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Storage of human milk and the influence of procedures on immunological components of human milk

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The storage of human milk for use later by the mother's own infant or an unrelated recipient has an impact on its constituents. These effects involve the storage container, heating, cooling and freezing the milk. Overall, glass is the least destructive container. Milk can be safely refrigerated for 72 h with little change. Freezing destroys cellular activity and reduces vitamins B_6 and C. Boiling, in addition, destroys lipase and reduces the effect of immunoglobulin A and secretory immunoglobulin A. The nutrient value of human milk is essentially unchanged, but the immunological properties are reduced by various storage techniques. □ *Donor milk, freezing, human milk, pasteurization, storage*

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The storage of human milk was investigated initially because of the need for storing milk by human milk banks who wished to provide the milk of donors. It has equal significance, however, for mothers who collect their own milk for later feeding. The possible influences on the stability of the properties of the milk include the effect of the container, the temperature of storage and the possibility of sterilization prior to storage. This paper will address specifically the impact on the immunological components of human milk, including the living cells (macrophages and lymphocytes), immunoglobulins, antimicrobial proteins, enzymes and fat globules.

The immunological constituents of human milk are numerous and were reviewed by Goldman (1). Host defenses were originally considered to be limited to leukocytes, macrophages, T cells and neutrophils and their products, but are now known to include proteins such as lactoferrin, lysozyme, fibronectin, secretory IgA, C_3, mucins, oligosaccharides and lipids. The anti-inflammatory factors in human milk include cytoprotectives, epithelial growth factors, maturational factors, enzymes and antioxidants. Immunomodulating agents in human milk include the cytokines interleukin-1β, (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (see Table 1).

What is the fate of these important components of human milk during collection and storage when the milk is collected for feeding later to the mother's own infant or an unrelated recipient? The potential for alteration of the constituents begins with the mode of collection and the nature of the collecting and storage vessel (2). The temperature for storage, i.e. room temperature, refrigeration or freezing, and pasteurization or sterilization have an impact. The process of thawing and warming the feeding entails possible microwaving or active heating. Finally, the process of administration by cup, bottle or tube can influence what the infant actually receives. The discussion addresses the issues by the process employed.

Collection

The infant is the most effective pump (2). Manual expression for some women is very successful, although few can achieve the fat content potential of the infant or a good electric pump. Drip milk collected while the infant suckles at the other breast or via a nesty cup worn over the breast inside the brassiere between feeds is notably contaminated and low in fat. Pumping with a small hand pump (the bicycle horn pump) that allows milk to wash back over the breast also causes excessive contamination (2).

The ideal artificial collection agent is an electric pump that cycles the negative pressure with a rhythmic action simulating suckling that will provide good fat content. The breast flange and tubing must at a minimum be dishwasher sterilized to keep bacterial contamination negligible.

The storage container can influence the cell content of the milk, as the cells adhere to the walls of a Pyrex glass container but not to polyethylene or polypropylene containers (3). Glass is rigid, tolerates heating and

Table 1. Antimicrobial agents and immunomodulating factors in human milk.

Agent	Primary function
Proteins	
Lactoferrin	Fe^{2+} chelation
Lysozyme	Degrades peptidoglycans
Fibronectin	Opsonins
Secretory IgA	Antigen binding
Mucin	Fragments are opsonins
C_3	Anti-rotavirus
Oligosaccharides	Receptor analogs
Lipids	Disrupt enveloped viruses
Cytokines	
IL-1β	Activates T cells
IL-6	Enhances IgA production
TNF-α	Enhances SC production
TGF-β	Enhances 150 type switching to IgAB cells

Adapted from Goldman (1).

freezing and can be used to feed the infant. Rigid polypropylene can also be used for pasteurization and freezing. Water-soluble constituents and immunoglobulin A (IgA) remain stable in both glass and polypropylene, which are easier to handle and do not leak. Glass has the potential for breakage, however. Table 2 illustrates the impact of the container. It should also be noted that the cell count after 24 h of storage is increased over the count at 4 h, presumably because the cells no longer are adherent to the glass. The cell function is considerably reduced the longer the storage time.

