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Short Note

Optimalization of Fluorescence *in situ* Hybridization Conditions in Mare Oocytes and Mouse Embryos*

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The aim of the study was to optimize hybridization conditions of molecular probes specific for X sex chromosomes of the domestic horse in mare oocyte chromosomes. Mare oocytes, recovered from slaughterhouse ovaries by scraping the granulosa layer, were cultured *in vitro*. Metaphase II mature oocytes were treated with hypotonic solution and fixed, followed by hybridization of the molecular probe specific for the X chromosome of the domestic horse. Hybridization of probes specific for mouse heterosomes on mouse oocytes and early embryos was performed to verify the FISH technique. Of 438 oocytes analysed, 29% reached metaphase II. Despite many changes in the composition of hypotonic solutions and modification of the FISH protocol, the fluorescence signal was observed in mouse oocytes and embryos but not in mare oocytes.

Key words: Mare, mouse, oocytes, FISH technique.

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Research on embryo production *in vitro* has been of essential importance for the development of reproductive biotechnology in animals. *In vitro* oocyte maturation and *in vitro* fertilization methods allow for a better use of the reproductive potential of mares. *In vitro* maturing oocytes, or embryos produced are increasingly used in assisted reproduction research.

The many failures associated with high mortality of embryos cultured *in vitro* may be due to chromosomal abnormalities. The identification of these abnormalities may be facilitated by rapid advances in cytogenetics, which has contributed to the development of modern techniques for identification of chromosomal abnormalities. The combination of classical cytogenetics and DNA recombination technology gave rise to a new discipline known as molecular cytogenetics, which makes it possible to analyse chromosomes using fluorescence *in situ* hybridization.

Abnormal segregation of chromosomes during spermatogenesis or oogenesis results in a gamete with an abnormal chromosome set. When such a gamete participates in fertilization, the developing embryo will carry a mutation. Mutations include changes in the number of chromosomes or genes (genome mutations), changes in chromosome structure (chromosome mutations) or changes of a gene to create a new allele (gene mutations). In animal breeding, mutations, especially genome and chromosome mutations, are undesirable and often lead to death, congenital malformations or reduced fertility of the carriers. Therefore, the aim of the present study was to optimize hybridization conditions of molecular probes specific for the X sex chromosome of the domestic horse in mare oocyte chromosomes.

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Material and Methods

Oocytes recovered from slaughterhouse ovaries of 114 mares were investigated. Mouse oocytes and early embryos were used for verification of the FISH technique.

Oocyte collection and culture

Oocytes were recovered from ovaries by scraping the granulosa layer. Primary oocytes at the germinal vesicle stage were detected with a stereoscopic microscope. The oocytes were classified according to the criteria of CHOI *et al*. (1993) by evaluating the cohesion of cumulus oophorus and corona radiata cell layers as well as by cytoplasm morphology. The first three classes of oocytes were used for *in vitro* culture. Degenerated oocytes were rejected.

Oocyte culture was performed in maturation medium (TCM-199 with Earle's salts) with 20% FBS, 0.1g/ml L-glutamine, 5μg/ml FSH, 1μg/ml pyruvate, 1μg/ml 17β-estradiol, and a mixture of PSA antibiotics. Oocytes were cultured in an incubator for 30-40 h at 38°C under 100% humidity and 5% CO_2.

Preparations from mature oocytes

Mature oocytes deprived of cumulus cells were treated with hypotonic solution causing the cells to swell and thus increasing chromosome dispersion. The following types, combinations and incubation times of hypotonic solutions were tested:

1. 1% sodium citrate, for 10-30 min at 38°C or at room temperature;

2. 0.88% sodium citrate, for 10-30 min at 38°C or at room temperature (OCANA-QUERO 1999);

3. 0.75% sodium citrate, for 10-30 min at 38°C or at room temperature;

4. 0.05 mM KCl, for 10-30 min at 38°C or at room temperature;

5. 160 μl 0.5M HCl + 8 μl Tween20 + 10 ml distilled water solution, for 10-30 min at 38°C or at room temperature (RAMBAGS 2005);

6. 17mM sodium citrate + 30 mM TRIS + 50 mM saccharose + 15mM EDTA + 0.5mM DTT, for 15-30 min at 38°C or at room temperature.

