Asian Journal of Andrology (2017) 19, 52–56

www.asiaandro.com; www.ajandrology.com

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ORIGINAL ARTICLE

Sperm Biology

Seasonal fluctuation in the secretion of the antioxidant melatonin is not associated with alterations in sperm DNA damage

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A high sperm DNA fragmentation index (DFI) is associated with reduced fertility. DFI is influenced by the balance between reactive oxygen species and antioxidants. A circannual variation in melatonin, an antioxidant and free radical scavenger, could thus impact semen quality and fertility. The association between the major melatonin metabolite, urine 6-sulfatoxymelatonin (aMT6s), and DFI was analyzed in 110 Oslo men (south of the Arctic Circle) and 86 Tromsoe men (north of the Arctic Circle). Two semen analyses, summer and winter, and four urine samples (early/late summer; early/late winter), were analyzed. The associations between aMT6s in urine and DFI were characterized in a cross-sectional and longitudinal manner using correlation analysis and linear regression. Regardless of season and location, no significant correlations between aMT6s and DFI were observed. The correlation coefficients for associations between changes over time (early winter–early summer) in aMT6s and DFI were for the total cohort: rho = –0.08 (*P* = 0.322), for the Oslo cohort: rho = –0.07 (*P* = 0.485), and for the Tromsoe cohort: rho = –0.14 (*P* = 0.273), respectively. Similar results were seen when comparing late winter and late summer. There was no any statistically significant correlation between changes over time in aMT6s and DFI for men with DFI below and above the median value (10%), respectively. The seasonal variation in melatonin excretion seems not to have any impact on DFI.

Asian Journal of Andrology (2017) **19**, 52–56; doi: 10.4103/1008-682X.186870; published online: 14 October 2016

Keywords: male infertility; melatonin; semen quality; sperm DNA index

INTRODUCTION

A conventional semen analysis is not a sufficient tool for assessment of male fertility status,[1] and evaluation of sperm DNA damage provides additional information about the fertilizing capacity of the sperm.[2] It has been shown that a high level of sperm DNA damage leads to reduced fertility *in vivo*.[3,4] Evidence suggests that reactive oxygen species (ROS)-mediated damage to spermatozoa is a significant contributing factor in a substantial number of male infertility cases.[5] ROS can be generated or originate from various sources, both endogenous and exogenous.[6] Studies suggest that ROS attack the integrity of DNA in the sperm nucleus by causing base modifications, DNA strand breaks, and chromatin cross-linking. Due to the complex packaging arrangement of DNA, spermatozoa have limited defense mechanisms against oxidative stress.[7]

Furthermore, under normal conditions, spermatozoa generate low levels of ROS to stimulate proper sperm function[6] such as capacitation, hyperactivation, and sperm-oocyte fusion.[7] Seminal plasma serves as a natural reservoir of antioxidants, removes ROS, and consequently reduces oxidative stress.[8] Disturbances in the balance between ROS and antioxidant factors can damage spermatozoa impacting their fertilization capacity and disrupting their genetic integrity.[9,10] Impairment of sperm DNA can be analyzed and quantified by measuring DNA fragmentation index (DFI).[6,11]

Melatonin is a well-known powerful wide spectrum free-radical scavenger, believed to be the most effective natural lipophilic antioxidant. It can easily cross cell membranes and protect intracellular macromolecules and DNA by acting as a direct scavenger. Melatonin is a daylight-regulated hormone produced by the pineal gland with the highest secretion during the dark periods and transferred to semen through blood. The concentration of melatonin in testis tissue is in the same range as in other organs, e.g., in kidney and heart. Although extrapineal melatonin production has been suggested, it has so far not been proven.[8]

Birth rates vary over the year,[12] which could be due to a possible seasonal variation in DFI contributing to periodical changes in male fertility. It has also been suggested that daylight-dependent fluctuations in secretion of melatonin could impact male reproductive function.[13] Short-term exposure of spermatozoa to melatonin has been shown to improve semen quality *in vitro*, and higher melatonin and antioxidants levels have been studied in recently proven fathers.[5] A melatonin-related circannual variation in oxidative stress could, thus, have an impact on the quality of semen, and thereby affect birth rates.

