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# Chromatin Configuration Within the Germinal Vesicle of Horse Oocytes: Changes Post Mortem and Relationship to Meiotic and Developmental Competence[1]

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## ABSTRACT

**We evaluated the relationship of initial chromatin configuration to time of oocyte recovery and to nuclear maturation after culture in horse oocytes having compact (Cp) and expanded (Ex) cumuli. In addition, we evaluated the effect of oocyte type, time of recovery, and duration of culture on blastocyst development after intracytoplasmic sperm injection. In oocytes collected within 1 h of slaughter, fibrillar and intermediate chromatin configurations were more prevalent in Cp than in Ex oocytes (68% and 12%, respectively). In Cp oocytes collected after a 5- to 9-h delay, the proportions in the fibrillar and intermediate configurations decreased significantly, and the proportions of degenerating and homogeneously fluorescent configurations increased. When cultured, 20% of oocytes classified as having fibrillar chromatin resumed meiosis, whereas 82% of intermediate and 81% to 86% of condensed chromatin oocytes did so. Meiotic resumption was higher in oocytes recovered immediately after slaughter, but these oocytes took longer to mature. Duration of maturation significantly affected blastocyst development rates in Cp oocytes recovered after a delay (13% and 38% for oocytes matured 24 and 36 h, respectively). Oocytes recovered after a delay had higher blastocyst development rates than did those collected immediately after slaughter. We conclude that the fibrillar and intermediate chromatin configurations may degenerate during ovary storage, resulting in decreased maturation rates, especially of Cp oocytes. Time of oocyte recovery and duration of maturation significantly affect the rate of blastocyst development. Oocytes with Cp and Ex cumuli have similar developmental competence to the blastocyst stage.**

*assisted reproductive technology, chromatin, embryo, horse, in vitro fertilization, oocyte development*

## INTRODUCTION

Much current research is focused on identifying oocyte characteristics associated with meiotic and developmental competence. The endpoints for these competencies are, respectively, maturation of the oocyte to metaphase II (MII), and embryonic or fetal development, typically as measured by blastocyst development in vitro. However, these endpoints do not depend only on the intrinsic character of the oocyte. Ovary and oocyte handling procedures before the onset of maturation are known to affect the proportion of oocytes that are capable of subsequent maturation and blastocyst formation [1–3]. It has recently been suggested that the prematuration environment can also alter the kinetics of maturation. Bovine oocytes held in meiotic suppression with butyrolactone and subsequently matured reached MII 6 h earlier than did controls [4]. Similarly, bovine oocytes held in meiotic suppression before maturation seemed to attain developmental competence early: compared with 22-h matured controls, blastocyst development was similar when suppressed oocytes were matured 16 h, but development was lower when they were matured 22 h [5]. We found that horse oocytes recovered from ovaries after a 5- to 9-h holding period appeared to reach MII in culture more rapidly than did oocytes recovered immediately postmortem [6]. Most studies evaluating differences in oocyte competencies have used only one standardized maturation duration (e.g., cattle [7–10]). The above findings suggest that different classes of oocytes may have different optimum maturation periods.

In the horse, the low number of existing abattoirs can necessitate ovary or oocyte transport over long distances, thus the question of the effect of the prematuration environment on oocyte meiotic and developmental competence is a particularly important one. Factors affecting the competence of horse oocytes are poorly understood, and many reports in these areas appear contradictory. Horse oocytes from follicles that are viable (as determined on histological section) are marked by having compact (Cp) cumuli, whereas oocytes from atretic follicles are marked by having expanded (Ex) cumuli [11]. Some laboratories report high rates of maturation for Ex oocytes (~65%) and low rates of maturation for Cp oocytes (~30%) [11–14], whereas other laboratories report a high maturation rate for Cp oocytes (88% [15]; 63% [16]). It has been reported that Ex horse oocytes take 24 h to reach maximum proportions at MII, whereas Cp oocytes take more than 30 h [17, 18]. However, there is no standard classification or selection system for horse oocytes, and the duration of culture used for in vitro maturation of horse oocytes has ranged from 24 h [15, 19, 20] to 40 h or more [21, 22].

Oocyte meiotic competence in the horse, as in other species, is associated with the chromatin configuration within the germinal vesicle (GV) before culture [11, 13, 23–25]. Chromatin condensation in equine oocytes is especially marked, similar to the karyosphere reported in human oocytes [26, 27]. Condensed chromatin is more prevalent in Ex oocytes, whereas Cp oocytes have a higher proportion of diffuse chromatin within the GV [12, 18, 28]. In our laboratory, the most common diffuse chromatin configuration has been homogeneous fluorescence throughout the GV on Hoechst staining (fluorescent nucleus; FN) [18]. The proportion of Cp oocytes having the FN configuration has

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been repeatable over many studies in our laboratory, and has been reported by others [11, 12, 18, 29]. In contrast, some laboratories have not recognized this configuration in GV horse oocytes, finding instead that equine GV stage oocytes have fibrillar chromatin [30, 31]. The prevalence of the FN configuration in equine oocytes has been reported to increase with increased duration between slaughter and oocyte collection [29] or by holding oocytes at room temperature after collection [32]. This suggests that the FN configuration may be an artifact of chromatin degradation. To our knowledge, the effect of delayed recovery from the ovary on oocyte chromatin configuration has not been detailed in any species.

While the rate of maturation of cultured horse oocytes has been compared with the rates of initial chromatin configuration of fixed oocytes from the same population [11, 13, 18, 33], the direct relationship between chromatin configuration and meiotic competence in horse oocytes has not been evaluated. In the mouse, chromatin configuration has been recorded after Hoechst staining of live oocytes and standard fluorescence microscopy using brief (<3 sec) UV exposure [34]. After being imaged, these oocytes subsequently matured successfully in culture. In the current study, we evaluated whether a similar technique could be used to determine the direct relationship between GV chromatin configuration and meiotic competence in the horse oocyte.

