Research Article

# Cytological Alterations in Buccal Mucosa and Molecular Interaction of Nicotine with XRCC1 In Young Male Gutkha Chewers: A Cross-Sectional and in Silico Study

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**Abstract**

**Background:** Smokeless tobacco products such as gutkha are widely consumed in South Asia and are strongly linked to oral potentially malignant disorders and oral cancer. Nicotine, the principal addictive alkaloid in tobacco, is absorbed through the oral mucosa and may compromise DNA repair capacity, yet the combined cytological and molecular evidence linking gutkha use to early genotoxic change in young users remains limited.

**Objective:** To assess awareness of the harmful effects of tobacco among young male gutkha users, to characterise cytological alterations in exfoliated buccal epithelial cells, and to evaluate the molecular interaction between nicotine and the DNA-repair protein X-ray repair cross-complementing protein 1 (XRCC1).

**Methods:** A cross-sectional study was conducted among 60 male gutkha users aged 18–25 years and 60 age-matched non-tobacco-using controls. A structured, pre-validated questionnaire captured knowledge, attitude and practice (KAP) related to tobacco use. Buccal smears were stained by the Papanicolaou (PAP) and May–Grünwald Giemsa (MGG) methods and screened for nuclear and cytoplasmic aberrations. Molecular docking of nicotine with XRCC1 was performed in AutoDock 4.2 following Lamarckian genetic algorithm optimisation; ligand toxicity was profiled with ProTox-3.0.

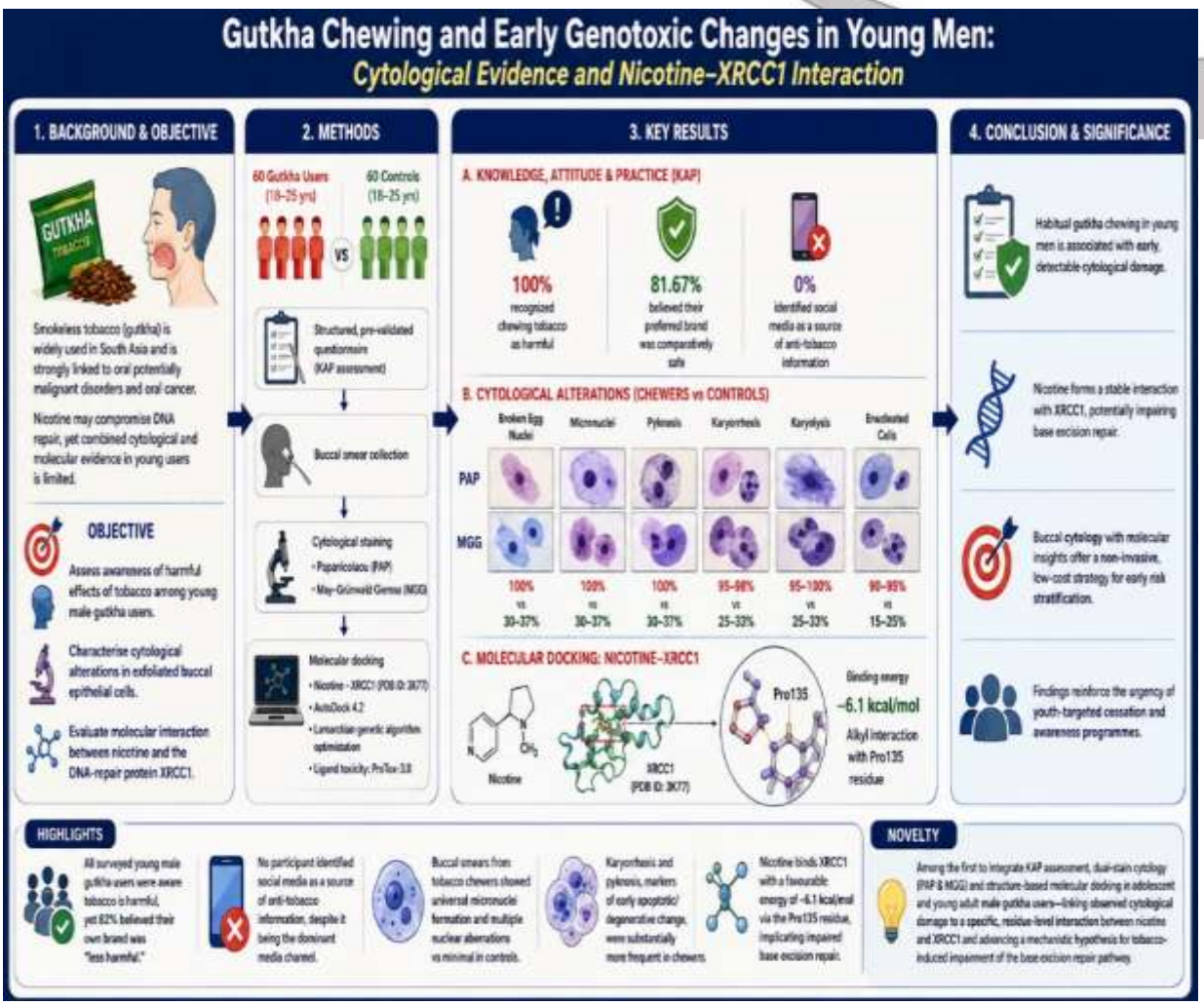
**Results:** All participants (100%) recognised chewing tobacco as harmful, yet 81.67% believed their preferred brand was comparatively safe, and none cited social media as a source of anti-tobacco information. Cytological screening revealed a marked excess of broken egg nuclei, micronuclei, pyknosis, karyorrhexis, karyolysis and enucleated cells in tobacco chewers relative to controls across both staining methods (e.g micronuclei present in 100% of chewer smears vs 30–37% of control smears). Molecular docking demonstrated a thermodynamically favourable interaction between nicotine and XRCC1, with a binding energy of −6.1 kcal/mol, mediated by an alkyl interaction with the Pro135 residue.

**Conclusion:** Habitual gutkha chewing is associated with early, detectable cytological damage in the buccal mucosa of young men, and nicotine forms a stable interaction with XRCC1 that may impair base excision repair. Buccal cytology, combined with molecular insight into nicotine–DNA-repair-protein interactions, supports a non-invasive, low-cost strategy for early risk stratification and reinforces the urgency of youth-targeted cessation programmes.

**Novelty Statement**

This study is among the first to combine field-based knowledge–attitude–practice assessment, dual-stain (PAP and MGG) exfoliative cytology, and structure-based molecular docking in a single investigative framework focused specifically on adolescent and young adult male gutkha users. By anchoring the observed cytological damage to a specific, residue-level molecular interaction between nicotine and XRCC1, the study moves beyond descriptive cytopathology toward a mechanistic hypothesis for tobacco-induced impairment of the base excision repair pathway in a high-risk, under-studied population.

**Graphical Abstract**




# 1. Introduction

**Background**

Smokeless tobacco (SLT) use continues to represent one of the most pervasive and under-addressed public health challenges in South and Southeast Asia. Chewing tobacco products, prominently gutkha—a masticatory blend of areca nut, slaked lime, catechu and powdered tobacco—are consumed by hundreds of millions of people across the Indian subcontinent, with initiation frequently occurring in adolescence.[1,2] Nicotine, the principal psychoactive alkaloid in tobacco, does not itself account for the full spectrum of tobacco-related tissue injury; rather, its primary biological role is to sustain compulsive use, thereby prolonging and intensifying cumulative exposure to the mixture of genotoxic and cytotoxic constituents present in SLT products.[1,2]

**Global Burden**

The global burden associated with SLT is substantial. Chronic exposure has been linked to immune dysregulation, cardiovascular disease, cerebrovascular events and multiple malignancies, with oral cancer representing the most extensively documented outcome.[3] In India specifically, gutkha and related areca-nut–tobacco combinations account for a disproportionate share—over 80%—of oral cancer cases attributable to SLT use, a pattern reinforced by large-scale epidemiological analyses spanning more than 125 countries.[3,4,5] The convergence of high prevalence, early age of initiation, and strong dose–response relationships with oral potentially malignant disorders makes SLT-related oral pathology a priority area for both surveillance and mechanistic research.

