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Genotoxicity evaluation of metformin and glimepiride by micronucleus assay in exfoliated urothelial cells of type 2 diabetes mellitus patients

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ABSTRACT

Micronucleus (MN) assay was performed on the exfoliated urothelial cells to detect the genotoxic effects of the anti-hyperglycemic drugs, metformin and glimepiride in T2DM patients and to use it as a biomarker for DNA damage by assessing the frequency of micronuclei in the exfoliated urothelial cells. A total of 201 subjects (147 T2DM patients & 54 Normal cases) were selected from diverse age groups (25–75 years) and the mean MN frequency was examined per 1000 cells in all the subjects. Relative to the control group (5.02 ± 1.01), an increased MN frequency was observed in females (26.15 ± 2.15) when compared to males (23.08 ± 2.09) in T2DM patients. Further analysis showed that there was a profound increase in the number of MN in the patients using metformin alone (23.02 ± 4.44), or combination of metformin & glimepiride (24.98 ± 2.87) than to the subjects using glimepiride alone (17.52 ± 3.28). It has been proven by this simple, reliable and non-invasive method that metformin has a potential role in causing genotoxicity and that the MN observed in exfoliated urothelial cells could be used as a reliable biomarker in monitoring the genotoxic risk of the anti-hyperglycemic drugs.

1. Introduction

Type 2 diabetes (non-insulin-dependent diabetes/T2DM) is a serious health concern all over the world. WHO (World Health Organization) estimates that about 90% of people around the world are affected with Type 2 diabetes (Alberti and Zimmet, 1998). The main treatment option available to control diabetes is by lowering the blood glucose level, and most of the drugs developed aim towards this. Metformin (dimethyl-biguanide), an oral anti-hyperglycemic drug, is the most commonly prescribed drug as the first line of treatment worldwide (Van Staa et al., 2012). Generally, metformin is prescribed along with sulfonylureas for efficacy in the treatment of T2DM. One such recent sulfonylurea drug is glimepiride, which has lower cardiovascular risk compared to other sulfonylurea drugs and is also effective in treating T2DM (Rendell, 2004; Nissen et al., 2008; Schotborgh and Wilde, 1997). Metformin in combination with glimepiride plays a significant role in suppression of hepatic glucose production, and thus could also be used in pre-diabetic conditions (Viollet et al., 2012). Although widely used, metformin can cause minor incidents of gastrointestinal upsets such as dysphagia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting, and diarrhea (Wolosin and Edelman, 2000) but no major side effects have been reported. However, it has been observed that metformin induces DNA damage either by increasing the levels of reactive oxygen species and reducing aconitase activity (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3313505/Anedda et al., 2008) or by increasing the cumene hydroperoxide (CumOOH) content which in turn induces fragmentation (Onaran et al., 2006).

The ability to detect genotoxicity in exfoliated cells has proven to be the most reliable and affordable method to investigate the effects caused by malignancies (Benner et al., 1994), pesticides (Bortoli et al., 2009), smoking (El-Setouhy et al., 2008) and several drugs. Recently urine samples has been considered as an ideal source for performing genotoxicity analysis since the number of urothelial cells in an individual remains remarkably constant (Fontana et al., 2001). DNA damage of epithelial cells can be observed by performing the micronucleus test, which is one of the most well-established techniques to detect DNA damage (Obiakor et al., 2014).

This study aimed to assess the frequency of micronuclei in

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exfoliated urothelial cells as a biomarker for DNA damage. Here, we investigated the genotoxic effect caused by metformin and glimepiride in combination by observing the exfoliated urothelial cells of patients with Type 2 diabetes so as to establish a simple, reliable and noninvasive method for detection of genotoxic effects caused by anti-diabetic drugs.

2. Materials and methods

2.1. Subjects

147 patients (86 females and 61 males) who were treated with metformin and/or glimepiride for Type 2 diabetes and 54 healthy volunteers, matched to the age and sex of the patients, as controls, were selected. Also blood samples were collected from a small subset of 38 volunteers (27 patients of T2DM and 11 healthy individuals) to study the significance of MN formation in peripheral blood mononuclear cells (PBMC). The patients were aged between 25 and 75 years (51.67 ± 1.29). All the subjects involved in the study were nonsmokers and nonalcoholic, with no history of urinary tract infection or cancer. Informed consent was obtained from the subjects participating in the study.

2.2. Urine & blood sample collection

The first morning samples of the subjects were voided and they were requested to wash their urethral area extensively with flushable pre-moistened wipes in order to avoid microbial and squamous cells contamination, particularly in females. Mid-stream urine samples (50–100 mL) were collected in a sterile container. All the samples were processed within 2 h or stored at 4 °C for later use (Koss and Melamed, 2005). Heparinized venous blood were collected from donors and processed immediately for PBMC isolation using Ficoll–Paque method as per manufacturer's instruction (HI Media Laboratories, India).