Developmental competence of horse oocytes has only recently started to receive attention, as intracytoplasmic sperm injection (ICSI) has become efficient [35, 36] and methods for in vitro culture to the blastocyst stage have been developed [36, 37]. The optimum duration of oocyte maturation for developmental competence has not been defined in the horse, and may vary according to oocyte cumulus type: we found that cleavage rates at 48–96 h after ICSI varied differentially with maturation duration for Ex and Cp oocytes [38]. Information is not available on the effects of ovary storage, cumulus type, or duration of oocyte maturation on blastocyst development in horses. In cattle, reports vary on the effect of ovary storage on developmental competence. In one report, there was no effect of storage of cow ovaries for up to 11 h at 25°C on blastocyst formation after in vitro maturation, in vitro fertilization, and in vitro culture [1]. Storage of cow ovaries for 4 h at 30°C was reported to increase blastocyst development after in vitro maturation and in vitro fertilization [3], whereas others reported no effect of storage at 35°C for 3 h [39]. Storage of bovine ovaries for longer times, at cooler temperatures, or both, has been associated with either depression of development, or with no effect [1, 39–41].

The aims of the present study were to 1) compare the chromatin configuration of horse oocytes recovered immediately after slaughter to those recovered after 5–9 h ovary storage; 2) examine the direct relationship of initial chromatin configuration to meiotic competence in horse oocytes; and 3) evaluate the interactions among time of oocyte recovery, oocyte type, and duration of culture on the meiotic and developmental competencies of horse oocytes.

## MATERIALS AND METHODS

### *Experiment 1: Effect of Oocyte Recovery Time on Horse Oocyte Chromatin Configuration*

Chromatin configuration of GV-stage horse oocytes was evaluated in oocytes collected from the ovary either within 1 h postmortem (immediate-fix) or after a delay of 5–9 h postmortem (delay-fix), which is the standard operating procedure in our laboratory. To evaluate chromatin configuration of oocytes in the immediate-fix treatment, horse ovaries were processed and the oocytes fixed at the abattoir. Each pair of ovaries was placed in a separate plastic bag marked with the time of removal from the mare. Oocytes were recovered from ovaries within 45 min of removal from the mare; any ovary not finished processing at that time was discarded. To recover oocytes, all visible follicles on and within the ovaries were opened with a scalpel blade, and the granulosa layer of each follicle was scraped using a 0.5-cm bone curette. The contents of each follicle was washed from the curette into an individual Petri dish using holding medium (Hepes-buffered TCM-199 with Hanks salts [Gibco Life Technologies, Inc., Grand Island, NY] plus ticarcillin, 0.1 mg/ml [SmithKline Beecham Pharmaceuticals, Philadelphia, PA]).

The contents of the Petri dishes were examined using a dissection microscope at 10–20× magnification. Oocyte-cumulus complexes were classified as compact, expanded, or degenerating depending on the expansion of both mural granulosa and cumulus as described previously [11, 13]. Oocytes with any sign of expansion of either the cumulus or the mural granulosa (from having individual cells visible protruding from the surface to having full expansion with copious matrix visible between cells) were classified as Ex oocytes. Oocytes having both compact cumulus and compact mural granulosa were classified as Cp oocytes. As each oocyte was identified, it was denuded of cumulus by pipetting in a solution of 0.25% trypsin and 1 mM EDTA in Hanks salts without $CaCl_2$, $MgCl_2$, and $MgSO_4$ (Gibco). Oocytes remained in this solution only as long as it took to denude them (~1 min). After cumulus removal, oocytes were fixed in phosphate buffered saline containing 4.75% formaldehyde.

For the delay-fix treatment, ovaries were recovered at the abattoir (during a 2-h collection period) and were transported to the laboratory (4 h transport time) in a plastic bag at 22 to 25°C. At the laboratory, oocytes were collected from all visible follicles and classified as described above. Oocytes were placed in holding medium at room temperature until all ovaries had been processed (1–3 h), and were then denuded and fixed as described above. Total time from slaughter to oocyte fixation was 5–9 h.

Fixed oocytes were labeled for chromatin evaluation by placing them on a glass slide with 6.5 μl of mounting medium (9:1 glycerol:PBS containing 2.5 μg/ml Hoechst 33258). The oocytes were evaluated using a fluorescence microscope with a 365-nm exciter filter. Chromatin configuration was classified as previously described [31, 32]. Photomicrographs of oocytes in each classification are presented in Figure 1. The classifications of GV chromatin used were 1) fibrillar, having distinct strands of intertwined chromatin visible throughout the germinal vesicle; 2) intermediate, having chromatin strands or irregular masses of chromatin spread over approximately half the area of the germinal vesicle; 3) loosely condensed chromatin (LCC), having chromatin in one dense mass with an irregular outline, usually surrounding a nonfluorescent area interpreted as being the nucleolus; 4) tightly condensed chromatin, (TCC), having one dense, circular to oval mass of chromatin, again usually surrounding a nucleolus; and 5) fluorescent nucleus (FN), in which the entire GV fluoresced homogeneously with or without small aggregations of chromatin. Other classifications were MI, MII, and degenerating (i.e., no chromatin, chromatin spread throughout the cell, or abnormal chromatin configurations).

The proportions of oocytes in the different chromatin configurations were compared between immediate-fix and delay-fix groups and between Ex and Cp oocytes by chi-square analysis, with the Fisher exact test used when a value of less than 5 was expected for any parameter.

