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ARTICLE

# The analysis of the histomorphometric characteristics and ultrastructural features of the liver in response to environmental cycle desynchronization in the wild desert rodent (*Gerbillus tarabuli*)

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**ABSTRACT** The architecture of the hepatic tissue in the nocturnal wild desert rodent (*Gerbillus tarabuli*) subjected to a shifted LD (Light/Dark) cycle was investigated using transmission electron microscopy and histomorphometry. Investigation involved two groups: the control group (n = 5), exposed to the regular LD cycle (LD: 12:12), and the shifted group (n = 5), exposed to the LD regimen with a prolonged light phase of 8 h, in a day by day alternation with regular LD cycle (LD: 12:12 / LD: 20:4) during 12 weeks. Histological examination showed in the shifted group disorganization of hepatocyte trabeculae, sinusoidal dilatation, and inflammatory cell infiltration around the portal tracts. Morphometric results revealed hypertrophy of hepatocytes with a size of 29.83 ± 1.69 µm vs. 17.52 ± 0.81 µm and of their nuclei diameter with 8.69 ± 0.25 µm vs. 6.55 ± 0.15 µm. This increase is highly significant ($p<0.001$) and represents an increase of 70.17% and 32.67%, respectively. We also noticed a significant increase in the number of binucleated cells compared to the control group with an average of 9.73 ± 0.98 vs. 5.53 ± 0.38 binucleated hepatocytes per area unit, respectively. Ultrastructural analysis corroborated the histological findings, confirming the installation of inflammatory foci, characterized by portal and intraparenchymal infiltration of leukocytes, proliferation of Kupffer cells and stellate cells, as well as an expansion of collagen fiber deposits. Electron microscopy also revealed in the shifted group some hepatocytic lesional features represented by vacuolar or liquefied cytoplasm associated with degenerative mitochondria and a rarefaction of organelles in addition to an abundance of lysosomes. Additionally, strong glycogen depletion was observed at the metabolic level. This study showed that 12 weeks of lengthening the light phase of the LD cycle, corresponding to the gerbil's resting period, had a discernible impact on hepatocellular activity, making *G. tarabuli* an interesting model to study the influence of artificial light at night as a circadian rhythm disruptor.

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## INTRODUCTION

The use of artificial light at night (ALAN) has become almost universal in the modern world, raising the issue of light pollution (Hicks et al. 2020). Although there is an ongoing debate about how these changes may affect human well-being and health, there is no doubt that exposure to ALAN negatively influences the circadian system and is considered as circadian rhythm disruptor (Lee and Kim 2019). The internal clock that governs a large number of essential biological functions, particularly our sleep rhythm, needs to synchronize itself with a high level of luminosity during the day and total darkness at night. However, current lifestyles increasingly tend to disrupt this circadian rhythm through intense light exposure in the evening and during the night via artificial lighting and screens, with a current increased rate of over 6% per year (Hölker et al. 2010).

Aside from purely economic considerations such as 24/7 industries, every individual can be awake and active at any time of the night with artificial lighting, which runs counter to and disrupts our normal daytime physiology. Furthermore, exposure to even low intensities of ALAN has been shown to disrupt circadian rhythms over by suppressing or reducing melatonin release (Dominoni et

al. 2013; de Jong et al. 2016; Grubisic et al. 2019; Ziegler et al. 2021; Meléndez-Fernández et al. 2023).

It is therefore not surprising that modern lifestyle factors and exposure to ALAN (e.g., time shifts induced by trans-meridian travel, night work, and shift work) result in circadian misalignment (Falcón et al. 2020). In particular, they can generate environmental light pollution which can also induce, in the short term, fatigue and sleep loss (Lunn et al. 2017) and cause, in the long term, through changes in our metabolism, diseases such as obesity, diabetes and certain cardiovascular diseases (Dominoni et al. 2016; Chellappa et al. 2019), inflammatory and liver diseases (Reinke and Asher 2016; Derbouz Rouibate et al. 2020) even brain disorders (Logan and McClung 2019).

