

## Chapter 10

# Transgenerational Effects on Calf Spermatogenesis and Metabolome Associated with Paternal Exposure to the Fukushima Nuclear Power Plant Accident



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**Abstract** The Fukushima nuclear power plant (FNPP) accident raised worldwide attention to the health risk of radiation exposure and to its potential transgenerational effects. Here, we analysed transgenerational effects on calf spermatogenesis and on blood plasma metabolome in order to detect alterations associated with paternal exposure to low-dose-rate (LDR) radiation. Sperm was collected from a bull exposed to radiation for 2 years abandoned in the ex-evacuation zone of the FNPP accident (the abandoned bull) and was used for artificial insemination (AI) into a non-radiocontaminated cow. Haematoxylin and eosin stained sections of the testis of a 13-month-old calf revealed spermatogonia, spermatocytes, spermatids, and sperm in normal morphology. Nuclear and acrosomal morphology of sperm

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was generally normal. Metabolomic profiling of plasma using capillary electrophoresis–mass spectrometry resulted in 104 peaks of candidate compounds suggestive of paternal exposure. A calf was delivered by AI using sperm from the abandoned bull. Regarding glycolysis, the contents of nucleotide sugars tended to be lower in the delivered calf than in the control calf. Among energy carries, AMP and ATP showed different tendency between non-radiocontaminated and delivered calf. In conclusion, there were no apparent transgenerational effects on both spermatogenesis and blood plasma metabolome in a calf obtained by AI using sperm from the abandoned bull exposed to LDR in the ex-evacuation zone of the FNPP accident for about 2 years.

**Keywords** Calf · Fukushima Daiichi Nuclear Power Plant accident · Low-dose-rate · Metabolome · Spermatogenesis

## 10.1 Introduction

Following the Fukushima Daiichi Nuclear Power Plant (FNPP) accident, large amounts of radioactive substances, particularly volatile elements such as radioactive iodine ( $^{131}\text{I}$ ,  $^{132}\text{I}$  and  $^{133}\text{I}$ ), cesium ( $^{134}\text{Cs}$ ,  $^{136}\text{Cs}$  and  $^{137}\text{Cs}$ ), tellurium ( $^{132}\text{Te}$ ), and inert gases (e.g.,  $^{133}\text{Xe}$ ), were released into the environment [1]. We have established an archive system composed of livestock and wild animals in a 20-km radius around FNPP, that is, the ex-evacuation zone of the FNPP accident [2–5]. This system provides critical information for the understanding of radioactive contamination, environmental pollution, biodistribution and metabolism, and the biological effects of internal and external exposure in association with dose evaluation. Recently, we

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reported organ-specific deposition of the individual radionuclide in abandoned cattle following the FNPP accident. Radioactive caesium ( $^{134}\text{Cs}$  and  $^{137}\text{Cs}$ ) is notably detected in all organs examined [6–8]. Further, we reported that spermatogenesis occurred normally in the reproductive organs of bulls and boars that were abandoned in the ex-evacuation zone, following chronic low-dose-rate (LDR) radiation [9, 10]. However, to date there is no clear evidence whether transgenerational effects of radiation exposure in livestock animals associated with the FNPP accident exist or not.

Here, we analysed transgenerational effects on the calf spermatogenesis, and blood plasma metabolome using capillary electrophoresis–mass spectrometry (CE-TOFMS) with paternal exposure to chronic LDR radiation by staying for 2 years in the ex-evacuation zone.

