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ORIGINAL ARTICLE

Genotoxicity and cytotoxicity of copper oxychloride in cultured human lymphocytes using cytogenetic and molecular tests

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Abstract The genotoxicity of copper oxychloride was investigated in human lymphocytes using chromosome aberration (CA) and micronucleus (MN) tests and the randomly amplified polymorphic DNA-polymerase chain reaction technique. The lymphocytes were treated with 3, 6, and 12 µg/mL of copper oxychloride for 24 and 48 h. Copper oxychloride increased CA and abnormal cells in a dose-dependent manner. The frequency of MN and micronucleated binuclear cells also increased at all concentrations and treatment periods. However, copper oxychloride cytotoxicity, observed through lower mitotic and nuclear division index, was significantly lower only at the higher concentrations (6 and 12 µg/mL). Copper oxychloride increased the polymorphic bands and decreased genomic template stability. In conclusion, in this study it was confirmed that copper oxychloride has genotoxic potential for human lymphocytes in vitro. Additionally, caution is advised for its use as a fungicide, because it may increase the risk of exposure through the food chain.

Keywords Copper oxychloride · Genotoxicity · Chromosome aberration · Micronucleus · RAPD-PCR

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Introduction

Copper oxychloride [$3Cu(OH)_2 \cdot CuCl_2$] is a common broad spectrum fungicide used to protect vegetables and fruits against diseases such as *Venturia inaequalis*, *Taphrina deformans*, *Coryneum beijerinckii*, *Xanthomonas campestris* pv, *Vesicatoria*, *Phytophthora infestans*, *Alternaria solani*, and *Deuterophoma tracheiphila* (Pérez-Rodríguez et al. 2013; Al-Assiuty et al. 2014).

Copper is a heavy metal that can be accumulated in various tissues of all living species (Snyman et al. 2004; Matache et al. 2013; Hurwitz et al. 2014). Its continuous application may lead to copper accumulation in crops, soil, and water (Chen et al. 1997; Masaka and Muunganirwa 2007) that consequently leads to human and animals to be exposed to copper through the food chain (La Pera et al. 2008), causing diseases such as kidney dysfunction, hepatocellular neoplasia, and hemolytic anemia (Waheed et al. 2013; Hurwitz et al. 2014; Ciji and Bijoy Nandan 2014).

Chronic copper poisoning can also cause weakness, abdominal pain, headache, dizziness, leg and back pain, anemia, and chronic kidney and liver damage in humans (Gunay et al. 2006; Hloch and Charvát 2012).

There are a few studies that have confirmed the genotoxic and cytotoxic effects of copper oxychloride in test animals using chromosome aberration and molecular toxicity tests (Pirtskhelani et al. 2008) or random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR) (Atienzar et al. 2001; Gupta and Sarin 2009). To our best knowledge, so far none has examined its genotoxic effects on human lymphocytes as the most relevant markers of human exposure (Madle et al. 1993; Buyukleyla and Rencuzogullari 2009; Sevindik and Rencuzogullari 2014). The aim of this study was to fill this gap by investigating the genotoxic effects of copper oxychloride using widely adopted methods to detect DNA damage in humans, namely chromosome aberration (CA), micronucleus (MN), and RAPD-PCR tests (Albertini et al. 2000; Norppa and Falck 2003; Atienzar and Jha 2006; Kocaman and Topaktaş 2010).

Materials and methods

The test substance copper oxychloride was obtained in the commercial formulation that contains min 95 % copper oxychloride with min 51.4 % of the suspended active ingredient Cu (514 g Cu/L) (Tarem Inc., Delaware, DE, USA). The formula and other properties of the product are given in Table 1.

Chromosome aberration and micronucleus test

Chromosome aberration test was applied following the method described by Evans (1984) and the MN test was used as described by Fenech (2000) and Kirsch-Volders et al. (2003) with minor modifications. We also followed the IPCS guidelines (Albertini et al. 2000) and obtained the approval from the Ethics Committee of Adiyaman University (registered under no. 19.03.2013/03-01.1) in accordance with the Declaration of Helsinki.

