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Gender differences in antioxidant capacity of rat tissues determined by 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays

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Abstract

Differences in susceptibility to oxidative stress between males and females have been postulated. Several methods have been developed to assess the total antioxidant capacity of human serum or plasma, but just recently some of them were employed for measurement of antioxidant capacity of tissues. In this study, we measured and compared antioxidant capacity of heart, kidney, liver and brain tissues of male and female rats. Antioxidant capacity was determined using 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. In the same samples, lipid peroxidation products of these tissues were analysed using thiobarbituric acid reactive substances (TBARS) assays. Antioxidant capacity of heart, kidney and liver tissues was higher in female than male rats for both FRAP and ABTS assays. We found positive correlation between FRAP and ABTS values for all tested tissues. FRAP and ABTS proved to be comparable, simple and quick methods for antioxidant capacity scanning in tissues. TBARS levels differed only for brain tissue, being higher in males. These results indicate stronger defense against oxidative damage in females for all observed tissues. These finding may account for the longer lifespan of females.

Keywords: ABTS; Antioxidant capacity; Brain; FRAP; Gender; Heart; Kidney; Liver; TBARS

1. Introduction

Production of reactive species, including free radicals, is an integral part of mammalian metabolism. Living organisms have developed complex antioxidant systems to counteract reactive species and to reduce their damage. These antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin; and an array of small molecules, including ascorbic acid, alpha-tocopherol, carotenoids, polyphenols, ubiquinol-10, reduced glutathione (GSH), methionine, uric acid, and bilirubin (Yu, 1994). Oxidative damage occurs when this system is overwhelmed. Because of the high potential to damage vital biological systems, reactive species have been incriminated in aging and in numerous disease state including diabetes, cancer, arthritis, cardiovascular disease, macular degeneration, Alzheimerw's disease and Parkinson' disease (Halliwell et al., 1992; Ames et al., 1993).

Differences in susceptibility to oxidative stress between males and females have been postulated. Halliwell and coworkers found that the level of oxidative damage to DNA is higher in males than in females (Proteggente et al., 2002). Studies in vitro have demonstrated that estrogens have significant antioxidant properties, although exact mechanisms by which estrogens act as antioxidants remain unknown (Yagi and Komura, 1986; Nakano et al., 1987; Sugioka et al., 1987; Subbiah et al., 1993; Ruiz-Larrea et al., 1997). It is possible that other antioxidant-related factors also contribute to gender-related differences in antioxidant protection. Higher levels of vitamin E and elevated activity of

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glutathione peroxidase have been found in females (Salminen et al., 1988; Chen et al., 1992; Tiidus et al., 1999; Yamamoto et al., 2002). Finally, recent studies reported that mitochondrial oxidant production and oxidative damage to mitochondrial DNA are significantly lower in female than in male rats (Sastre et al., 2002; Borras et al., 2003).

Accumulation of oxidative stress markers is a function of many parameters beside antioxidant defense, like tissue composition (lipids being the most sensitive to oxidative stress), elimination and repair strategy (Terman, 2001). Lipid peroxidation is one of the most important organic expressions of oxidative stress (Yagi, 1987). Following peroxidation of ω-6 and ω-3 polyunsaturated fatty acids (PUFAs), relatively unstable fatty acids hydroperoxides may be converted into more stable carbonyls, among others malondialdehyde (MDA). In fact, MDA formation, often assayed with thiobarbituric acid (TBA) assay, is the most widely used index of lipid peroxidation in human and animal studies (de Zwart et al., 1999). Increased levels of MDA in humans were found in disease like myocardial infarction, diabetes mellitus, Alzheimer's disease and others (de Zwart et al., 1999). However, next to MDA, several other compounds (e.g., bilirubin, hemoglobin, some products of lipid peroxidation) are also reactive towards TBA, which is the main reason that the level of lipid peroxidation is usually expressed as thiobarbituric acid reactive substances (TBARS; Janero, 1990; Valenzuela, 1991). In the 90s, a group of prostaglandin (PGF_2)–like compound known as F_2-isoprostanes–were introduced as novel markers of lipid peroxidation (Morrow et al., 1990). F_2-isoprostanes are formed in situ in phospholipids by free radical-catalysed peroxidation of esterified arachidonic acid and subsequently are released in free form. Increased levels of isoprostanes in human plasma and/or urine were found in smokers, pancreatic diseases, diabetes, cardiac ischemia/reperfusion injury and others. These compounds are promising biomarkers for free radical damage in vivo, however, complex analytical methodology and need for laborious sample pre-treatment limit their general applicability (de Zwart et al., 1999).

