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Genotoxic evaluation of morphine, buprenorphine, pentazocine, and noscapine by micronucleus and comet assay in albino mice

Lakshman Kumar Puli, P. A. Patil

ABSTRACT

Objectives: The present study was planned to explore the genotoxicity of morphine, buprenorphine, pentazocine, and noscapine.

Materials and Methods: Bone marrow micronucleus assay and single cell gel electrophoresis assay were employed after 24 h (single dose) and 72 h (three doses) of treatment with clinically equivalent doses of opioids in albino mice. Percentage of micronucleated polychromatic erythrocytes and comet tail length were determined and the results were analyzed by one-way ANOVA and Student's 't' test.

Results: Only morphine and noscapine showed significant (P < 0.01) increase in both the number of micronucleated polychromatic erythrocytes and comet tail lengths in acute (24-h) as well as subacute (72-h) studies.

Conclusions: These results clearly indicate the genotoxic potential of morphine and noscapine at the clinically equivalent doses.

KEY WORDS: Buprenorphine, comet assay, micronucleus assay, morphine, noscapine, pentazocine

Opioids, commonly used for their analgesic and antitussive effects, are known to cause several adverse effects, including dependence. Of late, the importance of genotoxicity has been realized and it is now mandatory that a new molecule be screened for its genotoxic potential before it is released for clinical use.^[1] Drugs that have been in clinical use since ages, before enforcement of such regulation, need to be reappraised for their genotoxic potential. Although there is abundant literature about the clinical uses and adverse effects of opioids, information regarding their genotoxicity appears to be scanty. Heroin, though not used clinically, has been reported to possess genotoxicity.^[2] Morphine, a well-known drug for pain management has been shown to be a clastogenic in murine lymphocytes.^[3,4] Similarly, buprenorphine has been reported to induce DNA fragmentation and apoptosis in NG108-15 nerve cells.^[5] Noscapine, a commonly used opioid antitussive, has been shown to induce an euploidy in vitro, [6,7] but there are no reports of genotoxicity from *in-vivo* studies. Pentazocine, a partial *k*-agonist has been reported to possess weak antitumor and cytotoxic activity;^{18]} it is well known that cytotoxic drugs are usually genotoxic, but there is paucity of such information regarding pentazocine. Therefore, the present study was planned to confirm the genotoxic activity of morphine as well as that of noscapine and to explore the genotoxic potential of pentazocine and buprenorphine by micronucleus and comet assay in Swiss albino mice.

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Materials and Methods

Drugs

Morphine, buprenorphine, pentazocine, and cyclophosphamide were procured from K.L.E.S's hospital pharmacy (Belgaum, India). Noscapine was obtained as a gift sample from Gland Pharma Ltd. Low-melting agarose (LMA), normal-melting agarose (NMA), and fetal bovine serum were obtained from HIMEDIA. Histopaque was obtained from Sigma (St. Louis, MO, USA). All other chemical reagents used were of analytical grade.

The clinically used maximum doses were computed to per-kilogram-body-weight mice equivalents with the help of the table devised by Paget and Barnes;^[9] the values were 8 mg for morphine, 0.2 mg for buprenorphine, 47 mg for pentazocine, and 15 mg for noscapine. Based on earlier reports, cyclophosphamide was used in the dose of 40 mg/kg. Except for noscapine, which was given by the oral route, the other drugs were administered intraperitoneally in a volume of 8 ml/kg.

Swiss albino mice of either sex, weighing 20-25 gm. procured from the National Institute of Nutrition, India, were used in the present experiments. Animals were maintained on standard rodent feed (Amrut Feeds), with drinking water ad libitum, and were acclimatized for 1 week to laboratory conditions. They were housed, handled and sacrificed at the end of the experiment in accordance with the guidelines of

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CPCSEA and the study was approved by the institutional animal ethical committee. In the acute study, blood and bone marrow were collected 24 h after treatment, whereas in the subacute study, two more doses of the drug were repeated every 24 h and samples were collected at the end of 72 h (24 h after the third dose). Both in acute and subacute studies, in addition to four treated groups (n = 5 in each), there were two more groups: one treated with cyclophosphamide (positive control) and another with normal saline (negative control).

