Imbalance between Oxidative stress and Antioxidants in human Skin Carcinoma

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ABSTRACT

Background: Skin cancer belongs to the most frequent types of malignancies, that actually constitutes an important public health concern. Oxidative stress is an essential element and probably a driving factor for many chronic diseases, encompassing diabetes, Alzheimer and cancer. Malondialdehyde (MDA), Catalase (CAT) and superoxide dismutase (SOD) considered the most widely employed biomarkers for oxidative stress. **Aim of the study:** To assess the oxidative stress imbalance between tumor and normal tissues in Egyptian patients, suffering from skin cancer. **Patients and method :** The present study is regarded as a pilot case-control study constituting twenty skin tumor tissues and their adjacent normal tissues extracted from patients with human basal cell (BCC) and squamous cell (SCC) carcinomas, so as to evaluate the difference in the levels of MDA, CAT and SOD biomarkers between normal and cancer tissues, using a spectrophotometry method. The *P-value* is considered significant if its value is less than 0.05. **Results :** Tumor tissues had mean MDA levels of 1256±391.6 nmol/ml, which are higher than normal tissues' mean values 828.5± 117.8 nmol/ml. However, the mean SOD level of tumor tissue was decreased (100.6 ± 16.66 nmol/ml) compared to normal tissues (174.2± 14.69 U/L). With a *P*-value <0.05, the mean CAT level was lower in tumor tissue (101.0 ± 21.92U/L) than in normal tissues (182.9± 17.52ng/ml). **Conclusion:** MDA, SOD and CAT levels could be utilized as significant predictors or screening tests for detecting the population more likely to develop certain forms of skin cancer.

Key Words:

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1. INTRODUCTION

Skin malignancies have become a major public health issue due to their rising prevalence, difficult therapy, and the necessity to regularly observe patients in order to identify relapses or new cases [1]. Non-melanoma skin cancers (NMSCs) and melanoma are two main categories for skin cancer. NMSCs further subdivided into squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). So far, up to 90% of skin cancer patients die from malignant melanoma. Excessive sun exposure and an active outdoor

lifestyle that have emerged in recent years are associated with an increased prevalence of melanoma [2] . Cancer is a genetic disorder caused by incrementally accumulation of genetic mutations. The DNA of skin cells experiences endogenous damage and replication errors, making human tumors genetically unstable [3]. Collectively, these lesions may cause a spectrum of genomic changes, involving somatic single base substitutions, rearrangements, deletions or insertions, and copy number changes, thereby resulting in diseases, such like cancer [4]. Overproduction of reactive oxygen species (ROS) in the cell, contributes to enhancing cellular damage. And hence, antioxidant defense systems are present in cells to counteract elevated ROS and preserve the redox equilibrium, both of which are essential for cell survival [5]. In general, when the balance between antioxidants and oxidants is disturbed, the resultant Oxidative stress (OS) can trigger several pathologies in approximately every human organ [6]. OS is the major contributor of cell structural damage, including membranes' components, lipids, proteins and particularly DNA. With the production of secondary reactive species, such damage can impair cell activities and endanger the health of cells by provoking a range of cellular processes. It is a crucial process connected to serious chronic diseases, such as cancer, hepatic, cardiovascular, and neurological complications [7]. Certain enzymes, namely primary enzymes can either directly prevent the production of free radicals, or indirectly supporting other endogenous antioxidants (AOs) enzymes. An example, for such effective AO enzymes are superoxide dismutase (SOD), glutathione peroxidase (Gpx) and catalase (CAT) [8]. Oxidative stress can, biologically, increase the propensity to inflammatory diseases and disorders as well as the risk of cancer development [9]. ROS are more abundant in cancer cells than in normal cells, and they in turn exerts a vital role in regulating the tumour initiation, development, and progression [10]. There for, in this study, we aimed to evaluate the role of MDA and antioxidants in different types of skin cancer.

2. PATIENTS

The present study is regarded as a pilot case-control study that was conducted on 20 patients, evaluating the tumors and their adjacent normal tissues. Patients' ages ranged from 40 – 80 years (mean: 68 ± 10); 11 out of them were SCC and 9 were of BCC (table 1), composing 11 males and 9 females They were randomly selected from the Oncology center at Mansoura University in Egypt. After obtaining consent form, samples were collected from all patients before starting the project. the study protocol was approved by the Ethics and Scientific Committees of Mansoura University in Egypt.

