Chromosomal numeric abnormalities on early bovine embryos derived from in vitro fertilization: effect of the oocyte quality, maturation environment and incubation time.

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Abstract

Chromosomal aberrations are one of the major causes of embryo failures in the mammals. It is also been demonstrated that the occurrence of this kind of abnormalities is higher on *in vitro* produced (IVP) embryos. This study was conducted to investigate the effect of oocyte morphology and maturation conditions on chromosomal abnormality rates in bovine preimplantational embryos. To this end, 790 early cattle embryos derived from oocytes with different morphology and maturated in different conditions (maturation period: 24 h *vs* 36 h; maturation media: five different serum supplements in TCM-199) were cytogenetically evaluated in three sequential experiments. Rates of normal diploidy and abnormal haploidy, polyploidy and aneuploidy were determined in each embryo. Throughout all experiments, the rate of chromosomal abnormalities were significantly ($p<0.05$) influenced by the oocyte morphology and maturation conditions (maturation time and culture media). We find that a lower morphological quality is connected with high rates of numerical chromosome abnormalities ($p<0.05$). Moreover, we also find that polyploidy is associated with increased maturation time ($p<0.01$), whereas maturation media significantly ($p<0.05$) affected the rates of haploidy and polyploidy. In general, the use of ECS or FCS as serum supplements produced ~~the~~ higher rates of chromosomal aberrations ($p<0.05$) compared to the other serum supplements. According to our results, we can conclude that morphological quality of oocytes and maturation conditions affect the chromosomal abnormality rates of the subsequent IVP cattle embryos.

Keywords: Chromosomal abnormalities, *in vitro*, bovine embryo, oocyte maturation.

1. Introduction

The bovine *in vitro* fertilization (IVF) industry has been constantly improving during the last decade. At present time the number of *in vivo* and *in vitro* produced (IVP) embryos is similar in some countries (Pontes, Silva *et al.* 2010). Despite the vast researches performed during the last years, the overall efficiency of IVP cattle embryos still remains low. Only 30% of cultured oocytes reach the blastocyst stage (Lonergan 2007). It is well known that *in vivo* derived embryos have higher quality and viability to term than those produced by *in vitro* maturation, fertilization and culture (Ulloa Ulloa, Yoshizawa *et al.* 2008b), even when the same donor cow was used (Pontes, Nonato-Junior *et al.* 2009). While the causes of this inefficiency are likely numerous and complex, the oocyte morphological quality at the beginning of the IVP process and the oocyte maturation protocols have been described as major contributing factors on this issue. Several authors demonstrated that oocytes with higher morphological quality (class "A" and "B") have shown improved developmental rates to term in the subsequently formed embryos (Hawk and Wall 1994). In this sense, maturation media (Bilodeau-Goeseels 2006; Räty, Ketoja *et al.* 2011), maturation protocols (Yang, Jiang *et al.* 1993) and serum supplementation (Fukui and Ono 1989; Pereira, MacHado *et al.* 2010) are also described as potential causes of these differences. For this reason different *in vitro* oocyte maturation media, maturation protocols and media supplementation patterns are being developed and tested constantly in order to meet the metabolic requirements just as *in vivo* conditions (Purpera, Giraldo *et al.* 2009; Rizos, Ward *et al.* 2002).

Multiple causes have been described as being responsible for increased developmental failure on IVP embryos (Van Soom, Vandaele *et al.* 2007; Vandaele and Van Soom 2011).

In this sense, chromosomal abnormalities are thought to be a major contributor and are associated with reduced developmental potential (King, Coppola *et al.* 2006), impaired embryo viability, (Kawarsky, Basrur *et al.* 1996) and embryonic death and abortion (King 1990). In cattle, it has been reported that IVP embryos have a higher rate of chromosome abnormalities than their *in vivo* counterparts (Viuff, Rickords *et al.* 1999). Similar results have been found in pig (Ulloa Ulloa, Yoshizawa *et al.* 2008a), horse (Rambags, Krijtenburg *et al.* 2005), goat (Villamediana, Vidal *et al.* 2001) and sheep (Alexander, Coppola *et al.* 2005). It has been demonstrated that the percentage of chromosomal abnormalities appears to be significantly influenced by maturation environment in oocytes (A'Arabi, Roussel *et al.* 1997) and by post-fertilization culture conditions on IVP embryos (Lonergan, Pedersen *et al.* 2004).

