Effects of Various Conditions Related to Circadian Rhythm Disturbances on Plasma and Erythrocyte Lipids in Rats: A Peroxisomal Perspective

Hüsniye Gül Otlu
Inonu Universitesi Tip fakultesi

Yılmaz Uğur
Inonu Universitesi

Azibe Yıldız
Inonu Universitesi Tip fakultesi

Selim Erdoğan
Inonu Universitesi

Saim Yoloğlu
Inonu Universitesi Tip fakultesi

Nigar Vardı
Inonu Universitesi Tip fakultesi

Tayfun Güldür (tayfun.guldur@inonu.edu.tr)
Inonu Universitesi Tip fakultesi https://orcid.org/0000-0002-1623-2880

Research

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Abstract

Background: It was aimed to investigate effects of various conditions known to cause circadian rhythm disturbances (i.e. calorie restriction, time-restricted feeding, constant light exposure) on various peroxisomal parameters and to compare those effects with that of fenofibrate, a PPARα agonist, in rats.

Methods: Plasmalogens and some fatty acids in erythrocyte lysates were analyzed by GC. Peroxisomal metabolites including very long chain fatty acids as well as phytanic and pristanic acids in plasma were measured by GC-MS.

Results: Unlike calorie restricted feeding, fenofibrate treatment yielded lower level of plasma phytanic acid concentration implying higher peroxisomal α-oxidation rate. However both calorie restriction and fenofibrate treatment exhibited lower plasmalogen, DHA and arachidonic acid contents of erythrocyte lysates.

Conclusion: Shared effects of conditions associated with circadian rhythm disturbances and peroxisomal induction by fenofibrate on erythrocyte membrane lipids might indicate a link between them.

Introduction

Circadian rhythm includes rhythmic metabolic processes as well as physiological and behavioural activities occured in an organism during 24 hour period. Clock machinery is composed of transcriptional, translational and posttranslational feedback loops associated with clock genes (i.e. *Clock, Bmal1, Cry and Per*). Two clock mechanisms take part in generation of rhythmicity. The central clock resides in suprachiasmatic nucleus (SCN) of hypothalamus which is entrained by light. Whereas the periferal clocks are suggested to occur at various tissues which can be entrained by food. Two clocks are believed to be connected via hormonal and neuronal means. Daily oscillations in secretions of melatonin and cortisole or corticosterone, are regulated by SCN through which propagation of the rhythmicity to periferal tissues occurs (1–6). As a result of modern life style, circadian rhythm disturbances caused by night-shift working conditions, crossing time zones in a very short period of time (jet-lags), late night eating, exposure to blue light during night are all reported to be linked with chronic diseases such as obesity, diabetes (7). Since the foods are robust circadian zeitgebers, alterations in feeding regimens results in circadian rhythm disturbances (8). Restricted feeding (7 days) causes a phase-shift in circadian expression of the major oscillator genes and their downstream targets in adipose tissues (9, 10). Peripheral oscillators become uncoupled from the master SCN under restricted feeding conditions. Intermittent fasting in mice causes arrythmicity and phase advance in some clock genes when the food was introduced during the day (passive phase of nocturnal mice), not in night, since the effect of fasting on circadian rhythm depends on feeding time (11). Calorie restriction is also known to affect the temporal organization of the SCN clockwork and circadian outputs under light/dark cycle (12). It is well established that continuous light exposure also alters circadian rhythmicity and circadian clock genes in both SCN and peripheral tissues whereby changing metabolic functions (13, 14).

Clock genes are known to regulate metabolic processes through nuclear transcription factors including PPARs and REV-ERBα (15). Agonists of PPARs, such as fenofibrate, can not only induce peroxisomal growth and proliferation (16) but also is able to reset rhythmic expression of *Bmal1, Per1, Per3* and *Rev-erbα* in mouse hepatocytes (17).

Sleep restriction is one of the condition known to disturbe circadian rhythmicity. Sleep restriction in both human and rats resulted in elevated plasma phospholipids. Seven plasmalogen species were reported to be raised in the rat under acute or chronic sleep restriction conditions. As the plasmalogens are partly synthesized in peroxisomes, the authors pointed out the induction of peroxisome proliferator-activated receptors and disruptions of the circadian clock (18). The most altered plasma lipids as a function of sleep restriction are various glycerophospholipids including plasmalogens that were reported to exhibit circadian oscillation (19–23). However, effects of circadian rhythm disturbances caused by conditions other than sleep restriction on plasmalogen and some other peroxisome-related lipid levels (e.g. plasmalogens, long chain fatty acids, pristanic acid, phytanic acid) have not yet been considered. To this end, restricted feeding, calorie restriction and continuous light exposure conditions known to interfere with either central or periferal clock rhythm mechanisms in rats were tested for their final effect on some peroxisome-related parameters in blood and liver tissue samples obtained at the termination of 2 weeks exposure. In order to evaluate the effects of these conditions linked to circadian rhythm disturbances on peroxisomes as well as peroxisomal lipids, an additional approach was implemented in which rats were fed with fenofibrate (a PPARα agonist) supplemented feeds in order to provoke peroxisomes. Then the results were compared with those of circadian rhythm disturbances tested. The results indicated some similarities as well as differences between the conditions related to circadian rhythm disturbances and fenofibrate administration.

Methods

Experimental design

Seventy five Sprague Drawley male rats weighing 360–380 g were used. Animal procedures were performed in accordance with the guideline set by İnönü University Scientific Ethical Committee on Animal Experimentation (Protocol number: 2017/A-29). During the adjustment period for one week, the rats were assigned to five groups ,15 of each, and kept at 21–22 ^{o}C under 12:12 hours light-dark cycle and fed ad libitum. Following this period, in the case of time restricted feeding group (TRF), the rats were allowed access to food (rat chow pellet) only between 8:00 a.m. and 11:00 a.m. for two weeks. The rats were allowed daily access to 60% of their normal daily calorie consumption starting at 08:00 am every day in the case of calorie restricted feeding (CRF) experimental condition. Because the effect of fasting on circadian rhythmicity in mice depends on feeding time, in the current work, feeding time was implemented during day time (11). Rats in continuous light exposure (CLE) group were kept under continuous light. Additionally, another fifteen of rats were fed with chow pellet contaning 0.1% fenofibrate (Lipanthyl, Reciparm Fontaine or Alembic Pharmaceuticals) (FSD: Fenofibrate Supplemented Diet) for

two weeks. Finally, control group (CTR) was kept under the conditions same as described for the adjustment period.

In our work, we aimed to investigate the effects of two weeks (long term) exposure to conditions known to be associated with circadian rhythm disturbances on some peroxisome related lipid parameters. Blood samples were taken at the end of two weeks period. The current work did not aim to indicate daily oscillation of lipid parameters.

