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ARTICLE *in* INTERNATIONAL JOURNAL OF DAIRY SCIENCE · FEBRUARY 2008

DOI: 10.3923/ijds.2008.86.92

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## Changes in Milk Protein of Lactating Mothers Following Breast Massage

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**Abstract:** The effect of breast massage on mother milk protein according to the Oketani method was studied. Milk samples were obtained from 39 healthy breast feeding Japanese mothers during day 1 to 11 months. Samples were divided into three groups based on the lactation period. The obtained results showed that whey protein concentration was increased and casein was decreased by breast massage. Whey protein/casein ratio was increased in early lactation compared with late lactation. The SDS-PAGE revealed some new protein bands after massage with high and low molecular weight compared to before massage. This could be due to protease activity which was stimulated by the connective tissue massage.

**Key words:** Breast massage, oketani method, milk protein, casein, SDS-PAGE, lactating mothers, human milk

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### INTRODUCTION

Breast feeding is an important factor in the physical and developmental wellbeing of children and that it is very important for the mother. Breasts organs are changed by hormones and nerves, in the postpartum period, breasts can easily become abnormal because of the rapid hormonal changes following delivery (Kobayashi, 1989). Even if the mother wishes to breast-feed swollen hard breasts are painful for her and difficult for the baby to suck, to dissolve this problem breast massage should be used such as Oketani massage (Oketani, 1992). The Oketani method is a connective tissue massage, for which special training and technique are essential. This connective tissue massage encourages breast health by manual separation of adhesions between the breast base and the major fascia of the pectoral muscles with the aim of helping to restore and maintain natural breast contour and normal breast function. The breast base consists of connective tissue and is affected to various degrees by the conditions of the mother's life. The massage process of the Oketani method is said to increase the mammary depth and the elasticity of the base is improved (Kobayashi and Oketani, 1982). Proponents have claimed that performing Oketani breast massage before lactation increases the quantity of the excreted milk and stimulates the physical and mental development of infants, improves both their mood and sleep patterns (Kobayashi, 2000). A previous study by Foda *et al.* (2004) showed considerable differences in lipids, protein, lactose and the gross energy, which increased significantly by the Oketani breast massage. But, the physical and mental benefits for the infant cannot be explained solely in terms of quantitative changes in chemical components. It is likely that other functional materials such as opioid and other small peptides are enhanced through massage and then excreted to the breast milk. So, the aim of this research was to study the changes in milk protein of lactating mothers after Oketani breast massage during different lactation times.

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## MATERIALS AND METHODS

### Collection and Classification of Milk Samples

Thirty nine healthy, exclusively breast-feeding Japanese mothers participated in this study and were divided into three groups, based on the lactation time after delivery. Group 1, from 1 to 15 days ( $n = 5$ ). Group 2, from 21 days to 3 months ( $n = 16$ ) and Group 3 from 3 to 11 months ( $n = 18$ ). Breast massage of lactating mothers was performed following the Oketani method (Oketani, 1992). Milk samples were obtained freshly within 30 min before massage and immediately after massage. Usually, lactating mothers ate breakfast and then visited the maternity clinic. A midwife trained and licensed in the Oketani massage method performed the massage for 15 min. Each mother provided only one pair of samples before and after massage in this study and did not participate again as late lactation mothers. The samples before and after massage were collected by the mother herself. We did not make any intervention for the milk sample collection. Milk was expressed by hand until the entire breast was emptied, then collected in small plastic vials and stored at  $-20^{\circ}\text{C}$  for later analysis.

### Milk Analysis

All samples were thawed and stirred before analysis, then defatted by centrifugation at 4,000 g for 30 min at  $4^{\circ}\text{C}$ . In order to separate whey protein and casein, 10 mL milk sample were centrifuged using Ultracentrifuge, (CP 100 MX, Hitachi Koki CO., Ltd., Tokyo, Japan), at 105,000 g for 30 min at  $20^{\circ}\text{C}$ . Supernatant (whey protein) was collected and the sediment (casein) was dissolved in saline (5 mL), adjusted to pH 8.0 with sodium hydroxide (Lönnerdal and Forsum, 1985). Whey protein and casein concentrations were determined using the method of Bradford (1976) and bovine serum albumin as standard.

