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Research Article

The Interaction of Oxidative Stress Response with Cytokines in the Thyrotoxic Rat: Is There a Link?

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Oxidative stress is regarded as a pathogenic factor in hyperthyroidism. Our purpose was to determine the relationship between the oxidative stress and the inflammatory cytokines and to investigate how melatonin affects oxidative damage and cytokine response in thyrotoxic rats. Twenty-one rats were divided into three groups. Group A served as negative controls. Group B had untreated thyrotoxicosis, and Group C received melatonin. Serum malondialdehyde (MDA), glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), and nitric oxide derivates ($NO^{\bullet}x$), and plasma IL-6, IL-10, and TNF-alpha were measured. MDA, GSH, $NO^{\bullet}x$, IL-10, and TNF-alpha levels increased after L-thyroxine induction. An inhibition of triiodothyronine and thyroxine was detected, as a result of melatonin administration. MDA, GSH, and $NO^{\bullet}x$ levels were also affected by melatonin. Lowest TNF-alpha levels were observed in Group C. This study demonstrates that oxidative stress is related to cytokine response in the thyrotoxic rat. Melatonin treatment suppresses the hyperthyroidism-induced oxidative damage as well as TNF-alpha response.

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1. Introduction

Hyperthyroidism is associated with an increased metabolic rate due to increments in the rate of oxygen consumption in target tissues [1]. Acceleration of aerobic metabolism by thyroid hormones enhances the generation of oxidative stress [2]. Oxidative stress is regarded as a pathogenic factor in hyperthyroidism. Clinical and experimental studies reported increased levels of free oxygen radicals and a decreased antioxidant status in thyrotoxicosis [3–6].

Free oxygen radicals are general mediators of signal transduction pathways, which are able to induce cytokine production from various cell types [7–10]. Studies attesting to the role of antioxidants, of which potentially inhibit the activation of oxidant-mediated transcription factors, reported that these antioxidants also prevent the transcriptional activation of inflammatory cytokines [11–13]. These studies suggest that antioxidants may play a role in decreasing the immune response by suppressing the oxidative stress. Since these findings may be of clinical relevance in the thyrotoxic patient, herein we aimed to investigate (1) whether oxidative stress affects the production of cytokines IL-6, IL-10, and TNF-alpha and (2) whether these alterations are inhibited by melatonin-based antioxidative therapy.

2. Methods

Twenty-one 12-week-old male Wistar albino rats with a body weight of 250–300 g were included in the study. All were housed in groups of 7 in identical wire-bottomed cages with a 12 hour day-night cycle, at a constant room temperature of 24 ± 1°C. The animals were acclimatized to these conditions for 10 days before the experiment. Standard rat food and water was freely available. All rats were treated according to the UFAW Handbook on the Care

and Management of Laboratory Animals (Blackwell, 7th ed, Blackwell Science, Iowa, 1999) and Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985). The protocol was approved by Ege University Ethics Committee of Experimental Studies and Ege University , Department of Experimental Surgery.

Rats were randomized to the following groups according to the treatment to which each was subjected and were sacrificed at the end of the 3-weeks treatment:

(1) Group A ($n = 7$), the sham group, was not subjected to any procedure, except receiving daily intraperitoneal injections of 0.9% saline solution;

(2) Group B ($n = 7$), the untreated (control) group, underwent intraperitoneal L-thyroxine administration for induction of hyperthyroidism, for 21 days and at a daily dose of 0.2 mg/kg. L-thyroxine was dissolved using 0.01 N NaOH, and the final solution was prepared with 0.9% saline solution;

(3) Group C ($n = 7$), the treatment group, underwent L-thyroxine as in Group B and besides, received daily (each 24 hours) 3 mg/kg melatonin for 21 days (Ilsan Iltas Laboratories, Istanbul), intraperitoneally.

