

## Evaluation of cytotoxicity and mutagenicity of the benzodiazepine flunitrazepam *in vitro* and *in vivo*

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Flunitrazepam (FNZ) is a sedative benzodiazepine prescribed for the short-term treatment of insomnia. However, there are concerns regarding possible carcinogenic or genotoxic effects of this medicine. Thus, the aim of this study was to evaluate the cytotoxic, clastogenic and aneugenic effects of FNZ in hepatoma cells from *Rattus norvegicus* (HTC) *in vitro* and in bone marrow cells of Wistar rats *in vivo*. These effects were examined *in vitro* following treatment with 0.2, 1.0, 5.0 or 10 µg/mL FNZ using a micronucleus test with a cytokinesis block or *in vivo* using a chromosomal aberration test following treatment with 7, 15 or 30 µg/mL/kg body weight. The results showed that the benzodiazepine concentrations tested were not cytotoxic, aneugenic or clastogenic. However, considering the adverse effects of using this benzodiazepine, more studies are required.

**Uniterms:** Flunitrazepam/cytotoxic effects. Flunitrazepam/clastogenic effects. Flunitrazepam/aneugenic effects. Sedative benzodiazepine/adverse effects/*in vitro* and *in vivo* study. Chromosomal abnormalities/medicines effects.

Flunitrazepam (FNZ) é um sedativo benzodiazepínico prescrito para o tratamento da insônia em curto prazo. Entretanto, existe a preocupação com relação aos possíveis efeitos carcinogênicos ou genotóxicos causados por este fármaco. Então, o objetivo deste estudo foi avaliar os efeitos citotóxicos, clastogênicos e aneugênicos do FNZ em células de hepatoma de *Rattus norvegicus* (HTC) *in vitro* e em células de medula óssea de ratos Wistar *in vivo*. Foram testadas as concentrações de 0,2, 1,0 e 10 µg/mL de FNZ pelo teste do micronúcleo com bloqueio de citocinese *in vitro* e 7, 15 e 30 µg/mL/kg de peso corpóreo para o teste de aberração cromossômica *in vivo*. Os resultados mostraram que as concentrações do benzodiazepínico testadas não foram citotóxicas, aneugênicas ou clastogênicas. Entretanto, considerando os efeitos adversos do uso deste benzodiazepínico, mais estudos são necessários.

**Unitermos:** Flunitrazepam/efeitos citotóxicos. Flunitrazepam/efeitos clastogênicos. Flunitrazepam/efeitos aneugênicos. Sedativo benzodiazepínico/efeitos adversos/estudo *in vitro* e *in vivo*. Anormalidades cromossômicas/efeitos de medicamentos.

### INTRODUCTION

Flunitrazepam (FNZ) is a potent benzodiazepine agonist primarily used as a hypnotic and preanesthetic agent across Europe (Woods, Winger, 1997; Druid, Holmgren, Ahlner, 2001). FNZ is a sedative benzodiazepine that is prescribed for the short-term treatment of insomnia at a recommended dose of 0.5-1.0 mg, with a maximum dose

of 2.0 mg (Ohshima, 2006). FNZ has an appreciable abuse potential (Druid, Holmgren, Ahlner, 2001; Ohshima, 2006) and is among a cluster of “club drugs” misused at raves and in other social settings, including in the United States, where this drug is not legally available (Woods, Winger, 1997; Smith *et al.*, 2002; Britt, Mc-Cange-Katz, 2005).

Similar to other cerebral modulating drugs, FNZ can produce pharmacological effects that include sedation, memory impairment, and behavioral disinhibition, which is described as an increase in the probability of behaviors that typically occur at low frequencies due to social and

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interpersonal constraints, such as sexual misconduct. These disinhibitory and amnesic effects might result in increased risky behavior and altered decision-making (e.g., violence, other drug use and high-risk sexual activity) (Wu, Schlenger, Galvin, 2006; Lane, Cherek, Nouvion, 2008).

