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Oxidative stress in heroin administered mice and natural antioxidants protection

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Abstract

The oxidative stress of heroin administered mice via intraperitoneal injection, and the therapeutic effects of exogenous antioxidants on the restrain of the oxidative damage of biomolecules and withdrawal syndrome were studied. After administered with heroin, mice showed decrease of total antioxidant capacity in blood, increase of reactive oxygen species production in white blood cells, and increase of oxidative damages of protein and lipid in brain and liver, but not in heart. On the other hand, exogenous antioxidants could restrain the oxidative stress, even alleviate withdrawal syndrome.

Keywords: Heroin addiction; Oxidative stress; Antioxidant; Withdrawal syndrome

Introduction

Heroin addiction is a phenomenon with complex physiological and social causes and consequences. Despite a great deal of research, the exact mechanisms of dependence and withdrawal remain unclear. Most of phenomena occurring in heroin addicts, such as aging, abscesses, arthritis, other rheumatologic disorders and immunity disorders are related to degenerative diseases (Oliveira et al., 2002). There are

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some indirect evidences showed the relationship between heroin addiction and the products of free radical reaction. Heroin is able to induce oxidative cell injury in neuronal cells (Oliveira et al., 2002) and neurodegeneration (Spina and Cohen, 1989). A metabolite of heroin, morphine, could directly affect the formation of superoxide in glomerular mesangial cells (Singhal et al., 1994). Besides, substantial pain relief and reduction of dosage or complete cessation of analgesie opioids were found in cancer patients treated orally with antioxidants (Evangelou et al., 2000). Megadoses of vitamin C could prevent the development of tolerance and physical dependence on morphine in mice (Khanna and Sharma, 1983). High doses of Ascorbic acid administered orally can ameliorate the withdrawal syndrome of heroin addicts (Evangelou et al., 2000).We propose that oxidative damage may be an important pathological factor in heroin addiction, and antioxidant maybe a useful agent in the release of withdrawal syndrome.

Uric acid (Hooper et al., 2000), quercetin (Molina et al., 2003), rosmarinic acid (Lamaison et al., 1990) and ascorbic acid (Som et al., 1983; Bodannes and Chan, 1979) are antioxidants and were used in some degenerative diseases. In the present paper, we focused on the oxidative stress in heroin addiction and the release of withdrawal syndrome by antioxidants.

Methods

Subjects

Male and female (in half) Kunming mice (Experimental Animal Center of Lanzhou Medical College), 6–7 weeks old, weighting 30.0 ± 2.5 g, were housed and maintained on a 12 h light-dark cycle. Food and water were available ad libitum in the home cage.

Materials

Heroin was provided by Police Bureau of Gansu Province, China, containing 83.26% of diacetylmorphine, 6.41% of acetylmorphine and 10.33% of acetylcodeine. Ascorbic acid, uric acid, quercetin, rosmarinic acid and thiobarbituric acid (TBA) were obtained from Sigma (St. Louis, MO) and dissolved in 0.9% NaCl solution immediately before injection, and injected at the volume of 1.0 ml/kg. Quercetin was dissolved first in 0.9% NaCl solution, and then adjusted the pH to 7.4 with NaOH for solubilization; rosmarinic acid was dissolved in hot 0.9% NaCl solution and was kept at room temperature at least 10 minutes for balance temperature. The rosmarinic acid solution is prepared freshly for each time. Control animals were given saline injection at a corresponding volume. All other agents were of analytical quality.

Induction of heroin dependence

Heroin was dissolved in saline and administered according to references (Li et al., 2001; Geoffrey et al., 1983) with modifications. Mice were randomly assigned to heroin administrated and saline control groups. Prior to and during treatment, the animals were housed in plastic cages in a room at 21±1 °C. Mice were pretreated with saline (control) or heroin at an interval of 12 h for 15 days with the increasing dosage, and the total injection times is 30 (Fig. 1). On the day 16th, heroin treated mice were randomly assigned to 9 groups, each group has 20 mice. (1) heroin alone, (2) heroin + LAA (low concentration of