It is better known today that the excessive development of artificial lighting implies harmful consequences on the fauna, not only at the level of a given species but also for the ecosystem. It structurally modifies the movements of the fauna, by diminishing and fragmenting its natural habitats. It can thus impact the distribution of certain species on the national territory (Azam et al. 2015). Moreover, an inverse correlation has been found between illuminated surfaces and mammalian species richness (Duffy et al. 2015; Ciach and Fröhlich 2019). Mice (Rotics et al. 2011) and small mammals in tropical forests (Bengsen et al. 2010) are less active under exposure to ALAN, which could lead to significant changes in the wildlife habitats (Dominoni et al. 2020).

The repercussions of light pollution, fueled by expanding urbanization, are more disastrous for the ecosystems of nocturnal species where the circadian misalignment disrupts species activities, potentially hastening their extinction or ecosystem collapse. All these arguments make Tarabul's gerbil (*Gerbillus tarabuli*), a nocturnal wild desert rodent, an interesting model for studying ALAN effects on humans. Moreover, this species holds significant value in biological research (Hamidatou Khati et al. 2022).

The purpose of the present study is to describe the ultrastructural and morphometrical changes in the liver of Tarabul's gerbil submitted to a prolonged light phase of LD cycle.

## MATERIAL AND METHODS

### *Animal experiments*

For this study, 10 mature *G. tarabuli* were used, with a mean body weight of 42 g. The gerbils were trapped in April in the desert of Béni-Abbès region (240 km southeast of Béchar and located 1200 km southwest of Algiers, 30° 07' N 2° 10' W). They were housed in individual cages under ambient temperature of 22 ± 2 °C under 12:12 h light-dark conditions (lights on at 00:00), fed on barley grains *ad libitum* without water. Four weeks later, gerbils were placed in chronobiotic rooms and were randomly assigned into 2 experimental protocols for 12 weeks: the control group (n = 5) was exposed to the regular LD cycle (LD: 12:12) (lights on at 00:00, lights off at 12:00) and the shifted group (n = 5) was exposed to the LD regimen with prolonged light phase of 8 h, in a day by day alternation with regular LD cycle (LD: 12:12 / LD: 20:4) (Fig. 1). All experiments were carried out in compliance with the European Union Directive (2010/63/EU) for animal experiments and the study was performed in conformity to the recommendations of the "Association Algérienne des Sciences en Expérimentation Animale (AASEA) (http://www.aasea.asso.dz/).

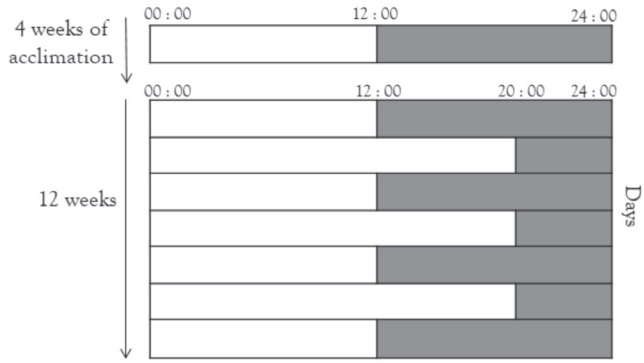


**Figure 1.** Experimental design displaying LD cycle with a prolonged light phase of 8 h, in a day-by-day alternation with regular LD cycle during a period of 12 weeks. White and black bars: duration of the light and dark phases, respectively.

### *Light microscopy*

Livers of both animal groups were collected after anesthesia during the light phase. Every liver tissue sample was divided into two specimens. The first was immersed in Bouin's fluid and embedded in paraffin blocks. Sections of 4 μm were stained with hematoxylin and eosin (H&E) for morphological studies (Martoja and Martoja-Pierson 1967). All paraffin sections were examined with a standard light-microscope Zeiss Axoplan and photographed with High-Resolution Optics Microscope Camera (MA88-500/Premiere®, 5.0 Megapixels with a 1280 x 1024 resolution) using TSView 6.2.4.5 software. None of the collected images were manipulated.