Individuals who abuse FNZ often commit sexual assaults and violent acts (Almeida *et al.*, 2010). The increasing abuse of both FNZ and ketamine hydrochloride has particularly been observed in young people in social settings, such as clubs. Frequently, young people take FNZ in conjunction with alcohol and exhibit euphoria, agitation and amnesia (Druid, Holmgren, Ahlner, 2001; Smith *et al.*, 2002). FNZ is becoming increasingly abused by young people who use this drug in combination with alcohol and then commit violent crimes (Almeida *et al.*, 2010).

In addition to these harmful effects, there are concerns regarding possible genotoxic or carcinogenic effects caused by this medication or its derivatives (Assaf, Abdel-Rahman, 1999; Brambilla, Carrozzino, Martelli, 2007), thereby stressing the importance of cytogenetic testing with FNZ.

Short duration test systems are widely used to detect environmental mutagens, such as ionizing radiation or chemically synthesized substances. These systems include cultures of mammalian cells grown *in vitro* as well as cultures of human lymphocytes and cell lines from Chinese hamsters and rats (*Rattus norvegicus*). In turn, *in vivo* test systems, such as rats, respond in a manner similar to that observed *in vitro*. Additionally, a close correlation exists between the biological metabolism of rats and that of humans, which justifies the use of this animal in the identification of substances that may possess mutagenic potential in humans (Waters *et al.*, 1996; Natarajan, 2002).

The aim of this study was to evaluate the cytotoxic, aneugenic and clastogenic potential of FNZ through the induction of micronuclei in cultured hepatoma cells *in vitro* and through the appearance of chromosomal aberrations in *R. norvegicus* bone marrow cells *in vivo*.

## MATERIALS AND METHODS

### *In vitro*

#### Cell Line – HTC

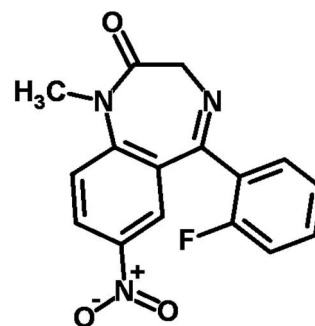
HTC cells that were derived from a *Rattus norvegicus* hepatoma were provided by Dr. Mário Sérgio Mantovani, State University of Londrina – Paraná – Brazil. Cells ( $2 \times 10^6$ ) were grown in 25 cm<sup>2</sup> culture flasks containing 5 mL DMEM (Gibco), which was supplemented with

10% fetal bovine serum (Gibco), incubated at 37 °C. Accordingly, the lineage cell cycle is approximately 24 hours.

#### Treatment Solution

For the negative control, 20 µL of phosphate buffered saline (PBS)/mL was added to the culture medium (DMEM). The positive control was doxorubicin (DXR – Ackros) at a final concentration of 0.2 µg/mL DMEM.

Treatments with benzodiazepine FNZ (Rohypnol®) (Roche), with the molecular structure shown in Figure 1, were performed with four different concentrations of this medicine: 0.2 [1], 1.0 [2], 5.0 [3] or 10.0 [4] µg/mL DMEM.



**FIGURE 1** – Fluorine molecule of flunitrazepam. Source: www.rsc.org (2013).

#### Cytokinesis Block Micronucleus assay (MNCtB)

Cells were incubated for 24 hours with the treatment solutions and with cytochalasin B (Sigma, 3.0 µg/mL DMEM) to obtain binucleated cells to evaluate their cytotoxicity and mutagenicity, as described by Fenech (2000). Cells were harvested according to a previously published protocol by Oliveira *et al.* (2002). Briefly, cells were trypsinized (500 µL trypsin-0.025% EDTA (Gibco) at 37 °C); centrifuged (5 min at 1,000 rpm); hypotonized (1.5 mL of 1% sodium citrate) and fixed (5 mL of a 3:1 mixture of methanol:acetic acid).

All of the experiments were performed in triplicate; 3,000 binucleated cells were analyzed per treatment to assess the frequency of micronuclei (MN) and 1,500 cells were counted to determine the cytokinesis block proliferation index (CBPI). The process for selecting binucleated cells, identifying micronuclei and calculating the CBPI was followed as described by Fenech (2000). The percentage of cytostasis was calculated by the following formula (OECD, 2010):

$$\% \text{ Cytostasis} = 100 - 100 \left[ \frac{(\text{CBPI}_{\text{Treatment}} - 1)}{(\text{CBPI}_{\text{Control}} - 1)} \right]$$

Statistical analysis was performed using an ANOVA and Tukey's test ( $p < 0.05$ ).