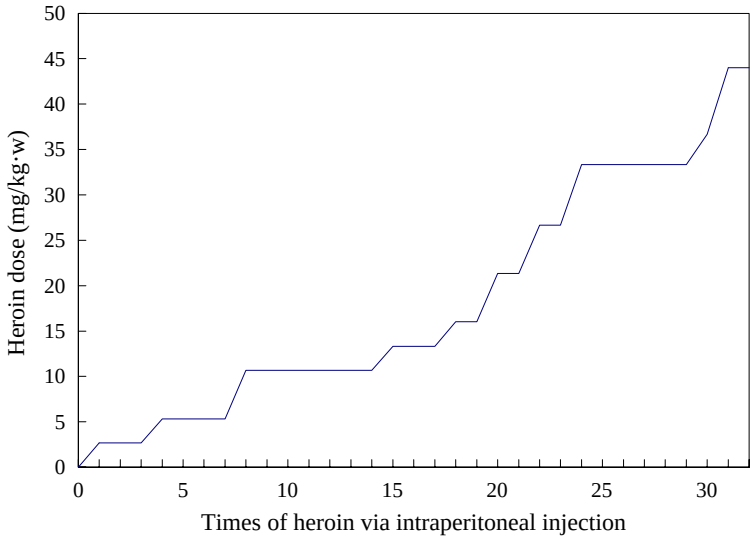

Fig. 1. The process of heroin dose administered to mice via intraperitoneal injection. Mice were randomly assigned to heroin administrated and saline control groups. The heroin administrated mice were i.p. heroin twice daily for 15 days. The dosage is gradually increased to the indicated levels.

ascorbic acid, AA, 10 mg/kg, ip) , (3) + HAA (high concentration of AA, 50 mg/kg, ip), (4) + LUA(low concentration of uric acid, UA, 10 mg/kg, ip), (5) + HUA (high concentration of UA, 50 mg/kg, ip), (6) + LQU (low concentration of quercetin, QU, 10 mg/kg, ip), (7) + HQU (high concentration of QU, 50 mg/kg, ip), (8) + LRA (low concentration of rosmarinic acid, RA, 10 mg/kg, ip) and (9) + HRA (high concentration of RA, 50 mg/kg, ip). From the day 16th to 20th, antioxidant was injected once every day in antioxidant therapy groups for 5 days, while corresponding volume of solvent was injected to the heroin alone group and saline control group. On the day 21st, blood was collected and used for assay of total antioxidant capacity (TAC) and ROS at once. Tissues were collected and kept at −80 °C until analysis. All experiments were conducted strictly in conformity with the National Institute of Health Guide for the Care and Use of Lab Animals.

Measurement of the withdrawal syndrome

On the day 16th, the withdrawal syndromes were measured. Experiment was carried out in a quiet room and the animals were not acclimatized to test situation beforehand. Naloxone was given 1 h after the last heroin injection. Different kind of antioxidants was given 30 min before naloxone, and the naloxone-induced withdrawal behavior was determined. Three previously identified behavioral characteristics of the mice opiate abstinence syndrome, jumping, shaking and exploring, were recorded over a period of 30 min (Rasmussen et al., 1996).

Assay for total antioxidant capacity (TAC)

TAC was measured using a kit test (Najing Jiancheng Bioengineering Institute) based on the method (Benzie and Strain, 1996) with a minor modification. This assay measures the ferric-reduction ability of

plasma. The stable color of the Fe^{2+}-*o*-phenanthroline complexes due to the overall reducing agents in plasma reduced Fe^{3+} to Fe^{2+}, which reacted with substrate *o*-phenanthroline and was measured at 520 nm. The final result of TAC was expressed as 1 ml of blood led absorbency (OD_{520} nm) value increase 0.01/min at 37 °C as one unit (U/ml). In theory, the total antioxidant capacity is a sum of the activities of the various antioxidative substances (Young, 2001). This method has been used frequently for TAC determination in scientific research and gives comparable results (Benzie and Strain, 1996).