**Current Knowledge**

Oral carcinogenesis is multifactorial, shaped by the interplay of genetic susceptibility, oral hygiene status, concurrent smoking, areca nut chewing and other lifestyle exposures.[6,7] Nicotine absorbed through the buccal mucosa is a key contributor to sustained tissue-level harm, both through direct cytotoxic effects and through modulation of inflammatory signalling pathways in oral epithelial and osteogenic tissue.[8] While histopathological evaluation of biopsy tissue remains the diagnostic gold standard for oral cancer,[9] it is invasive, resource-intensive and impractical as a population-level screening tool. Exfoliative cytology of the buccal mucosa offers a minimally invasive alternative capable of detecting early, subclinical cellular alterations—including micronuclei, karyorrhexis, karyolysis, pyknosis, binucleation, nuclear fragmentation and broken egg nuclei—that frequently precede histologically overt dysplastic change.[10,11,12] These nuclear anomalies, particularly micronuclei, are established biomarkers of chromosomal instability and DNA damage resulting from chronic genotoxic exposure.[10,11,12]

At the molecular level, genomic stability is safeguarded by coordinated DNA repair systems, among which the base excision repair (BER) pathway plays a central role in correcting small-scale base lesions and single-strand breaks characteristic of oxidative and alkylating damage.[13,14] BER capacity is itself an important determinant of cancer susceptibility, since impaired repair allows genotoxic lesions to persist and accumulate.[15,16] X-ray repair cross-complementing protein 1 (XRCC1) is a scaffolding protein indispensable to BER, coordinating the sequential recruitment of DNA polymerase β, DNA ligase III and poly(ADP-ribose) polymerase at sites of base damage.[17] Polymorphisms and functional alterations in XRCC1 have been repeatedly associated with altered cancer risk and impaired repair efficiency, including in head and neck and oral cavity malignancies.[18,19,20]

**Research Gap**

Despite this substantial body of literature, few studies have simultaneously examined behavioural risk factors, cytological damage and molecular-level interactions between tobacco constituents and DNA-repair machinery within a single, coherent investigative framework—particularly in young, otherwise healthy male gutkha users. Most prior cytological studies have compared broad tobacco-user categories without close attention to age-specific consumption patterns in adolescents and young adults, while most XRCC1-related work has focused on genetic polymorphism–disease association rather than direct biophysical characterisation of nicotine–XRCC1 binding. This leaves an important gap: whether nicotine itself, independent of polymorphic variation, can physically interact with XRCC1 in a manner plausibly compatible with impaired base excision repair.

**Rationale**

Addressing this gap has direct translational relevance. If nicotine can be shown, through structure-based computational modelling, to interact favourably with a key BER protein, this would provide a mechanistic complement to the cytological evidence of genotoxic and cytotoxic damage observed in tobacco users, strengthening the biological plausibility of buccal cytology as an early-warning screening tool. Furthermore, characterising the awareness and consumption behaviour of the specific population under cytological and molecular study allows the biological findings to be interpreted within an actionable public health context.

**Study Aim**

The present study was therefore designed to integrate three complementary lines of evidence in a single cohort of young male gutkha users: (i) behavioural and awareness data obtained through a structured questionnaire, (ii) cytomorphological evidence of nuclear and cytoplasmic damage in exfoliated buccal epithelial cells using dual staining techniques, and (iii) in silico molecular docking evidence characterising the interaction between nicotine and XRCC1.

**Objectives**

Specifically, the study aimed to: (1) assess the level of knowledge, attitude and practice regarding tobacco-related harm among young male daily gutkha users; (2) identify and quantify cytological aberrations—broken egg nuclei, micronuclei, pyknosis, karyorrhexis, karyolysis and enucleation—in buccal smears of tobacco chewers relative to non-tobacco-using controls, using Papanicolaou and May–Grunwald Giemsa staining; and (3) computationally evaluate the binding interaction between nicotine and the DNA-repair protein XRCC1 using structure-based molecular docking.

## 2. Materials and Methods

### 2.1 Study Design

This investigation combined a cross-sectional observational component (behavioural survey and comparative exfoliative cytology) with an in silico structural biology component (molecular docking). Reporting of the observational component followed the general principles of the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) checklist for cross-sectional studies.

### 2.2 Study Setting

The study was conducted within the Programme of Medical Laboratory Technology, Faculty of Allied & Healthcare Sciences, Assam down town University, Guwahati, Assam, India.

### 2.3 Study Duration

Sample collection, cytological processing and questionnaire administration were carried out over a defined study period at the host institution; molecular docking and validation were performed subsequently on the selected ligand–receptor pair.( Jan 2024- July 2024)

### 2.4 Study Population

The study population comprised male students and young adults aged 18–25 years. The tobacco-exposed (Study) group consisted of 60 self-reported daily gutkha chewers, and the comparator (Control) group consisted of 60 age-comparable healthy males with no history of tobacco, areca nut or smokeless-tobacco use.

### 2.5 Inclusion Criteria

- Males aged 18–25 years.
- For the Study group: self-reported daily gutkha chewing for a minimum sustained period, with willingness to disclose consumption history.
- For the Control group: no current or past history of any form of tobacco or areca nut use.
- Willingness to provide informed consent/assent and to undergo buccal smear sampling.

### 2.6 Exclusion Criteria

- History of concurrent smoking or dual tobacco use in the control group.
- Presence of clinically diagnosed oral mucosal lesions, active oral infection, or recent oral surgical intervention that could confound cytological interpretation.
- Refusal to consent or incomplete questionnaire response.

### 2.7 Sample Size

A total of 120 participants were enrolled (60 tobacco-chewing cases and 60 non-tobacco-using controls), based on feasibility and comparable prior cytogenotoxicity studies in buccal mucosa; this sample size was considered adequate to detect gross differences in the frequency of nuclear aberrations between groups.

### 2.8 Sampling Method

Participants were recruited by purposive/convenience sampling from the eligible student and young-adult population meeting inclusion criteria, with group allocation determined by self-reported tobacco use status.

### 2.9 Ethical Approval

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Informed written consent was obtained from all participants prior to questionnaire administration and sample collection. Institutional ethical clearance was obtained from the Institutional Ethics Committee of Assam down town University prior to commencement of the study (Memo No.: AdtU/Ethics/stdnt-lett/2024/153)

**2.10 Data Collection Procedure**

A structured, interviewer-administered questionnaire was used to capture demographic details, chewing history (age of initiation, frequency, daily packet consumption), knowledge and awareness of tobacco-related harm, perceived brand safety, quitting intentions and barriers, sources of anti-tobacco information, and perceived effectiveness of existing tobacco-control campaigns.[21]

**2.11 Laboratory Methods**

**2.11.1 Chemicals and Reagents**

All chemicals used were of analytical grade. Reagents for Papanicolaou (PAP) staining—absolute ethanol, Harris haematoxylin, Orange G-6 (OG-6) and Eosin Azure 50 (EA-50)—and xylene were obtained from Sigma-Aldrich. May–Grünwald and Giemsa stains were procured from Sisco Research Laboratory (SRL), India. Methanol (fixative), buffer solutions, cotton swabs, glass slides, mounting media and coverslips were used as consumables.

**2.11.2 Sample Collection**

Buccal swab samples were obtained from the buccal mucosa of each participant using a sterile cotton swab following informed consent and structured interview. Cells were transferred immediately onto a clean glass slide and fixed for subsequent staining.

**2.12 Staining Procedures**

**2.12.1 Papanicolaou (PAP) Stain**

Fixed slides were stained using the Papanicolaou method,[22] employing sequential application of Harris haematoxylin, Orange G-6 and Eosin Azure 50, with graded ethanol and xylene steps for dehydration and clearing. Stained slides were examined under bright-field light microscopy for cytological abnormalities in epithelial cells.

**2.12.2 May–Grunwald Giemsa (MGG) Stain**

Cells were fixed in methanol and stained sequentially with May–Grunwald (eosinate-based) and Giemsa (basic/acidic dye mixture) solutions.[23] Basic dye components preferentially stained nuclei and basophilic granules, while acidic components stained eosinophilic elements, enabling clear resolution of nuclear and cytoplasmic morphology. Slides were examined microscopically for cellular components and aberrations.

**2.12.3 Cytological Scoring**

For each stained smear, cells were screened and the number of aberrant cells per 100–200 cells scored was recorded across six morphological categories: broken egg nuclei, micronuclei, pyknosis, karyorrhexis, karyolysis and enucleated cells. Findings were stratified into aberration-count bands (0, 1–5, 6–10, 11–15 cells) for each parameter, separately for PAP- and MGG-stained preparations.

