Oxidative Stress Markers in Chemically Induced Oral Premalignant Lesions

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In our study we induced oral premalignant lesions using 4 nitro-quinoline-1-oxide (4NQO) topically applied on oral mucosa for 24 weeks. We detected an oxidative stress marker malondialdehyde (MDA) significantly increased ($p<0.05$) and an antioxidant/detoxifying activity marker, glutathione (GSH), significantly decreased ($p<0.05$) in the oral induced epithelial dysplasias. We appreciated that issue MDA and GSH could serve as diagnostic, screening and evaluation of response to therapy tools in oral premalignacy.

Keywords: oral premalignant lesions, oxidative stress, malondialdehyde, glutathione

Oral cancer is the result of various DNA changes that can lead to the progress of a normal cell into a potentially malignant or a premalignant cell [1]. DNA mutations occur spontaneously, especially via oxidative stress (OS) [2]. OS is defined as *an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage* [3]. Oxidants (ROS), when at low concentrations have physiological [4] and they are scavenged by complex antioxidant systems. When ROS are produced in excess or the capacity of organism to fight them is overwhelm, they accumulate and produce pathological insults to biomolecules like lipids, proteins and nucleic acids [4]. These insults result in various pathological conditions including oral cancer [2, 5-7]. Dysplasia precedes oral cancer therefore early diagnostic, adequate treatment and identification of the lesions at high-risk of malignization is the main goal in oral cancer management [8].

Wistar rats were used in the carcinogenesis experiments because of the similarities between the morphology of their oral mucosa and human oral mucosa. 4 nitro-quinoline-1-oxide (4NQO), is a complete carcinogen, induces sequentially the phases of carcinogenesis and produces morphological and biochemical alteration that mimic those in humans during cancer development [9]. 4NQO is a quinoline that possesses a heterocyclic aromatic structure and a C9H7N chemical formula. Although it may naturally occur in the environment it is typically manufactured for research purposes [10].

In this study we aimed to induce oral premalignant lesions using 4NQO in Wistar rats and to evaluate two oxidative stress biomarkers, malondiadehyde (MDA) and reduced glutathione (GSH) in the induced oral dysplastic lesions.

Experimental part

Reagents

4NQO, 2-thiobarbituric acid, o-phthalaldehyde, polyethylene glycol, Bradford reagent, trichloroacetic acid were purchased from Sigma Aldrich Chemicals GmbH (Germany) and absolute ethanol (ST113) and n-butanol (ST266) from Chimopar (Bucharest). All reagents were of high grade purity. 4NQO was prepared 0.5% wt/vol in polyethylene glycol and used in 25μL doses.

Animals and experimental design

In the Physiology Department Biobase, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania 20 Wistar male albino rats (220±20 g, 8 weeks old) were housed 5 per cage, at a constant temperature of 21±2°, 70±4% humidity, 12h dark/ 12h light cycle and fed standard pellet laboratory food and water ad libitum. They were acclimated one week and then divided in two groups (n=10): the tested group and the control group (fig. 8). At the end of the experiment, the rats were anaesthesised with a ketamine xylazine cocktail injected i.p. Oral mucosa was macroscopically inspected, histopathological examined and biochemical assayed (fig. 1). The rats were sacrificed by cervical dislocation.

All experiments were performed according to the approved animal care protocols of The Ethical Committee on Animal Welfare of the Iuliu Hatieganu University 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.

