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Equine oocyte quality depending on original follicle population

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Summary

In vitro embryo production is not successful in the horse and only a few foals have been born by using this technology. While the rate of chromosomal maturation of in vitro cultured horse oocytes is high, fertilization rates in vitro are still not efficient. This could be due to an impaired cytoplasmic maturation in vitro. The aim of the present study was to characterize the cytoplasmic and chromatin quality of oocytes depending on different follicle populations in mares. Therefore, 14 Mecklenburger Warmblood mares underwent repeated transvaginal ultrasound guided follicle aspiration. Aspiration sessions were performed 44 times during the heat of the mare to obtain oocytes from preovulatory and subordinate follicles and 79 times oocytes were recovered from progressive follicle populations before a dominant follicle had developed. Steroid analysis of the follicle fluid confirmed clinical deviation of the follicle populations. Every single oocyte was analysed for cumulus morphology, chromatin configuration, glucose-6-phosphate dehydrogenase activity as well as the mitochondrial activity and aggregation. Our results showed no impact of the follicle population upon the glucose-6-phosphate dehydrogenase activity in the oocytes, measured by brilliant cresyl blue staining. We observed that progressive follicle populations contained significant more oocytes with a fibrillar diplotene configuration and a higher mitochondrial activity, while subordinate follicle populations had more oocytes with a condensed diplotene and a lower mitochondrial activity. In conclusion, the follicle population, originating the oocyte, significantly influences parameters of its quality. Therefore, the origin of the oocyte should be more noticed in programmes of in vitro embryo production in the future.

Keywords: oocyte quality, follicle population, chromatin configuration, glucose-6-phosphate dehydrogenase status, mitochondria status, horse

Eizellqualität in Abhängigkeit von der Follikelpopulation bei Stuten

Obwohl es in den letzten 10 Jahren eine rasche Entwicklung bei den assistierten Reproduktionstechniken bei Pferden gab, ist die In-vitro-Erzeugung von Embryonen bei dieser Spezies noch sehr ineffizient. Bis heute wurden weltweit erst wenige Fohlen auf diese Weise erzeugt. Obwohl nach der In-vitro-Reifung von equinen Eizellen Kernreifungsraten (Metaphase II der Meiose) von 60-70% erreicht werden, bleibt die Entwicklung eines Embryos nach konventioneller In-vitro-Fertilisation (IVF) eher ein sporadisches Ereignis. Als ein Grund für das Fehlschlagen der IVF beim Pferd wird auf der maternalen Seite eine unzureichende zytoplasmatische Reifung der Eizelle in vitro neben der erfolgreichen Kernreifung diskutiert. Durch das dynamische Follikelwachstum auf den Ovarien von Stuten bieten sich dem Tierarzt zu verschiedenen Zyklusabschnitten differente Populationen von Follikeln für die Eizellgewinnung an. Ziel unserer Untersuchungen war es, chromosomale und zytopasmatische Qualitätsparameter von Oozyten aus physiologisch unterschiedlichen Follikelpopulationen zu untersuchen. Die Oozytengewinnung erfolgte durch wiederholte transvaginale ultraschallgeleitete Follikelpunktionen (OPU), die in 120 Follikelpunktionssitzungen an 14 Mecklenburger Warmblutstuten während einer Zuchtsaison vorgenommen wurden. Die Eizellgewinnung erfolgte zunächst bei rossigen Stuten, denen 24 Stunden zuvor ein hCG-Präparat appliziert worden war. Die Follikelaspirate wurden getrennt nach präovulatorischen Follikeln und subordinanten Follikeln gesammelt. Nachdem alle sichtbaren Follikel in der Rosse aspiriert worden waren, wurde die Stute nach Ablauf einer Woche einer zweiten Follikelaspiration unterzogen, noch bevor sich in der neu gewachsenen Follikelpopulation ein dominanter Follikel entwickelt hatte. Zur genaueren Charakterisierung der verschiedenen Follikelgruppen erfolgten Analysen der Estradiol- und Progesteronkonzentration in den Follikelflüssigkeiten. Jede einzelne Oozyte wurde auf ihre Kumulusmorphologie, Chromatinkonfiguration, Aktivität der Glucose-6-Phosphat Dehydrogenase (G6PDH), sowie auf ihre mitochondriale Aktivität und mitochondriale Aggregation untersucht. Dazu wurden die Kumulus-Oozyten-Komplexe (KOK) nach der Gewinnung unter einem Stereomikroskop beurteilt, in Brillant-Kresyl-Blau-Lösung (BCB) unter Kulturbedingungen inkubiert (Bestimmung der G6PDH-Aktivität) und anschließend parallel mit MitoTracker Orange (Bestimmung der mitochondrialen Aktivität und Aggregation) und Hoechst 33342 (Bestimmung der Chromatinkonfiguration) gefärbt. Es wurden keine signifikanten Unterschiede bei der G6PDH-Aktivität der Oozyten in Abhängigkeit zu den untersuchten differenten Follikelpopulationen beobachtet. Die Morphologie der gewonnenen KOK sowie die Chromatinkonfiguration und die mitochondriale Aktivität und -Aggregation in den Oozyten standen jedoch im Bezug zur follikulären Herkunft der Oozyten. Wachsende Follikelpopulationen enthielten gehäuft kompakte KOK und Oozyten mit fibrillärem Diplotän-Chromatin, sowie hoher mitochondrialer Aktivität; während subordinante Follikelpopulationen gehäuft Oozyten mit kondensiertem Diplotän-Chromatin und geringerer mitochondrialer Aktivität aufwiesen ($p<0.05$). Höchste Werte für die mitochondriale Aktivität wurden in Oozyten aus präovulatorischen Follikeln gemessen, deren Mitochondrien alle ein grobkörniges Aggregationsmuster aufwiesen. Zusammenfassend kann festgestellt werden, dass chromosomale und zytoplasmatische Qualitäten von equinen Oozyten von der follikulären Herkunft beeinflusst werden. Die klinischen Informationen, die der Tierarzt über den physiologischen Zustand der Follikelpopulationen zum Zeitpunkt der Follikelaspiration erlangt, sollten deshalb in Zukunft mehr Beachtung bei der Durchführung der Eizellgewinnung für die In-vitro-Embryonenerzeugung bei Pferden finden.

