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Influence of physical exercise on β-amyloid, α-synuclein and tau accumulation: an *in vitro* model of oxidative stress in human red blood cells

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ABSTRACT

A common pathological feature of neurodegenerative disorders (NDs), such as Alzheimer's (AD) and Parkinson's (PD) diseases, is the accumulation and misfolding of specific proteins, primarily α-synuclein (α-syn), β-amyloid$_{1-42}$ (Aβ) and tau, in brain and in peripheral tissues too. Oxidative stress has been proved to be involved in NDs at various levels and, in particular, in such protein alterations, on the contrary physical activity is emerging as a counteracting factor in NDs.

In the present work, the content of Aβ, α-syn and tau in red blood cells (RBCs) derived from ten endurance athletes (ATHL) and ten sedentary volunteers (SED) were compared before and after in vitro oxidative stress treatment.

Total Aβ, α-syn and tau were quantified in RBCs (isolated from the subjects) by immunoenzymatic assays. Oxidative stress was induced by in vitro H_2O_2 administration to RBCs.

H_2O_2 treatment was confirmed to significantly enhance ROS accumulation in RBCs. Total Aβ content in RBCs was lower in the ATHL subgroup with respect to the SED one. In the SED subgroup, but not in the ATHL one, total Aβ levels were increased by oxidative stress. Total α-syn content was lower in the ATHL subgroup with respect to the SED one and α-syn levels were increased by oxidative stress in both subgroups, with the percentage of increase higher in SED. Total tau content was comparable in both ATHL and SED and it was not affected by oxidative stress.

Our data confirm previous findings evidencing that both oxidative stress and sedentary style contribute to aberrant folding and accumulation of NDs-related proteins, pointing to the importance of both anti-oxidant therapies and exercising in the prevention and treating of such diseases.

Key words

Oxidative stress • Protein accumulation • Neurodegenerative diseases • α-synuclein • β-amyloid • Tau • Physical exercise

Introduction

Oxidative stress is induced by an excess of formation of Reactive Oxygen Species (ROS), caused by an imbalance between their generation and elimination by the biological system (Ienco et al., 2011). Among its various effects, oxidative stress plays a key role in aging (Poon et al., 2004) and in its related neurodegenerative disorders (NDs) (Gandhi and Abramov, 2012; Patten et al. 2010), as an excess of Reactive Oxygen Species (ROS) can lead to cellular damage and death (Fulda et al., 2010), as well as to mitochondrial dysfunction in neurons (Fulda et al., 2010; Federico et al., 2012; Navarro and Boveris, 2010). As a consequence, antioxidant therapies are becoming increasingly important in the treatment

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of age-related NDs (Feng and Wang, 2012; Singh, 2015; Ienco et al., 2011).

In NDs, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis, there is pathological evidence of a progressive neuronal loss (Parihar and Hemnani, 2004; Bartels and Leenders, 2009; Román, 1996) and reactive gliosis (Dalle-Donne et al., 2006; Klein et al., 2004) in specific brain areas, in which aggregating misfolded proteins form high-ordered insoluble fibrils in neurons and/or glial cells (Dalle-Donne et al., 2006; Klein et al., 2004; Ugalde et al., 2016). For example, accumulation of α-synuclein (α-syn) has been established to form Lewy bodies and neurites in PD and dementia with Lewy bodies (DL) and glial cytoplasmic inclusions in multiple system atrophy (Goedert et al., 2013). In AD, there are senile plaques containing extracellular deposits of Aβ and intra-neuronal neurofibrillary tangles composed of hyperphosphorylated tau protein (Maurer et al., 1997). However, postmortem evaluation has shown a mixed pattern of proteinopathies, commonly accompanied by signs of chronic cerebrovascular disease pathology. The potential contribution of α-syn to AD pathogenesis is emerging (Larson et al., 2012; Marsh and Blurton-Jones, 2012), with 30-40 % of AD cases presenting Lewy bodies and Lewy neurites (Trojanowski, 2002). Literature data suggest that Aβ, tau and α-syn promote the accumulation and/or aggregation of one another (Lee et al., 2004). Alterations in protein folding related to NDs are not restricted to the brain, thus extensive research has been conducted on biological changes and putative biomarkers in other tissues or in cerebrospinal fluid (CSF) (Growdon, 1999; Garlind et al., 1999; Hock et al., 2000; Hampel et al., 2001; Andreasen et al., 2001; Giacomelli et al., 2017). The use of blood has gradually emerged, due to its availability and low cost and to the time effectiveness of the method (Reiber, 2003).