Collection of the samples

The rats were weighed before and after the experimental period in order to follow up changes in body weights. Following the two weeks experimental period, anaesthesia of the rats of any group commenced at 7:00 pm onward and blood samples obtained from the bifurcation of the femoral artery were collected in blood collection tubes containing EDTA. Then the blood samples were centrifuged at 3.000 rpm for 10 min. at 15 ^{0}C. The resultant plasma were kept at -80 ^{0}C until analysed.

For plasmalogen analysis, plasma and buffy coat layer were removed by centrifugation as above and the resultant erythrocyte pellet was washed with an equal volume of saline (0.9% NaCl). The pellet were placed in an Eppendorf tube containing 100 µL of butylated hydroxytoluene (BHT) dried under the nitrogen stream and kept at -80^{0}C until analysed.

Histopathological Analyses

2–3 mm thick liver specimens were taken from the same lobe of the rat liver were fixed in 10% formalin and was embedded in paraffin. Tissue sections were cut at 4 *µm*, mounted on slides, stained with hematoxylin-eosin (H-E) for general liver structure. Hydropic changes in the liver was assessed in 10 randomly selected fields on each section. Alterations in structure were evaluated using a histopathological score as follows: 0, normal; 1, mild; 2, moderate; 3, severe (24).

Immunohistological Analyses

4 µm thick tissue sections were deparaffinized and rehydrated and placed in antigen retrieval solution (citrate buffer, pH 6.0) and boiled in a pressure cooker for 20 minutes and cooled to room temperature for 20 min. Then the sections were washed with phosphate-buffered saline (PBS, pH 7.4). For blocking endogenous peroxidase activity, the slides were incubated in 0.3% hydrogen peroxide solution for 15 min at room temperature and washed in PBS. After the blocking of non-specific antigen-binding sites with protein block, primary catalase antibodies (Boster catalog no: PB9925, China) were applied for 60 minutes at room temperature. Having rinsed with PBS, sections were incubated with biotinylated secondary antibody and streptavidin peroxidase for 10 minutes at room temperature. Samples were visualized with the chromogenic substrates AEC, counterstained with hematoxylin and mounted in glass slide. According to the diffuseness of the staining, the sections were graded as 1 = 0–25% staining; 2 = 25–50% staining; 3 = staining 51–75%; 4 = staining 76–100%. According to the staining intensity, the sections were graded as follows: 0 = no staining; 1 = weak but detectable staining; 2 = distinct; 3 = intense staining. Total staining score was obtained as (diffuseness)X(intensity) (25).

Analyses of C16:0 and C18:0 plasmalogens, arachidonic acid (AA) and docosahexaenoic acid (DHA) content in erythrocyte lysates

By adding methanolic HCl to erythrocyte lysates and heating the mixture in a closed vial at 90 °C for 4 h, fatty acid glycerol esters are transmethylated resulting in the formation of fatty acid methyl esters whereas the alkyl-1-enyl ether linkage in plasmalogens is cleaved with acidified methanol, leading to the quantitative formation of the fatty aldehyde dimethyl acetals. After cooling the sample, the fatty acid methyl esters and the dimethylacetals are extracted with hexane. Then 1 µl of the resulting hexane solution was injected to GC (26).

Instrumentation: Chromatographic analyses were achieved on a Shimadzu 2010 gas chromatography (GC) (Shimadzu Technologies, Kyoto, Japan) consisting of autosampler, in-line degasser and a flame ionization detector (FID). The instrumental configuration and analytical conditions were summarized in the following; Shimadzu 2010 GC-FID instrument equipped with a RT-2560 capillary column (100 m x 0.25 mm x 0.20 µm, RESTEK Scientific) under the following temperature program: 140 °C for 5 min followed by an increase to 240 °C at a rate of 20 °C/min for 45 min. The injector and flame ionization detector temperatures were set at 240 °C. Carrier Gas: Helium, 20 cm/sec., 150 ^{o}C, Detector: FID, 240 °C. Liner: 4 mm I.D split, cup design. AA and DHA content were expressed as percentages of total fatty acid methyl esters in erythrocyte lysates.

The determinations of fatty acid methyl esters (FAME) was carried out by comparing the retention time with those of the reference standard mixture (37-Component FAME Mix, Supelco) analyzed under the same analytical conditions. The plasmalogens are eluted adjacent to their corresponding fatty acid methyl esters. The plasmalogen values are not expressed in absolute values, but as a percentage of the level of the corresponding fatty acid. Hence, the C16:0 dimethylacetal and C18:0 dimethylacetal were compared with the C16:0 fatty acid methyl ester and C18:0 fatty acid methyl esters respectively (26) (Fig. 1.).

Very long chain fatty acids, pristanic acid and phytanic acid analyses in plasma

Following alkaline and acid hydrolysis, plasma very long chain free fatty acids, phytanic and pristanic acids extracted with hexane were derivatized with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide and 1% tert-butyldimethylchlorosilane to tertiarybutyl-dimethylsilyl derivatives (26). For the quantitative analysis, stable isotopes for C26:0, C24:0, C22:0, phytanic acid and pristanic acid were used (C26:0-d_4 (3,3,5,5-$^2H_4$-hexacosanoic acid), C24:0-d_4 (3,3,5,5-$^2H_4$-tetracosanoic acid), C22:0-d_4 (3,3,5,5-$^2H_4$-docosanoic acid), pristanic acid-d_3 (2-methyl-$^2H_3$-6,10,14-trimethyl) pentadecanoic acid), phytanic acid-d_3 (3-methyl-$^2H_3$-7,11,15-trimethyl) hexadecanoic acid). The resultant sample dissolved in hexane were injected into GC-MS (Agilent 6850 GC/Agilent 5977E MS) equipped with a column (Agilent HP5ms, 30 m

x 0.25 mm x 0.25 µ) and column oven (Agilent 5890B). These analysis were conducted in Synlab Turkey Laboratory (Ankara/Turkey).

Plasma melatonin analyses

Plasma samples were preextracted through SPE column (C-18, Supelco HLB 30 mg / 1 mL SPE tube) with dichloromethane in order to remove substances which may interfere with the subsequent melatonin analysis. The extract dried under nitrogen gas stream was resuspended in acetonitrile: water (1:1, v/v) prior to injection to HPLC system (Shimadzu HPLC with SPD-M 20 a fluorescence detector) which was equipped with C18 (250 mm x 4.6 mm) ODS-2 reverse phase Kromasil-100-5 column. The analysis was performed at an excitation wavelength of 275 nm and emmision wavelength of 345 nm. A calibration curve of set of melatonin standards of known concentrations (0.2 pg/mL – 200 pg /mL) was used for quantitative evaluation of plasma melatonin levels. Mobile phase was composed of the mixture of 720 mL of 75 mM sodium acetate and 280 mL of acetonitrile, final pH adjusted to 5. The flow rate was set at 1 mL /1 min (27, 28).

