IN VITRO FERTILIZATION OF BUFFALO OOCYTES MATURED IN VITRO IN THREE DIFFERENT MEDIA

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ABSTRACT

In order to evaluate the effect of media on *in vitro* maturation (IVM; 24 hours) and subsequent fertilization (IVF; 24 hours), culturable grade buffalo oocytes (n=1058) were matured (Expt. 1, n=517) or matured and fertilized (Expt. 2, n=541) *in vitro* in three different media (Ham's F-10, Waymouth MB and TCM-199) with same supplements (5µg/ml FSH, 5µg/ml LH, 1µg/ml estradiol, 25mMHepes, 0.25mMpyruvate and antibiotics) to record two end points; IVM and IVF. The overall mean culturable grade oocyte recovery was 3.12±0.20. At the end of Expt. 1 significantly higher (P<0.01) proportion of oocytes were matured *in vitro* in Waymouth compared to Ham's F-10. In TCM-199, IVM rates were non-significantly higher compared to Ham's F-10 and non-significantly lower compared to Waymouth MB medium. At the end of Expt. 2 the proportion of oocytes that fertilized were 10%, 17% and 16.4% in Ham's F-10, Waymouth and TCM-199 respectively which were non significantly different. It was concluded that Way mouth MB medium is the most appropriate medium for *in vitro* maturation of buffalo oocytes followed by TCM-199 and Ham's F 10 however the IVM media had a negligible effect on the subsequent fertilization of buffalo oocytes.

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Key words: Buffalo, In vitro maturation, In vitro fertilization, Media, Oocytes.

INTRODUCTION

The birth of the first buffalo calf following *in vitro* fertilization (Madan *et al.,* 1991) generated interest for *in vitro* production of buffalo embryos owing to research and commercial applications. *In vitro* embryo production (IVEP) would be an effective technique to improve the efficacy of transferable embryo production (Drost, 2007).

Buffalo oocyte IVM rates were low in initial studies, but improved with the addition of buffalo serumor hormones (Hammam *et al.,* 2010) in the culture medium. Addition of cumulus cells alone did not improve the oocyte maturation rate (Das *et al.,* 1997). Expensive components of *in vitro* maturation (IVM) medium, such as fetal calf serum and hormones, were successfully replaced by steer serum and follicular fluid (Nandi *et al.,* 2002). The effect of supplements in the medium such as cysteamine, growth factors and PMSG was considered useful to promote *in vitro* maturation and subsequent fertilization of buffalo oocytes. A large number of variables that influence *in vitro* maturation of buffalo oocytes such as follicular size from which the oocytes are recovered, oocyte recovery procedures, presence or absence of cumulus cells, supplements in the media have been explained and recently (Mahmoud and El-Naby, 2013). Inspite of several attempts to improve the *in vitro* maturation and subsequent fertilization of buffalo oocytes the results obtained had been modest and inconsistent (Hegab *et al.,* 2009). The composition of media appears to be an important regulator of sequential *in vitro* maturation and subsequent development of follicular oocytes (Hammam *et al.,* 2010). The commonly used media for IVM of buffalo oocytes is TCM 199 (Deneke *et al.,* 2013) however, since this media is complex other culturable media such as Ham's F 10 (Hammam *et al.*, 2010) DMEM (Hegab *et al.,* 2009) Ferti Cult medium (Hegab*et al.,* 2009), Ham's F 12 have been experimented with variable results. The use of defined synthetic medium such as Way mouth MB medium have shown some

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advantage over TCM 199 when supplemented with growth factors (Purohit*et al.*, 2005). The *in vitro* maturation and subsequent development of bubaline oocytes in different media with the same supplements is sparsely studied. In this study we evaluated TCM-199, Waymouth MB medium and Hams F-10 for *in vitro* maturation and subsequent fertilization of buffalo follicular oocytes.

MATERIALS AND METHODS

Buffalo ovaries (n=368) were collected from a local abattoir in warm normal saline and brought to the laboratory. Surface follicles from the ovaries were aspirated to collect the oocytes. The culturable grade oocytes (n=1058) were randomly allocated to three different media (Ham's F-10, Waymouth MB and TCM-199) to record two end points *in vitro* maturation (Experiment 1) and *in vitro* fertilization (Experiment 2) in two separate experiments. In experiment 1, all oocytes that were matured for 24 hours were fixed and evaluated for nuclear maturation (proportion reaching Metaphase- II). In experiment 2 all oocytes were first matured *in vitro* and after 24 hours were fertilized with prepared buffalo spermatozoa. After 24 hours of sperm oocyte co-incubation the presumptive zygotes were fixed and evaluated for *in vitro* fertilization. All media and chemicals were from Sigma chemical company USA.

The oocytes were divided into three groups of approximately equal number of oocytes and cultured in either TCM-199, Waymouth MB media or Hams F-10 with the same supplements (5µg/ml FSH, 5µg/ml LH, 1µg/ml estradiol,25mMHepes, 0.25mM Pyruvate and antibiotics) in 50-100µl maturation media covered by sterile paraffin oil for 24 hours at 38±1°C and 5%CO_2 in humidified air in a CO_2 incubator for *in vitro* maturation. After 24 hours of maturation, all oocytes from different groups were collected and fixed separately for staining. The surrounding cumulus cells were removed by vortexing for 1 minute in TCM 199 with hyaluronidase (0.3%). The oocytes were placed in the center of an area delineated by two paraffin wax bars on a clean grease free glass slide. The denuded oocytes were compressed gently with a cover slip to hold and were fixed for 24 hours in acetic acid and methanol [1:3(v: v)] and stained with 1% Giemsa stain for evaluation of nuclear status. Oocytes were considered mature if they were at metaphase-II.

