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ORIGINAL ARTICLE

Circadian rhythms of hemostatic factors in tetraplegia: a double-blind, randomized, placebo-controlled cross-over study of melatonin

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Study design: This is a double-blind, randomized, placebo-controlled cross-over study of melatonin in complete tetraplegia.
Objectives: Tetraplegic patients have an increased risk of venous thrombosis despite prophylaxis, blunted variations in melatonin and altered circadian variation of several hemostatic markers. To examine whether melatonin could modify the regulation of hemostasis, we measured plasma melatonin and several markers of hemostasis in tetraplegic subjects with or without melatonin supplement.
Setting: The study was conducted in the Section for Spinal Cord Injury, Sunnaas Hospital, Nesoddtangen, Norway.
Methods: Six subjects with long-standing complete tetraplegia were included in this cross-over study with 2 mg of melatonin or placebo given 4 days before sampling. We also included six able-bodied men without any intervention. Plasma samples were then collected frequently during a 24-h awake/sleep cycle. The plasma concentrations of melatonin and the various markers were analyzed using linear mixed models.
Results: The 24-h profiles of prothrombin fragment 1+2 and von Willebrand factor, but not D-dimer, activated FVII, tissue factor pathway inhibitor and plasminogen activator inhibitor type 1, differed ($P < 0.05$) between tetraplegic patients and able-bodied subjects. The absolute plasma concentration of activated FVII was higher ($P < 0.05$) among the able-bodied compared with the tetraplegic groups. Supplementation of melatonin had no impact on these findings.
Conclusions: We found differences in circadian variation of several hemostatic markers between able-bodied and tetraplegics. These differences were apparently unrelated to fluctuations in the melatonin concentrations, suggesting little or no role of melatonin in the regulation of hemostasis in tetraplegia.
Sponsorship: Financial support was provided from the Throne Holst Foundation.
Spinal Cord (2015) **53**, 285–290; doi:10.1038/sc.2014.243; published online 3 February 2015

INTRODUCTION

Spinal cord injury (SCI) results in a reduction or lack of autonomic control that is directly related to the level of injury, which affects most of the body organs including the cardiovascular system,[1] giving rise to acute cardiovascular complications,[2] as well as hypotension and altered vascular regulation. Patients with SCI currently have a life expectancy that is approximately the same as able-bodied subjects, and they are therefore susceptible to the same chronic conditions across the life span. In fact, cardiovascular disease is one of the leading causes of mortality in both able-bodied subjects and in patients with SCI.[3] Whereas in able-bodied subjects there is an increased cardiovascular disease risk in the few hours after waking up in the morning, postulated to be owing to the early-morning surge in blood pressure, this is apparently not the case with SCI patients.[4] Moreover, in able-bodied subjects, the incidence of both venous and arterial thrombotic disorders seems to peak in the morning.[5,6] In line with this, the plasma concentrations of several hemostatic factors vary according to specific circadian patterns, including certain coagulation factors and markers of coagulation activation, the coagulation inhibitors protein C, protein S and tissue factor pathway inhibitor (TFPI), as well as the fibrinolytic inhibitor plasminogen activator inhibitor type 1 (PAI-1).[7–9]

The biological mechanisms causing these circadian variations are poorly understood. Experiments in transgenic mice and *in vitro* studies demonstrated that variations of coagulation factor VII were driven at the transcriptional level through the recruitment of circadian transcription factors.[10,11] Furthermore, deletion or mutation of circadian transcription factors in mice changed the time to thrombotic vascular occlusion.[12] In addition, circadian variations of mRNA expression of coagulation and fibrinolytic factors have been demonstrated in several murine organs.[13] These studies indicate that blood coagulation is influenced by endogenous biological clocks and daylight.

Despite recommendation-based preventive use of antithrombotic medication, the incidence of venous thrombosis (VT) is unacceptably high in patients suffering from paraplegia or tetraplegia, with about 1

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Received 12 August 2014; revised 3 December 2014; accepted 17 December 2014; published online 3 February 2015

in 4 being affected.[14,15] It is therefore of interest that in subjects with tetraplegia the circadian rhythm of several hemostatic factors varies either more (for example, PAI-1) or less (for example, D-dimer and TFPI) than in able-bodied subjects.[7] Moreover, the mean plasma concentrations differ between able-bodied and tetraplegic subjects (for example, elevated fibrinogen and PAI-1 and reduced von Willebrand factor plasma levels in tetraplegia).[7]

