



Metformin (dimethyl-biguanide) induced DNA damage in mammalian cells

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Abstract

Metformin (dimethyl-biguanide) is an insulin-sensitizing agent that lowers fasting plasma-insulin concentration, wherefore it's wide use for patients with a variety of insulin-resistant and prediabetic states, including impaired glucose tolerance. During pregnancy it is a further resource for reducing first-trimester pregnancy loss in women with the polycystic ovary syndrome. We tested metformin genotoxicity in cells of Chinese hamster ovary, CHO-K1 (chromosome aberrations; comet assays) and in mice (micronucleus assays). Concentrations of 114.4 µg/mL and 572 µg/mL were used in *in vitro* tests, and 95.4 mg/kg, 190.8 mg/kg and 333.9 mg/kg in assaying. Although the *in vitro* tests revealed no chromosome aberrations in metaphase cells, DNA damage was detected by comet assaying after 24 h of incubation at both concentrations. The frequency of DNA damage was higher at concentrations of 114.4 µg/mL. Furthermore, although mortality was not observed in *in vitro* tests, the highest dose of metformin suppressed bone marrow cells. However, no statistically significant differences were noted in micronuclei frequencies between treatments. *In vitro* results indicate that chronic metformin exposure may be potentially genotoxic. Thus, pregnant woman undergoing treatment with metformin should be properly evaluated beforehand, as regards vulnerability to DNA damage.

Key words: metformin, DNA damage, chromosome aberration.

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Introduction

Metformin (dimethyl-biguanide) is an insulin-sensitizing agent that lowers fasting plasma insulin concentrations by increasing the peripheral uptake of glucose and decreasing hepatic glucose output (Nisbet *et al.*, 2004). It is prescribed for patients suffering from a variety of insulin-resistant and prediabetic states, including impaired glucose tolerance, obesity (Tankova *et al.*, 2003; Rotella *et al.*, 2006; Hirsch *et al.*, 2009) and metabolic abnormalities associated with HIV disease (Hadigan *et al.*, 2000; Johnson *et al.*, 2005; Diehl *et al.*, 2008). Furthermore, metformin is also used regulating anovulation, and induce and sustain pregnancy in polycystic-ovary syndrome patients (Jakubowicz *et al.*, 2002; De Leo *et al.*, 2006; Lilja and Mathiesen, 2006; Brassard *et al.*, 2008).

The toxicity of metformin is well-known in cases of metabolic impairment due to liver and kidney diseases, its toxicity, by being mainly associated with lactic acidosis,

can occur through both acute and chronic exposure (DePalo *et al.*, 2005; Strack, 2008). Secondary to metformin-associated lactic acidosis, patients may undergo alterations in the central nervous system, cardiovascular collapse, renal failure, and eventually, death (Snyder and Berns, 2004; Spiller and Sawyer, 2006). Nonetheless, non-clinical or metabolic outcomes, possibly associated with genotoxic effects, have not received due consideration. Results from the few experimental *in vivo* and *in vitro* studies so far undertaken are controversial. While some reports indicated no genotoxic effects (Attia *et al.*, 2009), others (Janjetovic *et al.*, 2011) have assumed that metformin can produce oxidative stress due to DNA fragmentation. Onaran *et al.* (2006) proposed that high concentrations of metformin increased cumene hydroperoxide (CumOOH)-induced DNA damage. Furthermore, Anedda *et al.*, (2008) showed that metformin induces oxidative stress in white adipocytes, by|through increasing the levels of reactive oxygen species, and reducing aconitase activity.

Various drugs are capable of damaging the DNA in living cells. If not repaired, or if produced in excessive amounts, DNA damage can initiate a cascade of biological effects (cellular, organic, individual), the most outstanding

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consequence being carcinogenesis (Moustacchi, 2000; Barash *et al.*, 2010). Therefore, on considering the enhanced association between the chronic use of certain drugs and the increase in DNA damage, the aim of the current study was to evaluate metformin genotoxicity.

Materials and Methods

Chemicals

Metformin was commercially obtained as metformin hydrochloride 500 mg (Merck S.A.). Before using, the drug was dissolved in Dulbecco modified Eagle medium (DMEM) at determined experimental concentrations.

In vitro test

Cell and culture conditions

Chinese hamster ovary (CHO-K1) cells were grown at 37 °C, in an atmosphere of 5% of CO₂ in DMEM, (GIBCO-BRL), at pH 7.4, and supplemented with 10% fetal calf serum. The CHO-K1 cells (3.5 x 10⁵) were seeded into 25 cm² tissue culture flasks and stabilized for 24 h. All the experiments were run in triplicate.