## 10.2 Materials and Methods

### 10.2.1 Ethics

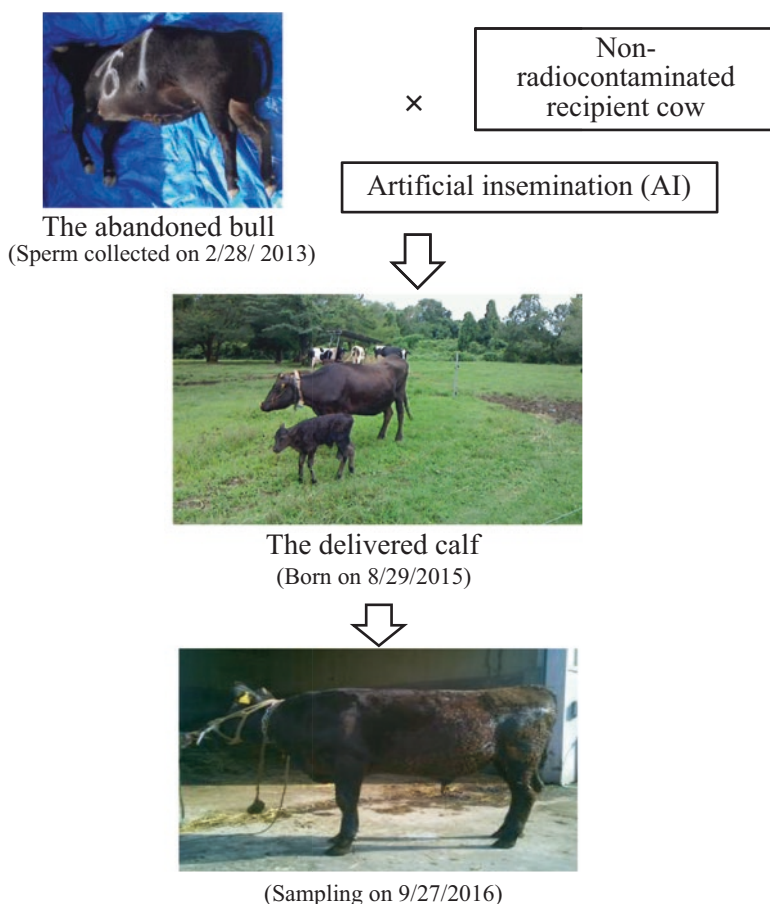
The Japanese government ordered Fukushima Prefecture to euthanize cattle in the ex-evacuation zone on May 12, 2011 to prevent radio-contaminated beef products from entering the human food chain. We collected organs and tissues from the euthanized cattle by the combined unit of veterinary doctors belonging to the Livestock Hygiene Service Center of Fukushima Prefecture and those belonging to the Ministry of Agriculture, Forestry and Fisheries, Japan. This study was approved by the Ethics Committee of Animal Experiments, Niigata University, Japan (Regulation No. 27-83-3).

### 10.2.2 Animals

We collected sperm from the testis of an euthanized Japanese black bull (the abandoned bull) at Tomioka Town, located 7 km south of FNPP, on February 28, 2013, as described previously [10]. Immediately after collection, the sperm was diluted with a Triladyl freezing extender containing egg yolk (Mini Tube, Germany); freezing protocol was performed as described previously [11]. On sampling date, ambient dose equivalent rate was  $1.7 \mu\text{Sv/h}$  (using a NaI (Tl) Scintillation Survey Meter), and the time elapsed since the FNPP accident (March 11, 2011) was almost 2 years. Dose rate of both internal and external exposure to  $^{134}\text{Cs}$  and  $^{137}\text{Cs}$  was estimated according to a modified method as previously described [3].

### 10.2.3 Artificial Insemination (AI)

Using the sperm of the abandoned bull, a non-radiocontaminated recipient black cow underwent AI at the experimental farm of Niigata University. A male Japanese black calf (the delivered calf) was born on August 29, 2015 (approximately 10-month pregnancy period) from which the testis, caudae epididymides, sperm, and blood plasma were sampled on September 27, 2016 (at approximately 13 months old) after euthanasia. As non-radiocontamination control, blood plasma was also collected from a non-castrated male Japanese black calf at almost the same age as the delivered calf from Niigata Prefecture, at the same latitude as Fukushima but not affected by the FNPP accident. The relationship between each cattle and artificial insemination is shown in Fig. 10.1.



**Fig. 10.1** The delivered calf obtained by artificial insemination using frozen sperm from an abandoned bull in the ex-evacuation zone of the FNPP accident

### **10.2.4 Histological Analysis**

The delivered calf testis was fixed in Bouin's solution. Sections were stained with haematoxylin and eosin (HE) and analysed microscopically as previously described [12].

### **10.2.5 Evaluation of Sperm Acrosomal Integrity**

The acrosomal integrity of the delivered calf sperm was assessed by staining with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Wako, Japan) according to the procedure described previously [13].

