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In vitro fertilization using sperm activated by ML-2–3 isolated from *Morinda lucida* Bentham leaves

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Abstract

Purpose: ML-2–3 is a novel tetracyclic iridoid derived from *Morinda lucida* Bentham leaves. This compound has anti-trypanosomal and anti-leishmanial effects. In this study, the authors investigated effects of ML-2–3 on in vitro fertilization (IVF) rates, motility, and acrosome reaction of the mouse sperm.

Methods: IVF was performed using sperm from BALB/cByJJcl mice treated with ML-2–3. Computer-assisted sperm analysis (CASA) was performed on the sperm of C57BL/6J mice to investigate sperm motility. The effect of ML-2–3 on the acrosome reaction was examined by observing the fluorescence of sperm labeled with the acrosin-EGFP transgene.

Results: ML-2–3 improved IVF in BALB/cByJJcl mice with low fertilization rates. The optimum concentration of ML-2–3 in sperm pre-culture medium was 20 μM, and no significant toxicity of ML-2–3 was observed in developing embryos at this concentration. ML-2–3 affected sperm motility but not the acrosome reaction. ML-2–3 increased the IVF rate of mouse sperm that had been refrigerated for 3 days.

Conclusions: ML-2–3 can improve the outcome of IVF and motility without inducing the acrosome reaction in mice. These effects of ML-2–3 on sperm behaviors are different from those of the similar drugs.

KEYWORDS
acrosome reaction, computer-assisted sperm analysis, infertility, refrigerated sperm, tetracyclic iridoids

1 | INTRODUCTION

In Japan, one in five couples struggle with infertility. Approximately 50% of infertility cases are attributed to male infertility, which may be due to low sperm production, poor sperm motility, abnormal sperm morphology, or a combination of these factors.[1,2] It is generally difficult to identify the cause of infertility. Surgery, hormone therapy, and medications are used as treatments.[3] If these treatments do not lead to pregnancy after a reasonable period, patients often choose assisted reproduction technology (ART), in which human eggs, sperms, or embryos are used to establish a pregnancy.[4] in vitro fertilization (IVF), a type of ART, is a technique whereby eggs are fertilized in a laboratory dish. The IVF pregnancy rate depends largely on sperm motility, with poor

sperm motility significantly decreasing the success of IVF.[5,6] As one of the methods to improve the IVF pregnancy rates without harming embryogenesis, pharmacological treatments of the sperm have been evaluated using a number of drugs. In our previous studies, we demonstrated that the aqueous extract of licorice roots (*Glycyrrhiza* spp.) improved the fertilization ability of mouse sperm using IVF.[7] Isoliquiritigenin and formononetin, two flavonoids isolated from licorice root, promoted improving the success rate of IVF.[8] As part of our ongoing research for the discovery of new sperm motility activators, we examined the effect of 10 compounds and 30 extracts from medicinal plants on sperm motility. Of the materials tested, ML-2–3 significantly improved sperm motility (Preliminary data, data not shown).

ML-2–3, a novel tetracyclic iridoid isolated from *Morinda lucida* Bentham (Rubiaceae), has anti-trypanosomal and anti-malarial activities in vitro.[9–13] In a previous study, we established a new method for the quantitative and qualitative analysis of tetracyclic iridoids, including ML-2–3.[13] We reported an efficient, rapid, and easy method for isolation and purification of this tetracyclic iridoid (Figure 1).[13] In this study, we demonstrated that ML-2–3 increased the rate of IVF and improved sperm motility in mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Female ICR mice (6 weeks old) were purchased from SLC Japan, Inc., and used at approximately 7–12 weeks of age. Male C57BL/6J, BALB/cByJJcl, and pseudopregnant female ICR mice were also purchased from CREA Japan, Inc.. C57BL/6-TgN(acr3-EGFP)Osb17 mice were bred to be specifically pathogen free in the animal room at Nagasaki International University.[14] The animals were sacrificed by cervical dislocation just before the experiments. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals, and they were approved by the Institutional Committee of Laboratory Animal Experimentation of Nagasaki International University (Approval Number: 128). The mice were kept under controlled temperature (18–26°C) and lighting (12:12 h light/dark cycle) conditions throughout the experiments and were provided food and water *ad libitum*.