Schlüsselwörter: Oozytenqualität, Follikelpopulation, Chromatinkonfiguration, Glucose-6-Phosphat-Dehydrogenase-Status, Mitochondrienstatus, Stute

Introduction

Development of assisted reproduction techniques in the horse based on in vitro procedures of embryo production have emerged in recent years and parallel to it, more and more horse breeders and breed associations have became interested in these techniques. However, the in vitro embryo production is not very successful in the horse and only few foals have been born by using this technology (*Galli* et al. 2007).

While the rate of chromosomal maturation of in vitro cultured horse oocytes is high, fertilization rates in vitro are still not efficient (*Hinrichs* et al. 2005). This could be due to an impaired cytoplasmic maturation of these oocytes. In contrast to compact cumulus-oocyte-complexes (COC), equine oocytes with expanded cumulus cells reach M2-stages of meiosis sooner in vitro (*Torner* et al. 2007) and provide higher fertilisation rates after conventional IVF (*Alm* et al. 2001) or ICSI (*Hinrichs* et al 2005). These expanded COCs show a consistent increase of mitochondrial activity in oocytes during in vitro maturation in contrast to compact COCs (*Torner* et al. 2007).

Although previous studies support the notion that oocyte competence depends on multiple factors, it remains difficult to draw clear and reliable parameter for oocyte selection during ultrasound guided follicle aspiration in vivo. The most important criteria for the maturation environment for horse oocytes in vivo like follicle size, health of follicle or phase of follicular wave have still not been described. Nevertheless, the link between follicle quality and oocyte quality is of great practical interest for the development of reproductive biotechniques in mares. The dynamic of follicle growth and atresia during the ovarian cycle are the cause, that follicles with different quality in different proportions are available for oocyte recovery during the cycle. At the end of the heat, all subordinate follicles show different stages of atresia in contrast to the still viable dominant follicle (*Watson* et al. 2002). Very short intervals between follicle aspiration sessions enhanced the proportion of viable follicles, especially when a dominant follicle had not developed between the sessions (*Gastal* et al. 1997, *Kanitz* et al. 2000).

