Cytokine and microRNA levels during different periods of paradoxical sleep deprivation and sleep recovery in rats

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Abstract

Background. Sleep has a fundamental role in the regulation of homeostasis. The aim of this study was to assess the effect of different periods of paradoxical sleep deprivation (PSD) and recovery on serum levels of cytokines and miRNAs related to inflammatory responses. **Methods**. Male Wistar rats were submitted to a PSD of 24, 96, or 192 hours, or of 192h followed by 20 days of recovery (192h PSD+R). The concentrations of corticosterone, cytokines (IL-6, TNF, IL-10, Adiponectin) and miRNAs (miR-146a, miR-155, miR-223, miR-16, miR-126, miR-21) in serum were evaluated. **Results.** At PSD 24h a significant increase of IL-6 and decrease of IL-10 were observed. At PSD 96h adiponectin increased. At 192h of PSD IL-6 increased significantly again, accompanied by a threefold increase of IL-10 and an increase of serum corticosterone. After 20 days of recovery (192h PSD+R) corticosterone, IL-6 and TNF levels increased significantly, while IL-10 decreased also significantly. Regarding the miRNAs at 24h of PSD serum miR-146a, miR-155, miR-223, and miR-16 levels all increased. At 96h of PSD miR-223 decreased. At 192h of PSD decreases in miR-16 and miR-126 were observed. After recovery serum miR-21 increased and miR-16 decreased. **Conclusion.** PSD induces a dynamic response likely reflecting the induced cellular stress and manifested as variating hormonal and inflammatory responses. Sleep deprivation disturbed corticosterone, cytokine and miRNA levels in serum related to the duration of sleep deprivation, as short-term PSD produced effects similar to those of an acute inflammatory response and long-term PSD induced long-lasting disturbances of biological mediators.

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Methods. Male Wistar rats were submitted to a PSD of 24, 96, or 192 hours, or of 192h followed by 20 days of recovery (192h PSD+R). The concentrations of corticosterone, cytokines (IL-6,

37   TNF, IL-10, Adiponectin) and miRNAs (miR-146a, miR-155, miR-223, miR-16, miR-126, miR-
38   21) in serum were evaluated.

39   **Results.** At PSD 24h a significant increase of IL-6 and decrease of IL-10 were observed. At PSD
40   96h adiponectin increased. At 192h of PSD IL-6 increased significantly again, accompanied by a
41   threefold increase of IL-10 and an increase of serum corticosterone. After 20 days of recovery
42   (192h PSD+R) corticosterone, IL-6 and TNF levels increased significantly, while IL-10 decreased
43   also significantly. Regarding the miRNAs at 24h of PSD serum miR-146a, miR-155, miR-223,
44   and miR-16 levels all increased. At 96h of PSD miR-223 decreased. At 192h of PSD decreases in
45   miR-16 and miR-126 were observed. After recovery serum miR-21 increased and miR-16
46   decreased.

47   **Conclusion.** PSD induces a dynamic response likely reflecting the induced cellular stress and
48   manifested as variating hormonal and inflammatory responses. Sleep deprivation disturbed
49   corticosterone, cytokine and miRNA levels in serum related to the duration of sleep deprivation,
50   as short-term PSD produced effects similar to those of an acute inflammatory response and long-
51   term PSD induced long-lasting disturbances of biological mediators.

52

53   **New & Noteworthy**

54   This is the first study to show that paradoxical sleep deprivation induces distinctive changes in
55   circulating microRNAs related to inflammation and that after 20 days of recovery from 192h of
56   paradoxical sleep deprivation the inflammatory response is not fully restored.

