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Effect of Recombinant Human Follicle Stimulating Hormone on Meiotic Competence of *In Vitro* Grown Nili Ravi Buffalo Oocytes

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***Abstract*—**In the present study, the response of Nili Ravi buffalo oocytes to recombinant human follicle stimulating hormone (rhFSH) (Organon) on meiotic maturation *in vitro* was examined. Oocytes were matured in vitro in medium containing either 0 or 0.05 IU/ ml rhFSH and the stage of nuclear maturation recorded after 24 hours. The percentage of oocytes in the control group undergoing germinal vesicle breakdown (GVBD) observed after 24 hours of culture was 29 % whereas as in rhFSH group the percentage was 10 % were at this stage (P< 0.001).Thus in the presence of rhFSH, a significantly greater number of oocytes had progressed to the more advanced stages of nuclear maturation. Indeed, the maturation of GV (Germinal Vesicle) stage oocytes to the metaphase II (M II) stage after 24 hours was significantly (P< 0.0001) increased by the addition of rhFSH (82 % VS 47 %). The percentage of degenerated oocytes after 24 hours of culture was 24 % in control group, whereas in rhFSH group the percentage was 8 % after 24 hours. Degeneration of the oocytes after 24 hours was not significantly (P = 0. 9361) decreased.

***Keywords*—**Buffalo, in *vitro*, oocytes, recombinant FSH.

I. Introduction

RECENTLY, recombinant gonadotrophins have become commercially available, these are very pure source of hormones that can allow the individual role of FSH and LH to be investigated without the hormone cross contamination of pituitary, serum or urinary preparation.[1]. Recombinant gonadotrophins have been demonstrated to stimulate ovulation maturation and sterodogeneis in rate [2,3] however; studies utilizing recombinant gonadotrophins for in-vitro maturation of immature oocytes are limited largely to studies in rodent oocytes [4] and the effect of the recombinant gonadotrophins on embryonic development is largely unexplored.

There is also experimental evidence that FSH can modulate the meiosis arresting activity of bovine granulose cell. Recent evidence has shown that maturation condition (oocyte mature *in vivo* or *in vitro*) has a significant influence on the number of embryo developing to blastocyst stage [5]. This suggest improvement in maturation media and protocols still could be made that would improve competence and developmental rate of buffalo oocytes. Gonadotrophins are often added to maturation media to induce cytoplasmic maturation, cumulus expansion, and improve embryonic development [1]. Efficient systems that support the growth of ruminant oocytes in vitro are required to provide a valuable means of increasing, the number of embryos produced in vitro from animals of agriculture interest and to enlarge the population of transgenic or endangered animals. However, oocyte growth in ruminants is very slow and this may explain the limited success obtained in these species compared with that in mice. Throughout folliculogenesis, mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase, or germinal vesicle (GV) stage. Meiotic competence is defined as the ability of an oocyte to undergo germinal vesicle breakdown (GVBD), progress to metaphase I (M I), extrude the first polar body and reach metaphase II (MII). Meiotic competence is sequentially acquired during the final phase of oocyte growth. Oocytes initially acquire the ability to undergo GVBD, arresting at about MI. During further development, they acquire the ability to reach MII, and thereby became meiotically competent. In mammalian ovarian follicles committed for ovulation, two major events occur following the pre-ovulatory surge of gonadotropins. The residing oocytes resume and complete meiotic maturation and cumulus cells, representing several layers of granulose cells surrounding the oocytes, undergo a process of expansion [6].

When immature germinal vesicle (GV) stage mammalian oocytes are artificially released from the follicular environment and culture in-vitro, they are able spontaneously to meiotically mature to metaphase II (M II) [7] as the in-vitro maturation (IVM) of oocytes has shown to be a visible physiological phenomenon closely mimicking the in-vivo process [8]. In-vitro fertilized bovine embryo derived from in vitro matured oocytes obtained from slaughterhouse ovaries can be used for beef animal production and other biotechnological purposes [8].

During the maturation of bovine oocytes, the hormonal environment is important for subsequent fertilization and development [10] The present study was conducted to see the effect of rhFSH on meiotic competence of in-vitro grown buffalo oocytes.