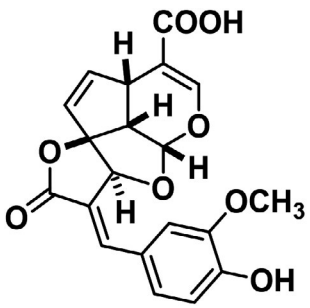

FIGURE 1 Chemical Structure of ML-2–3

2.2 | Reagents and chemicals

Paraffin oil, bovine serum albumin (BSA), and hyaluronidase were purchased from Nacalai Tesque Inc. Human tubal fluid (HTF) medium was purchased from LifeGlobal® Media; IVFonline. Pregnant mare serum gonadotropin and human chorionic gonadotropin were purchased from ASKA Pharmaceutical Co., Ltd. Potassium simplex optimization medium was purchased from Zenith Biotech. Polyvinyl alcohol and methyl-beta-cyclodextrin were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from FUJIFILM Wako Pure Chemical Corp (Osaka, Japan). ML-2–3 was isolated from ethanol extracts of *M. lucida* leaves. Details on the isolation method and the method for determining the chemical structure of ML-2–3 were reported in previous papers.[9–13] *M. lucida* leaves were collected in Mampong, Ghana, in January 2016. ML-2–3 was dissolved in DMSO and 2 mg/mL of ML-2–3 was used as a stock solution. The stock solution was stored at −20°C and used for the experiments without dilution. DMSO was properly added to the control samples to equalize the solvent concentration among all samples. The final concentration of DMSO was 0.5% (vol/vol).

2.3 | IVF

BALB/cByJJcl mice (12–16 weeks) were sacrificed by cervical dislocation just before collection of the caudal epididymis. Mature caudal epididymal sperm (~8×10^6) from each mouse were incubated in 200 μL HTF medium without BSA covered with paraffin oil. After 5 min, 15 μL of the sperm suspension was transferred to 50 μL conditioned HTF medium (HTF medium with 4% BSA) containing ML-2–3 at several doses (0, 10, 20, 40, or 80 μM) and maintained at 37°C in a humidified incubator under 5% CO_2/95% air (~10 000/μL of the motile sperm concentration).[7,8] After 55 min, 16–20 μL of the sperm suspension from each conditioned medium was used for insemination (final motile sperm concentration =150/μL). Motile sperm swimming at the periphery of each drop were used for insemination as described previously.[7,8]

ICR mice (7–12 weeks) were superovulated by an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin, followed by 5 IU human chorionic gonadotropin after 46–48 h, and euthanized 14–16 h later. Ovaries with oviducts were collected from the mice just after euthanization and transferred to a 30 mm dish filled with paraffin oil. Cumulus–oocyte complexes were obtained from the ampullae of uterine tubes and transferred to dishes, each containing 200 μL HTF medium covered with paraffin oil, and observed under a stereomicroscope. Two to four cumulus–oocyte masses were transferred to 200 μL HTF medium covered with paraffin oil for insemination. A sperm suspension cultured in conditioned medium was transferred to the insemination drop containing the cumulus–oocyte masses. The fertilization rate was determined as the percentage of two-cell stage embryos among all the oocytes at 24 h after insemination. After incubation in potassium simplex optimization medium

(KSOM) for an additional 60 h, the blastocysts were transferred into the uteri of pseudopregnant females.

2.4 | Analysis of sperm motility

C57BL/6J mice (10–12 weeks) were sacrificed by cervical dislocation just before the start of the experiments, and the caudal epididymis was collected. Mature sperm from the caudal epididymis were incubated in 400 μL HTF medium without BSA. After 8 min, 40 μL of the sperm suspension was transferred to 160 μL of each conditioned HTF medium containing 20 μM ML-2–3 and maintained at 37°C in a humidified incubator under 5% CO_2/95% air (~10 000/μL of the motile sperm concentration). Then, 30 μL of the solution was removed from the medium at various times (0.5, 1, 2, 3, and 5 h) and placed in a pre-warmed Makler counting chamber (Sefi Medical Instruments,). Sperm movement was analyzed using computer-assisted sperm analysis (CASA) with the software HTM-CEROS v12.3 (Hamilton Thorne Research,). At least 100 sperm in three different fields were counted to evaluate the percentage of total motile, rapid-speed (VAP >10.0 μ/s), medium-speed (5 μ/s <VAP <10.0 μ/s), slow-speed (VAP <5.0 μ/s or VSL <0.0 μ/s), and static-speed sperm (sperm not moving at all), VAP, VSL, VCL, ALH, LIN, STR, and BCF.