57   **Introduction**

58   Sleep is a natural, reversible physiological state characterized by a reduction in the response to
59   external stimuli (Jones, 1998). Numerous hypotheses have been posited to explain the function of
60   sleep, but most authors consider it a homeostatic process involved in energy conservation, in
61   nervous system recuperation, thermoregulation, and the immune function (Siegel, 2005) Sleep
62   deprivation constitutes a relevant health problem in modern society (Tobaldini et al., 2017).
63   Research on sleep has paid special attention to the rapid eye-movement (REM) phase or
64   paradoxical sleep because deprivation of this sleep stage has been shown to induce various effects

65 in the brain, some of which are so significant that they can induce morphological changes, that

66 include reduced proliferation of neuronal precursors in the adult hippocampus (Sahu et al., 2013),

67 and modifications of the neurochemical, hormonal and inflammatory media (James M. Krueger,

68 Frank, Wisor, & Roy, 2016).

69 Both, at systemic and cerebral levels cytokines participate as mediators of various complex

70 physiological processes, including sleep regulation (Lorton et al., 2006). Depending on the amount

71 and/or duration of the loss, sleep deprivation produces multiple physiological effects, including

72 elevations in plasma cortisol or corticosterone levels (Deborah Suchecki, Tiba, & Tufik, 2002),

73 suggesting that PSD induces a stress response (McEwen, 2006). PSD also induces alterations of

74 inflammatory markers such as IL-1β, IL-6, IL-17 and TNF (Irwin, 2006), excessive daytime

75 secretion of IL-6 (Vgontzas et al., 1999), and modifications in coagulation and cell adhesion

76 molecules in humans (Grandner, Sands-Lincoln, Pak, & Garland, 2013) and in animal models

77 (Yehuda, Sredni, Carasso, & Kenigsbuch-Sredni, 2009). Inflammation itself can be regulated for

78 example by adiponectin, which induces a reduction in the secretion of the cytokines IL-6 and IL-

79 8 (Dietze-Schroeder, Sell, Uhlig, Koenen, & Eckel, 2005), and by MicroRNAs (miRNAs). Indeed,

80 some miRNAs play a significant role in sleep regulation and may be expressed at distinct moments

81 of sleep and in different brain structures, including cortical areas that regulate sleep duration

82 (Davis, Clinton, & Krueger, 2012). One recent study demonstrated that some plasmatic miRNAs

83 can be altered as part of the physiopathology of central hypersomnia (Holm et al., 2014),

84 obstructive sleep apnea (K. Li, Wei, Qin, & Wei, 2017) and sleep deprivation (Matos et al., 2014).

85 Six miRNAs (miR-146a, -155, -223, -16, -126 and -21) have been described as potential

86 biomarkers for at least nine non-neoplastic diseases that are associated with important

87 inflammatory processes (Haider et al., 2014). However, to the best of our knowledge, no studies

88 have yet related different periods of PSD and recovery from it to circulating levels of these

89 inflammation related miRNAs.

90 Considering that PSD is, in itself, a stressor and that there is scarce information about the effects

91 of different periods of PSD on inflammation related mediators, and the duration of these

92 alterations, the objectives of the present study were: 1) to determine the effects of different periods

93 of PSD (24h, 96h, and 192h) on serum levels of: a) corticosterone; b) IL-6, TNF, IL-10, and

94 adiponectin; and c) miRNAs related to the inflammatory response: miR-146a, -155, -233, -16, -

95   126 and -21; and 2) if a long-lasting PSD (192h) has long-term homeostatic consequences, as

96   determined by the same mediators, considering a recovery period of 20 days.

Method

98   The study utilized 3-month-old male Wistar rats weighing approximately 250 gr that were raised

99   in the animal facilities at Universidad Autónoma Metropolitana -Iztapalapa (UAMI) under the

100 conditions stipulated in the official Mexican norm (NOM-062-ZOO-1999, 2001 revision). The

101 experimental protocol was approved by the Ethics Committee of UAMI (Session 1514, verified 3-

102 15-14-2010-2018). The rats were deprived of paradoxical sleep for 24h (PSD 24h), 96h (PSD 96h),

103 and 192h (PSD 192h); a fourth group was also deprived for 192h, but measurements were done

