Aneuploidy detection and mitochondria quantification in bovine embryos with different cleavage onset using Next- generation sequencing-based protocol

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Abstract

Bovine embryos are now routinely used in agricultural systems as a means of disseminating superior genetics worldwide, ultimately with the aim of feeding an ever-growing population. Further investigations common for human IVF embryos are thus a priority to improve cattle IVF and one such area is aneuploidy (abnormal chromosome number). Although the incidence and consequences of aneuploidy are well documented in human preimplantation embryos, they are less well known for the embryos of other animals. To address this, we assessed aneuploidy levels in 30 2-cell bovine embryos derived from early and late cleaving zygotes. Contemporary approaches (whole genome amplification and next generation sequencing) allowed aneuploidy assessment for all chromosomes from oocyte donors aged 4-7 years. We also quantified mitochondrial DNA levels in all blastomeres assessed, thereby testing the hypothesis that they are related to levels of aneuploidy. Overall incidence of aneuploidy in this cohort of bovine embryos was 41.1%, significantly correlated to timing of cleavage (77.8% late cleaved vs. 31.7% early cleaved). Moreover, based on mtDNA sequence read counts, we observed that median mtDNA quantity is significantly lower in late cleaving embryos. The findings further reinforce the use of the bovine system as a model for human IVF studies.

Keywords

aneuploidy, cattle, embryo, mitochondria, Next- generation sequencing

Introduction

Both human and non-human studies indicate that chromosome aneuploidy occurs frequently in early mammalian embryos and leads to embryo developmental arrest, abortions, stillbirths or a birth of affected offspring [Munné et al., 2007; King, 2008]. In pigs, the frequency of aneuploidy in preimplantation embryos varies from 14.3 to 37.3% based on screening of all chromosomes by comparative genomic hybridization (CGH) [Hornak et al., 2009; Hornak et al., 2015]. Studies mainly focus on polyploidy and mixoploidy in cattle, revealing that mixoploidy occurs in 25–90% of bovine embryos [Viuff et al., 1999; Jakobsen et al., 2006; Garcia-Herreros et al., 2010]. It was estimated using FISH that aneuploidy is presented in at least 30% of bovine oocytes [Nicodemo et al., 2010] and 27% of porcine oocytes [Vozdova et al., 2001]. Combined detection of diploidy and aneuploidy by FISH showed that chromosome imbalance might reach 60% in porcine oocytes [Lechniak et al., 2008]. Current evidence suggests that the overall frequency of aneuploidy is higher in *in-vitro* produced (IVP) embryos compared to embryos generated *in-vivo* across all farm animal species thus far studied [Viuff et al., 1999; Rambags et al., 2005; Coppola et al.,2007; Lechniak et al., 2007; Ulloa et al., 2008; Hornak et al., 2015]. This, in turn, indicates that sub-optimal oocyte maturation media, maturation and embryo cultivation protocols and/or media supplements may induce aneuploidy at this early stage of development [Demyda-Peyrás et al., 2013].

We implemented Next-generation sequencing (NGS) to detect aneuploidy of all chromosomes in farm animals, which is applicable to single cell. The main advantage of this approach is that it might be universally applied to any organism with an available reference genome sequence assembly.

Bovine oocytes contain approximately 2.6×10^5 copies of mitochondrial DNA (mtDNA), which is 100 times higher than in somatic cells [Michaels et al., 1982]. This is achieved by approximately 45-fold increase of mtDNA copy number from primordial cell to preovulating oocyte [Smith and Alcivar, 1993]. Mitochondrial maturation, distribution, ATP production and its accumulation are important factors describing oocyte competence, which in turn condition successful embryo development and blastocyst rates [Stojkovic et al., 2001]. It has been established that the quantity of mitochondria correlates with fertilization outcome and serves as an important marker of oocyte quality, explaining fertilization failure in some cases [Reynier et al., 2001; Shourbagy et al., 2006; Santos et al., 2006]. It was shown that *in vitro* maturation might significantly change distribution pattern of mitochondria in oocytes and was described that higher levels of mtDNA in cumulus cells were observed in good-quality embryos derived from corresponding oocytes [Pawlak et al., 2016; Ogino et al., 2016]. Recently, it was also demonstrated that altered levels of mtDNA are associated with female age, aneuploidy, and provide an independent measure of embryonic implantation potential in human IVF programme [Fragouli et al., 2015].