2.3. Extraction of urothelial exfoliated cells

The urine samples were transferred to fresh centrifuge tubes (50 mL). The tubes were centrifuged at 3000 g for 15 min at 30 °C. The pellets were dissolved in 1× PBS (137 mM/L NaCl, 2.7 mM/L KCl, 10 mM/L Na_2HPO_4, 1.8 mM KH_2PO_4) and centrifuged at 1500 g for 10 min at 30 °C. The supernatant was discarded without disturbing the pellet that contained the urothelial cells. The pellets were fixed in Carnoy I fixative solution (methanol and glacial acetic acid, 3:1) and stored at 4 °C for further use (Lehucher-Michel et al., 1996).

2.4. Slide preparation and MN scoring

The stored samples were brought to room temperature and smeared on a clean, grease-free microscopic slide. The smeared samples were air dried and stained with 1% giemsa staining solution for 10 min. The excess stain was removed by 1× PBS and air dried. The slides were screened for nucleated cells at 40× magnification on a light microscope (Olympus Microsystems, CX21i) and cells containing an intact micronucleus were scored per 1000 urothelial cells. All MNs were confirmed by a second observer. The presence of micronucleus was further confirmed by performing propidium iodide staining (1.5 M) using fluorescent microscopy. All the criteria described by Fortin et al. (2010) to distinguish micronucleus in urothelial cells were taken into consideration.

2.5. Statistical analysis

Statistical analysis of the mean values of cells with MN in T2DM patients and controls were analyzed by student's t-test using the Graph pad online software (www.graphpad.com/quickcalcs/ttest1). To assess the correlations between different staining methods in order to rule out the cell anomalies, Spearman rank correlation coefficients were computed. The statistical tests were performed at a significant level of $p < 0.05$.

3. Results

The characteristics of the study population were grouped based on the age, sex and type of drugs used. As smoking and alcohol consumption causes cellular damage, all the cases selected were nonsmokers and nonalcoholics. The mean age of the patients and control groups were 51.67 ± 1.29 years and 48.45 ± 4.97 years, respectively. Thus, the age group of the patients and control selected for the study were almost identical (Table 1). The phase contrast and fluorescent microscopic images of the urothelial cells of T2DM patients containing MN and a normal cell with no MN is shown (Fig. 1). The frequency of cells with MN was higher in the T2DM patient group (24.98 ± 2.87) than in the control subjects (5.02 ± 1.01). In T2DM patients, females (26.15 ± 2.15) had a significant increase in MN frequency compared to men (23.08 ± 2.089; $p < 0.001$) (Fig. 2). Although there was a profound increase in MN frequency in patients using glimepiride alone (17.52 ± 3.28) in comparison with controls (Fig. 3), it was even higher in patients using either metformin alone (23.02 ± 4.44) or metformin and glimepiride in combination (24.98 ± 2.87). Further analysis on the MN frequency of PBMC showed that MN population was significantly high in T2DM patients using the drugs compared to that of the control (Fig. 4). Our study also showed that the duration of metformin and/or glimepiride treatment does not seem to have a significant effect on the formation of micronucleus (Fig. 5).

4. Discussion

Metformin is considered as the first drug of choice for treatment of T2DM patients, and is particularly suitable in the treatment of T2DM patients who are overweight and have hypoglycemic conditions. Based on the clinical characteristics of the patients, a combination of drugs, normally metformin with any sulfonylurea drug is preferred. One such new generation sulfonylurea drug is glimepiride, which has lower cardiovascular risks compared to other conventional drugs. Zhu et al. (2013) performed a meta-analysis to compare the efficacy of metformin and glimepiride in the treatment of T2DM. The analysis showed that metformin was more effective than glimepiride in controlling the levels of total cholesterol, low-density lipoprotein (LDL), and triglycerides (TG), whereas glimepiride was better in the overall efficacy, controlling the levels of HbA1c, postprandial blood sugar (PPBS), fasting plasma insulin (FINS), systolic and diastolic blood pressures (SBP and DBP). This confirms that, based on the clinical characteristics of subjects, a combination of drugs is generally prescribed.

The MN assay is a process used to detect clastogenic and aneugenic effects. Micronuclei are acentric fragments or complete chromosomes that fail to attach to the mitotic spindle during cytokinesis and are excluded from the nuclei due to the effect of chemicals. Apart from chemicals, pesticides and pollutants, several other factors such as smoking and alcohol consumption also have a direct impact on DNA damage. Stich and Rosin (1983) demonstrated a strong synergistic effect of smoking and alcohol consumption on the elevated levels of MN formation. According to Naderi et al. (2012), the mean number of micronuclei in the buccal mucosa cells of nonsmokers was significantly lower than that in smokers. In order to avoid such interference, all the subjects involved in the study were nonsmokers and nonalcoholics with no