

ORIGINAL ARTICLE

Circadian variation in haematological toxicity of the immunosuppressive agent “Mycophenolate Mofetil” in rats

Ichrak Dridi¹, Wafa Ben-Cherif¹, Karim Aouam¹, Mohsen Hassine², Mossadok Ben-Attia³, Alain Reinberg⁴, Naceur Abderrazak Boughattas¹

¹Laboratory of Pharmacology, Faculty of Medecine, University of Monastir, Tunisia

²Laboratory of Hematology, CHU Fattouma Bourguiba, Monastir, Tunisia

³Laboratory of Biomonitoring of the Environment (LR01/ES14), Faculty of Science of Bizerta, University of Carthage, Bizerta, Tunisia

⁴Unit of Chronobiology, Foundation A. de Rothschild Paris, France

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Summary

Because of biological rhythms, drug efficiency and toxicity vary according to the time of administration of the drug. This study investigates whether the haematological toxicity of the immunosuppressive agent Mycophenolate Mofetil varies according to the circadian dosing-time in rats. 300 mg/kg of Mycophenolate Mofetil was injected by *i.p.* route to different groups of animals at six different circadian stages (1, 7, 13, and 19 hours after light onset, i.e., HALO). Mycophenolate Mofetil treatment induced a significant decrease at 7 HALO in red blood cells (–18%), in haemoglobin rate (–15%) and in white blood cells (–54%). These parameters followed a circadian rhythm with an acrophase located at the end of the light-rest phase. A significant thrombocytopenia was observed according to Mycophenolate Mofetil the circadian dosing-time. In the controls, the number of platelets followed a circadian rhythm. Mycophenolate Mofetil modified this rhythm which became an 8-h ultradian rhythm. The data indicate that, the Mycophenolate Mofetil-induced haematological toxicity was maximum when the drug was administered in the middle of the light-rest phase, which is physiologically analogous to the end of the activity of the diurnal phase in human patients.

Key words: circadian rhythm; immunosuppressive agent; MMF; hematological toxicity; rat

INTRODUCTION

Chronobiological studies have been of great help in almost all medical fields but especially in chronopharmacology. The chronobiology studies

examine the relationship between the temporal factor and living beings (Reinberg 1992, Berger 2011). The temporal changes in drug effects include variations in both the desired (chroneffectiveness) and undesired (chronotoxicity) effects (Ben-Cherif et al. 2012). These biological responses to various drugs follow circadian rhythms in experimental animals as well as in human beings. Many drugs vary in potency and/or toxicity associated with the rhythmicity of biochemical, physiological, and behavioural processes. The choice of animal models is a revolutionary tool for chronobiological studies that provide some obvious benefits (Ohkura et al. 2007a, b). Experiments, which would not be feasible on humans, can be conducted on animals and the genetic background as well as most environmental

✉ Ichrak Dridi, Laboratory of Pharmacology, Faculty of Medecine, University of Monastir, 5019 Monastir, Tunisia

✉ dridi.ichrak@yahoo.fr

☎ + (216)73462200

☎ + (216)73460737

factors can be controlled. Such models have served as powerful tools for understanding the circadian rhythms of several biological variables.

The success of Mycophenolate Mofetil (MMF) in the field of solid organ transplantation (Neumann et al. 2003, Laskari et al. 2010) has made this drug attractive for the treatment of several autoimmune diseases (Sollinger 1995, Laskari et al. 2010). It is an ester prodrug of mycophenolic acid (MPA). Indeed, MMF is rapidly hydrolyzed in the gastrointestinal tract to MPA, which is quickly and almost completely absorbed (Bullingham et al. 1998). MPA, the major metabolite and active component of MMF, is a potent, reversible, non competitive inhibitor of inosine monophosphate dehydrogenase, the key rate-limiting enzyme involved in the *de novo* purine biosynthesis of guanosine nucleotides (Eugui and Allison 1993, Cantarovich et al. 2011). B and T lymphocytes rely on this *de novo* pathway for the generation of guanosine nucleotides.

