



# Clumpy Novel Mitochondrial Signatures in Irradiated Human Diabetic Buccal Cells: A Case Control Study

## *Işınlanmış İnsan Diyabetik Bukkal Hücrelerinde Kümelenmiş Yeni Mitokondriyal Signatürler: Bir Olgu Kontrol Çalışması*

Surraj SUSAI<sup>1</sup>, Mrudula CHANDRUPATLA<sup>1</sup>, Sumitra SIVAKOTI<sup>2</sup>, Govindrao N. KUSNENIWA<sup>3</sup>,  
Anand K. PYATI<sup>4</sup>

<sup>1</sup>All India Institute of Medical Sciences, Department of Anatomy, Bibinagar, India

<sup>2</sup>All India Institute of Medical Sciences, Department of Pathology, Bibinagar, India

<sup>3</sup>All India Institute of Medical Sciences, Department of Community medicine, Bibinagar, India

<sup>4</sup>All India Institute of Medical Sciences, Department of Biochemistry, Bibinagar, India

### ABSTRACT

**Objective:** This study aimed to determine the whole free mitochondria in type-2 diabetic buccal epithelial cells using a supravital stain called "Janus Green B" and assess their behavior after exposure to near infrared light. The researchers observed microscopic mitochondrial load and its intracellular spatial behavior after exposure to near-infrared rays, bridging the gap in understanding mitochondrial orientation in diseases like diabetes. The aim of this research was to find out the quantitative involvement and electrostatic intracellular spatial patterns of whole mitochondria in diabetes.

**Methods:** Exfoliated buccal cell wet mounts, supravitaly stained using Janus green, and excited using infrared rays, were observed using an advanced bright field Axiocam microscope, and the images of whole mitochondria within the cells were photographed and analyzed using the ZEN 2.0 cell sense software. The migration patterns of mitochondria were observed.

**Results:** A quantitative decrease in mitochondria was noted in diabetic cells. Signatures of clumpy peripheral shifts in mitochondria were observed in diabetic buccal cells post radiation.

**Conclusions:** Advanced glycation end products of diabetes combined with oxidative stressors influenced the free mitochondria to clump peripherally and produce a characteristic signature. The decreased mitochondrial load contributed additional evidence to the reduced respiratory capacity of cells, which forced mitochondria to emit a detectable signature when irradiated.

**Keywords:** Peripheral clumps, infra red rays, power houses, intracellular, shift, comets

### ÖZ

**Amaç:** Bu çalışmanın amacı, tip-2 diyabetik bukkal epitel hücrelerindeki tüm serbest mitokondriyi "Janus Green B" adı verilen supravital bir boya kullanarak belirlemek ve yakın kızılötesi ışığa maruz kaldıktan sonraki davranışlarını değerlendirmektir. Araştırmacılar, yakın kızılötesi ışınlar maruz kaldıktan sonra mikroskopik mitokondriyal yükü ve hücre içi uzamsal davranışını gözlemleyerek diyabet gibi hastalıklarda mitokondriyal yönelimin anlaşılmasındaki boşluğu doldurmuşlardır. Bu araştırmanın amacı, diyabette tüm mitokondrinin kantitatif katılımını ve elektrostatik hücre içi uzamsal modellerini bulmaktır.

**Yöntemler:** Janus yeşili kullanılarak supravital olarak boyanmış ve kızılötesi ışınlar kullanılarak uyarılmış eksofoliyeli edilmiş bukkal hücre ıslak numuneler, gelişmiş bir parlak alan Axiocam mikroskobu kullanılarak gözlemlendi ve hücrelerdeki tüm mitokondrilerin görüntüleri ZEN 2.0 hücre algılama yazılımı kullanılarak fotoğraflandı ve analiz edildi. Mitokondrilerin göç modelleri gözlemlendi.

**Bulgular:** Diyabetik hücrelerde mitokondride kantitatif bir azalma kaydedildi. Radyasyon sonrası diyabetik bukkal hücrelerde mitokondride toplanmış periferik kayma belirtileri gözlemlendi.

**Sonuçlar:** Diyabetin ileri glikasyon son ürünleri oksidatif stres faktörleriyle birleşerek serbest mitokondrilerin periferik olarak kümelenmesini ve karakteristik bir signatür üretmesini etkilemiştir. Mitokondriyal yükün azalması, hücrelerin solunum kapasitesinin azaldığına dair ek kanıtlar sunmuş ve bu düşüş mitokondriyi ışınlandığında tespit edilebilir bir signatür yaymaya zorlamıştır.

