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Assessment of Micronuclei Frequency in Buccal Mucosal Cells among Diabetic Patients in Shendi, Sudan: A Cross-Sectional Case-Control Study

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Abstract

Diabetes Mellitus (DM) prevalence has been increasing, with over 415 million cases worldwide and a mortality rate of 2.5–5 million each year. Micronuclei (MN), which are cytoplasmic chromatin masses, have recently been suggested as a biomarker for cancer risk. An elevated micronuclei frequency is associated with DM. The oral cavity is considered a mirror reflecting an individual's health. This study aims to investigate the genotoxic effects of diabetes mellitus on human oral cells. This is a case-control study conducted in Shendi town from October 2021 to March 2022 at diabetic clinics. Forty-three mouth rinse samples were collected from diabetic patients and seventeen from non- diabetic individuals. All samples were centrifuged, and the sediment was smeared onto clean glass slides. It was then immediately fixed in 95% ethanol and stained with Papanicolaou stain and the Feulgen reaction to detect and calculate the rate of MN formation among the study groups. Data were analyzed using SPSS version 28.0. Cytology screening showed the MN rate among diabetics was 5.17 MN/100 cells with a standard deviation of 5.01, while among non- diabetics it was 2.29 MN/100 cells with a SD of 1.49. The difference between the two means was statistically significant (*P-value* 0.024). In this study, MN formation in diabetic patients' mucosal cells was correlated with control of DM (*P-value* 0.02) and metformin intake (*P-value* 0.045), but it was not correlated with either age or duration of the disease. Other cellular changes observed alongside micronuclei in diabetic oral mucosa included nuclear anomalies (95. 3%), hyperkeratosis /metaplasia (42%), inflammatory cells (76.7%), megalo-plastic changes (53.5%), nuclear atypia (72.1%), and binucleation (67.4%). No cellular anomalies were observed in non-diabetic oral cells. Papanicolaou stain demonstrated higher sensitivity (87.1%) for detecting MN formation and lower specificity (10%) compared to the Feulgen reaction. DM and its treatment with metformin exhibit a genotoxic effect on oral cells. The study recommends implementing oral cytology and genetic screening programs for diabetic patients, utilizing the Feulgen reaction as the most effective method for MN detection.

Keywords: *Drug Therapy, DM,* Micronuclei*,* Metformin, Oral Cytology, Buccal Mucosa.

1. INTRODUCTION

Diabetes mellitus (DM) is an endocrine metabolic disorder characterized by an abnormal, elevated concentration of glucose in plasma (hyperglycemia) that, when not treated, can lead to ketoacidosis and chronic degenerative diseases of the heart, kidneys, eyes, and nerves. Besides self-resolving gestational DM, two other variants of DM can be defined based on the insulin hormone: DM1 is associated with low levels of insulin, and DM2 is associated with insulin resistance. DM2, comprising almost 90% of DM cases, is the result of a combination of environmental and genetic factors [1, 2]. DM prevalence has been increasing with more than 415 million cases worldwide and a mortality rate of 2.5–5million each year [3]. Chronic high levels of glucose during DM are thought to increase oxidative stress and the formation of free radicals that, in turn, damage cells [2-4]. Reactive oxygen species (ROS) chemically attack cellular components, altering metabolism, inflammatory mediators, and antioxidant

defense mechanisms, overall favoring the pathogenesis of the disease and the persistence of genetic damage [5]. Elevated micronuclei frequency is associated with DM2 [6], and DM1 [7]. In all DM patients, it is commonly reported in blood cells [6]. DM2 has been associated with elevated levels of DNA damage, increased susceptibility to mutagens, and a decreased efficacy of DNA repair [8]. Causing genomic instability and consequently cancer [9]. However, the mechanism underlying this association is unclear. The number of micronuclei (MN) has been mainly used as a biomarker in peripheral blood lymphocytes to evaluate genotoxic risks in the work environment [10]. MNs are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosomal fragments or intact whole chromosomes that lag at the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis [10], and has recently been suggested as a biomarker for cancer risk [11]. The conceptual basis for this application is the idea that increased cytogenetic damage reflects an enhanced cancer risk [12]. Oral mucosa often reflects disease changes; it is the first contact with many pollutants like tobacco or alcohol, and its affection can also be indicative of a systemic condition or side effects due to chemotherapy or radiotherapy administration [13, 14]. This study aimed to assess micronuclei in oral mucosal cells among diabetic patients in Shendi, Sudan.

