Study of Serum and Saliva Biochemical Levels for Copper, Zinc and Cooper-Zinc Imbalance in Patients with Oral Cancer and Oral Potentially Malignant Disorders and their Prostetical and DSSS (Disfunctional Syndrome of Stomatognathic System) Treatment

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The significant increase of the prevalence of oral cancer and oral potentially malignant disorders determined a lot of countries to integrate this pathology amongst the main public health problems of dental medicine. Apart from the essential role of copper and zinc in the functions of human body, it seems that changes in the serum and saliva levels of zinc and copper may play a role in the pathogenesis of oral cancer. The aim of this study was to measure the serum levels of copper and zinc in patients with oral cancer and oral potentially malignant disorders. In conclusions, the serum and saliva levels of copper in oral cancer were significantly higher than in healthy controls subjects.

Keywords: public health dentistry, copper, zinc, oral cancer, oral potentially malignant disorders prosthetical and DSSS treatment

In the past two decades, a significant increase of the incidence of oral cancer (around 300,400 new cases globally every year) was registered worldwide, which determined a lot of countries to place it in information and identification campaigns, and integrate it in the main public health problems of Dental Medicine [1].

On a European level, Romania occupies 5-th place regarding oral cancer, having for male gender a total of 3.320 new cases and a standardized incidence rate of 29.6 new cases at 100,000 inhabitants and for female gender 240 new cases and a standardized incidence rate of 3.3 new cases at 100,000 inhabitants (in 2012) [2]. Although, 12.88% new more cases of oral cancer illness were identified in our country than in the previous years, many of these cases were discovered in late stages of evolution. This determined a high standardized mortality rate (of 17.9 at 100,000 inhabitants for male gender and respectively 1.6 for female gender) [3].

Concerning the evolution of the standardized mortality rate (SMR) through oral cancer (oral cavity, lips and pharynx) on age groups, the data offered by the International Agency for Research on Cancer (IARC) reveal a slightly increasing tendency for the female gender in Romania, while the trend for the male gender is moving upward [4].

Oral Cancer Foundation calls attention to the fact that, when it discovered in early stage, oral cancer has an 80 to 90 % survival rate. Unfortunately at this time, the majority of new cases are found as late stage cancers, and this accounts for the very high death rate of about 43% at five years from diagnosis (for all stages and combined at time of diagnosis), and high treatment related morbidity in survivors [5]. These are few of the arguments for which oral cancer is considered to be one of the most important public health problems in Dental Medicine [6].

From a quantitative point of view, Zinc (Zn) represents the second trace element in human body, after Iron, which does not make deposits, requiring permanent exogenous intake [7]. Zinc is found in a total quantity of 1.5-2.5 g in the human body [8]. Zinc can be found in all organs and body tissues (intracellular especially), as well as in all body fluids. The majority of the total quantity of Zn can be found in bones and skeletal muscles (90%), and the rest can be found in tissues, organs and body fluids (blood plasma contains only 0.1% from the total quantity of Zn [9].

Zinc meets important structural and functional roles (catalytic and regulatory) in human body. Zinc helps to maintain intracellular homeostasis and contributes to signal transduction in most cells. As such, Zn directly affects tumour cells through its regulatory role in gene expression and cell survival, both of which are controlled at least in part by tumour-induced alterations in Zn transporter expression, and influences tumour cells indirectly by affecting the activation, function, and/or survival of immune cells [10].

Biochemical levels of Zn in serum and malignant tissues of patients with cancer are abnormal, supporting the involvement of Zn in cancer development [11].

The activities of many enzymes and transcription factors that require Zn to function are affected by the altered Zn concentrations found within the cancer microenvironment [12].

Oxidation-reduction reactions in tumours and surrounding tissues influence intracellular free Zn concentrations and Zn levels may be an early intracellular *reporter* of reactive oxygen species and subsequent biologic responses [13].

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Copper (Cu) is a trace element that can be found in majority of body tissues and organs. Although bones and muscles contain reduced concentrations of Cu, it is estimated that these contain 60% of the total quantity of Cu in the human body. It is estimated that in the body of an adult person there can be found a total quantity of 100–150 mg of Cu, the serum level of Cu being 92-123μg/dL, and the level of free Cu being 8μg/dL[14]. The main circulating form is α_2 copper-globulin (ceruloplasmin), which has an important antiperoxidative role and protects against the action of the peroxides released by phagocytes [15].

