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SPECTROMETRIC DETERMINATION OF ROSUVASTATIN AND EZETIMIBE IN TABLETS BY MULTIVARIATE CALIBRATION APPROACH

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ABSTRACT

Two multivariate calibration-prediction techniques, principal component analysis (PCR) and partial least squares (PLS) were applied to the spectrometric multicomponent analysis of the drug containing rosuvastatin (ROS) and ezetimibe (EZE) without any separation step. The selection of variables was studied. A series of synthetic solution containing different concentrations of ROS and EZE were used to check the prediction ability of the PCR and PLS. The results obtained in this investigation strongly encourage us to apply these techniques for a routine analysis and quality control of the two drugs.

1. INTRODUCTION

Many drugs belonging to this class are registered with the Food and Drug Administration (FDA) and differ in terms of potency and pharmacological profile. One of them is rosuvastatin (ROS) (Figure 1b), which represents a new class of synthetic statins, with enantiomeric purity (Campo, Carvalho, 2007). Rosuvastatin ((3R,5S,6E)-7-[4-(4-fluorophenyl)-6-(1-methylrthyl)-2-[methyl(methylsulfonyl)amino]-5-pyrimidinyl]-3,5-dihydroxy-6-heptenoic acid) was approved in 2003 by the FDA and is sold by AstraZeneca as Crestor®. It is sparingly soluble in water and methanol and slightly soluble in ethanol. Some methods have been developed for the quantitation of rosuvastatin in pharmaceutical formulations (Ramadan, Mandil and Alshelhawil, 2014) developed a spectrophotometric method based on a derivatization reaction of rosuvastatin with iodine, as well as an electrochemical method based on differential pulse polarography using a dropping mercury electrode (Ramadan, Mandil, Ghazal, 2015). In addition, some methods based on chromatographic separation have also been proposed (Beludari, Prakash, Mohan, 2013).

Alzoman *et al.* (2013) and Wani *et al.* (2013) developed and applied a spectrophotometric method based on the charge transfer reaction between calcium rosuvastatin and π acceptors. El-Bagary also proposed a method for the determination of this drug (El-Bagary, Elkady, Kadry, 2012) in pharmaceutical preparations based on spectrophotometric determinations.

Ezetimibe (EZE) (Figure 1a) is also an oral antilipidaemic agent and is chemically 1-(4-fluorophenyl) -(3R)- [3-(4 fluorophenyl)- 3S)-hydroxyphenyl]-4S-(4-hydroxyphenyl)-2-azetidinone. It is not official in any pharmacopoeia. HPLC (Sistla RM, et al.,2005) and LC-MS (Kosoglu T *et al.,* 2004) methods have been reported for the estimation of EZE in pharmaceutical formulations and in plasma. Also, HPLC (Shivshankar *et al.*, 2007, Lingeswara Rao Punati *et al.*, 2006) methods were reported for the simultaneous estimation of EZE in combined dosage forms.

In the present study, spectrophotometric method was suggested for direct and simultaneous determination of EZE and ROS using full-spectrum multivariate calibration methods, partial least-squares (PLS) and principal component regression (PCR) without any pre-separation step. The principal component regression (PCR) is a principal component analysis followed by a regression step. These methods are helpful chemometric techniques for the calculation of one component concentration in multicomponent mixtures. The offered methods are sensitive, simple, accurate, time saving, well-organized and are suitable for routine quality-control experiments. In this study, simultaneous determination and drug-dissolution monitoring was performed using PLS and PCR as most widely used multivariate calibration methods in binary mixtures of EZE and ROS as well as their combined tablet dosage form. It is noteworthy that simultaneous determination of EZE and ROS by the chemometrics-assisted methods has not been reported by other researchers.

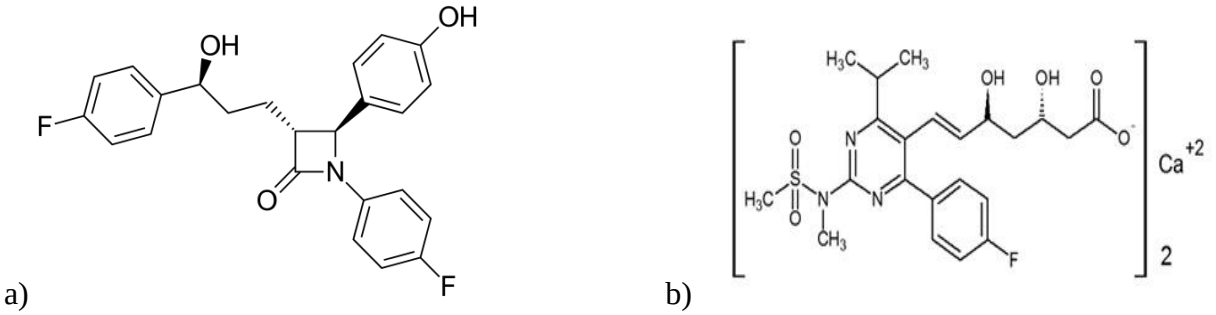

Figure 1. Structure of the drugs a) EZE b) ROS

The multivariate calibration techniques use full spectrum, full automation, multivariate data analysis and the reduction of noise and the advantages of the selection calibration model. In addition, these multivariate calibrations do not need any separation procedure, they are very cheap, very easy to apply and very sensitive. For these reasons these multivariate techniques are popular today.

In this study two chemometric methods were applied to analyse the synthetic mixtures and tablets consisting of EZE and ROS in the presence of interferences of the absorption spectra. The application of chemometrics allows the interpretation of multivariate data and is vital to the success of the simultaneous determination of the clinical drugs.

2. MATERIAL AND METHODS

2.1. Apparatus

A Shimadzu (Model UV-1700) UV-Visible spectrometer (Shimadzu, Kyoto, Japan), equipped with 1cm matched quartz cells was used for spectrometric measurements.

2.2. Standard solutions

All materials used were of analytical grade. Stock solutions of 10 mg/250 mL ROS and EZE were prepared in methanol. The solutions were stable for the least two weeks if they had been stored in a cool (< 25ºC) and dark place.

2.3. Pharmaceutical preparations

The stock solution of clomipramine hydrochloride was diluted with ultra-pure--deionized water to obtain various clomipramine hydrochloride concentrations (changed concentrated to dilute). Under the optimum conditions described in the experimental section, a linear calibration plot was constructed in the clomipramine hydrochloride concentration range $5x10^{-6}$-$3x10^{-5}$ mol $L^{-1.}$ The repeatability, accuracy, and precision were determined.

2.4. Procedure for dosage forms

An accurately weighed pulverized tablets equivalent to 100 mg of the studied drugs was extracted with 10 mL of M methanol, diluted with water, and sonicated for about 15 min. The extracts were filtered into 100 mL volumetric flasks then washed and diluted to volume with distilled water. Aliquots these solutions were transferred into a series of 10 mL volumetric flasks and the analysis were completed as spectrometric procedure. All the techniques were applied to the final solution.

2.5. Chemometrics Methods

PLS and PCR are factor analysis method, based on a two stage procedure; a calibration step, in which a mathematical model is built by using component concentrations and spectral data from a set of references, followed by a prediction step in which the model is used to calculate the concentrations unknown sample from its spectrum. These methods are also called factor methods because they transform the original variables into a smaller number of orthogonal variables called factors or principal components (PCs), which are linear combinations of the original variables. When multivariate calibration approaches are applied in spectrophotometric multi component analysis, a relationship between spectral and concentration data from reference samples, representing the variables of the system, is established. A new matrix constituted by the new variables PCs and scores is built. The calculation of this new matrix is planned by algorithm specific to the regression method adopted. The major difference in the predictive abilities of these two methods is that PLS seems to predict better than PCR when there are random linear baselines or independently varying major spectral components which overlap with the spectral features of the analysis. The optimal of calibration method depend on the particular experimental conditions. However, PLS seems to a reasonable choice over a wide range of conditions.

3. RESULTS AND DISCUSSION

Figure 1 shows the absorption spectra for ROS and EZE and their mixture in methanol.

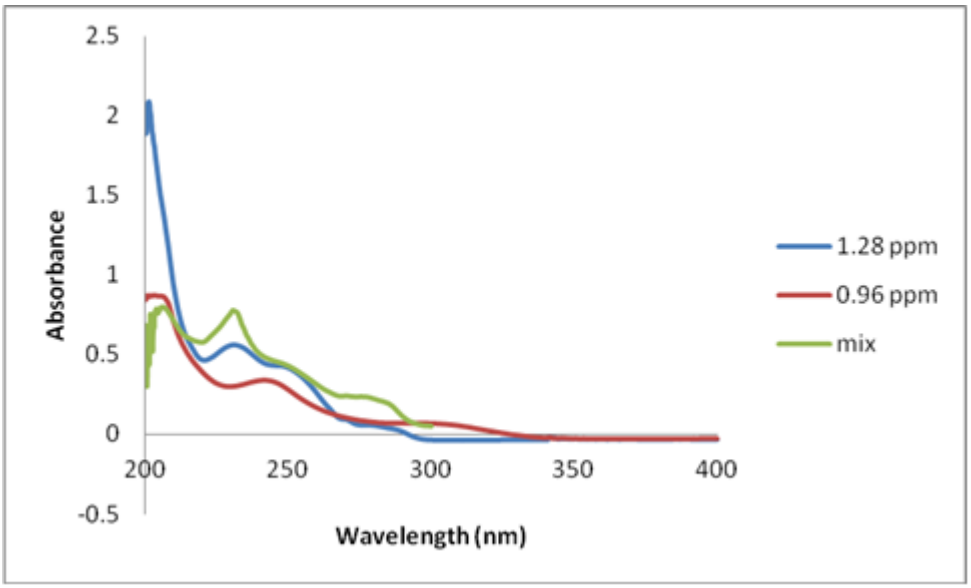

Figure1. Original absorption spectra of 1.28 µg/mL EZE 0.96 µg/mL ROS and their mixture in methanol

In order to build the two chemometric calibration, a training set was randomly prepared by using the standard mixture solution containing 0.48 - 0.96 µg/mL ROS and 0.32 - 1.60 µg/mL EZE in the variable proportions as shown in Figure 2.

The absorbance data matrix were obtained by measuring at the 13 wavelengths with the intervals $\Delta\lambda$ = 5 nm in the 200 – 400 nm spectral region. The prepared calibrations of three techniques using the absorbance data sets were used to predict concentration of the unknown values of ROS and EZE in their mixture. Linearity range was 0.48 - 2.40 µg/mL for ROS and 0.64 - 3.20 µg/mL for EZE in the multivariate calibration proposed.

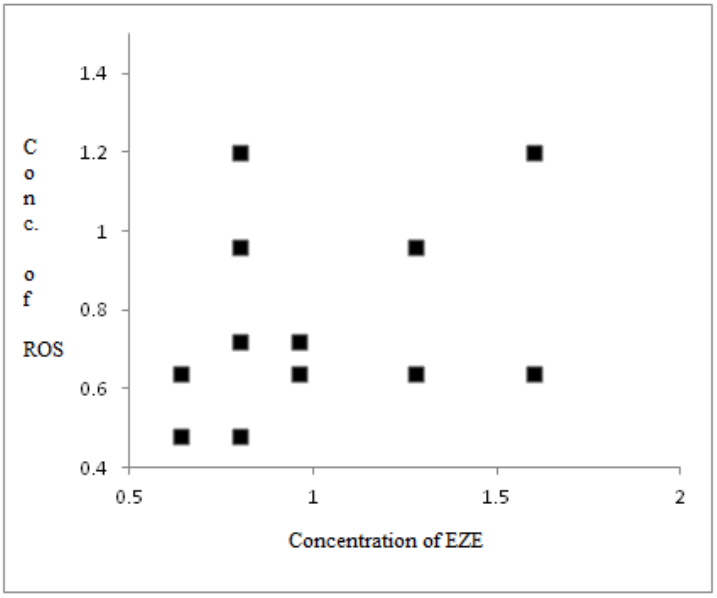

Figure 2. Concentration set design for the preparation of PCR and PLS calibrations

A calibration for each technique was computed in the MINITAB 16.0 and PLS Toolbox 4.0 software by using set consisting of two drugs and their absorbance data. The multivariate calibrations of three techniques were used to predict the unknown concentrations of ROS and EZE in the samples.

Some statistical parameters were given for the validation of the constructed calibrations for the training set and synthetic binary mixtures of both drugs.

The application competence of a calibration model can be explained in several ways. We can also examine these results numerically. One of the best ways to do this by examining the predicted residual error sum-of-squares or PRESS. To calculate PRESS we compute the errors between the expected and predicted values for all the samples, square them, and sum them together.

$$\text{PRESS} = \sum_{i=1}^{n} (C_i^{added} - C_i^{found})^2$$

Strikingly speaking, this is not a correct way to normalize the PRESS values when not all of the data sets contain the same number of samples. If we want correctly compare PRESS values for data sets that contain differing numbers of samples, we should convert to standard error of prediction (SEP), which is given by following formula.

$$\text{SEP} = \sqrt{\frac{\sum_{i=1}^{n} (C_i^{added} - C_i^{found})^2}{n-1}}$$

Where C_i^{added} the added concentration of drug is, C_i^{found} is the found concentration of drug and n is the total number of the synthetic mixtures. The SEP can provide a good measure of how well, on average, the calibration model performs. Often, however, the performance of the calibration model varies depending on the analyte level.

In the application of two chemometric techniques to the synthetic mixtures containing two drugs in variable compositions, the mean recoveries and relative standard deviations for PCR and PLS were found to be 99.98%, 0.01 and 100.04%, 0.02 respectively for EZE and 100.00% and 0.04, 99.99% and 0.09 respectively for ROS (Table 1 and 2).

Table 1. Results obtained for EZE and ROS indifferent synthetic mixtures by using PCR technique

Mixture (µg/mL)				Recovery(%)	
EZE	**ROS**	**EZE**	**ROS**	**EZE**	**ROS**
0.64	**0.72**	**0.63**	**0.72**	**99.97**	**100.05**
0.64	**0.96**	**0.63**	**0.96**	**99.98**	**100.03**
0.64	**1.20**	**0.63**	**1.20**	**99.98**	**100.02**
0.96	**0.48**	**0.95**	**0.48**	**99.97**	**100.08**
0.96	**0.96**	**0.95**	**0.96**	**99.98**	**100.02**
0.96	**1.20**	**0.95**	**1.20**	**99.99**	**100.01**
1.28	**0.48**	**1.27**	**0.48**	**99.98**	**100.08**
1.28	**0.72**	**1.27**	**0.72**	**99.98**	**100.04**
1.28	**1.20**	**1.27**	**1.20**	**99.99**	**100.01**
1.60	**0.48**	**1.59**	**0.48**	**99.98**	**100.07**
1.60	**0.72**	**1.59**	**0.72**	**99.98**	**100.04**
1.60	**0.96**	**1.59**	**0.96**	**99.99**	**100.02**
	$\bar{X}$	**99.98**	**100.04**		
	RSD*	**0.01**	**0.02**		

Table 2. Results obtained for EZE and ROS indifferent synthetic mixtures by using PLS technique

Mixture (µg/mL)				Recovery(%)	
EZE	**ROS**	**EZE**	**ROS**	**EZE**	**ROS**
0.64	**0.72**	**0.63**	**0.72**	**99.93**	**100.03**
0.64	**0.96**	**0.64**	**0.95**	**100.07**	**99.95**
0.64	**1.20**	**0.64**	**1.19**	**100.01**	**99.96**
0.96	**0.48**	**0.95**	**0.48**	**99.97**	**100.04**
0.96	**0.96**	**0.96**	**0.96**	**100.00**	**100.01**
0.96	**1.20**	**0.96**	**1.20**	**100.01**	**100.07**
1.28	**0.48**	**1.27**	**0.47**	**99.97**	**99.80**
1.28	**0.72**	**1.28**	**0.71**	**100.06**	**99.83**
1.28	**1.20**	**1.27**	**1.19**	**99.95**	**99.88**
1.60	**0.48**	**1.60**	**0.48**	**100.00**	**100.08**
1.60	**0.72**	**1.60**	**0.72**	**100.01**	**100.01**
1.60	**0.96**	**1.59**	**0.96**	**99.98**	**100.05**
		$\bar{X}$	**100.00**	**99.99**	
		RSD*	**0.04**	**0.09**	

According to the added concentration and the concentration found in samples, the SEP and PRESS values of PCR and PLS techniques were calculated $8.99.10^{-3}$, $9.62.10^{-3}$ and $1.21.10^{-3}$, $1.38.10^{-5}$ respectively for EZE, $1.90.10^{-2}$, $3.4.10^{-2}$ and $5.45.10^{-3}$, $1.73.10^{-4}$ respectively for ROS (Table 3).

Table 3. Statistical parameters in the calibration-prediction

Parameter	Method	EZE	ROS
PRESS	PCR	$1.21.10^{-3}$	$5.45.10^{-3}$
	PLS	$1.38.10^{-5}$	$1.73.10^{-4}$
SEP	PCR	$8.99.10^{-3}$	$1.90.10^{-2}$
	PLS	$9.62.10^{-3}$	$3.4.10^{-2}$
r	PCR	1.0000	0.9999
	PLS	1.0000	0.9996
Intercept	PCR	7.10^{-5}	0.0025
	PLS	0.0001	0.0016
Slope	PCR	1.0000	0.9994
	PLS	1.0000	0.9996

Accuracy and precision for the analysis of ROS and EZE substances in the prepared synthetic mixtures at three different concentration levels (0.64, 0.96 and 1.28 g/mL for EZE and 0.48, 0.72 and 0.96 g/mL for ROS) in intra-day (n=6) and inter-day (n=6), was tested for the applicability of the proposed chemometric methods. The calculated results for percent relative error, standard deviation and relative standard deviation were presented in table 4 and 5. Good accuracy and precision were observed for the results obtained by PCR and PLS calibrations.

Table 4. Accuracy and precision results for PCR

Added (µg/ml)		EZE					ROS				
EZE	ROS	Found	SD	% BSS	% RE	Recovery(%)	Found	SD	% BSS	% RE	Recovery(%)
0.,64	0.48	0.64023	2.94E-03	2.94E-05	0.03592	100.0359	0.481268	3.98E-02	3.97E-04	0.264213	100.2642
0.96	0.72	0.960256	2.99E-04	2.99E-06	0.026631	100.0266	0.721314	4.54E-02	4.53E-04	0.182444	100.1824
1.28	0.96	1.280282	5.81E-04	5.81E-06	0.022062	100.0221	0.961344	3.16E-02	3.16E-04	0.140019	100.14
					x̄	100.0282				**x̄**	100.1956
					SD	0.007062				**SD**	0.063127
					BSS	7.06E-05				**BSS**	0.00063
					LOD	0.023302				**LOD**	0.208299
					LOQ	0.070612				**LOQ**	0.631208

Intra-day (n=6)

Added (µg/ml)		EZE					ROS				
EZE	ROS	Found	SD	% BSS	% RE	Recovery(%)	Found	SD	% BSS	% RE	Recovery(%)
0.64	0.48	0.639877	3.23E-05	3.23E-07	-0.01916	99.98084	0.480404	4.39E-05	4.38E-07	0.084107	100.0841
0.96	0.72	0.959861	5.58E-05	5.58E-07	-0.01452	99.98548	0.720331	3.50E-05	3.0E-07	0.04592	100.0459
1.28	0.96	1.279844	6.46E-05	6.46E-07	-0.01222	99.98778	0.960278	5.00E-05	5.00E-07	0.028979	100.029
					x̄	99.9847				**x̄**	100.053
					SD	0.003535				**SD**	0.028238
					% BSS	3.54E-05				**% BSS**	0.000282
					LOD	0.011666				**LOD**	0.093203
					LOQ	0.035351				**LOQ**	0.282434

SD: Standard deviation, RE: Percent relative error

Table 5. Accuracy and precision results for PLS

Inter-day (n=6)

Added (µg/ml)		EZE					ROS				
EZE	ROS	Found	SD	% BSS	% RE	Recovery(%)	Found	SD	% BSS	% RE	Recovery(%)
0.64	0.48	0.640119	1.00E-03	1.00E-05	0.018659	100.0187	0.479889	0.087302	8.72E-04	-0.02302	100.0729
0.96	0.72	0.959843	7.00E-04	7.00E-06	-0.01634	99.98366	0.719971	0.033081	3.31E-04	-0.00403	99.93277
1.28	0.96	1.279961	7.72E-04	7.72E-06	-0.00303	99.99697	0.960427	0.064689	6.47E-04	0.044474	99.99183
					x̄	99.99976				**x̄**	99.97605
					SD	0.017666				**SD**	0.070367
					BSS	0.000177				**BSS**	0.000704
					LOD	0.058297				**LOD**	0.232212
					LOQ	0.176659				**LOQ**	0.703673

Intra-day (n=6)

Added (µg/ml)		EZE					ROS				
EZE	ROS	Found	SD	% BSS	% RE	Recovery(%)	Found	SD	% BSS	% RE	Recovery(%)
0.64	0.48	0.640051	7.29E-02	7.29E-04	0.007975	100.008	0.479889	0.153317	0.001534	-0.02302	99.97698
0.96	0.72	0.959954	2.03E-04	2.03E-06	-0.00482	99.99518	0.719971	0.050142	0.000501	-0.00403	99.99597
1.28	0.96	1.279951	6.12E-04	6.12E-04	-0.00386	99.99614	0.960427	0.083267	0.000832	0.044474	100.0445
					x̄	99.99976				**x̄**	99.97605
					SD	0.007127				**SD**	0.034808
					BSS	7.13E-05				**BSS**	0.000348
					LOD	0.023518				**LOD**	0.114867
					LOQ	0.071268				**LOQ**	0.348083

The linear regression analysis of the added concentration and the concentration found in the synthetic mixtures were realized for each drug and for each calibration technique. In this regression analysis, the correlation coefficient (r), intercept, slope and relative standard deviation values were found satisfactory for the proposed chemometric techniques in Table3. As can be seen, all the statistic values indicated that all techniques are convenient for the determination of two drugs in synthetic mixtures.

A summary of the assay results for the pharmaceutical formulation is given Table 6. The results of all methods were very to each other as well as to the label value of commercial drug formulation.

Table 6. Assay results for the pharmaceutical formulation (mg/tablet)

Drug	**PCR**	**PLS**
EZE		
Mean ± SD*	9.928± 0.02	10.017± 0.02
ROS		
Mean ± SD*	10.00±0.36	10.06± 0.54

Results obtained are average of six experiments for each technique.

*SD: Standard deviation *

4. CONCLUSIONS

Two chemometric technique in spectrometric analysis, PCR and PLS, were proposed for the simultaneous determination of EZE and ROS in their binary mixtures. These techniques were applied with great success to two commercial pharmaceutical tablets. The resolution of highly overlapping drug mixtures was achieved by the use of PCR and PLS techniques. A selection of working wavelength having high correlation values with concentration due to interference coming from matrix sample or additional analytes outside the working range. According to the obtained results, it was observed that the PCR method gave more accurate results than the PLS method in this combination of two drugs. The proposed chemometric techniques can be applied for the routine analysis of two drugs in the tablet formulation without any a priori chemical separation and without time consuming.

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STABILITY OF METAL OXIDE SUSPENSION IN THE CATIONIC POLYACRYLAMIDE PRESENCE

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ABSTRACT

In this study, electroanalytical technique was developed for the quantitative analysis of clomipramine hydrochloride from its commercial tablet dosage forms based on its oxidation behavior. The electrochemical determination of clomipramine hydrochloride was easily carried out on glassy carbon electrode (GCE) by two voltammetric techniques. The electrochemical measurements were carried out on GCE surface in different buffer solutions in the pH range from 2.00 to 12.00 by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques. The effect of pH on the anodic peak current and peak potential was investigated. Phosphate buffer (pH 6.50) was selected for analytical purposes. The diffusion-controlled nature of the peak was obtained. A linear calibration plot for DPV analysis was constructed in the clozapine concentration range from $5x10^{-6}$ mol L^{-1} to $3x10^{-5}$ mol L^{-1}. Limit of detection (LOD) and limit of quantification (LOQ) were obtained as $2.8x10^{-7}$ mol L^{-1} and $8.4x10^{-7}$ mol L^{-1} respectively.

1. INTRODUCTION

The main reaction of cationic polyacrylamide (PAM) synthesis is free radical copolymerization of acrylamide with the cationic monomer (Jiang and Zhu, 2014). The monomer containing positively charged functional groups used for synthesis of polymer applied in this study is dimethyldiallyl ammonium chloride (DMDAAC). As a result, the quaternary amine groups are introduced to the macromolecules. Contrary to polymer amide groups, these amine groups undergo ionization in aqueous solutions with the changes in solution pH value and are the source of positive charge of polymeric chains.