Cell viability analysis with trypan blue exclusion testing was to determine the metformin dosage necessary to kill 50% of the cells (lethal concentration 50, LC50). On considering previous studies, lower dosages of LC50, viz., 15% (114.4 µg/mL) and 80% (572 µg/mL), were employed for evaluating genotoxicity.

Experimental groups consisted of cells treated with metformin (114.4 and 572 µg/mL). Negative controls consisted of untreated cultures and positive control (cyclophosphamide at 6 µg/mL) groups. After treatment for 24 h, each culture was washed, harvested, re-suspended in PBS and used for the comet assay and chromosome aberration testing.

Comet assay

The comet assay (alkaline) described by Singh *et al.* (1988) was used with certain modifications. Briefly, 30 µL of cell suspension (10⁵-10⁶ cells) were mixed with 120 µL of low melting point agarose (0.5% in PBS buffer) and added to slides that had been previously covered with a layer of type II agarose (1.5% in PBS buffer). The slides (without coverslips) were immersed in cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris and 1X Triton X-100) for 1 h at 4 °C. They were then placed into an electrophoresis solution (300 mM NaOH, pH 13, 1 mM EDTA,) for 20 min, at 25 V and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris buffer (pH 7.5), stained with 50 µL ethidium bromide (20 µg/mL; Sigma), and analyzed using a fluorescence microscope (Carl Zeiss) at 400X equipped with exciting (515-560-nm) and barrier (590-nm) filters.

Approximately 300 randomly chosen cells per experimental point (100 per replicate slide) were analyzed. DNA damage category was assigned to five classes (0-4), based on the visual aspect of the comets, and considering the extent of DNA migration in accordance with Visvardis *et al.* (1997). Nucleoids with a bright head and no tail were classified as class 0 (undamaged nucleoids), whereas highly damaged ones, with a small head and a long diffuse tail, as class 4. Those presenting intermediate characteristics were classified as classes 1, 2 or 3. Index of DNA damage (IDD) was quantified using the equations described by Jalszynski *et al.* (1997):

$$IDD = \frac{(n_1 + 2n_2 + 3n_3 + 4n_4)}{(\Sigma / 100)}$$

where IDD is DNA damage in arbitrary units, $n_1 - n_4$ the number of class 1 to 4 comets and Σ the total number of scored nucleoids, including class 0. IDD values ranged from 0 (all undamaged nucleoids) to 400 (all maximally damaged nucleoids) arbitrary units, each of which was converted to a percentage of damaged DNA, with the total score of 400 representing 100% damage.

Metaphase preparation

Chromosome aberrations were carried out according to our own routine procedures (Almeida Santos *et al.*, 2005). Briefly, in all the treatments, colchicine 4% (10⁵ M) was added to the culture 2 h before harvesting. After harvesting, the cells were treated with a 0.075 M KCl hypotonic solution for 20 min at 37 °C, fixed three times in methanol-acetic acid (3:1), and slides prepared. These were stained with 10% Giemsa in a phosphate buffer (pH 6.8) for 10 min. 1556 metaphases (200 metaphases from each parallel culture) were analyzed per treatment. Blind analysis was through cytogenetic assaying. Cells with chromosome aberrations, such as gaps, breaks and acentric fragments, *i.e.*, fragments of chromosomes without centromeres, were recorded. The standard cytogenetic procedures established by the Chatham Bars Inn Conference (CBIC system) were adopted, *i.e.*, a chromatid lesion whose length was less than the diameter of the chromatid itself was classified as a chromatid gap, whereas, that whose length was equal to or greater than the diameter was regarded as a chromatid break.

In vivo test

All animal handling and procedures were carried out according to the international practices for animal use and care, and approved by the Animal Ethics Committee of the University of Brasilia. Female Swiss albino mice (10-12 weeks old) weighing 25-30 g were obtained from the Central Animal Facility of the University of Brasilia. 100 animals were acclimatized to laboratory conditions for one week prior to starting the study; they were provided with Purina mice chow and filtered water *ad libitum*. Following

acclimatization, they were randomly divided into groups (10 each) for acute toxicity testing to determine the median lethal dose (LD50), 7 days after an intraperitoneal (i.p.) injection. The Trimmed Spearman-Kärber method was used, whereby LD50 value was defined as 477 mg/kg. The metformin concentrations represented respectively 20%, 40%, and 70% of the LD50, and were given in the form of i.p. injections in a 0.5 mL volume.

For the genotoxicity experiment, 40 animals were randomly divided into five groups (8 in each). Three of these received metformin in the concentrations 95.4, 190.8, and 333.8 mg/kg body weight, respectively, representing 20%, 40%, and 70% of LD50, in the form of i.p. injections in a 0.5 mL volume. The remaining two groups served as controls, one receiving the same volume in saline solution (negative control) and the other cyclophosphamide (Endoxan) 30 mg/kg (positive control).