### SDS Polyacrylamide Gradient Gel Electrophoresis

To compare the individual human milk protein fraction before and after massage, the same volume of the protein and standard was applied for electrophoretic separation. Low Molecular Weight, Calibration Kit, Amersham, Pharmacia, Biotech, Sweden, (phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor,  $\alpha$ -lactalbumin), was used as standard for the determination of the MW of the denatured protein.

SDS-PAGE of milk protein samples was carried out as described by Laemmli, (1970), performed in 5-20% polyacrylamide gels containing 1% SDS (Ready gels J; BIO-RAD), using Mini-PROTEAN 3 Cell, BIO-RAD, California, USA. After fixing the proteins, the gels were stained with 2D-silver stain II kit (DAIICHI Pure Chemicals Co., Ltd., Tokyo, Japan), according to the method of Hitchcock and Brown (1983). Quantitation of protein bands on SDS gels was carried out by densitometry scanning with FUJIFILM LAS-1000 and FUJI-Image analyzer (Fujifilm Co., Ltd., Japan). The effect of breast massage on milk protein fractions was expressed as ratio of after/before, values of more than 1.0 being the increased band. All measurements were replicated three times for each sample.

### Data Analysis

Statistical analyses were carried out using the SPSS Base 10.0 (1999). A paired comparison t-test was used to compare the means of all variables before and after massage within each group in this study. Significant differences were considered at less than  $p < 0.05$ .

### Ethics

The study protocol and detailed description were reviewed and approved by the Ethics Committee of Siebold University of Nagasaki, Japan.

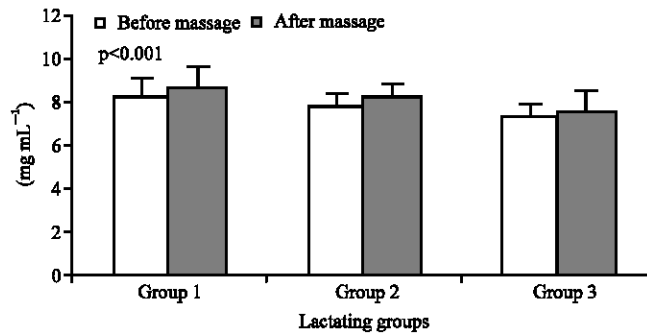


Fig. 1: Effect of Oketani breast massage on whey protein concentration for three groups of lactating mothers from day 1 to 11 months. Group 1 (1-15 days) n = 5, Group 2 (21 days to 3 months) n = 16, Group 3 (3-11 months) n = 18. Values are mean $\pm$ SD

## RESULTS AND DISCUSSION

### Whey Protein Concentration

It could be noticed that whey protein concentration before massage decreased as the time of lactation increased (Fig. 1). These results are in agreement with those obtained by Allen *et al.* (1991) who reported that the concentration of protein in mothers milk decreased by 6 months of lactation and Mitoulas *et al.* (2002) found same results, in addition to significant differences ( $p = 0.0001$ ) between mothers. Figure 1 shows the effect of breast massage in whey protein concentration which caused significant increase ( $p = 0.001$ ) in Group-1 (1-15 days) compared with before massage. Foda *et al.* (2004) reported that the whey protein concentration of pre-massage milk decreased significantly ( $p < 0.05$ ) from 0.8 to 0.73 g/100 mL between the early lactating period (before 3 months) and the late lactating period (after 3 months) and that of post-massage milk had similar decrease ( $p < 0.01$ ). Also, they found that breast massage caused a slight decrease (0.84 to 0.82 g/100 mL) in whey protein level in early lactation milk. These differences could be due to the individual variations between women.

### Casein Content

There is a marked increase in casein concentrations before and after massage during lactation (Fig. 2). In addition, massage showed a significant decrease in casein concentration in early lactation up to three months (groups 1 and 2).

