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ORIGINAL ARTICLE

Oxidative stress and autonomic nervous system functions in restless legs syndrome

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ABSTRACT

Background Oxidative stress has been implicated in over 100 disorders in recent years; however, the situation in restless legs syndrome (RLS) has not been studied yet.

Methods Fifty patients with RLS not medicated for RLS and 50 sex- and age-matched, healthy controls and controls with no pathology except mild iron deficiency or iron deficiency anaemia were enrolled. Patients with secondary RLS other than iron deficiency were excluded. Total oxidant status (TOS), total antioxidant status (TAS), oxidative stress index (OSI), arylesterase (ARE), paraoxonase (PON), stimulated paraoxonase (stim-PON), lipid hydroperoxides (LOOHs), acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE) were measured. Heart rate variability (HRV) analysis was performed.

Results TOS, ARE and AChE were increased ($P = 0{\cdot}018$, $P < 0{\cdot}001$ and $P < 0{\cdot}001$, respectively), whereas LOOHs were decreased ($P < 0{\cdot}001$) in RLS group. TAS, OSI, PON and stim-PON were comparable. Erythrocyte sedimentation rate (ESR) and mean platelet volume (MPV) were increased ($P = 0{\cdot}021$ and $P = 0{\cdot}037$, respectively) in RLS group. HRV triangular index (HRVi) was lower ($P = 0{\cdot}012$) in RLS group. Other HRV parameters were similar.

Conclusions Increased AChE and decreased LOOHs, which were influenced by increased PON1, were considered as indicators of efforts towards the protection of dopaminergic activity in central nervous system in RLS group. Increased ESR, MPV and low HRVi indicate elevated sympathetic activity in RLS group. Elevated sympathetic activity might be beneficial in relieving RLS symptoms, also causing increases in TOS. The evidence we found regarding oxidative stress and autonomic nervous system might be seminal in RLS treatment.

Keywords Acetyl cholinesterase, butyryl cholinesterase, erythrocyte sedimentation rate, heart rate variability, lipid hydroperoxide, mean platelet volume, oxidative stress, PON1, restless legs syndrome.

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Introduction

Restless legs syndrome (RLS) manifests with symptoms of spontaneous, continuous leg movements with unpleasant paresthesias. Symptoms occur only at rest and are relieved by movement [1].

Mild RLS symptoms occur in 5–15% of population [1]. Although its pathophysiology has not yet been elucidated, dopaminergic dysfunction in central nervous system (CNS) is the most supported argument [1,2]. RLS is classified as idiopathic or secondary. Iron deficiency, pregnancy and chronic renal failure (CRF) are well-documented causes of secondary RLS [1].

Oxidative stress (OS) implies increased oxidant production in animal cells characterized by the release of free radicals, resulting in cellular degeneration. The imbalance between excess free radical production and antioxidant defence causes cellular damage resulting in lipid peroxidation [3]. Studies on OS have made a surge recently, and OS has been implicated in over 100 disorders [4].

Because separate measurement of different oxidant molecules [hydrogen peroxide, superoxide radical anion, lipid hydroperoxides (LOOHs), etc.] is not practical and their oxidant effects are additive, we measured total oxidant status (TOS) in serum [4]. Likewise, we measured total antioxidant status (TAS), instead of measuring antioxidant molecules (vitamin C, trolox, uric acid, etc.) separately [5].

PON1, a well-known antioxidant, is an enzyme produced in the liver and associated with high-density lipoprotein particles. PON1 has three known enzymatic molecules; paraoxonase (PON), arylesterase (ARE) and dyazoxonase. PON acts together with ARE to function as a single enzyme. PON1 hydrolyses organophosphates and aromatic esters and also reduces the accumulation of lipid peroxidation products [6]. Through its peroxidase activity, PON also protects acetyl cholinesterase (AChE) from oxidative stress to which it is highly susceptible [7].

Changes in PON1 activity are because of the polymorphisms at the gene locus encoding the enzyme [8]. Amino acid change at the codon 192 in the PON1 gene leads to three different phenotypes; the low-activity homozygous QQ, the medium-activity QR and the high-activity RR groups [8,9].

