Experimental Animals
Exp. Anim. 69(3), 345–353, 2020

Original

Physiological and pharmacological evaluation of oxytocin-induced milk ejection in mice

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Abstract: Oxytocin, a posterior pituitary hormone, causes the contraction of the mammary myoepithelial cells that surround the acini. This ejects milk from the acini into the primary mammary ducts. The milk ejection responses by oxytocin have not yet been exactly evaluated in mice. Thus, we present a novel method for quantitatively evaluating oxytocin-induced milk ejection in anesthetized lactating mice. We cannulated the mammary duct, administered oxytocin intraperitoneally or intravenously, and collected and measured the ejected milk. Intraperitoneal oxytocin administration (150 mU) induced continuous but oscillatory milk ejection. Repeated intravenous administration of 1.5 mU of oxytocin elicited repeated transient milk ejection. The volume of the ejected milk as a proportion of the stored volume just before each ejection (rather than ejection volume itself) was an expedient and reliable parameter representing the potency of ejection. The oxytocin sensitivity of mice at day 18 of lactation was determined from a sigmoidal dose–response curve as $ED_{50} \approx 2.69$ mU. Based on this dose–response relationship, the specific activity of the oxytocin receptor agonists (Thr^4, Gly^7)-oxytocin and WAY 267464 were estimated as 976 and 6.87 U/mg, respectively. The assay presented here could be useful for physiological and pharmacological investigations of oxytocin-induced milk ejection.
Key words: bioassay, milk ejection, mouse, oxytocin,

Introduction

Oxytocin (OT)-induced milk ejection is the final, essential step in milk secretion [10, 18]. After milk is produced by mammary acinar cells, it is stored in the acini rather than being immediately secreted to the outside of the body. When pups suckle the dam's nipples, this stimulus is neurally transmitted to magnocellular neurons at the hypothalamic periventricular and supraoptic nuclei, where it induces the intermittent release into the blood of OT from the axonal end of the neurons located in the posterior pituitary [10]. This results in the contraction of myoepithelial cells around the acini, and alveolar milk is ejected into a primary duct beneath the nipple. The milk is finally secreted, assisted by the negative pressure from the pup suckling. This reflex is known as the milk "let-down" or "ejection" reflex. The pathway for the reflex has been investigated using sophisticated *in vivo* assays with anesthetized experimental animals, including rabbits, guinea pigs, and rats [2, 5, 25–27]. In these assays, a cannula connected to a manometer or pressure transducer was inserted into the mammary duct, and the increase in intramammary pressure was monitored as an outcome of milk ejection activity.

Although the intramammary pressure measurement is a refined tool for the study of the milk ejection reflex and OT-induced milk ejection, it has some disadvantages for the study of physiological milk ejection. First, the normal intramammary pressure is low, so it is only possible to measure the intramammary pressure sensitively when the milk is fully or excessively stored in the mammary gland. Therefore, an assay solution is sometimes added into the mammary duct before the experiment to increase the sensitivity [2, 24, 26]. Second, the

(Received 24 September 2019 / Accepted 1 March 2020 / Published online in J-STAGE 26 March 2020)
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Supplementary Figure: refer to J-STAGE: https://www.jstage.jst.go.jp/browse/expanim

inserted cannula does not have an outflow route due to the installed transducer, so the milk is retained in the mammary gland even after the OT-induced myoepithelial contraction. This experimental condition differs somewhat from the physiological state, where the milk is usually removed by pups and the remaining milk volume gradually decreases. Third, the mouse mammary duct is too small to insert the cannula for the pressure measurement, so the intramammary pressure measurement has never been successfully applied to mice [7].

We have established a novel method to investigate the properties of OT-induced milk ejection in anesthetized mice. In this method, the ejected milk in OT-challenged lactating mice is collected through a ductal cannula and the volume of injection is directly and chronologically measured. The results of using this method have revealed new aspects of the physiological features of milk ejection. Moreover, by taking advantage of the quantitative nature of this method, we were able to estimate the specific activity of oxytocin receptor (OTR) agonists *in vivo*.

Materials and Methods

Animals

The experimental procedures and animal care were performed in accordance with the Regulations on Management and Operation of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine and were approved by the University's Animal Care and Use Committee (No. 18-27, 18-31, 18-143, 29-32).

C57BL/6JJmsSlc mice were obtained from Nihon SLC (Shizuoka, Japan). The female mice were mated, and the lactating mice after parturition were used for the assay. The mice were housed at 23 ± 2°C with a 12 h: 12 h light: dark cycle, and given food and water *ad libitum*. The basic characteristics of the mice utilized in each experiment, including the number of tested mice, age, body weight, duration of lactation, and litter size, are summarized in Table 1.