These results could be reflected to the effect of breast massage on the progesterone level of the mother. Lönnerdal and Atkinson (1995) reported that casein synthesis is low or absent in early lactation, increases rapidly and then decreases; that is casein synthesis is expected to be high in very early lactation when progesterone levels have fallen. Additionally, Rosen *et al.* (1986) noted that the term casein includes a group of milk-specific proteins characterized by ester-bound phosphates, high proline content and a species-specific amino acid composition. Moreover, progesterone is known to inhibit its synthesis. Also, Rosen *et al.* (1986) reported that milk protein gene expression is regulated by hormones and therefore, the rapid changes in circulating hormones that accompany early lactation necessarily affect the concentration of different milk proteins. Thus, milk proteins that are synthesized by the mammary gland are expected to be more affected by the length of the period of lactation than proteins in milk that originate from serum and are passively transferred into the milk.

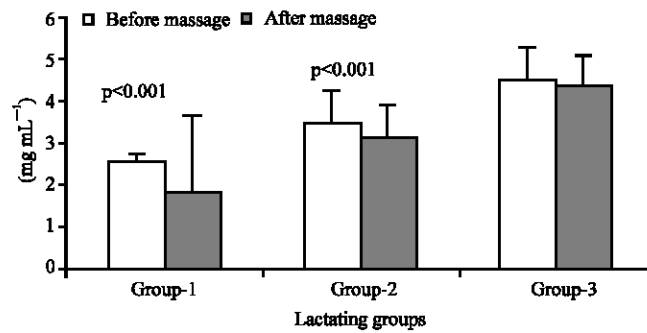


Fig. 2: Effect of Oketani breast massage on casein concentrations for three groups of lactating mothers from day 1 to 11 months. Group 1 (1-15 days) n = 5, Group 2 (21 days 3 months) n = 16, Group 3 (3-11 months) n = 18. Values are mean±SD

Table 1: Whey protein/casein ratio in human milk of lactating mothers before and after Oketani breast massage from day 1 to 11 months

Lactating period	Whey protein/Casein ratio	
	Before massage	After massage
Group 1 (1-15 days)	76/24±0.35	28/18±1.24 **
Group 2 (21-3 months)	70/31±0.61	72/28±0.75 **
Group 3 (3- 11 months)	61/39±0.16	63/38±0.03 **

Group 1 (n = 5) Group 2 (n = 16), Group 3 (n = 18), Values are mean±SD, \*\*: p<0.001 by student t-test

### Whey Protein/Casein Ratio

Table 1 shows that breast massage was found to change the ratio significantly ( $p < 0.001$ ) in all groups, early or late lactating period compared with before massage. Present observations on the patterns of concentration of casein and whey protein during lactation clearly showed that these proteins do not remain at fixed levels but vary during the lactation period. It should also be mentioned that there is considerable individual variation among mothers.

Kunz and Lönnerdal (1992) found the ratio of whey protein/casein was 90:10 in early lactation and 60:40 in mature milk. In a later study, Lönnerdal and Atkinson (1995) reported, generally high protein concentration of colostrum is largely due to the very high concentration of secretory IgA and lactoferrin, while, in contrast, casein is absent or present only very low concentrations during early lactation.

### SDS-PAGE

It could be noticed that milk protein fractions after massage (lane, 3 and 5) during lactating times 7 and 9 days had new bands with both high and low MW compared with before massage (Fig. 3). These results were confirmed in Fig. 4, which illustrated the effect of massage on milk protein bands as a ratio after/before in group 1 (1 to 15 days).