In an attempt to further understand male reproductive physiology and the mechanism behind changes in birth rates, we have previously (2001–2002) studied the impact of daylight on sperm production.[14] We compared seasonal variation of reproductive

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Received: 07 February 2016; Revised: 18 May 2016; Accepted: 11 July 2016

parameters in Norway by comparing a group of men living in Oslo, in the Northern Hemisphere but south of the Arctic Circle, with men in Tromsoe, north of the Arctic Circle. In both locations, a substantial variation in photoperiod is seen over the year, with extreme variation in Tromsoe, resulting in 2 months of total darkness in the winter and a corresponding period of 24 h daylight in the summer. This is in contrast to Oslo with less pronounced light variation.

Despite this study design, no significant seasonal variation was found in spermatozoa concentration, total spermatozoa count, semen volume, or spermatozoa motility. However, a seasonal variation in melatonin levels was seen in both locations, most pronounced in Tromsoe. The same was true for testosterone levels in Oslo but not in Tromsoe. In the former location the levels of this hormone were also statistically significant correlated to urinary excretion of the major melatonin metabolite, urine 6-sulfatoxymelatonin (aMT6s).[13]

The antioxidant properties of melatonin and the relatively large variation in melatonin levels in the present study make it feasible to investigate the hypothesized association between melatonin and DFI levels.

MATERIALS AND METHODS

Subjects and study design

A total of 204 men were included in the present study, 112 from Oslo and 92 from Tromsoe. The participants were recruited to the study by advertising in local media. Criteria for being included in the study were as follows: 19–40-year-old men who had lived in respective area for a minimum of 1 year before the study start and were to remain in the area, except a maximum of 5 weeks holiday, during the year of the study. All except one participant answered a questionnaire concerning reproductive history and diseases that could influence their reproductive function.[14] The mean age of the participants was 27.5 years in Oslo and 29.8 years in Tromsoe. The mean body mass index (BMI) was 24.4 kg m^{-2} in Oslo, 25.3 kg m^{-2} in Tromsoe, and 24.8 kg m^{-2} for the total cohort, respectively.

For forty participants, one of two semen samples and one, two, or three of four blood samples were missing. There was no significant difference in age and semen parameters (total sperm count, sperm concentration, sperm motility, or semen volume) when comparing results from the 167 men who had measurements done at all four occasions compared to the above-mentioned forty cases ($P > 0.20$).

Tromsoe is located at 69°42'N, 348 km north of the Arctic Circle (66°33'N) and Oslo (59°56'N), 739 km south of the Arctic Circle. The distance between the two locations is approximately 1150 km. In both locations, a substantial variation in photoperiod is seen over the year.

The periods for urine and semen sample collections were based on the time of sunrise and sunset in the respective area. Overnight urine samples for analyzing the urinary excretion of the aMT6s were collected at two occasions during summer and two during winter. The first sample was collected approximately 70 days (i.e., the duration for spermatogenesis) before the second sample, and at the latter occasion, a semen sample was also collected. We have previously reported that there was a moderate agreement between DFI in the first and in the second semen sample from the same case.[15]

The first period was summer and the second period was winter. In Tromsoe, urine samples were collected close to the polar day (May 18), the second time, 2–3 months later (July 30 to August 10, 2001), the third time during winter, close to the polar night (November 28), and the fourth time, 2–3 months later after polar night (January 21 to February 1, 2002). In Oslo, urine samples were collected close to the days of solstices (June 21 and December 21, 2001) and 2–3 months thereafter (September 3 to 14, 2001 and March 4 to 15, 2002).

The subjects received a compensation of 1000 NOK for their participation. The study was approved by the Ethics Committee of Lund University, Sweden, and Regional Committee for Medical Research Ethics, Southern Norway.

Semen analysis

Before delivering a semen sample, a period of abstinence of 2–3 days was recommended. However, for each case, the length of abstinence was recorded. The semen samples were analyzed according to the recommendations of the World Health Organization 1999.[16]

Sperm chromatin structure assay

The principles and procedures to measure DNA damage by flow cytometry semen chromatin structure assay (SCSA) are described in detail elsewhere.[17] In brief, the semen sample is exposed to low pH that denatures DNA at sites of single- or double-strand breaks whereas normal, double-stranded DNA remains intact. Thereafter, the sperm cells are stained with a fluorescent DNA dye, with which intact (double-stranded) DNA emits green fluorescence whereas denatured (single-stranded) DNA emits red fluorescence. Sperm chromatin damage is quantified in a flow cytometer (5000 cells counted in an FACSort [Becton Dickinson, San Jose, CA, USA]) and displayed as red versus green fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as DFI, i.e., the ratio of red to total fluorescence intensity, i.e., the level of denatured DNA over the total DNA, and analyzed using the SCSA-Soft software (SCSA Diagnostics, Brookings, SD, USA).