Current studies in the literature have thus far concentrated mostly on human embryos and, to a lesser extent, porcine ones. Bovine embryos have however been relatively under-studied but they are nonetheless important in that they are used extensively in animal breeding regimes and have been established as an excellent model for human IVF. The purpose of this study was therefore to provide an overview of both aneuploidy levels and mtDNA content in early bovine embryos, establishing the validity of an NGS-based approach to achieve this. We also tested the hypothesis that there are significant differences in aneuploidy and mtDNA content in early vs late cleaving embryos in order to establish whether this early stage of

morphokinetics may have a predictive value of the future health of the embryo and subsequent foetus.

Materials and methods

The chemicals used for early embryo production were purchased from Sigma-Aldrich Chemicals Co. (Prague, Czech Republic) unless otherwise stated. The all culture procedures were carried out at 39°C in a humidified atmosphere and 5% CO_2.

Oocyte maturation and fertilization

Slaughtered Holstein dairy cows (n = 16), aged 4 to 7 years, in the growth/stagnation or regression phases of folliculogenesis were used as oocyte donors. The oocytes were recovered from 3 to 9 mm sized follicles by ovarian cortex slicing. Only the oocytes with homogenous cytoplasm and compact homogenous cumuli were used for maturation. They were matured in 500 µl of TCM-199 medium (Earle's salt) supplemented with 0.20 mM sodium pyruvate, gonadotropins (P.G. 600, 15 IU/ml, Intervet, Boxmeer, Holland), 5% estrus cow serum (ECS, Sevapharma Prague, Czech Republic) and antibiotics (50 IU/ml penicillin, 50 µg/ml streptomycin) in four-well plates (Nunclon Intermed, Roskilde, Denmark) for 24 hours. In our conditions, 92.6% of the oocytes reached MII stage. Only those extruded the first polar body were used for fertilization (n = 139/150).

The motile spermatozoa were separated from frozen-thawed semen of the IVF tested bull by the swim-up method using modified Tyrode's medium (SP-TALP). The oocytes were inseminated with 1×10^6 spermatozoa/ml using modified Tyrode's medium (IVF-TALP) with 10 µg/ml heparin. The adequate number of inseminated oocytes was checked at 24 hours for fertilization efficiency. They were fixed with 2.5% glutaraldehyde solution (v/v) at 4^oC overnight, stained with 33258 Hoechst in citrate buffer for 10 minutes and examined by epifluorescence at a magnification of 400×.

Embryo cultivation and cleavage assessment

The preliminary zygotes were denuded from cumulus cells by vortex, rinsed in Dulbecco-PBS and cultured in 500 µl of MEM-medium supplemented with 5% ECS. The two-cell embryos were collected either at 26–31 h (early onset cleavage) or 32–36 h (late onset cleavage) after insemination. Only equally cleaved embryos were used for aneuploidy and mtDNA content examination. They were incubated in PBS with 0.1% pronase and 0.1% polyvinylalcohol to dissolve the zona pellucida and to separate mechanically individual blastomeres. The blastomeres were washed in several droplets of PBS to prevent DNA contamination. The each blastomere was individually transferred into PCR tube in 1 µl of 1 × PBS without Ca & Mg^{2+} and stored at -80°C until examination.

NGS validation

In order to validate a new NGS based protocol we reanalysed the WGA product of the aneuploid porcine embryos previously examined by CGH in the recent study [Hornak et al., 2015]. In total, 22 chromosome abnormalities observed in 7 aneuploid embryos by CGH were reanalysed by NGS protocol and we observed full concordance for chromosome losses and gains between both methods (Supplementary Fig. S1).