**2.13 Molecular Docking**

**2.13.1 Selection of Compound**

Nicotine was selected as the ligand of interest. Its two-dimensional/three-dimensional structure and physicochemical properties were retrieved from the PubChem compound database (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov). Predicted toxicity was assessed using the ProTox-3.0 web server.[24]

**2.13.2 Ligand Preparation**

The nicotine structure was downloaded in Structure Data Format (SDF) and converted to Protein Data Bank (PDB) format using OpenBabel. The ligand was subsequently prepared and energy-optimised for docking using AutoDock 4.2.[25,26]

### 2.13.3 Receptor Preparation

The three-dimensional crystal structure of XRCC1 (PDB ID: 3K77) was retrieved from the RCSB Protein Data Bank.[28] The structure was refined in PyMOL and its stereochemical quality validated using PROCHECK via Ramachandran plot analysis; structural visualisation was additionally performed in RasMol.[27,28,29] (Figure 1)

### 2.13.4 Docking Simulation

Protein–ligand docking was performed in AutoDock 4.2, with the receptor held rigid and the ligand treated as flexible. Polar hydrogens and Gasteiger partial charges were assigned to both protein and ligand, and PDBQT files were generated using AutoDockTools (v1.5.6) within the Graphical Library (GL) software suite. Docking poses were generated using the Lamarckian Genetic Algorithm (LGA) with 100 independent genetic algorithm runs and default parameters for all other settings. A grid box of dimensions X = 84.036 Å, Y = 96.9474 Å, Z = 84.7623 Å was centred at X = 14.4186 Å, Y = 40.5927 Å, Z = 24.0555 Å to encompass the relevant ligand-binding region. Binding poses and interaction diagrams were analysed and visualised using UCSF Chimera v1.14 and Discovery Studio Visualizer (Dassault Systèmes BIOVIA, 2017).[30]

### 2.14 Quality Control Measures

Freshly prepared reagents were used for each staining batch, and known positive/negative reference smears were included as internal controls to verify staining performance. All slides were independently screened, with ambiguous findings cross-checked by a second observer. For the docking workflow, receptor stereochemical quality was verified by Ramachandran plot analysis prior to docking, and docking parameters (grid box dimensions, LGA settings) were fixed a priori and applied uniformly.

### 2.15 Statistical Analysis

Cytological aberration data were summarised using frequency distribution analysis, with results expressed as counts and percentages (n, %) of samples falling within each aberration-count band for each staining method and cellular parameter. Descriptive comparisons between the tobacco-chewing and control groups were performed using proportions; where appropriate, categorical comparisons were evaluated at a two-sided significance level of $\alpha = 0.05$. Continuous/ordinal questionnaire responses are reported as percentages of respondents. Molecular docking output is reported as binding free energy (kcal/mol) for the lowest-energy, most thermodynamically favourable docking pose. Data were compiled and tabulated in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

## 3. Results

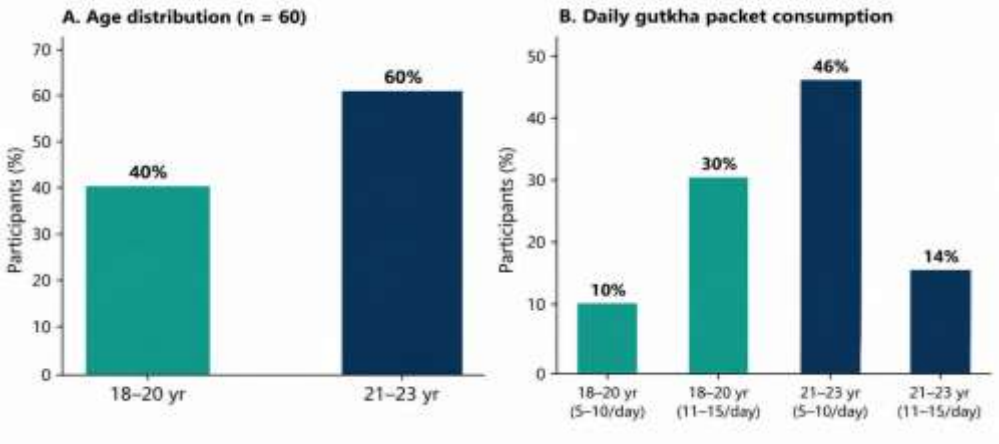
### 3.1 Participant Characteristics and Demographic Analysis

Sixty male daily gutkha chewers aged 18–25 years were enrolled and compared with 60 age-comparable non-tobacco-using controls. Within the tobacco-chewing group, 40% (n = 24) were aged 15–20 years and 60% (n = 36) were aged 21–25 years (Table 1, Figure A). Daily consumption intensity varied by age band: among 21–25-year-olds, 46% consumed 5–10 packets/day and 14% consumed 11–15 packets/day, whereas among the younger 15–20-year band, a disproportionately high 30% already consumed 11–15 packets/day and 10% consumed 5–10 packets/day, indicating an early and rapidly escalating pattern of heavy use (Table 1).

**Table 1. Age distribution and daily gutkha packet consumption among tobacco-chewing participants (n = 60)**

| Age group (years) | Participants, n (%) | Daily packets consumed | Participants, n (%) |
|---|---|---|---|
| 18–20 | 24 (40.0) | 5–10 | 6 (10.0) |
| | | 11–15 | 18 (30.0) |
| 21–25 | 36 (60.0) | 5–10 | 28 (46.7) |
| | | 11–15 | 8 (13.3) |

**Figure A. Demographic profile of tobacco-chewing participants. (A) Age distribution of the study group (n = 60). (B) Daily gutkha packet consumption stratified by age band, showing disproportionately heavy use (11–15 packets/day) among 18–20-year-olds.**




**3.2 Knowledge, Attitude and Practice (KAP)**

All participants (100%) recognised chewing tobacco as harmful to health. Oral cancer was identified as the principal health risk by 81.67% of respondents, while 18.33% cited stroke or cardiovascular events. Oral lesions were correctly recognised as a tobacco-related oral change by 68.33% of respondents, whereas 26.67% were uncertain. Notably, 81.67% believed their preferred gutkha brand was comparatively "less harmful," reflecting persistent brand-related misconceptions. Most respondents (91.67%) expressed a desire to quit, although 76.67% considered quitting difficult, chiefly due to fear of relapse (65%) and withdrawal symptoms (13.33%). All participants had received anti-tobacco information, predominantly via television (85%) and campaign material (15%); none reported social media as an information source. Existing anti-tobacco campaigns were rated as "somewhat effective" by 71.67% of respondents, "ineffective" by 8.33%, and 20% were unsure (Table 2, Figure B).

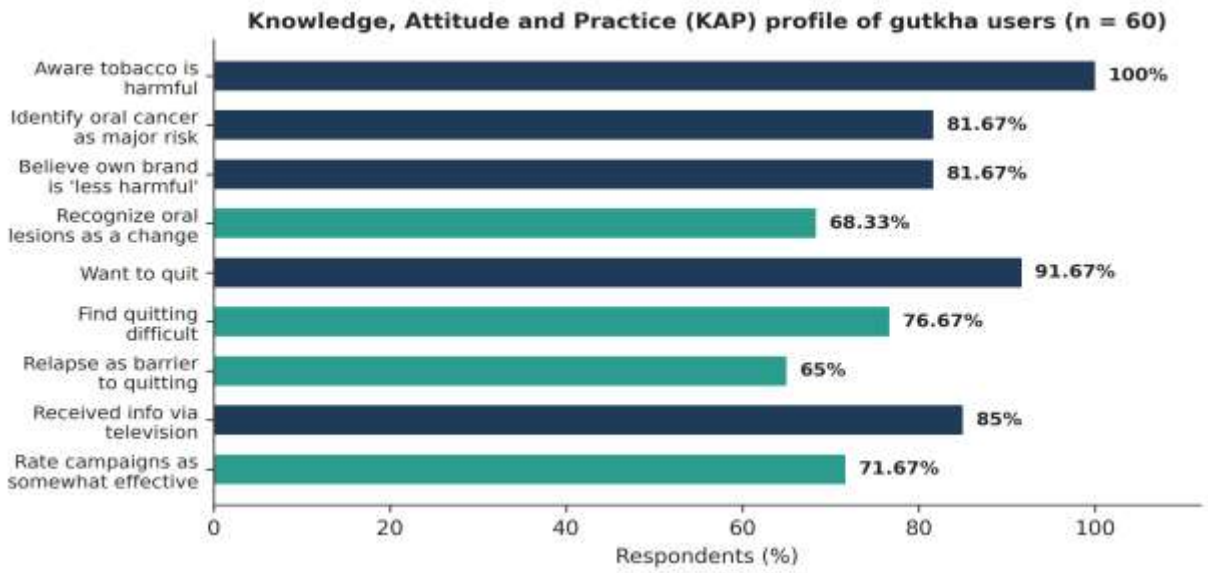
**Table 2. Knowledge, attitude and practice (KAP) profile of gutkha users regarding tobacco-related harm (n = 60)**

