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Full Length Research Paper

Investigation of the effects of constant darkness and light on blood serum cholesterol, insulin and glucose levels in healthy male rats

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This study was designed to investigate the effects of constant darkness and light on changes of serum cholesterol, insulin and glucose levels in healthy male rats. In this study, healthy male rats (n = 30) were divided into 3 groups of tens and kept at various light/dark conditions: Control 12:12 light/dark (LD); constant darkness (DD), and constant light (LL) groups for 2 weeks. Blood samples were obtained from retro-orbital sinus before start of experiment and on the 7th and 14th days of the experimental period. The serum cholesterol and glucose levels were measured by the enzymatic method and insulin levels were measured using insulin kit by enzyme-linked immunosorbent assay (ELISA) method. The results of the study showed that the levels of serum cholesterol and glucose on the 7th and 14th days of the experimental period in DD group significantly decreased compared to the LD and LL groups ($p < 0.05$). On the 14th day of experiment, we observed significant decrease of serum insulin level in the constant darkness group compared with the two other groups ($p < 0.05$). The study showed that on the 7th and 14th days of experiment, constant light significantly increased serum glucose level without having any significant effects on serum cholesterol and insulin levels. Also, the long period of time (14 days) was found to be more effective in the serum of these metabolic parameters changes than the short period (7 days).

Key words: Constant darkness, light, cholesterol, glucose, insulin, healthy male rats.

INTRODUCTION

The suprachiasmatic nucleus (SCN) is the master clock of mammals. In vertebrates, the clock mechanism that governs the rest of the organism is expressed at three Distinctive sites: The retina, the hypothalamic SCN and the pineal gland. The SCN are primarily synchronized to photic information provided by the daily alternation of day and night (Takahashi et al., 2001). Circadian clocks govern the timing of development, behavior, physiology, endocrinology and biochemistry, as well as photoperiodic events (Forster et al., 2001). Also, circadian clocks and energy metabolism are linked because mutation in the clock gene leads to metabolic syndrome in mice (Turek et al., 2005). Recent studies have linked circadian rhythms and metabolism. The best cases to evaluate the link between metabolism and circadian rhythms are the liver and brain. The circadian clock reportedly regulates meta-

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Abbreviations: SCN, Suprachiasmatic nucleus; **LD,** control group; **LL,** constant light group; **DD,** constant darkness group; **ELISA,** enzyme-linked immunosorbent assay; **LDL,** low-density lipoprotein; **FDP,** fructose-1, 6-diphosphatase; **PFK,** phosphofructokinase; **SNS,** sympathetic nervous system; **PNS,** parasympathetic nervous system.

bolism and energy homeostasis in the liver and other peripheral tissues by mediating the expression or activity of various metabolic enzymes and transport systems involved in cholesterol, glycogen, and glucose metabolism (Green et al., 2008; Zhang et al., 2006; La Fleur, 2003).

In addition, Challet et al., (2004) observed that blood glucose increased during exposure to light and decreased during darkness in rats. It has been shown that total cholesterol, lipids, and phospholipids in dogs did not respond to a 6-h delay in light onset (Bertolucci et al., 2008). Recent studies have demonstrated that constant darkness elevated 5' adenosine monophosphate (5'-AMP) in the blood of mice. Hence, 5'-AMP is a pivotal metabolic signal whose circulatory level determines the balance of the peripheral organ energy supply between glucose, glycogen and fat (Zhang et al., 2006). It is known that insulin secretion is not affected by photoperiod in cattle (Zinn et al., 1986; Peters and Tucker, 1978). On the other hand, there is evidence of relationship between the pineal gland and physiological regulation of carbohydrate, insulin secretion and lipid metabolism. In mammals, SCN activity is modulated by several neurotransmitters or neurohormones including melatonin (Armstrong et al., 1986). Melatonin is a pineal hormone with well-known actions on the neuroendocrine reproductive axis and circadian system as well as being an antioxidant (Pandi-Perumal et al., 2006). However, the results of studies investigating melatonin effect on insulin secretion, glucose and lipid metabolism in experimental animals are controversial, such as increase of blood glucose in rats (Csaba and Barath, 1971) or, on the contrary, reduction of blood glucose in rats (Lizuka, 1996). The antilipidemic effect of melatonin has been demonstrated in experimental animals (Sener et al., 2004) and humans (Wakatsuki et al., 2001). On the other hand, Some studies suggest that melatonin has no effect on insulin secretion and glucose homeostasis (Feldman and Lebovitz, 1972), whereas other results show either an inhibitory (Bailey et al., 1974; Dobozy and Csaba, 1976) or a stimulatory effect on insulin secretion (Diaz and Blazquez, 1986; Lizuka, 1996). Furthermore, in rats on high-fat diet, melatonin administration attenuated atheromatous changes in arteries along with the normalization of blood glucose and improvement of antioxidant capacity and lipid profile (Hussein et al., 2007). Therefore, little is known about variations of blood glucose, cholesterol and insulin levels under constant light and dark conditions. Also, the mechanisms participating in central effects of constant darkness and light on variation in these biochemical parameters are not yet known completely. Therefore, this study was designed to elucidate the effects of constant darkness and light on changes of blood serum cholesterol, glucose and insulin levels in rats. Also, the purpose of the present study is to determine whether these metabolic parameters were altered in constant dark and light exposure conditions at a short or long period of time in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (n = 30) weighing 200 - 250 g were obtained from the Pasteur Institute of Iran. Animals were housed individually under standard laboratory conditions in a 12 h/12 h light/dark cycle and at a temperature of 21 - 23°C. Rats had free access to food and water. The rats were maintained under these housing conditions for 10 days before the experiments.

