

ORIGINAL RESEARCH

Low incidence of chromosome aberrations in spermatozoa of fertile boars

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SUMMARY

Chromosomal imbalance in gametes and embryos is one of the factors contributing to early embryonic mortality. Although the rate of chromosomally abnormal sperm cells is low and usually does not exceed 1%, there is no clear indication of fertilizing potential of such gametes. The aim of the experiment was to investigate the type and incidence of numerical chromosomal aberrations in spermatozoa produced by fertile boars used in artificial insemination (AI). We used the protocol of fluorescent in situ hybridization (FISH) on sperm interphase nuclei with molecular probes for porcine chromosome pairs 1 and 10. Altogether 12 348 sperm cells were examined. Disomy was observed in spermatozoa of all seven AI boars whereas only one diploid cell was identified in all screened sperm cells. The average rate of chromosomally unbalanced sperm was 0.105% (13/12 348) with an inter-individual variation from 0.048% to 0.194%. Among abnormal sperm cells, both disomy (0.097%) and diploidy (0.008%) were detected. Nullisomy was not included into calculations. The estimated aneuploidy rate calculated by doubling the number of disomic cells was 0.194%. Chromosome pair 10 was significantly

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more often involved in non-disjunction (75%, 9/12 aneuploid sperm cells) than chromosome pair 1 (25%, 3/12). We have shown for the pig that the rate of disomic cells falls into a range presented by other authors, whereas that of diploid spermatozoa appeared to be lower in the present study. In conclusion, numerical chromosome aberrations were present in spermatozoa of all AI boars analyzed in this study. Therefore, it can be assumed that the presence of unbalanced spermatozoa at the level observed in fertile males does not significantly affect their reproductive potential. *Reproductive Biology* 2011 11 3: 224-235.

Key words: FISH, disomy, diploidy, pig, sperm

INTRODUCTION

Over 90% of ovulated porcine oocytes undergo successful fertilization *in vivo*. The extent of the early embryonic mortality however is high (38.5% of fertilized ova) with the main loss (26.3%) occurring before the day 10 of gestation [9]. There are several identified factors of maternal and paternal origin affecting this phenomenon (e.g. breed, age, quality of gametes, fertilization failures). Although chromosomal aberrations contribute to the early embryonic loss in domestic animals, the average rate of unbalanced porcine embryos developing in the uterus is estimated at 5%, so this factor exerts rather minor effect in the pig [7, 13]. Moreover, the contribution of chromosomal imbalance from the sperm and the oocyte is not equal, with a significantly higher rate of aberrant oocytes (*in vivo* >20%, *in vitro* >30%) in comparison to a rate of less than 1% in spermatozoa of fertile boars [8, 10, 16]. In the pig, a significant share of unbalanced embryos results from polyspermic fertilization which is relatively high in this species. It has been suggested however that polyspermy is not a physiological phenomenon but rather is a consequence of delayed insemination and oocyte aging [6, 23]. It is yet unknown what makes the spermatozoa more prone to polyspermic penetration and whether the chromosomally abnormal sperm participate in this phenomenon more often.

Although the rate of chromosomally abnormal sperm cells is low, there is no clear evidence on a relationship between fertilizing performance of a sperm and its chromosomal make-up. Ducos *et al.* [5] investigated *in vitro* fertilizing potential of spermatozoa from two bulls with normal sperm parameters but significantly reduced fertility

rates *in vivo*. One of the bulls was a diagnosed carrier of a reciprocal translocation, and the analyzed parameters (*in vitro* cleavage and blastocyst rates) for this bull were of average levels. However in case of the second normal bull both parameters were significantly reduced. The majority of published evidence concerning chromosomal constitution of spermatozoa is focused on individuals with reduced fertility or carrying chromosomal aberrations [1, 2, 15]. However, in order to define a border range of chromosomally aberrant spermatozoa in normal males it is necessary to collect data on fertile males. This will make the estimation of an increase in number of aberrations in infertile individuals possible. The aim of the present study was to evaluate the incidence of chromosomally unbalanced spermatozoa in ejaculates of fertile boars used in artificial insemination (AI boars) in western Poland.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals and reagents used in this study were purchased from Sigma-Aldrich. Seven AI boars of various breeds (2 Polish large white, 2 pietrain, 2 duroc, 1 crossbred) and proven fertility were included in the experiment. Their ejaculates met all the requirements for AI boars requested in Poland (volume 150-250 ml, concentration 150×10^6 sperm/ml, 70% progressive movement, 85% of sperm with normal morphology). Sperm samples were collected and commercially processed by the Center of Animal Breeding and Reproduction in Gostyn, Poland (European Commission registration number 30042301). Samples for DNA extraction were frozen in liquid nitrogen and stored in -80°C until analysis.

