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Circadian gene expression in peripheral blood of *Bos taurus* under different experimental condition

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ABSTRACT

The aim of the study was to investigate clock gene expression in *Bos taurus* and the alteration of that during two pathological conditions, evaluating the daily expression pattern of four clock genes (*Per2*, *Cry2*, *Bmal1*, *Clock*) in peripheral blood cells. Five healthy cows, five affected by Brucellosis (BR) and five affected by Bovine Viral Diarrhoea-Mucosal Disease (BVD-MD) were housed in indoor stalls under natural spring conditions, blood samples were collected at 4 h intervals over a 24 h period. Statistical analysis showed rhythmic expression of clock genes mRNAs in healthy cows. Cows affected by BR did not show any rhythmic expression of clock genes mRNAs, cows affected by BDV mRNA levels of *Bmal1*, *Clock* and *Cry2* changed during the day. These findings highlighted that circadian system could be involved in homeostasis alteration and that clock genes could be considerate as regulatory genes or early response genes during inflammation, so, their regulation should be evaluated in health research and treatment.

Introduction

The endogenous molecular mechanism that keeps track of time and allows the organism to anticipate upcoming daily environmental changes is termed circadian clock (Reppert and Weaver, 2002; Ko and Takahashi, 2006; Berger, 2007; Giannetto and Piccione, 2009). In mammals, the circadian timekeeping system is composed of a central pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus and of a set of peripheral oscillators in most of tissues and organs such as skin, liver, kidney and blood (Dibner et al., 2010). The clock mechanism in the SCN and the peripheral oscillators is similar at the molecular level (Nagoshi et al., 2004; Welsh et al., 2004; Brown et al., 2005), and consists of a network of transcriptional–translational feedback loops of core clock components (Reppert and Weaver, 2001; Stanewsky, 2003; Gachon et al., 2004; Lowrey and Takahashi, 2004). Core clock components (i.e. clock genes and proteins) are indicated as "positive" elements that induce the expression of "negative"

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elements, and "negative" elements that inhibiting the transactivity of positive elements (Takahashi, 2004; Zhang et al., 2009). The positive elements include members of the basic helix-loop-helix (bHLH)/PER-ARNT-SIM (PAS) transcription factor family, *Clock* and *Bmal1*. *Clock:Bmal1* heterodimer binds to E-box (CACGTG) promoter elements to mediate the transcription of many genes including *Periods* (*Per1*, *Per2*, and *Per3*) and *Cryptochromes* (*Cry1* and *Cry2*). *PERs* and *CRYs* are part of the negative feedback loop (Bellet and Sassone-Corsi, 2010; Baumann et al., 2013). They form oligomers that, after shuttling from the cytoplasm to the nucleus, repress their own transcription by inhibiting *CLOCK:BMAL1* activity (Griffin et al., 1999; Jin et al., 1999; Kume et al., 1999). The suppressed E-box driven transcription leads to a decline in *PER*, *CRY* and *REV-ERBα*protein levels, which finally re-activates *CLOCK:BMAL1* driven transcription.

Temporal variations of clock genes have been previously showed in peripheral blood of different mammals such as rats (Oishi et al., 1998), dogs (Ohmori et al., 2013) and humans (Boivin et al., 2003; Kusanagi et al., 2008). Interestingly, in human peripheral blood clock gene expression is affected by acute inflammation (Logan and Sarkar, 2012). In cows, two common infectious diseases are the Brucellosis (BR) and the Bovine Viral Diarrhoea-Mucosal Disease (BVD-MD). BVD-MD and BR are endemic in many countries resulting in high rates of animal death, economical refuse, abortion and infertility (Otachel-Hawranek, 2004; McDermott et al., 2013). The BR is caused by the gram-negative bacterium *Brucella abortus*, a highly contagious, zoonotic pathogen with worldwide distribution that determined high incidences of abortions (Lindberg et al., 2006; Christopher et al., 2010). BVD-MD, a viral disease of cow sand other ruminants caused by the bovine viral diarrhoea virus, is a widespread problem to beef and dairy herds that reduces productivity and increase death loss (Smith et al., 2014).

The aim of the present study was to investigate whether the clock gene expression in peripheral blood cells of cows is affected by infectious disease. To this purpose, we evaluated the daily expression pattern of *Per2*, *Cry2*, *Bmal1*, and *Clock* in the peripheral blood cells of healthy cows and in cows affected from BR or BVD-MD.

