Abstract. Background/Aim: The aim of the present study was to investigate the biological effects of subacute crack cocaine exposure in rat liver. Material and Methods: A total of 32 rats were distributed into four groups (n=8): Experimental group 1 (G1) and Experimental group 2 (G2): rats received 18 mg/kg of body weight (b.w) of crack cocaine for 5 days, once a day, group G2 remained 72 h without exposure after the experimental period (5 days)(abstinence); Experimental group 3 (G3): rats received 36 mg/kg of body weight (b.w) of crack cocaine for 5 days, once a day; Control Group (CTRL): rats received only the vehicle (DMSO) administered by the intraperitoneal (i.p) route for 5 days, once a day. Results: All groups exposed to crack cocaine had an increase in the number of micronucleated hepatocytes and binucleated cells only in the highest tested dose (36 mg/kg). Karyolysis had an increase in the 18 mg/kg dose, in the abstinence group (G2), and 36 mg/kg group (G3); whereas pyknotic nuclei had an increase in the G2 group. The group exposed to 18 mg/kg of crack cocaine also showed high 8 OHdG expression. The p-NF-κB p65 protein decreased in the groups exposed to crack cocaine at doses of 18 and 36 mg/kg, as well as in the abstinence group. MyD88 was also found decreased in the group exposed to crack cocaine at 18 mg/kg. Conclusion: Crack cocaine inhibited toll like signaling pathway whilst being associated with genomic instability in rat liver cells.

Crack cocaine is cocaine in the most harmful form, due to the presence of such remnants (adulterants), which are continuously used through refinement process (1). Usually, crack cocaine is presented in the form of base. The name “crack” is frequently used due to the onomatopoeic expression that refers to the crackling sound generated during pyrolysis, when the drug is consumed by smoking route (2).

Crack cocaine was first seen in Brazil in the early 90s, where it gained great popularity. Nowadays, the largest use and dissemination of crack cocaine across the country have been seen as a major public health problem, due to the severe dependence caused by using the illicit drug. As a result, risk behaviors and acts of violence are frequently observed in crack cocaine users. Such behaviors affect society as a whole, putting the health and safety of the population at risk (3). There is a high prevalence of crack cocaine users visiting health services around the world. In the United States of America (USA), crack cocaine is responsible for the largest number of visits to health services due to the use of illicit drugs. In Brazil, the same condition exists, with the attendance at these services being increasingly frequent due to the indiscriminate use of crack cocaine (4).

To date, few studies have reported the toxicity induced by crack cocaine in multiple organs and tissues. Previous studies conducted by our research group have demonstrated that acute doses of crack cocaine are able to induce genetic damage in multiple organs of rodents (5, 6). In humans, crack cocaine was able to increase the number of micronucleated cells of exfoliated oral mucosa cells as a result of chromosome breakage or loss (7, 8).

Toll like signalling pathway plays a pivotal role in the activation of immune system being responsible for protecting the living organism. Initiation of the toll like signalling pathway, occurs by MyD88 activating the interleukin receptor-associated kinases IRAK1 and IRAK4, which in turn stimulates the tumour necrosis-associated factor TRAF-
Materials and Methods

Animals and experimental design. All procedures were conducted according to the International Research Standards for Animals, and approved by the Animal Ethics Committee of the Federal University of Sao Paulo, UNIFESP, SP, Brazil (protocol no. 7038080219). The crack cocaine sample used in the present research has already undergone a chemical analysis in previous studies by our research group (5). A total of 32 Wistar male rats (Rattus norvegicus) weighing 250 g on average, and 9 weeks of age were distributed into four groups (n=8), as follows: Control (CTRL); Experimental 1 (G1); Experimental 2 (G2) and Experimental 3 (G3). The experimental groups G1 and G2 received 18 mg/kg body weight (b.w.) of crack cocaine administered by intraperitoneal route (i.p.) per 5 days, once a day. The experimental group G3 received 36 mg/kg body weight (b.w.) of crack cocaine administered by intraperitoneal route (i.p.) per 5 days, once a day. Crack cocaine was diluted in dimethylsulfoxide (DMSO). The choice of using DMSO as a vehicle was based on the fact that it is a solvent and a chelating agent, with a high diffusing capacity, easily penetrating all tissues of the organism. To standardize the amount of i.p. injection, the final volume used was 1 ml/kg for all animals. The dose levels used of crack cocaine correspond to 25% (18 mg) and 50% (36 mg) of the cocaine lethal dose to 50% (LD50) in rodents (12). The animals of groups G1 and G3 were euthanized 5 days after i.p. injection. The animals of group G2 were euthanized 72h after the 5 days of i.p. injection.