Table 1: Patient s' ccharacteristics (number, age & sex):

Characteristic	Statistics
Number	20
Age (mean ± SD, range, years)	68 ± 10 (40- 80)
Sex (male/female)	11/9

3. MATERIALS AND METHODS

3.1 Sample collection and analysis:

Tissue specimens (normal and tumor) were collected freshly after surgery between January 2020 and August 2021 and stored at -20 °C in 15 ml sterile tube till use, They were randomly selected from the Oncology center at Mansoura University at Egypt. The tissue specimens had to be converted from the solid phase into the liquid phase in order to be analyzed using the spectrophotometric technique. After resection, frozen samples of the tumor and normal tissues were thoroughly cleaned in cold normal saline

0.9% to remove all any remnants of blood. Afterward, samples in a 10 times volume of ice-cold, sterile distilled water, precisely weighed and homogeneously ground by tissue homogenizer. The lysate was then centrifuged in a cooling centrifuge (at 4 °C) for 5 minutes at 4,000 rpm. Finally the supernatants were collected and stored at at -80°C until used. The obtained liquid samples were then subjected to spectrophotometric analysis. Notably, a quick process for collecting and analyzing the specimens is preferable, since environmental conditions can easily influence the peroxidative activity. In addition to being quantitative and simple to use, the spectrophotometer is a time-saving approach for such purpose. Although melanoma is the most lethal skin cancers, this type of skin carcinoma was not included in the current research, since the tumor biopsy was not large enough and because melanomas often have far more severe features than other skin tumors ,Additionally the period of sample collection coincided with the COVID-19 epidemic, which has become the center of everyday clinical practice, impeded access to healthcare facilities, and delayed diagnosis of patients with cutaneous melanoma (CM).

3.1.1 Determination of malondialdehyde:

The content of MDA (nmol/mL) in the supernatants of tissue homogenate was estimated according to colorimetric method [11] by using Bio-diagnostic kit (Bio-diagnostic, Giza, Egypt).Before the experiment began, the chemicals and tissue supernatant were permitted a half-hour to come to room temperature. Each sample (0.2 ml) and standard (0.2 ml) received 1.0 ml chromogen, which was well mixed before being heated in a boiling water bath at 95°C for 30 minutes. Then, the mixture was left to cool, and the absorbance was measured at 534 nm versus blank utilizing a UV-200-Rs spectrophotometer. Simultaneously, the standard was also analysed at the same procedure.

3.1.2 Determination of superoxide dismutase (SOD) activity:

SOD was estimated in the homogenates of BCC and SCC tissues according to the colorimetric method [12]. Reagents for the kit (Bio-diagnostic, Giza, Egypt) include R1: 50 mM/L Phosphate buffer pH (8.5), R2: 1 mM/L Nitroblue tetrazolium (NBT), R3: 1 mM/L NADH, R4: 0.1 mM/L Phenazine methosulphate (PMS), and R5: Extraction reagent. In a test tube, 1.0 ml of supernatant was mixed with 0.5 ml of ice-cold extraction solution. After that, the tubes were then stirred by a vortex for nearly 30 seconds and centrifuged at 4000 rpm and 4°C for additionally 10 minutes. We prepared working solution for the reagents before starting as following: 10 µL of R4 was immediately diluted by 10 ml deionized water. Following that, the reaction mixture tube was made as follows: To create the control and sample, 1 ml of the working reagent was added. Moreover, 0.1 ml of the sample and 0.1 ml of distilled water were added to each cuvette. Thereafter, the reaction was started by adding 0.1 ml of R4 to both of them after they had been well mixed. At 25°C, record the increase in absorbance at 560 nm over the course of 5 minutes for the control ($\Delta A_{control}$) and the sample (ΔA_{sample}).

3.1.3 Determination of catalase (CAT) activity:

According to a colorimetric analysis, using Bio-diagnostic kit method (Bio-diagnostic, Giza, Egypt) CAT activity in tissue homogenate was assessed [13], through which CAT reacting with a measured quantity of H_2O_2. Using CAT inhibitor, the reaction is terminated after exactly one minute. Reagents components as follows: R1: 100 mM / L Phosphate buffer at pH 7.0, R2: 500 mM / L of H2O2 substrate or standard and diluted in 1000 times before using. R3 (Chromogen): Peroxidase over 200/L Enzyme in addition to 2mMol /L 4–Aminoantipyrine as preservative. R2 (H_2O_2): was used after being diluted 1000 times (10 µl + 10 ml distilled Water). Throw away after usage, whatever required dilution of the sample. Sample Blanks, samples, Standard Blanks and Standard were prepared as follows: a) 0.05 ml of sample was added to 0.05 dist. H_2O and 0.5 ml R1 to make sample blank. b) 0.05 ml of sample was added to 0.50 ml of R1 and 0.1 ml of R2 to make sample. c) 0.10 ml of dist. H_2O was added to 0.50 ml of R1 to make standard blank. d) 0.05 ml of dist. H_2O was added to 0.5 ml of R1 and 0.1 ml of R2 to make standard. 5-Incubate all exactly one min at 25 °C. e) Add 0.2 ml 0f R3 to all above (1-4). f) Add 0.5 ml of R4 to all

above (1-4). G) Read the sample (A_{sample}) against the sample blank after 10 minutes of incubation at 37 ^{o}C, and the standard (A $_{standard}$) versus the standard blank at 500-520 nm.

