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EFFECTS OF MARIJUANA AND DIAZEPAM ON LIPID PEROXIDATION, NA^+, K^+ ATPASE, AND LEVELS OF GLUTATHIONE AND 5-HTP IN RAT BRAIN

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Our aim was to evaluate the effects of marijuana (Mar) and diazepam (Dz) on lipid peroxidation (TBARS), Na^+, K^+ ATPase activity, levels of glutathione (GSH) and 5-hydroxytryptophan (5-HTP). Male Wistar rats were given a single dose per group: extract of Mar (100 µL/kg), Dz (5 mg/kg), Mar plus Dz, and NaCl for control. Sixty mins after treatment, animals were sacrificed, and their brains extracted and homogenised to measure GSH, TBARS and 5-HTP levels. Na^+, K^+ ATPase and total ATPase activities. GSH and TBARS did not show differences respect to controls. Na^+, K^+ ATPase activity was similar as well. However, groups treated with Mar, total ATPase activity decreased significantly ($p < 0.05$). Levels of 5-HTP decreased significantly ($p = 0.0001$) in rats treated either with Mar and or Dz. Mar and Dz induced biochemical effects on the serotonergic metabolism, which can alter the development and function in rat brain, because it has also been involved in scavenging free radicals present there.

Keywords: Brain – diazepam – glutathione – marijuana – rat

INTRODUCTION

Marijuana is a plant that contains more than 460 different substances, including abundant cannabinoids, such as the tetrahydrocannabinol, which induce biochemical alterations in the central nervous system (CNS) [37], including a possible neuroprotective effect during ischemic events [16]. For this reason, the use of marijuana with therapeutic purposes is officially authorised [31] in a few cities in the United States, and in some European countries; however, in several countries its use as well as its distribution and sale are considered illegal.

Marijuana can interact with some drugs that are currently used to improve people's health. The substances contained in this plant have been associated to disturbances in the central nervous, cardiovascular, respiratory and immunological systems. In spite of it, marijuana has been found among the most frequently consumed drugs of abuse. According to this questionnaire, people who use drugs, frequently combine marijuana with other psychotropic drugs that require medical prescription, such as benzodiaze-

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pins. The combination of these drugs has been used in chronic treatment to elucidate the probable mechanism of the observed antianxiety effects of cannabis. This suggests that such effect is mediated through central benzodiazepine receptors [32].

Diazepam belongs to this group of drugs and it is employed as a muscular relaxant, it is also indicated to patients who suffer from anxiety, and is found among the most illegally consumed drugs.

This psychotropic drug is known to disturb the metabolism of serotonin [29], an important neurotransmitter whose biosynthetic pathway can be altered in the decarboxilation step of its precursor 5-hydroxytryptophan (5-HTP) [5]. The clinical use of this precursor has been proposed to improve neurological functions and the maturation of the central nervous system of children with neurologic disorders [11].

Serotonin (5-HT) is necessary for the development and function of the CNS, and it has also been involved in scavenging free radicals present in damaged tissues [9], which can be increased as a consequence of oxidative metabolism in the presence of diverse neuroactive substances [8]. It is well known that the long-term use of drugs has been related to the levels required to reach analgesia. This phenomenon, defined as tolerance, is given by the cellular adaptation to the presence of those drugs [24], which are employed in any hospital service [26].

The μ opioid receptor plays an important role in the addiction to narcotics, in combination with the synaptic function [28], and those drugs also interfere with the activity of the neuronal enzyme Na^+ K^+ ATPase, which is responsible of the ionic exchange across the plasma membrane, so that it can be used as a metabolic probe to study the effect of those drugs in rat brain [35], although its mechanism of action remains to be completely elucidated.

CNS is particularly vulnerable to oxidative damage, whose control depends upon the presence of various antioxidants, such as reduced glutathione (GSH) [19], the molecule most frequently involved in the regulation of redox equilibrium that protects the tissues against the action of free radicals, mainly against those that attack cell membrane [1]. This cell compartment is composed by more than 100 different types of lipids, although one single type can form a lipid bilayer due to its polymorphism, in different tissues [2], being these lipids the main target of the free radicals [1].