### *Morphometric study*

The morphometric study of histological sections was performed using AxioVision 4.8 (Carl Zeiss MicroImaging, Germany). Analysis consisted in taking 16 repeated measurements of hepatocytes's and nuclear diameters on the photos taken from several sections from each group

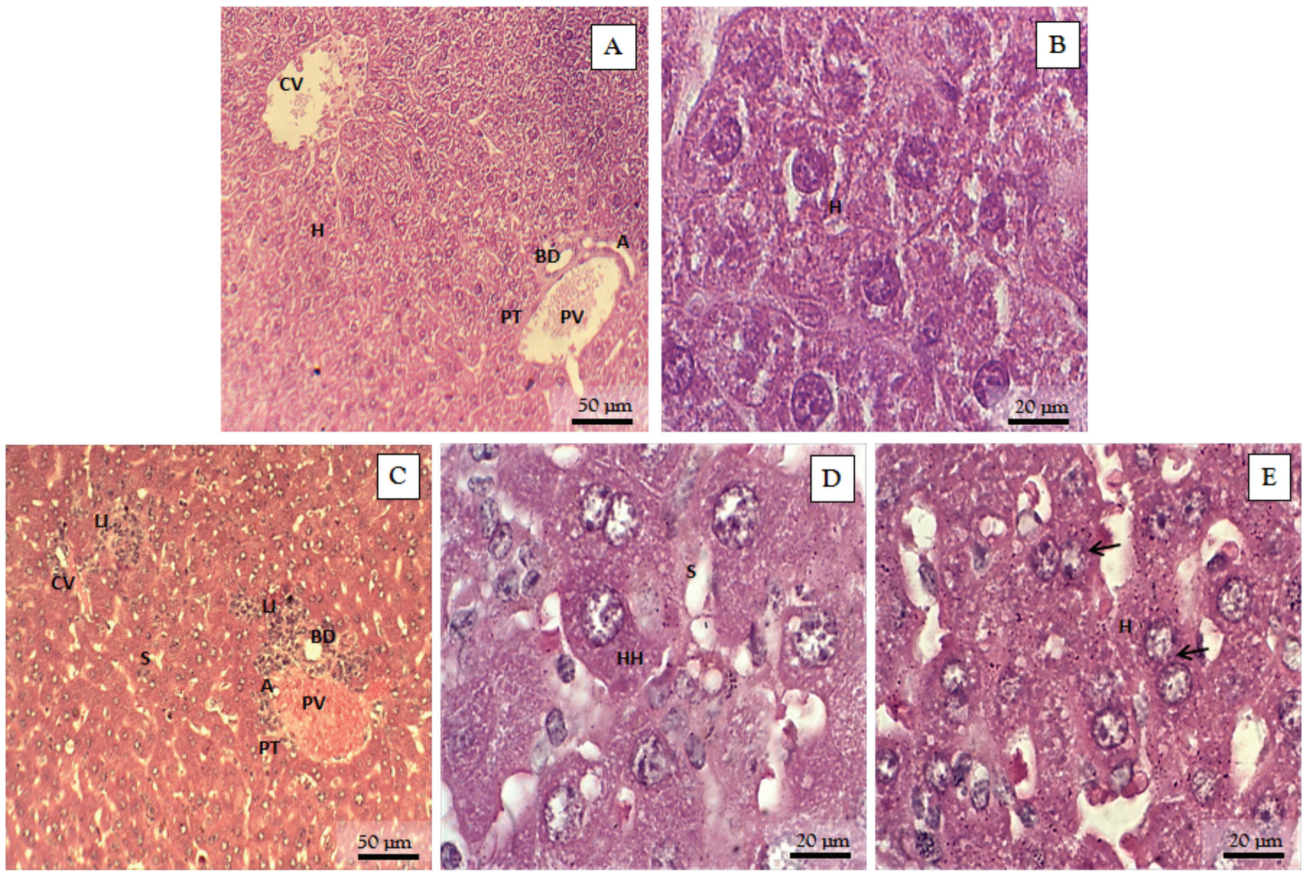


**Figure 2.** Light photomicrograph of liver sections of both groups of Tarabul's gerbils. A and B: control group, C to E: shifted group. **(A)** Normal architecture of hepatic tissue showing radiating cords of hepatocytes (H), anastomosing network of sinusoids (S), and central vein (CV) surrounded by portal tract (PT); portal vein (PV), hepatic artery (A) and bile duct (BD). **(B)** High magnification shows that hepatocytes (H) are polyhedral-shaped cells with a central rounded nucleus and granulated cytoplasm. **(C)** Light photomicrograph of liver sections from the shifted group showing disturbed normal architecture of hepatic cords, dilated sinusoids (S), congested central vein (CV) and portal vein (PV) and leukocyte infiltration (LI) with connective tissue extension. **(D)** Note in this group, the frequency of hypertrophied hepatocytes (HH). **(E)** Several of them are binucleated (arrow) or with hypertrophied nuclei. (A, B, C, D and E: H&E stain), Scale bar: 50 µm (A and C); 20 µm (B, D, and E).

at x100 magnification.