With the urgent need for establishing non-invasive and non-perturbing means for oocyte selection, the brilliant cresyl blue (BCB) staining test has been successfully used to differentiate oocytes with different developmental capacity in various species and at least in the horse, a relationship between BCB-staining of oocytes and their further developmental competence was found (*Mohammadi-Sangcheshmed* et al. 2009). BCB is a dye that can be degraded by the ooplasmic enzyme glucose-6-phosphate dehydrogenase (G6PDH; *Ericsson* et al. 1993, *Tian* et al. 1998). The activity of this protein is increased during the growth of the oocyte, which happens in early stages of the follicle development. With completing the growth phase, the oocytes achieve their developmental competence (*Wassarman* 1988, *Tian* et al. 1998). Oocytes that have finished their growth phase show a decreased G6PDH activity and exhibit cytoplasm with a blue colouration (BCB+), while growing oocytes are expected to have a high level of active G6PDH, which results in colourless cytoplasm (BCB-). Another prominent marker for cytoplasmic maturation is the activity of mitochondria. Mitochondria play a vital role in the oocyte to provide ATP as a mayor source of energy for processes at the time of fertilization and for pre-implantation embryo development. Data in human and bovine oocytes suggest that the level of mitochondrial respiration in oocytes is closely correlated to the rate of embryo development after fertilization (*Stojkovic* et al. 2001, *Wilding* et al. 2001).

Based on our previous investigation on cytoplasmic maturation of horse oocytes in vitro (*Torner* et al. 2007) the aim of the present study was to characterize the cytoplasmic and chromatin quality of oocytes depending on different follicle populations in mares.

Materials and Methods

To recover COCs in vivo, 14 Mecklenburger Warmblood mares underwent repeated transvaginal ultrasound guided follicle aspiration during one breeding season, as previously described (*Kanitz* et al. 1995). Follicle aspiration sessions were performed 44 times during the heat of the mare, 24 hours after they had achieved an injection of 2500 IU hCG and the aspirates of the preovulatory follicles and the subordinate follicle populations were separated. Further more, 79 follicle aspiration sessions were done one week after a total ablation of all follicles bigger than 0.5cm, before a dominant follicle (> 2.3cm) had developed in the new grown, progressive follicle population. Simultaneously random samples of follicle fluid from the different follicle populations were taken and analysed for their steroid content by a 3H-RIA later on. Every single oocyte was analysed for the chromatin and cytoplasmic quality. Therefore the oocytes of the different follicle populations were divided into groups depending upon their cumulus morphology and incubated under culture conditions with brilliant cresyl blue stain immediately, to evaluate the glucose-6-phosphate dehydrogenase activity (G-6-PDH). Briefly, a total of 178 cumulus–oocyte complexes (COCs) were subjected to 26 M BCB (B-5388, Sigma-Alderich) diluted in mDPBS for 90 min at 38.5 °C in humidified air atmosphere. The stained COCs were examined under stereomicroscope and categorised into two groups according to their cytoplasm colouration: oocytes with any degree of blue colouration in the cytoplasm (BCB+) and oocytes without visual blue colouration (BCB-).

After BCB-staining, the same oocytes were processed for fluorescence labelling of mitochondria according to the procedure described previously for porcine and horse oocytes (*Torner* et al. 2004, 2007). Briefly, COCs were incubated for 30 min in PBS containing 3% (w/v) BSA and 200 nM MitoTracker Orange-fluorescent tetramethylrosamine (M-7510, Sigma-Aldrich) under culture conditions. The mitochondrial-specific fluorescent and cell-permeant probe MitoTracker Orange CMTM Ros is readily sequestered only by actively respiring organelles, depending upon their oxidative activity. In the next steps the oocytes were mechanically denuded, washed in PBS without BSA and fixed for 15 min at 37 °C using freshly prepared 3% (v/v) paraformaldehyde in Hank's balanced salt solution. Immediately after fixation, the same oocytes were prepared for the further staining of the chromatin. They were washed again in PBS and then mounted between slide and cover slip together with three drops of a glycerol-PBS-solution, containing 2.5 mg/ml of the DNA stain bis-benzimide (Hoechst 33342, B-1155, Sigma-Aldrich). The slides were kept for 4-6 weeks at 4 °C in darkness until the oocytes were analyzed for chromatin configuration, mitochondrial aggregation and activity by an epifluorescence microscope (Jenalumar, Carl Zeiss, Jena) and a photometry system (Nikon P 100, Nikon, Düsseldorf), as previously detailed described (*Torner* et al. 2007).