### **10.2.6 Measurement of Metabolites**

Metabolome analysis of blood plasma was carried out by a facility service, Human Metabolome Technologies Inc. (Yamagata, Japan), according to the conditions described elsewhere [14–16]. A capillary electrophoresis system, Agilent 6210 Time-of-Flight LC/MS (Agilent Technologies, Waldbronn, Germany), was used for capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS). The spectrometer scanned from  $m/z$  50 to 1,000. Peak information included  $m/z$ , migration time (MT) and peak area. Peaks were obtained using an automatic integration software MasterHands (Keio University, Tsuruoka, Japan) [17]. Signal peaks corresponding to isotopomers, adduct ions and other product ions of known metabolites were excluded, and the remaining peaks were annotated with putative metabolites from the HMT metabolite database based on their  $m/z$  and MTs values determined by TOF-MS. The tolerance range for peak annotation was configured at  $\pm 0.5$  min for MT and  $\pm 10$  ppm for  $m/z$ . In addition, peak areas were normalised against those of the internal standards, and then the resultant relative area values were further normalised by the sample amount.

Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed by our proprietary software, PeakStat and SampleStat, respectively. Detected metabolites were plotted on metabolic pathway maps using VANTED (Visualization and Analysis of Networks containing Experimental Data) software [18].

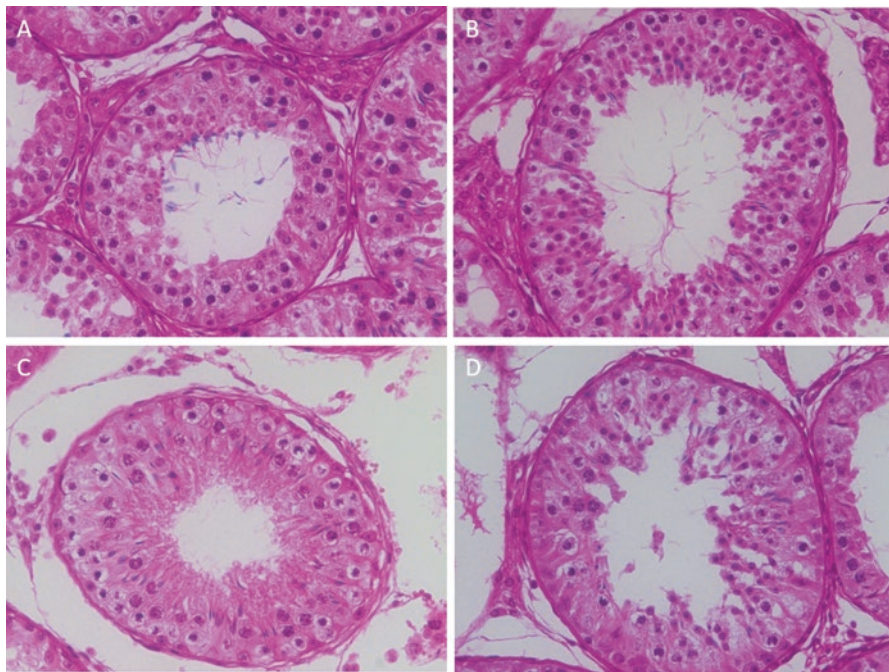
Compounds with a relative area ratio smaller than 0.66 or bigger than 1.5 were defined as having different levels between the delivered calf and the non-radiocontaminated control.