104 after 20 days of recovery (192h PSD+R). These intervals were chosen because many prior studies

105 of sleep deprivation also used a 96h evaluation period (Machado et al., 2004, Andersen et al., 2005,

106 Machado, Tufik, & Suchecki, 2010), but we decided to observe two additional, extreme periods

107 (24 and 192h) for comparative purposes. The rationale for adding a prolonged recovery period is

108 that this interval may allow the body to recover from acute PSD-induced alterations experienced

109 during 192 h. Given the aims of our study, we considered the 20-day period to acknowledge long-

110 term consequences of long-lasting PSD.

111 Each group consisted of 5 rats. The control group was housed in normal boxes in the same space

112 at the animals facility. All rats were kept under a light-dark cycle (9am-9pm) at a temperature of

113 approximately 24°C. The method of euthanasia by decapitation is approved by NOM-062-ZOO-

114 1991.

115 *Paradoxical sleep deprivation Method*

116 The modified multi-platform method was used to induce PSD (D Suchecki & Tufik, 2000). It

117 involves placing the rats in an acrylic box (127 cm long x 44 cm wide x 45 cm high) with a base

118 that has 14 platforms measuring 6.5 cm in diameter x 3 cm high with 10-cm separations. Water is

119 added to the base of the platform to a depth of approximately 1 cm. This method is based on the

120 loss of muscle tone that characterizes the REM sleep condition. When an experimental animal falls

121 into the water, it experiences a sudden loss of the sleep cycle. This REM sleep deprivation method

122 has been studied previously and is considered reproducible (Machado et al., 2004). Studies have

123 demonstrated that using this method of inducing PSD, animals not only lose REM sleep entirely,

124 but also approximately 37% of slow wave sleep (SWS) (Machado et al., 2004).

125 Although the modified multi-platform method, has been widely used and endorsed for PSD 96h

126 (Suchecki & Tufik, 2000, Machado et al., 2004), there is no information regarding the effect of

127 this method with other periods of PSD on loss of REM sleep and non-REM sleep. Thus, in order

128 to acknowledge the brain activity in each of the studied periods of PSD, polysomnography is

129 warranted. Another handicap is that recovery was not studied for shorter periods of PSD.

130 *Quantification of serum corticosterone and cytokines levels*

131 To evaluate corticosterone and cytokine concentrations, sera were obtained by decapitation,

132 ensuring that euthanasia was always carried out at the exact same hour: 9:00 am. Blood was drawn

133 in vacutainer tubes with separation gel, serum samples were aliquoted and preserved at -70°C until

134 they were used. To measure corticosterone concentrations, an ELISA Test Kit Mouse/Rat was

135 employed (Alpco, Salem, NH) following the manufacturer's instructions. (sensitivity 6.1ng/ml).

136 To measure cytokine concentrations, determinations were conducted also by ELISA: For IL-6 we

137 used Prepro Tech (Rocky Hill /NJ) (range 62- 8000pg/mL), for IL-10 and TNF we used Thermo

138 Fisher Scientific kits (Rockford, IL, U.S.A.) with sensitivity <3pg/ml, and 11.0pg/ml respectively;

139 and with an Alpco kit (Salem, NH) for adiponectin, with sensitivity of = 0.08 ng/ml.

140 *Serum miRNA quantification*

141 RNA extraction was performed using the miRNeasy Serum/Plasma kit (Qiagen). The miRNAs

142 were reverse-transcribed with the TaqMan MicroRNA Reverse Transcription Kit (Applied

143 Biosystems, Thermo Fisher Scientific, U.S.A). The RT reaction was incubated for 30 minutes at

144 16°C, 30 minutes at 42°C, and 5 minutes at 85°C. The miRNAs were then amplified using a

145 TaqMan MicroRNA RT kit (Applied Biosystems, Thermo Fisher Scientific, U.S.A), and detected

146 with TaqMan TM MicroRNA Assays hsa/mus miR-146a, miR-155, miR-233, miR-16, miR-126

