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Original Research

Quantification of circadian rhythm in mitochondrial DNA copy number in whole blood, and identification of factors that influence it

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Abstract: Mitochondrial DNA (mtDNA), the genetic material in mitochondria, encodes key genes related to the respiratory chain and ATP production. To accurate quantification mtDNA content in whole blood is important for various disease states. Absolute quantitative Real-time PCR and platelet contamination erase method were used for mtDNA copy number analysis in whole blood. In the quantitative study of mtDNA content, it was found that whole blood mtDNA copy number showed a fluctuating rhythm during a 24-h period due to dynamic changes in white blood cells combined with platelets. However, when isolated white blood cells were used, or absolute whole blood mtDNA was calculated, the circadian rhythm pattern of mtDNA disappeared. In this study, a feasible method that can accurately quantify mitochondrial DNA in small blood samples was established, and it was found that two factors which greatly influenced mtDNA copy number were sampling time and platelets in blood.

Key words: mtDNA copy number; Circadian rhythm; Platelets; Sampling time.

Introduction

Mitochondria are double-membrane-bound organelles which contain their own circular DNA as genetic material. The primary function of mitochondria in eukaryotic cells is to generate energy. In addition, mitochondria are involved in many other biological processes such as calcium homoeostasis (1), inflammation (2), steroid biosynthesis (3), signal transduction (4-6), and apoptosis (7). The functions of mitochondria are related to human diseases such as cardiac dysfunction (8-10), hearing impairment (11), systemic lupus erythematosus (12), amyotrophic lateral sclerosis (13-15), and autism (16, 17).

Mitochondrial DNA (mtDNA) contains only 37 genes in humans, and its translation products participate mainly in cellular respiration. Distinct from nuclear DNA, mtDNA may have up to one thousand copies in a single cell, depending on different tissues and cell types. Diseases associated with mitochondria dysfunction are thought to be caused by mutations in mtDNA or changes in mtDNA copy number in specific cells or tissues (18-22). Indeed, numerous researchers have reported correlations between blood mtDNA and many types of diseases such as metabolic syndrome (23-25), atrial fibrillation (26), cancer (27-35), neurodegenerative disease (36-39), HIV infection (40) and sepsis (41). In these studies, the quantification of mtDNA copy number was carried out using the relative copy number between mtDNA and nuclear genome. However, few universal standards for blood sample preparation existed prior to mtDNA quantification. In some extant literature, whole blood DNA was used for quantification of mtDNA copy number (42, 43), while in other studies, a buffy coat or peripheral blood mononuclear cell (PBMC) was used instead (23, 40, 41, 44). Although blood samples are easily accessed in patients, it is important to improve blood-based mtDNA quantification methods.

In this study, we compared mtDNA copy number in blood using different sample preparation methods and cell types, and provided a valuable procedure for quantifying mtDNA copy number with a small sample size. Interestingly, it was found that the mtDNA copy number of whole blood showed a fluctuating rhythm within 24 hours.

Materials and Methods

Ethical statement

The present study was approved by the Committee on the Ethics of Animal Experiments of China Agricultural University (Beijing, China). All the procedures used were in strict compliance with the Guiding Principles for the Care and Use of Laboratory Animals. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize pain and suffering in the animals.

Mouse blood collection and genomic DNA extraction

Male Balb/c mice aged 6–8 weeks were purchased

from Beijing Vital River Laboratory (Beijing, China) and housed in a well-controlled 12-h/12-h light/dark environment with feed and water available *ad libitum*. All blood samples collected at 10:00 AM, and circadian rhythm related sampling time started at 10:00 AM with 4 hours interval for 24 hours. For whole blood samples, 10-30 µl mouse tail blood was collected with 1/10 volume(s) of 0.05 M EDTA (Solarbio, Beijing, China). The blood samples were used for DNA extraction using FlexGen Blood DNA Kit (CWbio, Beijing, China) in accordance with the manufacturer's protocol. Blood routine examination was performed at the Health Science Center of Peking University. To decrease platelet contamination in whole blood, anticoagulated blood samples were added to 5 volumes of red blood lysis buffer (Solarbio, Beijing, China), centrifuged at 200 g for 5 min, and the supernatant was carefully aspirated to obtain low platelet-contaminated blood samples. In buffy coat sample preparation, 1 ml anticoagulated blood sample was collected through cardiac puncture and pooled to obtain a volume of 10 ml. Using the 10 ml of blood, buffy coat was separated into three tubes following the procedure of whole blood DNA extraction.