We could notice highly positive significant changes in some bands (unknown protein) with 200, 135, 57 and 41 kDa. In addition, some protein fraction bands insignificantly increased such as the band with 147 kDa (unknown protein), 79 k Da which was identified in the literature as lactoferrin, the band with 67 k Da identified as serum albumin and the band with 60 kDa as S IgA- heavy chain. On the other hand, Kunz and Lönnerdal (1992) reported that casein subunits range in molecular weights from 27,000 to 40,000. Also, Nommsen *et al.* (1991) and Lawrence and Lawrence (1999) found that casein subunits range in molecular weight between 24,000 and 40,000. Present results are in agreement with these findings. A slight decrease in protein band range at molecular weight 25 to 33 kDa was noticed; the polyacrylamide gel shows dense protein bands with molecular weights less than 33 000 (Fig. 3).

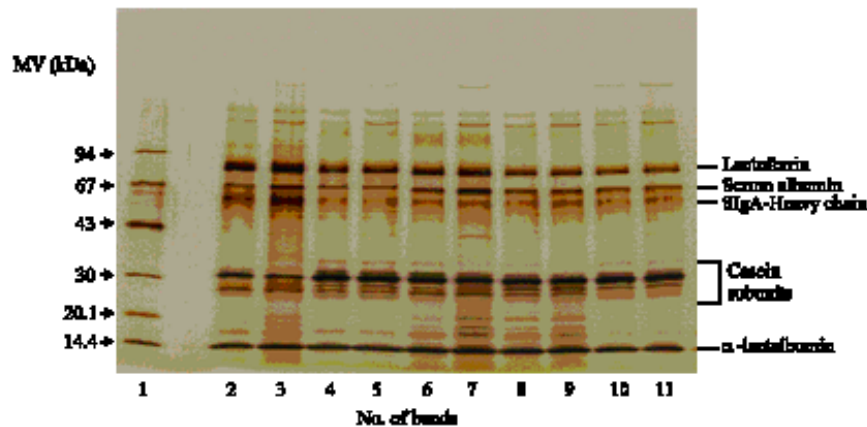


Fig. 3: Silver stained electrophoretogram (SDS-PAGE) of human milk protein samples during different lactating times before and after Oketani breast massage; Lane 1: Low molecular weight marker kit; Lane 2 and 3: (7 days) before and after massage; Lane 4 and 5: (9 days) before and after massage; Lane 6 and 7: (one month) before and after massage; Lane 8 and 9: (2 months) before and after massage and Lane 10 and 11: (3 months) before and after massage

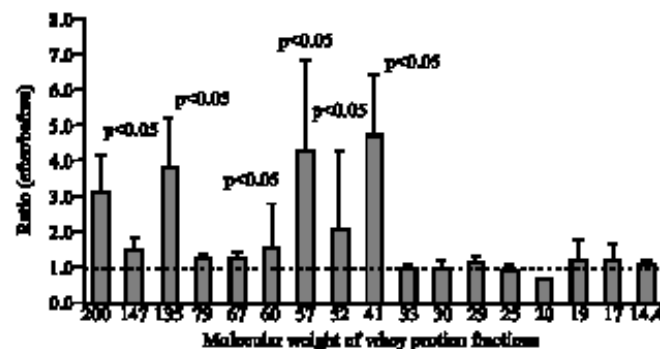


Fig. 4: Effect of Oketani breast massage, on milk protein in Group 1(1-15 days, n = 5), which was expressed as ratio after/before massage. Values more than 1.0 are the increased bands,  $p < 0.05$  means significantly changed by paired student t-test

Figure 5 shows the effect of Oketani breast massage on milk protein fraction in group 2 (21 days 3 months) in period of lactation, massage was effective on milk protein fractions and more significantly increased bands appeared at MW 19 and 20 kDa compared with group-1. SDS-PAGE (Fig. 3, 5) confirmed these results showing that lane (7) and (9), which represent milk protein fractions after massage during 1 and 2 months of lactation, respectively, had new bands with MW lower than 20.1 kDa compared with before massage.

Milk protein fractions were increased significantly with the same MW as group-2 (200, 135, 41 and 20 kDa), but with lower ratio (2.0). Casein subunits with MW 30, 29 and 25 kDa were almost similar to Group 2. Protein with low MW (20 kDa) was increased by massage in contrast with protein (19 kDa). In all groups the molecular weight of  $\alpha$ -lactalbumin (14.4 kDa) did not change before and after massage (Fig. 6).