Genome Amplification and next-generation sequencing (NGS) to detect aneuploidy

One of two individually tubed blastomeres underwent Whole Genome Amplification (WGA) using the Single cell WGA kit (New England Biolabs) according to the manufacturer's protocol. Amplified samples were checked on 1.5% agarose / 0.5 × TBE buffer for the presence of a 200-2000bp DNA smear, which indicated successful amplification. Stringent precautions against DNA contamination during the whole WGA process were taken; WGA

was performed in a UV-illuminated PCR box with a dedicated set of sterile PCR tubes, pipettes and filter tips. Amplified DNA was quantified using Qubit dsDNA HS (High Sensitivity) Assay Kit (Life Technologies). DNA library containing sequencing adaptors and unique indices (barcodes) was prepared for each sample using Nextera XT DNA Sample Prep Kit and Nextera® XT Index Kit (Illumina) according to manufacturer´s manual without any modification. Up to 48 individual samples were barcoded and pooled. The final pooled library was cleaned up using Agencourt AMPure XP magnetic beads (Beckman-Coulter) and requantified with Qubit dsDNA HS Assay Kit (Life Technologies). The library was diluted to 12.5 pM using HT1 hybridization buffer (Illumina) and sequenced on MiSeq sequencing system (Illumina, Inc.). A sequencing run with optimal cluster density (1.200–1.400k/mm^2) produced typically 25–30 millions of 36 bp-long reads with Q30 base quality ≥95%.

Bioinformatic pipeline for aneuploidy detection

A raw bcl files were demultiplexed by their barcodes and FASTQ files were created for each sample. Each FASTQ file was trimmed by Phred Quality Score which falls under Q=30 threshold. FASTQ files were aligned to reference genome of Bos taurus and Sus scrofa (SusScr3 and BosTau8 UCSC build; downloaded from Illumina´s iGenomes). Alignment was performed using Burrows-Wheeler Aligner algorithm [Li and Durbin, 2010], allowing a maximum of gaps opens [parameter = 1], Mismatch penalty [parameter = 3]. Output BAM files were filtered strictly by mapping quality [parameter = 15]. Unmapped reads and multi-mapped reads were filtered out. In final filtration step PCR and Optical duplicates were removed. The programming language AWK [Aho et al., 1987] was used for read counting and GC content calculation after each filtration were use. Then we split chromosomes into 10 Mb bins and calculate GC content and number of reads in each bin using bedtools and programming language AWK. Bin read counts were normalized to the total read count for

each sample and were compared to the bin read counts in an amplified male euploid reference samples. Sample:reference bin read counts ratio >1.3 and <0.7 was indicative of chromosomal region gain and loss, respectively. All the NGS results were plotted in statistical computing and graphics program R (freeware, available from https://www.r-project.org/). Standard library packages and functions were used for plotting in R.

Mitochondrial DNA quantification in early embryos

A quantification of mtDNA in one blastomere using NGS involved determination of the number of DNA sequence reads corresponding to the mitochondrial genome. Then the quantity of mtDNA was calculated as a fraction of mtDNA sequence reads related to the total reads obtained from the sample.

Statistical analysis

The data were analyzed with the chi-square test, Student´ s t-test using ANOVA and the Fischer's exact test, SPSS Version 11.5 for Windows (SPSS, Inc. Chicago, IL, USA).

Results

Early embryo development

In order to validate efficiency of fertilization and cleavage in our conditions, these parameters were at first assessed using oocyte control group. The mean proportions of fertilized and cleaved oocytes from those inseminated in control group are shown in Table 1. Start of embryonal development was different in terms of the first cleavage onset of embryos. The significantly higher (chi-square test; $P < 0.01$) proportion of embryos was cleaved from 26 to

31 h (early cleavage) than from 32 to 36 h (late cleavage). On the basis of first cleavage time, the 2-cell embryos were divided into two categories: “early cleaved” and “late cleaved.”

Aneuploidy in 2-cell bovine embryos

After successful validation of the NGS protocol we analysed copy numbers of each one of chromosomes in 31 single blastomeres from both the early and late cleaved 2-cell embryos (Table 2). The overall incidence of aneuploidy when merging both embryo categories was 41.1%. The significantly lower proportion of embryos with aneuploidy was found in the early cleaved embryos compared with the late cleaved embryos (Fischer’s exact; $P < 0.05$). The aneuploidies in both embryo categories were described. No significant difference in ratio of male and female sex was found between the early and late cleaved embryos (Table 2). Examples of aneuploidies detected in embryos by NGS are shown in Fig. 1.