3.2 Statistical analysis:

Using the tool GraphPad Prism ver. 5.0 (California, USA), all statistical analyses were carried out. Results, regarding sample size (n=6), are expressed as mean ± standard error of the mean. One-way analysis of variance (Anova), followed by the Neuman-Keule post-hoc test, was used to generate statistical comparisons. [14].   When the P-value was less than 0.05, the difference was declared significant, and also any higher significance threshold also reported.

4.   RESULTS

4.1 Levels of MDA As displayed in table 2 and figure 1**,** for each patient, tumor tissue had a significantly higher MDA concentration (1256±391.6 nmol/ml) compared to normal tissues (828.5± 117.8 nmol/ml, P< 0.05).

4.2 Levels of antioxidants (SOD and CAT) in table 2 and figure 1, results were statistically significant (P< 0.05). Incline in the activity of SOD and CAT in tumor tissues (100.6±16.6 U/l) and (101.0±21.9 U/l) compared to normal tissues (174.2±14.6 U/l) and (182.9±17.5 U/l) respectively.

4.3. Histopathology examination of basal and squamous cell carcinoma tissues compared to normal skin tissue:

Normal and tumor tissues biopsies were removed from patients and examined for histopathological changes by light microscopy (figure 2). Normal skin section showing normal mucosa and sub mucosa layers (figure 2 A). Skin section from **BCC** stained with hematoxylin-eosin stain, showing focal skin ulceration with infiltration by tumor tissue. The tumor is formed of sheets of atypical basaloid cells with focal sheets showing central squamous differentiation & keratinization. The atypical cells showed moderate atypia & pleomorphism, the surrounding stroma is fibrotic. The tumor is seen focally infiltrating the depth (Figure 2 B).   Whereas, **SCC** display ulcerated mucosa with related invasive carcinoma formed of solid groups of moderately keratinized squamous cells exhibiting moderate anaplasia and mitosis (Figure 2 C).

5. DISCUSSION

The skin serves as a barrier between the human body and the external milieu. Inducing ROS generation in the skin predominantly occurs as a result of environmental elements. At the skin's surface, keratinocytes generate the majority of ROS, however virtually all skin cell types are capable of producing ROS in response to different signals from aggressors including pollution, UV radiation, medicines, or biomolecules like cytokines and growth hormones. [15]. During oxidative stress events, ROS interact with lipids found in the stratum corneum layer, leading to the generation of lipoproxides that impair the redox state of the cell, and thus stimulate the fragmentation and alterations in the collagen fibers structure [16]**.** Actually, there is a pretty effective antioxidant system in the skin layers, which includes antioxidant enzymes; CAT, SOD and GPX and antioxidant substances (glutathione, vitamins and uric acid, etc.) that resist the destructive impact of the oxidative stress. Accordingly, countless pathologic disorders may arise when the equilibrium between oxidants and antioxidants is upset [15]. Oxidative stress in humans is also linked to biological diversity and numerous characteristics such as age, gender, ethnicity, and BMI. [17]. Nevertheless, due to a lack of sufficient sample number, these potential correlations were not involved in our current work. The incidence of NMSC varies widely from across countries based on the environmental carcinogens and population sensitivity. A little more than 4% of all malignancies in Egypt were caused by skin cancer. More precisely, the National Population-Based Cancer Registry Program