### *Experiment 2: Relationship of Initial Chromatin Configuration to Meiotic Competence*

To directly assess the relationship between chromatin configuration and meiotic competence, ovaries were collected from six mares on separate occasions immediately after they were killed by barbiturate overdose as part of another study. Work with these mares was performed according to the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training,* (National Institutes of Health, 1985) and was approved by the Laboratory Animal Care Committee at Texas A&M University. The ovaries were processed, and recovered oocytes were classified by cumulus morphology as described above. After classification, oocytes were held in holding medium with 10% fetal bovine serum (Hold+FBS) until all oocytes had been recovered. On occasions in which 15 or more oocytes were recovered (3 replicates), ½ to ⅔ of the oocytes were kept in Hold+FBS, while the remainder were stained and imaged to serve as controls for the effect of the holding delay and single-oocyte culture on maturation rate. A maximum of 18 oocytes was used for imaging in each replicate to limit the delay before initiation

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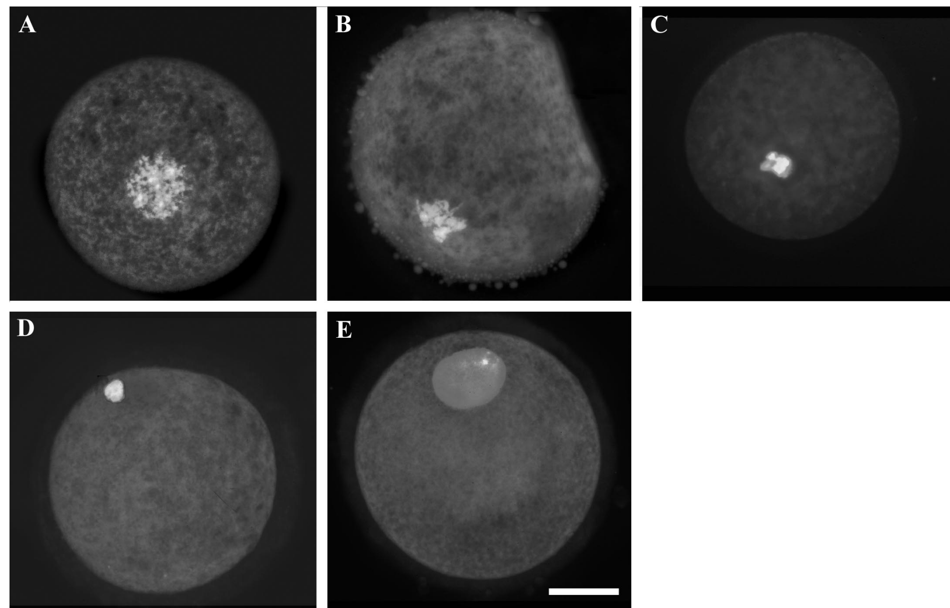


FIG. 1. Photomicrographs of horse oocytes stained with Hoechst 33258 and evaluated under fluorescence microscopy, demonstrating (**A**) fibrillar, (**B**) intermediate, (**C**) loosely condensed, (**D**) tightly condensed, and (**E**) fluorescent nucleus chromatin configurations. Bar = 50 μm.

of culture. If fewer than 15 oocytes were recovered (3 replicates), all oocytes were processed for imaging. Oocytes assigned to be imaged were denuded of cumulus by gentle pipetting in holding medium, then transferred back to Hold+FBS. Trypsin and EDTA were omitted from the denuding medium to avoid any potential effects of these components on subsequent oocyte maturation. Recovered cumulus cells were placed into Hold+FBS. The denuded oocytes were stained by transferring them to holding medium containing 1 to 2 μg/ml Hoechst 33342 and incubating at 37°C for 3 min. After staining, oocytes were washed in Hold+FBS, then mounted individually on a plain glass slide in approximately 10 μl of Hold+FBS medium. A small amount of paraffin was placed on each edge of an 18 × 18 mm coverslip, and the coverslip was placed over the droplet containing the oocyte. The coverslip was pressed against the slide until the oocyte was slightly flattened.

The slide was numbered and placed on the stage of a fluorescent microscope, and the oocyte was located on the slide using light microscopy. At 400×, the approximate location of the GV was estimated by its displacement of cytoplasmic granules. The oocyte was then exposed to fluorescent light long enough to visualize the chromatin configuration (<5 sec); the duration of exposure was timed using a stopwatch. Chromatin was classified as it was in experiment 1. After the chromatin had been visualized, the slide was removed from the microscope and the end of the slide containing the oocyte was immersed in a 100-mm Petri dish containing holding medium. Under the dissection microscope, the coverslip was lifted from the slide using a 1.5-inch, 20-gauge needle. The oocyte was recovered, washed in clean Hold+FBS, and held in a microdroplet of this medium at 37°C under light white mineral oil (Sigma Chemical Co., St Louis, MO) until the remainder of the oocytes had been imaged.

When all oocytes intended for imaging had been processed, the control and imaged oocytes were washed twice in maturation medium (TCM-199 with Earle salts [Gibco], 5 mU/ml FSH [Sioux Biochemicals Inc., Sioux Center, IA], 10% FBS, and 25 μg/ml gentamycin). Oocytes were cultured individually in 10-μl droplets of maturation medium under light white mineral oil at 38.2°C in 5% $CO_2$ in air for 24 h. Cumulus cells were added to droplets containing imaged oocytes to approximate the numbers of cumulus cells in the controls. After culture, control oocytes were denuded of cumulus as described for experiment 1, and all oocytes were fixed and stained for chromatin evaluation.