These results are in agreement with those obtained by Robert *et al.* (1995) who reported that several bands were found between  $\alpha$ -lactalbumin and the casein subunits (molecular weights between 14,400 to 25,000). These may be cleavage products caused by the action of proteases. These bands

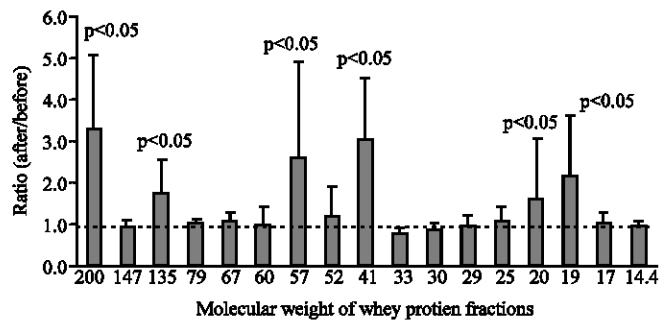


Fig. 5: Effect of Oketani breast massage on milk protein in Group 2 (21 days 3 months, n = 16) expressed as ratio after/before. Values more than 1.0 are the increased bands, p<0.05 means significantly changed by paired student t-test

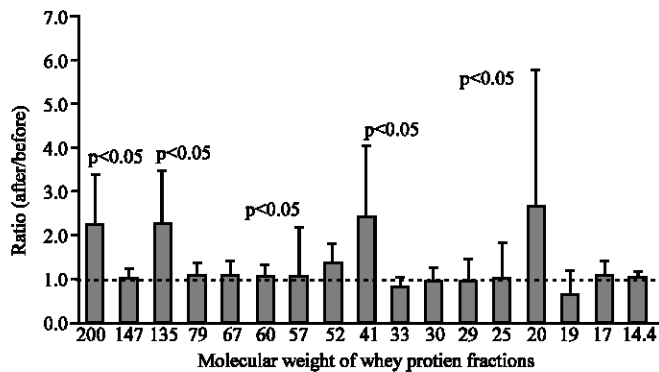


Fig. 6: Effect of Oketani breast massage on milk protein in Group 3 (from 3 to 11 months, n = 18), expressed as ratio after/before. Values more than 1.0 are the increased bands, p<0.05 means significantly changed by paired student t-test

did not change in Group 3 milk from a later stage of lactation. We suggest that this occurrence might depend on the immature assembly of casein subunits in the mammary gland because of phosphorylation and glycosylation, which are post translational events and may be immature in early lactation leading to lower molecular weights (Robert *et al.*, 1995). Another possibility would be the presence of high protease activity in early milk. The increase in casein content during lactation is pronounced and is likely to be caused by the maturation of the mammary gland; however, the exact mechanisms are not known. It is obvious that the synthesis of caseins and whey proteins is regulated by different mechanisms because most whey proteins decrease in concentration during lactation (Neville, 1995). Ohno (2001) found that the Oketani massage increased by 22% the average amount of small peptides (molecular weight, less than 10,000 kDa) in 11 of 15 lactating mothers. Nevertheless, it appears that a specified protease activity may be stimulated by the connective tissue massage and that the small peptide may thus be converted from large peptide or protein. Ohno (2001) also demonstrated that lipoyxygenase activity was significantly decreased by the Oketani massage method in 23 of 27 subjects. His results suggested this method may induce an antiallergic effect.

Although present study demonstrated the change of milk proteins after breast massage by the Oketani method, further study is needed to isolate the small peptides and identify the unknown proteins as well as evaluate their physiological effects, in addition to study the proteolytic activity in the milk.

## ACKNOWLEDGMENTS

The authors would like to thank Prof. Michiko Kobayashi, Nursing Department, Faculty of Nursing and Nutrition, for providing the mother milk samples and Mr. Takaaki Kawashima, Nutrition and Health Sciences Department, Siebold University of Nagasaki for his kind assistance.

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