**Anahtar kelimeler:** Periferik kümeler, kızıl ötesi ışınlar, güç santrali, hücre içi, kayma, komet

**Address for Correspondence:** Surraj Susai, All India Institute of Medical Sciences, Department of Anatomy, Bibinagar, India

**E-mail:** surraj18@gmail.com **ORCID ID:** orcid.org/0000-0001-5472-6841

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## INTRODUCTION

Though type-2 diabetes is a complex disorder with bio-molecular imbalances, its lab diagnostic investigations to date have focused only on sugar substrates, glycated products, or urinary sediment patterns. This is with the exception of a few studies that have focused on intracellular changes in cellular organelles, such as mitochondrial DNA, without giving impetus to the estimation of whole membrane-bound free mitochondrial count<sup>1,2</sup>. Mitochondria, the energy turbines of cells, are susceptible to glycation in type-2 diabetes, and there is no consensus neither on their intracellular behaviour nor on their count within the diabetic cells due to the paucity of microscopic studies on the same in contrast to the numerous studies that have been done on mitochondrial DNA<sup>3-5</sup>. Hence, this study aimed to quantify whole free mitochondria in type-2 diabetic buccal epithelial cells using the supravital stain "Janus Green B"; it also assessed their spatial behaviour after exposure to near-infrared light. Previous studies had only determined varied expressions of mitochondrial DNA, which may not reflect the disease's severity or prognosis<sup>3-5</sup>. The researchers in this study used supravital stained wet mount smears of infrared-irradiated inner cheek cells to assess the spatial arrangement of mitochondria, which helped them understand the spatial alignments of these organelles in response to the disease's course<sup>6</sup>. This approach overcame the problem of invasive needle insertion for obtaining blood samples for mitochondrial DNA estimation. This research is the first of its kind to bridge the gap in understanding the intracellular orientation of mitochondria in diseases like diabetes<sup>2,3,6</sup>. This research also opens a new area to study the electrostatic repulsive behaviour of adapted mitochondria within buccal cells in response to infrared rays. This is also the first study to have observed specific whole mitochondrial signatures in human buccal cells in response to oxidative stress and glycosylation, and to determine their mitochondrial number and distribution. Previous similar studies had been done only in the myocytes, neurons, or blood cells of biopsied diabetic-induced rats or other lower mammals, but not in humans<sup>7,8</sup>.

## Methodology

### Study Design

Informed consent was obtained from all patients. This was a case-control study conducted at a tertiary care institute wherein the departments of anatomy, pathology, biochemistry, and community medicine were collaboratively involved. The case subjects for the study were chosen from the population that visited

the non-communicable disease clinic at the outpatient department. Cases included only patients with confirmed pure diabetes in the age group of 30 to 60 years, without other co-morbid stressors and having the disease for a minimum duration of at least 3 years. Controls included healthy volunteers who had visited the Out Patient Department for unrelated reasons. This study excluded subjects with history of smoking, betel nut chewing, other oxidative stress illnesses (except diabetes), poor oral hygiene, and oral diseases. Pre-diabetics, diabetics with hypertension, and diabetics with other co-morbid illnesses arising due to oxidative stress were excluded from the study. Forty cases and forty controls were included in this study, which was performed over 18 months. Cases and controls were age and sex matched to avoid bias. Approval was obtained from the Institutional Ethics Committee for this study (reference number: AIIMS/BBN/IEC/OCT/2022/224, date: 08.10.2022).

### Sample Size Estimation

The sample size for this study was calculated using the clincalc sample size calculator available free to all online using the website [www.clincalc.com/stats/sample](http://www.clincalc.com/stats/sample). The primary variable for assessing the outcome in both cases and controls was the mitochondrial load per field of microscope, which was expressed as mean  $\pm$  standard deviation for both groups. The means and standard deviations of mitochondrial load volume in case and control groups from a previously done related study on primate choroidal epithelial cells, which were 13 $\pm$ 1.9 and 11.5 $\pm$ 1.2 respectively, were then fed into the software<sup>5</sup>. It was estimated by the software that 38 patients in each group would be required to detect a mean difference of 12.25 in mitochondrial load between the two groups of cases and controls. The calculations were based on 80% power,  $\alpha=0.05$ , and a two-sided 95% confidence interval. Keeping an attrition factor of 2, the total sample size for both groups together in this case-control study was calculated to be 80, that is, 40 cases and 40 controls.

