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The change of bile salt stimulated lipase during 6 months and the correlation with macronutrients in Chinese human milk

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Abstract

Bile salt stimulated lipase (BSSL) is the most abundant lipase in human milk which plays a pivotal role in new-born fat digestion, especially in the first six months of life. Lipid as the main energy supply for infant was very depending on BSSL activity for obtaining optimal lipid absorption under immature digestion track and pancreatic system condition. Due to the important role of BSSL, this research was addressed to study the effect of gender and lactation stage on BSSL activity and the correlation of BSSL with macronutrients content in Chinese human milk. Cross sectional and longitudinal study were used to investigate the BSSL activity and concentration pattern during 6 months of lactation. A declining pattern of both BSSL activity and concentration was observed as lactation stage progressed. The BSSL concentration significantly declined from 138.56 to 97.07 µg/ml during 6 months. Significant differences of BSSL activity and concentration were also observed in human milk for different gender babies. Male babies had breastmilk with less BSSL rather than female babies. Protein had strong correlation with BSSL activity and concentration, while carbohydrate had non-significant negative coefficient correlation. However, no correlation observed between fat content and BSSL.

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Bile Salt Stimulated Lipase, Human Milk, Lactation Period, Gender, Macronutrients.

1. Introduction

Lipid, mainly triglyceride, is the main source of newborn energy since the first day of birth. The lipid utilization of infants is different from that of adults because of immature digestion track and system, especially in the pancreatic organ. Human milk, as the primary infant nutrition, has the complete nutritional composition needed by infants, including lipase for digesting lipids. Human milk bile salt stimulated lipase (BSSL), also called carboxyl ester lipase (CEL), is reported that it has a beneficial impact in breastfed for degrading lipids more efficiently rather than in formula-fed infants (1). BSSL is reported as a lipase that is available in human milk serum and secreted by the human mammary gland. This enzyme can lipolyze various sorts of substrates, including triacylglycerols, diacylglycerols, monoacylglycerols, cholesteryl esters, fat-soluble vitamin esters, phospholipids, galactolipids and ceramides (2). Previous studies showed that the absence of BSSL can lead to incomplete triglyceride utilization in human milk. Inoptimal absorption of triglycerides resulted in slower weight gain

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of new-born, which is undesirable for infants, particularly for preterm ones (3). BSSL in human milk is switching the low amount of pancreatic lipase in newborn, in the end, the lipid can be digested optimally as the main source of nutrition (1). Due to the relative lack of substrate specificity, this lipase has no clear positional selectivity when hydrolyzing triglycerides, which are good for infant nutritional absorption regarding the uncommon distribution of palmitic acid in the sn-2 position in human milk. In the end, the presence of BSSL can help in the optimal absorption of milk triglycerides which brought approximately 50% of energy substrates for the newborn. The BSSL in human milk synergizes with phospholipase-related protein 2 (PLRP 2), resulting in a larger amount of glycerol during digestion (4).

BSSL in human milk was mentioned to have a different function beyond optimizing lipid utilization. It has the ability to bind dendritic cell-specific intercellular adhesion molecules and inhibit the transfer of human immunodeficiency virus type 1 to CD4+T cells (5). However, BSSL was heat labile that can be denatured by heating, including pasteurization. This is the reason for the reduced fat absorption in pasteurized human milk-fed infants (6). Currently, some alternatives have been explored to improve the pasteurized human milk quality, especially in maintaining the activity of BSSL in human milk. One mentioned as a possible method to preserve BSSL activity was high-pressure processing at 550 MPa at 5 min (7).

The significant role of BSSL lead the researchers to create recombinant human milk bile salt stimulated lipase (rhBSSL) as the feeding strategy for the preterm infant (8,9). Though, the last study in preterm infants resulted insignificance improvement in growth velocity, but it significantly improves the growth of small gestational-age infants (10).