Isolation of lymphocytes and granulocytes

Whole blood was treated twice with red blood cell lysis buffer, and lymphocytes and granulocytes were sorted using flow cytometry at a low speed to avoid platelet contamination. The T cells and B cells were isolated from the lymphocytes ($2–5\times10^5$ cells) using 1 µl Dynabeads Mouse Pan T (Catalog No. 11443D) or Dynabeads Mouse Pan B (Catalog No. 11441D) beads (Thermo Fisher Scientific, Baltics, Lithuania). The T cells and B cells were digested with protein K (CWbio, Beijing, China) at 56 °C overnight, and total DNA was extracted using ethanol precipitation.

Quantification of mouse mtDNA copy number

The mtDNA copy number quantification primers and standard curves were prepared according to a previously described protocol (55). The PCR products were purified and diluted 10 times from a copy number of 10^8 to 10^3 in 2 µl DNase- and RNase-free water, and used in preparation of a standard curve. The mtDNA copy number was determined using qRT-PCR in a total reaction volume of 10 µl containing 5 µl of KAPA SYBR Fast Mix (Kapa Biosystems, Woburn, MA, U.S.A.); 0.2 µl of 10 µM forward and reverse primers, 2 µl template DNA (1–10 ng), and 2.6 µl DNase-free water. The qPCR reaction was conducted in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) according to the following protocol: pre-denaturation at 95 °C for 3 min (one cycle), denaturation at 95 °C, annealing at 60 °C and extension at 72 °C for 10 sec each (40 cycles); melt curve analysis was done with the default program. The R^2 of the standard curve was greater than 0.96, and standard deviations of ct values between replicates lower than 0.25 were accepted.

Calculation of absolute mtDNA copy number

The absolute mtDNA copy number of white blood cells was calculated taking into consideration the counts of lymphocytes and granulocytes in blood, and the average mtDNA copy number in various cell types. Whole blood absolute mtDNA copy number was determined as the sum of white blood cell mtDNA copy number and platelet mtDNA copy number (calculated from the platelet count and average mtDNA copy number in platelets).

Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical analysis was performed using two-tailed Student's *t*-test (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Statistical significance was set at $p<0.05$.

Results

Different cell types or isolation methods influenced blood mtDNA copy number

To estimate the potential influence of sample preparation methods on the quantification of blood mtDNA copy number, mtDNA copy number was quantified in pooled mouse blood. The results showed that mtDNA copy number was highest in whole blood samples (Figure 1). Buffy coat, the main source of clinical samples, had a relatively higher mtDNA copy number than specific blood cells (Figure 1). In addition, lymphocytes presented more mtDNA than granulocytes. There were no differences in mtDNA copy number between T cells and B cells, the major components of lymphocytes (Figure 1).

Circadian rhythm in mtDNA copy number in mouse whole blood samples

Changes in different cell types in human blood have been demonstrated to occur within a 24-h period (45, 46). Data from this study revealed that different cell types of mouse blood contained varied mtDNA copy numbers (Figure 1). Thus, it may be speculated that, in mouse whole blood samples, mtDNA quantification results might change at different time points. To test this hypothesis, mouse whole blood samples were continually collected at 4-h intervals for 24 hours to track dynamic changes in mtDNA contents. The results of mtDNA quantification revealed an obvious fluctuation rhythm in mtDNA copy number in blood (Figure 2A),

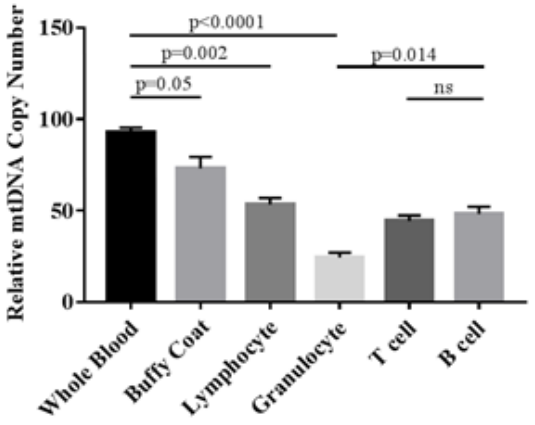

Figure 1. Variations in mtDNA copy number in different blood samples as a function of cell types or isolation methods. Buffy coats and different cell types isolated from pooled whole blood were subjected to quantification of relative mtDNA copy number.