Several methods have been developed to assess the total antioxidant capacity of human serum or plasma because of the difficulty in measuring each antioxidant component separately and the interactions among them (Glazer, 1990; Miller et al., 1993; Cao et al., 1993; Ghiselli et al., 1995; Benzie and Strain, 1996; Chapple et al., 1997; Re et al., 1999). The measured antioxidant capacity of a sample depends on which technology and which free radical generator or oxidant is used in the measurement. Therefore, comparison of different analytical methods is helpful for better understanding and interpretation of the results.

Similar to the methods for assessment of antioxidant capacity of plasma, it would be useful to have a simple and reliable method to quantify overall oxidative buffering capacity of tissues and organs. Recently, it has been demonstrated that the Trolox equivalent antioxidant capacity (TEAC) assay, based on 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) oxidation, can be utilised for quantification of antioxidant activity of brain tissue homogenates (Shea et al., 2003). Similarly, the ferric reducing/antioxidant power (FRAP) assay, based on ferric to ferrous iron reduction, was recently used to determine antioxidant capacity of lettuce leaf tissue (Kang and Saltveit, 2002). ABTS and FRAP assays use different technology for measuring antioxidant capacity and this fact must be kept on mind when interpreting the obtained results. ABTS decolorization assay is an inhibition method: a sample is added to a free radical generating system; the inhibition of free radical action is measured and this inhibition is related to the antioxidant capacity of the sample (Re et al., 1999). The FRAP assay measures the iron reducing ability of a sample (Benzie and Strain, 1996). Both these assays are quick and do not require sophisticated equipment, like fluorescence detector or GC-MS, which make them suitable for analyses of multiple tissue samples. ABTS assay has been found to correlate well with levels of endogenous glutathione (Kang and Saltveit, 2002), while FRAP assay accurately reflected plasma levels of ascorbic acid, uric acid and α-tocopherol (Benzie and Strain, 1996).

In order to elucidate inter-gender differences in susceptibility to oxidative stress, we measured and compared male and female antioxidant capacity of heart, kidney, liver and brain tissues in rats using FRAP and ABTS assays, and quantified lipid oxidation products of these tissues using TBA assay.

2. Materials and methods

2.1. Materials

Thiobarbituric acid (TBA) and *n*-dodecylsulfat natrium-salt were obtained from Merck (Darmstadt, Germany). Ferric chloride hexahydrate; 2,4,6-tri-(2-pyridil)-*s*-triazine (TPTZ); 1,1,3,3-tetrametoxy propane; 2,2′-azinobis(3-etil-benzotiazolin-6-sulfonic acid dominium salt; ABTS), Trolox, and potassium persulfate (di-potassium peroxidisulfate) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). All other chemicals were of reagent grade and were used without further purification. Spectrophotometric measurements were performed by UV–VIS spectrophotometer (double-beam) Specord 200 Analytik Jena GmbH, Germany.