Colostrum was found to be remarkably stable in all of these parameters in all containers (4).

Temperature

The effect of temperature on the stability of human milk is different for different constituents.

Storage at 15 °C (cool), 25 °C (mild) and 38 °C (warm climates) has been studied by Hamosh et al. (5), who reported that the pH decreased by 2 units within 24 h at all temperatures in samples collected at early (1 mo) and late (5–6 mo) lactation. Proteolysis was minimal at 15 °C and 25 °C for 24 h and measurable at 38 °C only after 24 h. Lipolysis, in contrast, was rapid, beginning within the first hour and increasing to 8% in 24 h. Free fatty acid content rose to 440% at 1 h and to 710% of levels in freshly expressed milk at 24 h at 38 °C. This was confirmed by Lavine and Clark (6), who reported that free fatty acids in human milk increase over time when stored at 25 °C, with a greater proportion of fatty acids 18:1 and 18:2 being released as the rate of release of others decreases (6).

Digestive enzymes, lipase and amylase were stable for 24 h at all three temperatures (7, 8). Bacterial growth was minimal at 15 °C throughout the 24 h, was low at 25° for 4–8 h, but was considerable at 38 °C at 4 h. Storage at 15 °C for 24 h and at 25 °C for 4 h was considered safe by Hamosh et al. (7). Storage at 38 °C is not considered safe at all.

Refrigeration at 0–4 °C has been studied by an number of investigators who examined a wide range of constituents of the milk (9–14). The results can be summarized as follows (see Table 3).

Bacterial growth of lightly and heavily contaminated milk decreases over 24 h at 4 °C (9, 10). Refrigeration had a significant inhibitory effect on bacterial growth, unlike the effect after freezing. It is suggested (9) that storage at 0–4 °C for up to 8 d is acceptable when milk is carefully collected.

Cellular activity is greatly reduced at 0–4 °C for 48 h. Macrophages and neutrophils were decreased in number but lymphocytes were unchanged (11). Lactose, lipids and IgA remained stable at 0–4 °C (9).

The creamatocrit measurement of fat content decreases within hours at room temperature but is stable for 14 d at 4 °C (12).

Table 2. Effect of container type on milk constituents after 4 h and 24 h storage.

	Pyrex	Polypropylene	Polyethylene bags	Rigid
Colostrum	NC	NC	NC	NC
Volume	NC	NC	NC	NC
Mature milk				
Cells				
Numbers	↑	↑	NC	NC
Functions	↓	↓	↓	↓
Proteins				
Lactoferrin	(↓)	(↓)	NC	NC
Lysozyme	↓	NC	↓	↓
sIgA	NC	NC	NC	NC
Total IgA	NC	NC	NC	NC
Antibodies to *E. coli*	NC	NC	↓	↓
Water-soluble vitamin C	NC	↓		
Fat-soluble vitamins	NC	NC	NC	NC

NC: no change.
Adapted from Goldblum et al. (3) and Lawrence, p. 60 (2).

Table 3. Impact on immunological properties.

	Storage (°C) 0–4	−20	Heat-treated 56 °C (30 min)
IgA (9, 16)	NC	NC	Stable (at 62 °C ↓)
sIgA (16)	NC	NC	Stable (at 62 °C ↓)
Lactoferrin (16)	NC	NC	NC (at 62 °C ↓)
Lysozyme (16)	NC	NC	NC (at 62 °C ↓)
Fibronectin	NC		
Mucid			
C_3 complement (16)	NC	NC	NC
Bifiduum factor (oligosaccharide) (20)	NC	NC	Stable
Glycoproteins (20)			Stable
α-Tocopherol (15)	Stable	Stable	
Cell count (11)			
Number	NC	↓	↓↓
Function	NC	↓	↓↓
Bacterial growth (9, 10)	NC	NC	↓↓↓
Inhibition of *E. coli* (31)	Adherence to HEp-2 cells	NC	NC

Cytokinesis: no studies.

