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Effect of different methods of pasteurization on bactericidal action of human milk: A prospective observational study

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***Background & objectives*: Pasteurization involves not only inactivation of pathogens, but also loss of immunological functions and bactericidal action of human milk. Hence, this study was aimed to explore the stability of such bactericidal action after subjecting human milk samples to thermal pasteurization under different condition of time and temperature.**

***Methods*: In this observational study 48 human milk samples were analyzed over a period of three months. The effect of holder and flash methods of pasteurization on bactericidal action against *Escherichia coli* was evaluated compared to the control sample before and after 72 h of storage at −18°C.**

***Results*: Both holder and flash methods of pasteurization showed significant reduction in the *E. coli* growth to 46.4 and 25.5 per cent, respectively, after 24 h of incubation ($P<0.001$). The bactericidal activity was significantly more in samples subjected to holder method compared to flash method before and after 72 h of storage (46.41±15.38 vs. 25.50±30.74, $P<0.001$ and 42.27±20.38 vs. 18.33±28.55, $P<0.001$).**

***Interpretation & conclusions*: Our results showed that the bactericidal activity of human milk was better preserved by the holder method of pasteurization. Further well-powered and well-designed randomized trials are needed to confirm the findings.**

Key words Bactericidal action - high temperature short time - human milk - low temperature long time - pasteurization

Human milk provides adequate nutrition, immune factors, growth factors, digestive enzymes, hormones and other bioactive factors for optimal growth and development of the infant[1-3]. Lactoferrin present in human milk has antimicrobial activity against a large number of bacteria, fungi and viruses[3]. Lysozyme inhibits the growth of Gram-positive bacterial species by disrupting the bacterial cell wall and also inhibits selected yeasts[2-4].

Banked donor human milk is the next best alternative after biological mother's breast milk[5]. Successful pasteurization of donor human milk involves not only inactivation of pathogens but also preservation of milk components. It is, therefore, essential that any new pasteurization system achieves the required inactivation of pathogenic microorganisms along with retaining the highest possible level of immune components[6]. Pasteurization

treatments at 62.5°C for 30 min (low temperature long time pasteurization/holder method) and at 72°C for 15 sec (high temperature short time pasteurization/ flash method) are the conventional methods used. Most human milk banks utilize holder method of pasteurization which is more convenient compared to flash method which requires high-tech equipment and high volume of milk[7].

Although pasteurization assures the microbiological safety of human milk, the mechanism of thermal inactivation of bacteria is detrimental to the bioactivity of the milk, since many proteins will denature when exposed to heat[8]. Studies have shown that human milk pasteurization alters the biochemical and immunological composition of human milk with significant decrease in lactoferrin, immunoglobulin and antibacterial components[9-11]. Holder pasteurization reduces to some extent the activity of important immunomodulating components of milk[11,12]. Hence, thermal treatment may not only impair the beneficial antibacterial properties of human milk but may also increase its susceptibility to subsequent bacterial contamination[13]. The effect of thermal treatment on bactericidal capacity against *Escherichia coli* was evaluated by Silvestre *et al*[14] and they found that the bactericidal capacity was better preserved by low temperature, long time pasteurization than high temperature and short time pasteurization.

This study was aimed to compare human milk's bactericidal capacity following two modalities of thermal pasteurization: (*i*) low temperature long time pasteurization/holder method (62.5°C for 30 min), and (*ii*) high temperature short time pasteurization/flash method (72°C for 15 sec), and also to find the effect of storage on bactericidal action of pasteurized human milk.

Material & Methods

This prospective observational study was conducted over a period of three months (April to August 2017) in the department of Neonatology, Seth GS Medical College & KEM Hospital, Mumbai, India.

The study included lactating mothers of term and preterm babies who were admitted in the NICU (neonatal intensive care unit) and completed seven days postpartum. This included 28 mothers of preterm and 20 of term infants who were clinically stable and expressing adequate milk. All infants enrolled in the study were clinically stable and on full enteral feeds. Mothers with evidence of mastitis and on antibiotic therapy were excluded.

Study design: Prior approval of the study protocol was obtained from the Institutional Ethics Committee and milk samples were collected after obtaining written informed consent. Mothers were taught hand expression of breast milk and 20 ml of milk sample was collected manually from one breast in one session. The time elapsed between collection to processing was less than three hours in all cases. Milk samples were stored in human milk bank.

Each human milk sample was divided into two aliquots of 10 ml each corresponding to each condition studied (Figure). To limit the bias due to the study methodology and to give permissible blinding, the milk samples were taken for two types of pasteurization from a common pooled raw milk sample. Pasteurization was done using Armada pasteurizer (Ashutosh Export International, New Delhi).

(*i*) Low temperature long time pasteurization/holder method: 10 ml of milk sample was pasteurized at 62.5°C for 30 min. Each sample thus treated was divided into two sub-aliquots (5 ml each). After thermal processing 5 ml sample was sent to microbiology laboratory and inoculated with *E. coli*, the remaining 5 ml sample was stored under −18°C for 72 h in human milk bank. After completion of storage for 72 h, milk sample was sent to microbiology laboratory and inoculated with *E. coli.*

(*ii*) High temperature short time pasteurization/flash method: 10 ml milk sample was pasteurized at 72°C for 15 sec. Each treated sample was divided into two sub aliquots (5 ml) each. After thermal processing 5 ml sample was sent to microbiology laboratory and inoculated with *E. coli*, remaining 5 ml sample was stored under −18°C for 72 h in human milk bank. After completion of storage for 72 h, milk sample

