Fluoxetine Decreases Glutathione Reductase in Erythrocytes of Chronically Isolated Wistar Rats

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Abstract

Alterations in the antioxidative defense parameters upon chronic stress are considered critical for pathophysiology of stress related psychiatric disorders, and their status in blood serves as biomarker for effects of pharmacological treatments. The present study was designed to investigate the modulation of erythrocyte antioxidant enzymes (AOEs): CuZn superoxide dismutase (CuZnSOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GLR) activities and their protein expression in Wistar male rats subjected to chronic psychosocial isolation and/or to pharmacological treatment with fluoxetine. Chronically isolated animals exhibited decreased levels of serum corticosterone, as opposed to other chronic stress paradigms. In addition to that, SOD, CAT and GPx status was not altered either by chronic psychosocial isolation or by fluoxetine treatment. In contrast, GLR activity and its protein level were both markedly reduced by fluoxetine. Since, GLR is crucial for overall oxido-reductive balance through maintaining optimal ratio of reduced/oxidized glutathione level (GSH/GSSG) in erythrocytes, these results could indicate that in spite of numerous beneficial effects of fluoxetine, it may compromise both haemoglobin function and oxygen transport.

Keywords: Fluoxetine, glutathione reductase, erythrocytes, chronic isolation, Wistar rat

1. Introduction

Oxidative stress is defined as the imbalance between production of reactive oxygen species (ROS) and the activity of antioxidant defense system.[1,2] ROS include free radicals that are highly reactive atoms or molecules with unpaired electron in their outer orbital and also may include nonradical compounds with high oxidative properties such as hydrogen peroxide (H_2O_2). ROS can be generated by a variety of processes in biological systems including external stimuli (UV light, toxins, drugs) and also in many physiological processes in the course of aerobic metabolism. Antioxidant defense system comprises of antioxidant enzymes (AOEs) which are capable of neutralizing or transforming particular ROS species and altogether create a powerful detoxification system.

During chronic exposure to neuroendocrine stress, alterations in antioxidant defense are considered to be one of critical conditions promoting pathophysiology of stress induced psychiatric disorders.[3,4] Compromised antioxidant defense and enhanced lipid peroxidation in blood plasma have been described in patients with depression.[5,6] Administration of different antidepressants have been shown to influence gene expression and activity of the important antioxidant enzymes such as blood superoxide dismutases (SODs).[7,8] There are increasing evidences showing that treatment with fluoxetine (selective serotonin reuptake inhibitor, SSRI) can reverse and prevent psychological stress induced oxidative damage, due to elevation in superoxide dismutase (SOD) and other components of antioxidant system.[9,10] Thus, alterations of antioxidant parameters in blood can serve as valuable biomarkers to follow effects of chronic stress

and/or pharmacological treatment of stress induced psychiatric disorders.[11]

Mature erythrocytes are terminally differentiated cells that are devoid of nucleus, as well as, of mitochondria and other organelles.[12] Due to lack of nucleus these cells cannot produce new proteins in response to stress so they have to rely on proteins synthesized earlier in development to protect themselves from damage by ROS and ensure their own survival.[13] Erythrocytes are readily exposed to free radicals which are endogenously generated *via* the auto-oxidation of hemoglobin from plasma, particularly to nitric oxide and hydrogen peroxide.[14] Therefore, red blood progenitor cells are supplied with extensive array of antioxidants to cope with ROS, including antioxidant enzymes such as SOD, catalase (CAT) and glutathione peroxidase (GPx) and those that continuously produce reducing agents through glutathione system such as glutathione reductase (GLR).[15] Coordinated activity of all these enzymes is necessary to prevent any type of erythrocyte damage caused by free radicals. Therefore, the activity of SOD which catalyses the dismutation of superoxide anion radical to hydrogen peroxide (H_2O_2) is followed by the corresponding activity of peroxidases, CAT or GPx converting H_2O_2 to water.[16] Both, cytoplasmic CuZn-SOD and CAT are highly abundant in erythrocytes.[17] In detoxification reactions GPx uses reduced glutathione (GSH) as an electron donor and GSH is oxidized to glutathione disulfide (GSSG),[18] while regeneration of GSH is accomplished by GLR activity.[19]

Antioxidant enzymes (AOEs) status in erythrocytes has been widely used to evaluate systemic state of oxidative stress under different metabolic disease conditions and it was also recently applied to follow states of neuroendocrine stress elicited mood disorders.[20,21,22,10]

Therefore, the present study was designed to investigate the modulation of antioxidant defense enzymes CuZnSOD, CAT, GPx and GLR, as well as, changes in their synchronized activities in erythrocytes of Wistar male rats that were subjected to chronic social isolation solely, or in stressed animals that were chronically treated with pharmacological dose of SSRI antidepressant fluoxetine.

