Circadian (about 24-hour) variation in malondialdehyde content and catalase activity of mouse erythrocytes

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Lipid peroxidation is a part of normal metabolism that may cause biological molecule damage leading to the formation of several specific metabolites that include aldehydes of variable chains, such as malondialdehyde (MDA). These biological effects are controlled *in vivo* by a wide spectrum of enzymatic and non-enzymatic defense mechanisms among which catalase (CAT) is considered as an important regulator of oxidative stress. The present study aimed to investigate the possible relationship between the temporal patterns of the formation of MDA and the activity of CAT in the erythrocytes of mice. Twenty-four-hour studies were performed on male *Swiss albino* mice, 12 weeks old, synchronized to a 12:12 light: dark cycle for 3 weeks. Different and comparable groups of animals ($n = 10$) were sacrificed at an interval of 4 hours (1, 5, 9, 13, 17, and 21 hours after light onset (HALO)). The levels of erythrocyte MDA concentration and CAT activity both significantly (analysis of variance: $F = 6.4$, $P < 0.002$) varied according to the time of sampling under non-stressed conditions. The characteristics of the waveform describing the temporal patterns differed between the two studied variables, e.g. MDA content showing one peak ($\cong 21$ HALO) and CAT activity showing three peaks ($\cong 9$, 17, and 21 HALO). Cosinor analysis revealed a significant (adjusted Cosinor: $P \leq 0.018$) circadian ($\tau \cong 24$ hours) rhythm in MDA level and no statistically significant rhythmicity in CAT activity. The differences and the absence of correlation between the curve patterns of erythrocyte MDA content and CAT activity under physiological conditions are hypothesized to explain that variation in lipid peroxidation may depend on several factors. Moreover, the identification of peak/trough levels of MDA accumulation in erythrocytes may reflect the degree of oxidative stress in these blood cells. In addition, the observed significant time-of-day effect suggests that, in both clinical and scientific settings, appropriate comparison of MDA production and CAT activity levels can only be achieved on data obtained at the same time of day.

Keywords: Catalase, Circadian rhythm, Erythrocyte, Malondialdehyde, Mouse

Introduction

Reactive oxygen species (ROS) are continually generated as by-products of aerobic metabolism in animals.[1–3] Thus, oxygen metabolism generates hydroxyl radical ($^{\bullet}OH$), superoxide radical ($O_2^{\bullet-}$), and non-radical hydrogen peroxide (H_2O_2). Transition metal ions such as iron and copper ions catalyze the reaction between $O_2^{\bullet-}$ and H_2O_2, generating $^{\bullet}OH$ (Haber–Weiss reaction).[4] These cytotoxic species may cause oxidative damage that manifests as the peroxidation of membrane polyunsaturated fatty acid chains, modifications of DNA, and carbonylation and loss of sulfhydryl in proteins.[5,6] Oxidative stress develops when there is an increase in the production of ROS or when the antioxidant systems are unable to contain the former.[7,8] On the other hand, the erythrocyte (RBC, red blood cell) is one of the main cells used as an oxidative stress marker in living animals, including humans, because their cell membranes as well as their antioxidant enzymes are sensitive to the presence of ROS.[8–10] Furthermore, because of their function as O_2 carriers, RBCs are constantly exposed to ROS, which lead to lipid peroxidation (LPO) of their membranes.[11] LPO refers to the oxidative degradation of lipids that consists of three major steps:

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initiation, propagation, and termination. The most common initiators in living cells are $^{\bullet}$OH and HOO$^{\bullet}$. During this oxidative process, several oxidized products, including malondialdehyde (MDA), are formed.

Despite the limited biosynthetic repertoire available to mature RBCs, they are resilient to oxidant-induced damage. Clearly, antioxidants in the form of scavengers and detoxifying enzymes provide an important protective system in RBCs,[4] but our knowledge of the molecular mechanisms that regulate the expression of antioxidants in RBCs is limited. Under normal circumstances, RBCs are protected against oxidative damage with a complement of antioxidant enzymes (superoxide dismutase, glutathione peroxidase (GPx), and catalase (CAT)) and other molecules (e.g. vitamin E, reduced glutathione (GSH)). These antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. Since these enzymes are known to scavenge ROS, any change in enzyme activities should provide indirect information about ROS production. Mammalian RBCs are endowed with extraordinarily high activities of CAT. Because CAT has a low affinity for H_2O_2, others have suggested that GPx clears most H_2O_2 within the erythrocyte and that CAT is of little importance.[12] However, inhibition of erythrocyte CAT abrogates the protective effect and the addition of purified CAT (not GPx) restores it.[13] Thus, the enzyme CAT, which detoxifies H_2O_2 (and, therefore, $^{\bullet}$OH) to oxygen (O_2) and water (H_2O), plays a crucial role in protecting erythrocytes from oxidative stress.[14] Thus, this enzyme that has been considered as an important regulator in protection against H_2O_2-mediated LPO may contribute to the modulation of MDA level in erythrocyte membranes.[15]

