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Determination of Photosynthesis-Related and Ascorbate Peroxidase Gene Expression in the Green Algae (*Chlorella vulgaris*) Under High-Temperature Conditions

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Abstract: Increasing water temperatures because of climate change resulted in population shifts and physiological responses in aquatic environments. In this study, short-term high-temperature condition effects on green algae *Chlorella vulgaris* were investigated at transcriptional and physiological levels. The photosystem II D1 protein (*psbA*) gene, a large unit of Rubisco (*rbcL*) gene and chloroplastic ascorbate peroxidase (*cAPX*) gene expressions were quantified using semi-quantitative real time-PCR. The *psbA* gene transcription level at 45˚C for 48 and 72 h was reduced by approx. 2.22 and 2.86-folds, respectively. The *rbcL* gene transcription level was also reduced by 1.54 relative to the control at 72 h. Our *APX* gene transcriptional level results indicated that the transcription of this gene was significantly increased at 35˚C at 24, 48, and 72 h. In contrast, the *cAPX* mRNA transcript level was reduced by approx. 2 times compared with the control. Our data demonstrated that alteration *cAPX* gene expression could play an essential role in high-temperature acclimation in *C. vulgaris*.

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1. INTRODUCTION

Temperature stress can be counted as a critical abiotic factor due to stimulated changes in some physiological processes like membrane stability, development, photosynthesis, plant growth, and respiration (Sinsawat et al., 2004). The high temperature also inhibits Calvin cycle activity by decreasing the activation state of Rubisco enzyme (Weis, 1981; Feller et al., 1998; Law & Carfts-Brandner, 1999). Photosystem II (*PSII*) also displays susceptible responses to increasing temperatures and heat-inhibition of photosynthesis. The inhibition of electron transport in photosynthetic organisms has been attributed to the thermal accumulation ability of *PSII*, which occurs in the formation of reactive oxygen species (*ROS*) from water (Allakhverdiev et al., 2007).

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The PSII reaction center includes two main proteins, *D1*, and *D2* proteins. *D1* proteins in the thylakoid membranes are known to be susceptible to many environmental factors (Giardi et al., 1997). *D1* proteins are generally affected by oxidative stress, and they can be degraded (Prasil et al., 1992) and leads to *PSII* photodamage. Photosynthetic organisms get typically harmed by the instability of synthesis/degradation balances of *D1* protein during stress conditions. *D1* protein is encoded by the *psbA* gene, which has a role in the replacing damaged *D1*. During the repair of damaged *PSII*, firstly, the damaged *D1* protein is removed then the new *D1* protein is synthesized instead of the damaged one. As a result, a new *D1* protein was added to the *PSII* system.

It has been demonstrated by previous studies some environmental stress factors such as metal and salt stress restrain the *PSII* repairment by the inhibition of *psbA* gene transcription and translation (Nishiyama et al., 2004; Allakhverdiev et al., 2008; Qian et al., 2009). The electron transport system is the primary basis of *ROS* in chloroplasts. Besides, the location of *ROS* generation changes according to stress types (Foyer & Noctor, 2003; Mittler et al., 2004). The *ROS* production is highly detrimental for the protein and lipid metabolisms and leads to the inhibition of algal growth (Sainju et al., 2001; Tang et al., 2007). Many photosynthetic organisms have robust antioxidant systems, embracing antioxidant enzymes and antioxidants. Antioxidant enzymes have played an essential role in reducing oxidative stress. Ascorbate peroxidase (*APX*) enzyme is fundamental in the ascorbate-glutathione cycle. They are found in green plants and algae and catalyze the transformation of H_2O_2 into the water using ascorbate as an electron donor (Asada, 1999). Photosynthetic organisms with escalated tolerance to several environmental stresses, comprising temperature stress, achieve such tolerance through the excited expression of *APX* genes. *APX* gene expression was induced after potato tubers were exposed to low temperatures (Kawakami et al., 2002). In chloroplasts, the over-expression of *APX* has a vital role for detoxification of H_2O_2. Up-regulation of *APX* could alleviate photooxidative depredation during temperature stress. Researches conducted with transgenic plants demonstrated that they have higher photochemical efficiency of *PSII* compared with wild-types under cold stress (Sun et al., 2010). Du et al. (2013) demonstrated that the transcript levels of cytosolic (cyt) *APX* were significantly higher in heat-tolerant *Poa pratensis* L. under long-term heat stress. However, there is less available data on the effects of high-temperature stress on *psbA, rbcL*, and chloroplastic *APX* transcription levels, and these different genes interact. The aims of this study are (i) to understand the tolerance capacity of green algae *C. vulgaris* by analyzing growth rate, chlorophyll quantity and chlorophyll degradation rate under high-temperature stress; (ii) to determine the effects of high-temperature stress on the transcription levels of *psbA*, *rbcL*, and chloroplast *APX* genes in by semi-quantitative real time-PCR.

