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Do CO_2 and oxidative stress induce cancer?: a brief study about the evaluation of PON 1, CAT, CA and XO enzyme levels on head and neck cancer patients

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ABSTRACT

Head and neck cancer (HNC) is one of the most common malignancies in the world. HNC is a group of cancers that starts in the mouth, nose, throat, larynx, sinuses, or salivary glands. According to this section of the body parts; induction of cancer can be associated with CO_2 and oxidative stress. The aim of this study is to assess the activities of carbonic anhydrase (CA), catalase (CAT), paraoxonase1 (PON1), and xanthine oxidase (XO) activities in 89 HNC patients and 115 healthy volunteers. Paraoxonase1 activity was found lower in HNC cancer patients. There is no statistically significant difference between patients and controls for catalase, carbonic anhydrase, and xanthine oxidase enzyme levels. According to this results, paraoxonase1 levels could be a candidate as an oxidative marker in HNC patients, but further studies are needed to investigate the other type of cancer related PON1 and the other enzyme levels.

Introduction

Head and neck cancers (HNCs) are found in the oral cavity, pharynx, nasal cavity, paranasal sinuses, larynx, and salivary glands[1]. The five-year survival rate is only 30–35% in HNC types[2].

Risk factors for HNCs are; using tobacco, high-volume alcohol, salt and processed food consumption, improper storage of food, nutritional deficiencies and inadequate oral hygiene[3,4]. All these risk factors related with inhalation may also affect enzyme denaturation since enzymes have numerous different functions in our bodies. Because of this reason we aimed to evaluate carbonic anhydrase (CA), catalase (CAT), paraoxonase1 (PON1) and xanthine oxidase (XO) levels in HNC patients. Another related effect of cancer is oxidative stress[5] which makes a wide contribution tissue destruction to give an imbalance between reactive oxygen species (ROS) and antioxidants to repair the resulting damage. ROS and antioxidants are very important in the development of treatment of various diseases[6]. On the other hand, the increase or decrease of the ROS level may affect the disease characteristics and the treatment effect[7].

Several functions such as cell proliferation, gene instability, invasion, and metastasis are affected by oxidative stress in cancer cells[8]. These cells are capable of producing too much ROS compared with normal cells and therefore they inhibit antioxidative defence system. In this mechanism, ROS plays an active role on the pathogenesis of different diseases such as cancer, and the processes of carcinogenesis. All can be induced by redox imbalance affected by ROS[9].

Cigarette smoke is a mixture of many chemical compounds, including various free radicals and toxic compounds[10]. That is why, smoking is a serious risk factor for the development of head and neck mucosal squamous cell carcinoma (SCC)[11]. Smoking changes endothelial function, inflammation, the redox state and global DNA methylation[12]. Smoking causes oxidative/antioxidant imbalance and causes oxidative stress. All of these are accompanied by lipid peroxidation, oxidative DNA damage, damage to macro- and micro-molecule cells, and antioxidant defensive disorders that can initiate a malignant process. Due to tobacco consumption, it influences the pH of body fluids as well as the formation and stabilisation of free radicals[13].

Different alcoholic beverages are differentially associated with HNC risk[14]. For a better understanding, alcohol-induced carcinogenesis is needed to adequately investigate the alcohol consumption which is a major risk factor. According to this, ethanol may additionally influence cancer risk, within this, HNC patients are directly affected by alcohol consumption. The risk is even greater in those people who smoke[15]. Even small amounts of alcohol can increase cancer risk, especially the risk of cancers of the mouth, pharynx, larynx, oesophagus, breast, bowel, and liver[16].

Today's concern is food safety for everybody since informal food production and marketing system is still a problem in many countries[17]. Diets in many countries have numerous increases in consumption of ultra-processed foods[18]. Definitely it is more crucial to examine the relative effect of the various dimensions of processing such as nutritional composition, food additives, and contact materials related with HNC risk[19,20].

