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Comparison of melatonin concentrations in raw and processed cow's milk

Vergleich der Melatoninkonzentrationen in Rohmilch und verarbeiteter Kuhmilch

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Summary

Processing-dependent changes of the melatonin concentration in milk were determined using a commercial melatonin ELISA. A total of 120 milk samples, comprising ultra-high temperature processed milk (UHT), ultra-pasteurized milk with extended shelf life (ESL-HE), microfiltered milk with extended shelf life (ESL-MF), high-temperature short-time pasteurized milk (HTST), raw milk (RAW), and a commercial milk powder claiming to be melatonin-rich (night-time milk powder, NTMP) were included in the study. Raw milk and night-time milk powder had the highest melatonin concentrations, while UHT milk had the lowest. The mean melatonin concentration in raw milk was 13.6 pg/ml, but levels varied strongly between individual cows (1.3–25 pg/ml). Finally, although all processed cow milks contained statistically significant less melatonin than NTMP and raw milk they were still in the same dimension (pg/ml).

Keywords: melatonin, night-time milk powder, night-milking, ELISA

Zusammenfassung

Temperaturabhängige Veränderungen der Melatoningehalte in der Milch wurden mit dem Melatonin ELISA bestimmt. Insgesamt 120 Milchproben, einschließlich ultrahocherhitzter Milch (UHT), hocherhitzter Milch mit längerer Haltbarkeit (ESL-HE), mikrofiltrierter Milch mit längerer Haltbarkeit (ESL-MF), Frischmilch (HTST), Rohmilch und ein als „Melatonin-reich“ deklariertes Nachtmilchpulver (NTMP) wurden in diese Studie einbezogen. Die Rohmilch und das Nachtmilchpulver enthielten den signifikant höchsten Melatoningehalt und ultrahocherhitzte Milch den geringsten. Der mittlere Melatoningehalt in der Rohmilch betrug 13,6 pg/ml, wobei die Konzentrationen stark zwischen den einzelnen Kühen variierten (1,3–25 pg/ml). Auch wenn die verarbeitete Kuhmilch signifikant weniger Melatonin enthält als das Nachtmilchpulver und die Rohmilch, so sind sie doch alle in derselben Dimension (pg/ml).

Schlüsselwörter: Melatonin, Nachtmilchpulver, Nachtmelken, ELISA

Introduction

Melatonin, N-acetyl-5-methoxy tryptamine, is a neurohormone secreted by the pineal gland. The main function is synchronizing circadian rhythms in many vertebrates (Kayumov et al., 2005) and it also acts as a free radical scavenger and antioxidant (Berra and Rizzo, 2009). Melatonin production is crucial for sleep duration and sleep quality in humans (Brzezinski et al., 2005; Valtonen et al., 2005). A low or irregular melatonin production can cause sleep and mood disorders or jet lag. The mean nocturnal melatonin plasma concentration in young healthy subjects is 54.4 pg/ml with high interindividual variations (SD = 54.7 pg/ml) (Fourtillan et al. 2001). The melatonin production clearly declines with age in both men and women (Sack et al., 1986). The EFSA (European Food Safety Authority) confirms that oral administration of 1 mg or 0.5–5 mg of melatonin per person is effective in the reduction of sleep onset latency (EFSA, 2011), and the reduction of subjective feelings of jet lag and the improvement of sleep quality (EFSA, 2010). For therapeutic effects melatonin is usually supplemented at daily doses between 0.5 and 5 mg per person and is effective in preventing or reducing jet lag (Herxheimer and Petrie, 2002) and improving sleep (Brzezinski et al., 2005). However, supplementation of even ultra low doses of melatonin (daily intake of 5–20 ng) may benefit the elderly by increasing daytime activity, maybe caused by the improved sleep quality (Valtonen et al., 2005), whereas these concentrations are surprising and strongly below the evaluations of melatonin doses made by the EFSA (EFSA, 2010 and 2011). The characteristics of melatonin, especially its lipophilicity, allow its rapid entrance from the pinealocytes into the blood and all other body fluids, even milk (Reiter, 1991). Milk melatonin levels reflect blood concentrations with a short delay (15–30 min) (Reiter, 1991). In bovine blood, melatonin levels range from 10 to 90 pg/ml (Berthelot et al., 1990; Burchard et al., 1998; Rodriguez et al., 2004; Muthuramalingam et al., 2006). Compared to that, raw milk melatonin concentrations of 3.9–40 pg/ml were reported (Eriksson et al., 1998; Valtonen et al., 2003; Valtonen et al., 2005; Kollmann et al., 2008; Milagres et al., 2013). The concentration in milk depends on the milking time since the hormone is only produced at night. Already light intensities above 50 lux and short wavelength lighting (between 470 and 525 nm) are adequate to abolish the normal melatonin surge that occurs within the early darkness hours (Lawson and Kennedy, 2001; Kayumov et al., 2005). Several methods for the production of milk or milk products with increased melatonin content were established (Gnann, 2010; Kangas et al., 2001; Haigh, 2003). Recently, a so-called night-time milk powder (NTMP) with a declared melatonin content of about 1800 pg/12 g is commercially available as food. Although the product does not use any legal nor illegal claim according to Regulation (EC) No 1924/2006, it is suggestive of being able to improve sleep by its' appearance. Furthermore, it comes along with a brochure claiming it contains up to 100 times more melatonin than conventional milk. However, it is not clarified if conventional milk in this context is raw milk or any kind of processed milk. In fact, there exist some data on melatonin in raw milk (Eriksson et al., 1998; Valtonen et al., 2003; Valtonen et al., 2005; Kollmann er al., 2008; Milagres et al., 2013), but with the exception of the study of Valtonen et al. (2005), who used pasteurized cow milk with melatonin concentration of about 13 - 15 pg/ml, there are no publications on melatonin concentrations in differently processed cow's milk. Therefore, the aim of this work was to measure and compare the concentrations of melatonin in raw milk, commercially available processed cow's milk and the melatonin-rich night-time milk powder.

