ROS

RESEARCH ARTICLE

Body Mass Index and Age-Related Changes of ROS Production and Oxidative Stress Biomarkers in Healthy Subjects

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ABSTRACT | In this study, age-related changes in reactive oxygen species (ROS) production and markers of oxidative stress were investigated specifically addressing the role played by the body mass index (BMI). In two groups of healthy subjects, old (aged 72.6 ± 4.7 years) and young (aged 18.9 ± 1.6 years), ROS production rate, products of lipid peroxidation (as thiobarbituric acid-reactive substances, TBARS), protein oxidation (as total protein carbonyls, PC), and total antioxidant capacity (TAC) were assessed. BMI was also determined, and the subjects were classified into normal weight, overweight, and obese. In both groups (young and old), significant increases in ROS production rate and levels of systemic oxidative damage biomarkers (TBARS and PC) and decreases in TAC levels were shown to correlate with BMI. When comparing data in the same BMI subgroup, significant differences (lower ROS production rate, PC and TBARS levels, and higher levels of TAC) were observed in the young group versus the old group. In conclusion, the study showed that oxidative stress biomarkers were elevated in obese subjects and old age was associated with increases in oxidative stress markers and decreases in TAC.

KEYWORDS | Age; Body mass index; Oxidative stress; Reactive oxygen species; Total antioxidant capacity

ABBREVIATIONS | BMI, body mass index; PC, protein carbonyl; ROS, reactive oxygen species; TAC, total antioxidant capacity; TBARS, thiobarbituric acid-reactive substances

CONTENTS

1. INTRODUCTION

Oxidative stress is implicated in the pathogenesis of many chronic progressive diseases, such as inflammation, cancer, and neurodegenerative disorders [1]. In addition, oxidative stress plays a potentially deleterious role in obesity-associated metabolic syndrome, diabetes, hypertension, dyslipidemia, and atherosclerosis [2]. Oxidative stress, referred to as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses [3–5], occurs when the net amount of ROS exceeds the antioxidant systems [6]. Thus, oxidative stress can be regarded as a consequence of a general increase in ROS generation, a depression of the antioxidant systems, or both. ROS (e.g., superoxide, hydroxyl radical, and H_2O_2) as well as reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, are produced by all aerobic cells and play an important role in aging and hence in age-related diseases [7]. It should be noted that, low ROS levels are indispensable in several biochemical processes, including intracellular signaling, cellular differentiation, growth arrest, apoptosis, and immunity and defense against microorganisms [8].

Numerous studies have shown that ROS overproduction can be involved in various diseases [7, 8] via lipid peroxidation and protein oxidation, leading to cell dysfunction and cell death [9]. Therefore, a redox status assessment becomes quite important in order to prevent complications caused by oxidative stress-related disorders both in children and adults. Moreover, increased oxidative stress may play an important role in obesity-related complications, including hypertension and atherothrombosis [8, 10]. Nowadays the prevalence of obesity and overweight has become one of the greatest challenges to health in many developing and industrialized countries [11]. Obesity and lack of exercise are the key components of a dangerous condition known as metabolic syndrome, which puts an individual at risk of developing serious health problems like atherosclerosis, hyperglycemia, dyslipidemia, and hypertension [12]. Age is another independent risk factor. Oxidative stress level may be found different in the elderly, since the levels of antioxidant enzymes, such as superoxide dismutase, decrease with age [13]. The free radical theory of aging holds that as an organism ages, it is less equipped to fend off oxidative stress from endogenous and exogenous sources [14, 15].

Direct measurements of ROS production are very difficult due to their high reactivity and low steady-state concentrations [16]. The most commonly adopted techniques for oxidative stress quantification are based on the determination of specific end products of the damage resulting from the interaction of ROS with most vulnerable biological targets (e.g., proteins, membrane lipids, and DNA) [16, 17]. However, all of these methods give an indirect ROS determination. In contrast, the electron paramagnetic resonance (EPR) spectrometry is the only technique capable of providing direct evidence of the "instantaneous" presence of ROS in a sample [18]. The aim of the present study was to investigate how age- and body mass index (BMI)-related changes influence oxidative stress patterns. The role of the two components (age and BMI) was separately examined. Whole-body oxidative stress was evaluated throughout the measurements of (i) ROS production rate in the blood by EPR; and (ii) ROS-induced modifications to plasma proteins and lipids as well as the total antioxidant capacity by using enzymatic assays.