2. Experimental

2. 1. Animal Care and Treatment

All experiments were performed in adult (2.5–3 months old) Wistar male rats (body mass 330–400 g), housed four per standard size cage and offered food and water *ad libitum*. Light was kept on, between 07:00 am and 07:00 pm, and room temperature (RT) was kept at 20 ± 2 °C. All animal procedures were approved by the Ethical Committee for the Use of Laboratory Animals of the VINCA Institute of Nuclear Sciences, according to the guidelines of the EU registered Serbian Laboratory Animal Science Association (SLASA).

For the experiments, animals were divided into four experimental groups: group I consisted of unstressed animals (control group); group II animals were subjected to chronic isolation stress, by housing them individually for 21 days; group III was vehicle group subjected to chronic isolation stress for six weeks and given distilled water (three weeks isolation+three weeks vehicle treatment-distilled water), group IV was treated with fluoxetine and also subjected to chronic isolation for six weeks (three weeks isolation+fluoxetine treatment during the last three weeks).

Fluoxetine-hydrochloride, (*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine), was dissolved in distilled water and administered intraperitoneally at a daily dose of 5 mg/kg body mass at 09:00 h during a 21-day period.

2. 2. Determination of Serum Corticosterone Concentration

Animals were sacrificed immediately after the termination of the stress procedure by decapitation with a guillotine (Harvard-Apparatus, USA). Blood was immediately collected and serum was prepared by 15 min centrifugation at 3000 rpm. Serum corticosterone (CORT) level was determined using the OCTEIA Corticosterone EIA kit according to manufacturer's instructions (American Laboratory Products Co.). Absorbance at 450 nm (reference 650 nm) was determined by microplate reader (Wallac, VICTOR^2 1420, PerkinElmer). CORT concentration (ng/ml) was determined using a standard curve.

2. 3. Preparation of the Erythrocyte Lysates

Trunk blood from each animal was taken after decapitation and collected in tubes containing EDTA as anticoagulant, diluted 1:1 with phosphate-buffered saline (PBS, pH 7.4) and layered over Lymphoprep Separation Medium (ICN Biomedicals, Costa Mesa, Calif, USA). After centrifugation for 30 minutes at 1300 rpm, the lymphocyte layer was removed and the obtained erythrocyte pellet was lysed by the addition of three volumes of ice-cold distilled water. Cell membranes were removed by centrifugation at 1000 g for 20 min and the supernatant was used as the erythrocyte lysates. Protein concentrations in the erythrocyte lysates were determined by the method of Lowry *et al.*[23]

2. 4. Determination of Antioxidant Enzymatic Activities

Superoxide Dismutase Activity
Total SOD activity in erythrocytes lysates was determined using a commercial kit (Randox Laboratories, Crumlin, UK). Briefly, this method uses xantine and xan-

tine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. One unit of SOD activity is that which causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride. The SOD activity was expressed as unit per mg of protein.

Catalase Activity

Catalase activity was determined according to Claiborne.[24] Decomposition of H_2O_2 was monitored by a decrese in absorbance at 240 nm. One unit of catalase activity is defined as the amount of enzyme that degrades 1µmol of H_2O_2 per minute per milligram of protein.

Glutathione Peroxidase Activity

The activity of glutathione peroxidase was assayed at 340 nm, using t-butil hydroperoxide and GSH as substrates according to Maral *et al.*,[25] and the activity was expressed as unit per mg of protein.

Glutathione Reductase Activity

Glutathione reductase activity was measured according to Glatzle *et al.*,[26] by following the oxidation of NADPH used for reduction of GSSG, and the activity was again expressed as unit per mg of protein.