## MATERIALS and METHODS

Informed consent was obtained from the patients prior to the start of this study. The study was done only after obtaining approval and clearance from the institute research council, institute scientific advisory committee, and institute ethics committee. The grants and funds that were required for purchasing the reagents and materials for this study were released by the institute where the authors work after ethics approval. This was an intramurally funded research. Two wet mount buccal epithelial cell smears were obtained from each of the cases and controls and smeared onto two clean glass

slides for each subject. The first smeared slide was stained directly with Janus green staining solution without subjecting the slide to infrared rays. It was then observed under the Zeiss Axiocam bright field microscope at 40X with a specialized in-built zoom camera that magnified the image of the buccal cells to 100X with visible mitochondrial dots, and the images were photo-captured with a computer-attached DSLR camera. The second smeared slide, on the other hand, was subjected to near-infrared radiation (600 nm wavelength at 65 cm distance from the central convex point of the infra-red lamp) for 7 seconds and then stained with Janus green and observed under the microscope. The slides were thoroughly examined from one corner of the smear to another corner by three independent researchers, and the number of buccal cells in a smear was counted using the image J software. The buccal cell density per smear was determined by the software and then the area of maximum density was marked for counting the number of dark colored mitochondrial spots within each of the buccal cells and a quantitative measurement of the same was made using the "ZEN BLUE cell sense-cell counting software" that picked up the mitochondria dots and estimated the number of mitochondria. The slide fields under the Axiocam microscope were observed from left to right from top to bottom in a zig-zag fashion by three independent researchers so that no mitochondrial dots were missed and the observer bias would be eliminated. The number of mitochondrial dots per buccal cell in cases was noted before and after radiation and compared with their blood HbA1C levels. The mitochondrial load was expressed as the aggregated mean of the mitochondrial dots per buccal cell for the cases and was then compared with the mitochondrial load of controls.

### Statistical Analysis

Descriptive and inferential statistics were used to analyze the data. The baseline characteristics were analyzed by descriptive statistics. The data on the quantity of mitochondria and levels of HbA1C were expressed as the mean with standard deviation, and an attempt was made to find out if an association existed between the mitochondrial load in cases compared with controls. The unpaired Student's t-test was used to compare the means between two groups. In contrast, the one-way analysis of variance (ANOVA) test was used to compare three groups at a time. The mitochondrial levels were correlated separately with the blood HbA1C levels by applying linear regression. All statistical analyses were carried out at a 5% level of significance, and the p-value <0.05 was considered significant. The GraphPad Prism

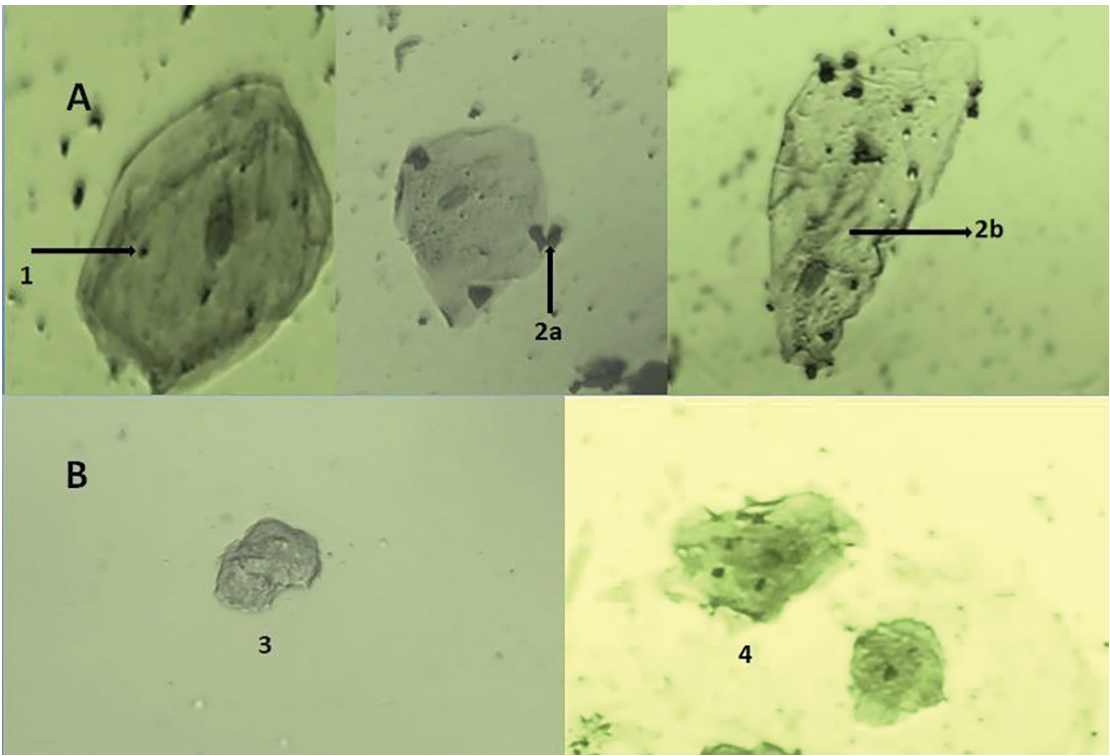
software version 2.0 was used for statistical analysis. The tables were plotted using Microsoft Office Excel 2010.