2. METHODOLOGY

This case-control study was conducted in Shendi town, Sudan, to evaluate cytological changes in the oral mucosa of diabetic and non-diabetic individuals. The study took place from September 2021 to March 2022. A total of 60 participants were included: 43 diabetic patients and 17 healthy controls. All participants were over 18 years old and included both genders. Diabetic patients were assessed based on their medical history, medication type, oral health status, and demographic and socioeconomic data. Control subjects were evaluated through clinical history and confirmed to have no personal history of diabetes mellitus. Oral mucosa samples were collected from all participants using a sterile cytobrush. The exfoliated cells were immediately transferred to clean slides and fixed. The slides were then processed and examined at the Histopathology and Cytology Laboratory, Shendi University. Two staining techniques were used: Papanicolaou stain for cytological evaluation, and Feulgen reaction stain to detect nuclear abnormalities like micronuclei (MN). To ensure accuracy and minimize confounding factors, specific inclusion and exclusion criteria were applied. Inclusion criteria: diagnosed cases of diabetes mellitus. Exclusion criteria: individuals with conditions or habits that could affect oral cytology independently of diabetes, such as hypercholesterolemia, arthritis, cancer, tobacco smoking, alcohol use, regular coffee consumption, certain medications, and exposure to local anesthetics.

Study Sample

Sample size was calculated using GPower software (α=0.05, power=80%), based on MN frequency data from Martínez-Pérez *et al*., [16]. Buccal and lingual smear samples (via mouth rinse technique) were collected from each participant to detect the presence of micronuclei (MN) and other nuclear abnormalities.

Sample Size

A total of sixty exfoliative oral mucosa samples were obtained—one from each participant, comprising both diabetic and non-diabetic individuals.

Data Collection Tool

A structured questionnaire was used to collect demographic, clinical, and oral health-related data from all participants. The gathered data were then organized into a master sheet for further analysis. Glycemic control was defined as HbA1c <7% (controlled) vs. ≥7% (uncontrolled) according to ADA guidelines.

Study Variables and Methods of Detection

Micronuclei and other nuclear abnormalities in the exfoliated oral epithelial cells were identified using Papanicolaou staining and the Feulgen reaction. Additional analytical and socio-demographic data were processed and interpreted using appropriate statistical analysis methods.

Sample Collection and Processing

Each participant was first asked to rinse their mouth with water to reduce contamination. Oral mucosal samples were then collected by having the participants rinse their oral cavity with a 0.9% NaCl saline solution prepared in distilled water. The rinse was centrifuged at 800 rpm for 5 minutes, and the supernatant was discarded. The resulting cell pellet was smeared onto clean glass slides, which were immediately fixed in 95% ethanol for a minimum of 15 minutes. Two staining techniques were applied for cytological examination: Papanicolaou staining and Feulgen/Fast Green staining. A total of 100 cells per subject were examined, and the results were expressed in percentages. All slides were analyzed under a light microscope.

Papanicolaou Staining Technique

Fixed smears were sequentially rehydrated in 95% ethanol, 80% ethanol, and distilled water (2 minutes each). Slides were then stained with Harris's hematoxylin for 5 minutes, briefly differentiated in 0.5% acid alcohol, and blued under running tap water for 2 minutes. Following this, slides were rinsed in 95% ethanol and stained with Orange G6 for 2 minutes, washed again in 95% ethanol, and counterstained with EA50 (Eosin Azure) for 3 minutes. The slides were then dehydrated in absolute ethanol, cleared in xylene, and mounted using DPX (Dibutyl Plasticizer and Xylene). Finally, the slides were screened under a light microscope.