Plasma corticosterone analysis

Plasma corticosterone levels of rats were analysed by LC-MS/MS (Agilent 1290 Infinity II UHPLC /Agilent 6460 Triple Quadrupole). The analysis was operated with JASEM Steroid Hormone Kit according to the manifacturer’s instruction in JASEM Sem Laboratuar Cihazları Pazarlama Sanayi ve Tic. A.Ş. (İstanbul /Turkey).

Quantitative analysis of plasma triacylglycerol

The analysis was carried out spectrophotometrically by Abbott Triacylglyceride Kit via Abbott Architect c16000 otomatic analyser according to the manufacturer’s instruction. A calibration curve was prepared using two triacylglycerol standards with concentrations of 94 mg/L and 450 mg/L (MCC, Architect, USA) with which quantitative evaluation was performed.

Statistical Analyses

Immunohistochemical findings of the study were analyzed using statistical software SPSS for Windows version 17. Shapiro-Wilk test was used to determine whether or not the data fit the normal distribution. Mann-Whitney U test was used for comparisons between the groups. All the data were expressed as median (min-max), $p < 0.05$ was considered statistically significant level. R version 3.5.0 and IBM SPSS Statistics 22.0 software were used for the statistical analyses of triacylglycerol, VLCFA, plasmalogen, corticosterone and melatonin levels and the rats’ body weight measurements. The data were summarized using median, minimum value, maximum value and interquartile range (IQR) statistics. Shapiro-Wilk test was used to determine whether or not the data fit the normal distribution. Kruskal-Wallis H test was used for comparisons between independent groups. Multiple comparison tests were performed with Conover test. Wilcoxon test was used for comparisons between dependent groups. $p < 0.05$ was considered to be statistically significant level.

Results

Body Weight Changes in Rats

Body weight of rats exposed to various experimental conditions known to influence circadian rhythm including time-restricted feeding (TRF), calorie-restricted feeding (CRF), continuous light exposure (CLE) and feeding diet supplemented with fenofibrate (FSD) for two weeks were recorded at the commencement and cessation of the experimental procedure. Body weight changes among experimental groups were compared (Table 1). In the calorie restricted group, rats were allowed to feed on only 60% of average daily feed intake and as a consequence, they lost average 9.6% of their body weight at the end of the experimental period. The decrease was found to be significant. Body weight changes recorded in other groups were found to be insignificant.

Table 1
Comparison of average body weight in each group of rats between initiation and completion of the experiment.

Average Body Weight (g)						
Group	**1st Day Median**	**IQR**	**15th Day Median**	**%Changes**	**IQR**	p
CTR	370	31.5	370	0	32.5	0.073
TRF	390	21.0	384	-1.5	14.0	0.393
CLE	373	41.0	377	+ 1.1	35.0	0.551
FSD	381	42.5	379	-0.5	31.0	0.802
CRF*	374	43.5	338	-9.6	34.5	0.001

Data are given as median and IQR (interquartile range). Significance of differences between initiation and completion of the experiment in each group are indicated as $^{*}p < 0.05$ (n = 15). CTR : Control group, TRF : Time restricted feding group, CLE : Continuous light exposure, FSD: Fenofibrate supplement diet group, CRF : Calorie restricted feeding group.

Effects of Experimental Conditions Influencing Circadian Rhythm on Some Blood Lipid Parameters

Plasma Triacylglycerol Levels

Plasma triacylglycerols of experimental groups are shown in Table 2. In plasma of rats fed on diet supplemented with 0.1% fenofibrate, which is known for its lipid lowering effect, average triacylglycerol concentration was found to be decreased by 66% compared to that of average control values. Whereas calorie restriction in rats resulted in a 45% decrease in average plasma triacylglycerol level compared to the control values. As both fibrate treatment and calorie restriction are well known to cause lower plasma triacylglycerol, these two conditions used in the current work appeared to be effective under experimental

conditions. Plasma triacylglycerol levels of TRF and CLE were also lower compared to controls albeit insignificant. Plasma triacylglycerols levels of groups can be arranged in descending order as follows : CTR > CLE > TRF > CRF > FSD (Table 2).

Table 2
Average serum triacylglycerol levels in each group of rats at the completion of the experimental period.

Triacylglycerol (mg/dL)					
Group	**Median**	**Minimum**	**Maximum**	**IQR**	p
CTR	69	33	95	56.8	0.0003
CLE	52	35	103	39.3	
TRF	41	31	72	10.5	
FSD*	23.5	16	41	12.5	
CRF*	31	22	35	9.0	

Values are given as median, minimum and maximum and IQR. *$p < 0.05$; compared with CTR group. n = 8 for each group.

Erythrocyte Lysate C16:0 and C18:0 Plasmalogen Levels

Gas chromatographic analysis of fatty acid methyl esters and plasmalogen dimethylacetals in erythrocyte lysates were carried out. The plasmalogens are eluted adjacent to their corresponding fatty acid methyl esters. The plasmalogen values are expressed as a percentage of the level of the corresponding fatty acid. The C16:0 dimethylacetal was compared with the C16:0 fatty acid methylester whereas C18:0 dimethylacetal with C18:0 fatty acid methyl ester.

Percentage C16:0 plasmalogen level in erythrocyte haemolysates of FSD rats were found to be slightly lower compared to that of control values. Whereas plasmalogen levels in other groups were similar to that of control values. In the case of percentage C18 plasmalogen levels in erythrocyte lysates, all but TRF group exhibited significantly lower level in comparison to that of control group. The decrease in all the groups varied from 6% to 10%. FSD and CRF groups showed highest level of decrease in erythrocyte plasmalogen level (Fig. 2).

Erythrocyte Lysate Long Chain PUFA Compositions

Arachidonyl and docosahexanoyl acyl chains are the most common at the sn-2 position of ether lipids (29). Arachidonic acid (C20:4 (n-6)) and docosahexaenoic acid (22:6 (n-3), DHA) percentages in erythrocyte haemolysate were found to be significantly decreased by 11% and 35% in FSD group respectively compared to that of control group. Both fatty acids were also lower in CRF group however in the case of DHA, the difference was not significant (Fig. 2).

Plasma Concentrations of Very Long Chain and Branched Chain Fatty Acids

Catabolism of the phytanic acid, a branched-chain fatty acid, by alpha-oxidation yields pristanic acid which is further broken down by peroxisomal beta-oxidation. In addition to very long chain fatty acids, either phytanic acid or pristanic acids are known to accumulate in some peroxisome related diseases (30).

Comparison of C22:0, C24:0 and C26:0 very long chain fatty acids concentrations made among groups or between control group and either of the experimental groups indicated no significant difference (Table 3). However slightly higher C22:0 and C24:0 levels (2–3 nmol/L) was discernable in CRF group compared to the others. In the case of branched chain fatty acids, calorie restriction produced higher level of plasma phytanic acid concentrations whereas fenofibrate treatment yielded lower concentrations compared to the average control value. However, plasma pristanic acid concentrations were found to be similar among all the groups (Table 3).