## RESULTS

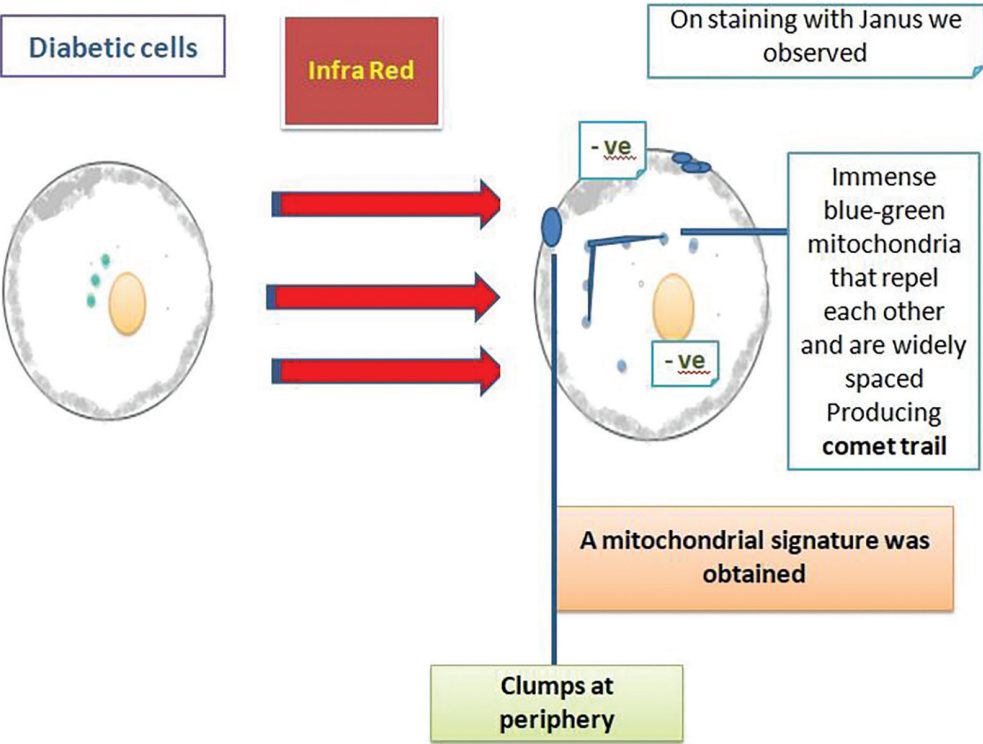
After staining the buccal smears with Janus green B stain prior to radiation, it was observed that the mean number of mitochondria per buccal cell in cases was fewer than the mean number of mitochondria per buccal cell in controls (Table 1). On subjecting the buccal cells of cases to near-infrared rays and then staining them with Janus green, it was observed that the contours of diabetic buccal cells became enlarged and distorted after radiation (Figure 1). The mitochondria present within the diabetic buccal cells became organized into clumps and shifted towards the periphery of the cell. Also, in those diabetic cells with a mean HbA1C value greater than 8 mg/dL, the mitochondria clusters moved away from the part of the cell that was close to the nucleus and migrated to the periphery of the cell, producing comet-like trails within the cell (Figures 1 and 2). Such a phenomenon was not observed in control cells after radiation, and there was no change in mitochondrial dots except that the cell outlines became distorted and the mitochondrial dots became brighter (Figure 1). It was observed that there was a significant association between the mean number of irradiated mitochondrial clumps per buccal cell of cases and the mean HbA1C values in blood (Table 2). The mean number of mitochondrial dots per buccal cell in cases before radiation, the mean number of irradiated mitochondrial clumps per buccal in cases after radiation, and the mean HbA1C values were compared using the one-way ANOVA test, and a significant association between each of the three groups was obtained (Table 3). Furthermore, the post-hoc Tukey HSD beta test (true post-hoc significant difference test) was applied to the three groups to validate the findings of the one-way ANOVA, and a significant Q-value was obtained between each of the three groups (Table 3). A positive linear correlation was obtained between the mean HbA1C levels and the mean number of irradiated mitochondrial clumps per cell in cases, further adding strength to this study (Table 2).