On the other hand, recent proteomic results revealed a noticeable amount of BSSL in human milk or human milk fractions (11). This milk enzyme can differentiate human milk from other milk sources such as cow, goat, and yak (12). And the concentration of BSSL was observed to decline significantly with the increase of baby age (13). The declining pattern of BSSL was priorly found by Lindquist to substitute the low pancreatic lipase in the newborn. By the maturity of pancreatic organ, the concentration of BSSL was reduced since the pancreas could work properly to secrete lipase (4).

To our knowledge, none of the research investigated the correlation of BSSL with macronutrients and gender. Due to the content variation either of macro- or micronutrients in each individual mother that can affect the differences of milk lipase content, it is possible to find correlation between BSSL and the macronutrient content of human milk. Meanwhile, due to the differences of growth patterns between genders, the utilization of lipids might be different between boys and girls because of the variation of BSSL in mothers' milk (14). Furthermore, to our knowledge, no data were available for BSSL activity and concentration of Chinese human milk. Hopefully, our results can be used as a guidance for the development of infant formula for Chinese infants in the aspect of lipid utilization and help in designing reasonable BSSL dosage used in either clinical trials or further the infant formula to optimize the function of BSSL in infant nutrition.

2. Materials and Methods

2.1. Sample Collection and Macronutrients Analysis

This research had been clinically registered in ClinicalTrials.gov with registration identifier NCT03675204. Sample collection was divided into two ways: cross-sectional and longitudinal study. For the cross-sectional study, 91 samples were obtained from the hospital and four mothers were tracked for longitudinal study. Cross-sectional study was used for

correlating the BSSL activity-macronutrient content, BSSL activity-gender, and the pattern during lactation periods, while the latter was used to observe BSSL content and activity pattern during six months period of lactation.

In cross-sectional study, 91 tubes of human milk (HM) from different mother from 1st until 6th month of lactations were obtained from different hospitals' human milk bank around Beijing, China. The obtained sample proportion for male and female babies in cross-sectional study was 42 and 49, respectively. Around fifteen samples were available in each period of lactation. The carbohydrate, protein, and fat contents of human milk collected samples were analyzed by using a human milk analyzer (MEIDII medical DMR-0822) and the rest of the volumes were stored in -80°C for further analysis.

Meanwhile, in longitudinal study, as many as 4 HM were tracked each month at the same date during 6 months of lactation. The HM was collected by breastmilk pump, then directly collected in the ice box for transportation to the lab and stored at -80°C. The tracked longitudinal human milk samples were used to observed the pattern of BSSL activity and concentration during lactation period.

2.2. BSSL Activity

BSSL activity was measured in triplicate using previously described method with minor modification (14). In brief, 370 µl 25 mM Tris HCl pH 9.0, 20 µl 0.25 mM 2-methoxyethanol, 5 µl 0.53 mM p-nitrophenyl myristate in dimethyl sulfoxide,5 µl 5 mM sodium cholate, were added to 10 µl diluted human milk (1:100). Then, the samples were incubated in room temperature for 15 minutes, and stopped with stop solution consisting of 200 µl n-heptane and 500 µl acetone. Centrifugation with 6000 g at 4°C for 2 minutes was conducted to separate the liquid phase from the stop solution. The 200 µl bottom layer was pipetted to microwell plates and read under 405 nm. Blanks were made with the same formulation as samples without incubation time.

2.3. BSSL Concentration

The concentration of BSSL was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Catalog No: DL-BSDL-Hu, DEVELOP, Shanghai, China). In brief, 100 µl of standard, blank and samples were added into wells and incubated for 2 hours at 37°C. The liquid was removed and 100 µl of primary antibody was added in each well, then incubated for 1h. The liquid was aspirated and washed with 300 µl of 1x washing solution. A secondary antibody was added and followed by 1 h incubation. Ninety microliters substrate solution was added for 15 minutes reaction and then 50 µl stop solution was added. The measurements of solution's wavelength were conducted at 450 nm immediately. The analysis was replicated twice.