Materials and methods

Samples

In total, 120 milk samples were examined. They were assigned to one of six groups based on their processing. These groups were ultra-high temperature processed milk (UHT), ultra-pasteurized milk with extended shelf life (ESL-HE), microfiltered milk with extended shelf life (ESL-MF), high-temperature short-time pasteurized milk (HTST), raw milk (RAW), and a commercial milk powder claiming to be melatonin-rich (night-time milk powder, NTMP). Every group comprised 20 samples except ESL-HE and HTST, which included 19 and 21 samples, respectively. The processed milk samples with different identification markings were purchased from supermarkets in four German federal states. Products were analyzed for melatonin at the day of purchase and stored at conditions following the declarations. Raw milk samples of ten dairy cows (Holstein-Friesian cattle) were obtained in two consecutive weeks. All cows were in the second lactation, lived among standardized conditions at the same farm and were fed by mixed ration, hay and individual concentrated feed dependent on the respective milk yield. The clinical status of every single cow was evaluated at the beginning of the study and onwards twice daily. None of the ten cows showed any signs of illness during the study. Each sample comprised 200 ml mixed milk from each udder quarter after foremilk was rejected and the vials were sterile, screw-capped polypropylene tubes. Raw milk was gained during the morning milking at 03:30 at both sampling days to obtain comparable values for individual cows' comparisons as well as for appropriate comparison with the NTMP values. Immediately before milking brightness was recorded by a digital luxmeter (Conrad MS-1500; RE 128802) which was positioned at a level of 1.5 meters at six different spots in the cote. The mean light intensity was 12 lux and 16.5 lux at the first and second sampling days, respectively. The raw milk samples were maintained at a temperature below 10 °C during transport to the Institute of Food Hygiene and were analyzed at the same day. Moreover, melatonin was quantified in 20 portions of NTMP. Respectively four portions (1800 pg melatonin/portion of 12g) of five packages, with same identification markings and three different lot numbers (1; 2 = 3; 4 = 5) were chosen since more heterogeneous samples were not available. Based on the manufacturer's information, the cow milk used for the production of this powder was gained at night under specific conditions and light regimes (Gnann, 2010). The powder (12 g) was dissolved in 200 ml distilled water to obtain a typical serving.

Melatonin ELISA

The Melatonin direct Saliva ELISA (IBL-International GmbH, Hamburg, RE 54041) was originally designed for examination of saliva. However, it was also shown to be applicable for detection of melatonin in other matrices like serum, whole blood (Ackermann et al., 2010) as well as milk (Milagres et al., 2013). We used this test for quantitative determination of melatonin in milk according to Milagres et al. (2013), only changing the milk sample dilution to 1:5. Based on the included standard dilution series a standard curve was developed and its equation was described by nonlinear fitting of the sigmoidal curve using Prism 4.00 (GraphPad Software Inc., USA) for every plate. The equation was used to calculate melatonin concentration of the samples. The robustness of the assay was tested by five replicate analyses of each one HTST and one ESL-MF milk sample. In addition, the effect of storage time on the melatonin levels was studied by repeated weekly analysis of four HTST milk samples over a period of 3 weeks.