## DISCUSSION

Eldarov et al.<sup>7</sup> found that mitochondria within the rat cells, when subjected to artificially induced diabetes, had fused with each other and then scattered away from the nucleus on subsequent application of stressors such as lipid peroxidation and intense light. A similar observation was noted by Brivio et al.<sup>8</sup> and Raza et al.<sup>9</sup>, wherein they had shown that mitochondria within the cells of stress-induced diabetic mice fused with each



**Figure 1.** Buccal cell Mitochondria (A-cases, 1-normal before IR, 2a-clumps after IR, 2b-comets after IR, B-controls, 3-before IR, 4-after IR) Janus Green B stain, 100X



**Figure 2.** Schema showing the formation of mitochondrial signatures post infra red radiation in diabetic buccal cells

**Table 1. Comparison between mean number of mitochondria per buccal cell in cases and controls prior to radiation.**

| Sl. no             | Cases (n=30)                            |                         | Controls (n=30)                         |                         | t-test*<br>p-value <sup>s</sup> |
|--------------------|---|-------------------------|---|-------------------------|---------------------------------|
|                    | Mean no.of mitochondria per buccal cell | Standard deviation (SD) | Mean no.of mitochondria per buccal cell | Standard deviation (SD) |                                 |
| 1                  | 10.33                                   | 0.47                    | 20.67                                   | 0.47                    | 0.00019                         |
| 2                  | 9.67                                    | 0.41                    | 20.67                                   | 0.36                    |                                 |
| 3                  | 9.33                                    | 0.41                    | 22                                      | 0.35                    |                                 |
| 4                  | 7.66                                    | 0.41                    | 20.67                                   | 0.47                    |                                 |
| 5                  | 8.33                                    | 0.41                    | 20.67                                   | 0.37                    |                                 |
| 6                  | 8.67                                    | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 7                  | 9                                       | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 8                  | 9.33                                    | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 9                  | 10                                      | 0.49                    | 20.67                                   | 0.45                    |                                 |
| 10                 | 9.33                                    | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 11                 | 8                                       | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 12                 | 8.67                                    | 0.47                    | 22                                      | 0.38                    |                                 |
| 13                 | 9                                       | 0.21                    | 22                                      | 0.38                    |                                 |
| 14                 | 8.67                                    | 0.29                    | 22                                      | 0.38                    |                                 |
| 15                 | 8.33                                    | 0.31                    | 22                                      | 0.38                    |                                 |
| 16                 | 8                                       | 0.29                    | 22                                      | 0.38                    |                                 |
| 17                 | 10                                      | 0.29                    | 22                                      | 0.38                    |                                 |
| 18                 | 9.67                                    | 0.27                    | 22                                      | 0.39                    |                                 |
| 19                 | 10                                      | 0.29                    | 22                                      | 0.39                    |                                 |
| 20                 | 10                                      | 0.29                    | 19                                      | 0.39                    |                                 |
| 21                 | 11.33                                   | 0.27                    | 19                                      | 0.39                    |                                 |
| 22                 | 11.33                                   | 0.31                    | 19                                      | 0.39                    |                                 |
| 23                 | 10                                      | 0.31                    | 19                                      | 0.45                    |                                 |
| 24                 | 10.67                                   | 0.31                    | 19                                      | 0.45                    |                                 |
| 25                 | 10                                      | 0.33                    | 19                                      | 0.45                    |                                 |
| 26                 | 10.67                                   | 0.31                    | 20.67                                   | 0.45                    |                                 |
| 27                 | 10.33                                   | 0.31                    | 20.67                                   | 0.45                    |                                 |
| 28                 | 10.33                                   | 0.31                    | 20.67                                   | 0.45                    |                                 |
| 29                 | 10                                      | 0.33                    | 20.67                                   | 0.45                    |                                 |
| 30                 | 10.33                                   | 0.31                    | 20.67                                   | 0.45                    |                                 |
| 31                 | 10.33                                   | 0.47                    | 20.67                                   | 0.47                    |                                 |
| 32                 | 9.67                                    | 0.41                    | 20.67                                   | 0.36                    |                                 |
| 33                 | 9.33                                    | 0.41                    | 22                                      | 0.35                    |                                 |
| 34                 | 7.66                                    | 0.41                    | 20.67                                   | 0.47                    |                                 |
| 35                 | 8.33                                    | 0.41                    | 20.67                                   | 0.37                    |                                 |
| 36                 | 8.67                                    | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 37                 | 9                                       | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 38                 | 9.33                                    | 0.47                    | 20.67                                   | 0.45                    |                                 |
| 39                 | 10                                      | 0.49                    | 20.67                                   | 0.45                    |                                 |
| 40                 | 9.33                                    | 0.47                    | 20.67                                   | 0.45                    |                                 |
| Aggregated mean±SD | 9.47                                    | 0.38                    | 20.75                                   | 0.42                    |                                 |