Nucleotides: appear to be stable as they have been used to fortify formulae without destruction.

Serum-stimulated and serum-independent lipolytic activity was significantly decreased in milk stored at 25 °C and at 4 °C. Accumulation of free fatty acids appears to be a balance between rates of triglyceride hydrolysis and fatty acid consumption at 4 °C and higher temperatures (13).

Levels of α- and γ-tocopherol are stable at room temperature and refrigeration for 72 h (14).

The effect of room-temperature storage is of significance for the mother who is saving her milk for her term infant day by day, when she and her baby are separated because of work or school. It appears that room temperature (25 °C) is safe and there is minimal loss of nutrients or protective properties for 8 h (5). Refrigeration at 0–4 °C is safe from the standpoint of bacteria and lipolysis for 48–72 h and even longer (2). When the milk is being stored for use by a sick or premature infant, fresh refrigerated milk stored for 72 h continues to keep most constituents intact and has minimal bacteria if it was collected clean. When the milk must be stored for longer periods, fresh freezing is utilized when the milk is to be used by the mother's infant. When it will be banked as donor milk, it must be pasteurized before being frozen.

Rancid or off-flavored milk is in part a result of short- to medium-chain fatty acids (C_4–C_{12}) produced predominantly by the action of bacterial lipases on milk triglycerides. The bacteria-producing lipases are able to grow at 7 °C (*Pseudomonas fluorcens* and *P. fragi*) (13).

Freezing

The effect of freezing has also been studied by many investigators. Freezing has been carried out at −20 °C, which is within the range of a home freezer, and at −70 °C, the range of a laboratory freezer (17). Home freezers with automatic defrost cause a freeze-thaw phenomenon and may cause formation of ice crystals that may damage some milk components, although the effect has not been studied. A summary of the documented effects is provided in Table 4, taken from reports in the literature (17–21). Essentially, freezing inflicts no change not precipitated by previous contamination, the container or exposure to light energy, except for possible lipolysis, demulsification and protein denaturation when thawed (19). Freezing breaks the emulsion between milk fat globules and the aqueous fraction or the lipid may adhere to the container and is not recovered (20). Slow freezing is simpler than quick freezing and affords essentially the same protection of constituents (18).

Milk can be safely frozen for 12 mo at −20 °C or indefinitely at −70 °C with changes only in the cell count and activity and some alteration in the fat globule (20).

Heat treatment

Heat treatment or pasteurization has the greatest potential for altering composition and has been studied extensively for use by human milk banks since they are now required to process donor samples. It is well known that pasteurization reduces the bacteria count. The other effects are outlined in Table 5 (21–25).

The process accepted by the Human Milk Banking Association of North America (HMBANA) with consultation from the Federal Drug Administration (FDA) is as follows: after proper preparation in a tightly closed glass container, the container is submerged in a well-agitated or shaking water bath preheated to a minimum of 56 °C and maintained for 30 min (21).

Evaluation of premature infants fed raw, pasteurized or "boiled" human milk for 3 consecutive weeks revealed a reduction in fat absorption and amount of nitrogen retained when the milk was heated. Mean weight gain was one-third greater when fed raw milk (22). There was no change in the retention of Ca, P or Na.

Heat treatment poses some threat to immunological protection, with a reduction in cells and in secretory IgA (sIgA), and some reduction in enzymic activity, according to some investigators (23–25). Other mechanical stresses of stored and artificially fed human milk involve the effect of photoenergy through exposure to light and the effect of settling of fat in a feeding tube or syringe en route to the premature infant (23, 26, 27).

