METHODS

Method of Cytogenetic Assay of Mouse Oocytes

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We developed an original method of isolation and analysis of cytogenetic micropreparations of mouse oocytes including treatment with buffered hypotonic saline, paraformaldehyde fixation, and fluorescent staining. The method had several advantages, including high quality of sections and low labour intensity.

Key Words: *oocytes, meiosis; cytogenetic assay; aneuploidy; mice*

Programs of testing of chemical substances for mutagenicity include a complex of methods allowing recording various types of genetic processes in various cell types.

The method of induction of dominant lethal mutations in embryo cells is commonly used for estimation of potential genotoxicity, but this test is imperfect and has serious disadvantages. In light of this, the development of new approaches remains an urgent problem, *e.g.* new methods are necessary for estimation of potential aneugenicity in embryo cells, because chromosome abnormalities contribute to the formation of congenital defects, spontaneous abortions, and other disorders of reproductive functions.

Much attention is paid to well-known cytogenetic methods. Dry-air method proposed by A. K. Tarkovskii (1996) was used for obtaining of micropreparations of mouse oocytes [12]. This method was modified several times due to the high labour intensity and low quality of micropreparations [2,5,7,11]. A. P. Dyban proposed using cold hypotonic solution and ice-cold fixative for preventing chromosome loss during metaphase fixation on slides [2]. A variant of this method included soft gradual fixation [5]. However, analysis of experimental data obtained by these methods showed that these techniques did not solve the main problem and did not prevent artifact variability of spontaneous and induced aneuploidy in mouse oocytes.

Here we developed the alternative method for obtaining and analysis of cytogenetic micropreparations of mouse oocytes.

MATERIALS AND METHODS

The experiment was performed on 6-8-month-old female hybrid mice (CBA×C57Bl/6)F_1 (Stolbovaya Breeding Center, Russian Academy of Medical Sciences). The animals were kept under vivarium conditions at V. V. Zakusov Research Institute of Pharmacology, Russian Academy of Medical Sciences (10-12 specimens per cage) at 12-h light/dark cycle.

They had free access to pelleted food (MEST) and water. The rules of animal management and care were consistent with the standards of "Principles of good laboratory practice" (Federal Standard R 53434-2009). The procedures of animal welfare were performed in accordance to the Standard Operating Procedures (SOP) of the laboratory.

Hormone-induced superovulation. Superovulation was modeled by intraperitoneal injection of 5 U pregnant mare serum gonadotropin (PMSG; Folligon) and 5 U chorionic gonadotropin (CG; Moscow Endo-

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crine Plant) with a 48-h interval between injections. Aneugenesis was induced by intraperitoneal administration of colchicines (0.2 mg/kg) simultaneously with CG. The experiments were conducted in series (5 animals per series) for the estimation of method reproducibility.

Oocyte isolation. The animals were sacrificed by cervical dislocation 17 h after CG injection. The ovarian tubes were isolated and put into a drop of heated to 37°C EKO1 Ooklin medium (PanEko) in a Petri dish. The ampulla of ovarian tubes was disrupted with a preparation needle under a Stemi DV4 stereo microscope (Carl Zeiss). Cumulus-oocytecomplexes were put in a medium containing 150 U/ml hyaluronidase (type 2, Sigma). After 20 min, oocytes free of cumulus cells were collected with a micropipette (EZ-Grip, RI) and washed in 6-8 drops of fresh medium for removing enzymes and follicular cells. Zona pellucida was removed by 20-min incubation with 0.02% collagenase (type 1, Sigma) at room temperature. Then oocytes were put in fresh medium for collagenase removal (as described previously).

Micropreparation obtaining. Hypotonic treatment was performed in three variants: treatment with 0.3% sodium citrate, 0.55% KCl, and buffered 0.4% KCl solution (10 mM HEPES-KOH pH 7.2; 1 mM $MgCl_2$). In all variants, the isolated oocytes were put into a drop of hypotonic solution in a Petri dish and incubated for 15 minutes at room temperature. Then, oocytes were transferred on a slide (20-25 oocytes per well) with 35 µl fixative (1% paraformaldehyde, 0.15% Triton X-100, and 3 mM dithiothreitol, pH 9.2). We used 3-well slides (well diameter 9 mm) with hydrophobic Teflon surface (Immuno-cell). Then micropreparations were put in a humid chamber for 2 h and exsiccated at 37°C.