It is known that cannabinoids can interact with the endogenous opioid system, besides, previous studies have shown that after the central or peripheral administration of opioids, the levels of GSH decrease in peripheral organs [12]. On the other hand, nitric oxide (NO) can play an important role in the development of tolerance to the opioids [20], which can be controlled by the presence of melatonin, an important biomolecule that arises from the serotonergic metabolism [7], which exerts a significant antioxidant effect within the CNS.

Due to the lack of related studies, the aim of the present work is to evaluate the possible oxidative damaged induced by Mar and Dz on rat brains, by means of the determination of GSH, 5-HTP, TBARS, Na^+, K^+ ATPase and total ATPase as indicators of biochemical disturbances.

MATERIAL AND METHODS

Twenty four male Wistar rats weighing 250 g were used, and were kept in closed cages with water and food *ad libitum,* under a twelve-hour photoperiod [12 hours light/12 hours darkness). Animals were grouped randomly in 6-rat groups, with the following treatment sets: Group I NaCl 0.9%; group II Mar extract 100 μL/kg; group III diazepam 5 mg/kg and group IV Mar+Dz 100 μL/kg+5 mg/kg. For dosages were considered those previously used by Wall et al. [36]. All the substances were administered intraperitoneally (i. p.), in a single dose. All experimental procedure was done with authorization of the Laboratory Animals Use and Care Committee of our institution.

Sixty minutes after administering their corresponding doses, the animals were sacrificed by decapitation, and then we proceeded to obtain the brains and put them in a NaCl 0.9% solution at 4 °C. The brains were sliced into two sections sagitally. The left portion was homogenised in a 0.05 M tris-HCl buffer, pH 7.2 to measure lipid peroxidation (TBARS), Na^+/K^+ ATPase and total ATPase, and the right portion was homogenised in 0.1 M perchloric acid ($HClO_4$) to measure GSH and 5-HTP. All the samples were kept at –20 °C until analysed.

The levels of reduced glutathione (GSH) were determined in the supernatant of homogenised tissue in $HClO_4$ after centrifuging it at 5000 rpm for 10 min (Hettich Zentrifugen, Mikro 12-42 model, Germany) by the technique modified from Hissin and Hilf [17]. An aliquot from the supernatant in $HClO_4$ was mixed with 1.8 mL of phosphate buffer pH 8.0, 0.2% ethylendiamine tetraacetic acid and 100 μL of orthophthaldehyde, 1 mg/mL in methanol, in a test tube. The mixture was incubated during 15 minutes at room temperature and protected from light, and afterwards samples were read at 350 nm/420 nm, excitation/emission wavelengths in a Perkin Elmer LS-55, England, spectrofluorometer FL Win Lab versión 4.00.02 software was used for the analysis.

The levels of 5-HTP were determined in the supernatant of the $HClO_4$-homogenised tissue, as mentioned above for GSH, by the technique developed by Calderón et al. [4]. Samples were filtered through a Millex HV (Sep-Pack C18 de Millipore) filter, and 20 μL were injected to the HPLC equipment to be analysed.

HPLC equipment: High performance liquid chromatographer with turbochrom system version 4.1 (Perkin Elmer) equipped with variable wavelength detector Spectra System UV1000 (Termo Separation Products), manual injector Rheodyne, four-gradient pump LC-1150 (GBC Scientific Equipment), double-channel interphase Perkin Elmer, and 3.9 × 150 mm Nova Pack C_{18} column (Waters).

Chromatographic conditions: 0.01 M sodium acetate mobile phase pH 4.0, Methanol (85 : 15, v/v), 1 mL/min flux velocity, 254 nm wavelength.

Reagents: Sodium acetate. $3H_2O$ and perchloric acid (reagent grade, Merck), methanol (HPLC, Caledon Laboratories), 5-HTP standard (Sigma) and deionised water.

Measurement of lipid peroxidation. TBARS determination was carried out through the technique modified from Gutteridge and Halliwell [14], as outlined herein: Each tissue sample was homogenised in 5 mL of phosphate buffer pH 7.4, from which,