Copper plays the role of a co-factor in numerous enzymatic systems: Cu-Zn Superoxide Dismutase (Cu-Zn SOD), cytochromoxidase, monoaminooxidase, monoxigenase, etc. [16]; Cu-Zn SOD protects proteins, lipids and nuclear DNA from oxidation [17]. Copper is also essential to a number of enzymes involved in energy production by the mitochondria.

The decrease in the level of Zn and the decrease of the Cu-Zn serum balance were identified as predisposing factors for the reduction of immunity and appearance of malignant tumours [18].

A rational approach towards Zn and Cu supplementation and modulation may ultimately emerge in the context of preventing or treating oral cancer [19].

Experimental part

Materials and methods

The *aim* of this study was to highlight possible variations of serum and salivary concentrations of Zn and Cu in patients with oral cancer and oral potentially malignant disorders by comparison to a control group comprised of healthy volunteers.

The present study settled the following *objectives* in order to reach the proposed aim:

-determining serum biochemical levels for Zn, Cu, and serum Zn/Cu *ratio*;

-determining saliva biochemical levels for Zn, Cu and saliva Zn/Cu *ratio*;

-testing statistic significance of the noticed differences related to variation of the analyzed biochemical parameters;

-highlighting possible correlations (by means of calculating the coefficients of correlation) between the analyzed biochemical parameters.

Out of 342 new clinical cases of oral cancer reported in 2015, for the area of Moldavia, there was chosen a representative group (cases 1), which was comprised of 35 patients. A group (cases 2) which included 28 patients with oral potentially malignant disorders was comprised in order make the comparison. 43 healthy volunteers who freely and being previously informed expressed their consent regarding the particpation to the study were enrolled in the control group. The average age for the control group was 51.95±17.13 years.

Out of the total oral cancer reported cases, there have been considered for study only those new clinical cases of illness, which at the time the diagnostic was established, they were in the first or middle stages of evolution (TNM II, respectively III) and for which the *inclusion criteria* were met: adult patients who expressed their consent in writing, to participate to the study.

Patient exclusion criteria were:

-patients who were administered medication containing minerals or medication which alter significantly the homeostasis of Zn and Cu, one month prior to participation to the study;

-patients who were administered diuretics (prolonged use of diuretics could deplete Zn tissue levels and increase urinary Zn excretion [20]) one month prior to participation to the study;

-persons suffering from diseases (chronic or acute) which may interfere with homeostasis of Zn and Cu (renal failure, liver diseases, heart failure, diabetes mellitus, bleedings).

The reported disease cases, which were also coded from C01-C09 according to the International Classification of Diseases (ICD 10, version 2013) [21] were comprised in cases 1 group, with oral cancer. The calculated average age for group cases 1 was 55.07±16.35 years, the minimum age was 29 years, and the maximum age was 78 years.

The same approach was applied to group cases 2, for oral potentially malignant disorders. The average age for cases 2group was 52.07 ± 15.45 years, the minimum age being 30 years, and the maximum age being 80 years.

Detailed history and clinical examination was done for each of the study subjects. The diagnosis was based on the medical history and physical exam, and the certainty diagnosis was established based on the histopathology examination.

Ethical clearance for the study was obtained from the institutional ethical committee.

Standard pre-investigative protocol was followed for the collection of biological material (saliva and blood) in order to perform dosing of Zn and Cu (no consumption of food and no smoking for an hour before saliva collection) [22]. Blood was collected by venipuncture and saliva was collected by Holmes method, which involves aspiration (for 5 min), 2 mL saliva was collected from cases and controls.

The collected saliva samples suffered a cold centrifugation process for 10 min , then the supernatant was pipetted in clean tubes, which were stored in the freezer (la -20°C), until the moment the respective tests were done.

Biochemical levels of Zn and Cu in serum and saliva were determined using the same protocol and method for healthy subjects from the control group, as well as for patients from the two groups considered for study.

Atomic absorption spectroscopy (AAS) [23] was used as a method for testing serum and saliva biological samples, for dosing Zn and Cu. The assays were performed in an accredited laboratory.

A database was generated using Microsoft Excel 2010 for Windows, in order to perform the statistical processing of data. The data were analyzed using SPSS 18.0 version for Windows. Main statistical indicators were calculated using the descriptive statistics module: mean value, standard deviation, and confidence interval (CI 95%). Data were expressed as mean ± standard deviation. Statistical comparisons were done by paired „t" test and Mann-Whitney U. The variability factors were also taken into consideration.