Mutagenicity and cytotoxicity. The tissues were stained with Feulgen-Fast Green for evaluating mutagenicity and cytotoxicity. For this purpose, the following metanuclear changes were recorded: micronucleated cells, binucleated cells, pyknosis, karyorhexis and karyolysis. A total of 2,000 cells were evaluated per animal.

Immunohistochemistry for 8 OHdG. The anti-8-hydroxy-2-deoxyguanosine (8 OHdG, Santa Cruz Biotechnologies Inc.™, MO, USA) antibody was used at 1:100 dilution. The analysis was based on scores, taking into account the presence or absence of immunopositive cells as well as the extent of the stained sections as follows: no staining [0], weak staining [1], moderate staining [2], and strong staining [3] (13).

Western blot of Toll like signaling pathway (MyD88, TRAF-6 and NFkB-p65). Western blot was conducted, as described by Yujra et al. (14). The antibody against pNfκBp65 (sc-101744) was purchased from Santa Cruz Biotechnologt™ Inc. (Santa Cruz, CA, USA). The antibodies against MyD88 (ab2064), TRAF6 (ab33915) and β-actin (ab9484) were obtained from ABCAM™ (Cambridge, UK). The intensities of each band sample were quantified by the ImageJ™ software (Image J™, National Institute of Health, MD, USA). All numerical values were normalized using β-actin levels in the respective membrane.

Statistical analysis. Data were expressed as mean±standard deviation. All analyzes were evaluated by the Kruskal-Wallis non-parametric test followed by Dunn’s test. p<0.05 was considered to be significant.

Results

Mutagenicity and cytotoxicity. The groups exposed to crack cocaine had an increase in the number of micronucleated hepatocytes only in the highest tested dose (36 mg/kg). Statistically significant differences (p<0.05) were detected in the G3 group in relation to the control. The same occurred in the evaluation of the total number of binucleated cells, i.e. significant statistically differences (p<0.05) were detected in the G3 group in relation to the CTLR group. Regarding cytotoxicity, karyolysis had an increase in the dose of 18 mg/kg followed by abstinence (G2), with statistically significant difference (p<0.05) when compared to the CTRL group (p<0.05). The group exposed to 36 mg/kg of crack cocaine also showed an increase in karyolysis, with a statistically significant difference (p<0.05) when compared to the groups CTRL and G1 (p<0.05). Pyknotic hepatocytes had a higher average in the G2 group when compared to the CTRL group, with a statistically significant difference (p<0.05). Karyorhexis did not show any changes among groups. These findings are shown in Table 1.

Immunohistochemistry for 8 OHdG. Immunohistochemical expression of 8 OHdG was detected both in the cytoplasm and in the nucleus of liver cells. The results showed that G1, which was exposed to 18 mg/kg, increased the expression of this immunomarker when compared to the CTRL group. Statistically differences (p<0.05) were detected between the groups evaluated (p<0.05). The abstinence group (G2) did not show any difference (p>0.05) when compared to the G1 and CTLR groups (p>0.05). The same occurred for the G3 group. Results are shown in Figure 1.

Protein content of Toll like signaling pathway (MyD88, TRAF-6 and NFkB-p65). In the hepatic tissue, the p-NF-kB p65 protein showed a decrease in the groups exposed to crack cocaine in relation to the CTRL group.
MyD88 protein also showed a decrease in the groups exposed to crack cocaine at the dose of 18 mg/kg (p ≤ 0.05). Crack cocaine was not able to modulate the expression of TRAF-6 (p > 0.05) in liver for all groups studied. These findings are demonstrated in Figure 2.

Discussion

The aim of the study was to evaluate cytogenetic damage, genotoxic oxidative stress and toll like signaling pathway in rat liver exposed to crack cocaine. First, our results demonstrated that crack cocaine at 36 mg/kg dose was able to induce mutagenicity by means of increased numbers of both micronucleated and binucleated cells in rat liver. Moreover, our results revealed that karyolysis was increased by crack cocaine at 36 mg/kg. In mice, necrotic areas in hepatic tissue as well. In this study, the mice received 30 mg/kg of by i.p route, once a day during 3 days. Our results are fully in line with previous reports since the presence of karyolysis in rat liver has been closely related to cellular death by necrosis.

In humans, it has been demonstrated that crack cocaine increases the total number of micronucleus in buccal mucosa cells (7, 8). The same results were found in human lymphocytes (17). Recently, others have assumed that crack cocaine altered the proliferative activity of buccal mucosa cells as a result of decreasing the number of AgNORs positive cells (18). However, our experimental group that remained 72h without the administration of crack cocaine after the 5 days of treatment (G2) increased the total number of pyknotic cells when compared to control group. Taken as a whole, it seems that the abstinence period after crack cocaine exposure was able to induce cell death as a result of apoptosis. This finding is particularly important, because it is consistent with notion that damaged hepatocytes are disposed from the liver parenchyma by apoptosis after finishing the crack cocaine exposure.