Among the side effects of immunosuppressive agents are haematological toxicity such as anaemia, due to bone marrow suppression or haemolysis (Danesi and Del Tacca 2004), thrombocytopenia and leukopenia which is an important adverse event associated with MMF administration (Mackie et al. 1996, Virji et al. 2001).

The present work is aimed at investigating whether murine haematological MMF toxicity varies according to circadian dosing-time.

MATERIAL AND METHODS

Animals and synchronization

A total of 80 male Wistar rats aged 6 to 8 weeks (SIPHAT, Tunisia) were synchronized for 3 weeks prior to the beginning of experiments in two air-conditioned rooms. The rooms were specially designed for chronobiological investigations by having an inverted light regimen to explore several circadian stages during the usual diurnal work span.

The animals were synchronized with an alternating 12 h light (L) and 12 h dark (D) cycle (light: 07:00 h to 19:00 h and dark: 19:00 h to 07:00 h). The room

temperature was maintained at 22±2 °C and the relative humidity was about 50–60%.

All experiments were performed according to the guidelines for the care and use of laboratory animals.

During all experiments, a standard rat diet (Purina Rat Chow; SICO, Sfax 3000, Tunisia) and water were provided *ad libitum*. The animals were randomly divided into 4 groups of 20 rats each, which corresponded to the four explored circadian stages denoted as 1, 7, 13 and 19 Hours After Light Onset (HALO).

The synchronization of animals was checked by assessing the circadian rhythm in rectal temperature which was measured with a digital thermometer. The acrophase (peak time) was used as a marker rhythm index.

Drug

MMF is a white crystalline powder. It was kindly provided by Medis laboratories (Nabeul, Tunisia). MMF has an empirical formula of $C_{23}H_{31}NO_7$.

It was freshly prepared on each day before injections by adding an adequate volume of sterile distilled water to obtain the desired concentrations. The drug was administered to the rats by *i.p.* route in a fixed fluid volume (10 ml/kg *b.w.*).

Study design

The study design is summarized in Table 1. A total of 80 rats were treated at four different circadian stages (1, 7, 13 and 19 HALO). The 20 control rats (5 rats per time point) received a saline injection. The 60 MMF-treated rats (15 rats per time point) were injected a 300 mg/kg dose by *i.p.* route.

Blood cell counts

The blood samples were taken three days after injection (maximum toxicity). All blood samples were withdrawn by cardiac puncture carried out under anaesthesia, taking into account the circadian injection time. Blood was collected in EDTA-treated tubes. The count of red blood cell (RBC), white blood cell (WBC) and platelets (PLT) per μ l was performed using an automatic counter (Medonic 650). Haemoglobin concentration (HGB) in g per l and haematocrit (%) was calculated automatically.

Table 1. Main characteristics of the study investigating hematological chronotoxicity of Mycophenolate Mofetil in male Wistar rats.

Drug	Dose	Number of rats	Circadian stages (HALO)	Hematological variables
Control (NaCl)	0.9%	20 (5 per stage)	1, 7, 13, 19	WBC, RBC, PLT, HGB, hematocrit
MMF	300 mg/kg	60 (15 per stage)		

Statistical analysis

Animal synchronization was verified by the Cosinor analysis (based on the least-squares method) of the 24 h body temperature marker rhythm to validate the circadian rhythm (with a trial period $t \frac{1}{4} 24$ h). A rhythm is validated by the rejection of the hypothesis whose amplitude is null. Rhythm detection was considered statistically significant at the significance level $2\alpha=0.05$. The rhythm is then characterized by the following parameters: i) the amplitude (A), ii) the mesor (M: the 24 h rhythm-adjusted mean) and iii) acrophase (\emptyset : the peak time of the cosine function) (Halberg 1969, Nelson et al. 1979). A, M, and \emptyset are given with their 95% confidence limits when rhythm detection is statistically significant (Sani et al. 2011).

All results were expressed as the mean values (m) with \pm standard deviation (SD). Significance was

calculated by one way-ANOVA at the significance level $2\alpha=0.05$.