Sperm samples (two from each boar) were thawed at room temperature (RT; 1-2 min) and washed twice in 0.5 ml of 6 mM EDTA in PBS. Each washing was completed by centrifugation at $400 \times g$ for 5 min. Decondensation of sperm nuclei was performed according to the protocol of Rubes et al. [16] and comprised of sample incubation (40 min, 0.5 ml of 2 mM DTT in PBS, RT) and double washing (PBS). Finally, sperm samples were fixed with methanol:acetic acid (3:1), vortexed and stored overnight in refrigerator. A small amount of fixed sperm suspension ($\sim 10 \mu\text{l}$) was placed on a clean slide (Menzel, Braunschweig, Germany) and left to dry.

Two-color FISH was performed with probes corresponding to the centromeric regions of the porcine chromosome pairs 1 and 10 [16]. The probe for chromosome 1 was labeled with Spectrum Green (Abbott Laboratories, Illinois, USA) while that for chromosome 10 with Spectrum Orange (02N33-050). Slides with decondensed sperm nuclei were denatured in 70% formamide at 73°C for 5 min and passed through a cold ethanol series (70%, 85%, 100%). The hybridization mix composed of both probes (30 ng each), 60% formamide, 10 µg salmon sperm DNA, and 10% dextran sulfate was denatured at 73°C for 10 min on a thermoblock and applied on each slide under a 24×32 mm coverslip. Slides were incubated overnight in a humidified dark chamber at 37°C. After a careful removal of the coverslips, the slides were washed in 2×SSC and incubated in 0.7×SSC/0.3% Tween 20 at 73°C for 2 min. The final steps involved washing in 2×SSC, drying and mounting with 0.2 µg/ml DAPI/antifade (Vector Laboratories, Burlingame, USA). Cytogenetic analysis was done under fluorescence microscope (Axiovert 200 with DAPI/FITC/Rhodamine filter sets; Zeiss AG, Gottingen, Germany) with the use of AxioImage Observer Software.

Analysis of sperm nuclei subjected to FISH procedure (the number and arrangement of signals from molecular probes) was based on a system described by Rubes et al. [16] and is presented in Figure 1. All samples were analyzed by three independent scorers. Only clear, strong spots were scored and signals found in a close proximity to each other were classified as a single spot. A multiple domain was identified when spots were of equal size and intensity. Since nullisomy caused by a hybridization failure can not be distinguished from that resulting from non-disjunction, such cells were not included into calculations. Therefore, the rate of aneuploidy was estimated by dividing the double number of disomic sperms by the number of haploid cells. The rate of diploidy was calculated by dividing the number of diploid sperms by the number of all examined cells (haploid and diploid).

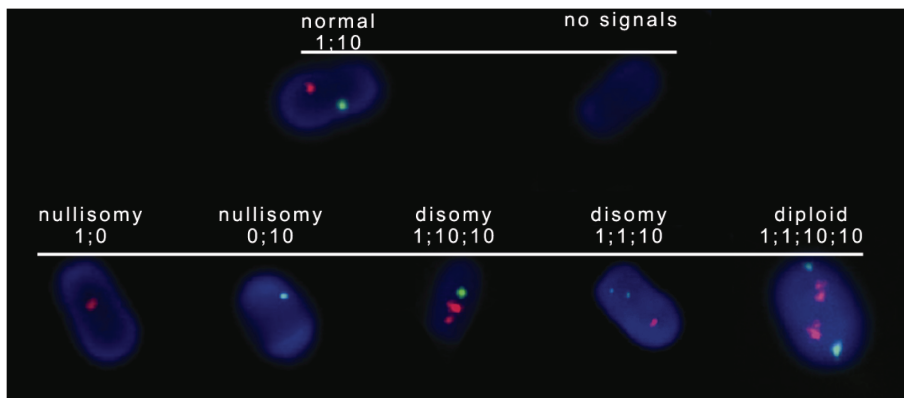


Figure 1. Analysis of sperm nuclei subjected to fluorescence in situ hybridization (FISH) with locus-specific molecular probes for the porcine chromosome pairs 1 and 10: 1/ normal sperm (one signal for each of the molecular probes; signal arrangement: 1,10); 2/ nullisomy (a single signal for one probe and no signal for the other one; signal arrangements: 1,0 or 0,10); 3/ disomy (a single signal for one probe accompanied by two signals for the second probe or vice versa; signal arrangements: 1,10,10 or 1,1,10); 4/ diploidy (two signals for both probes; signal arrangement: 1,1,10,10).

RESULTS

Chromosomal abnormalities were observed in spermatozoa of all seven AI boars of proven fertility investigated in this study. Frequencies of numerical aberrations for chromosomes 1 and 10 were assessed in a total of 12 348 sperm cells (from 1229 to 2478 sperm cells per male). Nullisomic cells were considered as artifact and were not included into the final calculations. The average rate of chromosomally unbalanced sperm was 0.105% with an inter-individual variation from 0.048% to 0.194% (tab. 1). Altogether 13 spermatozoa were carriers of chromosomal aberrations.