The type of interdependency between the studied variables, and the intensity of the respective correlation were highlighted by means of calculating the Pearson *r* coefficient of correlation.

The study observed the methodology of the case-control studies [24].

Results and discussions

Trace elements like Zn and Cu play a role in the anti-carcinogen defense system of the human body [25].

Serum Biochemical levels for zinc and copper

Modifications of trace elements levels appear in the neoplazic process, not only from a quantitative point of view, but also alterations of balance and interdependence between them.

Table 1

SERUM BIOCHEMICAL LEVELS FOR ZINC AND COPPER

Indicators	Zinc (in µg/dl)			Copper (in µg/dl)			Zinc / Copper *ratio*		
	Cases 1 Oral Cancer	Cases 2 Oral potentially malignant disorders	Controls	Cases 1 Oral Cancer	Cases 2 Oral potentially malignant disorders	Controls	Cases 1 Oral Cancer	Cases 2 Oral potentially malignant disorders	Controls
Absolute No. (n)	35	28	43	35	28	43	35	28	43
Mean value	93.91	101.72	105.57	100.07	88.89	83.85	0.93	1.15	1.26
Standard deviation	±27.06	±35.62	±29.81	±24.09	±20.64	±21.82	±0.32	±0.35	±0.38
Minimum value	63.83	67.81	70.49	76.91	66.80	69.60	0.6231	0.7340	0.7395
Maximum value	137.11	146.81	160.09	152.36	135.45	129.61	2.3140	2.1957	2.6667
Coefficient of variation (%)	28.81	35.01	28.84	24.07	23.22	26.02	34.41	30.43	30.16
95% Confidence Interval (95% CI)	89.51-97.13	101.80-109.76	101.84-113.31	72.41-109.07	84.71-95.08	77.33-90.37	0.9125-1.3402	1.2775-1.5636	1.2392-1.5264
p value (*Statistically Significant)	Cases 1 vs. Controls $p=0.031$* Cases 2 vs. Controls $p=0.027$* Cases 1 vs. Cases 2 $p=0.050$*			Cases 1 vs. Controls $p=0.021$* Cases 2 vs. Controls $p=0.017$* Cases 1 vs. Cases 2 $p=0.010$*			Cases 1 vs. Controls $p=0.011$* Cases 2 vs. Controls $p=0.005$** Cases 1 vs. Cases 2 $p=0.001$**		

Reference values for serum zinc in adults: 46-150 ì g/dl

Conversion factor: ìg/dl x 0.153 =µmol/; µmol/ x 6.54 = µg/d

Reference values for serum copper in adults: – Female: 76-152 ìg/dL; – Male: 70-140 ìg/dL

Conversion factor: vg/dL x 0.157 =µmol/L;µmol/Lx6.37=µg/dL

The main statistical indicators were calculated in order to evaluate serum levels of Zn and Cu in the study groups, the results being presented by comparison to the controls. From the results presented in table 1, the following can be noticed:

-statistically significant differences (p=0.031) regarding serum levels of the studied trace elements (of -6.73% for Zn and +19.34% for Cu) were highlighted in group cases 1 with oral neoplasias, by comparison to the control group;

-statistically significant differences (p=0.017) regarding serum levels of the studied trace elements (of -3.65% for Zn and of +6.01% for Cu) were highlighted for group cases 2 with oral potentially malignant disorders, by comparison to the control group.

The serum level of Zn is reversely correlated with the tumour development, being in opposition to the serum Cu level. A significant decrease of serum Zn may be considered an element of prediction for unfavorable evolution, and it can be noticed in patients found in late stages of the disease (III and IV).

Serum levels of Cu is rising significantly in patients with oral cancer (regardless of the location: lip, tongue, gingival and jugal mucosa etc.), the mean value being 101.12 ± 31.08 4 µg/dL, and in patients with adenocarcinoma, the mean value is 113.78 ± 34.24 µg/dL, by comparison both to patients with oral potentially malignant disorders and to the controls.

The statistical calculation of Pearson r coefficient reflects a mean negative correlation (r being -0.48 for oral cancer and -0.52 for oral potentially malignant disorders), statistically significant (p<0.05) between serum Cu and serum Zn.

The results in figure 1 represent the mean value of Zn and serum Cu and they reveal the decrease of serum Zn in the oral cancer cases by comparison to oral potentially malignant disorders and the controls.

