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Chapter 5

Cryopreservation of Cattle, Pig, Inobuta Sperm and Oocyte after the Fukushima Nuclear Plant Accident

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1. Introduction

After the Great East Japan Earthquake on 11 March 2011, the Fukushima Daiichi Nuclear Power Plant (FNPP) accident led to a discharge of a tremendous amount of radioactive substances [1, 2]. On 22 April 2011, the evacuation zone was set to a 20-km radius surrounding the FNPP, leaving approximately 3,400 cows, 31,500 pigs, and 630,000 chickens behind within the zone. On 12 May 2011, the Government of Japan ordered Fukushima prefectural government to euthanize unleashed livestock within the evacuation zone. Abandoned animals now have formed an invaluable model for studying the effects of chronic radionuclide intake. A comprehensive assessment of the effect of long-term exposure to internally deposited radionuclides on surviving domestic animals in the evacuation area is therefore urgently needed for the benefit of the livestock industry, as well as for human health. Radiobiological data from the FNPP accident could help to develop a set of internationally harmonized measures to protect domestic animals in the event of a future nuclear or radiological emergency.

Exposure to a large dose of ionizing radiation can cause irreparable damages to multiple organ systems, particularly those with highly proliferative cells, such as the skin, the hematopoietic and gastrointestinal system [3]. The testis and ovary are relatively radiosensitive organs [4], composed of a series of spermatogenic cells such as stem cells, spermatogonia, spermatids,

spermatocytes, sperm, and oogonium, primary oocyte, secondery oocyte and ovum, respectively. These different types of germ cells differ remarkably in their susceptibility to radiation-induced effects according to their level of reproductive activity [5]. The effect on reproductive organs and behaviour by chronic exposure to radionuclides is one of major concerns. Furthermore, radiation-induced genomic changes, occurring in germ cells may have hereditary effects, including carcinogenesis, congenital malformation and growth retardation in offsprings. A germ cell is the only cell that can produce next-generation. Therefore, greater use of cryopreservation of germ cells provide an essential resource to preserve their genetics and foetuses obtained by fertilization using those of the freezing sperm and oocytes for further studies on the effect of ionizing radiation on the next generations.

We have collected and cryopreserved the sperm and oocytes from three spices of domestic animals in the FNPP evacuation zone between 27 September 2011 and 31 March 2013. In this chapter, we introduce approaches to cryopreserve germ cells from the cattle, the pig, and the inobuta which is a mongrel of the wild boar and the pig for further research of radiobiology.

2. Reporting studies of Chernobyl for domestic animals and human

Data used for estimating the risk associated with exposure to ionizing radiation have been primarily obtained from epidemiological studies of survivors of the atomic bombing of Hiroshima and Nagasaki [6], the Chernobyl nuclear accident [7], and some complementary animal experiments [8–10]. However, reports of the effect of chronic low-dose radiation on livestock animals are limited.

Direct radiation injury to animals was reported only in local areas within the 30-km exclusion zone in Chernobyl nuclear power plant [11]. In some cases, chronic dose rates may have reduced the fertility of some animal species inside the zone. Recently, review of Russian language studies on radionuclide behavior in agricultural animals has been published [12, 13]. There are several important animal pathways for radionuclide transfer to the diet of humans. The important for many contamination scenarios for radiologically important radionuclides (^{90}Sr, ^{131}I and ^{137}Cs) is muscle (meat) consumption. The information presented this review has reported values are for Cs due to the Chernobyl accident in cattle, sheep, goat, rabbits, and chicken.

On the other hand, irradiation damages the ovaries and testes as direct effects of radiation, and indirectly affect through hormonal disruption. In human and wild animals, several studies of the functional changes in the reproductive tract have been made as a result of Chernobyl accident, abnormalities in spermatozoa and reproductive failures have been described [7]. Additionally, Weinberg et al. reported some genetic changes of unclear importance in offspring of Chernobyl accident liquidators [14, 15]. Although there are some claim that its changes is caused by the psychological factors (stressful conditions), it does not yet have enough information to explain all of the serious changes.