Melatonin is an important signaling molecule for circadian rhythms of many biological processes, but its circadian variation is almost totally blunted in tetraplegic subjects.[16] Notably, in able-bodied subjects, we previously found that the plasma concentrations of free TFPI and melatonin varied with seemingly opposite rhythms: when plasma melatonin reached its nadir values, plasma TFPI free antigen peaked and vice versa.[17] In tetraplegia, however, the plasma concentrations of neither free TFPI nor melatonin displayed any circadian rhythm.[17] We next showed that melatonin stimulated vascular endothelial cells grown in vitro to selectively secrete TFPI without altering transcription of the TFPI gene.[18] If melatonin increases TFPI release in a similar manner in vivo as in vitro, this could have potential clinical implications in both prophylaxis and treatment of thrombo-embolic events. Hence, we have now conducted a double-blind, randomized placebo-controlled cross-over study and measured various hemostatic factors in blood sampled repeatedly throughout a 24-h cycle in tetraplegic subjects with or without supplementation of melatonin.

MATERIALS AND METHODS
Subjects and design of study
The study was approved by the Regional Committee for Medical Health Research Ethics in Norway (no. 2010/295) and the Norwegian Medicines Agency (EUDRACT no. 2010-021212-24), and it is registered with Clinical-trials.gov unique identifier NCT 01741389.

The study was designed as a double-blind, randomized cross-over study of six tetraplegic men conducted at Sunnaas Hospital in December 2012. We also included a control group of six able-bodied men. Hence, we studied three groups: tetraplegic men with placebo, tetraplegic men with melatonin and able-bodied men (Table 1). The intervention in the tetraplegic group was capsules with placebo (Kragerø Tablettproduksjon AS, Kragerø, Norway) or 2 mg melatonin (Circadin; Neurim Pharmaceuticals, Zug, Switzerland). The able-bodied group received no intervention. The tetraplegic men were randomized to receive melatonin or placebo (daily at 22:00 h) for 4 days before they were subjected to a 24-h period of blood sampling. Randomization was performed by a person not involved in the study and using the software program www.randomization.com. and using sealed envelopes. Blood was drawn 10 times during a 24-h cycle (at 7, 12, 16, 18, 20, 22, 24, 2, 4 and 7 h). Then, the tetraplegic men were crossed over, and thus the three tetraplegic men who first received melatonin received placebo for 4 days and the three tetraplegic men who first received placebo received melatonin for 4 days before both groups went through a new period of 24-h sampling. The values measured at 07:00 h on day 1 did not differ significantly from those measured at 07:00 h on day 2. The 'wash-out' period lasted for 4 days before the cross-over, which is

assumed to be sufficient because the half-life of melatonin is about 35–50 min, thus ensuring minimal, if any, carry-over effect. The able-bodied men were subjected to a similar 24-h period of blood sampling without any intervention and with normal sleep. All the participants received standardized meals at regular time points. The only restriction was no alcohol intake, and a maximum of two cups of coffee were required by the participants during the study period. They slept only between 23:00 and 06:00 h (daylight from about 09:00 till 15:00 h).

Blood sampling
Venous blood samples were collected in 5-ml Vacutainer vacuum tubes containing 0.5 ml of buffered sodium citrate (0.129 M) (Becton-Dickinson, Plymouth, UK), and in 4.9-ml Monovette tubes containing potassium-ethylenediaminetetraacetic acid (Sarstedt AG, Nümbrecht, Germany). Citrated blood was kept at room temperature and immediately centrifuged at 2000 g for 15 min. Platelet-poor plasma aliquots and EDTA–blood were stored at − 70 °C until they were assayed.

Assays
Activated factor VII (factor VIIa), free TFPI antigen, von Willbrand factor (VWF) and D-dimer were analyzed using commercial ELISA assays (Staclot VIIa-rTF, Asserachrom Free TFPI, AsserachromVWF:Ag and Asserachrom D-DI) from Diagnostica Stago (Asnières, France), PAI-1 activity was analyzed with the Zymutest PAI-1 activity kit from Aniara Corporation (West Chester, OH, USA) and prothrombin fragment 1+2 (F_{1+2}) was analyzed with a kit from Siemens Healthcare (Erlangen, Germany). The lowest detection values are 6 mU ml^{-1} for FVIIa, 3 ng ml^{-1} for free TFPI, 5% for VWF, 60 ng ml^{-1} for D-dimer, 0.6 ng ml^{-1} for PAI-1 activity and 20 pmol ml^{-1} for F_{1+2}. The intra-assay coefficients of variation were < 5.2% for all the assays, whereas the inter-assay coefficients of variation were between 2.9 and 11.2%.