Results

In 120 follicle aspiration sessions 1058 follicles were aspirated resulting in 178 oocytes. Steroid analyses of random samples of follicle fluids from the different follicle populations showed significant differences between the oestradiol content ($p < 0.05$) while the content of progesterone was not statisti-

Table 1 Morphology of equine COCs related to their follicle population of origin In columns: a:b $p<0.05$; c:d $p=0.06$
Morphologie von equinen COC in Abhängigkeit von der follikulären Herkunft in Spalten.

		Cumulus-oocyte-complexe-morphology in % (LSM ± SE)		
Follicle population	Number of	Compact	Expanded	Corona radiata
subordinate	101	26.7 ± 4.4[a]	27.7 ± 4.5	45.6 ± 5.4[c]
progressive	75	44.0 ± 5.7[b]	24.0 ± 5.0	32.0 ± 5.0[d]
preovulatory	2	0	0	100

cally different. Lowest oestradiol concentrations were found in subordinate (54.4 ± 155.9 ng/ml), higher in progressive (885.6 ± 120.6 ng/ml) and highest concentrations were found in preovulatory follicles (1911.7 ± 185.5 ng/ml), which resulted in significant differences of the oestradiol:progesterone ratio between the three follicle groups (Fig. 1; $p > 0.001$). As demonstrated in Table 1; after evaluation of the cumulus-oocyte-complex morphology, significantly more oocytes with compact cumuli were found in the progressive follicle population ($p < 0.05$), whereas the subordinate follicle population tended to contain more corona radiata oocytes ($p = 0.06$). Oocytes, recovered by follicle aspiration of

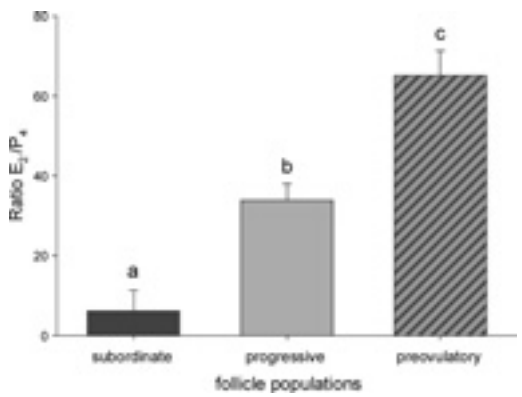

Fig. 1 Oestradiol:progesterone ratio (E2/P4, LSM ± SE) in equine follicle fluids of subordinate, progressive and preovulatory follicle populations. a:b; a:c; b:c $p < 0.001$
Quotient aus Oestradiol- und Progesteronkonzentrationen (E2/P4, LSM ± SE) in equinen Follikelflüssigkeiten von subordinanten, wachsenden und präovulatorischen Follikelpopulationen)

tene were found more in oocytes from the progressive follicle population ($p < 0.05$), while the amount of the diffuse diplotene did not differ between both these groups. In each follicle population were significantly more oocytes with a low activity of the G6PDH (BCB+; $p < 0.05$) and no differences between the follicle populations were seen. The mitochondrial activity was increased significantly in oocytes of subordinate follicles as compared to oocytes from progressive follicles ($p < 0.05$) and the highest value of mitochondrial activity (fluorescence intensity/oocyte) was reached in oocytes from preovulatory follicles. If the mitochondrial activity is related to both - the G6PDH activity and its origin of follicle population, no diffe-

