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Full Length Research Paper

Application of *in vitro* production-embryo transfer in the protection and development of lactational potential of superior cows

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This study was carried out to investigate if the technology of *in vitro* embryo production can be used for protection and development of milk productivity potential of the superior cattle via ablation. The ovaries of the superior cattle selected for ablation were collected immediately after slaughter. Bovine immature oocytes were then used for *in vitro* maturation/fertilization and the presumptive zygote were then cultured for about eight days in Vero-TCM culture medium before being transferred into previously synchronized heifers of low genetic merits. From 21 culled superior cattle, the highest causes of culling were related to reproductive problems (33.3%), lowest milk production was related to systemic diseases (7824), highest average of offspring production expected and number of offspring production were 9.2 and 7.4 in old cows, respectively and the highest average of unproduced offspring were three in reproductive problem cows. The highest average oocytes collection was related to old cows and rate of cleavage was significant in each four groups. Systemic diseases group was significantly lower (34.2%) than the reproductive problems (69.6%) and mastitis (60.6%) groups in 5 to 8 cells stage. Systemic diseases group was significantly lower (36.6) than the reproductive problem (73.9) groups in 8 to 16 cells. Morula rate was 71.8% in the reproductive problems group and the rate of blastocysts old cows was significantly higher (34.2%) than those of the other groups. Two established pregnancies (25%) and one viable offspring (50%) resulted from eight blastocysts transferred. The advances of assisted reproductive technology (ART) can effectively protect and multiply the superior genes of lactation and reproduction within the dairy farms.

Key words: *In vitro* production, embryo transfer, genetics potential, dairy cattle.

INTRODUCTION

Since the birth of the first calf derived from *in vitro* fertilization (IVF) in 1981, considerable progress has been made in the development of techniques for *in vitro* production (IVP) of bovine embryos for both research and commercial purposes (Brackett et al., 1982). IVP bovine embryos have emerged as a reliable alternative method to conventional ovulation induction techniques and an important tool to study pre-implantation embryo development. However, there is growing evidence that the bovine is better for the human embryo; with regards

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Abbreviations: TCM, Tissue culture medium; **ART,** assisted reproductive technology; **IVF,** *in vitro* fertilization; **IVP,** *in vitro* production; **MOET,** multiple ovulation embryo transfer; **COCs,** cumulus–oocyte complexes; **FCS,** fetal calf serum.

to the timing of genome activation, intermediate metabolism, and interaction with the culture medium (Wrenzycki et al., 2001). Considerable progress has been made in the development of techniques for IVP of bovine embryos for both research and commercial purposes, but the success rates in terms of blastocyst yield remain modest and range between 30 and 40% which is still lower than that obtained from embryos produced *in vivo*. Furthermore, the quality of IVF embryos is inferior to that of embryos produced *in vivo* as judged by morphology, increased susceptibility to cryo-injury and poor implantation and viability. Various factors including the quality of oocyte, protein source, somatic cells, culture media, oxygen tension, number of embryos per culture unit (embryo density), and energy substrate, may affect preimplantation embryo quality and its competence in further development *in vivo* (Nasr-Esfahani et al., 2008; Ferré et al., 2002; Moulavi et al., 2006).

Considering the genetical progress in the industrial husbandry particularly in animal's production and reproduction, high genetic merit cows were culled in industrial herd suddenly and these may be due to mismanagement, reproductive problems and diseases such as infertility, mastitis, lameness, infectious and metabolic disease. Although the mentioned cases caused the cull, ovary potential could be utilized in culling cow by using laboratory techniques. In this relation, we can protect and multiply high genetic merit cows that would be selected for culling by the application of the current techniques of ART (Assisted Reproduction Technology) and methods such as MOET (Mapletoft and Hasler, 2005; Lambert, 2003). The objectives of this study were to evaluate the reason of culling in high genetic merit cows and also, the use of *in vitro* production-embryo transfer technique (IVP-ET) in industrial husbandry for the protection and development of the genetic merit of superior cows.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma chemical Co. (St. Louis, Mo, USA), unless otherwise indicated**.**

Culture media

The media used for maturation of oocytes and development of zygotes *in vitro* were based on tissue culture medium (TCM) 199 supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). For maturation, TCM 199 was enriched by routinely used hormones FSH (10 µg/ml), LH (10 µg/ml) and 17-β-oestradiol (1 µg/ml).

Establishment and maintenance of Vero cell monolayer

For the establishment and maintenance of Vero cell monolayer, the method described by Moulavi (2006) was used.