(2008–2011) reported that the incidence of NMSC represented 1.35% of all cancers in men and 1.24% of all cancers in women [18]. Ultraviolet radiation (UVR) regarded as a key carcinogen in the development of skin cancer. The consequence of UV exposure on different forms of skin cancer is multifaceted and differs [19]. Around 60% of non-melanoma skin cancer (NMSC) cases in Egyptians might be traced to sun exposure, and over 45% to skin color [20]. , The majority of NMSC identified in the current study were discovered on sun-exposed skin, mostly on the head and neck (65%), and largely in farmers and employees in welding industries. In fact, the risk of skin cancer rises with age [21] and has an effect on the body's capacity for rebalancing between oxidant and antioxidant mechanisms inside the organism [22]. The average age of the participants in the research was 68 ± 10 years, and about 70.0% of the NMSC patients were above 50. The majority of cancers showed increases in oxidative stress, particularly lipid peroxidation (LPO) products, which were altered according to the diversity of tumour biological characteristics, including aldehyde metabolizing enzymes, the levels of lipid peroxidable substrates, such as the existence of inflammatory cells, which can raise the amount of diffusible aldehydes from the tissues around the tumor, and unsaturated fatty acids (UFAs) in cell membranes [17]. Moreover, in clinical samples from cancer patients, the ROS level is much greater in tumour masses compared to the precancerous tissues, or in similar specimens extracted from healthy people [23]. In this study the concentration of MDA in tumor tissue was significantly elevated compared with the normal tissue used as controls. Although MDA level did not significantly change among NMSC types, higher quantities of polyunsaturated fatty acids was observed, that in turn suggested MDA to be the robust mutagenic byproduct of LPO [24].Our findings are in line with those of Rahmani et al. [25], particularly in those who are at risk of developing BCC, whereby increased peroxidative activity in cancer skin lesions was observed and that predicts the presence of a potential tumour response against local or systematic antioxidants processes. Our results revealed that mean MDA concentrations were significantly ($p < 0.001$) higher in malignant skin than in unaffected or healthy skin. Despite the disparities in the research (Nuszkiewicz et al. [26], Maurya et al. [27], Tan et al. [28], and Khan and Malik et al. [29]), these studies reported in many experiments that the mean MDA concentrations in cancer tissues were significantly higher (*p<0.05*) than those in precancerous or healthy tissues. MDA might therefore be used as a trustworthy marker to study lipid peroxidation in tissues. In the study done by Fekecs et al [30] study, that constituted of 16 NMSCs patients collected after solid organ transplant, it was observed that patients without NMSC had MDA plasma levels higher than those of healthy control or the non-NMSC group ($P < 0.05$), however, MDA in tissue hemolysate was a little bit increased. Human skin is equipped with a network of antioxidants, including enzymes and low molecular weight compounds, to combat the harmful effects of ROS. SODs, CAT, thioredoxin reductase, glutathione peroxidases (GPxs) and glutathione-S-transferase are the primary enzymes that serve as the body's first line of defense against ROS. The activity of theses enzymes is reliant on certain trace elements suchlike copper and zinc (SOD) or selenium (GPx) [31]. In the present study, results showed a significant decrease in SOD and CAT activities in tumour tissues compared to normal tissues and the findings regarding catalase activity are compatible with those of **Fekecs et al** [30] in patients with NMSC group ($P < 0.05$). Whereas SOD activity was slightly lowered but not significantly. A study performed by **Sander et al** [32] reported that in human melanoma biopsies, a significant over-expression of the antioxidant enzymes, copper–zinc SOD, manganese superoxide dismutase and CAT, was observed when compared with surrounding non-tumor tissue, while, on the other hand, have a significantly disrupted antioxidant balance with decreased antioxidant enzymes in non-melanoma tumors.

6. Conclusions

In patients at-risk of cancer, Antioxidant activity in skin cancer types (SCC and BCC) was evaluated in the present study, which may predict the potential tumors response to local or systemic antioxidants. Additionally, MDA, SOD and CAT levels are significant predictors and screening tests for detection of the population at higher risk for developing certain types of skin cancer.

