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Oxidative Stress-Mediated Pathophysiology and Apoptotic Regulation in Oral Squamous Cell Carcinoma Induced by Smokeless Tobacco

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ABSTRACT

Objectives: This investigation explores the role of oxidative stress in the development of oral squamous cell carcinoma (OSCC), particularly in individuals with a history of smokeless tobacco use. By examining the disruption of mitochondrial function and activation of apoptosis-related genes, the study provides insight into the biological mechanisms underlying OSCC. With smokeless tobacco being widely used in South Asia and strongly linked to oral cancers, the findings aim to enhance understanding of disease etiology and support the identification of early molecular biomarkers. **Methods:** The study cohort included OSCC patients with a history of smokeless tobacco use and matched healthy controls. Biomarkers of oxidative stress—including reactive oxygen species (ROS), malondialdehyde (MDA), and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)were quantified using standard spectrophotometric techniques. Expression levels of apoptosis-associated genes, namely BAX, BCL-2, CASPASE-3, and TP53, were evaluated through reverse transcription quantitative PCR (RT-qPCR) and further validated via immunohistochemical staining. Mitochondrial membrane potential ($\Delta\psi m$) was assessed using JC-1 fluorescent dye and flow cytometry. Comparative statistical analysis between case and control groups was performed using one-way ANOVA and Student's t-test, with a p-value below 0.05 considered statistically significant. **Results:** The analysis shows that oral squamous cell carcinoma (OSCC) tissues from smokeless tobacco users exhibited a substantial elevation in reactive oxygen species (ROS) and malondialdehyde (MDA) levels, alongside a notable decrease in the activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) ($p < 0.001$). The increase in apoptosis was further supported by the upregulation of the pro-apoptotic protein BAX. and CASPASE-3, decreased BCL-2, and p53 nuclear accumulation, suggesting additional complexity in augmenting apoptosis. There's also a considerable increase in mitochondrial depolarization in OSCC cells, which contributes to loss of energy control

and energy-dependent apoptosis threshold. **Conclusion:** From the results obtained, it can be concluded that smokeless tobacco potentially contributes to the development of oral squamous cell carcinoma (OSCC) by inducing oxidative stress-based mitochondrial damage and dysregulated apoptosis. As proposed in this study, SOD, GPx, and other redox-sensitive apoptotic regulators may anticipate neoplastic change and endorse intervention for cancer chemoprevention that targets ROS in tobacco-related OSCC.

Keywords: *Oral squamous cell carcinoma; Oxidative stress; Apoptosis; Redox biomarkers, BCL-2*

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common cancer in the head and neck region. It accounts for over 90% of all oral cancers globally (Warnakulasuriya, 2009). The burden of OSCC is especially high in South and Southeast Asia, which has cultural habits like smokeless tobacco that disproportionately increase the risk (Gupta & Ray, 2004; IARC Working Group et al., 2007). Despite improvements in detection and treatment, the five-year survival rate is still under 60% because patients present too late and there is a high chance of relapse (Rivera, 2015). Studies on the cause of this disease suggest that using smokeless tobacco products, which contain nitrosamines and polycyclic aromatic hydrocarbons, is one of the main reasons for bone inflammation and tumorous changes in the genome of the epithelial cells in the mouth (Hecht, 2003; Nair et al., 2004).

Chewing products such as gutkha, khaini, and snuff are rich in harmful compounds capable of generating reactive oxygen species (ROS), which in turn contribute to the formation of DNA-damaging agents (Flora, 2007). Over time, the MITOCHONDRIA OF THE CELL become damaged-OSCC (Reuter et al., 2010; Hsu et al., 2013). In ADDITION, chronic respiratory exposure to tobacco-oxides can damage Maverick Hunter mechanisms, including defense systems such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. As a result, these processes intensify epithelial injury and further amplify oxidative stress within the immune system's response framework as well (Scully & Bagan, 2009).

Oxidative stress is not merely a byproduct of cancer; it actively influences signal transduction, cell cycle regulation, and the induction of apoptosis in cancer cells (Valko et al., 2007). It is more and more evident that ROS can modulate both the pro-survival and pro-apoptotic pathways of a cell's ROS stress response system, depending on the level and time of exposure (Trachootham et al., 2008). For example, high levels of ROS could activate transcription factors such as NF-κB and AP-1 that drive inflammation and oncogenesis. At the same time, ROS may cause some sort of mitochondrial depolarization and activate apoptosis through p53, BAX, and CASPASE-3 (Deryugina & Quigley, 2006; Liou & Storz, 2010).