Oxidative stress is also involved in aberrant protein aggregation in NDs (Chen et al., 2012), as Aβ deposition in AD participates in a positive feedback loop in which oxidative stress leads to increased Aβ generation and, conversely, Aβ polymerization generates oxidative stress (Zhang et al., 1997; Zhao and Zhao, 2013); in addition, ROS promote tau phosphorylation (Zhao and Zhao, 2013).

It has been demonstrated that regular exercise benefits brain function and cognition by exerting effects at multiple levels, from neurons to inter-regional cerebral pathways (Voss et al., 2013), including an increase in capillarization and proteolytic degradation of toxic oligomers (Johnson and Mitchell, 2003; Power et al., 2013). Physical activity has been demonstrated to up-regulate the enzymatic antioxidant system and to enhance the physiological response to oxidative damage, thus leading to a recovered redox state of brain cells (Radak et al., 2016). Exercising not only improves cognitive functions in healthy subjects (Kramer et al., 2003; Colcombe et al., 2004; Weuve et al., 2004; Winter et al., 2007; Hindin and Zelinski, 2012; Dishman et al., 2006) but also reduces the risk of developing age-related neurodegenerative diseases like dementia or AD (Laurin et al., 2001; Abbott et al., 2004; Larson et al., 2006; Paillard et al., 2015) and PD (Paillard et al., 2015). In addition, it seems to slow down the progress of such diseases, thus constituting a potential nonpharmacological therapeutic treatment (Kwok et al., 2016; Cusso, Donald and Khoo, 2016; Jakowec et al., 2016; Bernardo et al., 2016). It has been demonstrated that physical activity reduces beta-amyloid levels and amyloid deposition in transgenic mice (Lazarov et al., 2005) and recent work has shown that voluntary running counteracts Aβ deposition and tau phosphorylation in a mouse model of AD (Tapia-Rojas et al., 2016). In humans, it has been found that performers of higher levels of physical activity have lower Aβ plasma levels and brain depositions (Brown et al., 2013).

On the whole, literature data have proved there is a link between oxidative stress, neurodegeneration and physical exercise. Recently, our data demonstrated the correlation among plasma antioxidant capability (AOC), the primary marker of oxidative stress in aging-related pathologies (Pandey and Rizvi, 2010), physical exercise and the peripheral levels of α-syn, tau and Aβ in a cohort of young and older athletes and sedentary subjects (Daniele et al., 2017). Herein the accumulation of such ND-related proteins was investigated in a RBC model of oxidative stress. The cellular response to oxidative stress was compared among SED and ATHL in order to verify if physical exercise could exert a protective effect. RBCs constitute a good model to study aging-related biochemical alterations, including protein misfolding (Brown et al., 2013; Kiko et al., 2012; Wang et al., 2015). The contents of Aβ, α-syn and tau in RBCs derived from ten endurance athletes and ten sedentary volunteers were compared before and after *in vitro* oxidative stress treatment.

Methods

Materials

Recombinant human Aβ, α-syn were obtained from Sigma Aldrich, Milan, Italy. Antibodies against Aβ, α-syn and tau were from Santa Cruz Biotechnology.

Study population and setting of the study

The study population consisted in ten endurance athletes (ATHL, mean age 33.6±3.4 years), recruited from the Sport Medicine Unit of the Department of Clinical and Experimental Medicine of the University of Pisa, and ten age-sex-matched sedentary volunteers (SED, mean age 36.7±3.5 years) were studied (Table 1). Athletes performed endurance exercise more than three times/week and were also active in national road-running races.

All subjects were healthy, as assessed by clinical history, physical examination, basal and stress electrocardiography, blood chemistry, hematology and urine analysis. Major criteria for inclusion were as followed: total plasma cholesterol ranging from 3.1 to 5.8 mmol/L, HDL cholesterol from 0.67 to 1.9 mmol/L, plasma triglycerides from 0.34 to 1.7 mmol/L, body mass index lower than 30 kg/m^2, diastolic arterial blood pressure lower than 90 mmHg and systolic arterial blood pressure lower than 140 mmHg.

The level of intensity of physical activity for each participant was evaluated by the 15-point Borg RPE scale (Borg, 1982; Hamer and Slocombe, 1997). The scale ranges from 6 to 20, with 6 corresponding to no exertion at all, 7.5 to extremely light, 9 to very light, 11 to light, 13 to somewhat hard, 15 to hard, 17 to very hard, 19 to extremely hard, and 20 to maximal exertion.