Table 3
Levels of plasma very long chain fatty acids, pristanic acid and phytanic acid in various circadian disorder conditions of rats.

nmol/mL						
	Group	Median	Minimum	Maximum	IQR	p
C22:0	CTR	6.73	5.34	10.01	1.95	0.22
	CLE	7.37	3.12	15.30	6.27	
	TRF	5.92	3.87	10.22	4.28	
	FSD	6.17	3.12	10.60	4.60	
	CRF	8.51	5.72	12.56	4.21	
C24:0	CTR	15.28	9.32	21.37	3.50	0.275
	CLE	17.3	4.84	29.30	10.9	
	TRF	14.74	9.59	22.46	8.61	
	FSD	13.14	6.76	26.30	9.26	
	CRF	18.13	13.36	27.05	9.17	
C26:0	CTR	0.07	0.05	0.10	0.02	0.49
	CLE	0.06	0.05	0.10	0.03	
	TRF	0.07	0.05	0.60	0.02	
	FSD	0.06	0.05	0.61	0.02	
	CRF	0.06	0.04	0.09	0.02	
Phytanic Acid	CTR	0.44	0.32	0.59	0.12	0.0001
	CLE	0.48	0.26	0.96	0.30	
	TRF	0.43	0.29	0.71	0.16	
	FSD*	0.22	0.12	0.36	0.13	
	CRF*	0.58	0.42	0.72	0.16	
Pristanic Acid	CTR	6.46	4.76	11.50	3.41	0.571
	CLE	6.45	3.05	11.38	3.23	
	TRF	5.59	4.5	9.42	2.88	

Data are presented as median, minimum, maximum and IQR. Significance of differences compared with CTR group was indicated as *$p < 0.05$. n = 12 for each group.

nmol/mL				
FSD	5.49	3.16	11.10	3.50
CRF	5.97	5.15	9.54	1.64

Data are presented as median, minimum, maximum and IQR. Significance of differences compared with CTR group was indicated as *p < 0.05. n = 12 for each group.

Blood Parameters Associated with Master (Central) Circadian Clock: Plasma Concentrations of Melatonin and Corticosterone

The cortisol is the primary glucocorticoid in humans, but also in many other mammals, however, rats and mice have predominantly corticosterone. Plasma corticosterone levels in rats are under master circadian clock control and exhibit a robust daily oscillations (31) therefore used as an indicator of circadian phase in experimental groups. At the end of the experimental period, blood samples were obtained from rats of control and experimental groups at 7 p.m. onwards simultaneously with the collection of other specimens. The resulting serum samples were analysed by LC-MS for the determination of corticosteron concentrations. Comparisons were made among groups results of which are shown in Table 4.

Table 4
Plasma corticosterone concentrations in various circadian disorder conditions tested in rats.

Corticosterone (ng/mL)					
Group	**Median**	**Minimum**	**Maximum**	**IQR**	p
CTR	187.20	165.50	197.10	17.5	0.498
CLE	205.45	165.07	224.09	70.6	
TRF	231.66	140.67	239.86	49.1	
FSD	220.56	148.50	249.60	0.0	
CRF	193.36	159.70	209.32	30.4	

Plasma corticosterone levels were analysed by LC-MS/MS. Data are presented as median, minimum, maximum and IQR. No significant difference was found between any two groups. n = 5 for each group.

Although slightly higher average levels of plasma corticosterone levels in experimental groups compared to that in the control group are noticeable, the differences were found to be insignificant.

In mammals the pineal hormone melatonin is involved not only in transduction of a photoperiodic information, but also in modulation of the phase of the circadian system as well (31). In order to evaluate any phase shift of circadian oscillation in the experimental groups, plasma melatonin levels in rats exposed to various conditions known to cause circadian rhythm disturbances were analysed by HPLC

and the concentrations were compared among the groups (Table 5). Fenofibrate treatment resulted in a substantial decrease in plasma melatonin concentrations compared to other experimental groups. Average plasma melatonin level in FSD group was approximately one tenth of that either in contol or most experimental groups. The average melatonin level of TRF group was somewhat higher compared to other groups except FSD group however this difference was insignificant.

Table 5
Plasma melatonin concentrations of rats subjected to various conditions associated with circadian disorders.

Melatonin (pg/mL)					
Group	**Median**	**Minimum**	**Maximum**	**IQR**	p
CTR (n = 5)	214	156	223	51.8	0.0314
CLE* (n = 6)	219.5	155	276	245	
TRF*(n = 6)	362	180	446	67	
FSD (n = 7)	18	10	50	0	
CRF*(n = 7)	248	212	508	168	

Plasma melatonin levels were analysed by HPLC following preextraction with SPE column. *$p < 0.05$; compared with FSD group.

Morphology of the Liver

Liver tissue sections stained with hematoxylin-eosin (H-E) were evaluated for general liver structure. Hydropic changes were assessed in 10 randomly selected fields in each liver section and scored. The liver sections of CTR group were normal in their histological appearance. Hepatocyte cordons were radially organized around the central vein in an orderly way. Sinusoids associated with hepatocyte cordons were open. In portal areas around liver lobules, arterial, venal and bile canalicular structures were prominently observed. Hepatocytes displayed an eosinophilic cytoplasm with round euchromatic nucleus (Fig. 3). Hepatocytes of CRF and TRF groups however, showed marked hydrophobic changes ($p = 0.001$) (Fig. 3. B and C), whereas hydrophobic changes in that of CLE group was less noticeable. These changes were found to be significant in comparison to histological findings of control liver sections. Fenofibrate supplementation produced no noticable changes in hepatocyte histology. Histopathologic scoring of hydropic changes can be seen in Fig. 3.

Immunoreactivity of Catalase in Liver Tissue Sections of Rats

The liver sections were incubated with catalase antibody and then visualized by chromogenic substrates AEC. According to the intensity of the staining, the sections were graded. Total staining score were used an an indication of catalase concentration. Catalase localization was observed in the cytoplasm of

hepatocytes. The highest catalase immunoreactivity was observed in liver sections of CRF group whereas the lowest in that of fenofibrate supplemented group. Catalase immunoreactivity of control group had homogeneous distrubition throughout the liver section and was scored as 8 (4.0–12.0). Catalase scores of CRF, TRF and CLE groups were found to be 12.0 (12.0–16.0), 12.0 (4.0–16.0) and 10.0 (4.0–12.0) respectively being significantly higher as compared to that of control group. As in the case of control group, catalase immunoreactivity in liver sections of these groups exhibited a uniform distribution. On the other hand, heterogenous distrubition of catalase immunoreactivity in liver sections from fenofibrate supplemented group was observed. The treatment with fibrates induced stronger proliferation of peroxisomes in zone 3 (pericentral hepatocytes) than in zone 1 hepatocytes. Since 10 randomly selected fields on each section was assessed according to the staining intensity, average catalase score of the selected fields became lower than it actually was in fenofibrate treated liver sections. Scores of catalase immunoreactivity in hepatocytes from the groups are displayed in Fig. 4.

