

## Cytogenetic Analysis of Cattle Oocytes Remaining Uncleaved after *in vitro* Fertilization

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**Abstract:** A cytogenetic study was performed on uncleaved cattle oocytes after *in vitro* fertilization. To assess the contribution of chromosome anomalies to failure of bovine *in vitro* fertilization, 1044 uncleaved oocytes were investigated cytogenetically after *in vitro* fertilization. Uncleaved oocytes were selected after 72 h of culture post IVF and fixed for chromosomal analysis. A total of 744 oocytes were fixed on slides. Successful chromosome analysis was carried out on 472 oocytes; the remaining 272 oocytes could not be evaluated. Eight (1.1%) were immature oocytes in the germinal vesicle stage. Another 44 (5.9%) in anaphase and 52 (7.0%) in Telophase I were considered immature oocytes. About 27.4% of oocytes had a normal haploid chromosome complement; 48 (6.4%) were hypohaploid; 20 (2.7%) were hyperhaploid and 4 (0.5%) were diploid. Ninety-two (12.4%) oocytes showed prematurely condensed chromosomes of the G<sub>1</sub>-phase from sperm, as well as a set of maternal anaphase I or metaphase II chromosomes. The sperm nuclei showed a remarkable variation in the degree of condensation. In conclusion, oocyte immaturity and premature condensation of sperm head chromosome caused considerable fertilization failure. The high rate of chromosomal abnormalities in the uncleaved oocytes represents natural selection that occurs in IVF when oocytes/zygotes with chromosome abnormalities fail to cleave.

**Key words:** Cattle oocytes % Fertilization failure % Sperm chromosome condensation % Aneuploidy

### INTRODUCTION

Despite the continuous improvement of *in vitro* fertilization (IVF) techniques, fertilization failure is a recurrent phenomenon in both human and animals. Chromosomal analysis of unfertilized oocytes produced from *in vitro* fertilization may improve understanding oocyte maturation and fertilization [1]. Oocyte meiosis is very sensitive to endogenous and exogenous factors that could result in oocytes with chromosomal abnormalities. Aneuploidy may be affected by factors that appear in the *in vitro* maturation (IVM) and *in vitro* fertilization system. Media used for *in-vitro* oocyte maturation may cause maturation delay and aneuploidy [2]. Aneuploid germ cell may be attributed to the chromosome non-disjunction at the first or second meiotic division during gametogenesis or anaphase lag [3]. Numerical aberrations of chromosomes in oocytes occur due to nondisjunction and anaphase lagging [4] and contribute to reproductive failure. However, there are also reports of aneuploid bull

primary spermatocytes that arose because of a non-disjunction process in mitotic cleavage of spermatogonia [5].

During metaphase meiosis or mitosis, the chromosomes become condensed under the influence of factors that appear in the cytoplasm. This cytoplasmic control of chromosome behavior has been investigated by Clarke and Masui [6] who found that cytoplasm of the maturing oocyte contains a powerful activity that can stimulate the translocation of many nuclei to a metaphase condition. The primary functions of mitotic chromosome condensation in eukaryotic cells are to reduce chromosome arm lengths so that they avoid truncation during cell division and to facilitate proper separation and segregation of sister chromatids [7]. During normal *in-vitro* fertilization sperm oocyte fusion is followed by incorporation in the cytoplasm of a demembrated sperm nucleus which immediately becomes accessible for ooplasmic factors, e.g. the maturation promoting factor [8, 9]. It has been well established in normal IVF that

cytoplasmic chromosome condensing factors of an oocyte arrested at metaphase II can induce a premature chromosome condensation in the penetrating sperm nucleus [10]. Prematurely condensed sperm chromosomes in the G<sub>1</sub> phase (G<sub>1</sub>-PCC) are not only observed in standard IVF, but also in intracytoplasmic sperm injection with an overall rate, varying between 4 and 28% [10-13].

Previously, failure to fertilize following *in vitro* fertilization had been thought to be due to a lack of sperm penetration through the zona and/or lack of membrane fusion with oolemma. With the introduction of intracytoplasmic sperm injection, the zona and oolemma barriers have been bypassed [14]. However, the normal cleavage rate for intracytoplasmic sperm injection fluctuates between 50-70%, no higher than the *in vitro* fertilization rate for non-male patients, indicating that the presence of a spermatozoon does not guarantee fertilization. Much cytogenetic research has been done on animal *in vitro* matured oocytes including buffalo [15-17], camel [18], cattle [19-21], goat and sheep [22] and pig [23] as well as unfertilized human oocytes [24-26]. However, there is a shortage in literature of cytogenetic data about animal unfertilized oocytes, especially cattle. According to the above information, this study aimed to analyse the chromosomes of unfertilized cattle oocytes to search for possible reasons for lower fertilization as determined by cleavage.