2.5 | Observation of the acrosome reaction

C57BL/6-TgN(acr3-EGFP)Osb17 mice (10–12 weeks) harboring sperm labeled with the acrosine-EGFP transgene were sacrificed by cervical dislocation just before collection of the caudal epididymis. Mature sperm from the caudal epididymis were incubated in 400 μL HTF medium without BSA. After 5 min, 40 μL of the sperm suspension was transferred to 160 μL of each conditioned HTF medium containing 20 μM sample and was maintained at 37°C in a humidified incubator under 5% CO_2/95% air (~10 000/μL of the motile sperm concentration). The sperm were removed from the medium at various times (1, 2, 3, 5, 7, and 9 h) and spotted onto microscope slides. At least 50 sperm in each sample were counted under a fluorescence microscope. The percentage of acrosome reaction-positive sperm was evaluated as the number of acrosome-reacted sperm (GFP-negative) among the total number of sperm.[7,8]

2.6 | Refrigerated sperm collection for IVF

C57BL/6J mice (10–12 weeks) were sacrificed by cervical dislocation at 3 days before the start of the IVF experiments. The epididymis of each mouse was removed and stored at 4°C in paraffin oil. Mature caudal epididymal sperm (~ 8×10^6) from each mouse were incubated in 200 μL HTF medium without BSA covered with paraffin oil. After 5 min, 15 μL of the sperm suspension was transferred to 50 μL HTF medium containing 1 mg/mL polyvinyl alcohol, 1.0 mM methyl-beta-cyclodextrin, and 20 μM ML-2–3. The conditioned medium was kept at 37°C in a humidified incubator under 5% CO_2/95% air (~10 000/μL of the motile sperm concentration).[7,8] After 55 min, 16–20 μL sperm from each conditioned medium were used for insemination (final motile sperm concentration =150/μL). Motile sperm swimming at the periphery of each drop were used for insemination as described previously.[7,8]

2.7 | IVF using refrigerated sperm

IVF using refrigerated sperm was assessed as above but with minor modifications. After cumulus–oocyte complexes were obtained from the ampullae of uterine tubes and transferred to dishes, they were treated with 0.01% hyaluronidase. After 5 min, 10–20 cumulus-free oocyte masses were transferred to 200 μL HTF medium covered with paraffin oil. A sperm suspension cultured in conditioned medium was transferred to the insemination drop containing the cumulus-free oocyte masses. The fertilization rate was determined as the percentage of two-cell stage embryos among all oocytes at 24 h after insemination.

2.8 | Statistical analysis

All data were derived from at least three independent experiments. The results are expressed as means ± standard deviation (SD) for each condition. Differences between the experimental and control conditions were determined using the two-tailed paired *t*-test, and a p value of <0.05 or <0.01 was considered statistically significant.

3 | RESULTS

3.1 | Fertilization rate

We evaluated the effect of ML-2–3 on the fertilization ability of sperm from BALB/cByJJcl mice. The fertilization rates of these mice tend to be lower than those of C57BL/6J mice, and they have an unusually high proportion of morphologically abnormal sperm.[15,16] ML-2–3 was added to the sperm pre-culture in human tubal fluid (HTF) medium, and the sperm was then used to inseminate eggs. In the first experiment, the fertilization rate of sperm cultured in HTF medium without ML-2–3 was 66.7%, while that of sperm cultured in HTF medium with ML-2–3 (20 μM) was 100.0%. The fertilization rate in the second, third, fourth, and fifth experiments increased from 36.4% to 50.0%, from 66.7% to 83.3%, from 60.0% to 100.0%, and from 14.3% to 60.0%, respectively (Figure 2). These results demonstrate that ML-2–3 significantly increased the fertilization rate. Next, to identify the optimal concentration of ML-2–3, the dose dependency of the effect on IVF was examined. As shown in Figure 3, the fertilization rates of sperm cultured in HTF medium with ML-2–3 at a concentration of 0, 10, 20, 40, or 80 μM were 46.6 ± 20.8%, 63.5 ± 17.8% ($p < 0.05$), 73.3 ± 22.6% ($p < 0.05$), 74.6 ± 16.5% ($p < 0.05$), and 69.3 ± 13.2% ($p > 0.05$), respectively.

These results indicate that the optimal concentration of ML-2–3 was 20 μM. Furthermore, we examined the viability of embryos that developed following sperm culture with ML-2–3. Female mice became pregnant and delivered young ones after transplantation. The results shown in Figure 4 indicate that ML-2–3 significantly increased the fertilization rate without significant toxicity to embryogenesis.