### ***In vivo***

#### *Wistar Rats*

Six Wistar rats, three males and three females for each group, were obtained from the Central Vivarium – State University of Maringá (UEM). Experiments were performed on 35 days old rats weighing approximately 100 g body weight (bw). Animals were maintained during the period of experimentation in the Central Vivarium of the Department of Cell Biology and Genetics/UEM under controlled conditions of temperature  $\pm 25$  °C, humidity  $\pm 50\%$  and with a photoperiod of 12 hours light/dark, according to the standards established by the Ethics Committee on Experimentation with Laboratory Animals/UEM (process number: PRO 046/2010).

#### *Treatment Solution*

For the negative control, rats were treated with 1 mL of water/100 g bw (body weight), via gavage, for 24 hours. The positive control (CO<sup>+</sup>) was 1.5 mg of cyclophosphamide clastogenic drug/1 mL water/100 g bw, intraperitoneally administered for 24 hours to assess the responsiveness of the lineage.

Treatments with the benzodiazepine FNZ (Rohypnol®) (Roche) were performed by treating rats with three concentrations of this medicine: 7 [1], 15 [2] or 30 [3]  $\mu\text{g}/\text{mL}$  of water/kg, via gavage, as calculated by an extrapolation of the concentrations used by humans to the body weight of rats.

#### *Chromosomal Aberration Test*

The chromosomal aberration test was performed to obtain bone marrow cells of Wistar rats using the Ford and Hamerton method (1956), with some modifications. Mitotic cells were interrupted in metaphase with the intraperitoneal administration of 0.5 mL/100 g bw of colchicine (0.16%) half an hour before euthanasia.

For animal euthanasia, 0.5 mL/100 g bw of anesthetic (1 g sodium thiopental/25 mL of distilled water) was intraperitoneally administered.

Bone marrow was removed from the femurs with 5 mL of hypotonic solution (0.075 M potassium chloride). The suspension was incubated at 37 °C for 12 minutes, centrifuged for 5 minutes and then the supernatant was discarded. The material was fixed with 5 mL of methanol solution, 3:1 acetic acid and centrifuged for 5 minutes. The supernatant was discarded and the fixative was changed at least twice. The preparation was performed with a drop of

suspension on clean slides containing a film of distilled ice water. The coloration of the slides was performed using a drop from a film of Giemsa solution in phosphate buffer (0.12 M Na<sub>2</sub>HPO<sub>4</sub>, x 12 H<sub>2</sub>O and 0.06 M KH<sub>2</sub>PO<sub>4</sub>) at a ratio of 1:30 at pH 6.8.

The analysis of the slides was performed using a light microscope, analyzing 100 metaphases per animal, which totaled 600 metaphases for the control and treatment groups, and assessing the appearance of alterations, such as gaps, breaks, fragments, etc. The results were expressed as a percentage of total aberrations.

The cytotoxic evaluation of the mitotic index (MI) was calculated from 5,000 cells by sex, totaling 10,000 cells per group. The MI calculation, as a percentage, was performed using the number of dividing cells divided by the total number of cells present in the fields.

Statistical calculation was performed using the chi-square test ( $\alpha = 0.05$ ).

## **RESULTS AND DISCUSSION**

When examining the cytotoxicity of the benzodiazepine FNZ at various concentrations (0.2, 1.0, 5.0 and 10.0  $\mu\text{g}/\text{mL}$ ) *in vitro* (Table I), the results showed that this substance did not affect cytokinesis-block proliferation and showed no cytotoxic action in hepatoma cells from *R. norvegicus*. The addition of FNZ did not cause the inhibition of cell proliferation but stimulated cell proliferation, as shown by the percentage of cytostasis (Table I) at all concentrations evaluated: 11.76% at a dose of 0.2  $\mu\text{g}/\text{mL}$ ; 10.29% at a dose of 1.0  $\mu\text{g}/\text{mL}$ ; 11.76% at a dose of 5.0  $\mu\text{g}/\text{mL}$  and 14.70% at a dose of 10  $\mu\text{g}/\text{mL}$ .