Experimental design

Animals were randomized and divided into three groups of ten animals as follows: Control group (LD)-12 h/12 h light/dark cycle; constant light group (LL)-24 h light on (100 lux); constant darkness group (DD)-24 h darkness. All rats were kept in standard cages and were given *ad libitum*_standard laboratory meal and tap water (Challet et al., 2004).

On the day before the experimental period, blood samples were collected from retro-orbital sinus of three groups. After this, the DD group was transferred to constant darkness for 2 weeks. LL rats were exposed to a white light pulse (100 lux during 2 weeks). LD rats were maintained under 12 h/12 h light/dark conditions for 2 weeks of the experimental period.

On the 7^{th} and 14^{th} days as the days before, blood samples were collected separately from the three groups to determine the levels of serum cholesterol, glucose and insulin levels in the three groups of rats.

Biochemical analysis

Serum was obtained by high speed centrifugation at 3500 rmp for 10 min, and stored at 70°C until analysis. The concentrations of cholesterol and serum glucose were measured by enzymatic colorimetric methods with commercial kits (pars Azmone, IRI) on an automatic analyzer (Abbott, model Alcyon 300, USA) and serum insulin level was determined by enzyme-linked immunosorbent assay (ELISA) method using insulin kit (DRG, international, Inc, USA).

Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey tests were used to characterize the effects of constant darkness and light statistically using the Statistical Analysis System (SAS) software (version 9.1). P-values less than 0.05 were considered to be statistically significant.

RESULTS

Serum cholesterol

On the day before the experiment, there was no significant difference in the serum cholesterol levels among the three groups of rats ($p > 0.05$). On the 7^{th} day of experiment in the DD group, the concentration of serum cholesterol significantly decreased compared to the light

Table 1. Effects of constant darkness and light on serum cholesterol level (mg/dl) in healthy male rats for 2 weeks of experiment.

Group	Day		
	0	7	14
Constant light (LL)	63.8±4.849	64.00±3.91[a]	63.50±6.62[a]
Constant darkness (DD)	59.00±7.616	56.10±5.06[b]	53.40±3.17[b]
Control (LD)	64.5±13.616	65.20±8.257[a]	65.10±7.218[a]

N=10 (in every group); (mean ± SD). Different letters (a, b and c) within a column represent significant differences between various groups ($p < 0.05$) and the same letters (or no letter) shows no significant difference.

Table 2. Effects of constant darkness and light on serum insulin level (µg/Li) in healthy male rats for 2 weeks of experiment.

Group	Day		
	0	7	14
Constant light (LL)	1.26 ± 0.267	1.19 ± 0.39	1.35 ± 0.59[a]
Constant darkness (DD)	1.23 ± 0.302	1.03 ± 0.26	0.92 ± 0.10[b]
Control (LD)	1.17 ± 0.389	1.21 ± 0.42	1.35 ± 0.46[a]

N=10 (in every group); (mean ± SD). Different letters (a, b and c) within a column represent significant differences between various groups ($p < 0.05$) and the same letters (or no letter) shows no significant difference.

and control groups ($p < 0.05$). There was no significant difference in serum cholesterol between the LL and LD groups ($p > 0.05$). On the 14th day of experiment in the DD group, the level of cholesterol significantly decreased compared to the LD and the LL groups ($p < 0.05$). There was no significant difference in serum cholesterol between LL and LD groups after 2 weeks of experiment ($p > 0.05$). These results indicated that constant darkness decreased serum cholesterol level whereas continuous light exposure had no effects on serum cholesterol level throughout the experimental period. The results are shown in Table 1.