2. MATERIAL and METHODS

2.1. Culture Conditions

C. vulgaris culture was obtained from Ege University Microalgae Culture Collection (EGEMACC). Organisms were stored in Rudic Medium (RD) (Rudic & Dudnicenco, 2000) at 25°C in laboratory conditions until experiments. Five flasks containing 100 ml of *C. vulgaris* were used for the experiment. The culture was grown in RD at 25°C (as control), 35°C and 45°C. Aeration was provided to the culture flasks continuously by bubbling air via a blower.

2.2. Cell Density

The absorbance at 663 nm was determined with a UV-Vis spectrophotometer (Pharo 300, Merck) at 24, 48, and 72 h. Specific growth rate μ was calculated using the equation described by Guillard (1973) as follows (1):

$$\mu=\ln(X_t/X_0)/t \quad (1)$$

X_0 indicates the initial cell density, X_t indicates the cell density after t hours.

2.3. Determination of Chlorophyll a Degradation

Chlorophyll degradation detected according to dimethyl sulfoxide (DMSO) extraction protocol (Wellburn, 1994). 20 mg of cells was extracted with 3 ml DMSO in one hour at 65°C under unilluminated conditions. Polyvinylpyrrolidone was added to DMSO to prevent chlorophyll degradation during incubation. To determine the chlorophyll degradation, extracts were read at 665 and 649 nm in the spectrophotometer (Pharo 300, Merck). Chlorophyll a, b, and a/b were calculated via specific absorption coefficients.

2.4. RNA Isolation and Reverse Transcriptase-PCR

The material ground in liquid nitrogen and 1 mL of TRIZOL Reagent (Thermo Fisher Scientific, cat# 15596026) was added into the fine powder. For the homogenization, chloroform (Sigma-Aldrich, cat# 650498) was inserted into the mixture and centrifuged at 10000 x g for 15 min. at 4°C. After the incubation for 10 min. at 15 to 30°C, the samples were centrifuged at 10000 x g at 4°C for 10 min. After washing with 75% ethanol, the pellet was air-dried for 15 min (Poong et al., 2017). The quality and quantity of obtained RNA were measured by spectrophotometer (Pharo 300, Merck). Manufacturer's instructions of cDNA Reverse Transcription Kit (Invitrogen, cat# 4398814) were followed for Reverse Transcriptase-PCR.

2.5. Semi-Quantitative RT-PCR

The oligonucleotide primers were designed from the *C. vulgaris psbA*, *rbcL*, and *cAPX* gene sequences using the PerlPrimer open source PCR primer design programme (Marshall, 2004). PCR reactions were also performed with GAPDH primers as internal control. The following sequences were used for *psbA* forward (5´-GATGAGTGGTTATACAATGGTGG-3´) and reverse (5´-GTGAGTTGTTGAAAGAAGCGT-3´), for *rbcL* forward (5´-TAACTTACTACACTCCTGAC-3´) and reverse (5´-AAGAAGACCATTATCACGAC-3´), and for chloroplastic *APX* forward (5´-CCTTTCATCCCTCTACGGCT-3´) and reverse (5´-GTCCTCTGCATACTTCTCTCGG-3´) primers. The semi-quantitative RT-PCR was performed using 5 ng cDNA, 2.5 mM PCR buffer (10X), 10 mM dNTP mix, 10 µM primers, and 1U Taq DNA polymerase enzyme (Thermo Scientific, cat # EP0402). Each PCR cycle consists of 95°C of 60 sec. denaturation, 49°C (*psbA*), 53°C (*cAPX*), and 56°C (*rbcL*) of 75 sec. annealing, 72°C of 75 sec. elongation cycles. After 32 cycles the amplification ended with a 10 min. final elongation step at 72°C (Sen et al., 2014). Each Primer set was a number of PCR cycles optimized to ensure the linearity requirement for semi-quantitative RT-PCR analysis.