## MATERIALS AND METHODS

**Chemicals:** Chemicals and media were purchased from Sigma (St. Louis, MO) unless otherwise stated.

**Oocyte Recovery and Maturation:** Oocytes were aspirated from 3- to 8-mm follicles of abattoir-derived ovaries from feedlot heifers. Oocytes were matured in chemically defined medium (CDM) [27] supplemented with 0.5% fatty acid free bovine serum albumin (FAF-BSA; Sigma Chemical Co., St. Louis, MO, USA; A6003), 15 ng/mL NIDDK-oFSH-20, 1 mg/mL USDA-LH-B-5, 0.1 µg/mL E2, 50 ng/µL epidermal growth factor (Sigma E9644) and 0.1 mM cysteamine for 23 h at 38.5°C and 5% CO<sub>2</sub> in air.

**In-Vitro Fertilization:** Sperm were separated through a Sperm-Talp [28] Percoll (Sigma P1644) gradient and coincubated (0.5 X 10<sup>6</sup> sperm/mL) with matured oocytes in CDM fertilization medium supplemented with 0.5% FAF-BSA, 2 mM caffeine and 0.02% heparin (Sigma H9399) for 18 h at 38.5°C and 5% CO<sub>2</sub> in air. After fertilization, presumptive zygotes were vortexed for 60 s to

remove cumulus cells and then transferred into CDM1 which contained 0.5 mM glucose, 0.5% FAF-BSA, 10 µM EDTA and only nonessential amino acids (Sigma M7145). Embryos were cultured in 500 µL medium under paraffin oil containing 50 embryos in a 38.5°C, 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>, humidified incubator for 72h. Cleavage was assessed after 72 h of culture (day 0 = day of insemination). Unfertilized oocytes were defined as oocytes which failed to cleave after the day 3 of fertilization.

**Chromosome Preparation:** Degenerated and fragmented oocytes were excluded from this study. Slides of chromosomes were prepared according to the procedure described by Tarkowski [29]. Briefly, Each oocyte was transferred to 1% hypotonic sodium citrate solution for 10 min and then placed on a microscope slide with a minimal amount of hypotonic solution. Three drops of fixative (methanol: acetic acid, 3:1) were dropped onto the oocytes. Subsequently, the fixed material was stained with 1% orcein.

**Evaluation of Nuclear Status:** The state of chromosome was determined as described earlier by Mahmoud [30]. Germinal vesicle breakdown (GVBD) was considered to have taken place, when the chromatin material started condensing and was observed as isolated shrunken bodies. Separation of 2 chromosome sets towards opposite poles (anaphase I), completion of separation of 2 chromosome sets (telophase I) and MII emission of first polar body resulting in haploid set of chromosomes were reported. Abnormalities in metaphase II as aneuploidy (hypohaploid, hyperhaploid) and diploidy were recorded. The oocytes with less than 30 chromosomes were scored as hypohaploid. The oocytes with more than 30 chromosomes were scored as hyperhaploid. The oocytes with 60 chromosomes were scored as diploid oocytes. Prematurely condensed sperm head was a prematurely condensed chromatin mass. The observed degree of condensation varied between oocytes with all three degrees reported by Schmiady *et al.* [12], i.e. highly condensed, slightly condensed and decondensed structures observed.

## RESULTS

The outcome of our *in vitro* fertilization from where the failed fertilized oocytes were obtained is shown in table 1. The cleavage rate was 51.3% (1101/2145) including 51.8% good embryos and 48.7% poor embryos.

Table 1: Outcome of *in vitro* fertilization providing the source of uncleaved oocytes

Category	Number	%
Inseminated oocytes	2145	
Cleavage	1101	51.3
Good embryos	531	48.2
Poor embryos	570	51.8
Uncleaved	1044	48.7

Table 2: Summary of the cytogenetic status of 1044 uncleaved cattle oocytes that failed to cleave *in vitro*.

