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Original Research

Enucleation of Buffalo Oocytes: A Comparison of Methods

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Abstract

A comparative study of three enucleation methods; enucleation by pushing out small amount of cytoplasm beneath the first polar body, enucleation by bisectioning of oocytes, and enucleation by aspiration were carried out using the oocytes of Indian buffaloes. The statistical analysis of the results revealed that, there is no significant difference between the three enucleation methods. This information would be helpful for optimization of enucleation of recipient oocyte during somatic cell nuclear transfer.

Keywords: Buffalo; cloning; enucleation; nuclear transfer; oocyte

Introduction

The possibility of generating genetically identical offspring in livestock has evolved considerably during last few decades from the first embryo splitting experiments (Willadsen, 1979) up to the results obtained after the birth of Dolly, achieved with somatic cell cloning (Wilmut *et al.*, 1997). Since then, cows (Wells *et al.*, 1999) and mice have been cloned from adult cell donors. Enucleation of recipient oocyte is an important step affecting the developmental ability of cloned embryos and time consuming procedure in animal cloning (Peura *et al.*, 1998; Kawakami *et al.*, 2003; Savard *et al.*, 2004). The enucleated oocytes, after the removal of genetic materials, are used as the cytoplast recipient for nuclear transfer (NT). Presence of cytoplasmic lipids hinders visualization of metaphase chromatin in the unfertilized oocytes with the standard microscopy. Hence, the enucleation of oocytes has generally been accomplished by blind aspiration of cytoplasm near the first polar body, keeping the first polar body as a marker with a limited success rate (Prather *et al.*, 1987; Robl *et al.*, 1987; Bondioli *et al.*, 1990). The bottleneck of this approach is due to the fact that, in a number of cases, the metaphase plate is not close to the first polar body and removal of cumulus cells prior to the oocyte manipulation disrupts the relationship between the metaphase-II spindle and first polar body. Therefore, there is a necessity for the optimisation of enucleation methods.

In light of the above discussion, the present study was aimed to evaluate the performance of three enucleation methods using the buffalo oocytes. which included enucleation by pushing out small amount of cytoplasm beneath the first polar body, enucleation by bisectioning of oocytes, and enucleation by aspiration.

Materials and methods

The instrument and tools used in this study were prepared as reported in the literature (Wolfe and Kraemer, 1992).

Collection of Ovaries and Oocytes

Ovaries from the recently slaughtered buffaloes were collected from the slaughterhouse in Delhi, India. After thorough cleaning and washing, ovaries along with the fresh saline in thermos flask at 32-35°C were transported to the laboratory within 4-5 hours. The ovaries were again thoroughly washed in normal saline and dried by blotting. All the surface follicles of 3-10 mm diameter

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were aspirated in phosphate buffered saline with albumin (PBSA) medium using 5 ml plastic syringe (tissue culture grade) and a 19 gauge needle.

Gradation of Oocytes and Maturation of Oocytes

Gradation, maturation and cleaning of zona pellucida were done as described in the literature (Singla *et al*., 1997). The oocytes were graded into four grades: i) Grade A: Oocytes with a compact cumulus complex and 3 or more layers of cumulus cells; ii) Grade B: Oocytes with compact cumulus complex and 1-3 layers of cumulus cells; iii) Grade C: Oocytes with expanded cumulus complex and partially denuded cumulus cells; and iv) Grade D: Oocytes without cumulus cells.

Enucleation methods of buffalo oocytes

Method-I: Enucleation by pushing out small amount of cytoplasm beneath the first polar body

a) Zona cutting

The selected oocytes collected after washing were transferred to a 35 mm petri dish in such a way that they lie on one side of the microtool which can be pushed to the other side of the tools after processing. The oocytes were secured with the help of the holding pipette and the cutting needle was advanced through the middle of the holding pipette. The suction of the holding pipette was released and the cutting needle was rubbed against the edge of the holding pipette to cut a rent at an angle of 50°-60° in the zona pellucida. The cut in the zona pellucida was made near the fist polar body and, after zona pellucida cutting the oocytes were pushed to the other side to avoid its mixing with the unprocessed oocytes.

b) Bisectioning of oocytes

The oocytes after zona pellucida cutting were placed in PBSA containing 5 µg of cytochalasin-D (Sigma Chemicals Co., USA) per ml of medium (PBSA) and allowed to remain for few minutes prior to micromanipulation. Subsequently, the oocytes were held firmly with the help of the holding pipette and pushed with the help of a bisectioning rod, so that the cytoplasm oozed out and formed a figure of digit eight. When about half of the cytoplasm was out of the zona pellucida, the pressure through the bisectioning rod was released and the oocytes were bisected into two halves; one half remained within the zona and the other half came out and discarded. The bisected oocytes were transferred to PBSA without any cytochalasin-D (Singla *et al*., 1997).