Modified Feulgen Staining Technique

Feulgen staining was performed with the following modifications: Slides were hydrolyzed by immersion in 1N HCl at room temperature for 1 minute, then in 1N HCl at 60°C for 10 minutes, and again in 1N HCl at room temperature for 1 minute. After hydrolysis, the slides were immersed in Schiff's reagent for 90 minutes, rinsed with distilled water, counterstained with Fast Green for 30 seconds, and rinsed again. Under the microscope, nuclei appeared pink to orange, while the cytoplasm appeared blue.

Interpretation of Results

Slides were evaluated under a light microscope, and micronuclei (MN) were identified based on Fenech's criteria, including:

a) A diameter between 1/16 and 1/3 of the main nucleus,
b) Non-refractile appearance,
c) No connection or overlap with the main nucleus,
d) Similar staining intensity to the nucleus, and
e) Location within the cytoplasm.

In addition to MN, other nuclear abnormalities were identified, such as binucleation, multinucleation, megaloblastic changes (e.g., macrocytes, nuclear grooves), and anomalies like pyknotic nuclei, condensed chromatin, karyorrhexis, karyolysis, nuclear buds, and nuclear atypia (including high nuclear-to-cytoplasmic ratio, irregular contours, and prominent nucleoli).

Quality Control Measures

To ensure sample integrity and staining quality, all materials used were sterile. Oral rinse solutions were prepared using sterile 0.9% normal saline, and sterile disposable centrifuge tubes were used for cell precipitation. Smears were prepared directly to avoid air-drying artifacts and fixed immediately in ethanol. All staining reagents were filtered before use, and Coplin jars were cleaned before and after each use. The quality of staining solutions was checked before application. During processing, all jars and dishes were securely covered to prevent evaporation and contamination, including during the mounting and cover slipping procedures.

Data Analysis

After examination of the sections, the results of the laboratory investigation, as well as the demographic data from the patient's records, were processed using the Statistical Packages for Social Sciences (SPSS) computer program. Frequency, mean, and chi-square test values were calculated at <0.05 and considered statistically significant.

3. RESULTS

The cytological analysis of 43 diabetic patients and 17 non-diabetic controls revealed significant genomic alterations in the oral mucosa of individuals with diabetes. As shown in Table 1, micronuclei (MN) were detected in 87.8% of diabetic samples using Papanicolaou (Pap) stain and 75.6% with Feulgen stain, compared to minimal findings in controls (mean MN frequency: 5.17 vs. 2.29 per 100 cells; P = 0.024, 95% CI: 3.61–6.73 for diabetics, 1.52–3.06 for controls). Nuclear anomalies were common in 95.3% of diabetics, including binucleation (67.4%), karyolysis (60.4%), and micronuclei (60.4%), whereas no abnormalities were observed in controls (Table 2). Notably, MN formation strongly correlated with metformin therapy (93% positivity in users vs. 65% in non-users; P = 0.045) and poor glycemic control (HbA1c ≥7%; P = 0.02), but showed no association with age (P = 0.18) or disease duration (P = 0.9) (Tables 3–4). The Pap stain demonstrated high sensitivity (87.1%) but low specificity (10%) for MN detection, while Feulgen offered better specificity despite technical complexity (Table 5).

Table 1: Frequency of Cytological Abnormalities in Diabetic Patients

Abnormality	Frequency	Percentage (%)
Micronuclei (Pap)	36	87.8
Micronuclei (Feulgen)	31	75.6
Nuclear anomalies	41	95.3
Megaloblastic changes	23	53.5
Inflammatory cells	33	76.7
Binucleation	29	67.4
Nuclear atypia	31	72.1

Table 2: Nuclear Anomaly Subtypes in Diabetics

Anomaly	Frequency	Percentage (%)
Bi/Multinucleation	30	69.7
Karyolysis	26	60.4
Karyorrhexis	16	37.2
Pyknotic nuclei	17	39.5

Table 3: MN Association with Clinical Factors

Factor	MN+ (n)	MN– (n)	*P-value*
Metformin use			0.045
Yes (n=15)	14	1	
No (n=26)	17	9	
Glycemic control			0.02
HbA1c <7% (n=17)	16	1	
HbA1c ≥7% (n=24)	15	9	

