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Chromosomal unbalancements in sperm and oocytes of two Italian cattle breeds as determined by dual color fluorescent *in situ* hybridization (FISH)

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ABSTRACT - Aneuploidy is one of the most important causes of embryonic and foetal mortality in mammals. In order to assess the possible risk of chromosomal abnormalities in germ cells of domestic animals we investigated the aneuploidy rates on partially decondensed sperm and *in vitro* matured oocytes in two cattle breeds, Italian Friesian (I.F.) and Italian Brown (I.B.), by using FISH with chromosome-specific painting probes (chromosomes X-Y for sperm and chromosomes X-5 for oocytes). For each bull, more than 5,000 sperm were analyzed, for a total of 52,586 and 51,342 sperm cells for the two breeds, respectively. Aneuploid and diploid sperm had, respectively, a frequency of 0.110% and 0.050% in the I.F. and 0.078% and 0.062% in the I.B. breeds. Out of 100 *in vitro* matured oocytes for each breed, on the average, diploidy affected 11.2% and 18.4% in the I.F. and I.B., respectively, whereas disomy for chromosome X-5 had a frequency of 2% in the I.F. and 2.5% in the I.B. breeds. Further studies are needed to expand our knowledge on frequency of aneuploidy in sperm and oocytes of domestic animals, in order to assess their impact on productive and reproductive efficiency, also in relation to climatic changes and environmental hazards.

Key words: Chromosome aneuploidy, Sperm, Oocyte, FISH.

Introduction – Germ cell aneuploidy is one of the most important causes of embryonic and foetal mortality in mammals. Despite its great impact on fertility, limited information is available so far on the frequency of aneuploidy in sperm and oocytes of domestic animal species, and even less information is available on breeds or genetic types. Sperm chromosome studies in domestic animals have been mainly focused for detecting the efficiency of the various X-Y sperm separation procedures for embryo sexing predetermination. For this purpose, male specific DNA probes were first used in pig (Kawarasaki *et al.*, 1995) and in cattle (Hassanane *et al.*, 1999), soon followed by chromosome specific painting probes which have been produced by using chromosome micro-dissection followed by Degenerate-Oligonucleotide-Primed-PCR (DOP-PCR) in pig (Rubes *et al.*, 1999) as well as in cattle (Di Berardino *et al.*, 2004). Even though these methods have proven to be reliable for analyzing the aneuploidy frequency within the sperm population of individual sperm donors, specific information concerning the variability of the aneuploidy frequency 'among individuals' and, possibly, 'among breeds' or 'genetic types' is still lacking. The present study intends to fulfil, at least partially, this lack of knowledge by providing specific data on this topic. Thus, we decided to study X-Y and X-5 chromosome segregation in the sperm and oocytes of the Italian Friesian (I.F.) and Italian Brown (I.B.) breeds respectively, and to developed a dual color FISH method for evaluating the baseline level of aneuploidy for these two breeds, that could be used as reference for further investigations, also in relation to the genotoxicity of environmental mutagens and bio-hazards.

Material and methods – *Semen samples* - Cryopreserved semen samples belonging to I.F. and I.B. cattle breeds were used (10 animals for each breed). Decondensation of sperm nuclei was performed according to Han *et al.* (1992). *In vitro oocyte culture* - Cumulus-Oocyte-Complexes (COCs) were collected through aspiration with needles from ovaries of slaughtered cows. They were washed in TCM-199 and matured in the same medium, supplemented with 10% FBS, 10 mg/ml FSH, 10 mg/ml LH and 1 mg/ml β-estradiol, then incubated at 38.5°C for 24 h in a 5% CO_2 wet atmosphere. After maturation, COCs were exposed to a hypotonic Na-citrate solution (0.8% w/v) for 3 min, followed by 75 mM KCl treatment for 3 min. Fixation was carried out using cold methanol/glacial acetic acid (3:1). *Preparation of DNA probes and labelling* - Chromosome micro-dissection, PCR amplification, labelling of probes and FISH were performed as described by Di Berardino *et al.,* 2004. 'Xcen' and 'Y' probes were hybridized simultaneously on decondensed sperm, as well as 'Xcen' and '5' on individual oocytes. *Fluorescence analysis and scoring* - Probe signals were visualized with a LEICA fluorescence microscope and images were captured by LEICA Q4000 software. Scoring was carried out using strict scoring criteria (Robbins *et al.*, 1995). Relative proportions of X and Y bearing spermatozoa were determined in each sample, with aneuploid and diploid configurations. The χ^2 test was used for analyzing interspecific differences.