To improve fixative penetration, some oocytes were placed for 10-20 min in 0.5% pronase solution to dissolve the zona pellucida.

Oocytes were then fixed in one of the following solutions:

1. Carnoy's fixative (ice-cold acetic acid + methanol 3:1), for 5 min to 24 h, at room temperature, at -20°C, 60°C or 80°C (TARKOWSKI 1966);

2. ice-cold acetic acid + methanol 1:1, for 5 min to 24 h (OCANA-QUERO 1999);

3. 1% paraformaldehyde + 0.15% Triton X100, for 5-20 min at room temperature, followed by incubation in 0.4% Photo-Flo200 for 30 s.

Cytogenetic evaluation of preparations

To perform cytogenetic analysis, preparations were stained with 5% Giemsa for 15 min and examined with an Opton Axiophot microscope (Zeiss). Images of selected metaphase spreads were archived on a computer using LUCIA software (Laboratory Imaging LTD, Prague, Czech Republic), taking into account the exact locations of metaphases relative to the x and y axes of the microscope stage. Before the next phase, preparations were stained with 70% ethanol. Some preparations were analysed under phase contrast without prior staining. Selected preparations with visible metaphases were stored frozen at -20°C for further FISH analysis.

Fluorescence *in situ* hybridization (FISH) of mare oocytes

Just before hybridization, oocyte preparations were dehydrated in 100% ethyl alcohol. They were then digested with pepsin at 37°C for 10 min and washed twice for 1 min in 2xSSC (0.3M NaCl and 0.03M $Na\text{-}_3C_6H_5O_7$ x $2H_2O$). Preparations were dehydrated in an ethanol series (70%, 80%, 95%) and tempered for 15 min at 80°C. During this time, the horse X chromosome probe was denatured at 70°C for 10 min.

Preparations were denatured in 70% formamide in 2xSSC at 70°C for 2.5 min. Immediately after denaturation, they were passed through an ice-cold alcohol series (70%, 80%, 90% and 100%). A probe was applied to dried preparations, which were covered with a cover glass and sealed with Fixogum. Hybridization was performed in a moist chamber at 37°C for 1 to 3 days.

Hybridization for a dozen or several dozen hours was followed by a series of washes at 43°C, twice for 5 min in 50% formamide in 1xSSC buffer and in pure 1xSSC buffer. Preparations were then placed in an equilibration solution (Detergent Wash Solution, 100 ml 20xSSC + 400 ml distilled water + 250 μl Tween20) for 10 min at room temperature and then transferred to a preincubation buffer (4xSSC, 0.05% Tween20, 3% BSA-Albumin Bovine Serum) for 20 min at 37°C.

Signal was detected using avidin conjugated with FITC (fluorescein isothiocyanate). The fluorescence intensity was amplified using a biotinylated anti-avidin antibody.