Discussion

Blood samples were taken at the end of two weeks exposure to various conditions including different feeding regimens, continuous light exposure as weel as fenofibrate supplement. Plasma/erythrocyte lipids were analysed on the end point samples. Daily oscillations of the lipid parameters can be found elsewhere (32, 33). Effects of acute (one day) and chronic (5 days) sleep deprivation on plasma lipids of humans and rats were studied by Weljie *et al.* (18). They analysed serum lipids after the sleep restriction period. They also analysed end point samples.

Recent works indicate that sleep restriction is associated with disturbances in circadian rhythmicity (34). Higher plasma plasmalogen levels (seven plasmalogen species) after 5 days sleep restriction in both humans and rats led to conclusion that chronic sleep restriction may prompt PPARs' induction and disruption of circadian clock (18). The most altered plasma lipids as a function of sleep restriction are various glycerophospholipids including plasmalogens that were reported to exhibit circadian oscillation (19–23). In the current study, percentage of C18 plasmalogen level in erythrocyte lysates were found to be lower in all but TRF group. Lower level of C16 plasmalogen level was also detected in FSD group. However in humans, circadian rhythm disturbances induced by sleep restriction, higher plasma plasmalogen levels were reported (18). This discrepancy can be attributed. There are numerous situations that can possibly affect plasmalogen levels. Plasmalogens are highly susceptible to oxidation(35) and are consumed in this reaction (36). It is also possible that erythrocyte membrane plasmalogen levels might have been decreased by increased myeloperoxidase which was reported to react with vinyl ether bond of cellular plasmalogens by its ROS (36–38). Another possibility in alteration of plasmalogen levels can be associated with plasmalogen specific phospholipase A2, activity of which on membrane plasmalogens produces lysoplasmalogens (39).

Biochemically, various peroxisome dependent parameters are abnormal in peroxisome deficient mice which include accumulation of VLCFA (impaired VLCFA oxidation), lack of plasmalogen, abnormal bile acids, accumulation of phytanic acid, lower DHA in erythrocytes (30). In our work, none of the conditions

of circadian rhythm disturbances tested led to significant variations in plasma levels of very long chain fatty acids (C22:0, C24:0 and C26:0). In the case of plasma phytanic acid, calorie restriction produced higher levels. This might suggest a slowing in β-oxidation of peroxisomes due to calorie restriction. On the other hand, fenofibrate supplementation resulted in lower plasma levels of phytanic acid and triacylglycerol levels indicating occurence of peroxisomal induction by fenofibrates as expected. Overall, our data points out that calorie restriction might lower peroxisomal oxidation whereas fenofibrates enhance it. Calorie restriction and fenofibrates appear to have diverse effects on peroxisomal oxidations. Hepatocytes of CRF and TRF groups showed marked hydrophobic changes, whereas hydrophobic changes in that of CLE group was less pronounced. It is possible that excess lipolysis resulting from calorie restriction might have caused lipotoxicity(40–42) and subsequent hydropic changes in liver sections. On the contrary, fenofibrate supplementation produced no noticable hydropic changes in liver tissue sections. Since PPAR agonists inhibit inflammatory gene expression, down regulate acute phase protein and ROS production (43), it is possible therefore that, damaging effect of hydrogen peroxide by-product of peroxisomal oxidation activity migh have been served as an offset through antioxidant and antiinflammatory action of fibrates.

The catalase activity is largely or completely located in peroxisomes (44). Calorie restriction or body weight loss have been reported to be associated with enhanced catalase activity (43, 45, 46). Increased peroxisomal β-oxidation and as a result, increased production of hydrogen peroxide at peroxisomal acyl CoA oxidase stage might have been the cause of high catalase activity seen in the experimental groups other than FSD in our work. It has been reported that treatment with fibrates induced stronger proliferation of peroxisomes in zone 3 rather than in zone 1 hepatocytes (47) which is in line with our findings in FSD group. The present results might imply that altered feeding regiments and fenofibrates might influence different subpopulation of peroxisomes. However, data on the effects of fibrates on catalase activity are far from conclusive. Some of the work indicates an increase in catalase activity or expression (48, 49) following fenofibrate treatment whereas other found reduced or no effect (48, 50, 51).

With the exception of fenofibrate supplement, all the conditions associated with circadian rhythm disturbances tested in our work resulted in no significant alterations either in serum melatonin or corticosterone levels. Although the differences especially in melatonin levels among the groups were expected because of possible phase shift of melatonin oscillation induced by circadian rhythm disturbances, this was not the case. Some workers too reported no difference in either serum melatonin or corticosterone levels following various experimental conditions associated with circadian rhythm disturbances. Prolonged (10 days) constant light exposure in rats had no effects on serum melatonin level. Authors suggested that continuous light suppresses the activity of pineal gland. However, it would return to normal after certain period of time due to compensatory function of other melatonin producing and secreting organs (52). Moreover, in mice after 7 days restricted feeding period, measurement of circadian oscillations for serum melatonin did not achieve significance (9). Additional factors might also have contributed to high variations in serum melatonin levels such as age and wavelength of light (53, 54). On the other hand, serum glucocorticoid concentrations, which is an another parameter exhibiting daily oscillation, have been reported to be increased, reduced or not affected following nighttime light

exposure in rodents (13). We postulate that the significance could possibly have been reached with larger sample size.

In the case of fenofibrate supplementation, average plasma melatonin level was much lower compared to control or experimental groups. The decrease might indicate a prominent shift in melatonin synthesis/secretion or interference of fenofibrate with melatonin synthesis. The former is less likely because fibrates were reported to be able to reset rhythmic expression of circadian clock genes in peripheral tissues rather than in SCN (17, 55). The latter might be more likely. Fibrates, in particular fenofibrate, are known to increase homocysteine levels in plasma (56). This effect was mediated by PPARα (57). An inverse correlation between the plasma levels of melatonin and homocysteine has been reported (58, 59). Taken together the preceeding data it can be argued that the fenofibrate treatment might have interfered with melatonin synthesis. However, understanding of the exact mechanism requires further research.