Material and methods

Animal and experimental design

The study was carried out in Sicily (Italy, latitude 38°7′N, longitude 13°22′E) during the mild dry season of March–May. Fifteen not pregnant and not lactating Italian Brown cows (5 years old and weighted 650 ± 50 kg), housed in indoor stalls under natural spring conditions (sunrise 06:30 and sunset 19.00, ambient temperature of 16–21 °C, relative humidity of 40–50%) were enrolled in the study. Healthy and ill cows were selected by a routine screening for the detection of endemic bovine diseases such as brucellosis, chlamydiosis, leptospirosis, Q fever, neosporosis, toxoplasmosis, theileriosis, babesiosis, anaplasmosis, bovine viral diarrhoea infection, infectious bovine rhinothracheitis, virus respiratory syncytial bovine infection, parainfluenza-3 and herpes virus 4. Five healthy cows were free of signs of diseases and without history of significant pathologies. Among ill cows we choose cows affected by BR ($n = 5$) or by BVD-MD ($n = 5$). For the detection of BR antibodies anti-*B. abortus*, the sera were tested with Rose Bengal and Complement Fixation tests, the official tests used in the EU countries. Moreover, for measuring antigen/antibody interaction, the fluorescence polarization assay was performed as a control test. For the detection of BVD specific antibodies, serum samples were analyzed using an indirect ELISA test, following the manufacturer's instructions (Svanova, Biotech AB). All animals, subjected to the same type of management, were fed with hay (Triticosecale 40%, barley 40% and oats 20%) and water ad libitum. Professional staff not associated with the research team carried out the general animal care. All housing and care conformed to the standards recommended by the Guide for the Care and Use of Animals and Directive 86/609 CEE. Blood samples were collected at 4 h intervals over a 24 h period (starting at 10:00 on day 1 and finishing at 10:00 on day 2) via a jugular intravenous catheter into PAX gene Blood RNA Tube (Qiagen) and stored at −80 °C until processing.

Real-time RT-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was purified directly from whole blood samples collected from healthy and sick cows, using a PAX Gene Blood RNA kit (Qiagen), according to the manufacturer's instructions and resuspended in 80 μl of Elution Buffer. Reverse Transcription was carried out immediately, using the Superscript VilocDNA Synthesis Kit (Invitrogen), in a final volume of 20 μl, containing 3 μl of total RNA, a 5X Vilo Reaction mix (including random hexamers, $MgCl_2$, and dNTPs) and a 10X SuperScript Enzyme mix. An initial step at 25 °C for 10 min was followed by a reverse transcription step at 42 °C for 1 h. The resulting cDNA was stored at −20 °C prior to further analysis by RT-qPCR. Gene specific primers (Table 1) were designed using Primer3 software to amplify fragments of *Bos taurus* clock genes (*Cry 2*: XM_585942, *Per 2*: NM_001192317, *Clock*: XM_001254267 and *Bmal1*: XM_001251227). All reactions (in triplicate) were performed in a 20 μl of final volume, containing 2 μl of cDNA product, 1X Buffer Sybr green (Fast Sybr green master mix – Applied Biosystems) and 1 μM of each primer. The thermal profile was: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 1 min. Melting curve cycles were set as follows: 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. We verified the efficiency of the primers by doing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. Gene expression levels of selected bovine clock genes were tested together at GADPH, a gene previously used as reference (Robinson et al., 2007). The relative levels of each RNA were calculated by the $2^{-\Delta\Delta CT}$ method (CT standing for the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen, 2001). Each CT value used for these calculations is the mean of three replicates of the same reaction.

Statistical analysis

Nonparametric Friedman test, followed by Wilcoxon test post hoc test, was used to assess variations in gene expression

Table 1 – Nucleotide sequences and positions of primers used in RT-qPCR.

Gene	Acc no.	Direction	Sequence (5′ → 3′)
CRY2	XM_585942	F	TTCCTGTGTGGAAGACCTCAGCAA
		R	TCTGCTGCTTCCAGTTTGCGCTT
PER2	NM_001192317	F	ACAGAACTGCTCCCGGACTAAGAA
		R	AGGCTTGACACGTTTGGACTTCAG
CLOCK	XM_003584711	F	TCAAGCCCAGAATGTCTGGAAGCA
		R	TGATCCTTCATCCACACGCTGAGA
BMAL1	NM_001191170	F	ATGCAAGGGAAGCTCACAGTCAGA
		R	ACAGCCATCCTCAGCACAGTAAGT
GAPDH	NM_001034034	F	TGTTATATCCTTGCGGAGCTT
		R	AGCACTGCGGGAGAGTAGTAAACT

during the day. *P* values, in post hoc, were adjusted with Bonferroni/Sidak criteria. Only $P < 0.05$ were considered statistically significant. The RT-qPCR mRNA levels data were analyzed using Prism 7.0 (Graph Pad Software, San Diego, CA) to assess the rhythmicity of clock genes expression was maintained or was altered by experimental conditions, and all the results were expressed as mean ± standard error (SEM).