### 10.3 Results and Discussion

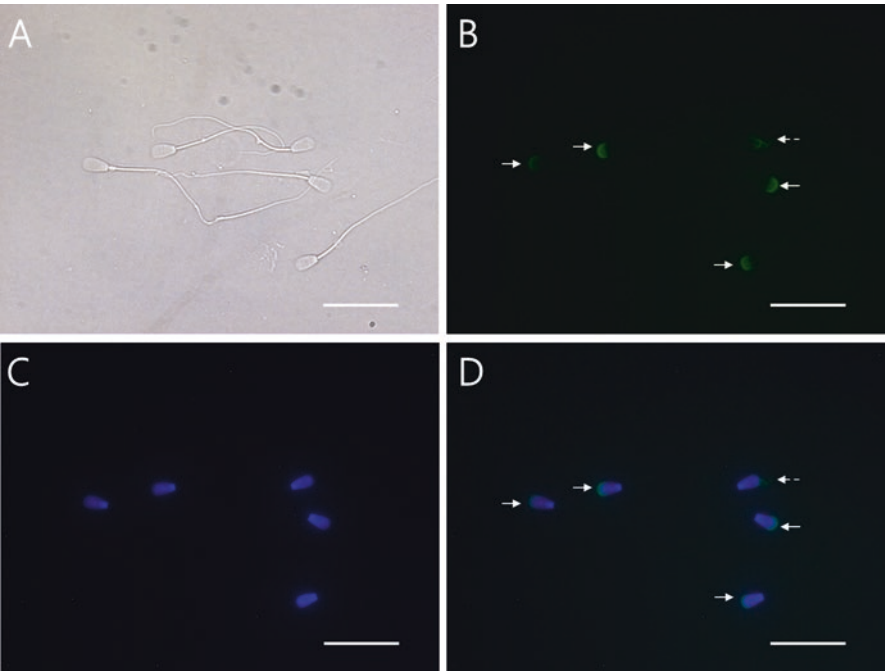
Total dose-rate in the testis of the abandoned bull attributed to radioactive Cs was 33.9  $\mu\text{Gy/day}$  (2.7  $\mu\text{Gy/day}$  for internal exposure and 31.2  $\mu\text{Gy/day}$  for external exposure).

Histology of the testis from the delivered calf at 13 months old revealed no remarkable changes in spermatogonia, spermatocytes, spermatids and sperm (Fig. 10.2). Sperm nuclear and acrosomal morphology was generally normal (Fig. 10.3). Abnormal sperm morphology, featuring partly absent acrosome, was observed at a rate of 7.0% (Table 10.1). These results show that, in the present study, paternal exposure to LDR radiation persistently did not affect the spermatogenesis process for calf.

CE–TOF–MS-mediated metabolic profiling of plasma resulted in 104 peaks of candidate compounds associated with exposure to chronic LDR radiation (hereafter “detected candidate” compounds). Among energy carries, AMP (ratio = 0.5) and ATP (ratio = 1.6) showed different levels between the delivered calf and the non-radiocontaminated control, whereas ADP did not (Table 10.2; Fig. 10.4). Detected candidate compounds associated with the energy supply system are shown in Table 10.3 and Fig. 10.3. Regarding glycolysis, the present study showed that contents of nucleotide sugars such as G1P, G6P, DHAP and 3-PG were lower in the



**Fig. 10.2** Histological sections of the seminiferous tubules of the delivered calf testis. Scale bar, 100  $\mu\text{m}$  in **a** and **d**; magnification, 200 $\times$



**Fig. 10.3** Epididymal sperm nuclei and acrosomes of the delivered calf stained with 4',6'-diamino-2'-phenylindole (DAPI) and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). (a) Phase contrast, (b) DAPI-stained, (c) FITC-PNA-stained and (d) DAPI+FITC-PNA-stained images. Scale bar, 50 μm; magnification, 400×

**Table 10.1** Rates of sperm acrosome normal or abnormal morphology

	Sperm acrosome morphology		Total
	Normal (%)	Abnormal <sup>a</sup> (%)	
Number of sperm	186 (93.0)	14 (7.0)	200

<sup>a</sup>Abnormal sperm morphology, featuring partly absent acrosome

**Table 10.2** Detected candidate energy-carrier compounds

Energy carrier	Relative area		Comparative analysis <sup>a</sup>
	Control calf	Delivered calf <sup>b</sup>	Ratio
ADP	7.0E-04	7.5E-04	1.1
AMP	4.5E-04	2.1E-04	0.5
ATP	3.4E-04	5.5E-04	1.6

<sup>a</sup>Control relative area served as denominator

<sup>b</sup>The delivered calf with paternal exposure to radiation (*n* = 1)