3.2 | Sperm motility

The motility of sperm incubated with ML-2–3 was analyzed using computer-assisted sperm analysis (CASA), which can provide sperm motility parameters objectively and repeatedly. In this experiment, we used 20 μM, which is the optimal concentration of ML-2–3. A percentage of motile sperm cultured in HTF medium containing ML-2–3 was significantly maintained after 2, 3, and 5 h of incubation (Figure 5A). Furthermore, ML-2–3 maintained a higher percentage of rapid-speed sperm after 3 h of incubation. A higher percentage of sperm with medium speed was also maintained at 3 h and 5 h after incubation (Figure 5B and Table 1). ML-2–3 maintained a lower percentage of static-speed sperm at 3 h and 5 h after incubation (Figure 5C). In contrast, ML-2–3 had little or no effect on the other

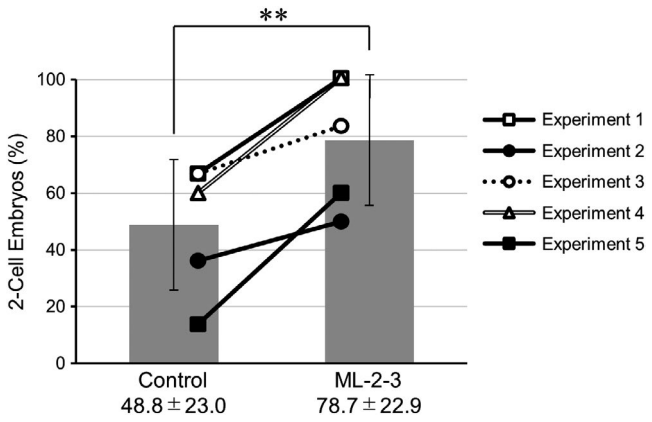

FIGURE 2 Effects of ML-2–3 on the Rate of Fertilization. Sperm from BALB/cByJJcl mice were pre-incubated in HTF medium with or without ML-2–3 (20 μM). Addition of ML-2–3 to the medium increased the percentage of two-cell embryos. The data are presented as means ± SD of five independent experiments. $^{**}p < 0.01$ compared with the control group

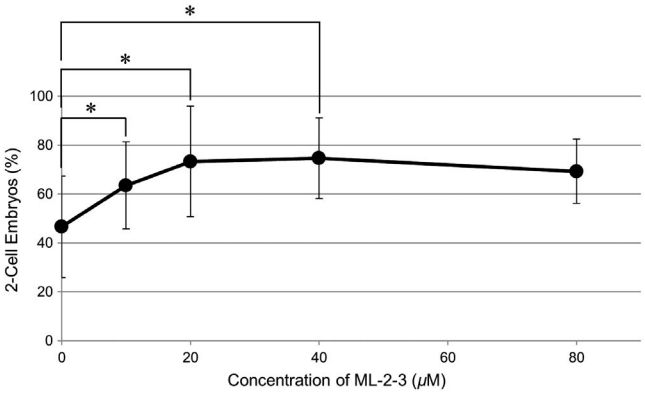

FIGURE 3 The Effect of Different Concentrations of ML-2–3 on the Rate of Fertilization. Sperm from BALB/cByJJcl mice were treated with ML-2–3 at various concentrations. The fertilization rate was determined by counting the number of two-cell embryos. The optimal concentration of ML-2–3 in the pre-culture medium for fertilization was 20 μM. The data are presented as means ± SD of three independent experiments. $^{*}p < 0.05$ compared with the control group