Assay for reactive oxygen species (ROS) in white blood cells

Activated white blood cells are the important source of ROS, which may impose on oxidative changes to plasma constituents and neighboring cells, such as circumulating red blood cells (Alice et al., 2001). The level of intracellular accumulation of ROS was determined by the alteration of fluorescence resulting from oxidation of 2′, 7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Sigma) (Lebel et al., 1992; Bass et al., 1983; Ishige et al., 2001). In the presence of ROS, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), DCFH is rapidly oxidized to highly fluorescent 2′–7′-dichlorofluorescein (DCF). Thus, the DCF assay, which detects all of the oxidizing species, provides a global approach to evaluating the production of ROS. DCFH-DA was dissolved in DMSO to a final concentration of 20 mM before use. Activated white blood cells are important sources of ROS. White blood cells were separated and then incubated with 10 μM DCFH-DA at 37 °C for 30 min, the excess DCFH-DA was washed with RPMI-1640 medium. The intensity of fluorescence was recorded using a fluorescence spectrophotometer, with an excitation filter of 485 nm and an emission filter 535 nm. The ROS level was calculated as a ratio: ROS=mean intensity of exposed cells/mean intensity of unexposed cells.

Assay for thiobarbituric acid reactive substances (TBARS) content in tissue

TBARS, the marker of oxidative damage of lipid, contained in liver, brain and heart was determined using thiobarbituric acid (TBA) method with modification (Heath and Packer, 1965). Briefly, tissue homogenate (prepared in 0.5 ml of PBS with 1% SDS) was mixed with 2.5 ml of 20% trichloroacetic acid (TCA) and 1 ml of 0.67% TBA, (0.67 g TBA dissolved in 50 ml double distilled water with 0.5 g solid NaOH and 50 ml glacial acid), and the tubes were covered with foil, heated at 95 °C for 30 minutes, and cooled subsequently to ambient temperature. In order to extract the TBARS, 2.5 ml N-butanol was added to each sample after cooling. The tubes were vortexed vigorously for 10 seconds, centrifuged at 5000 g for 10 minutes. The upper N-butanol layer with the extracted TBARS was transferred to a glass tube. The absorbance of the butanol phase was read at 532 nm. The TBARS content was expressed as $nmol \cdot mg^{-1}$ protein.

Assay for carbonyl content in tissue

Protein carbonyl, the marker of oxidative damage of protein, in tissue homogenate was measured according to method described in the literature (Levine et al., 1990). In each experiment, a 10% tissue homogenate was prepared in 5 mM PBS (pH 7.5) containing protease inhibitors leupeptin (0.5 μg/ml), aprotenin (0.5 μg/ml) and pepstain (0.7 μg/ml) and 0.1% Triton X-100, using a glass homogenizer at 0□. The homogenate was centrifuged at 700×g and 500-μl aliquots of the resulting supernatant

containing 1.6–2.0 mg protein were administered with 300 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl, or with 2 M HCl alone in the control. Samples were then incubated for 1 h at room temperature with vortexing every 10 min, then 20% TCA was added to final concentration 10%, centrifuged the tubes at 11,000 ×g for 3 min, and the supernatant was discarded. The pellet was washed 3 times with 1 ml ethanol-ethyl acetate (1:1) to remove free reagent. The sample was stood 10 min before centrifugation and the supernatant was discarded each time. The precipitate was redissolved in 0.6 ml guanidine solution (6 M, with 20 mM potassium phosphate, adjusted to pH 2.3 with trifluoroacetic acid) for 15 min at 37 °C. The solution was centrifuged at 11,000 × g for 3 min. HCl 2 M was added into the supernatant instead of 2, 4-dinitrophenylhydrazine as a blank. Spectrum absorbance at 370 nm against complementary blank was read. The carbonyl content was calculated with coefficient of 22 000 M^{-1} cm^{-1}.

Assay for protein content

Protein content was measured by Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard.

Data analysis

The scores of withdrawal symptoms and other data are expressed as mean ± SD, and individual comparisons within the group were made by the two-tailed Dunnett's test. The data between two groups were subject to two-tail Student's *t* test. Statistical differences at $P<0.05$ were considered significant. Spearman correlation test was used for the correlations between oxidative stress and withdrawal behaviour with MSExcel statistical software.