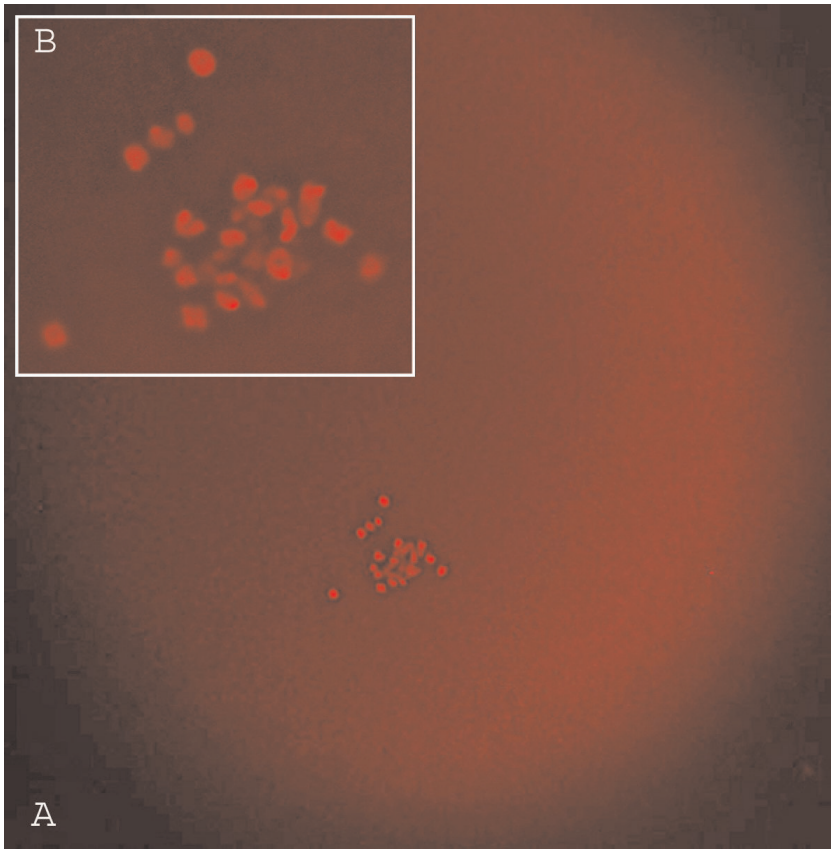

Fig. 1. Metaphase II oocyte. A – FITC staining; B – enlarged metaphase spread after application of the FISH technique, no signal from the horse X chromosome painting probe.

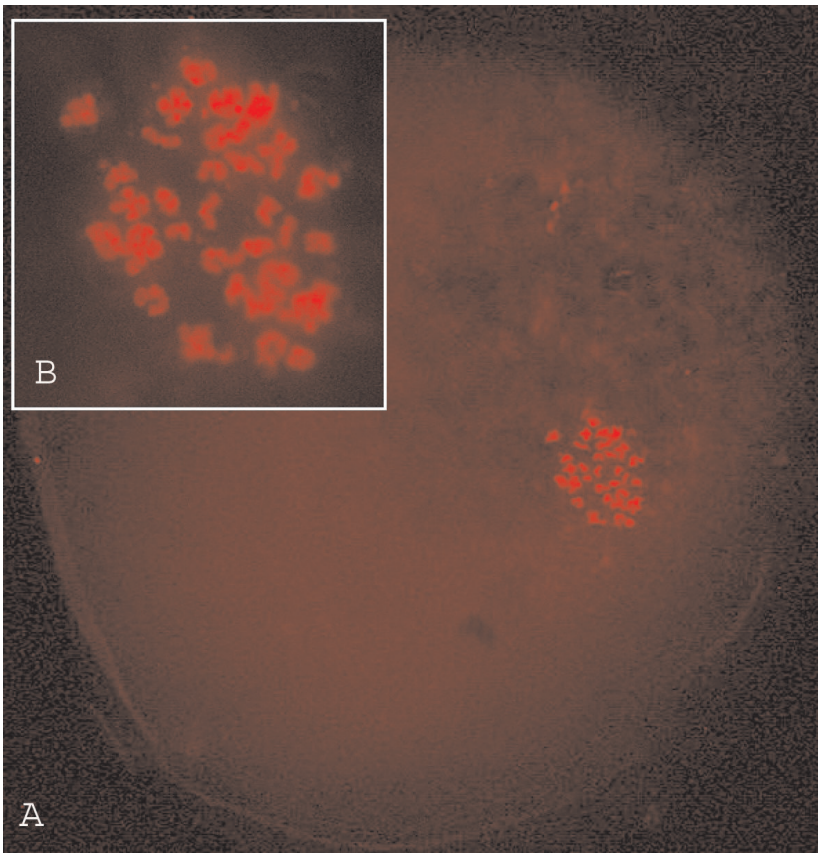

Fig. 2. Metaphase II oocyte. A – FITC staining; B – enlarged metaphase spread after application of the FISH technique, no signal from the horse X chromosome painting probe.

