Original Article

Effect of Mechanical Micro-Vibrations on The Efficiency of Leopard Inter-Species Somatic Cell Nuclear Transfer

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Abstract

Objective: Scarcity of oocytes for assisted reproduction in endangered species can be bypassed by interspecies somatic cell nuclear transfer (iSCNT). In Felids, domestic cat (*Felis catus*) oocytes can serve as recipients for the nucleus of the endangered Persian leopard (*Panthera pardus saxicolor*). However, *in vitro* oocyte maturation is still suboptimal in cats, whereas it has been reported to benefit from micro-vibration in non-felid species. Therefore, the present study is aimed to determine whether micro-vibration, applied during *in vitro* maturation (IVM), improves the embryogenic potential of cat oocytes transplanted with fibroblast nuclei of the Persian leopard.

Materials and Methods: In the experimental study, cat cumulus-oocyte complexes (COCs) were randomly assigned to the treatment group (micro-vibration) or control group (static culture). Resultant metaphase II (MII) oocytes were enucleated and reconstructed with nucleus transplants from leopard fibroblasts, followed by artificial oocyte activation and embryo culture under the same condition (static) for 7 days.

Results: While cumulus cell expansion and oocyte maturation profited from micro-vibration (P<0.05), the quantity and quality of blastocysts were significantly lower in micro-vibration than in the control group (P<0.05). The total number of blastocyst cells tended to be lower in the micro-vibration than in the control group (P=0.075). Nevertheless, the proportion of ICM and TE cells did not differ between the micro-vibration and control groups (P>0.05).

Conclusion: The present study indicated that micro-vibration at a frequency of 44 Hz for 5 secs per hour enhanced nuclear maturation and cumulus cell expansion of cat oocytes. However, exposure to micro-vibration during IVM impaired the survival rate of reconstructed oocytes during the iSCNT process and their developmental competence toward the blastocyst stage.

Keywords: Domestic Cat, *In Vitro* Maturation, *Panthera Pardus Saxicolor*, Somatic Cell Nuclear Transfer, Vibration

Introduction

According to the red list of the international union for conservation of nature, the population of the wild members of the Felidae family has dramatically dwindled over the past decades and all of them are presumed to be in jeopardy of extinction (1). This phenomenon has been attributed to various issues, including genetic, demographic, environmental, and human-associated factors restricting wild felids territory and rendering wildlife habitats uninhabitable (2, 3). In this regard, the Persian leopard (*Panthera pardus saxicolor*), which is one of the eight subspecies of leopard species (*Panthera pardus*), is mostly distributed in Iran and considered endangered; hence, protection of this subspecies is of utmost importance (4).

In the context of wildlife conservation, assisted reproductive technologies can be applied to protect endangered animals (5) and there are numerous successful reports in leopard cats (6), African lions (7), and black-footed cats (8). In this regard, interspecies somatic cell nuclear transfer (iSCNT), in which the nucleus of one species is transferred to the enucleated oocyte of another species, could serve as a promising technique, particularly in wild cats affected by oocyte scarcity (9). Indeed, iSCNT using domestic cat oocytes has been applied to rescue a number of endangered feline species such as the African wild cat (10), sand cat (11), tiger (12), and cheetah (13). In these cases, the domestic cat oocyte was utilized as the recipient for the nucleus since it is the only feline species which is not considered endangered (10, 13). Nevertheless, iSCNT technique in feline species suffers from limitations. Chief among them is the fact

that despite major progress over recent years, rates of *in vitro* maturation (IVM) of domestic cat oocytes are still unsatisfactory for large-scale applications in iSCNT (14).

To improve IVM rate in cats, most studies have relied on chemical modifications of culture media, for instance, incorporation of growth factors or antioxidants (15). However, the possibilities are not exhausted with chemistry, and physical parameters are an option as well. Dynamic *in vitro* culture (IVC) using micro-vibration has been proposed to resemble *in vivo* conditions more closely, supporting improved oocyte maturation and development of resultant embryos (16-20). In validation of this notion, evaluating the effect of micro-vibration during IVM and IVC of embryos in pig, Mizobe et al. (17) observed that application of micro-vibration during IVM enhanced development of embryos up to the blastocyst stage, irrespective of the micro-vibration treatment during IVC, and the beneficial effect of micro-vibration was more pronounced during IVM than IVC. Also, micro-vibration has shown positive effects on embryo development in mice, cattle, pigs, and humans in the frequency range of 20-44 Hz.

