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Holding equine oocytes in a commercial embryo-holding medium: New perspective on holding temperature and maturation time

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ABSTRACT

In the present study, we examined the effect of holding equine oocytes in Syngro embryo holding medium (EHM) overnight at either 4 °C, 17 °C, or 22 °C to 25 °C, on the time to maturation and developmental competence. We also examined the effect of placing denuded oocyte without extruded polar body back in maturation condition on subsequent maturation rate. In experiment 1, cumulus-oocyte complexes (COCs) were recovered postmortem and placed in EHM at 22 °C to 25 °C for 18 to 20 hours (OH) or placed directly in maturation (DM). The maturation rate was assessed after 22, 24, or 28 hours of culture. After denuding cumulus cells at 22 or 24 hours, oocytes without obvious polar body were placed back into culture and reassessed at subsequent time points. At 22 hours, a higher proportion of oocytes placed in OH achieved nuclear maturation than those placed in DM (63% and 37%, respectively, $P = 0.008$). At 24 and 28 hours, no significant differences in the % MII stage oocytes were observed between OH and DM. The nuclear maturation rate for OH oocytes was similar at 22, 24, and 28 hours, indicating that the maximum maturation rate was reached at an earlier time than that in DM. Oocytes fertilized by intracytoplasmic sperm injection resulted in a 7.1% and 6.3% blastocyst rate for OH and DM, respectively. Denuding oocytes after 22 hours or more of culture did not have an adverse effect on the final nuclear maturation rate. After 28 hours of culture, the same nuclear maturation rate (MII) was reached for nondenuded oocytes and oocytes denuded after 22 hours of 24 hours of culture. In experiment 2, COCs were held overnight at room temperature in EHM, then placed in maturation for 20, 22, and 28 hours. Nuclear maturation rate was significantly lower at 20 hours than 22 and 28 hours of culture and was similar at 22 and 28 hours, suggesting that at least 22 hours of culture is required to reach maximal maturation rate for stored oocytes (43%, 62%, and 65% at 20, 22, and 28 hours, respectively. $P < 0.001$). In experiment 3, COCs were either placed directly in culture or held at 22 °C to 25 °C, 17 °C, or 4 °C overnight. After 24 hours of culture, maturation rate was similar for all groups, suggesting that COCs can be stored in conventional 4 °C transport condition or 17 °C. In preliminary studies, COCs were held at 4 °C followed by 24 hours of culture, and mature oocytes were fertilized using intracytoplasmic sperm injection. Twenty-three injected oocytes yielded four blastocysts. In conclusion, we reported that oocytes can be placed in a commercial EHM and stored overnight without a detrimental effect on maturation rates or blastocyst development. Oocytes held in holding medium require less time to reach the MII stage than oocytes placed in culture directly. Removing the cumulus cells from oocytes that have been cultured for at least 22 hours does not seem to alter the final nuclear

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maturation rate. Finally, we observed no detrimental effect of storing oocytes at 4 °C for up to 18 hours, and oocytes appeared to maintain developmental competence and blastocyst potential.

1. Introduction

Intracytoplasmic sperm injection (ICSI) is currently being used to successfully produce equine embryos *in vitro* [1–6]. However, owing to the complexity of the ICSI procedure, there are currently only a few centers worldwide reporting acceptable results with clinical ICSI in horses. The availability of efficient transport methods to ship harvested, immature oocytes from an ovum pick up (OPU) center to a laboratory proficient in *in vitro* production of embryos is essential for continuing the growth of this field [6].

Immature equine oocytes can be effectively maintained in meiotic arrest without the use of a meiotic inhibitor by simply holding the oocytes at room temperature [6–8]. Different types of holding media have been used to store equine oocytes, with holding times varying between 15 and 63 hours [6,8,9]. Although it has been proposed that oocytes are relatively robust during overnight holding, it is likely that different media will affect subsequent maturation differently [10,11]. The ability to use commercial embryo holding media to ship oocytes would increase the ease of transport from the site of collection to the ICSI laboratory.