In vivo micronucleus assay

At the appropriate sampling time, the animals were sacrificed by excess ether anesthesia and bone marrow was collected by the procedure of Schimdt.^[10] Briefly, bone marrow that was aspirated with the help of a syringe as a fine suspension in 0.2 ml of fetal bovine serum (FBS) was centrifuged at 1000 rpm for 5 min and the isolated pellet was suspended in 1-2 drops of FBS. Smears of bone marrow, prepared in duplicate on clean glass slides, were air dried and stained with undiluted May-Gruenwald (MG) stain for 3 min, followed by staining for 2 min with diluted MG stain (1:1; with distilled water). Later, the slides were stained with diluted Giemsa stain (1:6; with distilled water) for 10 min. The slides were then rinsed in distilled water and the reverse of the slide was cleaned with methanol before they were air dried and mounted permanently with cover glass. The coded slides were examined under a microscope (oil immersion) for the presence of micronuclei in 2000 polychromatic erythrocytes (MnPCE) per animal. Bone marrow toxicity was assessed by the incidence of polychromatic erythrocytes (PCE) per 200 total erythrocytes.

Comet assay

At the end of treatment period about 0.5 ml of venous blood was collected from the tail after anesthetizing the mice. Lymphocytes were separated using Histopaque 1077.^[11] By trypan blue exclusion method, samples with less than 90% viable lymphocytes were rejected. The slides were prepared according to the standard procedure as described by Rojas et al., with slight modification in the time of electrophoresis.^[12] The slides were coated with three layers of agarose: the bottom layer was composed of 0.67% NMA; the second (middle) layer consisted of 0.5% LMA, in which lymphocytes were suspended; and finally, there was 0.5% LMA to form the third and top layer. The slides were then kept in lysis buffer [2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 10% DMSO (added freshly) and 1% Triton X100 (added freshly); with pH adjusted to 10] for 2 h. Slides were incubated for 20 min in electrophoresis buffer (100 mM EDTA, 300 mM NaOH; pH > 13) prior to electrophoresis at 25 V and 300 mA for 1 h. After electrophoresis, the slides were washed thrice with neutralization buffer (0.4 M Tris-HCl; pH > 7.5) and were air dried. Subsequently, silver staining was carried out by the procedure of Nadin et al.[13] Briefly, air-dried slides were immersed in fixing solution (75 gm trichloroacetic acid, 25 gm zinc sulphate and 25 gm glycerol in 500 ml distilled water) for 10 min and washed with double-distilled water 6-8 times before they were air dried for about 1 h. Sixty-eight milliliters of staining solution B (100 mg ammonium nitrate, 100 mg silver nitrate, 500 mg tungstosilicic acid, and 250 μ l of formaldehyde in 500 ml double-distilled water) was mixed with 32 ml of staining solution A (25 gm sodium carbonate in 500 ml double-distilled water) and poured into a Coplin jar. The slides were dipped vertically into these Coplin jars until a grayish color developed on the slides. Staining was stopped by dipping the slides in stopping solution (1% glacial acetic acid) for 5 min. The whole procedure was carried out in a dim light to minimize artificial DNA damage.

Microscopically ($45\times$), a minimum of 100 comets were scored per animal (50 in each of two replicate slides). All the slides were coded before evaluation. Comet head diameter and total comet length were measured using an ocular micrometer which was calibrated with the help of a stage micrometer, and tail length was calculated by the formula:

comet tail length (μm) = total comet length – head diameter.

The slides were decoded at the end of the study.