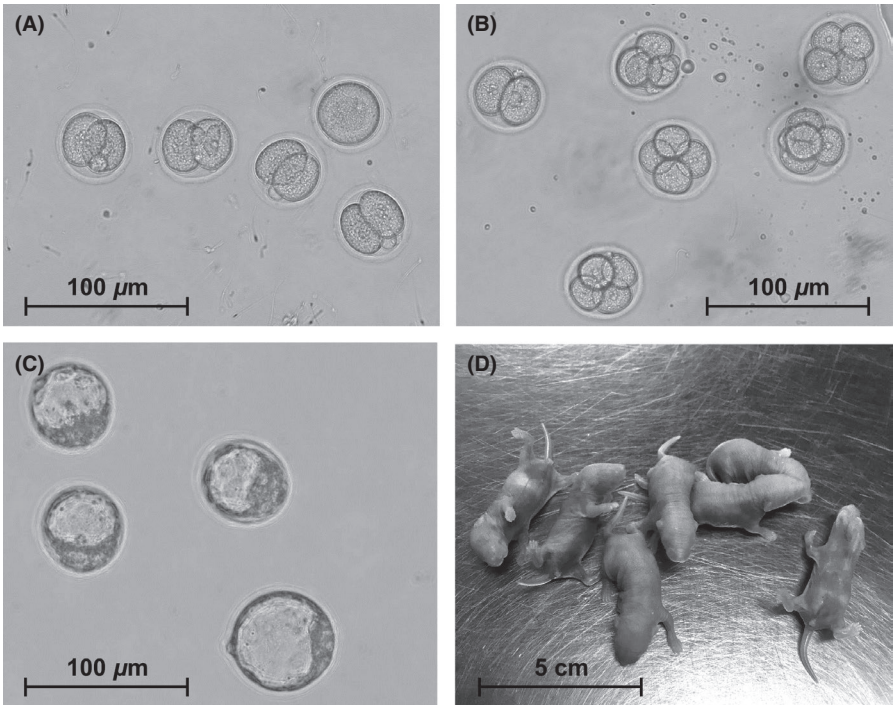

FIGURE 4 Progeny from Oocytes and Sperm Incubated with ML-2–3. Two-cell stage embryos (A) that developed from sperm pre-incubated in HTF medium with ML-2–3. Four-cell stage embryos (B), blastocysts (C), and newborn mice delivered from the embryos after transfer to pseudopregnant female mice (D)