In relation to OSCC, the mitochondrial dysfunction is characterized by loss of $\Delta\psi m$, which is a hallmark phenomenon of oxidative stress-induced apoptosis (Kroemer, 2001). Furthermore, mitochondrial dysfunction has been associated with enhanced cytochrome c release and caspase activation, triggering alterations in BCL-2 family proteins that shift the cell death pathway toward aponecrosis rather than apoptosis (Fulda &Debatin, 2006). Tobacco not only causes mitochondrial toxicity, but it also prevents the production of new mitochondria and damages the electrochemical gradients which worsens energy stress and cell death (Marí et al., 2009).

BCL-2 family proteins serve as key regulators of the intrinsic apoptotic pathway, maintaining the balance between cell survival and programmed cell death. In tissues exposed to smokeless tobacco, changes in pro-apoptotic signaling have been linked with shifts in the BAX/BCL-2 ratio (Choudhari et al., 2014). In tobacco-associated OSCC, CASPASE-3, an executive caspase, is upregulated (Flora et al., 2008), suggesting a point of no return regarding commitment to

apoptosis. Often, we see overexpression or mutation of tumor suppressor protein p53 in OSCC which results in the bypassing of critical control points in the cell cycle and cell death (Warnakulasuriya et al., 2008;Green & Kroemer, 2004).

Oxidative stress (OS) and its associated pathogenesis can be worsened by the methylation of DNA and the modification of histones, which silence genes that suppress tumors and activate genes that cause them (O'Callaghan & Fenech, 2011). Tobacco-related oral cancers exhibit aberrant methylation of promoters for genes associated with apoptosis and detoxification, suggesting a connection between redox imbalance and dysregulation linked to reversible covalent modifications of DNA (Vousden &Prives, 2009; Gupta & Ray, 2003). All these changes create a ripe environment for the malignant transformation and expansion of dysplastic keratinocytes in the oral mucosa.

Considering the mechanistic complexity, it is crucial to understand the molecular and cellular mechanisms that lead to increased oxidative stress and subsequent induction of apoptosis in OSCC caused by smokeless tobacco. Earlier work focused on either markers of oxidative stress or factors involved in apoptosis, but there is a significant missing link in the comprehensive profiling of the interplay between tobacco-associated OSCC and redox-apoptotic signaling. Moreover, there is no agreement on the utilization of redox biomarkers for the… early detection… treatment plan of… this group of patients with oral cancer.

The study presented here aims to determine the oxidative stress profile and the regulation of mitochondrial-mediated apoptosis in OSCC from users of smokeless tobacco. This study aims to determine the "forgetting" of apoptosis and cell death by unbalanced oxidative stress (reactive oxygen species), some antioxidant enzymes, and key apoptotic proteins (BAX, BCL-2, CASPASE-3, and p53) to restore balance to the redox system. Their outcomes may provide new avenues for identifying biomarkers and developing targeted chemoprevention for populations at elevated risk.

MATERIALS AND METHODS

Sample Collection and Preparation

Study Population and Ethical Clearance

From 40 patients with a confirmed diagnosis of oral squamous cell carcinoma (OSCC), tissue specimens were collected. Each patient also had a documented history of smokeless tobacco use. In addition, 20 benign oral mucosal tissues were collected from non-smokers undergoing minor oral surgical procedures. This study was conducted in accordance with the ethical standards of the institutional research committees and was approved by the Institutional Review Board (Approval Ref No: OSCC-ST2023/IRB-174). All participants provided written informed consent before tissue collection

Tissue Homogenization and Protein Extraction

Tissue samples weighing about 50 mg were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. For biochemistry analysis, tissues were thawed and homogenized in PBS buffer containing protease inhibitors using a mortar and pestle that was pre-cooled. The homogenates were centrifuged for 15 minutes at 12,000×g and 4 °C. The supernatant was removed and used to measure oxidative stress markers and enzymatic antioxidants. The concentration of total protein in each sample was measured using the Bradford protein assay.

