Contribution of light and electron microscopy in the identification of morphological alterations in large Japanese field mouse (*Apodemus speciosus*) testes exposed to low-dose-rate radiations

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SUMMARY

Ionizing radiation affects biological systems, resulting in an increased risk of cancer and mutagenesis. Male reproductive function is sensitive to ionizing radiation, with implications connected to infertility. Following the Nuclear Power Plants accident of Fukushima in 2011, there was great attention regarding exposure damage to low-dose-rate (LDR) radiation on the reproductive system. This preliminary study aimed to evaluate the role of light (LM) and transmission electron microscopies (TEM) to identify the potential effects of LDR radio-exposure on the morphology of large Japanese field mouse (*Apodemus speciosus*) testes living in the Fukushima Daiichi Nuclear Power Plant (FDNPP) ex-evacuation area. After collection samples were subjected to the standard preparative for LM and TEM. The testicular parenchyma was characterized by numerous seminiferous tubules, delimited by a thick and continuous basal lamina. Basally, the germinal epithelium presented round and pale spermatogonia, primary spermatocytes; while, more adluminally, round and elongated spermatids were at different phases of development. Pale and irregular Sertoli cells were interspersed among germ cells. Occasionally, cytoplasmatic holes interrupted the nuclear membrane integrity in spermatocytes and spermatids. Residual bodies were seen at the luminal surface. In conclusion, this study suggests that LM and TEM analysis are useful in evaluating potential morphological features in the male reproductive system after LDR exposure.

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Introduction

Exposure to long-term ionizing radiation impacts human and animal health. Ionizing radiation is known to induce genomic changes, injuries to the nucleotide bases, DNA and DNA-protein crosslinks, DNA single- and double-strand breaks (DSBs). These lead to mutagenesis, congenital malformation, and carcinogenesis (Koturbash *et al.,* 2006; Fukunaga *et al.,* 2017). Cytogenetic and genetic studies reported an increase of chromosomal aberration and aneuploidy after low chronic radiation exposure (Cardoso *et al.,* 2001; Kumar *et al.,* 2013). A growing risk of hematological malignancies was revealed on health workers occupationally exposed (Kumar *et al.,* 2013).

As in most multiple organ systems, the male reproductive system is sensitive to ionizing radiation (Otala *et al.,* 2004; Chute, 2012). The International Committee on the Radiological Protection (ICRP) report confirmed that radiation cause *azoospermia* and estimated the weighting factor of the gonads as 0.08 (ICRP, 2007). The germinative epithelium is composed of germ cells at different maturation stages, with different vulnerability to radiation-induced effects due to numerous factors, including the reproductive activity level (Liu *et al.,* 2006; Yamashiro *et al.,* 2013). Animal studies revealed that radiation exposure could damage the spermatogenesis by increasing reactive oxygen species (ROS) production, with DNA damage (Sabeti *et al.,* 2016; Fukunaga *et al.,* 2017), increased spermatogonial apoptosis (Hamer *et al.,* 2003) and stem cells reserve reduction (Grewing *et al.,* 2015). Available data on the effects of low doses of radiation exposure on testicular function results from current clinical practice, such as radiotherapy. In humans, treatment with 1 Gy radiation showed a relevant decline in spermatocytes' count. In rodents, irradiation with 2 or 4 Gy and 1 Gy caused a decrement in the spermatogonial number (Ahmad and Agarwal, 2017; De Felice *et al.,* 2019). Further data concerning the risk associated with exposure to low-chronic ionizing radiation come from epidemiological studies of environmental and occupational exposure. Evidence from exposed health workers showed alterations in sperm motility, abnormalities in sperm morphology, sperm DNA fragmentation, and hypermethylation (Kumar *et al.,* 2013; Zhou *et al.,* 2016).

The nuclear plant accidents of Chernobyl in 1986 and Fukushima in 2011 caused a dramatic dispersion of radioactive substances. The long-term effect of this exposure on health become a primary worldwide concern. Few studies have been carried out, to date, to investigate the effects of ionizing radiations on the ultrastructure of the male reproductive system. Available data reported sperm ultrastructural defects affecting male fertility, as head vacuoles, in Chernobyl subjects exposed to radiation (Fischbein *et al.,* 1997). Recently, increased cellularity, in terms of spermatogonia, spermatocytes, and spermatids number was reported in exposed raccoon testis (Komatsu *et al.,* 2020). This may be due to a slower spermatogenic regression in control raccoons compared to Fukushima raccoons, which delayed the reproductive season in control raccoons. Differently, wild mice and bull testis collected from the Fukushima area did not show morphological changes (Yamashiro *et al*., 2013; Okano *et al*., 2016), even if others reported increased spermatogenesis in mice (Takino *et al*., 2017). Since the use of electron microscopy is essential to characterize morphological changes in testis (Baccetti *et al.,* 2004; Moretti *et al.,* 2016; Leão *et al.,* 2020), we here report preliminary data to evaluate the contribution of LM and TEM in the characterization of possible LDR radiation effects on wild large Japanese field mice testis.