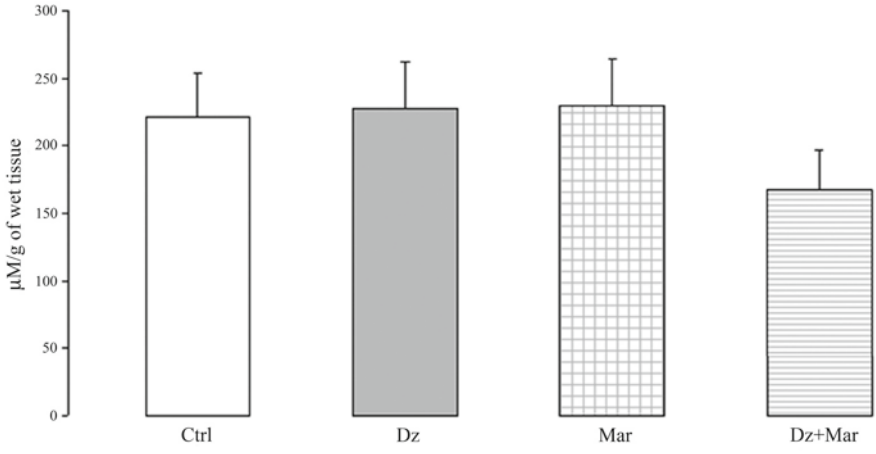

Fig. 1. GSH levels in wistar rat brain after single doses of marijuana and diazepam. Values were inferred from a standard curve and were expressed as nanomoles of GSH per gram of wet tissue. Ctrl = control, Dz = diazepam, Mar = marijuana. Average values (mean) ± D.E.

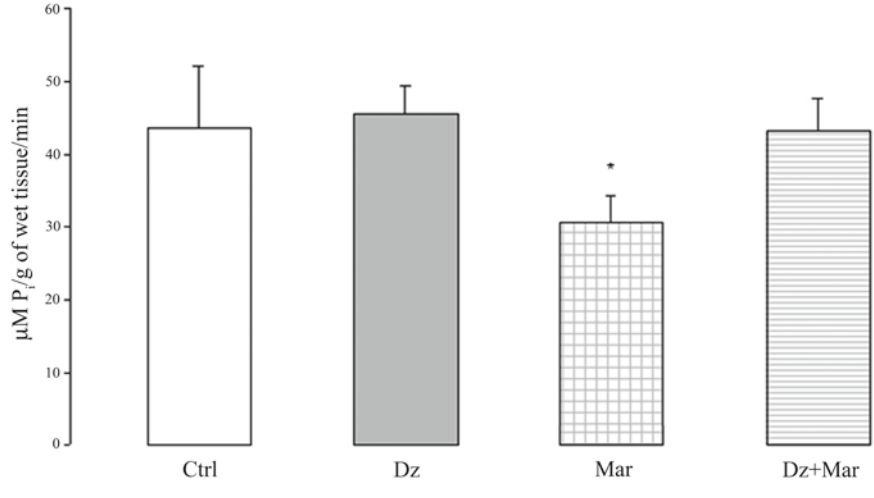

Fig. 2. Total ATPase activity in rat brain after single doses of marijuana and diazepam. Values were expressed as µM of inorganic phosphate (P_i) per gram of wet tissue per minute. Ctrl = control, Dz = diazepam, Mar = marijuana. Mean ± D.E. ANOVA. * $p < 0.05$

1 mL aliquot was taken and added 2 mL of a thiobarbituric acid (TBA) solution, which contained 1.25 g of TBA, 40 g of trichloroacetic acid (TCA) and 6.25 mL of concentrated hydrochloride (HCl), dissolved in 250 mL of distilled water, and was heated to 80 °C in a Thermomix 1420 equipment for 30 minutes. Afterwards, samples were put in an ice bath for 5 minutes and then centrifuged at 3000 g for 15 minutes (Sorvall RC-5B, Dupont centrifuge). Supernatant absorbance was read in a three sample set at 532 nm in a spectrophotometer (Helios-α, UNICAM).

Determination of Na^+, K^+ ATPase activity. The method proposed by Calderón et al. [6], was carried out. Approximately 1 mg aliquots of 10% homogenised brain tissue in 0.05 M tris-HCl, pH 7.4 were incubated for 15 minutes in a medium containing the following: 3 mM $MgCl_2$, 7 mM KCl, 100 mM NaCl, with or without 0.06 mM ouabain. After this time, 4 mM tris-ATP was added and incubated for another 30 minutes at 37 °C in a Dubnoff Labconco shaking bath. Reaction was stopped by adding 100 μL of 10% TCA.