2.6. Statistical Analysis

Statistical significance was assessed using a student's t and ANOVA test (SPSS, for Windows, Version 11.0). A $p<0.05$ value was considered statistically significant. All experiments were repeated three times.

3. RESULTS and DISCUSSION

According to our results, specific growth rates gradually decreased over four days at 45˚C, whereas growth rates did not change significantly at 35˚C. Figure 1 demonstrates the high-temperature effects on growth ratio. Besides, maximum cell densities and the growth rates of *C. vulgaris* at 45˚C showed a significant reduction ($p< 0.05$) after 72 h compared to the control group and cultures at 35˚C. Bajguz (2009) demonstrated that high-temperature stress leads to inhibition of photosynthetic oxygen evolution, and decreased cell division in *C. vulgaris*. Temperature optima for many commercial microalgae changes between 20-30˚C (Sánchez-Luna et al., 2007). The previous study showed the inhibition of *C. vulgaris* growth above 30˚C

(Converti et al., 2009). Sorokin and Krauss (1962) demonstrated that at 45˚C, no constant growth was observed in *C. pyrenoidosa.*

Figure 1. The effects of different temperatures (35ºC and 45ºC) on the growth rate of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared with the control, (**) represents a statistically significant difference of $p<0.01$.

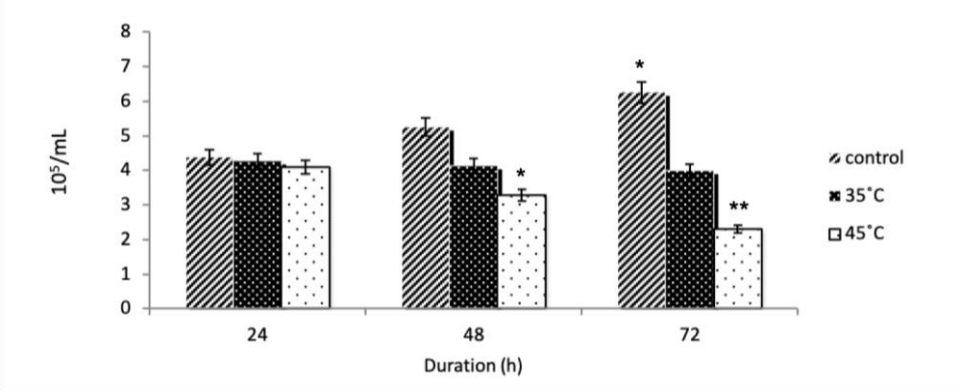


Table 1. Chlorophyll a, and b content and Chla/b rate of the algae *Chlorella vulgaris* cultivated with a growth 25 ºC (control), 35 ºC and 45ºC temperature.

