Assessment of Serum Lipid Peroxidation and Redox Thiol System Status in Different Stages of Oral Carcinogenesis

IOANA SCROBOTA1,2,3, CAMELIA ALB1, HORIA CALNICEANU1,2,3, GRIGORE BACIU1, IOANA BERINDAN NEAGOE1, DOINA ONISEI1, AMINA RAMONA POPOVICI1,4, ROXANA BUZATU5, POMPEI BOLFA7,8

1“Tuliu Hatieganu” University of Medicine and Pharmacy, Department of Maxillofacial Surgery and Radiology, 37 Cardinal Iuliu Hossu St., 400029, Cluj Napoca, România
2University of Oradea, Department of Dental Medicine, Faculty of Medicine and Pharmacy, 10, 1 Decembre Sq., 410073, Oradea, România
3Oncology Institute "Prof. Dr. I. Chiricuta", Departments of Radiobiology and Tumour Biology, 34-36 Republicii Str., 400015, Cluj-Napoca, România
4“Tuliu Hatieganu” University of Medicine and Pharmacy, Department of Dental Propaedeutics, Aesthetics, 32 Clinicilor Str., 400006, Cluj-Napoca, România
5“Victor Babes” University of Medicine and Pharmacy, Faculty of Dental Medicine, Department of Periodontology, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania
6“Victor Babes” University of Medicine and Pharmacy, Faculty of Dental Medicine, 2 Eftimie Murgu Sq., 300041 Timisoara, Romania
7Department of Biomedical Sciences, Ross University School of Veterinary Medicine Basseterre, St. Kitts, West Indies
8University of Agricultural Sciences and Veterinary Medicine, Department of Pathology, 3-5 Calea Manastur, 400372, Cluj-Napoca, Romania

In this study we aimed to monitor serum lipid peroxidation and thiol redox status as indicators of oxidative stress (OS) at different moments during oral carcinogenesis. We evaluated serum malondialdehyde (MDA) and reduced glutathione (GSH) at 12 weeks and 24 weeks of 4NQO induced oral carcinogenesis in Wistar rats. MDA was significantly elevated and GSH significantly reduced (p<0.05) after 12 weeks of carcinogen exposure indicating the installation of OS. From week 12 to week 24 only serum GSH was modified significantly (p<0.05) probably used by the carcinogenetic cells to improve their viability. This modification indicates the disruption in the thiol redox status and persistence of OS even in the absence of increased MDA. Biochemical alterations were associated with oral mucosal dysplastic lesions. Our results support the recent “redox theory” of oxidative stress (OS) and emphasizes the necessity for clarifying the networks involved in OS-mediated carcinogenesis. Such evidence could set the basis in developing new targeted therapeutic protocols to improve the prognostic of oral cancer.

Key words: oral carcinogenesis, lipid peroxidation, redox thiol system status

Numerous discoveries were made in oral cancer diagnoses and treatment but the overall survival rates did not ameliorate [1]. A deeper understanding of the mechanisms involved in the etiology and biology of this disease could improve its prognostic [2].

Oral carcinogenesis is a multifactorial process in which a normal cell is transformed into a neoplastic one. It develops through three steps namely initiation, promotion and progression [3]. Endogenous and exogenous factors mediate the molecular and cellular events in oral carcinogenesis. Among them, reactive oxygen species (ROS) interfere with all stages of malignant transformation. Moreover, tobacco, alcohol, and Human Papilloma Virus, significant etiological risk factors in oral carcinogenesis [4], participate to oral carcinogenesis by ROS-mediated mechanisms [5].

ROS are intermediates of oxygen reduction and include free radicals (FR) and nonradical oxidants. FR are small molecules possessing an unpaired electron that confers them high instability and reactivity. They are generated endogenously as a common event during normal metabolism and exogenously by different xenobiotic sources [6]. The steady-state level of FR in organism is maintained by antioxidant systems that equilibrate FR production and elimination. Oxidants increase, antioxidants or antioxidant enzymes decrease, or a combination of the above factors, can alter this balance, and determine FR to accumulate, and OS to install. This OS causes DNA damage, protein modification, and/or lipid peroxidation [6 - 8].