The proportion of Cp and Ex oocytes in each of the initial chromatin configurations, and the rates of resumption of meiosis (MI plus MII) and maturation to MII were compared among groups by chi-square analysis, with the Fisher exact test used when a value of less than 5 was expected for any parameter.

## *Experiment 3: Effect of Postmortem Holding Time and Culture Duration on In Vitro Maturation Rate and Development to Blastocyst after ICSI*

Oocytes were collected either at the abattoir within 45 min of slaughter (immediate group), or at the laboratory 5–9 h after slaughter (delay group), as described for experiment 1. For the immediate group, after classification of recovered oocytes as Cp or Ex at the abattoir, oocytes were placed into a correspondingly labeled dish of holding medium at room temperature. Every 30 min, oocytes that had been recovered and classified during that period were placed into 1-ml borosilicate glass tubes containing 1 ml of maturation medium previously equilibrated at 5% $CO_2$ in air, and placed in a portable incubator at 38.2°C. After all oocytes were collected, the incubator was transported back to the laboratory. Oocytes were recovered from the tubes and placed into microdroplet culture in maturation medium at 10 μl/oocyte under oil at 38.2°C in 5% $CO_2$ in air. Total time from slaughter to placement into maturation culture at the abattoir was 15 min to 1.25 h; time in culture within the portable incubator until placement in microdroplet culture at the laboratory was 4 to 7 h.

Oocytes in immediate and delay groups were cultured for 24, 30, or 36 h. The start of culture was considered to be the time of placement into maturation medium; for the immediate group, placement into tubes at the abattoir; for the delay group, placement into culture at the laboratory. Because the rate of maturation to MII in the immediate 24-h groups was anticipated to be low [6], these groups were allotted twice the number of oocytes as were placed in the 30- and 36-h groups, to allow adequate numbers of MII oocytes for ICSI and comparison of embryo development. A minimum of four replicates were performed for each group.

After culture, oocytes were denuded of cumulus by pipetting in 0.05% hyaluronidase in CZB-M [42] to aid dispersal of the mature expanded cumuli. Those oocytes having a polar body were placed in CZB-H [42] and returned to the incubator until being used for ICSI. Oocytes not having a polar body were fixed and stained as described above, and evaluated to determine their chromatin configuration.

## *Sperm Preparation*

Semen was collected from one stallion and frozen in a skim-milk glucose extender. Work with this stallion was performed according to the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training,* (National Institutes of Health, 1985) and was approved by the Laboratory Animal Care Committee at Texas A&M University. Semen straws were thawed at 37°C for 30 sec, and 200 μl of semen was placed at the bottom of a 5-ml tube containing 1 ml of Sp-CZB [42] for swim-up. After 20 min of incubation at 38.2°C in an atmosphere of 5% $CO_2$ in air, 0.6 ml of medium was collected from the top of the tube, and centrifuged at 327 × $g$ for 3 min in a 1.7-ml polypropylene tube. Then the sperm pellet was resuspended and washed once with the same medium.

## *Intracytoplasmic Sperm Injection*

The ICSI procedure was conducted as previously described [35] except that the media used were modified as given below. Briefly, the outside diameter of the pipette used for ICSI was 7–8 μm. For holding oocytes, a 120–140 μm (outside diameter) pipette was used. Immediately before

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TABLE 1. Chromatin configurations in oocytes fixed at different times after slaughter.

| Oocyte Type | n | Chromatin configuration | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | FN | Fibrillar | Intermediate | LCC | TCC | MI | MII | Degenerating |
| Cp-Immediate | 50 | 2 (4%)[a] | 20 (40%)[a] | 14 (28%)[a] | 9 (18%) | 0 | 1 (2%) | 0 | 4 (8%)[a] |
| Cp-Delay | 124 | 18 (15%)[b] | 11 (9%)[b] | 9 (7%)[b] | 25 (20%) | 7 (6%) | 1 (1%) | 0 | 53 (43%)[b] |
| Ex-Immediate | 78 | 1 (1%)[a] | 2 (3%) | 7 (9%) | 37 (47%) | 7 (9%) | 4 (5%) | 4 (5%) | 16 (21%) |
| Ex-Delay | 140 | 10 (7%)[b] | 0 | 4 (3%) | 52 (37%) | 27 (19%) | 16 (11%) | 1 (1%) | 30 (21%) |

[a,b] Within columns and within cumulus type, values with different superscripts are significantly different ($P < 0.05$).

injection, 1 μl of sperm suspension was placed in a 3-μl droplet of Sp-CZB containing 10% polyvinylpyrrolidone (Sigma) under oil. Oocytes were placed in a separate 50-μl drop of CZB-M containing 10% FBS, and ICSI was performed in this droplet. Each spermatozoon was immobilized by applying a few pulses with a Piezo drill (Prime Tech Ltd., Ibaraki, Japan) to the tail immediately before injection. All manipulations were performed at room temperature. Groups of injected oocytes were placed in CZB-H containing 10% FBS, returned to the incubator, and held until all oocytes had been manipulated.

Injected oocytes were then transferred to microdroplets of Dulbecco modified Eagle medium (DMEM)/F-12 medium (Sigma) containing 10% FBS [37] at a ratio of 5 μl medium per oocyte, in a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.2°C. Medium was completely refreshed at 48 h, at which time uncleaved embryos were removed, and the medium was completely refreshed again at 96 h culture. At the conclusion of culture (7.5 days), embryos were evaluated using a dissection microscope, then fixed and stained with Hoechst 33258 as described above, and examined under fluorescence microscopy. Embryos were classified as morulae if they contained more than 32 cells but did not have an organized outer rim of cells; and as blastocysts if they contained more than 64 cells and had started organizing an outer presumptive trophoblast layer [37]. Only nuclei considered to be normal were included in the nucleus number; nuclei showing signs of degeneration (vesiculation, pyknosis, or chromatin disaggregation) were disregarded.