Results

Established heroin-dependent model in mice via intraperitoneal injection

Heroin-administered mice exhibited significant withdrawal signs, jumping, shaking and exploring, were increased to 6.8, 3.5 and 28.3 counts/30 min comparing with control mice 2, 1.7 and 12.2 counts/30 min respectively (Table 1). Therefore, the heroin-dependent model in mice via intraperitoneal injection was established.

Table 1
Different withdrawal syndromes in heroin administered mice

	Jumping	Shaking	Exploring
		counts/ 30 min	
Control	2.0 ± 0.25	1.7 ± 0.3	12.2 ± 2.1
Heroin alone	6.8 ± 0.77[#]	3.5 ± 0.5[#]	28.3 ± 4.1[##]

[#] $p<0.05$.
[##] $p<0.01$.

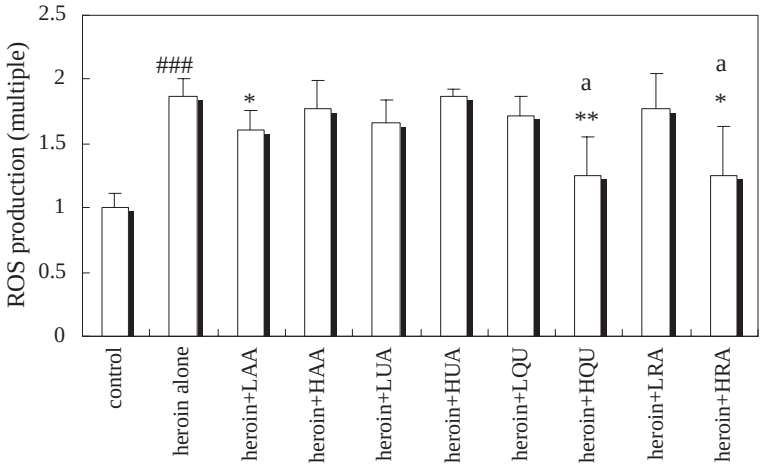

Fig. 2. ROS production of white blood cells in mice was stimulated by heroin and inhibited by some antioxidants. Mice were pretreated with saline (control) or heroin twice daily for 15 days. From the day 16th to 20th, different antioxidants were injected once every day in antioxidant therapy groups, and solvent was injected to the heroin alone group and control group. On the day 21st, white blood cells were collected and used for assays of ROS at once. The level of blood ROS was measured by the alteration of fluorescence resulting from oxidation of 2′, 7′-dichlorofluorescein diacetate. Data plotted are means ± *SD*. $^{*}p<0.05$ and $^{**}p<0.01$ vs heroin alone; $^{\#\#\#}p<0.001$ and $^{a}p>0.05$ vs control (Student's *t* test).

Increase of ROS production and decrease of total antioxidant capacity (TAC) level in heroin addicted mice

After treated with heroin for 15 days, ROS production in white blood cells of mice increased significantly up to 1.86-fold of control (Fig. 2), meanwhile, TAC decreased 69.4% from 330.5 U/ml

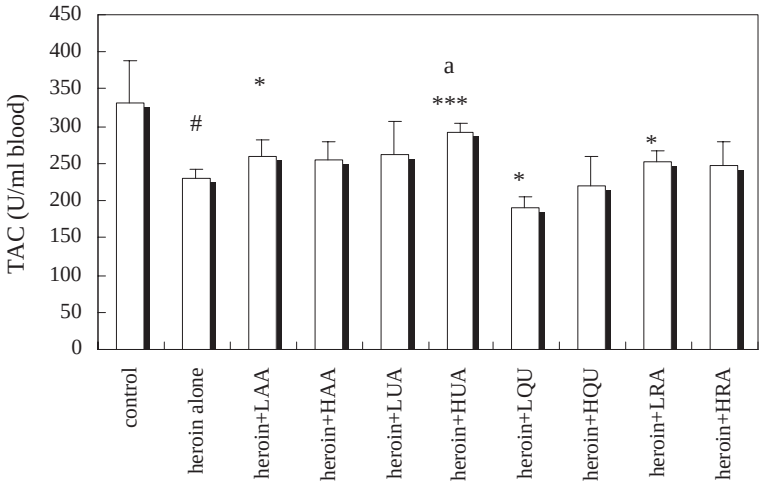

Fig. 3. Total antioxidant capacity (TAC) of blood in mice was inhibited by heroin and increased by some antioxidants. The treatments are same as Fig. 2. The TAC was measured by the alteration of the ferric-reduction ability of blood. Data plotted are means ± *SD*. $^{*}p<0.05$ and $^{***}p<0.001$ vs heroin alone; $^{\#}p<0.05$ and $^{a}p>0.05$ vs control (Student's *t* test).