These results differ from those results found by Assaf Abdel-Rahman (1999), who showed that a 0.16 mM solution of FNZ (50  $\mu\text{g}/\text{mL}$ ) significantly reduced the number of viable rat liver cells after 2 hours of exposure. However, although the base concentration used in this study was extrapolated to the total weight of the culture medium (5 g) based on the highest concentration consumed by humans (2 mg) and although concentrations up to 50 times higher than this concentration were also used, the concentrations used in this study are much smaller than the 50  $\mu\text{g}/\text{mL}$  concentration that was used in Assaf Abdel-Rahman's (1999) work, which may explain the lack of cytotoxicity observed in the HTC cells.

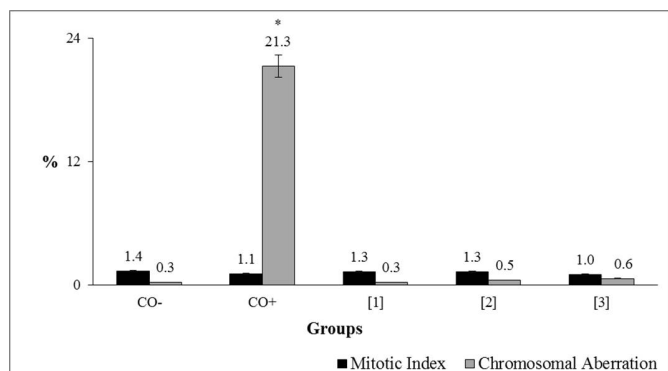
Non-cytotoxic results were also observed *in vivo* in the bone marrow cells of Wistar rats (Figure 2) treated with concentrations of 7, 15 or 30  $\mu\text{g}/\text{mL}/\text{kg}$  FNZ, which suggest that FNZ did not affect the cell division index. Moreover, in the case of an acute treatment with the extrapolated doses commonly consumed by humans

(0.5, 1.0 and 2.0 mg), the absence of toxicity of FNZ is noticeable. However, studies that have used high doses of FNZ have shown divergent results. One example is Namera *et al.* (2012), who showed that FNZ has acute toxic effects in high doses because FNZ caused the death of a woman who ingested high doses of FNZ and triazolam (another anxiolytic and sedative).

**TABLE I** - The mean cytokinesis block proliferation index (CBPI) of 1,500 control cells compared with cells that were treated with flunitrazepam

Group	Total cells analyzed	CBPI $\mu\pm$ SD	% Cytostasis
CO-	1,500	1.68 $\pm$ 0.09	-
CO+	1,500	1.84 $\pm$ 0.02	23.52
[1]	1,500	1.76 $\pm$ 0.09	11.76
[2]	1,500	1.75 $\pm$ 0.06	10.29
[3]	1,500	1.76 $\pm$ 0.00	11.76
[4]	1,500	1.78 $\pm$ 0.09	14.70

CO-: Negative control; CO+: Doxorubicin; FNZ: [1]: 0.2  $\mu$ g/mL; [2]: 1.0  $\mu$ g/mL; [3]: 5.0  $\mu$ g/mL; [4]: 10  $\mu$ g/mL.

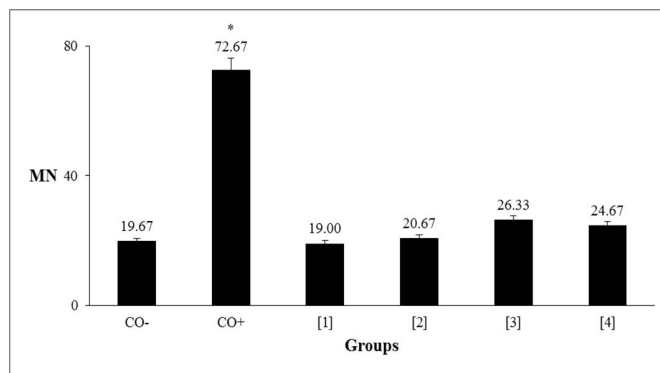


**FIGURE 2** - Percentage of the mitotic index and chromosomal aberrations for the negative (CO<sup>-</sup>) and positive (CO<sup>+</sup>) control groups and for groups treated with concentrations of 7 [1], 15 [2] or 30 [3]  $\mu$ g/mL/kg of flunitrazepam *in vivo*. \*Statistically significant result compared with the negative control ( $p < 0.001$ ).