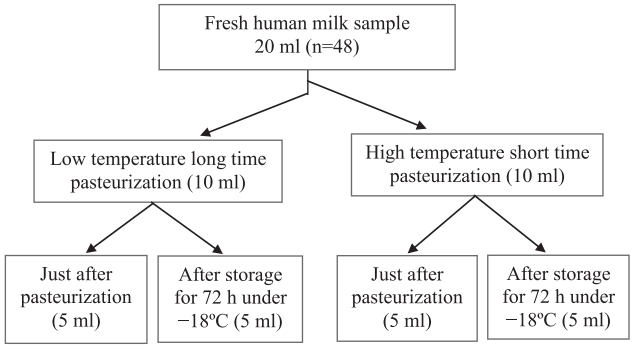

Fig. 1. Flow chart showing study methodology.

was sent to microbiology laboratory and inoculated with *E. coli.*

The degree of bacteriolysis was calculated in each milk sample by the following formula[14]:

$$\frac{\textit{E. coli} \text{ count in control sample} - \textit{E. coli} \text{ count in milk sample}}{\textit{E. coli} \text{ count in control sample}}$$

E. coli ATCC 25922 strain (Microbiologics Inc, USA) was used as the bacterial agent for the study. *E. coli* strain was grown in the blood culture medium overnight. The inoculum was counted manually in the log phase and adjusted to turbidity equivalent to McFarland 1 which corresponded to 3×10^8 cfu/ml. This was diluted to 1 in 100 and 0.2 ml of the inoculums thus obtained was added to 2 ml of milk sample and peptone broth (control). Further, 0.1 ml of this sample was inoculated on enriched medium and incubated at 37°C (3×10^6 cfu/ml). *E. coli* count was checked at 4, 8 and 24 h. Percentage reduction in *E. coli* growth as compared to control sample was noted in each milk sample[14].

Statistical analysis: The results were presented as percentages of control sample counts (% reduction in *E. coli* growth). Increased bactericidal activity in milk was associated with an increased percentage reduction in the growth of *E. coli*. The difference between values in term and preterm mothers after each sample treatment was assessed using Mann-Whitney U-test. Data were also analyzed by the least significant difference test for highly significant differences, using the Statgraphics Plus v5.1 statistical package (Statpoint Technologies, Inc., USA).

Results & Discussion

The milk samples were subjected to both methods of pasteurization and reduction in bacterial growth was evaluated. Low temperature and high temperature pasteurized milk showed a significant ($P<0.001$) reduction in the growth of *E. coli* (46.41 and 25.50%, respectively) after 24 h of incubation (Table I).

The percentage reduction in bacterial growth in the two different methods of pasteurization with two subgroups *i.e.*, inoculation immediately after pasteurization and after storage for 72 h at −18°C was analyzed (Table II). The bactericidal activity was more in samples subjected to holder method after incubation period for 24 h. The difference between the two methods showed significant difference ($P<0.001$). Thus, the samples exposed to flash method presented a greater decrease in the percentage reduction of *E. coli* growth after 24 h of incubation than the samples subjected to holder method, indicating a comparatively greater loss of bactericidal capacity. Confluent growth of *E. coli* was seen in 28.89 per cent samples subjected to holder method and 40 per cent samples subjected to flash method of pasteurization 12 h post-inoculation.

Table I. Percentage reduction in bacterial growth by different methods of pasteurization (n=48)

Per cent reduction in bacterial growth	Method of pasteurization	
	Low temperature long time (immediately after pasteurization)	High temperature short time (immediately after pasteurization)
After 4 h (mean±SD)	91.56±1.52	94.77±1.20
After 8 h (mean±SD)	90.97±1.43	94.43±1.04
After 24 h (mean±SD)	46.41±15.38***	25.50±30.74***

***$P<0.001$ compared to respective 4 and 8 h

Table II. Effect of storage on bactericidal action of milk processed by two different methods of pasteurization (n=48)

Reduction in bacterial growth	Method of pasteurization	
	Low temperature long time (holder method) (with 72 h storage)	High temperature short time (flash method) (with 72 h storage)
After 4 h (mean±SD)	91.41±1.40	95.88±12.72
After 8 h (mean±SD)	91.18±1.04	94.76±10.04
After 24 h (mean±SD)	42.27±20.38***	18.33±28.55

***$P<0.001$ compared to flash method

In our study all the milk samples demonstrated bactericidal action against *E. coli*, which was comparable to a few other studies[15,16]. The bactericidal action of breast milk was more preserved after storage of milk pasteurized by holder method.

Pasteurization reduces the activity of protective components present in breast milk such as lysozyme, lactoferrin and immunoglobulin, whereas components such as growth factors and interleukins remain stable[6,17]. Impact of pasteurization on bactericidal action of milk was addressed earlier by Van Gysel *et al*[13]. The study compared the bactericidal action of unpasteurized milk and pasteurized milk (by holder method) against *E. coli* and *Staphylococcus aureus*. There was significantly higher growth inhibition of both *E. coli* and *S. aureus* in unpasteurized milk compared to corresponding portion of pasteurized milk[13]. Silvestre *et al*[14] compared the effect of high and low temperature pasteurization on bactericidal action of human milk. Untreated milk, low and high temperature-pasteurized milk showed reduction in the growth of *E. coli* by 70.10, 52.27 and 36.39 per cent, respectively. The bactericidal action of the breast milk post pasteurization remained unchanged before and after storage. These results were comparable to our results.

In conclusion, human milk possesses antibacterial activity which is partially lost as a result of thermal treatment. Holder method of pasteurization preserves the bactericidal action of human milk better than flash method. A well-designed and powered randomized controlled trial which checks the effects of different methods of pasteurization on different bacterial strains is required.

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