Materials and Methods

Animals and treatments

The study protocol followed laboratory animal care guidelines; all procedures were conducted according to the Ethics Committee for Care and Use of Laboratory Animals for Research of Niigata University, Japan. Large Japanese field mice were captured using traps in April 2017, in Namie Town, adjacent to the ex-evacuation zone of the FDNPP. Before sample collection, the captured mice were sacrificed by cervical dislocation and both testes excised and cut in half or quarter.

Light microscopy (LM) and transmission electron microscopy (TEM)

Testes fragments were immediately fixed in 2.5 % glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS for at least 48 h at 4°C before processing for LM and TEM as previously described (Palmerini *et al.,* 2017). Briefly, samples were rinsed with PBS, post-fixed with 1 % osmium tetroxide (Sigma-Aldrich) in PBS and rinsed again in PBS. They were then dehydrated in ascending ethanol grades (Carlo Erba Reagents, Milan, Italy, immersed in propylene oxide for solvent substitution, embedded in epoxy resin EMbed-812 (Electron Microscopy Sciences, 1560 Industry Road, Hatfield, PA, USA). Sectioning utilized a Reichert-Jung Ultracut E ultramicrotome. Semi-thin sections (1 mm thick) were stained with Toluidine Blue, analyzed using LM (Zeiss Axioskop), and photographed using a digital camera (Leica DFC230). Ultrathin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids, stained with saturated uranyl acetate and lead citrate (SIC, Rome, Italy), before being examined and photographed using Philips TE CM100 electron microscopes operating at 80 kV (Belli *et al.,* 2019; Taghizabet *et al.,* 2018).

Results

Morphology of large Japanese field mice testes was evaluated by toluidine blue staining of seminiferous tubules semithin sections using LM. Seminiferous tubules, delimited by a thick and continuous basal lamina, showed good overall preservation (Figure 1A). Type A spermatogonia were recognized by their intensely stained nucleus, and intermediate spermatogonia were identifiable by their heterochromatin. Type B spermatogonia were adherent to the basal lamina. Near to the type B spermatogonia, numerous spermatocytes at the preleptotene stage were visible. More luminally, numerous spermatocytes were at the pachytene stage together with round-to-elongated spermatids. In some elongated spermatids, nuclei showed intensely stained chromatin (Figure 1B).

Preliminary data from TEM evidenced that spermatogonia

showed a central nucleus with condensed patches of chromatin. Numerous electron-dense lipid droplets of different dimensions were visible in Sertoli cells. Adjacent to the basal membrane, roundish spermatogonia were found. Spheric spermatocytes composed the second line of spermatogenic cells. They had large nuclei with no prominent nucleoli and a rich mitochondria pool, appearing often aggregated and vacuolated. Round-to-elongated spermatids were found at different phases of acrosome biogenesis. It was fre-