Oxidative stress has been studied in many neurologic diseases including Parkinson's disease where dopaminergic deficiency is obvious, but it has not been investigated in RLS up to date [7,10,11]. We measured TOS, TAS, oxidative stress index (OSI), PON, stim-PON, ARE and LOOH levels and performed PON1 phenotypings. We also measured serum AChE and BuChE levels and performed routine blood analysis. The relation between autonomic dysfunction and oxidative stress is well known [12]. The alterations of cardiac autonomic function were measured by heart rate variability (HRV) analysis using 24-h ambulatory ECG recordings.

Material and methods

Subjects

Subjects were chosen among patients who presented to Vakif Gureba Hospital (VGH) neurology outpatient clinics and who were not taking any RLS medications. Because iron deficiency is very frequent in Turkey, patients with RLS with iron deficiency or iron deficiency anaemia were included. Patients with all other causes of secondary RLS were excluded. Controls were chosen among healthy subjects and subjects who had no pathological finding except mild iron deficiency or iron deficiency anaemia through medical history, physical examination and blood analyses. Fifty patients with RLS (34 women, 16 men), and age- and sex-matched 50 controls (35 women, 15 men) were enrolled. The four basic criteria developed by International RLS Study Group in 1995 were used for RLS diagnosis [1]. Our patients with RLS had all of these four criteria.

Thorough medical history was taken, medication use was queried, and physical examination was performed. Thyroid functions, routine blood chemistry, postprandial blood sugar, HbA1C, CBC, urinanalysis, erythrocyte sedimentation rate (ESR), sensitive c reactive protein (CRP), ferritin, vitamin B12 and folate, anti-HCV, HBsAg, anti-HIV 1–2 were tested. Patients using drugs that influence oxidative stress parameters (statins, fenofibrates, antioxidant vitamins, oral contraceptives etc.) were excluded. Additional criteria for exclusion were neuropathy, CRF, pregnancy, lactation, menorrhagia and/or metrorrhagia, haematuria, melena, hematochezia, gastrectomy, pronounced gastric complaints, recent alteration in defecation habits, strict vegetarian diet, diabetes, hypothyroidism, ischaemic heart disease, peripheral arterial disease, lower extremity varices, chronic venous insufficiency, chronic low back pain or lumbar disc herniation, heart failure, alcohol abuse, malignancy, use of neuroleptics or antidepressants, severe hyperlipidemia, chronic hepatitis, liver cirrhosis, nephropathy, COPD, bronchial asthma, collagen tissue diseases, local or systemic infections and persistent transaminase elevation. Subjects taking antihypertensives with significant antioxidant properties (carvedilol, nebivolol, zofenopril and captopril) were also excluded.

Smoking subjects and patients with controlled hypertension (HT) were included; however, HRV analysis was not performed in patients taking beta-blockers or nondihydropyridine group calcium channel blockers.

Informed consents were obtained from patients and controls prior to the study. VGH ethical committee approved the study protocol and procedures, which were in accordance with the Helsinki Declaration of 2008. Reporting of the study conforms to STROBE along with references to STROBE and the broader EQUATOR guidelines.

Samples

All blood samples were collected in the morning after an overnight fast; serum samples were stored at −70 °C until assay for TAS, TOS, PON1, ARE, stim-PON1, LOOHs and BuChE. Serums obtained after overnight fasting were stored at 2–8 °C for 4–5 h until AChE assay.

Measurements

Serum TOS and TAS were measured using a novel automated colorimetric measurement method developed by Erel [4,5]. The ratio of TOS to TAS was accepted as the OSI.

Paraoxonase and ARE activities were measured using paraoxon and phenylacetate substrates [9]. Paraoxonase phenotype distribution was determined by a double-substrate method measuring the ratio of paraoxonase activity to ARE activity [9].

Serum LOOH levels were measured by the ferrous ionoxidation – xylenol orange (FOX-2) method [13]. BIOASSAY SYSTEM brand AChE commercial kit was used for AChE measurement according to the manufacturer's instructions [14]. CHE Gen.2 Cobas C Integra brand (Roche Diagnostics Corporation, Indianapolis, IN, USA) commercial kit was used for pseudocholinesterase measurement.