Temporal coordination of biological processes with an ~24 hours cycle (circadian) is common in animals and humans.[16] Under physiological conditions, the use of O_2 by cells varies according to the time with regard to the 24 hours light/dark (LD) cycle, and ROS damage to biological molecules presents rhythmic changes. Circadian rhythms of antioxidants have been the subject of considerable interest in recent years[17] and significant 24-hour fluctuations of oxidants and antioxidants have been reported in animals[18–20] and humans.[21] The importance of the protective mechanisms of RBCs is evident from a consideration of human hemolytic disorders due to a variety of enzyme deficiencies involving pathways that maintain intracellular reductive molecules.[4] Deficiencies compromising the capacity to detoxify oxidant molecules such as H_2O_2 and O_2 radicals result in oxidant-induced denaturation of intracellular molecules and premature destruction of RBCs. Expression of a variety of genes encoding antioxidant enzymes is mediated partly by a *cis*-active DNA element designated the antioxidant response element (ARE). Although several proteins bind the ARE, factors mediating the expression of genes controlled by the ARE have not been identified. Among possible factors, Nrf1 and Nrf2 were implicated as playing a part in ARE-mediated regulation of antioxidant gene expression. Several different transcription factors, including basic leucine zipper (bZIP) proteins, activator protein 1, and other novel factors, were shown to bind the ARE.[22,23] However, erythrocytes are considered as passive 'reporter cells' for the oxidative status of the whole organism and circadian variations of LPO and antioxidant enzymes are not well studied in the mouse erythrocyte. Hence, here, mice were studied under non-stressed conditions for the formation of a LPO endproduct (MDA) and the variation of antioxidant enzyme CAT activity in relation to the 24-hour scale in erythrocytes. The current study aimed to assess rhythmic patterns of MDA production and CAT activity and to explore the possible relationship between these two physiological parameters under normal conditions.

Materials and methods
Animals and housing
These experiments were carried out at the Faculty of Sciences of Bizerte (Tunisia), Laboratory of Toxicometry and Chronobiometry from September to November 2007. For both studies, 120 male Swiss albino mice (12 weeks of age) were randomly divided into 12 groups (10 per group) and kept 5 per cage. The animals were obtained from the Central Animal House (Institut Pasteur 1000, Tunis, Tunisia) and were maintained for 3 weeks[24] after arrival in two air-conditioned rooms specially designed as a chronobiological animal facility with food and water available *ad libitum*. The chronobiological facility was equipped with temperature-controlled (22 ± 1°C) compartments, a relative humidity-controlled (50–60%) system, and provided with independent 12 hours L: 12 hours D reversed cycles (in one room, the lights were on from 07:00 to 19:00 hours; in the other room, the lights were from 19:00 to 07:00 hours). The inverted light regimen helps to explore several circadian stages during the usual diurnal work span. Time is referenced relative to hours after light onset (HALO) in these nocturnal active animals. In this study, all the experiments were conducted in accordance with the local ethics committee of Tunis University for the use and care of animals and according to the ethical and methodological standards for animal chronobiology research.[25]

Study designs and samples collection
The experiment comprised of two parts. For lipid peroxide assay (Study 1), 10 mice at each of six different

circadian stages (1, 5, 9, 13, 17, and 21 HALO) were sacrificed by decapitation and blood samples were collected into test tubes containing ethylenediaminetetraacetic acid (1.34 mM) plus GSH (0.65 mM). For CAT activity measurement (Study 2), 10 animals per timepoint were sacrificed at the same respective circadian stages and blood samples were collected into heparinized tubes. Whole blood was centrifuged at 3000 rpm for 15 minutes at 4°C to separate plasma from erythrocytes.