The rates of meiotic resumption (maturation to MI or MII), maturation to MII, and development to the blastocyst stage were compared among groups using chi-square analysis, with the Fisher exact test used when a value of less than 5 was expected for any parameter.

## RESULTS

### *Experiment 1: Effect of Oocyte Recovery Time on Chromatin Configuration of Horse Oocytes*

For the immediate-fix group, 59 ovaries were processed at the abattoir and 50 Cp, 78 Ex, and 7 degenerating oocytes were recovered. Time from ovary removal from the mare to the end of oocyte collection from that ovary was 32 ± 12 min (mean ± SD). For the delay-fix group, 143 ovaries were processed at the laboratory (5 replicates). From these, 109 Cp, 218 Ex, and 31 degenerating oocytes were recovered, of which 140 Ex oocytes and 79 Cp oocytes were fixed, 4 Ex and 10 Cp oocytes were broken during denuding, and the remainder were used in separate projects. Because of the lower proportion of Cp oocytes recovered, an additional 86 ovaries (3 replicates) were processed in the laboratory and 45 Cp oocytes were fixed, for a total of 140 Ex and 124 Cp in the delay-fix groups.

The chromatin configurations of immediate-fix and delay-fix horse oocytes are presented in Table 1. Both Cp and Ex oocytes had significant differences in chromatin configuration associated with delayed collection. Delayed collection of Cp oocytes was associated with a significant decrease in the proportions in the fibrillar ($P < 0.001$) and intermediate ($P < 0.001$) configurations, and a significant increase in the proportions in the FN ($P < 0.05$) and degenerating ($P < 0.001$) configurations. Delay-fix Ex oocytes also had significantly higher proportion in the FN ($P < 0.05$) configuration than did immediate-fix Ex oocytes; however, this represented only 7% of this group.

There were significant differences in chromatin configuration between Cp and Ex oocytes within both immediate-fix and delay-fix treatments. In the immediate-fix treatment, a significantly higher proportion of Cp than Ex oocytes were in the fibrillar ($P < 0.001$) and intermediate ($P < 0.001$) configurations, whereas a significantly higher proportion of Ex than Cp oocytes were in the LCC ($P < 0.05$) and TCC ($P < 0.05$) configurations. For delay-fix oocytes, a significantly higher proportion of Cp than Ex oocytes were in the fibrillar ($P < 0.001$) and degenerating ($P < 0.001$) configurations, whereas a significantly higher proportion of Ex than Cp oocytes were in the LCC ($P < 0.01$), TCC ($P < 0.001$), and MI ($P < 0.01$) configurations.

### *Experiment 2: Relationship of Initial Chromatin Configuration to Meiotic Competence*

A total of 63 Cp, 66 Ex, and 19 degenerating oocytes were recovered from the six pairs of ovaries. Of these, 39 Cp and 32 Ex oocytes were imaged and then cultured, and 24 Cp and 34 Ex were cultured as controls. Oocytes were collected within 1 h of removal of the ovaries from the mare in five cases, and within 2 h of ovary removal in one case; the total time from removal of the ovaries to placement of oocytes in culture was 3.4 ± 0.37 h (mean ± SD). The duration of exposure to UV light during imaging was 1.7 ± 0.5 sec. Chromatin could not be adequately visualized within 5 sec in two Ex oocytes, and these were disregarded. The initial chromatin configuration of imaged oocytes is presented in Table 2. Only one imaged oocyte was in the FN configuration. The fibrillar ($P < 0.001$) and intermediate ($P < 0.001$) configurations were observed only in Cp oocytes, and significantly more Ex than Cp oocytes were in the TCC configuration ($P < 0.001$). Additionally, significantly more Ex oocytes had already resumed meiosis (i.e., they were in MI) at the time of collection from the follicle ($P < 0.05$).

Two imaged oocytes (Ex) were lost during recovery from the slide. The overall rates of meiotic resumption (maturation to MI or MII) after 24 h of culture were not significantly different between imaged and control oocytes ($P > 0.1$); these were 23/39, 59% and 13/24, 54% for imaged and control Cp, respectively; and 21/28, 75% and 26/34, 77% for imaged and control Ex, respectively. Similarly, the rates of maturation to MII within the 24-h culture period were not significantly different between imaged and control oocytes ($P > 0.1$); these were 11/39, 28% and 7/24, 29%, for imaged and control Cp, respectively; and 14/28, 50% and 15/34, 44%, for imaged and control Ex, respectively.

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TABLE 2. Initial chromatin configurations of oocytes and stage of meiosis achieved after 24 h culture.

| Oocyte type | Initial chromatin | n | Lost before culture | Chromatin configuration after culture | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Degenerating | GV | MI | MII | Meiotic resumption (MI + MII) |
| Cp | FN | 0 | | | | | | |
| | Fibrillar | 15 | | 3 | 9 | 2 | 1 | 3 (20%)[a] |
| | Intermediate | 11 | | 2 | 0 | 5 | 4 | 9 (82%)[b] |
| | CC | 10 | | 2 | 0 | 4 | 4 | 8 (80%)[b] |
| | TCC | 3 | | 0 | 0 | 1 | 2 | 3 (100%)[b] |
| | MI | 0 | | | | | | |
| | Degenerating | 0 | | | | | | |
| Ex | FN | 1 | | | 1 | | | 0 |
| | Fibrillar | 0 | | 0 | 0 | 0 | 0 | |
| | Intermediate | 0 | | 0 | 0 | 0 | 0 | |
| | LCC | 5 | 1 | 0 | 0 | 1 | 3 | 4 (100%)[b] |
| | TCC | 19 | 1 | 1 | 3 | 4 | 10 | 14 (78%)[b] |
| | MI | 4 | | 1 | | 2 | 1 | NA[c] |
| | Degenerating | 1 | | 1 | | | | 0 |

[a,b] Within columns, values with different superscripts differ significantly ($P < 0.05$).
[c] NA, not applicable; meiosis had resumed at time of collection.