| KAP domain | Response category | Respondents, n (%) |
|---|---|---|
| Awareness that tobacco is harmful | Aware | 60 (100.0) |
| Perceived major health risk | Oral cancer | 49 (81.7) |
| | Stroke/cardiovascular disease | 11 (18.3) |
| Recognition of oral lesions as a tobacco-related change | Recognised | 41 (68.3) |
| | Unsure | 16 (26.7) |
| Perceived brand-specific safety | Believes brand is "less harmful" | 49 (81.7) |
| Desire to quit | Yes | 55 (91.7) |
| Perceived difficulty in quitting | Difficult | 46 (76.7) |
| | — mainly due to fear of relapse | 39 (65.0) |
| | — mainly due to withdrawal symptoms | 8 (13.3) |
| Source of anti-tobacco information | Television | 51 (85.0) |
| | Campaign material | 9 (15.0) |
| | Social media | 0 (0.0) |
| Perceived effectiveness of current campaigns | Somewhat effective | 43 (71.7) |
| | Unsure | 12 (20.0) |
| | Ineffective | 5 (8.3) |

Note: Percentages calculated relative to the total tobacco-chewing group (N = 60)

**Figure B. Knowledge, attitude and practice profile of gutkha users (n = 60). Bars are colour-graded by response magnitude (navy ≥ 80%, teal 60–79%, orange < 60%) to visually emphasise the coexistence of near-universal risk**

**awareness with persistent misconceptions about brand-specific safety and a complete absence of social-media-based risk communication.**




### 3.3 Laboratory and Cytological Findings

Buccal smears from 60 healthy non-tobacco users (Control group) and 60 tobacco-chewing participants (Study group) were evaluated cytologically following PAP and MGG staining (Figures 2–5). The full frequency distribution of aberrant cells for the control and tobacco-chewing groups is presented in Tables 3 and 4, respectively.

**Table 3. Frequency distribution of cytological aberrations in buccal smears of healthy non-tobacco users (Control group, n = 60)**

| Aberrant cells (n) | Broken egg nuclei (PAP) | Broken egg nuclei (MGG) | Micronuclei (PAP) | Micronuclei (MGG) | Pyknosis (PAP) | Pyknosis (MGG) | Karyorrhexis (PAP) | Karyorrhexis (MGG) | Karyolysis (PAP) | Karyolysis (MGG) | Enucleated (PAP) | Enucleated (MGG) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 54 | 56 | 42 | 38 | 28 | 32 | 35 | 40 | 44 | 48 | 50 | 52 |
| 1–5 | 6 | 4 | 18 | 22 | 31 | 27 | 25 | 20 | 16 | 12 | 10 | 8 |
| 6–10 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11–15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

PAP: Papanicolaou stain; MGG: May–Grünwald Giemsa stain. Values represent number of smears (n = 60) falling within each aberrant-cell-count band.

**Table 4. Frequency distribution of cytological aberrations in buccal smears of daily tobacco chewers (Study group, n = 60)**

| Aberrant cells (n) | Broken egg nuclei (PAP) | Broken egg nuclei (MGG) | Micronuclei (PAP) | Micronuclei (MGG) | Pyknosis (PAP) | Pyknosis (MGG) | Karyorrhexis (PAP) | Karyorrhexis (MGG) | Karyolysis (PAP) | Karyolysis (MGG) | Enucleated (PAP) | Enucleated (MGG) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1–5 | 60 | 60 | 41 | 51 | 43 | 47 | 60 | 56 | 49 | 39 | 60 | 60 |
| 6–10 | 0 | 0 | 17 | 9 | 17 | 13 | 0 | 4 | 11 | 20 | 0 | 0 |
| 11–15 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |

PAP: Papanicolaou stain; MGG: May–Grünwald Giemsa stain. Values represent number of smears (n = 60) falling within each aberrant-cell-count band.

**Figure 2. Papanicolaou (PAP)-stained buccal smears of daily tobacco chewers, showing representative nuclear aberrations including micronuclei, pyknosis and karyorrhexis.**

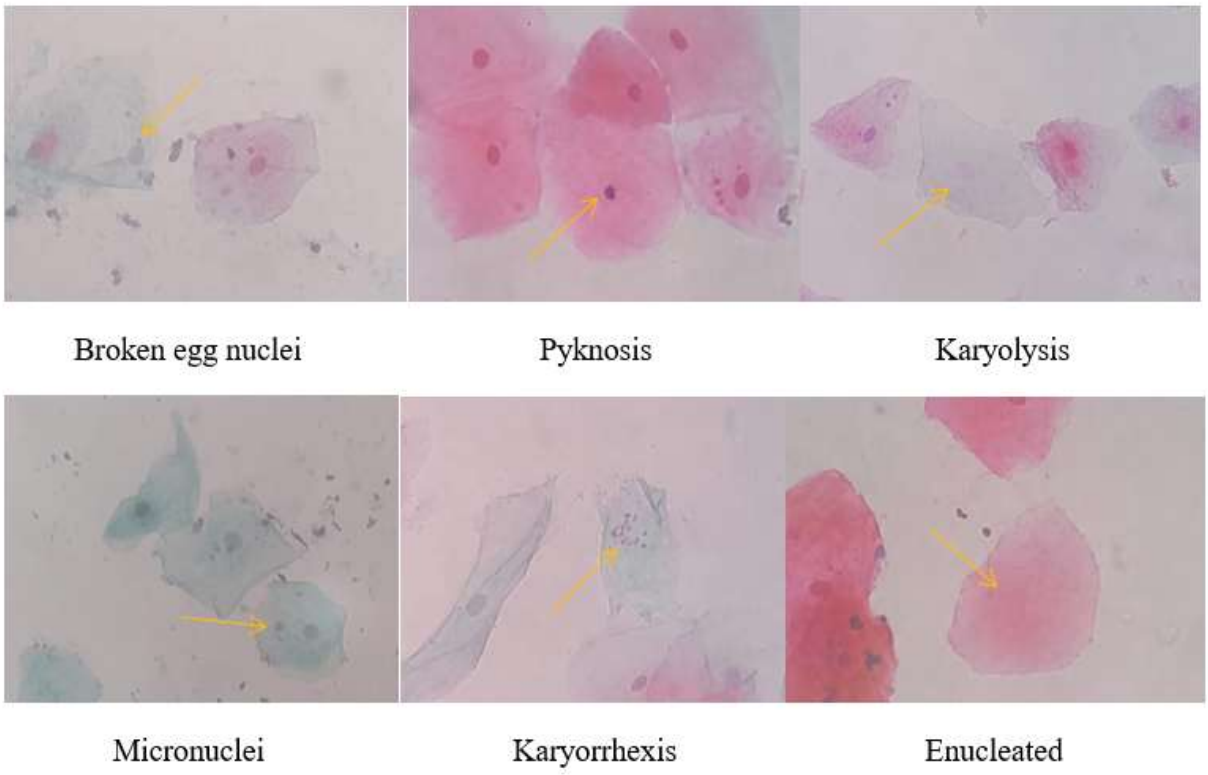



**Figure 3. Papanicolaou (PAP)-stained buccal smears of non-tobacco chewers (controls), showing predominantly normal epithelial cell morphology with intact nuclei.**