2.2. Animals and tissue homogenates

Following Ethical Committee approval, 10 Wistar rats (*Rattus norvegicus*) of each gender (weight range, 290–310 g) were used for this study. Age range was 101±4 days for male and 105±6 days for female rats. They had access to food and drinking water ad libitum. After injection of urethane (1.2 g/kg intraperitoneally), rats became unrespon-

sive to noxious stimulation after which they were decapitated with guillotine. Heart, kidney, liver and brain were immediately removed from the animal. The organs were chilled by immersing into ice-cold 1.15% solution of KCl, and immediately processed for biochemical analysis. Fresh tissue samples were weighed and homogenates (10% w/v) were prepared using a glass homogenizer with ice-cold 1.15% KCl. The ability of tissue homogenate to resist oxidative damage was determined as reducing ability, by FRAP assay, and as free radical scavenging ability, by ABTS assay. The level of lipid oxidation products was measured by TBA assay. The adequate amount of freshly prepared tissue homogenate was immediately used for the determination of TBARS. The rest of each tissue homogenate was centrifuged (10 min×1500 rpm), after which aliquots were used in FRAP and ABTS assays.

2.3. FRAP assay

The reducing ability of biological sample was determined by FRAP assay of Benzie and Strain (1996). FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue coloured Fe^{II}-tripyridyltriazine compound from colourless oxidised Fe^{III} form by the action of electron donating antioxidants. The working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L acetate buffer, pH 3.6, with 1 volume of 10 mmol/L TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40 mmol/L hydroclorid acid and with 1 volume of 20 mmol/L ferric chloride. Aqueous solutions of known Fe^{II} concentration, in range of 100–1000 μmol/L ($FeSO_4x7H_2O$) were used for calibration. All solutions were used on the day of preparation.

Freshly prepared FRAP reagent (1.5 mL) was warmed to 37 °C and a reagent blank reading was taken at 593 nm ($A_{\text{reagent blank}}$; t=0 min). Subsequently, 50 μL of sample and 150 μL of deionized water was added to the FRAP reagent. The reaction mixture was incubated at 37 °C. The absorbance at 4 min after starting the reaction (adding the sample) was selected as final reading (A_{sample}). Sample blank reading, using tissue homogenate and adequate volume of acetate buffer, was taken too ($A_{\text{sample blank}}$). The difference between A_{sample} and $A_{\text{sample blank}}$ was calculated as $A1_{\text{sample}}$. The change in absorbance, between $A1_{\text{sample}}$ and $A_{\text{reagent blank}}$ was selected for calculation of FRAP value. In the FRAP assay the reducing ability of the tissue homogenate under the test was calculated with reference to the reaction signal given by a Fe^{II} solution of known concentration. The values were expressed as micromole of Fe^{II} equivalents per gram wet tissue weight. All measurements were done in four repetitions for each tissue homogenate.

2.4. ABTS assay

This assay is based on the inhibition of the absorbance of the radical cation of 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) which has characteristic long-wavelength absorption spectrum showing maxima at 660, 734 and 820 nm (Re et al., 1999) by tested antioxidant. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study $ABTS^{\cdot+}$ solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. After addition of 2.0 mL of diluted $ABTS^{\cdot+}$ solution to 20 μL of biological sample, or Trolox standard, the reaction mixture was incubated for 6 min in a glass cuvette at 30 °C. The decrease in absorbance at 734 nm was determined exactly at 6 min after initial mixing for all samples. The absorbance of the $ABTS^{\cdot+}$ without sample, i.e. the control, was measured daily. All measurements were performed in four repetitions. The percentage inhibition of $ABTS^{\cdot+}$ by the sample was calculated according to the formula:

$$\%\ \text{Inhibition} = \left[\left(A_{C(0)} - A_{A(t)}\right)/A_{C(0)}\right] \times 100$$

were $A_{C(0)}$ is the absorbance of the control at t=0 min; and $A_{A(t)}$ is the absorbance of the antioxidant (biological sample or Trolox standard) at t=6 min.

The free radical scavenging capacity of the biological sample, calculated as percentage inhibition of $ABTS^{\cdot+}$, was equated against a Trolox standard curve (1.5–30 μmol/L, final concentrations). The results are expressed as Trolox equivalents per gram wet tissue weight, that is, the amount of Trolox (μmol) with an equivalent antioxidant potential to 1 g of the tissue under investigation.