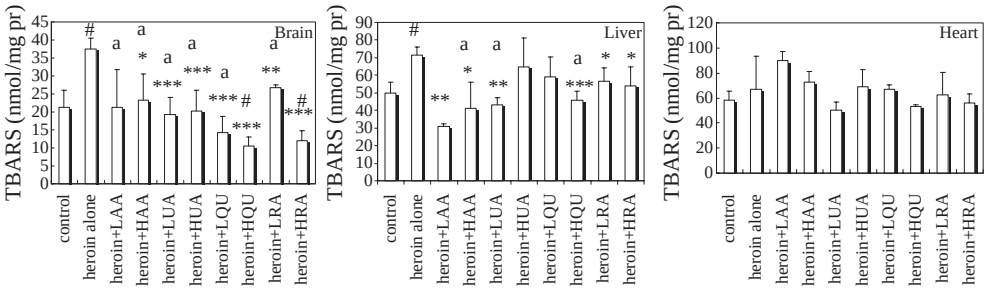

Fig. 4. TBARS content in mice was stimulated by heroin and inhibited by some antioxidants. The treatments are same as Fig. 2. On the day 21st, animals were sacrificed and tissues were collected and kept at -80 °C until analysis. TBARS in liver, brain and heart were determined using thiobarbituric acid (TBA) method with modification. Data plotted are means ± *SD*. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. heroin alone; $^{\#}p<0.05$ and $^{a}p>0.05$ vs. control (Student's *t* test).

blood of control to 229.4 U/ml blood of heroin group (Fig. 3). It is suggested that heroin addicted mice were suffered from a serious oxidative stress, and their antioxidative defense systems were destroyed during the course of heroin addiction.

Increases of TBARS contents of brain and liver but not of heart in heroin addicted mice

After administered with heroin for 15 days, the TBARS contents of liver and brain increased 1.43-fold and 1.76-fold respectively from 50.09 and 21.33 nmol/mg pr to 71.55 and 37.47 nmol/mg pr, but no significantly increase in heart (Fig. 4). Result showed brain is more sensitive to; oppositely, heart is more resistant to lipid oxidative damage induced by heroin.

Increased carbonoxyl contents in heroin addicted mice

After administered with heroin for 15 days, the products of protein oxidative damage elevated 1.50-fold and 1.31-fold respectively in brain and liver from 1.21 and 3.90 nmol/mg pr to 1.81 and

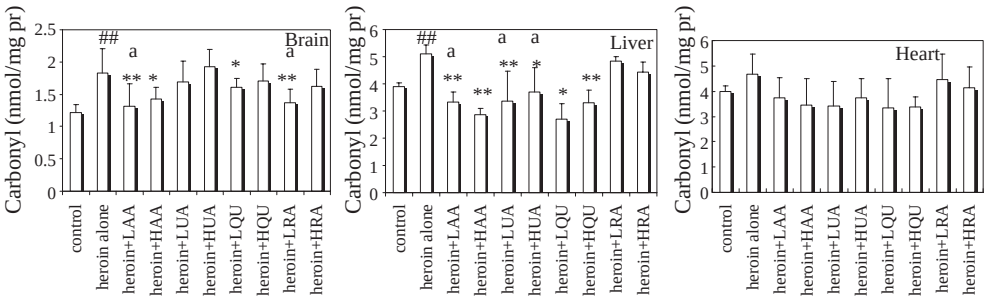

Fig. 5. Carbonoxyl level in mice was stimulated by heroin and inhibited by some antioxidants. The treatments are same as Fig. 2. On the day 21st, animals were sacrificed and tissues were collected and kept at -80 °C until analysis. Protein carbonyl in tissue homogenate was measured according to 2,4-dinitrophenylhydrazine (DNPH) method with modification. Data plotted are means ± *SD*. $^{*}p<0.05$ and $^{**}p<0.01$ vs heroin alone; $^{\#\#}p<0.01$ and $^{a}p>0.05$ vs control (Student's *t* test).