2.4. Statistical Analysis

The statistical differences for lactation period and genders were analyzed using One-way ANOVA (IBM SPSS 23). Macronutrients and BSSL correlations were run in R software package version 2.13.2 (The R Foundation for Statistical Computing, Vienna, Austria) with a 5% significance level.

3. Results and Discussion

3.1. Chinese Human Milk Macronutrients Concentration

Overall, no significant change was observed in macronutrient content of Chinese human milk during lactation (Table 1). Fat content was increasing from the first to the fifth month and slightly decrease in the sixth month, but the change was not statistically significant. Protein content was also not significantly different, while it had decreasing pattern. A slight fluctuate pattern was observed in the carbohydrate content of Chinese human milk during 6 months of lactation, but not statistically different.

Table 1. Macronutrients content of Chinese human milk in each month of the lactation.

Macronutrients (Mean±SD)	1st	2nd	3rd	4th	5th	6th	Average	*P-value*
Fat (g/dl)	3.47±1.01	3.66±0.83	3.69±1.52	4.00±1.13	4.07±1.56	3.48±1.27	4.04±0.30	0.35
Protein (g/dl)	1.23±0.37	1.19±0.29	1.13±0.26	1.22±0.38	1.05±0.29	1.04±0.15	1.14±1.33	0.46
Carbohydrate (g/dl)	5.54±1.89	5.93±1.23	6.20±1.33	5.79±1.25	5.96±1.07	5.88±1.18	5.89±1.37	0.88

The concentration of human milk macronutrients was different in individuals tailoring the needs of babies. The average amount of fat in 100 ml of Chinese human milk was higher than that of Korean human milk which only accounted for 3.0 g, but the carbohydrate and protein content of Chinese human milk was lower (14). Compared to the earlier study in Inner Mongolian breast milk, the average concentration of fat, protein, and carbohydrate in the present study was not significantly different (15). On average, the content of carbohydrates in Chinese human milk was relatively low compared to other countries including the Philippines (7.31 g/dl), Gambia (7.74 g/dl), Australia (6.14 g/dl), Bangladesh (8.08 g/dl), Sweden (6.70 g/dl), USA (7.40 g/dl), and Japan (7.58 g/dl) (16–19). In contrast, the protein and lipid content of Chinese human milk were highest among the countries. The different results above can be possible due to the variation of many factors such as lactation period and dietary intake. Daily consumption influences the human milk profile in Chinese human milk (20). For instance, high dairy diet resulted in a higher concentration of fat in milk or a higher protein diet led to a higher protein concentration (21,22). Moreover, eating habits varied much from one to others in each country (23).

The finding of this present research was consistent with previous studies that showed fat and carbohydrate content did not change from the first until the 6th months of lactation. However, in their results, the concentration of protein increased by about 2 g/dl in the 6th month (24). In contrast, Elwakiel et al. observed the declining protein concentration in human milk serum during 6 months period of lactation (25). The variation of change patterns for macronutrients in different studies could be because of the dissimilar sampling approach and analytical technique.

3.2. Lactation Period Affects BSSL Activity and Concentration

Declining pattern was observed both in activity and concentration during 6 months of lactation. The activity and concentration of BSSL were significantly different among lactation months ($p<0.001$) (Figure 1 (a) and (b)). From the beginning, the activity of BSSL was about 15 U/ml and progressively decreased until the end of the time frame. Meanwhile, the concentration has almost similar depletion trend with the activity and significantly changed from the first to the last month of lactation.