\*Unpaired t-test was used, <sup>s</sup>statistically significant, SD: Standard deviation



**Table 2. Comparison between the mean number of irradiated mitochondrial clumps per buccal cell in cases with their blood HbA1C levels.**

| Sl. no                 | Cases (n=30)                                    |                         |                                    |                    |                              | Linear regression                                   |
|------------------------|---|-------------------------|------------------------------------|--------------------|------------------------------|---|
|                        | Mean no.of mitochondrial clumps per buccal cell | Standard deviation (SD) | Mean HbA1C values of last one year | Standard deviation | t-test* p-value <sup>§</sup> |   |
| 1                      | 2.33  | 0.37                    | 7.1                                | 0.37               | 0.0031                       | y=1.53x+3.97<br>positive@ correlation,<br>p=0.0001, |
| 2                      | 1.33  | 0.37                    | 6.1                                | 0.31               |                              |   |
| 3                      | 3   | 0.37                    | 9.1                                | 0.31               |                              |   |
| 4                      | 2   | 0.37                    | 7.1                                | 0.31               |                              |   |
| 5                      | 1.33  | 0.37                    | 6.1                                | 0.31               |                              |   |
| 6                      | 1.33  | 0.45                    | 6.2                                | 0.31               |                              |   |
| 7                      | 1.33  | 0.45                    | 6.2                                | 0.31               |                              |   |
| 8                      | 1.33  | 0.45                    | 6.2                                | 0.31               |                              |   |
| 9                      | 1.33  | 0.45                    | 6.2                                | 0.29               |                              |   |
| 10                     | 1.33  | 0.39                    | 6.2                                | 0.29               |                              |   |
| 11                     | 1.33  | 0.33                    | 6.2                                | 0.29               |                              |   |
| 12                     | 2.33  | 0.39                    | 7.2                                | 0.29               |                              |   |
| 13                     | 2.33  | 0.33                    | 7.2                                | 0.29               |                              |   |
| 14                     | 2.33  | 0.33                    | 7.2                                | 0.29               |                              |   |
| 15                     | 2.33  | 0.33                    | 7.2                                | 0.29               |                              |   |
| 16                     | 2.33  | 0.33                    | 7.2                                | 0.33               |                              |   |
| 17                     | 2.33  | 0.33                    | 7.2                                | 0.33               |                              |   |
| 18                     | 3   | 0.33                    | 9.2                                | 0.22               |                              |   |
| 19                     | 3   | 0.33                    | 8.6                                | 0.22               |                              |   |
| 20                     | 1   | 0.33                    | 6.2                                | 0.22               |                              |   |
| 21                     | 2   | 0.33                    | 7.4                                | 0.22               |                              |   |
| 22                     | 2   | 0.33                    | 7.4                                | 0.22               |                              |   |
| 23                     | 3   | 0.33                    | 8.9                                | 0.22               |                              |   |
| 24                     | 3   | 0.33                    | 8.9                                | 0.22               |                              |   |
| 25                     | 2   | 0.45                    | 6.2                                | 0.22               |                              |   |
| 26                     | 2   | 0.45                    | 7                                  | 0.22               |                              |   |
| 27                     | 2   | 0.45                    | 7                                  | 0.44               |                              |   |
| 28                     | 1   | 0.45                    | 6.2                                | 0.44               |                              |   |
| 29                     | 2.33  | 0.45                    | 7.1                                | 0.44               |                              |   |
| 30                     | 2   | 0.45                    | 7.1                                | 0.44               |                              |   |
| 31                     | 2.33  | 0.37                    | 7.1                                | 0.37               |                              |   |
| 32                     | 1.33  | 0.37                    | 6.1                                | 0.31               |                              |   |
| 33                     | 3   | 0.37                    | 9.1                                | 0.31               |                              |   |
| 34                     | 2   | 0.37                    | 7.1                                | 0.31               |                              |   |
| 35                     | 1.33  | 0.37                    | 6.1                                | 0.31               |                              |   |
| 36                     | 1.33  | 0.45                    | 6.2                                | 0.31               |                              |   |
| 37                     | 1.33  | 0.45                    | 6.2                                | 0.31               |                              |   |
| 38                     | 1.33  | 0.45                    | 6.2                                | 0.31               |                              |   |
| 39                     | 1.33  | 0.45                    | 6.2                                | 0.29               |                              |   |
| 40                     | 1.33  | 0.39                    | 6.2                                | 0.29               |                              |   |
| Aggregated mean and SD | 1.92  | 0.39                    | 6.99                               | 0.31               |                              |   |