Chemicals

OT (lyophilized powder, Cat No. O4375, Lot No. 103H05241V, 16.4 U/mg) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in water to prepare a 100 U/ml solution. A peptidic OT analog, (Thr^4, Gly^7)-oxytocin (TGOT) (Cat No. H-7710, Lot No. 1062174), obtained from Bachem (Bubendorf, Switzerland), was dissolved in water at a concentration of 5 mg/ml. A nonpeptidic OTR agonist, WAY 267464 dihydrochloride (Cat No 3933, Batch No 4), obtained from Tocris Bioscience (Bristol, UK), was dissolved in DMSO at a concentration of 50 mg/ml. These solutions were divided into aliquots and stored at −80°C until use. Before the assay, the stored solutions were diluted into the appropriate concentrations with PBS.

Surgical preparation for the assay of milk ejection

Lactating mice on days 10–19 of lactation were used for the milk ejection assays. The pups were removed from the dam and killed by inhalation of carbon dioxide approximately 4 h before the start of recording to allow milk to accumulate in the mammary glands. The dam was anesthetized by intraperitoneal administration of 75 mg/kg ketamine and 1 mg/kg medetomidine. One-fifth volume of an initial anesthetic was intraperitoneally injected as needed. For the experiments involving intravenous injections of OT, a catheter was inserted to the jugular vein. A cannula for monitoring the milk ejection was inserted into the duct of an abdominal mammary gland (Figs. 1A–D). To do this, under a stereomicroscope, a small incision was made on the primary duct beneath the nipple of an abdominal mammary gland, and

Table 1. Characteristics of the mice used in the experiments

	ip exp.	iv exp.					
	OT	OT, 1 min interval	OT, 5 min interval	OT, 10 min interval	OT, Dose-response	TGOT	WAY267464
Number	n=7	n=5	n=6	n=5	n = 7	n = 5	n = 5
Dam weight (g)	31.8 ± 1.4	32.6 ± 0.9	32.1 ± 2.5	32.3 ± 1.9	31.5 ± 2.0	33.0 ± 1.3	32.1 ± 1.6
Dam age (wks)	14.7 ± 1.4	18.6 ± 0.5	16.5 ± 1.6	16.8 ± 1.5	16.9 ± 0.7	15.6 ± 0.9	18.6 ± 1.3
Litter size	6.0 ± 1.6	7.2 ± 1.3	6.8 ± 1.7	6.4 ± 1.3	7.0 ± 1.5	7.2 ± 0.8	6.4 ± 1.3
Pup weight (g)	5.6 ± 0.9	6.8 ± 0.9	6.7 ± 0.5	6.8 ± 0.9	7.0 ± 1.5	6.7 ± 0.1	7.5 ± 0.7
Lactation (day)	13.9 ± 3.1	15.6 ± 1.0	14.3 ± 0.9	15.8 ± 1.3	18 ± 0	15.0 ± 1.0	16.6 ± 1.7
AM weight (mg)	-	366.9 ± 17.9	332.3 ± 45.1	365.4 ± 48.3	303.7 ± 44.9	338.2 ± 11.2	372.0 ± 90.3
Total milk (μl)	221.0 ± 87.0	212.3 ± 32.3	214.6 ± 69.6	223.1 ± 30.8	208.4 ± 39.3	215.8 ± 15.4	216.3 ± 92.2

The table summarizes the body weight and age of the dams, the litter size, the average body weight per pup, the duration of lactation, the weight of the tested abdominal mammary gland (AM), and the total volume of milk from the tested gland in the experiments that investigated milk ejection induced by intraperitoneal (ip) and intravenous (iv) treatments of oxytocin (OT) and OT agonists. Body weights were measured before the experiment and the weights of the AMs after it. The AMs were not weighed in the intraperitoneal experiment. Values represent means ± SD.