All SCSA measurements were performed on semen, which on the day of analysis was quickly thawed and analyzed immediately. For the flow cytometer setup and calibration, a reference sample from a normal donor ejaculate retrieved from the laboratory repository was used.[17] The same reference sample was used for the whole study period. A reference was run for every fifth sample. The intralaboratory CV for the DFI analysis was 4.5%. A single SCSA measurement was made for each reference sample. All analyses were conducted at the same location.

Melatonin analysis

aMT6s was analyzed using a commercially available method kit (Immuno-Biological Laboratories, Hamburg, Germany). Urinary aMT6s concentrations were standardized for intra- and inter-individual variation in nocturnal diuresis by dividing the concentration of aMT6s (nmol l^{-1}) by urinary creatinine levels (mmol l^{-1}); the ratio is referred to as aMT6s throughout this study. Creatinine was analyzed on a Synchron LX20 (Beckman Coulter, Brea, CA, USA) using the Jaffé method. All assays were performed at the Department of Clinical Chemistry, Skane University Hospital, Malmö, Sweden. Overnight (12 h) urine samples were collected at four occasions for each participant: early summer, late summer, early winter, and late winter, as described above.

Statistical analysis

Initially, the correlations between early and late aMT6s summer excretion and summer DFI were investigated using the nonparametric Spearman's test. All analyses were performed for the total cohort as well as separately for the Oslo and the Tromsoe cohort. Corresponding analyses were performed for the correlations between early and late aMT6s winter excretion and winter DFI.

To evaluate whether time-related variations in aMT6s were associated with similar variations in DFI, we investigated whether the difference in aMT6s (i.e., early winter aMT6s–early summer aMT6s or late winter aMT6s–late summer aMT6s) correlated with the difference in DFI (i.e., winter DFI–summer DFI). Furthermore, with the same setup, the association between differences in aMT6s (i.e., early winter aMT6s–early summer aMT6s or late winter aMT6s–late summer aMT6s) was also analyzed. To investigate whether the association between aMT6s and sperm DNA fragmentation level was dependent on the extent of sperm DNA damage, separate analyses were done for those with DFI below and above the median value (10%).

Finally, a *post hoc* statistical power analysis based on the available sample size was done. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS (IBM SPSS Statistics 22, Chicago, IL, USA).

RESULTS

Summer and winter semen parameters, DFI and excretion of aMT6s

The mean DFI for the Oslo cohort was 12% (s.d. 7.4%) in summer and 11% (s.d. 6.1%) in winter. The corresponding values for Tromsoe were 12% (s.d. 8.8%) in summer and 12% (s.d. 7.5%) in winter. For the entire cohort, these values were 12% (8.0%) in summer and 11% (6.8%) in winter. The median DFI value in all samples was 10% and 6 of the men presented with a mean DFI above 30% – such high levels were reported to be associated with very low probability of conception *in vivo*.[18] The aMT6s values and DFI are presented in **Table 1**.

The intraindividual summer-winter coefficient variation for DFI was 14.8% and the corresponding value for aMT6s (early summer, late summer, early winter, and late winter) was 24.4%. When taking the summer (early and late) and winter (early and late) values separately for aMT6s within the same man, the coefficient variation for summer was 17.3% and for winter was 15.8%, respectively.

As previously reported, differences in standard sperm parameters between summer and winter were not statistically significant at either location, nor were they significant when subjects from both cities were analyzed as one group.[14] The results for the sperm parameters are given in **Table 2**. As shown in **Table 3**, there was a statistically significant negative correlation between DFI and progressive sperm motility (A + B), both summer and winter in Oslo, Tromsoe and when analyzing Oslo and Tromsoe together. In the Oslo group, there was a statistically significant negative correlation in early summer between DFI and sperm concentration.

Association between aMT6s and DFI

There were no significant correlations between aMT6s and DFI, neither for the winter/summer comparisons nor for the separate cohorts. The correlation coefficients varied between −0.18 and 0.11 (**Table 4**).