Serum insulin

As shown in Table 2, the day before experiment and after 7 days of experiment, no significant differences was found among the DD, LL and LD groups ($p > 0.05$). However, our data showed that on the 14th day of experiment in the DD group, the level of insulin significantly decreased compared to the LL and the LD groups ($p < 0.05$). There was no significant difference in serum insulin between LL and LD groups after 14 days of experiment ($p > 0.05$) (Table 2).Therefore, the results of the present study showed that constant darkness decreased serum insulin level after 2 weeks of experiment (darkness condition), but constant light did not show any significant change in the serum insulin levels in rats.

Serum glucose

Table 3 demonstrates the changes of serum glucose levels in the three groups of rats throughout the experimental period. On the day before the experiment, there was no significant difference in serum glucose levels between DD, LL and LD groups ($p > 0.05$). On the 7th day of experiment, the concentration of serum glucose significantly increased in the LL group and decreased in the DD group compared to the LD group ($p < 0.05$). After 2 weeks of experiment, the level of serum glucose significantly increased in LL rats and decreased in DD rats compared to LD rats ($p < 0.05$) (Table 3). The data of the present study showed significant differences between the DD and LL group in the level of serum glucose after 7 days of experiment ($p < 0.05$). Also, significant differences were observed in the serum glucose between the DD and LL groups compared to the control on the 7th and 14th days of experiment ($p < 0.05$).

DISCUSSION

This is the first study that showed that constant darkness significantly decreased the level of serum cholesterol in healthy male rats on the 7th and 14th days of experiment (Table 1). Based on the data of the present study, it seems that the decrease of cholesterol level in the DD group may be due to melatonin effect on lipid parameters.

Table 3. Effects of constant darkness and light on serum glucose level (mg/dl) in healthy male rats for 2 weeks of experiment.

Group	Day		
	0	7	14
Constant light (LL)	76.80±8.93	114.10±11.84[a]	130.00±18.86[a]
Constant darkness (DD)	83.30±13.09	78.30±9.59[c]	70.00±10.13[c]
Control (LD)	85.30±9.55	92.10±9.73[b]	93.40±11.40[b]

N=10 (in every group); (mean ± SD). Different letters (a, b and c) within a column represent significant differences between various groups ($p < 0.05$) and the same letters (or no letter) shows no significant difference.

The result of the present study is supported by earlier reports that indicated the importance of melatonin in the regulation of serum cholesterol. These importance include decrease of plasma and liver cholesterol levels in hypercholesterolemic mice (Sener et al., 2004), inhibition of low-density lipoprotein (LDL) receptor activity and cholesterol synthesis in human mononuclear leucocytes (Muller-Wieland et al., 1994), reduction of serum cholesterol levels and lipid profile normalization (Wakatsuki et al., 2001), and the effect on cholesterol metabolism via influence on cytokine secretion from macrophages for example, interleukin 2 (Morry et al., 1994; Garcia-Maurino et al., 1997, 1998). Perhaps, the antilipidemic effect of melatonin in the DD rats may be partly due to the antioxidant properties of melatonin. It is well understood that potent antioxidant ability can be explained by the potential to scavenge hydroxyl radical (Bromme et al., 2000). On the other hand, we found that pinealectomy was associated with several metabolic alteration including hypercholesterolemia in type 2 diabetic rats (Nishida et al., 2003). Interestingly, our findings showed that constant light had no significant effect on the level of serum cholesterol after 2 weeks of experiment (Table 1). Data obtained for DD group showed that the concentration of serum cholesterol on the 14th day of experiment was lower than it's concentration on the 7th day of experiment. Thus, the long period of darkness (14 days) was found to be more effective in the serum cholesterol level change than the short period (7 days) in rats. Regarding the effect of circadian clock on biochemical parameters, it is worthy to note that in homozygous mice with mutation in circadian clock gene, the metabolic syndrome characterized by obesity, hyperlipidemia, hyperleptinema, hyperglycemia, and hyperinsulinemia developed (Turek et al., 2005). Therefore, it seems that melatonin and circadian clock may be responsible for the variation in serum cholesterol level in animals.