HRV analysis

We obtained 24-h ambulatory electrocardiograms using NORAV MODEL NO: DXP1045 digital Holter recorder

(Norav Medical Ltd., Yokneam, Israel). HRV analysis was performed with the special NORAV package software. HRV indices comprised the mean heart rate, the standard deviation of all RR intervals (SDNN), the HRV triangular index (HRVi), the root mean square of successive differences (RMSSD), the low-frequency (LF) and the high-frequency (HF) component, as well as the LF/HF ratio [15].

Statistical analysis

Data were evaluated by SPSS 13 version (SPSS Inc., Chicago, IL, USA). Student's *t*-test, Mann–Whitney *U* test, univariate two-way variance analysis, chi-square test and bivariate correlation tests were employed. Two-sided *P*-values were used, and $P < 0{\cdot}05$ value was accepted as significance level.

Results

Patient and control groups had the same number of subjects. Female/male ratio, age, BMI, hypertension and smoking status were similar (Table 1). Based on the threshold criteria of ferritin level < 20 ng mL^{-1} and/or transferrin saturation < 20%, 19 subjects in the RLS group and 18 in the control group had iron deficiency. Five subjects in the RLS group and eight in the controls had Hb levels ranging 10–12 g dL^{-1}.

WBC, Hb, Hct, Plt, sensitive CRP, ferritin, transferrin saturation rate (TSR), vitamin B12 and folate levels were similar in both groups. ESR and mean platelet volume (MPV) were significantly increased in RLS group (Tables 2 and 3).

Serum total cholesterol, HDL, LDL and triglyceride levels were similar. Groups were not statistically different in terms of TAS, OSI, PON and stim-PON. TOS and ARE were increased, but LOOHs were decreased significantly in patients with RLS (Tables 2 and 3).

AChE was significantly increased (Table 3), and BuChE was increased with a value approaching significance (Table 2) in the RLS group. Univariate two-way variance analysis was used to explore the impact of gender (female/male) in addition to RLS (absent/present) independent variable on the variables ferritin, TSR, TOS, ARE, LOOHs and AChE. TOS, ARE, PON, LOOHs and AChE were not affected by gender; however, ferritin and TSR were significantly decreased in women ($P < 0{\cdot}001$, for both).

Bivariate correlation was sought among age and ferritin, TSR, TOS, ARE, PON, LOOHs and AChE. In the RLS group, age had a moderate negative correlation only with ARE and PON ($r = -36/P = 0{\cdot}013$ and $r = -0{\cdot}31/P = 0{\cdot}034$, respectively); in the controls, age had a weak negative correlation only with PON ($r = -0{\cdot}28/P = 0{\cdot}048$) (Figs 1 and 2).

PON1 phenotype distribution, which was not performed in two subjects because of insufficient serum samples, was similar in RLS and control groups (Table 4). Ten of the study participants were excluded from HRV analysis; four because of intolerance to ECG recorder, four used verapamil or bisoprolol and two had to start RLS treatment because of intense complaints. HRVi was decreased significantly in RLS group. The groups did not differ in terms of other HRV parameters (Table 5).

Table 1 Demographic characteristics of patients

Parameters	RLS	Controls	*P* value
Patients, *n*	50	50	
Male/female, *n/n*	16/34	15/35	
Hypertensive subjects, *n*	9	7	
Smoking subjects, *n*	11	12	
	Mean ± SD	Mean ± SD	
Age (years)	44·6 ± 12·9	43·1 ± 10·03	0·49
BMI, kg m^{-2}	27·98 ± 4·1	27·18 ± 3·9	0·32

RLS, restless legs syndrome.
Chi-square test and Student's *t*-test were used in the statistical analyses shown in this table.

Discussion

In patients with RLS, we found serum ARE and AChE levels higher and LOOH levels lower than the controls. Paraoxonase levels were similar in both groups.

The reason for increased ARE in patients with RLS might be the stimulation of PON1 enzyme production in the liver. However, the reason for not observing an increase in paraoxonase as found in ARE might be its consumption through the process aimed at protecting AChE from oxidative stress and preventing lipid peroxidation [16,17]. Increased TOS in patients with RLS can also decrease PON [18]. Both increased ARE and the significant difference in the levels of products that are affected by this enzyme (increased AChE, decreased LOOHs) in RLS group support the assumption that PON1 generation is stimulated.