8 OHdG is one of the markers used to identify oxidative damage to DNA, because among the various by-products generated during oxidative damage to DNA, changes in nitrogenous bases are synthetized in vivo and can be evaluated after DNA damage (19). The formation of 8 OHdG occurs due to the oxidation of the nitrogenous guanine base, with the addition of HO• in the 8-position carbon, giving rise to a DNA adduct, C8-hydroxy-guanine (8 OHGua). After this process, this molecule ends up losing electrons, giving rise to 8 OHdG (20). Our results demonstrated that immunoexpression for 8 OHdG increased in the group exposed to crack cocaine at 18 mg/kg only. In humans, a study conducted by Bacchi et al. (20), found that regular cocaine users had an increase of 8 OHdG in urine. In rats, previous studies by our research group showed that a single dose of 18 mg/kg of crack cocaine was able to induce the formation of 8 OHdG in hepatocytes (5). The groups G2 (abstinence) and G3 (36 mg/kg) did not show remarkable differences when compared to control. It is important to

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Table I. Metanuclear changes in rat liver induced by crack cocaine.

| Groups (n=08) | Micronucleus | Binucleation | Pyknosis | Karyorhexis | Karyolysis |
|--------------|--------------|--------------|----------|-------------|------------|
| CTRL         | 0.3±0.5      | 92.1±30.2    | 9.8±8.9  | 0.7 ±0.8    | 192.3 ±71.0|
| G1           | 0.5±0.7      | 100.5±11.7   | 15.6±8.3 | 0.6 ±1.1    | 354.8±95.5 |
| G2           | 0.6±0.5      | 127.7±12.5*  | 22.2±7.6*| 0.5 ±0.7    | 421.8 ±59.6*|
| G3           | 1.3±1.0*     | 172.1±17.5   | 2.2±6.8  | 1.5±2.7     | 563.7±58.9*|

Values are shown as mean±S.D. Control group (CTRL); Crack cocaine 18 mg/kg group (G1); Crack cocaine 18 mg/kg and 72 h after exposure (abstinence) group (G2); Crack cocaine 36 mg/kg group (G3). *p≤0.05 when compared to CTRL.

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Figure 1. Scoring of 8 OHdG immunoexpression in rat liver exposed to crack cocaine. Control group (CTRL); Crack cocaine 18 mg/kg group (G1); Crack cocaine 18 mg/kg at 72 h after exposure (abstinence) group (G2); Crack cocaine 36 mg/kg group (G3). *p≤0.05 when compared to CTRL.
Figure 2. Toll-like signaling pathway (MyD88, TRAF-6 and p-NF-κB p65) in rat liver exposed to crack cocaine. Control group (CTRL); Crack cocaine 18 mg/kg group (G1); Crack cocaine 18 mg/kg and 72 h after exposure (abstinence) group (G2); Crack cocaine 36 mg/kg group (G3). *p≤0.05 when compared to CTRL.

Figure 3. Pathobiological effects of crack cocaine in the toll like signaling pathway as a result of genomic instability in liver cells.
highlight that increased levels of 8 OHdG are correlated with the development of several pathologies, including cancer.

Regarding Toll like signaling pathway, our findings demonstrated, for the first time, that MyD88 protein expression decreased in the group exposed to 18 mg/kg of crack cocaine. With regard to NFkB-p65 protein expression, it also decreased in all experimental groups exposed to crack cocaine (18 mg/kg and/or abstinence and 36 mg/kg groups). Therefore, we assume that crack cocaine inhibited the toll like signaling pathway denoting the immunosuppressive effects in hepatocytes. Conversely, some authors showed that cocaine significantly increased the levels of MyD88, IRAK1, and TRAF6 in mice microglial cells (21, 22). Further studies are required to elucidate the issue, especially to clarify the real impact of toll like signaling proteins in different tissues and organs exposed to crack cocaine.

In conclusion, our results demonstrated that crack cocaine inhibited toll like signaling pathway whilst being associated with genomic instability in rat liver cells (Figure 3).

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors’ Contributions

DVS, BAR and ITM performed the experimental design. DVS, ITM and BAR performed immunohistochemistry experiments. DVS and DAR evaluated the micronuclei. DVS, LVM and LPP performed western blot experiments. All Authors interpreted the results and wrote the manuscript.

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