A common ground among food restriction, fibrate administration and circadian rhythm could be PPARα. During the fasting period, PUFAs released from peripheral tissues are ligands for PPARα. Fenofibrates such as fenofibrate are also ligands for PPARα (60–62). PPARα has direct interaction with the core clock genes (60). PPARα mRNA is induced during fasting in wild type mice (63). PPARα plays an important role in mediating the action of calorie restriction. Overlap of genes influenced by calorie restriction and by a compound activating PPARα were reported (62). Both calorie restriction and fenofibrate treatments were reported to have induced changes in fatty acid and phospholipid compositions in tissues compositions (49, 61, 64–67). It was found that several plasmalogen phosphatidylethanolamines significantly decreased with acute calorie restriction in humans (67). This supports our results in which both calorie restriction and continuous light exposure as well as fenofibrate treatment resulted in lower C18:0 plasmalogen levels in erythrocytes. Moreover, fenofibrate supplementation also caused reduction in C16:0 plasmalogen levels. The sn-2 position of o-alkyl or o-alkenyl ether lipids generally contain arachidonyl and docosahexanoyl acyl chains (68). In our work, arachidonic acid and docosahexaenoic acid contents in erythrocytes were found to be lower in both FSD and CRD groups compared to that of control group. Some workers also reported alterations in arachidonic acid or docosahexaenoic acid in calorie restriction as well as in fenofibrate treatments. Calorie restriction was found to induce a decrease in the relative amount of arachidonic acid and increase in linoleic acid in membrane phospholipids (64, 65). Treatment with PPAR agonists (fibrates) was reported to increase peroxisomal catabolism of VLCFAs and subsequent oxidation in the mitochondria may induce shifts in fatty acid composition (69). In grass carp, fasting increased EPA and DHA content in tissues, whereas fenofibrate treatment decreased EPA and DHA contents in tissues (49). Taking together, it can be argued that both fasting and PPAR agonist treatments may induce changes in FA composition in tissues which are both reported to be able to have an impact on circadian transcriptomes (17, 60, 70, 71). On the other hand, catalase activity was also reported to be altered in fasting (43, 45, 46) and fibrate treatment (48–50) both of which are known to affect circadian rhythmicity (9, 12, 17). Our results also indicate an increased levels of catalase in all experimental groups.

Conclusions

In conclusion, common effects of the various conditions of circadian rhythm disturbances and fenofibrate inducement on the erythrocyte membrane lipids point out a link between them. Evaluation of fatty acid and phospholipid compositions in the membranes could be a new tool for the diagnosis of circadian rhythm disturbances in the future.

Abbreviations

PPARα
Peroxisome proliferator-activated receptor α; DHA:Docosahexaenoic acid; EPA:Eicosapentaenoic acid; SCN:Suprachiasmatic nucleus; TRF:Time restricted feeding; CRF:Calorie restricted feeding; CLE:Continuous light exposure; FSD:Fenofibrate supplemented diet; CTR:Control; BHT:Butylated hydroxytoluene; FAME:Fatty acid methyl esters; PUFA:Polyunsaturated fatty acids; VLCFA:Very long chain fatty acids.

Declarations

Ethics approval and consent to participate

All the procedure in the study was approved by the Research Ethics Committee of İnönü University of Medicine.

Consent for publication

All authors approved and agreed to publish the final version of the manuscript.

Availability of data and materials

The datasets generated and analyzed for this study are available from the corresponding author upon reasonable request.

Competing interests

All authors declare that there are no conflicts of interest.

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Authors' contributions

TG designed the study; HGO collected samples and prepared samples for analyses; AYand NV made the histological analyses; SE and YU achived melatonin and plasmalogen measurement; SY statisticaly

analysed the results. TG wrote the manuscript. All authors read and approved the final manuscript.

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Figures

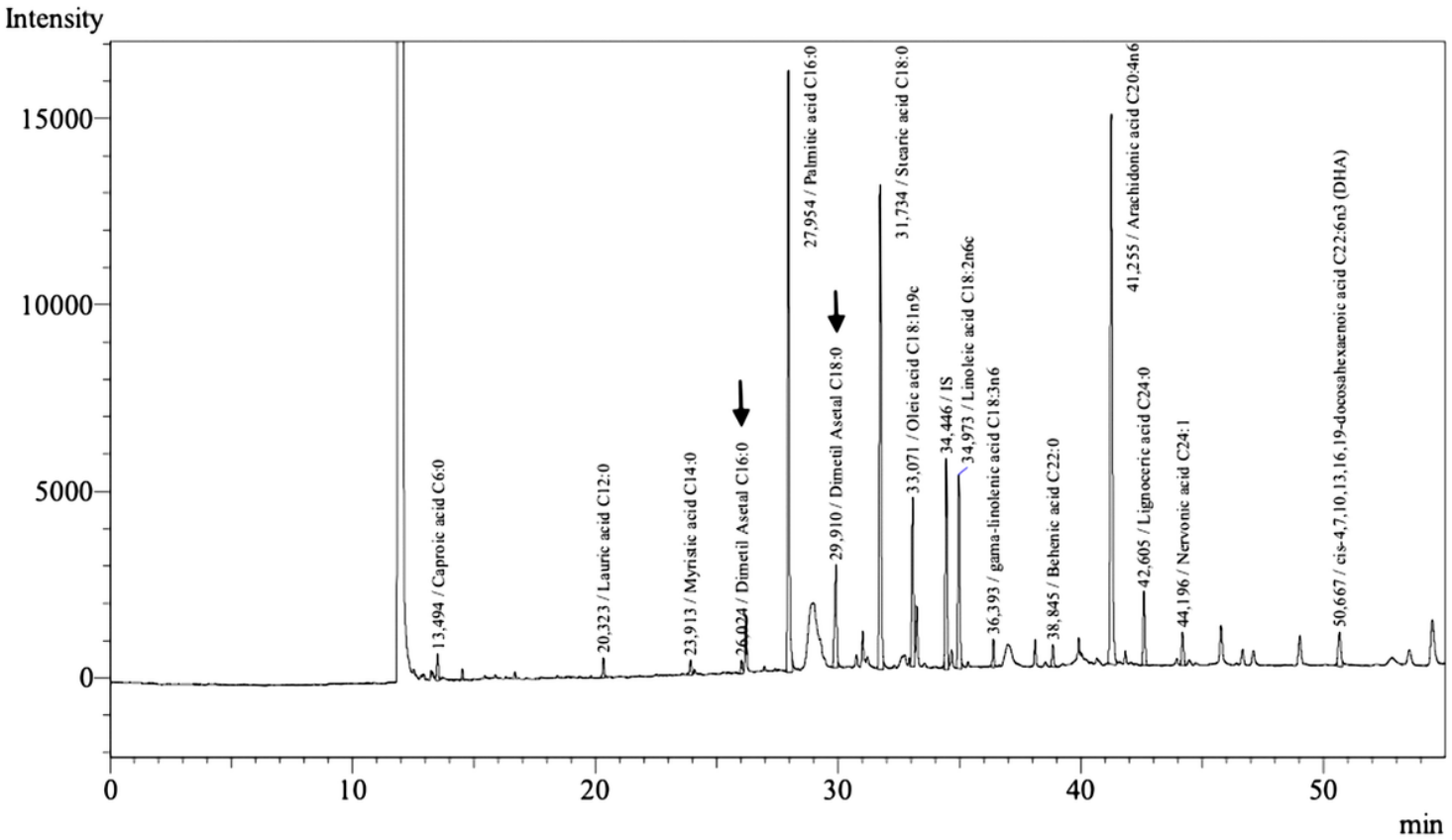


Figure 1

GC chromatogram overlay of fatty acid methyl esters and plasmalogen dimethylacetals in erythrocyte lysates. The plasmalogens can be distinguished next to their corresponding fatty acid methyl esters. Arrows indicate C16:0 or C18:0 plasmalogen dimethylacetals.