In RLS, dopaminergic insufficiency is thought to occur because of a functional insufficiency, not because of a neurodegenerative process [19]. Dopamine (DA) synthesis or dopamine receptors might be reduced [2,19,20]. Dopaminergic insufficiency is supposed to occur in the regions of basal ganglia, substantia nigra and supraspinal A11 dopamine cell group [2,19,20].

There are regions in the brain with low ACh levels, however, with disproportionately increased AChE levels. The recognized ones are cerebellum, hippocampus, hypothalamus, locus coeruleus, substantia nigra and striatum [21]. This finding gave rise to the argument that AChE might have activities in CNS other than breaking acetylcholine [21]. A soluble form of AChE is released by the dendrites of dopamine-containing neurons

Table 2 Selected blood tests, OS parameters and serum BuChE (normal distribution)

	RLS	Controls		
Parameters	**Mean ± SD**	**Mean ± SD**	**Normal range**	***P* value**
WBC, *n* per μL × 10^3	6·99 ± 1·6	6·77 ± 1·3	4·6–10·2	0·434
Hb, g dL^{-1}	13·2 ± 1·4	13·1 ± 1·2	12·2–18·1	0·593
Hct, %	39·2 ± 3·7	39·2 ± 3·4	37·7–53·7	0·994
Plt, *n* per μL × 10^3	269·2 ± 61·5	260·1 ± 61·8	142–424	0·462
MPV, fl	8·86 ± 1·47	8·31 ± 1·09	0·00–99·9	0·037
ESR, mm h^{-1}	18·6 ± 15·2	12·5 ± 10·3	5–20	0·021
Transferrin sat, %	24·26 ± 12·1	25·6 ± 11·1	20–40	0·327
Folate, ng mL^{-1}	9·1 ± 2·6	8·5 ± 2·3	4·5–32·2	0·205
Total cholesterol, mg dL^{-1}	205·8 ± 41·8	195·7 ± 34·4	< 200	0·190
HDL cholesterol, mg dL^{-1}	50·9 ± 11·5	52·7 ± 14·1	> 55	0·465
LDL cholesterol, mg dL^{-1}	128·4 ± 33·7	117·4 ± 29·9	60–150	0·087
Paraoxonase, U L^{-1}	140·50 ± 81·54	146·61 ± 96·54	Undetermined	0·740
Arylesterase, U L^{-1}	302·68 ± 76·31	229·32 ± 97·19	Undetermined	< 0·001
Stimulated paraoxonase, U L^{-1}	431·36 ± 275·9	420·70 ± 235·8	Undetermined	0·837
BuChE, U L^{-1}	8436·3 ± 1764·3	7773·2 ± 1827·7	Varying by age	0·060
Subjects studied, *n*	50 (stim. paraoxonase test was not performed in one subject)	50 (stim. paraoxonase test was not performed in one subject)		

RLS, restless legs syndrome.
Student's *t*-test was used in the statistical analyses shown in this table.

Table 3 Selected blood tests, OS parameters and serum acetyl cholinesterase (AChE) (non-normal distribution)

	RLS			Controls				
Parameters	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	**Normal range**	***P* value**
S CRP, mg dL^{-1}	0·29	0·01	1·60	0·26	0·01	1·70	0–0·8	0·397
Ferritin, ng mL^{-1}	31·8	2·6	285	41	5·7	258	30–400	0·370
Vit. B12, pg mL^{-1}	295·4	105	1098	289	56·7	1350	191–663	0·512
Triglycerides, mg dL^{-1}	110	43	452	100	34	397	< 200	0·470
TAS, mmol Trolox, Eq L^{-1}	1·123	0·156	1·899	1·146	0·644	10·60	Undetermined	0·817
TOS, μmol H_2O_2, Eq L^{-1}	3·331	1·617	12·904	2·909	0·883	9·684	Undetermined	0·018
OSI ratio	0·287	0·032	0·914	0·255	0·059	0·687	Undetermined	0·065
LOOH, μmol L^{-1}	0·477	0·154	2·551	1·241	0·292	16·80	Undetermined	< 0·001
AChE, U L^{-1}	2212	525	5058	1727	218	3661	Undetermined	< 0·001
Subjects studied, *n*	50			50				

RLS, restless legs syndrome; TAS, total antioxidant status; TOS, total oxidant status.
Mann–Whitney *U* test was used in the statistical analyses shown in this table.