Melatonin was first extracted from the blood plasma samples using a vacuum manifold procedure, as described in the Direct Saliva Melatonin ELISA kit obtained from Buhlmann Laboratories AG (Basel, Switzerland). The extracted melatonin concentrations were then quantified using the Buhlmann Laboratories ELISA kit, which was based on a melatonin biotin conjugate, an enzyme label and a tetramethyl benzidine substrate. The product of the substrate was measured spectrophotometrically at 450 nm. The assay sensitivity range was 1–60.6 pg ml^{-1}.

All analyses were performed examiner-blind, and the samples were run in-batch using a balanced setup with equal number of cases and controls in each run.

Statistics
Formal power calculation was considered impossible owing to lack of relevant information. We reasoned that the tetraplegic subjects with and without melatonin supplementation would differ with respect to the circadian variation of the studied hemostatic markers. The statistical analyses were performed with SPSS version 18.0 (IBM Corporation, Chicago, IL, USA) and the MedCalc Software (Ostend, Belgium). We considered P-values ⩽ 0.05 to indicate statistical significance. Values are given both as mean absolute plasma concentrations with s.e.m. and as mean percent change (s.e.m.) from the starting point at 07:00 h on day 1. Circadian variations, as well as differences in the absolute plasma concentrations of the various coagulation parameters between the three study groups, were evaluated with linear mixed models.

RESULTS
Figure 1 shows that, as expected, the plasma concentrations of melatonin remained quite low and with virtually no significant circadian pattern among the tetraplegic subjects who received placebo. When they were given melatonin, a circadian pattern of plasma melatonin appeared that resembled that of the reference group of able-bodied subjects, although with a peak concentration considerably above that for the able-bodied subjects, probably as a result of the 4-day treatment with melatonin before the blood-sampling period.
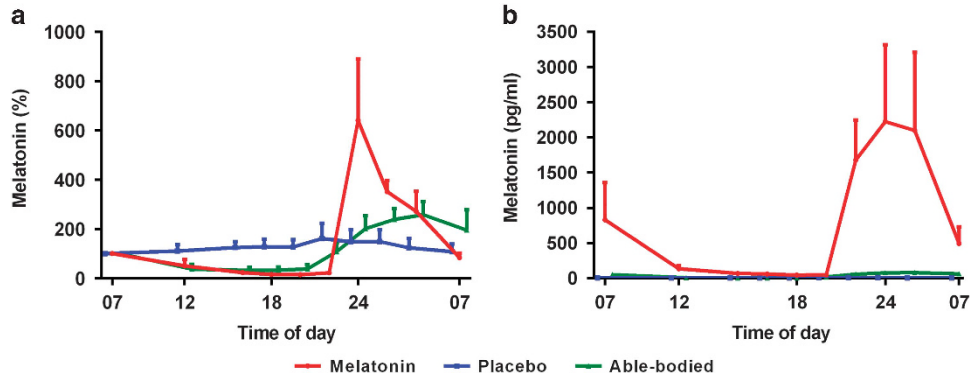
Table 1 Characteristics of the study subjects

Parameter	Tetraplegia (n = 6)	Able-bodied (n = 6)
Age (years)	45 (27–60)	42 (34–54)
Level of injury	Cervical 5—Cervical 8	—
Time since injury (years)	16 (3–43)	—
BMI (kg m^{-2})	25.4 (23.8–26.6)	26.6 (20.1–35.3)

Abbreviation: BMI, body mass index.
Values are medians (range).