Results

mRNA levels of positive (*Clock* and *Bmal1*) and negative (*Per2* and *Cry2*) elements of the circadian molecular clockworks in the peripheral blood of healthy cows were evaluated by means RT-qPCR. The findings showed that *Cry2* and *Per2* mRNAs were statistically significant rhythmically expressed (Fig. 1A). Statistical significance rhythmic expressions were also found for *Clock* and *Bmal1* (Fig. 1B). Both positive and negative clock elements peaked during the dark phase of the natural photoperiod between 20:00 and 24:00 ($P < 0.05$) and showed the lowest levels during the light phase ($P < 0.05$).

Cows affected by BR did not show that clock genes mRNAs was statistically significant rhythmically expressed (Fig. 1C and D). Conversely, in cows with BDV mRNA levels of *Bmal1*, *Clock* and *Cry2* changed during the day (Fig. 1E and F) with statistically significant peaks at the end of night (4:00), whereas *Per2* mRNA levels were not rhythmically expressed (Fig. 1E).

Discussion

Our data showed mRNA expression of *Bmal1*, *Clock*, *Per2* and *Cry2* in the peripheral blood of healthy cows, with peaks in the early night. The expression profiles of clock genes in the peripheral blood considerably changed from different species. For instance, in healthy dogs the peripheral blood mononuclear cells expressed rhythmically *Per1* but not *Per2*, *Clock*, *Bmal1* and *Cry1* (Ohmori et al., 2013). Differently, in human peripheral blood mononuclear cells all *Per* genes were rhythmically expressed, where as *Bmal1* and *Cry1* mRNA expression did not change during the day (Boivin et al., 2003; Kusanagi et al., 2008). In peripheral mononuclear leukocytes of rats *Per2* is expressed in circadian manner (Oishi et al., 1998). In another domestic species, the horse, no significant differences in daily expression of *Per2*, *Bmal1* and *Cry1* were detected in the peripheral blood (Murphy et al., 2007). The blood is not a homogenous tissue, and the failure to detect the circadian expression of some core clock genes could be due to the different cell types, which were not synchronized among them. This fact could also justify the absence of an anti-phase expression of positive (*Clock* and *Bmal1*) and negative (*Cry1* and *Per2*) clock elements showed in many peripheral circadian oscillators (Gachon et al., 2004). The atypical expressions patterns of circadian clock, that the authors had found, in agree with Nebzydoski et al. (2010), could be to a lack of neural regulation in peripheral tissue, like blood, in bovine.

Interestingly, acute systemic inflammation by lipopolysaccharide (LPS) administration induced *Per2* and *Bmal1*mRNA expression in equine peripheral blood (Murphy et al., 2007). This result prompted us to investigate the effect of systemic inflammation that altered the normal internal homeostasis in the blood. It is known that in peripheral blood, during BVD-MD or BR chemokines are overexpressed to attract inflammatory cells or to coordinate cell trafficking during homeostasis (Weiner et al., 2012). Circadian clock genes expression was abolished in cow affected to BR, where as the BVD did not abolish the circadian expression but change the phase of the peak. These results point out to different effects of these pathologies on the clock gene expression in the blood cells. The disappearance of the circadian clock gene expression in the blood cells of cow affected to BR could depend to an alteration of the synchronization mechanism, whereas the shift of the *Bmal1*, *Clock* and *Cry2* expression pattern could due to an alteration of daily resetting mechanism of the circadian clock. Furthermore, our results indicate that the temporal variations in the blood cells are not linked to external synchronization agents as light–dark cycle or food availability. The function of these modifications in the circadian pattern of clock genes expression in the blood cells during a systemic inflammation remains unclear, but the authors hypothesized that, in agree with studies conducted on mice (Yamamura et al., 2010), Brucella's LPS could reduce the expression of clock genes. This reduction can be due to a TNFα serum levels increment. Instead, BVD-MD virus do not affect the clock rhythmicity because, was observed that Faviviruses Toll-like receptor 9 (TLR 9) could not interfere with clock genes system of the host (Silver et al., 2011). The effect of two different pathologies of different origins (parasite and bacterium) on the expression of these genes in the blood cells could depend on a different physiological response to immune challenges.