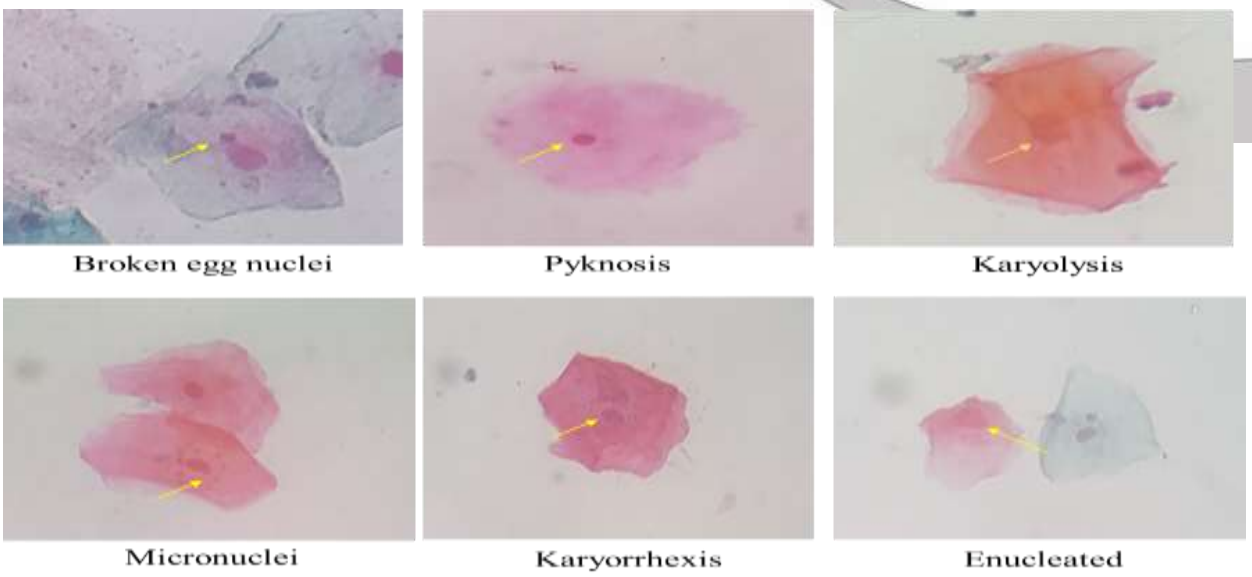



**Figure 4. May–Grunwald Giemsa (MGG)-stained buccal smears of daily tobacco chewers, demonstrating nuclear and cytoplasmic aberrations consistent with genotoxic and cytotoxic damage.**

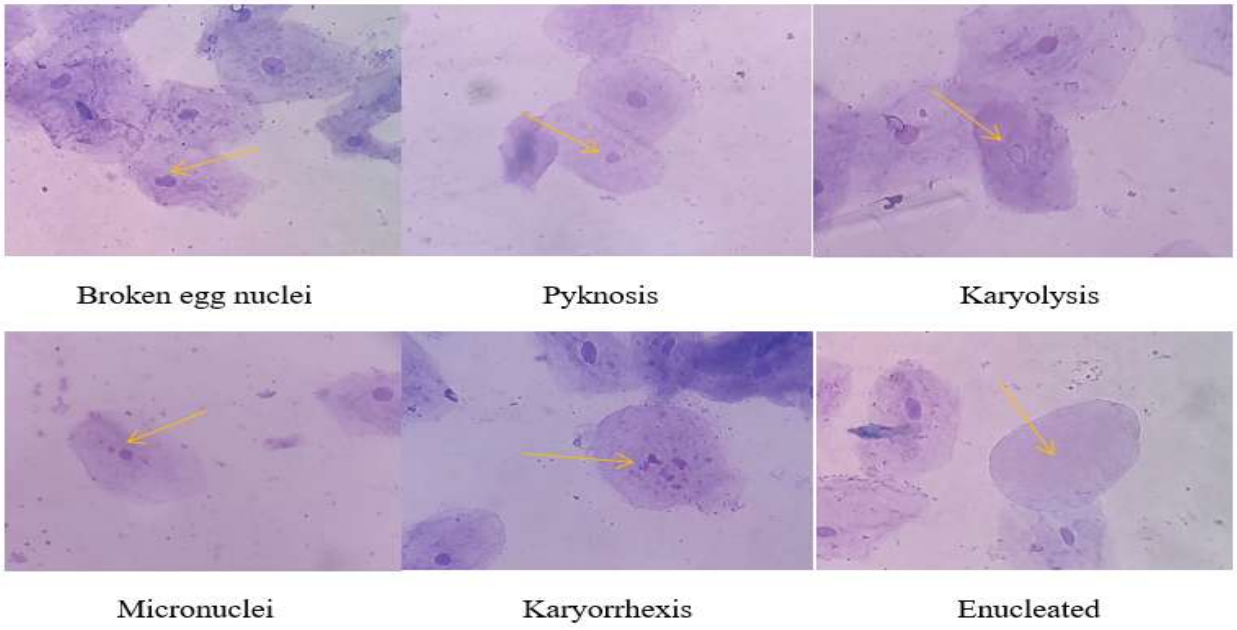
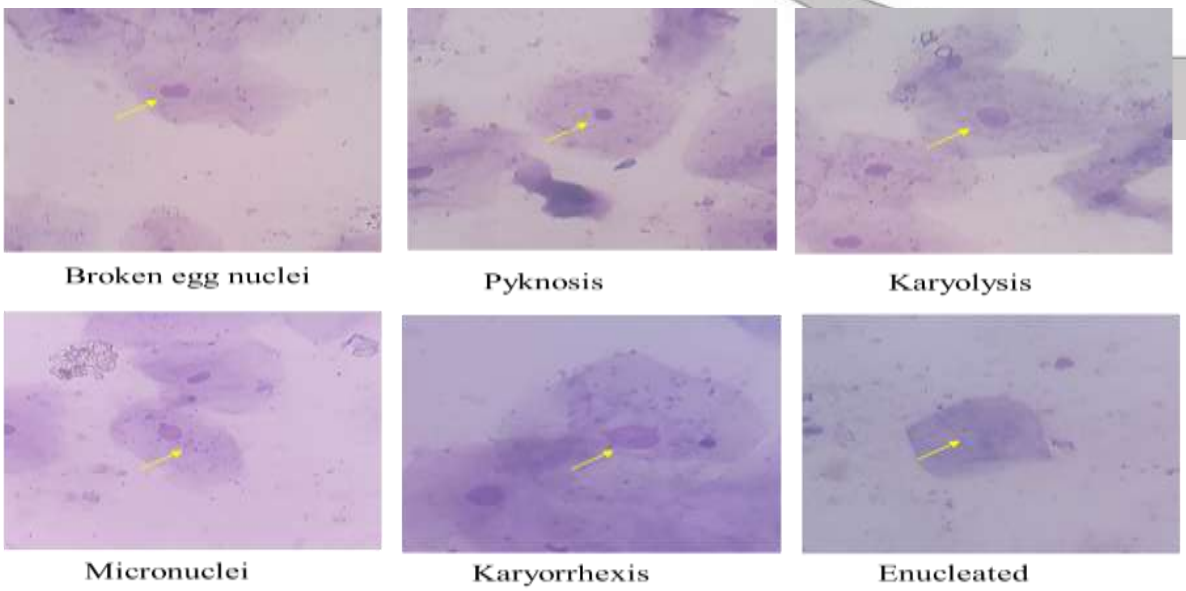



**Figure 5. May–Grunwald Giemsa (MGG)-stained buccal smears of daily non-tobacco chewers (controls), showing predominantly normal cytomorphology.**




**3.4 Comparative Analysis**

Direct comparison of the two groups showed a consistent and substantial excess of aberrant cells among tobacco chewers across every cytological parameter examined and in both staining methods (Table 5, Figures C–D). In the control group, nuclear integrity was largely preserved: broken egg nuclei were entirely absent (0 aberrant cells) in 54/60 (90%) PAP-stained and 56/60 (93%) MGG-stained smears, and only 1 control smear per stain showed as many as 6–10 pyknotic cells, with no control sample reaching the 11–15 band for any parameter. By contrast, in the tobacco-chewing group, zero-aberration smears were entirely absent for every parameter and stain combination (0/60), broken egg nuclei and karyorrhexis were present (1–5 cells) in all 60/60 smears on both stains, and micronuclei reached the 6–10 and 11–15 bands in 17/60 and 2/60 PAP smears, respectively.