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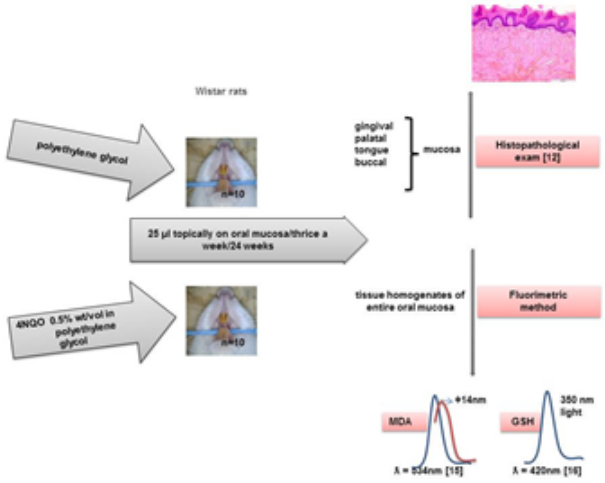

Fig. 1. Experimental design. 20 Wistar male albino rats were randomly divided in two groups. To one group oral premalignant lesions were induced with 4NQO as the carcinogen. The control group received vehicle alone in the same quantity as the tested group. At the end of the experiment, under anaesthesia, the oral cavities were opened and examined grossly; oral mucosa was immediately collected and used for histopathological examination

Histological examination

Cross sections from oral gingival, palatal, lingual and buccal mucosa in each rat were cut and tissues were fixed in freshly prepared paraformaldehyde, embedded in paraffin and microtome sectioned, deparaffinized, rehydrated, stained with hematoxylin-eosin (H&E) and histopathological examined.

Oxidative stress parameters

Briefly, oral mucosal tissues were homogenized, centrifuged [11] and the protein content in samples was measured with Bradford method [12].

MDA was measured fluorimetrically using 2-thiobarbituric acid [13]. GSH was measured fluorimetrically using o-phtalaldehyde [14]. MDA and GSH concentration were determined using standard curves made with known concentrations of MDA and GSH respectively, processed in the same way. Results were expressed as nmoles/mg protein.

Statistical analysis

The statistical analysis was performed with SPSS (version 13.0) and Microsoft Excel. Shapiro–Wilk test was used to evaluate the normal distribution of values. Mann-Whitney U test was applied in the case of not normal distribution, and, one-way ANOVA test in the case of normal distribution of data. P significance was threshold set at 0.05.

Results and discussions

In our carcinogenicity study, all control and 4NQO-treated Wistar rats survived the whole experiment.

No gross lesions were visible at the end of the 24-week 4NQO treatment in the gingival, palatal, lingual or buccal regions.

According to the 2005 WHO classification of oral precursor lesions [15] the epithelia were classified into five types and a score was given to each type: 0 - normal, 1 - epithelial hyperplasia, 2 - mild dysplasia, 3 - moderate dysplasia, 4 - severe dysplasia.

The 4NQO treated group showed microscopic evidence of dysplastic changes in all four areas examined, whereas no histological changes were observed in the control rats [15].

As illustrated in figure 2 the scores corresponding to the histoarchitectural disturbances varied depending on the oral area investigated.

Gingival, palatal, lingual and buccal mucosa showed a very clearly defined epithelium with a single layer of basal cells in the control group (fig. 3, A-D). In the 4NQO treated group, in all four oral areas studied, the microscopical changes were mainly endophytic. Gingival mucosa

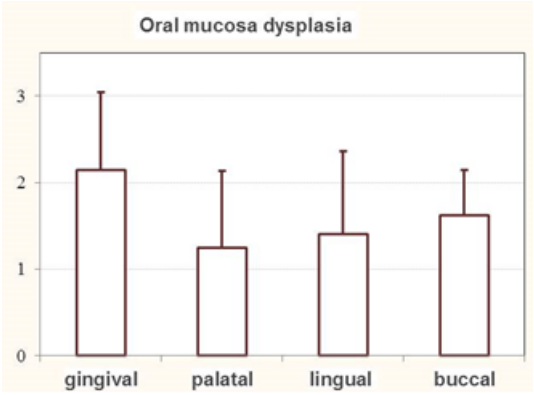

Fig. 2.Scoring of oral cavity dysplastic changes following 4NQO treatment for 24 weeks in Wistar rats (mean±SD), where: 0-No change, 1-Hyperplasia, 2-Mild dysplasia, 3-Moderate dysplasia. The highest lesional scores were found in gingival mucosa and the lowest in lingual and palatal mucosa. No statistically significant differences were depicted between scores in the four areas examined. Statistical analysis was done by Shapiro-Wilk test followed by one-way ANOVA test ($p<0.05$).

