

## ORIGINAL ARTICLE

# Assessment of Biochemical Variables of Medical Importance in Patients of Oral Squamous Cell Carcinoma (OSCC) Using Betel Nuts

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## ABSTRACT

**Background:** Betel nut is considered one of the leading causes of oral cell carcinoma. After caffeine, alcohol and tobacco, the betel nut mastication is the fourth most used drug in the world.

**Aim:** To evaluate the fluctuating levels of oxidative stress markers in patients of oral squamous cell carcinoma (SCC) using betel nuts.

**Study design:** Case control study

**Place and duration of study:** University of Lahore Teaching Hospital, Lahore from 21<sup>st</sup> January 2021 to 10<sup>th</sup> July 2021.

**Methodology:** One hundred participants recruited and divide into two groups. First group consisted of confirmed cases of oral SCC on histopathological evidence and fifty age and sex matched normal healthy subjects were taken as a control in group B. Oxidative stress markers malondialdehyde (MDA), Isoprostanes, 8- hydroxy deoxyguanosine (8-OHdG), and 4-hydroxynonenal (4-HNE) were measured in both groups by using ELISA and TBRAS methods.

**Results:** Significant difference in oxidative stress markers in oral cell carcinoma patients and control group. The *p* value differed significantly for MDA, Isoprostanes, 8-OHdG and 4-HNE in serum (*p*= 0.021, 0.033, 0.001, and 0.000 respectively) as well as in saliva samples (*p*= 0.011, 0.021, 0.011 and 0.001 respectively).

**Conclusion:** The oxidative stress markers like MDA, Isoprostanes, 8-OHdG and 4- HNE are significantly elevated in patients of oral squamous cell carcinoma as compared to the normal healthy individuals.

**Key words:** Betel nuts, Oxidative stress marker, Oral cells carcinoma, MDA, 8-OHdG, Isoprostanes, 4-HNE

## INTRODUCTION

Cancer is a condition caused by an uncontrolled division of abnormal cells in any part of the body. Oral cancers are malignant and affect the structures or tissues surrounding the mouth.<sup>1</sup> They can be primary from the mouth, secondary from metastasis from a remote site, or from an extension from a nearby region<sup>2,3</sup>. This cancer is the 6th most widely distributed neoplasm in the world. About 90% of them are squamous cell carcinoma (SCC). Squamous cell carcinoma in the areas over the lips and oral cavity<sup>4</sup>. It is origin is epithelial and malignant whereas other tumors, like angiosarcoma, are mesenchymal and invasive. Squamous cell carcinoma generally affects the tongue and certain sites such as the base of the mouth, the mucosa of the cheeks, palate, gums, or lips<sup>5</sup>.

There are approximately 275,000 new cases of oral squamous CA cells worldwide each year, but their incidence is highly diverse. In the southern Asia & Latin American countries the incidence of OSC carcinoma is 20 times more as compared to Northern Europe, and accounts for the most common neoplasm in the male gender in risky areas of Asia<sup>6</sup>. In South Asia, Pakistan and India have higher prevalence of OSCC as compared to the western countries, which can be attributed to the same cultural practices and habits<sup>7</sup>. The prevalence of this neoplasm in Pakistan is about 10%. A maximum number of the cases have been identified in middle and old age population, but in the past few years, studies revealed an increased number of new cases among young population also. Epidemiological studies have shown a male preponderance of OSCC with female with a ratio of 2:1<sup>8</sup>.

Oral squamous cell carcinoma is a major cause of disability and mortality due to malignant cancers. The risk factors for OSCC are age, gender, race, tobacco, alcohol, betel, diet and nutrition. Historical evidence indicates that betel nuts play a major role in the

development of the OSCC. After caffeine, alcohol and tobacco, the beta nut mastication is the fourth most used drug in the world<sup>9</sup>. Chewing usually begins at a young age and later becomes common, regular use of betel nut that develops over many years. Polyphenols, which are tannins and alkaloids, are the most common chemicals in betel nuts. Arecoline is the primary type of alkali which is a muscarinic acetylcholine receptor agonist having cholinergic response on the PNS and is considered psychoactiv.1.0

