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Effects of verbascoside and luteolin on oxidative damage in brain of heroin treated mice

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Extensive but fragmentary studies have shown: (i) heroin, morphine and opiates are able to induce reactive oxygen species (ROS) formation in several cells, (ii) they decrease the antioxidant defense system including enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and antioxidants, glutathione (GSH), Se, and vitamins. This study is to investigate the oxidative damage to DNA, proteins, and lipids in brain of mice administered heroin *via* intraperitoneal injection, and the effects of verbascoside and luteolin on this damage. All the indices of oxidative damage, such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), protein carbonyl group and malondialdehyde (MDA) contents increased significantly compared to those of controls in the brains of heroin-administered mice, while the indices related to the *in vivo* antioxidative capacity, such as the ratio of GSH and oxidized glutathione (GSSG), and activities of SOD, CAT and GPx in the brain, and total antioxidant capacity (TAC) in serum significantly decreased. When heroin-dependent mice were treated with verbascoside or luteolin, oxidative stress status was limited.

1. Introduction

Although there is no direct evidence for the production of ROS in heroin abusers, heroin has been shown to elevate synaptic dopamine (DA) levels (Di Chiara and Imperato 1988). It is well known that an increase in DA oxidative metabolism leads to an increase in ROS formation (Desole et al. 1995; Spina et al. 1989). Therefore, ROS have been frequently associated with neuronal cell death due to damage to carbohydrates, amino acids, phospholipids, and nucleic acids. Moreover, the rise in ROS levels can be followed by a decrease in the levels of reduced glutathione (GSH), which can lead to the inhibition of mitochondrial respiratory chain activity and, subsequently, to oxidative damage. In fact, it has been demonstrated *in vitro* that heroin is able to induce the production of ROS, and leads to oxidative cell injury in PC12 cells (Oliveira et al. 2002). Furthermore, morphine, a metabolite of heroin, can directly affect the formation of superoxide in glomerular mesangial cells (Singhal et al. 1994).

On the other hand, some exogenous antioxidants, such as ascorbic acid (Evangelou et al. 2000) and melatonin (*N*-acetyl-5-methoxytryptamine) (Reiter et al. 2002; Raghavendra and Kulkarni 1999) are able to attenuate heroin or morphine-withdrawal symptoms, and sodium selenite has also been found to be able to protect the liver against injury induced by heroin and morphine (Nagamatsu and Hasegawa, 1993).

Therefore, we surmise that heroin may cause oxidative stress and lead to oxidative damage of biomolecules, which could have pathophysiological relevance in the organic injuries of heroin addiction. Whether oxidative damage of two very important biomolecules, DNA and protein, occurs in heroin abuse has not been studied, although lipid peroxidation caused by heroin has been reported (Zhou et al. 2000).

In the present study, we investigated the oxidative damage of DNA, proteins and lipids as well as other indexes related to oxidative stress, in the brain of heroin-administered mice, and we also studied the effects of the exogenous antioxidants, verbascoside and luteolin, to confirm our hypotheses, while also seeking a mechanism for treatment of heroin abusers.

2. Investigations and results

As shown in Fig. 1, the content of 8-OHdG, carbonyl groups and MDA increased in the brain of heroin administrated mice with prolonged administration time and increasing dose.

As shown in Fig. 2, the TAC in serum and the activity of the anti-oxidative enzymes, GSH/GSSG in the brain finally decreased in heroin administrated mice. The activities of SOD and CAT, and TAC were higher at the beginning of the test period than those in controls, and all of them eventually decreased with prolonged administration time and increasing dose. The activity of GPx and GSH/GSSG significantly declined over the whole test period.