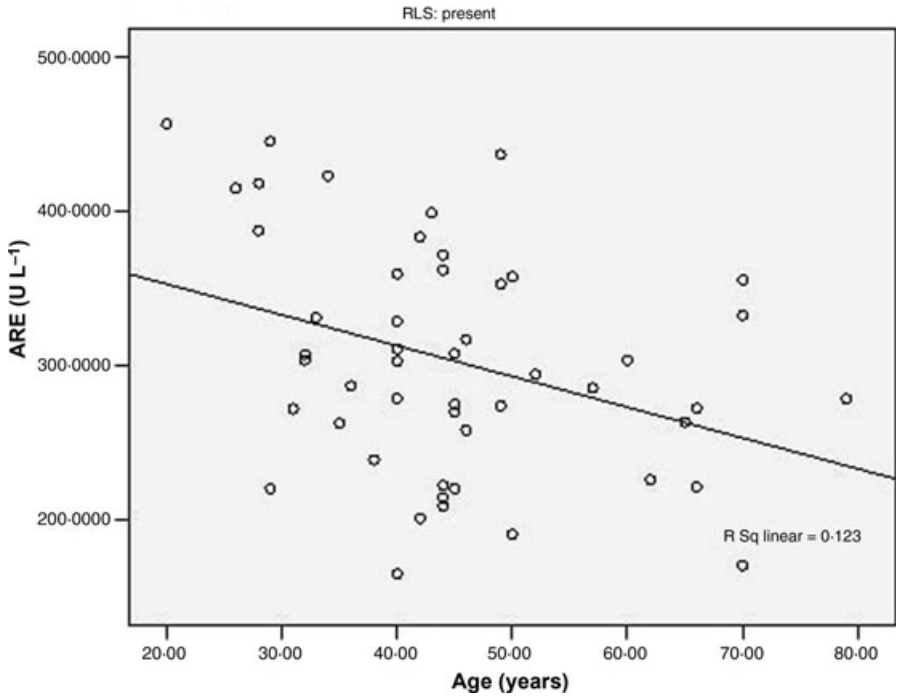

Figure 1 Scatter plot showing a negative moderate correlation between age and arylesterase in restless legs syndrome group.

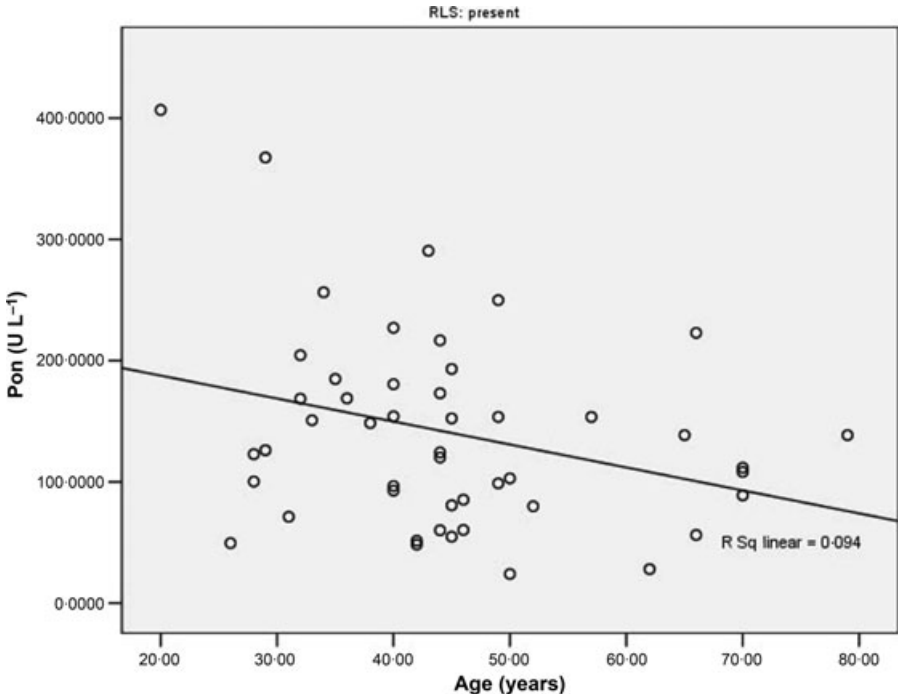

Figure 2 Scatter plot showing a negative moderate correlation between age and PON in restless legs syndrome group.