This study was approved by the Ethics Committee of the Great North West Area of Tuscany (271/2014 to F.F.) and it was carried out in accordance with the Declaration of Helsinki. All subjects gave informed consent to participate in the study. Fully informed consent was obtained from each subject entering the study.

RBCs collection

Whole blood (total volume: 6 ml) was collected from the subjects into a tube containing EDTA as an anticoagulant. RBCs were separated from plasma by a centrifugation at 200 × g at 4 °C for 10 minutes and the RBC pellet was centrifuged at 1000 x g for 10 min and washed three times with PBS, then frozen at -20 °C until use. For immunoenzymatic assays, the RBC pellets were resuspended to have 40 mg total protein/100 µl.

Detection of total Aβ

Aβ levels in blood samples were measured using an immunoenzymatic assay, as described previously (Pesini et al., 2012). The plate was pre-coated o.n. at 4°C with a specific antibody to Aβ (Santa Cruz, sc-9129). After extensive washing with PBS-T, non-specific sites were blocked with 1% BSA. RBCs (0,2 mg/100 µl) were added to each well and incubated at 25°C for one hour. After extensive washing with PBS-T, samples were detected using the polyclonal antibody to Aβ (sc-5399, Santa Cruz Biotechnology). The standard curve was constructed using recombinant human Aβ solutions at eight different concentrations.

Detection of total α-synuclein

Total α-synuclein was detected in RBCs following literature's protocols (Foulds et al. 2011). Briefly, wells were pre-coated o.n. at 4°C with a full length polyclonal antibody to α-syn (sc-10717, Santa Cruz Biotechnology), and non-specific sites were blocked using Bovine Serum Albumine (BSA) for 1 h at 37°C. RBCs (0,150 mg/100 µl) were captured on wells for 2h at 25°C. Purified recombinant protein standards of α-syn were assayed in parallel with human samples to generate a standard curve. After extensive washing, samples were probed with a mouse monoclonal antibody to α-syn (Santa Cruz, sc-12767), and subsequently with an anti-mouse-HRP antibody. The wells were then washed 4 times with PBS-T (phosphate buffered saline containing 0.01% Tween 20), before adding the enzyme substrate TMB (3,3',5,5'-tetramethylbenzidine, Thermo Scientific) and leaving the colour to develop for 30 min at room temperature. Absorbance values at 450 nm.

Detection of total tau

Tau levels in blood samples were measured using an immuno-enzymatic assay, as described previously (Pesini et al., 2012). The plate was pre-coated overnight at 4°C with a specific antibody to tau (Santa Cruz, sc-32274). After extensive washing with PBS-T, non-specific sites were blocked with 1% BSA. RBCs (0,5 mg/100 µl) were added to each well and incubated at 25°C for one hour. After extensive washing with PBS-T, samples were

Tab. 1. - Descriptive analysis of the total population and of the subgroups. The data are the mean±SD.

	Number of subjects (N)	Age (y)	BMI	Heart rate	Physical activity level
ATHL	10	33.6±3.4	23,6±1,9	52,0±3,0	14,2±2,2***
SED	10	36.7±3.5	24,8±1,3	60,1±5,3	6,0±0,5

BMI, Body Mass Index; ATHL, Athletes, SED, sedentary. * **P < .001 vs sedentary subgroups.

detected using the polyclonal antibody to tau (sc-5587, Santa Cruz Biotechnology). The standard curve was constructed using recombinant human tau solutions at eight different concentrations.

In vitro model of oxidative stress

RBCs were isolated from whole blood 8total volume: 6 ml) of the healthy subjects as described above. Cells were collected by centrifugation at 1100g for 5 min, and suspended in DMEM-F12, containing 2 mM L-glutamine, 2 mM penicillin-streptomycin and 10% Foetal Bovine Serum. RBCs were counted using an automatic cell counter. Cells (2 x 10^6 cells/sample) were seeded and treated with 1.5 mM H_2O_2 for 16 h. Following incubation, the cells were collected and resuspended to a final concentration of 80 mg/100 µl.

Levels of total Aβ, α-syn and tau were quantified by immunoenzymatic assays reported above.