The animals were sacrificed by cervical dislocation, twenty four hours after receiving the i.p. injections. Bone-marrow cells were then collected and washed with 3 mL of a phosphate buffer saline (PBS) solution containing 5 IU heparin/mL. After fixing the harvested cells three times in methanol-acetic acid (3:1), slides were prepared. These were then stained with 10% Giemsa. Two thousand polychromatic erythrocytes (PCEs) and two thousand normochromatic erythrocytes (NCEs) were examined per animal. Micronuclei were only scored in PCEs. The PCE/NCE relationship was recorded as a sign of cytotoxicity. Micronucleus assaying was according to MacGregor *et al.* (1987).

Statistical analysis

Statistical analysis of data from the *in vivo* test was based on micronucleated cells/group and analyzed by Chi-square test using Yates correction. Comet-assay data were analyzed taking into account two parameters, namely i) frequency of DNA damaged cells and ii) frequency of DNA damage (DD). Prior to statistical analysis, data were transformed into arcsine x (x = number of cells with DNA damage) or $\log x$ (x = IDD). Transformed data (arcsine or $\log x$) underwent variance analysis (ANOVA). The Fischer test was used in the event of statistically significant differences ($p < 0.05$).

Table 1 - DNA damage in CHO-K1 cells exposed to metformin, as measured by comet assaying.

Treatments	Cell with DNA damage (%)	DNA damage (%)
Negative control	24.53 ± 13.08 ^a	16.26 ± 7.18 ^a
Positive control	95.88 ± 1.06 ^b	89.43 ± 5.79 ^c
Metformin 114.4 µg/mL	86.77 ± 4.72 ^b	64.12 ± 7.03 ^{bc}
Metformin 572.0 µg/mL	73.77 ± 12.46 ^b	41.74 ± 9.23 ^{ab}

^{a, b, c}Different superscripts in the same column denote significant differences ($p < 0.05$).

Results

Compared to the control group, metformin at the lower concentration induced DNA damage in CHO-K1 cells (Table 1). However, although treated with both concentrations, the frequency of those with increased levels of DNA damage was higher when they were not dose-related. No chromosome aberrations were observed in metaphase cells at the treatment-levels used (Table 2).

The *in vivo* tests showed there to be no bone-marrow cells at the highest concentration (333.9 mg/kg), probably as a result of acute cytotoxicity, although without any clinical outcome, such as morbidity or mortality. Nonetheless, at the lower concentrations (95.4 mg/kg and 190.8 mg/kg), there was neither an increase in micronuclei nor a decrease in PCE frequency in viable cells (Table 3).

Table 3 - Micronuclei in polychromatic erythrocytes of Swiss mice treated with metformin*

Treatments	MN – PCE (%)	PCE/NCE (%)
Negative control	3.5 ± 2.70 ^a	45.51 ^a
Positive control	23.3 ± 12.80 ^b	34.88 ^a
Metformin 95.4 mg/kg	7.7 ± 7.20 ^a	46.11 ^a
Metformin 190.8 mg/kg	11.3 ± 10.30 ^a	46.03 ^a

MN: micronuclei; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes *Metformin at concentrations of 334.7 mg/kg – there are no bone-marrow cells, probably as result of acute cytotoxicity. ^{a, b}Different superscripts in the same column denote significant differences ($p < 0.05$).

Table 2 - Chromosome aberrations in metaphase CHO-K1 cells exposed to metformin.

Treatments	Examined cells (N)	Structural chromosome aberrations (%)			Total structural aberrations (%)	Number of damaged cells
		Breaks	Gaps	Acentric fragments		
Negative control	400	0.50 ± 0.50	1.25 ± 1.06	2.75 ± 0.35	4.5 ± 0.00 ^a	18
Positive control	356	2.56 ± 1.35	-	9.24 ± 0.60	11.82 ± 0.74 ^b	42
Metformin 114.4 µg/mL	400	0.75 ± 0.35	0.75 ± 1.06	5.50 ± 0.70	6.50 ± 2.82 ^{ab}	28
Metformin 572.0 µg/mL	398	1.76 ± 1.07	0.25 ± 0.35	4.02 ± 0.73	6.03 ± 1.46 ^a	32

^{a, b}Different superscripts in the same column denote significant differences ($p < 0.05$).

Discussion

According to comet assaying, metformin was shown to induce DNA damage in CHO-K1 cells. However, this *in vitro* genotoxicity was not noted in chromosome aberrations and *in vivo* tests (micronucleus). Micronucleus assaying, as of chromosome aberrations, is a cytogenetic form that measures chromosomal damage, thus only effective when both DNA strands are broken. On the contrary, comet assaying quantitatively measures DNA strand breaks in either or both strands (Slamenova *et al.*, 1997; Zeni and Scarfi, 2010).