2. MATERIALS AND METHODS

2.1. Subjects

Two groups of healthy, free-living, non-smoking men and women were recruited to participate in the study: the young group (Y) consisted of 16 females and 26 males, aged 18.9 ± 1.6 years; the elderly group (O) was composed of 17 females and 24 males, aged 72.6 ± 4.7 years. The exclusion criteria were: acute illness or severe chronic disease, diabetes, hypertension, angina pectoris or previous myocardial infarction or peripheral vascular disease, thyroid dysfunction, smoking, hormonal treatment, lipid-lowering medication, or vitamin or iron supplementation in the last 6 weeks before entry. The study was

conducted according to the guidelines laid down in the Declaration of Helsinki and all the procedures have been approved by the Institutional Review Ethical Board. The informed consent was obtained from all participants, aware of the purpose and implications of the study.

Weight and height measures were utilized to calculate the body mass index (BMI, kg/m^2). To avoid variability in the values, weight measurement was taken after 12 h of rest in subjects refrained from excessive eating and drinking the day before the analysis. Subjects were classified into three groups, according to Caucasian classification [19, 20]: BMI between 18.5 and 24.9 kg/m^2, normal weight (nw); BMI between 25 and 29.9 kg/m^2, overweight (ow); BMI ≥ 30 kg/m^2, obesity (ob).

2.2. Experimental Procedures

2.2.1. Blood Sampling

For either group of subjects (Y and O), blood was collected, and the experiments were conducted in parallel. After an overnight fast, 5 ml of blood was drawn into a heparinized vacuum tube. The collected blood was centrifuged at 3,000 *g* for 5 min at 4°C to separate the plasma, which was immediately stored in multiple aliquots at –80°C until analysis. For determination of ROS production rate by electron paramagnetic resonance (EPR) spectrometry, 50 µl of capillary blood was taken from the fingertip and collected in heparinized capillary tubes (Cholestech LDX, Germany).

2.2.2. Assessment of Plasma Oxidative Stress Biomarkers

Oxidative stress biomarkers were assessed by enzymatic methods using a microplate reader spectrophotometer (InfiniteM200, Tecan, Austria). All determinations were carried out in duplicate and the inter-assay variation coefficient was within the range indicated by the kit manufacturer.

To determine the amount of lipid peroxidation in the plasma, the malondialdehyde (MDA) levels were analyzed spectrophotometrically using the modified thiobarbituric acid-reactive substance (TBARS) method. The measurement of TBARS by a commercial assay kit (Cayman Chemical, Ann Arbor, MI, USA) allows a rapid photometric detection at 535 nm of the thiobarbituric acid-MDA (TBA-MDA) adduct. A linear calibration curve was computed from pure MDA-containing reactions.

The protein carbonyl (PC) content, index of protein oxidation, was determined by means of a commercial kit (Cayman Chemical) through the reaction of 2,4-dinitrophenylhydrazine (DNPH) and carbonyls. This reaction forms a Schiff base producing the correspondent hydrazone. The latter was spectrophotometrically analyzed, reading the absorbance signal in the 360–385 nm range. Values were normalized to the total protein concentration in the final pellet (absorbance reading at 280 nm) in order to take into account possible protein loss during the washing steps.

Plasma total antioxidant capacity (TAC) was measured by means of an enzymatic kit (Cayman Chemical). This assay is based on the ability of the antioxidants present in the plasma to inhibit the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline) sulfonic acid (ABTS) to the radical cation $ABTS^{+\bullet}$ by a peroxidase: the antioxidants concentration is proportional to the suppression of the absorbance signal at 750 nm. TAC was evaluated by a trolox standard curve and expressed as trolox-equivalent antioxidant capacity concentration.