2.1. Laboratory Measurements. All evaluations were done by a single biochemist blinded to the study. Blood was collected through heart puncture to estimate thyroid function tests (TSH, FT_3, and FT_4), oxidative stress markers (malondialdehyde [MDA], glutathione [GSH], glutathione reductase [GR], glutathione peroxidase [GPx], and nitric oxide [$NO^{\bullet}$]), and cytokines (IL-6, IL-10 and, TNF-alpha). Blood samples were centrifuged at 2500 g for 5 minutes at room temperature. The serum was removed and stored at −80°C for later studies. Free T_3 (triiodothyronine) (FT_3), free T_4 (thyroxine) (FT_4), and TSH (thyroid stimulating hormone) analyses were carried out with a chemiluminescent enzyme radioimmunometric assay by using the IMMULATE 2000 automated hormone analyzer (Diagnostic Products, Los Angeles, Calif, USA). The reproducibility of the methods, established before the study by conducting 2 additional independent experiments, was good with clear normative limits and a 95% agreement.

2.2. Measurement of MDA. Lipid peroxidation (LPO) is frequently investigated in biomedical research, and the assays for thiobarbituric acid-reactive substances (TBARSs) are more widely used than any other index of LPO in biological samples. Thiobarbituric acid reacts with LPO aldehydes, such as malondialdehyde (MDA). Therefore, assessment of TBARS is a useful index of oxidative deterioration and LPO determination in body fluids. MDA levels were determined spectrophotometrically at 532 nm by the method of Ohkowa et al. [14]. MDA formed a colored complex in the presence of thiobarbituric acid, which was detectable by measurement of absorbance at 532 nm. Absorbance was measured with Shimadzu UV-160 spectrophotometer. 1,1′,3,3′ tetraethoxypropane was used as a standard. Levels were calculated as nmol/mL.

2.3. Measurement of GSH. Total glutathione content was measured according to the method of Tietze [15]. In brief, 0.5 mL sample or standard solution was mixed with 0.25 mL of 1 mol/L sodium phosphate buffer (pH 6.8) and 0.5 mL 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.8 g/L in the phosphate buffer) for 5 minutes. Then, the absorbance was measured at 412 nm using a Shimadzu UV-160 spectrophotometer. The GSH concentration was determined using standard aqueous solutions of GSH. Results were expressed as mg/dL.

2.4. Measurement of GR. Glutathione reductase was assayed by following the oxidation of NADPH at 340 nm at 37°C [16]. The reaction was initiated by the addition of 50 μL supernatant to 1 mL of assay mixture containing 50 mmol/L Tris, pH 7.6, 100 μmol/L Na_2EDTA, 4 mmol/L GSSG, and 120 μmol/L NADPH. A blank cuvet was prepared in which the sample was replaced with water. The reaction was linear for 2 to 3 minutes. Hemoglobin in erythrocyte lysates was estimated using the method of Drabkin and Austin [17]. Activity in samples was normalized for hemoglobin content and is expressed in U/g Hb.

2.5. Measurement of GPx. Glutathione peroxidase activity was determined by a minor modification of the method of Paglia and Valentine [18]. A 50 μL supernatant was transferred to a 1 mL quartz cuvet, containing 950 μL of the reaction mixture (Tris buffer, 50 mmol/L, pH 7.6, containing per liter, 1 mmol of Na_2EDTA, 2 mmol of reduced glutathione, 0.2 mmol NADPH, 4 mmol sodium azide, and 1000 U of glutathione reductase). The mixture was incubated 5 minutes in 37°C. Then, the reaction was initiated by adding 25 μL of H_2O_2, 8.8 mmol/L (% 30). The decrease in NADPH absorbance at 340 nm was followed for 3 minutes. The nonenzymic reaction rate (blank) was determined by substituting water for the supernatant. The decrease in NADPH absorbance was recorded. Hemoglobin was evaluated as described above. Activity in samples was normalized for hemoglobin content and is expressed in U/g Hb.

2.6. Measurement of $NO^{\bullet}$ Derivative Content. $NO^{\bullet}x$ (nitrite + nitrate) was assayed by a modification of cadmium-reduction method as mentioned by Navarro-Gonzàlves [19]. The nitrite produced was determined by diazotization of sulphanilamide and coupling to naphthlethylene diamine. For the measurement of $NO^{\bullet}x$, a 400 μL sample was denatured by adding 80 μL 30% $ZnSO_4$ solution, stirring and then centrifuging at 10 000 xg for 20 minutes at 4°C. First, we activated Cd granules using $CuSO_4$ solution in glycine-NaOH buffer. Then, 100 μL of deproteinized samples and standards were added. This reaction, using pretreatment of samples to reduce nitrate to nitrite, can be accomplished by catalytic reactions using enzyme or Cd. The samples were analyzed spectrophotometrically using a microplate reader and quantified automatically against KNO_3 standard curve. Results were expressed as μM/L.