Samples were centrifuged at 3500 rpm for 5 minutes at 4 °C, and a supernatant aliquot was used to measure inorganic phosphate (P_i) according to the method proposed by Fiske and Subbarow [10]. Supernatant absorbance was read at 660 nm using a UNICAM Helios-α spectrophotometer and the difference between the absorbance of both samples with and without ouabain, was considered as the Na^+/K^+ ATPase activity, expressed as μM of inorganic phosphate (P_i) per gram of wet tissue per minute, whereas the total ATPase activity was obtained in the absence of ouabain.

Statistical analysis

In order to represent the data, the graphs considered mean ± standard deviation, and the strategy for the analysis of interference consisted in comparing these values of the studied variables GSH, 5HTP, TBARS, total ATPase and Na^+, K^+ ATPase. The results were analyzed by one-way ANOVA or Tukey test. Contrasts and comparisons for all pairs were obtained by Dunnett and Kramer`s HSD statistical tests, respectively. Values of $p < 0.05$ were considered statistically significant. The analysis was carried out by using the JMP V 7.0 de SAS Institute software (SDS 2003) [33].

RESULTS

Regarding to the levels of GSH in rat brain after treatment with Mar and Dz (Fig. 1), the combination of Mar and Dz induced a small decrease in this naturally occurring antioxidant; however, each one separately, did not induce any appreciable difference with respect to the control group.

Total ATPase activity decreased substantially ($p < 0.05$) in the group that received only Mar extract (Fig. 2), whereas the animals given Dz alone or in combination with Mar extract showed an activity akin to that of the control group. As is shown in Figure

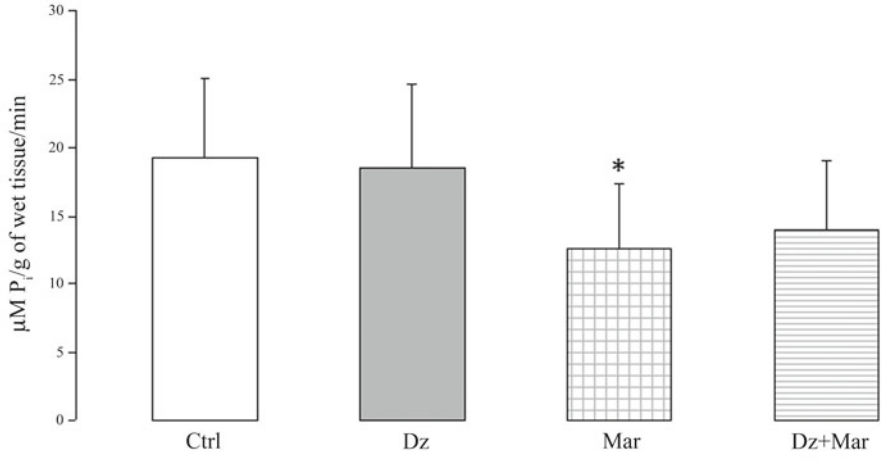

Fig. 3. Na^+, K^+ ATPase activity in rat brain after single doses of marijuana and diazepam. Values were expressed as µM of inorganic phosphate (P_i) per gram of wet tissue per minute. Ctrl = control, Dz = diazepam, Mar = marijuana. Mean ± D.E. ANOVA

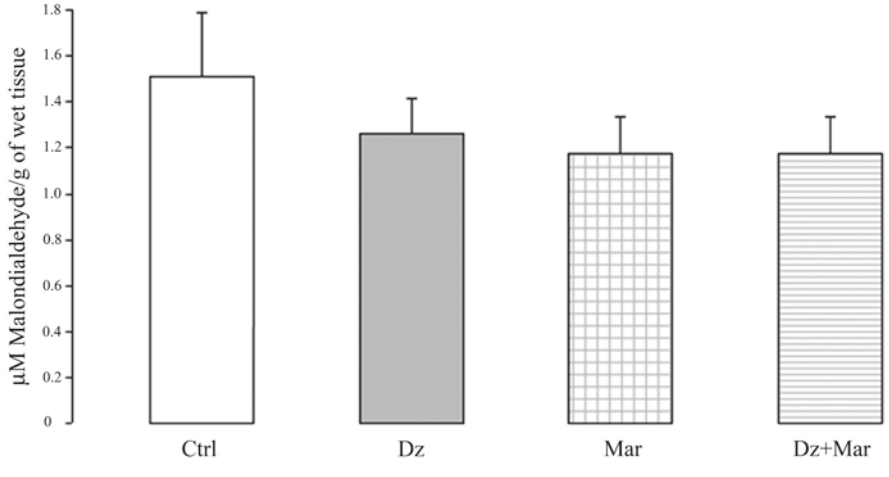

Fig. 4. Lipid peroxidation products in rat brain after single doses of marijuana and diazepam. Levels of thiobarbituric acid reactive substances (TBARS) were expressed in micromoles of malondialdehyde per gram of wet tissue. Ctrl = control, Dz = diazepam, Mar = marijuana. Mean ± D.E. ANOVA