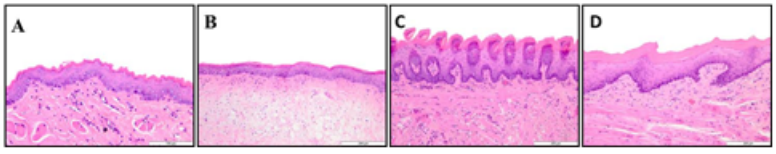

Fig. 3. Normal squamous epithelium of the gingival (A), palatal (B), lingual (C) and buccal (D) mucosa of a control rat (H&E).

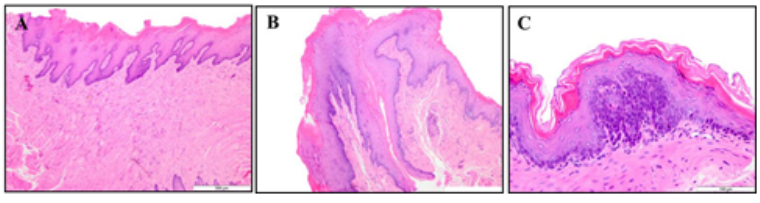

Fig. 4. Pathological evidence of gingival dysplastic changes in Wistar rats after 4NQO treatment by topical application (Experimental part) for 24 weeks (H&E): gingival changes varied from hyperplasia with marked orthokeratotic hyperkeratosis (A) to mild dysplasia, mainly basal changes with discrete nuclear pleomorphism and mild cytologic disorganization (B) and moderate dysplasia, with architectural changes extending to the middle third of the epithelium, dyskeratotic cells (C).

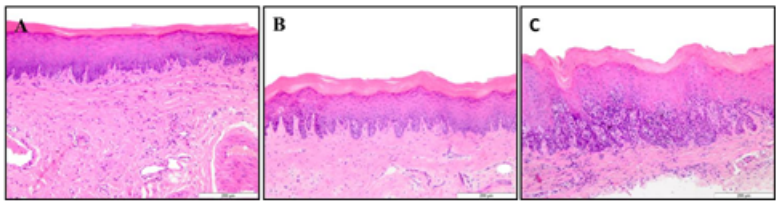

Fig. 5. Pathological evidence of palatal dysplastic changes in Wistar rats after 4NQO treatment by topical application (Experimental part) for 24 weeks (H&E): palatal mucosa showed either no pathological change (A), or epithelial hyperplasia with mild orthokeratotic hyperkeratosis (B), or mild dysplasia, located in the lower third, with increased number of mitosis, anisonucleosis, anisocytosis, nuclear and cellular pleomorphism (C)

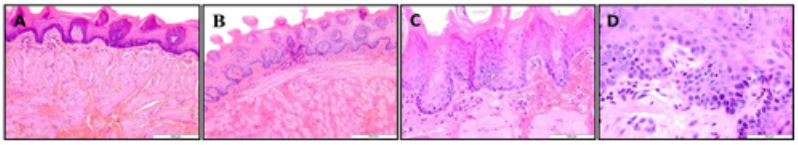

Fig. 6. Pathological evidence of lingual dysplastic changes in Wistar rats after 4NQO treatment by topical application (Experimental part) for 24 weeks (H&E): in the lingual mucosa, there was either no change (A), or hyperplasia (B), mild dysplasia, with increased number of suprabasal mitosis, and mild cytological atypia (C) and moderate dysplasia with changes mainly located in the lower third, but some mitoses and cytological atypia into the middle third (D)