Experimental design

After coordination with two industrial husbandry complexes, superior cows exposed to cull were transferred to the slaughter-house. Ovaries of superior cow were collected at abattoirs immediately after slaughter and transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (200 IU/ml), and streptomycin (200 µg/ml) at 35°C, within 2 to 3 h. At the laboratory, the cumulus–oocyte complexes (COCs) from 2 to 8 mm follicles were collected by Slicing method with a scalpel blades and a disposable syringe containing H-TCM and FCS (10 µg/ml) + heparin (10 IU/ml) at 38.5°C. Only oocytes having a homogenous evenly granulated cytoplasm were surrounded by a compact cumulus oophorus with more than two layers that were selected under stereomicroscope and washed in H-TCM+FCS (10 µg/ml) medium three times, after which they were transferred into maturation medium containing TCM199 supplemented FCS (10 µg/ml), HMG (0.1 IU/ml) and 17-β-Estradiol (1 µg/ml). Every five COCs were placed in 100 µl maturation droplets over Vero cells (Moulavi et al., 2006). Maturation was carried out in 90% humidity at 38.6°C and 5% CO_2 in air in an incubator (Labotect C200, Germany).

Determination of maturation status

At the end of the maturation period, oocytes maturation was determined by physiologic parameters such as expansion and modified cumulus cells (Mori et al., 1988).

Sperm preparation and IVF

Commercially distributed frozen semen from Holstein bulls with proven fertility was used throughout this study. IVF COCs were washed twice and transferred to groups of 25 to 30 per 200 µl drop of fertilization medium under mineral oil. The IVF medium consisted of fert-TALP supplemented with heparin (10 µg/ml), penicillamine (20 µM), hypotaurine (10 µM) and epinephrine (1 µM). After thawing semen in 37°C water, motile spermatozoa were obtained by swim up procedure and were added to the fertilization drop in a final concentration of 1×10^6/ml. Spermatozoa and oocytes were co-incubated for 20 h at 38.6°C with 5% CO_2 in humidified air.

In vitro culture and embryo evaluation

Following fertilization, presumptive zygotes (note that in bovine embryos, the two-pronuclear stage is not observed, so cleavage is presumed to indicate fertilization) derived from matured oocytes were washed and then the presumptive zygotes were cultured over Vero cells in TCM 199 plus 10% FCS at 90% humidity, 38.6°C in 5% CO_2 and 5% O_2 for a period of 8 to 9 days (day 0 is considered as the day of insemination). During the whole period of embryo culture, the embryos were transferred daily to the new culture dish containing established Vero cells. This transfer was for the reason that the Vero cells were exposed to trypsinization shock and released stress non-specific proteins that do not have beneficial effects on the ability of survival of embryos.

Evaluation of embryos

Embryos were evaluated daily and scored as cleaved, 8 to 16 cell, compact morula and early/ expanded blastocyst stage on day 2, 3, 5 and 6 to 9, respectively. The data were presented as percentage of cleavage rate and calculated by dividing the number of presumptive embryo on day 2 to the number of inseminated oocytes. The percentage of the embryo developmental stages was

Table 1. The characteristics of superior cows culled.

Reason of cull	Number of donor cow (%)	Average of production/whole lactation (kg)	Average of age (year)	Average of expected production offspring (calf)	Average of produced offspring (calf)	Average of non- produced offspring (calf)
Reproductive	7 (33.3)	9714	6.8	5.8	2.8	3
Udder system	4 (19.1)	10875	6.5	5.5	4.5	1
Systemic diseases	5 (23.8)	7824	5.4	4.4	3.2	1.2
Age	5 (23.8)	9500	10.2	9.2	7.4	1.8

calculated by dividing the number of embryos in that stage with the total number of presumptive embryos. The blastocysts were graded to 1 and 2 on the basis of morphology and cell appearance and were then transferred to low genetic merit Heifers.

Embryo transfer

Heifer's recipient was taken from low genetic merit and was estrous synchronized by Ovsynch method (Youngquist et al., 2007). The high quality blastocysts were transferred to the uterus on the basis of active corpus luteum in ovary (Rivera et al., 2005). The pregnancy diagnosis was evaluated in different stages (28th and 42nd day, and 3rd and 7th month) by ultrasonography and palpation of embryonic vesicle via the rectum.

Evaluation of superior cows (donor oocytes)

In this study, 21 superior cows were culled and categorized on the basis of culling, causing four groups of reproductive problems, udder system problems, systemic diseases and age. According to the recorded characteristics, milk production, age, average number of expected production offspring, number of produced offspring and number of non-produced offspring were evaluated. However, average number of expected production offspring was estimated as:

$$\frac{\text{Average age of cow at the time of cull- 1 (age at first insemination)}}{\text{Needed time to produce a calf and enter to the next cycle for calf production}}$$

Statistical analysis

The data were registered in excel software and analyzed by using the Genmod logistic regression for the statistical package SAS (1997). Then, the least squares mean were compared for various groups by chi square test at 5% level.