147 and miR-21 primers and probes (Applied Biosystems, Thermo Fisher Scientific, U.S.A). The

148 cycling conditions were: initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for

149 15 s, at 60°C for 40 s, and at 72°C for 10 s. PCR was performed with a LightCycler TM 480 II

150 System (Roche Applied Science, Basel, Switzerland) and the LightCycler 480 Probes Master kit

151 (Roche Applied Science). miRNA concentrations were normalized with the concentrations of

152   miR-39. The miRNeasy serum/plasma Spike-In Control was added to samples after the addition

153   of QIAzol lysis reagent (2μL of $1.6X10^8$ copy/μL of cel-miR-39), which can control for varying

154   RNA isolation yields and amplification efficiency. ΔCt values were calculated with problem Ct

155   *i.e.*, the reference Ct at which miR-39 is taken as the reference gene, represented as $2^{-\Delta Ct}$ (Dehoux,

156   van Beneden, Fernández-Celemín, Lause, & Thissen, 2003).

157

158   *Statistical analysis*

159   All serum cytokine concentrations and serum miRNA levels were analyzed by a one-way

160   ANOVA, followed by a post hoc Tukey-Kramer test. NCSS software was used for these analyses.

161   Statistical significance in relation to the control group and between groups was set at $p < 0.05$

162   **Results**

163   *Corticosterone concentrations increased after long periods of PSD and stayed elevated after*

164   *recovery.*

165   In order to assess the effects of increasing lengths of sleep deprivation on the stress response,

166   corticosterone levels were measured. Short periods of PSD had no significant effect upon serum

167   corticosterone, but it was found sharply elevated after PSD 192h as compared to the control group

168   [$F_{(4,25)} = 17.18$, $p < 0.0001$]. After recovery (192h PSD+R) the levels of corticosterone were

169   significantly lower than after PSD 192h but were still significantly higher than those found in

170   controls (Figure 1).

171   *Circulating cytokines in rats were modified by deprivation of paradoxical sleep for different*

172   *periods and were maintained altered after recovery.*

173   The effects of different periods of sleep deprivation on serum pro- and anti-inflammatory cytokines

174   levels were measured. With the exception of PSD 96h the levels of IL-6 were significantly higher

175   than in controls in all PSD groups, including those measured after 192h PSD+R [$F_{(4,25)} = 17.39$,

176   $p < 0.0001$] (Fig. 2A). PSD did not modify TNF concentrations as compared to controls and a

177   significant increment was only seen after recovery, at 192h PSD+R, [$F_{(4,25)} = 8.717$, $p = 0.0003$]

178   (Fig. 2B). IL-10 levels were significantly lower than in controls at PSD 24, raised significantly

179   after PSD 192h and dropped again below controls levels after PSD+R [$F_{(4,25)} = 32.84$, $p < 0.0001$]

180 (Fig. 2C). Finally, circulating levels of adiponectin showed a significant increase only after PSD

181 96h [F (4,25) = 4.741, p=0.0074] (Fig. 2D).

182 *Changes in miRNA levels were found after the different periods of PSD and recovery*

183 Serum levels of miRNA related to the inflammatory response were measured at the different

184 periods of PSD and after recovery. At PSD 24h three miRNA were found significantly increased

185 compared to controls: miR-146a [F (4,25) =9.538, p=0.0002] (Fig. 3A), miR-155 [F (4,25) =5.895,

186 p=0.0027] (Fig. 3B), and miR-223[F (4,25) =21.75, p<0.0001]. The latter was observed decreased

187 at PSD 96h (Fig. 3C), as well as miR-16, which was found also decreased at PSD 192h and

188 PSD192+R compared to controls [F (4,25) =23.29, p<0.0001] (Fig. 3D). miR-126 was decreased

189 at PSD 192h [F (4,25) =3.669, p=0.0213] (Fig. 3E), and miR-21, showed no significant changes