 doi: 10.1538/expanim.19-0126

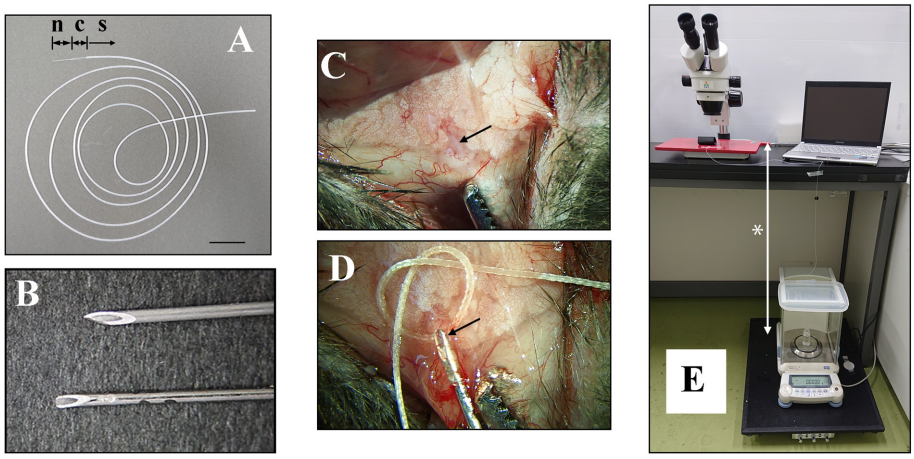

Fig. 1. Preparation for milk ejection analyses in mice. *A*: A mammary ductal cannula and milk collecting tube. A blunted 27-G needle (n) as a ductal cannula and a thin and long silicon tube (s) for milk collection was connected with a short connection tube (c). The bar indicates 2 cm. *B*: The processed tip of the cannula. The tip of the 27-G needle (top) was blunted to prepare the cannula (bottom). *C, D*: Insertion of the mammary ductal cannula. The primary duct beneath the nipple of an abdominal mammary gland was exposed (*C*, arrow) and the cannula was inserted to the duct from the small incision (*D*). *E*: An overview of the apparatus for milk ejection analysis. The operated mouse was placed on a heater on a bench. The open end of the milk collection tube was immersed in the bottle in the balance that was placed on a floor. The level difference (*) was approximately 80 cm.

the cannula was inserted a few millimeters and tied into position with thread (Figs. 1C and D). The cannula was made from a 27G needle (NN-2719S; Terumo, Tokyo, Japan); this was cut to approximately 15 mm long and the tapered tip was blunted with a rasp (Figs. 1A and B). A 1-cm perfluoroalkoxy tube (internal diameter 0.3 mm, outer diameter 0.5 mm; EXLON PFA tube; IWASE, Kanagawa, Japan) was used to connect the cannula to a silicon tube for milk collection; this was approximately 120 cm long (internal diameter 0.5 mm, outer diameter 1.0 mm; Cat. No. 100-00N; Kaneka Medix, Osaka, Japan) (Fig. 1A). The cannula and tubes were filled with PBS, and the open end of the silicon tube was immersed in water in a glass bottle mounted on an electrical balance. The open end of the tube on the balance was positioned approximately 80 cm below the level of the mammary gland, resulting in negative pressure inside the cannula due to the weight of fluid inside the tube (as with the siphon principle) (Fig. 1E). Without this negative pressure, we could not detect milk ejection, probably because of the low ejection pressure. During the measurement, the anesthetized mouse was laid on its cannulated side, and warmed with a heater at 35°C. With this arrangement, the ejected milk entering the tube through the cannula forced the pre-filled PBS into the bottle on the balance, allowing the milk ejection to be monitored as a weight increase. The weight change was recorded every 3 s. After the experiment, mice were killed by cervical dislocation without ever gaining consciousness, and the tested mammary gland was collected and weighed.

OT administration

In the experiment of intraperitoneal administration, OT was administered two times (at 0 and 20 min) and the recording was continued until 20 min after the second administration. In this experiment, 150 mU of OT dissolved in 150 μl of PBS was injected as this dosage was enough to induce the ejection of substantial proportion of the stored milk.

In the experiment of intravenous administration, OT at an appropriate concentration dissolved in 10 or 50 μl PBS was rapidly injected through the jugular vein catheter. This was followed by an injection of ~50 μl of PBS supplemented with 10 U/ml heparin to flush the drug inside the catheter. Repeated OT stimulations were given at intervals of 1, 5, or 10 min, and the ejected milk was collected with additional aspiration by manually squeezing the collecting silicone tube because the constant negative pressure generated by the fluid inside the tube was sometimes insufficient to aspirate all the milk ejected in the mammary duct within the short reaction period. At the end of each experiment, we injected 20 mU of OT twice to collect the all the available milk inside the mammary gland and to measure the total volume of milk (V_{total}).

Data analyses

For the data analysis and/or graphical display, the digital data were processed with Excel (Microsoft, Redmond, WA, USA) and Igor Pro (WaveMetrics, Lake Oswego, OR, USA).

Before analyzing, the evaporation of water from the bottle on the balance, which resulted in a slight background drift, was compensated for by subtracting the weight change during the pre-stimulation period of each experiment. Then, the data were used to calculate the ejection speed, ejection volume (V), and mammary ejection fraction (mEF) induced by each stimulation. The value of mEF induced by the n-th OT stimulation (mEF_n) was determined as follows:

$$mEF_n\ (\%) = 100 \cdot V_n / V_{remained,n} = 100 \cdot V_n / (V_{total} - \sum_{i=1}^{n-1} V_i),$$

where, V_n and $V_{remained,n}$ represent the milk volume ejected by the n-th OT stimulation and the stored milk volume in the mammary gland just before the n-th stimulation, respectively.

To determine OT sensitivity, 0.005–20 mU of OT was intravenously administered to a single lactating mouse in increasing doses at 5-min intervals, and the values of V and mEF at each dose of OT were measured. The relationship between mEF and the challenge OT dose was plotted and fitted with the following equation:

$$mEF(x) = mEF_{min} + (mEF_{max} - mEF_{min}) / (1 + (x/ED_{50})^h),$$

where x and ED_{50} represent the OT dose administered (in units per mouse) and the OT dose at which half of the stored milk was ejected, respectively, and h is the Hill coefficient. The minimum value of mEF (mEF_{min}) was 0% and the maximum value (mEF_{max}) was assumed to be 100%.