5.10 nmol/g pr, while had no change in heart (p>0.05) (Fig. 5). Result showed brain is more sensitive to; oppositely, heart is more resistant to protein oxidative damage induced by heroin.

The protections of exogenous antioxidants on ROS production, total antioxidant capacity and oxidative damages in heroin addicted mice

Of the serious oxidative stress induced by heroin, exogenous antioxidants (ip) exhibited some good effects. ROS production of white blood cells, total antioxidant capacity of blood, TBARS and carbonyl contents in brain and liver, but not in heart were all amended in different degrees by 4 antioxidants. LAA, HQU and HRA could obviously decrease the ROS production in white blood cells; moreover HQU and HRA were able to decrease ROS to the normal level (Fig. 2). The above three antioxidants and HUA also could increase the total antioxidant capacity of blood remarkably (Fig. 3). All four antioxidants no matter low or high concentration could almost decrease TBARS content in brain and liver, but no in heart, to normal level, however, there are two exceptions, HUA and LQU exhibited no significant effect on TBARS content in liver (Fig. 4). For carbonyl groups, LAA, HAA, LQU and LRA decreased carbonyl groups in brain. Besides LAA, HAA, LQU and LRA, additionally HQA and HUA decreased carbonyl groups in liver too (Fig. 5). These results demonstrated that exogenous antioxidant could reduce the oxidative stress induced by heroin in an all-round way.

The protections of antioxidants on heroin withdrawal behavior

Three obviously signs of heroin withdrawal behavior were measured during the first 30 min after administration with naloxone due to most severe withdrawal behaviors were observed in this period (Lu

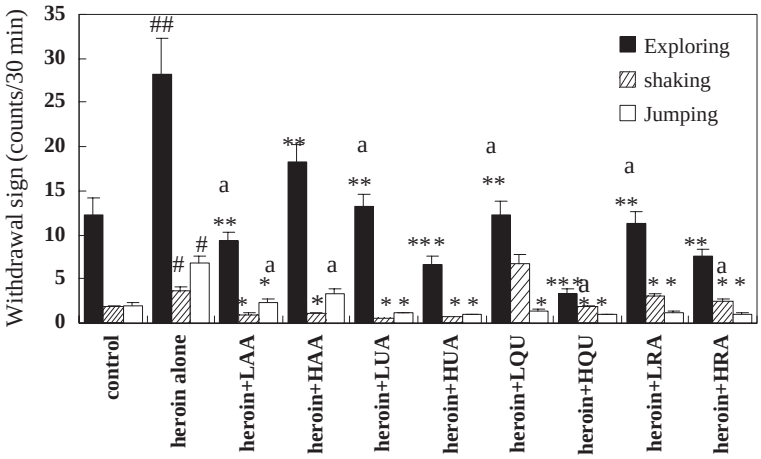

Fig. 6. Effect of exogenous antioxidants pretreatment on heroin withdrawal signs. Mice were pretreated with saline (control) or heroin twice daily for 15 days in the increasing dosage. On day 16, heroin treated mice were randomly assigned to 9 groups. Naloxone (4 mg/kg) was given 1 h after the last heroin injection. Different antioxidants were administered 30 min before naloxone. Three previously identified behavioral characteristics of the mice opiate abstinence syndrome, jumping, shaking and exploring, were recorded over a period of 30 min. Data plotted are means ± *SD*. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs heroin alone; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{a}p>0.05$ vs control (Student's *t* test).