Currently, it is not possible to evaluate the chromosomal complements of matured oocytes previous to fertilization in the IVF laboratory by simple and non-invasive techniques (Coticchio, Sereni *et al.* 2004). Therefore, oocytes with normal maturation parameters can have an abnormal chromosomal composition, producing higher rates of fertilization failures and development impairment. Despite of their potential economic impact on livestock production, only a few cytogenetic studies of IVP embryos have been reported (Garcia-Herreros, Carter *et al.* 2010). Moreover, moreover they did not focus ~~they have not been focused~~ on the influence of oocyte quality and maturation environment as potential causes of these alterations.

The present study was therefore designed to examine the effect produced by: 1) the oocyte morphological quality, 2) oocyte maturation time, and 3) oocyte maturation media (serum supplementation), on the incidence of numerical chromosomal abnormalities in early IVP bovine embryos.

2. Materials and methods

2.1 Media and reagents

Chemicals were purchased from Sigma-Aldrich, Spain, unless otherwise is indicated. Percoll were purchased from GE Healthcare, Spain. Nunclon™ Plastic dishes were purchased from Termofischer Scientific, Germany. Sterilization filters used in culture media were purchased from Millipore Corporation, Spain. Oocyte and embryo were manipulated with 20 µl micropipette tips purchased from Eppendorf Iberica, Spain. Estrus cow serum (ECS) were obtained according to our previous protocols (Ocana-Quero, Pinedo-Merlin *et al.* 1999b). Briefly, sterile complete blood samples were obtained by jugular venopunction of six estrous cows. Pooled serum was obtained by centrifugation (200 x G, 15 min), inactivated by heat (56ºC, 30 min) and sterilized with a 0.22 µm membrane filter. Aliquots were frozen and stored at -20ºC until its use. Anestrus cow serum (ACS) was obtained following the same protocol by collecting blood samples from 6 different anestrous cows.

2.2 In vitro embryo production

Bovine embryos were produced according to previous studies performed in our laboratory (Ocana-Quero, Pinedo-Merlin *et al.* 1999a), with the following modifications:

2.2.1 Collection of ovaries and oocytes and in vitro maturation

Ovaries were collected from slaughtered cows in the local abattoir and transported to the laboratory within the next 2 h after collection. The ovaries were washed thrice in warm

physiological saline solution supplemented with kanamycin (25 mg/ml). Cumulus oophorus complexes (COCs) were obtained from follicles between 4 and 8 mm through aspiration with 18 G needle and placed into a 15 ml conical tube with 2 ml of warm H-TALP media. After 1 h sedimentation at 38.5ºC, oocytes were poured on Petri dishes and examined under a stereomicroscope with a warm plate. In Experiment 1, oocytes were classified according to their morphology in four groups (A, B, C and D) (Cetica, Dalvit *et al.* 1999; Hawk and Wall 1994). Based on the outcome of Experiment 1, and to eliminate the possible detrimental effect of morphological quality, only class "A" oocytes (compact-intact cumulus cell layers and good morphology) were used for Experiments 2 and 3 of~~n~~ this study. Selected COCs were washed twice in H-TALP and cultured in groups of 100 in 500 µl modified bicarbonate-buffered TCM199 media, supplemented with 10% of fetal calf serum (FCS); 0.4 mmol/l glutamine; 0.2 mmol/l sodium pyruvate; 0.5 mg/ml FSH, 5 mg/ml LH; 1 mg/ml estradiol and gentamicin (50 mg/ml) for 24 h, at 38.5ºC in 5% CO_2.

2.2.2 Sperm capacitation and in vitro fertilization

Frozen semen straws from different bulls of the Retinta breed with a history of proven fertility were thawed in a water bath at 38ºC during 1 min. Viable spermatozoa were selected by centrifugation through a discontinuous Percoll gradient (45 and 90% (v/v) in Sp-TALP) and washed twice in warm equilibrated Sp-TALP media. The final sperm concentration of 1 x 10^6 sperm cells/ml was adjusted in IVF-TALP, supplemented with bovine serum albumin (BSA; 6 mg/ml) and heparin (100 mg/ml). Groups of 60 oocytes were washed in 500 µl of equilibrated IVF-TALP and incubated with sperm at 38.5ºC in 5% CO_2.

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135 *2.2.3 Culture of zygotes*

136 After 20–24 h of incubation, groups of 100 presumptive zygotes were denuded by vortex in
137 15 ml tubes during 3 min. Thereafter, it were transferred to synthetic oviduct fluid (SOF),
138 supplemented with BME amino acid solution and MEM non-essential amino acid solution
139 and 5% (v/v) FCS and were cultured in 50 µl droplets under mineral oil in groups of 25 for
140 72 h at 38.5ºC in 5% CO_2.