The evaluation of the aneugenic and clastogenic potential of FNZ, which was performed by the *in vitro* micronucleus test in HTC cells (Figure 3) and by the *in vivo* chromosomal aberration test in the bone marrow cells of Wistar rats (Figure 2), showed that the numbers of micronuclei and chromosomal aberrations were not statistically different from the negative control at all concentrations evaluated. These data suggest that FNZ does not possess aneugenic or clastogenic activity. Furthermore, the percentage of chromosomal aberrations

did not differ according to sex, indicating that mutagenic activity does not occur in both males and females.

However, in general, one can observe a dose-dependent effect of FNZ both *in vivo* and *in vitro*. As the dose increased, the average percentage of chromosomal aberrations (7  $\mu$ g/mL/kg = 0.3%, 15  $\mu$ g/mL/kg = 0.5% and 30  $\mu$ g/mL/kg = 0.6%) increased *in vivo*, and the average formation of micronuclei (0.2  $\mu$ g/mL = 19, 1.0  $\mu$ g/mL = 20.67, 5.0  $\mu$ g/mL = 26.33 and 10  $\mu$ g/mL = 24.67) increased *in vitro*. Staiano *et al.* (1984) also observed a dose-responsive effect for FNZ mutagenicity using the *Salmonella*/microsome mutagenicity test. Peterka *et al.* (1992) also demonstrated that the proportion of living, malformed embryos of White Leghorn chickens gradually increased when treated with increasing doses of FNZ (0.6, 6.0 and 20  $\mu$ g).



**FIGURE 3** - The average number of micronuclei (MN) for the negative (CO<sup>-</sup>) and positive (doxorubicin) control groups and for groups treated with concentrations of 0.2 [1], 1.0 [2], 5.0 [3] and 10 [4]  $\mu$ g/mL of flunitrazepam *in vitro*. \* Statistically significant result compared with the negative control ( $p < 0.001$ ).

In this study, FNZ treatment did not result in mutagenicity; these findings are similar to those results obtained by Degraeve *et al.* (1985), who investigated the clastogenicity of this medicine by the chromosomal aberration test in the bone marrow cells of male mice intraperitoneally treated with 850  $\mu$ g/kg for 12 to 72 hours. In addition, other studies have indicated that FNZ was not able to reversely mutate the *Salmonella typhimurium* TA100 strain when treated with a concentration of 5,000  $\mu$ g/plate and did not cause the formation of mutations in the 15178Y gene in mouse lymphoma cells (Brambilla, Carrozzino, Martelli, 2007; Brambilla, Martelli, 2009). Additionally, FNZ treatment did not damage the single-stranded DNA of rat liver cells *in vivo* (Carlo *et al.*, 1989) and did not cause chromosomal aberrations in the bone marrow cells of mice *in vivo* (Brambilla, Carrozzino, Martelli, 2007; Brambilla, Martelli, 2009).

Although the results of this study have not shown any aneugenic, clastogenic or cytotoxic effects of FNZ, subchronic studies with FNZ should be performed to evaluate the long-term effects of daily consumption, which is typical in humans, and of higher doses to evaluate the effects of acute high concentrations, such as in situations of abuse.

## CONCLUSIONS

The results of this study show that the concentrations of the benzodiazepine flunitrazepam tested *in vitro* (0.2, 1.0 and 10 µg/mL) and *in vivo* (7, 15, and 30 µg/mL/kg) were not cytotoxic, aneugenic or clastogenic by micronucleus test or by the chromosome aberration test, respectively. Thus, the extrapolation of daily doses consumed by humans showed no potentially harmful effects in HTC cells or in the bone marrow cells of Wistar rats. However, considering the adverse effects of use of this benzodiazepine, further studies should be performed.

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## CONFLICT OF INTEREST

THE AUTHORS DECLARE NO CONFLICT OF INTEREST.

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