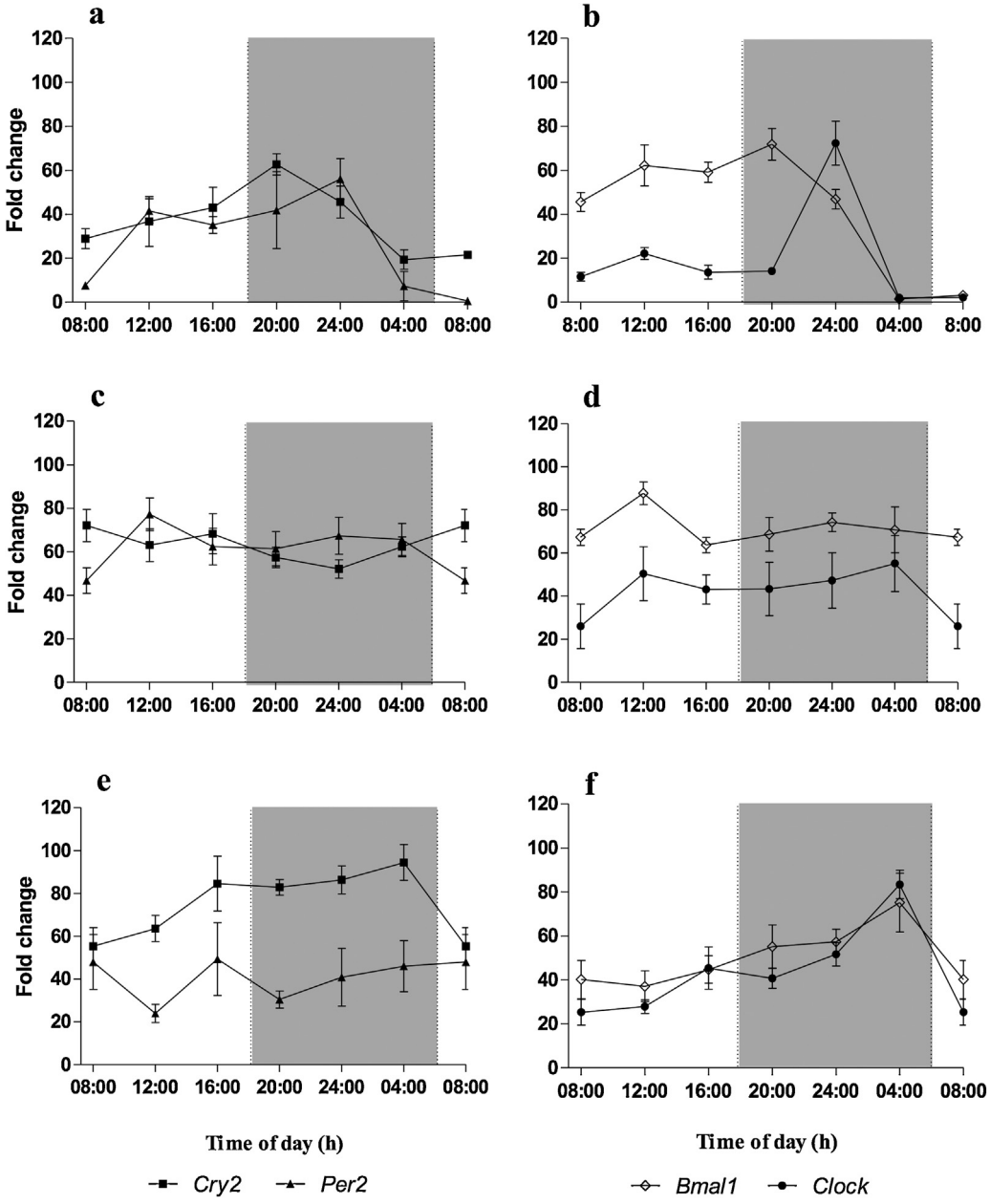

Fig. 1 – Clock gene expression in cows. RT-qPCR analysis of four endogenous clock gene expression for 24 h in healthy cow (a, b) and in cow affected to by BR (c, d) or by BDV (e, f). Grey areas indicate the scotophases of the natural light–dark cycles. On the Y-axes are plotted relative expression levels, while on the X axes time the hours of the day. For all panels, each point represents the mean ± SEM.

In conclusion, the synchronization of clock gene expression in peripheral blood of cows highlights the possible involvement of the circadian system in restoring homeostasis in this dynamic tissue during inflammatory challenge. So, it might be of importance to consider clock genes as regulatory genes and/or early response genes and to assess their regulation on a routine basis in health research and treatment.

Conflict of interest

The authors declare they have no conflict of interest.

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