2. 5. Western Blot Analysis of Antioxidant Enzymes in the Erythrocyte Lysates

For Western blot, erythrocyte lysates were prepared with denaturing buffer according to Laemmli (1970),[27] boiled for 5 min at 100 °C, and 30 µg of protein were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Subsequently, proteins were transferred onto PVDF membrane (Immobilon-P membrane, Millipore) using a blot system (Transblot, BioRad). The membranes were incubated in appropriate primary and secondary antibodies. Rabbit polyclonal anti-β-actin (ab8227, Abcam) was used to detect β-actin, as a loading control, and anti-CuZnSOD (Stressgen), anti-catalase (CAT) (Calbiochem), anti-GPx (Santa Cruz Biotechnology), anti-glutathione reductase (GLR) (Santa Cruz Biotechnology), were used to detect CuZnSOD, CAT, GPx and GLR, respectively. Blots were developed with the secondary goat anti-rabbit IgG-HRP conjugate. The signal was developed using enhanced chemiluminescence reagent (ECL, Pierce) and exposed to X-ray film (Agfa). The quantification was performed by Image J PC software analysis.

2. 6. Statistical Analysis of Data

Data are presented as mean ± SD of 10–11 animals per group. For establishing significant differences data were analyzed by the One-way ANOVA followed by the Tukey *post hoc* test. Values were considered statistically significant if the p value was less than 0.05 and in order to simplify presentation of data, all statistically significant differences are presented by single asterisk.

3. Results and Discussion

3. 1. Corticosterone

To assess the effectiveness of stress, we first measured serum corticosterone (CORT) levels and found that it was significantly decreased in animals subjected to chronic psychosocial isolation (Figure 1, *p = 0.048). This finding is in accordance with our previously published data and with data of other authors showing HPA axis hypoactivity in Wistar rats under similar conditions.[28,29] The treatment of CPSI animals either with vehicle (CPSI+Veh) or with fluoxetine (CPSI+Fluox) did not alter their CORT levels. Namely, the CORT levels in CPSI+Veh and CPSI+Fluox rats were close to the control group (Figure 1) and without significant differences between these experimental groups, indicating that fluoxetine does not influence the CORT concentration.
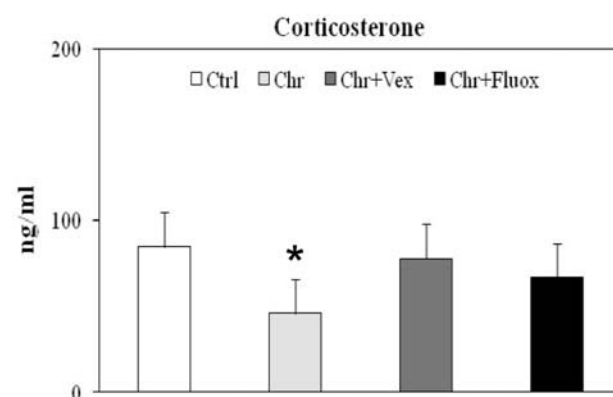
Figure 1. Serum corticosterone concentration (ng/ml) in the control (Ctrl), chronically isolated (CPSI), chronically isolated and vehicle treated (CPSI+Veh) and chronically isolated and fluoxetine treated groups (CPSI+Fluox) of male Wistar rats. Data are presented as mean±SD (n = 10–11). Asterisks indicate significant differences between the respective treatment group and unstressed controls *p = 0.048, obtained from One-way ANOVA analysis followed by *post hoc* Tukey test.

In our opinion the level of CORT found in CPSI+Veh and CPSI+Fluox rats could rather be ascribed to intraperitoneal injections than to the application of antidepressant drug,[30] since they were observed in both groups of animals. Also it is well known that only acute administration of the majority of antidepressants lead to activation of the HPA axis in both humans and laboratory animals, while long-term administration of antidepressants lowers ACTH and CORT levels in the blood.[31]

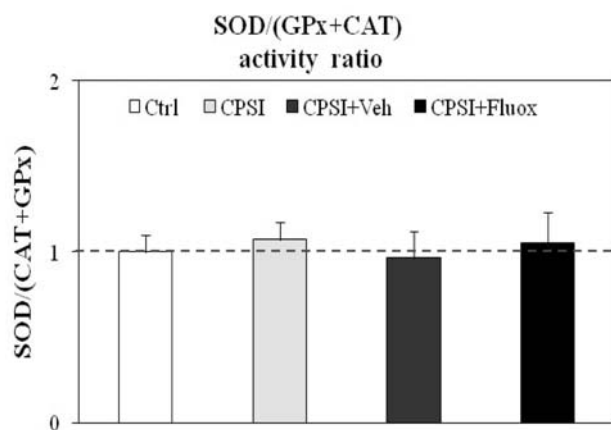## 3. 2. Antioxidant Enzymes Activities in Rat Erythrocytes in Response to CPSI and Fluoxetine Treatment

Since, the antioxidant enzymes (AOEs) status in erythrocytes has been widely used to evaluate systemic state of oxidative stress under states of neuroendocrine stress elicited mood disorders,[20,21,22,10] the current study was designed to investigate AOEs status in rat erythrocytes in response to CPSI and/or fluoxetine treatment.