With regard to the present study, our data demonstrated that in the constant darkness group the level of insulin significantly decreased on the 14^{th} day ($p < 0.05$) (Table 2), but constant light and control groups did not show any significant variation on the serum insulin level throughout the experimental period ($p > 0.05$). This study thus shows that there is a relationship between long period of darkness (14 days) and decrease insulin levels (Table 2). In addition, the findings of the present study showed that after 7 days of experiment, the concentration of serum glucose significantly increased in the constant light group and decreased in the constant darkness group when compared to the control group ($p < 0.05$) (Table 3). With regard to this, it seems that insulin secretion and the variation in serum glucose levels were affected by circadian clock and melatonin. Sakaguchi et al. (1988) suggested that in rats, diurnal insulin secretion may be controlled directly by a circadian oscillator that influences pancreatic secretion and glucose metabolism through a neural signal modulated by the suprachiasmatic nucleus. Based on previous research, data concerning changes in the circadian levels of plasma insulin and plasma glucose are rather controversial. These include decrease of insulin secretion in the evening compared to morning (Caroll and Nestel, 1973) in contrast to an increase of insulin secretion at night (Penicaud and Magnen, 1980), increase of glucose level during the day compared to night in rats (Jolin and Montes, 1973) contrasting an increased glucose level at night compared to its level during the day (Pauly and Scheving, 1967). Interestingly, the present study results are in agreement with recent studies that indicated that constant darkness decreased blood glucose in mice (Zhang et al., 2006). They found one possible mechanism that showed a relationship between serum glucose and increase of 5' –AMP during dark conditions in mice. They observed that increase of 5' –AMP in the blood of mice subjected to darkness caused the activity of fructose-1, 6-diphosphatase (FDP) to be inhibited and that of phosphofructokinase (PFK) to be enhanced. Consequently, the rate of gluconeogenesis was reduced but that of glycolysis was enhanced, leading to depletion of the blood glucose pool. The findings of the present study are in agreement with previous studies which suggest that constant light increased the level of blood glucose in rats (Challet et al., 2004). It is interesting to note that Nagai et al. (1994) after carrying out SCN lesions in rats obtained decreased level of blood glucagon but increased blood insulin level. There also were

changes in the blood glucose concentration after insulin injection into the SCN was eliminated and alteration in activity of autonomic efferents to peripheral organs on light exposure. It is possible that some neurons in the SCN play a role in photic modulation of blood glucose. Besides, SCN activity is modulated by several neurotransmitters or neurohormones including melatonin, which is synthesized during the night by the pineal gland (Armstrong et al., 1986). Based on results of the present study, decrease of insulin level and serum glucose in the DD group may be related to melatonin function in the dark. There are a number of documents which strongly support the importance of melatonin in the regulation of circadian function. Its influence on insulin secretion and the level of blood glucose include inhibition of insulin secretion (Mazpa et al., 2000), governing of the shift of sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) (La Fleur et al., 2001), influence on beta cells activity which may be indirect since cortisol and epinephrine are known to influence both insulin secretion and blood glucose (Goodman, 1994), the presence of high affinity melatonin receptors in the SCN and simulation of the condition of altered photoperiod (Sankaran and Subramanian, 2006), reduction of oxidative load (Tan et al., 2007) and attenuation of sympathetic tone by direct activation of melatonin receptors (Girouard et al., 2003). Therefore, there is a relationship between melatonin and variation in biochemical parameters such as serum insulin, glucose and the cholesterol levels in animals. In this regard and according to our findings, melatonin and circadian clock have important role on variation in serum glucose, insulin and cholesterol levels in animals. Although results of investigation on variation in serum insulin level in the circadian rhythms are rather controversial, the data of the present study showed that constant light had no effect on serum insulin level. On the other hand, while both constant darkness and light showed significant effect on the serum glucose on the 7^{th} and 14^{th} days during the experimental period, in the DD group, the concentration of the serum glucose on the 14^{th} day was lower than on the 7^{th} day. In the LD group, the concentration of serum glucose on the 14^{th} day was higher than on the 7^{th} day (Table 3). Therefore it is clear that long period of constant darkness and light was found to be more effective in the serum glucose changes than the short period of time during the experiment.

In conclusion, the results obtained suggest that constant darkness decreased serum cholesterol, insulin and glucose levels. In contrast, constant light increased serum glucose but did not affect serum cholesterol and insulin levels in healthy male rats. According to the results of this study, further studies will be necessary to investigate the effects of constant darkness and light on biochemical parameters in human and other animal and to confirm the data of the present study as well as evaluate the role of melatonin and circadian clock in the regulation of these metabolic parameters.

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