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.

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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 (Violet 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...
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 Na2HPO4, 1.8 mM KH2PO4) 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. 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, CX211) 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 ± 2.08; 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...
Innumerable studies in the past decades indicate that the high frequency of MN observed in T2DM patients could be due to the elevated level of oxidative stress (Relative oxygen species ROS production) (Blasiak et al., 2004), downregulation of insulin producing genes (Merkel et al., 2003), and intake of certain anti-diabetic drugs that decrease vitamin B12 levels and increase the levels of homocysteine, perhaps by inhibiting its absorption, and thus increasing the frequency of MN (Fenech, 1999). In fact, all these parameters are interrelated and could be due to the long-term intake of anti-diabetic drugs, particularly metformin and glimepiride, which may increase ROS production causing cellular damage and eventually DNA, protein modification, and peroxidation-induced micronucleus (Narayanan et al., 2003; Sawada et al., 2008).

Fig. 1. Microscopic image of exfoliated urothelial cells of (A) & (B). Phase contrast image of a normal urothelial cell with no micronucleus (A), Urothelial cell with micronucleus in T2DM patients treated with metformin and glimepiride in combination (B) (40× mag). (C) & (D) fluorescent images of a normal urothelial cell and an urothelial cell possessing MN respectively (40× mag).

Fig. 2. Average number of MN/1000 cells in T2DM patients using metformin and glimepiride in combination, and in control.

Fig. 3. Average number of MN/1000 cells on control cases and T2DM patients using metformin, glimepiride, and a combination of both metformin and glimepiride.
invasive, less time-consuming and can be easily performed on interphase cells with no requirement for cell culture or metaphase preparations (Holland et al., 2008). Buccal cells could be another source for analyzing the genotoxicity as it is less invasive and easy to extract (Thomas et al., 2009). Performing the MN assay on exfoliated urothelial cells has several advantages than on blood lymphocytes and buccal epithelial cells, as it is a noninvasive method. Further, the epithelial cells in the basal layer of the buccal mucosa are renewed every 1–3 weeks and, hence, any recent genetic damage to the layer might not be inferred through the tests, as the exfoliated cells are removed from the basal layer. This strengthens the option of using exfoliated urothelial cells as a suitable biomarker in evaluating the genotoxic damage caused by any clastogens. Due to the potential artifacts generated by Giemsa staining we have evaluated some of the samples using a fluorescent (PI) staining and a good correlation was observed, which would confirm the consistency of our data (Fig. 6).

In this study, the assay was performed on exfoliated cells from the bladder of T2DM patients taking metformin and/or glimepiride. Subsequently, the patients had higher micronucleated cells than those in the control group, which signifies the genotoxic level of the compounds. This study also reveals a comparison between metformin and glimepiride in MN formation. Although glimepiride can also induce genotoxic damage, the effect caused by metformin on the cells is relatively higher (Fig. 3). In vitro results indicate that chronic exposure to metformin and glimepiride may be potentially genotoxic (Amador et al., 2011). In vivo studies by Gurbuzel et al. (2014) state the potential genotoxic effect of glimepiride in Drosophila. Similarly, Shaik et al. (2010) performed the MN assay in T2DM patients treated with a combinational therapy of pioglitazone and glimepiride on buccal cells and found an increased frequency of MN in patients exposed to long-time usage of the drugs. We also performed a comparative analysis of MN frequency in PBMC and urothelial cells to study the significance of using urothelial cells as a potential marker. Apparently, the frequency of MN formation in urothelial cells was 0.5 times higher when compared with peripheral blood mono-nucleated cells. Since the BNMN frequency was very less, it was not taken into consideration and further work was focused only towards MN frequency in urothelial cells (Fig. 4). However, in our study we were not able to find any significant differences between the duration of metformin/glimepiride use and MN formation (Fig. 5). However, controversially some studies using experimental rats/mice have shown that metformin treatment did not stimulate genotoxicity in both normal and diabetic rats (Attia et al., 2009) and also there was no evidence of carcinogenicity (Sant’Anna et al., 2013). Similarly, Rabbani et al. (2009) showed that the drugs do not attenuate nuclear damage, suggesting the dosage and duration of co-exposure to drugs may also have an influence on the micronuclei frequency. Conclusive information about the action of metformin in causing genotoxicity is under debate, as several genetic and environmental factors are associated with genotoxicity. Therefore, this preliminary study
performed on exfoliated urothelial cells of T2DM patients suggests that acute or chronic exposure to anti-hyperglycemic drugs could be one of the prime factors involved in increasing the frequency of micronucleated cells.

5. Conclusion

From this study it can be inferred that exposure to anti-hyperglycemic drugs (metformin and/or glimepiride) increases the frequency of micronucleated cells compared to controls. Moreover, observing MN in exfoliated urothelial cells can be an invaluable tool in bio-monitoring and can be used as a marker for bladder mutagenic agents. Taking into consideration the results from this study, it can be concluded that the MN assay in urothelial cells can be used as a reliable and simple method for detection of cytogenetic damage, which reflects an enhanced risk of cancer. However, further studies are required to understand the genetic factors associated with the mechanism of the action of drugs in causing genotoxicity.

Conflict of interest

The authors declare that there are no conflicts of interest.

References