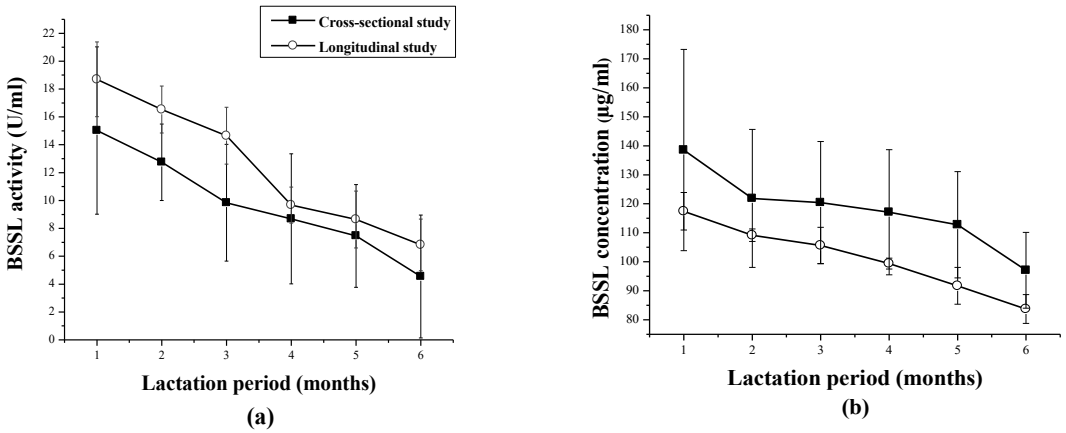

Figure 1. (a) Cross sectional and longitudinal study of BSSL activity and (b) BSSL concentration.

In the cross-sectional approach, the BSSL concentration declined during first 6 months of lactation period (Figure 1 (a) and (b)). The concentration of the first month was 138. 56 µg/ml then gradually decreased until 97.07 µg/ml in the sixth month. The similar decreased also happened in longitudinal study that begin with 117.71 µg/ml and end up in 83.71 µg/ml.

The similar decreasing pattern was noticed between cross sectional and longitudinal study (Figure 1 (a) and (b)). Both activity and concentration during lactation period declined. The graphs plotted by the BSSL activity of longitudinal study consisted of the mean values of all four mothers. This research revealed clear declining pattern of BSSL concentration and activity as the increase of lactation stage using large population in cross sectional approaches as well as using tracking samples in in longitudinal study.

Based on the data obtained in the BSSL concentration, it was comparable with the previous study in Western human milk that mentioned concentration of BSSL was about 100-200 µg/ml (26). Moreover, from the pattern of present result, the trend matched with the former study that mentioned the BSSL concentration ultimately decreased during the first 6 months (12). The declining pattern was also comparable to the recent proteomics result which reveals the significant decreasing of BSSL protein from first week lactation until the end of 6 months in Chinese and Dutch human milk (25). Regarding to its function for helping immature infant digestion, the activity was decreased by the maturation of pancreatic organ. Pancreatic bile salt-dependent lipase (BSDL), which is identical enzymes with BSSL of lactating mammary gland, consecutively replaced the role of human milk BSSL in fat utilization (12). By the increasing age towards the end of exclusive breastmilk period, bile salt secretion increases because of the maturation of liver function, then PLRP2, BSDL and BSSL became less important. At that time, children were ready to take their solid food (27).

Even the identical pattern was observed between random and longitudinal study, the standard deviation in random sample were quite high. It means the individual BSSL activity remained highly various. The variation of BSSL can be a sign that each infant can have the different efficiency of lipid digestion.

3.3. Gender Bias in BSSL Activity and Concentration

In Figure 2 (a) and (b), there was no significant difference observed in both macronutrients and energy content between male and female. Milk for female contained

slightly higher carbohydrate and protein in average, but milk for male had higher fat than that for female, resulting in the comparable energy calculation for both genders.