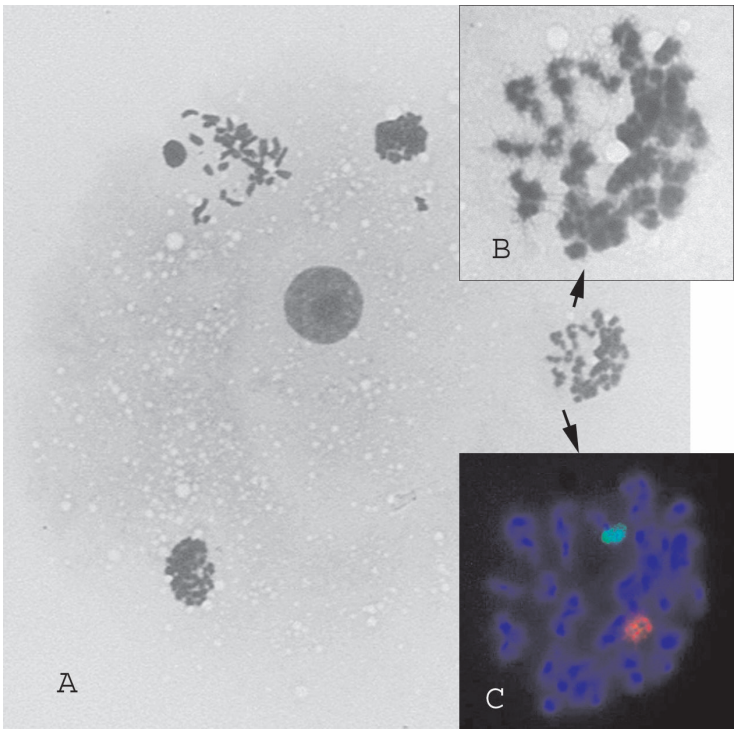

Fig. 3. FISH with sex chromosome probes on a 4-blastomere mouse embryo. A – embryo with 4 metaphase spreads and polar body, Giemsa staining; B – enlarged metaphase spread, Giemsa staining; C – FISH with X (green) and Y chromosomes (red).

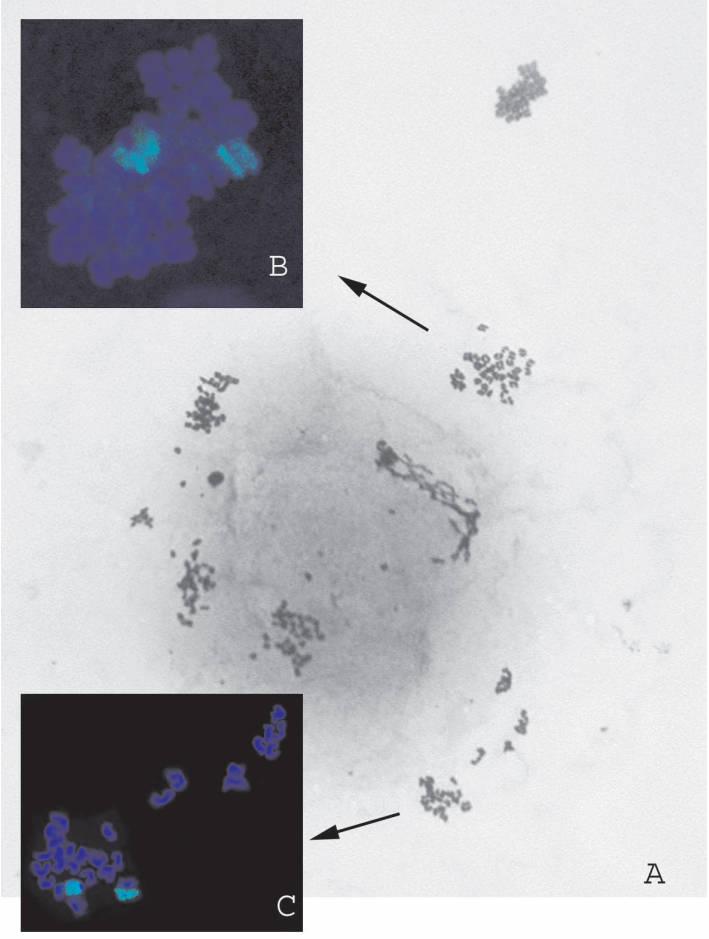

Fig. 4. FISH with sex chromosome probes on an 8-blastomere mouse embryo. A – 8-blastomere embryo, Giemsa staining; B-C – metaphase spreads with two X chromosomes (green), FISH technique.