190 during PSD, but an increase was observed at 192h PSD+R compared to controls and to PSD 192h

191 [F (4,25) =15.48, p<0.0001] (Fig. 3F)

192

193 Discussion

194 We found that different periods of PSD modify corticosterone, IL-6, IL-10, and adiponectin levels,

195 depending on the duration of PSD. Most of these alterations in cytokines did not return to basal

196 values even after 20 days of recovery: only adiponectin did. TNF, which was not modified during

197 the different PSD periods, was found elevated only after recovery. Related to miRNAs, except for

198 miR-21 which was increased only during recovery, the levels of all the other inflammation related

199 miRNAs (miR-146a, miR-155, miR-223, miR16, miR-126) were also modified by PSD. Notably,

200 differentiating the miRNAs response to that of the cytokines, all these miRNAs but miR-16 which

201 persisted in decreased levels, had returned to base-line levels after the recovery period. These

202 findings suggest that PSD induces a dynamic response pattern that reflects cellular stress that is

203 manifested as hormonal and inflammatory responses in different stages, that could be long-lasting.

204 REM sleep deprivation may induce a stress response (McEwen, 2006) that involves not only

205 behavioral responses, but is also associated with major modifications in brain biochemistry and in

206 the endocrine and immune systems, that may be dependent on the duration of REM sleep

207 deprivation in a response related to acute or chronic stress. After PSD 24h, elevated levels of the

208 inflammation related molecules IL-6 and miR-146a, miR-155 and miR-223 along with a decrease

209   in the levels ofhe anti-inflammatory cytokine IL-10 were observed. Increased expression of miR-

210   146a has been previously reported in the cerebral cortex of rats that were sleep-deprived for 24h

211   (Matos et al., 2014). Particularly, since miR-146a is upregulated as NF-kB is activated and controls

212   cytokine signaling (Taganov, Boldin, Chang, & Baltimore, 2006), this increase suggests that a

213   regulatory response is induced by short-term PSD (that is further lost after longer periods of PSD).

214   Also, it seems that the increase of these miRNAs at 24h may be part of a rapid response to oxidative

215   stress and endotoxemia induced by sleep deprivation (Hirotsu, Matos, Tufik, & Andersen, 2013)

216   processes that foster the secretion of proinflammatory cytokines, as in the case of IL-6 (Matos et

217   al., 2014 ;   )Novotny et al., 2008). Furthermore, during acute stress a polymorphic region related

218   to the gene of the 5HT transporter modulates the response of inflammatory cytokines (Yamakawa,

219   Matsunaga, Isowa, & Ohira, 2015).

220   During the "resistance phase" of stress, organisms manifest a process of adaptation to those

221   demands during a defined time, and activate a series of mechanisms in a stress response that aims

222   to restore homeostasis (Goldstein & Kopin, 2007) which is what apparently happens when PSD-

223   induced stress is prolonged. During 96h of PSD, only miR-223, miR-16 and adiponectin were

224   modified, where the level of miR-223 and miR-16 decreased, but adiponectin increased. These

225   changes may be due to a compensatory response that seeks to limit tissue damage or dysfunction

226   related to both the metabolic and inflammatory alterations involved in this phenomenon

227   (Villarreal-Molina & Antuna-Puente, 2012).

228   After a chronic sleep restriction (192h) we found increased serum levels of corticosterone, IL-6,

229   and IL-10. The stress and anti-inflammatory cytokines responses seem to be at their peaks. An

230   increase in the levels of IL-10 after long-term sleep deprivation (18h/day for 21 days) has been

231   previously reported (Venancio & Suchecki, 2015). The increase in IL-6 after PSD 192h may be

232   part of this anti-inflammatory responses, since   IL-6 has been described to play both   pro-

233   inflammatory and anti-inflammatory roles because in some scenarios it stimulates IL-10 synthesis

234   (Petersen & Pedersen, 2006), and inhibits TNF and IL-1 production (Wolkow et al., 2015). At this

235   point of PSD it seems that an effort to closely regulate inflammation is in process in order to control

236   its negative effects and the impairments associated with it in terms of performance, cognition,

237   sleepiness (Yirmiya & Goshen, 2011) and the incidence of degenerative disorders such as diabetes

238   (Petersen & Pedersen, 2006).