Method-II: Enucleation by bisectioning of oocytes

After zona pellucida cutting the oocytes were carefully transferred to a 35 mm petri dish containing PBSA with 5 ug of cytochalasin-D per ml of PBSA and allowed to remain few minutes prior to micromanipulation. The cleaned and cut-extra-zona were added to the petri dish. The oocytes were secured by the holding pipette and, then, the bisection/suction pipette was introduced into the rent, which was positioned against the oolema. Bisection was accomplished by slow aspiration; if the pipette does not remain in complete contact with the oolema, aspiration may result in loss of small piece of ooplasm rather than clean bisection. When half of the ooplasm entered inside the bisection pipette, the pipette was removed from the perivitalline space and rubbed against the cut edge of zona pellucida to complete bisectioning. The demi-oocyte of the bisectioning pipette was transferred to an empty zona and both the demi and enucleated oocytes were set aside.

Method-III: Enucleation by aspiration

After complete maturation and zona cleaning, the oocytes having intact oolema were kept in 30 ul of TCM-199 containing 10% fetal bovine serum under paraffin oil for few minutes. The oocytes were held one by one by the holding pipette; then the sharpened beveled aspiration pipette was advanced and punctured through the zona pellucida near the first polar body. About half of the cytoplasm was removed and the pressure on the holding pipette was released, wherein the oocytes were set aside to avoid any mistake.

The Hoechst-33342 staining and fluorescent microscopy was used to ensure enucleation in the bovine oocytes (Westhusin *et al*., 1990).

Results

The total number of oocytes subjected to enucle-

Table I. The success rate of enucleation of in-vitro matured buffalo oocytes by different methods considered in the present study

Sl. No	Enucleation Method	Number of oocytes subjected to enucleation	Number of oocytes enucleated (%)
I	Pushing out small amount of cytoplasm	315	247 (81.8 ± 3.7)
II	Bisectioning after zona pellucida cutting with the help of the bisectioning pipette	318	253 (80.7 ± 2.8)
III	Aspiration of small amount of cytoplasm beneath the first polar body	299	249 (83.5 ± 2.6)

ation and the number of oocytes successfully enucleated is presented in Table-I. Among 315 demi-oocytes subjected to screening, 247 demi-oocytes were successfully enucleated (method-I), with a success rate of 81.8±3.7% (60%-93.6%). Similarly, out of 318 demi-oocytes, 253 demi-oocytes were successfully enucleated (Method-II). This method performed with the success rate of 80.7±2.8% (60%-90.7%) enucleation. Further, out of 299 demi-oocytes screened for enucleation, 249 oocytes were successfully enucleated (Method-III), with success rate of 83.5±2.6% (71.7% to 93%). Statistical analysis reveals that, at 5% level of significance, there is no significant difference between the three enucleation methods described herein.

Discussion

About 90% ooplasm of oocytes, after zona cutting, were bisected in which a success rate of 75% was achieved (Willadsen, 1986). Enucleation by aspirating ooplasm adjacent to the first polar body depends upon the amount of cytoplasm aspirated, i.e., when a half of the cytoplasm was removed as compared to a small amount of cytoplasm by using the sharpened beveled pipette, the probability of enucleation was higher in the former case than the later. About 70% of success of enucleation can be achieved by using the sharpened beveled pipette was reported (Prather *et al*., 1987; Yang *et al*., 1992). There was no significant difference in the percentage of embryo developing to the compact morula and early blastocyst, when the effect of reduction of the amount of cytoplasm available for an early embryonic development was evaluated (Westhusin *et al*., 1990). However, as compared to the result reported elsewhere (Prather *et al*., 1987; Yang *et al*., 1992; Chen *et al*., 2006), our result showed a significantly higher percentage of enucleation rate. Further, pushing down on the zona after zona cutting to expel some amount of cytoplasm along with the first polar body could also perform enucleation of oocytes. By this procedure, a considerable high rate (80%) of enucleation was achieved (Takano *et al*., 1993). In buffalo oocytes 88.2% of enucleation was reported (Singla *et al*., 1997). Conversely, this study showed 81.9% of enucleation rate which is comparable to the results as reported elsewhere (Yang *et al*., 1992), however, lower than the result as reported in buffalo (Singla *et al*., 1997). On the other hand considerable high rate (95%) of enucleation was achieved by spindle imaging system (Chen *et al*., 2006).

Conclusion

There is no significant difference in the success rate of enucleation of in-vitro matured buffalo oocytes by pushing out small amount of cytoplasm beneath the first polar body, bisectioning of oocytes, or aspiration. Further investigation is required to test the suitability of these methods in terms of ability of the enucleated oocytes to support embryonic development.

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