The frequency of cells with DNA damage was higher when CHO-K1 cells were treated with both metformin concentrations, although the frequency of DNA-strand breaks was only perceived at the lower concentration (Table 1). It is presumed that cells treated with the higher concentration were severely damaged in the first cycle. Thus, the second cycle resulted in fewer cells capable of successfully repairing the damage. In fact, only moderate alterations were noted (comet type 1 and 2). Under stress conditions, such as DNA damage, p53 blocks the cell-cycle, thus facilitating DNA repair or inducing apoptosis. Therefore, CHO-cell deficiency in p53 might lead to the inefficient repair of cells damaged by substances, such as metformin. Furthermore, the failure to eliminate DNA damaged cells might give rise to morphologically and functionally altered cell-transformed cell clones. Buzzai *et al.* (2007) suggested that metformin is selectively toxic to p53-deficient cells. On other hand, the lower concentration did not induce cell-death in the first cycle. Thus, the expected changes were apparent in cells in the second cycle, *i.e.*, the accumulated damage from both cycles showing 3 and 4 comet-types.

DNA damage can have biological consequences, such as transcription and/or replication inhibition, ultimately leading to cell-death (Olsen *et al.*, 2005; Hales, 2005). Accumulation of DNA damage and unrepaired DNA lesions can cause cell-death or permanent cell-cycle blockage (Hassa and Hottiger, 2005). Therefore, it is reasonable to assume that those cells surviving the first cycle modulated the observed type of DNA damage. The association between drug exposure and DNA damage (and eventually apoptosis induction) has been established. Kefas *et al.* (2004) showed that longer metformin exposure (> 24 h) resulted in a progressive increase in apoptotic rat β -cells, the effects of metformin being both dose- and time-dependent. In fact, low concentrations of metformin are capable of inducing AMP-activated protein kinase (AMPK) mediated alterations in β -cells (Kefas *et al.*, 2004). On the other hand, Ben Sahra *et al.* (2008) showed that, although not apoptosis inductive, metformin was effective in blocking the cell-cycle in G₀/G₁.

So far, there has been no indication of chromosome aberrations induced by metformin in bone-marrow cells. According to the *in vivo* study, no micronuclei were ob-

served in mice bone-marrow cells at concentrations of 95.4 mg/kg and 190.8 mg/kg, although the higher concentration induced cytotoxicity. Twenty four hours after administration of metformin at 333.9 mg/kg, no cells were found for micronucleus analysis. It is noteworthy that not only the dose, but also the type of tissue, can modulate genotoxicity, some substances possibly presenting pro- and anti- genotoxic effects. Bone marrow, easy to isolate and process, is routinely used in chromosome aberration tests. This highly vascular tissue has a population of rapidly cycling cells, thereby increasing vulnerability to toxic drugs. On the other hand, the lower metformin concentrations (95.4 mg/kg and 190.8 mg/kg) did not induce micronucleated cells. It is probable that, under the prevailing experimental conditions, metformin induces more breaks in a single strand of DNA. Thus, in the absence of double strands, no damage was detected by cytogenetic tests.

The mechanism of metformin effects on DNA molecules is unknown. Notwithstanding, these might possibly be mediated through its activation of AMPK, thereby increasing nitric oxide synthase (Zhou *et al.*, 2001; Davis *et al.*, 2006; Za'tara *et al.*, 2008). Zou *et al.*, (2004) speculated that mitochondria-derived reactive-nitrogen-species mediate AMPK activation by way of metformin. Depending on the dose, nitric oxide is capable of inducing beneficial effects by playing a role in the gene regulation and signal transduction pathways possibly involved in defensive mechanisms against oxidative stress (Miyamoto *et al.*, 2003). Nevertheless, high levels (nanomoles) can damage macromolecules, such as lipids, proteins and DNA, thereby leading to mutagenesis and carcinogenesis (Bishop and Cashman, 2003). Moreover, it has been shown that nitric oxide can induce mutation by impairing the repair-enzyme system (Jaiswal *et al.*, 2001). Metformin is known to accumulate in tissues. Studies have indicated that doses insufficient for lowering hyperglycemia in diabetic animals concentrate in tissues (Wilcock and Bailey, 1994). Hirsch *et al.*, (2009) showed that low doses of metformin inhibits cellular transformation and selectively kills cancer-stem cells in four genetically different types of breast cancer. The authors propose combining metformin with chemotherapy as a novel treatment, not only for breast cancer, but possibly others. Furthermore, *in vitro* lymphocytes challenged with CumOOH showed that high concentrations of metformin potentially induce DNA damage (Onaran *et al.*, 2006). Taken together, the data suggest that chronic metformin exposure may be potentially genotoxic. Thus, pregnant woman under treatment with metformin should be properly evaluated as to vulnerability to DNA damage.

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