\*Unpaired t test was used, <sup>§</sup>statistically significant, @both parameters were deviating positively in one direction with good correlation, SD: Standard deviation

**Table 3. Comparison between no. of mitochondria, mitochondrial clumps and blood HbA1C levels in cases.**

| Parameter  | Cases (n=30) |  |                          |   |
|--|--------------|--|--------------------------|---|
|  | Mean±SD      | f-ratio value between the group parameters | One way ANOVA (p-value)* | Post-hoc tukey HSD@ (beta) to validate the ANOVA (p-value)* |
| Aggregated mean no. of mitochondrial dots per buccal cell in cases   | 9.47±0.38    | 593.706                                    | 0.00011                  | 0.00121   |
| Aggregated mean no. of mitochondrial clumps per buccal cell in cases | 1.92±0.39    |  |                          |   |
| Mean blood HbA1C levels in cases (mg/dl)                             | 6.99±0.31    |  |                          |   |

\*Significant ANOVA, @true post-hoc significant difference, SD: Standard deviation, ANOVA: Analysis of variance

other and exhibited a metabolomic signature by moving slightly away from the nucleus upon the application of external stressors. In this study, we showed that mitochondria within the buccal cells of diabetic humans formed clumps on being subjected to near infrared radiation and shifted towards the periphery of the cell, sometimes even distorting the cell's outline. The authors would like to term this phenomenon "mitochondrial signatures" exhibited by the mitochondria inside the diabetic buccal cells when they receive a radiation boost. Goh et al.<sup>10</sup> and Li et al.<sup>11</sup> observed that guinea pig cells under severe oxidative states such as diabetes, when subjected to laser, formed an oscillatory pattern that shifted away from the center of the cell. Romanova et al.<sup>12</sup> observed similar findings in ovarian cells of diabetic Chinese hamsters when subjected to oxidative stress, wherein the mitochondria underwent a peripheral shift away from the nucleus. In our study, we found that the mitochondria within the buccal cells of those diabetic patients in whom the blood HbA1C levels were more than 8 mg/dL formed comet-like trails along with peripheral clumps during their migration towards the periphery of the cell away from the nucleus. On the other hand, those mitochondria within the diabetic buccal cells of patients whose HbA1C values were less than 8 mg/dL did not show a comet trail; rather, they just formed clumps at the periphery of the squamous cell.