Statistical analysis

Results were expressed as mean ± S.E.M. In the *in-vivo* micronucleus assay, chromosomal damage was measured as the percentage of MnPCE per 2000 PCE (%MnPCE). Bone marrow cytotoxicity was assessed in terms of the percentage of PCE in 200 total erythrocyte count. DNA damage proportional to tail length was expressed as mean ± SEM, while cell viability of lymphocytes was reported as the percentage of viable cells. Statistical comparison of the cyclophosphamide group with the saline-treated group in micronucleus as well as comet assay was done with the help of the unpaired Student's 't' test. To compare different groups with the saline group, one-way analysis of variance (ANOVA) followed by Dunnet's *post hoc* test was done. $P \le 0.05$ was considered to be significant.

Results

As expected, the incidence of micronuclei was comparable to that in the negative control group. The cyclophosphamide group (positive control) showed significant (P < 0.0001) increase in %MnPCE in both acute and subacute studies, with mean values of 3.48 ± 0.073 and 4.190 ± 0.236 , respectively, as compared with that of saline-treated (negative control) animals [Table 1]. In the treated groups, morphine and noscapine showed a significant (P < 0.01) increase in the incidence of micronuclei in the acute studies, with the mean values of 7.605 ± 0.1925 and 6.023 ± 0.3000 , respectively. Similarly, in subacute studies, significantly (P < 0.01) increased MnPCE values were 1.070 ± 0.075 in the morphine-treated and 0.6800 ± 0.104 in noscapine-treated groups. However, buprenorphine and pentazocine failed to show such increase. Induction of MnPCE by morphine and cyclophosphamide appears to be dose and duration dependent, since the incidence of MnPCE in both the treated (with three doses) groups was significantly increased as compared to that after single-dose treatment; on the other hand, in case of noscapine the incidence of MnPCE is irrespective of its duration and treatment [Table 1]. In both acute as well as subacute studies, no significant bone marrow toxicity (as assessed by %PCE/total erythrocytes) was observed [Figure 1].

Silver-stained lymphocytes subjected to SCGE were studied for the presence of comets. SCGE (comet assay) helps to determine DNA damage and genotoxicity by calculating the comet tail length. It is well known that tail length is directly proportional to the extent of DNA damage. Both in acute as well as subacute

Table 1

|--|

| Treatment group (mg/kg) (n = 5) | Acute study | | Subacute study | | | |
|---|--------------------------|---------------------------|---------------------------|----------------------------|--|--|
| | %MnPCE Mean \pm S.E.M | Comet tail length (µm) | %MnPCE Mean \pm SEM | Comet tail length (µm) | | |
| Normal saline (8 ml/kg) | 0.120 ± 0.025 | 3.358 ± 0.3328 | 0.1300 ± 0.025 | 3.579 ± 0.1346 | | |
| Cyclophosphamide (40 mg/kg) | $3.48 \pm 0.073^{**}$ | $23.03 \pm 0.6695^{**}$ | $4.190 \pm 0.236^{**}$ | $28.24 \pm 0.6539^{**}$ | | |
| Morphine (8 mg/kg) | $0.610 \pm 0.079^{*}$ | $7.605 \pm 0.1925^{\ast}$ | $1.070 \pm 0.075^{*}$ | $7.537 \pm 0.6039^{*}$ | | |
| Buprenorphine (0.2 mg/kg) | 0.190 ± 0.033 | 3.155 ± 0.1387 | 0.1500 ± 0.027 | 3.791 ± 0.3054 | | |
| Pentazocine (47 mg/kg) | 0.200 ± 0.035 | 3.473 ± 0.1724 | 0.1200 ± 0.025 | 3.362 ± 0.3631 | | |
| Noscapine (15 mg/kg) | $0.610 \pm 0.064^{\ast}$ | $6.023 \pm 0.3000^{\ast}$ | $0.6800 \pm 0.104^{\ast}$ | $6.635 \pm 0.4289^{\star}$ | | |
| ANOVA followed by Dunnet's test: *P < 0.01; Student's 't' test: **P < 0.0001 compared to saline-treated group | | | | | | |

Figure 1: Incidence of micronucleated polychromatic erythrocytes in acute and subacute study. *P < 0.01, **P < 0.0001



studies the tail length of comets was significantly increased in the cyclophosphamide-, morphine-, and noscapine-treated groups [Table 1]. There was no significant change in comet tail lengths in the groups treated with buprenorphine and pentazocine as compared to that in the saline-treated group [Figure 2]. From the findings of present study it appears that noscapine genotoxicity is comparable to that of morphine, while cyclophosphamide is more genotoxic than morphine. There was no mortality observed in any of the treated groups or in the normal saline (control group) or cyclophosphamide groups.