Assay for the OT activity of OTR agonists

To assess the OT activity of OTR agonists *in vivo*, we examined the effect on milk ejection of the intravenous administration of the agonists. After administering the control OT (1 or 1.5 mU), 2.5 ng of TGOT or 0.21 μg of WAY 267464 was injected intravenously, and then an excess of OT (20 mU) was administered twice to measure the total milk volume. To minimize errors arising from interindividual difference, the OT sensitivity (ED_{50} value) of each mouse was estimated from the mEF value resulting from the control OT, assuming h in the equation to be 1.1. The OT activity of each agonist was calculated from the mEF values obtained.

Statistics

Data are expressed as means ± SD. The ejection activities resulting from the repeated administration of the same dose of OT were analyzed using repeated-measures ANOVA followed by post hoc testing with the Bonferroni correction for multiple comparisons. Welch's test was used for comparisons between two groups. Correlations between factors were analyzed with Pearson's correlation analysis. A value of $P<0.05$ was considered as statistically significant.

Results

Direct measurement of milk ejection induced by intraperitoneal OT administration

The milk ejection volume induced by intraperitoneal OT (150 mU) administrations was directly measured over time in anesthetized mice (n=7). The ejection started at 1.5 ± 0.7 min after the administration, and was oscillatory and lasted for about 10 min with a maximum speed of 91.9 ± 47.9 μl/min (Fig. 2A). Half of the total volume of milk had been ejected at 5.5 ± 2.1 min after the OT injection (Figs. 2B and C). The oscillation peaks were observed at a frequency of 2.3 ± 0.3 per min. During the 20 min after the injection, 209 ± 91 μl milk (91.1 ± 8.5% of the total volume) was ejected (Figs. 2B and C). An additional injection of OT (150 mU/mouse) induced only a little extra ejection. In total, 221 ± 87 μl of milk was ejected.

Direct measurement of milk ejection induced by intravenous OT administration

Next, we measured milk ejection after a rapid injection of OT (0.5 mU/mouse) via the jugular vein of mice (n=6). This resulted in the transient ejection (Fig. 3A) of 31.4 ± 14.7 μl of milk (Fig. 3B). The repeated administration of 0.5 mU of OT at 5 min intervals induced repeated transient milk ejection, with the volume of ejected milk gradually decreasing (Figs. 3A and B). Subsequently, the repeated injection of 1.5 mU of OT also induced repeated ejections (Fig. 3A), albeit with a significant reduction in ejected volume. This reduction may have been the result of the reduction in the available milk remaining in the mammary gland. We therefore evaluated the ejection volume as a proportion of the volume of milk remaining before the ejection (mammary ejection fraction: mEF). There were no significant differences between the mEF values for the first, second, and third injections of 0.5 or 1.5 mU of OT (Fig. 3B). The average value of mEF for the 1.5 mU injections (34.2 ± 10.2%) was significantly greater than that for the 0.5 mU injections (14.3 ± 6.7%) (n=6, $P<0.05$, Welch's t-test). These results suggest that mEF values resulting from a given dose of OT remained approximately constant even when the residual milk in the mammary gland decreased with