Table 4 PON1 phenotyping of subjects

PON1 phenotype	RLS *n* (%)	Controls *n* (%)	Average normal range, %	*P* value
QQ	22 (44)	21 (42)	48	0·694
QR	21 (42)	20 (40)	42	
RR	6 (12)	8 (16)	11	

RLS, restless legs syndrome.
Chi-square test was used in the statistical analyses shown in this table.

Table 5 Heart rate variability (HRV) analysis parameters

Parameters	RLS Mean ± SD	Controls Mean ± SD	*P* value
Mean heart rate, n min^{-1}	76·1 ± 8·1	75·3 ± 7·7	0·667
SDNN, ms	259·9 ± 309·4	210·4 ± 146·8	0·335
SDANN, ms	636·8 ± 2470	345·5 ± 1386	0·492
RMSDD, ms	300·3 ± 516·9	190·4 ± 232·4	0·198
HRV triangular index	21·56 ± 8·43	26·53 ± 10·17	0·012
HF, ms^2	151·68 ± 42·20	145·33 ± 33·03	0·434
LF, ms^2	142·85 ± 36·91	142·33 ± 52·65	0·957
VLF, ms^2	225·1 ± 81·15	265·72 ± 192·2	0·195
ULF, ms^2	78·48 ± 39·70	91·09 ± 33·57	0·115
LF/HF	1·06 ± 0·49	0·99 ± 0·36	0·535
Subjects studied (F/M), n/n	33/12	32/13	

RLS, restless legs syndrome.
Student's *t*-test was used in the statistical analyses shown in this table.

at the nigrostriatal region, independent of cholinergic transmission [21]. Independent of its classic actions, AChE enhances sensitivity of nigrostriatal neurons to synaptic inputs [21]. Additionally, AChE was shown to protect dopamine from oxidative destruction [22]. Thus, while PON1 protects AChE from oxidative stress, AChE itself protects dopamine from oxidative stress. Holmes *et al.* [23] showed that monomer and dimer forms of AChE (the abundant form found in the developing nervous system) increased the outgrowth and survival of midbrain dopaminergic neurons in mice.

We measured AChE in serum. It is believed that erythrocytes release 46%, and brain or autonomic ganglia or neuromuscular junction releases 54% of AChE in blood [24]. If we accept the theory of reduced dopaminergic activity in basal ganglia and substantia nigra as a base for RLS pathophysiology, the increase in serum PON1 activity can elevate dopaminergic activity by increasing AChE through the paths mentioned earlier. Dopaminergic and cholinergic systems are in a dynamic balance in CNS [25]. Disruption of this balance leads to neurologic, psychiatric and drug addictive disorders [25].

Striatum is a region where intrinsic cholinergic and extrinsic dopaminergic innervation and functional DA and acetylcholine interaction are of importance [25]. ACh decreases its own release through muscarinic autoreceptors (M2 and/or M4) on the cholinergic interneurons, while it can increase striatal dopamine release via M4 and M5 receptors or decrease via M3 receptors. DA, on the other hand, increases ACh release from cholinergic interneurons via D5 receptors and decreases via D2 receptors [26]. In RLS, our organism might respond to decreased dopaminergic activity by breaking ACh more intensively. Increased AChE might be beneficial through this route as well.

Oxidative stress in the brain easily leads to lipid peroxidation because of high concentrations of polyunsaturated fatty acids, such as docosahexaenoic acid and arachidonic acid (AA) present in the brain. The highly unsaturated status of fatty acids makes them susceptible to peroxidation, and LOOHs are generated. Hydroperoxide species may cause dopamine to form amide linkage dopamine adducts. Dopamine adducts lead to dopamine loss and one particular type (hexanoyl dopamine) leads to death of dopaminergic cells [26]. In RLS, reduced LOOHs might be of benefit in improving dopaminergic insufficiency.