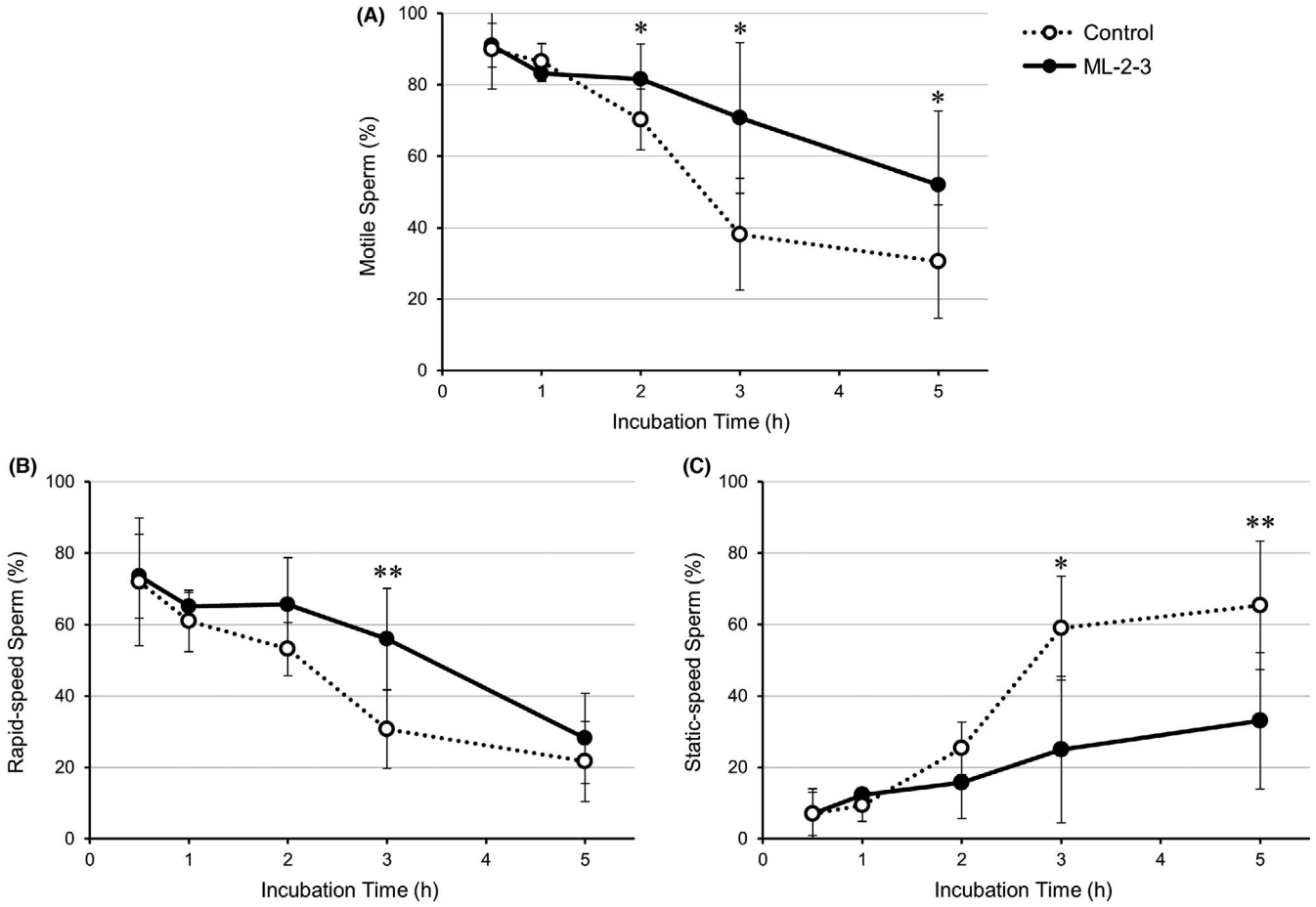

FIGURE 5 Incubation with ML-2–3 Activated Sperm Motility. Sperm motility was evaluated by CASA. Sperm from C57BL/6J mice were treated with ML-2–3 (20 μM) in HTF medium for 0.5, 1, 2, 3, or 5 h. (A) Motile sperm (%), (B) rapid-speed sperm (%), and (C) static-speed sperm (%) in the sperm motility parameters. Values represent means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control group

parameters in CASA (Table 1). These results indicate that ML-2–3 maintained sperm speed under standard IVF conditions.

3.3 | Acrosome reaction

As sperm approaches the egg, the membrane surrounding the head of the acrosome fuses with the plasma membrane to enable the fusion with the egg.[17,18] This is called the acrosome reaction, which is necessary for the sperm to penetrate the zona pellucida and is an important step in fertilization.[17–19] The timing of the acrosome reaction and the percentage of acrosome-reacted sperm affect the success of IVF.[20] We examined the effect of ML-2–3 on the acrosome reaction using sperm from C57BL/6-TgN(acr3-EGFP)Osb17 mice harboring acrosomes that accumulate EGFP protein.[14] Acrosome-reacted sperm lose their fluorescence. In this experiment, as in the previous section, the concentration of ML-2–3 was set at 20 μM. To determine the effect of ML-2–3 on the acrosome reaction, we counted the acrosome-reacted sperm among the total sperm. As shown in Figure 6, no significant difference in acrosome reaction rates was observed between the ML-2–3-treated sperm and the control. Thus, we concluded that ML-2–3 had no effect on the acrosome reaction.

3.4 | Refrigerated sperm

We further examined the effect of ML-2–3 on sperm that had been refrigerated for 3 days (Figure 7). The fertilization rate of refrigerated sperm cultured without ML-2–3 was 4.7 ± 4.8%, while that of refrigerated sperm cultured with ML-2–3 was 10.9 ± 8.1% ($p < 0.05$).

4 | DISCUSSION

When epididymal sperms pass through the female reproductive tract or the appropriate medium, they exhibit progressive motility and the ability to bind to the zona pellucida.[21] This is called capacitation. Sperms in the capacitation stage show progressive motility, which is characterized by symmetric and low-amplitude flagellar bends. However, after capacitation, sperms show asymmetric and large-amplitude flagellar bends, leading to more rotational and less

TABLE 1 Time-course analysis of sperm motility in the presence of ML-2-3[a]