Mitochondrial DNA (mtDNA) quantification in early embryos

Based on mtDNA sequence read counts in all 31 blastomeres examined for aneuploidy we also quantified mtDNA. When mtDNA was calculated for early and late cleaved embryo group, we have observed that median mtDNA quantity was significantly decreased (Student´s t-test; $P < 0.01$) in the late cleaved embryos compared with the early cleaved embryos (Fig. 2).

Discussion

Next-generation sequencing protocol

Cattle (*Bos taurus*; 2n = 60) with 29 acrocentric autosomes and one sex chromosome pair represent significant challenge for standard karyotyping. Several studies have focused on aneuploidy detection in bovine embryos however most only yield information about polyploidy or mixoploidy [King et al., 1987; Kawarsky et al., 1996; Viuff et al., 1999]. Other studies used FISH in order to detect aneuploidy of individual bovine chromosomes, however, in those studies only a few chromosomes were analysed [Nicodemo et al., 2010]. Using NGS-based protocol however we were able to detect copy number of all bovine chromosomes from single blastomere. Every chromosome was sub-divided into approximately 10Mb intervals (bins), which were plotted individually at NGS charts. This allowed us to increase resolution of the platform and detect segmental chromosomal abnormalities. By direct comparison with arrayCGH technology we demonstrated that NGS offers increased sensitivity when embryonic biopsies were analysed during preimplantation genetic diagnosis [Fiorentino et al., 2014; Yang et al., 2015]. We subsequently performed a validation study of our NGS-based protocol by a comparison of metaphase-CGH and NGS results from the same WGA product of the previously analysed porcine blastomeres using metaphase-CGH [Hornak et al., 2015]. The metaphase-CGH and NGS charts are shown in Supplementary material (S1). From the validation study we conclude that, for whole chromosome aneuploidy, the sensitivity of both platforms is comparable, however for segmental chromosome abnormalities the NGS analysis was more accurate and offered better resolution. NGS technology is perceived as expensive one, however this is a relative statement. For example, NGS might be coupled with barcoding technology, where different samples might be multiplexed with a use of specific indices before sequencing run. In our study up to 48 samples were barcoded and sequenced in a massively parallel fashion. The final cost per sample was comparable with the metaphase-

CGH technology used in our previous study [Hornak et al., 2015]. The disadvantage of NGS technology is its inability to detect polyploidy.

Aneuploidy frequency in bovine embryos

As well as in humans, in cattle and pigs, timing of the first cleavage is also a valuable indicator of intrinsic quality of early embryos and their developmental competence [Lonergan et al., 1999; Lonergan et al., 2000; Lundin et al., 2001; Brevini et al., 2002]. Early cleaved porcine embryos are more likely to develop to blastocysts than their late cleaved counterparts [Isom et al., 2012]. The timing of first zygotic cleavage is associated with many factors including transcript level of developmentally important genes and chromosomal aberrations [Lechniak et al., 2008]. In the present study we established that bovine early cleaved embryos show lower levels of aneuploidy than late cleaved ones (31.8% vs 77.8%). Our findings thus provide strong evidence that embryo selection based on the time of zygotic cleavage may increase proportion of euploid embryos. Our data are supported by time lapse studies of human IVF embryos showing that any delay in cleavage times, incorrect divisions or lower morphology grade are linked with higher aneuploidy levels [Chawla et al., 2014; Coates et al., 2015].

Aneuploidy frequency varies, between indiviudals of the same species and is probably affected by the different age of animals used, the protocols employed and/or whether *in-vitro in-vivo* production is used. Here we report that the frequency of aneuploidy in 2-cell stage IVP bovine embryos obtained from 4–7 years old oocyte donors employing whole chromosome screening by NGS-based protocol is 41%. This frequency is comparable to similar study focusing on porcine 2-cell stage IVP embryos where we observed 37% of the embryos to be aneuploid [Hornak et al., 2015]. Nicodemo et al., [2010] reported that