Among abnormal sperm cells, disomy (0.097%) and diploidy (0.008%) were detected. In the majority of analyzed males (85.7%, 6/7) only aneuploid complement was identified. In one boar, one aneuploid spermatozoan was accompanied by one diploid germ cell that was the only one detected in this study. The estimated aneuploidy rate calculated on the double number of disomy was 0.194%. The chromosome pair 10 was significantly more often involved in non-disjunction (75%, 9/12 aneuploid sperm cells) than the chromosome pair 1 (25%, 3/12 aneuploid sperm cells; tab. 1).

Table 1. Distribution of chromosomally unbalanced (diploid and aneuploid) spermatozoa in ejaculates of seven AI boars of proven fertility

Boar	Number and percentage of sperm cells					
	Analyzed	Abnormal (%)	Diploidy 2n (%)	Disomy n+1 (%)	Estimated aneuploidy* (%)	Nullisomy** n-1 (%)
1	2478	2 (0.081)	1 (0.04)	1 ^{ch10} (0.04)	2 (0.081)	2 (0.081)
2	2050	2 (0.097)	0	2 ^{ch10} (0.097)	4 (0.195)	2 (0.097)
3	1036	2 (0.193)	0	0.193 1 ^{ch1}	4 (0.386)	2 (0.193)
4	2080	1 (0.048)	0	(0.048) 1 ^{ch10}	2 (0.961)	3 (0.144)
5	1412	1 (0.071)	0	(0.071) 1 ^{ch1}	2 (0.141)	3 (0.212)
6	2063	4 (0.194)	0	4 ^{ch1(1), ch10(3)} (0.194)	8 (0.388)	0
7	1229	1 (0.081)	0	1 ^{ch1} (0.081)	2 (0.163)	2 (0.163)
Total	12 348	13 (0.105)	1 (0.008)	12 (0.097)	24 (0.194)	14 (0.113)

FISH with locus-specific probes for chromosome pairs 1 and 10 was performed in the study; data on individual boars are related to the number of sperm cells analyzed per male; total rates are calculated in relation to all analyzed spermatozoa; *estimated aneuploidy rate: double number of disomic spermatozoa divided by the number of haploid cells; **nullisomic sperm cells were considered as artifact and were not included into calculations;

^{ch1, ch10}: chromosome pairs involved in non-disjunction; AI: artificial insemination

DISCUSSION

Chromosomal aberrations of *de novo* origin result from disturbances in mitotic and/or meiotic divisions that may occur during spermatogenesis. Therefore, abnormal sperm cells have been observed in ejaculates of fertile males of various species. In order to assess an increase in the rate of aberrant spermatozoa in individuals with reduced fertility, adequate data from fertile males is necessary. We have shown that sperm samples of all AI boars investigated in this experiment contained abnormal spermatozoa. The observed rate of disomic cells falls into a range presented by other authors for the pig whereas that of diploid spermatozoa appeared to be lower (tab. 2).

Disomy dominated among the chromosomal abnormalities observed in this study, and this result generally confirmed previously described tendencies (tab. 2). Aneuploidy (trisomy) occurs at a low

frequency (1.8%) in porcine embryos [24]. Besides, it can not be excluded that some of the embryos diagnosed as trisomic were in fact mixoploid, since not all of the blastomeres could be analyzed. Considering the incidence of aneuploidy in porcine gametes (sperm 0.24%, oocytes 1.7%) and embryos (1.8%) investigated for the same chromosome pairs (1 and 10), the authors assumed that the rate of trisomic embryos reflects the sum of disomic spermatozoa and oocytes [16, 22].

Table. 2 Distribution of numerical chromosome aberrations in spermatozoa of normal males

Males	Number of sperm cells	Average % of spermatozoa with			FISH probes for chromosome pairs**	Reference
	Total examined	Disomy	Diploidy	Estimated disomy per chromosome*		
Boars						
AI boars (n=17)	171 116	0.236	0.177 0.022	0.078	1, 10 10, Y	[16]
Control boar normal karyotype	50 555	0.103	0.09 0.02 0.05	0.045 0.01 0.025	1,10 11,18 13,X,Y	[2]
Control boar normal karyotype	10 099	0.05	0.02	0.025	3,18	[15]
AI boars (n=7)	12 348	0.097	0.008	0.0485	1, 10	present study
Bulls						
Control bull normal karyotype	10 004	0.11	0.05	0.055	1,29	[1]
AI bulls (n=47)	478 711	0.077	0.038	0.026	6,X,Y	[18]
Stallions						
Normal stallions (n=4)	8850	0.26	0.034	0.087	4,X,Y	[3]
Men						
Normal men (n=10)	225 494	0.27 0.42	0.16	0.135 0.21	1,12 X,Y	[12]
Normal men (n=10)	200 000	0.162	0.248	0.081	13,21	[20]
Normal men (n=25)	125 000	0.348	0.277	0.116	18,X,Y	[4]