Oral hygiene is one of the many factors, which together with tobacco and alcohol have related effects on oral cancer such as having the result of HNC[21]. Nutrition is critical to the oral health of the individual.

All cancers have an appointment in the DNA sequence of the genomes of cancer cells. This indicates that chaperones and protein homeostasis play an important role in cancer formation[22], and thus, appreciating the role of enzymes may lead to having more opportunities to understand their effects for therapeutic intervention.

For the purposes of this study, HNC cases were evaluated by enzyme activity relations to have a better understanding of CO_2 attraction by CAs and oxidative stress which is stimulated by CAT, PON1, and XO levels related with HNC. According to their importance a consequence summary of their roles are determined as follows.

When we use oxygen our bodies constantly produce free radicals. Free radicals are chemically unstable molecules or atoms. With this ability, they also make other molecules or atoms in the body very unstable resulting in damage of proteins, cell membranes, and even DNA structure. High levels of H_2O_2 may be a risk factor for damaged cells and various diseases[23]. Catalase transforms harmful superoxide radicals into hydrogen peroxide as an antioxidant enzyme, catalyses the reaction of hydrogen peroxide decomposition to water and oxygen.

PON 1, an ester hydrolase bound to calcium, is a member of free radical-scavenging system in metabolism. PON1 has high antioxidant capacity against lipid peroxidation resulting from free radicals. PON1 plays a role in the metabolism of oxidised, biologically active lipids and responsible for HDL-mediated reduction of LDL oxidation[8].

XO, an enzyme containing iron and molybdenum, catalyses the xanthine oxidation of hypoxanthine and the uric acid oxidation of xanthine[24]. XO is usually found in the liver, lung, kidney, heart, brain, and plasma[25,26] and causes the formation of reactive oxygen species by the production of superoxide anion radicals[27].

Different isoforms of CAs are known in human which belong to α-class[28] and CA-I and CA-II are two major cytosolic isoenzymes. CAs catalyse the convertible hydration of carbon dioxide to carbonic acid and place an active role for physiological and biological processes in human such as cell-cell adhesion and cell proliferation[29,30]. CA-II is associated with cancer and it modulates the regulation of pH balance between cancer cells and tumour microenvironment[31].

According to their cancer relations, CAs, XO, PON1, and CAT levels were evaluated in this current study.

Materials and methods

Eighty nine HNC cases (77 male, 12 female) in the mean age of 59.3 (±9.2) and 115 healthy volunteers (96 male, 21 female) in the mean age of 57.6 (±11.3) were included in the study in the Department of Otolaryngology, Diskapi Yildirim Beyazit Research and Training Hospital, Ankara, Turkey. The patients had the following diagnoses: larynx ($n = 74$) and oral cavity ($n = 15$) cancer (Table 1). This study was also approved by Diskapi Yildirim Beyazit Research and Training Hospital Ethics Committee (30 May 2016, 30/37). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

All chemicals were of analytical grade and obtained from Sigma-Aldrich (Taufkirchen, Germany) and Merck (Darmstadt, Germany).

Table 1. Demographic findings of the HNC patients.

	Patient ($n = 89$)	Control ($n = 115$)	Total ($n = 204$)
Age [mean ± sd]	59.3 ± 9.2	57.6 ± 11.3	58.3 ± 10.4
Gender [n (%)]			
Male	77 (86.5)	94 (81.7)	171 (83.8)
Female	12 (13.5)	21 (18.3)	33 (16.2)
Stage [n (%)]			
T1	15 (16.9)	–	–
T2	24 (27.0)	–	–
T3	31 (34.8)	–	–
T4	19 (21.3)	–	–
Cancer type [n (%)]			
Larynx	74 (83.1)	–	–
Oral cavity	15 (16.9)	–	–

Blood samples

Blood samples were taken from the Department of Otolaryngology, Diskapi Yildirim Beyazit Research and Training Hospital, Ankara, Turkey. Venous peripheral blood samples (5 ml) were collected and placed on ice.