After mice were treated with heroin for 40 days, the heroin-administrated mice were divided into two groups. One group was injected with verbascoside or luteolin for an-

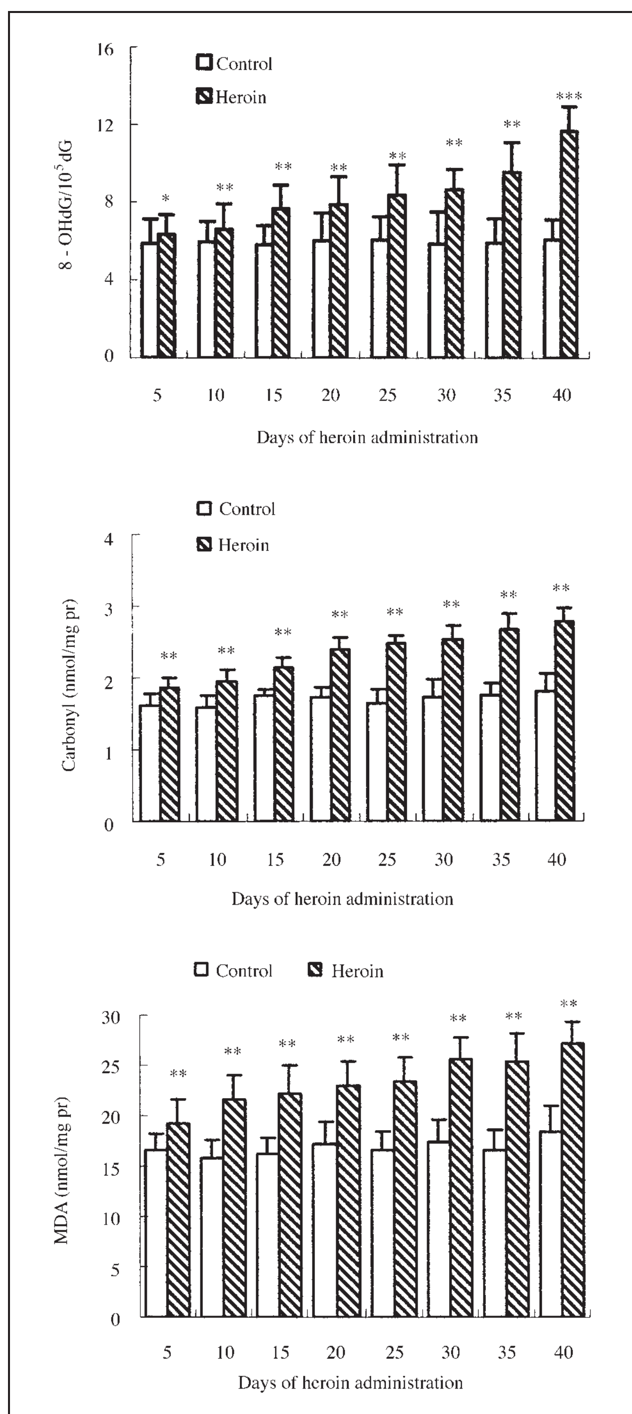

Fig. 1: Increase in 8-OHdG/10^5dG and contents of carbonyl group and MDA in brain of heroin administered mice with longer time and increasing dose. ** $P < 0.01$, *** $P < 0.001$ vs. control, n = 15

other 5 days; while the other group was injected with the same volume of solvent instead of antioxidants. The exogenous antioxidants, luteolin-7-glucoside and verbascoside, decreased the content of 8-OhdG, carbonyl groups, and MDA in the brain of heroin administrated-mice (Fig. 3).

3. Discussion

After administration of heroin, mice showed decreased capability of their antioxidant defense system, such as reduction of GSH/GSSG, TAC, SOD, CAT and GPx in serum or brain, whereas the production of oxidative damage increased, reflected by increased levels of 8-OHdG/10^5dG, carbonyl groups and MDA. Furthermore, antioxidants including verbascoside and luteolin were able to limit oxidative stress in heroin-administered mice. The convincing evidence outlined above powerfully demonstrates that there was serious oxidative stress in heroin-administered mice. In fact, some data reported by others also support our results.

First, the opiate was able to induce the production of ROS. 1. It has been demonstrated that heroin or morphine are able to induce the production of ROS in several cells, such as, PC12 cells (Oliveira et al. 2002), and glomerular mesangial cells (Singhal, et al. 1994); 2. It has been demonstrated that morphine is able to induce an increase in DA and xanthine oxidative metabolism (Desole et al. 1996; Enrico et al. 1997). It is well known that ROS can be generated during both DA (Spina and Akil 1991) and xanthine oxidase (XO) metabolism (Becker et al. 1993), xanthine can be oxidized by XO and produce superoxide anion ($O_2{}^{\bullet-}$), and dopamine can be oxidized by $O_2{}^{\bullet-}$ through monoamine oxidase-B (MAO-B) in humans, giving rise to dihydroxyphenylacetic acid (DOPAC) and H_2O_2 (Olanow and Tatton 1999). H_2O_2 can interact with transition metal ions and produce the highly toxic hydroxyl radical ($^{\bullet}$OH) *via* the Fenton-Haber Weiss reaction (Sevanian et al. 1991).