FR-mediated macromolecular damages have been considered the main events in OS. Recently, disruption of thiol redox circuits has emerged as a possible mechanism [9, 10]. Protein thiols are implicated in cell signaling and redox homeostasis and are controlled by two major thiol-containing systems: thioredoxins and glutathione (GSH). Nonradical oxidants continuously oxidize protein thiols and maintain a stability in the thiol pathways but not an equilibrium. Therefore, modification of nonradical oxidants generation could lead to OS by altering thiol redox state and signal transduction pathways. Nonradical oxidants include peroxides, aldehydes, quinones, and epoxides and can result from endogenous and exogenous sources. Although not radicals by definition they are extremely reactive [9, 10].

In oral cancer, direct FR damaging effects to macromolecules play an important role in initiation, while ROS and cellular redox status, seem be responsible for promotion and progression [11].
Many researchers studied the implications of OS in oral cancer or precancer. As a logical followup different targeted chemopreventive antioxidant therapies were elaborated with various results. Little data exist though regarding OS status not at a certain moment but at different moments during oral carcinogenesis [12, 13, 14].

In this study we aimed to evaluate the OS implications in oral carcinogenesis by monitoring serum lipid peroxidation and redox thiol system status at different periods of time during disease development.

**Experimental part**

**Subjects**

Preliminary *in vitro* studies evidenced the role of OS in oral carcinogenesis [15, 16]. We chose an *in vivo* model because we wanted to evaluate the selected biomarkers in the same group at different moments in time.

Several animal models were used to study carcinogenesis *in vivo* [17]. Wistar rats are considered an ideal model because the alterations in their oral mucosa during carcinogenesis showed similarities to human oral mucosa [18].

20 Wistar male albino rats (8 weeks old and weighing a mean±deviation 220±20 g) were obtained from the Animal Department of “Iuliu Hatieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania and transferred to the Physiology Department Biobase. They were housed 5 per cage, at a constant temperature of 21±2°, 70±4% humidity, under 12h dark/ 12h light cycle, receiving standard pellet laboratory food and water *ad libitum*. The rats were acclimated one week prior the start of the experiment.

**Reagents**

To induce oral carcinogenesis we used 4-Nitroquinoline 1-oxide (4NQO) because: it is a powerful complete chemical carcinogen; acts by direct binding to DNA and by generating large amounts of endogenous ROS [19]; and generates genetic, molecular and cytological modifications similar to the ones in humans [19, 20].

4NQO, 2-thiobarbituric acid, o-phthalaldehyde, polyethylene glycol, Bradford reagent, trichloroacetic acid were purchased from Sigma Aldrich Chemicals GmbH (Germany) and absolute ethanol and n-butanol from Chimopar (Bucharest).

4NQO was dissolved 0.5% wt/vol in polyethylene glycol and used to induce oral carcinogenesis.

**Experimental design**

Rats were randomly divided in two groups (n=10) - group 1 (control group) and group 2 (tested group). Group 2 received 25μL of 4NQO three times a week, topically applied on the oral mucosa, and group 1 the same volume of polyethylene glycol (vehicle only). After each topical application the rats were prevented from eating and drinking water for two hours.

Blood biochemical parameters were measured in the tested group at 12 weeks and 24 weeks of 4NQO administration and compared with the control group. Histopathological exam of oral mucosa was performed in both groups.

All the procedures in this study were approved by The Ethical Committee on Animal Welfare of „Iuliu Hatieganu” University of Medicine and Pharmacy in accordance with European Convention for the Protection of Vertebrate-Animals used for Experimental and other Scientific Purposes, Council of Europe No 123, Strasbourg 1985.

**Assays**

After the rats were anesthetized, 1.5 mL blood samples were drawn from the retro orbital vein and centrifuged at 3500rotations/min for 5 min. In the resulted serum total malondialdehyde (MDA) [21] and reduced GSH [22] were assessed. The results were expressed in nmol/ml.