Sample preparation

The collected blood samples were centrifuged at 2208 g for 20 min. and after the centrifugation, the serum and the buffy coat were collected. Serum samples were used for determining the activities of XO and PON1. The packed red cells were washed twice with NaCl (0.9%), the erythrocyte lysates were prepared by putting the cells into three freeze-thaw cycles in dry ice with the addition of five volumes of ice-cold distilled water. The hemolysates were used to determine the activity of CAT[23].

Determination of enzyme levels

Cytosolic carbonic anhydrases (CA-I and CA-II) Activity
Carbonic anhydrase activity was measured by the hydration of CO_2 according to the method of Wilbur and Anderson[32] which is based on the determination of the time required for the pH of solution decreasing from 10.0 to 7.4 owing to the hydration of CO_2. Assays were performed at least twice on each lysate[23].

Catalase activity
CAT activity in erythrocytes was determined by using spectrophotometric UV assay method on a Biotek (Winooski, VT, USA). This method is based on monitoring CAT-catalyzed decomposition of H_2O_2 for 5 min. by measuring a decrease in absorbance at 240 nm, at 25 °C. The results were expressed as units per litre (U/L)[33].

Paraoxonase activity
PON1 activity towards paraoxon as a substrate was quantified spectrophotometrically by the method described by Ganet al[34]. The reaction was followed for 2 min. at 37 °C by monitoring the appearance of p-nitrophenol at 412 nm on a Biotek (Winooski, VT, USA). The final substrate concentration during enzyme assay was 2 mM, and all rates were determined in duplicate and corrected for the non-enzymatic hydrolysis. A molar extinction coefficient (ϵ) of 17,100 $M^{-1} cm^{-1}$ for p-nitrophenol at pH 8.0 in 100 mMTris–base buffer was used for the calculation. One unit of PON1 activity is defined as 1 μmol of p-nitrophenol formed per minute under the above assay conditions[35]. Enzyme units were calculated from the absorbance change[36].
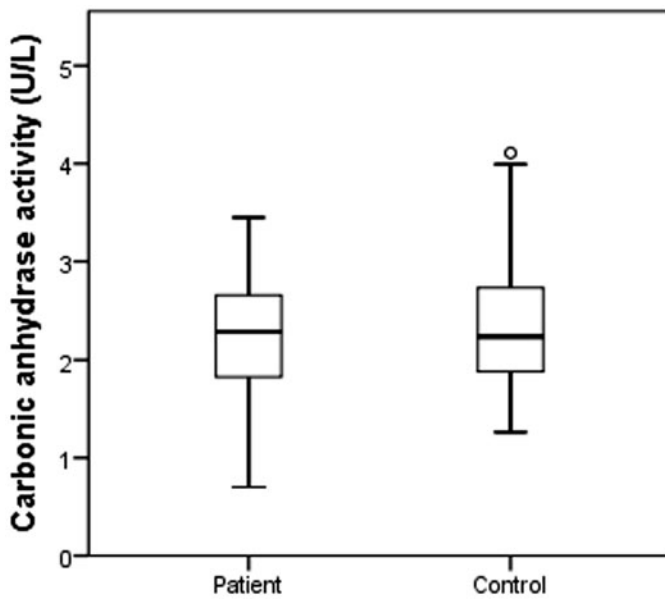
Figure 1. Carbonic anhydrase (CA I + CA II) enzyme activity levels on HNC cases and control group.

Xanthine oxidase activity

XO activity was analysed essentially according to the method described by Roussos[37]. The change in absorbance was recorded at 290 nm at 15 s interval for 1 min on a Biotek (Winooski, VT, USA). Suitable control was run simultaneously. Roussos[37] defined 1 U of activity as change in absorbance at 290 nm in 1 min by 1 ml enzyme preparation[33].

