious support, to Claudio Gamboz (Centro di Microscopia Elettronica, Dipartimento di Scienze della Vita, University of Trieste) for TEM sample preparation and to James Morris for the linguistic support.

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