In this study, we hypothesized that micro-vibration during IVM would result in cat oocytes that are more supportive of iSCNT. We conducted the present study to test this hypothesis, using oocytes from the domestic cat as recipients and fibroblasts from the Persian leopard as nucleus donors.

Materials and Methods

All procedures were performed according to institutional guidelines on animal experimentation and care and approved by the Ethical Committee at Royan Institute (IR.ACECR.ROYAN.REC.1397.188). Unless otherwise mentioned, all materials used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA).

Oocyte collection and IVM

Ovaries were collected from queens following routine ovariohysterectomy in veterinary clinics and kept in phosphate-buffered saline (PBS, Sigma-Aldrich, USA) at 4°C during transportation to the laboratory. After transportation, ovaries were washed (3 times) using PBS supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin at room temperature. Each ovary was sliced by a scalpel blade to release cumulus-oocyte complexes (COCs) into a washing medium (WM) containing HEPES tissue culture medium 199 (HTCM 199) supplemented with 10% fetal bovine serum (FBS, Gibco, Ireland). COCs with good morphology and quality (uniform in morphology with intact and dark ooplasm and more than three layers of cumulus cells) were selected for IVM (13). Maturation medium consisted of tissue culture medium

199 (TCM 199) supplemented with 2.5 mM sodium pyruvate, 1 ng/ml estradiol, 1 mM L-glutamine, 3% bovine serum albumin (BSA, Sigma-Aldrich, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin, 25 ng/ml EGF, 100 ng/ml IGF1. The selected COCs were cultured in IVM medium droplets (5 to 10 COCs in each 50 μL droplet), which were placed on tissue culture dishes and covered with mineral oil, at 38°C in 5% CO_2 for 24 hours. In the control (static) group, COCs received no more treatment, whereas, in the treatment (dynamic) group, COCs were subjected to mechanical micro-vibration at the frequency of 44 Hz for 5 seconds per hour using Viboviduct 1500 (SimSoTec GmbH, Cologne, Germany) (17, 18). Following IVM, cumulus expansion area was measured in 12 COCs using Fiji software (National Institutes of Health, Bethesda, MD, USA) as an image processing program, in which system units were changed to μm^2 by calibration. Afterward, cumulus cells were removed by treatment with hyaluronidase (300 IU/ml in HTCM199 containing 10% FBS) for 3 minutes, resulting in denuded oocytes. Oocyte maturation to metaphase II (MII) was scored by the presence of a polar body using a phase-contrast inverted microscope (Olympus CKX41, Tokyo, Japan), and the MII stage was confirmed by Hoechst staining (0.5 mg/ml) of 5 oocytes randomly selected from each group of denuded oocytes. Subsequently, the matured oocytes were deprived of the zona pellucida by incubation in pronase (2.5% in HTCM199 also containing 10% FBS) for 30 seconds on a warm plate and then allowed to recover for 15 minutes in 20% FBS.

Isolation and preparation of leopard fibroblast cell line

The skin biopsy from the Persian leopard was provided to the Royan Institution by Tehran Zoo in April 2018. This step was implemented based on the methodology described previously by Dalman et al. (21). In brief, a biopsy from the ear of a Persian leopard was punched. After washing three times with PBS containing antibiotics (penicillin G 250 U/ml and streptomycin 250 mg/ml), the dermis was dissected into small pieces. After washing, the explants were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% FBS, 1% penicillin and streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate at 38°C in a 5% CO_2 incubator. After 10 days of culture when the cells reached confluency (80-90%), tissue explants were removed and cells were dissociated in trypsin (0.25% trypsin/EDTA) and centrifuged at 1200 rpm for 5 minutes and seeded. Early cell passages were cryopreserved in a medium containing 10% Dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and stored in liquid nitrogen. For use in iSCNT experiments, cryopreserved fibroblasts were thawed, passaged 3 to 5 times in DMEM supplemented with 15% FBS at 38°C in a 5% CO_2 incubator, and