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3. Cryopreservation of cattle, pig, inobuta sperm

The Japanese government ordered Fukushima prefecture to euthanize cattle in the evacuation zone on 12 May 2011 to prevent radio-contaminated livestock products from entering the human food chain. We obtained testes and ovaries from the euthanized cattle, pigs and inobutas collected by the combined unit of veterinary doctors belonging to the Livestock Hygiene Service Center of Fukushima prefecture.

Almost bulls and boars were castrated. Therefore, we could only collect testes from 11 euthanized Japanese black beef bulls, 3 boars, and 1 male inobuta between 29 August 2011 and 28 February 2013 (Figure 1). Testes were collected in Kawauchi village located 15 km southwest of FNPP: the air dose rate was 0.5 μSv/h, Naraha town located 17 km south of FNPP: the air dose rate was 2 μSv/h, Tomioka town located 7 km south of FNPP: the air dose rate was 20 μSv/h.

Figure 1. Animals in the evacuation zone of the Fukushima Daiichi Nuclear Plant. A: Japanease black beef cattle B: Pig C: Inobuta

In bull, sperm from two caudae epididymides were collected. Immediately after collection, sperm were diluted with a Triladyl freezing extender containing egg yolk at natural temperature (Mini Tube, Germany). The tubes containing sperm were transferred to the Niigata University within 6-8 h after collection. Semen samples were cooled to 5-10°C during transferring. Aliquots of 0.5 ml of sperm suspension were individually placed in straws and ends were sealed. The straws were then placed in liquid nitrogen vapor for 10 min and then plunged directly into liquid nitrogen. In boars and male inobuta, freezing protocol was performed as described above. The semen extender, Modena extender containing egg yolk was used for freezing epididymal sperm. Total number of frozen sperm was 507 straws from bulls, 160 straws from boars and 83 straws from inobutas (Table 1).

Animal	Number of animals	Number of cryopreserved sperm
Bull	11	507
Boar	3	160
Inobuta	1	83

Table 1. Total number of cryopreserved straws contained sperm from bulls, boars, and male inobuta in the evacuation zone of the Fukushima Daiichi Nuclear Plant.

4. Cryopreservation of cattle, pig, inobuta oocytes

Oogenesis is associated closely with folliculogenesis in mammals. Oogenesis begins in the fetal ovary when the primordial germ cells arrive in the gonad of a genetic female and become oogonia. These cells proliferate via mitosis during fetal development. When proliferation ceases and the cells enter meiosis either before birth (human, cows, sow) or shortly thereafter (mice, rats, hamster) [16, 17], they are defined as primary oocytes arrested in the first meiotic prophase. Primordial follicles are formed in which the primary oocytes are surrounded by single layer of flattened granulose cells. Although the primordial follicles remain in this state of suspended animation for long time, oocytes and follicle resume the development near the time of ovulation. When follicles enter the development phase, they develop into primary and subsequent secondly follicles, along with proliferation of granulose cells and the oocytes growth. Primary and secondly follicle have cuboidal single layer and multiple layers of granulose cells, respectively. During the next phase, a fluid-filled cavity is formed adjacent to the oocyte in the follicle defined as antral follicle. Finally, one follicle growth rapidly and become the ovulatory follicle (maturation). In most mammals, the oocytes resume and complete the first meiotic division at ovulation.

Ionizing radiation may affect infertility or genetic disorders in subsequent generation induced by DNA damage in the germ cells and follicular cells. Many experiments have shown that radiosensitivity of follicle/oocyte varies widely according to the developmental stage of them and species [18]. In mice, the genetic sensitivity of oocyte in early stages of follicle development is a relatively high, and which decreases during the last week before ovulation. However, the sensitivity increases around the time of ovulation again. In contrast, oocytes in primordial follicle show a very low genetic sensitivity, and which increase with follicle development thereafter in guinea pigs. However, the knowledge in livestock is limited. In order to reveal the affect of exposure to low-dose of radiation on germ cells, it is necessary to study carefully at long term, including the influences on subsequent generations.