Analysis of AOE activities of superoxide dismutase (CuZnSOD), catalase (CAT) and glutathione peroxidase (GPx) in rat erythrocytes did not show any significant changes either in stressed or in vehicle or in fluoxetine treated animals (Table 1). The only significant change was observed in decreased activity of glutathione reductase (GLR) in chronically stressed animals treated with fluoxetine, both in respect to the control and to chronically stressed animals that received distilled water (Table 1, *p = 0.0037, *vs*. control, #p = 0.0072 *vs*. chr+veh, respectively).

In accordance, we calculated that quotient SOD/GPx+CAT (Figure 2) which indicates that neither CPSI nor fluoxetine treatment did not influence tightly synchronized relationship between SOD on the one side, and GPx and CAT on the other side, probably implying absence of cellular damages by free radicals in rat erythrocytes.
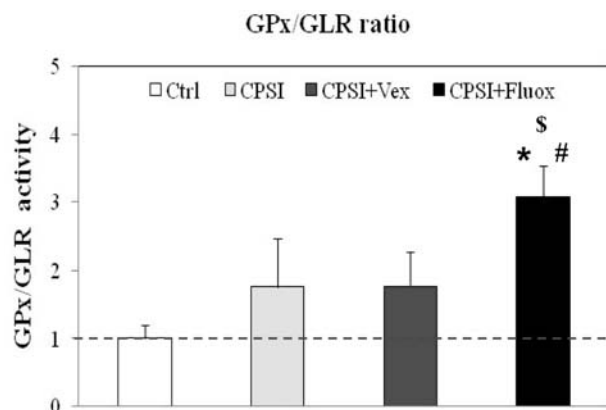
Our findings regarding the SOD, CAT and GPx activities in response to CPSI may therefore, at least in part, be interpreted to be due to low level of CORT.[35] Namely, the unchanged SOD, CAT and GPx activates in response to CPSI could be a consequences of low CORT level, since it is known that glucocorticoids (GCs) are important inducers of AOEs and a glucocorticoid response element has been reported in the gene for human CuZnSOD.[36] This statement is supported by evidence that GCs increase the toxicity of oxygen radical generators,[37,38] and may increase the basal level of ROS produced in cells.[35,39] This increase could result from a decrease in antioxidant capacity in peripheral tissues.[40,41] It is therefore possible that the levels of protective AOEs were unaffected in response

Table 1. Antioxidant enzyme activities in the control and treated animals.

Enzyme	Control	CPSI	CPSI+Veh	CPSI+Fluox
CuZnSOD	15.7 ± 3.1	18.0 ± 3.8	15.4 ± 3.1	16.5 ± 3.0
Catalase	233.8 ± 32.8	239.8 ± 34.5	215.1 ± 20.8	225.2 ± 46.8
Glutathione peroxidase	149.2 ± 16.3	172.4 ± 29.2	176.7 ± 32.5	166.0 ± 30.5
Glutathione reductase	56.5 ± 7.1	46.6 ± 2.9	52.1 ± 6.22	29.3 ± 3.7[*#]

In addition, the sensitivity of a cell to free radicals apparently depends on the relationship between SOD, CAT and GPx rather than on absolute concentrations of an individual AOE.[32] Moreover, it has been suggested that the alteration in the SOD/GPx+CAT quotient is an important indicator of cellular damage rather than the absolute concentrations of AOE.[33,34] This is because the changes in this quotient correlate well with increases in lipid damages.[34]

to CPSI, since the GCs levels under CPSI condition were low.

Our results also suggest that since CPSI of animals did not alter their AOEs status in erythrocytes, it does not generate significant systemic state of oxidative stress, as opposed to some other chronic stressors, like restraint stress which is characterized by elevated CORT.[42] In addition, the lack of majority of AOEs response to fluoxetine treatment in our opinion could be a consequence of their high abundance in erythrocytes and relatively low dose of fluoxetine applied (5 mg/kg/day). Alterations in the SOD, CAT and GPx activities might possibly be expected provided either the higher doses of fluoxetine,[43] or prolonged period of its administration (6 weeks).