Uncleaved oocytes	Number	%
oocytes fixed on slide	744	71.3
Absence of chromosomes	124	10.9
Undefined chromosomes	148	19.9
Chromosomes suitable for examination	472	45.2

Table 3: Chromosomal status of metaphase in 472 uncleaved cattle oocytes

Metaphase II chromosomes	Number	%
1- Premature oocytes	104	14.0
Germinal vesicle breakdown (GVBD)	8	1.1
Anaphase I (AI)	44	5.9
Telophase I (TI)	52	7.0
2- Mature oocytes		
Haploid MII	204	27.4
3- Aneuploid oocytes	68	9.1
Hypohaploid	48	6.5
hyperhaploid	20	2.7
4- diploid oocytes	4	0.5
5- Premature chromosome condensation of sperm	92	12.4
Total number of oocytes	472	

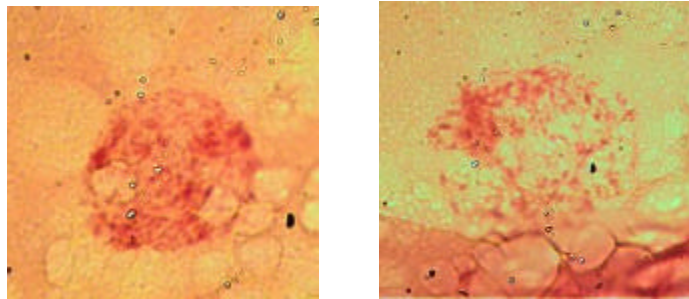


Fig. 1: Unfertilized bovine oocytes at germinal vesicle break down with different degree of chromatin condensation

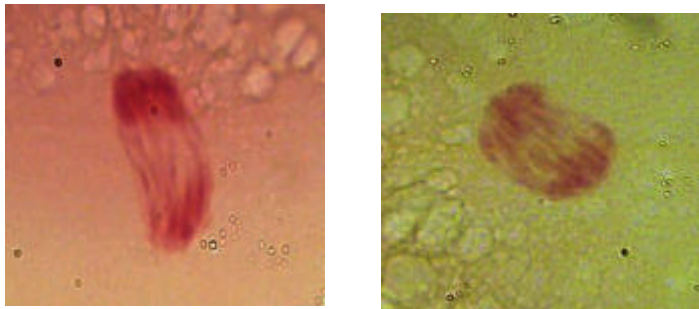


Fig. 2: Unfertilized bovine oocytes at anaphase I stage showing complete homologous segregation of chromosomes

A total of 1044 uncleaved oocytes were processed, of them 744 oocytes were fixed on the slides, but only 472 were analyzable. The remaining oocytes were lost during fixation. There were 148 oocytes (19.9%) that could not be analysed because of inferior quality. 124 (10.9) oocytes showed absence of chromosomes mainly due to degeneration (Table 2).

Details of the chromosome analysis are given in table 3. Photographs of chromosomes were obtained in 472 oocytes, eight (1.1%) were considered immature oocytes in the process of germinal vesicle breakdown (Figure 1). Another Forty- four (5.9%) oocytes were in anaphase of metaphase II (Figure 2) and fifty- two

(7.0%) showed Telophase I (Figure 3). About 27.4% of oocytes had a normal haploid chromosome complement (Figure 4-A); 48 (6.5%) were hypohaploid; 20 (2.7%) were hyperhaploid and 4 (0.5%) were diploid (Figure 4-B).

Ninety-two (12.4%) oocytes showed prematurely condensed chromosomes of the G<sub>1</sub>-phase from sperm, as well as a set of maternal anaphase (Figure 5.A) and metaphase II chromosomes (Figure 5.B). There were more than one set of sperm condensed chromosome in oocytes (Figure 6). Sperm nucleus with varying degrees of condensation termed highly condensed, slightly condensed and decondensed were observed in figure 7.

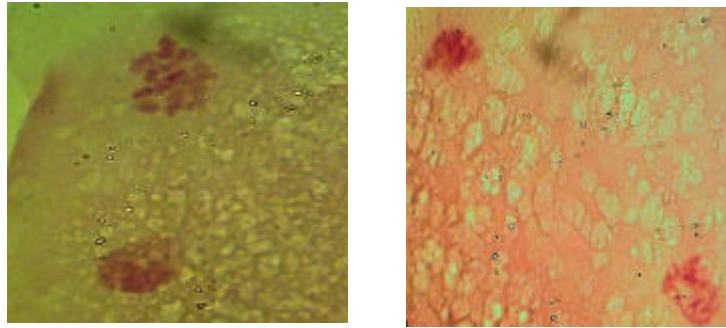


Fig. 3: Unfertilized bovine oocytes at Telophase I stage showing two groups of equally spread homologous chromosomes.