arrested in G0 by culturing them 48 hours in DMEM with 0.5% FBS (Fig.1).
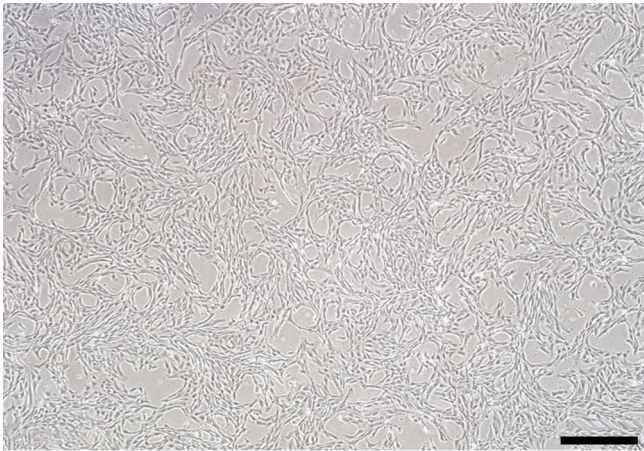
Fig.1: Cultured leopard fibroblast cells (scale bar: 200 μm).

Enucleation of oocytes

Enucleation was performed manually using a pulled Pasteur pipette as described previously by Hosseini et al. (22). In brief, zona-free oocytes were cultured in TCM199 supplemented with 0.41 μg /ml demecolcine, 10% FBS, 10 mg/ml BSA, and 3 mg/ml polyvinyl alcohol (PVA) for 1.5 to 2 hours to induce cytoplasmic protrusion. Thereafter, manual enucleation of the oocyte was implemented under a stereomicroscope by a pulled Pasteur pipette. Before nuclear transfer, enucleated oocytes were maintained for 30 minutes in TCM199 supplemented with 10% FBS, 10 mg/ml BSA, and 3 mg/ml PVA.

Interspecies somatic cell nuclear transfer

To place leopard cells in the vicinity of an enucleated oocyte, the population of fibroblast cells (about 500 cells) was transferred to 50 μl drops of HTCM199 supplemented with 10 mg/ml Phytohemagglutinin (PHA-P). Each enucleated oocyte was dropped on top of an intact and viable fibroblast and the two cells were pushed against each other with a Pasteur pipette. The resultant aggregates were placed in drops of HTCM199 supplemented with 1 mg/ml PVA before fusion. The electrodes of the fusion chamber, lying 0.5 mm apart, were overlaid with a fusion medium consisting of TCM199, 0.3 mM mannitol, and 0.05% BSA. The aggregates were aligned manually and the cell membranes were fused by applying two pulses of direct electric current (1.75 kV/cm for 80 seconds and 1-second delay, Cryologic, Australia). Resultant reconstructed oocytes were maintained in HTCM199 supplemented with 3 mg/ml PVA for 30 to 40 minutes to assess fusion success, before proceeding to activation.

Artificial oocyte activation and *in vitro* embryo culture

Reconstructed oocytes were incubated for 5 minutes in drops of activation medium composed of HTCM199, 1 mg/ml BSA, and 10 μg/ml calcium ionophore A23187. After brief washing in a drop of TCM199 supplemented with 10 mg/ml BSA and 1 mg/ml PVA, reconstructed oocytes were incubated 5 hours in 6-Dimethylamino purine (6-DMAP, 5 μg/ml). Given that zona-free oocytes were used for iSCNT; we chose a 'well of well' (WOW) culture system so as to prevent the embryos from sticking to each other while allowing for diffusion of embryo-derived growth factors between embryos (23). Six reconstructed oocytes were co-cultured in each WOW overlaid with 20 μl of synthetic oviductal fluid (SOF) medium with oil on top, at 39°C, under an atmosphere of 5% O_2, 5% CO_2, and maximum humidity. *In vitro* culture conditions were identical between control and micro-vibration groups.

Differential staining of embryos

Differential staining was used to identify inner cell mass (ICM) and trophectoderm (TE) cells by means of two DNA-specific fluorochromes, namely, propidium iodide (PI) and Hoechst 33342 (24). To this end, day-7 blastocysts were transferred to 2.5% Triton X100 in HTCM plus 5 mg/ml BSA for 15 seconds, and then, they were transferred into HTCM-BSA medium containing 30 μg/ml PI for 45 seconds and washed in HTCM-BSA medium. Afterward, blastocysts were transferred into 800 μl of Hoechst 33342 (10 μg/ml in Ethanol) for 15 minutes and were further mounted on glass slides and examined under a fluorescence microscope (Nikon Eclipse 50i, Tokyo, Japan). Counting of cell nuclei was performed on microphotographs taken with a digital camera. Following enumeration of cells in ICM and TE, the proportion of ICM and TE cells relative to total blastocyst cells (ICM+TE cells) were calculated for further analysis.