Oocytes were matured *in vitro* in the three different media utilizing the same procedures as above, and after 24 hours of maturation they were fertilized with prepared buffalo spermatozoa.

Frozen thawed buffalo semen was prepared for IVF using a discontinuous Percoll density gradient to separate highly motile live spermatozoa as per Grant *et al.* (1994). Briefly 4 ml of 90% isotonic Percoll was layered in a 15 ml centrifuge tube beneath 4 ml of 40% isotonic Percoll. The sperms were washed initially in TALP-BSA by centrifugation at 250 g for 4 minutes. The sperm pellet was resuspended in 1 ml of the medium. The washed sperm pellet was layered on the top of Percoll gradient and centrifuged at 300 g for 35 min. The resultant pellet was removed from the bottom and washed twice in TALP-BSA by centrifugation.

The sperm pellet was re-suspended in TALP to give a final concentration of 1-2 million sperms. This was incubated for 2-3 hours in a CO_2 incubator. The matured oocytes were transferred to another dish containing Fert-TALP medium (TALP supplemented with 30 µg/ml penicillamine, 15 µmol/ml hypotaurine, 10 µg/ml Heparin and 1 µmol/ml adrenaline) under paraffin oil. They were inseminated with prepared sperms in a volume, so as to give a final concentration of 1-2 million sperms.

Following co-incubation for 20-24 hours with sperms, all the oocytes from each group were washed with fresh medium and vortexed for 1 minute to separate the cumulus mass. They were processed for fixing and staining in the same way as oocytes were fixed after IVM. Oocytes were considered fertilized if they revealed 2 pronuclei or a swollen sperm head along with M-II plate as described previously (Purohit*et al.*, 2005).

The arcsine transformed data of the proportion of oocytes matured or fertilized was compared by one way ANOVA.

RESULTS AND DISCUSSION

The overall mean number of oocytes recovered per ovary was 3.66 ± 0.24 whereas the overall mean number of culturable oocyte recovery per ovary was 3.12 ± 0.20. Similar recovery rates were observed in a few studies on buffalo (Mistry and Dhami, 2009). However a large number of previous studies had recorded a lower culturable grade oocyte recovery rates varying from 0.4-2.17. The reasons for differences in the oocyte recovery rates are diverse and include reproductive status of the animal from which they are retrieved, presence or absence of CL, season of recovery and recovery procedure adopted (Mehmood *et al.,* 2011).

In vitro maturation of buffalo oocytes in Expt 1 and Expt 2 revealed that significantly higher ($P<0.01$) proportion of oocytes matured *in vitro* (reached M-II stage) in Way mouth MB media compared to Ham's F 10 media. The number of oocytes that matured *in vitro* was non-significantly higher ($P>0.01$) in Way mouth MB media compared to TCM 199 suggesting better performance of oocytes in Way mouth MB media. The overall maturation rates (all three media) obtained during the present study were 70.01 percent. Similar maturation rates were recorded in many previous studies on buffalo oocytes matured *in vitro* (Leal *et al.*, 2010).

Previous studies on buffalo oocyte maturation *in vitro* have shown TCM-199 to be better over Hams F-10 (Hammam *et al.*, 2010). The beneficial effect of TCM-199 on IVM may be related to some factors in its composition such as essential amino acids and glutamine that stimulate DNA and RNA synthesis and enhance cell division (Mahmoudand El-Naby, 2013).

Waymouth medium was found to support *in vitro* maturation of buffalo oocytes even better to TCM-199. Xu *et al.* (1992) have previously shown that Waymouth medium yielded better cleavage rates compared to TCM-199 during bovine *in vitro* embryo development. A previous study on buffalo oocytes (Purohit*et al.,* 2005) had recorded comparable *invitro* maturation rates both with TCM-199 and Waymouth medium.

In Expt 2, in vitro maturation in Way mouth MB media resulted in subsequent higher fertilization rates (17%) compared to Ham's F 10 (10%) and TCM 199 (16.4%) however the differences were non-significant.

The fertilization rates recorded during the present study are similar to a few of previous study that recorded fertilization rates varying from 11-24% for buffalo oocytes (Hammam *et al.*, 2010). However many other studies recorded higher *in vitro* fertilization rates (43-82%) for buffalo oocytes (Mehmood *et al.*, 2011). This could be because of the difference in the supplements used, the initial quality of oocytes utilized and other variables.

Although differences in the *in vitro* fertilization rates for buffalo oocytes across various media used for their *in vitro* maturation have been recorded (Kumar *et al.,* 2008) however it is a general consensus based on analysis of many reports that the supplements are much more important and concur with the findings of the present study.

Comparison of *in vitro* maturation and fertilization of buffalo oocytes across the three media revealed that *in vitro* maturation was better in Waymouth and TCM media compared to Hams F-10 in both experiment 1 and experiment 2 and although the fertilization rates were also higher yet the differences across media are more operative during *in vitro* maturation of oocytes. It was concluded that Way mouth MB medium is the most appropriate medium for *in vitro* maturation of buffalo oocytes followed by TCM-199 and Ham's F 10 however, the IVM media had a negligible effect on the subsequent fertilization of buffalo oocytes.

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