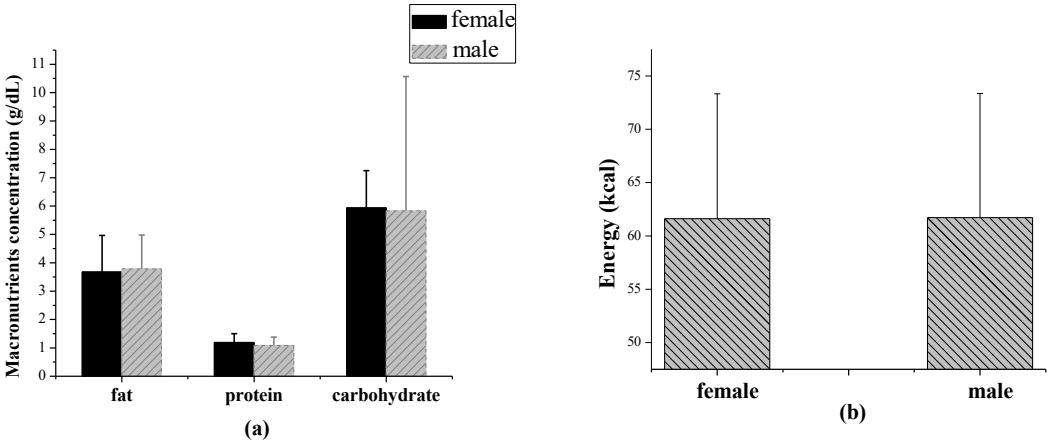

Figure 2. (a) Macronutrients and (b) energy of human milk in different gender.

Based on Figure 3(a), milk for boy and girl showed remarkable different activity of BSSL with p-value of 0.027. Breastmilk for female babies were containing higher activity of lipase enzyme by 2.26 U/ml rather than those for male. In Figure 3(b), the BSSL concentration in milk for female was also significantly higher than that for male (p=0.024). The BSSL concentration in human milk for girls was 10 µg/ml higher than boys.

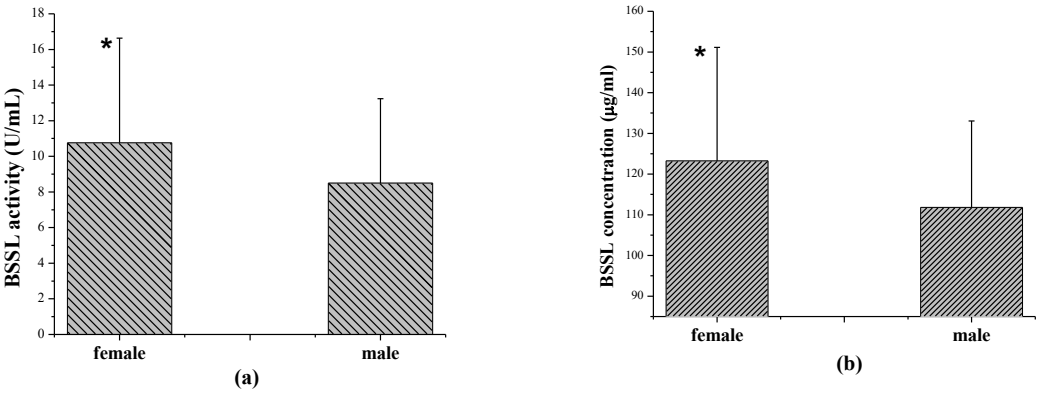

Figure 3. (a) BSSL activity and (b) concentration in different gender.

Gender bias in primates is becoming issue in human biology. Several researches were conducted observation in the milk of different primate species, such as kangaroos, macaques, and humans. Most of the research found that mothers produce richer milk either in protein or energy for male offspring (28,29). Meanwhile, the other study conducted in South Korea revealed higher carbohydrate and energy content in milk for female babies (30). It was supported by Fujita and co-workers that in Kenyan milk for low socioeconomic female babies tended in having higher fat than that for male babies (31).

No gender bias finding in this result was consistent with the prior result conducted in Filipino breast milk, no evidence for sex biases in milk macronutrients and energy output (16). However, the specific mechanism of newborn energy production especially from fat digestion was not described yet. The significant difference in greater activity of BSSL in human milk for

girls might be a strategy for efficient lipid utilization in energy provision. However, the effect of higher BSSL activity and concentration in milk for female babies needs to be further investigated.