Statistical analysis

There were three biological replicates in each experimental group. Continuous data, including COC expansion (size) and the number of blastocyst cells, were initially tested for normality using the Kolmogorov-Smirnov test, and as they had normal distribution, they were analyzed by t test. Non-continuous data, including rates (of IVM, reconstructed oocytes formation, degeneration, cleavage, and blastocyst formation) as well as proportions (of ICM and TE cells) were analyzed using logistic regression (GENMOD procedure), which generated odds ratios (ORs), as the strength of the difference between groups, and 95% confidence intervals (95% CIs). All analyses were conducted in SAS version 9.4 (SAS, 2013). Data are presented as mean ± SEM. Differences at P<0.05 were considered statistically significant.

Results

The effect of micro-vibration on domestic cat oocyte IVM

Based on the projection area of the COCs (Fig.2A), micro-vibration during IVM supported greater expansion of the cumulus cells (9.48E+04 ± 1.37E+04 μm², n=12)

compared to the control group (4.74E+04 ± 0.18E+04 μm^2, n=12) (P=0.0007, Fig.2B). Based on the presence of a polar body (Fig.3A), a higher proportion of cat oocytes progressed to MII in the micro-vibration group (91.70 ± 1.77 %, n=383) compared to the control group (84.95 ± 2.85 %, n=383, OR=1.816, 95% CI=1.152-2.865, P=0.010, Fig.3B).

A

B
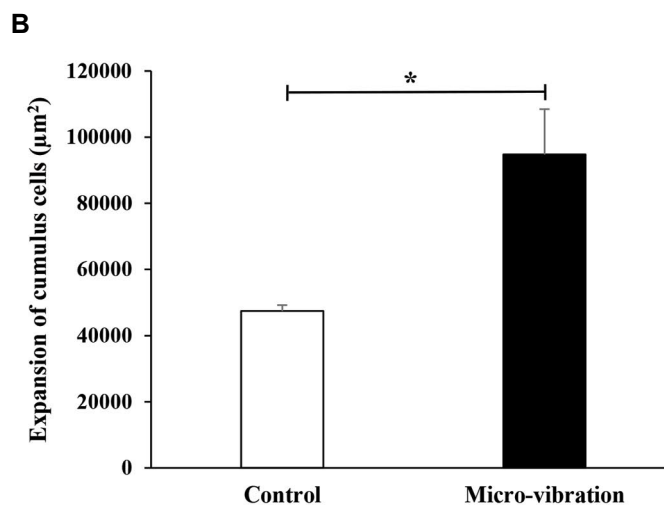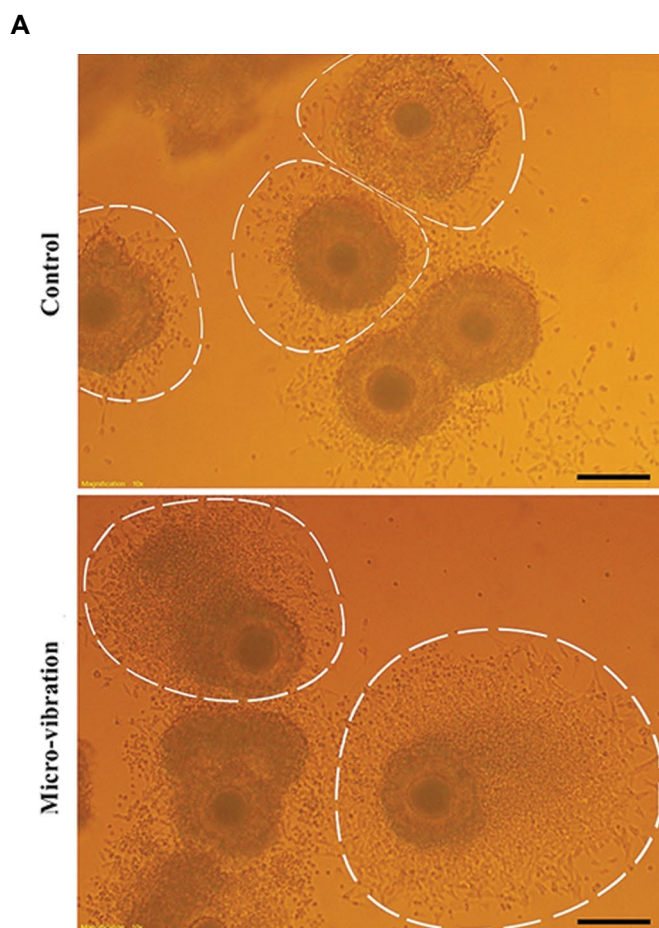
Fig.2: Expansion of cumulus cells. **A.** Expansion of cumulus cells was highlighted by the dashed circles in control and micro-vibration groups (scale bar: 100 μm). **B.** Comparison of cumulus cell expansion in control (n=12) and micro-vibration (n=12) groups. *; P<0.05.