Frequent mastication results in chronic irritation and inflammation of the epithelium of the buccal cavity. Arecoline and Areca nut compositions may induce pro-carcinogenic properties which includes nitrosamines production and reactive O<sub>2</sub> radicals (O<sup>-</sup>), changeover of matrix metalloproteinases (MMPs) and their tissue blockers, inhibition of collagenases and increased number of collagen cross linkage, up-regulation of heat-shock proteins (HSPs) and integrins and increased expression of cytokines which are inflammatory, including, TNF-alpha, interleukin 8, interleukin-6, interleukin-1-β, Micro-nucleus genesis and increased sister-chromatid exchange is seen in cultured peripheral lymphocytes of betel nut users<sup>11</sup>. This inflammatory response also increased in the oxidative stress markers like malondialdehyde, isoprostanes, 8-hydroxy-deoxyguanosine and 4-hydroxynonenal<sup>12</sup>.

In this study we analyzed the pathological effect of different biochemical variable like oxidative stress markers in patients of oral squamous carcinoma among smokers and betel nut chewers visiting University of Lahore Teaching Hospital, Lahore.

## MATERIALS AND METHODS

Current study included fifty patients having diagnosis of OSCC and fifty (n=50) healthy subjects' taken as control group, serum, and saliva were taken to assess the oxidative stress markers in the study. All of the work done for experiment was approved from the Research Ethical Committee of the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore. 5 ml of blood

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was drawn & serum was separated with the help of centrifuge machine within the 1 hour of withdrawal and kept at  $-70^{\circ}\text{C}$  until used. MDA was estimated by the simple method devised by Ohkawa *et al* (1979). For which about 200  $\mu\text{l}$  sample was placed in the test tube then about same amount of 8.1% SDS along with 1.5ml of acetic acid (20%) and 1.5ml of TBA was add up and then tubes were allowed to heat for an hour. At last, it was cooled down and 4ml of n-butanol was added and then centrifugation was done at 3000rpm for 10mins. The topmost layer was then taken, and absorbance was recorded at 532nm against the blank. Estimation of isoprostanes, 8-Hydroxydeoxyguanosine (8-OHdG) and 4-Hydroxynonenal (4-HNE) were evaluated with the help of commercially available ELISA kits. The t test was used to assess the significance of variance between the controls and cases. The data was analyzed by using software SPSS version 25 and the  $p$  value less than 0.05 remained the significant for our study.

## RESULTS

The male to female ratio in subjects were 32:18 and in control group it was 21:29. In betel nuts users 88.4% participant were using betel nuts for more than 10 years and 8.6% were more than 5 years and rest of the participating betel nuts for less than 5 years.

Table 1 showed the OSCC patients, the mean serum level of MDA was recorded as  $1.89 \pm 0.049$  nmol/ml and in normal individuals it was  $0.99 \pm 0.009$  nmol/ml displaying the raised level of MDA in OSCC patients as compared with normal and statistically significant ( $p=0.021$ ). The data analysis of isoprostanes and 8-OHdG showed statistically significant enhanced levels in OSCC group  $7.33 \pm 0.651$  pg/ml and  $1.83 \pm 0.29$  pg/ml in comparison with normal subjects'  $1.78 \pm 0.018$  pg/ml and  $0.49 \pm 0.008$  respectively. The data interpretation of 4-HNE has shown highly significant raised levels in OSCC group ( $4.59 \pm 0.319$   $\mu\text{mol/ml}$ ) as compared with normal subjects ( $1.89 \pm 0.025$   $\mu\text{mol/ml}$ ).

The results represented in table 2 reflecting the activity of investigated lipid peroxidation products MDA (Malondialdehyde), isoprostanes, 8-OHdG (8-hydroxy-deoxyguanosine) and 4-HNE (4-hydroxynonenal) in saliva sample of normal subjects and their altered behavior in oral-sq-cell CA. The facts are found to be statistically highly significant. The significant increasing trend of MDA was recorded in OSCC patients in contrary to normal individuals. The isoprostanes and 8-OHdG in OSCC patients are displaying the increasing levels as compared to normal individuals and is statistically significant. Saliva 4-HNE level increases in OSCC individuals'  $0.159 \pm 0.087$   $\mu\text{mol/ml}$  was highly significant ( $p=0.001$ ).