Figure 1 Plasma concentrations of melatonin. Values (mean+s.e.m.) are expressed as percentages (**a**) or as absolute plasma concentrations (**b**). Data are from tetraplegic subjects receiving either placebo (blue line) or melatonin (red line), and from a reference group of healthy able-bodied subjects (green line). The lines are somewhat mutually offset for illustration purposes.
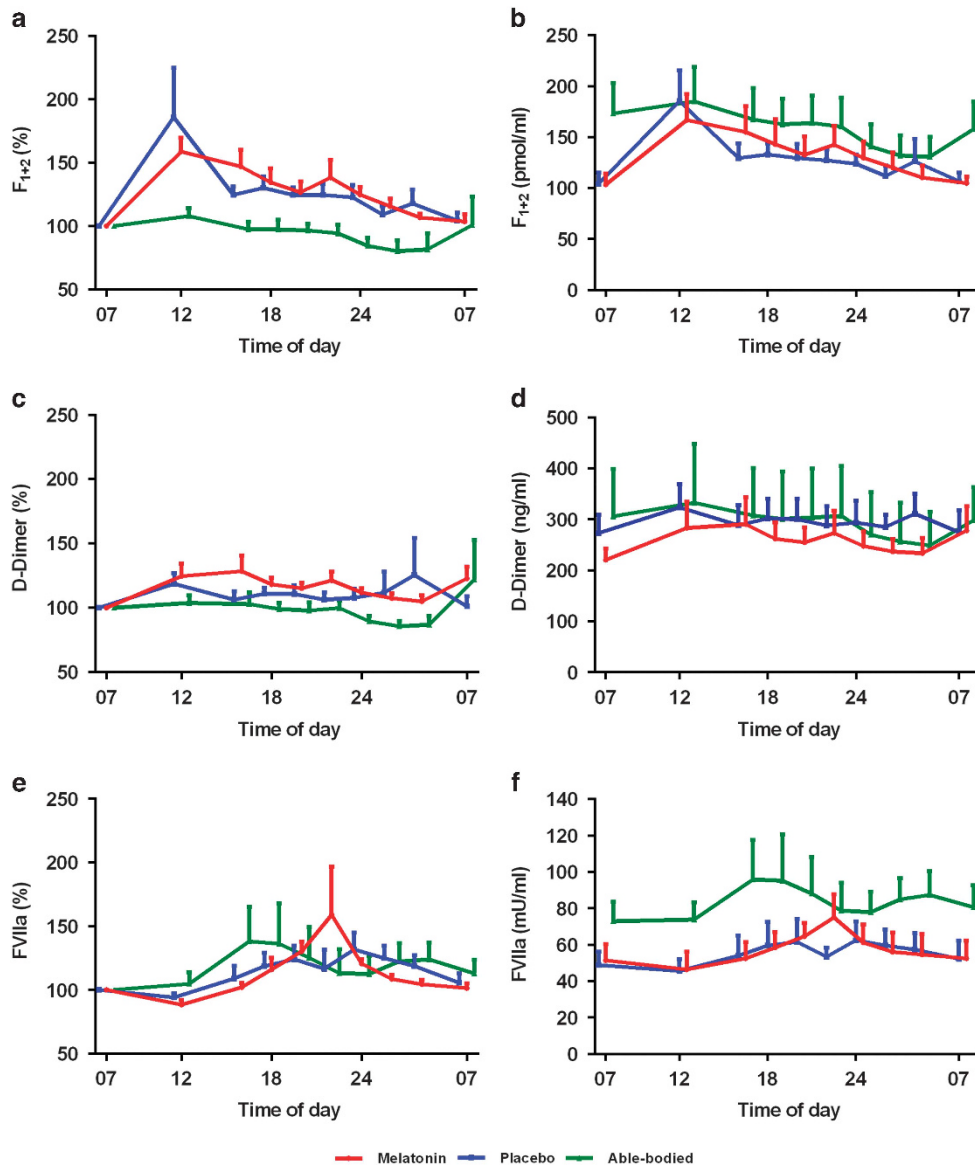