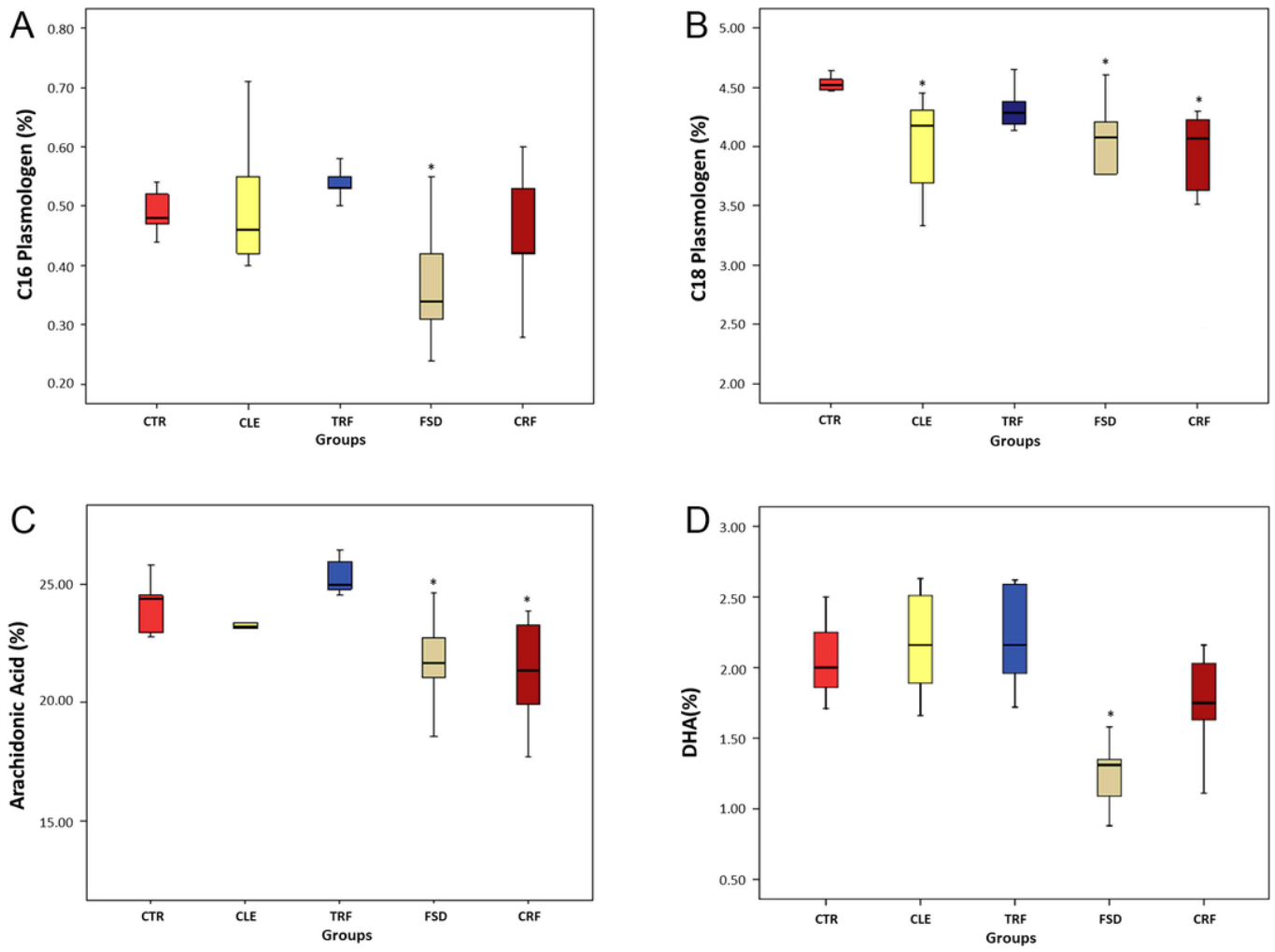
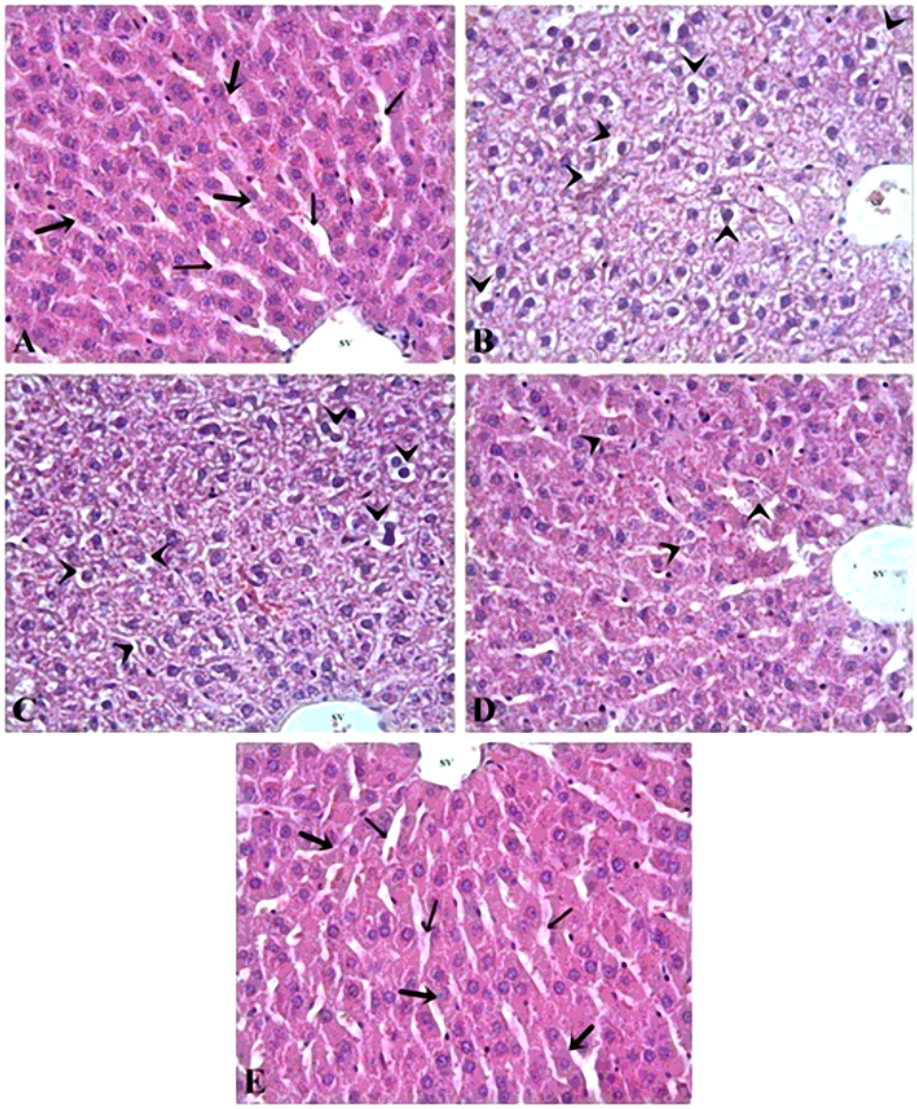