2.2.3. ROS Production Rate Assessment by EPR Spectrometry

All EPR measurements were carried out on a X-band spectrometer (Escan, Bruker BioSpin, GmbH, Billerica, MA, USA) equipped with a temperature and gas controller "BIO-III" to measure ROS production rate under an in vivo condition at 37°C. Based on a previously described method [21–23], for each recruited participant, 50 µl of capillary blood was incubated with CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) probe solution (1:1). 50 µl of the obtained solution was put in a glass EPR capillary tube (Noxygen Science Transfer & Diagnostics, Elzach, Germany) placed inside the cavity of the EPR spectrometer for data acquisition. EPR signals generated by the reaction of the probe with the blood radicals were acquired and the spectra sequentially recorded for about 5 min allowed us to calculate the ROS production rate. EPR signal is proportional to the unpaired electron numbers and can, in turn, be transformed in the absolutely produced micromoles level (µmol min^{-1}). To this aim, the stable $CP^{\bullet}$ (3 carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) radi-

cal signal was recorded in a separate session and used as an external reference [21–23]. Spectra were recorded and analyzed by using the Win EPR software (version 2.11) supplied by Bruker.

2.3. Statistical Analysis

Data were expressed as mean ± SD. Statistical analysis was performed using the GraphPad Prism package (GraphPad Prism 8.0, GraphPad Software Inc., San Diego, CA, USA). Experimental data were analyzed using repeated Shapiro–Wilk test and compared by variance analysis, ANOVA, with Bonferroni multiple comparison test to further check the among-groups significance. A p value < 0.05 was considered statistically significant. Pearson's correlation coefficient (r) with 90% confidence intervals (CI) was used to examine the relationships between selected parameters.

3. RESULTS

In both groups, increased levels of oxidative damage markers and decreased levels of TAC, with increasing BMI, were observed (**Figure 1**). Significant ($p < 0.0001$) direct correlations were observed between BMI and: (i) capillary ROS production rate in young ($r = 0.77$) and old ($r = 0.74$) (**Figure 1A**); (ii) plasmatic PC (Y: $r = 0.75$; O: $r = 0.63$) (**Figure 1C**); and (iii) TBARS concentrations: (Y: $r = 0.87$; O: $r = 0.74$) groups (**Figure 1D**). Significant ($p < 0.0001$) indirect correlations were observed between BMI and plasmatic TAC values in Y ($r = 0.75$) and O ($r = 0.73$) groups (**Figure 1B**).

Results of anthropometric parameters showed that for the young subjects, about 24% was in the normal weight (Y-nw) group; 29% was overweight (Y-ow); and 47% was obese (Y-ob). Sixty-six percent of the old subjects were in the normal weight group (O-nw), 17% was overweight (O-ow) and obese (O-ob). Increasing BMI ranges was associated with increases in the capillary ROS production rate and the plasma PC and TBARS levels, and decreased TAC in both young and old groups. Moreover, when comparing the data belonging to the same BMI class, significant differences (range from $p < 0.05$ to $p < 0.0001$) of young versus old in the levels of all oxidative stress biomarkers were demonstrated. A lower ROS production rate (**Figure 2A**), lower plasmatic PC (**Figure 2C**) and TBARS (**Figure 2D**) concentrations, and higher levels of TAC (**Figure 2B**) were observed in the young group compared with the old group. At the same time, when comparing the data obtained from different BMI classes (i.e., normal weight vs. overweight) in the same age group, significant differences (range from $p < 0.05$ to $p < 0.0001$) were observed for all the investigated parameters with overweight/obese subjects showing increased oxidative stress markers and decreased TAC (**Figure 2A–2D**).

4. DISCUSSION

Overweight and obesity represent a growing social problem worldwide [11]. Due to excessive fat accumulation, the increase in body weight leads to several diseases including metabolic syndrome, diabetes, cardiovascular diseases, chronic inflammatory disorders, and cancer [20], the leading causes of death. Several mechanisms are involved in the development of overweight and obesity, among which oxidative stress and proinflammatory processes have been demonstrated to be strongly related [24].

The data collected in the present study gave further evidence that overweight and obesity per se may induce oxidative stress and that fat accumulation closely correlates with the systemic oxidative stress biomarkers (**Figure 1**). Indeed overweight and obesity have been reported to be directly associated with increased oxidative damage to macromolecules [25–27] as well as with inadequate antioxidant defences [28, 29]. Our data are in good agreement with studies suggesting that systemic oxidative stress correlates with BMI [26, 30]. Our study combined EPR spectrometry with determination of oxidative stress biomarkers. EPR is the only technique allowing us to perform an absolute quantitative determination of free radicals. This is the main novelty of the present research that in turn reinforces the previously reported results.