The most important field of cationic polyacrylamide application is the flocculation process of undesirable solids characterized by high dispersion in aqueous solutions. Long polymeric chains (with molecular weight of the order of several and dozen million Daltons) have ability to form polymeric bridges between the solid particles and their effective aggregation (easily sediment flocs are formed). Moreover, PAM is widely used in removal of polyvalent metal ions, organic compounds (i.e. dyes) and microorganisms from aqueous solutions. For such reasons ionic polyacrylamide finds application in the procedures of industrial waste water purification and drinking water treatment (Entry and Sojka 2000, Amuda *et al.* 2006, Barakat 2011). Cationic polyacrylamide is also used as the rain erosion control agent is agriculture (Sojka *et al.* 2007). By binding loose solid particles the polymer improves considerably the consistency of the soil (simultaneously maintaining surface roughness and continuity of pores).

Thus, such long polymeric chains containing charged functional groups are effective modifiers of the surface properties of solid particles dispersed in liquid medium. The specific structure of polymeric layers covering colloidal particles determines directly such systems stability.

Suspensions of metal oxides are colloidal systems very often encountered in practice. Metal oxides play an important role in adsorptive removal of a great variety of hazardous substances (Wawrzkiewicz *et al.* 2015). They are also used in production of cosmetics, pharmaceuticals and mineral pigments (Simpson *et al.* 2011, Vargas-Reus *et al.* 2012). To obtain the required surface properties of metal oxide particles they were often modified by adsorbed polymers. The specific conformation of polymer chains adsorbed at the solid-liquid interface can be obtained by appropriate selection of among others polymer kind (ionic, nonionic) and metal oxide type, temperature, solution pH, as well as supporting electrolyte type and its concentration (Wiśniewska 2012).

The main aim of studies presented in the manuscript was comparison of stability of three selected metal oxides suspensions (chromium(III) oxide – Cr_2O_3, zirconium(IV) oxide – ZrO_2 (zirconia) and aluminum(III) oxide – Al_2O_3 (alumina) in the presence of cationic polyacrylamide (PAM). The obtained changes in these systems stability (turbidimetry method) after polymer addition were explained based on the analysis of adsorption (spectrophotometry method), surface charge density of the solid (potentiometric titrations) and zeta potential of metal oxide particles (laser microelectrophoresis technique) data.

2. MATERIAL AND METHODS

2.1. Adsorption system characteristics

In the experiments three metal oxides were used as adsorbents - chromium(III) oxide: Cr_2O_3 (*POCh*), zirconium(IV) oxide: ZrO_2 (*Sigma-Aldrich*) and aluminium(III) oxide: Al_2O_3 (*Merck*). They were characterized by the specific surface area (S_{BET}) equal to: 7.12, 21.7 and

155 m^2/g, respectively. S_{BET} was determined using the low-temperature nitrogen adsorption-desorption isotherm method (*Micrometritics ASAP 2405 Analyzer*).

The cationic polyacrylamide – PAM (*Korona*) with the weight average molecular weight 7 000 000 Da and the content of quaternary amine groups 50% was applied as an adsorbate (Fig. 1). The pK_b value of this polymer is equal to 9.9 and was determined using the potentiometric titration method. Knowing pK_b value, the ionization degree (α) of the PAM cationic groups can be calculated (Wiśniewska *et al.* 2015). At pH 3 α=99.9 %, at pH 6 it assumes the value 99.9 %, whereas at pH 9 the ionization degree is equal to 88.8 %.

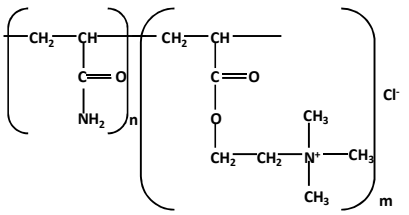

Figure 1. Chemical formula of cationic polyacrylamide.

All measurements were made in the pH range 3-9 at 25°C in the presence of NaCl (as supporting electrolyte) with the concentration $1 \cdot 10^{-2}$ mol/dm^3.

2.2. Stability measurements

The stability measurements of metal oxide suspensions in the absence and presence of PAM were performed using the turbidimetry method (Terpiłowski *et al.* 2015). For this purpose, the apparatus Turbiscan Lab^{Expert} with the cooling module TLAb cooler (*Formulaction, France*) was applied. The suspension of 0.2 g of oxide in 20 cm^3 of NaCl solution (or PAM-NaCl, C_{PAM} 100 ppm) was prepared. Each experiment of suspension stability lasted 15 h and every 15 min the respective data (light transmission and backscattering level) were collected. TLab EXPERT 1.13 and Turbiscan Easy Soft computer programs enabled calculations of stability coefficients TSI (*Turbiscan Stability Index*). The limit values of TSI are 0 (very stable suspensions) and 100 (extremely unstable systems). Moreover, knowing the aggregate migration velocity (on the basis of the changes of transmission or backscattering in time), the aggregate size was calculated using the general law of sedimentation – Stokes law extended on concentrated dispersions (Snabre and Mills 1994).

2.3. Adsorption measurements

Adsorption measurements were performed using the static method in the polymer concentration range 10-150 ppm. The reaction of cationic polyacrylamide with a saturated solution of bromine, sodium formate and starch (in acetate buffer) (Scoggins and Miller 1975) was applied. It enables determination of the polyacrylamide concentration in the solution after the adsorption process (equilibrium concentration). The formed complex gives blue colour of the solution of different intensity depending on the polymer concentration. The solution absorbance was measured after 5 min with the UV-VIS spectrophotometer (Carry 1000; Varian) at 585 nm. Using the calibration curve, the amount of the adsorbed polymer (in unit's mg/m2 and mg/g) was determined from the difference between the PAM concentration in the solution before and after the adsorption process.

2.4. Solid surface charge density determination

The potentiometric titration method (Janusz 1999) was used for the solid surface charge density determination. The appropriate mass of metal oxide was added into the thermostated (*thermostat RE204, Lauda*) Teflon vessel containing 50 cm^3 of polymer solution (100 ppm) in the NaCl electrolyte (or only to NaCl electrolyte solution). Such systems were titrated with base NaOH with the concentration $1 \cdot 10^{-1}$ mol/dm^3 using the automatic burette Dosimat 665 (*Methrom*). The changes in the pH values during titration were monitored with the pH-meter 71 pHmeter (*Beckman*). The special program Titr_v3 (author W. Janusz) was applied to solid surface charge density calculation from the difference in the base volume added to the suspension and the supporting electrolyte solution that leads to the specific pH value. These measurements enable also determination of point of zero charge (pzc) values for all examined systems.

2.5. Zeta potential determination

The electrophoretic mobility of metal oxide particles (without and covered with polymer) was measured using the Zetasizer Nano ZS with the universal dip cell and MPT-2 titrator (*Malvern Instruments*). The Doppler laser electrophoresis technique was applied and the zeta potential value was calculated with the special computer program using the Henry equation (Hunter 1981). Metal oxide suspensions (without and with PAM) were prepared adding appropriate mass of the solid to 100 cm^3 of NaCl solution or PAM-NaCl solution (polymer concentration 10 ppm). After the sonification process the obtained suspension was divided into 6 parts of the same volume. Then in each of them the pH value was adjusted to: 3, 4.5, 5.5, 6.5, 8.5 and 10±0.1, respectively and their electrophoretic mobility were measured. The values of isoelectric points (iep) of metal oxides were determined from obtained curves presenting dependencies of zeta potential as a function of solution pH.

3. RESULTS AND DISCUSSION

As can be seen in Fig. 2 cationic polymer has the great impact on the metal oxide suspension stability at pH 6.

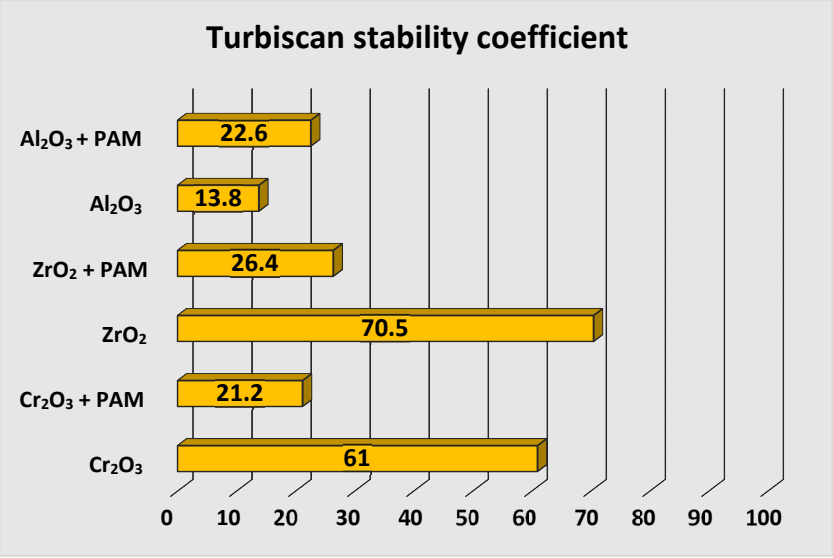

Figure 2. TSI stability coefficients of the metal oxide suspension without and with polyacrylamide at pH 6.

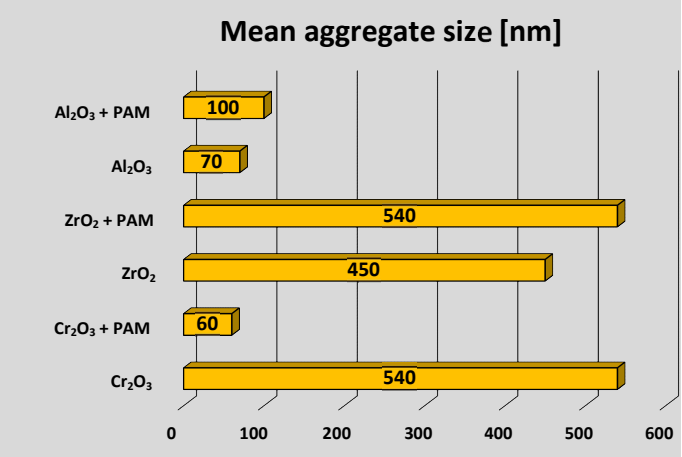

Figure 3. Mean aggregate sizes formed in the metal oxide suspension without and with polyacrylamide at pH 6.

In the case of Cr_2O_3, ZrO_2 systems without PAM and ZrO_2 system with PAM , the suspensions are unstable (high TSI values) and large aggregates are formed – sizes in the range 450 nm – 540 nm, respectively, for chromium(III) oxide and zirconium(IV) oxide (Fig. 3). The polymer addition causes considerable improvement of stability conditions in these systems (TSI values decrease). In the case of Al_2O_3, suspension without cationic PAM is relatively stable and in the polymer presence insignificant decrease of its stability is observed. This is reflected in much smaller sizes of formed aggregates – not exceeding 100 nm (Fig. 3).

In order to explain the obtained changes in solid suspension stability after cationic polyacrylamide addition the analysis of adsorption and electrokinetic results is necessary. Figure 4 presents the PAM adsorbed amounts on the surfaces of applied metal oxides expressed in mg/m^2 (polymer/metal oxide surface, Fig.4a) and mg/g (polymer/metal oxide mass, Fig.4b), examined at three solution pH values.

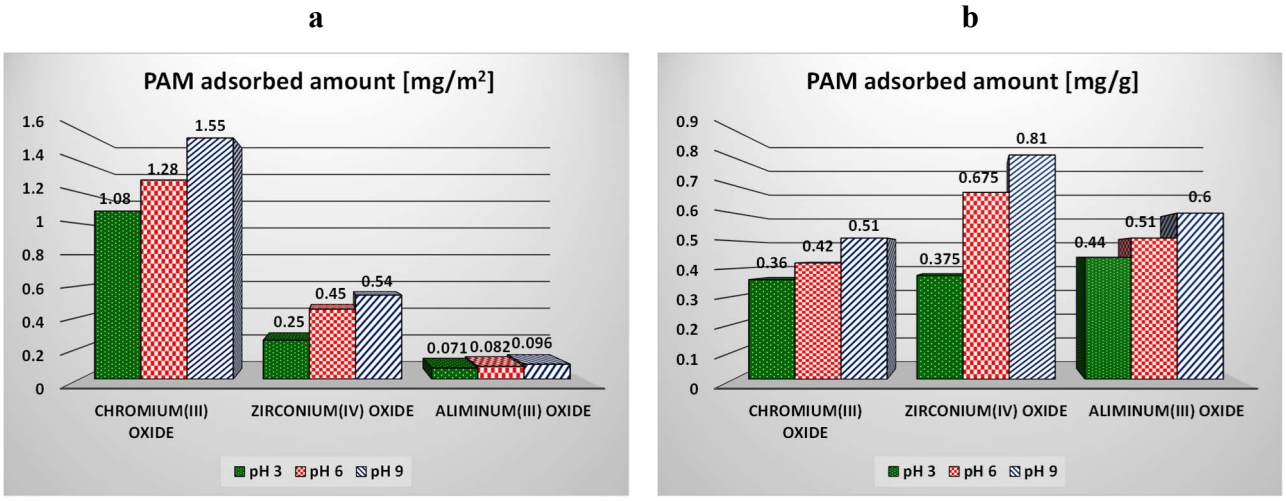

Figure 4. Adsorbed amounts of cationic PAM on the surfaces of examined metal oxides at pH 3, 6 and 9.

The analysis of the data presented in Fig. 4 indicated that adsorption of cationic polyacrylamide increases with the pH increase for all examined metal oxides. Moreover, adsorbed amounts of the polymer per unit area of the solid is the greatest in the case of chromium(III) oxide (Fig. 4 a). It is probably connected with concentration of different types of solid hydroxyl groups on the unit of solid surface area (Chibowski and Wiśniewska 2001). In the case of Cr2O3 the concentration of these groups must be the highest and the polymer adsorption is the greatest. Due to the fact that Cr2O3 is characterized by the smallest specific surface area the adsorbed amounts of the polymer per unit weight of the solid is the smallest (Fig. 4 b), but comparable with those obtained for Al2O3 (with the highest specific surface area). This proves the high adsorption affinity of the ionic polyacrylamide to the surface of chromium (III) oxide compared to other examined adsorbents.

The PAM adsorption changes with the solution pH increase is the result of changes in both the solid surface charge and ionization of the PAM carboxyl groups with the pH rise. The total surface charge of the solid assumes zero value (point of zero charge - pzc) at specific pH for each system containing metal oxide. The determined values of pHpzc points for all examined systems are presented in Fig. 5. Below pH_{pzc} the solid surface is positively charged and above this value, the solid surface assumes negative charge. Thus, the most favorable electrostatic conditions for adsorption of cationic polymer on the surface of given metal oxide is above its pH_{pzc} value.

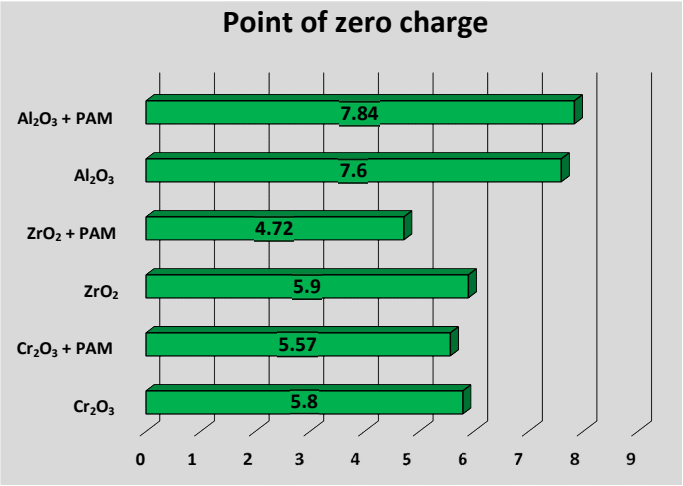

Figure 5. pH_{pzc} values of the metal oxide systems without and with cationic polyacrylamide.

As can be seen in Fig. 5 the points of zero charge for Cr_2O_3, ZrO_2 and Al_2O_3 oxides are equal to 5.8; 5.9 and 7.6, respectively. For this reason, the greatest adsorption of positively charged PAM macromolecules is observed at pH 9 at which the electrostatic attractions between adsorbate and adsorbent takes place. The polyacrylamide adsorption has the lowest level at pH 3 due to the repulsion between the totally ionized PAM macromolecules and the positively charged surface of the solid. The polymer binding with the metal oxide surface undergoes through the hydrogen bridges under these disadvantageous conditions. Although the charged active solid sites can contribute to formation of these bonds, the neutral ones are mainly responsible for it. This results from the fact that $\equiv MeOH^0$ surface groups are much more numerous than $\equiv MeOH_2^+$ and $\equiv MeO^-$ ones (Chibowski and Wiśniewska 2001).

Very important parameter related to solid suspension stability is electrokinetic potential zeta. The specific pH value at which potential zeta of solid particles is equal to zero is called isoelectric point (iep). As can be seen in Fig. 6, the isoelectric points of metal oxides without the polymer are located at pH values 6, 6.4 and 8.4 for Cr_2O_3, ZrO_2 and Al_2O_3, respectively. Some differences between the pH_{pzc} and pH_{iep} values of applied oxides are a result of overlapping of the electrical double layers formed in the pores present in the applied solids structure (Skwarek 2015).

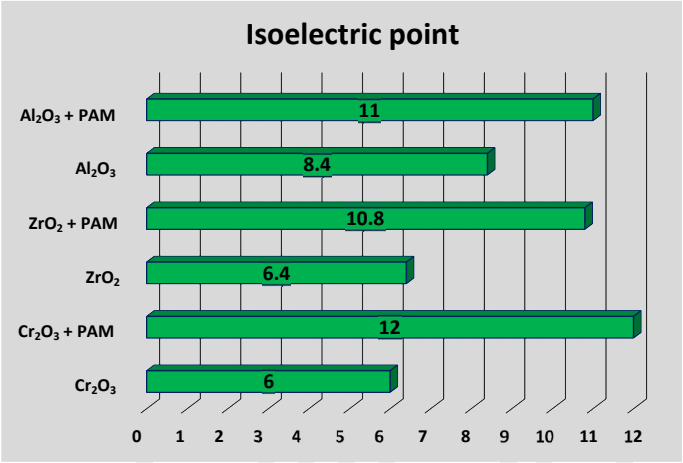

Figure 6. pH_{iep} values of the metal oxide systems without and with cationic polyacrylamide.

The presence of cationic polymer layer around the solid particles results in a significant increase of the zeta potential values and the considerable shift of the pH_{iep} towards the higher pH values. The main reason for zeta potential increase in the cationic polyacrylamide presence is the occurrence of positively charged functional groups of the adsorbed polymer macromolecules in the area of slipping planes around the solid particles (Wiśniewska *et al.* 2016). These groups belong to the polymeric segments located in the loop and tail structures of the adsorbed macromolecules and are not directly bound with the solid surface. They are present in large quantities in the border of the stiff and diffusion parts of the electrical double layers formed on the solid particle surfaces. As a result, such great shift of pH_{iep} values for systems with PAM in comparison to the system without PAM is observed.

Taking adsorption and electrokinetic data into consideration the most probable mechanism of solid suspension stability in the cationic polyacrylamide presence can be proposed. The ionic PAM addition at pH 6 causes considerable improvement of chromium(III) oxide and zirconium(IV) oxide systems stability. These suspensions without polymer are unstable at pH 6 due to the fact that such pH value is very close to points of zero charge and isoelectric points obtained for Cr_2O_3 and ZrO_2 (Figs 5 and 6). The adsorption of completely ionized polymer chains on the solid surface at pH 6 results in appearance of electrosteric repulsion of PAM adsorption layers covered metal oxide particles. The adsorbed polymeric layers prevent the aggregation of solid particles not only due to the steric hindrance presence (PAM macromolecules) and also electrostatic repulsion coming from positively charged groups of the polymer. In the case of Al_2O_3 the polymer addition at pH 6 causes insignificant improvement of solid suspension stability. The alumina system without polymer is relatively stable in comparison to other examined oxides. Under such conditions bare Al_2O_3 particles are positively charged and repulse each other preventing their rapid aggregation. The polymer

adsorption layer presence makes connection of solid particles even more difficult (for electrosteric reasons).

4. CONCLUSIONS

Presented results indicated that high-molecular cationic polyacrylamide, commonly referred to as a polymeric flocculant, can also act as effective stabilizer of aqueous suspensions of metal oxide particles. At specific solution pH value (pH 6) and PAM concentration (100 ppm) it causes considerable improvement of chromium(III) oxide and zirconium(IV) oxide suspensions stability. The total ionization of functional groups of adsorbed macromolecules results in electrostetic repulsion between solid particles covered with polymeric layers. Moreover, it was shown that ionic polymer adsorption changes considerably structure of electrical double layer formed at the solid-liquid interface which is reflected in changes of the surface charge density and zeta potential of metal oxide particles dispersed in aqueous solution.

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DETERMINATION OF THE RELATIONSHIP AMONG RAW COW MILK COMPOSITION AND SOMATIC CELL COUNT OBTAİN FROM MILK COLLECTION CENTRES

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ABSTRACT

This study investigates protein, amount of fat, lactose, dry matter, point of freezing and number of somatic cells (SCC) as well as the relationship among these mentioned components of milk collected from milk collection tanks from three different collection centers in Biga Town in Canakkale Province by using 825 raw cow milk sample. Mastitis is a high cost sickness in dairy cattle production industry and number of somatic cells (SCC) in the milk is an indicator of the problem. SCC also cause loss in the productivity of raw milk and increase the production costs of raw milk at the same time. Increased number of somatic cells also affect content of lactose, protein and dry matter in the milk to a great extent. This study aims to identify the relationship among SCC identified in raw cow milk and dry matter, non-fat dry matter, fat, protein and lactose contents.

1. INTRODUCTION

Raw milk according to Turkish Food Codex Communiqué on Raw Milk and Heat Processed Drinking Milk is defined as the mammary gland secretion obtained by milking of one or more than one cow(s), Goat(s), sheep, buffalo(s); except the colostrum not having been heated above 40°C treated by any other process having equivalent effect (Anonymous, 2000). In addition to growth and development; it has many other important properties in its life cycle such as vitamins and minerals in protein and peptide constituents such as physiologically important immunoglobulins, enzymes, enzyme inhibitors, growth hormones and other hormones, growth factors and antimicrobial agents, as well as fatty acids (Besler and Ünal, 2006). In a healthy udder, the milk in the cistern is sterile. However, after reaching the canal, it is contaminated by microorganisms. These bacteria are also the bacteria that have previously reached the mammary gland or the streak canal. This is described by Kielwein (1985) as "contamination of raw milk during secretion." (Kielwein, 1985). Thus, the investigator reports that during secretion the first milk should be discarded without being used (Kurweil and Busse,

1973; Tolle,1980; Alişarlı *et al.*,2003). Nowadays, somatic cell count (SCC) is used as an important criterion in the determination of raw milk quality and udder health. The somatic cell is a name given to any cell of a living organism other than the reproductive cells. Somatic cells in milk consist of epithelial cells, large squamous cells, epithelial cell debris and non-nucleated cells, red blood cells (erythrocytes), plasma cells, colostrum corpuscles, and leukocytes. Somatic cells can be used as a criterion for diagnosing udder health and subclinical mastitis (Dohoo and Meek, 1982; Rice and Bodman, 1997; Manlongate *et al*., 1998). In the case of a bacterial infection in the udder or any trauma, the SCC in the milk begins to increase (Kaya *et al*., 2011).

Legally, in many countries, the SCC in the milk is used as an indicator to determine milk quality standards and determines the level of milk payments made to the producer. According to Turkish Food Codex Communiqué on Raw Milk and Heat Processed Drinking Milk (No: 2000/6) published the Ministry of Agriculture and Rural Affairs, the limitation to SCC found in milk has been determined as ≤500.000 per milliliter (Anonymous, 2000). The European Union (EU), New Zealand, Australia and a few other countries have adopted a standard for maximum allowable cell counts in their Grade A type milk of 400,000 cell/ml. In Canada it is at 500,000 while it is 750,000 in the USA (Anonymous, 1998).

SCC in a healthy cow milk should be less than 200,000 cell/ml (Anonymous, 2006). However, SCC can be below 100,000 cell/ml in herds with good management or after the first lactation. SCC of more than 250,000 to 300,000 per ml is usually indicative of abnormal milk and a potential exposure of the udder to an infection (mastitis) leading to a decrease in milk yield and quality (Rice and Bodman, 1997; Atasever *et al*., 2010; Anonymous, 1998).

2. MATERIAL AND METHODS

In the research, 275 raw milk samples from the group I region, 275 samples from group II region and 275 samples from group III region, totaling 825 samples (sampling techniques of raw milk from cooling tanks were applied) were collected from milk collection centers from January to December 2015. Samples were taken to the laboratory in 100 ml sterile cups preserving cold chain (+4°C) and protein, fat ratio, lactose, dry matter, freezing point and somatic cell counts were examined on the same day. Flow cytometry (Bactocount IBCm, Bentley Instrument, USA) was used for somatic cell counts, protein, fat ratio, lactose, dry matter, freezing point (Anonim, 2013). Monthly average values obtained from weekly counts were used as data.

In the statistical study, statistical analyzes were performed after lactose, protein, total dry matter, freezing point, somatic cell counts of the milk samples were determined. The SPSS 19 package program was used to calculate the coefficient of correlations.