A

B
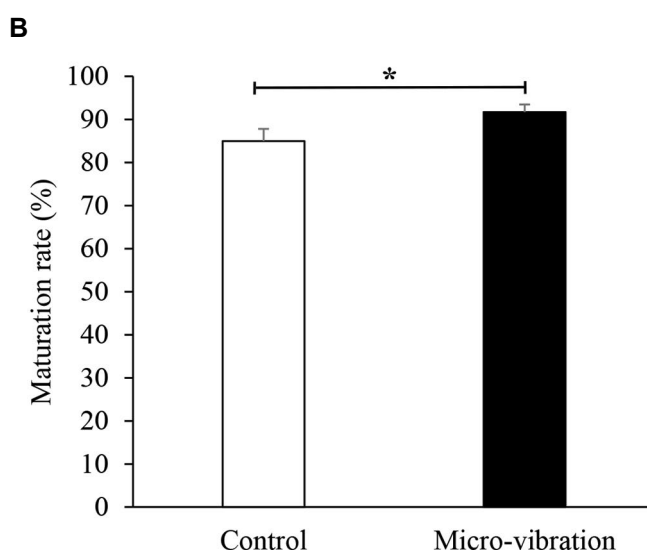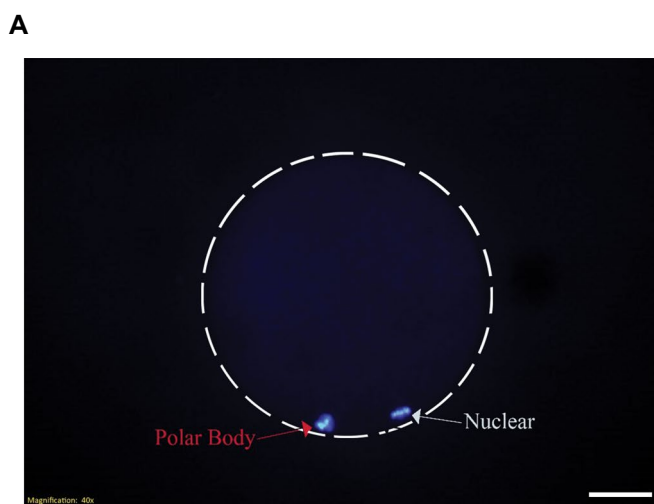
Fig.3: Oocytes maturation. **A.** Extrusion of the first polar body was substantiated by Hoechst staining in a subset of MII-stage oocytes (five oocytes per replicate) (scale bar: 20 μm). **B.** Maturation rate of domestic cat oocytes subjected to *in vitro* maturation (IVM) under control (n=383) and micro-vibration (n=383) conditions. *; P<0.05.