The number of mitochondrial dots that representing whole free mitochondria was found to be reduced in human diabetic buccal cells, as compared to normal cells in this study. Our findings allude to Kwak et al.<sup>1</sup> and Sivitz and Yorek<sup>2</sup> which showed that the mitochondria in the cells of mammals subjected to diabetes would undergo dysfunction due to dysregulation of the genes coding for mitochondrial proteins. The increase in mitochondrial clumps after the infrared stimulation in

diabetic buccal cells suggested the massive influx of calcium from the mitochondrial membranes, and the shift of mitochondria to the periphery was possibly due to the release of electrons from their membranes. Latti et al.<sup>13</sup>, had theorized the importance of buccal cells as indicator tools in diabetes and this study also proves the same as buccal cells provided a clear image of the free mitochondrial behaviour in diabetes. Ravindran et al.<sup>14</sup> have shown that the mucins between the buccal cells increased in diabetics and postulated that the number of mitochondria in diabetic buccal cells may have an impact due to the mucins and also found an association between salivary glucose and mucins. This study partly alluded to the findings of Ravindran et al.<sup>14</sup>, due to the observations which revealed that the number of mitochondria within the buccal cells in this study correlated well with the number of peripheral mitochondrial clumps and the levels of HbA1C seen in diabetic individuals. Hence, it can be said that an association exists between the levels of advanced glycation and the levels of mitochondria and that the end products of glycation could have induced the mitochondria to emit the peripheral clumpy shift signatures, along with comet trails, in response to a sudden flow of negatively charged electrons when bombarded with near infrared rays (Figure 2). The schematic diagram in Figure 2. explains the reason for mitochondrial clumps due to a negative charge boost of electrons, which were observed in response to oxidative stressors such as diabetes. The possible reasons could be the oxygen glucose deprivation, as observed by Yu et al.<sup>15</sup>, in the cortical neurons of lower mammals, or could be due to the rapid calcium influx in response to glycation end products excited by infrared rays, as observed by Barrett et al.<sup>16</sup> in lower mammals. The possible explanations behind the formation of clumps and shifts in mitochondria within the diabetic buccal epithelial cells could either be due to dense electron-induced apoptotic

trigger mechanisms that initiated when the mitochondria try to align themselves to the infra-red rays to counter-balance Earth's magnetic field, or due to the triggering of mitochondrial signaling pathways. These pathways are initiated by upstream mitochondrial regulatory proteins in response to glycosylation end products and oxidative stress<sup>17,18</sup>. The findings in our study partly alluded to the excitatory findings of mitochondria in diabetic mammals observed by other researchers. The clumpy peripheral mitochondrial signatures with comet-like trails observed by us in human diabetic cells after radiation were quite unique, and could be used along with HbA1C levels in inspecting the course of type 2 diabetes, as it is non-invasive too.

## CONCLUSIONS

Mitochondrial signatures in the form of peripheral clumps were observed in diabetic buccal cells after exposure to infrared rays. Intracellular mitochondrial comet trails were noticed in advanced diabetic cells, post-radiation. There was an overall decrease in free mitochondrial count in diabetic buccal cells as compared to controls. This spatial shift of mitochondrial dots could serve as a guide to indicate the progression of diabetes in collaboration with HbA1C levels.

## Ethics

**Ethics Committee Approval:** Approval was obtained from the Institutional Ethics Committee for this study (reference number: AIIMS/BBN/IEC/OCT/2022/224, date: 08.10.2022).

**Informed Consent:** Was obtained from patients

## Footnotes

## Author Contributions

Surgical and Medical Practices: S.S.M.C., S.S., G.N.K., A.K.P., Concept: S.S.M.C., S.S., G.N.K., A.K.P., Design: S.S.M.C., S.S., G.N.K., A.K.P., Data Collection and/or Processing: S.S.M.C., S.S., G.N.K., A.K.P., Analysis and/or Interpretation: S.S.M.C., S.S., G.N.K., A.K.P., Literature Search: S.S.M.C., S.S., G.N.K., A.K.P., Writing: S.S.M.C., S.S., G.N.K., A.K.P.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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