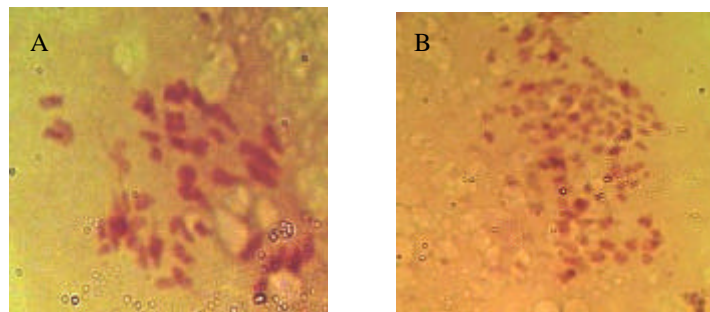


Fig. 4: Unfertilized bovine oocytes at Metaphase II Note the normal (A), diploid number (B).

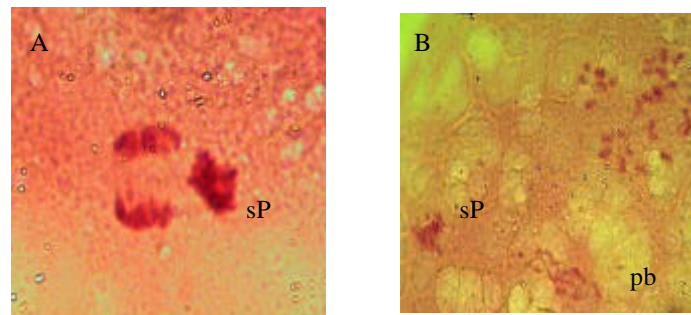


Fig. 5: Unfertilized bovine oocytes at (A) anaphase I chromosomes are shown beside condensed sperm chromosome, and (B) Metaphase II with polar body chromatin and condensed sperm chromosome.

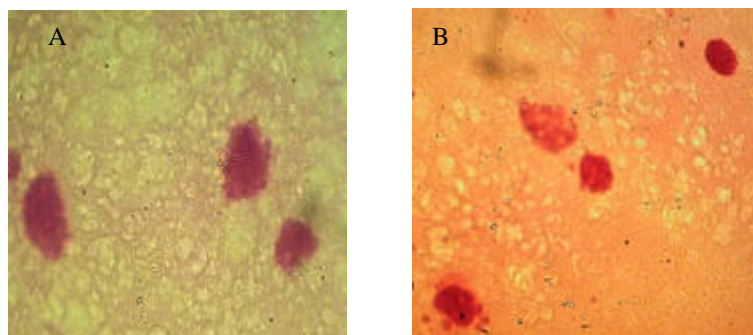


Fig. 6: Unfertilized bovine oocytes penetrated by three sets (A) and four sets (B) of sperm showing different forms of chromosome condensation

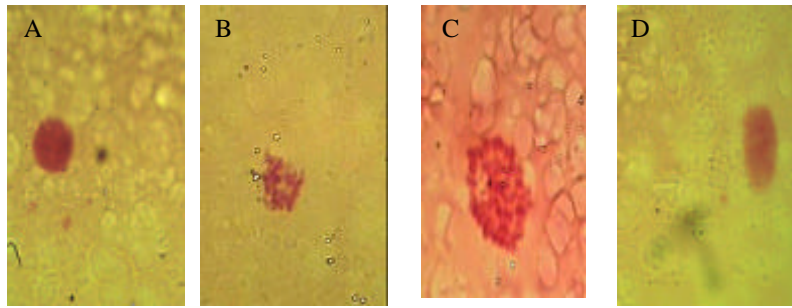


Fig. 7: Different forms of premature condensed sperm chromosomes (G1-PCC) of the sperm nucleus in unfertilized bovine oocytes showing varying degrees of condensation ; highly condensed (A), slightly condensed (B) , decondensed (C), intact sperm head; sp (D).

### DISCUSSION

In our study, the total cleavage rate on day 3 was 51.3%. Similar cleavage rate in cattle was obtained by Eckert and Niemann [31, 32]. Our percent of cleavage was lower than value reported by Lonergan *et al.* [33]. About 48.7% of oocytes remained unfertilized during *in vitro* fertilization, so it is important to examine this high percentage of unfertilized oocytes cytogenetically to search for possible reason for fertilization failure. During the cytogenetic analysis, about one third of oocytes were lost during fixation on the slides.

The configuration of meiotic chromosomes at the time of co-culture of oocytes with spermatozoa for *in vitro* fertilization has a direct influence on the final success of fertilization. The MII stage, which is also known as the second phase of meiotic arrest, is considered ideal for fertilization. In our study 14.0% of oocytes was immature. Windt *et al.* [34] reported a failure of fertilization by intracytoplasmic sperm injection in immature human oocytes arrested at MI and the injected sperm nucleus was arrested in the middle of the chromatin decondensation process, with no visible nuclear envelope reformation. The absence of complete cytoplasmic activation after sperm injection could also be due to a biochemical defect of the ooplasm unrelated to meiosis resumption [35].