The effect of micro-vibration on iSCNT rate and embryo development

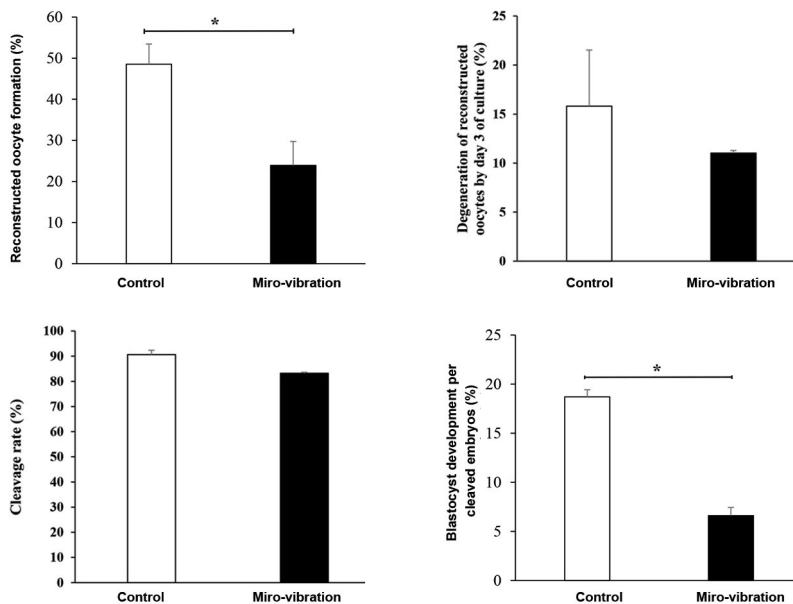Rate of reconstructed oocyte formation was less in the micro-vibration group (n=350, 23.92 ± 5.82%) compared to the control group (n=327, 48.53 ± 4.90%, OR=0.334, 95% CI=0.240-0.465, P<0.0001, Fig.4). Following activation, similar proportions of reconstructed oocytes degenerated in the two groups (11.02 ± 0.26%, n=81, and 15.81 ± 5.72%, n=15, in the micro-vibration and control groups, respectively). Likewise, cleavage rates were similar in the two groups (83.19 ± 0.44%, n=72, and 90.63 ± 1.73%, n=130; in the micro-vibration and control groups, respectively) (P>0.10, Fig.4). However, the proportion of cleaved embryos that developed to the blastocyst stage after seven days of culture was lower in the micro-vibration (n=60, 6.61 ± 0.82%) than in the control group (18.70 ± 0.72%, n=118, OR=0.312, 95%

CI=0.102-0.951, P=0.041, Fig.4).

The effect of micro-vibration on iSCNT blastocysts quality

Based on the absolute cell counts after differential staining (3 blastocysts in each group) (Fig.5A), the number of ICM cells was lower in the micro-vibration (19.67 ± 1.20) than in the control (25.33 ± 0.88) group

(P=0.019, Fig.5B), while the number of TE cells (70.00 ± 5.77, 86.33 ± 5.24) and total blastocyst cells (89.67 ± 6.94, 111.87 ± 6.01) were not significantly different in the micro-vibration and control groups, respectively (P>0.05, Fig.5B). According to the differential staining method, the ICM cell number did not differ between micro-vibration and control groups (22.00 ± 0.54% and 22.75 ± 0.66%, respectively), so did the TE cell number (78.00 ± 0.54% vs. 77.25 ± 0.66 %) (P>0.05, Fig.5B).