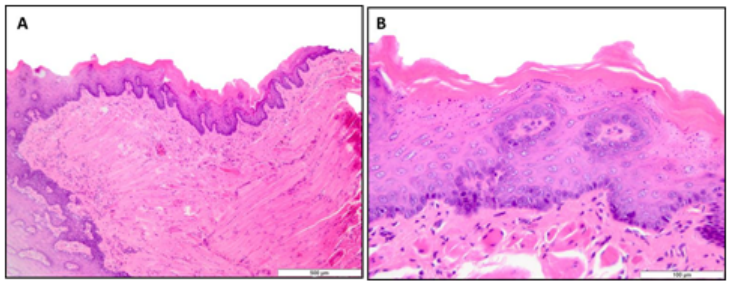

Fig. 7. Pathological evidence of buccal dysplastic changes in Wistar rats after 4NQO treatment by topical application (Experimental part) for 24 weeks (H&E): all rats showed some changes of the buccal mucosa, which were considered as either epithelial hyperplasia (A) or mild dysplasia, with hyperplastic epithelium, increased number of mitoses in the lower third and some degree of cytological atypia (B)

represented the area with most prominent dysplastic changes. No rat had a histologically normal gingival mucosa. The gingival changes varied from simple epithelial hyperplasia to mild and moderate dysplasia (fig. 4, A-C). Palatal mucosa showed less prominent architectural disturbances, varying from no change to epithelial hyperplasia and mild dysplasia (fig. 5, A-C). In the lingual mucosa (mainly the dorsal surface), microscopical changes varied from no change to epithelial hyperplasia to mild and moderate dysplasia (fig. 6, A-D). There was less variation in the buccal mucosa. All rats showed microscopical changes in this area, categorized as either epithelial hyperplasia or mild dysplasia (fig. 7, A-B).

MDA significantly increased ($p=0.0001$) and GSH significantly decreased ($p=0.036$) in oral mucosa of 4NQO treated rats compared with controls (fig. 8).

In the current study, we induced premalignant lesions of oral mucosa by topically application of 4NQO on oral mucosa for 24 weeks.

Saliva in the oral cavity of rats and its growth factors were found responsible for delayed malignant transformation presumably because of their interacting with the carcinogen, the epithelial response and the stromal reaction [16]. In our case, none of the animals developed any oral tumor, nor there any animals with severe dysplastic oral changes. This aspect has turned into an advantage in our case, as we aimed to study precursor lesions.

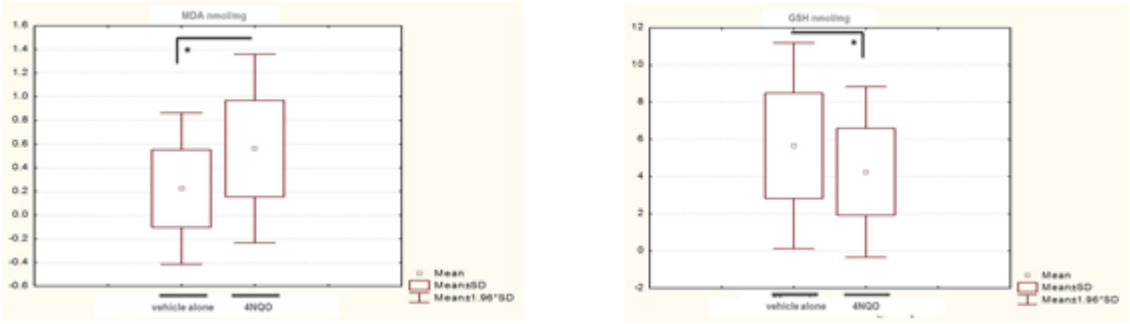

Fig. 8. Tissue parameters of oxidative stress (MDA) and antioxidant capacity (GSH) in 4NQO induced oral dysplasias. Two groups of Wistar rats (n=10) were oral topically applied vehicle alone (polyethylene glycol) - control group, and 4NQO – oral dysplastic rats group, for 24 weeks, as described in the experimental design. Oral tissue from entire mucosa was sampled and MDA and GSH were assayed by fluorimetric methods. MDA levels significantly increased and GSH levels significantly decreased in oral mucosa of rats treated with 4NQO compared with controls. Statistical analysis was done by Shapiro-Wilk test followed by Mann-Whitney U test (*$p<0.05$).