271 approximately 30% of IVM bovine oocytes are aneuploid, by applying FISH for the X
272 chromosome and chromosome 5, and extrapolating data to the rest of the karyotype. The
273 incidence of aneuploidy in porcine and bovine cleavage embryos seems to be lower compared
274 to human embryos evaluated by preimplantation genetic screening, where aneuploidy
275 typically affects more than 50% of IVF produced embryos [Hellani et al., 2008; Mantzouratou
276 and Delhanty, 2011; Chavez et al., 2012]. However, it is important to emphasize that the
277 majority of human IVF embryos derives from infertile couples and such a cohort of embryos
278 might, in general, display elevated frequency of chromosomal abnormalities.
279
280 Chromosomal differences in length, centromere position, pericentromeric and other repetitive
281 sequences, recombination patterns and chromatin characteristics might all be related to a
282 differential susceptibility to aneuploidy [Warburton and Kinney, 1996]. In human oocytes and
283 embryos, there is shown tendency that chromosomes equal to or smaller than chromosome 13
284 are involved most frequently in aneuploidy [Fragouli et al., 2010; Gabriel et al., 2011;
285 Alfarawati et al., 2011]. It is important to stress that within the above-mentioned
286 chromosomes frequently found in aneuploidy are involved all human acrocentric
287 chromosomes. Similarly, in porcine embryos, the acrocentric chromosomes were relatively
288 frequently involved in aneuploidy [Hornak et al., 2012]. In the present study of bovine
289 embryos, no striking difference between the size of chromosome was noticed (Table 1),
290 moreover all bovine autosomes are acrocentric. Thus, the observation on the type of
291 chromosomes involved in aneuploidy might indicate that not the smallest, but rather
292 acrocentric chromosomes are more prone to error during meiotic/mitotic divisions. However,
293 from human and animal studies it is well documented that aneuploidy can affect any
294 chromosome [Hassold et al., 2007; Hornak et al., 2012].
295

Mitochondrial content in early bovine embryos

In the present study, single blastomeres derived from 2-cell stage bovine embryos were analysed, thus the ratio between the read count mapped to mtDNA and the total read count reflects relative difference in number of mitochondria in analysed embryos. The majority of DNA sequence reads were derived from the nuclear genome and the mtDNA represented approximately 1.5–2% of all DNA sequence reads. We observed that in a group of late cleaved embryos there was significantly lower numbers of mitochondria compared to the early cleaved group. This might be explained by decreased ATP reserves in embryos coming from oocytes with lower mitochondria counts. Indeed, in a cohort of human oocytes there was observed that oocytes with higher ATP content displayed increased potential for continued embryogenesis and implantation [Blerkom et al., 1995]. However, in our study late embryos displayed significantly higher level of aneuploidies compared to early ones. This observation indicates that the aneuploid 2-cell stage embryos contain less mtDNA compared to euploid ones. As a possible explanation it was shown that ATP levels influence spindle formation and chromosome alignment during meiosis [Zhang et al., 2006; Johnson et al., 2007]. In contrast, comparable study using NGS technology showed that, on average, biopsy specimens derived from aneuploid blastocysts contained significantly greater amounts of mtDNA than samples from embryos that were euploid [Fragouli et al., 2015]. These contradictory findings between present study and the study of Fragouli et al., [2015] very probably relates to different stages of embryos analysed. In the present study the mtDNA reflects copy number of mitochondria coming from oocytes, but in the latter study the trophectoderm biopsies of human blastocysts were analysed. It is known that in the trophectoderm of blastocyst stage embryos the replication of mitochondria is initialized in order to fulfil ATP demands of preimplantation embryos [John et al., 2010]. So the increased mtDNA in trophectoderm of aneuploid embryos might reflect enhanced energy requirements of chromosomally abnormal embryos, which

might be caused, for example, by overexpression of DNA repair signalling pathway genes in such embryos [Bazrgar et al., 2014]. However, whether and what relationship between aneuploidy and mtDNA exist cannot be clearly answered at the moment and there are more studies needed to clarify the issue.

The role of mitochondria seems to be important also for fertilization outcomes. In porcine oocytes, data suggest that a correlation exists between increased cytoplasmic volume and increased mtDNA content, and that appears to be factors positively influencing fertilization outcomes [Shourbagy et al., 2006; Santos et al., 2006]. However, to confirm this observation was beyond the scope of the research reported here.

Declaration of interest

The authors declare that there is no conflict of interest and have nothing to disclose.