Because the results were not positive, the following changes were made to the original protocol:

1. Additional RNase digestion (100 μg/ml in 2xSSC) was applied at 37°C for 1 h.

2. The RNase concentration was doubled to 200 μg/ml in 2xSSC and pepsin digestion time was increased from 10 to 30 min.

3. The temperature of preparation denaturation was increased from 70°C to 80°C and its time was increased from 2.5 to 5 min.

4. The hybridization time was extended to 72 h.

5. Milder washing was used after hybridization (2xSSC instead of 1xSSC) and formamide was also diluted in 2xSSC.

6. The composition of Detergent Wash Solution was changed from 100 ml 20xSSC + 400 ml distilled water + 250 μl Tween20 to 160 ml 20xSSC + 636 ml distilled water + 4 ml Tween20).

7. A directly labeled probe was used.

Fluorescence *in situ* hybridization (FISH) of mouse oocytes and embryos

Hybridization of a molecular probe specific for mouse X and Y sex chromosomes on mouse oocytes and early embryos was performed to verify the FISH technique using preparations with metaphase II oocytes. Both mouse oocytes and early embryos were treated with a hypotonic solution (0.05M KCl) for 10 min and fixed with methanol and acetic acid at 3:1 and left to dry.

Immediately before hybridization, oocyte and embryo preparations were dehydrated in 100% alcohol. They were then digested with RNAse and pepsin and dehydrated in an ethanol series (70%, 80%, 90%). Preparations were then tempered for 15 min at 80°C. During this time, commercial probes for mouse X and Y chromosomes (Cambio) were denatured at 70°C for 10 min and placed in an incubator. The X probe was labeled indirectly with biotin, wheras the Y probe was directly labeled with the Cy3 fluorochrome.

Preparations were denatured in 70% formamide in 2xSSC at 80°C for 5 min. Immediately after denaturation, preparations were passed through an ice-cold alcohol series (70%, 80%, 90%, 100%). Probes were applied to dried preparations, which were covered with a cover glass and sealed with fixogum. Hybridization was performed in a moist chamber at 37°C for 3 days.

Hybridization for several dozen hours was followed by a series of washes at 42°C, twice for 5 min in 50% formamide in 2xSSC buffer and twice for 5 min in pure 2xSSC buffer. Preparations were then placed in an equilibration solution (Detergent Wash Solution, 100 ml 20xSSC + 400 ml distilled water + 250 μl Tween20) for 4 min at 42°C.

Post-hybridization staining of preparations and microscopic examination

Preparations were stained with propidium iodide or DAPI (4'-6-Diamidino-2-phenylindole). All preparations were examined with an Opton Axiophot microscope (Zeiss). Images were captured by a computer-linked camera and analysed using Lucia software (Laboratory Imaging LTD) and archived.

Results

Collection and cytogenetic analysis of *in vitro* maturing oocytes

Preparations were made from 438 oocytes (74.34%; 481/647). Further oocytes were destroyed during removal of the corona radiata cells or were lost during transfer to a hypotonic solution and fixing on slides. Moreover, during the FISH procedure, 9.82% of the oocytes (43/438) dislodged from the slides.

An X chromosome signal was not detected in mare oocytes despite the observation of metaphase spreads in mare oocytes, many changes in the composition of the hypotonic solutions and modification of the FISH protocol with the horse X chromosome painting probe (Figs 1-2).

Fluorescent signals were obtained after applying the FISH technique with mouse sex chromosome painting probes to fertilized oocytes and 2-, 4- and 8-blastomere embryos (Figs 3-4).