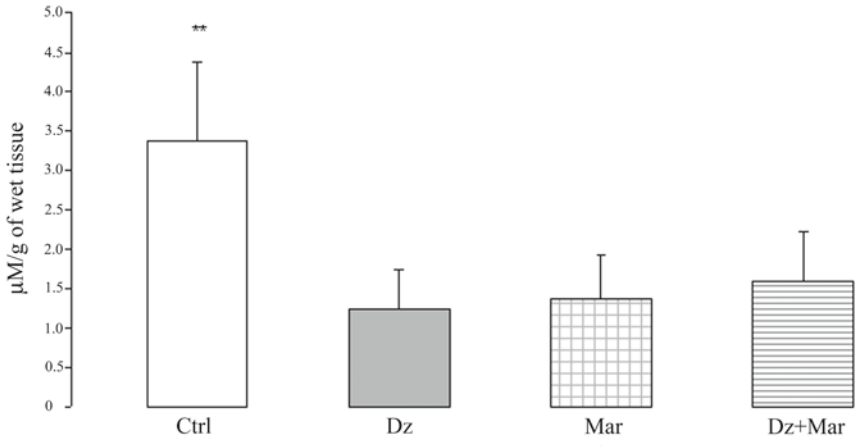

Fig. 5. 5-Hydroxytryptophan levels in rat brain after single doses of marijuana and diazepam. Values were inferred from a previously constructed standard curve and then expressed as micromoles of 5-HTP per gram of wet tissue. Ctrl = control, Dz = diazepam, Mar = marijuana. Mean ± D.E. ANOVA. **p = 0.0001

3, Na^+, K^+ ATPase was found lower than the control values, this difference was statistically lower in those groups treated with Mar.

As is shown in Figure 4, thiobarbituric acid reactive substances (TBARS) had a lower concentration in the animals given either Mar, Dz and Mar + Dz, but the differences were statistically meaningless. A significant change was observed for 5-HTP levels, since in all the treated groups, regardless of administered drug, 5-HTP were smaller than the control values, these were statistically different ($p = 0.0001$) (Fig. 5).

DISCUSSION

Neurochemical mechanisms underlying development of drug dependency have not been fully clarified. The present experimental study provides evidence that biomarkers here analyzed may point at a common biochemical process.

GSH levels decreased after the administration of marijuana combined with diazepam, this effect suggests that the drugs of abuse fail to protect the brain against oxidative damage arising from its own endogenous metabolism [13], and these drugs leave the central nervous system (CNS) vulnerable to oxidative damage, induced by hydrogen peroxide and hydroxyl radicals, produced through the normal metabolic pathways [34], and probably due to the production of nitric oxide (NO), which also induces oxidative stress, and whose synthesis takes place within neurons during the opioid syndrome [18].

Na^+, K^+ ATPase and total ATPase activities decreased substantially after the administration of Mar, suggesting that the inactivation of the enzyme could be responsible for the effect induced by these substances [21, 22], having also an effect on the fluidity of plasma membrane due to the presence of intracellular sodium [3]. Although the Ca^{++} and Mg^{++} ions, which are also responsible for the total ATPase function, could be disturbed by the high density of opioid receptors produced in brain tissue [25].

On the other hand, lipid peroxidation products decreased weakly in those animals given only Mar. These results are in agreement with those reported by Hampson et al. [16], which suggest that the main components of marijuana are antioxidant. This effect of the drugs of abuse may be explained since their chemical structure contains importantly lipophylic functional groups, which confers them a strong electrophylic character toward the lipid-rich cells within the brain.