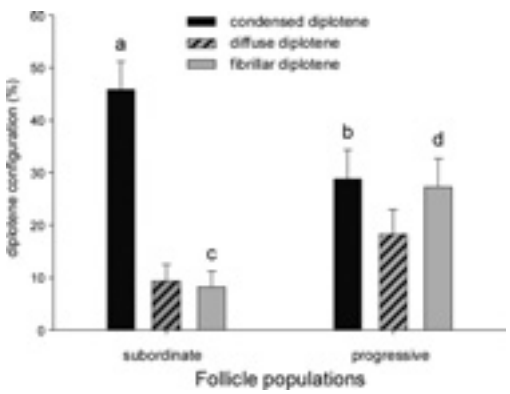

Fig. 2 Proportions of different diplotene configurations (%, LSM ± SE) of equine oocytes from subordinate and progressive follicle populations. a:b; c:d $p < 0.05$
Anteil der verschiedenen Diplotän-Konfigurationen (%, LSM ± SE) bei equinen Oozyten aus subordinanten und wachsenden Follikelpopulationen)

preovulatory follicles, were only surrounded by corona radiata cells in our study. While the nucleus of a few oocytes from each follicle population showed a progression of meiosis at the time of recovery, significant differences were observed between the different diplotene configurations (Fig. 2). In immature equine oocytes, three different chromatin configurations in the diplotene stage of meiosis before germinal vesicle break down could be observed. The proportion of oocytes with a condensed diplotene were higher in oocytes from the subordinate follicle population whereas the fibrillar diplo-

rences were found between BCB- and BCB+ oocytes from subordinate and progressive follicle populations (Tab. 2). Two different types of mitochondrial aggregation pattern were observed in the oocytes: fine aggregation of the mitochondria (Fig. 3) and granulated mitochondrial aggregation (Fig. 4). It is illustrated in Figure 5, that equal minor numbers of oocytes from subordinate and progressive follicle populations showed the granulated mitochondrial aggregation type, in contrast ($p < 0.05$) to the oocytes of preovulatory follicles, in which the granulated mitochondrial aggregation type was found only.

Table 2 Mitochondrial activity in horse oocytes depending on the follicular origin and G6PDH status
Mitochondriale Aktivität in Pferdeoozyten in Abhängigkeit von der follikulären Herkunft und dem G6PDH-Status

	Mitochondrial activity in µA/oocyte (LSM ± SE)	
Follicle population	G6PDH status of oocytes	
	BCB+	BCB-
subordinate	266.4 ± 44.6	189.8 ± 57.5
progressive	360.5 ± 46.9	402.8 ± 76.6
preovulatory	1025 ± 325.0	248.0 ± 325.0

Discussion

We expected to obtain COCs from mature follicles by aspirating preovulatory follicles, 24 hours after administration of hCG (*Ginther* et al. 2007) and COCs from follicles in different stages of atresia by aspirating subordinate follicles at the end of the heat (*Watson* et al. 2002). Also, we were interested in COCs from a greater number of young, viable follicles by aspirating progressive follicle populations before a dominant follicle had developed (*Gastal* et al. 1997, *Kanitz* et al. 2000). Steroid analysis of the follicle fluid could confirm the clinical deviation of the follicle populations. High concentrations of oestradiol and a high E2/P4 ratio are typical for viable and preovulatory follicles, while atretic follicles have a low synthesis of all steroids (*Belin* et al. 2000). Furthermore, the higher proportion of oocytes with compact cumulus cells in the progressive follicle population could be discussed as an indication that this follicle population consisted of a higher amount of viable and still growing follicles (*Kanitz* et al. 2000). Our results did not show an impact of the used different follicle populations of tertiary follicles (> 0.5 cm) upon the G6PDH-activity of the enclosed oocytes. This underlines the opinion that the G6PDH-activity of the oocytes is strongly linked to preantal and early stages of antral follicles (*Mangia* and *Epstein* 1975). We observed in our investigation that progressive follicle populations contained significant more oocytes with a fibrillar diplotene chromatin and a higher mitochondrial activity, while subordinate follicle populations had more oocytes with a condensed diplotene and a decreased mitochondrial activity ($P<0.05$). The fibrillar diplotene is discussed as an immature chromatin, while the condensed diplotene should be the stage in which the equine oocytes became developmentally competent (*Hinrichs* et al. 1997). However, in our study the condensed diplotene was linked to subordinate follicles, which contain oocytes with a low mitochondrial activity, so that this chromatin configuration could also be a first step of the degeneration of the oocyte. Our investigations of the mitochondrial status in equine oocytes in vivo agree with our previous in vitro studies on horse oocytes (*Torner* et al. 2007), that an increasing mitochondrial aggregation and activity is a process, that happens in the final maturation period of the horse oocyte.