2.7. Measurement of IL-6. Plasma IL-6 levels were determined via a commercial rat ELISA kit (IBL Co., Ltd.

Table 1: Comparison of thyroid function tests.

	n	Group A	Group B	Group C
FT_3 (pg/mL)	7	1.49 ± 0.26	8.68 ± 3.69*	3.28 ± 2.98**
FT_4 (ng/dL)	7	1.44 ± 0.44	4.62 ± 2.18*	1.94 ± 1.22**
TSH (μIU/mL)	7	0.25 ± 0.02	0.15 ± 0.01	0.20 ± 0.36

Values were expressed as mean ± standard deviation.
*$P < .01$; significant when compared with Group A.
**$P < .01$; significant when compared with Group B.

Table 2: Comparison of MDA, GSH, GR, GPx, and $NO^{\bullet}$ derivate levels.

Group	n	MDA (μmol/L)	GSH (mg/dL)	GR (U/g Hb)	GPx (U/g Hb)	NO (μM/L)
A	7	3.42 ± 1.47	0.26 ± 0.06	0.83 ± 0.34	4.02 ± 1.51	6.72 ± 1.55
B	7	4.80 ± 1.23*	0.43 ± 0.06*	1.05 ± 0.39	3.48 ± 0.76	66.0 ± 58.54*
C	7	2.31 ± 0.71**	0.35 ± 0.07**	0.78 ± 0.31	3.88 ± 1.17	47.11 ± 15.18**

Values were expressed as mean ± standard deviation.
*$P < .01$; significant when compared with Group A.
**$P < .01$; significant when compared with Group B.

Hamburg, Germany), catalogue and lot number 17196. The test results were calculated by bioelisa reader ELx800 using standard curve. The kit measurement range was 11.72–750 pg/mL. Interassay values were calculated for three different measurement values (34.83, 71.71, and 293.31 pg/ml), and CV values were found to be 2.3%, 1.9%, and 1.7%, respectively, ($n = 10$). Besides, intra-assay values determined three different measurement values (33.57, 70.45, and 293.45 pg/mL), and CV values were found to be 8.8%, 7.7%, and 4.9%, respectively, ($n = 10$). We did not use haemolysed samples.

2.8. Measurement of IL-10. IL-10 levels were determined via a commercial human ELISA kit (IMMUNOTECH; Beckman Coulter Company, Marseille Cedex, France). Test results were calculated by bioelisa reader ELx800 using standard curve. The kit measurement range was 0–2000 pg/ml. Kits' performance characteristics were performed. Intra-assay precision was determined by assaying sera 10 times, and CVs ranged between 3.3 and 4.0%. Interassay precision was determined by assaying samples 10 times in independent assays. CVs ranged between 5.6 and 8.6%. We did not use haemolysed samples.

2.9. Measurement of TNF-Alpha. TNF-α levels were determined via a commercial rat ELISA kit (IBL Co., Ltd. Hamburg , Germany), catalogue number 17194. Test results were calculated by bioelisa reader ELx800 using standard curve. The kit measurement range was 0.39–25 ng/mL. Kits' performance characteristics were performed. Interassay values were calculated for three different measurement values (0.36, 1.69, and 7.03 ng/mL), and CV values were found to be 8.8%, 3.0%, and 1.0%, respectively, ($n = 10$). Besides, intra-assay values determined three different measurement values (0.33, 1.69, and 7.03 ng/mL), and CV values were found to be 8.8%, 3.0%, and 1.0%, respectively, ($n = 10$). Besides, intra-assay values determined three different measurement values (0.33, 1.68, and 6.7 ng/mL), and CV values were found to be 9.1, 9.5, and 4.3, respectively, ($n = 10$). We did not use haemolysed samples.

2.10. Statistical Analysis. All analyses were performed by using SPSS Statistical Analysis software (release 10.0, Chicago, Ill, USA). All data are expressed as mean ± standard deviation. To search difference between the groups, Kruskal-Wallis one-way analysis was used. Depending on the homogeneity of the variances, Mann-Whitney U-test and Bonferonni post hoc test were used in pair comparison. Correlations between oxidative stress parameters and serum concentrations of cytokines were studied by regression analysis. P-values of less than .05 were considered to be significant.