Binucleated cells were counted on three random sections from each animal of each group. The counting was carried out by defining a surface area of 55433.54 $\mu m^2$ applied to all the sections analyzed at x40 magnification. All measurements were calibrated against a micrometer slide that enables us to obtain the measurement unit in µm instead of pixels.

Results were expressed as means ± standard measurement error (SEM). Comparisons were performed using Student's t-test. Statistical differences were considered significant when $p<0.05$.

### *Electron microscopy*

The second liver specimens were prefixed with 2.5% glutaraldehyde - 4% paraformaldehyde (pH 7.2-7.4) at 4 °C and subsequently post-fixed in 1% osmic acid ($O_SO_4$) solution for 2 h, then embedded in epoxy resin blocks for transmission electron microscopy (TEM). Ultrathin sections (60-70 nm; Ultramicrotome Leica EM UC7) mounted on slot grids (Ted Paella) were double contrasted with lead citrate and uranyl acetate (Reynolds 1963). The sections were examined using a transmission electron microscope (FEI Technai G2 at 80 kV) and photographed with a high-resolution camera (GATAN camera). This technique was performed at the BioVis EM, IGP, Uppsala University, Sweden.

## RESULTS

### *Histomorphometric observations*

The control liver sections H&E stained revealed a homogenous structure of hepatic parenchyma with radiating