Statistics

Means of each group of milk were compared by ANOVA followed by Bonferroni multiple comparison test. This was eligible since preceded normality tests showed Gaussian distribution of data. Tests as well as initial descriptive statistics were conducted using Prism 4.00 (GraphPad Software Inc., USA). Differences were considered to be statistically significant when $p < 0.05$. Raw milk samples were collected from ten different cows at two samplings. Since these 20 data points are not independent we computed means for each cow and used these ten mean values for further statistical comparisons (ANOVA, Bonferroni multiple comparison test).

Results

The robustness check of the melatonin assay revealed mean values of 6.28 pg/ml (SD = 0.92 pg/ml) and of 7.23 pg/ml (SD = 0.8 pg/ml) for the five replicate analyses of one HTST and one ESL-MF milk sample, respectively. So, results' variation is about 11.1–14.7 %.

No effect of storage was observed, since no decrease of melatonin concentration during the repeated weekly analysis of four HTST milk samples over a period of 3 weeks was noticed.

The mean melatonin concentrations and the obtained standard deviations of the six sample groups are depicted in figure 1. In raw milk, melatonin averages of 13.6 pg/ml (SD = 6.3 pg/ml) were detected. The processed milks showed mean melatonin contents of 7.6 pg/ml (SD = 1.9 pg/ml) in HTST, 7.0 pg/ml (SD = 1.1 pg/ml) in ESL-HE, 6.5 pg/ml (SD = 2.3 pg/ml) in ESL-MF and 4.5 pg/ml (SD = 1.7 pg/ml) in UHT milk. In NTMP, 12.4 pg/ml (SD = 2.8 pg/ml) was found, confirming to the declared concentration. The sample with the overall lowest melatonin concentration was UHT milk (0.6 pg/ml) while the one with most melatonin was raw milk (25.0 pg/ml).

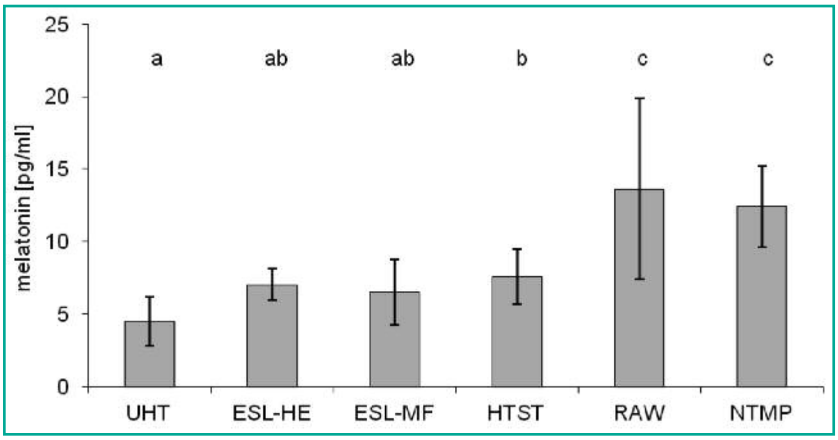

FIGURE 1: *Melatonin concentrations of UHT (n = 20), ESL-HE (n = 19), ESL-MF (n = 20), HTST (n = 21), RAW (n = 10), NTMP (n = 20 / dissolved in water). Means and standard deviations are plotted. Different letters (a–c) indicate statistically significant differences (p < 0.05).*

Raw milk and night-time milk powder showed significantly highest melatonin concentrations while UHT milk showed the lowest. However, no statistically significant differences were obtained between UHT and both ESL milk groups. As indicated by the high SD in the raw milk group, individual melatonin concentrations of the examined cows were very variable. The lowest melatonin content was 1.3 pg/ml, the highest level was 25.0 pg/ml. A follow-up examination showed very constant levels of melatonin in the milk of each individual cow as specified in table 1.

TABLE 1: *Melatonin concentrations in raw milk of ten dairy cows, drawn in a 2-week interval.*

cow	melatonin concentration [pg/ml] day 0	day 14
1	10.9	10.0
2	14.6	12.6
3	8.0	6.8
4	16.6	17.0
5	25.0	22.2
6	1.3	1.7
7	16.6	17.4
8	15.7	11.3
9	22.0	17.8
10	13.8	11.3