RESULTS

Rectal temperature

The rectal temperature rhythm was used as a marker of circadian synchronization of the animals. The analysis of the time series of rectal temperature by the Cosinor test revealed a statistically significant circadian rhythm with an acrophase which is usually located near the mid-dark activity span ($16.7 \text{ HALO} \pm 1.1 \text{ h}$) (Fig. 1). The characteristics of the 24-h pattern in rectal temperature confirmed the physiological synchronization of the animals to the environmental L/D: 12/12 schedule.

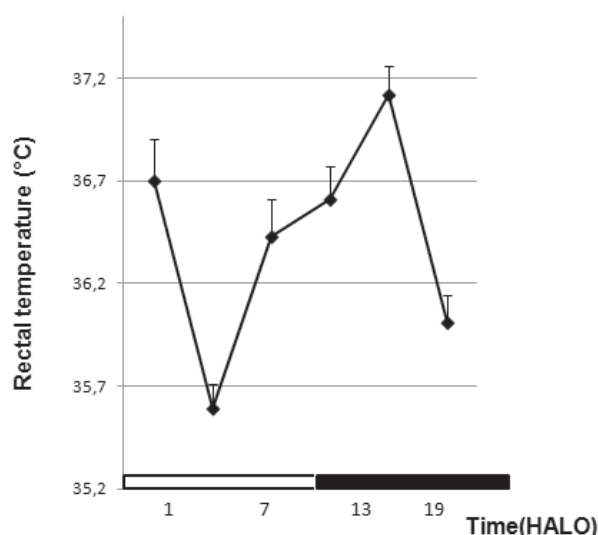


Fig. 1. Variation of temperature according to the circadian time before treatment.

MMF effect on the red blood picture

In controls, the maximum RBC counts ($7.37 \pm 0.15 \cdot 10^{12}/l$) and the HGB concentration ($1.53 \pm 0.01 \text{ g/l}$) were located at 7 HALO. The minimum ones were located at 19 HALO ($5.93 \pm 0.35 \cdot 10^{12}/l$) and ($1.3 \pm 0.02 \text{ g/l}$) respectively.

A similar circadian rhythm in RBC counts and HGB concentration was observed in controls with an acrophase located at $8 \text{ HALO} \pm 0.26 \text{ h}$ and $8.9 \text{ HALO} \pm 0.2 \text{ h}$ respectively. The 24 hr-means of RBC count and HGB concentration decreased in MMF-treated rats (Fig. 2, Table 2).

MMF induced a statistically significant decrease in RBC counts (-15% to -18%) and HGB concentration

(-13% to -15%) whatever the circadian time of MMF injection (Fig. 2a, b). These two parameters followed a statistically significant circadian rhythm ($\tau = 24 \text{ h}$). A circadian variation of HCT rate was validated significantly in controls (Fig. 2c) with the highest values observed at 13 HALO ($39.7\% \pm 0.6$) and the lowest ones at 19 HALO ($34.5\% \pm 0.5$). In treated animals, the HCT rate decreased significantly only at 1 and 19 HALO (-16.3% vs. -28.7%) [$F_{(79,76)} = 6.36$].

In controls and treated animals, the HCT rate followed a similar circadian rhythm with an acrophase located at the end of the light-rest phase ($10 \text{ HALO} \pm 0.24 \text{ h}$ vs. $9.9 \text{ HALO} \pm 0.15 \text{ h}$ respectively).