The serum level of ceruloplasmin is rising in the case of some clinical forms of oral cancer (42.76 ± 3.45 mg/dL) by comparison to oral potentially malignant disorders and the control group. Applying the «U» statistic test indicates significant statistical differences for ceruloplasmin, between oral cancer and the control group ($u_c = 10.17 > u_{t0.05} = 1.96$; $p < 0.05$).

The increase in the serum level of ceruloplasmin in oral cancer may be interpreted as a balancing reaction to the

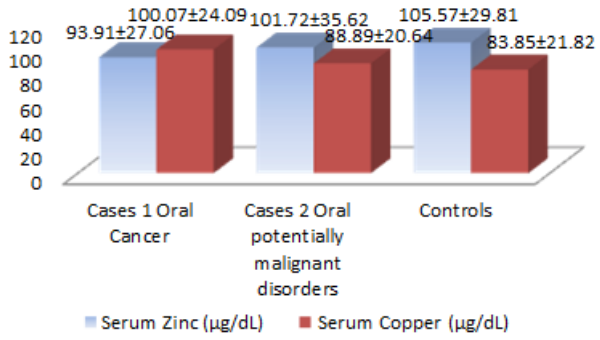

Fig.1. Mean values of serum levels for zinc and copper

production of reactive oxygen species.

By analyzing the relationship between ceruloplasmin and serum Cu, one may state that ceruloplasmin serum level can be considered a relatively faithful indicator of Cu concentration in serum in patients with oral cancer. On the other hand, the decrease of Cu-Zn SOD (Cu-Zn Superoxide Dismutase) would influence the excessive accumulation of superoxide anion with its role in stimulating the growth and pathologic cellular differentiation in patients with oral cancer.

Salivary biochemical levels for zinc and copper

The main statistic indicators were calculated in order to evaluate the salivary levels of the trace elements investigated in the two groups taken for study, the results being presented by comparison to the controls (table 2).

From the results presented in table 2 there can be noticed the following:

-in group cases 1, with oral cancer there were highlighted significant statistical differences (p=0.041), regarding salivary concentrations of studied cations (of -8.56% for Zn and of +28.48% for Cu) by comparison to the control group;

- for cases group 2 with oral potentially malignant disorders there have been noticed differences regarding salivary levels of studied cations (of -3.37% for Zn and of +8.97% for Cu) by comparison to the controls, and statistically significant differences (p=0.027).

The increased excretion of Cu through saliva can be seen as a mechanism involved in maintaining the homeostasis of this trace element and adjusting the copper-zinc imbalance

Table 2

SALIVA BIOCHEMICAL LEVELS FOR ZINC AND COPPER

Indicators	Zinc (în µg/dl)			Copper (în µg/dl)			Zinc / Copper *ratio*		
	Cases 1 Oral Cancer	Cases 2 Oral potentially malignant disorders	Controls	Cases 1 Oral Cancer	Cases 2 Oral potentially malignant disorders	Controls	Cases 1 Oral Cancer	Cases 2 Oral potentially malignant disorders	Controls
Absolute no. (n)	35	28	43	35	28	43	35	28	43
Mean value	22.01	23.26	24.07	73.71	62.52	57.37	0.36	0.37	0.39
Standard deviation	±7.12	±5.26	±8.32	±4.56	±5.49	±5.16	±0.13	±0.14	±0.15
Minimum value	9.97	16.81	1.06	47.91	50.81	44.04	0.31	0.32	0.02
Maximum value	46.08	34.81	47.02	76.36	72.32	71.06	0.72	0.59	0.83
Coefficient of variation (%)	33.45	29.47	24.05	13.71	14.75	12.65	36.11	37.84	38.46
95% Confidence Interval	20.13-25.02	21.80-24.17	21.44-26.42	62.41-75.07	60.17-67.08	58.95-63.07	0.3125-0.4028	0.3351-0.4173	0.3413-0.3957
p value (*Statistically significant)	Case 1 *vs.* Control $p = 0.041$* Case 2 *vs.* Control $p = 0.05$* Cases 1 *vs.* Cases 2 $p = 0.001$**			Case 1 *vs.* Control $p = 0.001$* Case 2 *vs.* Control $p = 0.027$* Cases 1 *vs.* Cases 2 $p = 0.01$*			Case 1 *vs.* Control $p = 0.015$* Case 2 *vs.* Control $p = 0.024$* Cases 1 *vs.* Cases 2 $p = 0.05$*		