Secondly, our results clearly indicate that heroin reduced the antioxidative defense system. On the one hand, to mop up excessive ROS induced by heroin will consume a great deal of antioxidants, e.g. heroin or morphine can decrease the level of GSH in brain (Goudas et al. 1999) and liver (Eklow-Lastbom et al. 1986), and vitamin C, vitamin E, and β-carotene in plasma of heroin abusers (Zhou et al. 2000). On the other hand, excessive ROS caused the structural alteration and functional inactivation of many enzymatic proteins including SOD, CAT and GPx (Davies 1988).

For elimination of excessive ROS, biological antioxidants including SOD, CAT, GPx and nonenzymatic antioxidants will react with most oxidants. However, when ROS levels exceed the antioxidant capacity, a deleterious condition known as oxidative stress occurs. Unchecked, excessive ROS can lead to the damage of cellular components including DNA, proteins and lipids, and ultimately cell death *via* apoptosis or necrosis, e.g., morphine can induce lymphocyte apoptosis (Wang et al. 2001; Singhal et al. 1999), which may result in compromised immune function, heroin and morphine can induce undifferentiated PC 12 cell apoptosis (Oliveira et al. 2002), which involves neurodegeneration induced by the drugs and ROS, and morphine promoted the content of MDA (Knight et al. 1988; Panchenko et al. 1999; Zhou et al. 2000). Our results not only confirmed the lipid oxidative damage, but also found that DNA and protein were seriously damaged.

Thirdly, our results showed that exogenous antioxidants, ascorbic acid, uric acid, glutathione, and mannitol were able to abate the oxidative damage to DNA, proteins and lipids caused by heroin. It has been reported that glutathione, alpha-tocopherol and the spin trapper 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) can protect lipid peroxidation (Di Bello et al. 1998), which is consistent with our results.

Fourthly, our results also showed that the exogenous antioxidants, luteolin-7-glucoside and verbascoside, were able to attenuate oxidative damage to DNA, proteins and lipids induced by heroin. Above all, from an other angle, they confirm our hypothesis that heroin abuse is associated with serious oxidative stress and damage.