Statistical analysis

The distribution of enzyme levels were examined visually with Shapiro–Wilk test. The enzyme levels were presented as median (min-max: minimum-maximum). Enzyme levels of patient and control groups were compared with Mann–Whitney U test. The relationship between disease stages and enzyme levels in the patient group was investigated by Polyserial correlation coefficient. Statistical significance level was accepted as $p < .05$. All reactions were performed in triplicate. Statistical analysis was carried out with the use of IBM SPSS Statistics 21.0 IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. Statistical significance was set at $p \leq .05$.

Results

All above concerning lead us to evaluate their enzyme activity levels of cytosolic CAs, CAT, PON1, and XO on HNC patients. According to the results, there was no significant difference among CA, CAT, and XO when the enzyme levels of patients and controls were compared ($p > .05$) (Figures 1–3). PON 1 levels were significantly higher in controls ($p = .010$, Table 2) and there is a respectable difference for PON 1 levels (Figure 4).

HNCs are composed of heterogeneous groups of tumours and it is one of the most common causes of cancer related deaths[38]. Free radicals have a crucial role in the pathogenesis of the carcinogenesis[39]. ROS is produced as a cell-mediated response in normal cellular process; however, when ROS is produced in excessive amounts, it damages cellular components such as DNA, lipids, proteins and causes harmful effects on the body[9]. ROS plays a dual role in the tumours; have the ability to support tumorigenesis and
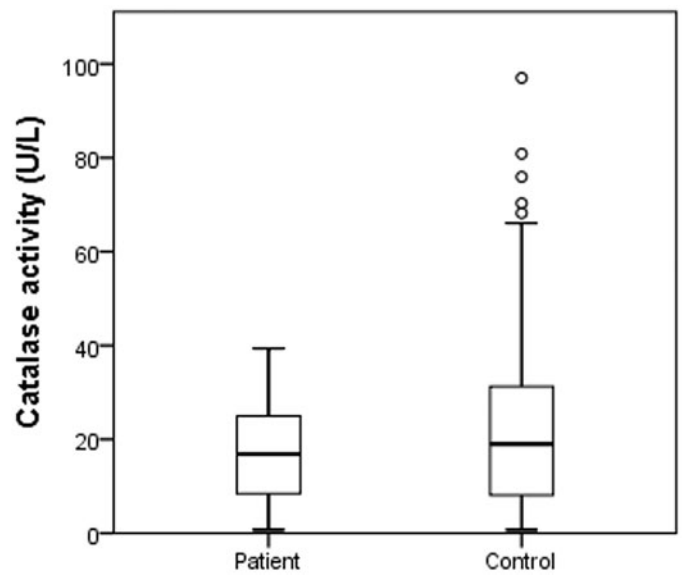

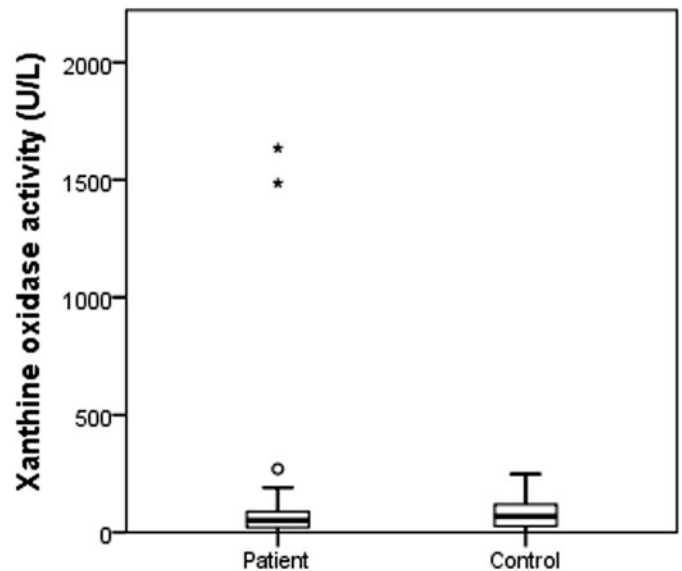**Figure 2.** Catalase enzyme (CAT) activity levels on HNC cases and control group.