141

142 2.3 Chromosome preparation

143 Chromosome preparation was performed according to our standard method (Ocana-Quero,
144 Pinedo-Merlin *et al.* 1999a). At the end of the culture period, colchicine (0.1 µg/ml) was
145 added to the culture media and embryos were cultured for up to 6 h. After that, embryos
146 were placed in a 500 µl hypotonic solution of 0.88% (w/v) tri-sodium citrate for 45–60 min.
147 Thereafter, embryos were fixed mildly by pouring about 500 µl of Carnoy´s 1:1 fixative
148 (one part acetic acid: one part methanol) for 10 min and placed in a 3:1 Carnoy´s fixative
149 overnight. Each embryo was then fixed onto a clean glass slide and blastomeres were
150 separated using acetic acid (100%, v:v). The nucleus from each blastomere was spread with
151 several drops of Carnoy´s 1:1 fixative. Samples were air-dried and stained with 5% Giemsa
152 solution for 10 min. Only those blastomeres containing an analyzable metaphase (intact and
153 non-overlapping chromosomes) were assessed under X 1250 magnification in an optical
154 microscope (Reichert-Jung Polyvar, Austria).

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156 2.4 Cytogenetic criteria

Only the embryos showing more than one analyzable metaphases were included in this study. Embryos with 60 chromosomes in all countable metaphases were defined as normal diploids (2n = 60). Those in which all analyzable metaphases contained 30 chromosomes were defined as haploids. If the embryo have shown more than two sets of chromosomes (3n = 90 or 4n=120) in all blastomeres were judged as polyploids. Finally, those that showed an abnormal chromosomal number were defined as aneuploids.

2.5 Experimental design

2.5.1 Experiment 1

The aim of this experiment was to examine the incidence of morphological oocyte quality on the appearance of chromosomal abnormalities in early IVP bovine embryos. A total of 249 oocytes were classified in four different groups (A, B, C and D) according to their subjective quality as described previously. Oocytes were fertilized, cultured and cytogenetically analyzed as described above.

2.5.2 Experiment 2

The aim of this experiment was to evaluate the incidence of oocyte maturation time on the appearance of chromosomal abnormalities in early IVP bovine embryos. In this study, a total of 157 embryos derived from class "A" morphological quality oocytes were classified into two groups according to their maturation period: 24 h and 36 h. Thereafter, oocytes were fertilized, cultured and cytogenetically analyzed as described above.

2.5.3 Experiment 3

This experiment was performed to investigate the effect of different serum supplementation strategies during *in vitro* oocyte maturation on the percentage of chromosomal abnormalities observed in early IVP bovine embryos. A total of 384 embryos derived from class "A" morphological quality oocytes were produced as described previously from oocytes matured in 6 groups of TCM-199 medium supplemented with: 10% ECS (n = 62; prepared as described previously); 10% FCS (n = 59; F2442, Sigma Aldrich, Spain); 10% ACS (n = 59; prepared as described previously); 10% of bovine amniotic fluid (BAF; n = 60; A5130, Sigma Aldrich, Spain); 0,6% (w/v) BSA (n = 74) and 10% of bovine steer serum (BSS; n = 70; A9433, Sigma Aldrich, Spain) used as control. After maturation, oocytes were fertilized, cultured during 72 h and cytogenetically analyzed as described previously.

2.6 Statistical analysis

Data were analyzed using the Chi-Square procedure of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). Differences observed among treatments were determined using a two factors expected proportion test. Data were significant at $p<0.05$.

3 Results

Significant ($p<0.05$) effects of oocyte morphological quality, oocyte maturation time and media serum supplementation were found on the percentage of abnormal chromosomal complements observed across all the analyzed embryos.

3.1 Experiment 1

A significant ($p<0.05$) relationship between the oocyte quality and the ploidy of the resulting embryos was found (Table 1). The number of normal diploid embryos significantly ($p<0.05$) increased with increasing oocyte morphological quality of the oocyte~~increased as increase the morphological quality of the oocyte~~. Therefore, the lowest percentage of total chromosomal abnormalities ($p<0.05$) were observed only in embryos derived from superior quality oocytes (class "A"). When analyzing separately each kind of chromosomal abnormalities, we found that the highest haploidy percentage ($p<0.05$) was observed in embryos derived from oocytes class "C" and "D". Similarly, the percentages of polyploidy and aneuploidy were statistically ($p<0.05$) lower in embryos derived from class "A" oocytes compared to those classified as class "B", "C" and "D".