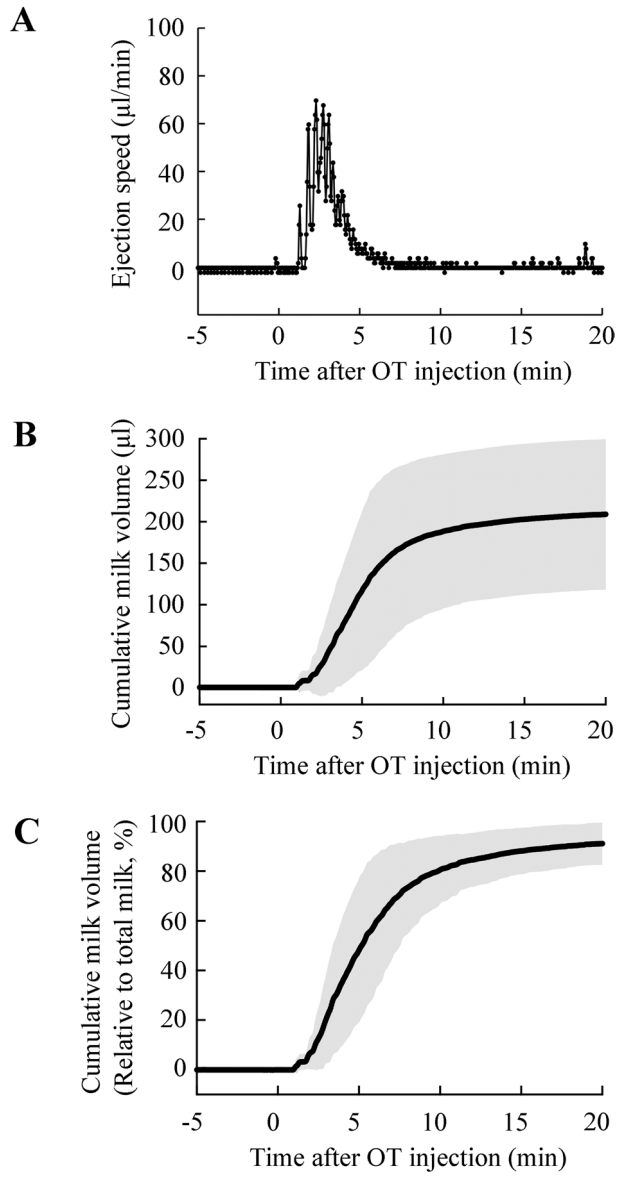

Fig. 2. Milk ejection induced by intraperitoneal injections of oxytocin (OT). *A*: A representative trace of ejection speed after the intraperitoneal OT administration (150 mU) at 0 min to anesthetized mice. The speed of milk ejection was recorded every 3 s. *B, C*: Cumulative volume of ejected milk after the intraperitoneal injection of OT, showing the averaged value of the cumulative volume of ejected milk (*B*) and the averaged value of the cumulative volume as a proportion of the total volume of ejected milk after the second OT injection (*C*). The lines and shadows indicate mean and SD values, respectively (n=7).

repeated OT challenges, and thus that the *mEF* value is a preferable indicator for evaluating the milk ejection activity in this method.

Influence of the interval time of repeated OT administrations on milk ejection

It is conceivable that the values of ejection volume and *mEF* induced by repeated OT injection may have been influenced by the length of intervals between repeated injections. To investigate this, we administered repeated challenges of 0.5 or 1.5 mU of OT with shorter (1 min) or longer (10 min) intervals (n=5, each) (Figs. 3C and D). In the experiment with 10-min intervals, the repeated challenge of both doses of OT showed decreasing ejection volumes and almost stable *mEF* values (Fig. 3D), as shown in the experiment with the 5-min intervals (Fig. 3B). At 1-min intervals, unlike at 5- and 10-min intervals, the repeated injections of 0.5 mU of OT did not induce significant reduction of ejection volume and showed significant increase of *mEF* values (Fig. 3C). However the value of *mEF* was decreased after third injection of 1.5 mU of OT at 1-min intervals (Fig. 3C). This decrease was presumably due to the depletion of the remaining milk. From these results, the *mEF* values obtained by the repeated OT administration at the intervals of 5 min or more would be preferable for stably evaluating the milk ejection activity.

Dose-dependency of OT-induced milk ejection

We evaluated the sensitivity of milk ejection to the dose of OT administered. The value of *mEF*, but not the ejection volume, was utilized as an indicator of the OT activity because it was less affected by the volume of milk remaining in the experiment of OT injection at 5-min intervals (Fig. 3B). On day 18 of lactation, OT was sequentially injected at increasing doses (from 0.005 mU to 20 mU) with 5-min intervals and the *mEF* values following each injection were determined (Figs. 4A and B). The injection of 0.005 mU of OT did not elicit milk ejection in any mouse tested (n=7). However, all the mice showed a significant peak in ejection after the 0.05 mU injection, with an average *mEF* of 6.1 ± 3.8%. These results demonstrate that the threshold dose in our mouse assay with intravenous injections was equivalent to or lower than that for the assays with the other rodents [2, 26]. The injection of a higher dose of OT resulted in higher *mEF* values. The relationship between the logarithmic value of the OT dose and *mEF* exhibited a sigmoidal curve with an ED_{50} of 2.69 ± 0.78 mU and h value of 1.07 ± 0.24).

Measuring the milk ejection activity of OTR agonists

We used our assay to determine the OT activity of peptidic OTR agonist, TGOT (Fig. 5A). In our method, to correct for individual differences in sensitivity to OT, OT was administered as a control before an injection of TGOT (2.5 ng). Using the estimated equation for the sigmoidal dose-response curve in each mouse, the specific activity of TGOT was estimated as 976 ± 79 U/mg (n=5). The activity of WAY 267464, a non-peptide OTR