Fig.4: Rate of reconstructed oocyte formation, degeneration rate of reconstructed oocytes by day three of culture, cleavage rate of intact reconstructed oocytes, and rate of blastocyst formation relative to cleaved embryos in control and micro-vibration groups. *; P<0.05.
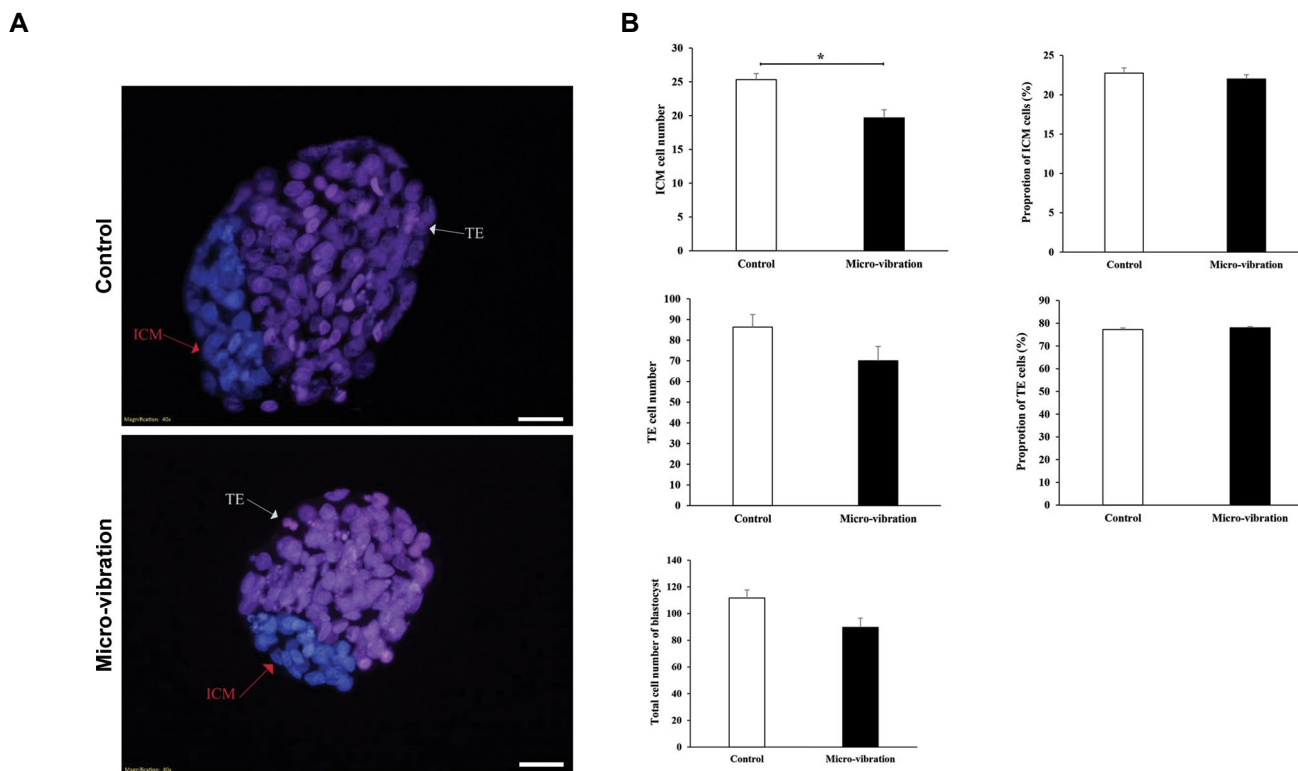
Fig.5: Interspecies somatic cell nuclear transfer (iSCNT) blastocysts. **A.** Differential staining of iSCNT-generated blastocysts in control and micro-vibration groups (scale bar: 20 μm). **B.** Number of embryonic cells and proportion of inner cell mass (ICM) and trophectoderm (TE) cells relative to the total number of blastocyst cells in the control (n=3) and micro-vibration (n=3) groups. *; P<0.05.

Discussion

An inadequate rate of IVM in the domestic cat impedes the success of iSCNT in wild felids (17, 18). The present study aimed to investigate the effect of micro-vibration, applied during IVM, on the maturation of domestic cat oocytes and their embryogenic potential after iSCNT from Persian leopard fibroblasts. The results showed that to apply micro-vibration with the frequency of 44 Hz for 5 seconds per hour, the maturation rate of oocytes was improved but the blastocysts formation rate was decreased.

Our results show that micro-vibration enhanced the expansion of COCs as well as the MII progression of oocytes in the domestic cat. This is consistent with reports in pigs (25, 28). In particular, Mizobe et al. (17) demonstrated a greater extent of cumulus cell expansion in porcine COCs exposed to mechanical micro-vibration during IVM, although these authors did not observe any significant influence of micro-vibration on the proportion of oocytes reaching the MII stage. Mechanical micro-vibration has also proven itself in somatic cell applications, augmenting the proliferation, differentiation, and secretion parameters (27-29). Since COCs are comprised of somatic cells encasing an oocyte, it seems logical that micro-vibration could enhance oocyte maturation through facilitation of expansion in cumulus cells, which govern the maturation and developmental competence of oocytes (30). As an expected result of the enhancement, the IVM oocytes could be better prepared for their biological tasks, including fertilization and embryonic development (31).