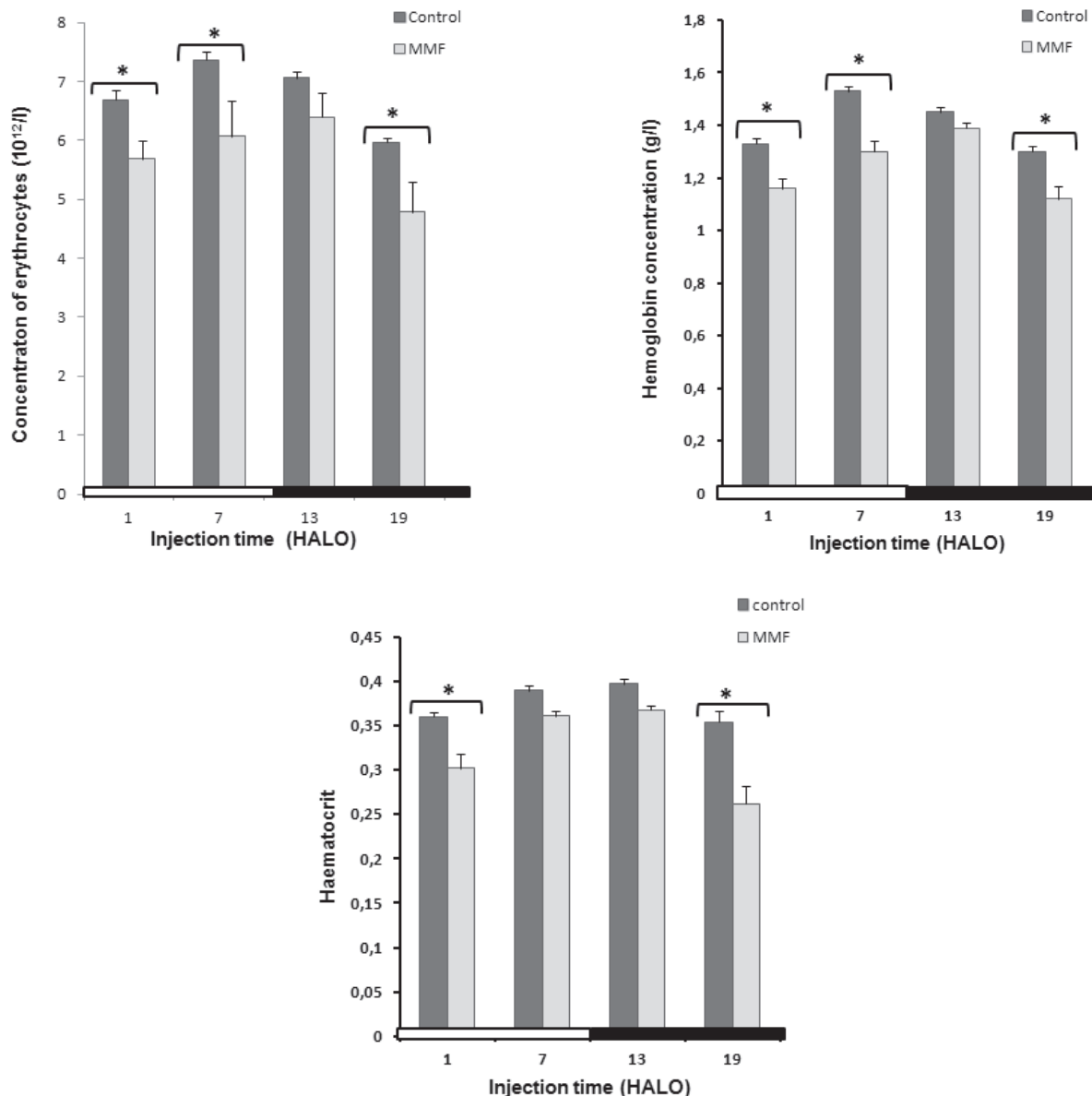


Fig. 2. **Effect of Mycophenolate Mofetil (300 mg/kg *i.p.*) on the red blood picture;** red blood cell counts (A) hemoglobin concentration (B) and hematocrit rate (C) of male rats treated at 1, 7, 13 and 19 HALO; * statistically significant as compared with controls.

MMF effect on leukocyte line

The circulating white blood cells (WBC) varied significantly according to a physiological circadian rhythm with an acrophase located in the middle of the light-rest phase ($\bar{\theta} = 6.9 \pm 0.39$) in control rats.

MMF treatment induced a decrease in the 24hr-mean of WBC counts in the treated rats ($6.96 \pm 1.2 \cdot 10^9/l$) as compared to the controls ($10.2 \pm 1.6 \cdot 10^9/l$).

The WBC count decrease varied according to the MMF circadian dosing-time. It was statistically

significant in treated animals at 7 (–54%) and 13 HALO (–42%) [$F_{(79,76)} = 5.22$].