Table 1: Lipid peroxidation products in serum sample

Variable	Subjects	Controls	p value
MDA (nmol/ml)	$1.89 \pm 0.049$	$0.99 \pm 0.009$	0.021
Isoprostanes (pg/ml)	$7.33 \pm 0.651$	$1.78 \pm 0.018$	0.033
8-OHdG (pg/ml)	$1.83 \pm 0.29$	$0.49 \pm 0.008$	0.001
4-HNE ( $\mu\text{mol/ml}$ )	$4.59 \pm 0.319$	$1.89 \pm 0.025$	0.000

Table 2: Lipid peroxidation products in saliva sample

Variable	Subjects	Controls	p value
MDA (nmol/ml)	$0.027 \pm 0.068$	$0.016 \pm 0.0019$	0.011
Isoprostanes (pg/ml)	$0.95 \pm 0.028$	$0.813 \pm 0.028$	0.021
8-OHdG (pg/ml)	$0.412 \pm 0.0019$	$0.25 \pm 0.041$	0.011
4-HNE ( $\mu\text{mol/ml}$ )	$0.159 \pm 0.087$	$0.089 \pm 0.0032$	0.001

## DISCUSSION

Squamous cell CA of oral cavity (OSCC) is the most important and regular occurring cancers in the world that has a effect on the structures and tissues around mouth.<sup>13</sup> It involves the tongue and other sites such as floor of mouth, gingiva, cheek lining, palate or lips.<sup>14</sup> In South Asian countries, Pakistan and India have elevated prevalence of OSCC as compared to western countries, which can be attributed to the same cultural practices and habits. The risk factors that attribute to OSCC are age, sex, race, diet, alcohol and

smoking while use of betel nut is considered most common cause of OSCC development.<sup>15</sup> There is historical proof that shows that the betel chewing may be a cause of developing OSCC. A study carried out on stress markers and reported an increase in the serum Malondialdehyde concentrations in several carcinomas even in the early stages.<sup>16</sup> A raised amount of the lipid peroxidation end products was established in the tissue of tumor itself; evidently pointing to source of MDA increased levels among carcinoma patients<sup>17</sup>. MDA is an extremely toxic aldehyde molecule and considered an ideal lipid peroxidation marker. The findings of our study showed raised level of malondialdehyde among patients than healthy people. The mean serum level of MDA among victims with OSCC was  $1.89 \pm 0.049$  nmol/ml while controls had  $0.99 \pm 0.009$  nmol/ml. Similar results obtained from the samples of saliva. The result was found statistically significant as the  $p$  value was 0.021. A recent study carried reported similar results and confirmed that OSCC patients showed raised level of malondialdehyde than healthy individuals<sup>18,19</sup>.

Isoprostanes are the prostaglandin-like compounds which are generated through free radical-mediated peroxidation of the polyunsaturated fatty acids. It is evident that F2-isoprostanes could be used as an indicator of lipid peroxidation caused by mechanism of the chemical stability and formation. A modified F2-isoprostanes generation has been observed in numerous diseases related to oxidative stress for example carcinoma<sup>20</sup>. It was found during study that patients had raised level of isoprostanes than healthy controls. The mean isoprostanes among OSCC patients was  $7.33 \pm 0.651$  pg/ml while among controls the mean level was  $1.78 \pm 0.018$  pg/ml. The result was found statistically significant ( $P=0.033$ ). Another study also confirmed that OSCC patients had raised level of isoprostanes than healthy individuals.<sup>21,22</sup> They reported that patients with OSCC had  $68.73 \pm 12.83$  pg/ml mean level of isoprostanes while controls had  $48.31 \pm 9.9$  pg/ml. The result was also notable when seen in light of statistics. ( $P<0.001$ ).

8-OHdG (8-Hydroxydeoxyguanosine) is most common oxidative DNA stable product damage caused from ROS and was reported to boost its levels in the body fluids as well as tissues during the inflammatory conditions<sup>23,24</sup>. They reported that patients with OSCC had enhanced level of 8-OHdG than controls because mean level of patients was  $1.83 \pm 0.29$  pg/ml and mean level of 8-OHdG among controls was  $0.49 \pm 0.008$  pg/ml. The results were found statistically significant ( $P=0.001$ ). A similar study carried out by Khadem-Ansari *et al*<sup>25</sup> highlighted that elevated level of 8-OHdG ( $157.08 \pm 13.61$  pg/mL) among patients was spotted when contrast with health controls ( $61.48 \pm 9.93$  pg/mL) which showed that levels of 8-OHdG among patients with OSCC were significantly raised than healthy controls ( $P<0.001$ ). Another study done on OSSC demonstrated raised level of 8-OHdG among patients than controls<sup>26</sup>. The mean 8-OHdG level among patients was  $2.05 \pm 2.02$  ng/ml while among controls mean level was  $2.01 \pm 1.11$  ng/ml.