Adipose tissue is metabolically active so that the great amount of tissue macrophages produces an array of signaling molecules. Under the presence of excessive adipose stores, the cellular milieu remodels to activate a number of stress pathways, including those that will increase oxidative stress [2, 31]. Indeed, adipose tissue is an endocrine organ secreting several hundreds of adipokines, that lead to the gen-

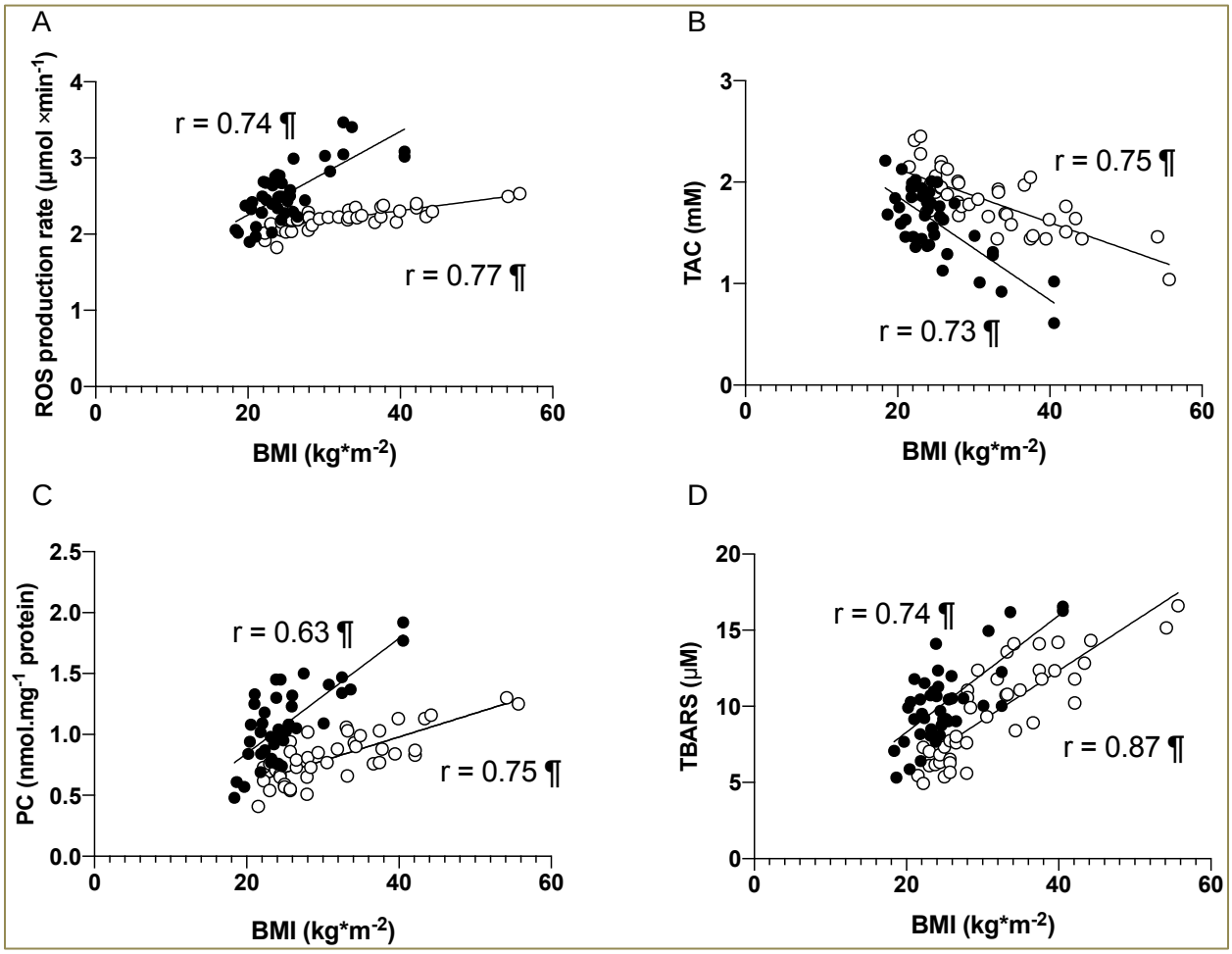

FIGURE 1. Correlations between various parameters and body mass index (BMI) in young (Y, empty symbols) and old (O, full symbols) subjects. (A): ROS production rate (µmol min^{-1}) vs. BMI; (B): total antioxidant capacity (TAC, mM) vs. BMI; (C): protein oxidation (PC, nmol.mg^{-1} protein) vs. BMI; (D): lipid peroxidation (TBARS, µM) vs. BMI. For each variable and group, the regression lines (solid lines) are indicated. The significance levels (r) are reported in the figure.

eration of ROS, and as such, this tissue can be regarded as an independent factor for systemic oxidative stress generation [27]. As demonstrated by the present study, the overproduction of ROS from accumulated fat leads to increased oxidative stress also in the blood of overweight (Y-ow, +7%; O-ow, +5%) and obese (Y-ob, +10%; O-ob, +32%) subjects, thereby hazardously affecting other organs including the liver, skeletal muscle, and vasculature. Thereafter the increased oxidative stress in accumulated fat has been proposed as an early instigator and one of the most important underlying causes of the obesity-associated metabolic syndrome [10]. The relationship between obesity and increased oxidative damage found in the present study can be seen as a consequence of the decreased TAC in the overweight (Y-ow, -6%; O-ow, -10%) and obese (Y-ob, -21%; O-ob, -37%) groups that would be the crucial factor in the development of a redox status imbalance inducing cell damage (**Figure 2**).

The free radical theory of aging, the other factor taken into account in the present study, suggests the