To confirm ROS activity upon H_2O_2 treatment, the fluorogenic dye 2,7-dichlorofluorescin diacetate (H_2DCFDA, Molecular Probes, Invitrogen) was used (La Regina et al., 2013; Daniele et al., 2016). RBCs were seeded in black 96-multiwell plates (10 × 10^3 cells/well), and incubated with H_2O_2 in DMEM without phenol red. One hour prior to treatment completion, 50 µM H_2DCFDA was added to the same media in the dark at 37 °C. The fluorescence intensity (excitation 485 nm and emission 520 nm) was normalized based on the number of cells stained with crystal violet (Daniele et al., 2016).

Statistical analysis

Analysis of variance was used to assess mean differences between subjects. Differences were considered significant at values of $p<0.05$. Data analysis from the *in vitro* model was performed using the t test and one-way analysis of variance (ANOVA) with Bonferroni's corrected t tests for post-hoc pairwise comparisons (Daniele et al., 2016).

Results

Descriptive statistics

The variables of the two groups are reported in Table 1.

The subjects did not present significant differences in body mass index (BMI) and age. As expected, ATHL showed a lower resting heart rate than the sedentary. The level of physical activity of in the ATHL subgroup was significantly higher than the one of the sedentary (Table 1).

In vitro model of oxidative stress

H_2O_2 (2 mM, 16 h) was utilised in RBCs as an *in vitro* model of oxidative stress. As depicted in Figure 1, H_2O_2 was confirmed to significantly enhance ROS accumulation in RBCs.

Total Aβ concentrations

Total Aβ levels were quantitatively measured in RBCs isolated from ATHL and SED.

As depicted in Figure 2A, the basal concentration of total Aβ in RBCs showed significantly lower values in the ATHL subgroup with respect to the SED one. In the sedentary subjects, total Aβ levels were significantly higher following oxidative stress, whereas in ATHL oxidative stress did not significantly affect Aβ accumulation.

These data suggest that the basal level of Aβ in RBCs is modulated *in vitro* by oxidative stress and that a regular physical activity may be a protective factor toward oxidative stress-induced responses.

Total α-syn concentrations

Total α-syn levels were quantitatively measured in RBCs isolated from the two subgroups.

As depicted in Figure 2B, total α-syn in RBCs showed significantly lower values in the ATHL subpopulation with respect to SED subjects, both in the presence and in the absence of oxidative stress.

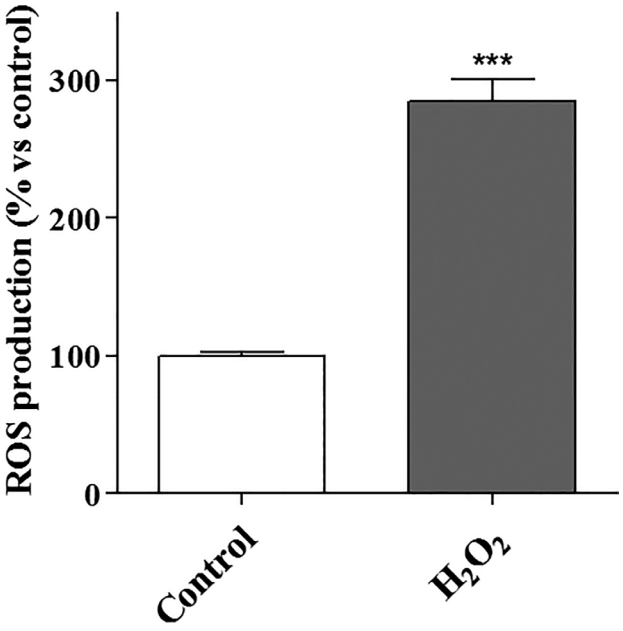

Fig. 1. - In vitro model of oxidative stress in RBCs obtained by H2O2 (2 mM, 16 h) administration. Comparison of ROS accumulation between control and H2O2-treated cells. ***P<0.001 H2O2 versus Control.

In both groups, total α-syn levels were significantly enhanced upon RBC treatment with H_2O_2.
These data suggest that RBC α-syn concentrations can be modulated by oxidative stress independently of the physical exercise.

Total tau concentrations

As depicted in Figure 2C, Total tau in RBCs showed comparable levels in both ATHL and SED, suggesting that it is not modulated by physical activity, at least in our small cohort.
Moreover, total tau content in RBCs was not significantly modulated by H_2O_2 in the two groups, suggesting that RBC tau is not affected by oxidative stress.