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Figure legends

Figure 1. Detection of chromosome aneuploidies in single blastomeres derived from 2-cell stage bovine IVF embryos by Next-generation sequencing-based protocol (NGS)

NGS charts showing copy number of each bovine chromosome. (A) a male euploid bovine embryo blastomere with 2 copies of all autosomes and 1 copy of chromosome X and Y; (B) afemale blastomere with monosomy of chromosome 6; (C) a male blastomere with

monosomy of chromosome 4 and partial gain of chromosome 13 (15–84Mb); (D) a female blastomere with monosomy of chromosome 19.

Figure 2. Comparison of mtDNA quantity between single bovine blastomeres derived from early and late cleaved embryos by Next-generation sequencing-based protocol (NGS)

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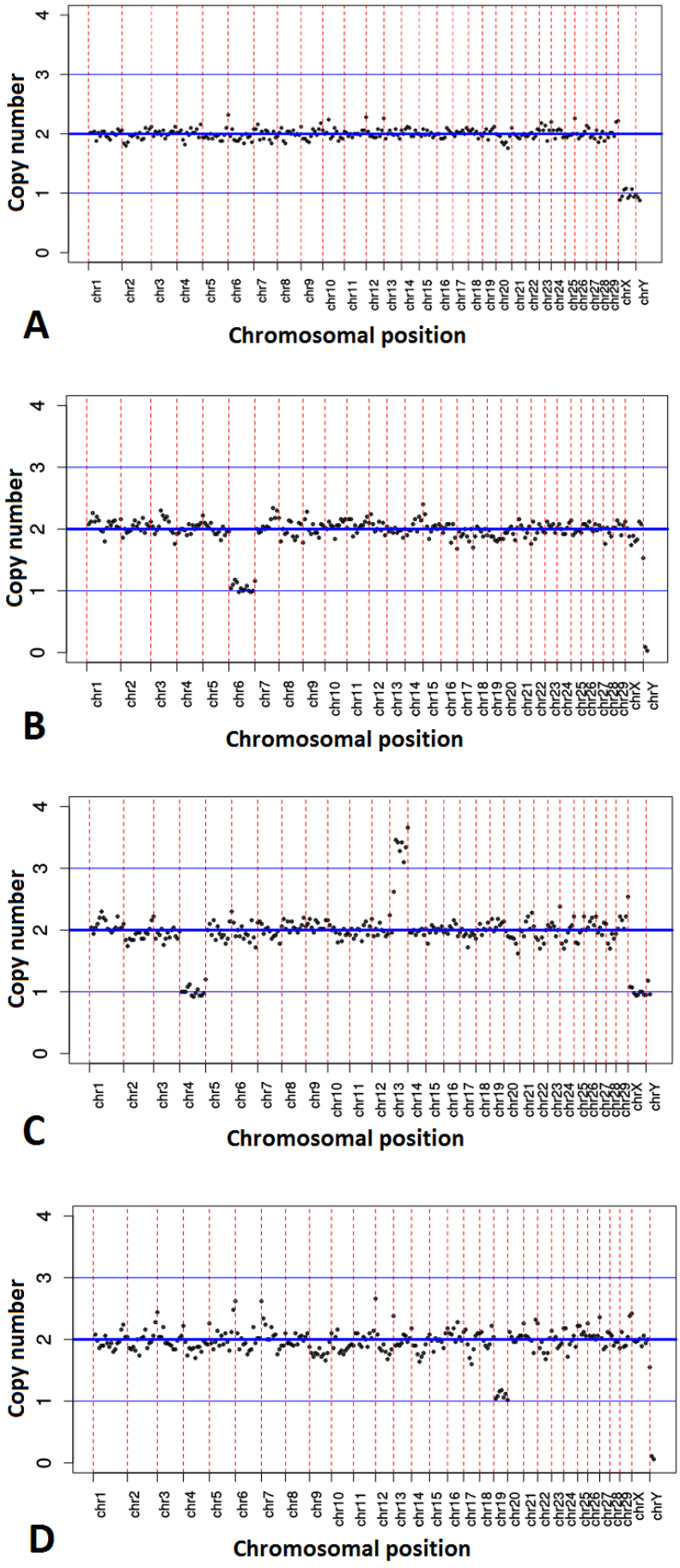
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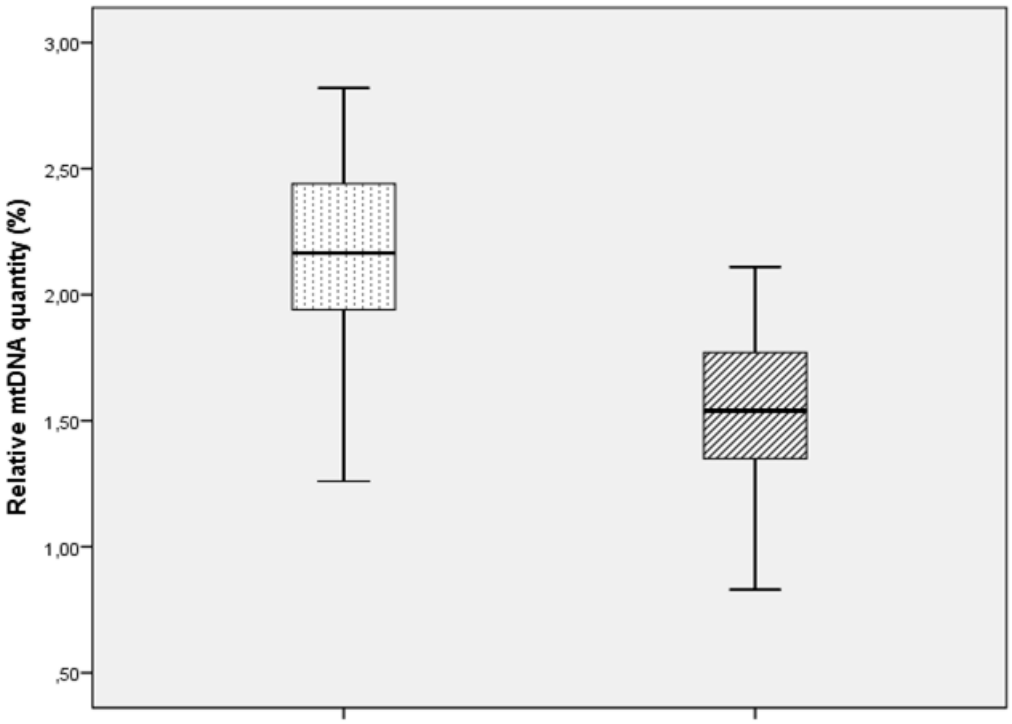
492 **Figure 1**