Table 1: DFI and aMT6s summer and winter in the Oslo and Tromsoe cohorts

	Mean (s.d.), median (range), n		
	Oslo + Tromsoe	*Oslo*	*Tromsoe*
DFI (%)			
Summer	12 (8.0), 9.6 (2.4–55), 198	12 (7.4), 10.1 (2.4–51), 110	12 (8.8), 9.5 (2.5–55), 88
Winter	11 (6.8), 9.2 (1.9–49), 193	11 (6.1), 9.5 (1.9–39), 108	12 (7.5), 8.8 (2.6–49), 85
aMT6s (nmol mmol^{-1} creatinine)			
Early summer	7.4 (4.7), 6.0 (0.6–25), 221	8.4 (4.8), 7.4 (1.3–25), 119	6.2 (4.3), 5.0 (0.6–22), 102
Late summer	8.5 (5.2), 7.3 (1.1–31), 221	9.6 (5.3), 8.7 (1.4–31), 119	7.1 (4.7), 6.0 (1.1–24), 102
Early winter	8.4 (5.0), 6.9 (1.4–25), 179	8.7 (4.9), 7.5 (1.4–24), 107	7.8 (5.1), 6.5 (1.4–25), 72
Late winter	8.8 (5.1), 7.3 (0.6–27), 221	9.9 (4.9), 9.2 (1.5–23), 119	7.4 (5.0), 6.0 (0.6–27), 102

s.d.: standard deviation; *n*: number; DFI: DNA fragmentation index; aMT6s: 6-sulfatoxymelatonin

Table 2: Seasonal variation in sperm parameters in Oslo and Tromsoe cohorts

	Mean (s.d.), median (range), n		
	Oslo + Tromsoe	*Oslo*	*Tromsoe*
Abstinence time (h)			
Summer	85 (48), 72.0 (12.0–504), 203	83 (51), 72.0 (12.0–504), 111	88 (45), 72.0 (24–336), 92
Winter	81 (37), 72.0 (12.0–336), 201	73 (32), 72.0 (12.0–168), 111	90 (40), 78.0 (36.0–336), 90
Semen volume (ml)			
Summer	3.9 (1.6), 3.8 (0.5–9.3), 204	3.8 (1.5), 3.8 (0.7–8.7), 112	3.9 (1.8), 3.8 (0.5–9.3), 92
Winter	3.8 (1.6), 3.6 (1.0–9.0), 201	3.7 (1.5), 3.6 (1.0–9.0), 111	3.9 (1.7), 3.7 (1.1–9.0), 90
Sperm concentration (×10^6 ml^{-1})			
Summer	70 (58), 57.0 (2.00–371), 203	73 (58), 59.0 (2.00–289), 111	65 (59), 55.0 (4.00–371), 92
Winter	67 (49), 58.0 (0.00–265), 201	66 (47), 53.0 (2.00–219), 111	68 (51), 61.0 (0.00–265), 90
Total sperm count (10^6)			
Summer	259 (228), 199.8 (5.200–1476), 203	269 (227), 210.0 (6.600–1476), 111	247 (229), 186.0 (5.200–1209), 92
Winter	251 (212), 202.8 (0.00–1380), 201	236 (183), 185.5 (5.00–882), 111	270 (244), 222.1 (0.00–1380), 90
Sperm motility A+B (%)			
Summer	46 (11), 48 (8.0–66), 203	46 (10), 48 (12–66), 111	47 (11), 49 (8.0–64), 92
Winter	47 (11), 48 (0.0–69), 201	47 (8.4), 48 (22–64), 111	46 (13), 50 (0.0–69), 90
Sperm morphology (%)			
Summer	7.0 (5.0), 6.0 (0.0–29), 147	7.6 (5.3), 6.0 (0.0–29), 83	6.2 (4.6), 5.5 (0.0–20), 64
Winter	8.0 (5.4), 7.0 (0.0–28), 154	6.5 (4.4), 6.0 (1.0–21), 86	10 (5.9), 10 (0.0–28), 68

s.d.: standard deviation; *n*: number. A: rapid progressive motility; B: slow/sluggish progressive motility

Table 3: The association between DNA fragmentation index (DFI) and semen parameters in Oslo and Tromsoe computed with a Spearman's correlation coefficient, rho (*P* value)