No significant leucopenia was observed when MMF was injected at 1 and 19 HALO.

In MMF-treated animals, the cosinor analysis revealed a circadian rhythm in WBC counts with an acrophase shifted to the beginning of the light-rest phase ($2.13 \text{ HALO} \pm 1.25 \text{ h}$) as compared to controls (Table 2).

Table 2. Circadian rhythm parameters of five hematological variables in controls and Mycophenolate Mofetil-treated rats. Data represents the estimated chronobiological parameters \pm SD.

	Treatment	Period	Mesor \pm SD	Amplitude \pm SD	Acrophase (HALO \pm h)
WBC ($10^9/l$)	Control	24	10.2 \pm 0.28	3.95 \pm 0.4	6.9 \pm 0.39*
	treated	24	6.97 \pm 0.48	2.11 \pm 0.69	2.13 \pm 1.25*
PLT ($10^9/l$)	Control	24	871 \pm 22.6	144 \pm 32	1.9 \pm 0.4*
	treated	8	663 \pm 34.5	175 \pm 10	11.4 \pm 0.2*
RBC ($10^{12}/l$)	Control	24	6.77 \pm 0.04	0.73 \pm 0.05	8 \pm 0.26*
	treated	24	5.74 \pm 0.1	0.73 \pm 0.14	8.9 \pm 0.76*
HGB (g/l)	Control	24	14.1 \pm 0.05	1.31 \pm 0.06	8.9 \pm 0.2*
	treated	24	12.5 \pm 0.1	1.47 \pm 0.15	10.5 \pm 0.4*
HTC (%)	Control	24	37.6 \pm 0.1	2.52 \pm 0.15	10 \pm 0.24*
	treated	24	33.1 \pm 0.13	4.74 \pm 0.2	9.9 \pm 0.15*

* Statistically significant

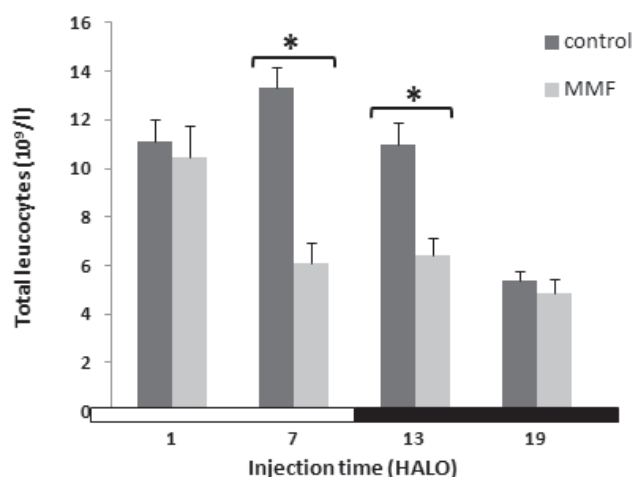


Fig. 3. Effect of Mycophenolate Mofetil (300 mg/kg *i.p.*) on white blood cell counts of rats treated at 1, 7, 13 and 19 HALO. * Statistically significant as compared with controls.

Impact of MMF treatment on thrombocyte line

The 24 hr-mean PLT count decreased significantly from 870.8 \pm 70.6 $10^9/l$ in controls to 645.1 \pm 66 $10^9/l$ in MMF treated rats [$F_{(79,76)} = 6.64$], irrespective of blood sampling-time.

In controls, the cosinor analysis of the physiological variation of PLT counts revealed a circadian rhythm with an acrophase located near the middle of the light-rest phase (4.9 HALO \pm 0.8 h) (Table 2).

MMF treatment induced a significant decrease in PLT counts at 7 HALO (–33%) [$F_{(79,76)} = 3.8$]. An absence of thrombocytopenia was observed when MMF was injected at the other circadian times. In

MMF-treated rats no significant 24 hr-rhythm was detected in PLT counts.