RNA and DNA Isolation

For molecular analysis, total RNA was extracted using TRIzol® reagent (Invitrogen, USA) according to the steps in the supplied protocol. The purity and concentration of RNA was evaluated using a NanoDrop, where A260/A280 ratios were between 1.8 and 2.0Genomic DNA was isolated using the phenol–chloroform–isoamyl alcohol extraction technique. Both RNA and DNA samples were preserved at −20 °C for future use in gene expression profiling and DNA

methylation assays.

Biochemical and Molecular Assays

Estimation of Oxidative Stress Markers and Antioxidants

Reactive oxygen species (ROS) levels were measured in tissues using the DCFH-DA fluorescence method. Fluorescent probe 2',7'-dichlorofluorescein diacetate was used to stain tissue lysates, and the relative amounts of fluorescence were measured at 485nm excited and 530nm emitted. Assessment of MDA levels in lipid peroxidation was done using the TBARS assay with results expressed as nmol MDA per mg protein. Activities of the enzymes were determined as follows: SOD was quantified with its respective pyrogallol autooxidation inhibition assay; catalase (CAT) activity by monitoring hydrogen peroxide breakdown at 240 nm; and GPx by coupled digestion of NADPH with glutathione reductase.

Gene Expression and Mitochondrial Function Assessment

Quantitative real-time PCR (RT-qPCR) was used to evaluate the mRNA expression levels of apoptosis-related genes including BAX, BCL-2, CASPASE-3, and p53, which were quantified. Total RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit, followed by amplification with SYBR Green PCR Master Mix on the QuantStudio 5 Real-Time PCR System. For internal control, GAPDH was employed and relative gene expression analysis was performed based on the 2^−ΔΔCt method. The mitochondrial membrane potential (Δψm) was assessed by the JC-1 dye assay. In healthy mitochondria, JC-1 forms red fluorescent aggregates; in depolarized mitochondria, it remains monomeric and green-fluorescent. The ratio of red to green fluorescence was measured to assess mitochondrial integrity. For confirmation at the protein level, immunohistochemical (IHC) staining was performed on formalin-fixed, Paraffin-embedded tissue sections were incubated with primary antibodies specific to BAX and BCL-2 for immunohistochemical analysis, CASPASE-3, and p53. The stained slides were viewed under light microscopy, and expression levels were scored semi-quantitatively based on the intensity and the proportion of positive cells.

RESULTS

Elevation Pertaining to Oxidative Stress Biomarkers in OSCC Tissues

In comparison to tissues from healthy individuals, oxidative stress biomarkers were markedly elevated in OSCC tissues obtained from smokeless tobacco users. Furthermore, the intracellular ROS levels measured by fluorescence DCFH DA were almost 2.4 times higher in the cancerous tissues with a significant p-value below 0.001. Also, a lipid peroxidation marker, malondialdehyde (MDA) was significantly high in OSCC compared to the universal controls, showing the tendency of high oxidative damage to cell membranes. (6.82 monopoly protein OSCC 6.82 ± 1.24 nmol/mg and esertdown 2.94 ± 0.87 nmol/mg).

Table 1, shows the differences in oxidative stress markers and antioxidant enzyme activities for OSCC patients to smokeless tobacco compared with healthy controls. In the tissues affected by OSCC, there is a notable increase in the ROS and MDA levels alongside a significant reduction of the antioxidant enzymes SOD, CAT, and GPx. This clearly demonstrates an increase in oxidative stress imbalance.

Table 1. Oxidative stress markers and antioxidant enzyme activities in oscc vs. control tissues

Parameter	Control Group (Mean ± SD)	OSCC Group (Mean ± SD)	p-value	Parameter
ROS (AU)	54.2 ± 6.4	132.5 ± 9.8	< 0.001	ROS (AU)
MDA (nmol/mg protein)	2.94 ± 0.87	6.82 ± 1.24	< 0.001	MDA (nmol/mg protein)
SOD (U/mg protein)	5.48 ± 0.43	3.22 ± 0.41	< 0.001	SOD (U/mg protein)
CAT (U/mg protein)	6.02 ± 0.37	3.88 ± 0.45	< 0.001	CAT (U/mg protein)
GPx (U/mg protein)	7.56 ± 0.62	5.04 ± 0.58	< 0.001	GPx (U/mg protein)