Staining of micropreparations. Cytogenetic micropreparations were stained with Hoechst 33258 fluorescent dye (0.5 µg/ml in PBS): 30 µl dye was put into wells and then the wells were covered with a cover glass. Microscopic examination was performed on AxioImager M2 epifluorescent microscope (Carl Zeiss) at ×400 and ×1000 (oil immersion). Digital images of metaphase plates were obtained using AxioCam MRm digital camera.

RESULTS

Depending on the mouse age and strain, 8-10 oocytes can be obtained from intact female laboratory mice and up to 40 oocytes can be obtained after superovulation [8]. Preliminary experiments showed that the optimal age of hybrid mice $(CBA\times C57Bl/6)F_1$ for the obtaining of maximum number of oocytes after superovulation is 6-8 weeks. During the experiment, 5243 oocytes were isolated (31.6±6.2 per mouse).

PMSG stimulating follicle growth and CG promoting resumption of meiotic division of oocytes from the dictyotene of meiotic prophase I are used for superovulation [8]. Stimulation of ovulation with gonadotropins has significant effects on the quality and maturity of oocytes [3]. According to our data, injection of hormones in doses >5 U was followed by ovulation of 3% oocytes at meiotic metaphase I (Fig. 1, *a*), while after administration of <5 U, oocyte yield was reduced. Thus, the dose of gonadotropins of 5 U is suggested to be optimal, because it minimizes the negative influence on oocyte maturation, but allows obtaining sufficient amount of oocytes for cytogenetic assay.

In experimental series I, 1353 oocytes were isolated and fixed and 771 of them (57%) were meiotic metaphase II oocytes (Fig. 1, *b*); 447 (58%) metaphases were suitable for cytogenetic assay and 40 of them (9%) had hypohaploid chromosome set (n=16-19). Uneven spreading and multiple overlap of chromosomes were observed in 13% metaphases, which complicated their analysis; 29% metaphase plates were damaged and fragments of one metaphase (several chromosomes) were located in different visual fields (Fig. 1, *c*). Fixation on chamber slides with hydrophobic surface and fluorescent staining of preparations excluded metaphase/chromosome loss from the glass surface allowed localization of all chromosomes of the metaphase plates on the slide. Individual chromosomes were seen on the slide, which probably suggests that hypohaploidy observed in 9% cases can be related to artifact loss of chromosomes from the metaphase plates during preparing the slides.

Hypotonic shock before fixation was aimed at increasing cell volume for chromosome separation with the following spreading on the microslide without coverings [1]. High percentage of fragmented metaphases and metaphases with excessive spreading (Fig. 1, *d*) indicated non-optimal conditions for hypotonic treatment.

In a special experimental series, hypotonic sodium citrate solution was replaced with 0.55% KCl that is normally used for preparing cytogenetic preparations of mouse bone marrow cells. In this series, 2431 oocytes were isolated and fixed, of them 1458 (60%) were meiotic metaphase II oocytes (Fig. 1, *b*); 86% metaphase plates were suitable for cytogenetic assay. Compact spreading and multiple overlap of chromosomes were observed in 14% metaphases (Table 1). Fragmented metaphase plates or isolated chromosomes were not found. Cytogenetic assay revealed no metaphases with hypohaploid chromosome set.

For improving micropreparation quality, hypotonic treatment with buffered KCl solution was used. The use of this hypotonic solution allows improving the quality of cytogenetic preparations of embryonic stem cells, the cells that are difficult to prepare for

TABLE 1. Effects of Hypotonic Treatment Conditions on the Quality of Cytogenetic Micropreparations of Oocytes

Conditions of hypotonic treatment	Isolated and fixed oocytes (per mouse)	Meiotic oocytes		Suitable for metaphase assay		Unsuitable for metaphase assay				Metaphases with chromosome number			
						with chromosome overlaps		fragmented		20		<20	
		abs.	%	abs.	%	abs.	%	abs.	%	abs.	%	abs.	%
Intact mice (0.3% sodium citrate)	1353 (31.4)	771	57	447	58	100	13	224	29	407	91	40	9
Intact mice (0.55% KCl)	2431 (31.1)	1458	60	1254	86	204	14	0	0	1254	100	0	0
Intact mice (buffered 0.4% KCl)	1152 (32.9)	703	61	668	95	35	5	0	0	668	100	0	0
Mice treated with 0.2 mg/kg colchicine (buffered 0.4% KCl)	307 (30.7)	190	62	177	93	13	7	0	0	162	92	15	8

Note. *Metaphases with chromosome number >20 were not found. In parentheses: mean number per mouse.

cytogenetic analysis [9]. In this series, 1152 oocytes were isolated and fixed, of them 61% were meiotic metaphase II cells. Compact spreading of metaphases with chromosome overlaps were observed in 5% of metaphases. Fragmented metaphase plates and isolated chromosomes were not found; 95% metaphase plates were appropriate for cytogenetic assay (Table 1). Cytogenetic assay did not observe metaphases with hypohaploid chromosome set.