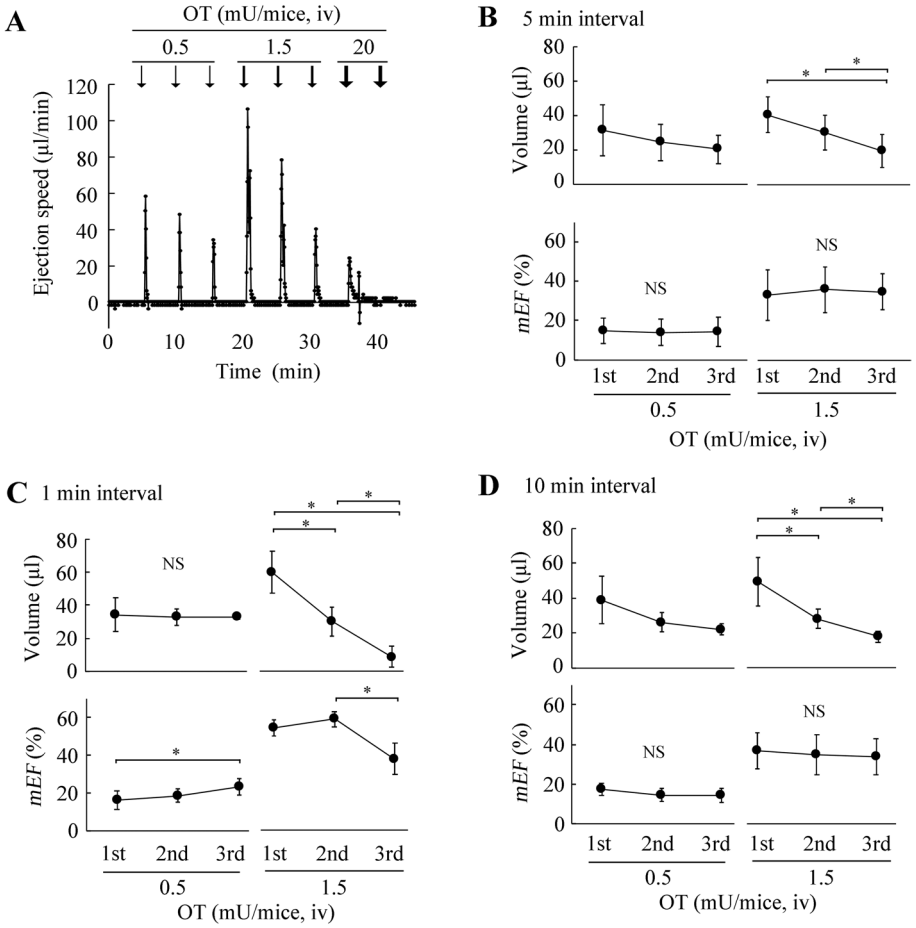

Fig. 3. Milk ejection induced by intravenous injections of oxytocin (OT). *A, B*: Milk ejection induced by intravenous injections of OT at 5-min intervals (arrows in *A*) at 0.5, 1.5, and 20 mU. The speed of milk ejection was recorded every 3 s. A representative trace of the change in milk ejection speed after repeated intravenous injections of OT is shown (*A*). The volumes of ejected milk and the mammary ejection fraction (*mEF*) induced by each OT injection were averaged (*B*). Each point in *B* represents the mean ± SD (n=6). *C, D*: Milk ejection induced by intravenous injections of OT with different intervals. OT was administered intravenously at 0.5, 1.5, and 20 mU in the same order as *A* at 1-min (*C*) or 10-min (*D*) intervals. The volume of ejected milk and *mEF* induced by each injection are summarized. Each point in *C* and *D* represents the mean ± SD (both n=5). Asterisks indicate significant differences between the indicated groups by ANOVA followed by post hoc testing ($P<0.05$). NS indicate a non-significant difference by ANOVA.

selective agonist, was also evaluated in a similar manner. The administration of WAY 267464 resulted in milk ejection (Fig. 5B), and its activity was estimated as 6.87 ± 2.59 U/mg (n=5).

Discussion

A report written more than 50 years ago describes the application of intraductal pressure measurement to mice to evaluate the OT-induced milk ejection, but that attempt failed because of the difficulty of cannulating the small diameter of the mouse mammary duct [7]. Milk ejection in mice has therefore been evaluated using indirect and semi-quantitative methods, such as the measurement of increases in pup weight, and morphological and histological observations of milk delivery to the ducts [6, 16, 19, 20, 23]. In the present study, we demonstrated that it is possible to directly measure the volume of ejected milk in response to exogenous OT.

We detected continuous and oscillated milk ejection in the experiment of intraperitoneal OT administration at a single dose of 150 mU (Fig. 2A). The maximum ejection speed (91.9 ± 47.9 µl/min) and the cumulative ejection volume (Fig. 2B) showed large variations (high SD values), possibly because these factors were also affected by the remaining milk volume in the mammary gland and the artificial flow resistance partly due to the position of the cannula tip. Thus, these indicators might not be the most favorable for assessing the milk ejection activity in the experiment of intraperitoneal administration. The volume of the ejected milk as a proportion of the total milk volume, as shown in Fig. 2C, would be a