Iron deficiency has been proposed to cause RLS either by slowing down the dopamine synthesis or by reducing the number of dopamine receptors [2,19,20]. PON1 activity is reduced in iron deficiency anaemia [27]. As explained earlier, PON1 can influence dopaminergic functions. Decreased PON1 might contribute to the development of RLS in iron deficiency. We found ARE higher in patients with RLS without iron deficiency than iron-deficient patients with this condition (P = 0·061).

Pregnancy and CRF are also well-known causes of RLS. Although the reason for the frequent occurrence of RLS in pregnancy and CRF is not known, an association with iron deficiency has been proposed [1,2,19,20]. Paraoxonase activity is significantly reduced in pregnancy and CRF [28,29]. Low paraoxonase activity might contribute to the development of RLS in pregnancy and CRF.

Both frequency and symptom severity of RLS increase during ageing [30]. PON1 activity is known to decrease with increasing age [31]. Low paraoxonase activity might contribute to the increase in RLS frequency and symptoms during ageing. We also found a weak negative correlation between age and PON in the controls. This correlation was stronger in the RLS group.

It was reported that QQ phenotype created a predisposition to Parkinson's disease in people living in agricultural areas [7].

We saw that PON1 phenotype distribution in patients with RLS was not different from the control group.

We found ESR increased in the RLS group. Other acute phase laboratory parameters and haemoglobin levels were similar. MPV was also higher in the RLS group. MPV increase is associated with increased platelet turnover, a tendency to coagulation and increased sympathetic activity [32–34]. The cause of increased ESR and MPV may be elevated sympathetic activity in RLS group [34,35].

Analysis of cardiac autonomic function revealed a low HRVi indicating an increased sympathetic and a diminished vagal activity in RLS group. LOOHs are among main components of TOS [4]. The prominent decrease in LOOHs in patients with RLS might have restricted the increase in TOS levels.

Stress and elevated sympathetic activity can lead to increased oxidative stress [12]. In patients with RLS, sympathetic activity elevation may be a beneficial compensatory mechanism in attenuating RLS symptoms. Movement, walking, excitement and mental activity translate into sympathetic activity elevation. These activities attenuate or resolve RLS symptoms [2,19,20]. Exercise increases the transmitters in the brain such as adrenaline, dopamine, noradrenaline and serotonin [36–38].

The interaction between sympathetic activity elevation and cortisol release mutually increases each other [39,40]. Cortisol increases dopaminergic activity in the brain [41,42]. In animals, after administration of corticosterone at a dose similar to stress-induced plasma concentrations, extracellular concentrations of dopamine in nucleus accumbens were increased, and this increase was augmented in dark phase [43]. Low-dose hydrocortisone infusion administered at night improved RLS symptoms in one study [44]. But cortisol levels in RLS and control groups were found to be similar in another study [45]. The small number of subjects in the latter might have led to this.

It is not exactly known what causes evening increase in symptom severity in RLS [46,47]. It is known that dopamine has a circadian pattern occurring in a manner contrary to the circadian pattern of RLS symptoms in human body, and this could be related to melatonin [46–48]. There are articles reporting that dopaminergic functions at midbrain dopaminergic neurons are influenced by the cortisol circadian rhythm [49,50]. A relation might exist between the autonomic nervous system and cortisol, and circadian pattern of RLS symptoms. Elevated vagal activity, lower sympathetic activity and decreased cortisol secretion in the evening might have an influence on the occurrence of RLS symptoms. Patients with RLS who cannot sleep throughout the night because of bothering sensation in the legs feel relieved and fall asleep at around 00 : 05 towards morning [2,20]. Cortisol release starts to increase towards morning, and sympathetic system gets activated [51]. Unfortunately, we could not compare the groups in terms of salivary cortisol.

We found PON1 generation increased in RLS. TOS was mildly but significantly increased. The increased serum AChE levels and decreased serum LOOH levels were considered as indicators of efforts in the body towards protection of dopaminergic activity in CNS. The increased ESR and MPV together with low HRVi indicated an elevation in sympathetic activity in RLS group. The elevated sympathetic activity might be beneficial in relieving RLS symptoms, also causing TOS to increase. Starting from here, we propose more in-depth investigations, which might lead to trials examining pharmacologic and non-pharmacologic interventions aimed at increasing PON1 activity in RLS.

Conflict of interest

We declare no conflicts of interest.

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