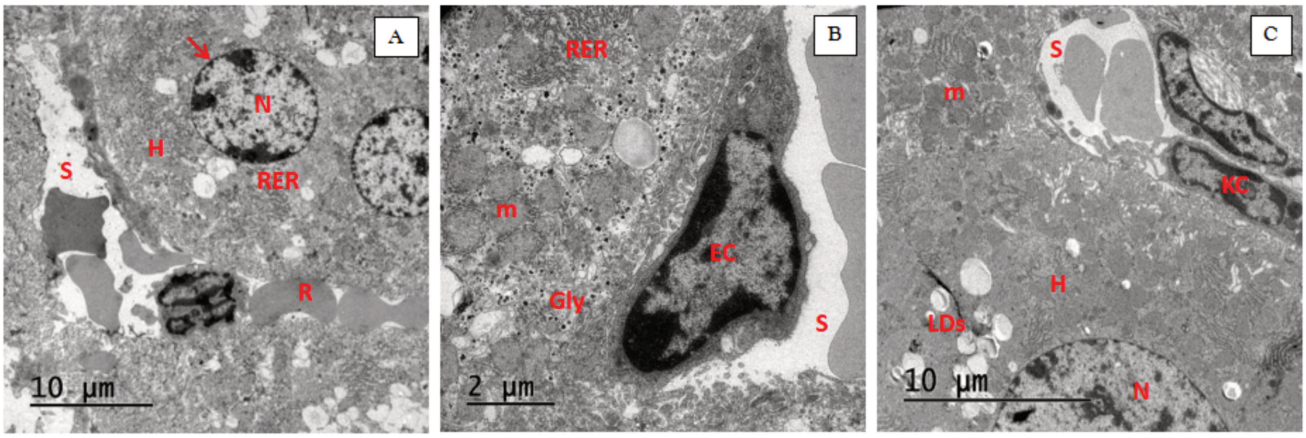


**Figure 4.** Transmission electron micrographs of liver sections from control group showing **(A)** normal hepatocyte (H) with round shaped nucleus (N). The cytoplasm is rich in rough endoplasmic reticulum (RER) and mitochondria (m). Note red blood cell (R) within hepatic sinusoid (S). In **(B)** Glycogen granules (Gly) in hepatocyte contacting an endothelial cell (EC) bordering sinusoid space (S). **(C)** Shows Kupffer cells (KC) and cytoplasmic lipid droplets (LDs).

cords of hepatocytes and an anastomosing network of sinusoids (Fig. 2A, B). Lobules are centered by a centrolobular vein and delimited with portal spaces (Fig. 2A). The hepatocytes exhibited a polyhedral shape with a rounded central nucleus (Fig. 2B).

In contrast, the shifted liver sections showed considerable alterations, manifested by highly dilated sinusoid capillaries disorganizing the lobules architecture. In addition, congested centrolobular and portal veins were accompanied by massive inflammatory cellular infiltration and extended connective tissue (Fig. 2C). We also noted hypertrophy of numerous hepatocytes and their nuclei (Fig. 2D) and several of them were binucleated (Fig. 2E).

Compared morphometric analysis showed an increase in hepatocytes and their nuclei sizes in shifted gerbils with average of 29.83 ± 1.69 µm *vs.* 17.52 ± 0.81 µm and 8.69 ± 0.25 µm *vs.* 6.55 ± 0.15 µm (Fig. 3), this increase is highly significant ($p<0.001$) and represents a percentage of 70.17% and 32.67%, respectively. Additionally, the number of binucleated hepatocytes was significantly increased in this group with 9.73 ± 0.98 *vs.* 5.53 ± 0.38 binucleated cells per area unit ($p<0.001$). For more detailed histological observations, refer to our previous study (Derbouz Rouibate et al. 2020).

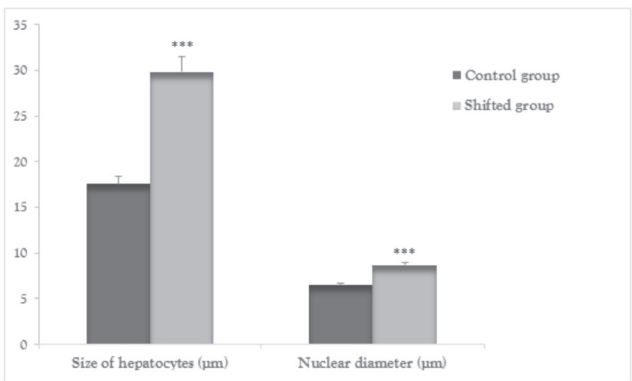


**Figure 3.** Average morphometric variations of *G. tarabuli*'s liver in control group (dark bars) and shifted group (gray bars). Values are expressed as mean ± SEM, (***$P<0.001$).