3. Results

3.1. Thyroid State. The thyroid state of different groups of animals was characterized by the data reported in Table 1. The values indicate significant rises in FT_3 and FT_4 levels after L-thyroxine administration ($P < .01$). It was also found that melatonin treatment significantly decreased the concentrations of FT_3 and FT_4 when compared to rats with hyperthyroidism in the untreated group ($P < .01$).

3.2. Oxidative Stress and Response to Oxidative Stress. MDA, GSH, GR, GPx, and $NO^{\bullet}$x levels of control and treatment groups are presented in Table 2. Comparison among the groups revealed that MDA, GSH, and $NO^{\bullet}$x levels in hyperthyroid rats were significantly higher than the negative control and treatment groups ($P < .01$).

3.3. Plasma Cytokine Concentration. Levels of both IL-10 and TNF-alpha were significantly enhanced after L-thyroxine administration ($P < .01$), while the rise in IL-6 was insignificant ($P < .05$). Melatonin treatment was associated with significant decreased TNF-alpha levels ($P < .01$)

Table 3: Comparison of plasma cytokine levels.

Group	n	IL-6 (pg/mL)	IL-10 (pg/mL)	TNF-alpha (ng/mL)
A	7	324.66 ± 26.28	33.53 ± 10.03	0.76 ± 0.04
B	7	345.99 ± 109.20	51.99 ± 17.43*	0.88 ± 0.1*
C	7	333.33 ± 38.92	56.00 ± 10.33	0.75 ± 0.05**

Values were expressed as mean ± standard deviation.
*$P < .01$; significant when compared with Group A.
**$P < .01$; significant when compared with Group B.

(Table 3). There was a significant correlation between IL-10 concentration and both oxidative stress parameters MDA and NO ($r = 0.48$, $P < .05$ and $r = 0.39$, $P < .01$, resp.). A significant correlation also existed between serum TNF-alpha and MDA ($r = 0.49$, $P < .01$) as well as TNF-alpha and NO level ($r = 0.69$, $P < .01$). No correlations were found between TNF-alpha and thyroid hormone levels ($P > .05$).

4. Discussion

The findings of the study suggest that (1) thyrotoxicosis stimulates the oxidative stress response and the production of inflammatory cytokines (IL-10 and TNF-alpha), (2) there is a relationship between oxidative stress parameters and inflammatory cytokines, (3) melatonin prevents the increase in FT_3 and FT_4 caused by chronic T_4 injection, and (4) melatonin-based antioxidative treatment inhibits thyroid hormone induced increases in MDA, GSH, $NO^{\bullet}$, and TNF-alpha levels. Meanwhile, to our knowledge, the latter observation, that melatonin acts on TNF-alpha levels, seems novel. We hypothesized that reactive oxygen intermediates, that were induced by thyrotoxicosis, act as a signal for the release of cytokines like IL-6, IL-10, and TNF-alpha. We documented the increased oxidative stress and antioxidant system capacity, together with response to allopurinol, in different time intervals, in another paper, which is soon going to be published elsewhere.

Previous studies have provided evidence that free radicals increase during hyperthyroidism [3–6]. Whatever the pathways are, it is clear that T_3-induced liver free-radical generation occurs in concomitance with enhanced respiratory burst activity and chemiluminescent response in both experimental and human studies [20, 21]. The evidence that oxidative stress occurs related to thyroid hormone induction is supported in the current study, since we have demonstrated significant increases in oxidative stress parameters like MDA and $NO^{\bullet}x$. Although some modalities have been suggested to prevent the development of oxidative stress during thyrotoxicosis, no management has yielded promising uniform success, clinically [22–24].

Oxidative stress is paralleled by impaired antioxidant mechanisms; glutathione is one of these important systems [25]. In one of the few studies related to GSH in hyperthyroidism, GSH was stimulated in an experimental model [26]. In these studies, it was concluded that free radicals appear to stimulate GSH production in prolonged oxidative stress. This explanation seems plausible, since biochemical data of our study showed an increase in GSH.