4-HNE (4-Hydroxynonenal) is primary  $\alpha/\beta$  unsaturated hydroxyalkyl which is generated through lipid peroxidation within the cells. It can bind to cysteine, histidine and lysine residues of the proteins through Michael-addition reaction. Hence, 4-HNE can enhance oxidation PTM (post translational modification) and then modify cell signalling and protein function. It leads to induction, phosphorylation as well as p53 nuclear accumulation. 4-HNE increases the growth of cells and differentiation. 4-HNE raised levels were also seen in invasive breast carcinoma. The results of our study showed highly significant raised levels of 4-HNE among OSCC patients ( $4.59 \pm 0.319$   $\mu\text{mol/ml}$ ) than controls ( $1.89 \pm 0.025$   $\mu\text{mol/ml}$ ) with statistically significant results ( $P=0.000$ ). A study done by Ahmad and associates<sup>27</sup> also found raised level of 4-HNE among patients than controls ( $3.265 \pm 0.124$  vs  $1.66 \pm 0.016$ ). But the findings of a study undertaken by Ma and associates<sup>28</sup> indicated that controls had raised level of 4-HNE than patients with OSCC ( $18.13 \pm 7.95$  vs  $13.93 \pm 7.50$ ).

Study revealed that patients OSCC had raised mean salivary level of MDA, Isoprostanes, 8-OHdG and 4-HNE as compared to controls. The mean salivary level of MDA among patients with oral

squamous cell carcinoma was  $0.027 \pm 0.068$  nmol/ml while among controls mean level was  $0.016 \pm 0.0019$  nanomol/ml. The results were found statistically significant ( $P=0.011$ ). A study performed by Metgud and Bajaj<sup>29</sup> verified that patients with OSCC had higher mean salivary level of MDA ( $32.75 \pm 3.03$  nmol/dL) than controls ( $19.98 \pm 0.81$  nmol/dL) showing significant results ( $P<0.001$ ). Study further disclosed that patients with OSCC had raised salivary level of isoprostanes as compared to healthy individuals. The mean level of isoprostanes among OSCC cases was  $0.95 \pm 0.028$  pg/ml while among controls the mean level was  $0.813 \pm 0.028$  pg/ml. The result was found statistically significant ( $P=0.021$ ). Ahmad and associates<sup>27</sup> also confirmed that OSCC patients had raised salivary level of isoprostanes than healthy controls ( $1.66 \pm 0.019$  vs  $0.616 \pm 0.017$ ) and the results was found statistically significant ( $P=0.000$ ). Study found that OSCC patients had enhanced mean salivary level of 8-OHdG than controls as the mean level of patients was  $0.412 \pm 0.0019$  pg/ml and mean level of 8-OHdG among controls was  $0.25 \pm 0.041$  pg/ml ( $P=0.011$ ). A recent study carried out by Tarrad et al<sup>30</sup> indicated that OSCC patients had enhanced mean salivary levels of 8-OHdG than controls as the mean level among patients was  $11.27 \pm 4.835$  ng/ml while mean level of 8-OHdG among controls was  $1.15 \pm 0.631$  ng/ml. In our study OSCC patients had raised mean salivary levels of 4-HNE than controls ( $0.159 \pm 0.087$  vs  $0.089 \pm 0.0032$   $\mu$ mol/ml,  $P=0.001$ ). Same results were also reported by Ahmad and associates<sup>27</sup> who confirmed that patients had elevated mean value than controls ( $0.66 \pm 0.15$  vs  $0.161 \pm 0.0091$ ,  $P=0.019$ ).

## CONCLUSION

Squamous cell carcinoma of oral cavity is an escalating problem of health not only in Pakistan but also among world countries. Present study assessed the oral squamous cell carcinoma among smokers and betel nut chewers. Study concluded that patients with OSCC had elevated levels of MDA, isoprostanes, 8-OHdG, 4-HNE in serum and saliva. Further studies are required to be conducted on large scale to evaluate oral squamous cell carcinoma among smokers and betel nut chewers.

**Conflict of interest:** Nil

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