Photodegradation has been noted to reduce the amount of vitamin C (44%) and B_6 (19%) (23, 26). Vitamins A, D and E are not affected. Exposure to

Table 4. Freezing of human milk: impact on constituents

	Temperature (°C)	
	−20	−70
Mature milk		
Volume	Stable	
pH	3% ↓	Stable
Total energy (14)	Stable	
Bacterial growth (9)	NC	NC
Cells (11)		
Number	↓	↓
Function	↓	↓
Lactose (9)	NC	
Lipid (9, 14)	NC: ↓ in contaminated milk	Stable: long-chain polyunsaturates
Creamatocrit	NC: freezing & thawing ×2: ↓	
IgA (9)	NC: ↓ in contaminated milk	
sIgA		
Proteins (34)	±denaturalization on thawing	
Water-soluble vitamins (biotin, niacin, folic acid) (18)	NC	
α- and γ-tocopherols (15)	NC	NC
Enzymes (8, 18)		
Lipase	Stable	Stable
Amylase	Stable	Stable
Lactoperoxidase	↓	
Serum-stimulated lipolytic activity (12)	NC	NC
Serum-independent lipolytic activity (6)	NC	NC
Lipolysis (18, 19)	Possible over long period	Possible

Table 5. Heat processing of human milk: impact on constituents

	Pasteurization (56 °C; 30 min)	Boiling
Volume (21)	(↓)	↓
pH		
Total energy (20)	(↓)	↓
Bacterial growth (21)	0	0
Cells		
Number	↓	0
Function	0	0
Lactose (16)	Stable	
Lipid (22)	Stable	↓
Creamatocrit (22)	Stable	↓
IgA (16)	±	↓
sIgA (16)	±	↓
Proteins (22)	Stable	↓
Water-soluble vitamins (thiamin) (23)	B_6 folacin C ↓	↓
Fat-soluble vitamins (A, D, E, heat resistant) (23)	Stable	(↓)
Enzymes (20)		
Lipase (BSSL) (24)	↓↓	↓↓↓↓
Amylase (24)	↓	↓↓
Lactoperoxidase (24)	↓	↓↓
Serum-stimulated lipolytic activity (20)	↓	↓↓
Serum-independent lipolytic activity (20)	↓	↓↓
Lipolysis (8% in 24 h at 15 °C) (20)	↓	↓↓

phototherapy further increased the loss of vitamin C to 53% (26).

An additional hazard of tube feeding is the loss of fat, which clings to the walls of the tube as the slow-moving flow of milk settles in the feeding system (27). Loss of protein has also been identified (28). This loss is greater when the feeding drips in by gravity compared with bolus feedings using a Holter pump. Ultrasonic homogenization has been evaluated by Martinez and coworkers (28, 29, 30), who found that it reduced the loss of fat by decreasing creaming and reducing the size of the fat globule. Because milk contains not only calories and macronutrients but also a great number of bioactive components (growth factors, hormone, immunoglobulins, enzymes, etc.), Hamosh (31) has expressed concern that this highly structured fluid will lose some unique immunological properties when the fat and aqueous fraction are no longer separate. The ability to inhibit the adherence of *Escherichia coli* to HEp-2 cells was unaffected by pasteurization, lyophilization and microwave radiation, however (32).

Microwave radiation, which is utilized by many but is not recommended for the warming of infant feedings, is a possible source of constituent loss (33). IgA is decreased by 98% and lysozyme by 96%. As a result, the level of *E. coli* is greatly increased, especially at higher microwave temperatures. Microwaving is also known to reduce vitamin C. Microwave radiation of human milk reduces the immunological properties of human milk (34).

Summary

The immunoprotective constituents of human milk are stable when stored at room temperature for 8 h, refrigerated at 0–4 °C for 3 d or frozen at −20 °C for 12 mo (35). They are also stable after pasteurization at 56 °C for 30 min (36). Sonification may well reduce the protective effects on IgA, sIgA and lysozyme and the ability to inhibit *E. coli*. The cells in human milk are reduced by storage, freezing, pasteurization, microwaving and sonification (6, 11). Cell activity is also reduced in the surviving cells.

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