3. RESULTS AND DISCUSSION

The results obtained from the I. group are shown in Table 1. According to statistical analysis results (pearson correlation), a strong and statistically significant relationship of the fat ratio with protein (r=0,545; P<0,001) and total dry matter rations (r=0.913; P<0.001) determined. These results indicate that cow milks with high-fat milk content also have high protein and dry matter values. According to Table 1, there is a weak but statistically significant relationship between fat content and freezing point of raw cow milk (r=0.192; P<0.01).
While weak but statistically significant positive correlations were determined between lactose proportion and dry matter (r=0.227; P<0001) and lactose proportion and freezing point (r=0,389; P<0.001), weak but statistically significant negative correlation (r=-0.359; P<0.001) was found between lactose proportion and SCC. This analysis suggests that animals with high lactose levels have low somatic cell counts. According to Table 1, there is a strong and

statistically significant positive correlation between protein content and total dry matter. As a result of this analysis, it was determined that animals with high protein value also had high dry matter value. There is a weak but statistically significant positive relationship protein proportion with freezing point and SCC.

Table 1. Coefficient of correlations among proportions of fat, lactose, protein, total dry matter (TDM), freezing point (FP) and somatic cell count (SCC) in group 1.

	Fat	Lactose	Protein	TDM	FP	SCC
Fat	-	-0,068 ^{NS}	0,545***	0,913***	0,192**	0,094 ^{NS}
Lactose		-	0,064 ^{NS}	0,227***	0,389***	-0,359***
Protein			-	0,758***	0,194**	0,154*
TDM				-	0,301***	0,029 ^{NS}
FP					-	-0,123 ^{NS}

^{NS}: P>0.05; *P<0.05; **P<0.01; ***P<0.001

The results obtained from group II region are provided in Table 2. According to Table 2, there is a positive and statistically significant correlation of fat proportion with total dry matter ratio (r=0.914; P<0.001), freezing point (r=0.222; P<0.001) and SCC (r=0.144; P<0.05). These results indicate that milk of cows with high values in terms of milk fat ratio have higher protein ratios, total dry matter ratios, freezing point and SCC. A strong positive correlation between protein ratio and total dry matter ratio (r=0.702; P<0.001) was determined.

While lactose ratio had a positive correlation with lactose ratio and total dry matter ratio (r=0.286; P<0.001) and freezing point (r=0.608; P<0.001), a negative correlation between lactose ratio and SCC (r=-0.274; P<0.001) was determined. This result indicates that as the lactose ratio in the milk increases, the SCC decreases.

It was observed that as the total dry matter ratio increased the freezing point also increased (r=0.364; P<0.001). There is a weak but statistically significant negative correlation between the freezing point and SCC (r=-0.128; P<0.05).

Table 2. Coefficient of correlations among proportions of fat, lactose, protein, total dry matter (TDM), freezing point (FP) and somatic cell count (SCC) in group 2.

	Fat	Lactose	Protein	TDM	FP	SCC
Fat	-	0,018 ^{NS}	0,511***	0,914***	0,222**	0,144*
Lactose		-	-0,043 ^{NS}	0,286***	0,608***	-0,274***
Protein			-	0,702***	0,049 ^{NS}	0,102 ^{NS}
TDM				-	0,364***	0,069 ^{NS}
FP					-	-0,128 *

^{NS}: P>0.05; *P<0.05; **P<0.01; ***P<0.001

The results obtained from III group region are provided in Table 3. According to Table 3; fat proportion had positive correlation with protein ratio (r=0.381; P<0.001), total dry matter ratio (r=0.856; P<0.001) and freezing point (r=0.152; P<0.05), and a negative but statistically significant correlation between fat and lactose ratio (r=-0.184; P<0.01). These results indicate that milk of cows with high values of milk fat ratio have higher protein ratios, total dry matter ratios and freezing point; while the lactose ratio is lower.

A positive correlation between protein ratio with total dry matter ratio (r=0.690; P<0.001) and freezing point (r=0.182; P<0.01) was determined.

While there was a positive correlation between lactose ratio with total dry matter ratio (r=0.163; P<0.01) and freezing point (r=0.438; P<0.001), there was a negative correlation between lactose ratio and somatic cell count (r=-0.127; P<0.05). This result indicates that as the lactose ratio in the milk increases, the total dry matter ratio and freezing point increase, while the number of somatic cells decreases.

It was observed that as the total dry matter ratio increased, the freezing point also increased (r=0.334; P<0.001).

Tablo 3. Coefficient of correlations among proportions of fat, lactose, protein, total dry matter (TDM), freezing point (FP) and somatic cell count (SCC) in group 3.

	Fat	Lactose	Protein	TDM	FP	SCC
Fat	-	-0,184**	0,381***	0,856***	0,152*	0,112 ^{NS}
Lactose		-	-0,085 ^{NS}	0,163**	0,438***	-0,127*
Protein			-	0,690***	0,182**	0,079 ^{NS}
TDM				-	0,334***	0,076 ^{NS}
FP					-	-0,014 ^{NS}

^{NS}: P>0.05; *P<0.05; **P<0.01; ***P<0.001,0

In a study conducted to determine the relationship between SCC and milk composition, it was shown that as milk SCC increases, fat, protein, lactose and total dry matter content decreases. (Felix *et al*., 2005).

Ayaşan *et al*., (2011), reported that while low or high SCC in milk effect milk fat, milk lactose, nonfat dry matter and density statistically (P <0.05) the effects on milk protein, milk casein, dry matter, acidity, free fatty acid, citric acid and freezing point are insignificant.

Şahin and Kaşıkçı (2014), investigated the two groups in their study and reported that in the group with high SCC, the relation between the number of somatic cells with protein (r=0.145), nonfat dry matter (r=-0.109) and lactose (r=-0.490) were significant (P<0.05), and the correlation of SCC with dry matter, nonfat dry matter, fat, protein and lactose contents were insignificant (P>0.05). They have specified that the relationship between fat content with protein and dry matter content were significant, while the relation between fat content and other milk components is insignificant (P>0.05). They have determined while the relation between milk protein ratio and somatic cell number in group I was negative and insignificant (P <0.05), it was positive and significant in the group II. In the same study, the correlation of nonfat dry matter content and SCC was not positive insignificant (P> 0.05) in group I, whereas the relation in question was negative and significant (P<0.05) in group II In a study conducted by Reis et al., (2013) similar results were found.

Lactose, which affects the freezing point of milk, causes an increase in milk density (Demirci *et al.*, 2010). Şahin and Kaşıkçı (2011) have determined milk lactose levels were 4.1% in the group with high SCC and 4.7% in the group with low SCC (P<0.05).

The relation between milk fat ratio and somatic cell counts in group II were negative and significant. In another study on Holstein cows, it was determined that the correlation between somatic cell count and fat content was 0.21 ($P<0.05$) (Konjačić *et al*., 2010).

Elevated levels of SCC in milk from cows with mastitis cause changes in milk enzymes, which causes the breakdown of proteins and fats. Plasmin, one of these enzymes, increases the breakdown of casein due to elevated SCC in milk. It, therefore, reduces the amount of casein in milk composition (Aytekin and Boztepe, 2014; Rajèevıè *et al*., 2003). In the study we have conducted, it was determined that the increase in the number of somatic cells in milk caused the lactose content to decrease. A similar result was obtained in studies conducted by Şahin and Kaşıkçı (2014). Yet in other similar studies, it has been reported that lactose content decreases with the increase of SCC and that this relation is significant (Reis *et al*., 2013; Cerón-Muñoz *et al*., 2002; Paura *et al*., 2002; Sawa and Piwezynski, 2002).

3. CONCLUSION

The quantity of milk is as important as the quality of milk. The most important criterion affecting the quality of milk is the SCC and the milk components. As SCC increases in the raw milk, milk components are also negatively affected. An emphasis on animal health and welfare in farms, good herd management and udder and equipment hygiene on a producer basis will prevent the increase of SCC, and as a result milk component will be affected positively, hence the amount of milk and healthy milk will increase. Therefore, as per the Turkish Food Codex Communiqué on Raw Milk and Heat Processed Drinking Milk (No: 2000/6) published the Ministry of Agriculture and Rural Affairs, the limitation on SCC found in milk has been determined as ≤500.000 per milliliter (Anonymous, 2000).

Today, pollution of water resources as a result of anthropogenic activities presents a great danger. With industrialization and urbanization, many wetlands are polluted, and this pollution directly or indirectly threatens the health of people and other living organisms. As a result of the studies and the different test methods used, it is clear that water and sediment are contaminated with many genotoxic compounds and that these compounds cause mutagenic effects on different organisms.

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RESEARCH OF COST EFFECT OF OPENNESS AND DECK MATERIAL IN SUSPENSION BRIDGES

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ABSTRACT

Recently, the following rapid development of the national economy and the advancement of communication and transportation, the construction of Long-Span Suspension Bridges have been increasing gradually. Long-Span Suspension Bridges are greatly preferred by engineering designers rather than other bridges equipped with construction supporting systems, not only for economic reasons, but also Long-Span suspension bridges are in the possession of advanced construction techniques when we deal with long distances, for Long-Span suspension bridges acquire supporting constructions features rigged with main cables and ropes. Long-Span suspension bridges are bridge constructions included with cable systems and under strong nonlinear factor effects, which the more the suspension increases, the more the bridges construction lightens, and the more the flexibility grows. There is a lot differences shown in the cost of the Suspension bridges according to different opening. The effect of the deck material is like the effect of the openness. The cost of the suspension bridge in the world is to be discussed too, so the cost is to investigate the effect of opening the drawbridge. The cost of suspension bridges in the world is very controversial, so investigating the financial effect of openness and deck material on suspension bridges is required. In this study, a three-dimensional model of the main cable, suspension rope, deck and tower elements of suspension bridge system by using CSI Bridge Program was constructed and numerical analysis was carried out. The standards used in analysis and design are used the AASHTO LRFD standard, which is common in the world. Suspension bridges of 250, 500, 750, 1000, 1500, 2000 meters were analyzed and designed in different deck material and openings. The material and cost of the suspension bridges have been investigated. The costs according to the openness and deck material of Suspension bridges have been shown by the obtained results.

1. INTRODUCTION

All the discoveries made by mankind are to sustain. It was first discovered places to live, found something to eat, especially invented new tools for hunting and has constructed buildings for shelter. Bridges are one of these examples. The first bridges were built from the throne over the years in 5000 BC to cross barriers or openings.

These structures, which seem to be easy at first, have become more complicated with time due to the increase of openings and the multiple openings by adding columns together.

The complexity and the size of the structure have brought social importance to be the forefront. Thus, bridges are not only structural but also as a social object are to be the forefront (Akca, 2009).

As countries and societies evolve, the importance of transportation is increasing and the need for transportation structures (bridges, viaducts, etc.) increases parallel to this situation, and these buildings become prestige structures. One of these prestige constructions built in developed and developing countries is the large span Suspension bridges (Boduroğlu, 2007).

Suspension bridges are the oldest steel bridge type, not the new inventions developed by the technique. In Eastern Asia, South America and Ecuador Africa, suspension bridges have been used in ancient times. The first suspension bridge was built by the Chinese using chain and ropes. There are only 100-year past of the suspension bridges used today. With the development of steel cables and the use as carrier system material, great developments have been made in suspension bridges (Günaydın, 2011).

The suspension bridge is a type of bridge that has been thought of since the early ages for pass through large openings. In the general, more than 100 m openings are passing, and the construction of suspension bridge instead of concrete and steel construction lattice systems is a more suitable solution (Cengizoğlu, 2006). Suspension bridges can easily be built in large openings with the help of development in engineering and construction technologies (Boduroğlu, 2007).

The suspension bridge is composed of the cables and ropes hanging deck carrying the main load. Since the cable and suspension elements are playful, additional rigidity elements are used to rig the bridge. Additional required rigidity is generally provided by exploiting the bridge deck.

The most important problem in suspension bridges is to eliminate the swing. Since this swing can not be completely removed, the remedy for lowering it to the smallest possible level has always been explored. Today, due to the developing technology possibilities, rigidity is ensured by using bridge decks. To reduce the effects of wind, bridge cross-section aerodynamically shape is formed (Cengizoğlu, 2006).

The main elements of carrying and supporting such bridges are deck system, H tower with suspension cables etc.; Among those factors, The H tower with suspension cables supports the total weight of bridges, even it supports the weight of vehicles, winds, and other heavy loads.

The working principle of the suspension bridges has been tried to be explained simply. Suspension bridges have two carrier edge feet, two main carrier cables and anchorages masses connected by main carrier cables. The road we said the deck where the vehicles pass is suspended to two main carrier cables with the help of cables called suspension rods and usually in vertical position (Karataş et al., 2000).

Suspension bridges, before being built tower and anchoring systems were built. After building the tower, the main cable is pulled. After the main cable is pulled, the hanger ropes are connected to the main cable and by means of a crane the pavers start gradually from the middle of the main opening to an equal number of mounting to the two ends (Günaydın, 2011).

2. MATERIAL AND METHODS

In this study; the analysis and design were carried out with the CSI Bridge program to investigate the cost effect of openness and deck material in suspension bridges commonly used in the passage of large openings. The AASHTO LRFD Specification, which is widely used in the world, was used in the analysis and design in the study (AASHTO, 2012).

The bridge openings covered in the study are as follows:

a. 250 meter
b. 500 meter
c. 750 meter
d. 1000 meter
e. 1500 meter
f. 2000 meter

The deck systems of the bridge considered in the study are as follows:

a. Concrete Deck Suspension Bridges
b. Steel Deck Suspension Bridges

2.1. Suspension Bridge

Bridge is one of the most important engineering structures that have made life easier since the existence of human beings. Since ancient times, bridges, streams, deep valleys, even continents have been making it easier for people to cross. In the past, bridges were made of masonry and wooden materials that could carry small, narrow spans and light loads; today, these bridges are replaced by reinforced concrete and steel bridges. For this purpose, nowadays, reinforced concrete and steel bridges are being built which can carry, large spans and heavy loads.

Suspended bridges are more economical engineering structures than similar ones in terms of passing large openings and using the areas under the bridge comfortably. Suspension bridges are also important in terms of engineering. Considering the high construction costs of these bridges and the logistical importance of the regions in which they are located, it turns out that the structural behavior of such engineering structures should be well defined. Because getting damage to this type of bridges, means that the loss of life and property as well as the lack of transport links (Atabey, 2011).

2.1.1. Basic Characteristics of Suspended Bridges

Suspension bridges are structures used to pass large openings of 550 meters. The edge openings are on the floor. If the distance between the floor and the edge opening is small, the edge openings can be formed by a single hanging clearance built on the floors sitting on the floor, which is a more economical solution from the material point of view. When it is desired to pass large openings, medium and edge openings can be hanged. In the case where the passage of much larger openings is the case, they are designed as four or more openings. Suspension bridge consists of different behavioral elements such as main cable, decks, towers, hanging bar, tower top saddle and anchor blocks (Mubuli, 2016).

2.1.2. Elements of Suspension Bridges

Suspension bridge consists of main elements such as tower, anchor, cable, hangers and decks. On the bases that constitute support for the towers, when it is necessary to construct in the water, they become important because they are expensive and difficult. So multi - span suspension bridge construction is not avoided unless it is necessary.

2.1.2.1. Towers

Suspension bridge towers are reinforced concrete structures built higher than the surface of the deck. Towers are subject to unequal tensile forces by the main cables of openings in the center and edge. However, to prevent overturning of the towers, care is taken to ensure that the horizontal components of the tensile forces are equal. The towers stand box girders forming generally are hollow. Elevators are provided in the towers to provide access to the support

saddles for road level and maintenance. Each tower is composed of two feet and these feet are connected to each other by horizontal or diagonal beams (Atabey, 2011).

2.1.2.2. Cables

The main cables take the load on the deck with hanger ropes and transfer it to the floor through the tower and anchor blocks. The main cables are usually made of galvanized steel wire with a diameter of 5 mm and strength of 160-180 kg / mm2. After the main cables are formed, they are primed and painted after they are covered with soft galvanized wire so that they can be protected from external influences (Atabey, 2011).

2.1.2.3. Hanger ropes

The hanger ropes transfer the load on the deck to the main cable. The hanger ropes transfer the load on the deck to the main cable. Each hanger consists of three elements, hanger-deck connection, hanger-cable connection and hanger itself. The hangers are formed from steel bars, may be in the twisted wire ropes or parallel wire ropes. In modern suspension bridges, the most commonly used type of hanger is those with twisted wire ropes (Atabey, 2011).

2.1.2.4. Deck

Decks are longitudinally structures that emit and carry moving car loads. The conveyor system of the deck to be used by the vehicles is made of reinforced concrete or steel with box section. As material, concrete has high pressure resistance and low tensile stress. The use of concrete is appropriate when the deck of the suspension bridges is reduced by tensile stress cables. Lifting is carried out by means of an itinerant crane arranged between the main cables. The concrete deck is happen large cross section and heavy, while the steel deck is happen smaller cross section and lighter. Concrete and steel decks are widely used in suspension bridges (Atmaca, 2012).

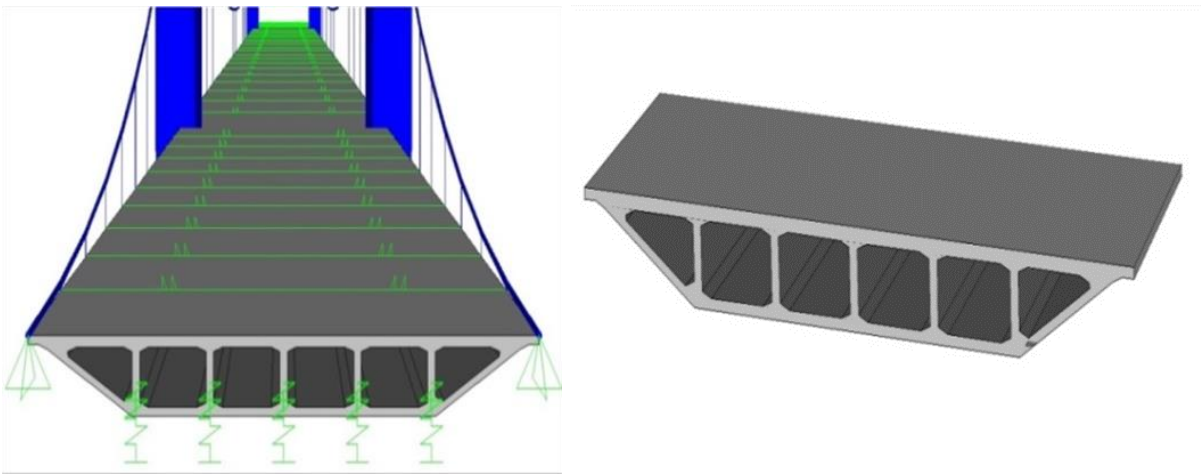

Figure 1 . Concrete Deck Used in Computer Model.

2.2. AASHTO LRFD Standards

AASHTO-LRFD allows for the design of bridges with force-based linear methods for certain bridges, considering the nonlinear, supra-elastic behavior of bridges in terms of material. This regulation replaced the AASHTO directive in the United States. The existing AASHTO regulation is based on the load factor method (LFD), AASHTO-LRFD adopted the design according to the load and strength factor (LRFD). Compared to these two regulations, safe design ranging from 3.2 to 4 times according to changing bridges openings in the LRFD regulations and safe design ranging from 1.5 to 4.5 times in AASHTO regulations (Uluğ, 2008).

2.3. Computer Model of Bridge

The computer model of the build was done using the CSI Bridge program. The bridge carrier system is modeled in 3 dimensions as main cable, hanger ropes, deck and tower and it is evaluated by solution under vertical loads and earthquake loads. In the description of the truck load on the deck, the moving load module of the CSI Bridge program is used. The weights and masses of the carrier elements are automatically calculated by the program. If the weight and mass of the non-bearing elements are (asphalt, railing, etc.), the prepared model is also entered (Uluğ, 2008).

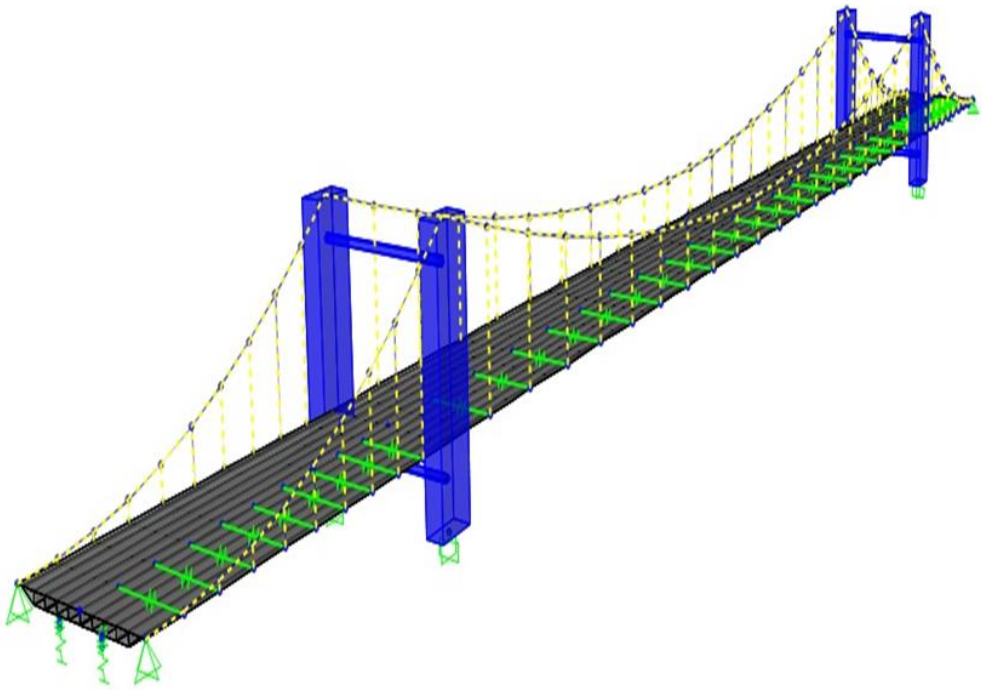

Figure 2. Computer Model of Steel Deck Suspension Bridge.

3. OBTAINED RESULTS

In this study, six different openings suspension bridge analysis were carried out. Suspension bridge at 250, 500, 750, 1000, 1500, 2000 meter openings were modeled using CSI Bridge computer program, also analysis and design was carried out. Sample images of the results of the analysis and design are given in the figures below.

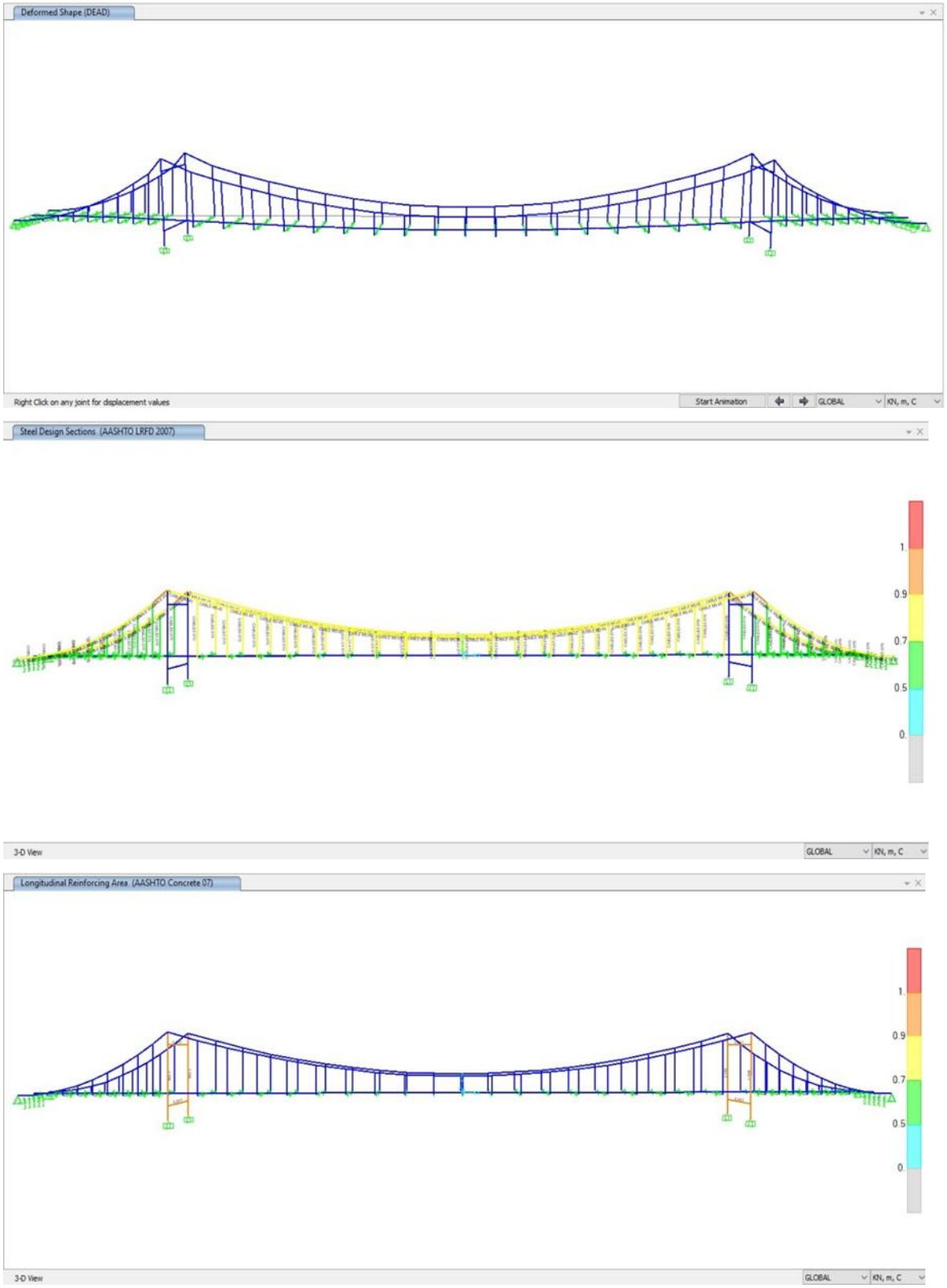

Figure 3- 5. Concrete Section Design

At the end of the design performed, weight and cost calculations of bridges were made. Cost calculations have been denominated in US dollars and using actual prices.