Preparation of erythrocyte membranes
Erythrocyte membranes were prepared according to the method of Dodge *et al.*[26] as modified by Quist.[27] The erythrocytes remaining after the removal of the plasma were washed three times with saline (9‰) and then with an isotonic solution of trishydroxymethylaminomethane (Tris)-HCl (310 mM, pH = 7.4). Hemolysis was performed by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes that contained 20 mM hypotonic Tris-HCl buffer (pH = 7.2). The erythrocyte membranes were sedimented in a high speed centrifuge at 20 000 g for 40 minutes. The supernatant was decanted and the erythrocyte membrane button was made up to a known volume using 200 mM isotonic Tris-HCl buffer (pH = 7.4), and then stored at −25°C until MDA or CAT activity measurements.

Assay for LPO
LPO level in the erythrocyte membranes was monitored by determining an endproduct of LPO, MDA, using the thiobarbituric acid (TBA) test as described by Buege and Aust.[28] Then, 0.5 ml of suspended erythrocyte membranes was added to the test tube containing 2 ml of 1% phosphoric acid (pH 2–3), 2.5 mM butylated hydroxytoluene in absolute ethanol, 0.1 ml of 8.1% sodium dodecyl sulfate, and 1 ml aqueous solution of 0.6% TBA, followed by 10 minutes heating at 80°C. The MDA concentration was determined by reference to standard curve. Erythrocyte MDA values were calculated using the extinction coefficient of the MDA–TBA complex at 530 nm = 1.56×10^5 and expressed as pmol/mg protein.

Assay procedure of erythrocyte CAT activity
CAT activity was measured by the method of Sinha[29] using H_2O_2 as substrate. Before the assay, further dilution of the suspended erythrocyte membranes was made in Tris-buffered saline (26 mM). Briefly, to the test tube containing 0.05 ml of diluted sample were added 1.55 ml of potassium buffer (pH 7) and 0.4 ml of H_2O_2 (0.2 M). The reaction was stopped by the mixed solution of acid acetic/dichromate after 60 seconds. All the reaction mixture (2 ml) was added followed by 10 minutes heating at 100°C and then cooling at room temperature. The absorbance of each mixture was spectrophotometrically read at 620 nm. For each experiment, a corresponding standard curve was fitted using a series of different standards, ranging from 2 to 30 μmol of H_2O_2. All measurements of standards were made using the same procedure as for samples. The sensitivity of the assay was reflected in the H_2O_2 standard curve, which was linear in the range of 2–30 μmol. The enzymatic activity was expressed as μmol of H_2O_2 per mg of protein and per minute of incubation (μmol H_2O_2/minute/mg protein).

Protein assay
The determination of protein content in erythrocyte membrane suspension was performed according to the method of Lowry *et al.*[30] as modified by Hartree.[31] Bovine serum albumin was used as standard.

Statistical analysis
When animals are synchronized to 12 L: 12 D, as in this study, a six timepoint study is recommended when no prior information is available concerning the rhythm under investigation.[32,33] Timepoint data were expressed as means ± standard error of the mean (SEM) and pertinent curves were drawn. The significance of the 24 hours variation in each parameter was analyzed first by one-way analysis of variance (ANOVA; InStat for MacIntosh, GraphPad Software, San Diego, CA, USA) to detect temporal difference between the timepoint means and thereafter by the iterative Cosinor method (chronobiological window), since the underlying frequency is not sufficiently known.[32,34] Linear least-square cosine regression was then applied with different test periods within the range of dominant period. For each frequency, Cosinor method[35] tests and confirms, for a detected rhythm, the statistical hypothesis of non-null amplitude, with the corresponding *P*-value. For a given trial period (i.e. $\tau = 24$ hours), the best fitting (least-square method) cosine function approximating all data of a given timeseries provides parameters to characterize a rhythm, namely *M* (MESOR: Midline Estimator Statistic Of Rhythm); 24 hours adjusted mean, *2A* (double amplitude, or difference between the peak and trough of the best-fitted cosine function), and Φ (acrophase, or peak time location). *A* and Φ were obtained with their 95% confidence limits when a rhythm was detected. This was achieved when *A* differed from zero (non-null amplitude *F* test) with $P \leq 0.05$. The period that corresponded to the highest amplitude (highest percentage rhythm, i.e. accounting for the greatest variance) and the lowest residual mean square (error) was considered the dominant period if $P < 0.01$. The peak time refers to the time of occurrence of the highest level of the variable. The