Table 4: MN Frequency by Demographics

Variable	MN+ (n)	MN– (n)	***P-value***
Age			0.18
36–60 years (n=25)	20	5	
>60 years (n=15)	11	4	
Disease duration			0.9
≤10 years (n=26)	20	6	
>10 years (n=15)	11	4	

Table 5: Diagnostic Performance of Staining Methods

Parameter	Pap Stain (%)	Feulgen Stain (%)
Sensitivity	87.1	100.0
Specificity	10.0	88.2
PPV	75.0	93.9

4. DISCUSSION

The findings of this study indicate a significantly higher frequency of micronuclei and nuclear abnormalities in the oral mucosal cells of diabetic patients compared to non-diabetic controls, suggesting increased genomic instability among diabetics. This aligns with previous studies that reported similar increases in micronuclei frequency due to oxidative stress and DNA damage associated with chronic hyperglycemia [2-15]. The mean number of micronuclei was significantly higher in diabetic individuals ($P = 0.024$), supporting the idea that diabetes mellitus contributes to cytogenetic damage in epithelial tissues [16-19]. The presence of nuclear anomalies such as bi/multinucleation, karyolysis, pyknotic nuclei, and nuclear budding, as observed in this study, shows disturbances in cell division and apoptosis. These features are consistent with the genotoxic environment created by high glucose levels, which can lead to chromosomal breakage and impaired DNA repair mechanisms [20-22]. Megaloblastic changes observed in metformin users may suggest vitamin B12 deficiency, a known side effect of long-term therapy [23–26], though serum B12 levels were not measured. Since vitamin B12 is essential for DNA synthesis, its deficiency can cause nuclear enlargement and abnormal nuclear morphology, as seen here [27, 28]. No significant link was found between age or duration of diabetes and micronuclei frequency ($P > 0.05$), suggesting that the genotoxic effects of diabetes may occur independently of these factors. However, a strong connection was observed between poor glycemic control and increased micronuclei formation ($P = 0.02$), highlighting the importance of metabolic regulation in reducing cellular damage [15-29]. This emphasizes the need for regular monitoring and effective management of blood glucose to lower long-term risks, including potential pre-cancerous changes in oral tissues. Furthermore, comparing Feulgen and Pap staining techniques showed that Pap stain had high sensitivity (87.1%) but low specificity (10%), which could lead to overestimating micronuclei due to misinterpreting keratin or apoptotic debris. Feulgen, being DNA-specific, provided more reliable results [30]. Therefore, Feulgen staining remains the preferred method for accurate micronuclei assessment, despite its technical complexity. Overall, this study demonstrates that exfoliative cytology combined with the micronucleus assay can be a valuable, non-invasive tool for detecting early genotoxic changes in diabetic patients. The results highlight the potential of using oral cytological markers as an early warning system for monitoring the impact of diabetes on cellular health and for identifying patients at risk of developing precancerous or dysplastic lesions [13-33].

Study Limitations

The small control group size (n=17) limits generalizability. Serum vitamin B12 levels were not assessed to confirm deficiency. Potential confounding factors (e.g., dietary habits) were not controlled.

5. CONCLUSION

Micronucleus screening could be prioritized for diabetics with poor glycemic control or long-term metformin use. Routine vitamin B12 monitoring is advised for metformin-treated patients.

6. RECOMMENDATION

Micronucleus testing should be considered for routine screening in diabetic patients, especially those on

long-term metformin therapy. Regular monitoring of vitamin B12 levels is advised. Further studies with larger cohorts are recommended to validate these findings and explore their clinical implications.

Consent: The patient's written consent has been collected.

Ethical Approval: The study was approved by the Department of Histopathology and Cytology in Medical Laboratory Sciences at Shendi University, and the study was matched to the ethical review committee board. Sample collection was done after signing a written agreement with the participants. Permission for this study was obtained from the local authorities in the area of study. The aims and the benefits of this study were explained with the assurance of confidentiality. All protocols in this study were done according to the Declaration of Helsinki (1964).

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