Figure 3. Xanthine oxidase (XO) enzyme activity levels on HNC cases and control group.

Table 2. Comparison of enzyme levels.

	Patient Median (min-max)	Control Median (min-max)	p
CA1	2.29 (0.70 – 3.45)	2.24 (1.26 – 4.11)	.969
CAT2	16.87 (0.70 – 39.37)	18.98 (0.70 – 97.03)	.194
PON3	65.70 (0.61 – 271.40)	103.16 (4.30 – 459.30)	**.010**
XO4	50.11 (0.00 – 1634.32)	67.90 (0.00 – 248.95)	.080

[1]$n = 76$ for patients; $n = 115$ for controls.
[2]$n = 64$ for patients; $n = 115$ for controls.
[3]$n = 88$ for patients; $n = 115$ for controls.
[4]$n = 88$ for patients; $n = 115$ for controls.

vascularisation. On the other hand, they may induce DNA damage, arrest cell cycle and induce apoptosis[40]. Reactive oxygen species and other free radicals contribute to the development of cancer through chronic oxidative stress and associated chronic inflammation. ROS takes place in three major stages in the classical carcinogenesis model: initiation, promotion, and progression[41]. With the knowledge of the roles of ROS species and when we have look on
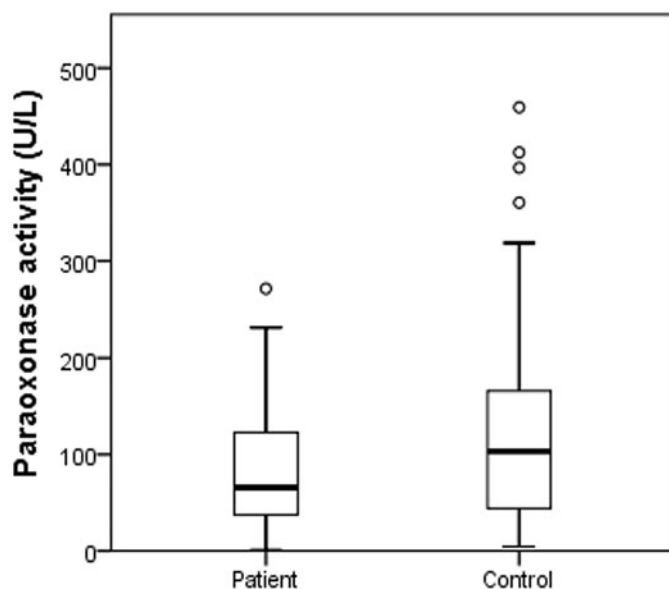
Figure 4. Paraoxonase (PON1) enzyme activity levels on HNC cases and control group.

the activity levels of CAT, PON1, and XO levels on HNC patients, the results have demonstrated a decreased PON 1 activity levels in patients. There were no statistically differences among CAT and XO enzymes according to the results.

CAs are important mediators of tumour cell pH by modulating the bicarbonate and proton concentrations for cell survival and proliferation[42].The pH of the tumour microenvironment drives to have a better understanding the relation between CO_2 and tumour microenvironment with CAs[43]. In the literature, CO_2 insufflations influences the growth of cultured human tumour cells[44].

Due to CAs broad roles in metabolism we have served as their ability to understand how they are effectively active on HNC patients. Thus CA activity levels on HNC patients weren't adequately present as statistically.

Discussion

The main role of CAs in cancer may be to act as pH regulating mediators[45]. According to the Frost's group, CAs are the driving force in pH regulation in primary tumour cells[46] and they also confirmed that the CAs have a tumour cell validity in the differential pH environment.

