

Comparative analysis of the effect of ultra-heat and ultraviolet treatment on milk exosomes and their miRNAs

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Abstract

Exosome-encapsulated miRNAs have been identified as the bioactive ingredient in milk, which provides immunological and nutritional advantages. Milk is processed through various treatments to ensure safe consumption, including pasteurisation, ultraviolet (UV) treatment, and ultra-heat treatment (UHT). Trends in the consumption of such processed milk are increasing across the world. In the present study, the effect of UV radiation as well as UHT processing on exosomes and their encapsulated miRNAs was investigated. The presence and size of exosomes in the UV-treated and UHT milk were confirmed by Zetasizer analysis. Bicinchoninic acid (BCA) protein assay showed that there was no significant loss of exosomes after UV radiation treatment, whereas after UHT processing, a significant loss was observed. As analysed by qPCR, a significant loss of exosomal miRNAs was observed after UV radiation as well as UHT processing, as compared to pasteurized milk. Since milk miRNAs have been shown to possess physiological effects, such loss due to UV and UHT processing could be of concern.

1. Introduction

Milk is a biological fluid with excellent nutritional value. Milk secretion has evolved to feed the mammalian infants (McClellan *et al.*, 2008). Gradually, over the years, there has been worldwide consumption of milk and milk products by all age groups for their nutritional benefits. Thus, for its consumption and commercialization on a large scale, milk undergoes various processing treatments (Tiedman, 1958). Milk processing involves various treatments like pasteurization, homogenization and ultra-heat treatment. Pasteurization involves heating at 72°C for 10-15 s to sterilize milk, whereas homogenization is a physical technique for reducing the size of fat globules to avoid cream formation (Qi *et al.*, 2015). Recently, commercialized milk processing technique, i.e. ultra-heat treatment (UHT) of milk, is the technique in which milk is heated to a temperature above 135°C for 1-4 s to ensure complete sterilization and increase its shelf life (Hansen *et al.*, 2017). The main concern in the dairy industry is to conserve the nutritional treasure of milk after its processing of milk. One of the emerging bioactive components of milk is microRNA (miRNA). Apart from being present in the other fractions, they are

encapsulated and stable in the lipid bilayered nano-vesicles called exosomes (Rani, Vashisht, Golla *et al.*, 2017). Milk exosomes enclose biological molecules like miRNAs, mRNAs and proteins. They encapsulate bioactive miRNAs in them, which can regulate the target gene expression when they get incorporated into cells. Different groups have identified and characterized the spectrum of miRNAs encapsulated in the exosomes. They play a significant role in providing immunological protection and prevention against diseases to the infants and the adult consumers of milk (Baier *et al.*, 2014). Considering the other aspect, some milk miRNAs like miR-148a and miR-200 family can also lead to the development of diseases like diabetes and inflammation (Belgardt *et al.*, 2015). A recent study showed that milk processing and storage cause considerable loss of miRNAs (miR-200c and miR-29b) from cow milk (Howard *et al.*, 2015). Although UHT-processed milk is commercially successful, ultra-heat treatment of milk changes the properties of milk. It can cause denaturation of milk proteins, loss of folate, vitamin C, vitamin B12 and thiamin (Morgao, 2003). Also, UV-treated milk is considered safer as it reduces the bacterial count while retaining the nutritional content (Christen *et al.*, 2013).

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However, the effect of UHT and UV processing on emerging bioactive components of milk, i.e., exosomal miRNAs, has not been studied. Thus, the present study aimed to study the effect of UHT and UV treatment on the abundance of exosomes and their encapsulated miRNAs in milk.

2. Materials and methods

2.1 Milk sample collection

Pasteurized milk and UHT milk were purchased from the local store of Amul Company, which is a prestigious Indian Dairy Cooperative brand. Both the milk types contained 3.0% fat and 8.5% minimum non-fat solids. Milk was immediately processed in the laboratory.