2.5. TBA assay

For measurement of the lipid oxidation products in tissue samples of the liver, kidney, heart and brain, the standard procedure using thiobarbituric acid (TBA) reaction under

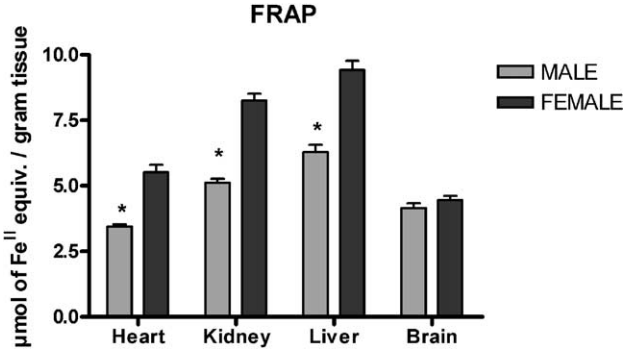

Fig. 1. FRAP values for heart, kidney, liver and brain tissue in male and female rats. Each column represents mean±S.E.M. of ten values corresponding to ten animals, each value being the mean of quadruplicate assays. *p<0.05 for MALE vs. FEMALE group.

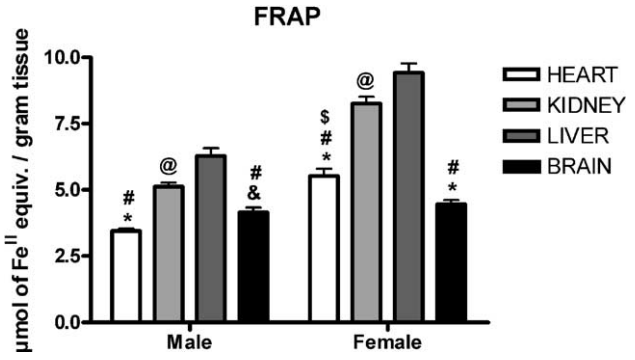

Fig. 2. FRAP values for heart, kidney, liver and brain tissue in male and female rats. Each column represents mean±S.E.M. of ten values corresponding to ten animals, each value being the mean of quadruplicate assays. *p<0.001 vs. KIDNEY; $^{\#}p$<0.001 vs. LIVER; $^{\$}p$<0.01 vs. BRAIN; $^{@}p$<0.01 vs. LIVER; $^{\&}p$<0.01 vs. KIDNEY.

the conditions specified by Yagi was used (Ohkawa et al., 1979). TBARS were expressed as malondialdehyde equivalents per gram wet tissue weight. All measurements were done in three repetitions for each tissue homogenate.

2.6. Statistical analysis

All data are expressed as mean±S.E.M. The effects of gender on antioxidant capacity and lipid peroxides in different organs were analysed by two-way ANOVA (organ and gender were the analysed factors) followed by Bonferroni test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA USA, www.graphpad.-com. Correlation of one antioxidant capacity measurement vs. another was also computed using GraphPad Prism. p<0.05 was considered to indicate statistical significance.

3. Results

We found significant inter-gender difference for reducing ability of tested tissues (Fig. 1). FRAP values for heart, kidney and liver tissues in female rats were higher in comparison to male. Only the values for brain tissue did not significantly differ between female (4.459±0.156) and male (4.142±0.188 μmol of Fe^{II} equiv./g tissue) rats. A hierarchy in antioxidant/reducing capacity of tissues could be observed as well: liver>kidney>heart>brain for females, and liver>kidney>brain=heart for males (Fig. 2).