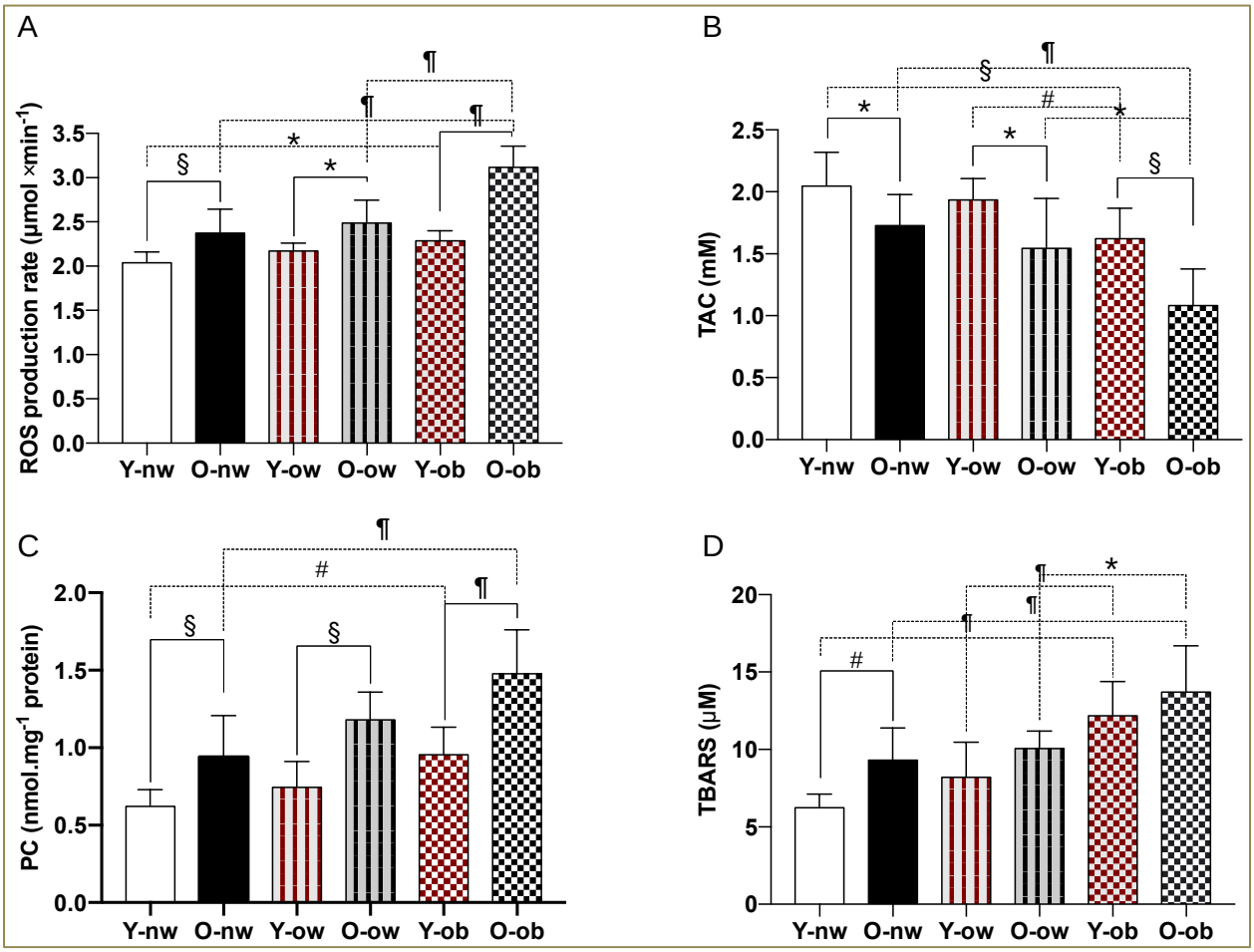

FIGURE 2. Oxidative stress parameters among different groups and subgroups. (A) ROS production rate (µmol min^{-1}), (B) total antioxidant capacity (TAC, µM), (C) protein oxidation (PC, nmol mg^{-1} protein), and (D) lipid peroxidation (TBARS, µM) were obtained from each age (young, Y and old, O) and BMI (normal weight, nw; overweight, ow; and obesity, ob) group. Each bar is calculated as the mean ± SD. *, $p < 0.05$; #, $p < 0.01$; §, $p < 0.001$; ¶, $p < 0.0001$.

oxidants coming from partial oxygen reduction or products of lipid peroxidation as the main endogenous mutagens [31]. An age-related rise in blood lipid peroxides has been reported in several studies, whereas the concentration of certain antioxidants was found decreased [2, 30, 31]. In addition to the elevated ROS levels and redox disbalance in aged organisms, our data highlighted a strong positive correlation between aging and increase in oxidative damage (**Figure 1**). Thus, the observed changes match the oxidative stress theory of aging where the oxidative damage products, such as protein carbonyls, lipofuscin, and lipid peroxidation-derived aldehydes, were found elevated also with advancing age, and this led us to hypothesize that oxidative stress and the related inflammation cause cellular and molecular damage when ROS overwhelm antioxidant defenses, leading to progressive deleterious changes over time [31]. Moreover, the ROS imbalance not only leads to structural damage to macromolecules, including lipids, proteins, and nucleic acids, but also activates transcriptional factors that could upregulate

proinflammatory cytokines leading to a chronic state of low-grade inflammation [31, 32].