Discussion

In the present study, we could show statistically significant differences between raw and processed milk with raw milk showing the highest and UHT milk the lowest melatonin content. Data varied from 0.6 pg/ml up to 25.0 pg/ml melatonin in one UHT milk and one raw milk sample, respectively. Relative standard deviation (RSD) of repeated measurements of one sample was about 11.1–14.7 %. However, RSD of samples within groups was higher (UHT = 37.8 %, ESL-HE = 15.7 %, ESL-MF = 35.4 %, HTST = 25 %, Raw milk = 46.3 %, NTMP = 22.6 %) indicating raw milk variation being the highest. However, also UHT and ESL-MF showed high relative standard deviations. The high absolute and relative standard deviation of raw milk can be explained by the fact, that the samples are cow individual samples, collected on the same dairy farm while processed milks are bulk tank samples from different farms, collected and mixed by the dairy. Kollmann et al. (2008) also showed large individual differences between the animals in milk and plasma melatonin concentrations of Brown Swiss dairy cows and heifers. Milagres et al. (2013) found a higher melatonin concentration (39.43 pg/ml) in raw milk of ten Holstein cows at 02:00 in the morning, than we did. They used the same analytical kit, but contrary to our test execution, fat was extracted and milk samples were diluted 1:10 with Standard A. Besides these technical and cow individual differences no information were given on milking and feed which could result in different melatonin concentrations. Muthuramalingam et al. (2006) detected plasma melatonin levels of 10–81 pg/ml, e.g. the peak is more than three-fold higher than we detected in raw milk. However, they used prepubertal Holstein heifers instead of lactating Holstein cows. Whereas those heifers were acclimated to a special indoor photoperiod (16 h light (200 lux) and 8 h dark (0 lux)), cows used in our study were not treated by any special light regime. Different sampling times may influence the melatonin levels, because melatonin production peaks with interspecies differences either in the late dark phase, in the mid dark phase (most pattern) or shows a prolonged peak during majority of dark phase (Reiter, 1981). Since we obtained raw milk samples at 03:30 in the morning, we assume to have sampled around the time of the highest melatonin production. Similar mean nocturnal melatonin concentrations in blood plasma samples of lactating, multiparous pregnant Holstein cows of 70.82 pg/ml were detected by Burchard et al. (1998). The cows were exposed to a combination of artificial and natural light (12 h light/12 h dark) and the study was carried out between March and August. Berthelot et al. (1990) measured the highest plasma melatonin concentration of about 90 pg/ml in the middle of the scotophase in pasture-bred, non-pregnant, dry French Friesian cows under natural photoperiod conditions in summer.

In contradiction, Rodriguez et al. (2004) detected serum melatonin concentrations during the light period of 12.4 pg/ml in lactating, pregnant Holstein cows subjected to short photoperiods (8 h light/16 h dark). The use of serum and the application of the radioimmunoassay to quantify melatonin concentration could account for the differences compared to our results in raw milk. In general, the lower melatonin content in processed cow milks, compared to raw milk and the night-time milk powder may be the result of the diluting effect occurring from mixture of day- and nighttime milk in the bulk tank, because melatonin will only be present in the milk obtained at night. We sampled raw milk only in the dark to obtain appropriate comparable values to those of NTMP. However, if we would have examined raw milk gained at day-time the differences to processed milk samples may have been smaller or even not existing. Furthermore, an explanation for the different melatonin concentrations within processed milks (HTST, ESL-HE, ESL-MF, UHT) could be heat-related effects. The melting point of melatonin of 117 °C (Lee et al., 1997) is exceeded in UHT and ESL-HE milk which are usually heated at 135 °C or up to 127 °C, respectively. The application of those temperatures might damage or destroy melatonin. Agarwal et al. (2008) tested the dry heat degradation of melatonin (at 80°C for three hours) and determined eight degradation products. However, further research is necessary to detect thermally introduced metabolites at 135 °C to confirm this. The NTMP was produced under vacuum and cooled down to –20 °C to protect melatonin against thermal degradation processes which could be the reason for the relatively high concentrations. To the author's knowledge to date there are no published examinations on the development of the melatonin concentration in raw cow milk over time. We have compared the melatonin concentrations in the raw milk of ten cows in a two-week interval. Although melatonin concentrations differed between cows, we found similar results at the examination two weeks later in the same cow. An explanation for the high individual differences in milk melatonin levels (cow 6: 1.3–1.7 pg/ml; cow 5: 22.2–25.0 pg/ml) may be the genetic influence on melatonin production (high heritability), already detected in the blood of ewes (Zarazaga et al., 1995). Kollmann et al. (2008) also showed varying melatonin plasma levels during nighttime (2.4–36.5 pg/ml) between single heifers. If the melatonin content in the raw milk depends on current lactation status, number of lactation or other milk quality factors is not known yet and should be matter of further research.

Altogether, data of melatonin concentrations in different processed, retail cow milk provide a first insight into the extent to which melatonin concentration is altered during processing.

Finally, although all processed cow milks contained statistically significant less melatonin than NTMP and raw milk they were still in the same dimension (pg/ml). So, if there are any effects of such low melatonin doses, that are way lower than necessary to claim any effect as proposed by EFSA (2010 and 2011), it can be assumed that these are also achieved from consumption of respective volumes of processed milk.

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Conflict of interest

We declare that there are no conflicts of interest.

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