239   Interestingly, at this point miRNAs seem not to be participating in an active control of

240   inflammation, but rather may reflect an exhaustion situation due to the long-term, sustained

241   wakefulness. At PSD 192h we found that serum miR-146a, miR-155, and miR-223 had returned

242   to base-line levels. However, low levels of miR-16 and miR-126 were observed. In other scenarios

243   –for example, under hyperglycemic conditions– these two miRNAs participate in the suppression

244   of inflammation and ROS production (Harris, Yamakuchi, Ferlito, Mendell, & Lowenstein, 2008;

245   Tang, Wang, Shao, Wang, & Zhu, 2017). Also, decreased serum expression of miR-126 (Chen et

246   al., 2016) and miR-16 in the brain (Li et al., 2010) has been associated with type II diabetes. Other

247   reports also indicate that miR-16 may play a role in the active coping mechanisms for stress

248   resilience (Zurawek et al., 2017). This miRNA is highly-expressed in serotoninergic (5-HT) and

249   noradrenergic neurons (NA) (Obrietan & Hansen, 2013), both of which show high activity during

250   wakefulness, and enhance their functioning during sleep deprivation (Hipólide et al., 2005).

251

252   Reestablishing homeostatic processes is important for organisms, as it induces a period of

253   recuperation and repair after subjection to a stressful condition. It is interesting to note that even

254   after a 20-day recovery period the consequences of a chronic sleep deprivation were present as

255   alterations in biological mediators. Serum IL-10 and miR-16 were found decreased, while

256   corticosterone, IL-6, TNF, and miR-21 levels were increased in the 192h PSD+R group compared

257   to controls. A chronic sleep deprivation is undoubtedly a stressful situation. Thus, it is plausible

258   that one possible mediator of this delayed return to a homeostatic state would be corticosterone

259   since high levels were found after chronic PSD and even after a recovery period of 20 days. This

260   situation adds to the reported alteration in glucose metabolism (Brianza-Padilla et al., 2016) after

261   PSD. A previous study of the effects of stress hormones on SD showed that some miRNAs can be

262   regulated by corticosterone in tissue under experimental conditions (Mongrain et al., 2010).

263   Even when the reported recovery period was clearly not enough to a full-return to homeostasis,

264   there are hints to suggest the presence of a repairing response. For instance, since miR-21 has been

265   associated with the protection of various organs from suffering lesions (Tong, Xiao, Zeng, &

266   Xiong, 2015, Xu et al., 2017), the elevated levels of miR-21 seen after the 20-day recovery period

267   posterior to 192h of PSD may well be reflecting a mechanism aimed to restore functionality.

268 **Conclusion**

269 Sleep deprivation may disrupt cytokine and miRNA production, seemingly related to stress and

270 inflammatory responses. Changes in cytokines and miRNAs in sera seem to be related to the

271 duration of sleep deprivation, as short-term PSD produced effects similar to those of an acute

272 inflammatory response. Although an anti-inflammatory response may appear as PSD duration

273 increases, the disruption of homeostasis probably leads to long-lasting variations of some set points

274 that may be involved in the development and/or progression of several diseases.

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Figure 1

Serum corticosterone levels during PSD.