With respect to the levels of 5-HTP, after the administration of either Mar or Dz decreased those substantially, probably due to the strong serotonergic activity [9], induced by marijuana, or to the inhibitory effect on the synthesis of 5-HT, through the GABAergic effects induced by diazepam [30]. These results suggest that the brain of people using marijuana and diazepam could be left unprotected due to decrease in 5-HTP, since this serotonergic neurotransmitter exerts an antioxidant effect within the brain [23]. On the other hand, since the marijuana extract-treated animals stopped eating normally, it may be interesting to consider the disturbances in, 5-HT and their corresponding metabolites, specially subtype 6 5-HT receptor, since these neurotransmitters play an important role in the control of appetite and hunger [15, 27].

Our results suggest that marijuana and diazepam are drugs that alter serotonergic metabolism, in rat brains, which can modify the development and function of the CNS, because it has also been involved in scavenging free radicals present there.

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REFERENCES

1. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., Freeman, B. A. (1994) Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxides. *Proc. Natl. Acad. Sci. USA. 87,* 1629–1634.
2. Ben de Kruijff (1987) Polymorphic regulation of membrane lipid composition. *Nature 329,* 587–588.
3. Brase, D. A. (1990) Is intracellular sodium involved in the mechanism of tolerance to opioid drugs? *Med. Hypotheses 32,* 161–167.
4. Calderón, G. D., Toledo, L. A., Hernandez, I. J., Barragán, M. G., Rodríguez, P. R., Hernandez, G. E. (2001) Determinación de triptófano en cerebro de ratas expuestas a ozono. *Arch. Neurosci. (Mex) 6,* 2–5.

5. Calderón, G. D., Hernandez, I. J., Espitia, V. I., Barragán, M. G., Hernandez, G. E., Juarez Olguín, H. (2004) Pyridoxine, regardless of serotonin levels, increases production of 5-hydroxytryptophan in rat brain. *Arch. Med. Res. 35,* 271–274.
6. Calderón, G. D., Espitia, V. I., López, D. A., Hernández, G. E., Huerta, G. B., Juarez Olguin, H. (2005) Effect of toluene and nutritional status on serotonin, lipid peroxidation levels and Na^+/K^+-ATPase in adult rat brain. *Neurochem. Res. 30,* 619–624.
7. Ebadi, M., Govitrapong, P., Phansuwan-Pujito, P., Nelson, F., Reiter, R. J. (1998) Pineal opioid receptors and analgesic action of melatonin. *J. Pineal Res. 24,* 193–200.
8. Enrico, P., Mura, M. A., Esposito, G., Serra, P., Migheli, R. (1998) Effect of naloxone on morphine-induced changes in striatal dopamine metabolism and glutamate, ascorbic acid and uric release in freely moving rats. *Brain Res. 797,* 94–102.
9. Fernstrom, J. D., Wurtman, R. J. (1972) Brain serotonin content: Physiological regulation by plasma neutral amino acids. *Science 178,* 414–416.
10. Fiske, C. H., Subbarow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem. 66,* 375–400.
11. García, C. A., Serrano, M., Perez, D. B., Gonzalez, V., Ormazabal, A., Pineda, M. (2007) Secondary abnormalities of neurotransmitters in infants with neurological disorders. *Dev. Med. Child Neurol. 49,* 740–744.
12. Goudas, L. C., Carr, D. B., Maszczynska, I., Marchand, J. E., Wurm, W. H., Greenblatt, D. J. (1997) Differential effect of central versus parenteral administration of morphine sulfate on regional concentrations of reduced glutathione in rat brain. *Pharmacology 54,* 92–97.
13. Goudas, L. C., Langlade, A., Serrie, A., Matson, W., Milbury, P., Thurel, C. (1999) Acute decreases in cerebrospinal fluid glutathione levels after intracerebroventricular morphine for cancer pain. *Anesth. Analg. 89,* 1209–1215.
14. Gutteridge, M. C., Halliwell, B. (1990) The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem. Sci. 5,* 129–135.
15. Halford, J. C., Harrold, J. A., Boyland, E. J., Lawton, C. L., Blundell, J. E. (2007) Serotonergic drugs: effects on appetite expression and use for the treatment of obesity. *Drugs 67,* 27–55.
16. Hampson, A. J., Grimaldi, M., Axelrod, J., Wink, D. (1998) Cannabidiol and (–) D9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc. Natl. Acad. Sci. USA. 95,* 8268–8273.
17. Hissin, P. J., Hilf, R. (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissue. *Anal. Biochem. 74,* 214–226.
18. Jhamandas, J. H., Harris, K. H., Petrov, T., Jhamandas, K. H. (1996) Activation of nitric oxide-synthesizing neurones during precipitated morphine withdrawal. *Neuroreport 7,* 2843–2846.
19. Keromov, B. F. (2004) Glutathione-deficient state of nervous tissues in starved animals intensifies lipid peroxidation and oxidation of protein SH-groups. *Ukr. Biokhim. Zh. 76,* 108–113.
20. Lue, W. M., Su, M. T., Lin, W. B., Tao, P. L. (1999) The role of nitric oxide in the development of morphine tolerance in rat hippocampal slices. *Eur. J. Pharmacol. 383,* 129–135.
21. Masocha, W., González, L. G., Baeyens, J. M., Agil, A. (2002) Mechanisms involved in morphine-induced activation of synaptosomal Na^+, K^+-ATPase. *Brain Res. 957,* 311–319.
22. Masocha, W., Horvath, G., Agil, A., Ocaña, M., Pozo, E., Szikszay, M. (2003) Role of Na^+, K^+-ATPase in morphine-induced antinociception. *J. Pharmacol. Exp. Ther. 306,* 1122–1128.
23. Muñoz, C. J., Montilla, P., Padillo, F. J., Bujalance, I., Muñoz, M. C., Muntané, J. (2006) Role of serotonin in cerebral oxidative stress in rats. *Acta Neurobiol. Exp. 66,* 1–6.
24. Ossipov, M. H., Lai, J., King, T., Vanderah, T. W., Porreca, F. (2005) Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolymers 80,* 319–324.
25. Pillai, N. P., Ross, D. H. (1986) Effects of opiates on high-affinity Ca^{2+}, Mg^{2+}-ATPase in brain membrane subfractions. *J. Neurochem. 47,* 1642–1646.
26. Puppala, B. L., Matwyshyn, G., Bhalla, S., Gulati, A. (2004) Evidence that morphine tolerance may be regulated by endothelin in the neonatal rat. *Biol. Neonate 86,* 138–144.
27. Prunet, M. B., Desbazeille, M., Bros, A., Louche, K., Delagrange, P., Renard, P. (2003) Melatonin reduces body weight gain in sprague dawley rats with diet-induced obesity. *Endocrinology 144,* 5347–5352.