2.2 Exosome isolation

Milk was processed to collect whey for exosome isolation as described previously with minor modifications (Vashisht *et al.*, 2017). Briefly, milk was centrifuged at 2000×g for 10 min at 4°C using Hettich Mikro 22 R centrifuge (Tuttlingen, Germany). Discarding the pellet of cells and the upper fat layer, the fluid fraction was again centrifuged at 21,500×g for 30 min at 4°C. Repeating the above procedure, the fluidic fraction was centrifuged at 21,500×g for 1 h at 4°C, removing residual fat and casein fraction. The milk whey was obtained and filtered through a 0.45 µm filter. The filtrate was used for exosome isolation. 1 mL of filtrate was mixed with 400 µL of exosome precipitation buffer provided in the miRCURY exosome isolation kit (Exiqon, Denmark). The above whey/exosome precipitation mixture was incubated for 3 h at 4°C. The incubated mixture was centrifuged at 10,000×g for 30 min at room temperature. The pellet was resuspended in 100 µL of resuspension buffer provided in the kit.

2.3 UV treatment

Milk exosomes isolated from the pasteurized milk were subjected to UV-light (254 nm) for 1 h at 4°C in an ultraviolet sterilizing PCR workstation.

2.4 Dynamic light scattering

The size of exosomes isolated from pasteurized, UV-treated, and UHT milk was analyzed using Zetasizer (Malvern Instruments Limited, UK). Exosomal solution diluted in a ratio of 1:100 was analyzed with the equilibration time of 120 s and at constant temperature (25°C). A laser beam of wavelength 632.8 nm was applied to the diluted exosomal suspension. The light scattered by the nano-particles in suspension was detected by an Avalanche Photodiode Detector at 173° and non-invasive back scattering optics. The size of

exosomes was determined by taking the average of three measurements (Baddela *et al.*, 2016).

2.5 Bicinchoninic acid protein assay

Total exosomal protein was estimated using Pierce™ BCA Protein Assay kit (Thermo Scientific, Rockford, USA). Exosomes were diluted in 1:10 ratio for analysis. For 10 µL of the exosomal solution, 200 µL of BCA working reagent was prepared by mixing Reagent A and Reagent B in the ratio of 1:50, respectively. BCA working reagent was mixed with diluted exosomal solution in the 96-well plate, followed by incubation for 30 min at 37°C. The absorbance of exosomal protein samples was measured at 562 nm using a microplate reader (BioTek Instruments, USA). The exosomal protein concentrations were measured using a standard curve ($r^2 = 0.997$) plotted with diluted BSA standards (Rani, Vashisht, Golla *et al.*, 2017).

2.6 Exosomal RNA isolation and qRT-PCR for miRNA analyses

Exosomes were isolated from 3 mL of milk whey of pasteurized, UV-treated and UHT milk in three tubes, with 1 mL in each tube, separately. Equal volumes of milk whey were taken, as exosomes are present in the whey fraction. Exosomal pellet was resuspended in 50 µL resuspension buffer in each tube and pooled to a final volume of 150 µL solution. Total RNA was isolated from the pasteurized, UV-treated and UHT milk using Trizol LS following the manufacturer's instructions. 450 µL of Trizol LS was added to the exosomal solution, followed by incubation for 10 min at room temperature. 100 µL of chloroform was added to the above mixture and incubated for 15 min. The mixture was centrifuged at 12,000×g for 15 min at 4°C, followed by the extraction of the upper aqueous layer in a separate tube. 250 µL of isopropanol was added to the aqueous layer and incubated for 1 h at -20°C. Then, centrifugation was done at 12,000×g for 15 min at 4°C. Discarding the solution, the pellet was washed with 75% ethanol. The pellet obtained after washing was allowed to air dry and resuspended in 20 µL nuclease-free water.