MESOR corresponds to half of the total rhythmic variability in a cycle. The 24-hour mean is the rhythm-adjusted mean; it is equal to the arithmetic mean for equidistant data covering a 24-hour period. If the confidence limits of the acrophase for a given trial period (e.g. i.e. $\tau = 24$ hours, 12 hours) are greater than ± 2 hours, quantification of the acrophase and amplitude is questionable[36] because the waveform pattern of the timeseries is likely to deviate from a sinusoidal pattern.[37] Therefore, the significance of both conventional and chronobiological statistics was needed for validating temporal changes as rhythms.[37] Since multiple tests (non-null hypotheses) were conducted, it is necessary to use a multiple testing correction to address the problem of the critical significance level (*P*-value cutoff). One of the simplest and most conservative approaches used is Bonferroni correction. This method allows adjustment of *P*-values derived from multiple statistical tests to correct for occurrence of false positives. For this method, the *P*-value of each test (provided by Cosinorwin computer Software Program) is multiplied by the number of performed tests ($n = 60$, in this case). If the corrected *P*-value is still below the error rate, the test will be significant: corrected *P*-value = *P*-value $\times 60 < 0.05$.

Results

Since before each chronobiological study the synchronization of animals is hardly required, timeseries of the
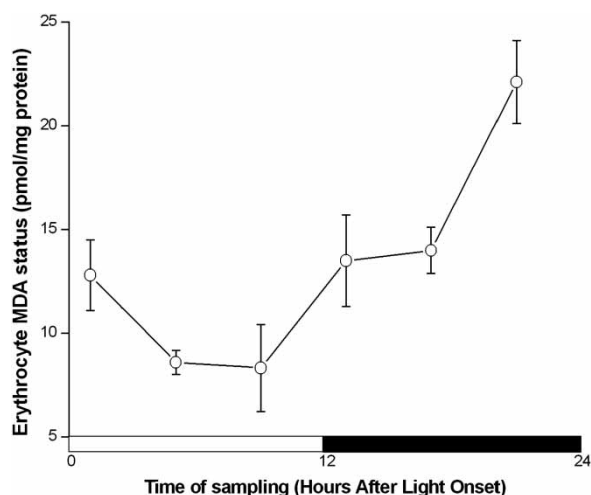
Figure 1 Circadian variation of erythrocyte MDA status (pmol/mg protein) in normal male mice. The curve represents the chronogram drawn from data (mean $\pm$ SEM) of six circadian time stages (1, 5, 9, 13, 17, and 21 HALO). Each timepoint value is the mean $\pm$ SEM of 10 independent values. The black bar corresponds to the dark period. MDA value was higher at the end of the activity ($\cong$21 HALO) compared to the end of the rest span ($\cong$9 HALO). One-way ANOVA further validated a statistically significant difference according to the sampling time ($F = 6.7$, $P < 0.0001$). A circadian ($\tau \cong 24$ hours) rhythm was confirmed by Cosinor analysis with correction for multiple testing ($P < 0.02$).

rectal temperature was used as the marker rhythm to ensure that the mice were (chrono-) physiologically healthy and synchronized to the environmental LD schedule. Thus, a circadian rhythm in rectal temperature was validated ($P < 0.006$) by adjusted Cosinor on the day before each experiment. The acrophase of this 24-hour rhythm was similar in both studies, occurring in the first half of the dark (activity) span (respectively, $\Phi = 16$ HALO ± 90 minutes and $\Phi = 16.4$ HALO ± 120 minutes) as expected. The characteristics of the 24-hour pattern in rectal temperature confirmed the synchronization of the mice to the LD entraining cycle.

Under physiological conditions, LPO is associated with metabolism and MDA production is observed. In this study, the MDA level, expressed as mean $\pm$ SEM values, significantly (one-way ANOVA: $F = 6.7$, $P < 0.0001$) varied according to the time of day (Fig. 1). A physiological circadian ($\tau = 24$ hours) rhythm was also validated by Cosinor analysis ($P < 0.02$; see Table 1) with the peak and trough times located at the end ($\cong$21 HALO) of the dark and the second half ($\cong$9 HALO) of the light span, respectively (see Fig. 1). One-way repeated measures ANOVA of CAT activity revealed significant ($F = 6.4$, $P < 0.002$) time-of-day-dependent changes (see Fig. 2). However, the Cosinor analysis detected no statistically significant (see Table 1) rhythm in that enzyme activity. Raw data (Fig. 2) showed that the curve pattern was somewhat non-sinusoidal, confirming that it differed from a regular cosine function. Moreover, MDA level and CAT activity were both greater in the dark than the light phase, as seen in Figs. 1 and 2.

