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Inherent inferior quality of follicular fluid in repeat breeder heifers as evidenced by low rates of *in vitro* production of bovine embryos

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ABSTRACT

The aims of the present study were to determine the effect of follicular fluid obtained from the ovulatory follicle of repeat breeder heifers on *in vitro* oocyte maturation (Experiment 1), fertilization (Experiment 2) and production of bovine embryos (Experiment 3). Holstein virgin heifers (VH, n = 5) with normal fertility or repeat breeder syndrome (RBH, n = 5) were used in the present study. Follicular fluid of VH and RBH was aspirated from ovulatory follicles and used as maturation medium. Bovine oocytes were aspirated from follicles of slaughterhouse ovaries and randomly allocated in three groups; in Group 1, oocytes cultured in TCM-199 supplemented with 10% heat-treated fetal calf serum and hormones (5 IU/mL hCG plus 0.1 IU/mL rFSH); in Group 2, oocytes cultured in TCM-199 supplemented with 10% filtered follicular fluid of VH without hormones; in Group 3, oocytes cultured in TCM-199 supplemented with 10% filtered follicular fluid of RBH without hormones. The mean (±SEM) percentage of matured oocytes was different between VH and RBH groups (72.2 ± 4.0 vs 56.4 ± 4.6%; $P < 0.05$, respectively). Further, the mean (±SEM) percentage of normal oocyte fertilization was higher in the VH than the RBH group (49.3 ± 2.1 vs 32.0 ± 4.2; $P < 0.05$, respectively). The mean percentage of embryos developed to the blastocyst stage was higher in the VH than the RBH group (12.0 ± 1.3 vs 7.0 ± 1.6; respectively; $P < 0.05$). In conclusion, our findings support our hypothesis that the ovulatory follicle microenvironment of Holstein repeat breeder heifers places their oocytes at a developmental disadvantage compared with Holstein fertile virgin heifers and that this suggest the existence of an inherent inferior quality of the ovulatory follicle microenvironment in repeat breeding Holstein heifers.

1. Introduction

Repeat breeding syndrome (RBS) is one of the most important reproductive disorders in dairy cows. Increased days open, more number of services per conception and high risk of early involuntary culling are important consequences of RBS in dairy cows. All these consequences significantly affect the profitability of dairy industry [1,2,36]. RBS cows are clinically healthy and have normal estrous cycles (18–24 days). These animals do not have palpable anatomical reproductive abnormalities or palpably detectable uterine infections, however they do not conceive after either at least three times continuous artificial inseminations or natural mating using semen from well-known fertility bulls [3]. Incidence of RBS in dairy cows has been reported from 9% in the UK [45] to 10% in Sweden [1], 25% in Spain [44], 14% in Japan [47], 22% in Michigan, the USA [46], and to 62% under tropical condition, in Indonesia [43]. Today's dairy cattle industries are faced with a high milk yield and concomitantly, deterioration in reproductive performance in different parts of the world [4,37]. Subclinical endometritis [5], nutritional deficiencies [6], abnormal heat behavior or improper heat detection [7,8], mis-management in artificial insemination [9], and endocrine dysfunctions [8] are considered as potential causes of the RBS in dairy cows.

In well managed dairy herds and particularly in dairy heifers, it would be difficult to find an explanation for the occurrence of RBS. Therefore, the reasons for subfertility in some dairy cows or heifers could be assumed to lie within the individual animal as intrinsic factors [10]. This assumption is further strengthened by the fact that based on the results of epidemiological studies [1,11]; some repeat breeder cows or heifers may eventually become pregnant; however there is a risk of the re-occurrence of RBS in subsequent lactations. Dairy heifers do not have the stress of milk production and its associated metabolic disorders, therefore, they are a suitable

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model for studying the possibility of the presence of intrinsic factors as the potential causes for the occurrence of the RBS. In a series of studies it was showed that suprabasal progesterone levels, abnormal follicular dynamics, delayed ovulation, and inferior quality of oocytes are responsible for subfertility in RBS Holstein heifers [10]. The role of follicular fluid in normal oocyte maturation has been of interest to many researchers in various species including mares [12], humans [13,26]; and cows [14,15]. However, there is no study to determine the effects of follicular fluid of ovulatory follicles of repeat breeder heifers on *in vitro* development of bovine embryos. Therefore, we hypothesized that follicular fluid of ovulatory follicles in repeat breeder heifers has an inherently inferior quality for *in vitro* development of bovine oocytes. The specific objectives of the following study were to determine the effects of the follicular fluid obtained from ovulatory follicles of virgin and repeat breeding Holstein heifers on *in vitro* oocyte maturation (experiment 1), *in vitro* fertilization (experiment 2) and *in vitro* development of bovine embryos (experiment 3).

2. Materials and methods

2.1. Experimental design

This study was approved by the ethical and research committee of the School of the Veterinary Medicine, Shiraz University (94GCU6M1251). Follicular fluid samples from the ovulatory follicles of clinically healthy virgin and repeat breeder Holstein heifers were collected 6–12 h after estrous detection. The aspirated follicular fluid samples were pooled and then used in the maturation medium for bovine oocytes in each experimental group. The experimental model used in the present study was based on bovine cumulus-oocyte complexes (COCs) recovered from follicles of Holstein cow ovaries obtained from a local slaughterhouse.