qRT-PCR analysis was done as described previously (Rani, Vashisht, Golla *et al.*, 2017). Briefly, 6 µL of total RNA was reverse transcribed to synthesize cDNA using the miScript II RT Kit (Qiagen, Germany). Subsequently, the diluted cDNA (1:20) was used for the Real Time PCR analysis of the exosomal miRNA, i.e. bta-miR-2478, using miScript SYBR® Green PCR Kit (Qiagen, Germany). The primer sequence used for its analysis was as follows: ATCCCACTTCTGACACCA. Real-time PCR conditions included an initial activation for 15 min at 95°C, followed by 40 cycles of

denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 70°C for 30 s in Light Cycler® 480 II Real Time PCR System (Roche Molecular Diagnostics, USA). All of the reactions, including the no-template controls, were run in duplicates. Ct values obtained for each miRNA with fixed threshold settings were used for analyses.

2.7 Statistical analysis

Data were analyzed using GraphPad Prism 5.0 software. The unpaired t-test was employed to compare the miRNA levels in the exosomes isolated from pasteurized milk, UV-treated and UHT milk. All the data values were represented as mean \pm standard error of the mean (SEM). Statistical significance was considered if $P \leq 0.05$.

3. Results

The presence and the size of exosomes in pasteurized, UV-treated and UHT milk were analyzed by Zetasizer analysis. The exosomes isolated from pasteurized milk, UV-treated and UHT milk were 74.32, 72.73 and 76.06 nm in diameter (Figure 1(a)) with polydispersity indices of 0.278, 0.261 and 0.267, respectively (Figure 1(b)). BCA protein assay was performed to estimate the total protein concentrations of exosomes isolated from equal volumes of whey of pasteurized, UV-treated and UHT milk. The concentration of exosomes was quantified in terms of total exosomal protein concentrations (Yu *et al.*, 2017). The total exosomal protein quantified was 14.85 ± 0.5919 $\mu\text{g}/\mu\text{L}$, 15.29 ± 0.78 and 6.147 ± 0.2469 $\mu\text{g}/\mu\text{L}$ in the 100 μL exosomal suspension isolated from pasteurized milk, UV-treated and UHT milk, respectively. There was a significant difference between the total protein concentrations of exosomes isolated from pasteurized and UHT milk, whereas there was none between

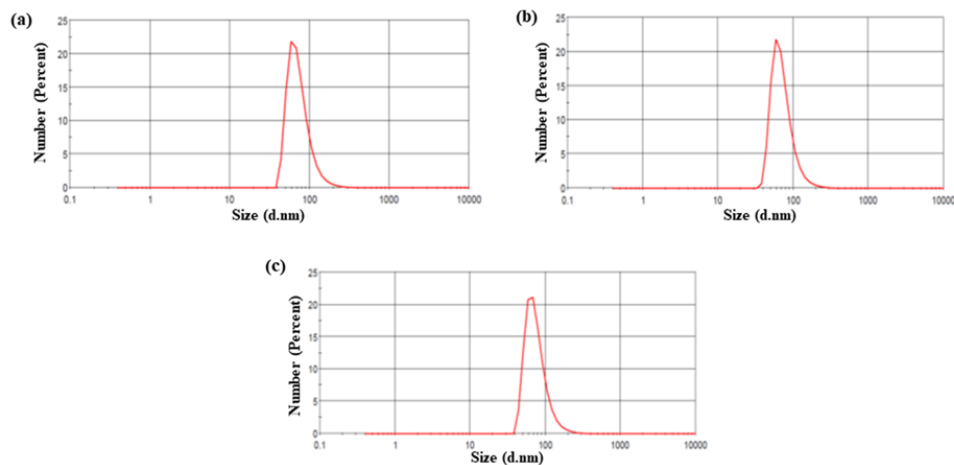


Figure 1. Characterization of exosomes. Zetasizer measurements for the size of exosomes (1 mL of exosomal suspension diluted (1:100) with PBS) isolated from (a) pasteurized milk, (b) UV-treated milk and (c) UHT milk.