Discussion

In the present work, the effects of oxidative stress and physical activity on the accumulation of ND-related proteins were evaluated in the RBCs of a group of twenty healthy subjects composed by ten endurance athletes and ten sedentary volunteers. Herein, it was confirmed that oxidative stress impacts Aβ and α-syn RBC contents *in vitro* and that the concentrations of these proteins could be differently modulated in relation to sedentary *versus* trained life style.
Oxidative stress is one of the putative common etiologies in various NDs, as it triggers mitochondrial dysfunction, cellular damage and DNA repair system impairment, all of which known to be key factors in accelerating both aging and ND development (Kim et al., 2015; Gandhi and Abramov, 2012; Patten et al., 2010). In particular, oxidative stress is related to the accumulation of misfolded proteins, such as Aβ, α-syn and tau, which constitutes the neuropathological hallmark of AD, PD and other neurodegenerative proteinopathies (Ugalde et al., 2016). For example, oxidative stress has been demonstrated to exacerbate Aβ production and aggregation, as well as to promote tau phosphorylation, potentially inducing a vicious cycle of pathogenesis in AD (Kim et al., 2015; Zhao and Zhao, 2013). Mitochondrial dysfunction related to oxidative stress has been strongly associated with α-syn accumulation and apoptosis of dopaminergic neurons in PD (Blesa et al., 2015).
Misfolded proteins related to NDs are hypothesized to accumulate in the brain even decades before the appearance of symptoms (Danev and St Stoyanov, 2010; Price et al., 2009). Recent studies have demonstrated a cell-to-cell transmission of pathologic Aβ and α-syn in anatomically interconnected areas of the brain (Luk et al., 2012; Ridley et al., 2006). Brain, CSF and blood concentrations of such protein aggregates seem to be in a dynamic equilibrium (Kawarabayashi et al., 2001; Ghersi-Egea et al., 1996), suggesting that increased production in the brain could be associated with increased concentrations in the blood as the result of protein oligomers transfer across the blood brain barrier (Zlokovic, 2004; Eisele et al., 2010; DeMattos et al., 2001). Among blood cells types, RBCs seem to be particularly sensitive to oxidative stress and misfolded proteins (Wang et al., 2015; Kiko et al., 2012; Mohanty et al., 2014), exhibiting damage to cell membranes and decreased cell deformability, which is necessary for effective oxygen transport and delivery (Mohanty et al., 2014). Based on these findings, recent efforts to study RBC concentrations of misfolded proteins and their relationship with oxidative stress or NDs have emerged, including our recent work (Wang et al., 2015; Kiko et al., 2012; Daniele et al., 2017).