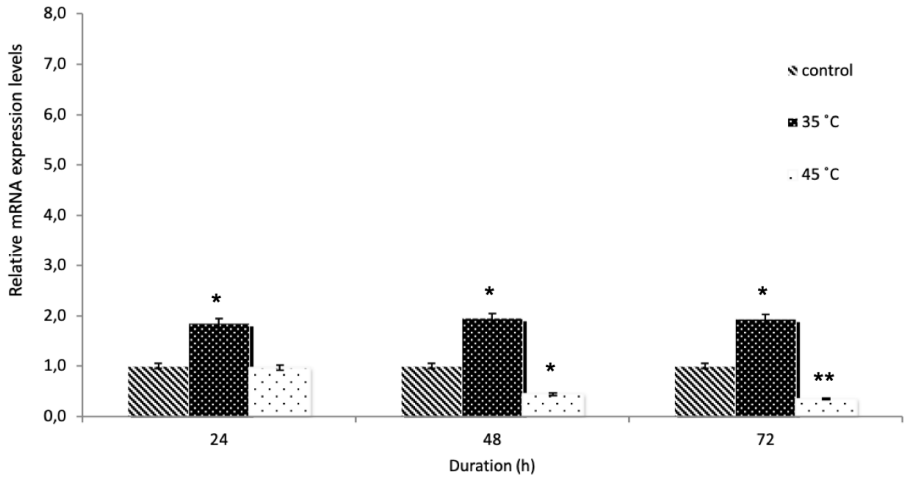
Groups	n	Chl a			Chl b			Chl a/b		
		24 h x±SD	48 h x±SD	72 h x±SD	24 h x±SD	48 h x±SD	72 h x±SD	24 h x±SD	48 h x±SD	72 h x±SD
Control	3	25.32 ±0.22	25.32 ±0.22	25.32± 0.22	7.69± 0.14	7.69± 0.14	7.69± 0.14	3.31± 0.1	3.31± 0.1	3.31± 0.1
35˚C cultures	3	24.09 ±0.2	21.81 ±0.28	19.17± 0.19	7.22± 0.19	8.75± 0.09	8.99± 0.11	3.34± 0.07	2.49± 0.06	2.09± 0.05
45˚C cultures	3	21.99 ±0.13	20.06 ±0.06	16.47± 0.06	8.58± 0.16	9.23± 0.13	11.23 ±0.08	2.54± 0.03	2.17± 0.04	1.46± 0.01

Values in bold are significantly different from control samples. Significance of differences ($p<0.05$) was checked by one-way analysis of variance (ANOVA). n=number of replicates, x=mean values, SD=standard deviations.

Chlorophyll amount is very important for photosynthesis since chlorophyll absorbs sunlight and synthesizes carbohydrates with the participation of CO_2 and water. Chlorophylls are susceptible molecules to a sense of stress-initiated oxidative stress (Puckett et al., 1973; Sandmann & Böger, 1980; Chettri et al., 1988). Under oxidative stress conditions, chlorophyll a is oxidized from the methyl group on ring II to the aldehyde groups and occurs in chl b formation (Chetri et al., 1988). For this reason, chlorophyll a/b ratio is more sensitive than chlorophyll a+b to modification. In the present study, we tested all chlorophyll parameters for understanding high-temperature effects on *C. vulgaris* culture. Based on our data, the pigment levels were not significantly different at both 35˚C and 45˚C for 24 h. Chl a, Chl b, and Chl a/b amount was significantly ($p< 0.05$) different when comparing the control group with at both 35˚C and 45˚C for 48 and 72 h (Table 1). It was observed that the chlorophyll a content and chlorophyll a/b ratio for the 72 h application period at 35˚C decreased by 20.34% and 36.56%, respectively, and at 45˚C decreased by 34.95% and 55.89%, respectively. Our present results also confirmed that a high temperature (45˚C) treatment for 72 h resulted in a significant increase in chl b and chl a consistent with the expedited conversion of one to the other (Table 1).

The high temperatures lead to *D1* protein damage and contribute to descended electron transport efficiency. Damaged *D1* protein could be immediately re-synthesis via *PSII* repair mechanisms for providing redox homeostasis in chloroplasts. Therefore, the replacement of new *D1* proteins in *PSII* needs to be the expression variations of the *D1* coding gene *psbA*. In the present study, transcription levels of two photosynthesis-related genes were analyzed by semi-quantitative RT-PCR and compared the chlorophyll degradation results under heat stress. The results revealed that the mRNA transcript level of *psbA* increased at 35˚C for 24, 48, and 72 h compared with control. The *psbA* mRNA level of *C. vulgaris* cultured at 35˚C for 24, 48, and 72 h was increased by 1.85, 1.95, and 1.94 times, respectively, as compared with the control group (Figure 2). In cultures subjected to 45˚C for 24 h, the *psbA* mRNA transcript level did not display significant differences as compared with the control group (Figure 2). However, the *psbA* mRNA transcript level was slightly decreased by 2.22 and 2.86 times, respectively, relative to control at 48 and 72 h under high temperature. Similarly, both salt stress and oxidative stress (Nishiyama et al., 2006; Allakhverdiev et al., 2008) prohibit the repair of photodamaged PSII by inhibiting the *psbA* gene transcription and translation. Qian et al. (2009) studied the effects of copper and cadmium stress on *C. vulgaris,* and the results proved that metal stress inhibits the expression of *psbA* and *rbcL* genes at the transcriptional level.