**Table 5. Comparative summary: proportion of smears with at least one aberrant cell, tobacco chewers vs controls (PAP stain, n = 60 per group)**

| Cytological parameter | Control group, n (%) affected | Tobacco chewers, n (%) affected | Absolute difference (percentage points) |
|---|---|---|---|
| Broken egg nuclei | 6 (10.0) | 60 (100.0) | +90.0 |
| Micronuclei | 18 (30.0) | 60 (100.0) | +70.0 |
| Pyknosis | 32 (53.3) | 60 (100.0) | +46.7 |
| Karyorrhexis | 25 (41.7) | 60 (100.0) | +58.3 |
| Karyolysis | 16 (26.7) | 60 (100.0) | +73.3 |
| Enucleated cells | 10 (16.7) | 60 (100.0) | +83.3 |

“Affected” is defined as any smear with ≥1 aberrant cell recorded for the given parameter (i.e., not falling in the “0” aberration band).

**Figure C. Comparative cytological aberration burden (PAP stain) between tobacco chewers and controls. Bars represent the number of smears (of 60 per group) showing at least one aberrant cell for each morphological parameter. Every parameter shows a marked excess of affected smears among tobacco chewers, with broken egg nuclei, karyorrhexis and enucleated cells reaching 100% prevalence in the exposed group.**

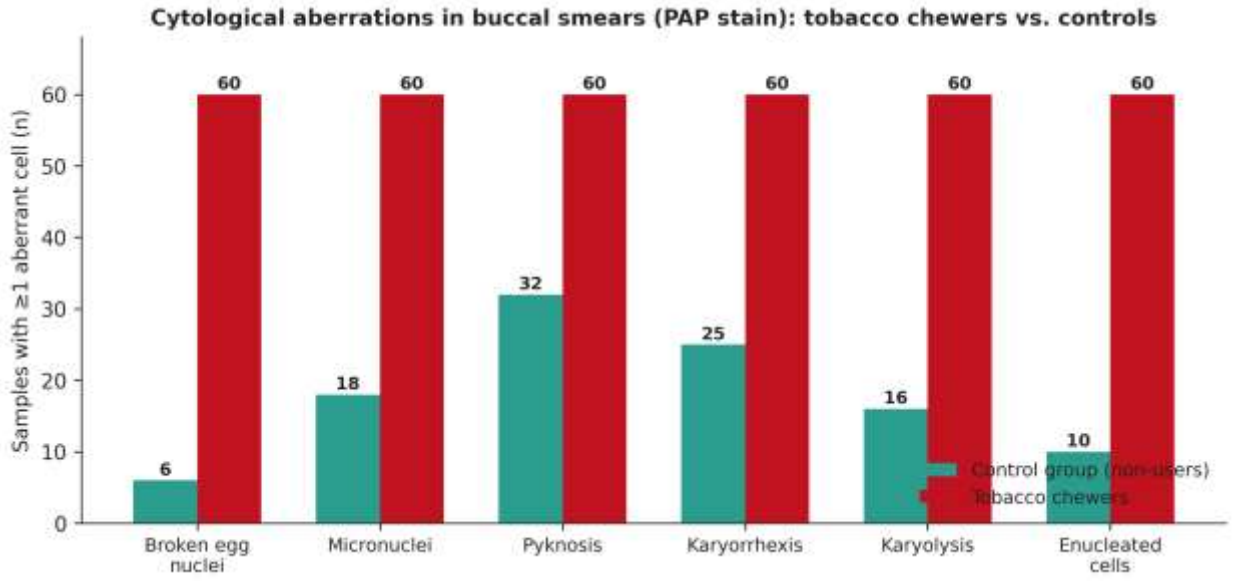
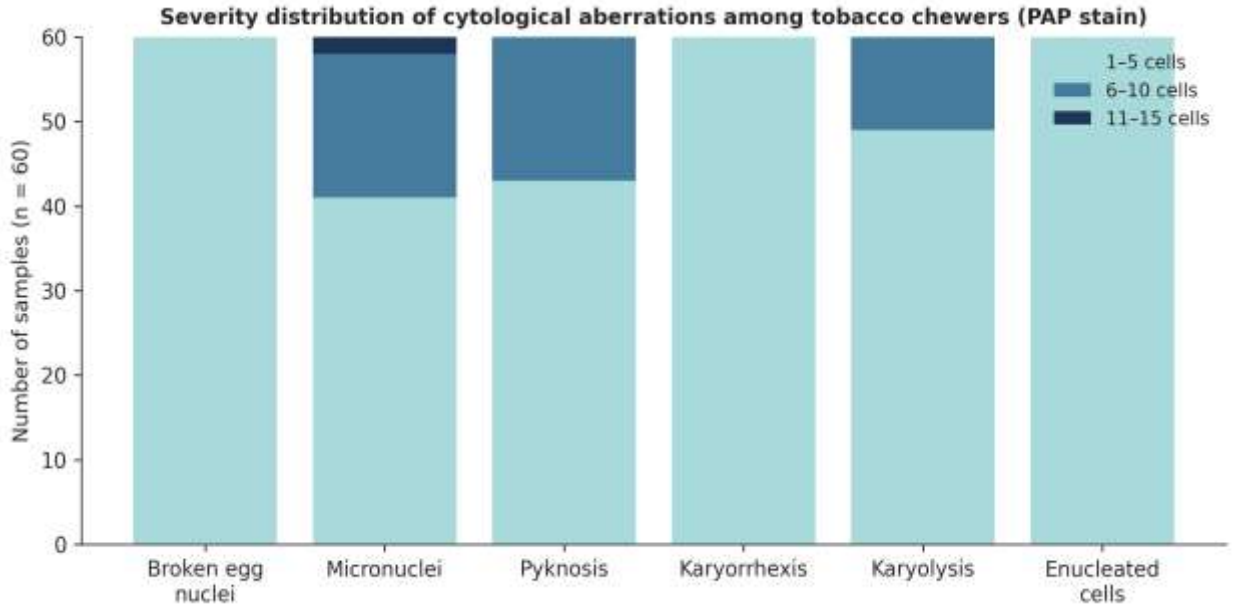



**Figure D. Severity distribution of cytological aberrations among tobacco chewers (PAP stain). Stacked bars show the number of smears (of 60) within each aberrant-cell-count band (1–5, 6–10, 11–15 cells). Micronuclei and pyknosis show the greatest proportion of smears in the higher-severity (6–10 and 11–15 cell) bands, indicating more extensive chromosomal instability fo these parameters.**




### 3.5 Molecular Docking Findings

Structural validation of the XRCC1 receptor (PDB ID: 3K77) confirmed acceptable stereochemical quality by Ramachandran plot analysis, with the majority of residues in favoured/allowed conformational regions (Figure 1). Docking of nicotine with XRCC1 using AutoDock 4.2 and the Lamarckian genetic algorithm yielded a lowest-energy binding pose with a binding free energy of −6.1 kcal/mol (Table 6), indicating a thermodynamically favourable and stable protein–ligand interaction. Interaction analysis identified an alkyl-type interaction between nicotine and the proline residue at position 135 (Pro135) of XRCC1 as the principal stabilising contact (Figure 6).

