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Genotoxicity and Oxidative Stress of Water Concentrates from Recreation Pools After Various Disinfection Methods

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Abstract: In this study, it was aimed to evaluate the pool water quality, bacterial contamination, genotoxicity and oxidative damage parameters according to different disinfection methods in regression swimming pools in tourist facilities in Antalya, Türkiye in March 2019. For this purpose disinfected pool water samples and erythrocyte solutions and lymphocytes were incubated at two hours. At the end of the incubation, erythrocyte lipid peroxidation by thiobarbituric acid reactive substances method and peripheral blood lymphocytes DNA damage by the single cell gel electrophoresis assay were determined. It showed that all recreational pool water samples disinfected with different disinfectants caused more DNA damage and oxidative stress than mains water. Genotoxicity and oxidative stress data showed that the silver-copper ionization method was the best disinfection method compared to other disinfection methods. Our findings support the potential genotoxic and oxidative stress effects of exposure to disinfectant residues due to different disinfectant uses in regression swimming pools. The positive effects of swimming on human health, continuous monitoring of the quality of pool water and the preference of silver-copper ionization for disinfection can reduce potential health risks.

Key words: Swimming pools, Cytotoxicity, Genotoxicity, Oxidative stress

1. Introduction

While swimming pools have long been a tool for exercise and relaxation around the world, it remains one of the most popular and attractive activities due to its positive effects on health [1,2]. Unhygienic pools harbor potentially accumulated harmful microorganisms and organic matter. Pathogenic microorganisms and environmental pollutants directly or indirectly enter pools and cause water-borne infections. Microorganisms that cause infections in pool water can be transmitted to swimming pool users through saliva, blood, urine, vomit and feces [3,4]. Disinfectants (such as chlorine, chloramines, ozone and UV) used in swimming pools to neutralize microorganisms and reduce the health hazard of pool users, also provide the formation of residual disinfectant

that harm the health of people using these pools [5]. It has been determined that the residual disinfectant formed after disinfection is cytotoxic, neurotoxic and genotoxic. In addition, residual disinfectant has been found to be mutagenic, carcinogenic and/or teratogenic [6]. Since chlorine, which is widely used in the disinfection of swimming pool water, creates many organohalogenated DBPs, research on alternative methods for disinfection (such as ozone, hydrogen peroxide, copper-silver ionization and UV) of swimming pool water is increasing [7, 8].

Reactive chemicals used in pool water disinfection cause cellular damage by directly or indirectly oxidizing DNA, protein/peptide and lipids. The resulting oxidation products, together with the reactive species produced by disinfection, bind inflammatory cells, causing oxidative stress [9, 10]. The disinfectant residue formed because of disinfection used in pool water can cause a general oxidative stress in cells and tissues [11]. Exposure to residual disinfectant has been found to induce several molecular signaling pathways in the cellular response to oxidative, genotoxic, and proteotoxic stresses. Exposure to residual disinfectants created by disinfection methods (such as ozone, hydrogen peroxide, copper-silver ionization and UV) used in pool water has been found to induce various molecular signaling pathways in the cellular response to oxidative, genotoxic and proteotoxic stresses [10-13]. The formation of residual disinfectants can cause cancer, degenerative conditions, and immune system dysfunction in somatic cells, while DNA damage in germ cells is linked to reproductive dysfunction, hereditary damage, and developmental defects Therefore, many studies have focused on the genotoxic effects of residual disinfectants after disinfection of pool water [14-17].

While previous researchers have focused on the concentrations of certain disinfectant residues in recreational waters and tried to correlate them with adverse health outcomes in humans, these efforts may fall short due to the wide variety of toxic components in these waters due to different disinfection methods. The main purpose of this study is to compare the genotoxicity and oxidative stress parameters of recreational waters obtained from a single source treated with different disinfectants (chlorine, ozone, hydrogen peroxide and copper-silver ionization), rather than trying to relate specific residual disinfectants or DBPs with genotoxicity and oxidative stress. In our study, we applied water samples collected from four regression pools with different disinfection methods to erythrocyte and lymphocyte cultures and compared genotoxicity and oxidative stress parameters with main water serving as a common source. Our results can help identify different disinfection treatments in a recreational water system and their impact on human health.