Figure 2. The effects of different temperatures (35ºC and 45ºC) on the relative expression of *psb*A of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared with the control, (**) represents a statistically significant difference of $p<0.01$.



It has been shown in previous studies the maximal transcript accumulation temperature was distinctly different for several photosynthesis-related genes. Kusnetsov et al. (1993) demonstrated that the maximum transcription level for *psbA, psbE* genes, *psbB, psbC, atpA* genes, and *psbA, psbD* genes were observed 38˚C, 40˚C, and 42˚C, respectively in higher plants. Similarly, the highest *psbA* mRNA transcript levels were found with *C. vulgaris* cultures at 35˚C at 48 and 72 h (1.95 and 1.94 times higher than the control group, respectively). The rise of the transcript levels might increase the corresponding enzyme and its activity. Thus, it might protect the electron transport in *PSI* and *PSII* under moderate high-temperature stress. According to Kusnetsov et al. (1993), the rate of electron transport decreased due to the inactivation of *PSII* acceptor side at temperatures below 40-42˚C.

Vierling and Key (1985) reported that the *rbcL* transcript level was slightly varied within the temperature range of 28 to 48˚C in soybean suspension cultures. In the present study, Figure 3 shows the mRNA transcript level of *rbcL* under high temperature. The transcript level of *rbcL* was significantly changed at 35˚C for 24, 48, and 72 h compared to the control group. According to our results at 35˚C for 24, 48, and 72 h, the transcript level of *rbcL* did not

significantly change; however, the transcript level of *psbA* increased dramatically after 24 and 48 h at 35˚C. However, compared to the control, the mRNA transcript level of *rbcL* was decreased significantly (1.07, 1.3, and 1.54, respectively) after exposure to 45˚C for 24, 48, and 72 h (Figure 3). In the present study, the mRNA transcript levels of *psbA* and *rbcL* decreased significantly after 72 h at 45˚C. The decrease in transcript levels might be the result of the prevention of normal electron transport in PSI and PSII and block carbon assimilation.

Figure 3. The effects of different temperatures (35ºC and 45ºC) on the relative expression of *rbcL* of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared to the control, (**) represents a statistically significant difference of $p<0.01$.

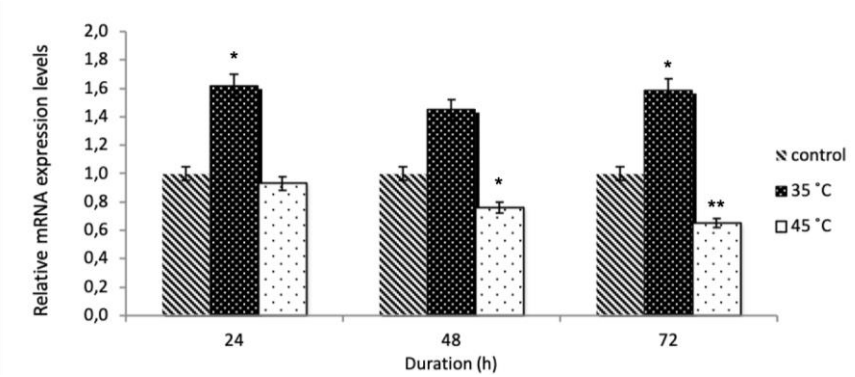
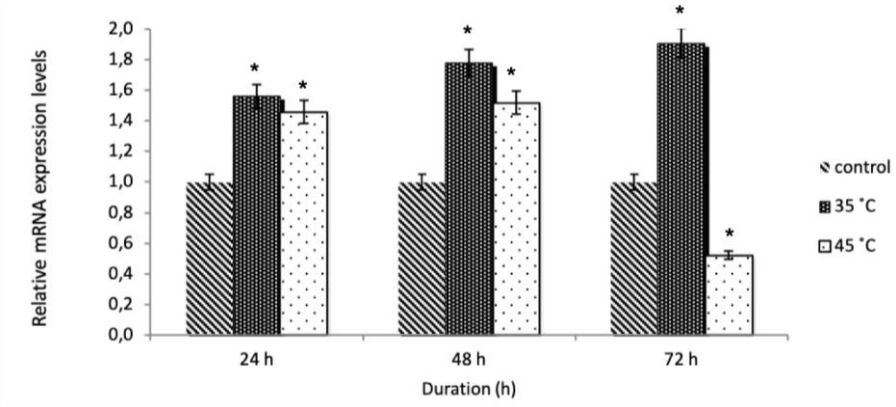