3.2 Experiment 2

Chromosomal abnormality rates were significantly ($p<0.01$) higher in oocytes matured for 36 h than those matured for 24 h (Table 2). Interestingly, only the polyploidy rates were significantly ($p<0.01$) different between treatments. However, no significant ($p>0.05$) differences were found between the other type of chromosomal ~~complements~~ abnormalities (haploid and aneuploid) and maturation time (24 h and 36 h).

3.3 Experiment 3

Supplementation strategies of maturation media with different serum significantly ($p<0.05$) influenced the incidence of chromosomal abnormalities of IVP bovine embryos (Table 3). In general, the use of ECS or FCS as serum supplements produced ~~the~~ higher rates of chromosomal abnormalities ($p<0.05$) compared to the other serum supplements. Moreover, the rates of haploidy and polyploidy were significantly ($p<0.05$) higher in those embryos

maturated in the presence of ECS. However, there was no statistical ($p>0.05$) differences between supplements for the rate of aneuploidy. ~~Moreover, the rate of haploidy was significantly ($p<0.05$) higher in those embryos maturated in the presence of BSA.~~

4. Discussion

Chromosomal abnormalities have been suggested as a major cause of embryonic failure on IVP embryos (King, Coppola *et al.* 2006). Previous reports demonstrated that the incidence of these abnormalities appears to be influenced by methods of embryo handling used during these procedures as well as suboptimal culture environments in which embryos must be developed (King, Coppola *et al.* 2006). The present study evaluated the impact of the quality of the oocytes and their *in vitro* maturation environment on the chromosome make-up of the subsequently produced embryos, which is one of those potential causes described above. In this way, our results showed that initial poor quality of oocytes as well as suboptimal maturation conditions produced a higher rate of chromosome abnormalities at earliest stages of embryonic development.

The first experiment was conducted to evaluate the importance of oocyte morphology in the incidence of chromosomal abnormalities in subsequent produced embryos. Previous studies demonstrated that the initial quality of the oocytes had greater impact on early embryonic survival, establishment and maintenance of pregnancy (Krisher 2004). In our study, the rate of chromosome abnormalities in embryos was significantly affected by the morphological quality of the oocytes. In this sense, it has been also demonstrated that the maturation rates and embryo development of the subsequent produced embryos are strongly related with the oocyte original morphology (Blondin and Sirard 1995; Long, Damiani *et al.* 1994). However, to our knowledge, there are no previous studies that evaluate the chromosomal

composition of the derived embryos of morphological classified oocytes as possible cause of these losses in IVP cattle embryos. In our study, we observed in matured oocytes that begin to multiply after fertilization higher rates of chromosomal abnormalities associated with low quality oocytes (pale and heterogeneous cytoplasm, class "D"). As a possible explanation, this type of oocytes has a low density of organelles to support the meiotic process appropriately that leads to higher rates of meiotic failures (Nagano, Katagiri *et al.* 2006). On the other hand, the lower incidence of morphologically normal spindles in the low quality oocytes leads to higher rates of chromosomal gains or losses during replication (Long, Damiani *et al.* 1994). In this way, embryos derived from lower-grade oocytes, with poor cy~~i~~toplasmatic ~~morphology~~ quality (granularity or discoloration of the cytoplasm, vacuolization, and incorporations such as refractile bodies), are related with pregnancy rates which are five times lower than those of embryos derived from normal quality oocytes~~rates 5 times lower than those with normal cytoplasm~~ (Ebner, Moser *et al.* 2003). We would like to highlight the fact that only high quality oocytes (class "A") showed a lower polyploidy rate. Recent studies have shown that the incidence of polyspermic fertilization, major cause of embryo polyploidy, is higher in low quality oocytes compared to those with optimal morphology (Wortzman and Evans 2005). This fact can be attributed to a delayed and incomplete exocytosis of the cortical granules (Wang, Hosoe *et al.* 1997) or to a premature cortical granules release in low quality oocytes (Suzuki, Saito *et al.* 2003), causing a weaker block to polyspermy.