In our study, the premalignant changes in all four areas of the oral mucosa had low variation, with the gingival area presenting the most severe lesions. This was maybe also influenced by the direct topical application in our case and drinking water [17]. We obtained similar results in a previous research after 12 weeks of 4NQO induced oral premalignant lesions [18]. The lingual mucosa showed the most marked variation regarding premalignant changes in the experiment. Histopathological changes varied from no change (two rats) to mild dysplasia (one rat). Palatal mucosa and buccal mucosa showed similar results, with just one rat presenting no change on the palate, and all the others showing either epithelial hyperplasia or mild dysplasia.

4NQO carcinogenetic mechanism of action is through ROS generation that induces intracellular oxidative stress [19]. A frequently used way to measure ROS is by quantification of their oxidative damage to biomolecules [20].

Lipid peroxidation acts by damaging the membranes structure and by generating secondary products [21]. Reactive aldehydes as secondary products of lipid peroxidation, including MDA, react with biomolecules like proteins, DNA and phospholipids [22] and contributes to the mutagenic and carcinogenic effects associated with oxidative stress-induced lipid peroxidation [2, 23].

The rats in the tested group showed significantly enhanced MDA levels in the dysplastic oral mucosa indicating an intense local lipid peroxidation. Our results are in concordance with other studies in which tissue MDA levels have been reported increased in oral premalignant lesions [24-26].

GSH is the most abundant intracellular nonprotein thiol antioxidant and it has multiple roles in cellular homeostasis. GSH can act as an antioxidant in the first line of defense against reactive species by scavenging ROS directly, or, indirectly, as a cofactor in regulating several enzymatic pathways. GSH is required in detoxification too, by forming conjugates with toxicants and suppressing apoptosis [27]. Besides its role as an antioxidant/detoxifying agent, GSH plays a critical role in maintaining the intracellular redox balance and the essential thiol status of proteins [2, 22, 27].

Decreased levels of GSH were associated with initial risk factors for cancer and explained by GSH capacity to detoxify carcinogens by conjugation [28] and high levels of dietary GSH showed protection against cancer development [28, 29]. On the other hand GSH was found enhanced in tumors and responsible for tumor cells viability and resistance to cancer treatment [30]. In one of the few studies evaluating the glutathiolated proteins in tumoral tissue during 8 weeks 4NQO induced oral carcinogenesis, at week 16 and week 32 GSH was found significantly increased [31]. We found decreased GSH levels in 4NQO induced oral premalignant lesions. This result could be due to GSH utilization as an antioxidant/detoxifying agent following continuous 4NQO exposure in our experiment [32]. Similar were found after 12 weeks of 4NQO delivery [18].

Although oral cancer arises in visible, accessible locations and progresses through well-characterized, recognizable premalignant lesions, still, in most of the cases, it is detected in advanced stages [33, 34]. Recently, new improved visual techniques emerged in oral cancer diagnostic but none with better results than an oral examination with conventional histopathology [1, 35]. New research in molecular biology revealed a number of tissues, blood or saliva molecular parameters proposed as biomarkers in oral premalignant lesions but they need to be validated before extensively used. They also imply complex tests and expensive costs that make them difficult to use in daily clinical practice [36]. Since OS is involved in oral carcinogenesis, evaluating the oxidative stress-associated molecular changes in oral premalignant lesions could improve oral premalignant lesions management with impact on oral cancer prognostic.

Conclusions

Tissue MDA was increased and tissue GSH was decreased in oral dysplastic lesions demonstrating the presence of the redox imbalance and the possible role of oxidative stress in the pathogenesis of oral premalignant lesions. Therefore we believe that the use of tissue MDA and GSH as oxidative stress biomarkers in oral carcinogenesis could enrich oral premalignant lesions diagnostic tools and screening methods, and help developing new therapeutic targets.

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