RESULTS

From large industrial herds in Esfahan province (city of Iran), the numbers of 21 superior and high genetic merit cows selected for culling were evaluated. Table 1 shows the characteristics of the superior cow's culled. The highest causes of cull were related to reproductive problems (7, 33.3%) and then, systemic diseases (5, 23.8%), udder system problems (4, 19.1%) and age group (5, 23.8%). Also, the table indicates that the average milk production in cows culled were the same in the reproductive problems, udder system problems and age groups approximately, but the average milk production was different in systemic diseases group (7824 kg) as compared to the other groups. However, the average age of cows culled in the reproductive problems, udder system problems, systemic diseases and age groups were 6.8, 6.5, 5.4 and 10.2, respectively.

Average of the expected production offspring in the reproductive problems, udder system problems, systemic diseases and age groups were 5.8, 5.5, 4.4 and 9.2, respectively; though the reproductive problems, udder system problems and systemic diseases groups were much lower than the age group.

Average of produced offspring was 2.8, 4.5, 3.2 and 7.4 for the reproductive problems, udder system problems, systemic diseases and age groups, respectively; while the average of non-produced offspring was 3, 1, 1.2 and 1.8 for the reproductive problems, udder system problems, systemic diseases and age groups, respectively. This expression has shown that udder system problems, systemic diseases and age groups can produce more calves relatively.

Table 2 shows embryo development of superior cow. In this table, it was observed that the highest number of oocytes was found in the age group (146), followed by the reproductive problems group (128), systemic diseases group (125) and the udder system problem group (51), respectively.

Cleavage rate was highest in the udder system problem group (39.2%) than the other groups, but there was no significant difference between all the groups.

The rate of 5 to 8 cells was lowest in the systemic diseases group and there was significant difference in the reproductive problems and udder system problem groups (34.2 vs. 69.6 and 60.0%). The rate of 8 to 16 cells was highest in the reproductive problem group and there was significant difference in the systemic diseases and age groups (73.9 vs. 36.6 and 39.5%), but there was no significance in the udder system problem group (73.9 vs. 55.0%).

Morula rate was significantly different in the reproductive problems group as compared to other groups (71.8 vs. 35.0, 19.6 and 10.6%) at day 5. However, in the

Table 2. Embryo development of superior cows.

Donor groups on the basis of cull reasons	Number of collected oocytes[1]	Embyo development stage (%)				
		Cleavage	5 - 8 Cells	8 - 16 Cells	Compact morula	Blastocyst
Reproductive	128	46(35.9) [a]	32(69.6) [a]	34(73.9) [a]	33(71.8) [a]	6(13.1) [b]
Udder system	51	20(39.2) [a]	12(60.0) [a]	11(55/0) [ab]	7(35.0) [b]	0(0.0) [c]
Systemic diseases	125	41(32.8) [a]	14(34.2) [b]	15(36.6) [b]	8(19.6) [bc]	4(9.7) [b]
Age	146	38(26.1) [a]	19(50.0) [ab]	15(39.5) [b]	4(10.6) [c]	13(34.2) [a]

[1]COCs collected from ovaries in each group. [a - b] Means within a row with different superscripts differ ($P < 0.05$); *donor groups on the basis of cull reasons.

udder system problem group, there was significant difference compared with the age group (35.0 vs. 10.6%).

Blastocyst rate was highest in the age group (34.2%) and there was significant difference as compared to the other groups. The rate of blastocyst was related to the reproductive problems and systemic diseases groups (13.1 and 9.7% respectively), and there was significant difference in the udder system problem group (0.0%). Altogether, 23 blastocysts were produced from 450 COCs (5.1%).

Following IVP, the eight high quality blastocysts were transferred to eight low genetic merit heifers and then, these resulted to two pregnancies. In this study, one pregnancy was aborted for an unknown reason at four months of gestational ages, and after 278 days, the other pregnancy led to parturition.

DISCUSSION

The results of this study showsthat embryo production technology can preserve the genetic potential of superior cows by COCs extraction of ovaries and production of embryo *in vitro*. Also, the results of IVF and embryo development show that this technology could produce blastocysts using the ovarian reserves of superior cows (Mapletoft and Hasler, 2005).

In this study, 16 cows from the 21 cows were exposed to culling for reproductive problems, udder system problem and systemic diseases. In fact, this expression showed that although the total cattle housing system was optimal, more than 75% of high genetic merit cows were involved in vital disease (Fourichon et al., 2000). Recent researches show that high production is a major factor in the long-term stress.

The long-term stress resulted in unstable animal health condition against pathogens and disease. The reproductive problems, udder system problem and age groups of average milk production manifested that stress exists in the cows and this is a very good record and important basis for decisions about the quality genetic cows. However, despite a very good pedigree about cattle genetic, low milk production in the systemic diseases group may be due to the negative effect of diseases (Moore et al., 1992; Dohoo and Martin, 1984).