Table 1. Material price list

Element	Unit Price
Cable	1500 $ / ton
Concrete	40 $ / m^3
Reinforcement steel	650 $ / ton
Profile steel	650 $ / ton

After getting the calculations results from the analyses and designs by using CSI bridge program. For the following tables, considering that deck materials that used in this study is concrete and steel, for clear view purposes, the results have been classified as tables for better way to understand it and to explain it according to deck materials and costs for multi bridge spans.

Table 2. Amount of material for the concrete deck suspension bridges

Name of the Bridge	Material Weight (Ton)			
	Total Concrete (Ton)	Total Concrete (m^3)	Total Reinforcement Steel (Ton)	Cable Steel(Ton)
ASM250-BE	37.042	15.434	3.246	765
ASM500-BE	133.085	55.452	13.861	2.700
ASM750-BE	287.464	119.777	27.302	5.823
ASM1000-BE	529.347	220.561	58.030	10.582
ASM1500-BE	1.255.556	523.148	137.949	22.991
ASM2000-BE	2.450.059	1.020.858	252.782	40.344

Table 3. Material Price and Bridge Price for the concrete deck suspension bridges

Name of the Bridge	Material Price ($)				
	Total Concrete Price ($)	Reinforcement Steel Price ($)	Cable Steel Price ($)	Total Price ($)	Bridge Unit Cost ($/meter)
ASM250-BE	617.365	2.110.164	1.146.988	3.874.518	15.498
ASM500-BE	2.218.084	9.009.332	4.050.330	15.277.746	30.555
ASM750-BE	4.791.069	17.746.616	8734349	31.272.034	41.696
ASM1000-BE	8.822.455	37.719.704	15.873.271	62.415.430	62.415
ASM1500-BE	20.925.934	89.667.063	34.486.346	145.079.343	96.720
ASM2000-BE	40.834.310	164.308.390	60.516.045	265.658.745	132.829

Table 4. Amount of material for the steel deck suspension bridges

Name of the Bridge	Material Weight (Ton)				
	Total Concrete (Ton)	Total Concrete (m^3)	Total Reinforcement Steel (Ton)	Profile steel (Ton)	Cable Steel (Ton)
ASM250-CE	10.172	4.239	997	5.260	300
ASM500-CE	47.324	19.718	5.395	10.520	1.056
ASM750-CE	120.556	50.232	13.728	15.779	2.253
ASM1000-CE	244.364	101.818	26.020	21.039	4.133
ASM1500-CE	751.816	313.257	80.094	52.893	12.394
ASM2000-CE	1.748.434	728.514	184.868	102.365	27.684

Table 5. Material Price and Bridge Price for the steel deck suspension bridges

Name of the Bridge	Material Price ($)					
	Total Concrete Price	Reinforcement Steel Price	Cable Steel Price	Profile Steel Price	Total Price ($)	Bridge Unit Cost ($/meter)
	($)	($)	($)	($)		
ASM250-CE	169.541	648.006	449.678	3.418.853	4.686.079	18.744
ASM500-CE	788.730	3.506.706	1.583.743	6.837.706	12.716.885	25.434
ASM750-CE	2.009.267	8.923.462	3.378.969	10.256.559	24.568.257	32.758
ASM1000-CE	4.072.733	16.912.980	6.199.843	13.675.413	40.860.969	40.861
ASM1500-CE	12.530.268	52.061.257	18.591.076	34.380.300	117.562.901	78.375
ASM2000-CE	29.140.575	120.164.342	41.526.227	66.537.503	257.368.647	128.684

The following graphics have been used to view the results comparison between the bridge systems of concrete deck and steel deck. These graphics shows the huge differences in the amount of these materials which affect the costs of the bridges in general.

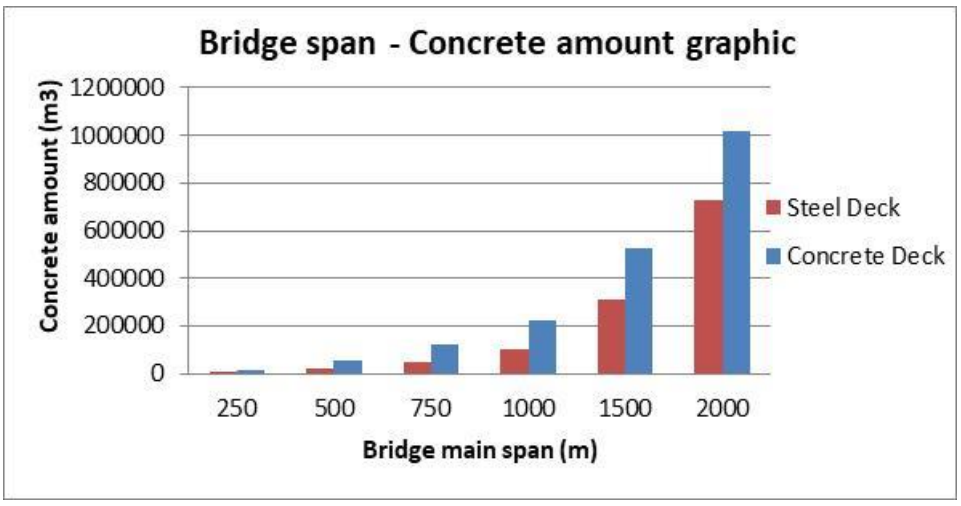

Figure 6. Bridge span - Concrete amount graphic

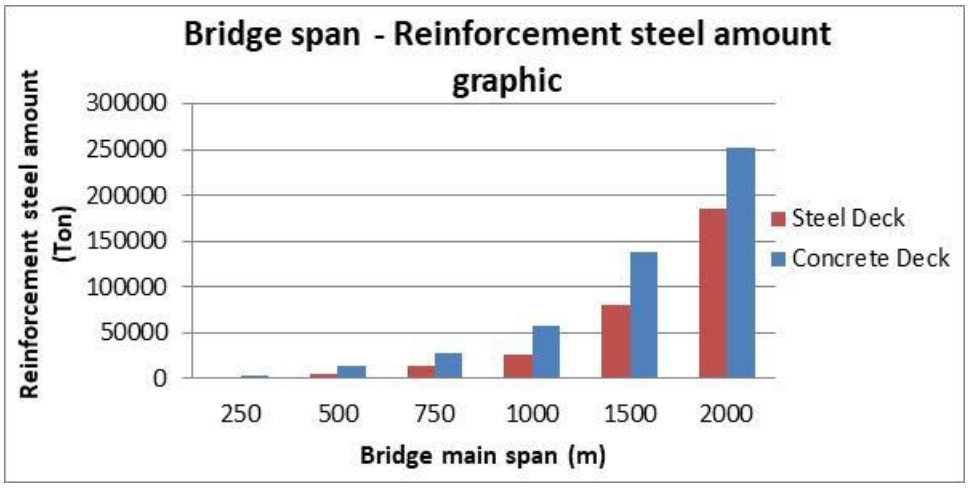

Figure 7. Bridge span - Reinforcement steel amount graphic

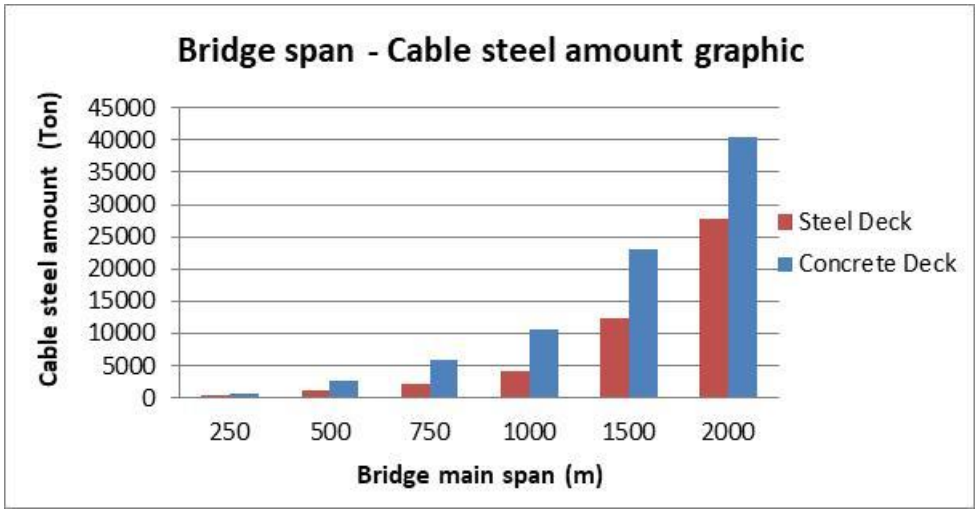

Figure 8. Bridge span – Cable steel amount graphic

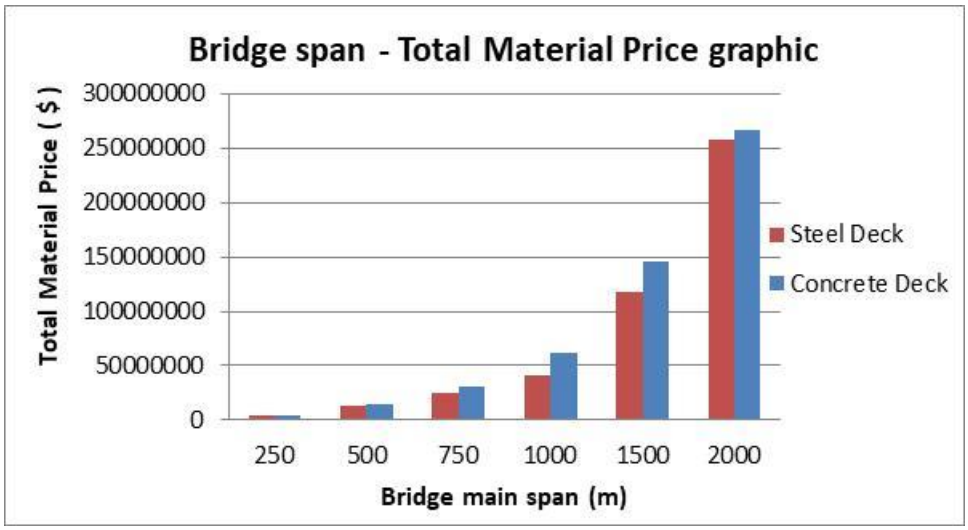

Figure 9. Bridge span - Total Material Price graphic

4. CONCLUSIONS AND RECOMMENDATIONS

At the end of the analysis and cost calculations made, the following results have been achieved.

- Total cost of construction is increasing considerably, is as the case in the span of bridge. However, the increase in the unit construction cost is much higher than the prevailing value of openness. The unit cost is increasing at the higher degree, not proportionally to the passed openness. Namely; despite the 8-fold increase in openness for a 2000-meter clearance with a 250-meter clearance the increase in total construction cost is approximately 69 times for reinforced concrete deck bridges and 55 times for steel deck bridges.
- While the reinforced concrete deck suspension bridge gives economic results for the opening of 250 meters, after this openness, steel deck suspension bridges are becoming more economical.
- With the new studies to be done, similar cost analysis can be done for different bridge building systems and the economics of different bridge building systems can be investigated according to the openings.

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CYTOTOXIC AND MOLECULAR MECHANISMS IN OTOTOXICITY OF CISPLATIN

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ABSTRACT

Ototoxicity is based on inner ear dysfunction creating hearing loss, balance disorder or both symptoms depending on the drug or chemical agent. Genetic and nongenetic risk factors, in addition to dose and time, play important roles in cisplatin ototoxicity. Although the changes firstly begin from first line of outer hairy cells on the Corti organ in the inner ear and then progress. Though the effect on the spiral ganglion and stria vascularis in addition to the Corti organ are well-defined, the molecular mechanisms that cause hearing loss are not fully understood. Cellular and molecular mechanisms and particularly apoptotic mechanisms explain cisplatin cytotoxicity leading to cochlea damage. DNA damage induced by cisplatin and ROS production seem to be mainly responsible for cisplatin toxicity.

Children treated with cisplatin are at risk of early or late hearing loss which could affect learning, communication, school performance, social communication and general quality of life. For this reason, many protective agents are used with cisplatin without changing its antitumoral efficiency. Studies of compounds to prevent ototoxicity may provide compounds for use in routine clinical practice and prevent one of the major dose-limiting side effects of cisplatin therapy, which will increase treatment efficacy and improve patient quality of life.

1. INTRODUCTION

A medication or chemical agent that causes inner ear dysfunction in the form of hearing loss, balance disorders or both symptoms together is said to cause ototoxicity. Ototoxicity occurs due to antibiotics, diuretics, anti-inflammatories, antimalarial medications, antineoplastic agents and some other medications.

The strong antineoplastic medication of cisplatin was first synthesized by Peyrone in 1845. In 1965 de Rosenberg and Cavalieri found platinum complexes that formed in the presence of ammonium and chloride ions. Cisplatin was used for clinical chemotherapy at the beginning of the 1970s as the most active platinum compound for experimental tumor systems (Rosenberg and Cavalieri 1965). Cisplatin (cis-diaminedichloroplatinum [II], CDDP) cisplatin (cis-diamminedichloroplatinum II) cisplatin (cis-diamminedichloroplatinum II) within the group called platinum compounds may be used in the treatment of many malignant diseases like head and neck squamous cell carcinoma, solid testis, ovarian, bladder, prostate, and cervix

tumors and non-small cell lung carcinoma and for pediatric malignancies like neuroblastoma, osteosarcoma, hepatoblastoma and germ cell tumors (Ali et al. 2013, Gunes et al. 2009, Langer et al. 2013, Rybak et al. 2009, Rybak et al. 2007, Sakamoto et al. 2000, Waissbluth and Daniel 2013). Serious side effects limiting clinical use include nephrotoxicity and ototoxicity causing dose limitations. There are many studies on the side effects of cisplatin (Daldal et al. 2007, Fetoni et al. 2004a, Van den Berg et al. 2006).

2. MATERIAL AND METHODS

The ototoxic effect of cisplatin is characterized by temporary tinnitus accompanied by irreversible, progressive bilateral, moderate or severe sensorineural hearing loss beginning at high frequencies and then affecting low frequencies important for perception of speaking (Cooley et al. 1994, Fetoni et al. 2004a, Rybak et al. 2007, Sakamoto et al. 2000, Sluyter et al. 2003, Van den Berg et al. 2006). Ototoxicity is frequently associated with the dose and duration of cisplatin, while some genetic and non-genetic risk factors play a role in this irreversible side effect. Among non-genetic risk factors affecting the incidence of ototoxicity are form of use, cumulative dose, high cumulative dose (> 400 mg/m2), age (children younger than 5 years), bolus injections, dietary factors, malnutrition, anemia, hypoproteinemia, serum protein levels, use with medications that are ototoxic agents especially aminoglycosides, furosemide, gentamicin, amphotericin B and vancomycin, noise, low renal function, previous hearing loss and history of cranial radiotherapy (Cooley et al. 1994, Sakamoto et al. 2000). Severity of hearing loss is related to young age when cisplatin is first administered, number of cycles and high cumulative dose (Allen et al. 1998).

It is well known that as much as side effects, the pharmacological efficacy of a medication is linked to the intake, metabolism, excretion and detoxification of the medication. Genetic factors may affect the metabolic activity of medication. Coding isoenzymes playing a critical role in protection against ototoxicity in tumor cells and shown as a risk factor for cisplatin, five glutathione S transferase (GST) genes were researched by Peters et al. (Peters et al. 2000) in a pharmacogenetic study in the pediatric population. Polymorphism was researched and it was found that GSTM3*B alelle had a protective effect against cisplatin ototoxicity. GST1, GSTT1 and GSTM1 gene polymorphisms are the most common abnormalities in ototoxicity formed by cisplatin. However, there are some debates related to the protective effect of GSTs linked to different tumor types, age, chemotherapy regimes, cumulative cisplatin doses, different analyses and statistical methods (Hirst and Robson 2010, Jamesdaniel et al. 2012b, Olgun et al. 2016a, Sanchez-Gonzalez et al. 2011, Tian et al. 2010, Wang et al. 2007). High megalin expression levels, expressed in marginal cells in the apical section of the inner ear stria vascularis, identified that single nucleotide polymorphisms (SNPs) rs2075252 and rs2228171 played a role in ototoxicity formed by cisplatin (Hirst and Robson 2010, Jamesdaniel et al. 2012b, Riedemann et al. 2008).

The use of chemotherapy combined with radiotherapy or radiotherapy combined with chemotherapy may be very effective on both the formation and degree of ototoxicity (Goldwein).

In spite of the histopathology of cisplatin ototoxicity being well-known, the hearing loss mechanism is not fully known. These mechanisms are still being defined in many studies (Cardinaal et al. 2000, Feghali et al. 2001, Hinojosa et al. 1995, Smoorenburg et al. 1999, Teranishi and Nakashima 2003, van Ruijven et al. 2005, van Ruijven et al. 2004). Ototoxicity has known cellular and molecular mechanisms.

Cellular mechanisms of ototoxicity

Outer hair cell injury

Support cell injury

Nerve cell injury in the stria vascularis

Spiralligament injury

Spiralganglioncellinjury

Molecular mechanisms of ototoxicity

Formation of reactive oxygen species (ROS)

Loss of antioxidant glutathione and formative enzymes

Increased lipid peroxidation rates

Oxidative modifications of proteins

Nucleic acid damage with caspase system activation

S-nitrosylation of cochlear proteins

Changes in protein expression (Chirtes and Albu 2014)

2.1. Cellular mechanisms of ototoxicity

As soon as cisplatin enters cells it is transformed to a highly reactive intermediate product - monohydrate complexes (MHC) – which causes formation of reactive oxygen products and DNA damage resulting in apoptosis and cell death by an “aquation reaction” (intracellular hydrolytic biotransformation). MHC is believed to be the most important cytotoxic agent as it enters reactions with DNA (Deng et al. 2006). Additionally, it is believed that MHC is responsible for the basic toxic side effects of cisplatin (Deng et al. 2010).

Though cytotoxic effects ease the reduction in tumor size and/or prevent tumor development, they limit the anticancer efficacy of cisplatin and significantly limit the quality of life of people struggling with cancer.

Ototoxicity of cisplatin is characterized by cochlea injury (cochleotoxic) especially (Nakai et al. 1982). It affects three areas of the Corti organ in the inner ear (external hair cells), spiral ganglion cells and outer wall (stria vascularis and spiral ligament) (Langer et al. 2013). Cisplatin causes progressive injury to external hair cells in the cochlea, sporadic destruction of internal hair cells, atrophy of the stria vascularis, collapse of the Reissner membrane and damage to support cells in the Corti organ (Laurell and Bagger-Sjoback 1991) .

When used at low doses, initially it causes damage to stereocilia tip-link connections, this is later followed by stereocilia fusion and disorganization (Comis et al. 1986, Fetoni et al. 2004b). At high doses, it causes mitochondrial and endoplasma reticulum damage, loss of stereocilia and hair cells, atrophy of the stria vascularis, collapse of Reissner membrane and damage to support cells (Estrem et al. 1981, Fetoni et al. 2004b, Goncalves et al. 2013).

Hair cells are mechanotransducers in the inner ear necessary for hearing and balance. Hair cell death commonly occurs following exposure to acoustic trauma and to ototoxins like aminoglycoside antibiotics and the antineoplastic agent cisplatin. Loss causes permanent sensorineural hearing loss, balance disorder or both together. To aid in developing therapeutic

strategies, it is necessary to better understand the molecular mechanisms underlying hair cell degeneration.

In response to cochlea trauma, hair cell death may occur through both necrosis and apoptosis routes. Apoptosis of hair cells is associated with caspases and secretion of inflammatory cytokines in response to trauma and MAPK/JNK pathways activated by oxidative stress. Description of pathways causing apoptosis provides therapeutic targets for protection of hearing (Abi-Hachem et al. 2010)

In the stria vascularis, reactive oxygen species (ROS) cause changes in permeability of the mitochondrial membrane and apoptosis of marginal cells. Degeneration of the stria vascularis is one of the first events in development of hearing loss due to cisplatin. Additionally, spiral ligament fibrocytes provide potassium (K+) for endolymph and thus keep the endolymph ionic concentration in balance with vital function for transformation of K+. They have a blood-cochlear barrier function to prevent ototoxic medication effects. Degradation of these fibrocytes has been shown to be strongly linked to hearing loss (Le et al. 2017).

2.2. Molecular mechanisms of ototoxicity

Despite good descriptions of changes initially beginning with external hair cells of the Corti organ in the inner ear and then advancing to other areas, and affecting the spiral ganglion and stria vascularis in addition to the Corti, the molecular mechanisms underlying hearing loss are not fully understood (Jamesdaniel et al. 2012a, Karasawa and Steyger 2015, Kuhlmann et al. 1997, More et al. 2010, Rybak et al. 2007).

CISPLATIN EXHAUSTS THE ANTIOXIDANT SYSTEM IN THE COCHLEA

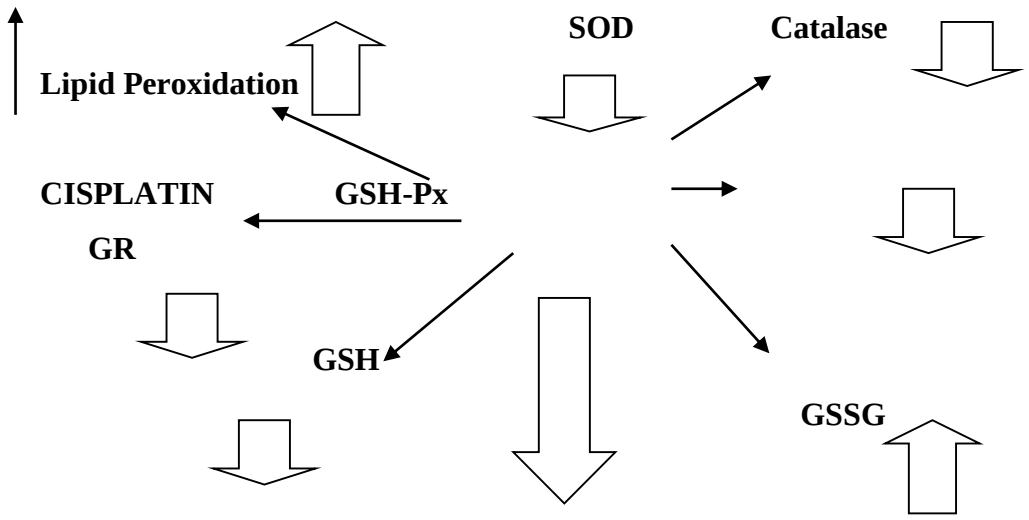

Figure 1. Effect of cisplatin on the antioxidant systems (Rybak et al. 2007)

Cisplatin and metabolites accumulate due to the cochlea being an anatomically closed system and metabolites being difficult to excrete (Ali et al. 2013, Deavall et al. 2012, Devarajan et al. 2002, Ding et al. 2012, Goncalves et al. 2013, Gunes et al. 2009, Langer et al. 2013, Rybak 2007, Rybak et al. 2009, Rybak et al. 2007, Sakamoto et al. 2000, Schacht et al. 2012, Waissbluth and Daniel 2013). The accumulating cisplatin is integrated into cellular DNA

causing dysfunction of protein synthesis including antioxidant enzymes. The cochlea antioxidant systems include glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, glutathione-S transferase and glutamyl cysteine synthetase (Waissbluth and Daniel 2013).Inhibition of antioxidant enzyme activities increases strong reactive oxygen species (ROS) responsible for ototoxicity of cisplatin like superoxide, hydrogen peroxide and hydroxyl radicals within the cochlea and allows the accumulation of toxic lipid peroxides (Clerici et al. 1995).Normally the cochlea has an endogenous antioxidant defense system related to the membrane scavenging ROS and preventing cell death caused by ROS. As a result of interaction of cellular components, the membranes experience damage such as aldehydic lipid peroxidation, oxidative modification of proteins and DNA lesions (Clerici et al. 1995, Giridharan et al. 2012, Ikeda et al. 1993, Mukhopadhyay et al. 2010, Raman et al. 2001).