However, a significantly higher proportion of metaphase configurations were considered to be abnormal in imaged oocytes than in control oocytes (19/44 vs. 1/39; $P < 0.001$), in that chromosomes were poorly aligned on the metaphase plate or were misshapen (elongated or bent).

For imaged oocytes, the relationship of the initial chromatin configuration of Cp and Ex oocytes to the chromatin configuration of these same oocytes after maturation culture is presented in Table 2. There were no significant differences between Ex and Cp oocytes in maturation rate of a given chromatin configuration. In Figure 2, these data are combined to show the relationship of GV chromatin configuration to both oocyte type and maturation rate. Combining Cp and Ex oocytes, those with fibrillar chromatin resumed meiosis significantly less frequently than did oocytes of the other GV chromatin configurations (3/15, 20% vs. intermediate: 9/11, 82%; LCC: 12/14, 86%; and TCC: 17/21, 81%; $P < 0.01$). Oocytes with fibrillar chromatin also had a significantly lower rate of maturation to MII within the culture period (1/15, 6%) than did oocytes in the LCC or TCC configurations (7/14, 50%, $P < 0.05$, and 12/21, 57%, $P < 0.01$, respectively). There were no significant differences in meiotic resumption or maturation to MII among oocytes initially having the intermediate, LCC, or TCC configurations.

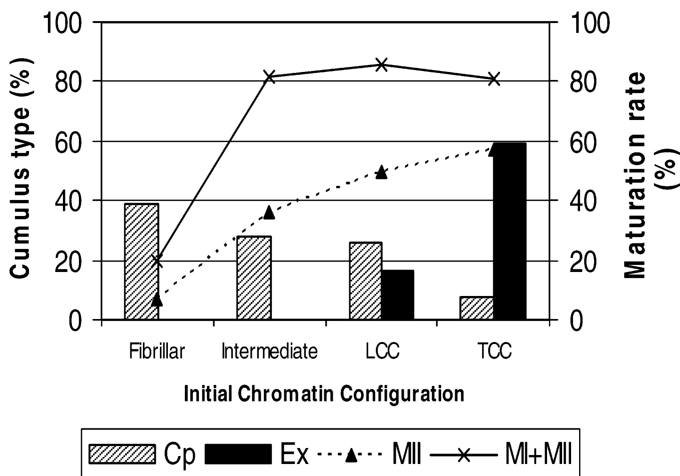


FIG. 2. Proportions of oocytes with expanded and compact cumulus having the different initial germinal vesicle chromatin configurations, and the relationship of chromatin configuration to rate of maturation in vitro (duration 24 h).

### *Experiment 3: Effect of Postmortem Holding Period and Duration of Culture on In Vitro Maturation Rate and Development to Blastocyst after ICSI*

For the immediate treatment, 246 ovaries were processed and 200 Cp, 487 Ex, and 48 degenerating oocytes were recovered. Nine Ex oocytes were lost or broken before evaluation. For the delay treatment, 292 ovaries were processed and 325 Cp, 532 Ex, and 78 degenerating oocytes were recovered. Of the Ex oocytes recovered, 151 were used in separate projects. An additional 81 ovaries were processed for Cp oocytes only, and 90 Cp oocytes were recovered. Two Cp and 3 Ex oocytes were lost or broken before evaluation, thus a total of 413 Cp and 378 Ex oocytes were evaluated after culture in the delay treatments (Table 3).

Within treatment (immediate or delay), culture duration did not affect the rate of resumption of meiosis (combined MI and MII) for either cumulus type (Table 3). When culture durations were combined within treatment, resumption of meiosis was significantly higher for Ex than for Cp oocytes for both immediate (399/478, 83% vs. 130/200, 65%, respectively) and delay (281/378, 74% vs. 126/413, 31%, respectively) treatments ($P < 0.001$). Within cumulus type, oocytes in the immediate-fix treatment group had a significantly higher rate of resumption of meiosis than did oocytes in the delay-fix treatment group for both Cp and Ex oocytes ($P < 0.01$; Table 3). However, oocytes in the immediate treatment group progressed to MII more slowly in culture, as at 24 h the proportion of oocytes at MI was significantly higher in immediate-fix than in delay-fix oocytes (37% vs. 5%, respectively for Cp; 48% vs. 8%, respectively for Ex; $P < 0.001$). Increased duration of culture was associated with a significant decrease in the proportion of oocytes at MI for immediate Cp and Ex oocytes ($P < 0.01$), and also for delay Ex oocytes, ($P < 0.01$), although in the latter group this represented a drop from only 8% to 1%. In the immediate-fix treatments, the proportion of oocytes reaching MII increased significantly between 24 and

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TABLE 3. Proportion of Ex and Cp oocytes, recovered from the ovary either within 45 min of slaughter or after a delay, reaching MI and MII after maturation culture for 24, 30, or 36 h.