Group	N	Min	Q1	Median	Q3	Max
Early cleaved	22	1.26	1.94	2.19	2.46	2.86
Late cleaved	9	0.83	1.285	1.54	1.83	2.11

493 **Figure 2**

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496 **Table 1. Early embryos developed from bovine oocytes fertilized *in vitro* (control group)**
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Oocytes examined n	fertilized/inseminated n (%)	cleaved/inseminated n (%)	Cleaved embryos early/total n (%)	Cleaved embryos late/total n (%)
139	27/31 (87.1)	92/108 (85.2)	75/92 (81.5)[a]	17/92 (18.5)[b]

498 Values with different superscripts are statistically different (a-b; P<0.01).
499 Early cleaved embryos: cleavage onset at 26–31 h post insemination.
500 Late cleaved embryos: cleavage onset at 32–36 h post insemination.
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Table 2. Chromosome abnormalities in early bovine embryos developed *in vitro*

Embryo cleavage category	Embryos examined	Aneuploidy	No. of embryo	Description of aneuploidy	Sex of embryo	
					XY	XX
	n	n (%)			n (%)	n (%)
early	22	7 (31.8%)[a]	1.	-20	13 (59.1)[a]	9 (40.9)[a]
			2.	-6		
			3.	+21		
			4.	-13,+17		
			5.	+8,-10,-19,+25,+26,-27		
			6.	-6		
			7.	-4,+13*part.(15-84Mb)*		
late	9	7 (77.8%)[b]	1.	+2*part.(65-141Mb)*	6 (66.7)[a]	3 (33.3)[a]
			2.	+23,-24		
			3.	-6,+11		
			4.	-14*part.(32-81Mb)*		
			5.	+20		
			6.	-5*part.(0-22Mb)*,+5*part.(22-125Mb)*		
			7.	-19		

Values with different superscripts are statistically different (a-b; $P<0.05$).
+/ – stands for trisomy or monosomy of particular chromosome
part. = gain or loss of chromosome part (length in megabases)

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