Live offspring produced is an important factor in the development of animal husbandry. In this context, generator cows at the end of their first year of life are prepared for artificial insemination and pregnancy. Also, it was expected that each generator cow with high genetic merit produce a live calf with 10 tons of milk at the end of each year.

According to Table 1, it can be seen that although the average milk yield of the superior animal examined in this study was 10 tons approximately, the average production of progeny was expected to be lower than that of the other groups.

On the other hand, in the reproductive problems group, the average of expected production offspring and average of production offspring was 5.8 and 2.8; indicating three calves low production at an economic long-life for each cow. However, udder system problem, systemic diseases and age groups produced 1, 1.2 and 1.8 calves less than expected, respectively. Due to attention treatment management strategy of cows in the industrial husbandry, the probable reasons for this marked difference in the number of expected production offspring and production offspring can be presented as follows:

1) In the reproductive problems group, drug therapy was done for the eradication of problems and it had a high successful level on the reproductive system problems. This led to frequent examinations and treatments; as such, the cow was not pregnant at the prolonged time until it was culled for infertility and reproductive inability (Fourichon et al., 2000). This result shows increases in the number of expected non-production offspring cows (three calves).

2) Udder system problems group are prevalent in high production cows; so, these cows need to be culled. Although this resulted to a reduction of the cows' economic age, they were exposed to pregnancy and culling period in the husbandry, and this reduced the number of expected non-production offspring cows (1 calf) (Moore et al., 1992, Dohoo and Martin, 1984). 3).

In the age group, there were relatively good average production offspring, but increasing "days open" and age

cows provided the cause for culling (Dohoo and Martin, 1984; Walti et al., 2004). However, the results of Table 1 indicate that despite optimal management of industrial husbandry, high production resulted to diseases; as such, these cows were culled (Fourichon et al., 2000). Thus, the IVP-ET technology can provide proper potential for beneficial reproduction of these cows.

Embryo developmental results in Table 2 indicate that the relative number of oocytes extracted was dramatically lower than that of all other groups in udder system problems. Although the exact cause is unknown, it appears that the high production increased the metabolism of reproductive hormones affecting ovarian activity and the occurrence of mastitis that resulted to low dry matter intake, which affected the estrous cycle. Therefore, the active capacity of ovaries was inactive and was found in a non-functional state (Santos et al., 2004). This result shows that despite the use of the slicing system for extracting the highest number of oocytes, the number of oocytes obtained was significantly lower than that of all the other groups.

In this study, the amount of cleavage in the groups was about 26.1 to 39.2 with a mean total of 33.5%, and was significantly lower than that of other researches. Although, the cause is not clear, it seems that the following two causes could be responsible for it:

1) In this study, oocytes was derived by slicing of ovaries, and therefore, it is possible that the extracted part of preantral oocytes, or oocytes in different phases of degeneration or without fertilization can be possible by this method (Rocha et al., 2006).
2) In this study, donor cows were exposed to chronic stress due to high production and it resulted in negative potential ovaries for embryo development (Mee et al., 2000).

Table 2 indicates that the rate of blastocysts was highest in the age group (34.2%), which was significantly higher than that of the other groups. Conversely, the rate of blastocysts was lowest in the udder system problems group. Although, the reasons for this difference are not clear, it seems that cows having the udder system problems were affected by the quality of reproductive and ovarian activity. Thus, it is not only the number of oocytes extracted that influenced the effect of cows found with the udder system problems which led to the loss of blastocysts' production in this group, but also the embryo development including cleavage, 5-8 cells, 8-16 cells and morula (Soto et al., 2003).

Embryo transfers of 8 blastocysts to recipient cows led to 25% pregnancy and 12.5% live born offspring. An overview of similar studies by other researchers showed that embryo transfer capability was 55 to 75% with live birth of offspring as 55 to 65%, indicating significant difference with our results in this study (Youngquist et al., 2007).

Among the various factors, two basic factors affected the low capability of IVP-ET in this study:

1) Lack of optimal selection systems of recipients suitable for embryo transfer: the recipient cows were chosen on the basis of oestrus apparent symptoms, so there was the possibility of error in precision timing oestrus; as such, it was chosen as the best recipient on the basis of uterine and ovarian examination (Youngquist et al., 2007).
2) *In vitro* studies showed that only about 55 to 65% of the embryos developed *in vitro* can have hatching capability, and so, there was no hatching capability for 35% of the embryos (Nasr-Esfahani and Johnson, 1991).

In conclusion, this study shows that IVP-ET technology can effectively protect and multiply the superior genes, and extra calves can be produced per one superior cow and could be used in traditional and semi-industrial husbandry.

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