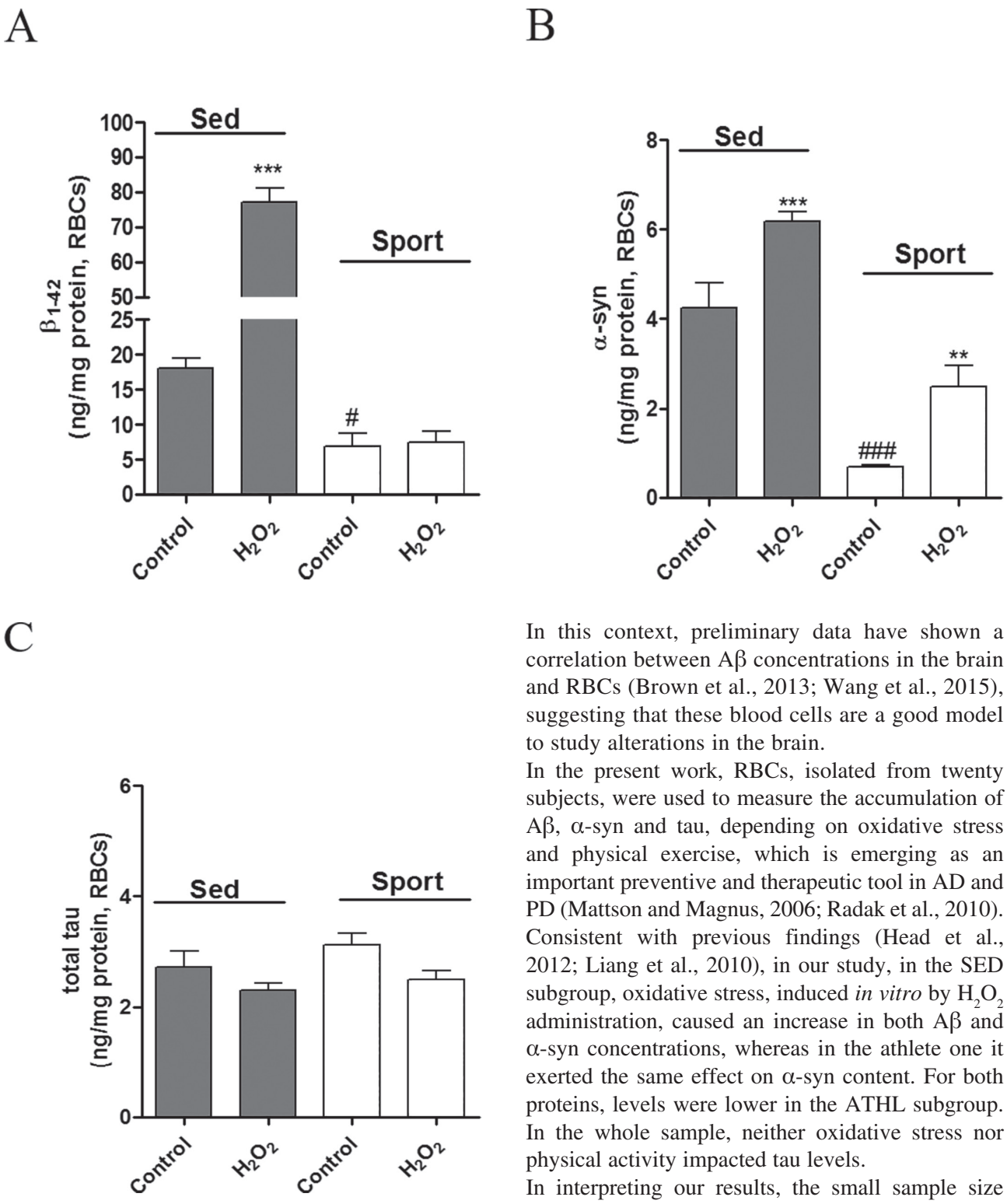

Fig. 2. - Accumulation of ND-related proteins in the in vitro model of oxidative stress. A-C RBC levels of total Aβ (A), α-syn (B) and tau (C) in a sedentary and athlete subject (mean ± SD). Lysates obtained from RBCs were subjected to specific immunoassay, as described in the Methods section. **P<0.01, ***P<0.001 H2O2 versus Control; #P<0.05, ###P<0.001 ATHL Control versus SED control.

In this context, preliminary data have shown a correlation between Aβ concentrations in the brain and RBCs (Brown et al., 2013; Wang et al., 2015), suggesting that these blood cells are a good model to study alterations in the brain.

In the present work, RBCs, isolated from twenty subjects, were used to measure the accumulation of Aβ, α-syn and tau, depending on oxidative stress and physical exercise, which is emerging as an important preventive and therapeutic tool in AD and PD (Mattson and Magnus, 2006; Radak et al., 2010). Consistent with previous findings (Head et al., 2012; Liang et al., 2010), in our study, in the SED subgroup, oxidative stress, induced *in vitro* by H_2O_2 administration, caused an increase in both Aβ and α-syn concentrations, whereas in the athlete one it exerted the same effect on α-syn content. For both proteins, levels were lower in the ATHL subgroup. In the whole sample, neither oxidative stress nor physical activity impacted tau levels.

In interpreting our results, the small sample size should be considered. Nevertheless, the present paper demonstrates the greater susceptibility of sedentary subjects *versus* athletes to oxidative stress, thus confirming the importance of investing effort in developing antioxidant therapies, as well as therapies based on physical exercise, in preventing

NDs and also in delaying these diseases' progress. In addition, our work further highlights RBCs levels of NDs-related proteins as putative disease biomarkers.

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Authors' contributions

CI drafted the manuscript. SD, UB and MLT were involved with the conception, design, and interpretation of data. SD, DP, CI and JF performed the experiments. SD, DP, JF, FG, MLT and FF were involved with data analysis. SD and DP collected the clinical material. CM, MLT and UB provided general overall supervision of the study and acquired funding. All authors contributed to the drafting and critical revision of the manuscript and have given final approval of the version to be published.

Abbreviations

Aβ, β-amyloid$_{1-42}$. α-syn, α-synuclein. ATHL, athletes subgroup. AD, Alzheimer's disease. NDs, neurodegenerative diseases. PD, Parkinson's disease. RBCs, red blood cells. ROS, reactive oxygen species. SED, sedentary subgroup.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Great North West Area of Tuscany (271/2014 to F.F.) and it was carried out in accordance with the Declaration of Helsinki. All subjects gave informed consent to participate in the study.

Availability of data and materials

All data generated during this study are included in this published article. The original datasets analysed during the current study are not publicly available due to individual privacy.

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