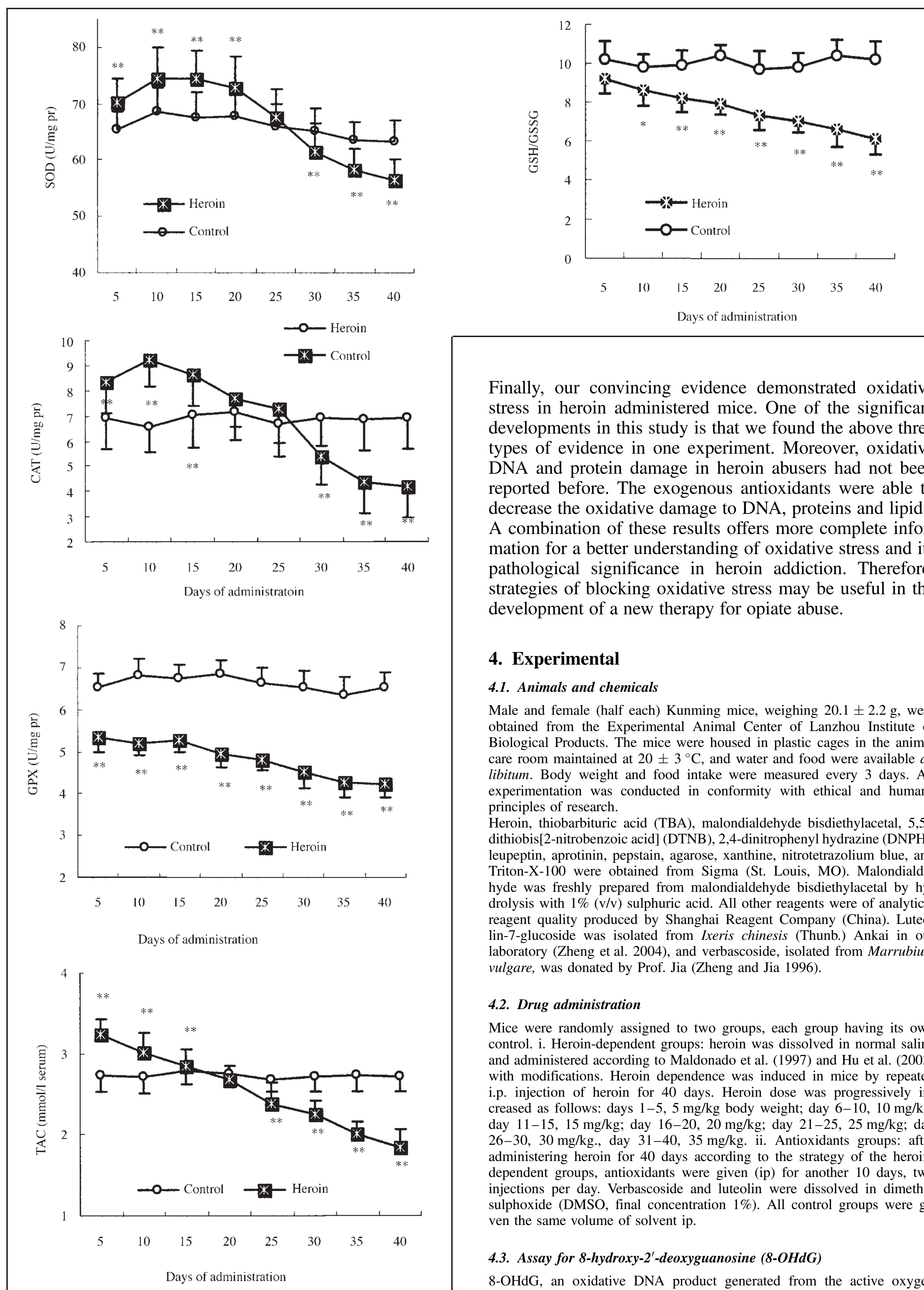

Fig. 2: The effects of heroin on total antioxidant capacity (TAC) in serum and the activities of SOD, CAT, and GPx, and GSH/GSSG in brain. ** $P < 0.01$ vs. control, n = 15

Finally, our convincing evidence demonstrated oxidative stress in heroin administered mice. One of the significant developments in this study is that we found the above three types of evidence in one experiment. Moreover, oxidative DNA and protein damage in heroin abusers had not been reported before. The exogenous antioxidants were able to decrease the oxidative damage to DNA, proteins and lipids. A combination of these results offers more complete information for a better understanding of oxidative stress and its pathological significance in heroin addiction. Therefore, strategies of blocking oxidative stress may be useful in the development of a new therapy for opiate abuse.

4. Experimental

4.1. *Animals and chemicals*

Male and female (half each) Kunming mice, weighing 20.1 ± 2.2 g, were obtained from the Experimental Animal Center of Lanzhou Institute of Biological Products. The mice were housed in plastic cages in the animal care room maintained at 20 ± 3 °C, and water and food were available *ad libitum*. Body weight and food intake were measured every 3 days. All experimentation was conducted in conformity with ethical and humane principles of research.

Heroin, thiobarbituric acid (TBA), malondialdehyde bisdiethylacetal, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), 2,4-dinitrophenyl hydrazine (DNPH), leupeptin, aprotinin, pepstain, agarose, xanthine, nitrotetrazolium blue, and Triton-X-100 were obtained from Sigma (St. Louis, MO). Malondialdehyde was freshly prepared from malondialdehyde bisdiethylacetal by hydrolysis with 1% (v/v) sulphuric acid. All other reagents were of analytical reagent quality produced by Shanghai Reagent Company (China). Luteolin-7-glucoside was isolated from *Ixeris chinesis* (Thunb.) Ankai in our laboratory (Zheng et al. 2004), and verbascoside, isolated from *Marrubium vulgare,* was donated by Prof. Jia (Zheng and Jia 1996).