Figure 2 Plasma concentrations of markers of coagulation. Values (mean+s.e.m.) are expressed as percentages (**a**, **c**, **e**) or as absolute plasma concentrations (**b**, **d**, **f**). The panels show data for F_{1+2} (**a**, **b**), D-dimer (**c**, **d**) and FVIIa (**e**, **f**). Data are from tetraplegic subjects receiving either placebo (blue line) or melatonin (red line), and from a reference group of healthy able-bodied subjects (green line). The lines are somewhat mutually offset for illustration purposes.
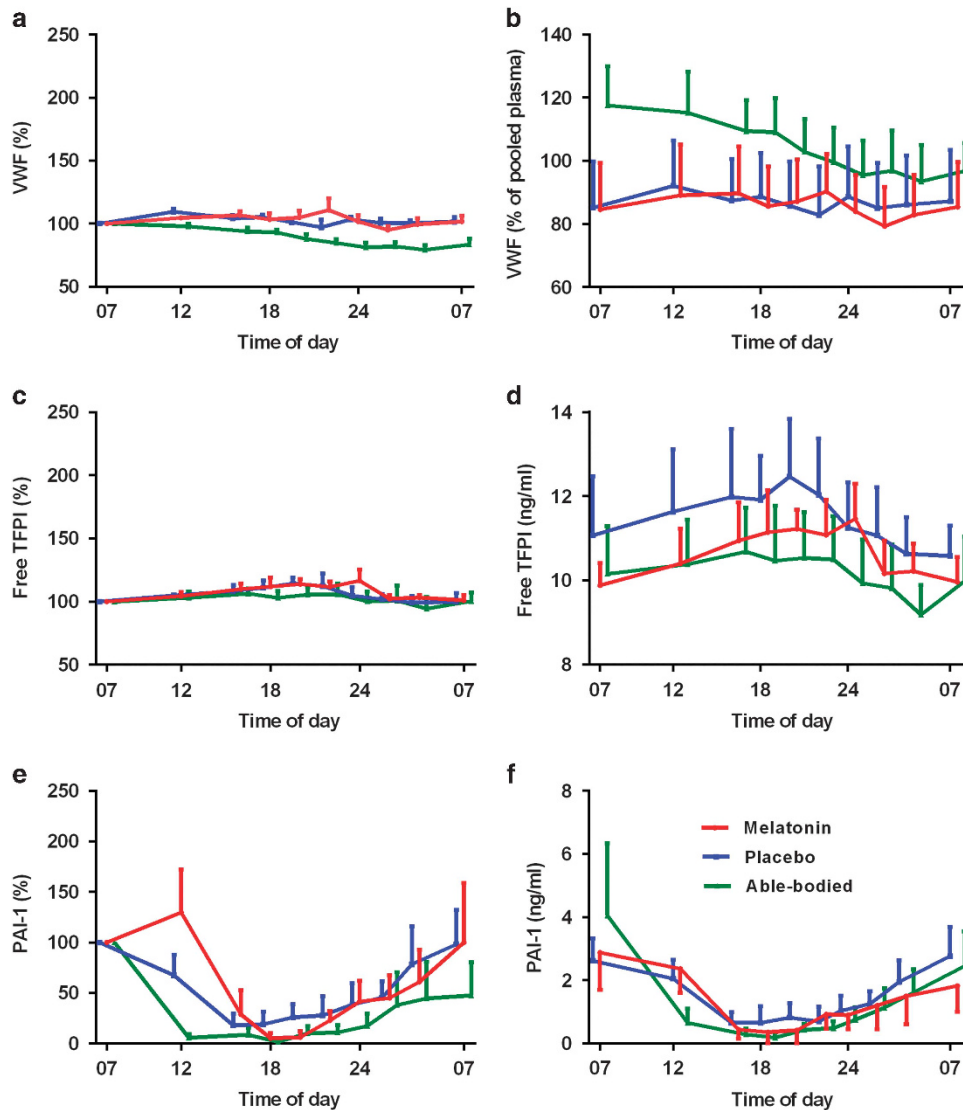
Figure 3 Plasma concentrations of various hemostatic markers. Values (mean+s.e.m.) are expressed as percentages (**a**, **c**, **e**) or as absolute plasma concentrations (**b**, **d**, **f**). The panels show data for VWF (**a**, **b**), free TFPI (**c**, **d**) and PAI-1 (**e**, **f**). Data are from tetraplegic subjects receiving either placebo (blue line) or melatonin (red line), and from a reference group of healthy able-bodied (green line). The lines are somewhat mutually offset for illustration purposes.

The tetraplegic subjects reported unchanged sleeping duration and sleeping quality irrespective of whether they received placebo or melatonin.

Having established that supplementation of melatonin in tetraplegia could mimic its endogeneous circadian variation, we next examined the 24-h plasma profiles of various hemostatic markers. The circadian patterns of the F_{1+2}, a marker of thrombin generation, and D-dimer, a marker of activated coagulation and fibrinolysis, were apparently similar ($P > 0.05$) between the tetraplegic groups, that is, irrespective of whether they were given melatonin (Figures 2a and c). Compared with the able-bodied group, the tetraplegic groups with or without melatonin supplementation showed an apparent increase in the circadian variation of F_{1+2} ($P = 0.01$ and $P < 0.001$, respectively), whereas the circadian pattern for D-dimer was similar ($P > 0.05$) across the three study groups. The absolute values for the plasma concentrations of F_{1+2} and D-dimer (Figures 2b and d) were similar ($P > 0.05$) in all three study groups.