| Oocyte type | Treatment | Culture (h) | n | MI (%) | MII (%) | MI+II (%) |
|---|---|---|---|---|---|---|
| Cp | Immediate | 24 | 100 | 37 (37%)[a] | 25 (25%)[a] | 62 (62%)[a] |
| | | 30 | 53 | 5 (9%)[b] | 28 (53%)[b] | 33 (62%)[a] |
| | | 36 | 47 | 4 (9%)[b] | 31 (66%)[b] | 35 (74%)[a] |
| | Delay | 24 | 119 | 6 (5%)[b] | 34 (29%)[a] | 40 (34%)[b] |
| | | 30 | 158 | 5 (3%)[b] | 33 (21%)[a] | 38 (24%)[b] |
| | | 36 | 136 | 5 (4%)[b] | 43 (32%)[a] | 48 (35%)[b] |
| Ex | Immediate | 24 | 227 | 110 (48%)[d] | 74 (33%)[d] | 184 (81%)[def] |
| | | 30 | 123 | 18 (15%)[e] | 89 (72%)[ef] | 107 (87%)[d] |
| | | 36 | 128 | 6 (5%)[fg] | 102 (80%)[e] | 108 (84%)[de] |
| | Delay | 24 | 114 | 9 (8%)[ef] | 77 (68%)[f] | 86 (75%)[ef] |
| | | 30 | 115 | 4 (3%)[fg] | 82 (71%)[ef] | 86 (75%)[ef] |
| | | 36 | 149 | 1 (1%)[g] | 108 (72%)[ef] | 109 (73%)[f] |

[a–g] Within columns within oocyte type, values with different superscripts differ significantly ($P < 0.05$).

30 h for both Cp and Ex oocytes; however in the delay treatments, there were no significant differences in the proportion of oocytes in MII among culture durations for either Cp or Ex oocytes.

Duration of maturation significantly affected blastocyst development within delay Cp oocytes: blastocyst development for the 24-h maturation group was significantly lower than that for 36-h maturation ($P < 0.05$; Table 4). There was a similar trend among maturation durations in immediate Cp oocytes (9%, 18% and 23% for 24 h, 30 h and 36 h, respectively), but these differences were not significant. There was no significant effect of culture duration on blastocyst development for Ex oocytes within immediate or delay treatment groups. Overall, blastocyst development for delay Ex oocytes was significantly higher than for immediate Ex oocytes (75/261, 29% vs. 39/251, 16%; $P < 0.001$). For Cp oocytes, while delay-fix Cp oocytes matured for 30 or 36 h tended to have a higher blastocyst development rate (35% and 38%, respectively) than did similarly matured immediate-fix Cp oocytes (18% and 23%, respectively), these differences were not significant.

When Ex and Cp oocytes were compared at each maturation duration within treatments, blastocyst development was significantly higher for delay-fix Ex than for delay-fix Cp at 24 h ($P < 0.05$) but there was no difference between cumulus morphologies when oocytes were matured for 30 and 36 h. There were no significant differences in blastocyst development between Ex and Cp oocytes within maturation durations in the immediate-fix groups. The highest rates of blastocyst formation obtained for Ex oocytes (32%; delay/30 h) and for Cp oocytes (38%; delay/36 h) were not significantly different.

## DISCUSSION

The results of this study show that the chromatin configuration of horse oocytes changes significantly during ovary storage, and that this is associated with a reduction in meiotic competence. After ovary storage, the proportion of Cp oocytes having the fibrillar or intermediate configuration fell from 68% to 16%; concomitantly, the proportions of Cp oocytes having the FN or degenerating configuration rose from 12% to 58%. There was no significant effect of storage on the proportion of oocytes having the condensed (LCC and TCC) configurations in either Cp or Ex groups. This suggests that the condensed configurations are resistant to change during ovary storage, whereas the more diffuse configurations (fibrillar and intermediate) are labile and undergo chromatin degradation to FN or degenerating over time. The virtual absence of the FN configuration in immediately fixed oocytes indicates that this configuration does not occur in vivo, supporting the recent findings of Pedersen and coworkers [29].

The direct comparison of initial chromatin configuration to nuclear status after culture (experiment 2) showed that the fibrillar and intermediate configurations differed in their meiotic competence. Oocytes in the fibrillar configuration had low meiotic competence, whereas oocytes in the intermediate configuration resumed meiosis at rates similar to those of oocytes with condensed chromatin. Both diffuse

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TABLE 4. Embryo development at 7.5 days after ICSI of oocytes recovered from the ovary either immediately after slaughter or after delay and matured for 24, 30, or 36 h before ICSI.

| Oocyte type | Treatment | Culture (h) | n | Cleavage rate (%) | Morulae (%) | Blastocysts (%) | Morulae + Blast (%) |
|---|---|---|---|---|---|---|---|
| Cp | Immediate | 24 | 23 | 19 (83%) | 3 (13%) | 2 (9%)[a] | 5 (22%) |
| | | 30 | 28 | 25 (89%) | 1 (4%) | 5 (18%)[abc] | 6 (21%) |
| | | 36 | 31 | 25 (81%) | 1 (3%) | 7 (23%)[abc] | 8 (26%) |
| | Delay | 24 | 32 | 22 (69%) | 1 (3%) | 4 (13%)[ab] | 5 (16%) |
| | | 30 | 31 | 24 (77%) | 1 (3%) | 11 (35%)[bc] | 12 (39%) |
| | | 36 | 37 | 33 (89%) | 0 | 14 (38%)[c] | 14 (38%) |
| Ex | Immediate | 24 | 73 | 60 (82%) | 8 (11%) | 8 (11%)[d] | 16 (22%) |
| | | 30 | 87 | 69 (79%) | 4 (5%) | 15 (17%)[de] | 19 (22%) |
| | | 36 | 91 | 76 (84%) | 3 (3%) | 16 (18%)[de] | 19 (21%) |
| | Delay | 24 | 75 | 64 (85%) | 7 (9%) | 22 (29%)[ef] | 29 (39%) |
| | | 30 | 81 | 69 (85%) | 8 (10%) | 26 (32%)[f] | 34 (42%) |
| | | 36 | 105 | 88 (84%) | 8 (8%) | 28 (27%)[ef] | 36 (34%) |

[a–f] Within columns within oocyte type, values with different superscripts differ significantly ($P < 0.05$).

oocyte types appear to be subject to damage during ovary storage. Thus, intermediate oocytes make a major contribution to the proportion resuming meiosis when oocytes are recovered immediately postmortem, but not when they are recovered after a delay.