	Rho (P), n	
	DFI summer	*DFI winter*
Oslo + Tromsoe		
Total sperm count (10^6)	–0.02 (0.837), 198	0.08 (0.270),193
Sperm concentration ($\times10^6$ ml^{-1})	–0.10 (0.162),198	0.02 (0.771),193
Sperm motility A+B (%)	–0.36 (<0.001**), 198	–0.36 (<0.001**),193
Sperm morphology (%)	–0.18 (0.027), 144	0.05 (0.578), 148
Semen volume (ml)	0.199 (0.005**), 198	0.16 (0.027*),193
Oslo		
Total sperm count (10^6)	–0.09 (0.334), 110	0.11 (0.255), 108
Sperm concentration ($\times10^6$ ml^{-1})	–0.21 (0.028*), 110	–0.01 (0.89), 108
Sperm motility A+B (%)	–0.26 (0.006**), 110	–0.39 (<0.001**), 108
Sperm morphology (%)	–0.26 (0.021*), 82	–0.01 (0.912), 83
Semen volume (ml)	0.25 (0.009**), 110	0.25 (0.009**), 108
Tromsoe		
Total sperm count (10^6)	0.05 (0.639), 88	0.07 (0.533), 85
Sperm concentration ($\times10^6$ ml^{-1})	0.01 (0.931), 88	0.08 (0.472), 85
Sperm motility A+B (%)	–0.44 (<0.001**), 88	–0.32 (0.003**), 85
Sperm morphology (%)	–0.13 (0.300), 62	0.09 (0.497), 65
Semen volume (ml)	0.14 (0.181), 88	0.05 (0.675), 85

DFI: DNA fragmentation index; rho: Spearman's correlation coefficient; *n*: numbers; A: rapid progressive motility; B: slow/sluggish progressive motility. * $P<0.05$; ** $P<0.01$

The correlation coefficients for associations between time-related differences (early winter–early summer) in aMT6s and in DFI were for the total cohort: rho = −0.08 ($P = 0.322$); for the Oslo cohort: rho = −0.07 ($P = 0.485$); and for the Tromsoe cohort: rho = −0.14 ($P = 0.273$) (**Table 4**). The pattern was very similar when the differences between late winter and late summer were compared.

Furthermore, there were no statistically significant correlations between time-related differences (early winter–early summer) for aMT6s and DFI or men with DFI <10% (rho = −0.068, $P = 0.53$), and aMT6s or for late winter–late summer (rho = 0.008, $P = 0.94$). The corresponding results for men with DFI ≥10% were (rho = −0.071, $P = 0.53$) for early winter–early summer and (rho = −0.13, $P = 0.21$) for late winter–late summer.

Post hoc statistical analysis

A *post hoc* statistical power analysis showed that the available sample size was sufficient to fined a correlation coefficient of 0.2 with a statistical power of 0.8 ($\alpha = 0.05$) (https://www.statstodo.com/SSizCorr_Pgm.php).

DISCUSSION

Despite the design of the study with significant differences in the length of the daylight period, in particular north of the Arctic Circle, the previously reported winter–summer variation in excretion of aMT6s, the major melatonin metabolite, was not associated with corresponding fluctuations in DFI levels in summer and winter. Neither were there any significant correlations when comparing differences in aMT6s with DFI for the participants with DFI below nor above the median value of 10%. In general, DFI >30% is considered to have a negative impact on male fertility *in vivo*;[18] however, due to an insufficient number of men with DFI >30%, the subjects were divided into two groups according to the median DFI value.

Based on the way of recruitment and also on the background characteristics for both groups of men, we assume that they represent healthy Norwegian men, and the lack of circannual variation in DFI is not due to an underlying pathological condition. Despite a rather young age, 27% and 49% of subjects in Oslo and Tromsoe, respectively, reported proven fertility. Fertility problems were claimed by 5% of men in Oslo and by 15% of men in Tromsoe, in which numbers also include a female factor being at an expected level in the general population. Chronic diseases had been diagnosed in only a few men.[14] Data on ethnicity were not included in this study. There may be genetic differences between the two groups, perhaps with a higher proportion of Sámi and Russian admixture in Tromsoe; however, since each participant served as his own control, the genetic impact might be of less importance.

Our sample size was sufficient to find, with a statistical power of 0.8, correlation coefficient of 0.2 for the association between aMT6s and DFI. Thus, if melatonin excretion has any impact on sperm DFI, <5% of the variation in the latter can be explained by fluctuations in melatonin levels.

