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Effect of antioxidant activity of Pulsed electric field processed donor mother's milk

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Abstract
Human milk bank facilitates the collection of mother's milk from the donor mothers, pasteurizes and distributes to the needy neonates. In human milk banks, donor mother's milk is holder pasteurized at 62.5 ℃ for 30 minutes. Heat sensitive vitamins, antioxidants, bioactive components and nutritional values significantly deteriorate during pasteurization. Hence the present study was envisaged to process the donor mother's milk using Pulsed electric field (PEF) technology - A novel non-thermal preservation method. The donor mother's milk samples were collected from donor mothers in and around Koduveli villages and human milk bank attached to Institute of child health and Hospital for children, Chennai. The donor mother's milk samples were subjected to different voltage gradients of 25kV/cm and 30kV/cm by varying the treatment time 3000 to 4500µs and number of pulses from 1200 to 1800 pulses. Total antioxidant activity and ascorbic acid content were analyzed immediately after PEF processing. The PEF processed mother's milk samples were compared with holder pasteurized and raw mother's milk samples. With the data obtained one way analysis of variance (ANOVA) was carried out and it was observed that there was highly significant difference (p<0.01) in total antioxidant activity and ascorbic acid content immediately after PEF processing when compared with holder pasteurization. About 95% of ascorbic acid was retained in PEF processed samples, whereas 43% of ascorbic acid was retained in holder pasteurized mother's milk. The raw, holder pasteurized and PEF processed milk samples were stored at 5 ℃ to conduct storage studies. Storage studies revealed that highly significant difference (p<0.01) was observed in both ascorbic acid content and total antioxidant activity during the storage period.

Keywords: Antioxidant, Pulsed, electric, donor, mother's milk

1. Introduction
Mother's milk contains biologically active substances such as antioxidants and antibodies in addition to macronutrients (proteins, lipids, and carbohydrates). Breast milk is the source of lipophilic antioxidants such as tocopherols, retinol and carotenoids and hydrophilic antioxidants such as ascorbate, polyphenolics, low molecular weight Thiols, casein and whey proteins (Lugonja *et al*., 2021) [1]. Antioxidants are excellent in preventing lipid peroxidation which protects the neonates against cellular oxidative stress. Antioxidants are necessary for the absorption of calcium which helps in development and bone mineralization of neonates (Ramirez *et al*., 2021) [2].

Donor human milk is a secondary option for women who are unable to feed their infants. Human Milk Bank collects, processes, preserves and distributes donor milk to infants. In human milk bank, donor mother's milk samples are holder pasteurized at 62.5 ℃ for 30 minutes. Holder pasteurization is efficient in inactivating the pathogenic microorganism and also reduces the inherent antioxidant activity of human milk. Reduction in ascorbic acid and vitamin E was observed in holder pasteurized mother's milk samples when compared to unpasteurized mother's milk samples (Nadal *et al*., 2008) [3].

Novel processing technologies such as Pulsed electric field has recently gained popularity in the food industry for better retention of nutritional and sensorial properties of milk. In pulsed electric field treatment, food is sandwiched between a series of parallel plate electrodes and short duration of electric pulses was applied with the voltage gradient in the range of microseconds. No heat is generated when food is exposed to high voltage gradient. Microbial organisms are inactivated by the electric field produced by the high voltage (Sujatha *et al*., 2021) [10]. Since pulsed electric field is a non-thermal processing method, the food can be preserved with minimal or no change in nutrient content of food.

The aim of this study was to process the donor mother's milk samples using pulsed electric field technology by varying the voltage gradient, number of pulses and treatment time and to analyze the total antioxidant activity and ascorbic acid content immediately after PEF processing and in holder pasteurization of mother's milk samples.

2. Materials and Methods

2.1 Collection of donor mother's milk

The human milk bank connected to the Institute of Child Health and Hospital for Children, as well as the donor mothers in and around the village of Koduveli were the sources of the mother's milk samples. Donor's mother's milk was collected in sterile containers and kept chilled.

2.2 PEF processing of mother's milk

The main components of pulsed electric field processing system are the high voltage pulse generator and PEF treatment chamber. Coaxial PEF treatment chambers are currently used extensively due to its structural construction. Coaxial chamber with capacity of 200mL was utilized for processing donor mother's milk. The hollow cylinders made of SS304 stainless steel act as electrodes and base of the chamber was made up of acrylic material. Two hollow cylinders were placed concentrically over the base. The distance between the two electrodes was fixed to be 10mm. The PEF system's pulse generator, which comprises of a pulse forming network (PFN) produces square pulses with a pulse width of 2.5µs. The voltage gradients of 25kV/cm and 30kV/cm were applied by varying the number of pulses and treatment time to the donor mother's milk samples. As shown in the table below, six different treatments were selected with different voltage gradient, pulse count and time duration to process collected mother's milk samples.