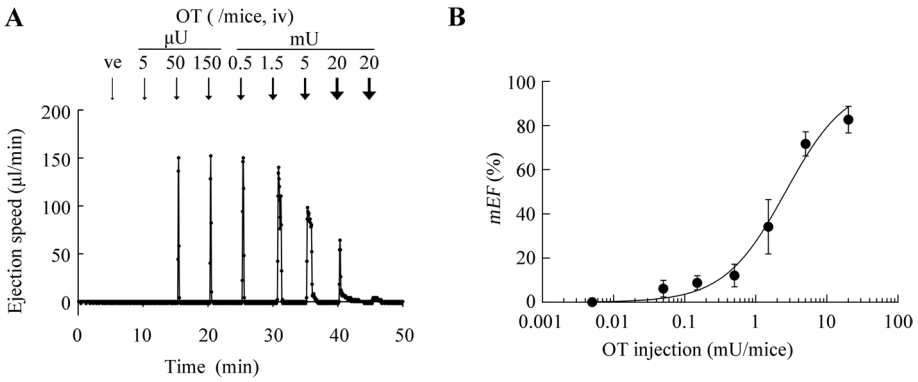

Fig. 4. Dose-dependency of oxytocin (OT)-induced milk ejection. *A*: A representative trace of the speed of milk ejection after intravenous (iv) injections of various doses of OT (0.005–20 mU) or vehicle (ve) at 5-min intervals on day 18 of lactation (n=7). The ejection speed was recorded every 3 s. The arrows indicate the times of the injections. *B*: The relationship between the dose of injected OT and *mEF*. Each point represents the mean ± SD (n=7). The plots were fitted to the Hill equation ($h \approx 1.07$, and $ED_{50} \approx 2.69$ mU).

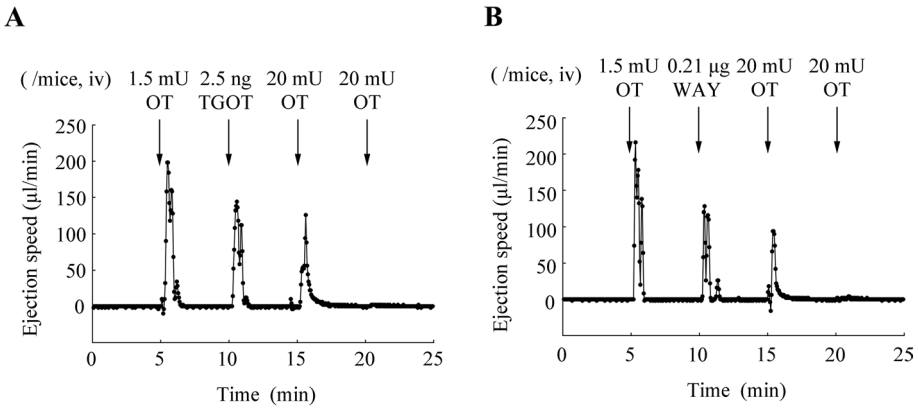

Fig. 5. Estimation of the milk ejection activity of oxytocin receptor (OTR) agonists. *A, B*: Representative traces of the speed of milk ejection in assays for estimating the activity of the OTR agonists TGOT (*A*) and WAY 267464 (WAY) (*B*), which were administered intravenously (iv) after a standard injection of oxytocin (OT) (1.5 mU). After the injection of the agonists, 20 mU of OT were administered before measuring the total milk volume. The speed of milk ejection was recorded every 3 s. The arrows indicate the times of the injections.

better indicator for that.

Milk ejection induced by the intraperitoneal OT administration demonstrated the oscillation at 2.2 peaks per min (Fig. 2). It has been reported that isolated mammary acini demonstrate oscillatory contraction after OT stimulation at a frequency of approximately four times a min [6]. This coordinated contraction of myoepithelial cells results from cellular communication via a gap junction and is crucial for milk ejection [16]. The oscillations of the ejection in the anesthetized mice in the present study were seemingly slower than those reported for isolated acini. Because the lactating mammary gland is composed of clusters of lobuloalveolar units including several lactating acini [1, 14, 21], there might be an inter-acinus and/or inter-lobuloalveolar mechanism for coordinating the OT-induced acinar contraction. Such a coordinated contraction might contribute to the effective milk delivery from acini to the nipple. The manner of the coordination and its mechanism should be addressed in the future to understand the physiological function of the mammary gland.

The experiment involving intravenous OT administration enabled the quantitative evaluation of OT-induced milk ejection and showed that *mEF* is a suitable indicator of milk ejection activity, unaffected by the volume of milk remaining (Fig. 3). It can be used to evaluate milk ejection activity even in the physiological state where the remaining milk continuously decreases.