After aneugenesis induction with colchicine (0.2 mg/kg), 307 oocytes were isolated and fixed, 62% of these were metaphase cells (Table 1), 15 oocytes (8%) of analyzed 177 oocytes were aneuploid (n=19, Fig. 1, *e*), 2 oocytes were polyploid (Fig. 1, *f*), and in 3 oocytes we observed premature sister chromosome disjunction.

Thus, hypotonic treatment serves as the key stage, which determines the quality of cytogenetic preparations of mouse oocytes. Treatment with buffered hypotonic KCl solution and paraformaldehyde fixation on chamber slides with hydrophobic surface allowed preparing high-quality metaphase preparations of oocytes, where >90% metaphases were appropriate for analysis. Cytogenetic analysis did not observe oocytes with aneuhaploid chromosome set in intact hybrid mice (CBA×C57Bl/6)F_1. Under normal conditions, no premature sister chromatid segregation was observed. At the same time, 5% metaphases in our experiment were inappropriate for cytogenetic analysis, and we can not assert that none of oocytes were aneuploid under normal conditions.

The critical moments affecting the quality of preparations were determined. It is known that zona pellucida is typical of mammalian oocytes. Removal of zona pellucida is necessary for obtaining high-quality micropreparations. Fixation of oocytes with intact zona pellucida retained high amount of cytoplasmic material and determined poor metaphase spreading. These problems complicated cytogenetic assay (Fig. 1, *g*). However, removal of the zona pellucida has to be optimized. Collagenase activity varies depending on the manufacturer and series. We used collagenase from Sigma (Type I from *Clostridium histolyticum*) with activity of 0.25-1.00 FALGPA/mg. Treatment with this enzyme in a dose >0.02% led to damage of some oocytes during hypotonic treatment. These data determine the necessity of titration of each enzyme series before experiments.

Another critical point is fixation. A decrease in pH of paraformaldehyde solution impaired morphology and staining of chromosomes. In addition, the volume and immobility of fixative drop are of crucial importance during manipulation with slides. Chamber slides with hydrophobic coating are optimal for these purposes.