Treatment parameters	Treatments
25 kV, 1200 pulses 3000 µs	T_1
25 kV, 1500 pulses 3750 µs	T_2
25 kV 1800 pulses 4500 µs	T_3
30 kV, 1200 pulses 3000 µs	T_4
30 kV, 1500 pulses 3750 µs	T_5
30 kV, 1800 pulses 4500 µs	T_6

After PEF processing the mother's milk samples were collected in sterile condition in sterile containers and total antioxidant activity and ascorbic acid content were analysed immediately after PEF processing. The PEF processed milk samples were compared with holder pasteurized and raw mother's milk samples. Storage studies were conducted by storing the PEF processed samples at 5 ℃.

2.3 Determination of total antioxidant activity: Antioxidant activity of PEF processed mother's milk was determined by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method according to Nogueira *et al*., 2020 [4]. 0.9mL of each HM sample was added with 1 mL DPPH in ethanol solution (0.06mM). After homogenization, the mixture was allowed to stand for 30 minutes in a water bath at 37 °C. Absorbance of the resulting solution was measured at 517nm by a UV–Visible spectrophotometer.

2.4 Determination of ascorbic acid content

The amount of ascorbic acid in the processed mother's milk was assessed using the indophenol method. Ascorbic acid, a potent reducing agent, transforms the dye 2, 6-dichlorophenol indophenols, A blue compound that turns red in acid solution, into a colorless leucobase. To achieve a light pink colour, 0.4 mg/ml of 2, 6-dichlorophenol indophenol dye was titrated against 10mL of sample using 25mL of 0.5 percent oxalic acid and 10mL of distilled water.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS and the descriptive data were reported as mean and standard error.

3. Result and Discussion

3.1 Effect of antioxidant activity of PEF processed mother's milk during storage

Total antioxidant activity of the PEF processed mother's milk samples is shown in table 1. The antioxidant activity of mother's milk is considered as importance mechanism for the prevention of lipid peroxidation and the scavenging capacity of free radicals. From Table 1 it was inferred that there was highly significant difference (p<0.01) in total antioxidant activity of PEF processed mother's milk when compared with holder pasteurized mother's milk as well as between treatments during the storage period. This result coincides with the findings of Parreiras *et al*., 2020 [4] who observed the significant reduction in antioxidant activity immediately after holder pasteurization (62.5 ℃ for 30mins). About 30.4% of antioxidant activity was decreased immediately after holder pasteurization (Nogueira *et al*., 2020) [4]. A previous study stated that there was no significant difference (p>0.05) in antioxidant activity immediately after pasteurization (Lorenconi *et al*., 2020). Highly significant (p<0.01) difference was observed in antioxidant activity during the storage period. This result is consistent with the findings of Paduraru *et al*., 2018 who observed reduction in total antioxidant activity when raw mother's milk samples stored at refrigerated temperature for 24 hrs. Prolonged storage up to 72hrs led to highest reduction of antioxidant activity. Both refrigerated storage and freezing reduced the antioxidant activity of mother's milk (Hanna *et al*., 2004) [7].

Table 1: Total antioxidant activity of the PEF processed mother's milk during storage at 5℃ (Mean±SE) @

Treatment	0th Day	3rd Day	6th Day	9th Day	12th Day	15th Day	18th Day	21st Day	F value
T_1	58.67 ± 0.84^{bC}	57.17 ± 0.48^{bB}	54.50 ± 0.43^{bA}	-	-	-	-	-	26.82**
T_2	58.83 ± 0.54^{bcC}	57.00 ± 0.40^{bBC}	54.83 ± 0.43^{bAB}	53.17 ± 0.95^{bA}	-	-	-	-	25.82**
T_3	58.50 ± 0.76^{bC}	57.67 ± 0.71^{bB}	54.17 ± 0.60^{bB}	54.17 ± 0.54^{bA}	50.50 ± 0.34^{aA}	-	-	-	20.54**
T_4	57.83 ± 0.54^{bD}	56.50 ± 0.85^{bCD}	54.83 ± 0.70^{bC}	52.67 ± 0.76^{bB}	51.67 ± 0.56^{bAB}	47.50 ± 0.89^{aA}	-	-	26.85**
T_5	59.33 ± 0.49^{bE}	56.83 ± 0.87^{bDE}	55.00 ± 0.86^{bCD}	53.83 ± 0.79^{bBC}	52.92 ± 0.82^{cB}	47.50 ± 0.96^{aB}	45.50 ± 0.67^{aA}	-	32.14**
T_6	59.50 ± 0.76^{bE}	58.50 ± 0.92^{bE}	56.00 ± 0.73^{cD}	55.00 ± 0.63^{cCD}	52.98 ± 0.93^{cC}	50.20 ± 0.45^{bA}	49.67 ± 0.92^{bB}	41.83 ± 0.48^{A}	47.92**

Hop	47.17±0.31^{aD}	44.83±0.40^{aC}	42.50±0.43^{aB}	39.83±0.25^{aA}	-	-	-	-	19.76**
RAW	61.00±0.52^{bB}	57.50±0.67^{bA}	-	-	-	-	-	-	17.09**
F Value	37.88**	31.64**	41.95**	39.39**	5.85*	5.18*	6.98*		