In Experiment 2, embryos derived from high quality oocytes (class "A") were differently matured and cultured *in vitro*. A higher rate of chromosomal alterations was observed when the oocyte maturation time was increased from 24 to 36 h. It has been widely demonstrated that longer maturation times decreases the outcome of IVP embryos (Gliedt, Rosenkrans Jr

et al. 1996) and stimulate higher nuclear maturation rates by a greater positive feedback from cumulus cells (Barrett and Albertini 2010). However, over-maturation for more than 24 h promotes an "aging" effect, showing signs of degeneration probably due to the excessive time in sub-optimal maturation environment (Máximo, Martins da Silva *et al.* 2012). An interesting observation was that only the rates of polyploidy were significant higher when the time of maturation was increased. This abnormality was noted as a major cause of developmental failure on IVP embryos in mammals. It has been previously suggested that maturation prepares the oocyte to interact with the fertilizing spermatozoa at a particular moment in time (Dale and Defelice 2011). If the oocyte is not fertilized, the maturation processes continue and the cell ages, leading to higher polyspermy rates (Tarín 1996). In a later study, Tarín, Trounson *et al.* (1999) also suggest that oocyte post-ovulation aging may leads to a series of ultrastructural changes related to the meiotic spindle integrity. Moreover, same authors suggest that these changes may produce higher rates of failure in the extrusion of the second polar body by the oocyte after sperm penetration, leading to higher polyplody rates. Polyspermy has been widely described as a major concern particularly in pig embryos (Niwa 1993). Rather, partial induction of the acrosome reaction of boar spermatozoa in IVF media and sperm fertilizing doses have been indicated as the primary causes in this species (Funahashi 2003).

Previous authors have demonstrated the influence of the culture system upon the ploidy of *in vitro* matured oocytes in human (Christopikou, Karamalegos *et al.* 2010) and domestic animals (Lechniak, Szczepankiewicz *et al.* 2005; Ocana-Quero, Pinedo-Merlin *et al.* 1999b; Ueno, Kurome *et al.* 2005). Furthermore, the same effect was observed in oocyte nuclear maturation rates and blastocyst yield of IVF-derived embryos in goat (Tajik and Esfandabadi 2003), porcine (Ott, Schernthaner *et al.* 2002) and cattle (Ocana-Quero,

Pinedo-Merlin *et al.* 1999b; Russell, Baqir *et al.* 2006). As a possible explanation, age related changes in oocytes due to oxidative damage associated with high cellular metabolism and genomic instability, (e.g., mitochondrial mutations and telomere shortening) can be triggered by the environmental culture conditions (Pan, Ma *et al.* 2008). Mainly, this can be due to a lack of energy supply by oxidative phosphorylation in the mitochondria within the oocyte (Eichenlaub-Ritter, Wieczorek *et al.* 2011). Our study is in agreement with theses hypothesis, since we found a clear influence of the culture media in the ploidy of the derived embryos. However, it has been described that matured oocytes appear fairly normal until resumption of maturation when they form aberrant spindles, without being able to distinguish them from the normal (Ebner, Moser *et al.* 2003). Since we cannot evaluate the chromosomal composition and meiotic spindle previous to fertilization, it can be assumed that oocyte with higher level of chromosomal abnormalities, produced by the suboptimal environmental conditions (A'Arabi, Roussel *et al.* 1997), will produce embryos with an higher rate of abnormalities that can have a normal development at very early stages (King, Coppola *et al.* 2006).

In Experiment 3, the higher rates of chromosomal abnormalities were observed in the culture medium supplemented with ECS. It has been demonstrated that this serum supplementation produces higher levels of FSH, LH and estrogens in the maturation media (Ott, Schernthaner *et al.* 2002). These hormones have been associated with oocyte aging (Liu, Feng *et al.* 2011), marked ultrastructural changes of embryonic cells and with higher rate of blastomeric apoptosis (Ott, Schernthaner *et al.* 2002) and chromosomal abnormalities (Vialard, Boitrelle *et al.* 2011). Indeed, embryos derived from these oocytes can develop aberrant mitotic spindles, leading to a failure of chromatid migration to the cell

poles during cell division, DNA replication without associated cytokinesis and consequently to an altered ploidy (Liu and Keefe 2002).

The lack of a significant difference in aneuploidy rates in the results of Experiment 2 and 3 suggests that maturation environment (oocyte maturation time and culture media supplementation) is less involved in the appearance of this kind of chromosomal abnormalities. In fact, aneuploidy have mainly been related to intrinsic factors of the humans (Frumkin, Malcov *et al.* 2008) and bovine oocytes (Lechniak, Świtoński *et al.* 1996; Nicodemo, Pauciullo *et al.* 2010).