Several previous reports have tried to address the contribution of thyrotoxicosis to the rise in inflammatory cytokines such as IL-1, IL-6, IL-10, and TNF-alpha [13, 27–34]. Although controversial results, almost all these studies suggest that cytokines may play important roles in the process of hyperthyroidism. The only report investigating the effect of TNF-alpha levels on thyroid hormone kinetics is the one by Ongphiphadhanakul et al. These investigators found that TNF-alpha might inhibit the peripheral generation of T_3 from T_4 and act as an autocrine factor by decreasing thyroidal generation and release of T_3 [33]. Our data is not in accordance with this finding, since no correlation was found between TNF-alpha levels and thyroid hormones. In the present study, we demonstrated a significant IL-10 and TNF-alpha release after thyroid hormone induction, while the increase of IL-6 was insignificant. Thus, our results do not support any role for IL-6 in the pathogenesis of thyrotoxicosis.

IL-6 acts as a systemic hormone [35] that may mediate the well-documented inhibitory effect of IL-1 on thyroid cell functions [36]. In regard to IL-6, our findings are in agreement with the data presented by Senturk et al. [29] and Burggraaf et al. [32], since we could not present a statistically significant difference between thyrotoxic rats and control subjects. We suggest that this may be attributed to the duration of thyroid hormone induction or the consequence that since IL-6 can be bounded to plasma proteins, which may camouflage IL-6 immunoreactivity in serum, as previously suggested [29].

In contrast to IL-6, IL-10 is one of the most potent anti-inflammatory cytokines [37]. IL-10 interferes with cellular immunity in several ways and is known to attenuate the expression and/or production of proinflammatory cytokines [38]. TNF-alpha is expressed as one of these cytokines [39]. Our data did not support this, since both IL-10 and TNF-alpha levels were elevated after T_4 induction. Interestingly, proinflammatory stimuli like TNF-alpha enhance its secretion without any influence of IL-6 [40]. There is a lack of evidence regarding IL-10 and nonautoimmune thyroid disorders. Data presented by de la Vega et al. supported that Il-10 levels in multinodular glands did not reach a significant difference versus normal subjects [41]. But the study population they studied was only 15, where each group consisted of 5 patients.

The link between free oxygen radicals and inflammatory processes in hyperthyroidism was studied previously by Fernández et al. [27]. They addressed that the effects of thyroid hormones on the expression of nuclear factor kappa

B (NF-κB) ligand are involved in the cytokine response seen in hyperthyroidism. There is evidence that T_3administration to rats show induced expression of NF-κB-responsive genes for TNF-alpha and IL-10 [13]. This was correlated with increases in the serum levels of the cytokines in the same study.

Even though deleterious effects of $NO^{\bullet}$ in oxidative processes [42], its production is accepted to be induced by proinflammatory cytokines [43]. In our model, thyrotoxicosis resulted in obvious increased $NO^{\bullet}x$ levels, and there was a correlation between $NO^{\bullet}x$ and cytokine levels. This correlation suggests a link between NO and cytokine response in the thyrotoxic rat. This might simply mean that while a probable contributory role for these cytokines is suggested, it seems not to be the only factor involved in the complex pathogenesis in thyrotoxicity-related oxidative stress.

Melatonin, after discovered as a free radical scavenger in 1993 [44], has been reported to be protective in various models of oxidative stress, both through its free radical scavenging effect as well as by directly increasing antioxidant activity [45]. Although melatonin has been proposed to be useful for the prevention of oxidative stress during hyperthyroidism [23, 46], to our knowledge, there has been no report studying the effects of antioxidative therapy on serum concentrations of IL-6, IL-10, and TNF-alpha in the thyrotoxic rat. The hypothesis that melatonin is protective against free oxygen radicals was further supported by our observation that after melatonin treatment, increased MDA, GSH, and $NO^{\bullet}x$ levels decreased significantly. Besides, thyroid hormone and TNF-alpha levels after melatonin treatment were lower than in untreated rats, suggesting an inhibitory effect of melatonin on T_3, T_4, and TNF-alpha. The decrement in TNF-alpha levels did not relate with the decrease of thyroid homone levels, suggesting a direct antioxidant effect of melatonin on TNF-alpha. Studies on the hypothesis of an inhibitory effect of melatonin on thyroid hormones documented contradictory results [47, 48]. In fact, melatonin is known with its stimulating effect on antioxidative enzymes, such as GR and GPx [49]. These enzymes convert oxidized glutathione to GSH, leading to increased GSH activity. However, in our study, melatonin administration did not result in increased GR and GPx levels. Moreover, it resulted in decreased GSH. This paradox might be attributed to differences in type and manner of melatonin administration. Melatonin suggests an immunotherapeutic potential, since it inhibits TNF-alpha. Whatever the mechanism is, the fact that melatonin counteracts the increase of TNF can help toward an understanding of the complex nature of TNF induction in hyperthyroidism.

