Method for collecting mouse milk without exogenous oxytocin stimulation

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It has been reported that breast-feeding more than 6 months strongly decreases the risk of allergy, diabetes, obesity, and hypertension in humans. In order to understand the mechanisms responsible for this benefit, it is important to evaluate precisely the composition of maternal milk, especially in response to environmental cues. Mouse models offer a unique opportunity to study the impact of maternal milk composition on the development and health of offspring. Oxytocin injection of the dam is usually used to stimulate milk ejection; however, exogenous oxytocin might have deleterious effects under some experimental conditions by modifying milk content as well as the physiology and behavior of the dam. Taking advantage of the natural stimulation of the mammary gland that occurs after the reunion of a dam that has been separated from her pups, we developed a new procedure to collect mouse milk without the injection of oxytocin. This method is easy to use, low-cost, and non-invasive. Moreover, it provides a sufficient amount of milk for use in a wide range of biological analyses.

In mammals, maternal milk is an ideal source of nutrients. It has been widely reported in observational cohort studies that breast-feeding more than 6 months strongly decreases the risk of allergy, diabetes, obesity, and hypertension in humans (1,2). In order to understand the mechanisms responsible for this benefit, it is important to be able to evaluate precisely the composition of maternal milk and, more

specifically, its variation in response to environmental cues (3).

Mouse models offer a unique opportunity for the detailed study of the impact of maternal milk composition on the development and health of offspring (4,5), and interest in mouse milk for analyses of proteins, hormones, nucleic acids, and fatty acids or other lipids has been growing. However, the usefulness of milk collected

from mice is limited by the small volumes collected, which impact the reliability of subsequent analyses (6,7).

In order to overcome this difficulty, published studies have used a milking process involving injection of the dam with a massive dose of oxytocin (0.2–2 IU) in order to stimulate milk ejection (6–8). Oxytocin is a nonapeptide hormone that, in addition to stimulating milk ejection, controls physiological functions such as sexual receptivity, maternal behavior, pair bonding, and uterine contractions (9,10). In the context of milk secretion, its release in mothers is initiated by the sensory perception of visual, auditory, and olfactory stimuli from infants (11–13). Oxytocin is naturally present in milk, and variations in its levels in milk following injection into the mother may thus impact offspring development (14,15). Moreover, injected oxytocin could also affect more broadly the biological content of milk (e.g., endocrine hormone profiles, microRNAs, concentrations of various milk constituents) (16) and the gene expression profiles of cells present in milk (e.g., leukocytes) (17). For all of these reasons, oxytocin injection might produce deleterious side effects under some experimental conditions by modifying the physiology and behavior of the mother as well as the composition of her milk (18,19).

Here we present a new procedure for collecting mouse milk without injection of oxytocin that provides sufficient amounts of milk for a wide range of biological analyses. Our milk collection approach was adapted from a traditional and unusual milking technique used for milking Salers cows in Cantal (France) (20), which will not give milk without their calves close by. The critical step that differentiates our method compared with previously used methods is that milk ejection is induced by the pups themselves (6).

Before milk collection it is important to create an appropriate environment for nesting during the gestation and lactation periods. Soft wood-chips and cotton were added to the cages, since stressful conditions negatively affect the yield of milk (21,22). Three- to six-month-old female BALB/c mice were mated with males, and the day of detection of a vaginal plug was considered

METHOD SUMMARY

Dams and their pups are separated for 5 hours, after which physical contact is re-established in order to naturally stimulate milk ejection. Dams are then anesthetized, and gentle finger massage around the mammary glands is used to stimulate milk ejection in order to collect drops of milk beading from the teat using a Pasteur pipet.

Figure 1. Procedure for milking mice without oxytocin. (A) Pups are isolated from their dams for 5–6 h in a box located in the same cage. (B) Detailed view of the isolation box allowing the exchange of olfactory and sound stimuli between dams and pups. (C) Upon opening of the box after 5–6 h of separation, the dam will search for her pups. (D) The dam quickly starts to feed the pups. Dam and pups are left together for 3–5 min. (E) The dam is removed from the pups' box and then anesthetized using a nose cone connected to an isoflurane gas flow. (F) The pups are kept in the separation box and placed close to the dam. (G) Cooling and milk collection system. (H) Collected milk in a microcentrifuge tube.

as day 0.5 of gestation. At the time of the milking procedure (~10 days post-parturition) dams and pups were separated for 5 to 6 h (Figure 1A and Supplementary Video S1). Pups were isolated in metal-meshed boxes located inside their dam's cage in order to maintain visual, auditory, and olfactory stimuli between the pups and the dam (Figure 1B). The maintenance of these sensory stimuli during the separation period increases the accumulation of milk in the mammary glands. During separation, pups are maintained in a warm environment by placing their cotton nest inside the isolation box. Separations longer than 8 h are not recommended, since mammary gland engorgement and inflammation and constriction of the lactiferous duct might occur (23–25).