2. Material and Method

2.1. Chemical and reagents

Sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), trichloroacetic acid (TCA), potassium dihydrogen phosphate (KH_2PO_4), triton-X-100 ($C_8H_{17}C_6H_4(OC_2H_4)_{9\text{-}10}OH$), thiobarbituric acid (TBA), sodium hydroxide (NaOH), hydrogen chloride (HCl) purchased from the company Merck. Sodium bicarbonate ($NaHCO_3$), citric acid ($C_6H_8O_7$), tris base ($NH_2C(CH_2OH)_3$), acetic acid ($C_2H_4O_2$), ethanol (C_2H_6O), tris aminomethane ($NH_2C(CH_2OH)_3$), sodium mono hydrogen phosphate ($Na_2HPO_4.\ _7H_{20}$) were supplied by SIGMA company.

2.2. Sampling and analysis

Swimming pool samples were collected from five different regression pools using the same mains water located in the city of Antalya (Türkiye), with an estimated length of 25 m, a width of 12.5 m, and a depth of 1.5-2.5 m. Water samples were taken in March 2019. Sampling in ponds was done 15 to 30 cm below the water surface. Each of the locations

was systematically sampled on alternating days at times when it was open to the public (between 12 and 14 hours). Samples were collected in sterilized vials and immediately transported to the laboratory. All samples were kept at +4°C until the time of study. Water sample characteristics are provided in Table 1.

Table 1. Characteristics of pool samples

Pool Code	Type	Location	Temperature (°C)	Disinfection Procedure
1	cold pool	indoor	25 – 27 °C	Chlorination
2	cold pool	indoor	25 – 27 °C	Ozonation
3	cold pool	indoor	25 – 27 °C	Hydrogen peroxidation
4	cold pool	indoor	25 – 27 °C	Silver-copper ionization
5	cold pool	indoor	25 – 27 °C	Mains water

2.3. Determination of physical–chemical parameters

The main purpose of physical and chemical research was to determine the chemical composition of water, with special consideration of substances hazardous to human health. All physical and chemical laboratory tests were done on the day of sample collection, according to the Water Environmental Federation, and American Public Health Association (APHA) [18] and the findings were compared with local guidelines. Swimming pool water temperature (°C) and pH values were measured using a Testo 106 brand temperature meter and WTW pH 330i brand pH-meter, respectively. Other chemical parameters were analyzed according to APHA, and the findings were compared with local guidelines [18].

2.4. Determination of microbial factors

The main goal of the microbiological research was to assessment the time-dependent variation of *Escherichia coli (E. Coli)* growth, which plays a dominant role in the control process of swimming pool water quality, regardless of the degree of contamination of the pool water with DBP using different disinfectants. Microbiological analysis according to time in regression pools with four different disinfections was performed according to the TS-EN-ISO-9308-1 method.

2.5. Determination of erythrocyte MDA levels

The levels of TBARS in erythrocytes were determined as described previously by Stocks and Dormandy with some modifications [19]. Blood was collected in vials containing heparin (10 U/mL), from healthy cows slaughtered at the slaughterhouse. Erythrocytes resuspended at 3 mg/dL hemoglobin in PBS containing 2 mM sodium azide as catalase inhibitor. This suspension was incubated at 37°C for 60 min in a shaking water bath to allow inactivation of erythrocyte catalase by the sodium azide. Freshly disinfected pool waters, main waters (control), positive control (50 µM t-BHP) and negative control (50 µM resveratrol) were equilibrated with PBS puffer (50 mM, pH=7.4). The 3 mL of each sample were taken and incubated with 1 mL previously prepared red cells suspension at 37°C for 120 min in a shaking water bath. The positive control contains 1.5 mM t-butylhyroperoxide (t-BHP) volume of in the place of the samples. After the incubation, 900 µL of the erythrocytes exposed to different compounds were mixed with 600 µL of 28% TCA and centrifuged over 10 min at 4500 rpm at room temperature. Thereafter, an aliquot of 800 µL of supernatant was added to 400 µL of thiobarbituric acid (0.069 M).