Collectively, data presented in this work support a link between thyroid hormone-induced oxidative stress and cytokine response. It provides new insights into the role of IL-10 and TNF-alpha in thyrotoxicosis. Therapy for increased oxidative stress with melatonin significantly reduces triiodothyronine, thyroxine, IL-10, and TNF-alpha levels. The fact that circulating cytokines might differ from those in different tissues is a limitation of this study. Although the expression of cytokines seems to be involved with thyrotoxicosis-induced oxidative stress, this relation remains to be studied in future qualitative studies.

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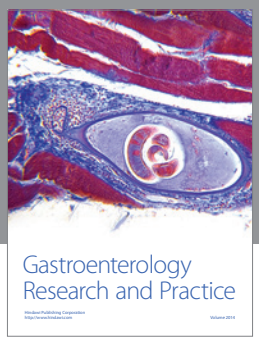

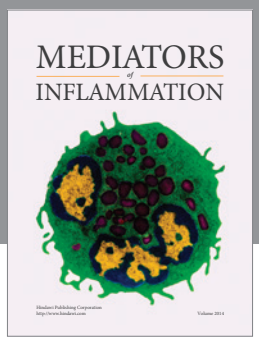



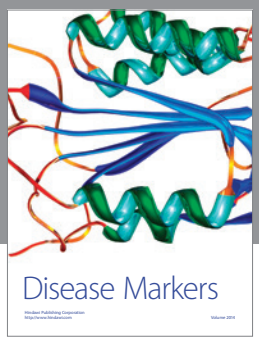











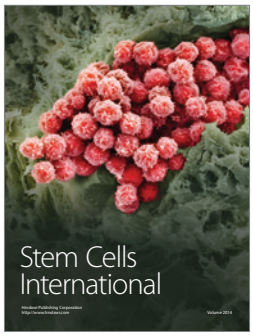

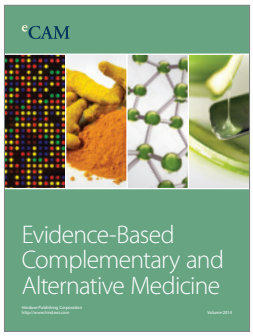



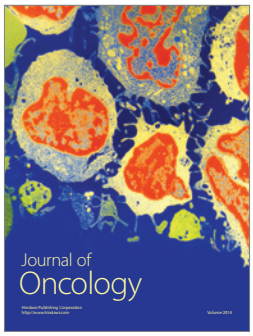

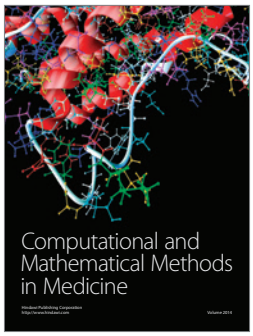

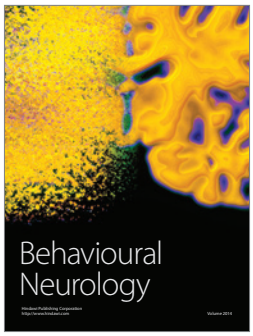



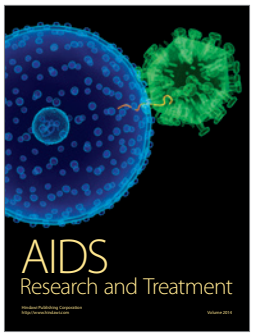

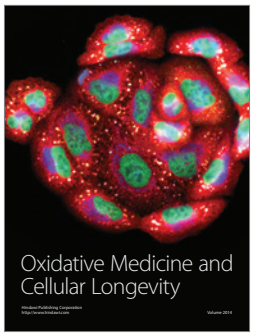

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