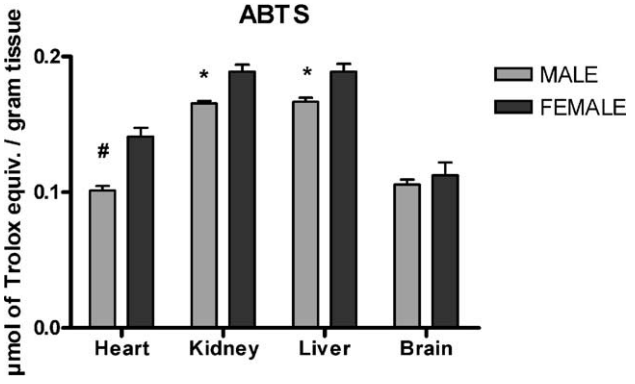

Fig. 3. ABTS values for heart, kidney, liver and brain tissue in male and female rats. Each column represents mean±S.E.M. of ten values corresponding to ten animals, each value being the mean of quadruplicate assays. *p<0.05 for MALE vs. FEMALE group; $^{\#}p$<0.01 for MALE vs. FEMALE group.

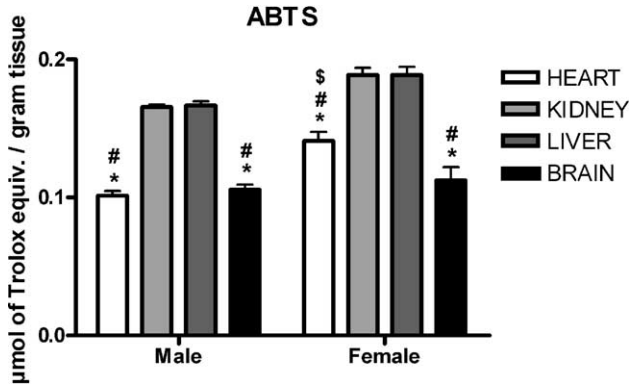

Fig. 4. ABTS values for heart, kidney, liver and brain tissue in male and female rats. Each column represents mean±S.E.M. of ten values corresponding to ten animals, each value being the mean of quadruplicate assays. *p<0.001 vs. KIDNEY; $^{\#}p$<0.001 vs. LIVER; $^{\$}p$<0.001 vs. BRAIN.

ABTS radical cation scavenging ability (Fig. 3) for heart, kidney and liver tissues in female rats was higher in comparison to male, matching results obtained by FRAP assay. Similarly, brain tissue values differed only slightly between females (0.112±0.010) and males (0.106±0.004 μmol of Trolox equiv./g tissue). The antioxidant activity for the tested tissues, established by ABTS assay, also showed hierarchy between tissues: liver=kidney>heart>brain for females and liver=kidney>heart=brain for males (Fig. 4).

There was a significant positive correlation between FRAP and ABTS values for tested tissues (r=0.815, p<0.0001; Fig. 5).

Level of lipid peroxide differed only for brain tissue, being higher in males (474.8±18.9 vs. 328.5±24.5 nmol

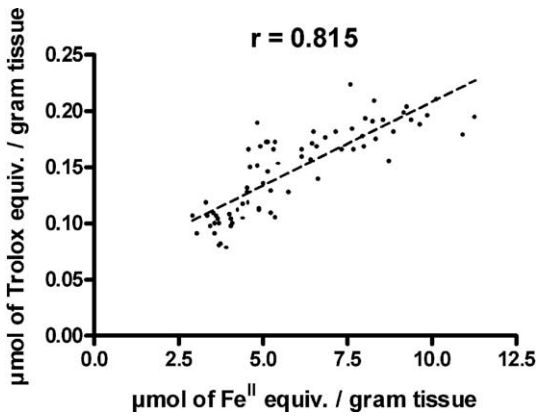

Fig. 5. Scatter plot of FRAP and ABTS values for all eighty tested tissues, each value being the mean of quadruplicate assays. There is a significant positive correlation.