@ Average of six trials (Different superscript in a same row and column differs significantly)
NS – Non Significant (p>0.05)
** Highly significant (p<0.01)
* Significant (p<0.05)
T_1 - 25 kV, 1200 pulses 3000 µs
T_2 - 25 kV, 1500 pulses 3750 µs
T_3 - 25 kV 1800 pulses 4500 µs
T_4 - 30 kV, 1200 pulses 3000 µs
T_5 - 30 kV, 1500 pulses 3750 µs
T_6 - 30 kV, 1800 pulses 4500 µs
HOP – Holder pasteurized mother's milk
Raw – Raw mother's milk
"-" indicates loss of stability

3.2 Effect of ascorbic acid of PEF processed mother's milk during storage

Ascorbic acid content of PEF processed mother's milk samples is shown in table 2. It was observed that there was highly significant difference (p<0.01) in ascorbic acid content of PEF processed mother's milk when compared to holder pasteurized mother's milk sample. About 43% of ascorbic acid content was retained in holder pasteurized mother's milk samples. This is because of the fact that ascorbic acid is heat-sensitive and thermal pasteurisation has caused ascorbic acid to change into dehydroascorbic acid which had resulted in a drop in ascorbic acid levels. This result is coincides with the findings of Abramovich *et al*., 2013 [8] who observed a reduction in vitamin C after holder pasteurization. No significant difference (p>0.05) was observed in ascorbic acid content of PEF processed mother's milk when compared with raw mother's milk samples. This findings correlates with Puigmarti *et al*., 2011 who observed no changes in vitamin C and ascorbic acid contents of milk after high pressure processing with respect to untreated samples as there was no rise in temperature during non-thermal processing.

Highly significant difference (p<0.01) in ascorbic acid content was observed during the storage period. Buss *et al*., 2001 observed a decreased tend in vitamin C content of raw mother's milk stored at refrigerated temperature. About 63% of vitamin C was reduced when raw mother's milk was stored at 4 °C for 96hrs (Nadal *et al*., 2008) [3].

Table 2: Ascorbic acid (mg/100mL) of PEF processed mother's milk during storage at 5°C (Mean±SE) @

Treatment	0th Day	3rd Day	6th Day	9th Day	12th Day	15th Day	18th Day	21st Day	F value
T_1	4.44±0.25^{bB}	4.00±0.25^{cB}	3.20±0.25^{bA}	-	-	-	-	-	5.83**
T_2	4.40±0.27^{bB}	4.00±0.25^{cB}	3.60±0.27^{bcA}	3.00±0.27^{bA}	-	-	-	-	5.86**
T_3	4.40±0.03^{bB}	4.10±0.20^{cB}	3.80±0.27^{cA}	3.00±0.27^{bA}	2.40±0.05^{aA}	-	-	-	8.63**
T_4	4.44±0.25^{bC}	4.00±0.25^{cC}	3.80±0.25^{cB}	3.50±0.25^{bB}	2.40±0.30^{aA}	1.60±0.25^{aA}	-	-	18.00**
T_5	4.45±0.25^{bC}	4.20±0.27^{cC}	3.80±0.20^{cD}	3.60±0.25^{bC}	3.10±0.05^{bC}	2.40±0.20^{bC}	1.60±0.25^{aB}		17.50**
T_6	4.45±0.25^{bE}	4.40±0.25^{cE}	4.20±0.27^{dC}	4.00±0.27^{cB}	3.20±0.30^{bB}	2.80±0.27^{bA}	2.40±0.27^{bA}	2.04±0.05^{A}	46.90**
HOP	2.05±0.25^{aC}	2.00±0.25^{aC}	1.12±0.05^{aB}	1.04±0.05^{aA}	-	-	-	-	8.51**
RAW	4.64±0.20^{bC}	3.80±0.40^{bA}	-	-	-	-	-	-	6.20**
F Value	13.35**	9.07**	18.22**	10.36**	9.71**	5.82*	4.99*		

@ Average of six trials (Different superscript in a same row and column differs significantly)
NS – Non Significant (p>0.05)
** Highly significant (p<0.01)
* Significant (p<0.05)
T_1 - 25 kV, 1200 pulses 3000 µs
T_2 - 25 kV, 1500 pulses 3750 µs
T_3 - 25 kV 1800 pulses 4500 µs
T_4 - 30 kV, 1200 pulses 3000 µs
T_5 - 30 kV, 1500 pulses 3750 µs
T_6 - 30 kV, 1800 pulses 4500 µs
HOP – Holder pasteurized mother's milk
Raw – Raw mother's milk
"-" indicates loss of stability

4. Conclusion

Breastfeeding and mother's milk is a crucial defense mechanism against oxidative stress, free radicals and oxygen reactive species. The donor mother's milk collected from donor mother's subjected to pulsed electric field treatment showed highest retention of total antioxidant activity and ascorbic acid content in PEF processed mother's milk when compared to holder pasteurized milk. Highly significant difference (p<0.01) was observed in both ascorbic acid and total antioxidant activity during the storage studies. Since pulsed field technology is a non-thermal preservation method it retained the quality of mother's milk without affecting the antioxidants and vitamins present in PEF processed mother's milk samples.

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