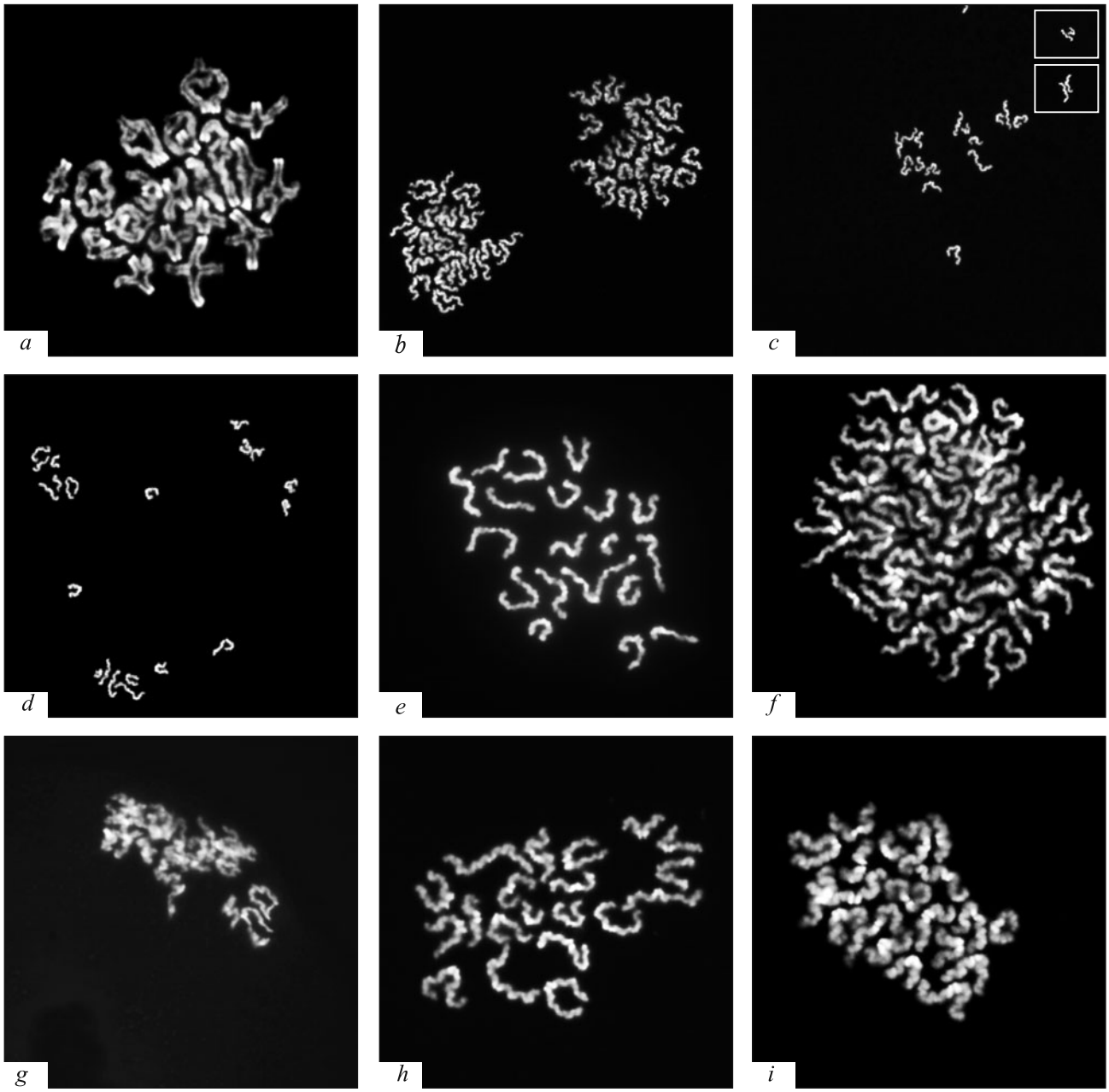

Fig. 1. Cytogenetic micropreparations of mouse oocytes. Stained with Hoechst 33258, ×400 (*b, c, d, g*), ×1000 (oil immersion; *a, e, f, h, i*). *a*) meiosis metaphase I; *b*) meiosis metaphase II; *c*) fragmented metaphase; in the brackets, metaphase chromosomes in the neighbor visual fields; *d*) metaphase with excessive spreading; *e*, metaphase with hypohaploid chromosome set (*n*=19); *f*) metaphase with polyhaploid chromosome set; *g*) oocyte metaphase plate fixed without *zona pellucida* removal; *h, i*) chromosome morphology under various conditions of terminal exsiccation.

The conditions of terminal exsiccation after fixation considerably affect metaphase morphology. After exsiccation of micropreparations at low humidity, chromosomes spread as long compact structures (Fig. 1, *h*), while high humidity promoted the formation of short diffuse structures (Fig. 1, *i*). Staining of pericentromeric regions of chromosomes was more pronounced at high humidity, which allowed quantitative analysis in some compact metaphase plates with chromosome overlaps.

Comparative analysis of obtained results with published data demonstrated significant advantages of the developed method. The procedure of direct transfer of isolated oocytes on chamber oslides with hydrophobic coating prevented their loss at the stages of hypotonic treatment and fixation, which constituted ~60% during standard methods [7]. As mentioned above, more than 90% metaphases on the obtained preparations are appropriate for assay. Published data indicate that this parameter significantly varied (from 20% to 80%)

[5,7,11]. Maximum oocyte output ensures high representativeness of the sample and improve data reliability. In addition, maximum output allows reducing the number of animals in experimental groups without reducing sample volume necessary for statistical analysis. The use fluorochrome Hoechst 33258 for preparation staining allowed detection of pericentromeric regions on metaphase plates of mouse cells due to their more intensive selective staining [10]. As distinct from routine differential C-staining, staining with Hoechst 33258 does not require complicated post-fixation procedures, which makes the assay more rapid and excludes the risk of metaphase plate/chromosome loss during various manipulations. One more advantage is the possibility of analyzing fresh preparations. Stained micropreparations can be stored for 6 months at -20°C without worsening of metaphase plate morphology.

Thus, we developed an original technique of preparing and analyzing cytogenetic micropreparations of mouse oocytes that has several significant advantages over standard methods.

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