In animals given ototoxic doses, malondialdehyde levels, an indicator of lipid peroxidation in cochlear tissues, showed reciprocal increase with a reduction observed in glutathione and antioxidant enzyme levels (Banfi et al. 2004, Clerici et al. 1995, Clerici and Yang 1996, Dehne et al. 2001, Kopke et al. 1997, Rybak et al. 2000). Lipid peroxidation in cochlear tissues may result in disrupted activities of antioxidant enzymes and glutathione reductase as much as a reduction in glutathione. The reduction in cochlear antioxidant enzyme activities may be linked to direct bonding of sulfhydryl groups in enzymes to cisplatin reduction in copper and selenium important for superoxide dismutase and glutathione peroxidase activities (DeWoskin and Riviere 1992) increased inactivation of organic peroxides by ROS and antioxidant enzymes (Pigeolet et al. 1990) reduction in NAPDH and glutathione (Somani et al. 2001) necessary for glutathione peroxidase and glutathione reductase activities (Fechter and Pouyatos 2005, Fetoni et al. 2004b, Teranishi and Nakashima 2003, Van den Berg et al. 2006) .

Reactive oxygen species molecularly activate signal transducer and activator of transcription (STAT1) transcription factor beginning inflammatory and apoptotic cascades in the cochlea (Avan et al. 2015, Jamesdaniel et al. 2008, Kaur et al. 2016). Reactive nitrogen species contribute to cisplatin ototoxicity (Giridharan et al. 2012, Manetopoulos et al. 2003) .

Superoxide radicals formed from hydroxyl radicals (OH-) react with unsaturated fat acids in the lipid layer of the cell membrane causing formation of the very toxic aldehyde 4-hydroxynonenal. The increase in aldehyde 4-hydroxynonenal causes increased calcium flow in external hair cells and apoptosis (Sanchez-Gonzalez et al. 2011)It may inactivate antioxidant enzymes and cause release of cytochrome C from damaged mitochondria and then caspase 9, 3 and 7 activation leading to migration of pro-apoptotic Bax protein to the cytosol.

The free radical of NO (nitric oxide) is formed by the nitric oxide synthase (NOS) enzyme in cells. NO production is linked to Ca^{2+}/CaM bonding and has broad distribution in cochlear cells (Fessenden et al. 1994, Hess et al. 1999) . One of the 3 isoforms of NOS described in mammals of nNOS plays a specific role in hair cell physiology (Zdanski et al. 1998) and eNOS is responsible for regulation of cochlear blood flow (Ren et al. 1997). Under normal conditions, iNOS is inactive or not expressed in the cochlea (Gosepath et al. 1997, Hess et al. 1999) and controls transcriptional levels and this isoform produces high rates of NO. NO is a very important gas structure material for hearing function (Fessenden et al. 1994, Gosepath et al. 1997, Hess et al. 1999). The effects of NO are essential for physiologic functions; however, as NO is a free radical excessive production is damaging for cochlear tissues (Raman et al. 2001, Takumida and Anniko 2001, Watanabe et al. 2001, Watanabe et al. 2000).

The 3 isoforms of the nicotinamide adenine dinucleotide phosphate oxidase enzyme (NOX3) (NOX3NAPDH oxidase pathway) only found in the inner ear contribute significantly

to ROS formation and are thought to play a clear role in cochlear oxidation (Watanabe et al. 2001, Watanabe et al. 2000). In cochlear tissue increased ROS causes peroxynitrite formation which enters reactions with nitric oxide (Kaur et al. 2016) ROS in cochlear tissue may increase expression of transient vanilloid receptor potential 1 ion channel (receptor transient potential V1), a member of the transient receptor family of ion channel proteins and expressed by small-diameter neurons and non-neuronal tissues (Corti) (Tian et al. 2010, Wang et al. 2007) This increase leads to calcium loading and increased calcium flow to cells contributing to apoptosis and cell death by caspase activation (Deng et al. 2006, Ross et al. 1983, Watanabe et al. 2001).

2.3. Apoptotic mechanism of cisplatin toxicity

Apoptotic cell death forms the basis of understanding cisplatin toxicity in healthy tissues like the cochlea (Biedler et al. 1978, Bollimuntha et al. 2005, Cheng et al. 2005). Apoptotic cell death is linked to the formation of ROS in the cochlea. Additionally, oxidative stress induced by the medication may trigger a cascade of intracellular reactions leading to apoptosis.

The first mechanism proposed for the cytotoxic mechanism of cisplatin is that it affects nucleophilic structures like guanine and adenine (complex interactions between cisplatin and damaged DNA) (Rausaria et al. 2011). Cisplatin binds covalently to guanine bases in DNA, inducing p53 by inter chain and intrachain crosslink formations, with aberrant genetic transcription preventing normal cell cycle progression and causing cell cycle arrest and apoptosis (Laurell and Bagger-Sjoback 1991). The 2nd proposed mechanism attracts attention to the much-more debated free radical formation and increased ROS (Teranishi and Nakashima 2003). Apart from this, cisplatin ototoxicity is associated with mitochondrial dysfunction (Fechter and Pouyatos 2005). and increased RNS (reactive nitrogen species) (Fetoni et al. 2004b) (37) formation. Reactive oxygen species (ROS) increase lipid peroxidation, change enzyme and structural proteins and cause apoptotic cell death. The medication induced oxidative stress may trigger an intracellular reaction cascade leading to apoptosis (Goncalves et al. 2013).

Studies in recent times have illuminated the roles of molecules containing and affecting the flows of MAPK (mitogen activated protein kinases) and p53. They may trigger mitochondrial pathway mediated apoptotic cascades (Ali et al. 2013, Gunes et al. 2009, Langer et al. 2013, Rybak et al. 2009, Rybak et al. 2007). With the reduction in antioxidant enzymes, increased aldehydes and total lipid peroxidases like malondialdehyde, aldehyde 4-hydroxynonenal and peroxynitrite increase calcium influx to cochlear cells and release of cytochrome c from mitochondria and caspase 9 and caspase 3 activation causes apoptosis (Ali et al. 2013, Deavall et al. 2012, Devarajan et al. 2002, Ding et al. 2012, Goncalves et al. 2013, Gunes et al. 2009, Langer et al. 2013, Rybak 2007, Rybak et al. 2009, Rybak et al. 2007, Sakamoto et al. 2000, Schacht et al. 2012, Waissbluth and Daniel 2013).In response to different ototoxic stimuli, degeneration mechanisms of sensory hair cells generally unite in a single route; caspase activation. The key molecules in the hair cell death mechanism are caspase-9 and -3. The Bcl-2 family is a group of proapoptotic and antiapoptotic molecules regulating caspase activation and flows. Caspase inhibition prevents or delays hair cell death and may protect hearing/balance functions (Cheng et al. 2005).

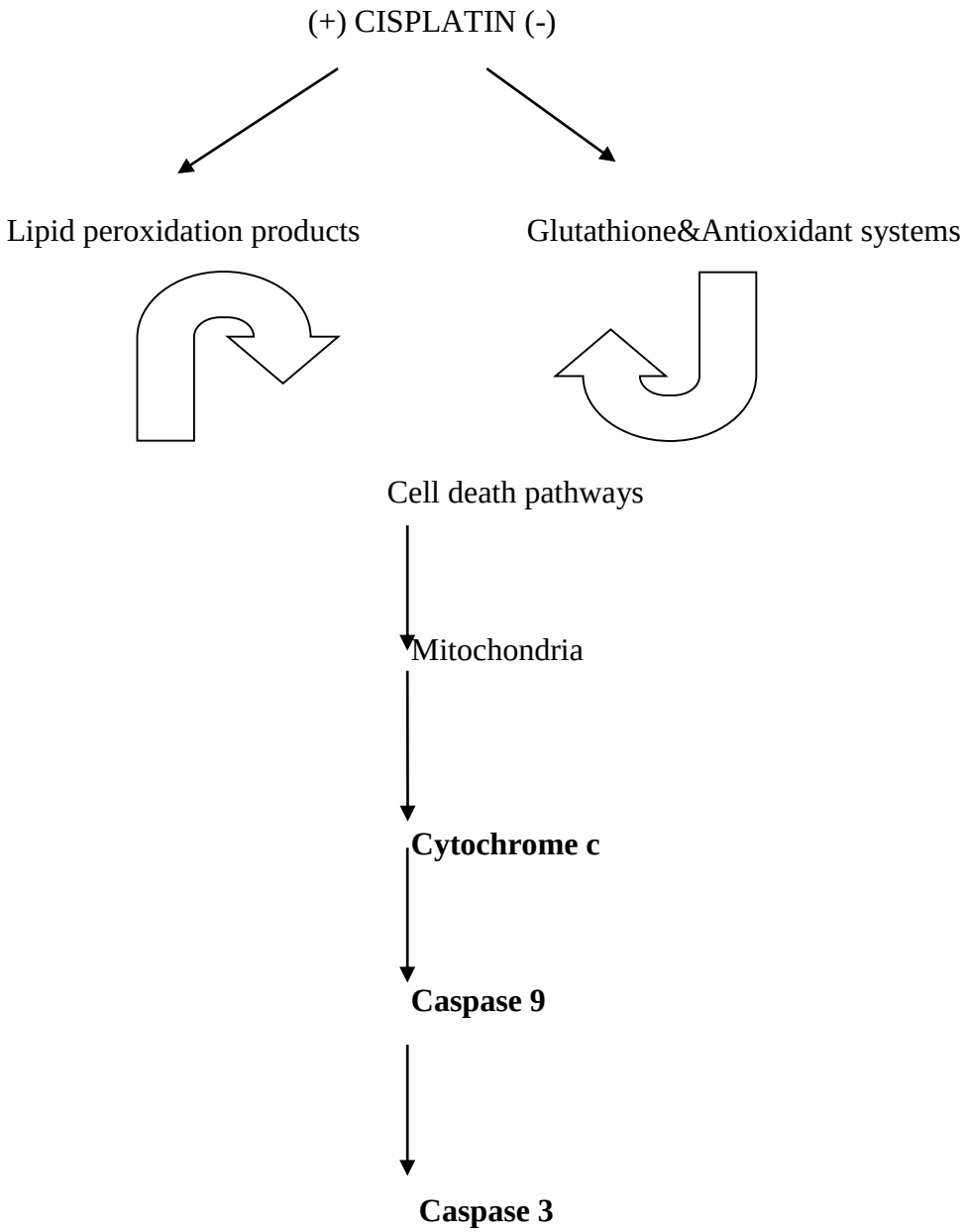

Figure 2. Apoptotic Mechanism of cisplatin toxicity

In short, the apoptotic process encompasses disrupted redox status, increased lipid peroxidation, formation of DNA products and increased production of proinflammatory cytokines (Kim et al. 2014). The role of cytokines in cytotoxicity of cisplatin has been shown by many studies. At cellular level, cisplatin ototoxicity includes a network of complex events including ROS formation, and activation of inflammatory cytokines and stress signal pathways. These events cause cell death due to apoptosis (Boulikas and Vougiouka 2003, Rybak et al. 2007). Apoptotic cell death is reported to form by expression and secretion of proinflammatory cytokines via ERK and NF-KB activation. In HEI-OC1 cells treated with cisplatin, it is noteworthy that there is an increase shown for proinflammatory cytokines of TNF-α, IL-1β, and IL-6with increased activation of MAPKS and NF-κB (Boulikas and Vougiouka 2003, Kim et al. 2014) .

As seen in apoptosis created by cisplatin, there are a variety of signal pathways. Definition of the signal pathways regulating the cytotoxic effects of cisplatin is necessary to discover targets that may be manipulated to prevent side effects among people fighting cancer.
Nitrosative stress-sensitive cells were revealed as an important factor affecting apoptotic signalization (Hirst and Robson 2010). Nitration and nitrosylation of proteins induced by cisplatin has been shown in the cochlea. Nitratified proteins are known cells targeted by cisplatin, but are especially localized in external hair cells (Jamesdaniel et al. 2012a, Jamesdaniel et al. 2012b).Increased nitrogen and nitrite levels (Giridharan et al. 2012, Jamesdaniel et al. 2008, Mukhopadhyay et al. 2010) regulate phosphorylation cascades in protein nitration, easing proteolytic degradation of nitratified proteins and disrupting protein functions and may cause vital changes in cell biological functions.

Behaving as a scaffold protein (Manetopoulos et al. 2003, Sum et al. 2002) and binding with many transcription factors to regulate signal formation as transcriptional regulator is necessary for normal development in both the cochlea and vestibule (Deng et al. 2006, Deng et al. 2010). LMO4 is defined as the most nitratified cochlear protein in ototoxicity induced by cisplatin (Le et al. 2017) (Jamesdaniel et al. 2012a)). In Wistar rat studies of LMO4 and the study by Rajamani et al. it was identified to play a role in easing the occurrence of ototoxic side effects of cisplatin (Jamesdaniel et al. 2012a) . There are studies explaining the regulation of cellular apoptosis with LMO4 (Tian et al. 2010, Wang et al. 2007) . Reduction in LMO4 inhibits epithelial cell proliferation and the increase is a toxic side effect of cisplatin. It was shown to play a potential role in easing cell death and apoptotic responses. Targeted inhibition of LMO4 nitration and/or LMO4 degradation in sensitive cells may be a potential therapeutic strategy to prevent side effects of cisplatin (Rathinam et al. 2015) .

While procedures including high energy radiation and especially doses above >30 Gy for pediatric head and neck tumors, especially, and neuroblastoma, hepatoblastoma, osteosarcoma or germ cell tumors affect the ear, commonly used radiotherapy platinum compounds (cisplatin and/or carboplatin) alone or in combination create significant risks for patients. Hearing loss in children may be more severe as radiotherapy is used more often for head/neck solid tumors compared to adults. Standard therapy for head/neck cancers is CRT (concurrent chemo-radiotherapy) (El-Sayed and Nelson 1996, Munro 1995, Pignon et al. 2005, Pignon et al. 2000, Pignon et al. 2007). Ototoxic effects generally occur on the second day after the start of treatment and may continue until seven days after treatment ceases (Cooley et al. 1994, Feghali et al. 2001, Janning et al. 1998, Rybak et al. 2007, Sakamoto et al. 2000, van Ruijven et al. 2005) The threshold value for hearing loss in patients receiving combined radiotherapy and chemotherapy treatment is 10 Gy (Hitchcock et al. 2009) At doses lower than 40 Gy, patients receiving radiotherapy for head-neck cancers will not have clinically significant hearing loss. However, when patients also receive 100 mg/m^{2}cisplatin, sensorineural hearing loss is high. Cranial radiation increases the possibility and severity of hearing loss (Hitchcock et al. 2009). With density-regulated radiotherapy, some authors have found administration of limited radiation into the inner or middle ear may protect hearing (Plowman 2002). Additionally, as the inner ear is included in the irradiation area, there is more danger of permanent sensorineural hearing loss as a result of radiotherapy including the cochlea. Sensorineural hearing loss forms after a latent period varying from 1.5 years to 5 years after conventional fractionated radiotherapy (Arora et al. 2009, Hua et al. 2008, Jereczek-Fossa et al. 2003, Low et al. 2006, Paulino et al. 2000, Pearson et al. 2006, Wang et al. 2004, Zhang et al. 2009).

This type of reduction in hearing appears to result in loss of cilia cells especially in the basal section and/or spiral ganglion damage in the cochlea. The etiology of these losses is thought to be related to insufficient vascularization, fibrosis and ossification of inner ear fluids (Chen et al. 1999, Zuur et al. 2009) causing atrophy and progressive degeneration of inner ear

sensory structures and abnormalities of the cochlear nerve. Hearing difficulties create negative effects and cause limitations or changes affecting the lives of cancer patients. Chen et al. (Chen et al. 1999) accepted that radiation doses higher than 6000 cGy was the most significant factor for the presence of sensorineural hearing loss. A study by Herman et al. (Herrmann et al. 2006) with 2000-3000 cGy doses to the cochlea found clear hearing loss in 50% of patients and ototoxic effects formed after radiotherapy 41.8% of patients with head and neck tumors. Bhandare et al. (Bhandare et al. 2007, Schultz et al. 2010) attributed complications to the dose of radiotherapy. The authors stated the incidence of ototoxic effects increased when the radiotherapy doses increased from 6000 to 6600 cGy. This study found a total dose of 6500 cGy was statistically significant for the presence of hearing loss.

Children treated with platinum compounds, especially cisplatin, are at risk of early or late hearing loss which may affect learning, communication, school performance, social communication and general quality of life. For this reason, many protective agents that do not change the antitumoral efficacy but reduce the injury caused by cisplatin are used together with cisplatin. Glucocorticoids (dexamethasone, prednisone, methylprednisolone, etc.) and vitamin E are promising potential medication groups for otoprotection(Cooley et al. 1994) . Corticosteroids have been shown to limit ROS formation in the inner ear (Fechter and Pouyatos 2005, Goncalves et al. 2013, Laurell and Bagger-Sjoback 1991).

Vitamin E is a vitamin including tocopherol and tocotrienol species. Due to the lipophilic property of α-tocopherol with highest antioxidant activity, it is a membrane-specific antioxidant forming the first line of defense for unsaturated fat acids in the structure of membranal phospholipids from the effect of free radicals. The lipid peroxyl radical is removed and the lipid peroxidation chain reactions end. Due to this property, it is known as the chain-breaking antioxidant (Rybak 2007, Rybak et al. 2007, Waissbluth and Daniel 2013) Teranishi and Fetoni et al. (Fetoni et al. 2004b, Teranishi and Nakashima 2003) showed that vitamin E has a protective effect against the ototoxicity of cisplatin (Rybak 2007, Rybak et al. 2007, Waissbluth and Daniel 2013) .

There are strong nucleophilic compounds containing sulfur that have the ability to enter interactions with the electrophilic structure of platinum linked to large density of electrons around the sulfur atoms. Amifostine, WR-1065, N-acetyl cysteine, acetyl-l-carnitine, d-methionine, sodium thiosulfate and erdostein thio are among these protective agents as they form complexes with cisplatin due to structure (Altun et al. 2014, Altun et al. 2016, Altun et al. 2010, Gunes et al. 2011, Olgun et al. 2013, Olgun et al. 2014, Tufekci et al. 2009) (Altun et al. 2014, Altun et al. 2016, Altun et al. 2010, Doğan et al. 2014, Gunes et al. 2011, Olgun et al. 2013, Olgun et al. 2016b, Olgun et al. 2014, Tufekci et al. 2009). As ROS formation is the most important factor starting ototoxicity, strategies to prevent ototoxicity include administering free radical scavengers (amifostine, acetyl cysteine, salicylate and vitamin E) to prevent ROS reactions with cellular protein, lipid and DNA. Other strategies include administering compounds that can induce endogenous antioxidant production (acetyl cysteine, sodium thiosulfate, salicylate, d-methionine and ebselen) and agents that prevent ROS formation (allopurinol, erdosteine and adenosine agonists).

Many experimental studies have reported a variety of compounds like acetyl-L-carnitine (Altun et al. 2014, Altun et al. 2016, Altun et al. 2010, Gunes et al. 2011, Tufekci et al. 2009) , resveratrol (Olgun et al. 2013, Olgun et al. 2014) Korean red ginseng (Olgun et al. 2016b) and recombinant human erythropoietin(Doğan et al. 2014) with significant chemoprotective effects on ototoxicity after administration of cisplatin.

3. CONCLUSION

The main factors responsible for toxicity of cisplatin are the induced DNA damage and ROS production. A variety of clinical approaches have been developed to prevent or reduce these effects. In light of results obtained from studies of compounds to reduce ototoxicity, there is no specific agent that can be transferred to routine clinical practice. In addition to the lack of consensus about agents that may be used for otoprotective aims, there is no definite consensus of the administration route for these agents. Today these results are carried to clinical practice by important animal models. The use of stem cells to repair cells and organs that do not have the ability to regenerate is hopeful for ototoxicity. In clinical practice the greatest potential is for amifostine, vitamin E, silymarin and NK-1 receptor agonists (Santabarbara et al. 2016).

One of these studied compounds may have protective ability against ototoxicity and thus may resolve one of the major dose-limiting side effects of cisplatin therapy. Possibly this will increase the efficacy of cisplatin treatment and improve the quality of life of a broad group of patients.

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iNOS IMMUNOREACTIVITY IN THE OVARIES OF RATS FEEDING WITH MUSSEL (Mytilus galloprovincialis)

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ABSTRACT

The ovary is an endocrine and exocrine gland that plays primary role in female development and reproductive activities. The seamen are collecting a lot of heavy metals by serving as filters in the seas. Since the last decades of the nineteenth century and until today, the study of the consequences of human exposure to heavy metals has risen as a central research area in the toxicological field. Among the group of metals with proven human toxicity aluminum (Al) and lead (Pb) are known to be highly neurotoxic. The Mediterranean mussel (Mytilus galloprovincialis) is a species of bivalve, a marine mollusc in the family Mytilidae. In our previous researches, we found Al, zinc (Zn) and iron (Fe) values were higher in mussels taken from Çamburnu region of the Dardanelles. In many tumors, inducible nitric oxide synthase (iNOS) expression is high, however, the role of iNOS during tumor development is very complex and quite perplexing, with both promoting and inhibiting actions having been described. The purpose of the study is to demonstrate the iNOS immunoreactivity in the ovarian tissues of rats which are fed with mussels that are collected from the Çamburnu region of the Dardanelles. The mussels given as food to the rats were removed from the Çamburnu region of the Dardanelles. Average 100±10 g weight were selected. After the beaks were overcooked, the meat broke off and the meat at 100 degrees was dried. Two groups of rats are included in the study, group 1 (n=6), control group fed with standard rat food, group 2 (n=6), 90% mussels and 10% standard rat food daily. To detect iNOS localization in the tissues, the LAB-SA Detection System was used. iNOS immunoreactivity was detected in the interstitial cell cytoplasm of the ovaries of rats fed with mussel. iNOS immunoreactivity was also observed in germ cell cytoplasm in some primordial follicles. There was statistically significant difference between the iNOS immunoreactivity of the interstitial cells in the ovarian parenchyma of the rats in the experimental and control groups (p> 0.05).

1. INTRODUCTION

Pb is a heavy metal with no known biological function in humans. On the contrary, it can damage various systems of the body including the hematopoietic, renal and skeletal systems with the central nervous system being its primary target [Wilson et all., 2000]. The susceptibility to Pb toxicity is influenced by several factors such as environmental exposure, age and nutritional status. Human exposure to Pb occurs via food, water, air and soil. Food

and water Pb sources include the use of Pb-containing ceramic dishware, metal plumbing, and food cans that contain Pb solder (White et al., 2007).

Oxidative stress, oxidative damage to cellular components, and the activation of oxidant sensitive transcription factor could in part underlie some of the toxic effects of Pb. The deleterious effects of Pb can involve both, ROS and reactive nitrogen species. Oxidative stress has been associated with Pb exposure in humans and in experimental animal models. In humans occupationally exposed to Pb, biomarkers of oxidative stress such as malondialdehyde, GSH status, glutathione peroxidase and catalase, exceeded the mean value of the control population (Costa et al., 1997; Garcon et al., 2004; Devi et al., 2007).

The interference with nitric oxide production might represent another mechanism accounting for Pb neurotoxicity. Pb affects neuronal nitric oxide synthase (nNOS) in the developing rat brain. Rats perinatally exposed to Pb had decreased nNOS protein levels and activity in cerebellum and hippocampus at PND 21 and 35 as compared to their age-matched controls (Chetty et al., 2001).

In general, perinatal rat exposure to low levels of Pb causes an increased sensitivity of brain dopamine D2 and D3 receptors (Cory-Slechta et all. 1992; Gedeon et al., 2001), increased hippocampal activity of tyrosine hydroxylase [Bielarczyket et all., 1998], produces higher levels of dopamine (Leret et all., 2002; Devi et al., 2005), and enhances catecholaminergic neurotransmission in cerebral cortex, hippocampus and cerebellum due to increased turnover of norepinephrine (Devi et al., 2005). On the otherhand, perinatal exposure to high Pb concentrations decreases norepinephrine, epinephrine and dopamine levels in the cerebral cortex, hippocampus and cerebellum (Devi et al., 2005; Dubas et al., 1978; Sidhu et al., 2003), and decreases the activities of acetylcholinesterase (Sidhu et al., 2003), monoamino oxidase (Devi et al., 2005), and tyrosine hydroxylase (Mc Intosh et al., 1989).

Lead intoxication has been shown to promote atherosclerosis in experimental animals. There are also indications that chronic lead exposure may affect systemic lipid metabolism (Reviset al., 1981). Current evidence on lead-induced oxidative stress has been based mostly on in vitro experiments (Ding et al., 2000) or studies conducted in animals (Fowler et al., 2004).

In this study, it was aimed to determine the iNOS immunoreactivity in the ovarian tissue of rats fed with mussels containing heavy metal salts.