Contrary to our expectations, cat oocytes subjected to micro-vibration during IVM were more vulnerable to iSCNT procedure, as measured by rates of reconstructed oocyte formation, which were inferior in the micro-vibration group compared to the control group. This observation implies that despite improved cumulus cell expansion and oocyte maturation to MII, the oocytes were probably damaged in other ways, thereby detracting from their ability to survive the subsequent procedures. Since the settings of intensity, duration, and frequency of micro-vibration used in this study were adopted from studies in other species (e.g. pig (17), human (18), mice (19), and cows (20), it is conceivable that the settings did not suit feline oocytes, reducing their ability to support iSCNT in terms of embryo development (17,18). In this context, we speculate that the plasma membrane may be key to understanding what happened in our study. The plasma membrane is the outermost cellular component that receives the shear stress induced by micro-vibration, and plasma membrane also plays a critical role as a signal transduction interface between external and intracellular environment, subject to modulation by its structural and biochemical properties, particularly its lipid composition and organization (32-34). It is of note that lipid composition and organization determine plasma membrane integrity, prerequisite for cellular homeostasis, and tolerance to extracellular pressures and manipulations (32, 33, 35).

Hence, the adverse effects of micro-vibration settings - adopted from other species - on cat oocyte survival during iSCNT could be attributed to the difference(s) between cat and other species in terms of oolemma' s physical and biochemical properties, including lipid composition (36).

After activation of the reconstructed oocytes, degeneration and cleavage rate did not differ between the two experimental groups, while the micro-vibration group produced fewer embryos with diminished proliferation. This negative effect of micro-vibration on embryonic development may have resulted from settings (e.g. duration and/or intensity of micro-vibration) which were inappropriate for the domestic cat oocyte. In this regard, while evaluating the effect of various durations of micro-vibration on IVM of porcine oocytes, Mizobe et al. (17) observed that prolonged exposure to mechanical micro-vibration reduced parthenogenetic development of oocytes up to the blastocyst stage. Moreover, aiming to ascertain the optimum frequency of micro-vibration for IVM and IVC of bovine oocytes and embryos, Takahashi et al. (20) found that an excessive micro-vibration intensity reduced development to the blastocyst stage as well as blastocyst cell proliferation. In the present study, the nuclear maturation rate was improved by micro-vibration, but cytoplasmic maturation remains to be assessed under static vs dynamic conditions. Therefore, the adverse effect of micro-vibration on oocyte developmental competence in the current study could have been mediated through the disruption of processes involved in oocyte cytoplasmic maturation. Indeed, normal development relies on coordinated nuclear and cytoplasmic maturation of oocytes (37, 38). Interference of micro-vibration with the oocytes' cytoplasmic maturation during IVM might have led to disruption of molecular mechanisms regulating proliferation of the future embryonic cells. Among these mechanisms, we envision, for example, the tissue growth factor β superfamily proteins (39) and the Hippo signaling pathway (40).

In this study, the most important limitation that we faced was the scarcity of cat ovaries and oocytes and it restricted the study from finding the optimal frequency for cat oocytes and iSCNT embryos.

Conclusion

Micro-vibration at the frequency of 44 Hz for 5 seconds per hour during IVM augmented cumulus cell expansion and meiotic maturation in domestic cat oocytes. However, the oocytes that matured under the influence of micro-vibration were less tolerant of iSCNT and produced lower numbers of reconstructed oocytes. In addition, these reconstructed oocytes were less capable of developing into blastocysts, which also contained fewer cells. Therefore, it appears that this field of research requires further studies to tailor the characteristics of micro-vibration during IVM of domestic cat oocytes for use in either intraspecies or interspecies purposes. In particular, it should be clarified if the low tolerance of micro-vibrated cat oocytes to iSCNT stemmed from the micro-vibration, or from the

specific settings used (intensity, duration, and frequency of micro-vibration). It would also be helpful to examine the expression of pluripotency and trophectoderm markers in the blastocysts like *OCT4, CDX2*.

Acknowledgments

Authors' Contributions

M.Sh., A.D.; Participated in the investigation, methodology, writing, review, and editing. V.A., P.E.-Y.; Funding acquisition, project administration, supervision, formal analysis, review, and editing. M.H.; Prepared cat's ovaries following ovariohysterectomy. M.V., M.H.N-E.; Provided critical revision of the study. N.T.V.; Contributed to all experimental work and data. All authors read and approved the final manuscript.

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