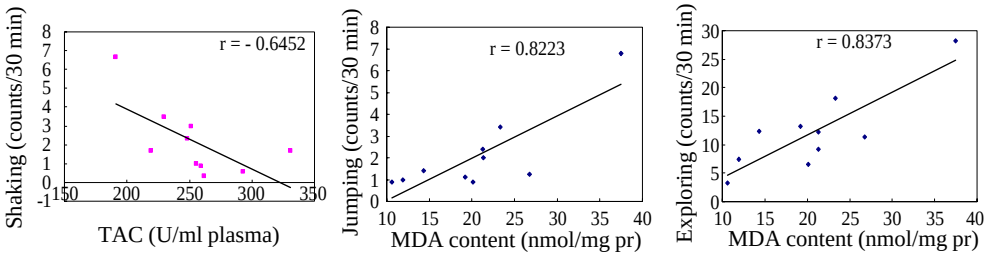

Fig. 7. Correlations between oxidative stress and withdrawal behaviour. The correlations between oxidative stress (ROS production, total antioxidant capacity, TBARS and carbonyl contents) and withdrawal behaviour were subject to CORREL of Office-Excel statistical software. Linear correlation coefficient was plotted as r (n=20, $p<0.05$).

et al., 2001). Significant increases in all of the three signs associated with naloxone-induced heroin withdrawal behavior were observed (Fig. 6).

Correlations between oxidative stress and withdrawal behaviour

To examine the relationship between 3 signs of withdrawal behavior and ROS production, total antioxidant capacity, TBARS and carbonyl contents, only three correlations were found: the TBARS content positively correlated with two withdrawal behaviors, jumping and exploring (the linear correlation coefficient r = 0.8223 and 0.8373 respectively); while the total antioxidant capacity negatively correlated with shaking (r=−0.6452) (Fig. 7). These results also approved that oxidative stress was involved in heroin addiction.

Discussion

Our study found that in heroin addicted mice the ROS production and oxidative damages of protein and lipid increased, as well as total antioxidant capacity decreased, while oxidative damages of biomolecules and withdrawal behavior can be protected by relatively lower concentration of exogenous antioxidants. Moreover, the correlation between withdrawal behavior and the oxidative stress were also found by us. All these results implied that heroin addicted mice are seriously suffered from oxidative stress.

A direct evidence for ROS being concerned with process of opiate dependence is that heroin can directly stimulated the formation of superoxide in glomerular mesangial cells in dose dependent manner, since superoxide has been demonstrated to cause mesangiolysis, it was suggested that ROS may play a role in the induction of mesangial injury in patients with opiate abuse (Singhal et al., 1994). A single heroin administration increased dopamine and xanthine oxidative metabolism with a consequent increase of ROS production (Enrico et al., 1997). In addition, heroin could also be metabolized into free radicals (Di Bello et al., 1998). On the other hand, a single morphine administration decreased antioxidants, such as 5-hydroxytryptamin and ascorbic acid in mice brain (Desole et al., 1996), urinary Se concentration in heroin abusers (Rodriguez et al., 1994), endogenous intracellular GSH in brain and peripheral organs of rodents and in cerebrospinal fluid in patients (Goudas et al., 1999). Thus, heroin caused an increase of ROS formation and a decrease of ROS defense in a vicious circle. When ROS level exceed the antioxidant capacity, a deleterious condition known as oxidative stress occurs.

Conclusion

Although previous studies have shown that heroin, morphine and opiate are able to (i) induce ROS formation in several cells (Oliveira et al., 2002; Sharp et al., 1985), (ii) decrease of the antioxidant defense system including enzymes (Davies, 1988) and antioxidants (Goudas et al., 1999; Rodriguez et al., 1994; Zhou et al., 2000), (iii)increase of lipid peroxidation (Zhou et al., 2000), (iv)prevent of etiology of heroin addiction by antioxidants (Raghavendra and Kulkarni, 1999; Evangelou et al., 2000). However, one of the significant developments in this study is that we found the above 4 respects of evidence in one experiment, and found that brain is more sensitive and heart is most resistant to the oxidative stress induced by heroin. Moreover, the report of protein oxidative damage in heroin abusers had not been found before. At the same time, the analyses of the correlation between oxidative stress and the withdrawal signs also demonstrated that the existences of the oxidative stress in the course of heroin addiction. In one word, strategy of blocking oxidative stress by natural antioxidants may be useful in the development of a new therapy for opiate abusing.

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