### *Electron microscopic observations*

Ultrastructural results of the control Gerbil's liver revealed polygonal hepatocytes with rounded euchromatic nuclei and cytoplasm rich in organelles such as mitochondria and rough endoplasmic reticulum. In addition, glycogen stored particles occupied an extended cell area (Fig. 4A, B, and C). Compared to control livers, the shifted gerbil's liver sections showed subcellular abnormalities represented by dilated sinusoids and pronounced binucleated and hypertrophied hepatocytes (Fig. 5A, B) characterized by glycogen particles depletion (Fig. 5B) and cytoplasmic vacuolation (Fig. 6A). Electron microscopic results also revealed signs of hepatocyte necrosis. Indeed, hepatocytes of this group exhibited two distinct phenotypes; some with homogenous cytoplasm and irregularly distributed organelles; others, more abundant, showed cytoplasm dissolution with rarified organelles, coalesced and degenerated mitochondria as well as extended smooth reticulum network (Fig. 5B).

Additionally, we noted numerous stellate cells in the Disse space (Fig. 5C), increased collagen fiber deposits (Fig. 5D), and abundant inflammatory Kupffer cells (Fig. 6A). Besides rarified organelles, we noted increased heterogeneous lysosomes (Fig. 6B). Also, electron microscopy showed in this group a massive circulating leukocyte in

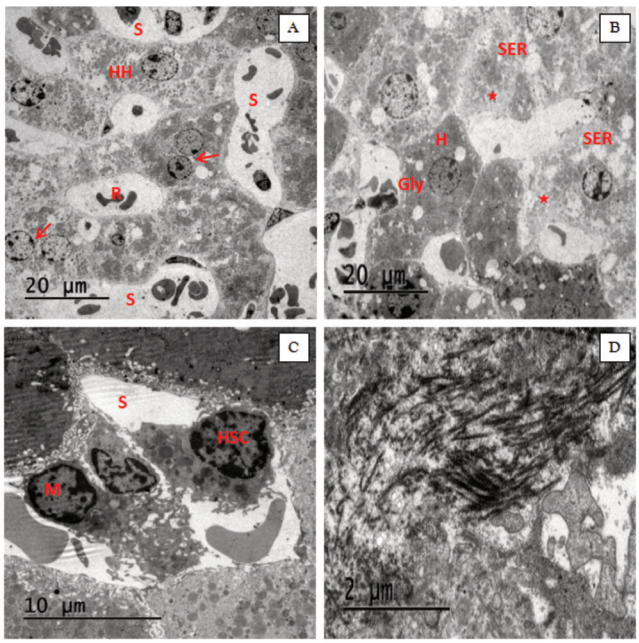


**Figure 5.** Photo electron micrographs of liver sections from shifted group showing **(A)** dilated sinusoid (S), hypertrophied hepatocytes (HH), and numerous binuclear hepatocytes (arrows). **(B)** Two types of hepatocytes: some with homogeneous cytoplasm (H) and others showed cytoplasm dissolution (stars) with extended smooth endoplasmic reticulum (SER). **(C)** Increased hepatic stellate cell (HSC). **(D)** Increased collagen fibers deposits.