In experiment 1, *in vitro* maturation of bovine oocytes was performed in the presence of follicular fluids either collected from the ovulatory follicle of VH or RB heifers. Cumulus cell expansion and oocyte nuclear maturation rates were determined at the end of maturation.

In experiment 2, oocytes matured *in vitro* in the presence of follicular fluid of either the ovulatory follicle of VH or RB heifers and then co-cultured with sperm cells for 18 h and subsequently the nuclear progression of the fertilized oocytes were determined using aceto-orcein staining.

In experiment 3, *in vitro* maturation and fertilization of the oocytes were performed as mentioned in experiments 1 and 2. Then, the presumptive zygotes were cultured for 8 days. The proportion of zygotes that cleaved to the 2- to 4-cell stage and developed to morula and blastocysts was recorded on days 2, 6, 7 and 8 after fertilization, respectively. In addition, blastocyst quality was examined by a double-staining procedure to count the total cell number.

2.2. Collection of follicular fluid from ovulatory follicles

All heifers used in the present study were selected from a high producing Holstein dairy herd with the same schedule of feeding and ration. Manual rectal palpation and trans-rectal ultrasonography did not reveal any detectable pathology in the reproductive tract of the heifers. The repeat breeding heifers (RBH; n = 5) had a history of at least five unsuccessful artificial insemination using semen from high fertile bulls. The reason for using repeat breeder heifers with such a high number of previous unsuccessful artificial inseminations was to rule out the possible causes of subfertility like improper heat detection, semen handling and management in artificial insemination. Virgin heifers (VH; n = 5) in good body condition score were also selected as control. The duration of the estrus cycle of heifers ranged from 19 to 21 days. The estrous cycle of Holstein VH and RBH was synchronized using two injections of PGF2α (500 μg Cloprostenol sodium, Estroplan Parnel Living Science) 11 days apart. Six to 12 h after detection of the standing estrus, ovarian ultrasonography was performed to assess the presence of an ovulatory follicle (12–17 mm diameter). Then, a sample of follicular fluid was aspirated trans-rectally using a long fine-needle covered by a hard plastic tube under the caudal epidural anesthesia (2% Lidocaine hydrochloride; 0.2 mg/kg). In addition, a blood sample from coccygeal vein was also collected to confirm estrus. Blood samples were centrifuged at 300×*g* for 10 min and then serum was separated. All samples of sera and follicular fluids were frozen at −20 °C until assayed for progesterone, estradiol-17β and lipopolysaccharides (LPS). All VH and four RBH were inseminated after our samplings in their next estrus. One of the RBH was culled from the herd. The VHs were diagnosed as pregnant after the first artificial insemination, however, the RBH did not become pregnant.

2.3. Progesterone and estradiol-17β assay

Serum and follicular fluid progesterone and estradiol-17β concentrations were determined using validated radio-immunoassay commercial kits (Immunotech kit, France). A parallelism test was designed to measure follicular fluid progesterone and estradiol-17β concentrations. Known concentrations of hormone standards were added into the samples and the recovery percentages were calculated. The sensitivity of the tests and the recovery rate for progesterone and estradiol-17β were 0.05 ng/mL and 85–98%, 0.5 pg/mL and 85.5–97%, respectively. Based on the results of the hormone assay, the mean (±SEM) serum progesterone and estradiol-17β concentrations were 0.48 ± 0.07 ng/mL and 65.7 ± 8.5 pg/mL in VH and 0.65 ± 0.09 ng/mL and 52.5 ± 7.2 pg/mL in RBH ($P > 0.05$), respectively. These results showed that all heifers used in this study were in estrus at the time of follicular fluid sampling and therefore, we separately pooled the follicular fluid samples of the ovulatory follicles in VH and RBH. The pooled follicular fluid of each group of heifers was centrifuged (300×*g* for 10 min) and then filtered and kept frozen at −20 °C until used in each replicate of *in vitro* oocyte maturation. The follicular fluid samples were all free of blood contamination.

2.4. Assay of LPS in follicular fluid

Follicular fluid LPS concentrations were determined using a commercial LPS ELISA assay Kit (Hangzhou, Eatpharm Co., LTD, China) following the manufacturer's guidelines. The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of LPS in serum, plasma and other related tissue liquid samples. All samples including the standard, blank and follicular fluid were assayed in duplicate. Internal recovery as determined using positively spiked follicular fluid samples was >80% and the intra- and inter-assay coefficients of variation were <10 and < 12%, respectively. The minimum detectable concentration of LPS was 5.25 EU/L.

2.5. Experiment 1: in vitro maturation of bovine oocytes

Bovine ovaries (*Bos Taurus*) collected at a local abattoir were transported to the laboratory within 2–3 h in sterile normal saline maintained at 34–36° C. Upon arrival at the laboratory, ovaries were washed three times in pre-warmed (37° C) sterile phosphate buffered saline. The COCs were recovered from healthy antral follicles (2–6 mm in diameter with smooth, transparent outer walls)