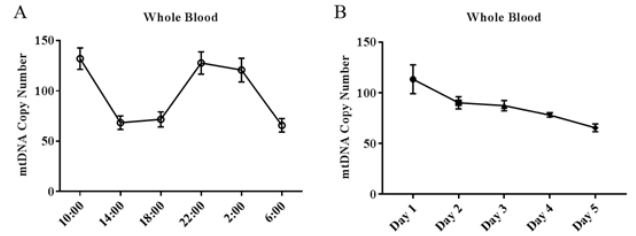

Figure 2. Circadian rhythms of mtDNA copy number were detected using whole blood samples of mice. Relative mtDNA copy number of mouse whole blood in 24 h at 4-h intervals (A) or for 5 consecutive days at 24-h intervals (B); n=5.

which was in accordance with expectation. The fluctuating rhythm appeared to exhibit a circadian cycle of at least 1 day. Subsequently, a study was carried out to determine whether a longer circadian rhythm in mtDNA copy number was present in blood. To this end, blood mtDNA copy numbers were determined for five consecutive days at the same sampling time point. The results showed no obvious circadian rhythm (Figure 2B).

Dynamic changes in different cell types in mouse blood during a 24-h period

Blood comprises various cell types which can be classified according to shape, function, and surface proteins. It is well known that the most abundant cell type in blood is red blood cell. However, mature red blood cells in mammalian animals contain neither nuclear genome nor mtDNA, and thus they will not influence blood mtDNA copy number quantification (47). Indeed, a verifiable explanation for the circadian rhythm in mtDNA copy number may be based on changes in the counts of different cell types containing mtDNA. In order to elucidate the main cause of changes in mtDNA copy number in whole blood during a 24-h period, major blood cell counts were measured. The results showed that platelet counts appeared stable, but white blood cells changed throughout the 24 h period (Figures 3A and 3B). Simultaneously, the ratio of lymphocytes to granulocytes also varied respectively, with changes in the two cells following opposite trends (Figures 3C-3D).

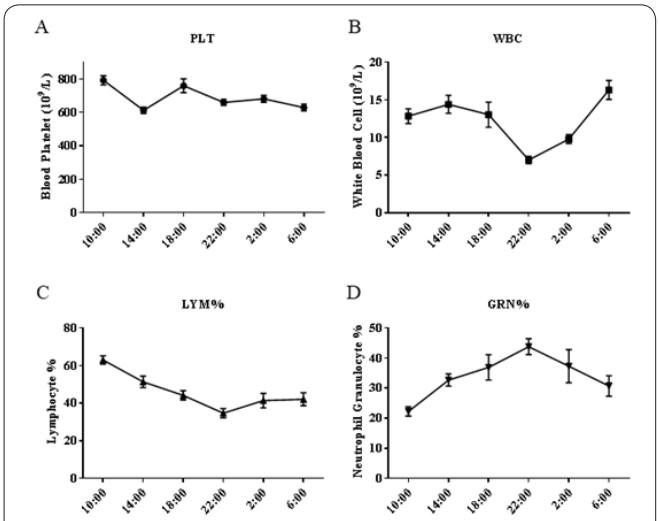

Figure 3. Dynamic changes in the main blood cell counts of mice during a 24-h period at 4-h intervals. D: daily dynamic changes in platelets (PLT), B: daily dynamic changes in white blood cells (WBC), C: % of lymphocytes (LYM), and D: % of neutrophil granulocytes (GRN).

Circadian rhythm in mtDNA copy number was due to dynamic changes in the ratio of white blood cells to platelets

In whole blood samples, platelet counts were 10–100 times higher than white blood cells in mouse blood (Figures 3A and 3B).Therefore, it is possible to approximately calculate absolute mtDNA copy number in whole blood or white blood cells based on the average mtDNA copy number of various cell types (Figures 4A and 4B). The results indicated that the mtDNA content of whole blood or specific blood cells was consistently stable during a 24-h period (Figures 4A and 4B).

However, when the ratio of platelet to white blood cell counts in whole blood samples in 24 h was calculated, it was found that it fitted well with the previous circadian patterns (Figure 2A; Figure 4C). To test whether the circadian rhythm in mtDNA copy number was present in white blood cells only, most of the platelets were eliminated using low-speed centrifugation of whole blood samples, prior to quantifying mtDNA copy number during a 24-h period. The results showed that there was no significant circadian rhythm in low platelet-contaminated white blood cells (Figure 4D). Thus, relative quantification revealed that the dynamic changes in white blood cells combined with platelets led to a circadian rhythm in mtDNA copy number in whole blood samples (Figure 2A; Figures 4C-4D).