DISCUSSION

The administration of immunosuppressive drugs may be associated with a great deal of haematological toxicity (Danesi and Del Tacca 2004). The present work aims to investigate whether murine haematological toxicity of the immunosuppressant agent MMF varies according to circadian dosing-time. The circadian rhythm in the number of circulating blood

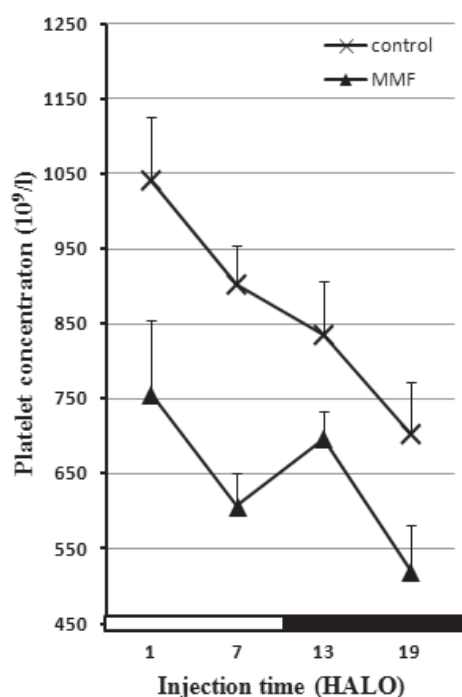


Fig. 4. Effect of Mycophenolate Mofetil (300 mg/kg *i.p.*) on the number of platelets of male rats treated at 1, 7, 13 and 19 HALO.

cells in mammals is highly regular and reproducible (Haus et al. 1983). However, the complex nature of this regulation has prevented the elucidation of the underlying mechanisms of circadian changes in circulating blood cells (Haus and Smolensky 1999).

The results obtained in the present study suggest that the circadian time at which MMF is administered is important.

In each study, the chronobiological synchronization of animals is an essential step. It was verified using the rectal temperature as a marker rhythm. The circadian peak of rectal temperature usually occurs in the middle of the dark-activity span of rats. The peak times of rectal temperature were localized at $16.7 \text{ HALO} \pm 1.1 \text{ h}$. This peak location coincides with the occurrence of the highest physical activity in mice, *i.e.* during the dark span (Khedhaier et al. 2003, Ben-Cherif et al. 2012).

This work has shown that MMF administration resulted in a change in the rates of various haematological parameters. It caused a decrease in the RBC count, HGB concentration and HCT level. This reduction was significant when the drug was injected at 1, 7 and 19 HALO for the RBC count and HG concentration. It was also significant at 1 and 19 HALO for HCT rate. This treatment caused anaemia at 1, 7 and 19 HALO, which is in accordance

with several previous studies that have shown MMF treatment induced haematological toxicity and could cause anaemia (EBPG 2002a).

The general mechanisms responsible for haematological toxicity following treatment with immunosuppressant agents, including MMF, range from cross-reactivities of targets on circulating elements (ATG/ALG, OKT3) to a direct lymphocytolytic effect (immunosuppressive corticosteroids) and inhibition of critical signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), AKR mouse T-cell lymphoma (AKT), mammalian target of rapamycin (mTOR)/p70S6 kinase (p70S6K), which are involved in the suppression of apoptosis, responses to growth factors and cellular proliferation (sirolimus). Finally, direct inhibition of nucleic acid synthesis may result in bone marrow suppression, as in the case of MMF and AZA. In particular, *de novo* purine and nucleic acid synthesis is suppressed by MPA, a selective, reversible, noncompetitive antagonist of inosine monophosphate dehydrogenase (IMPDH) activity of bone marrow cells and lymphocytes (Weber et al. 2002). IMPDH catalyses the rate-limiting step in the *de novo* synthesis of guanine nucleotides from inosine monophosphate (IMP). Therefore, the inhibition of IMPDH activity by MPA, a major metabolite of MMF, may result in the induction of apoptosis or inhibition of cell proliferation (Danesi et al. 2000).

In the controls, RBC counts, HGB concentration and HCT level follow a circadian rhythm. MMF treatment did not alter the circadian rhythmicity of these haematological parameters. This result suggests that the circadian rhythm in the number of blood cells is dependent on core components of the circadian clock. Several hormones such as steroid hormones play an important role in generating the circadian rhythm of circulating blood cells (Ottaway and Husband 1994) whose functioning are probably not affected by the immunosuppressive agent MMF, because it does not alter the rhythmicity of these haematological parameters.