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II. Material and Methods

A. Collection and Culture of Oocytes

Approximately 320 ovaries of Nili Ravi buffalo were collected from a local abattoir and transported to laboratory in warm saline (30-35C°) containing pencilline and streptomycine. On arrival within four hours of slaughter ovaries were washed and immersed in 0.9 % (Weight/Volume) NaCl, containing 10 IU pencilline /ml and 10 µg streptomicine (Zafa Pharma). Cummulus oocytes complexes (COCs) were recovered by aspiration from non-preovulatory follicles between 5-30 mm in diameter by using an 18-gauge needle attached with to a 10 ml syringe. The contents of any visible follicle were aspirated and the follicle was flushed up to a three times with phosphate buffer saline (PBS) containing 50 mg bovine serum albumen (BSA)/ ml (Sigma) and 25 IU heparin /ml (Leo pharma).

The recovered follicular fluid-PBS mixture was allowed to stand for about 25 minutes at 30 C°. After removing the supernatant the resulting pellet was washed twice in PBS. After sedimentation, the final pellet was collected and examined under a phase contrast inverted microscope. Complexes from all aspirated oocytes with compact cumulus vestments with 3-20 cell layers were selected. The selected COCs were than washed in maturation medium and culture in pre equilibrated 50µl drops of maturation media (bicarbonated- buffered) TCM-199 supplemented with 0.23m mol sodium pyruvate /lit, 4.0mg BSA /ml, (Sigma), 1mg/100ml Oestradiol (Merck) and 0.05 IU/ml rhFSH (puregon. Organon). COCs culturled without rhFSH served as control. The maturation drops were overlaid with mineral oil and incubated at 38.5 C° wit 5 % CO2 in humidified air for 24 hours.

B. Assessment of Nuclear Maturation

After in vitro maturation of oocytes the meiotic competence of the oocytes was assessed under inverted phase contrast microscope (Nikon). Oocytes with a single prominent nucleus were classified as being in the germinal vesicle (GV) stage as presented in Fig. 1(a). Oocytes that has underwent germinal vesicle breakdown but no polar body were classified as GVBD stage as presented in Fig. 1(b)and oocytes with a polar body were classified as being in the metaphase II (MII) stage of the maturation process as presented in Fig. 2 (a).

III. Statistical Analysis

The percentage of GVBD, MII and degenerated oocytes was examined by chi square test between control and rhFSH treatment group. A P value < 0.05 was considered significant.

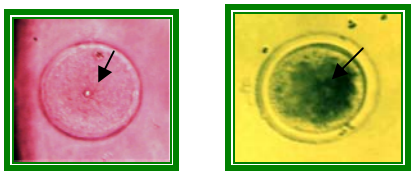
IV. Results

After 24 hours of incubation the percentage of the oocytes at GVBD stage in control and rhFSH treated group is presented in Table I. 29.03 % of the oocytes were still arrested at GVBD stage in control group. Whereas in rhFSH group only 10.11% of the oocytes were arrested at GVBD stage. The percentage of the GVBD stage oocytes was significantly (P=0.001) lower in rhFSH group compare to the control group as most of the oocytes had progressed to the advanced stage of nuclear maturation. After 24 hours of incubation the percentage of the oocytes at MII stage in control and rhFSH treated group is presented in Table I. The percentage of MII oocytes was 47.32 % in control group and in treated group, which was supplemented with rhFSH the percentage of MII oocytes, was 82.02%. The percentage of MII oocytes was significantly (P<0.0001) enhanced by the addition of rhFSH during IVM.

After 24 hours of incubation the percentage of degenerated oocytes in control and rhFSH treated group is given in Table I. The percentage of degenerated oocytes was 23.66 % in control group where as in rhFSH it is 7.86 %. The percentage of degenerated oocytes was decreased in treated group, which was supplemented with rhFSH, but there was not a significant (P = 0.9361) difference between the two experimental groups.

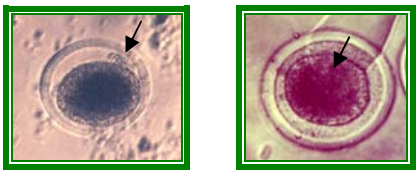
TABLE I
Effect of Human Recombinant Folicle Stimulating Hormone on Meiotic Competence of *In Vitro* Grown Nili Ravi Buffalo Oocytes

Group	Total No. of oocytes at GV stage before culture	Nuclear Stage of Oocytes after 24 hrs of Culture							
		GV		GVBD		M II		Degenerated	
		No.	%	No.	%	No.	%	No.	%
Control	93	--	0	27	29.03	44	47.31	22	23.65
Treatment (rhFSH)	89	--	0	09	10.11	73	82.02	07	07.86



(a) (b)