For FVIIa, no significant changes were observed for the circadian variation (Figure 2e) among the three study groups. The absolute plasma concentration was higher among the able-bodied subjects compared with the tetraplegic groups with ($P = 0.050$) or without ($P = 0.039$) melatonin (Figure 2f). With regard to VWF, which is a marker of endothelial function, the able-bodied subjects had a different circadian variation characterized by decreasing ($P < 0.001$) VWF concentrations during the night (Figure 3a). The absolute plasma concentration for VWF was, however, not different ($P > 0.05$) in the able-bodied reference group compared with tetraplegic subjects with or without supplementation of melatonin (Figure 3b).

The plasma concentrations of free TFPI, the only endogenous inhibitor of tissue factor induced coagulation, did not differ significantly with regard to circadian variations (Figure 3c) or absolute plasma concentration (Figure 3d) between the able-bodied and the tetraplegic subjects with or without supplementation of melatonin. Similarly, the plasma concentrations of PAI-1, a fibrinolytic inhibitor,

did not differ ($P > 0.05$) among the three study groups, with regard to circadian variations (Figure 3e) or absolute plasma concentration (Figure 3f).

DISCUSSION

We here show that melatonin, but not placebo, supplementation for 4 days to patients with stable, complete tetraplegia could nearly restore the 24-h profile of this major biological clock regulator. However, melatonin supplementation did not induce any major alterations in the circadian variation of a wide range of hemostatic markers when their plasma concentrations were compared with those in tetraplegic patients given placebo. Yet, we did demonstrate differences in the circadian variation of several hemostatic markers between tetraplegic and able-bodied subjects, suggesting that these differences might be unrelated to fluctuations in the melatonin concentrations.

The absolute peak concentrations of melatonin were considerably higher among the tetraplegic patients receiving melatonin compared with the endogeneous concentrations in the able-bodied reference subjects. Importantly, these concentrations apparently did not induce any adverse effects among the study patients, and the dose used (2 mg) is the one recommended for treating sleeping disorders, the most common indication for melatonin supplementation.

Even with recommended prophylaxis, for example, the use of low-molecular-weight heparins, patients suffering from SC are prone to VT. In line with this, over a median follow-up period of 36 months, Giorgi et al.[15] recently found that VT was diagnosed in 23.4% SCI patients despite thromboprophylaxis. Similarly, Chung et al.[19] reported adjusted hazard ratios of 2.46 and 1.57, respectively, for the risk of deep vein thrombosis and pulmonary embolism in SCI patients compared with the general population in a nationwide survey in Taiwan. Moreover, the risk for VT is highest in the acute phase following the SCI, suggesting that immobilization alone cannot explain the increased risk of venous thromboembolism. Collectively, the time-dependent decline in the risk of VT and the inadequate effect of current antithrombotic prophylaxis emphasize the need for a better understanding of the regulation of hemostasis in SCI patients.

The altered circadian variations in the concentrations of several hemostatic markers coupled with a dysregulated 24-h profile of melatonin concentrations in tetraplegia and a putative regulatory role of melatonin on vascular endothelial secretion of free TFPI in vitro[5,17,18] prompted us to examine the effect of melatonin supplementation on hemostatic markers in these patients. Our present findings indicate similar effects regarding the absolute plasma concentrations of F_{1+2} and D-dimer, as well both the 24-h profiles and absolute plasma concentrations of D-dimer, TFPI and PAI-1. On the other hand, the 24-h profile of the concentrations of F_{1+2} and VWF were different among the able-bodied subjects compared with those in the tetraplegic patients; however, melatonin supplementation had no apparent effect on the circadian pattern of F_{1+2} and VWF. With regard to FVIIa, the able-bodied subjects had apparently higher absolute plasma concentrations than both the tetraplegic groups, but similar 24-h profiles.

We cannot exclude the possibility that our repeated blood sampling may have been a stressor on the hemostatic factors. Saliva sampling might have overcome this possible confounder; however, this sampling technique was not available to us. Despite the low number of participants, our study was robustly designed and the study subjects were carefully monitored under standardized conditions during the 24-h study periods. Moreover, the tetraplegic group was rather uniform, as only men were included and all had a complete and stable, long-standing injury (>3 years). The tetraplegic and able-bodied subjects were well matched with regard to gender, age and body mass index. In addition, the level of injury, a factor that may affect the risk of venous thromboembolism, showed little variation (Cervical 5–Cervical 8).[20]

In conclusion, it appears that there are differences in the circadian variations of the coagulation system between tetraplegic and able-bodied subjects, as illustrated by the 24-h profiles of F_{1+2}, FVIIa and VWF; however, they are probably unrelated to melatonin. To gain further insight into the regulation of hemostasis in SCI, detailed studies of clotting formation and fibrin production should probably be conducted.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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