4.2. *Drug administration*

Mice were randomly assigned to two groups, each group having its own control. i. Heroin-dependent groups: heroin was dissolved in normal saline and administered according to Maldonado et al. (1997) and Hu et al. (2003) with modifications. Heroin dependence was induced in mice by repeated i.p. injection of heroin for 40 days. Heroin dose was progressively increased as follows: days 1–5, 5 mg/kg body weight; day 6–10, 10 mg/kg; day 11–15, 15 mg/kg; day 16–20, 20 mg/kg; day 21–25, 25 mg/kg; day 26–30, 30 mg/kg., day 31–40, 35 mg/kg. ii. Antioxidants groups: after administering heroin for 40 days according to the strategy of the heroin-dependent groups, antioxidants were given (ip) for another 10 days, two injections per day. Verbascoside and luteolin were dissolved in dimethyl sulphoxide (DMSO, final concentration 1%). All control groups were given the same volume of solvent ip.

4.3. *Assay for 8-hydroxy-2′-deoxyguanosine (8-OHdG)*

8-OHdG, an oxidative DNA product generated from the active oxygen species, was detected in brains. To minimize artificial 8-OHdG generation during sample preparation, DNA isolation from brain was performed according to the pronase/ethanol method described by Kendall et al. (1991). Briefly, DNA was extracted from brains by homogenization in 1 ml of

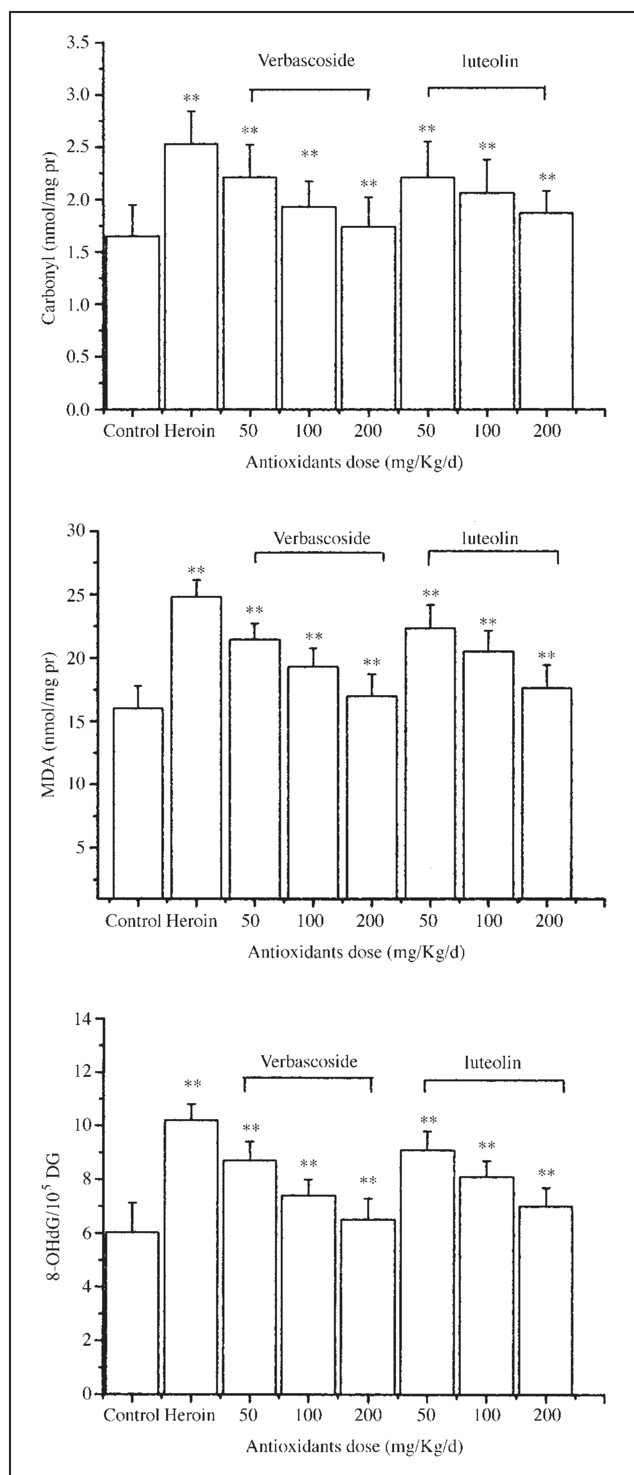

Fig. 3: Effects of verbascoside and luteolin on oxidative damage of DNA, protein and lipid in mouse brain induced by heroin, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. heroin; $^{++}$ $P < 0.01$, $^{+++}$ $P < 0.001$ vs. control, n = 15. Heroin group: after administration of heroin for 40 days, mice were treated ip with the same volume of solvent instead of verbascoside or luteolin for another 10 days