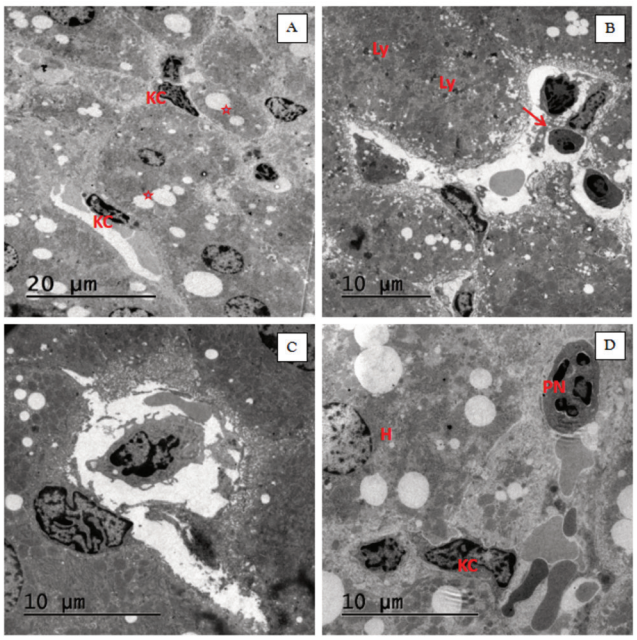


**Figure 6.** Electron micrographs of liver sections from shifted group showing **(A)** vacuolation of hepatocyte cytoplasm (stars) and inflammatory intraparenchymal Kupffer cells (KC). **(B)** Numerous intrahepatocyte lysosomal bodies (Ly), and massive circulating leucocytes (arrow) in portal space. **(C)** Transendothelial leucocyte migration and intraparenchymal infiltration in **(D)**. Polynuclear neutrophil (PN) and hepatocyte (H).

portal space (Fig. 6B), transendothelial leucocyte migration (Fig. 6C), and intraparenchymal infiltration of immune cells (Fig. 6D).

## DISCUSSION

Light and electron microscopic results indicate in the shifted group an alteration of tissue architecture characterized by enlarged sinusoid spaces with proliferative Kupffer cells and prominent intraparenchymal and periportal infiltrated leucocytes as well as extended connective tissue deposits. This pattern of damage reflects an inflammatory state following hepatocellular dysfunction.

The inflammatory response induces cytokines release to generate a specific immune response (Dufour and Clavien 2015). During liver inflammatory phenomenon, activated Kupffer cells can migrate into the Disse space and come into contact with hepatocytes (Losser and Payen 1996; Roberts et al. 2007) that which may explain the observed dilation of sinusoidal capillaries and Kupffer cells proliferation in these experimental conditions.

Moreover, the inflammatory response is characterized by increased levels of circulating proinflammatory cytokines and tissue infiltration by immune cells such as macrophages, neutrophils, and eosinophils (Weisberg et al. 2003; Xu et al. 2003). Neutrophils, in particular, play a pivotal role in the inflammatory response; they are attracted early by chemotactic factors and reach tissue sites through capillaries with increased permeability (Babior 2000).

Neutrophils are considered major effector cells in the tissue damage observed in many inflammatory diseases (Zimmerman et al. 1990; Heinzelmann et al. 1999). We have already demonstrated by IHC in these animals under the same experimental conditions an intense immunoreactivity of hepatic myeloperoxidase (MPO) a reliable indicator of inflammation confirming neutrophils' presence in liver tissue (Derbouz Rouibate et al. 2020), Indeed it is reported that neutrophils are the predominant inflammatory cells in liver injury (Voigt et al. 2018). Furthermore, inflammatory cells, along with Kupffer cells, secrete the cytokines that activate hepatic stellate cells (HCS), causing them to divide and perpetuate the synthesis of proinflammatory cytokines, leading to abundant extracellular matrix production (Patsenker and Stickel 2011). It is also reported that upon injury, stellate cells become the primary source of extracellular matrix production and accumulation in the liver (Friedman 2008; DeLeve et al. 2011).

Simultaneously, electronmicroscopic analysis of the shifted group in our study showed an increase in hepatic stellate cell numbers and expansion of collagen fiber deposits, reflecting the development of fibrosis. The activation of hepatic stellate cells is a critical step in the development of liver fibrosis (Jarnagin et al. 1994; Gressner and Bachem 1995; Bekheet et al. 2009; Abdel-Hamid et al. 2018). This process represents the tissue response to a persistent injury, promoting accelerated extracellular matrix generation (Enzan et al. 1995; Patsenker and Stickel 2011).

Our team revealed, by body temperature recording, that activity phase of *G. tarabuli* is at night (Issad et al. 2021). It seems that the phase-shifting protocol induced a lengthening of its rest phase leading to liver injury. It is worth noting that ALAN exposure is associated with a series of disruptive processes in the circadian rhythm system that induce inflammatory responses (Guan et al. 2022). It is also demonstrated that ALAN exposure in rodents consistently elevated basal inflammation (Walker et al. 2022) by upregulating TNF-α (tumor necrosis factor) and MAC1 (macrophage-1 antigen) genes expression in the liver (Fonken et al. 2013). Moreover, MAC1 is required for leukocyte recruitment in inflammation (Dunne et al. 2003; Fan and Ley 2015) that which support our structural and ultrastructural observations.