Previous authors suggest that post-fertilization culture environment of the developing embryo can affect the incidence and severity of chromosomal abnormality rates in the resulting blastocyst (Lonergan, Pedersen *et al.* 2004; Ulloa Ulloa, Yoshizawa *et al.* 2008b; Xu, Wang *et al.* 2008). However, all of these studies evaluated the chromosomal complements of derived embryos at blastocyst stage. Dieleman, Hendriksen *et al.* (2002) suggest that the ploidy of the resulted embryos is less affected at early stages of development by the culture environment. It has previously been described (Mermillod, Schmaltz *et al.* 2010; Tajik, Beheshti-Govij *et al.* 2008) that long-term incubation induce developmental failures in embryos due to their exposure to higher levels of toxic metabolites and oxidative stress in late culture developmental stages.

In conclusion, morphological quality of oocytes and maturation protocols affect the chromosomal abnormality rates of the subsequent IVP cattle embryos. Further studies are necessary to improve the oocyte maturation protocols currently used in the *in vitro* cattle embryo production industry to minimize the appearance of chromosomal abnormalities on the subsequent produced embryos.

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Table 1: Analysis of chromosomal complements of day 3 IVP bovine embryos derived from oocytes classified according their morphological quality

Oocyte Quality	Total number of analyzed embryos	Chromosomal complements											
		Normal Embryos		Abnormal embryos									
		Diploid		Total		Haploid		Polyploid		Aneuploid			
A	60	56	93,33% [a]	4	6,67% [c]	1	1,67% [bc]	2	3,33% [b]	1	1,67% [b]		
B	65	53	81,54% [ab]	12	18,46% [b]	2	3,08% [b]	6	9,23% [ab]	4	6,15% [ab]		
C	61	45	73,77% [bc]	16	26,23% [ab]	6	9,84% [ab]	6	9,84% [ab]	4	6,56% [ab]		
D	63	39	61,90% [c]	24	38,10% [a]	10	15,87% [a]	8	12,70% [a]	6	9,52% [a]		

On each row, values followed by different letters (a, b and c) show statisticall significant differences (P<0.05, Expected proportion test).

234x50mm (200 x 200 DPI)

Table 2: Analysis of chromosomal complements of day 3 IVP bovine embryos from oocytes maturated by two different periods

Oocyte maturation time (hs)	Number of embryos analyzed	Chromosomal complements									
		Normal Embryos		Abnormal embryos							
		Diploid		Total		Haploid		Polyploid		Aneuploid	
24	77	71	92,21% [A]	6	7,79% [B]	2	2,60% [a]	3	3,90% [A]	1	1,30% [a]
36	80	60	75,00% [B]	20	25,00% [A]	6	7,50% [a]	12	15,00% [B]	2	2,50% [a]

On each row, values followed by different capital letters (A and B) show highly significant differences (P<0.01, Expected proportion test).

Values followed by different lowercase letters (a and b) show significant differences (P<0.05, Expected proportion test).

206x45mm (200 x 200 DPI)

Table 3: Analysis of chromosomal complements of day 3 IVP bovine embryos from oocytes maturated in different culture media

Culture media Suplementation	Number of embryos analyzed	Chromosomal complements									
		Normal Embryos		Abnormal embryos							
		Diploid		Total		Haploid		Polyploid		Aneuploid	
BSS	70	66	94,29% [a]	4	5,71% [c]	2	2,86% [b]	1	1,43% [b]	1	1,43% [a]
ACS	59	54	91,53% [a]	5	8,47% [c]	1	1,69% [b]	3	5,08% [b]	1	1,69% [a]
BAF	60	54	90,00% [abc]	6	10,00% [bc]	2	3,33% [b]	2	3,33% [b]	2	3,33% [a]
BSA	74	66	89,19% [ab]	8	10,81% [bc]	5	6,76% [ab]	3	4,05% [b]	0	0,00% [a]
FCS	59	46	77,97% [bcd]	13	22,03% [b]	5	8,47% [ab]	6	10,17% [ab]	2	3,39% [a]
ECS	62	37	59,68% [d]	25	40,32% [a]	11	17,74% [a]	12	19,35% [a]	2	3,23% [a]

On each row, values followed by different letters (a, b and c) show statisticall significant differences (P<0.05, Expected proportion test).

BSS: bovine steer serum; ACS: anestrous cow serum; BAF: bovine amniotic fluid; BSA: bovine serum albumine; FCS: fetal calf serum and ECS: estrous cow serum

206x66mm (200 x 200 DPI)