After the separation phase, physical contact is reestablished between dams and pups by opening the isolation box for 3 to 5 min. Dams immediately show maternal behaviors such as licking, grooming, and breast-feeding of the pups (Figure 1, C and D), and this must be checked carefully as it is an essential step. This dam–pup interaction stimulates a natural increase of oxytocin in the dams and leads to strong milk secretion (26). Dams were then anesthetized with isoflurane gas vaporized into a hermetic chamber (Tec7 Vaporizer; GE Healthcare, Little Chalfont, Buckinghamshire, UK) (Figure 1E), removed from the chamber, and placed on the bench close to the pups (Figure 1F). The anesthetized state is maintained by positioning a nose cone connected to an isoflurane gas flow. Gentle finger massage around the mammary glands located in the inguinal region is used to stimulate milk ejection and also to guide

the milk through the lactiferous ducts. Drops of milk beading from the teat are collected using a fine glass Pasteur pipette kept cold in an ice-filled holder (Figure 1G). It is recommended to proceed gently and slowly to prevent injury or trauma to the tissue and inflammation of the lactiferous duct, all of which would stop milk secretion. In addition, it is recommended that milking be completed within 15 min. Collected milk is then quickly transferred to a microtube (Figure 1H).

We successfully collected 50 μ L to 420 μ L of milk from a single lactating female. This volume is sufficient for hormone assays

and constituent analyses. The best results in terms of yield were obtained using dams at 10–11 days after parturition ($130.5 \pm 25.1 \mu$ L, $n = 20$; for comparison, the classical method allows collection of roughly 200 μ L of milk). However, we also succeeded in collecting milk at day 3 even though the yield was lower ($55.6 \pm 7.0 \mu$ L; $n = 8$). After day 14, pups begin to nibble solid food, and milk production in dams progressively decreases. This method can also be used with other mice strains such as C57BL6.

In order to assess the advantage of our method, we quantified oxytocin in both plasma from the mother's blood and milk obtained using our stimulation procedure (SP), compared with that obtained by the classic procedure (CP). As shown in Figure 2, the oxytocin level is strongly increased in both plasma and milk (2.8- and 1.8-fold, respectively) of mothers injected with 0.3 IU oxytocin (CEVA-SA, Libourne, France) to induce milk ejection compared with our method.

Here we describe a new method for collecting mouse milk without oxytocin injection that provides sufficient milk for biological analyses. The advantage of our approach lies in the lack of a massive oxytocin injection and the use of basic equipment found in any laboratory. Our method should be of great interest to researchers studying the functional components of milk in relation to both maternal behavior and offspring development (2,3,27).

Author contributions

Y.M., P.F., and C.J. designed the study. Y.M. and L.P. conducted the research. J.A., A.T.,

Figure 2. Oxytocin quantification in plasma and milk of lactating dams with or without oxytocin injection. Plasma and milk were collected from dams using either our stimulation procedure (SP) or the classic procedure (CP) involving intraperitoneal injection of 0.3 IU of oxytocin as described in Reference 15. Blood was collected by intra-cardiac puncture immediately after milk collection. The oxytocin concentrations of 1:20 diluted samples were determined using the oxytocin ELISA kit from Enzo Life Science according to the manufacturer's instructions.

A.C.M., C.C. F.M., V.C., and A.B. provided essential reagents. J.A., A.C.M., A.B., and C.C. edited the manuscript; Y.M. analyzed the data; Y.M., P.F., and C.J. wrote the paper; All authors have read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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Hamamatsu Corporation | 37
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Lonza | 4
Millipore Sigma | 6
MP Biomedicals | Cover 4
NanoString | 42
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PerkinElmer | 10
Prior Scientific | 41
The State Bar of Wisconsin | 30
Tib Molbiol GmbH | 19

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