**Table 10.3** Detected candidate compounds involved in carbohydrate metabolism

Carbohydrate metabolite	Relative area		Comparative analysis <sup>a</sup>
	Control calf	Delivered calf <sup>b</sup>	Ratio
3-Hydroxybutyric acid	2.6E-02	3.8E-02	1.4
Citric acid	2.9E-02	2.5E-02	0.8
Lactic acid	1.1E-01	5.0E-02	0.5

<sup>a</sup>Control relative area served as denominator<sup>b</sup>The delivered calf with paternal exposure to radiation ( $n = 1$ )**Table 10.4** Detected candidate amino acids

Amino acid	Relative area		Comparative analysis <sup>a</sup>
	Control calf	Delivered calf <sup>b</sup>	Ratio
Ala	5.9E-02	4.7E-02	0.8
Arg	1.9E-02	2.7E-02	1.4
Asn	5.2E-03	6.2E-03	1.2
Asp	3.2E-03	1.3E-03	0.4
Cys	N. D.		
Gln	4.8E-02	7.1E-02	1.5
Glu	2.3E-02	1.1E-02	0.5
Gly	5.0E-02	4.9E-02	1.0
His	1.6E-02	1.7E-02	1.1
Ile	5.4E-02	6.3E-02	1.2
Leu	N. D.		
Lys	1.7E-02	2.4E-02	1.4
Met	7.8E-03	6.7E-03	0.9
Phe	2.4E-02	2.3E-02	1.0
Pro	2.9E-02	2.5E-02	0.9
Ser	1.1E-02	1.4E-02	1.3
Thr	2.0E-02	1.7E-02	0.8
Trp	1.1E-02	1.3E-02	1.1
Tyr	1.5E-02	1.5E-02	1.0
Val	1.0E-01	1.1E-01	1.1

<sup>a</sup>Control relative area served as denominator<sup>b</sup>Delivered calf associated with paternal exposure to radiation ( $n = 1$ )

delivered calf than in the control calf. Lactic acid, a final product of glycolysis under an aerobic conditions, featured lower-level tendency in the delivered calf than in the control calf (ratio = 0.5). 3-Hydroxybutyrate (3-HBA) is a ketone body used as an energy source, whereby it is converted to acetyl CoA and oxidised in the TCA cycle. 3-HBA showed also no difference (ratio = 1.4). Citric acid is a metabolite of the TCA cycle that showed no difference between the delivered calf and the control (ratio = 0.8). This indicates that, under anaerobic conditions, the energy use of the delivered calf has lower tendency than that of the non-radiocontaminated calf.

Amino acids catabolized to simple intermediate and oxidised in the TCA cycle showed almost no difference between the delivered calf and the control (Table 10.4),

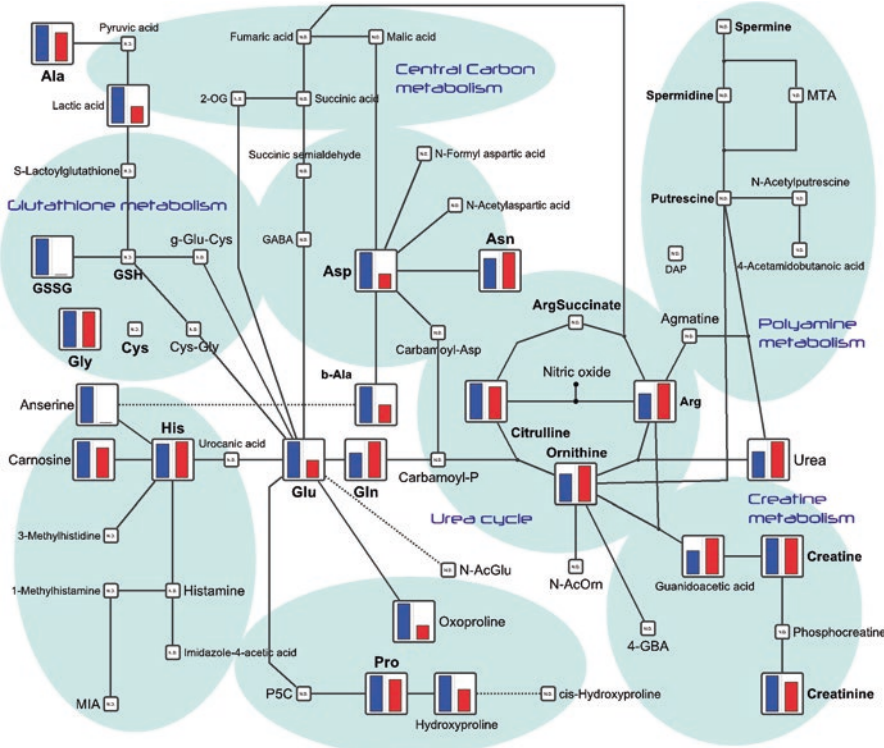
with the exception of aspartic acid (Asp; ratio = 0.4), glutamine (Gln; ratio = 1.5), and glutamine acid (Glu; ratio = 0.5). Glu collects amino groups from most amino acids, including Asp, followed by conversion to  $\alpha$ -ketoglutarate. Since Gln is converted to Glu by glutaminase, it is possible that higher levels of Gln in the delivered calf, as compared with the control calf, serve to produce Glu, which in turn activates amino acid catabolism. These may also explain the higher ATP levels detected in the delivered calf.