Discussion

Genotoxicity testing has always been a daunting task for both the industry and drug regulatory authorities all over the world. The *in-vivo* micronucleus test is a method devised primarily for screening chemicals for chromosome-breaking effects,^{110]} whereas the comet assay detects DNA single-strand breaks with a single cell approach. The power of this assay lies in its ability to evaluate DNA damage and repair in proliferating or nonproliferating cells and to provide insights into intercellular differences in response.¹¹²

Since many commonly used drugs were found to be positive in at least one genotoxicity test, there is immense pressure to screen the newer drugs for their mutagenic and carcinogenic potential. However, little attention is being paid to those drugs that were introduced into clinical practice long ago and are still in use. The present study is an effort to explore the genotoxic potential of the clinically used opioids. From the point of clinical relevance, therapeutically equivalent doses of these drugs were





administered in a single dose (24 h: acute study) and in three doses (72 h; subacute study). Morphine, in this study, showed significant increase in the number of micronuclei as well as DNA damage in both the acute and subacute studies. This finding is in concordance with an earlier report,^[14] in which morphine has been shown to induce micronuclei in a dose-dependent fashion. Buprenorphine failed to show either increased DNA or chromosomal damage as evidenced by insignificant change in the comet tail length and number of MnPCE, both in acute as well as subacute studies. The findings of the present study differ from an earlier report^[5] in which buprenorphine has been shown to induce DNA fragmentation and apoptosis. This discrepancy could be explained on the basis of the large dose tested in vitro on a nerve cell line (NG108-15) in the earlier study.^[5] Pentazocine also failed to produce genotoxicity in both the acute and subacute studies. There are no reports regarding the genotoxicity of pentazocine, though it has been reported to possess antitumor activity.^[8] Noscapine, a naturally-occurring opium alkaloid, at 15 mg/kg produced significant increase in MnPCE and comet tail length in acute as well as subacute studies. The positive genotoxic activity of noscapine in the present *in-vivo* study agrees with previous studies, in which noscapine has been reported to be a potent aneugen.^[6,7] All the above results show that only morphine and noscapine possess significant genotoxicity, while buprenorphine and pentazocine are devoid of genotoxic potential. It is well known that opioids act through their specific receptors, but it is not definitely known whether the genotoxicity of morphine is also mediated through one of these receptors; however, µ-receptor involvement in the mediation of morphine genotoxicity has been reported.^[3] The lack of genotoxicity of buprenorphine and pentazocine in the present study probably rules out the possibility of μ and κ receptors mediating genotoxicity.

Morphine is known to generate oxidative stress like opioid peptides.^[15,16] Similarly, like morphine, other opioids could be expected to generate reactive oxygen species (ROS), which are well known to cause DNA damage.^[17] Therefore, the genotoxicity of morphine could be mediated through ROS. Morphine has also been reported to inhibit GSH, and this effect of morphine may also contribute to its genotoxicity, since enzymes like GSH SODs etc., are well known to provide protection against such ROS-induced DNA damage.^[18] However, the inhibitory effect of morphine on peripheral GSH was reported to be mediated through central μ receptors. The inability of pentazocine to suppress GSH,^[18] as shown in the earlier study, could explain the lack of genotoxicity observed in the present study. However, it is very difficult to explain the inability of buprenorphine to induce genotoxicity though it is a more potent analgesic than morphine. Noscapine binds to tubulin and affects microtubule assembly, thereby arresting mitosis^[19] and this might mediate its genotoxicity. Irrespective of the mechanism of action, it is clear from the present study that morphine and noscapine are genotoxic and need to be used carefully, or avoided to the extent possible, in day-to-day clinical practice.

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