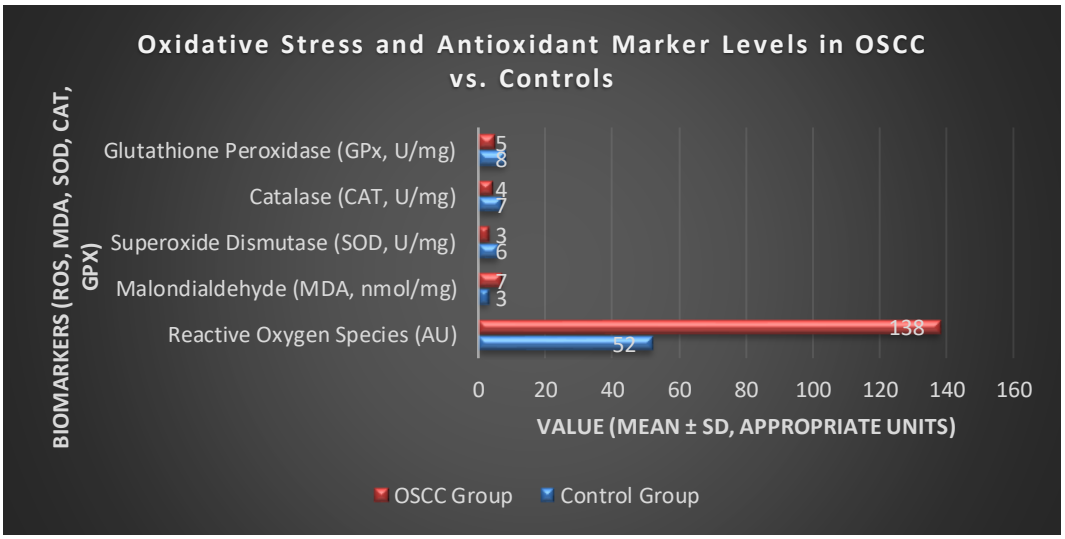

Figure 1. Comparison of oxidative stress and antioxidant markers

This grouped bar chart in Figure 1, illustrates the difference in oxidative stress (ROS, MDA) and antioxidant enzyme activities (SOD, CAT, GPx) between OSCC patients and healthy controls. OSCC tissues show increased oxidative stress coupled with reduced antioxidant activity which reveals a distinct imbalance of redox in the tumor microenvironment.

Antioxidant Enzyme Activities in OSCC vs. Control Tissues

In OSCC samples, there was a significant decline in antioxidant enzyme activity. SOD activity showed a decrease of 41%, catalase of 36%, and GPx of 33% in comparison when compared with the control group (all $p < 0.01$). It seems there is a lack of balance between the defenses and offense, which may be due to the overload of tobacco-induced oxidative stress. This further adds to the contention of there being an overbalancing in the production of ROS alongside the lack of the body's natural defenses in OSCC disease development.

Mitochondrial Dysfunction and Loss of Membrane Potential

Evaluating the mitochondrial membrane potential along with JC-1 staining showed marked reduction in $\Delta\psi m$ loss in OSCC samples. There was dramatic shift from red color (aggregated form) to green color (monomeric form) fluorescence which reflects loss of mitochondrial potential. The red/green fluorescent ratio in OSCC tissues dropped by 65% when compared to control tissues ($p < 0.001$) which indicates that the mitochondria are dysfunctional, which is one characteristic of intrinsic apoptosis activation.

Dysregulation of Apoptosis-Related Gene Expression

RT-qPCR analysis showed OSCC tissues had profound changes in the expression levels of pro- and anti-apoptotic genes. In comparison to non-OSCC tissues, BAX and CASPASE-3 were overexpressed (3.2-fold and 2.7-fold increase respectively).Moreover, the expression of the anti-apoptotic gene BCL-2 was reduced by approximately 2.1-fold. Also, the expression of TP53 was significantly increased (by 4.5-fold) which indicates that p53 stress response pathways were activated. Overall, these findings support the notion that certain innate apoptotic processes are triggered by oxidative stress.