Arg, Asp, citrulline, and ornithine participate in the urea cycle (Table 10.5 and Fig. 10.5), a pathway that eliminates ammonia outside the body by converting it to

**Table 10.5** Detected candidate compounds involved in urea metabolism

Urea metabolite	Relative area		Comparative analysis <sup>a</sup>
	Control calf	Delivered calf <sup>b</sup>	Ratio
Citrulline	1.6E-02	1.4E-02	0.9
Ornithine	1.2E-02	1.5E-02	1.3
Urea	3.8E-01	5.2E-01	1.4

<sup>a</sup>Control relative area served as denominator  
<sup>b</sup>Delivered calf associated with paternal exposure to radiation ( $n = 1$ )



**Fig. 10.5** Diagram of urea cycle of the delivered calf (red) and the non-radiocontaminated control (blue). The control relative area was served as the denominator

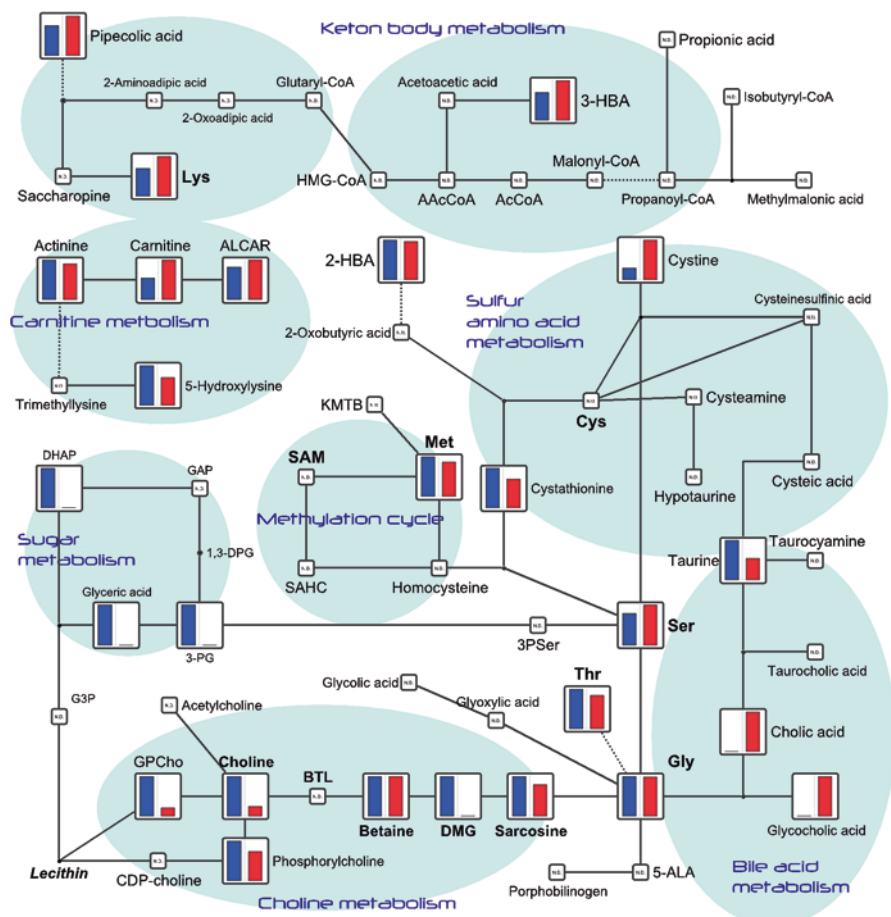
**Table 10.6** Detected candidate compounds involved in lipid metabolism