The resulting samples were heated at 100 °C for 60 min. After cooling, the intensity of pink color of the end fraction product was determined at 532 and 600 nm. MDA concentrations were calculated as follows:

$$MDA = (A_{532} - A_{600}) \times 900 = nmolMDA/g\ Hb.$$

2.6. The comet assay analysis

Cell viability was assessed by trypan blue staining, and samples with >90% viability were used. Lymphocytes were isolated by mixing bloods with Histopaque-1077 (Sigma, Germany) at a ratio of 1:1 and centrifuged at 2000 rpm for 10 min. at 4 °C. Pool waters and unconditioned water (negative control) samples buffered with 1 mL of PBS (50 mM PBS, pH=7.4) was added to lymphocytes and lymphocyte suspensions were incubated at 37°C for 1 and 2 h. Each sample was subjected to triplicate analysis.

The comet assays analysis was carried out under alkaline conditions, basically as described by Singh et al. with minor modifications [20]. Cell suspensions were centrifuged at 2500 rpm for 10 min. after incubation, supernatant was removed and centrifuged again at 2500 rpm for 10 min by adding 1:1 phosphate buffered saline (PBS). The supernatant was removed again and diluted with PBS depending on the density of the remaining cells. Approximately 50 µl of cell suspension (approximately $5x10^4$ cells) mixed with100 µL 0.75% low-melting agarose (LMA, Fisher Scientific), spread on slides that had previously been coated with 1.0% normal-melting agarose (NMA, Serva Electrophoresis GmbH), and covered with a coverslip. 5 min later the coverslip was removed without damaging the agarose gel and the slides were held in lysis solution (freshly added 2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, pH 10, 10% DMSO, and 1% Triton X-100) in the dark at 4°C for 120 min. Then, they were incubated in freshly prepared cold electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min in the dark at 4 °C. Then, the slides were placed in an electrophoresis tank containing electrophoresis solution, and electrophoresis was applied at 25 V (1.02 V/cm) for 30 min. Cells were then washed three times with neutralization buffer. Samples were stained with ethidium bromide and photographed with Zeiss Axiocam Icc 1 camera under Zeiss Imager A1 fluorescence microscope. An average of 100 cells per slide were evaluated automatically using Open Comet software [21]. Tail moment, tail length and tail density parameters were used to evaluate DNA damage.

2.7. Statistical analysis

Statistical analyses were evaluated using the SPSS version 11.5 (SPSS Inc., Chicago, Illinois, USA) statistical program. Differences between groups were assessed using analysis of variance (ANOVA) followed by Tukey multiple comparisons. Data are presented as mean ± standard deviation (SD) of the mean. A p values <0.05 was regarded as significant.

3. Results

3.1. Physical–chemical quality assessments

Our research results determined that all physical-chemical parameters of the examined swimming pools compliance with the Türkiye Swimming Pool Water Standards (Decree No. 28143/2011).

The physico-chemical results and national standard values of the water disinfected with different disinfectants in the regression pools examined are shown in Table 2. The recorded temperature for the five swimming pools studied in this study was kept constant

between 36 and 37°C. The pH value in the pool was found to be within the recommended range in the Türkiye National Standard (6.5-7.8) and the World Health Organization (WHO) directive (7.2-7.8). The mean alkalinity value was within the national standard range (30-80 mg/L) in all samples. All the samples showed the hardness values recommended in the national standards. It was determined that the other physical-chemical values of the pools were within the Türkiye National Standards.

Table 2. Physical-chemical analyzes of water samples taken from the examined swimming pools

	Parameters									
Pool Code	***Temperature (°C)***	***pH (units)***	***Total Alkanity (mg/L)***	***Biguand (mg/L)***	***Copper (Cu) (mg/L)***	***Ammonium (NH_3) (mg/L)***	***Nitrite (NO_2) (mg/L)***	***Nitrate (NO_3) (mg/L)***	***Aluminum (Al) (mg/L)***	***Hydrogen peroxide (H_2O_2)***
1	26–34	7.4	56	-	<0.1	<0.023	<0.014	18.56	<0.05	-
2	25–32	7.4	60	2	<0.1	<0.023	<0.014	16.79	<0.05	-
3	26–30	7.4	65	-	<0.1	<0.017	<0.017	22.6	<0.04	55
4	25–28	7.2	58	-	<0.1	<0.023	<0.014	13.05	<0.05	-
5	25–27	≥6.5 ≤7.8	180	-	-	0.50	0.50	50	200	-
National standard[1]	26 -28	6.5-7.8	30-180	2-30	ND-1	ND-0.5	ND-0.5	ND-50	ND-0.2	40-80
Method		*TS 3263 ISO 10523*	*SM 2320 B*	*Pool tester*	*SM 3500 Cu B*	*SM 4500 NH_3 F*	*SM 4500 NO_2 B*	*SM 4500 NO_3 (E)*	*SM 3500 Al B*	*DIN 38409-15*

1:Chlorination, 2:Ozonation, 3:Hydrogen peroxidation,4:Silver-copper ionization, 5:Mains water.