The timing of oocyte maturation after holding at room temperature has not been evaluated. According to Hinrichs et al. [12], the required duration of IVM culture is 24 to 30 hours for equine oocytes with an expanded cumulus and 30 to 36 hours for oocytes with a compact cumulus. When collecting oocytes *in vivo* (OPU), most of the oocytes recovered are partially denuded of cumulus cells because of the collection technique [13]. This makes the classification of the cumulus morphology (expanded or compact cumulus) difficult. Therefore, most protocols using aspiration of oocytes adhere to 28 to 30 hours of maturation for all harvested oocytes without making a distinction on the basis of cumulus morphology [3,6,8,14–16]. Jacobson et al. [17], reported that equine oocytes that were recovered by OPU and held overnight resulted in a similar maturation and blastocyst rate when they were matured for 24 or 30 hours. However, the required culture time to reach maximal maturation rate in oocytes that were held overnight was not compared with the culture time required for oocytes that were placed immediately in IVM culture. Therefore, in this study, it is difficult to distinguish between the effect of holding oocytes and maturation conditions on the culture time required to reach maximum maturation rate. Assuming that the shortened maturation time is an effect of holding, it is not clear what exactly the minimum required IVM culture time would be. In bovine oocytes, it has been demonstrated that holding immature oocytes at room temperature affects the required IVM culture time [11]. Oocytes that were stored for 16 to 18 hours yielded higher embryo development rates when inseminated after 18 hours of IVM culture compared with the oocytes that matured for 22 hours, which is considered to be the required maturation time for oocytes placed immediately in IVM culture. This indicates that holding bovine oocytes shortens the maturation time [11]. Accordingly, it has been reported that the developmental competence of bovine oocytes decreases significantly starting at approximately 5 hours after reaching the MII stage [18,19]. It is possible that equine oocytes mature at an earlier time point when IVM is preceded by a holding period, as described in the bovine. Previous studies have not thoroughly examined the effect of temporary storage on the nuclear maturation kinetics in the equine. Choi et al. [7], suggested that stored oocytes risk overmaturation if held in maturation media for too long.

With the number of OPU centers increasing worldwide and transport of oocytes to ICSI centers gaining in popularity, it is important to understand the effects of oocyte transport conditions. The use of more standardized transport systems, which keep the oocytes temperature constant regardless of season and ambient temperature, may offer a more reliable method for transporting oocytes. Martino et al. [8], evaluated equine oocytes, either placed immediately in IVM culture or held for 16 to 18 hours in M199-based medium at 25 °C, 30 °C, or 38 °C before IVM culture. Holding oocytes at 25 °C maintained the oocytes in meiotic arrest. However, higher temperatures (30 °C and 38 °C) were associated with resumption of meiosis during storage. The effect of holding equine oocytes at low temperatures has not yet been investigated. In porcine and bovine, a detrimental effect of exposing oocytes to temperatures lower than 25 °C has been demonstrated [20,21]. In contrast to bovine and porcine, Li et al. [22] successfully preserved ovulated mouse oocytes at 15 °C for 36 hours and at 5 °C for 24 hours without impairing developmental competence. In 2004, Preis et al. [23] shipped whole equine ovaries at 12 °C or 22 °C and did not detect an appreciable difference in the ability of the oocytes to subsequently form pregnancies.

The objectives of the present study were to evaluate the effect of holding oocytes in a commercial equine EHM on nuclear maturation. The specific hypotheses that we have tested in these experiments are (1) overnight storage of equine oocytes in a commercial embryo holding solution at various temperatures does not affect the nuclear maturation rate; (2) overnight holding of oocytes before culturing reduces the time in culture required to reach nuclear maturation; and (3) denuding immature oocytes after 22 hours of culture does not impede the further maturation of these denuded oocytes when returned to culture.

2. Materials and methods

2.1. Chemicals

All media and fetal calf serum (FCS) were purchased from Gibco Life Technologies (Merelbeke, Belgium). Hoechst 33342 was obtained from Molecular Probes (Ghent, Belgium). All other chemicals not otherwise listed were obtained from Sigma–Aldrich (Bornem, Belgium).