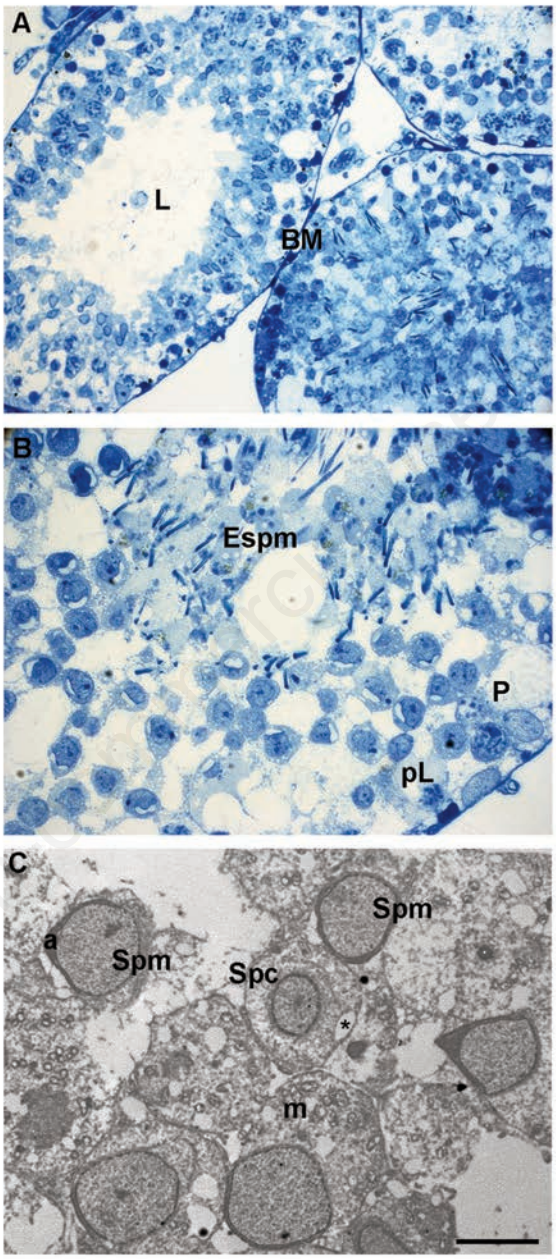

Figure 1. A) Representative image of a semithin section of mouse seminiferous tubule showing the germinative epithelium, delimited by the basement membrane (BM). Germ cells at different stages of maturation densely populate the lumen (L); LM, magnification: 40x. B) Higher magnification of a semithin section showing spermatocytes at preleptotene (pL) and pachytene (P) stage; elongated spermatids (Espm) are characterized by intensely stained nuclei; LM, magnification: 100x. C). Representative TEM micrograph of the adluminal compartment of a mouse spermatic tubule showing spherical spermatocytes (Spc) with evident nuclei, and numerous spermatids (Spm); vacuolated mitochondria (m) are visible in the cytoplasm of some round spermatids; a, a flattened acrosomal vesicle of a spermatocyte at the cap phase; the asterisk indicates an area of cytoplasmic detachment. TEM, bar: 5 µm.

quently possible to recognize the formation of the head cap-like structure at the cap phase or the elongation of the acrosome at the acrosome phase. Cytoplasmatic holes occasionally interrupted the nuclear membrane integrity in spermatocytes and spermatids from LDR exposed testis. Electron-negative vacuoles were found in the cytoplasm of spermatids and Sertoli cells. Numerous residual bodies and debris were present in the adluminal compartment (Figure 1C).

Discussion

This study aimed to assess TEM observations' potentiality on large Japanese field mice testes exposed to LDR radiations after the FDNPP accident. Clinical manifestation of ionizing radiation on living organisms depends on the size of the dose and type absorbed by different tissues (Mettler and Voelz, 2002). Effects on somatic and germ cells result in mutagenic effects leading to chromosomal aneuploidy, point mutations, and carcinogenesis (Little, 2000; Arruda-Neto *et al.,* 2009). Reproductive male system is hypersensitive to radiation exposure (Ahmad and Argawal, 2017; Fukunaga *et al.,* 2017). Indeed, evidence from accidental and clinical irradiation indicates that the testicular function can be impaired. Data from the current clinical practice in cancer patients showed that exposure to fractionated radiotherapy caused male gonadal toxicity, with spermatogenesis arrest and permanent azoospermia (Meistrich, 2013; De Felice *et al.,* 2019). After the nuclear accident in Chernobyl, 125 workers involved in clean-up operations showed relevant changes in sperm motility and a decrease in sperm count (Cheburakov and Cheburakova 1993). However, low-dose effects on male reproductive system remain unclear and controversies. Results from studies in endemic large Japanese field mice, raccoon and bull living in the FNDPP area observed an increasing number of spermatogenic cells, and no significant changes in sperm morphology and spermatogenesis in the testes (Komatsu *et al.,* 2020; Yamashiro *et al.,* 2013; Okano *et al.,* 2016; Takino *et al.,* 2017). Sperm ultrastructure and morphology is an important parameter, which can indicate the potential male fertility. To date, to our knowledge, there is only one study related to ultrastructure sperm characterization after the Chernobyl accident. The authors estimated significant malformations in spermatozoa heads of clean-up workers and controls. However, dose levels in the exposed subjects were not clearly indicated (Fischbein *et al.,* 1997).

In this preliminary study, no remarkable changes were identified by LM and TEM in mouse spermatogonia, spermatocytes, spermatids, and elongated spermatids. Occasionally, TEM observations showed cytoplasmic holes in germ cells, but further observations on a more representative number of samples will be performed; moreover, we cannot exclude artefacts connected to the sampling and preparative. In conclusion, LM and TEM are confirmed to be the gold-standard to identify morphological changes in cells or subcellular target organelles in large Japanese field mice testis exposed to ionizing radiation.

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