The repeated stimulation of 0.5 mU of OT at 5- and 10-min intervals demonstrated a gradual reduction in the ejected milk volume ($P<0.05$ by ANOVA) and a stability in the *mEF* value (Figs. 3B and D), while the re-

peated stimulation of 0.5 mU of OT at 1-min intervals showed a invariable ejected milk volume and a gradual increase in the *mEF* value (Fig. 3C). These intricate influences of interval times on ejected volume and *mEF* values might be explained by taking into consideration the factors including the remaining milk volume, OT clearance and desensitization. It was plausible that the ejection volume was affected by the milk volume remaining in the mammary gland because the first stimulation of 0.5 mU of OT (there was no influence of desensitization and OT clearance) tended to show a positive correlation between these two factors (R=0.407, P=0.117, n=16) (Supplementary Fig. 1A). The relationship between the ejected and remaining milk volumes induced by the repeated administrations of 0.5 mU of OT at 5- and 10-min intervals was similar to that of the first administration (R=0.419, P=0.015, n=33) (Supplementary Fig. 1B). Thus, the *mEF* values remained constant at 5- and 10-min intervals (Figs. 3B and D). This might suggest that the reduction of ejected milk volume by the repeated OT stimulation at 5- and 10-min intervals could be largely accounted for by the reduction of the remaining milk volume. However, the repeated administration at 1-min intervals tended to weaken the correlation between the ejected and remining milk volume (R=0.233, P=0.404, n=15) (Supplementary Fig. 1C) concomitant with the increase of the *mEF* value (Fig. 3C). This might indicate that the administration at 1-min intervals increased the ejection volume and counteracted the effect of the reduced remaining milk volume. It was considered that the administered OT had not completely disappeared within 1 min and the reaction was potentiated by next increase in OT, as it has been reported that the bioactivity of OT in blood declined with a half-life of 1.65 min in rats [11].

Therefore, we have speculated that desensitization might have a minor influence in the OT-induced milk ejection in our experimental condition. This speculation was inconsistent with some previous reports about OT desensitization. Conti *et al.* [4] reported that treatment with 10^{-7} M OT in culture media induced desensitization to OT in ~15 min through internalization of its receptors (~80%), possibly via an interaction with β-arrestin2 in OTR-transfected or OTR-expressing cultured cells; after the removal of OT, the receptors were recycled to the plasma membrane and resensitized within 4 h. Magalhaes *et al.* [15] demonstrated that the pretreatment of rat myometrial strips for at least 1 h with OT at 10^{-8} M, but not at 10^{-10} M, in perfusates, induced desensitization of the OT function. The simplest explanation for this discrepancy in findings might be that the concentration of OT in the blood in the present study was too low to induce the profound desensitization because the injection of 0.5 or 1.5 mU (0.8×10^{-12} or 2.5×10^{-12} mole, respectively) of OT would only elevate the concentration by 0.4×10^{-9} or 1.2×10^{-9} M at most, respectively, if the injected OT was equally distributed throughout the 2.1 ml of mouse blood. Alternatively, the myoepithelial cells in the mammary gland might have a mechanism to avoid desensitization, such as the rapid recycling of internalized receptors or interactions with molecules in the caveolae that rigidly tether the plasma membrane [4, 8]. Although further investigation is needed to reveal the mechanisms, it has been assumed that the weak desensitization to OT in milk ejection is beneficial for nursing, in which intermittent OT secretion is induced by the milk ejection reflex [12, 24].

Our method can be used for bioassays of OTR agonists (Fig. 5). In the present study, the milk ejection activity of TGOT was estimated to be 976 ± 79 U/mg, which was similar to the value reported in rats with the assay of intramammary pressure measurement (≈ 802 U/mg) [13], and was slightly more potent than purified OT (600 U/mg) [11, 17]. This result seems to contradict the *in vitro* binding affinity of TGOT with mouse OTR, which has been reported to be approximately 20-fold higher than that of TGOT with rat OTR and that of OT with mouse OTR [3]. The reason of this discrepancy remains unclear. The milk ejection activity of WAY 267464 was estimated to be 6.87 ± 2.59 U/mg. Previous *in vitro* evaluations have shown that, compared to OT, WAY 267464 bound to mouse OTR with a 130- to 980-fold lower affinity and exerted its function with a 21- to 98-fold lower potency in molar concentration [9, 22]. In the present study, we demonstrated for the first time that the *in vivo* activity of WAY 267464 was 151-fold less potent than that of OT.

In conclusion, this report describes a novel *in vivo* technique for evaluating OT-induced milk ejection in anesthetized mice. This technique enables researchers to quantify the milk ejection activity of OT and should be valuable for investigations of the regulatory mechanisms of milk ejection. Moreover, this technique is also useful for the bioassay for OTR agonists.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Funding

This work was supported in part by The Morinaga Foundation for Health & Nutrition, JSPS KAKENHI (Grant Number JP17K15552), and Grants-in-Aid for

Regional R&D Proposal-Based Program from Northern Advancement Center for Science & Technology of Hokkaido Japan (Grant Number T-2-9).

Author Contribution

A.K. conceived the project, designed the experiments, and wrote the manuscript. A.K. and J.S. performed the experiments and analyzed the data.

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