Regarding activities of GSH-dependant enzymes, we observed a significant decrease of glutathione reductase (GLR) in CPSI+Fluox group of animals, both in respect to the control and in respect to CPSI+Veh group (Table 1, *p = 0.0037, *vs*. control, #p = 0.0072, *vs*. CPSI+Veh). Despite diminished activity of GLR in CPSI+Fluox animals, their respective GPx activity was not altered by fluoxetine, indicating that in erythrocytes of these animals the concentration of GSH seemed to be sufficient for appropriate activity of GPx. Eventhough, the activity of GPx was not compromised by diminishment in GLR activity, the overall cellular level of GSH could be reduced, since

Figure 2. The SOD/GPx+CAT ratio as an indicator of cellular damage.

the changes in the activity of GLR can exhibit a greater potential impact on recycling of GSH than alterations in peroxidase activity.[44] Moreover, it is well known that the activity of GLR maintains a high reduced/oxidized glutathione ratio (GSH/GSSG) in normal states and that alteration in its activity could disturb optimal GSH/GSSG level and therefore may alter the erythrocytes redox environment.[45,46] In accordance with that, as an additional measure of alteration in GSH-dependent enzymes and consequently in GSH/GSSG level, we determined GPx/GLR ratio and observed its increasing trend in CPSI and CPSI+Veh groups (Figure 3). On the other hand, the GPx/GLR ratio was markedly increased in CPSI+Fluox group either in respect to the control or in respect to CPSI

and CPSI+Veh groups (Figure 3, *p = 0.00016, *vs.* Ctrl, $^\$$p = 0.0055 *vs.* CPSI and #p = 0.0059 *vs.* CPSI+Veh).
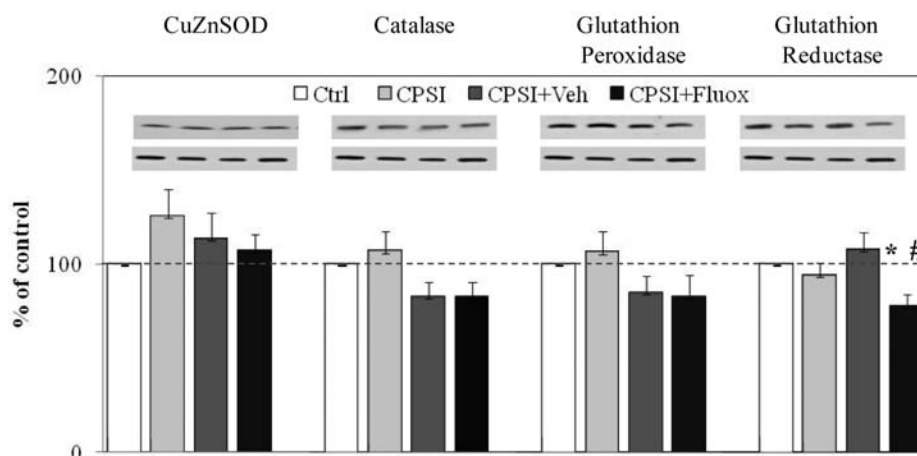
Although we did not measure the GSH levels directly, this level could be speculated from the GPx/GLR ratio. According to that, the GPx/GLR ratio indicate that CPSI itself might have disturb GSH-turnover, while chronic fluoxetine treatment of CPSI animals may have altered GSH recycling leading to higher prooxidative state.

3. 3. Antioxidant Enzymes Protein Levels in Rat Erythrocytes in Response to CPSI and Fluoxetine Treatment

In order to investigate the possible underlying cause in alterations of AOEs activities, especially under fluoxetine treatment, we followed AOEs protein levels. The quantification of protein expression of CuZnSOD, CAT and GPx in the erythrocytes did not reveal significant changes in the level of these enzymes (Figure 4), which was in accordance with unchanged AOEs enzymatic activities (Table 1).

Again, the main change was observed in the protein level of GLR in CPSI+Fluox group (Figure 4, *p = 0.0088, *vs.* Ctrl, #p = 0.0093 *vs.* CPSI+Veh) in which GLR level was decreased likewise its activity. This results clearly indicate that diminishment in the activity of GLR in response to chronic fluoxetine treatment is a direct consequence of decreased expression of GLR at the protein level.