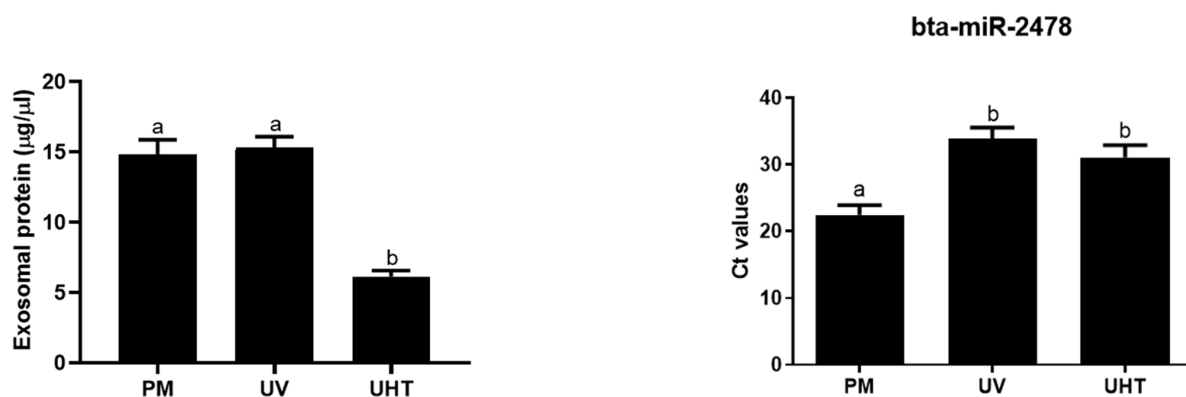


Figure 2. Quantification of exosomes in terms of total exosomal protein content. Exosomes were isolated from the same volume of pasteurized (PM), UV-treated (UV) and ultraheat-treated (UHT) milk whey, diluted in 1:10 ratio. The total exosomal protein was measured using the BCA protein assay taking the absorbance at 562 nm using microplate reader (BioTek Instruments, USA). The exosomal protein concentrations were measured using standard curve ($r^2 = 0.997$) plotted with diluted BSA standards. Bars with different notations are statistically significantly different ($P < 0.05$).

Figure 3. Analysis of abundant exosomal miRNA bta-miR-2478 present in milk exosomes. Total RNA was isolated from exosomes isolated from the same volume of pasteurized, UV and UHT milk whey using Trizol LS. cDNA of miRNAs was synthesized with the miScript II RT Kit. Real time PCR of abundant miRNAs was done using the miScript Syber Green kit. The Y-axis indicates Ct values obtained after real time PCR and X-axis represents pasteurized (PM), Ultraviolet (UV) and ultraheat-treated (UHT) milk. Bars with different notations are statistically significantly different ($P < 0.05$).

pasteurized and UV-treated milk (Figure 2). It suggests the number of exosomes present in the UHT milk was significantly lower than that of pasteurized milk, while there was no difference between pasteurized and UV-treated milk. The abundance of miRNA, i.e. bta-miR-2478, in the pasteurized, UV-treated and UHT milk exosomes isolated from equal volumes of milk whey was compared. This miRNA was abundantly present in the bovine milk exosomes. The miRNA was significantly less abundant in the exosomes isolated from equal volumes of UHT and UV-treated milk as compared to the pasteurized milk, comparing the Ct values using fixed threshold settings previously (Rani, Vashisht, Golla *et al.*, 2017) (Figure 3).