**Table 6. Binding energy and principal interacting residue of the nicotine–XRCC1 docking complex**

| S. No. | Protein–ligand complex | PubChem CID (ligand) | Binding energy (kcal/mol) | Principal interaction | Interacting residue |
|---|---|---|---|---|---|
| 1 | XRCC1 (3K77) – Nicotine | 157672 | −6.1 | Alkyl interaction | Pro135 |

**Figure 1. Three-dimensional cartoon structure of the DNA-repair protein XRCC1 (PDB ID: 3K77) (left) and the corresponding Ramachandran plot (right), confirming acceptable stereochemical quality of the receptor structure used for docking.**

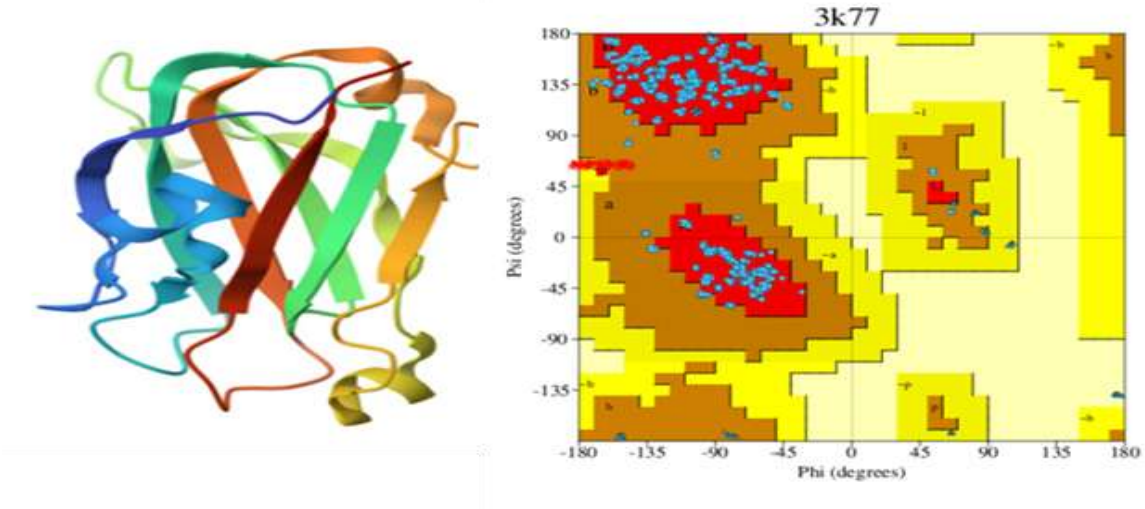
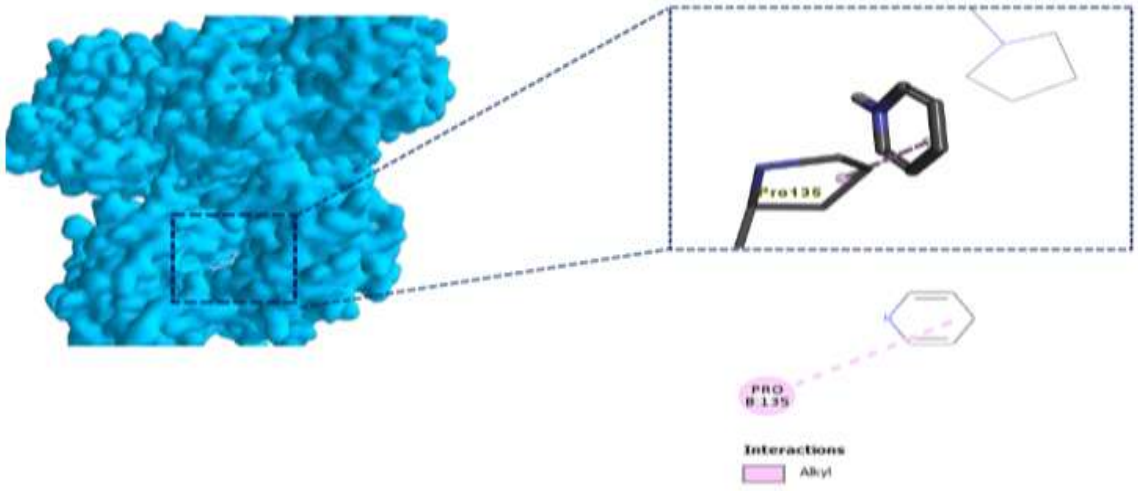



**Figure 6. (+)-Nicotine docking interaction with XRCC1 (PDB ID: 3K77). Surface representation of XRCC1 with the nicotine-binding pocket magnified (top), showing the ligand engaging the Pro135 residue via an alkyl interaction (pink dashed line, interaction legend shown at bottom).**




### 3.6 Summary of Key Findings

Collectively, the results demonstrate: (i) near-universal awareness of tobacco-related harm coexisting with persistent brand-specific safety misconceptions and a complete absence of social-media-based risk communication among young gutkha users; (ii) a consistent and substantial excess of nuclear and cytoplasmic aberrations—broken egg nuclei, micronuclei, pyknosis, karyorrhexis, karyolysis and enucleated cells—in the buccal mucosa of tobacco chewers relative to non-users, detectable by both PAP and MGG staining; and (iii) a thermodynamically favourable,

residue-specific interaction between nicotine and the DNA-repair protein XRCC1, providing molecular-level plausibility for tobacco-associated impairment of base excision repair.

# 4. Discussion

### 4.1 Principal Findings

This study integrated behavioural, cytomorphological and computational structural evidence to characterise the early biological impact of habitual gutkha chewing in young men. Three principal findings emerged. First, despite universal (100%) awareness that tobacco use is harmful, misconceptions regarding brand-specific safety were strikingly common (81.67%), and anti-tobacco messaging reached participants almost exclusively through television, with a complete absence of social-media-based communication—a critical gap given that social media is now the dominant information channel for this age group. Second, buccal cytology revealed a stark and consistent excess of nuclear and cytoplasmic aberrations among tobacco chewers relative to controls across every parameter examined and in both staining modalities, with several parameters (broken egg nuclei, karyorrhexis, enucleated cells) reaching 100% prevalence in the exposed group compared with 10–42% in controls. Third, in silico docking demonstrated that nicotine engages XRCC1 in a thermodynamically favourable interaction (−6.1 kcal/mol) centred on the Pro135 residue, offering a plausible molecular explanation for the cytological damage observed.

### 4.2 Comparison with Previous Studies

The behavioural findings are broadly consistent with prior knowledge–attitude–practice surveys of gutkha and areca-nut chewers in India, which have similarly documented high overall awareness of tobacco-related harm coupled with substantial gaps in risk perception, brand-specific misconceptions, and continued use despite a professed desire to quit.[31,32] The present study extends this literature by highlighting the complete absence of social media as a reported information source, a finding not always explicitly quantified in earlier KAP surveys and one that has become increasingly salient as digital media consumption among adolescents has grown substantially since many of the foundational KAP studies were conducted.

The cytological findings corroborate a substantial body of work demonstrating elevated micronuclei and other nuclear anomalies in the buccal mucosa of smokeless-tobacco and areca-nut users compared with non-users.[10,11,33,34,35] Motgi et al., and Yadav and Saini both reported significantly higher micronuclei frequencies in smokeless-tobacco users relative to non-tobacco controls, mirroring the near-universal micronuclei detection observed here.[10,11] Similarly, Sharma et al., and Joshi et al., documented a broad spectrum of nuclear anomalies—including karyorrhexis, karyolysis and pyknosis—in tobacco- and areca-nut-exposed buccal epithelium, consistent with the multi-parameter aberration profile observed in the present cohort.[33,34] Baxi and Gohil likewise reported a marked increase in nuclear anomalies among tobacco users compared with healthy controls using conventional cytological staining, reinforcing the reproducibility of this pattern across different study populations and settings.[35] Taken together, the convergence of the present findings with this literature strengthens confidence that the observed aberration burden reflects a genuine tobacco-associated cytotoxic and genotoxic effect rather than a population-specific artefact.

With respect to the molecular docking component, the present binding energy of −6.1 kcal/mol for the nicotine–XRCC1 interaction is consistent with previously described structural characterisations of the XRCC1 scaffold and its capacity to engage small-molecule and protein partners at discrete interface residues during coordination of base excision repair.[13,27] While direct nicotine–XRCC1 docking studies remain scarce in the literature, the broader association between XRCC1 functional status (including polymorphic variants such as Arg194Trp and Arg399Gln) and cancer risk—including head and neck squamous cell carcinoma—has been well documented.[18,19,20,36] The present computational finding is complementary to, rather than a replacement for, this genetic-epidemiological evidence: it suggests an additional, ligand-driven mechanism by which chronic nicotine exposure might functionally compromise XRCC1-mediated repair independent of the individual's underlying genotype, an angle not typically addressed in polymorphism-focused studies.

### 4.3 Biological Explanation

Mechanistically, the cytological aberrations observed here are recognised markers of distinct, though overlapping, cellular stress pathways. Micronuclei arise from acentric chromosomal fragments or whole chromosomes that fail to be incorporated into daughter nuclei during mitosis, and are a direct cytological correlate of chromosomal

instability and unrepaired DNA damage.[10,11,12] Karyorrhexis and pyknosis reflect nuclear fragmentation and chromatin condensation associated with apoptotic and pre-apoptotic cellular stress, while karyolysis represents a later, necrotic stage of nuclear dissolution.[12] Broken egg nuclei and enucleation are considered morphological indicators of severe nuclear membrane and cytoskeletal disruption. The co-occurrence of all six aberration types in the tobacco-chewing group suggests that gutkha exposure concurrently activates multiple, convergent pathways of cellular injury—genotoxic (micronuclei), apoptotic (karyorrhexis, pyknosis) and necrotic/structural (karyolysis, broken egg nuclei, enucleation)—rather than a single isolated mechanism.