2. MATERIAL AND METHODS

2.1. Ethics Statement

A total of 12 female Wistar albino rats, weighing 300±10 g, were used in the study. The study protocol was approved by the Çanakkale Onsekiz Mart University Ethics Committee for Animal Research (ÇOMÜ- HADYEK / 2011-08-08).

2.2. Animal Model

The rats were kept for 30 days under appropriate conditions of temperature/humidity and a 12-h light cycle while being provided sufficient water and feed. The rats were randomly selected and divided into 2 groups. The first group (n: 6) was the control group; standard rat diet. Experimental group (n: 6); 90% mussel + 10% standard rat diet. Rats were fed twice daily for 30 days at 15% of their weight every morning and evening at the same time. The mussels given as food to the rats were removed from the Dardanelles Çamburnu region (Photo 1). Average 100±20 g weight were selected. After the beaks were overcooked, the meat broke off and the meat at 100 degrees was dried.

It was weighed into each rat's weight and 10 mg/kg intraperitoenal ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey), and 20 mg/kg of alfazyne 2% (Rompun, Bayer Turkey Pharmaceutical Ltd., Istanbul, Turkey) were anesthetized and taken right ovary.

The rats, which had been hatchlings, were treated after the operation and delivered to the animal shelter for further investigations.

2.3. Histological evaluation

The ovarian tissues were maintained in immunofix (Leica) for 24 hours for histopathological examination. The paraffin embedded ovarian tissues were stained with hematoxylin and eosin (H & E) at a thickness of 5 microns. Immunohistochemical staining method was applied by cutting the paraffin embedded ovarian tissues 3 microns in thickness.
The LAB-SA Detection System, (Histostain-Plus Bulk Kit, Invitrogen) was applied to determine immunohistochemical localization of iNOS enzyme in tissues. Sections taken from paraffin blocks were deparaffinized and rehydrated. Subsequently, 3% H_2O_2 was applied to the sections to block endogenous peroxidase activity.

The sections were incubated in citrate buffer (0.1 M, pH: 6.0) in the microwave (800 watts, 10 min) for antigen retrieval, and the samples were washed with phosphate buffer solution (PBS, 0.1 M, pH 7.2). After the samples had been incubated in the blocking buffer for 10 min, they were washed with PBS. Next, slides were incubated with anti-iNOS (inducible nitric oxide synthase) antibody (anti-NOS2, Santa Cruz Biotechnology), which was diluted at 1: 400 for the ovarium, for an hour at room temperature, and they were then washed with PBS. Afterwards, biotinylated secondary antibody was applied to the samples for 30 min (Ultravision Detection System, Thermo Scientific, Fremont, USA). Then the samples were washed with PBS again and incubated with Broad Spectrum Antibody (Invitrogen, USA) for 30 min. After washing the samples, diaminobenzadine-tetrahydrochlorid (DAB, Invitrogen Corporation) was applied to them. Negative control was used to determine specific iNOS immunoreactivity, and hematoxylin stain was used as a nuclear counter stain.

Dye samples were evaluated on the Zeiss AXIO Scope 1 brand research microscope. Analysis of iNOS immunoreactive cells in the ovarium parenchyma was performed using the Leica LAS V3.8 image analysis system. The Mann Whitney U-test was used the Non Parametric tests to determine the differences between the experimental group and the control group.

3. RESULT

In the ovarian tissue samples from the rats in the experimental groups, extensive positive iNOS immunoreactivity was observed in the interstitial cell (Figure 1) and germ cell cytoplasm in the primordial follicle (Figure 2) when the anti-NOS2 antibody was applied.
iNOS immunoreactivity was not observed in the same tissue samples without iNOS antibody (Figure 3).

Positive iNOS immunoreactivity was not detected in the ovarian tissue samples from rats in the control group when anti-NOS2 antibody was applied (Figure 4).
Statistical Analysis

There was statistically significant difference between the iNOS immunoreactivity of the interstitial cells in the ovarian parenchyma of the experimental and control groups ($p > 0.05$).

4. CONCLUSION

In our study, positive iNOS immunoreactivity was detected in germ cell cytoplasm in interstitial cells and primordial follicles in the ovaries of rats fed with mussels containing heavy metal salts such as lead, copper, aluminum, zinc and iron.
Various in vitro and in vivo studies have explored the underlying mechanisms by which chronic low level lead exposure can raise arterial pressure, thereby cardiovascular disease (CVD) development. These studies have identified the involvement of oxidative stress and inflammation

(Vaziri et al., 1997), by promoting endothelial dysfunction (Vaziri et al., 1995), promoting vascular smooth muscle cells proliferation and transformation (Fujiwara et al., 1995), and impairing NO homeostasis (Ding et al., 1998).

iNOS catalyzes the production of NO, which has an important role in the maintenance of vascular regulation and immune system (Willerson et al., 2004).

Heavy metals are toxic because they may have cumulative deleterious effects that can cause chronic degenerative changes [Ibrahim et all., 2006], especially to the nervous system, liver, and kidneys, and, in some cases, they also have teratogenic and carcinogenic effects (IARC 1987). The mechanism of toxicity of some heavy metals still remains unknown, although enzymatic inhibition, impaired antioxidants metabolism, and oxidative stress may play a role. Heavy metals generate many of their adverse health effects through the formation of free radicals, resulting in DNA damage, lipid peroxidation, and depletion of protein sulfhydryls (e.g., glutathione) (Valko et al., 2005).

Although not fully proven, Al accumulation in the brain is proposed to be associated with neurodegenerative diseases, including Alzheimer's dementia, Parkinson's disease, amyotrophic lateral sclerosis, and dialysis encephalopathy (Gonçalves et al., 2007). Nutritional status is another significant risk factor for Pb intoxication and its effects. Iron, zinc and calcium deficiencies increase the retention of ingested Pb, which can also increase Pb gastrointestinal absorption (Goyer, 1996; Ruff et al., 1996), and affect the susceptibility to Pb neurotoxicity (Aimo et al., 2006). Human exposure to Al is mainly caused by environmental factors, such as soil contamination (Yokel et al., 2008). Al absorption are the gastrointestinal tract (Ittel, 1993).

Minerals play a critical role in iNOS expression. High levels of copper increase iNOS expression in lung, liver, and aorta (Cuzzocrea et al., 2003), thus demonstrating thsatexessive copper can have detrimental effects on both constitutive and inducible NO synthesis. The combined effects of increased iNOS expression and decreased eNOS activity in the same anatomical location could have profound consequences on inflammatory processes of cells within the cardiovascular bed. Iron either increases iNOS expression in macrophages and proximal tubules (Chen et al., 2001) or suppresses elevated iNOS protein levels in the hearth and kidney (Ni et al., 1997). Thus, iron excess or deficiency can impair immunlogical, cardiovascular, and renal function. In addition, zinc modulates iNOS expression in the small intestine, thereby preventing cytokine-induced diarrhea (Cui et al., 1997). In addition, chromium and lead inhibit, but nickel and cobalt incresae, iNOS expression in activated macrophages (Tian et al., 1996), suggesting an important role for NO in mediating the cytotoxic effects of environmental contamination by metals.

In pathological conditions, nitric oxide (NO) is a molecule of nitric oxide synthase (NOS) synthesized from L-arginine, which has a short potent free radical structure. As a result of high NOS production in pathological conditions, NO becomes neurotoxic and plays a role as cytotoxic molecule in the progress of secondary damage as a free radical.

Our previous researches have shown that some mollusc species such as mussels grown in the Dardanelles, sea grass, sea chestnut, aquiva, and heavy metals such as iron, zinc, aluminum, lead and copper are found in sea water (Demir et al., 2011; Gezen et al. 2011; Ozkurnaz et al., 2012; Gezen et al. 2011).

In our work, detection of positive iNOS immunoreactivity in germ cell cytoplasm of interstitial cells and primordial follicles in the ovaries of rats fed with mussels containing heavy metal salts such as lead, copper, aluminum, zinc and iron in our study suggests that heavy metal salts may promote iNOS release.

The ovary is one of the endocrine glands that plays an important role in the maintenance of the reproductive activities [Carr, 1998]. The presence of iNOS immunoreactivity in these cells is thought to impair the production of gonadal hormones secreted from these cells and may result in low fertility results.

Footnotes

**At the time of this research, Dr. Aslı MURATLI was working at Department of Pathology of Çanakkale Onsekiz Mart University.

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FIGURES

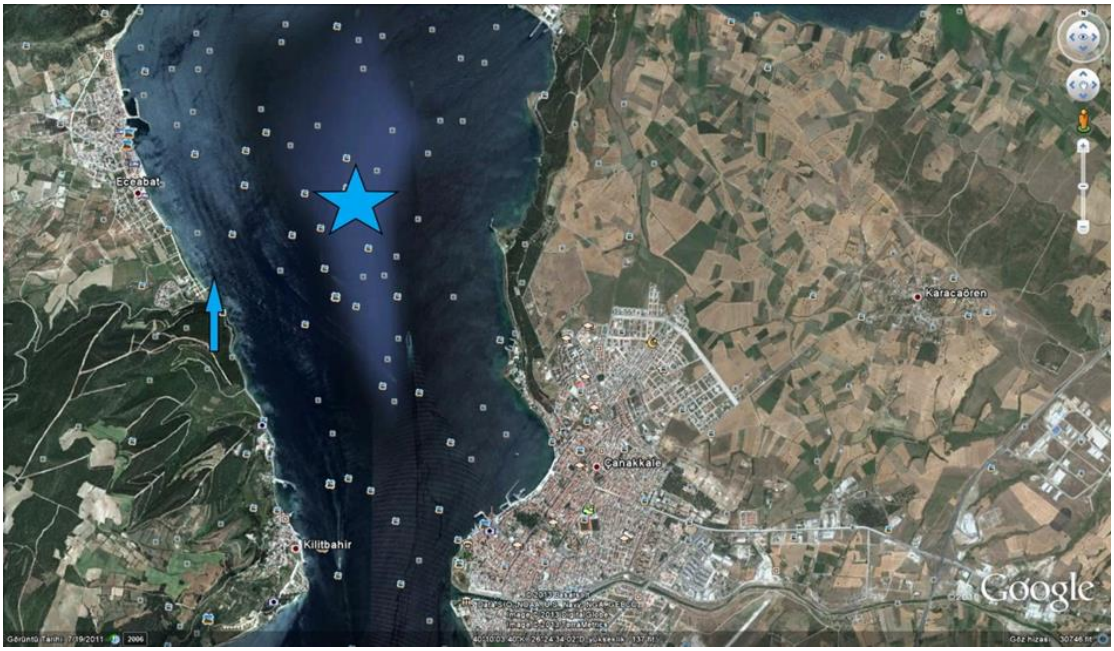

Photo 1. The area where the mussels are collected
Arrow: Çamburnu region (Çanakkale, Turkey)
Star: Dardanelles

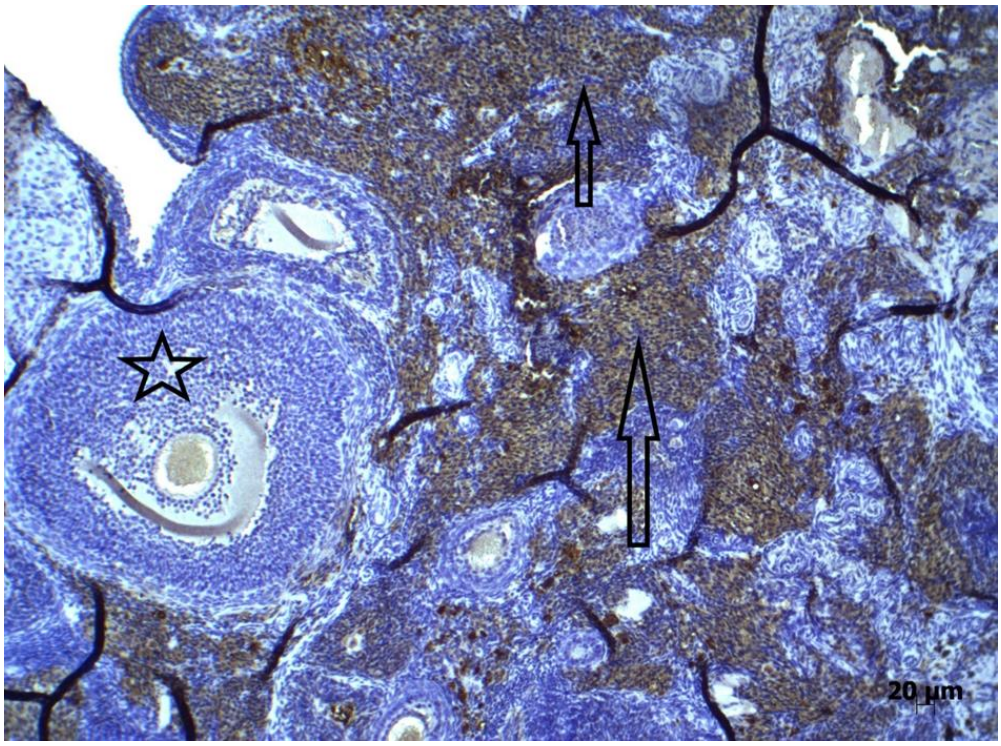

Figure 1. Experimental groups; 90% mussels + 10% standard rat diet standard rat feeds were given daily. Rat ovarium, (iNOS x10).
a. Arrow: Positive staining of iNOS in the cytoplasm of interstitial cells,
b. Star: Secondary follicle

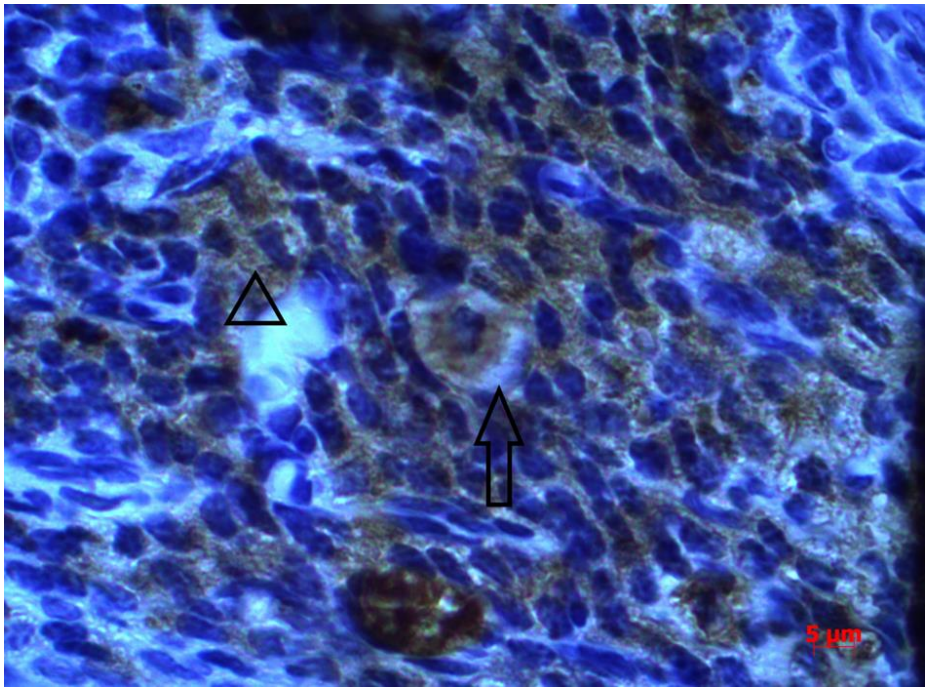

Figure 2. Experimental groups; 90% mussels + 10% standard rat diet standard rat feeds were given daily. Rat ovarium, (iNOS x100).
a. Arrow head: Positive staining of iNOS in the cytoplasm of interstitial cells,
b. Arrow: Positive staining of iNOS in the cytoplasm of primordial follicle,

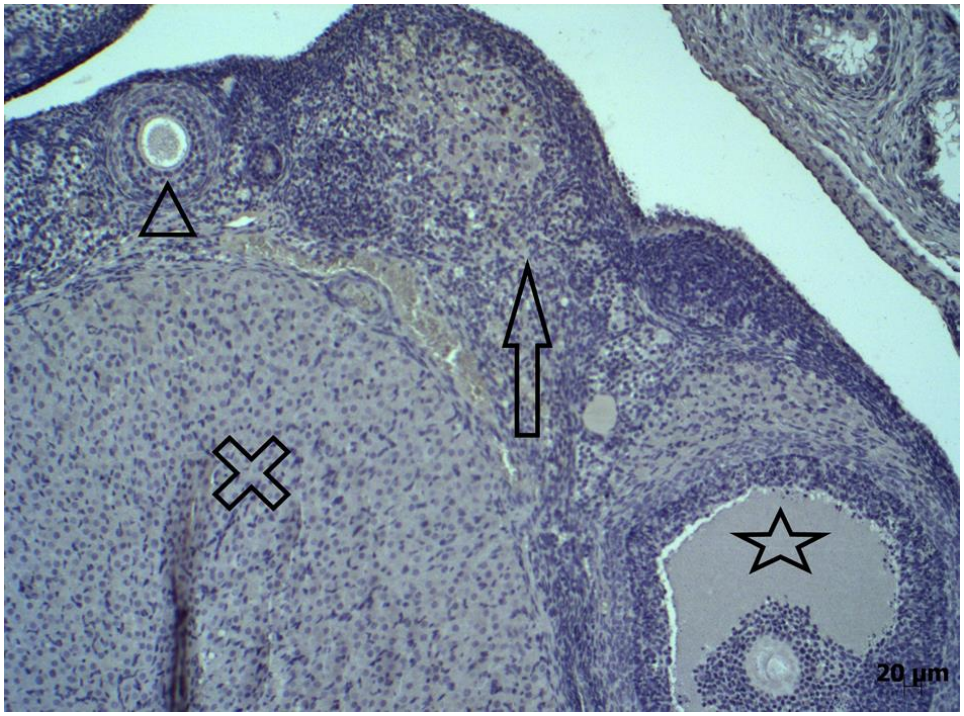

Figure 3. Experimental groups; 90% mussels + 10% standard rat diet standard rat feeds were given daily. Rat ovarium, Negative control staining of iNOS
a. Star: Secondary follicle,
b. Arrow: Interstitial cells,
c. Arrow head: Primary follicle,
d. Plus: Corpus luteum,

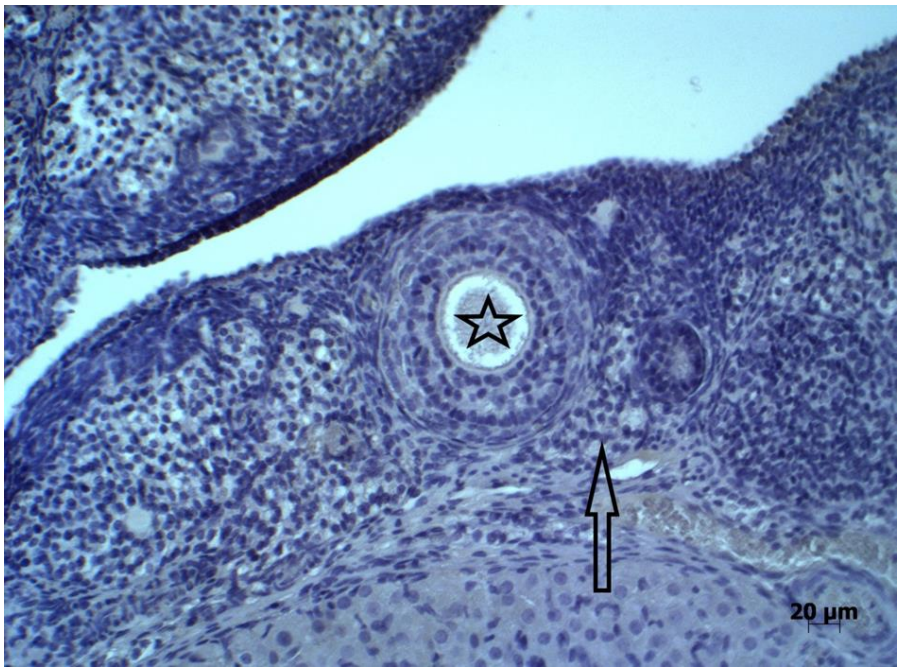

Figure 4. Control groups; 100% standard rat diet were given daily. Rat ovarium, (iNOS x20).
a. Arrow: Interstitial cells
b. Star: Primary follicle,

Abbreviation:

iNOS: inducible nitric oxide synthase, NOS: nitric oxide synthase, eNOS: endothelial nitric oxide synthase, CVD: cardiovascular disease, Al: aluminum, Pb: lead, H & E: hematoxylin and eosin, nNOS: neuronal nitric oxide synthase, NMDAR: N-Methyl-D-aspartate receptor

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HISTOPATHOLOGICAL CHANGES IN THE STOMACH TISSUE OF RATS FED WITH MUSSELS (Mytilus galloprovincialis)

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ABSTRACT

Dardanelles is exposed to environmental pollution for many years. The strait is important in terms of fish migration and shellfish farming. Fish and shellfish obtained from the strait is frequently consumed by local people. Mussels serve as useful indicators of temporal trends in environmental quality because they accumulate some contaminants in their tissues at levels many time higher than in the surrrounding water and quickly to changes in contamination. Although there are few data about the toxicity of seafood that is exposed to environmental pollution, there are no animal studies about the gastric toxicity of mussels grown in the Dardanelles. Our previous studies demonstrated that the water and mollusc from certain regions of the Dardanelles contained heavy metal salts. The purpose of the study is to demonstrate the histopathologic changes in the gastric tissues of rats which are fed with mussels that are collected from the Çamburnu region of the Dardanelles. Four groups of rats are included in the study, group 1 (n=6), control group fed with standard rat food, group 2 (n=6), 75% mussels and 25% standard rat food daily, group 3 (n=6), 75% mussels and 25% standard rat food every two days, group 4 (n=6), 75% mussels and 25% standard rat food every three days. After the routine histopathologic processing all gastric tissue samples are evaluated in terms of 8-hydroxy-2' -deoxyguanosine (8-OHdG) immunoreactivity with light microscopy and image analysis software. No histopathologic differences found in standard hematoxylin-eosin (H.E.) stained gastric tissue samples of the control group. In the second and third groups, a small amount of mononuclear cell infiltration was detected. In the fourth group, no significant difference was observed in mononuclear cells. In immunohistochemical staining, 8-OHdG immunoreactivity in gastric epithelial cells. 8-OHdG immunoreactivity was negative in gastric tissues in all groups. There was no statistically significant difference between the groups that were fed every day, every other day and every three days with clam (p>0.05).

1. INTRODUCTION

All heavy metals are potentialy harmfull to most organisms at some level of exposure and absorption. Aquatic animals are also exposed to elevated levels of heavy metals. Some trace metals are essential in low concentrations fort he metabolism of animals, but in the excess all trace metala are toxic (Rainbow 1997). Trace metals such as Cd, Pb, Hg, Zn accumulate mainly at the hepatopancreas, gonads and in the gills of molluscs and shellfishes. Therefore, mussels especially have been used as biological indicator organisms to monitor marine pollution by toxic heavy metals and potentially toxic chemicals due to their own properties of inhabitation (Pempkowiak et al., 1999; Hu, 2000). In recent years, heavy metal accumulation in fish and other aquatic organisms has been investigated along the coasta of Turkey. Sea of Marmara,

Aegean Sea, Black Sea and Mediterranean Sea of have been exposed to more heavy metal pollution depending on industrial pollution from the different facilities, took place around it (Unsal and Besiktepe, 1994; Egemen et al., 2004; Goksu, 2005; Turkmen et al., 2005). The accumulation and effects of the organism depend on the characteristics of the composition of the arsenic and can cause chronic effects, DNA damage or cancers in the embryo (Dons and Beck 1993, Berg et al..1997). Guevara et al. (2004) found that some heavy metals (Ag, As, Cr, Hg, Sb, Ba, Br, Ca, Co, Cs, Fe, Na, Sr, Zn), which are considered as potential contaminants, are in high amounts in the soft tissues of Diplodon chilensis (Gray 1828) type mussels. The level of heavy metal accumulation in water creatures depends on age, habitat and nutritional behavior. Heavy metal concentrations in coastal areas and closed seas are higher than open seas (Egemen 2000). Gezen (2011), Demir (2011) and Özkurnaz (2012) have detected heavy metals in sea water and many molluscan species that growing in the Dardanelles.

Heavy metals, such as mercury, cadmium, lead, copper, nickel, zinc, chromium and arsenic, have gained significance because they are toxic on certain concentrations and can increase their concentration during transition from one organism to another. Mussels are living organisms that are fed by filtering organic matter and phytoplankton in the water. The mussels can also filter toxic substances during water filtration. Pollution travels along food chains and can harm all living things, including humans (Julsham and Grahl-Nielsen 1996, Bat et.al. 1999).