The corticosterone levels increases bye effect of PSD at 192h and 192h+R. Values represent means ± SEM. ANOVA followed by Tukey's tests were performed *p<0.05 compared to control; φp<0.05 compared to the PSD 192h+R group.
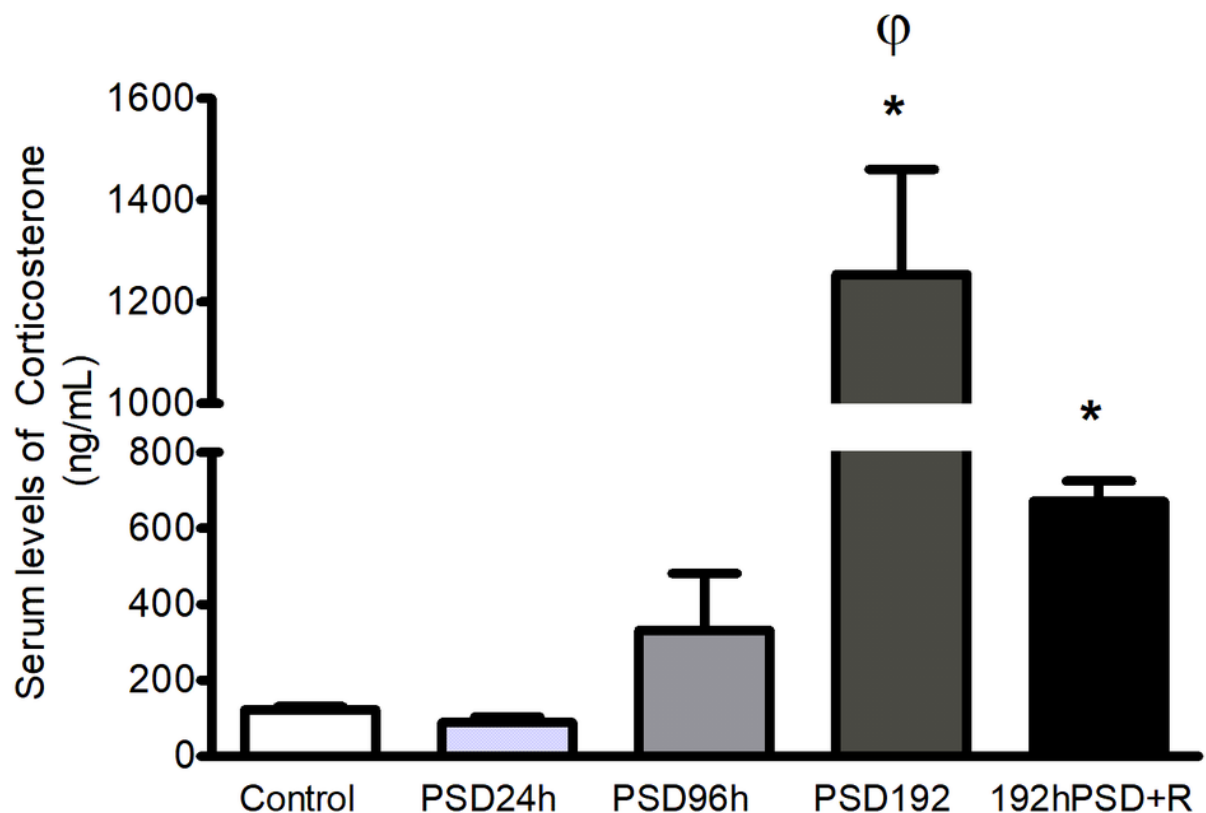
Figure 2

Effect of different periods of PSD on IL-6 (A), TNF (B), IL-10 (C), and adiponectin (D) concentrations