		Time after pre-incubation (h)				
Parameter	**Sample**	**0.5**	**1**	**2**	**3**	**5**
Motile sperm (%)	Control	90.0 ± 11.2	86.5 ± 5.1	70.3 ± 8.5	38.1 ± 15.7	30.6 ± 15.9
	ML–2–3	91.1 ± 6.16	83.2 ± 2.2	81.7 ± 9.7*	70.7 ± 21.1*	52.0 ± 20.7*
Rapid-speed sperm (%)	Control	72.0 ± 18.0	61.0 ± 9.0	53.0 ± 7.9	30.7 ± 11.1	22.0 ± 11.1
	ML–2–3	73.7 ± 11.5	65.0 ± 4.0	65.7 ± 13.3	55.7 ± 14.3**	28.3 ± 12.3
Medium-speed sperm (%)	Control	18.0 ± 8.7	25.7 ± 8.4	17.0 ± 2.6	7.7 ± 5.5	9.0 ± 5.3
	ML–2–3	17.3 ± 6.4	18.0 ± 4.4	16.3 ± 4.0	14.7 ± 8.1*	24.0 ± 7.9*
Slow-speed sperm (%)	Control	3.0 ± 4.4	4.0 ± 1.0	4.3 ± 1.2	3.0 ± 3.6	4.0 ± 2.6
	ML–2–3	2.0 ± 1.0	4.0 ± 2.6	2.7 ± 3.1	4.3 ± 1.5	15.0 ± 7.8
Static-speed sperm (%)	Control	7.0 ± 7.0	9.3 ± 4.5	25.3 ± 7.4	59.0 ± 14.5	65.3 ± 18.0
	ML–2–3	7.0 ± 6.1	12.3 ± 1.5	15.7 ± 10.0	25.0 ± 20.5*	33.0 ± 19.1**
Path velocity (μm/s)	Control	89.4 ± 12.9	79.6 ± 30.2	83.6 ± 4.1	76.6 ± 12.4	40.6 ± 15.5
	ML–2–3	86.8 ± 7.5	90.3 ± 16.7	94.4 ± 20.8	85.0 ± 8.3	39.7 ± 10.1
Progressive velocity (μm/s)	Control	63.0 ± 8.7	54.0 ± 21.0	53.4 ± 5.0	49.8 ± 11.8	21.2 ± 10.0
	ML–2–3	59.9 ± 7.9	56.0 ± 11.8	61.0 ± 15.5	51.3 ± 4.6	22.0 ± 9.0
Track speed (μm/s)	Control	165.0 ± 27.8	145.2 ± 48.7	156.4 ± 5.1	146.5 ± 21.3	99.1 ± 31.7
	ML–2–3	158.9 ± 8.4	169.8 ± 24.0	173.3 ± 36.4	153.3 ± 18.6	87.1 ± 14.5
Lateral amplitude (μm)	Control	8.9 ± 0.4	8.5 ± 0.9	8.9 ± 0.9	8.6 ± 0.9	6.9 ± 2.1
	ML–2–3	8.2 ± 0.2	9.4 ± 0.6	9.7 ± 1.1	8.8 ± 0.4	7.6 ± 0.6
Beat frequency (Hz)	Control	27.5 ± 1.3	27.1 ± 6.0	26.0 ± 2.7	28.9 ± 1.2	33.5 ± 6.1
	ML–2–3	27.5 ± 1.3	24.6 ± 1.6	24.6 ± 2.1	25.6 ± 2.5	32.7 ± 7.8
Straightness (%)	Control	66.3 ± 2.1	60.7 ± 1.5	60.7 ± 3.2	59.3 ± 6.7	51.3 ± 4.0
	ML–2–3	63.0 ± 3.6	58.3 ± 1.2	60.7 ± 3.2	54.7 ± 3.8	55.7 ± 10.1
Linearity (%)	Control	37.3 ± 1.2	34.3 ± 2.3	32.3 ± 2.5	32.7 ± 3.5	26.7 ± 2.1
	ML–2–3	36.0 ± 2.6	32.7 ± 1.5	34.7 ± 1.5	31.0 ± 1.7	28.6 ± 6.5
Elongation (%)	Control	53.0 ± 1.0	51.3 ± 3.1	52.3 ± 1.5	52.0 ± 9.2	51.7 ± 8.5
	ML–2–3	53.3 ± 4.6	52.0 ± 2.6	53.0 ± 1.7	50.3 ± 6.4	48.0 ± 4.0
Area (μm^2)	Control	10.5 ± 0.8	9.7 ± 0.6	9.8 ± 1.9	10.4 ± 1.1	7.9 ± 1.2
	ML–2–3	11.1 ± 1.9	9.4 ± 0.8	8.9 ± 0.5	9.1 ± 0.3	7.4 ± 0.5

*$p < 0.05$.

**$p < 0.01$ compared with the control group.

[a]Values represent means ± SD ($n = 3$).

progressive motility.[21] This is called hyperactivity, which physically directs the sperms to the zona pellucida. When hyperactivation is induced, changes in the specific analytical parameters of CASA are observed.[22] Specifically, path velocity (VAP), track speed (VCL), lateral amplitude (ALH), and beat cross frequency (BCF) increase, while progressive velocity (VSL), linearity (LIN), and straightness (STR) decrease.

The results of the sperm motility analysis indicated that ML-2–3 did not affect the above parameters or did it induce hyperactivation. On the other hand, ML-2–3 significantly maintained a higher percentage of motile and rapid-speed sperm and a lower percentage of slow-speed sperm. The acrosome reaction, which is a morphological change that follows capacitation, is an essential reaction for fertilization. In the acrosome reaction, the sperm cell membrane and the acrosome outer membrane undergo membrane fusion at multiple sites, and enzymes contained in the acrosome are released.[21] As shown in Figure 6, ML-2–3 did not affect the acrosome reaction. We therefore concluded that ML-2–3 maintained sperm motility without inducing hyperactivation. This result might be due to the large number of sperms whose hyperactivation was induced by the primary mechanism of sperm while maintaining sperm motility in IVF. Hochuekkito, pentoxifylline, coenzyme Q10, Maca (*Lepidium meyenii* Walpers), isoliquiritigenin, and formononetin have been reported to improve sperm motility.[23–28] Oral administration of Hochuekkito increases sperm concentration. Furthermore, Hochuekkito acts directly on sperm to improve motility.[23] Pentoxifylline is a xanthine derivative used for the treatment of peripheral and cerebrovascular diseases. The compound contributes to IVF success by enhancing the calcium ionophore concentration and inducing the acrosome reaction.[24–26]