There are limited studies in the literature regarding to cytosolic carbonic anhydrases related with HNCs. Demir et al. found that there is no relation between of oesophageal cancer patients and control group for CA activity levels[9]. From this point; it is clearly shown that high CA activity levels are related with CO_2 levels due to smoking. Despite mounting preclinical evidence to support the efficacy of CO_2 only a few clinical trials have been conducted on carcinogenesis. Supuran et al. have so many studies on tumour microenvironment related with CAs[47]. In our study, 89 HNC patients were smokers and all healthy volunteers were non-smokers, due to the results we demonstrated high cytosolic CA activity in HNC patients but it was not statistically significant. Further studies are needed to evaluate the other type of CA isoenzymes to indicate the correlation on HNC patients.

Oxidative stress is related to all aspects of cancer[48]. Cellular targets as DNA, phospholipids, proteins, and carbohydrates are affected by oxidative stress on the cell membrane. Insufficient antioxidant activity in the body is also considered a risk factor for

developing cancer. That is why we aim to evaluate the CAT, PON1, and XO activity levels on HNC patients.

PON1 is sensitive to oxidative damage. Many studies have reported the correlation between PON1 enzyme and several cancer types such as bladder[49], prostate[50], lung[51], gastric[52], and ovarian[53].

Metin et al. reported that the levels of PON1 activity were lower in oral squamous cell carcinoma than that of controls. Also, this group defended that there was no relation between serum PON1 activity level and tumour stage[54]. Another group showed a decreased serum PON1 enzyme activity in oesophageal squamous cell carcinoma patient[8]. Malik et.al also reported the low PON1 activities in oral squamous cell carcinoma patients. This group has suggested that PON1 levels may be a marker for oxidative stress in cancer[9]. In our study, we evaluated that PON1 activity levels decreased in HNC patients via control group ($p = .010$) (Figure 4). Therefore, according to our results PON1 may modulate an effective reaction to have a better understanding proper in carcinogenesis.

Cancer-associated XO induces oxidative stress, and XOR-derived ROS can activate genes associated with cancer[55]. Kalcioglu et al. found an increased XO enzyme activity in HNC patients[56].The hydrogen peroxide produced by superoxide dismutase (SOD) reacts with CAT to convert it into water and molecular oxygen. Gargouri et al. demonstrated the decreased CAT activity in erythrocytes of nasopharyngeal carcinoma patients[57]. Moreover, Demir et al showed that the activity levels of CAT were lower in the patients of oesophageal cancer[29] and Mrowicka et al. found a reduced CAT enzyme activity in HNC patients with a none significant difference[58]. In our study, XO and CAT enzyme levels were lower than control group enzyme levels and we found no significant value of these enzymes.

As a consequent of this part, the enzymes CAT, PON1, XO, and CA (I and II) have definitely different activities which also belong to the different groups. Thus, in the literature, all of these enzymes are related to ROS species. It is also well documented that ROS can activate cancer cells[59]. High levels of ROS suppress tumour growth and the interaction between ROS and enzyme levels through the inhibition of cell proliferation and induction of apoptosis[60] which is an important pathogenic factor for carcinogenesis. Within this, we aim to investigate the CAT, PON1, XO, and CA (I and II) enzyme levels according to their relations with cancer whether they have a common point or not.

Though there was no statistically significant difference among CAT, CA, and XO enzymes, there may be a relationship among the elevated level of oxidative stress on the lack of antioxidant protection and cancer development in HNC patients. Thus, further studies on mechanism and pathways of antioxidant defensive system will provide new insights into our future projects with the relation of CAs, XO, and CAT. Besides, our study has revealed significantly lower PON1 activity levels in HNC patients compared to control group. Decreased PON1 levels result may be a leading tool on oxidative stress pathways and it may be an effective agent in cancer-related complications. However, PON1's activity alone is not an adequate parameter for understanding the cancer development process but we highly suggest that our data also support the major role of oxidative stress in cancer.

Disclosure statement

There is no conflict of interest of this study.

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