buffer containing 1% sodium dodecyl sulfate, 10 mM Tris, 1 mM EDTA (pH 7.4) and overnight incubation in 0.5 mg/ml proteinase K at 55 °C. Homogenates were incubated with RNase (0.1 mg/ml) at 50 °C for 10 min and extracted twice with chloroform/isoamyl alcohol (24 : 1, v/v). The extracts were mixed (1 : 15 v/v) with 3 M sodium acetate (pH 7.0) and 2 vols of 100% cold ethanol to precipitate DNA at −20 °C for 1 h. The samples were centrifuged at 17,000 × g for 10 min. The resultant DNA pellets were washed twice with 70% ethanol, air-dried for 3 min and dissolved in 100 ml of 10 mM Tris/1 mM EDTA (pH 7.4) (Liang et al. 2000). DNA digestion was performed as described by Kasai et al. (1986). The oxidative DNA adduct, 8-OHdG, was measured by HPLC with electrochemical and UV detection (Kaneko et al. 1996) using a CoulArray system (Model 5600). Analytes were detected on two coulometric array cell modules, each containing four electrochemical sensors attached in series. UV detection was set at 260 nm. The HPLC was controlled and the data acquired and analysed using CoulArray software. The mobile phase was composed of 50 mM sodium acetate/5% methanol at pH 5.2. Electrochemical detection potentials for 8-OHdG were 120/230/280/420/600/750/840/900 mV (vs Pd) and the flow rate was 1.0 ml/min (31 °C). The amount of 8-OHdG was expressed as the number of 8-OHdG molecules for every 10^5 dG in DNA (Kaneko et al. 1996).

4.4. Assay for carbonyl content

Protein carbonyl content, the marker of oxidative protein damage, was measured in brain homogenate according to Levine et al. (1990). In each experiment, a 10% brain homogenate was prepared in 5 mM PBS (pH 7.5) containing protease inhibitor 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 °C. The homogenate was centrifuged at 700 × g and 500-μl aliquots of the resulting supernatant containing 1.6–2.0 mg protein were added to 300 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl, or to 2 M HCl alone in the controls. Samples were then incubated for 1 h at room temperature with vortexing every 10 min, then 20% trichloroacetic acid was added to a final concentration of 10%, the tubes were centrifuged 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 left to stand for 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. 2 M HCl were added to the supernatant instead of 2,4-dinitrophenylhydrazine as a blank. Spectrum absorbance at 370 nm against the corresponding blank was read. The carbonyl content was calculated with a coefficient of 22 000 M^{-1} cm^{-1}.

4.5. Assay for malondialdehyde (MDA) content

MDA, the marker of oxidative lipid damage, was measured to estimate the extent of lipid peroxidation. MDA content in brain homogenate was determined using an HPLC method (Ren et al. 1998). Briefly, brain tissue homogenate (prepared in 0.5 ml of PBS with 1% SDS) was centrifuged at 1800 × g for 10 min. The supernatant (400 μl) was vigorously mixed with 400 μl of acetonitrile and centrifuged at 1800 × g for 20 min. The supernatant was filtered through a 0.2-μm filter (Millipore). The Waters Associates HPLC system was equipped with a Model 6000 A solvent delivery system and an N60 injector. Separation was achieved on a Waters carbohydrate analysis column. The mobile phase consisted of acetonitrile in 0.03 M Tris buffer, pH 7.4 (98 : 2, v/v), at a flow rate of 0.9 ml/min. Detection of MDA was based on absorbence at 267 nm. The molar extinction coefficient is 31,800 M^{-1} cm^{-1}. Quantification of MDA was performed by electronic integration with a Varian CDS-401 digital integrator.