Figure 4. The effects of different temperatures (35ºC and 45ºC) on the relative expression of *Apx* of *Chlorella vulgaris* culture. (*) Represents a statistically significant difference of $p<0.05$ when compared to the control.



In living organisms, ROS accumulation occurs as a result of various stress conditions. Plants neutralize ROS by antioxidant systems, such as ascorbate peroxidase enzyme. *APX* encoding gene expressions are modulated by multiple environmental stresses, such as drought, salinity, extensive light, pathogens, and low temperature (Zhang et al., 1997; Yoshimura et al., 2000; Agrawal et al., 2003; Menezes-Benavente et al., 2004; Lin & Pu, 2010). Lin and Pu (2010) reported escalating cytosolic accumulation of *APX* transcripts in a salt-tolerant sweet potato. Goyary (2009) also demonstrated the increment of ascorbate content and *APX* gene expression in transgenic tomato plants compared to wild-type under cold temperatures. *APX* is known to have an important function against high temperatures by intercepting the oxidation of enzymes and the degradation of membranes. Previous studies reported that over gene expressions of *APX* enhanced the tolerance capacity and minimized photooxidative damage under temperature stress (Caverzan et al., 2012; Sato et al., 2011; Shi et al., 2001; Miller et al., 2007). Park et al. (2004) also emphasized the highly induced *cAPX* gene levels in sweet potato

leaves after high-temperature exposure. Moreover, Ma et al. (2008) showed the increment expression levels *APX* in apple leaves at 40˚C for 4 h exposure and decreasing afterward. In the present study, c*APX* gene was up-regulated at both 35˚C and 45˚C with different time periods, as shown in Figure 4. The c*APX* gene transcription level after exposure at both 35˚C and 45˚C for 24 h was significantly different from that of the control. According to our results, cultivation at 35˚C for 24, 48, and 72 h, the *cAPX* mRNA levels were increased by 1.56, 1.78, and 1.91 times, respectively, as compared with the control group (Figure 4). However, the c*APX* mRNA transcription level was also decreased by approx. 2 times in the *C. vulgaris* culture at 45˚C for 72 h.

4. CONCLUSION

Green alga *C. vulgaris* was used to determine the affects of the moderate and high temperature stress. The experiments were conducted with 3 different temperatures; 25ºC as control group; 35ºC as moderate temperature group and 45ºC as high temperature group. All measurements (cell density and growth rate) and analysis (*rbcL*, *psbA*, *cAPX* genes transcription levels) applied on 24th,48th and 72nd hours of the experiments. According to our results, moderate temperature does not show a significant affect on growth rate and cell density. However, at high temperature conditions growth rate decreased after 4 days. High temperature stress leads to inhibition of photosynthetic oxygen evolution, and decreased cell division in *C. vulgaris* as suggested in previous studies. Chl a content and chl a/b ratio decreased under moderate and high temperature stresses after 72 h. Besides pigment ratio changes, some differences are determined on stress genes transcription levels. For example, *psbA* gene transcription levels decreased at high temperature stress conditions after 48 hours. The *cAPX* levels of moderate and high temperature exposed groups were up-regulated after 24 hours. Our results suggest that the *cAPX* gene expression could mitigate high temperature-induced oxidative damage in *C. vulgaris*, depending on the application period, through increased *psbA* and *rbcL* transcript levels and decreased chlorophyll degradation. Future work will focus on how the *cAPX* interacts with the *psbA* and *rbcL* expression responses to high-temperature stress.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Inci Tuney Kizilkaya: Writing, editing, validation. **Sedef Akcaalan:** Laboratuary work. **Dilek Unal:** Experiment design, supervision, statistical analysis, validation.

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