Recent studies have suggested a close association between chronic inflammation and carcinogenesis in some organs. For example, patients with chronic viral hepatitis, inflammatory bowel disease, and Helicobacter pylori-associated gastritis are at higher risk for hepato-cellular carcinoma, colon cancer and gastric cancer, respectively. Although the precise mechanism sunderlying the development of malignant neoplasmsduring the course of chronic inflammation remain unclear, previous studies have suggested a possible involvement of oxidative DNA damage in this pro-cess (Seki et al.2002).

There is no research revealing histopathologic changes in the stomach tissues of living beings fed with mussels collected from the Çamburnu region in the Dardanelles (Çanakkale, Turkey).

The purpose of the study is to demonstrate DNA damage and the histopathologic changes in the gastric tissues of rats which are fed with mussels that are collected from the Çamburnu region in the Dardanelles (Çanakkale, Turkey).

2. MATERIAL AND METHODS

2.1. *Ethics Statement*

A total of 24 male Wistar albino rats, weighing 290–310 g, were used in the study. The study protocol was approved by the Çanakkale Onsekiz Mart University Ethics Committee for Animal Research (Protocol number: 2010/09-03).

2.2. *Animal Model*

The rats were kept for 30 days under appropriate conditions of temperature/humidity and a 12-h light cycle while being provided sufficient water and feed. The rats were randomly selected and divided into 4 groups. For the first study group (n: 6), was the control group; standard rat diet was given every days. For the second study group (n: 6), 75% mussels + 25% standard rat diet standard rat feeds were given daily. For the third study group (n: 6), 75% mussels + 25% standard rat diet was given every two days. Standard rat diet was given the other day. For the fourth group (n: 6), 75% mussels + 25% standard rat diet was given every three days. Standard rat diet was given the other two day.

Rats were fed twice daily for 30 days at 15% of their weight every morning and evening at the same time. The mussels given as food to the rats were removed from the Dardanelles Çamburnu region (Figure 1). Average 70 ± 10 g weight were selected. After the beaks were overcooked, the meat broke off and the meat at 100 degrees was dried.

It was weighed into each rat's weight and 10 mg/kg intraperitoenal ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey), and 20 mg/kg of xylazine 2% (Rompun, Bayer Turkey Pharmaceutical Ltd., Istanbul, Turkey) were anesthetized. The rats were anesthetized and then sacrificed. After the rats have received the stomachs other organs were also taken for further research.

2.3. *Histological evaluation*

The stomach tissues were maintained in immunofix (Leica) for 24 hours for histopathological examination. The paraffin embedded stomach tissues were stained with hematoxylin and eosin (H & E) at a thickness of 5 microns. Immunohistochemical staining method was applied by cutting the paraffin embedded stomach tissues 3 microns in thickness.

For 8-hydroxydeoxyguanosine (8-OHdG) staining, tissue samples were incubated at 37 ° C in Proteinase K (dilution 1: 30, Millipore Corporation) for 40 min. and then waited in the laboratory until the room was warmed up. Subsequently, tissue samples were resuspended in 0.2% Triton X 100 (Santa Cruz Biotechnology) solution prepared with Phosphate Buffer Saline (PBS, Invitrogen) for 5 min. were kept. This allowed better passage of solutions from the pores in the cell and nucleus membranes. The tissue samples confined to the Pap pen were washed three times with PBS for 3 min. Peroxidase block solution (Diagnostic BioSystems) was placed on each tissue sample to block the peroxidase enzyme in the cell and incubated for 30 min. waiting for a while. Subsequently, this solution was dissected and each tissue sample was washed three times with PBS for 3 min. (LAB-SA Detection System, Histostain-Plus Bulk Kit, Invitrogen) was placed on each sample and the remaining PBS solution was rinsed with the drying paper and incubated for 30 min. waiting for a while. Subsequently, each tissue sample was purged with drying paper by pouring serum blocking solution. Tissue specimens we separated as negative controls were loaded with serum blocking solution and polyclonal goat anti-8-Hydroxydeoxyguanosine (8-OHDG, dilution 1: 200, Millipore Corporation) primer antibodies on other tissue samples. Subsequently, each tissue sample was washed three times with PBS for 3 min. The PBS solution remaining around each sample was purged with the drying paper and a solution of 3,3'-Diaminobenzidene tetrahydrochloride (DAB, Invitrogen Corporation) was added as a chromogen. kept in dark place for a while. At the end of this period, DAB solution was poured over the tissue specimens and water was dewatered. Tissue samples were taken 2 times and 2 min. for 5 minutes in Mayer's Hematoxylin for counter-staining. and kept in the tap water for 10 minutes. Washed. Subsequently, xylene and graded alcohols were passed through and entrapped (Bio Mount, Bio-Optica).

Dye samples were evaluated on the Zeiss AXIO Scope 1 brand research microscope. Analysis of iNOS immunoreactive cells in the stomach tissue was performed using the Leica LAS V3.8 image analysis system. Five of the sections from the blocks containing the stomach tissues of all the rats in all groups were stained. From the stained sections, 1000 cells were counted and immunoreactive cells were identified among these cells. Tosun et al. (2006), Bakir et al. (1996), and Avunduk et al. (2000) for this purpose;

$$\frac{\text{Immune-positive cells}}{\text{Total cell count (1000)}} \times 100\ \% = \ldots\ldots\ldots\ldots\ldots\ \%$$ formula were used.

SPSS 15 version was applied for the statistical evaluation of the results obtained with the applied formula. Kruskal-Wallis Test was used for nonparametric tests to determine the differences between iNOS immunoreactivity groups. The difference between the groups was considered significant in the results of $p < 0.05$.

Study Area and Sampling Stations

The Karamenderes River rises in Kazdağı and passes near the Bayramic and Ezine districts of Çanakkale and flows into the Dardanelles near Kumkale. Karamenderes River is the only source of water that constantly flows into the region (Kayacan, 2008). Water and sediment samples were taken from 5 stations (1. Kumkale, 2. Sarımsaklı, 3. Ahmetçeli, 4. Mollahasan, 5. Karaköy) along Karamenderes River in November 2012 (Figure 1). The water samples were taken from the area where the current is not very high in amounts of 5 lt and sediment samples were obtained from the surface of the river bed in amounts of 250 g. The water samples were placed in polyethylene bottles (500 mL) and immediately transported to the laboratory.

3. RESULT

There was no significant change in the staining of the stomach of the rats in the first group with Hematoxylin Eosin (Figure 2). In the second group, a small amount of mononuclear cell infiltration was detected (Figure 3). In the third a small amount of mononuclear cell infiltration was detected (Figure 4) In the fourth group (Figure 5), no significant difference was observed in mononuclear cells. 8-OHdG immunoreactivity was negative in stomach tissues in all groups. No significant differences were detected between groups in immunohistochemical staining with 8-OHdG (Figure 6).

4. CONCLUSION

In this study, we found mononuclear inflammatory cell in gastric mucosa of rats fed with mussels containing heavy metal salts.

Gezen et al. (2011) has investigated the accumulation of heavy metals in the carpet shell clam, clam, sea snails and oysters from the Dardanelles Umurbey region. In this research, Zn in carpet shell clams, Zn and Mn in clams, Zn in oysters, Al, Zn, Fe, Cu and Mn in sea snails found the metals as high. If the same zone is in seawater, the Zn level is high. In sea chestnuts growing in Dardanells, the values of Al, Zn, and Fe in samples taken from Gelibolu Hamzakoy station are high. Al and Fe values were higher in samples taken from Çardak region. Al, Fe and Zn values were higher in samples taken from Umurbey region. Al, Fe and Zn values were higher in samples taken from Çamburnu region (Gezen et al. 2011). Demir et al. (2011) has investigated the accumulation of heavy metals in the carpet shell clam, clam, sea snails and oysters from the Dardanelles Karacaören region. In this research, Al, Zn and Fe in carpet shell clams, Zn and Mn in clams, Zn in oysters, Al, Zn, Fe, Cu and Mn in sea snails found the metals as high.

IARC (1987) has explained that heavy metals may affect and cause chronic degenerative changes and, in some cases, teratogenic and carcinogenic effects, especially by affecting the nervous system, liver and kidneys. In addition to the findings of other researchers, we have also found that heavy metal salts cause histopathological changes in the stomach.

Gezen (2017) stated that immunohistochemical staining methods are used to detect damage to cells and tissues. The positive cells (8-OHdG) were predominantly observed in the areas of active inflammation with prominent cell infiltration (Seki et al. 2002). In this study, we investigated whether or not to produce DNA damage by feeding the mussels containing heavy metal salts to rats. However, 8-OHDG was found to be negative in the gastric mucosa of all groups in our study. It is thought that heavy metals in low levels (NOEL) in the grains do not cause DNA damage.

It is a very consumed food material, especially if the people of the region are in need of mussel filling in many settlements which are coastal. Considering that mussel stuffs are consumed in large quantities at once, it is thought that serious digestive system diseases can occur when mussels are exposed to environmental factors such as heavy metals, peptides and waste water.

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FIGURES

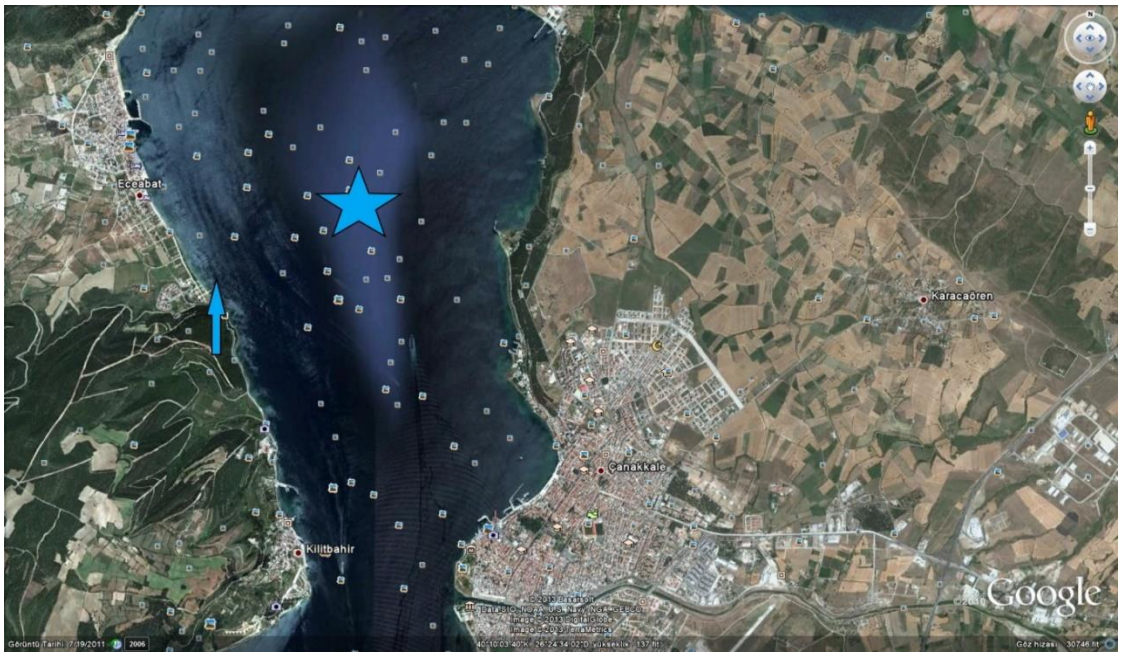

Figure 1: The area where the mussels are collected

Arrow: Çamburnu region (Çanakkale, Turkey)

Star: Dardanelles

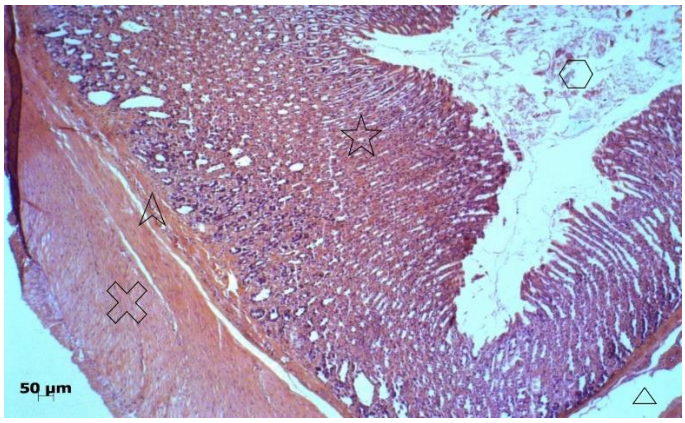

Figure 2. For the first study group was the control group; standard rat diet was given every days. Rat stomach, (H.E. x 5).

Star: Lamina propria mucosa

Pointed arrow: Lamina muscularis mucosa

Arrow head: Lamina submucosa

Crossed: Tunica muscularis

Hexagon: Gastric lumen

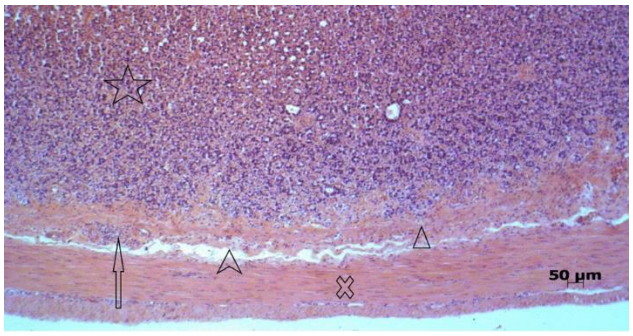

Figure 3. For the second study group; 75% mussel + 25% standard rat diet standard rat feeds were given daily. Rat stomach, (H.E. x10).

Star: Lamina propria mucosa

Arrow head: Lamina muscularis mucosa

Pointed arrow: Lamina submucosa

Crossed: Tunica muscularis

Arrow: Mononuclear inflammatory cells

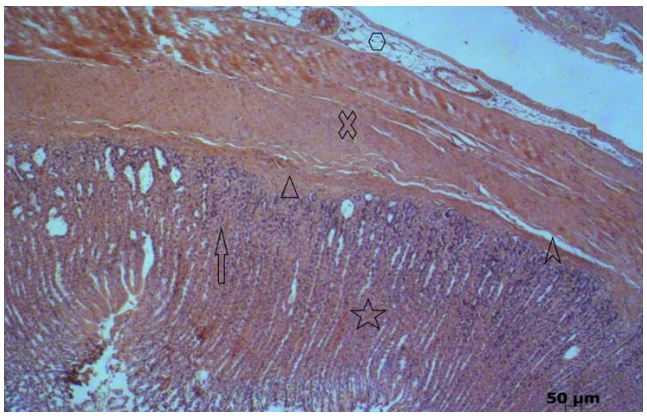

Figure 4. For the third study group; 75% mussel + 25% standard rat diet was given every two days. Standard rat diet was given the other day. Rat stomach, (H.E. x 5).

Star: Lamina propria mucosa

Arrow head: Lamina muscularis mucosa

Pointed arrow: Lamina submucosa

Crossed: Tunica muscularis

Hexagon: Tunica adventitia

Arrow: Mononuclear inflammatory cells

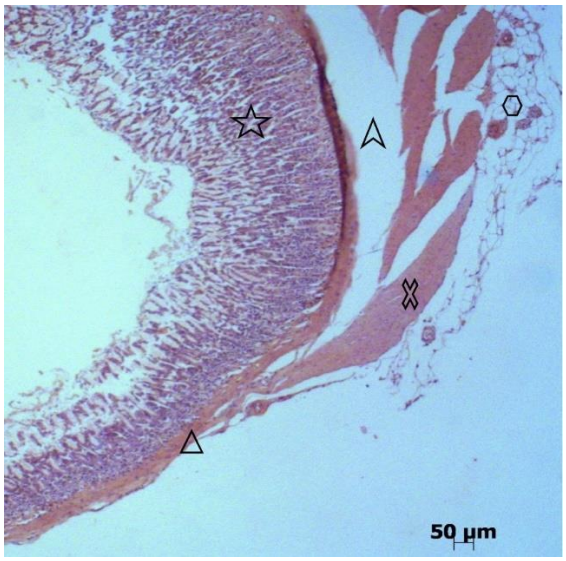

Figure 5. For the fourth group; 75% mussel + 25% standard rat diet was given every three days. Standard rat diet was given the other two day. Rat stomach, (H.E. x10).

Star: Lamina propria mucosa

Arrow head: Lamina muscularis mucosa

Pointed arrow: Lamina submucosa

Crossed: Tunica muscularis

Hexagon: Tunica adventitia

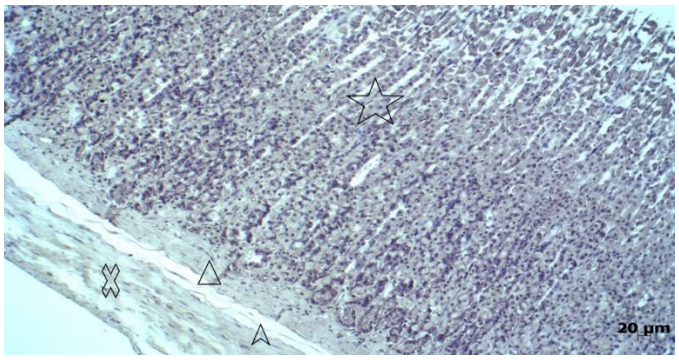

Figure 6. For the second study group; 75% mussel + 25% standard rat diet standard rat feeds were given daily. Rat stomach. (8-OHdG x 10)

Star: Lamina propria mucosa

Arrow head: Lamina muscularis mucosa

Pointed arrow: Lamina submucosa

Crossed: Tunica muscularis

Abbreviation:

H & E: hematoxylin and eosin,

8-OHdG: 8-hydroxy-2' –deoxyguanosine,

NOEL: no-observed-adverse-effect level,

IARC: International Agency for Research on Cancer

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POLLEN AND ACHENE MORPHOLOGY OF TARAXACUM F.H. WIGG. (ASTERACEAE) SPECIES DISTRIBUTED AROUND BURSA

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ABSTRACT

In this study, we extensively examined the pollen morphology and some important morphological characteristics of Taraxacum F.H. Wigg. (Asteraceae) species which have a wide distribution in and around Bursa. There are 7 Taraxacum species distributed in Bursa and its vicinity, 2 of which are endemic. Taxons are Taraxacum serotinum Poir., Taraxacum hyberniforme Soest, Taraxacum minimum Heldr. ex Nyman, Taraxacum aznavourii Soest, Taraxacum buttleri Soest, Taraxacum pseudobrachyglossum Soest and Taraxacum turcicum Soest. According to the palynological studies, T. turcicum pollens are moderate monad, their pollen type is three-zonocolporate rarely tetra-zonocolporate, pollen shape is subprolate, prolate-spheroidal, oblat-spheroidal and exine ornamentation is defined as spinulate. T. pseudobrachyglossum pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is subprolate, prolate-spheroidal, oblat-spheroidal; exine ornamentation is spinulate; spine and spinule. T. serotinum pollens are medium sized monad, pollen type is three-zonocolporate rarely tetra-zonocolporate, pollen shape is prolate-spheroidal, oblat-spheroidal; exine ornamentation is spinulate; spine and spinule. T. minimum pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is suboblate, prolate-spheroidal, oblat-spheroidal; exine ornamentation is spinulate; spine and spinule. T.hyberniforme pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is prolate-spheroidal, oblat-spheroidal; exine ornamentation is spinulate; spine and spinule. T. aznavourii pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is prolate-spheroidal, oblat-spheroidal; exine ornamentation is spinulate; spine and spinule. T.buttleri pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is suboblate, prolate-spheroidal, oblat-spheroidal, subprolate; exine ornamentation is spinulate; spine and spinule. T. turcicum, T.pseudobrachyglossum, T.aznavourii its achene has a strow coloured, 2,5-4mm achene lenght, T.minimum its achene has a yellowish or strow coloured, 3-4mm achene lenght, T.hyberbniforme its achene has a red-brown, 4-5,5mm achene lenght, T.buttleri its achene has a brown-red, 2,5-4,5mm achene lenght, T.serotinum its achene has a strow-brown, 2,5-3mm achene lenght. The aim of this study is to examine the morphological characteristics of the detailed pollen morphology and important characters of Taraxacum taxa belonging to the Asteraceae family of Bursa.

1. INTRODUCTION

The Asteraceae (Compositae) family is in the first place in terms of diversity having more than 1.100 genara and 20.000 species in the world. Family flora in Turkey has 134 genera and 1209 species (Davis et al 1988, Özhatay and Kültür 2006, Doğan 2007). It is found especially in tropical and subtropical semi-arid regions like the Mediterranean Region, Mexico and South Africa; in tropical forests of Africa, South America and Australia and in the stony and shrubland areas (Heywood 1978). They are less common in humid tropical forests (Kadereit and Jeffrey, 2007).

Asteraceae are entomophilous plants containing an annual, biennial or perennial herbaceous plants or schrubs and sometimes laticifiers. Leaves alternate or sometımes opposıte, exstıpulate (rarely stipuloid), entire, toothed, lobed or variously dissected. İndividual flowers usually numerous (rarely only), sessile and aggregated into a capitulum surrounded by a protective involucre of 1-many series of phyllaries (involucral bracts), rarely fused; capitula sometimes aggregated into a secondary capitulum-like head (pseudocephalium). Receptacle naked or bearing paleae (scales), long hairs or bristles. Flowers epigynous, either all hermaphrodite and protandrous, or female, male (at least functionally so), or neuter (sterile) (Davis et al.1975, Yıldız and Aktoklu, 2010).

One of the most important members of family is the *Taraxacum* F.H. Wigg. The *Taraxacum* F.H. Wigg. is represented by approximately 2500 species and 43 sections worldwide. The total number of species of *Taraxacum* (Compositae = Asteraceae) in our country is 54 taxons. It has a wide distribution in the hot regions of the northern hemisphere. Although it is a cosmopolitan species, it distributes especially in Europe, Central Asia, Turkey, Iran, Afghanistan, Pakistan and India. A number of studies have been conducted worldwide on this apomixis species whose taxonomy is problematic (Richards., John, 1968).

Taraxacum is a perennial plant which is glabrous or arachnoid. Distinguishing characteristics is to have a leafless stem that carries hollow, latex rising from the leaf on the bottom and carrying flowers at the top. The crown board is bare; achene is usually long-beaked rarely beakless. Pappus pluriseriate, finely scabrous, persistent (Davis et al.1975).

Bednorz & Maciejewska-Rutkowska (2010) have carried out a research on pollen morphology, exine structure, ornementation shape and morphological characteristics of the species using light microscope and scanning electron microscope (SEM) in their study of *Taraxacum pieninicum*. In addition, they investigated the outer surface of achene, spins, pappus and beak structure with scanning electron microscope (SEM).

Marciniuk et al. (2009) conducted a study on *Taraxacum scanicum* that shows the morphology of important characters such as pollen morphology, achene, outer bracts and flower. They studied showing the differences among the significant morphological characteristics of *Taraxacum scanicum*, *Taraxacum prunicolor* and *Taraxacum cristatum* species.

Marciniuk and Rudzińska-Langwald (2008) studied the diversity of pollen morphology of *Taraxacum* species in the ones which were selected according to chromosome level. They noted that there is a relationship between pollen morphology and reproduction pathways. In conclusion, the pollen of compulsory apomixis triploidal species shows variability and has characteristics of high rated deformed pollen grains. The optional apomixis tetraploids have relatively equal size and regular pollens. In the pentaploidal *Taraxacum mendax* and in species with unknown chromosome numbers (*Taraxacum portentosum* and *T. platyglossum*), these taxa have been shown as being optional apomixtis.

Jafari & Assadi (2007) investigated the morphological and anatomical features of *Taraxacum aurantacum* (ASTERACEAE) in the Iranian flora.

Gedik et al. (2014) studied two endemic species belonging to the *Taraxacum* species from the caryological perspective. There have been no previous studies on the karyology of *T. bellidiforme* and *T. revertens*; and the number of chromosomes and karyotype analysis of species have been presented for the first time in the science world.

The purpose of this study is to examine the morphological characteristics of the detailed pollen morphology and important characters of *Taraxacum* taxa belonging to the Asteraceae family of Bursa.

2. MATERIAL AND METHODS

Specimens belonging to T. serotinum, T. hyberniformе, T. minimum, T. aznavourii, T. buttleri, T. pseudobrachyglossum and T. turcicum were collected from different locations in Bursa in the months of April, May and June in 2017 during flowering and fruiting periods (Table 1). Plant samples taken from stations were pressed and dried in accordance with the herbarium rules and made ready for laboratory studies (Erdoğan et al. 2011, Günay. 2001). The samples are stored in the Uludağ University, Faculty of Sciences and Art, Department of Biology (BULU) herbarium.

For morphological studies, plant samples taken from 7 different stations were examined morphologically. Measurements were made for the individuals collected from each population using the criteria of plant height, achene length and width, cone, beak, pappus measurements, achene, measurements and positions of external bract. 10 to 30 measurements were made for each measurable character. OLYMPUS SZ 51 stereo microscope was especially used to be able to measure fruit parts and some micro characters in millimetric ocular.

Pollens from herbarium specimens were used for Palynological studies. Pollen slides prepared to examine in light microscope were prepared according to the methodology of Wodehouse (1935). The measurement and examination of pollens was conducted by using a microscope with Nikon Eclipse E100 including x40 and x100 lenses. Properties such as pollen diameter (AB), exine (E), intine (I), pore number, distance between pores, pore length (Plg), pore width (Plt) and spine length were taken into consideration in the microscopic measurement (Table2).