Discussion

One of the results obtained in this study revealed that platelet-derived mtDNA constitutes an important source of mtDNA in whole blood mtDNA, and is closely related to the circadian rhythm in mtDNA copy number. It is likely due to the higher number of platelet in mouse

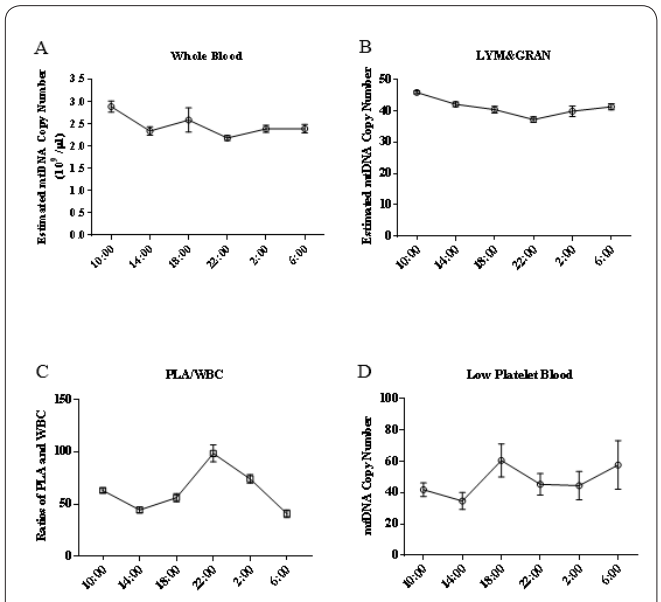

Figure 4. Effect of dynamic changes in nucleated blood cells combined with platelets on the circadian rhythm of mtDNA copy number in mouse whole blood. A: Absolute mtDNA copy number in whole blood; B: mtDNA copy number in lymphocytes (LYM) and granulocytes (GRAN); C: ratio of platelets to white blood cell counts (PLA:WBC) in whole blood during a 24-h period; D: relative mtDNA copy number quantified in low platelet-contaminated blood samples.

blood, which is consistent with previous reports on humans and mice (48, 49). Meanwhile, each blood platelet contains an average of 2-4 copies of mtDNA without genomic DNA (50). The profound role of platelets in whole blood mtDNA copy number is in accordance with extant literature which shows that platelets cause large variations in mtDNA copy number in human blood, as well as overestimation of same (51, 52). Many previous studies reported that human blood mtDNA content is correlated with disease states (22). However, most of these studies utilized buffy coats or whole blood samples as starting materials, and all the samples contained platelets. Consequently, under different platelet enrichment conditions, previous works on the correlations between blood mtDNA copy number and disease states should be carefully reconsidered. In addition, the proportion of different blood cell types in humans continually changes (45), which is in agreement with the results obtained in the present study. These changes in cell content might influence the results of quantification of mtDNA copy number in whole blood or buffy coats, due to different sampling times during a 24-h period. Furthermore, the use of different blood sampling times for patients in control and disease groups may complicate the conclusions from the research, even when a correction formula is employed to reduce platelet effects (53). Therefore, it is recommend that all studies on blood mtDNA copy number should carefully take platelet contamination and sampling time into account.

It is also worth noting that the mtDNA copy number in lymphocytes was two-fold higher than that in granulocytes, as shown in the results. A similar result was also reported in human samples from mtDNA sequencing data (54). It may be speculated that the differences in mtDNA copy numbers might be consistent with different energy demands of progenitor cells during differentiation. Accordingly, the use of two types of blood cells may be optimal for a research that focuses on the mechanisms underlying the regulation of mtDNA copy number. In this study, the mtDNA copy numbers in different cell types were analyzed with a view to ascertaining if there were any correlations amongst them. However, the mtDNA copy number of lymphocytes did not correlate with that of granulocytes, and this pattern was identical in the sub-populations between T cells and B cells, as revealed in supplementary Figure 1. Further analysis is necessary to confirm whether this pattern is present in more specific cell types other than general T cells or B cells. Overall, the results of the present study are expected to make researchers realize that using different cell types, even from the same individual, may lead to different conclusions regarding mtDNA copy numbers.

In summary, the present study has revealed that dynamic changes in nucleated blood cells combined with platelets constitute the major source of circadian rhythm in mtDNA copy number during a 24-h period. Quantitative analysis of mtDNA copy number in whole blood was carried out using real-time PCR. This study has established a feasible method for minimizing the adverse effect of platelets through the use of a small volume of blood sample. This novel approach provides a promising prospect for investigating the mechanisms underlying mtDNA copy number-related diseases using animal disease models. Moreover, since the study showed that mtDNA copy number differed between lymphocytes and granulocytes, future work should focus on relationships between mtDNA copy number of nucleated blood cells and various diseases.

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Conflicts of interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Junjie Luo; Yakun Wang, Yuanwu Liu, Yiqiang Zhao, Junjie Luo collected and analysed the data; Yakun Wang and Yuanwu Liu wrote the text and all authors have read and approved the text prior to publication.

Yakun Wang and Yuanwu Liu contributed equally to this work as co-first author.

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