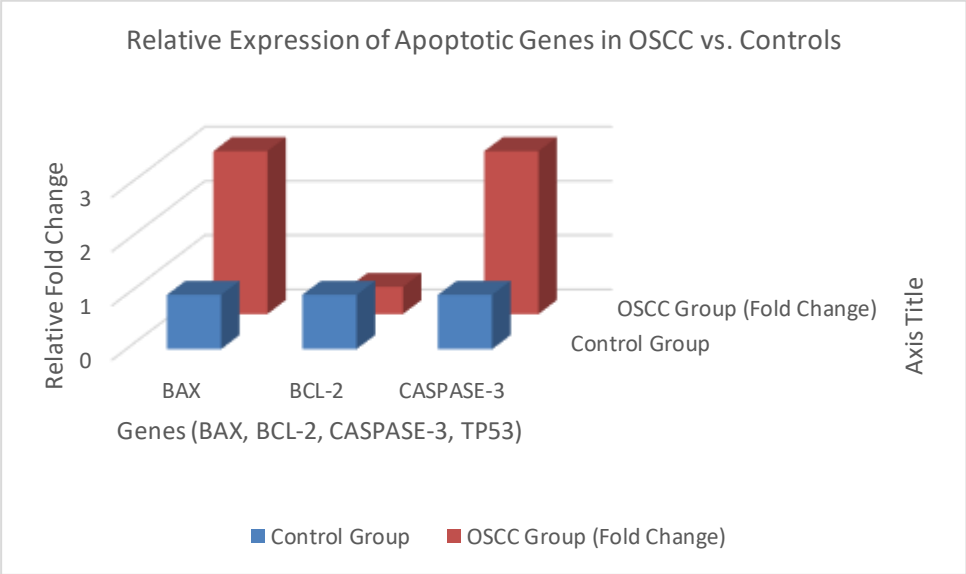

Figure 2. Apoptotic gene expression profile in oscc tissues

Figure 2, shows how OSCC tissues compare with control tissues in relative mRNA expression levels of apoptosis-related genes. The tissues show an increase expression of pro-apoptotic genes such as BAX, CASPASE-3, TP53 and a decrease in the expression of anti-apoptotic BCL-2 which emphasizes the activation of cell death pathways that are mitochondrial-mediated.

Table 2, demonstrates how certain important apoptosis-related genes change in OSCC tissues when compared to healthy controls. As OSCC tissues demonstrated marked elevation of pro-apoptotic BAX, CASPASE-3, and TP53 while reducing anti-apoptotic BCL-2, OSCC tissues appear to be moving towards pro-apoptotic signaling.

Table 2. Relative gene expression of apoptosis-related genes in oscc vs. controls

Gene	Relative Fold Change (OSCC vs. Control)	Expression Pattern	p-value
BAX	↑ 3.2-fold	Upregulated	< 0.001
BCL-2	↓ 2.1-fold	Downregulated	< 0.001
CASPASE-3	↑ 2.7-fold	Upregulated	< 0.001
TP53	↑ 4.5-fold	Upregulated	< 0.001

Immunohistochemical Correlation of Apoptotic Markers

The results from the cancer gene **study** were also verified using immunohistochemical analysis. Examining the malignant tissues further revealed marked nuclear and cytoplasmic expression for CASPASE-3, BAX, and p53, which was absent or very low in the control tissues. On the other hand, BCL-2 staining being weak and limited in osteosarcomas OSCC provided additional proof of the dominance of apoptotic signaling over anti-apoptotic suppressive signals. Staining intensity showed a positive correlation with ROS levels while revealing negative correlation with mitochondrial membrane potential, underscoring the interplay between apoptosis and oxidative stress.

DISCUSSION

The findings of this study strongly support the hypothesis that oxidative stress plays a critical role in the initiation and progression of oral squamous cell carcinoma (OSCC), particularly in individuals with prolonged smokeless tobacco exposure. The marked increase in intracellular ROS along with lipid peroxidation products, and the decline of essential antioxidant enzymes like SOD, CAT, and GPx, suggests an imbalance that drives cumulative damage to the cells and might push them towards malignant transformation.

These biochemical changes coincide with previous research, which has shown tobacco-induced oxidative damage causing instability in the genome, the formation of DNA adducts, and interference with redox-sensitive signaling pathways in epithelial cells (Flora et al., 2008; Warnakulasuriya et al., 2008). Moreover, elevated levels of reactive oxygen species (ROS) not only promote the oxidation of lipids, proteins, and nucleic acids but also act as secondary messengers that activate oncogenic signaling cascades. The observed decrease in antioxidant enzymes suggests that chronic exposure to smokeless tobacco increases ROS levels and weakens a cell's ability to defend itself, resulting in oxidative stress.