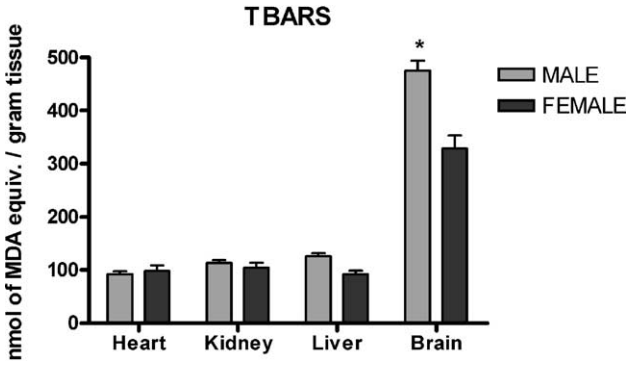

Fig. 6. TBARS values for heart, kidney, liver and brain tissue in male and female rats. Each column represents mean±S.E.M. of ten values corresponding to ten animals, each value being the mean of triplicate assays. *p<0.05 for MALE vs. FEMALE group.

MDA/g tissue). Values of TBARS (Fig. 6) for heart, kidney and liver tissue were similar for male and female rats. Lipid peroxides of brain tissue were markedly higher in comparison to other tissues for both genders.

4. Discussion

The antioxidant activity of a compound against a free radical does not necessarily match its reducing ability. However, we did find a strong correlation between values obtained with FRAP and ABTS assay (Fig. 5). This result is in contrast with a study that compared different analytical methods for assessing total antioxidant capacity of human serum (Cao and Prior, 1998) and where no correlation was found. This distinction may be due to different samples and sample preparation (animal tissue homogenates vs. human serum), and different antioxidant defenses in those samples (ascorbate and urate in serum, cellular antioxidants in tissue).

Using FRAP and ABTS assay we found that female rats have higher antioxidant capacity for heart, kidney and liver tissue. This finding is in accordance with previous studies that showed intrinsically higher activity of different antioxidant system in females for liver and heart tissue (Barp et al., 2002; Bureau et al., 2003). Female rats have greater mean hepatic and cardiac alpha-tocopherol levels, (Chen et al., 1992; Tiidus et al., 1999) total capacity of the cellular systems that detoxify reactive oxygen species or free radical-drug metabolites seems to be higher in female rat liver, (Julicher et al., 1984) as much as activities of hepatic and cardiac glutathione peroxidase and hepatic glutathione reductase (Pinto and Bartley, 1969; Salminen et al., 1988; Yamamoto et al., 2002). Several studies showed direct protective effect of estrogen against oxidative damage to heart and liver tissues (Yagi and Komura, 1986; Ruiz-Larrea et al., 1993; Persky et al., 2000; Busserolles et al., 2002; Bureau et al., 2003). Our study showed similar inter-gender difference for kidney tissue as well. The exact mechanisms for this difference in renal antioxidant capacity are yet to be clarified.

Interestingly, we found inter-gender difference in TBARS only in brain tissue, although a strong tendency was found in liver tissue (Fig. 6). The brain is exceptionally vulnerable to cytotoxic effects of oxygen-derived free radicals, (Kontos, 1989; Halliwell, 1992) and therefore probably most suitable for detecting inadequacy of antioxidant defense mechanisms. Brain is a highly oxygenated organ and it derives most of its energy from oxidative metabolism. There is a poor catalase activity and moderate amount of superoxide dismutase and glutathione peroxidase present in the brain. Also, brain membrane lipids are very rich in PUFAs, which are especially sensitive to free radical-induced peroxidation. Finally, high iron content of some brain areas (like substantia nigra) and hyperactivation of glutamate receptors (excitotoxicity) can directly promote formation of free radicals (Bondy and LeBel, 1993). In vitro studies showed that neuroprotection by estrogens and progesterone may involve multiple processes, including antioxidant properties, antagonistic effects to the glutamate NMDA receptor and genomic action (Weaver et al., 1997; Gridley et al., 1998; Nakamizo et al., 2000; Sawada and Shimohama, 2000; Roof and Hall, 2000). This could explain significant inter-gender difference in lipid peroxides in brain tissue but not in antioxidant capacity as well.

In summary, we have found inter-gender difference in antioxidant capacity for the observed organs. This may partially account for longer lifespan of female gender, observed in many species (Asdell et al., 1967; Vina et al., 2003).

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