Nevertheless, other studies report that ALAN exposure disrupts circadian rhythms and alters immune function (Mishra et al. 2019; Ziegler et al. 2021). Our results also showed the installation of fibrosis which could indicate an ineffectiveness of the immune responses in this species. Previous experiments on rodents suggested that animals kept under mostly constant light conditions for several weeks exhibit weaker immune responses (Kirby and Froman 1991; Moore and Siopes 2000; Durrant et al. 2020). In addition, alterations in circadian rhythms (e.g., clock gene mutation in mice or environmental disruption similar to shift work) lead to disturbed immune responses (Labrecque and Cermakian 2015).

In the current study, electronmicroscopy of hepatocytes from the shifted group showed images of hepatocyte cytoplasmic lysis areas and cytoplasmic vacuolation associated to degenerated and coalesced mitochondria as well as extended smooth reticulum network. These observations are most probably due to oxidative stress and lipid peroxidation. It is well documented that oxidative stress compromises cell organelles membranes, thus increasing their permeability (Shubin et al. 2016). Moreover, cytoplasm rarefaction may be due to proliferation of smooth endoplasmic reticulum (Lotowska et al. 2014).

In our previous results (Derbouz Rouibate et al. 2020), shifted gerbils showed perturbations in serum and hepatic metabolic parameters, including blood glucose, triglycerides, cholesterol, as well as hepatic glycogen and total lipids quantification, and here, the subcellular observations showed also depleted glycogen particles and vacuolated cytoplasms suggesting that these experimental conditions had led to an hepatocellular metabolic hypofunction.

Another interesting finding in shifted Gerbil's liver is the numerous binucleated hepatocytes exhibiting hypertrophied cytoplasm and nuclear area. These images suggest hepatocyte growth with compensatory regenerative purposes that could correct the hepatic necrosis. Liver regeneration is triggered in response to detected damage, with inflammation being one of the initial processes activated (Di-Iacovo et al. 2023). Indeed, damaged hepatocytes are recognized by leukocytes, which contribute to accentuating the inflammatory state (Canbay et al. 2003). Kupffer cells play a key role in inflammation activation via IL-6 and TNF-α (Selzner et al. 2003; Tang et al. 2022). In this phase, the liver's objective is to replace damaged cells primarily by increasing hepatocyte proliferation but also by activating ductal progenitors. Sinusoidal cells facilitate the generation of mitogenic signals such as HGF, promoting hepatocyte expansion (Ding et al. 2010). Indeed, hepatocytes interacting with Kupffer cells become sensitive to proliferative stimuli and contribute to the progression of the regeneration process (Zimmers et al. 2003). Tissue repair also involves of extracellular matrix deposition, primarily mediated by myofibroblasts, with hepatic stellate cells serving as their precursors. Activated by macrophages secreting TGF-β and TNF-α, hepatic stellate cells exhibit high replicative capacity and express collagen at high levels (Yavuz et al. 2020). Following the inflammatory response, recruited monocytes can also differentiate into myofibrocytes and contribute to regeneration (Brenner et al. 2012).

It seems that the phase-shifting protocol, had led to a circadian misalignment in gerbils resulting in an inflammatory response, along with a reparative process probably to correct the hepatic hypofunction.

## CONCLUSION

The main findings of this study indicate that a disruption of the environmental cycle affects hepatic tissue organization as well as its cytofunctional state. It appears clear that *G. tarabuli* provides an attractive model for studying the effects of ALAN exposure. Moreover, this study highlights the role of the photic signal in entraining circadian clocks, it is thus necessary for us to study the expression profiles of Gerbil's liver's clock genes under the same experimental conditions.

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