The aim of our studies is to examine of development of female germ cells in livestock within the evacuation zone and to preserve of female gametes for future studies. We collected ovaries from 36 cows, 12 sows, and 2 female inobutas. In cows, collected ovaries were washed and stored at 20°C in physiological saline containing antibiotics, and were transported to the laboratory within 7 h after the collection. Cattle cumulus-oocyte complexes (COCs) were

aspirated from small ovarian follicles and incubated in *in vitro* maturation (IVM) medium for 22 h. After maturation, the COCs were fertilized by co-incubation with thawed sperm. Then presumptive zygotes were cultured for *in vitro* development to the blastocyst stage. As a result, total 493 of morphologically normal COCs were recovered, and 40 blastocysts were yielded from 9 donors following *in vitro* embryo production. The bovine blastocysts produced were cryopreserved by vitrification using a nylon mesh method described in the previous report [19] (Table 2). On the other hand, pig and inobuta ovaries were transported to the laboratory at 37°C. Pig and inobuta COCs aspirated were carried out IVM for 44 h, and the presumptive matured oocytes were vitrified by similar method above. Total number of vitrified oocytes was 371 from sows and 64 from inobutas.

Animal	Number of animals	Developmental stage for cryopreservation	Number of cryopreserved egg
Caw	9	Blastocyst	40
Sow	12	Metaphase II	371
Inobuta	2	Metaphase II	64

Table 2. Total number of cryopresearved oocytes and embryos from caws, sows, and female inobutas in the evacuation zone of the Fukushima Daiichi Nuclear Plant.

5. Concluding remarks

Germ cells in the testis show one of the highest mitotic activities of any tissue in the body, so that in the human adult about 100 million new cells are produced each day [20]. Spermatogenesis is highly regulated, starting with spermatogonial stem cells and ending with differentiated, motile spermatozoa. The testis is one of the most radiosensitive tissues, with very low doses of radiation causing significant impairment of function. It is well known that immature cells are more radiosensitive to doses as low as 0.1 Gy, causing morphological and quantitative changes to the spermatogonia in human testis. Doses of 2–3 Gy result in overt damage to spermatocytes, leading to a reduction in the spermatid numbers. At doses of 4–6 Gy, the numbers of spermatozoa significantly decrease, implying damage to the spermatids. A recent study in mice showed that low-dose-rate radiation exposure (3.49 mGy/h) did not cause adverse effects at dose levels of ≤2 Gy, but the testis weight, sperm count and motility decreased at a dose of 2 Gy [21].

We needed to overcome a number of obstacles while working in the evacuation area, that is, dissections under the sun in the summer and snow in the winter, gathering the dissectors, as well as drive 400 miles a day. We also had restrictions on the time that we were allowed to stay in the area and the radiocontaminated materials to bring out of the area. We recently reported radionuclide deposition in organs of abandoned cattle following the FNPP accident. The deposition occurred in an individual radionuclide and in an organ-specific manner, and

radioactive Cs was detected in all the organs examined [22]. Discharge of ^{134}Cs and ^{137}Cs that emit γ-and β-rays is of primary concern, because they were released in a large amount and have a long half-lives [23]. Furthermore, we have investigated the effect of chronic radiation exposure on bull testes to ^{134}Cs and ^{137}Cs associated with the FNPP accident. Adverse radiation-induced effects, so far, have not been observed in bull testes following chronic exposure to the above levels of radiation for up to 10 months [24].

The paternal and maternal genomes are not equivalent and both are required for mammalian development. The difference between the parental genomes is believed to be due to gamete-specific differential modification, a process known as genomic imprinting, suggesting that DNA methylation may play a role in genomic imprinting. Lie et al, have examined the expression of these three imprinted genes in mutant mice that are deficient in DNA methyl-transferase activity [25]. Results demonstrate that a normal level of DNA methylation is required for controlling differential expression of the paternal and maternal alleles of imprint-ed genes. Few animal studies have investigated the possible link between paternal exposures and effects on genomic imprinting. Chronic treatment of male rats with 5-azacytidine, a drug that alters DNA methylation resulted in abnormalities in male germ cells and early embryo development but no increase in the incidence of congenital malformations [26]. This is an important area with potential consequences for the offspring of exposed males, and warrants further study [27].

In addition, questions regarding the effect of log-term exposure to radiation on the genetic damage to next generation are now being raised, but no clear evidence for this has been reported, except laboratory animals. Genetic analysis of foetuses obtained by fertilization using cryopreserved sperm and oocytes from cattle in the evacuation zone, is underway in our laboratory.

In conclusion, cryopreservation of germ cells has potential applications not only for production of next generations of animals but also for general reproductive biology including the field of radiation biology.

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