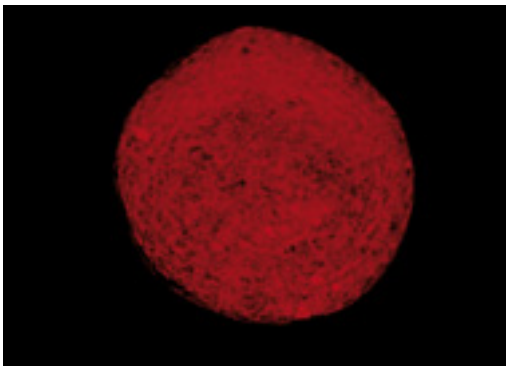

Fig. 3 Fine mitochondrial aggregation pattern in a horse oocyte from a compact cumulus-oocyte complex, originating from a progressive follicle population (250x, MitoTracker Orange CMTM Ros, M-7510 staining)
Feinkörniges mitochondriales Aggregationsmuster in equiner Oozyte von einem kompakten Kumulus-Oozyten-Komplex aus einer wachsenden Follikelpopulation (250x, MitoTracker Orange CMTM Ros, M-7510 Färbung)

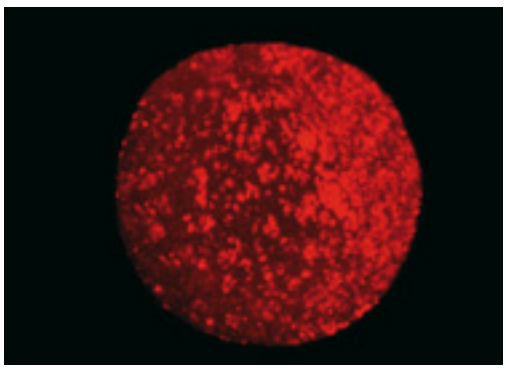

Fig. 4 Granulated mitochondrial aggregation pattern in a horse oocyte from a corona radiata oocyte, originating from a preovulatory follicle (250x, MitoTracker Orange CMTM Ros, M-7510 staining)
Grobkörniges mitochondriales Aggregationsmuster in equiner Oozyte von einer Corona radiata Oozyte aus einem präovulatorischen Follikel (250x, MitoTracker Orange CMTM Ros, M-7510 Färbung)

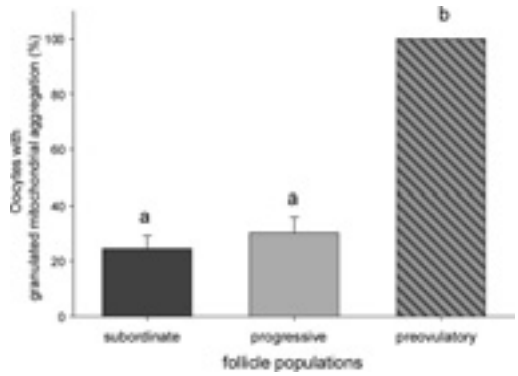

Fig. 5 Proportions of equine oocytes with granulated mitochondrial aggregation (%, LSM ± SE) from subordinate, progressive and preovulatory follicle populations. a:b $p < 0.05$
Anteil an equinen Oozyten mit granulierter mitochondrialer Aggregation (%, LSM ± SE) aus subordinanten, wachsenden und präovulatorischen Follikelpopulationen

In conclusion, the follicle population, originating the oocyte, significantly influences parameters of its quality, like mitochondrial and chromatin status. Therefore, the origin of the horse oocyte should be more noticed in programmes of in vitro embryo production in the future.

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