Discussion

In aerobic organisms, the use of O_2 to produce energy is associated with the production of ROS, which react with biological molecules to produce oxidized metabolites such as MDA. As the use of O_2 varies with cell type, it might be expected that damage induced by the incessant attack of these toxic molecules is cell membrane-related. Since RBCs (erythrocytes) contain large amounts of iron and operate in highly oxygenated tissues, their membranes encounter continuous oxidation. The present study focused on a final product of membrane LPO, MDA. This parameter, when expressed as mean values, exhibited statistically significant time-of-day variability differences. The results showed marked oscillations in MDA status over the 24 hours period, corresponding to a statistically significant circadian rhythm with a maximum level located at the end of the dark (activity) span. In our previous studies,[20] we also have shown that the LPO peak in plasma and kidney of mice occurred during the same (activity) span as well as in

Table 1   Characteristic parameters of Cosinor analysis of MDA and CAT physiological rhythms in the mouse erythrocytes

Variable	Period τ (hour)	MESOR ± SEM (pmol/mg of protein)	Amplitude ± SD (pmol/mg of protein)	Acrophase ± SD[b] (HALO) ± (min)	Adjusted P[a]
MDA	12	13 ± 0.9	2	10.0	NS
content	24	13 ± 0.8	5 ± 2	20.4 ± 96	0.018
CAT	12	80.7 ± 5.2	26.3	7.4	NS
activity	24	80.9 ± 5.2	26.2	16.4	NS

The results obtained by Cosinor analysis with a trial period ($\tau \cong$ 24 hours, 12 hours); confidence limits for amplitude and acrophase are given when rhythm detection is statistically significant at $P < 0.05$.
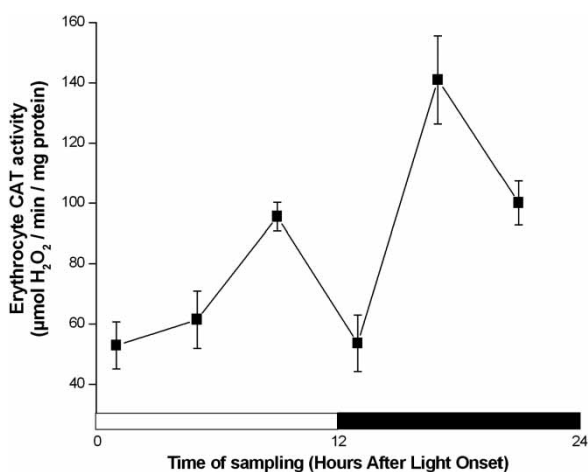[a]From an F test of the rejection of the null amplitude hypothesis.
[b]Time of maximum in fitted cosine function, in HALO.
NS, not significant ($P > 0.05$).

the erythrocytes. The lower level of MDA in erythrocytes compared to kidney and liver[4] can be explained by the fact that these two metabolically active organs are the main sites of xenobiotic detoxification and, so, are considered to be powerful ROS generators.[4] Nevertheless, the fact that the high level of erythrocyte MDA occurred at the same span as the kidney can be explained by the O_2-radical regulation of renal blood flow following the suprarenal aorta.[38] Moreover, our present findings match well with other studies that showed that the peak of lipoperoxides varied also according to season and was, in autumn, the highest in rat serum during the night.[39] Other studies realized in healthy human beings also revealed a circadian rhythm in plasma MDA with a peak time located during the activity (light) span.[40,41]

Since RBCs are continuously oxygenated and their membranes are the target for the high reactivity of

Figure 2   Circadian variation of erythrocyte CAT activity (μmol H_2O_2/minute/mg protein) in normal male mice. The curve represents the chronogram drawn from data (mean ± SEM) of six circadian time stages (1, 5, 9, 13, 17, and 21 HALO). Each timepoint value is the mean ± SEM of 10 independent values. The black bar corresponds to the dark period. Several high points of CAT activity are found during both the activity ($\cong$17 and 21 HALO) and the rest span ($\cong$9 HALO). One-way ANOVA further validated a statistically significant difference according to the sampling time ($F = 6.4$, $P < 0.002$). No statistically significant rhythm was detected by Cosinor analysis with correction for multiple testing.