In comparing the amplitudes of these parameters, the amplitude of RBC counts is less marked in both controls and treated. It's well known that the amplitude of the RBC rhythm is not physiologically large (Haus 1996). This might be related to the circulating RBC, which is determined not only by their clearance from the peripheral circulation in the spleen and the liver but also by their production in the bone marrow.

The acrophase of RBC counts, HGB concentration and HCT level are at the end of the resting phase in the controls and the animals treated with 300 mg/kg of MMF. These parameters follow a circadian rhythm with nocturnal acrophases in horses (Piccione

et al. 2005). There is evidence in the literature that the acrophase of the diurnal RBC rhythm is near the light-dark transition in nocturnal mice (Ohkura et al. 2007b). The acrophase of circulating RBC in diurnally active humans is also at the time of the light-dark transition (Haus et al. 1983).

MMF treatment caused a decrease in WBC number whatever the treatment time and the leucopenia index (%) depends on MMF injection in the 24-hr scale.

A statistically significant leucopenia of -54% and -42% was observed at 7 and 13 HALO respectively. This finding is also in accordance with several studies which showed that MMF caused leucopenia (EBPG 2002b). The subacute oral administration of a 10–20 mg/kg MMF dose for 2 weeks in male Lewis rats induced a significant reduction of RBC and also causes a significant leucopenia with a lymphopenic effect (Pally et al. 2001). Danesi and Del Tacca (2004) confirmed that MMF causes leucopenia.

The present work also showed that the number of white blood cells in controls and MMF treated animals followed a circadian rhythm with a peak at the rest period and a trough at the active phase.

In control and treated animals the acrophase is located at 6.9 HALO \pm 0.39 h and at 2.13 HALO \pm 1.25 h respectively.

The study of Ohkura et al. (2007a) showed that the circadian rhythm of WBC in nocturnal mice peaks at the beginning of the light period. On the other hand, the peak of circulating WBC in diurnally active humans is at the time of the light-dark transition (Haus et al. 1983). Thus, the difference of about 180° between circadian rhythms in diurnally active humans and nocturnally active rodents is essentially applicable to the rhythm of WBC numbers (Ohkura et al. 2007b).

Studies focusing on the relationship between the basal circadian rhythm of WBC numbers and immune rhythms using mouse models might help to establish immunomodulatory therapies on a chronobiological basis (Pally et al. 2001). The numbers of circulating WBC involved in the immune defense of organisms are subject to high-amplitude circadian rhythms (Haus and Smolensky 1999).

Tsutsumi et al. (1999) showed that the maximum of PLT counts is at 9:00 am (443 \pm 15.3 10⁹/l) in the male Minipigs and this haematological parameter follows a circadian rhythm.

The study of changes in platelet count has revealed a significant decrease whatever the treatment time with MMF. The index of the low platelets (thrombocytopenia) depends on MMF injection time. However, MMF treatment at 7 HALO resulted in a higher thrombocytopenia (-32%). This is in agreement with the work of several other authors' who

have shown that MMF may induce thrombocytopenia (Mackie et al. 1996, Virji et al. 2001).

Our study also showed that in control animals, the number of platelets followed a circadian rhythm with an acrophase located at the beginning of the resting phase ($\emptyset = 1.9 \text{ HALO} \pm 0.4 \text{ h}$). MMF treatment altered this rhythm which became an ultradian rhythm ($\tau = 8 \text{ h}$).

CONCLUSIONS

In conclusion, the administration of immunosuppressive drugs such as MMF may be associated with a great deal of haematological toxicity (anemia, leucopenia, thrombocytopenia) that could be mild and predictable coinciding with the time of MMF toxicity with an ultradian rhythm (8 h) shown in a preliminary study (Dridi et al. 2012).

The safe administration of immunosuppressive drugs requires therapeutic drug monitoring and the knowledge of potential drug interactions that may unpredictably be associated with the occurrence of severe haematological toxicity.

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