4.6. Assay for GSH and oxidized glutathione (GSSG) content

GSH and GSSG in brain tissue were determined according to Hissin and Hilf (1976) with minor modification. To 0.5 ml of 100,000 × g supernatant, 4.5 ml of phosphate-EDTA buffer, pH 8.0, was added. The final assay mixture (2.0 ml) contained 0.1 ml of the dilution tissue supernatant, 1.8 ml phosphate-EDTA buffer, and 0.1 ml of the *o*-phthaldehyde solution, containing 0.1 mg of *o*-phthaldehyde. After thoroughly mixing, the mixture was kept at room temperature for 15 min. For assay of GSSG, 4.3 ml of 0.1 N NaOH was added after the 0.5 ml sample was incubated with 0.2 ml of 0.04 M *N*-ethylmaleimide for 30 min. The solution for assaying GSH and GSSG as mentioned above was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with activation at 350 nm. GSH and GSSG contents were calculated from a standard curve of GSH or GSSG.

4.7. Assay for total antioxidant capacity (TAC) in serum

A standardized solution of Fe–EDTA complex reacts with hydrogen peroxide by a Fenton-type reaction, leading to the formation of hydroxyl radicals (•OH). These ROS degrade benzoate, resulting in the release of thiobarbituric acid reactive substances (TBARS). Antioxidants from the added serum samples cause suppression of the production of TBARS. This reaction can be measured spectrophotometrically and the inhibition of colour development defined as the TAC, a solution of 1 mM uric acid being used as standard. TAC was defined as mmol/l. Analytical procedures were carried out according to the methods described by Koracevic et al. (2001).

In theory, the total antioxidative capacity is a sum of the activities of the various antioxidative substances. Paradoxically, it is nearly impossible to measure TAC in a single assay (Young et al. 2001), and the Randox method has been used frequently for TAC determination in scientific research. This method gives comparable results (Koracevic et al. 2001).

4.8. Assay for activity of catalase (CAT)

Brain homogenate was prepared in 10 mM Tris-HCl, pH 7.5; 0.25 M sucrose, 1 mM EDTA, 0.5 mM DL-dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Triton X-100 was added to a final concentration of 1% and the sample was incubated for 30 min at 4 °C. After centrifugation at 3000 × g for 15 min, the CAT activity in the supernatant was assayed by the rate of disappearance of 15 mM hydrogen peroxide at 240 nm in phosphate buffer, pH 7.0 (Aebi 1984). The molar extinction coefficient of H_2O_2 is 43.6 $M^{-1} \cdot cm^{-1}$. The consumption of 1 μmol H_2O_2 per min was taken as 1 activity unit.

4.9. Assay for activity of superoxide dismutase (SOD)

The activity of SOD was measured at 550 nm by a spectrophotometer as the rate of suppression of the reduction of nitrotetrazolium blue when superoxide anion radical was generated during oxidation of xanthine by xanthine oxidase (Misra and Fridovich 1976). The reaction mixture contained 50 mM sodium carbonate dissolved in 50 mM K, Na-phosphate buffer (pH 7.8, at 25 °C), 0.1 mM EDTA, 0.1 mM xanthine, and 0.025 mM nitrotetrazolium blue. The activity of SOD was calculated according to the standard curve of SOD and expressed as $U \cdot mg^{-1}$ protein.

4.10. Assay for activity of glutathione peroxidase (GPx)

GPx activity was measured at 340 nm by spectrophotometer in a reaction medium containing 50 mM K, Na-phosphate buffer (pH 7.4, at 30 °C), 1 mM EDTA, 0.12 mM NADPH, 0.85 mM reduced glutathione (GSH), 0.5 U/ml yeast glutathione reductase, and 0.2 mM tert-butyl hydroperoxide as a substrate. The amount of the enzyme converting 1 μM GSH per min was taken as 1 unit (Wendel et al. 1975).

4.11. Assay for protein content

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

4.12. Statistical analysis

Quantitative differences between groups values were statistically analyzed using ANOVA (analysis of variance) with a multiple comparison post-test by the Bonferroni method. P values of < 0.05 were considered statistically significant. All values were expressed as mean ± SD.

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