*estimated disomy rate per one chromosome: total disomy rate divided by the number of chromosomes analyzed in the experiment (in the present study $0.097\%/2=0.0485\%$); **list of molecular probes applied to FISH procedure in the refereed experiments

However, the evidence on the rate of disomy in porcine oocytes analyzed by FISH involving the two chromosome probes is

not consistent. Higher incidence was reported by Lechniak *et al.* [10] and Pawlak *et al.* [14] when all examined oocytes were included into calculation (total rates of 5.6% [12/214] and 2.1% [10/471], respectively). Considering the three potential sources of aneuploidy in developing embryos (disomic spermatozoa or oocyte, chromosome non-disjunction during embryo cleavage resulting in a mosaic embryo) we can hypothesize that the impact of the spermatozoon is not significant. In addition, no information is available on the fertilizing potential of aneuploid sperm cells. It can be suspected that such spermatozoa do not or only occasionally penetrate oocytes.

Diploidy was the second numerical aberration observed in this study with a very low frequency of 0.008%. This anomaly was identified in one sperm cell only and was rare when compared with other data for the pig (0.02-0.177%; tab. 2). Diploid spermatozoa only occasionally are diagnosed in ejaculates of domestic animals and usually do not exceed the frequency of 0.05%. This result is in contrast with human data (0.16-0.28%). Thus, diploid spermatozoa participation in the fertilization process as well as the spermatozoa impact on the rate of unbalanced embryos should be very limited.

The FISH procedure used for analysis of sperm chromosome complements provides direct information only on those chromosome pairs that are recognized by the applied molecular probes. Moreover, the panel of molecular probes available for animals is limited in comparison to humans [17]. Despite these limitations, FISH is the only procedure available and successfully applied in this field [11]. In the refereed studies (tab. 2), the number of monitored chromosome pairs varied from 2 to 7 with an average of three chromosomes. In order to compare the data from different experiments it is necessary to estimate the disomy rate per one chromosome pair. In the porcine spermatozoa, this parameter varied from 0.01% to 0.078% depending on the chromosome pair. The disomy rate calculated for the chromosomes 1 and 10 in the present study varied from 0.045% to 0.078% (tab. 2). Generally, the rate of disomic sperm cells in fertile domestic animals is lower than that observed in humans. In ejaculates of normal men, the mean frequency of disomic spermatozoa per autosome was 0.15% and 0.26% for sex chromosomes [19].

FISH data on distribution of three fluorescent signals does not allow for precise evaluation of the overall rate of disomy, and it can be only estimated. Rubes *et al.* [16] estimated the overall frequency of

disomy in porcine sperm at 1.5%. This means that the frequency of aneuploidy ($2 \times$ disomy) in porcine sperm should be of approximately 3%. A similar calculations based on the present results (disomy rate per chromosome 0.0485%) showed the estimated overall rates for disomy and aneuploidy of 0.92% and 1.84%, respectively. The above estimation, however, has one flaw since it assumes a similar involvement of each chromosome pair in the non-disjunction process. Experiments on human spermatozoa revealed a non-equal distribution of disomy among chromosomes. Recently, Templado *et al.* [21] reported a diverse distribution of aneuploidy among autosomes and sex chromosomes in spermatozoa of normal men. The sex chromosomes were nine times more often involved in non-disjunction (0.27%) than chromosomes of the pair 8 (0.03%). With respect to the chromosome pairs analyzed in this study (1 and 10), the incidence of chromosome 10 disomy was three times higher than that of chromosome 1 (0.073% and 0.024%, respectively). However, in the previous study of Rubes *et al.* [16] who employed the same FISH protocol, no differences in distribution of the two chromosomes among aneuploid spermatozoa were observed. It is worth mentioning that in both experiments the sperm was collected from normal AI boars. In our opinion, the observed discrepancies in the non-disjunction rate of chromosomes 1 and 10 were mainly caused by the number of analyzed spermatozoa, which was significantly higher in the experiment of Rubes *et al.* [16] than in our experiment (171 116 vs. 12 348 cells, respectively). The tendency described by these authors (similar distribution for both chromosomes; [16]) may more closely reflect a biological trend.

In conclusion, numerical chromosome aberrations were present in spermatozoa of all AI boars analyzed in this study. Therefore, it can be assumed that the presence of unbalanced spermatozoa at a level observed in fertile males does not significantly affect their reproductive potential.

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