Lipid metabolite	Relative area		Comparative analysis <sup>a</sup>
	Control calf	Delivered calf <sup>b</sup>	Ratio
Betaine	4.0E-02	4.0E-02	1.0
Carnitine	3.0E-03	5.5E-03	1.8
Choline	2.9E-02	6.9E-03	0.2
Glycerophosphocholine	1.7E-03	3.5E-04	0.2
Acetylcarnitine (ALCAR)	1.3E-03	1.5E-03	1.2
Phosphorylcholine	1.2E-03	9.1E-04	0.7
Sarcosine	9.6E-04	7.6E-04	0.8
Taurine	3.3E-03	1.8E-03	0.5

<sup>a</sup>Control relative area served as denominator<sup>b</sup>Delivered calf associated with paternal exposure to radiation ( $n = 1$ )

urea. Urea levels were not different between the delivered calf and the control (ratio = 1.4), as was observed in Arg (ratio = 1.4), citrulline (ratio = 0.9) and ornithine (ratio = 1.3). Conversely, Asp showed lower levels in the delivered calf than in the control calf (ratio = 0.4). Although Asp gives a nitrogen atom to urea in the urea cycle, we assume that the difference in Asp levels had no influence on urea production in the delivered calf, since urea levels were not different between them.

Detected candidate compounds associated with lipid metabolism are shown in Table 10.6 and Fig.10.6. Carnitine is a carrier in the mitochondrial membrane responsible for transport of long-chain fatty acids into the mitochondrial matrix, where they are  $\beta$ -oxidised and used as the energy source in the TCA cycle. Carnitine levels were considerably higher in the delivered calf than in the control (ratio = 1.8). 5-Hydroxylysine, acetylcarnitine (ALCAR) and actinin, all associated with carnitine metabolism, were not different between them. Choline is a constituent of phosphatidylcholine and acetylcholine. It is converted to glycerophosphocholine, which is in turn converted to phosphatidylcholine. Glycerophosphocholine serves as a choline source. Choline and glycerophosphocholine were less abundant in the delivered calf than in control calf (ratio = 0.2), suggesting potential shortage in choline supply in the delivered calf as compared with the control calf. Choline is metabolised to betaine, then to sarcosine, and finally to glycine (Gly), but no difference in the levels of those metabolites, as well as that of phosphorylcholine, was observed between them. Taurine plays a role in conjugating bile acids, which are synthesised from cholesterol to form bile salts. Bile salts and acids are discharged from the body in the faeces. Taurine was less abundant in the delivered calf than in the control calf (ratio = 0.5), suggesting that bile-salt production tends to be delayed in the delivered calf.



**Fig. 10.6** Diagram of lipid metabolism of the delivered calf (red) and non-radiocontaminated control (blue). The control relative area was served as the denominator

## 10.4 Conclusions

In conclusion, there were no transgenerational effects on both of the spermatogenesis and on the blood plasma metabolome in calf obtained by AI using sperm from a LDR-radiated bull in the FNPP ex-evacuation zone. The area of the evacuation zone has been drastically reduced, and no more cattle have been euthanized since February 2014. It is therefore, difficult to investigate more animals with different annual doses. Here, we showed results of only one 13-month-old calf obtained by AI using sperm from a bull exposed to chronic LDR radiation for 2 years in the ex-evacuation zone of the FNPP accident (Fig. 10.1). This study is not a controlled experiment; however, we believe that our results give a framework not only for

estimating general effects of radiation in cattle but also contributing to the reconstruction of the Fukushima livestock industry and facilitating the improvement of food safety measures.

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**Conflict of Interest Statement** The authors declare no competing financial interests.

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