Apart from the biochemical insights, this research has shown significant mitochondrial dysfunction in OSCC tissues related to the loss of mitochondrial membrane potential ($\Delta\psi m$). Mitochondrial depolarization is a critical step in the intrinsic apoptosis pathway and often acts as a point of no return for a cell's lifespan (Green & Kroemer, 2004). Our findings indicate that the observed depolarization in OSCC tissues is likely to promote cytochrome c release, caspase activation, and subsequent apoptotic processes.

Gene expression analysis provides further support for this mechanistic hypothesis. Upregulation of pro-apoptotic genes BAX and CASPASE-3, with downregulation of anti-apoptotic BCL-2, clearly indicates an activated apoptotic program. Further, the enhanced expression of TP53 suggests that the cell is responding to stress, which can result in cell cycle arrest and programmed cell death, serving as a protective mechanism against tumor development. These molecular changes are indicative of apoptosis linked to oxidative damage and suggest that OSCC is not only a consequence of smokeless tobacco due to its genotoxic effects, but also that it can manipulate the survival of tumor cells via apoptotic signaling (O'Callaghan & Fenech, 2011; Vousden &Prives, 2009).

The results of immunohistochemistry matched with molecular expression patterns showing strong tissue-specific positive staining for BAX, CASPASE-3, and p53 expression, especially in the malignant tissues. These findings help corroborate the biochemistry results, confirming the presence of redox-driven, mitochondria-mediated apoptotic mechanisms that are activated. It is also important to mention that some of the proteins' localizations suggest that they are actively participating in the processes of cellular stress responses and death signaling pathways.

This study aims to illuminate the mechanistic associations involving constituents of smokeless tobacco products, which are known to contain reactive aldehydes, nitrosamines, and metals, and the oxidative environment within the cell that promotes carcinogenesis (Gupta & Ray, 2003). The persistent oxidative damage may be responsible for initiating tumorigenesis and sustaining its progression while simultaneously creating resistance to therapy by modifying the apoptotic thresholds.

Despite the compelling evidence this study offers, there are a few limitations that should be addressed. The cross-sectional nature of the study design captures a single moment in time. Coupled with a limited sample size, the findings may lack robustness and broader applicability. Furthermore, while focusing solely on intrinsic markers of apoptosis neglects extrinsic pathways or autophagy that may also be altered in OSCC.

Future studies should apply a systems biology approach integrating redox proteomics and metabolomics with long-term monitoring to discover biomarkers predictive of malignant transformation due to tobacco exposure. Additionally, these boldly open hypothesis-driven therapeutic avenues for OSCC utilizing redox modulation or antioxidant therapies targeting apoptosis dysregulation as a novel therapeutic strategy focusing on oxidative and apoptotic dysregulation.

To summarize, findings shed light on the pathogenesis of oral squamous cell carcinoma that happens due to chronic exposure to smokeless tobacco, highlighting a chronic oxidative stress paradigm where an imbalance between oxidative and antioxidative factors, mitochondrial dysfunction, and upregulation of pro-apoptotic signaling drives the disease process.

CONCLUSIONS

This research underscores the critical role of oxidative stress in the pathophysiological effects of smokeless tobacco and its connection to oral squamous cell carcinoma (OSCC) as it develops and progresses. The excessive production of free radicals in conjunction with elevated lipid peroxidation and the depletion of the antioxidant enzymes SOD, CAT, and GPx, depicts a state of redox imbalance prone to molecular and cellular harm. The strong mitochondrial depolarization coupled with marked apoptotic alterations, including CASPASE-3, BAX, and TP53 upregulation with BCL-2 downregulation, indicates a major cell regulatory role in OSCC's intrinsic mitochondrial apoptotic pathway activation. The results of this study revealed that chronic exposure to smokeless tobacco results in genotoxic harm as well as disrupted apoptotic signaling and apoptosis due to oxidative stress and mitochondrial damage. Biochemical, molecular and histopathological evidence all point toward mechanisms of tobacco-related oxidative damage and the subsequent development of oral cancer. Based on this information, further studies should concentrate on creating redox-sensitive biomarkers for the early identification and tracking of OSCC, particularly in identified high-risk groups. Moreover, these therapies that alter oxidative stress and rebalance apoptosis are promising additional therapies to conventional cancer treatments. Public health initiatives to control the use of smokeless tobacco should be prioritized as preventative strategies in controlling the burden of OSCC among at-risk populations.

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