3.4. Correlation of BSSL Activity with Protein and BSSL Concentration

Strong positive correlation of BSSL concentration and BSSL activity on Figure 4. Meanwhile, negative coefficient was figured out in BSSL activity and concentration with carbohydrates, but not significantly correlated. A fairly strong significant positive correlation was found between BSSL activity and protein content (p=0.0002). No significant correlation of fat with neither BSSL concentration nor activity.

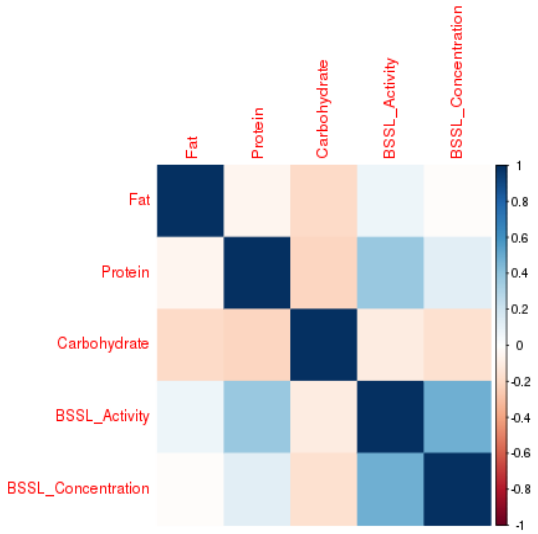

Figure 4. Correlation plot of macronutrients content with BSSL activity and concentration.

Correlation result supported the result of cross-sectional and longitudinal study in Figure 1 and Figure 2, that both of study showed dropping pattern either in activity or concentration of BSSL by the increase of lactation stage. A positive correlation of protein with BSSL concentration and activity indicated that the contents will be decreased by the increasing of time. Since BSSL contributes about 100-200 µg/ml in human milk (26), it can be possible this contribution also impacts in major protein concentration, supported by the positive correlation of BSSL concentration and protein. Other research related to human milk reveals the declining trend of total protein in human milk, particularly during 6 months or throughout one year (17,29). In present study, the decrease of protein content as the increase in lactation stage was also observed (Table 1), even it was not statistically significant.

Surprisingly, no significantly correlation was observed between BSSL activity and lipid content in human milk, even the function of BSSL itself was to digest the fat in human milk. This might indicate that there is other function of BSSL beyond its specific role to digest fat. It has been shown that BSSL also play roles in inhibition of pathogens and inflammation (5).

4. Conclusions

In case of Chinese human milk, beyond changing the composition of macronutrients, lactation period also affected the BSSL activity and concentration. In addition, gender also brought a significant impact in BSSL activity and concentration, human milk for female babies had higher BSSL activity and concentration than that of male babies. Protein had strong

positive correlation with BSSL activity, while carbohydrate had a negative correlation with both activity and concentration of BSSL. However, there was no correlation observed for fat with neither BSSL activity nor concentration. To our knowledge, this was the first study focused on the BSSL in Chinese human milk, which might help in better understand of the Chinese human milk and the variation of it from other countries. Meanwhile, the variation of BSSL activity and concentration observed in human milk for different genders might help in designing infant formula for boys and girls. Our observation also shed a light for better understanding of the BSSL function.

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Author Contributions

R.N.S.: methodology, formal analysis, investigation, data curation, writing original draft, visualization; J.L.: Conceptualization, methodology, validation, investigation, resources, review and editing, supervision, project administrator, funding acquisition; L.J.: supervision, project administrator, funding acquisition.

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Institutional Review Board Statement

The research had been clinically registered in ClinicalTrials.gov with registration identifier NCT03675204.

Data Availability Statement

Invalid.

Conflicts of Interest

Authors declare no conflict of interest in this research.

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