Figure 2

Effects of various experimental conditions associated with circadian disturbances on erythrocyte plasmalogens, arachidonic acid and docosahexaenoic contents. Effects of circadian disturbances on erythrocyte C16:0 plasmalogen content (A), on C18:0 plasmalogen content (B) , on arachidonic acid content (C) and on docosahexaenoic acid (DHA) (D). The plasmalogen levels are expressed as a percentage of the level of the corresponding fatty acid. The results of arachidonic acid and docosahexaenoic acid compositions are expressed as percentages of total fatty acid methyl esters . Error bars represent median (n= 10) . *$p<0.05$ compared with CTR group.

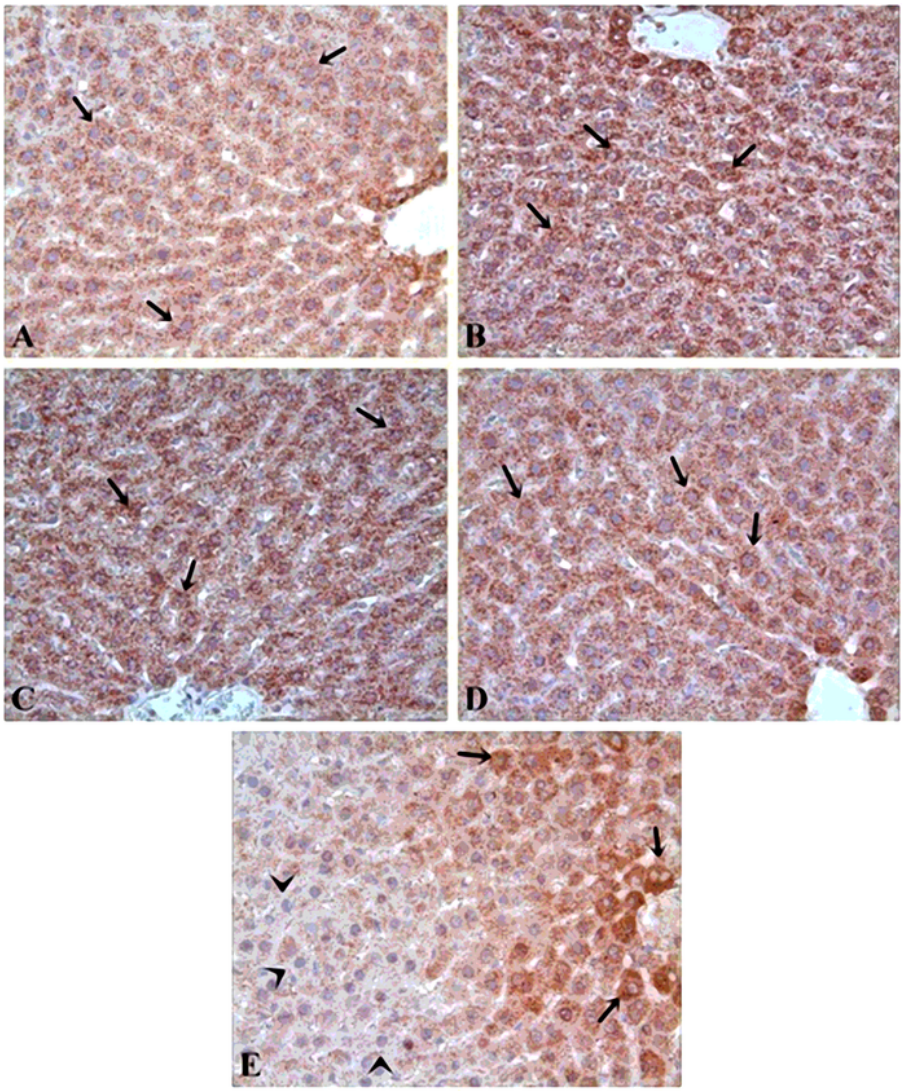


Scores of Hydropic Changes			
Group	**Median**	**Minimum**	**Maximum**
CTR	0.0	0.0	0.0
CLE*	1.0	0.0	3.0
TRF *	3.0	0.0	3.0
FSD	0.0	0.0	0.0
CRF *	2.5	1.0	3.0

Figure 3

Histopathological picture of H&E-stained sections and scores of hydropic changes in rat livers exposed to various conditions of circadian disruptions. Liver tissue sections were stained with hematoxylin-eosin for general liver structure. Hydropic changes was assessed in randomly selected 10 fields and scored. A. A nearly normal liver architecture with CTR group. Various degrees of hydropic changes can be discerned in CRF (B), TRF (C) and CLE (D) groups. Histological features of the liver from FSD group (E) was similar to

that of CTR group. SV : Central vein, thick arrows indicate hepatocyte cordons, thin arrows point sinusoids, arrowheads indicate hydropic changes.Magnification : x40. Scores of hydropic changes can be seen in the table below the pictures. ap ≤ 0.001, compared with CTR group. n= 10 for each group.



Scores of Catalase Immunreactivity			
Group	**Median**	**Minumum**	**Maximum**
CTR	8.0	4.0	12.0
CLE*	10.0	4.0	12.0
TRF *	12.0	4.0	16.0
FSD *	3.0	1.0	6.0
CRF *	12.0	12.0	16.0

Figure 4

Immunohistochemical reactivity for catalase in livers of rats subjected to various circadian perturbations. Liver tissue sections were incubated with catalase antibodies and visulaized by AEC. Catalase

immunoreactivity in liver sections of CTR group exhibites homogen distribution througout the liver sections (A). Catalase immunoreactivity of CRF (B), TRF (C) and CLE (D) were found to be higher compared to that of the CTR group. These groups also showed uniform distribution of catalase immunoreactivity. Heterogenous distribution of catalase immunureactivity in liver sections from FSD group are discernable (E) which were more prominent in zone 3. n=10 for each group. Arrows indicate catalase immunoreactivity. Arrowheads point hepatocytes without catalase immunoreactivity. Magnification : x40. According to the intensity of staining, the sections were graded. Total staining score were used as an indication for catalase concentration. The scores are displayed in the table below the pictures. bp $\leq$ 0.001, compared with CTR group.