Table 1. Plant samples collected from different localities of Bursa province.

Taxa	Localities	The date collected	Collected by	Identified by
T.turcicum	Bursa: Görükle; Around the lodgement	15.04.2017	Narmin ABDULLAYEVA	Gül KUŞAKSIZ
T.pseudobrachyglossum	Bursa: Uludağ University Campus; Around the Faculty of Agriculture	16.04.2017	Narmin ABDULLAYEVA	Gül KUŞAKSIZ
T.serotinum	Bursa: Subsection of Merinos, road sides	26.04.2017	Narmin ABDULLAYEVA	Gül KUŞAKSIZ
T. minimum	Bursa: Kumla; Şahintepe stony area and meadow	03.05.2017	Narmin ABDULLAYEVA Gül KUŞAKSIZ Betül UĞUR	Gül KUŞAKSIZ
T.hyberniforme	Bursa: Demirtaş the south parts of the Grass Skiing Facility	10.10.2017	Narmin ABDULLAYEVA Gül KUŞAKSIZ	Gül KUŞAKSIZ
T. aznavourii	Bursa: Gürsu exit-Dışkaya village	10.10.2017	Narmin ABDULLAYEVA Gül KUŞAKSIZ	Gül KUŞAKSIZ
T. buttleri	Bursa: Uludağ Wolfram mine upper regions	07.08.2003	R.GÜNAY Ö.YILMAZ	R.GÜNAY Ö.YILMAZ

3. RESULT

3.1. Pollen Morphology

The data obtained after the palynological studies carried out by the Wodehouse method for *T. turcicum* shows that pollen is medium sized monad, pollen type is three-zonocolporate rarely tetra-zonocolporate, pollen shape is subprolate, prolate-spheroidal, oblat-spheroidal; pollen diameter is A: 21,75-35,55µm, B: 21,5-33,83µm, A / B ratio 0.88-1.2; pore number is 5-(6) -7, distance between pores (mezoporium) is 15,03-24,53µm, pore length (Plg) is 4,43-7,02µm, pore width is (Plt) 3,07-6,16µm, Plg / Plt ratio is 1.18, subprolate, exine (E) is 0.91-2.25µm, intine (I) is 1.1-2.19µm; exine ornamentation is spinulate; spine length is 1,63-2,7 µm (Fig 1. 1-3).

According to the obtained data for *T. pseudobrachyglossum,* pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is subprolate, prolate-spheroidal, oblat-spheroidal; pollen diameter is A: 23.5-39.52µm, B: 20.04-38.45µm, A / B ratio is 0.9-1.2; pore number is 5- (6) -8, distance between pores (mezoporium) is 12,6-28,08 m, pore length (Plg) is 3,71-6,91 m, pore width (Plt) is 3,986,9 m, Plg / Plt ratio is 1.02, prolate-spheroidal, exine (E) is 1,14-2,39µm, intine (I) is 1,14-2,64µm; exine ornamentation is spinulate; spine and spinule length is 1,46-3,46 µm (Fig 1. 4-6).

According to the data obtained for *T. serotinum,* pollens are medium sized monad, pollen type is three-zonocolporate rarely tetra-zonocolporate, pollen shape is prolate-spheroidal, oblat-spheroidal; pollen diameter is A: 25,8937,27µm, B: 24,73-36,82µm, A / B ratio is 0.9-1,1; pore number is 6-(7)-8; distance between pores (mezoporium) is 15,9-24,48µm, pore length (Plg) is 3,79-7.06µm, pore width (Plt) is 3,37-5,64µm Plg / Plt ratio is 1.22, subprolate, exine (E) is 1.14-2.07µm, intine (I) is 1.3-2.23µm; exine ornamentation is spinulate; spin and spinule length is 1,63-3,27 µm (Fig 1. 7-9).

According to the data obtained for *T. minimum,* pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is suboblate, prolate-spheroidal, oblat-spheroidal; pollen diameter is A: 21,03-35,84µm, B: 23,32-37,18µm, A / B ratio is 0.8-1.09; pore number is 5 - (7) -9, distance between pores (mesoporium) is 2,89-23,98 µm, pore length (Plt) is 3,78-8,22µm, pore width (Plt) is 2,6 - 5,12 µm, Plg / Plt ratio is 1.36, prolate, exine (E) is 0.9-1.74µm, intine(I) is 1.18-2.43µm; exine ornamentation is spinulate; spine and spinule length is 1,47-3,16 µm (Fig 1. 10-11).

According to the data obtained for *T. hyberniforme,* pollens are medium sized monod, pollen type is three-zonocolporate, pollen shape is prolate-spheroidal, oblat-spheroidal; pollen diameter is A: 23,6731,38µm, B: 24,52-34,43µm, A / B ratio 0.9-1,1; pore number is 6- (7) -8, distance between pores (mezoporium) is 14,95-22,22µm, pore length (Plg) is 4,06-6,94µm, por width (Plt) is 3,94-5,31µm, Plg /Plt ratio is 1.2, subprolate, exine (E) is 0.86-1.63µm, intine (I) is 1.09-2.09 µm; exine ornamentationis spinulate; spine and spinule length 1,99-3,61 µm (Fig 1. 12-13).

According to the data obtained for *T. aznavourii,* pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is prolate-spheroidal, oblat-spheroidal; pollen diameter is A: 24,8130,24µm, B: 20,09-31,52µm, A / B ratio is 0.8-1,3; pore number is 5-(6)-7; distance between pores (mezoporium) is 18,14-22,92µm, pore length (Plg) is 3,67-6,71µm, pore width (Plt) is 2,94-5,1µm, Plg / Plt ratio is 1.14, subprolate, exine (E) is 0.83-1.62µm, intine (I) is 1.01-2.54 µm; exine ornamentation is spinulate; spine and spinule length 1,72-3,7 µm (Fig 1. 14-15).

Table 2. A comparison of pollen structures about *Taraxacum* taxa

Taxa	No. of Pores	A	B	A/B	Plg	Plt	Plg/Plt	Mesoporium	E	I	Spinulate
T.turcicum	6	30,55	29,3	1,04	5,36	4,55	1,18	20,19	1,52	1,7	2,15
T.pseudobrachyglossum	6	29,73	28,64	1,04	5,2	5,1	1,02	19,86	1,5	1,85	2,38
T.serotinum	7	30,14	29,9	1	5,38	4,42	1,22	19,99	1,51	1,73	2,43
T. minimum	7	27,61	26,51	1,04	5,18	3,81	1,36	17,73	1,27	1,71	2,21
T.hyberniforme	7	27,53	27,09	1	5,34	4,45	1,2	18,6	1,22	1,55	2,6
T.aznavourii	6	28,58	27,9	1,02	4,94	4,33	1,14	20,8	1,18	1,55	2,43
T.buttleri	6	26,47	25,77	1,03	4,34	4,12	1,05	20,11	1,18	1,43	2,33

A-pollen diameter (Long axis); B-pollen diametetr (Short axis); Plg- pore lenght; Plt-pore width; E-exine; I-intine.

According to the data obtained for *T.buttleri*, pollens are medium sized monad, pollen type is three-zonocolporate, pollen shape is suboblate, prolate-spheroidal, oblat-spheroidal, subprolate; pollen diameter is A: 22,98-22,54µm, B: 20,86-29,55µm, A / B ratio is 0.8-1,2; pore number is 6- (7) -8, distance between pores (mesoporium) is 17,63-25,8µm, pore length (Plg) is 2,95-5,61µm, pore width (Plt) is 2,886,1µm, Plg / Plt ratio is 1.05, prolate-spheroidal, exine (E) is 0.73-1.63µm, intine (I) is 1.01-2.19 µm; exine ornamentation is spinulate; spine and spinule length is 1,63-3,49µm (Fig 1. 16-17).

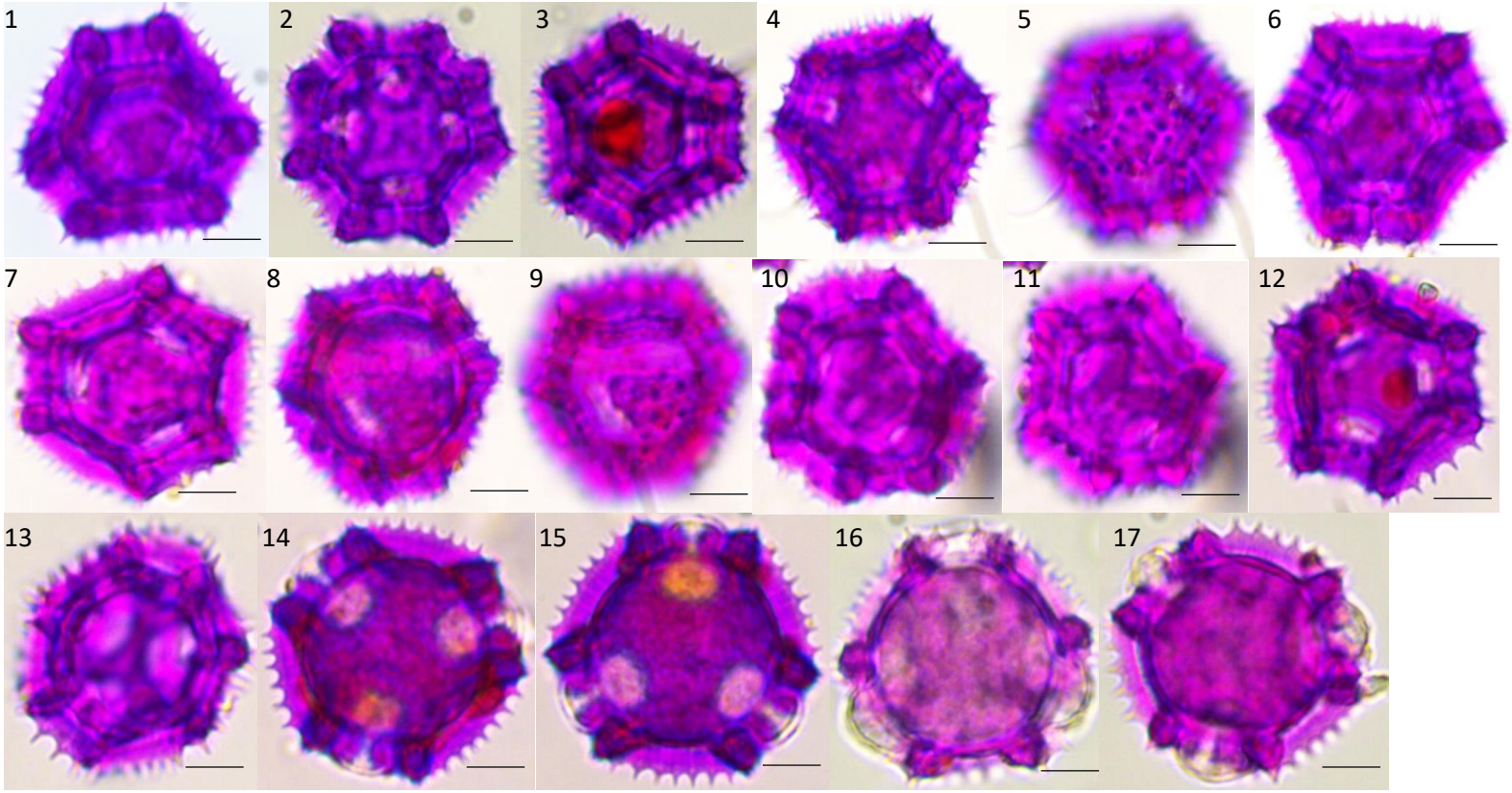


Figure 1. Pollen grains by light microscopy of *Taraxacum.* 1-3 *T.turcicum; 4-6 T.pseudobrachyglossum; 7-9 T.serotinum; 10-11 T.minimum;12-13 T.hyberniforme; 14-15 T.aznavourii; 16-17 T.buttleri* Scale bar 25 µm.

Tablo 3. Comparison of *Taraxacum* F.H. Wigg. morphological characters.

	T.turcicum	*T.pseudobrachyglossum*	*T.serotinum*	*T. minimum*	*T.hyberniforme*	*T.aznavourii*	*T.buttleri*
Achene color	strow coloured	strow coloured	strow brown	yellowish or strow coloured	red-brown	strow coloured	brown-red
Achene lenght	2,6-4,8mm	2,6-3mm	2,5-3mm	3-4mm	4-5,5mm	3-4mm	2,5-4,5mm
Achene width	0,8-1mm	1mm	0,5-1mm	1mm	1-1,5mm	1mm	0,5-1mm
Cone lenght	0,1-1mm	0-1mm	0,5-1mm	1-1,5mm	1-2mm	1mm	0,5-1mm
Beak lenght	5-12,6mm	7,5-11mm	5-10mm	8-11mm	4-8,5mm	3-6mm	2,5-5,5mm
Pappus lenght	6-8,2mm	5-8mm	6-7mm	5-6mm	5-7mm	3,5-5mm	3-5,5mm
The lenght of outer bracts	1-1,5cm	7-14mm	1,1-1,3cm	5-1,1cm	3,5-6mm	4-6mm	2-6mm
Position of outer bracts	spreading	recurved	recurved	spreading	spreading	recurved	recurved
Involucre lenght	1,3-1,9cm	1,1-1,8cm	1,1-1,9cm	1,1-1,8cm	1,2-1,6cm	1,1-1,3cm	7-12mm
Involucre hyaline margin	0,7-1,5mm	0,2-1mm	0,2-1mm	0,2-1mm	0,5-1mm	0,5-1mm	0,2-0,5mm
Height of the plant	11,5-25,3cm	12-32cm	17-36,5cm	12-19cm	5,5-15cm	8-19cm	5-13cm

3.2. Morphology and Achene Morphology

As a result of the researches, we saw similarities in the morphological characters among the species studied. However some characters show sharp differences from each other. The distinguishing characters are different in terms of achene colors, achene lengths, the position of the outer bracts and the edge width of the outer bracts. The differences between the examined morphological characters are shown in Table 3.

T. turcicum is a perennial herbaceous plant with a length of 11,5-25 cm. The plant floor is thick, its achene has a strow coloured and 4-6 mm in lenght (0,8-1 mm cone). The beak is 5 to 12 mm, it has off white pappus 6-8 mm. Outer bracts is ovate with 1-1,5 cm lenght, spreading. Involucre is 1,3 - 1,9 cm; it has hyaline margin of 0,7-1.5 mm.

T.pseudobrachyglossum is a perennial herbaceous plant with a length of 12-32 cm. Its achene has a strow coloured and it is 3,6-4,6 mm in lenght (1 mm cone); the beak is 7,5-11 mm long; it has off white pappus 5-8 mm. Outer bracts is 1-1,5 cm recurved. Involucre is 1,2-1,8 cm and it has a hyaline margin of 0,2-1 mm.

T. serotinum is a perennial herbaceous plant with a length of 17-36,5 cm. Plant is intensive arachnoid. Involucre is 1,1-1,9 cm in length, sometimes less or much. Outer bracts is brownish-reddish on the outside, linear or narrowly spandler, up to 1,1-1,3cm, recurved. Achene is pastel brown and has pedicellaria near the top. It is 3-4 mm in lenght with cone (0,5-1 mm). The beak is 5-10 mm; pappus is pastel brown and 6-7 mm long. Ligula is straw yellow and stigma is dirty yellow or greenish.

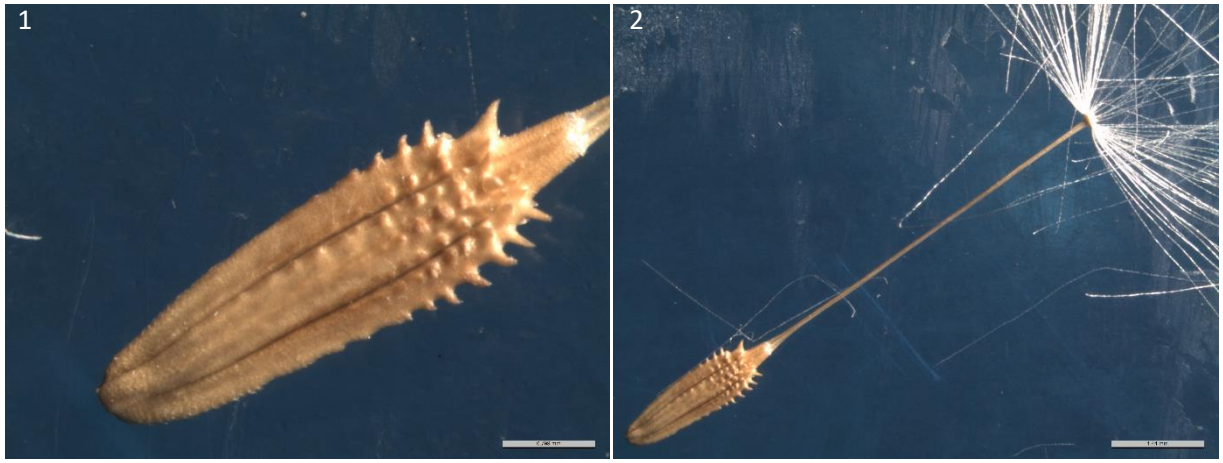
T. minimum is a perennial herbaceous plant with a length of 12-19 cm. Involucre is 1,1-1,8 cm; hyaline margin is 0,2-1 mm; ovate. Outer bracts is 0,5-1,1 mm ± spreading. Achene is 4-5 mm (1-1,5 mm cone included) in yellowish or strow colour. Beak is 8-11 mm in length, pappus is 5-6 mm. Stigma is greenish.

T.hyberniforme is a perennial herbaceous plant with a length of 5,5-15 cm. Outer bracts is 3,5-6 mm ± spreading, ovate, usually broad-edged, dark green with usually blackish median vein and dark humped. Involucre is 1,2-1,6 cm; it has a hyaline margin in 0,5-1 mm lenght. Ligula is gray-violet and externally striped. Achene is 5-7 mm (1-2 mm cone included in lenght), has red-brown coarse pedicellaria and thorny. The beak is 4-8,5 mm in length; pappus is 5-7mm long.

Taraxacum aznavourii is a perennial herbaceous plant with a length of 8-19 cm. Its achene has a strow coloured and it is small by having 3-5 mm lenght (1 mm cone). The beak is 3,5 to 5 mm, it has off-white pappus feathers of 3,5-5 mm. Outer bracts is 1-1,5 cm, lanceolate and its points are back-convoluted, humpless. Involucre is 1,1-1,3 cm and it has a hyaline margin of 0.5-1 mm.

Taraxacum buttleri is a perennial herbaceous plant with a length of 5-13 cm. The plant is small. Its achene has a dark brown-red color and 3-5,5 mm long (0,5-1 mm cone). The beak is 2,5 to 5,5 mm and it has off-white pappus feathers with 3,5-5 mm lenght. Outer bractea has 2-6 mm width and it is strictly back-convulated. Involucre is 0,7-1.2 cm. It has hyaline margin of 0,2-0,5 mm. Ligula is straw yellow and stigma is dirty yellow or greenish.

Figure 2. *Taraxacum* F.H. Wigg. Achene; Scale bar*: T. turcicum* 1 (1,44mm),2 (0.79mm) ; *T. pseudobrachyglossum* 3 (1.44mm), 4 (1.13mm); *T. serotinum* 5 (1.44mm),6 (0.707mm); *T. minimum* 7 (1.44mm),8 (0.801mm); *T. hyberniforme* 9 (1.44mm), 10 (0.801mm)*; T. aznavourii* 11 (1.44mm),12 (0.895mm); *T.buttleri* 13 (1.28mm),14 (0.801mm);

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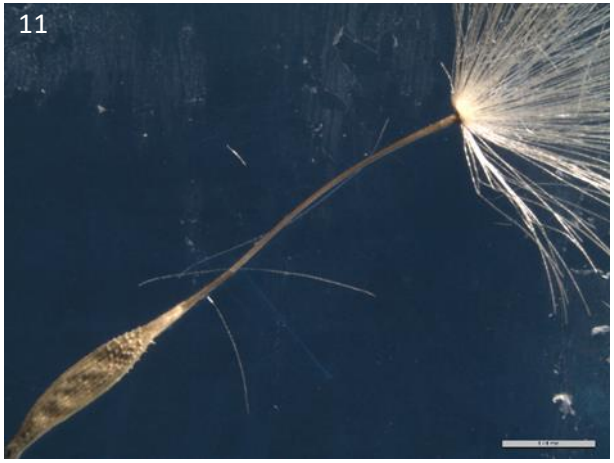
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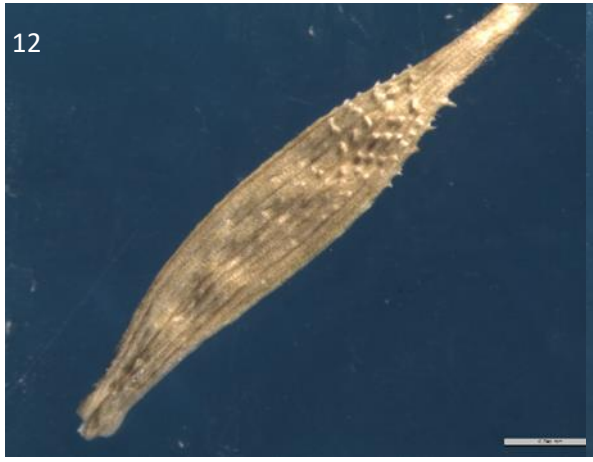
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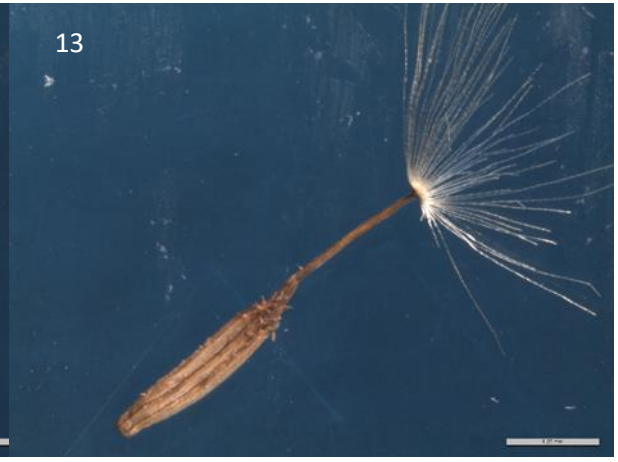
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4.Discussion

Palynological and morphological studies on *Taraxacum* taxa have revealed some differences when conpared with findings in this study. Bednorz & Maciejewska-Rutkowska (2010) according to the study about *Taraxacum pieninicum* taxon, pollen grains are mainly medium sized isopolar; the polar axis is 24 μm (range between 20-30 μm) and the equatorial axis (E) is 25 μm (range between 20-32 μm), the P / E value is from 0,98 to 1,16, the mean value is 1,06. Pollen shape is prolate spheroids, sometimes oblate spheroid and has circular ambles. Pollen grains are three-zonocolporate. Exine layer is determined having a thickness of 10-13μm (including the spines). The thickness of the exine layer is 4μm at maximum according to the data obtained in this study. Achene was determined as brownish purple and the fruit had an average height of 18 mm (15-20mm). The shape was elliptic. Achene is 5mm, cone is 2mm (in pyramidal shape) and the beak is 10mm. Achene also has ordered spines, ranging from 3 to 4 in each order.

Marciniuk et al (2009) *Taraxacum scanicum* achenes have differently ordered spins of 3,5-4 mm long and they are in red brown color. The outer bractea is green and sprawled. It has narrow edges of 0,1-0,2 mm. Palynological findings show that the average maximum pollen diameters of *Taraxacum scanicum* are 22.48 μm inside (without exine) and 33.83 μm outside. The variability is relatively low. The internal diameter range is 14,3 – 30,8μm. For the 78% of the pollen grains, it is determined as 20,1 - 25μm. In this study, the obtained data shows a difference between 27-30 μm.

Osman (2006) *Taraxacum turcicum* polar axis has been determined as 34 μm (range between 32 and 35 μm). Equatorial axis (E) is 37 μm (range between 35 and 43 μm) and P / E value is 0,92. Pollen shape is oblate-sferoidal and exine ornementation is spinelate. The pollen grains are three-zonocolpate; rarely tetra-zonocolporate. In the data obtained in this study, *Taraxacum turcicum* pollen type is three-zonocolporate rarely tetra-zonocolporate, pollen diameter is A: 21,75-35,55μm, B: 21,5-33,83μm, A / B ratio 0.88-1.2; pollen shape is subprolate, prolate-spheroidal, oblat-spheroidal; exine ornamentation is spinulate was found to be compatible with the work done.

The micromorphology of the most *Taraxacum* species has not been investigated. There are no information of achene microsculpturing of any dandelion species and there are only data of pollen morphology of a few species of this genus (Jones & Wilson 2001; Weryszko-Chmielewska & Chwil 2005; Osman 2006). That is why we can not entirely compare our results with the data from literature.

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