28. Sadee, W., Wang, D., Bilsky, E. J. (2005) Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. *Life Sci. 76,* 1427–1437.
29. Sakabe, T., Dahlgren, N., Carlsson, A., Siesjö, B. K. (1982) Effect of diazepam on cerebral monoamine synthesis during hypoxia and hypercapnia in the rat. *Physiol. Scand. 115,* 57–65.
30. Saner, A., Pletscher, A. (1979) Effect of diazepam on cerebral 5-hydroxytryptamine synthesis. *Eur. J. Pharmacol. 55,* 315–318.
31. Seamon, M. J., Fass, J. A., Maniscalco-Feichtl, M., Abu-Shraie, N. A. (2007) Medical marijuana and the developing role of the pharmacist. *Am. J. Health Sys. Pharm. 64,* 1037–1044.
32. Sethi, B. B., Trivedi, J. K., Kumar, P., Gulati, A., Agarwal, A. K., Sethi, N. (1996) Antianxiety effect of cannabis: involvement of central benzodiazepine receptors. *Biol. Psychiatry 21,* 3–10.
33. The Statistical Discovery Software SDS, Version 7.0 by SAS Institute Inc, 2003.
34. Sun, Y. (1990) Free radicals, antioxidants enzymes and carcinogenesis. *Free Rad. Biol. Med. 8,* 583–599.
35. Wan-Kan, O., Hosein, E. A. (1981) Synaptosomal Na^+, K^+ ATPase as a membrane probe in studying the *in vivo* action of morphine. *Can. J. Biochem. 59,* 687–692.
36. Wall, M. E., Brine, D. R., Perez, R. M. (1976) Metabolism of cannabinoids in man. In: Braude, M. C., Szara, S. (eds) *Pharmacol of Marijuana.* Raven Press, New York, pp. 93–116.
37. Wickelgren, I. (1997) Marijuana: harder than throught? *Science 276,* 1967–1968.