[1]: Türkiye National Standard (NO: 28143), swimming pools—general requirement.

ND: Standard value not defined.

3.2. Microbial assessments

Table 3 shows the results obtained regarding the time dependent growth of *E. coli* in the studied pools and comparing them with the Türkiye national standards Microbiological evaluation of pool water disinfected with different disinfectants according to time was made according to the TS-EN-ISO-9308-1 method. *E. coli* growth studied throughout the study varies over time in different disinfected pools. There was no growth in the four pool water samples that were disinfected on the first day of disinfection. At the end of the fifth day, it was found that *E. coli* growth in ozonated pool water was higher than other disinfectants.

Table 3. Time-dependent microbial quality of water samples collected from the examined swimming pools

Parameters	***E. coli (CFU/100 mL)***				
Pool Code	***Time (day)***				
	1	***2***	***3***	***4***	***5***
1	0/3	65/1.5	68/1.2	78/1.0	100/0.2
2	0/0.6	110/0.5	140/0.4	190/0.3	300/0.1
3	0/80	40/70	37/50	45/40	70/30
4	0/0.6	80/0.4	76/0.3	110/0.2	150/0.1
National standard[1]	< 200 CFU/ml				

CFU: colony-forming units.

1: Türkiye National Standard (NO: 28143).

1:Chlorination/ppm, 2:Ozonation/ppm, 3:Hydrogen peroxidation/ppm,4:Silver-copper ionization/ppm, 5:Mains water.

3.3. Biochemical result

In our study, free radical-mediated oxidative damage that may occur because of *in vitro* exposure of erythrocytes to residual disinfectant consisting of different disinfectants was evaluated by MDA determination method. The findings show that disinfectants used in disinfection of pool water oxidize erythrocyte membrane lipids and cause oxidative damage in lipids. In addition, lipid damage varies according to the type of disinfectant used.

In particular, the MDA level of disinfection with ozonation was found to be statistically significantly higher than the other groups except the positive control (t-BHP) ($p < 0.05$) (Table 4).

Table 4. Effects of different disinfection agents on lipid peroxidation level of erythrocytes

Group	***Erythrocyte MDA (nmol/g Hb)***
Main water	8.07±0.23
Hydrogen peroxidation (g/mL)	9.83±0.27^{a,b,c}

Ozonation (g/mL)	13.14±0.39[a,c]
Chlorination (g/mL)	11.75±0.72[a,b,c]
Silver-copper ionization (g/mL)	9.51±0.32[a,b,c]
t-BHP (g/mL)	14.36±0.34[a,c]
Resveratrol (g/mL)	7.17±0.18[a,b]

Data represented as mean X ± SD. Comparison between the groups analysis of variance (ANOVA) *p* value.

[(a)] :Compared with the control group,
[(b)] :Compared with the hydrogen peroxidation group,
[(c)] :Compared with the ozonation group,
[(d)] :Compared with the chlorination group,
[(e)] :Compared with the silver-copper ionization group,
[(f)] :Compared with the t-BHB group is significantly different.

Statistical differences between groups were made with the Tukey post-hoc multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

3.4. Comet assay assessments

DNA damage in pool water samples was determined according to different disinfection methods (Figure 1). Our analyzes showed that all disinfected pool water samples were more genotoxic than mains water. Chlorination method revealed statistically significantly higher DNA damage when compared to other disinfection methods ($p < 0.001$). The lowest DNA damage was detected in the disinfection of silver-copper ionization.