4. Discussion

Milk also contains lipid bilayered nano-vesicles called exosomes. They enclose bioactive cargo, including miRNAs within them. Exosomes participate in cell-cell communication by transferring their bioactive cargo to the target cells (Mathivanan and Simpson, 2010). It has been shown that the exosomal miRNAs are resistant to digestion and permeable to the intestinal barrier (Rani, Yenuganti, Shandilya *et al.*, 2017). In general, milk exosomes and their contents are stable against harsh conditions like low pH, boiling and enzymatic treatments (Baddela *et al.*, 2016). However, the effect of UHT processing on exosomal stability is not known yet. As the consumption and demand of UHT milk are increasing, it is important to know the effects of UHT processing on exosomes and their contents. Thus, the overall aim of this study was to investigate whether the UV treatment and UHT-processing of dairy milk affect the abundance of exosomes and their encapsulated miRNAs. The exosomes and their contents were analyzed in the UV-treated and UHT milk as compared to the pasteurized milk. Heating was done at ultra-high temperature, i.e., 135°C for 1-4 s for UHT milk as opposed to 72°C for 10-15 s in the case of pasteurized milk (Qi *et al.*, 2015). Ultra-heat treatment may lead to the denaturation of bioactive milk components like proteins, loss of folate, vitamin C, vitamin B12 and thiamin (Morgao, 2003). Zetasizer analyses indicate the presence of exosomes in the UHT milk and pasteurized milk. Analysis revealed that there was a homogeneous population of nanovesicles and the absence of microvesicles, as indicated by the polydispersity index. BCA protein assay revealed that the number of exosomes was significantly lower in UHT milk as compared with the equal volumes of pasteurized milk. Real-time PCR studies indicate the loss of miRNAs present in the exosomes isolated from the UHT milk as compared with exosomes isolated from equal volumes of pasteurized milk. While in the case of UV treatment, exosomes

remain intact, whereas miRNAs get degraded, as supported by the previous report (Srinivasan *et al.*, 2007). The results obtained in this study were well corroborated by the previous related studies. Recently, Howard *et al.* (2015) showed that the milk miRNAs are degraded during the processing and storage of raw milk. Milk miRNAs are significantly less abundant in the processed and stored milk compared to the raw milk. However, they have not analyzed exosomes or their contents in their study. Thus, it can be concluded that ultra-heat treatment leads to the partial degradation of exosomes and its contents. To the best of our knowledge, this was the first study demonstrating the effect of UV treatment and UHT processing on the milk exosomes and their contents. Milk exosomal miRNAs can provide immunological benefits to the adult consumer of bovine milk (Baier *et al.*, 2014). For instance, miR-155, which is abundant in milk exosomes, can regulate the proliferation and response of cytotoxic T cells, interferon signaling, antibody and memory response (Calame, 2007). Thus, taking UHT milk may have less nutritional value and provide fewer immunological benefits. On the contrary, milk also contains some miRNAs, which can lead to the development of disease if they get incorporated in the body. For instance, miR-200 family, which is abundant in the milk exosomes, is involved in the apoptosis of pancreatic beta cells and type 2 diabetes (Belgardt *et al.*, 2015). An epic study showed that the consumption of UHT milk is related to a lower incidence of diabetes, while UV-treated milk enhances the efficacy of milk (Melnik, 2015; Schaefer *et al.*, 2018). The results of the present study can explain the above fact, as a lesser abundance of milk exosomal miRNAs causing diabetes in UHT milk can ultimately lead to a lower incidence of diabetes. There is a recent report in which the milk consumption by women is directly related to the increased incidence of breast cancer (Fraser *et al.*, 2020). However, more focused studies with an appropriate experimental approach are required to be done to uncover the physiological effects of UHT-processing on exosomes and their bioactive contents. Exosomes are emerging as a novel and stable pharmacological vehicle to encapsulate the unstable drugs having anti-inflammatory, chemo-preventive and chemotherapeutic drugs like curcumin (Vashisht *et al.*, 2017). Milk is proposed to be the most suitable source of exosomes as a drug delivery vehicle for pharmacological research. The lower abundance of exosomes in UHT milk makes it unsuitable for use as the source for exosomes. Additionally, the separation of other fractions of milk for exosomes isolation is a bit more difficult in UHT-processed dairy milk.

5. Conclusion

In conclusion, significant loss of exosomal miRNAs occurs during UV treatment and UHT than the pasteurization of the milk. Such loss might be beneficial or detrimental to the consumers of UV-treated and UHT milk. For example, the consumers may not get health-promoting miRNAs, but they may be protected from health-detrimental milk miRNAs by UV and UHT processing.

Conflict of interest

The authors declare that they have no conflict of interest.

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