During over-nutrition, adipocytes release more ROS and pro-inflammatory cytokines, leading to a continuous state of "simmering" inflammation; when the inflammatory cascade of obesity is coupled with age-related inflammation, the net effect of increased catabolism and blunted anabolism is highly detrimental to muscle [32]. A number of observational studies have associated markers of oxidative stress or inflammation with measures of frailty such as slow gait speed [33, 34]. Moreover, it has been suggested that adiposity induces oxidative stress and grip strength [35]. Thereafter, it stands to reason that obesity in old subjects results in a particularly unfavourable condition.

There are some flaws in the present study design, such as the limited number of the participants in each of groups and subgroups, especially the subgroups. The authors are aware that this limitation could have partially affected the results and that improvement of the work would have been reached with more subjects in each subgroup.

5. CONCLUSION

In conclusion, the data collected in the present study allowed us to separately evaluate the influence of age and BMI on oxidative stress. The ROS production rate and the levels of oxidative stress biomarkers were more significantly elevated in obese than in non-obese subjects and increased as BMI increased. Moreover, age-associated increases in ROS production rate and oxidative stress markers and decrease in TAC were demonstrated implying that the deleterious effects of fat accumulation might be worsened in elderly subjects due to aging-related disbalance of the redox status.

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The authors declare no conflicts of interest.

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