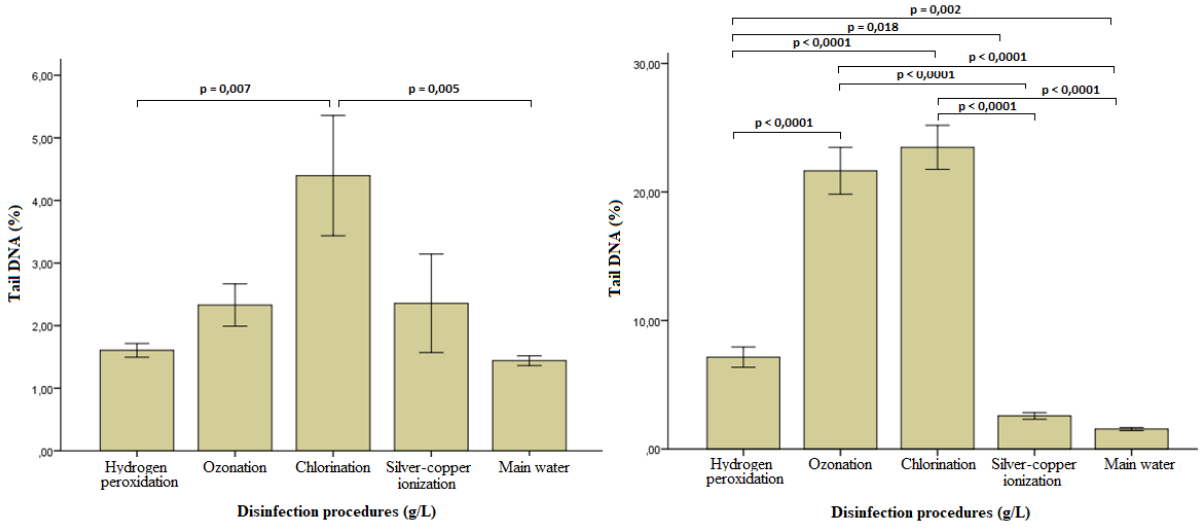

Figure 1. Comparison of time dependent genotoxicity of recreational pool water samples and mains water. First hour (A), Second hour (B). The columns in the graphs and the bars in the columns represent the mean ± standard error (X±SD)

4. Conclusion and Comment

In summary, the key findings in the current studies showed that the physico-chemical parameters of the regression pool water samples, in which we used different disinfection methods, were within acceptable limits recommended by national standards. Given the significant effects of regression pools on microbial safety and the health effects on users of these pools, physical-chemical parameters in regression pools should be constantly monitored. However, regression pool water samples containing *E. coli* or fecal coliform that did not meet the World Health Organization (WHO) standard indicated that pool water quality needed to be improved [22]. A comprehensive evaluation was made on the genotoxicity and oxidative stress of different disinfection types in recreational pools using the same mains water using the MDA parameter. Our data determined that all disinfected recreational pool water samples were more genotoxic than mains water. It was observed that especially disinfection with chlorination caused high genotoxicity. In our study, it was determined that the silver-copper ionization method used was the best disinfection method in terms of genotoxicity compared to other disinfection methods. It is thought that the application of different disinfection methods in the regression pool creates cytotoxicity in the cells and this causes oxidative stress. For this reason, the type of disinfection used in the pool water and the exposure time are very important. Our analyzes revealed that we should prefer silver-copper ionized pools, which have less negative effects on human health than the chemicals used in disinfection and the residual disinfectants formed by these chemicals compared to other disinfection types. In particular, our results highlighted the importance of using a combination of different bioassays to evaluate the oxidative stress and genotoxicity of water samples in different disinfection methods (chlorine, ozone, hydrogen peroxide and copper-silver ionization) commonly used in pool waters. Although swimming has anti-inflammatory, antioxidant and anti-apoptotic effects, it was concluded that the disinfectants used in regression pools and the residual disinfectants formed by them adversely affect human health.

Authorship contribution statement

Z. Bektaş: Fieldwork, Methodology, Investigation, Resource/Material Supply; **M. Şahin:** Validation, Formal Analysis, Investigation, Original Draft Writing, Review and Editing; **V. A. Toğay:** Data analysis, Formal Analysis, Investigation, Visualization; **U. Şahin:** Data Curation,Investigation, Visualization, Review and Editing; **M. Calapoğlu;** Methodology, Supervision/Observation/Advice, Project Administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Committee Approval and/or Informed Consent Information

As the authors of this study, we declare that we do not have any ethics committee approval and/or informed consent statement.

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