Values represent means ± SEM. ANOVA followed by Tukey's tests were performed; *p<0.05 compared to the control group; φ p<0.05 compared to PSD 192h+R.(n=5 for each group).
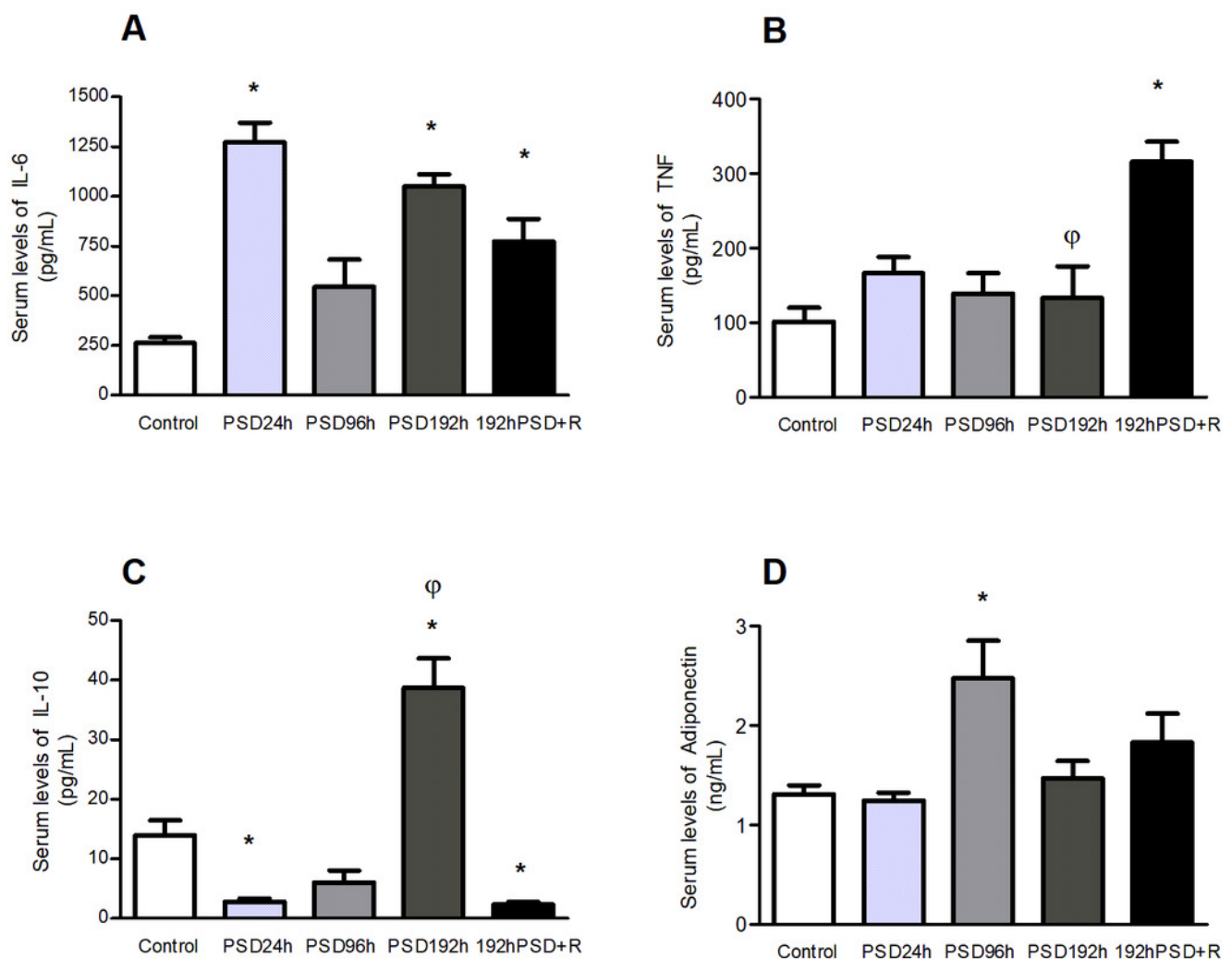
Figure 3

miRNAs levels in the sera of rats after different periods of PSD and recovery: miR-146a (A), miR-155 (B), miR-223 (C), miR-16 (D), miR-126 (E), and miR-21 (F)

Values represent means ± SEM. An ANOVA followed by a Tukey's test was performed.

*$p < 0.05$ compared to the control group, and φ $p < 0.05$ compared to the 192h PSD+R group.