Discussion

The development of new molecular biology techniques has improved our understanding of mechanisms behind chromosomal aberrations and molecular anomalies responsible for infertility and especially reproductive disorders. The development of methods for somatic cell chromosome analysis was paralleled by advances in meiotic chromosome research. This research, however, is not commonly used in cytogenetic diagnosis of horses. This results from difficulties in collecting material for research, because the number of slaughterhouses in Poland is small and even fewer slaughter horses. Ovaries are harvested from mares of unknown origin, age, condition and reproductive history. The material obtained is therefore very diverse and difficult to compare. Furthermore, reproductive physiology of mares differs considerably from that of other female farm animals. A significant impediment to mare oocyte research is the hardening of the zona pellucida,

which takes place during *in vitro* culture of oocytes and embryos (BIELAŃSKI & TISCHNER 1997). This phenomenon could indirectly hinder penetration of the hypotonic solution and make it difficult for the probe to reach chromosomes during the experiments described in the present paper. In addition, because the mare oocyte ooplasm contains many large and dense lipid droplets, the hypotonic solution was supplemented with detergent (Tween20) to neutralize them. The differences in morphology and the specific nature of the oocyte maturation process in mares are not without an influence on the efficacy of standard procedures that are successfully used with FISH for other cell types.

A method for making chromosome preparations from preimplantation mouse embryos was first described by TARKOWSKI (1966). This method was repeatedly modified by several researchers (GARSIDE & HILLMAN 1985), also when used in other species such as pigs (MCFEELY 1966) and cattle (KING *et al*. 1979). HSU (1952) and HSU and POMERAT (1953) confirmed the need for a hypotonic solution, which enables well-dispersed chromosomes to be obtained within metaphase spreads, thus improving the resolution of microscopic images. Placing cells in a hypotonic environment results in active absorption of water until the salt concentration levels off (HOLMOQUIST & MOTARA 1987). Chromosome dispersal is induced by disintegration of the mitotic spindle (HSU & POMERAT 1953) or loosening of intrachromosomal connections (KLASTERSKA & RAMEL 1978). Various hypotonic solutions have been used to visualize chromosome plates in embryos. 30% calf serum was used by KAMIGUCHI *et al*. (1976), 1% sodium citrate by TARKOWSKI (1966), and KCl by POPESCU *et al*. (1982) and LONG (1977). Some authors also used enzymes such as actinase (IWASAKI *et al*. 1988), trypsin (HARE *et al*. 1976; YOSHIZAWA *et al*. 1990) and pronase (ANGELL *et al*. 1983) to weaken the zona pellucida of oocytes, zygotes and embryos. In addition, OCANA-QUERO *et al*. (1998) applied a two-stage fixation process, using a combination of trypsin and sodium citrate, which is similar to the double fixation method of FUJIMOTO *et al*. (1975) or YOSHIZAWA *et al*. (1990).

Despite many modifications of the hypotonic solution and fixative as well as the FISH procedure itself, we failed to obtain a signal of the probe specific for X chromosome metaphase I spreads in mare oocytes. It seems that one of the reasons for the failure of the FISH technique are problems with penetration of the probe through remnants of the zona pellucida, which prevents hybridization of the molecular probe with chromosomes in the oocytes of this species. For this reason, it was also impossible to analyse X chromosome segregation abnormalities in reproductive cells of the mare.

Using standard banding methods, accurate identification of meiotic chromosomes in oocytes is not always possible due to the poor quality of metaphase spreads that is often observed after fixing. Few studies have used cytogenetic analysis of chromosomes (KING *et al*. 1987; KING *et al*. 1990; SOSNOWSKI *et al*. 2003). Chromosome painting using the FISH technique represents an efficient method for identification of one or several chromosomes and determination of aneuploidies in unfertilized oocytes (BENKHALIFA *et al*. 1992; BERGÈRE *et al*., 1995; BENZACKEN *et al*. 1998). However, based on literature data it can be stated that studies using FISH for these cells are difficult to carry out because of many technical problems (PELLESTOR 1991; DYBAN *et al*. 1996), which concurs with the observations made in the present study. Additional research with mouse oocytes has confirmed the efficiency of the FISH protocol used in other species. This shows that the FISH technique is correct but the preparation of material for further stages (e.g. hypotonization, fixing, enzymatic digestion) needs to be refined. It is appropriate to continue studies aimed at developing a FISH protocol for chromosomes in mare oocytes.

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