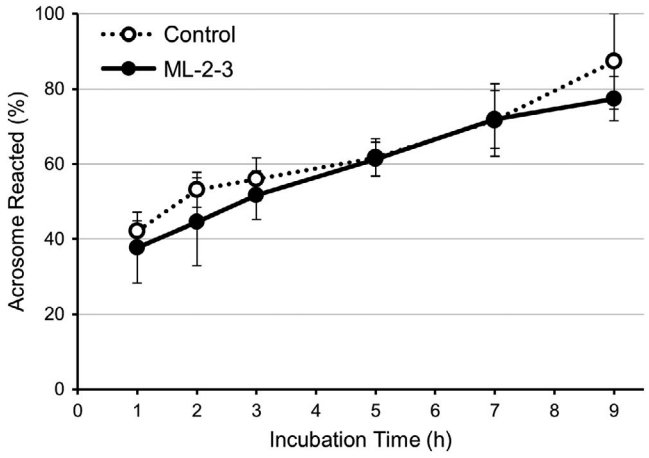

FIGURE 6 The Acrosome Reaction in Sperm Incubated with ML-2–3. Sperm from C57BL/6-TgN(acr3-EGFP)Osb17 mice were treated with ML-2–3 (20 μM) in HTF medium for 1, 2, 3, 5, 7, or 9 h. There were no differences between the control and ML-2–3 groups ($p > 0.05$). Values represent means ± SD of three independent experiments

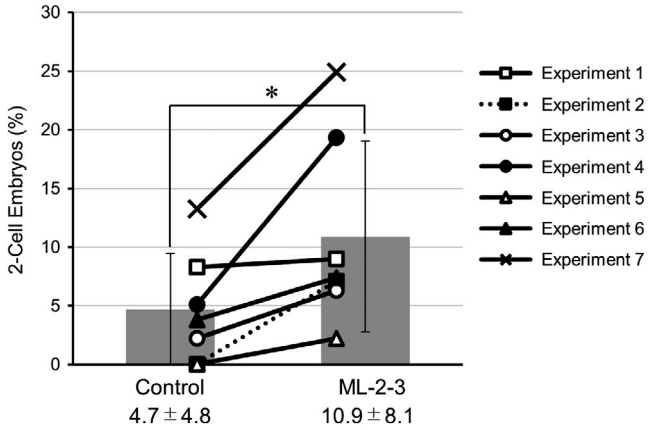

FIGURE 7 Effect of ML-2–3 on the IVF Rate when using Refrigerated Sperm. Epididymides from C57BL/6J mice were stored at 4°C in paraffin oil for 3 days. Sperm were removed and pre-incubated in HTF medium with or without ML-2–3 (20 μM). The medium containing ML-2–3 increased the percentage of two-cell embryos. The data are presented as means ± SD of seven independent experiments. $^*p < 0.05$ compared with the control group

Several studies have shown that coenzyme Q10 improves sperm motility by protecting sperm from oxidative stress via antioxidant effects.[27] Maca has been reported to increase IVF rates by inducing the acrosome reaction and improving sperm motility.[28]

In our previous study, the licorice-derived active flavonoids isoliquiritigenin and formononetin also slightly improved sperm motility and improved IVF rates in mice.[7,8] ML-2–3 improved the fertilization rate and increased the number of motile sperm without hyperactivation or induction of the acrosome reaction. This is the first compound to exhibit a mechanism that differs from those of the compounds tested previously. ML-2–3 also increased the IVF rate of sperm that had been refrigerated for 3 days. When refrigerated, sperm proteins gradually denature, significantly reducing the IVF rate. There are few reports of compounds that can improve the IVF rate of sperm refrigerated for 5 days.[29,30] Our result indicates the potential to further improve the refrigerated storage of sperm.

Elucidation of the molecular mechanism of ML-2–3, which appears to differ from those of other compounds, might be helpful in understanding sperm capacitation. Further studies are required to verify that ML-2–3 can be applied to human IVF. The results of this study imply that ML-2–3 significantly contributes to IVF.

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CONFLICT OF INTEREST

None of the authors declare competing financial interests.

ETHICAL APPROVAL

All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation and Research Ethics Committee of Nagasaki International University. This article does not contain any studies with human participants performed by any of the authors.

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