**Histopathological exam**

Oral mucosa was sampled from the rats and processed using the standard haematoxilin and eosin stain (H&E). The epithelial lesions found were classified into: normal, epithelial hyperplasia, mild dysplasia, moderate dysplasia and severe dysplasia, accordingly to the latest WHO classification of oropharyngeal tumors [23]. Tissue examination was done with a standardized Olympus system for image acquisition and analysis, an Olympus BX51 microscope and Olympus Cell B software.

**Statistical analysis**

Results were expressed as mean ± SD. Data normality was established using Kolmogorov-Smirnov test. Kruskall-Wallis test was used for overall group comparison and Mann-Whitney U’ Test for the comparison of two groups. Changes were considered significant for a p value < 0.05. Statistical analysis was done using SPSS 13.0 — Statistical Software Package (SPSS Inc, Chicago, Illinois, USA).

**Results and discussions**

12 weeks of 4NQO administration significantly increased MDA levels (p=0.003) and decreased serum GSH levels (p=0.05). The difference was maintained the same at week 24 (p=0.05). From week 12 to 24 MDA levels decreased but not significantly (fig.1).

![Fig. 1](image-url)
The principal macromolecular targets of FR are polyunsaturated fatty acids in cells membranes. The reaction is called lipid peroxidation and disturbs cells function by altering its metabolism, membrane permeability and transport. Moreover, lipid peroxidation products like aldehydes are extremely reactive acting like second messengers and increasing FR effects [24, 25, 26]. MDA is a final product of FR induced lipid peroxidation and is considered as a biomarker of FR oxidative damage because it is relatively stable compared with the other lipid peroxidation products and can diffuse to, and be assayed, in locations different from where it is produced [12, 27].

Increased serum MDA levels in our study were used to monitor the intensity of lipid peroxidation produced by 4NQO FR generation and accumulation beyond the capacity of the organism to scavenge them. Lipid peroxidation could be the oxidant-antioxidant imbalance apogee, and the main factor in cancer initiation and promotion [28, 29].

Besides lipid peroxidation, a dis-equilibrium in thiol pathways leading to altered cells function is considered an important mechanism of OS [30].

GSH, the most abundant thiol synthesized de novo in mammalian cells, is one of the factors that control the redox states of thiol systems [31]. It has an important role in antioxidant defense and drug detoxification too [10].

Serum GSH significantly decreased after 12 weeks of 4NQO administration (p=0.05) compared with controls, and decreased gradually (p=0.000) up to week 24. Within group 2 the difference was significant (p=0.024) between GSH levels at 12 and 24 weeks (fig. 1). This could be due to GSH participation in 4NQO detoxification, coenzyme in ROS scavenging and effort in maintaining redox homeostasis [20]. GSH depletion leading to a disruption of the redox circuit function might be one of the mechanisms implicated in the progression of oral cancer [9, 10, 32, 33]. Fig. 1. Serum MDA and GSH levels during 4NQO induced oral carcinogenesis in rats. GSH participation in 4NQO detoxification, coenzyme in ROS scavenging and effort in maintaining redox homeostasis [20]. GSH depletion leading to a disruption of the redox circuit function might be one of the mechanisms implicated in the progression of oral cancer [9, 10, 32, 33].

Conclusions

We can conclude that serum lipid peroxidation is increased and the thiol redox systems are compromised during 24 weeks of 4NQO induced oral carcinogenesis in Wistar rats. The installed OS is manifested by oral mucosa dysplasia ranging from epithelial dysplasia to moderate dysplasia. The rats in the control group presented no lesions (fig. 2).

For the classification of the lesions we applied the guidelines of WHO-2005, the head and neck epithelial dysplasias classification which is one of the most frequently used systems along with SIN (Squamous Intraepithelial Neoplasia) and SIL (Lubijiana Classification Squamous Intraepithelial Lesions). These systems have similar criterion (especially WHO and SIL) but they do not superpose or exhibit a direct correspondence [37]. Future discoveries, most likely in the molecular biology field, will enable the elaboration of a universal classification system of the upper aero-digestive intraepithelial lesions [38].

Although it was found that only a percent of oral dysplasias turn into cancer [39], these lesions have a temporal progression, corresponding to the sequences of carcinogenesis. Eventually mild dysplasia will progress to moderate, severe dysplasia and then cancer [39].
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