One of the weaknesses of the study is access to only a single measurement of sperm DFI for each of the two seasons. In this context, a key issue is the question of timing between variations in the oxidative stress and its effects on sperm DNA. Assuming a short lag period between changes in melatonin secretion, subsequent variations in oxidative stress level and in DFI, measurements of the major melatonin metabolite in urine were done at the same time as the semen sample collection. However, similar results were obtained when looking at melatonin levels 55–85 days before sample collection. One can speculate whether melatonin levels assessed at an intermediate time interval before semen analysis might show a stronger association with DFI.

Different mechanisms for sperm DNA damage have been described, and it seems most probable that it occurs at late stages of spermatogenesis and/or during the posttesticular period.[19] Spermatozoa are relatively deficient in ROS scavenging enzymes, and the degree of sperm DNA fragmentation in ejaculated spermatozoa can be higher than in those obtained from the testis and caput or corpus epididymis.[20] Therefore, the protective, antioxidative effect of melatonin in relation to sperm DNA integrity is expected to be rather immediate and should be seen when analyzing urine samples obtained just before delivery of ejaculates. The production and excretion of aMT6s follows an individual's circadian rhythm, such as melatonin, and the measurement of aMT6s in urine provides a noninvasive, integrated parameter by which these rhythms can be assessed.[21]

Melatonin has been shown to have antioxidative properties, and earlier studies have demonstrated the ability of melatonin to scavenge oxygen-derived free radicals.[22] Damaged sperm DNA measured as increased DFI has been shown to be an independent risk factor for decreased male fertility.[23] In a recent study, it was shown that melatonin supplementation (6 mg melatonin daily >45 days) to infertile men resulted in increased semen antioxidant capacity, a reduction in oxidative associated sperm DNA damage and improved *in vitro* fertilization treatment results.[22] According to Ortiz *et al*., recently proven fathers had higher melatonin and antioxidants levels compared to infertile men, and melatonin had beneficial effects on human spermatozoa *in vitro*.[5] This is in contrast to another recent study in infertile men where melatonin in seminal plasma was positively correlated with damaged sperm DNA, thus indicating that melatonin could have a negative impact on sperm function.[24]

Table 4: The association between DFI and aMT6s excretion in Oslo and Tromsoe computed with a Spearman's correlation coefficient rho (P)

	Rho (P), n		
	Oslo + Tromsoe	*Oslo*	*Tromsoe*
DFI summer versus			
aMT6s early summer	–0.06 (0.444), 195	–0.04 (0.681), 110	–0.16 (0.133), 85
aMT6s late summer	–0.05 (0.473), 196	–0.09 (0.346), 110	–0.09 (0.420), 86
DFI winter versus			
aMT6s early winter	0.07 (0.347), 171	0.11 (0.286), 103	–0.03 (0.786), 68
aMT6s late winter	0.01 (0.907), 191	0.08 (0.404), 106	–0.18 (0.106), 85
Δ-DFI winter–summer versus			
Δ-aMT6s early winter–early summer	–0.08 (0.322), 167	–0.07 (0.485), 102	–0.14 (0.273), 65
Δ-aMT6s late winter–late summer	–0.003 (0.967), 188	0.06 (0.514), 105	0.05 (0.632), 83

Rho: Spearman's correlation coefficient; *n*: numbers; DFI: DNA fragmentation index; aMT6s: 6-sulfatoxymelatonin

Seasonal variation in DFI could, thus, be a causative factor behind fluctuating birth rates. In the present study, we demonstrate that despite extreme daylight variation and seasonal differences in aMT6s values, no significant seasonal variation in DFI was seen, neither does the DFI values correlate with the excretion of aMT6s. The fact that there was a significantly negative correlation between sperm motility and DFI may suggest that DFI mainly reflects immotile or nonviable sperm.

CONCLUSIONS

Our study indicates that DFI does not display any seasonal variation and does not correlate with the seasonal variations in excretion of the major melatonin metabolite aMT6s, and the circannual variation in this sperm parameter is, therefore, hardly involved in the mechanism of seasonal variations in birth rate.

AUTHOR CONTRIBUTIONS

GM, AG, and TBH are responsible for study conception and design. GM wrote the first version of the manuscript. LR and GM performed statistical analyses and interpreted data. All authors approved of the final version to be published.

COMPETING INTERESTS

None of the authors declared competing financial interests.

ACKNOWLEDGMENTS

This research has been given grants from the Swedish Research Council (Grant 2014_3185); the Skane University Hospital Foundation, and the Swedish governmental funding for clinical research.

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