Degeneration of labile oocytes may occur rapidly during ovary storage. In previous studies in the horse in which all ovaries were stored at least 1.5 h, no difference was found in maturation rates among storage periods of 1.5 to 8.5 h [43] or 3 to 15 h [44]; however, the average maturation rate in these studies was lower than 50%. It is possible that damage to susceptible oocytes had already occurred after 1 to 3 h of storage. Storage of horse ovaries for >20 h may result in damage even to oocytes with condensed chromatin, as maturation rates of Ex oocytes stored for this longer period were essentially halved [45].

In experiment 3, oocytes that were recovered after being held within the ovary 5–9 h subsequently matured more rapidly, as evidenced by the significantly larger proportion of oocytes in MII at 24 h of culture in this group compared with the immediate group at the same time period. This suggests that prematurational changes may occur in horse oocytes held at room temperature within the ovary, and is similar to the finding of altered maturation kinetics after suppression of maturation with butyrolactone or butyrolactone + roscovitine in bovine oocytes [4, 5]. Thus, ovary handling procedures affect the observed period of culture needed for in vitro maturation of horse oocytes to reach MII, and may be a source of variation among laboratories.

When compared within treatment (immediate-fix or delay-fix), Cp and Ex oocytes had similar rates of blastocyst formation after ICSI. In a previous study, we found a higher rate of normal embryo cleavage after ICSI in Ex than in Cp oocytes [6]. However, in that study, all oocytes were matured for 24 h. In the present study, if oocytes are compared only at 24 h maturation, these two groups also tend to differ in blastocyst development (22/75, 29% vs. 4/32, 13%, respectively, $P = 0.1$). These results indicate that the duration of maturation needed for maximum developmental competence varies according to original oocyte cumulus type. When the optimum maturation duration for both cumulus types is compared, blastocyst development is equivalent (38% for delay Cp 36 h and 32% for delay Ex 30 h). Thus, although Ex oocytes originate from follicles in primary to tertiary atresia [11], it appears that their competence for postfertilization development to the blastocyst stage is not significantly impaired. Advanced cumulus expansion has been reported to either have a detrimental effect or no effect on nuclear and cytoplasmic maturation and on embryo development in cattle [46–51]; however, those studies compared oocytes at only one duration of maturation.

The maximum blastocyst development rates in this study (32% to 38%) are the highest yet reported for in vitro-produced horse embryos, and indicate that the system used (culture in DMEM/F-12 with FBS under mixed gas) supports development rates that may be comparable to those achieved in the bovine. Previously reported rates of blastocyst development in equine embryos cultured in other media alone (without coculture) have been lower than 10% [52–55]. We previously reported 15% blastocyst development for equine embryos cultured in DMEM/F-12 with FBS under 5% $CO_2$ in air [37].

Ovary storage was associated with an increase in developmental competence of horse oocytes, as reflected in the higher blastocyst development for each oocyte type at each maturation duration for oocytes collected after a delay (Table 4). It is notable that in Cp oocytes, while twice as many immediate-treatment than delay-treatment oocytes matured to MII, blastocyst development in the immediate group was about half of that in the delay group. Thus, the overall number of blastocysts obtained per oocyte cultured was similar between treatments. One interpretation of this data would be that while some fibrillar and most intermediate oocytes can mature to MII if placed into culture immediately, they are not developmentally competent. However, a similar trend was found in Ex oocytes, which have low proportions in the fibrillar and intermediate configurations. In the case of Ex oocytes, delayed recovery actually increased the proportion of blastocysts obtained per recovered oocyte, almost doubling it in the 30-h culture duration (12% [72% maturation × 17% blastocyst development] vs. 23% [71% maturation × 32% blastocyst development] for Ex immediate and delay 30-h treatments, respectively). This supports the conclusion of Blondin and coworkers [3] in cattle, that changes occurring in the oocyte within the follicle postmortem serve to increase cytoplasmic maturation and subsequent developmental competence.

The findings of this study help to clarify reasons for differences observed in GV chromatin configuration, maturation rates and, potentially, blastocyst development rates after ICSI of horse oocytes among laboratories. Laboratories that recover oocytes ex vivo, or immediately postmortem, and process them immediately would be expected to observe more oocytes in the fibrillar and intermediate configurations, fewer degenerating and FN oocytes, a higher maturation rate but slower progress during maturation, and a lower blastocyst development rate per MII oocyte than do laboratories that recover oocytes after ovary storage. In addition, both the proportion of oocytes at MII and the observed developmental competence of horse oocytes may vary depending on the criteria for selecting oocytes for culture and the duration of maturation used. Similar phenomena may be present in other species and should be considered when oocytes of different classes (e.g., those recovered ex vivo vs. those recovered from slaughterhouse tissue) are compared. Understanding these factors is essential to progress in the study of oocyte meiotic and developmental competence.

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