The incidence of aneuploidy in our study is much lower than reported in human. Plachot [36] found 13.3% hypohaploidy, 8.1% hyperhaploidy, 3.5% diploidy in human unfertilized oocytes. Also, Djalali *et al.* [37] reported 27% aneuploidy in unfertilized human oocytes. They attributed this chromosome aneuploidy to non disjunction or chromosome lagging. Chromosome elimination into the perivitelline space appeared to take place following anaphase lagging. According to Hamerton [38], the failure of inclusion of chromosomes in either

daughter cell is defined as anaphase lagging. However, as to the fate of lagging chromosomes, Kato and Sandberg [39] observed that in human somatic cells *in vitro*, some of them might be included in the daughter cells to form micronuclei during interphase and are transformed into pulverized chromosomes at the following metaphase. The frequency of aneuploidy noticed in the present study (6.5% for hypohaploid and 2.7% hyperhaploid) falls into the range of results published for mature cattle oocytes, 5.8% [19] and 7.1% [40]. Our investigation revealed that the percentage of hypohaploid chromosome was more frequent than hyperhaploid ones, although the observed excess of hypohaploid may be attributed to artificial loss during chromosome preparation. Our low percentage of aneuploidy disagree with Mahmoud [30] in buffalo and Plachot [36] and Zhivkova *et al.* [26] in human who reported the main abnormalities accompanied by fertilization failure was aneuploidy.

The present study showed lower frequency of chromosomal diploidy compared to previously reported results on chromosomal analysis of unfertilized human eggs [24, 37]. The higher percentage of diploidy in humans probably due to use of hormones to induce superovulation during human oocyte recovery. In this respect, Tarin and Pellicer [25] found that women with greater numbers of oocytes had more diploid oocytes than women with moderate or mild response to gonadotropins. Diploid oocyte is one documented cause of triploid embryos. The embryos formed as a result of fertilization of such oocytes would fail to develop [41]. McFadden *et al.* [42] demonstrated that 75% of the examined human triploid fetuses were of digynic origin while only 25% were diandric. The occurrence of unreduced oocytes is due to failure of first polar body extrusion, or disturbance of mitotic cleavage in the germ cell line resulting in the production of tetraploid oogonia followed by normal meiotic division [43].

In our study, 12.4% of unfertilized oocytes had prematurely condensed sperm chromosomes. This is contrast to the higher frequency (23%) following intracytoplasmic sperm injection reported by Gook *et al.* [13] and 16.9% reported following failure of conventional IVF by Ma *et al.* [24]. The higher incidence of PCC is a sign of oocyte immaturity [43]. The prematurely condensed sperm chromosomes in the G<sub>1</sub>-phase were first observed in human oocytes by Schmiady *et al.* [10]. They reported a frequency of 3 to 4%. This phenomenon is thought to represent the permanent arrest of the oocytes at Metaphase II after sperm penetration with the continuing presence of cytoplasmic chromosome condensing factors leading to the induction of premature chromosome condensation in the sperm nucleus. A progressive decondensation of chromatin has been observed as cells move through G<sub>1</sub>-phase [44]. This indicates that the degree of condensation of PCC is mainly influenced by the cell cycle stage and the degrees 'I, II and III' might correspond to 'early, mid and late' G<sub>1</sub>-phase. The appearance of G<sub>1</sub>-PCC can be associated with oocyte immaturity [11, 45]. In our study, failure of fertilization of cattle oocytes mainly due to premature sperm chromosome condensation of the sperm head is associated with delayed maturation of oocytes. Many oocytes containing condensed sperm heads were in anaphase. Another aspect concerning decondensation of the sperm nucleus might depend on sperm chromatin anomalies as described recently by Schmiady *et al.* [46]. Schmiady and Neitzel [47] reported oocytes with condensed maternal metaphase I chromosomes and premature chromosome condensation of the sperm nucleus in two sisters descended from consanguineous parents underwent unsuccessful IVF treatments.

In conclusion, oocyte immaturity and decondensation of sperm chromosomes may be the main causes of fertilization failure. The high rate of chromosomal abnormalities that occurred in the unfertilized oocytes represents the natural selection which occurs in IVF against morphologically abnormal oocytes and consequently against oocytes with chromosome abnormalities.

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