Results and conclusions - Preliminary results of I.F. and I.B. cattle aneuploidy scoring are summarized in Table 1. Regarding the analysis of the sperm aneuploidy, more than 100,000 cells were scored (range 4,794-6,002 per bull). The total frequency of aneuploidy was 0.160% in I.F. and 0.140% in I.B.; such difference was not statistically significant (Mann-Whitney test, U=63; α=0.05; p=0.35). The total frequency of disomic sperm was 0.110% in I.F. *vs.* 0.078% in I.B.; no significant differences were found (Mann-Whitney test, U=69.5; α=0.05; p=0.15); but in such case, the test showed a high trend to disomy incidence for the I.F. (p=0.15), thus, it is possible that increasing the number of examined sperm such difference could become significant. The disomic sperm cells could be distinguished by their smaller size from the diploid spermatozoa which occurred with a frequency of 0.050% and 0.063% respectively in I.F. and I.B. breeds. No significant differences were found even in this case (Mann-Whitney test, U=39; α=0.05; p=0.42). Comparison among the different types of chromosomal abnomalities between the breeds showed significant differences in two cases: the disomy XX with an average frequency of 0.044% for the I.F. and 0.022% for the I.B., and the disomy XY which was detected for the 0.031% in I.F. *vs.* 0.012% in I.B. Disomy YY was the most frequent abnormality found in I.B. (0.045%), followed by disomy XX for the I.F. (0.044%). The total frequency of aneuploidy found in this work for the I.F. is in agreement with the data shown by Hassanane *et al.* (1999) for the Swedish Friesian. On the contrary, the I.B. showed a different incidence for disomy and diploidy, thus it is reasonable to suppose inter-racial differences. Further studies are needed to expand our knowledge on this topic in order to establish whether this variability is specifically correlated to the breed. Regarding the analysis of the oocyte aneuploidies, a total of 257 COCs were analyzed, 143 for the I.F. and 114 for the I.B. (Table 1). A total of 37 diploid oocytes were detected (14.4%), 16 for the I.F. (11.2%) and 21 for the I.B. (18.4%). Such difference was not statistically significant (χ^2<3.84; df=1; α=0.05; p>0.05), but the p–value was 0.10, thus it is possible that increasing the number of examined cells such difference could become significant. About 51% of the investigated animals gave diploid oocytes, in particular 43% of I.F. showed one or more diploid oocytes *vs.* 62% detected for the I.B. The incidence of aneuploidy was assessed on a total of 179 oocytes, 100 for the I.F. and 79 for the I.B. The remaining 41 oocytes in MII fase out of 257 were excluded from the analysis because the first polar body was not clearly detectable. Four out of 179 oocytes were aneuploid (2.2%), in particular two COCs were disomic for chromosome 5 and two were nullisomic, the first one for chromosome 5 and the second one for chromosome X. Basically, chromosome 5 showed a frequency of aneuploidy higher (1.7%) compared to chromosome X (0.5%), but the chi-square test showed not statistically significant differences. The total incidence of aneuploidy was slightly higher in I.B. breed (2.5%) compared to I.F. (2%). In three oocytes (1.6%), a premature separation of the centromere (PCS) was observed. These COCs were excluded from the analysis of aneuploidy because this phenomenon not surely will give genetically unbalanced germ cells as consequence of the second meiotic division. These results show clearly that the incidence of chromo-

somal abnormalities in germ cells is different between sexes; in fact the abnormal sperm are 5-6% out of the total cells, whereas in oocytes the incidence of chromosomal abnormalities is ~40%. However, the analysed oocytes were maturated *in vitro* and this could be in part responsible for the higher incidence of abnormalities compared to the same data *in vivo*, as already shown for bovine (Viuff *et al.*, 1999), ovine (Coppola *et al.*, 2007), and equine embryos (Rambags *et al.*, 2005). Our results together with additional data on oocytes produced *in vivo*, could finally elucidate the real influence of *in vitro* colture on the total incidence of chromosomal abnormalities. In conclusion, further studies are needed to expand our knowledge on the frequency of aneuploidy in germ cells of our domestic animals, breeds or genetic types, in order to establish a baseline level, useful for monitoring future trends of aneuploidy, in relation to climatic changes and environmental hazards, thus rising the reproductive and productive efficiency.

Table 1. Main descriptive statistics for the detected chromosomal aberrations in sperm and oocytes of the Italian Friesian (I.F.) and Italian Brown (I.B.) cattle breeds.

I.F.	Sperm			Oocytes					
	Disomy	Diploidy	Aneuploidy	MII	Diploid	MII+PB	Aneuploid		PCS
	Tot	Tot	Tot	Tot			Nullisomic	Disomic	
Average	0.110	0.050	0.160	143	16	100	1	1	2
SD	0.055	0.032	0.053						

I.B.	Sperm			Oocytes					
	Disomy	Diploidy	Aneuploidy	MII	Diploid	MII+PB	Aneuploid		PCS
	Tot	Tot	Tot	Tot			Nullisomic	Disomic	
Average	0.078	0.063	0.141	114	21	79	1	1	1
SD	0.038	0.036	0.037						
			Tot	257	37	179	2	2	3

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