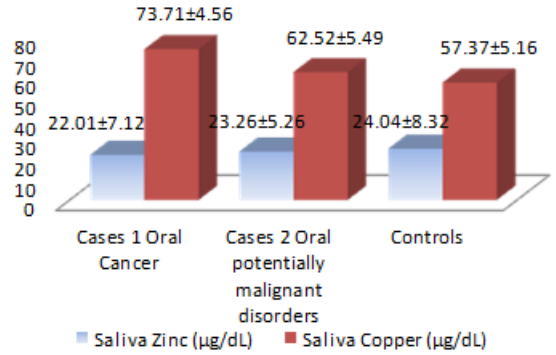

Fig.2. Mean values of saliva levels for zinc and copper

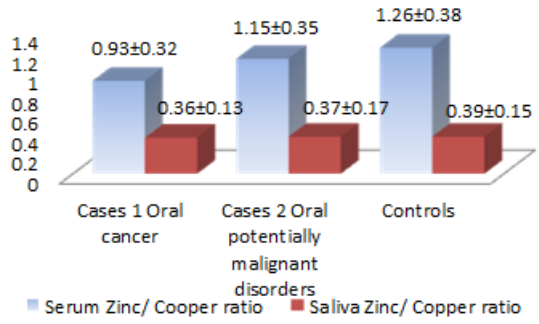

Fig.3. Copper - zinc imbalance

for patients with oral cancer and oral potentially malignant disorders.

The results stated comparatively in figure 2 represent the mean values of Zn and Cu in saliva and they reveal the decreased values of salivary Zn in oral cancer by comparison to oral potentially malignant disorders and the control group.

The calculation of Pearson *r* coefficient of correlation for the groups taken for study reflects the existence of a mean negative correlation (*r* being -0.31 for oral cancer and -0.47 for oral potentially malignant disorders), statistically significant ($p<0.05$) between salivary Cu and Zn.

Copper-Zinc imbalance

Copper and Zn are antagonists, and the balance between them is an example of biological dualism, aspect revealed by serum Zn/Cu *ratio*, which registers statistically significant decreases in the case of both groups of study (of -26.19% for oral cancer and respectively of -8.74% for oral potentially malignant disorders) *vs.* the control group (fig. 3).

Also in the case of salivary Zn/Cu *ratio* there were highlighted statistically significant decreases in both groups of study (of -7.69% for oral cancer and respectively of -5.13% for oral potentially malignant disorders) *vs.* controls.

In oral cancer, serum Cu/Zn *ratio* reflects the antagonism between them [26] inside the body, and the role they play in the neoplazic processes [27]. The association between increased serum level of Cu and oral cancer can be explained through its implication in the processes of cellular proliferation, in tissue damage through the influence of Cu over collagen and elastin, in oxidative stress as main inductor, in bringing injuries to genetic material, and probably through its ability to activate the apoptotic signaling channels into the tumour cells [28].

The correlations between serum and salivary levels of Zn and Cu and the location of the tumour did not prove to be statistically significant ($p>0.05$).

The correlation of the incidence of oral cancer with levels of Zn and Cu suggests the important role they have in the pathogenesis of oral cancer [29].

In these kind of patients it was established a prosthetic treatment in 48.57% of cases. Patients received treatment by fixed denture means, conjunct in 22.86% of the cases and the remaining mixed treatments, fixed and mobile in 14.28%, 11.43% only removable dentures, 11 % of patients were treated with oclusal splints ,the category of pacients with SDSS ,thus being rebuilt the morphological and functional dental arches thereby restoring the homeostasis of the stomatognathic system.[30-31]

Conclusions

Within the limitations of this study, the following conclusions were drawn:

Statistically significant differences were highlighted in the group comprised of patients with oral cancer by comparison to the control group, concerning serum concentrations, as well as salivary concentrations of Zn and Cu.

Statistically significant differences regarding serum and salivary concentrations of Zn and Cu were highlighted also in the group comprised of patients with oral potentially

malignant disorders, for the studied cations, by comparison to the control group.

The Zn/Cu *ratio* is an important indicator for the characterisation of these two trace elements in the human body. There were highlighted statistically significant decreases for Zn/Cu *ratio*, in serum, as well as in saliva, in both study groups *vs.* control group.

A statistically significant, mean pozitive correlation was highlighted in the case of the group comprised of patients with oral cancer, between serum Cu and salivary Cu.

Serum level of Zn is reversely correlated with the tumour growth, thus being in contrast with the level of serum Cu.

The variation of biochemical levels of Zn and Cu in serum and saliva can be used for monitoring the evolution of oral cancer and oral potentially malignant disorders.

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