The molecular mechanism underlying the fluoxetine-mediated decrease of GLR activity and expression remains to be investigated. At the moment, in literature there is no data linking the direct effect of fluoxetine on the activity or protein level of GLR. Besides that, based on the fact that mature erythrocytes are terminally differentiated cells which cannot produce new proteins,[12] it is likely that GLR depletion by fluoxetine may occur in the earlier stages of erythrocytes differentiation.[13] Even though

Figure 3. The GPx/GLR ratio in the control (Ctrl)-optimal activities of GPx and GLR presented as 1, in chronically isolated group (CPSI), chronically isolated and vehicle treated group (CPSI+Veh) and chronically isolated and fluoxetine treated group (CPSI+Fluox) of male Wistar rats-presented in respect to the control. Data are presented as mean ± SD (n = 10–11). Asterisks indicate significant differences between the respective treatment group and unstressed controls obtained from One-way ANOVA analysis followed by *post hoc* Tukey test (*p = 0.00016 *vs.* Ctrl, $^\$$p = 0.0055 *vs.* CPSI and #p = 0.0059 *vs.* CPSI+Veh).

Figure 4. Representative Western blot and relative quantification of CuZn superoxide dismutases (CuZnSOD), catalase, glutathione peroxidase, and glutathione reductase in the erythrocytes of control (Ctrl), chronically stressed (CPSI), chronically stressed and vehicle treated (CPSI+Veh) and chronically stressed and fluoxetine treated (CPSI+Fluox) Wistar rats. Data are presented as mean ± SD of 10–11 animals per group. (*p = 0.0088 *vs.* Ctrl, #p = 0.0093 *vs.* CPSI+Veh).

the GLR decrease may result in some cases from nutritional deficiency,[47] this is in our opinion highly unlikely to be the case, since the nutritional habits of our Wistar rats were not altered either qualitatively or quantitatively throughout the whole course of experiment. The decrease in GLR may therefore result as a possible consequence of GSH depletion, since decrease in GSH was previously reported to be capable to decrease GLR activity in some tissues by almost 40%.[48] The expression of GLR is also known to be inhibited by increase in H_2O_2.[49,50] Therefore, the causes of erythrocyte GLR diminishment in our study may be the factor affecting oxido-reductive disballance in favour of the former. It is tempting to propose that in chronically isolated Wistar rats that factor might be fluoxetine itself. If that be the case, the response of AOEs primarily that of GLR to fluoxetine treatment may be dependent of the type of chronic stress *ie.* level of CORT, since it seems to be opposite in isolated *vs.* restrained rats.[42]

The consequences of reduced level of GLR in rat erythrocytes under fluoxetine treatment may be multiple. Namely, GLR activity in erythrocytes was previously shown to be of special significance since it is required for the stability and integrity of the red cells, while its decrease was connected with drug-induced haemolytic anaemia and eye cataracts.[51] GLR is known to catalyze the reduction of hemoglobin-glutathione disulfides, thus that the reduction of its level/activity is expected to compromise haemoglobin function and consequently to affect oxygen transport.[52]

4. Conclusion

In conclusion, our study indicates that fluoxetine treatment of chronically psychosocially isolated male Wistar rats, with low or control levels of corticosterone, leads to significant reduction in the protein level and activity of erythrocyte glutathione reductase, an enzyme crucial for maintaining oxido-reductive balance in these cells. The results point out potential importance of determining glucocorticoids status before choosing fluoxetine for the therapy of stress-related disorders in clinical settings.

5. Acknowledgement

6. References

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Povzetek

Spremembe v obrambnih antioksidantih po kroničnem stresu so kritični del patofiziologije s stresom povezanih psihiatričnih obolenj, njihov status v krvi pa je pokazatelj učinka farmakološkega zdravljenja. Predstavljena študija proučuje spremembo v antioksidativnih encimih eritrocita (AOE): CuZn superoksidne dismutaze (CuZnSOD), katalaze (CAT), glutationske peroksidaze (GPx) in glutationske reduktaze (GLR). Proučevali smo aktivnosti in izražanje proteina v podganjih samcih Wistar, ki so bili podvrženi kronični psihološki izolaciji in/ali zdravljenju s fluoxetinom. V nasprotju s paradigmo stresa, kronično izolirane živali izkazujejo znižano količino serumskega kortikosterona. Tudi status encimov SOD, CAT in GPx ni bil spremenjen pri kronični izolaciji ali pri zdravljenju s fluoxetinom, medtem ko sta se aktivnost in količina GLR proteina s fluoxetinom signifikantno zmanjšali. GLR je ključni dejavnik celokupnega oksidoredukcijskega ravnotežja, saj v eritrocitu vzdržuje optimalne količine oksidiranega/reduciranega glutationa (GSH/GSSG). Predstavljeni rezultati nakazujejo, da fluoxetin, kljub številnim pozitivnim učinkom, lahko okvari funkcijo hemoglobina in transport kisika.