The docking data provide a plausible upstream molecular contributor to this convergent injury pattern. XRCC1 functions as a central scaffold within the BER pathway, coordinating DNA polymerase β, DNA ligase IIIα and PARP1 at sites of base damage; disruption of this scaffolding function—whether through polymorphic variation or through direct ligand engagement at a functionally relevant residue such as Pro135—could plausibly reduce the efficiency of repair for oxidative and alkylation-type lesions generated by other tobacco-derived genotoxicants (e.g., tobacco-specific nitrosamines, reactive oxygen species generated during areca nut metabolism).[13,14,17] Under this model, nicotine itself may act less as a direct DNA-damaging agent and more as a modulator that impairs the cell's capacity to resolve damage inflicted by co-exposures within the gutkha matrix, thereby amplifying the net genotoxic burden reflected in the micronuclei and other nuclear aberrations observed cytologically.

**4.4 Clinical Relevance**

Habitual gutkha use may cause measurable cellular damage as early as age 18, before visible oral lesions develop. Because exfoliative cytology is non-invasive, affordable, and simple, it could be used for early screening of high-risk adolescents and young adults in dental, primary-care, and educational settings. The identified Pro135 interaction with XRCC1 also suggests a potential future role for nicotine-exposure biomarkers and DNA-repair assays in assessing individual risk of DNA-repair impairment.

**4.5 Public Health Importance**

The findings show that general awareness of tobacco harms is high, but product-specific misconceptions remain widespread, suggesting that awareness alone does not change behaviour. Public health campaigns should directly challenge false claims that certain brands are safer. The absence of social media as an information source also highlights a major gap in reaching adolescents and young adults. Since 91.67% wanted to quit but 76.67% found quitting difficult, especially because of relapse fears, there is a strong need for youth-focused, digitally delivered cessation programmes emphasizing withdrawal management and relapse prevention.

**4.6 Strengths**

The study's main strength is its integrative approach, combining behavioural data, PAP and MGG cytology, and molecular docking in young male gutkha users. Using two complementary staining methods reduces the risk of stain-related artefacts, while a tobacco-naive, age-matched control group provides a reliable baseline for comparison. The identified nicotine–XRCC1 interaction at Pro135 further supports a potential mechanistic explanation for the observed cytological abnormalities.

**4.7 Age-Related and Dose-Related Patterns**

The study suggests early and rapid escalation of gutkha consumption, with 30% of participants aged 18–20 years consuming 11–15 packets daily, indicating substantial nicotine dependence soon after initiation. Heavy consumption at a young age may result in a disproportionately high cumulative genotoxic burden. However, the current study did not examine cytological abnormalities according to age or consumption intensity. Future analyses should investigate dose–response and duration–response relationships between gutkha use and cytological abnormalities, which could strengthen the evidence for causality.

**4.8 Interpreting the Cytological Pattern Across Both Stains**

The PAP and MGG staining results were largely concordant, supporting the reliability of the observed cytological abnormalities and reducing the likelihood of stain-specific artefacts. Minor differences, such as the higher detection of karyolysis by MGG, may reflect differences in dye affinity and visualization of degenerating nuclear material rather than methodological error. These findings support the value of dual staining, as using both techniques may provide a more comprehensive assessment of cytogenotoxic abnormalities than either stain alone.

### 4.9 Placing the Binding Energy in Context

The molecular docking analysis identified a −6.1 kcal/mol binding energy, suggesting a plausible moderate protein–ligand interaction between nicotine and XRCC1, with Pro135 as a key interacting residue. The interaction may provide a possible mechanistic explanation for DNA-repair effects, but experimental biochemical and functional studies are required to confirm whether nicotine actually disrupts XRCC1-mediated DNA repair in living cells.

### 4.10 Integrating the Three Evidence Streams

The study's behavioural, cytological, and computational findings complement one another, creating a coherent body of evidence. Behavioural data identify a high-risk population with early and frequent gutkha use, while cytology demonstrates measurable cellular damage in these individuals. Molecular docking further provides a plausible mechanism involving impaired DNA-repair capacity. Together, these findings suggest a progression from high-risk behaviour → early cellular injury → a potential molecular mechanism, although the computational mechanism requires experimental validation.

### 4.11 Future Research Directions

Future research should focus on longitudinal studies with biochemical exposure validation to determine how cytological abnormalities develop and whether they are reversible after cessation. The proposed nicotine–XRCC1 interaction should be experimentally validated using biophysical and functional DNA-repair assays. Molecular studies should also examine other gutkha constituents, such as arecoline, safrole, and tobacco-specific nitrosamines, and their combined effects on DNA-repair pathways. Finally, social-media-based counter-messaging and digital cessation programmes should be tested among young gutkha users to determine whether they reduce misconceptions, consumption, relapse, and cytological damage.

## 5. Conclusion

### Main Conclusion

This integrative study demonstrates that habitual gutkha chewing among young men is characterised by early initiation, high-intensity consumption, and persistent brand-specific safety misconceptions despite universal awareness of tobacco-related harm. Buccal exfoliative cytology revealed a consistent and substantial excess of nuclear and cytoplasmic aberrations—including micronuclei, pyknosis, karyorrhexis, karyolysis, broken egg nuclei and enucleated cells—in tobacco chewers compared with non-tobacco-using controls, indicating measurable genotoxic and cytotoxic mucosal injury already present in this young population. Molecular docking further demonstrated a thermodynamically favourable interaction between nicotine and the DNA-repair protein XRCC1 (binding energy −6.1 kcal/mol, mediated by the Pro135 residue), offering plausible molecular-level support for a mechanism by which nicotine may compromise base excision repair capacity and amplify the mutagenic impact of co-exposures within the gutkha matrix.

### Clinical Implications

The convergence of behavioural, cytological and computational evidence supports the use of buccal cytology as a practical, minimally invasive tool for early risk stratification in high-risk young populations, potentially enabling identification of subclinical mucosal injury well before clinically overt lesions develop. The identification of a specific nicotine–XRCC1 interaction residue provides a starting point for future mechanistic and biomarker-oriented research linking tobacco exposure to impaired DNA repair capacity.

### Recommendations

Public health strategies targeting young gutkha users should move beyond generic harm-awareness messaging to directly counter brand-specific safety misconceptions, incorporate social-media-based risk communication channels appropriate to adolescent and young-adult audiences, and expand accessible cessation support explicitly addressing relapse prevention and withdrawal management. Integration of buccal cytology into school- and college-based screening programmes should be considered as a low-cost adjunct for early detection of tobacco-induced cellular change in high-risk populations, alongside continued mechanistic research validating the nicotine–XRCC1 interaction identified in this study.

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## 8. Conflict of interest

The authors declare no conflict of interest.

## 9. Author contributions (CRediT Taxonomy)

Conceptualization: H.S.S., A.C., H.C.S. Methodology: H.S.S.,M.M.K. Software: H.S.S., H.C.S. Validation: M.M.K., H.C.S., S.N. Formal Analysis: H.S.S.,S.M, H.C.S. Investigation: H.S.S., A.C., H.C.S. Resources: H.S.S.,S.M, K.D.S., V.K., H.C.S., S.N. Data Curation: H.S.S.,S.M, W.W., H.C.S. Writing – Original Draft: H.S.S., A.C, H.C.S. Writing – Review & Editing: H.S.S., H.C.S., W.W., S.M. Visualization: H.C.S., S.N. Supervision: S.M., H.C.S., S.N.
All authors have read and agreed to the published version of the manuscript.

## 10. Ethical approval statement

The study was conducted in accordance with the Declaration of Helsinki and was reviewed and approved by the Institutional Ethics Committee of Assam down town University (Memo No.: AdtU/Ethics/stdnt-lett/2024/153). Written informed consent was obtained from all participants prior to enrolment.

## 11. Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

## 12. Consent statement

Written informed consent was obtained from all individual participants included in the study.

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