Fig. 1 Photomicrographs of a Nili Ravi buffalo oocytes. a) An intact germinal vesicle (indicated by arrow) with a single prominent nucleus arrested in dictyate stage of the first meiotic prophase. b) Oocyte undergoing GVBD during culture in vitro showing complete dissolution of the germinal vesicle membrane (indicated by arrow) with only a small remnant of the nucleolus



(a) (b)

Fig. 2 Photomicrographs of fully grown Nili Ravi buffalo oocytes during in vitro culture, a) that have completed meiotic maturation. Note that they have omitted a polar body (indicated by arrows) and have arrested at metaphase II stage. b) Degenerated oocyte showing shrinkage of ooplasm. Photographs were taken from an inverted phase contrast (Nikon) microscope (objectives: a-d, X20)

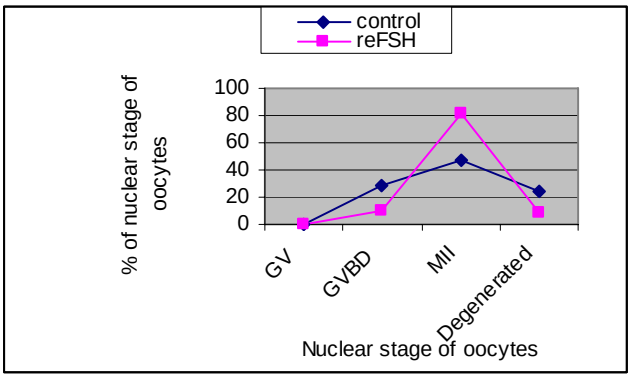


Fig. 3 Graphic representation of effect of recombinant human follicle stimulating hormone on meiotic competence of *in vitro grown Nili Ravi* buffalo oocytes culture in vitro

V. Discussion

The rhFSH supplementation significantly (P=0.005) decreased the percentage of oocytes at GVBD stage, as more number of oocytes had progressed to MII stage. The rhFSH supplementation significantly (P>0.001) increased the percentage of MII oocytes. [11] found that when the mouse oocytes were cultured in modified minimum essential medium (MEM initiated GVBD at 2 hours. At 3 hours, 62 % of the oocytes had already gone GVBD and after 5-6 hours nearly all oocytes 96% had resolved their nuclear membrane and resumed maturation. The fundamental role of FSH in in-vitro development of mammalian pre antral follicle is demonstrated by finding that a very high number of such follicles can be efficiently grown *in vitro* only under gonadotropins stimulation [12, 13]. [14] found that sheep oocytes can be efficiently mature *in vitro* under the effect of porcine FSH. Similarly in present study we have efficiently grown the buffalo COCs *in vitro* under the effect of recombinant human FSH. Non-recombinant FSH was also shown to enhance fertilization and embryo development of immature bovine oocytes [15, 16]. The present study also agrees with a study in which recombinant gonadotropins has been demonstrated to stimulate IVM of oocytes in rat [2, 3]. The study of recombinant recombinant gonadotropins on human oocytes showed that the addition of human recombinant gonadotropins increased the maturation of human oocytes to MII after 48 hours of maturation [17]. In present study rhFSH did significantly (P<0.0001) increased the percentage of oocytes reaching to MII after 24 hours of culture period. The present study is very similar to the other studies in which the effect of recombinant gonadotrophin was studied in human, sheep, rat, and equine [17, 18, 2, 3] In Nili Ravi buffalo, oocytes respond to rhFSH in a same way as these species responded.

FSH did stimulate the resumption of meiosis in oocytes cultured as COCs in absence of thecal tissue in previous study. Similarly in present study oocytes were cultured as COCs with recombinant gonadotropins, more number of the oocytes had resumed the nuclear maturation as compared to the control group.

Indeed, priming with gonadotropins has previously been demonstrated to improve meiotic maturation and evidence suggests that it may be due to the stimulation of cytoplasmic maturation [19]. So in present study the improvement in meiotic maturation by recombinant gonadotropins might be due to the stimulation of cytoplasmic maturation. Recombinant human FSH did significantly increase the proportion of reactivating the equine oocytes that reach MII stage during the 38 hours culture period [18]. At the end of the culture period the degenerated oocytes was 23.65% in control group and 7.86% in rhFSH treatment group. There was also a non-significant difference (P=0.9361) between the two groups but the percentage of degenerated oocytes remained low (7.86) in rhFSH-supplemented group after 24 hours of culture periods.

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