molecular O_2 and its reduction products, they possess various protective mechanisms to prevent the formation and the removal of various forms of ROS. These mechanisms are controlled *in vivo* by a wide spectrum of enzymatic[42,43] and non-enzymatic systems.[44–46] The antioxidant system protection depends on many factors such as hypoxia/reoxygenation[47] and this protection is more effective during hypoxia than during reoxygenation. Therefore, the observed circadian variation in erythrocyte MDA may also be the result of circadian rhythms of enzymatic and non-enzymatic detoxification.[48] There is growing evidence that the oxidative effects and the enzymatic antioxidant systems both are organized along the 24-hour time scale.[19,20] It seems that a temporal relation may be observed between the circadian rhythm in MDA and 24-hour variations of some antioxidant enzymes, including the CAT, that is considered as a key antioxidant in erythrocytes.[49,50] In the present study, the oscillation in CAT activity, although significant, exhibited only a low amplitude and the rhythm was not detected. The presence of more than two peak times (within the 24-hour scale) occurred both at the rest and the activity span, presumably indicating that the curve pattern is somewhat non-sinusoidal. The raw data (Fig. 2) confirm this; the curve pattern differs from a regular cosine function. Moreover, the observed variation in CAT activity (Fig. 2) did not correlate with the high-amplitude circadian rhythm of MDA status (Fig. 1), indicating that there was no temporal relationship between the two parameters. However, the temporal relation, in itself, does not mean that a causal relation is involved.[51] New investigations are needed to validate the latter and explore its biochemical aspects. In addition to LPO enzymes, it has been shown that the temporal pattern of MDA in serum depends on the nature of 24-hour rhythms of total lipids and detoxifying enzyme levels.[52] Our data showed that erythrocyte CAT activity and MDA status were both higher during the activity (dark) span, suggesting that the enzyme CAT does not probably counterbalance the production of ROS and, thus, damage to RBC membranes was observed as evidenced by the subsequent

increase in MDA concentration. Other previous findings suggested that the cap 'n' collar (CNC) factors might play essential roles in regulating the expression of genes that protect cells against oxidative stress. It has been found that p45NF-E2 (a member of the CNC-bZIP family of transcriptional activators)-deficient RBCs have increased levels of ROS and the susceptibility to oxidative-stress-induced damage. However, and in spite of the fact that MDA oscillation is modulated by various factors, our previous findings revealed a strong correlation between the low level of MDA[20] and the high level of CAT activity[19] both observed during the dark phase in the liver, suggesting the importance of this enzyme in this tissue. Therefore, it seems that the effectiveness of enzymatic or non-enzymatic antioxidant system protection against the generation of ROS and their oxidative effects may depend on the type of target cell. However, no experimental evidence to support this interesting hypothesis is yet available. Thus, the results observed in this study may deserve further investigation of the role of CAT in preventing oxidative stress in erythrocytes. However, others have found that CAT has an essential role in the detoxification of H_2O_2-derived radical species in the RBC.[53] A reduction in CAT activity would therefore be one important factor in the sensitivity of p45NF-E2-deficient RBCs to oxidant stress. Nevertheless, the findings from our study (analyzed by one-way ANOVA) revealed statistically significant time-of-day variability differences in both MDA content and CAT activity, indicating that the two studied parameters are dependent on the time of day at which testing is undertaken. Thus, the observed changes in the specific activity of the antioxidant enzyme CAT do not prevent damage to the erythrocyte membrane as evidenced by uncorrelated oscillations in MDA content. Therefore, despite the postulated crucial role of CAT in the protection of cell membranes against free radical-induced LPO, the erythrocyte membrane seems to be particularly susceptible to oxidative stress, perhaps due to its high content of polyunsaturated fatty acids.

Conclusion

This study points out that in mouse erythrocytes the MDA status exhibits a significant circadian rhythm but not the CAT activity. In addition, this study showed that during the rest span, erythrocytes have lower sensitivity to ROS-induced LPO damage. The findings from this study also revealed significant time-of-day dependence of both MDA content and CAT activity, suggesting that the erythrocyte protection against by-products of its own cargo (oxygen) varies over the day–night cycle. The absence of correlation between MDA production and CAT activity

does not exclude the capacity of this enzyme to protect erythrocytes against H_2O_2 damage under normal physiological conditions. Further research is needed to elucidate the mechanisms responsible for time-of-day differences in the occurrence of MDA production and to identify the role of CAT's capacity to scavenge H_2O_2 from blood across the 24-hour period.

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