7. Recommendations

 Further multicenter studies with a bigger sample size and sequential   blood collection will be required to ascertain the cutoff serum concentrations for their ultimate use as biomarkers in clinical practice.
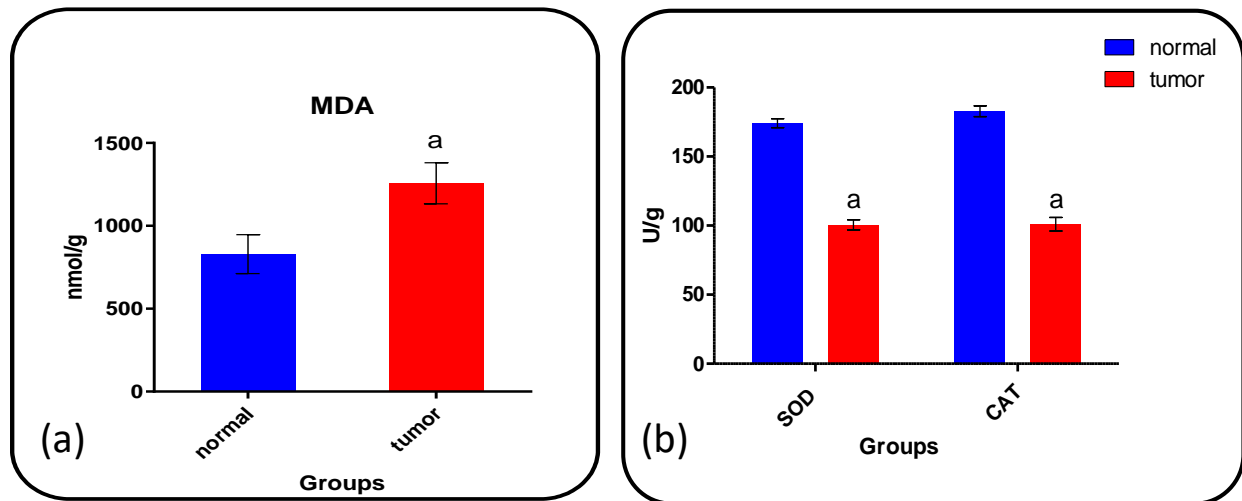
Figure 1: MDA   levels and antioxidants   activity (SOD & CAT) in patients **(a)** Concentration of malondialdehyde (MDA nmol/ml) **b)** activity of antioxidant enzymes (SOD U/l) & activity (CAT U/g) Data are presented as mean± SD.   (p -value <0.05 p < 0.05 vs. control group).
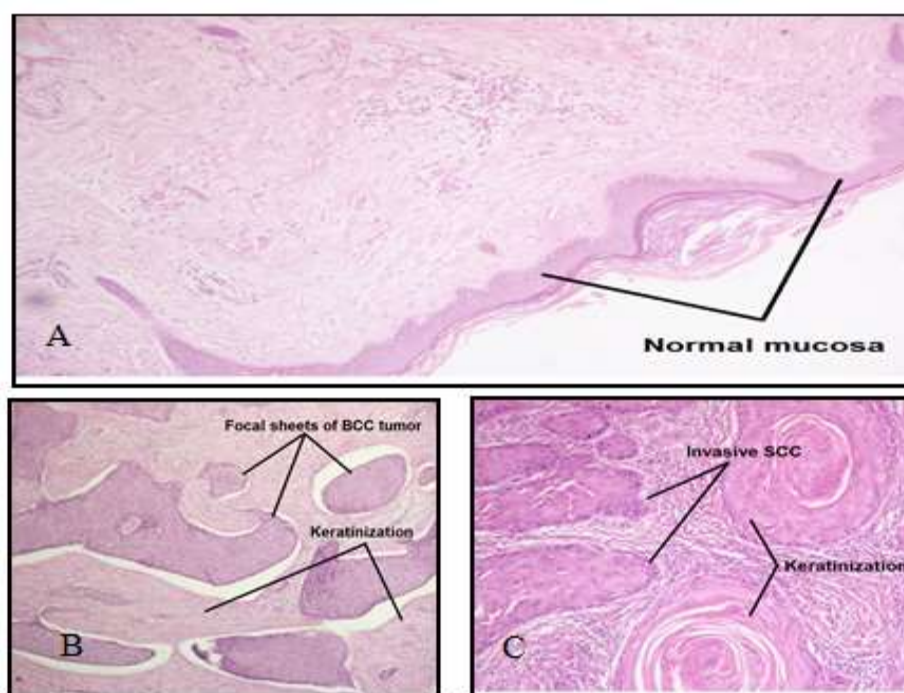
Figure 2: Representative histological sections of human skin **A**: **normal skin** tissue showing normal mucosa and sub mucosa layers. **B: basal cell carcinoma (BCC)** tumor, showing focal skin ulceration with infiltration showing central squamous differentiation & keratinization. **C: squamous cell carcinoma SCC** showing ulcerated mucosa with related invasive carcinoma with moderately keratinized cells (H&E, 100x).

Table 2: Biochemical measurements for oxidative stress in normal and tumor tissues

Variable	**Tumor tissue (n=20)** (Mean ±SD)	**Normal tissue(n=20)** **(Mean ±SD)**
Hemolysate MDA, nmol/mL	1256[a] ± 391.6	829.5 ± 117.8
SOD, IU/mL	100.6[a] ± 16.66	174.2± 14.69
CAT, U/mL	101.0[a] ± 21.92	182.9 ± 17.52

[a] Statistically significant differences in the activity ($P< 0.05$)

MDA: Malinodialdehyde, SOD: Superoxide dismutase and CAT: Catalase

8. Acknowledgement

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