To prepare the lymphocytes for CA testing, we added 0.2 mL of heparinized whole blood from five healthy, young donors (three females and two males, all non-smokers, aged 21–22) to 2.5 mL chromosome medium B (Biochrom, Berlin, Germany, F5023) and the culture was incubated at 37 °C for 72 h. The non-cytotoxic copper oxychloride concentrations were established in preliminary study. Cultured cells were treated with 3, 6, and 12 μg/mL concentrations of copper oxychloride which was dissolved in sterile distilled water as described by the manufacturer, for 24 and 48 h. Untreated blood samples and samples treated with 0.22 μg/mL ethyl methanesulfonate (EMS, Sigma, M0880) were used as negative and positive control, respectively. After colchicine treatment, 2 h before harvesting, the cells were treated with

Table 1 The formula and properties of copper oxychloride

Formula	OH HO–Cu Cl OH Cl–Cu OH HO–Cu HO–Cu
Product name	Copper oxychloride
Trade name	Cymoxanil (1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea)
Synonyms	Copper chloride, copper oxide hydrate, dicopper chloride trihydroxide
Chemical formula	$3Cu(OH)_2 \cdot CuCl_2$
CAS no	1332-40-7, 1332-65-6
Toxicity	Oral rat LD50: 1470 mg/kg
Fatal oral human dose	200 mg/kg body weight
Properties	Green powder, copper leaching and precipitation process, dry fluent
Chemical Properties	As solution: copper oxychloride min 95 % contains copper (Cu) min 50 %, arsenic max 50 ppm, lead max 200 ppm, mercury max 50 ppm, cadmium max 50 ppm, trace elements (iron, sodium, chloride)

a hypotonic solution of 0.4 % KCl for 5 min then fixed three times in a mixture of methanol and glacial acetic acid (3:1). Air dried slides were stained with 5 % Giemsa stain prepared with Sorensen's buffer for CA test following the standard methods (Evans 1984).

Chromosome aberration frequency and the percentage of abnormal cells (AC) were determined in 100 well-spread metaphases per donor (totaling 500 metaphases per concentration). Gaps were not counted as CA (Mace et al. 1978). Aberrations were classified according to the International System for Human Cytogenetic Nomenclature (ISCN) (Paz-y-Miño et al. 2002). The mitotic index (MI) was also determined by scoring 3000 cells from each donor. The MI detects delaying effects of chemicals on the G2 phase of the cell cycle (Evans 1984).

To score the number of micronuclei in binucleated cells, we used the cytochalasin B-induced cytokinesis block (Fenech 2000; Kirsch-Volders et al. 2003) by adding 0.2 mL of whole blood from each donor to the culture medium and treating the cultures with 3, 6, and 12 µg/mL of copper oxychloride solution for 24 and 48 h. Again, we used negative and positive control as described above. Six µg/mL of cytochalasin B (Sigma, St. Louis, MO, USA, C6762) was added to the cultures at hour of incubation in order to block cytokinesis. After 24-hour incubation with cytochalasin B at 37 °C, the lymphocytes were initially treated with 0.4 % KCl for 2 min then harvested by centrifugation (at 1200 rpm for 10 min). The harvested lymphocytes were fixed once with a mixture of methanol:glacial acetic acid (5:1) and an equal amount of 0.9 % NaCl, and then fixed twice with only methanol:glacial acetic acid (5:1). Air dried slides were then stained with 5 % Giemsa prepared in Sorensen's buffer (Rencuzogullari et al. 2004). For each subject, 2000 binucleated cells were scored for MN frequency and a total of 1000 viable cells for the frequency of cells with 1, 2, 3, or 4 nuclei to calculate the nuclear division index (NDI) using the formula: $NDI = (M1) + (2 \times M2) + (3 \times M3) + (4 \times M4)/N$ (Fenech 2000), where M1–M4 represents the number of cells with one to four micronuclei and N the total number of viable cells scored.

Statistical analysis

To determine the statistical significance for CA, AC, and MN counts we used the one-way analysis of variance (ANOVA). Correlation and regression coefficients were used to determine dose–response relationships. For all statistical analyses we used MINITAB 14 version (Minitab Ltd., UK). The P value <0.05 was considered significant.

Table 2 Primer sequences used for RAPD

Primers	Sequences (5′–3′)	G+C %	Cat. no.
PM4	5′-TTGGCACGGG-3′	70	OPD-07
PM6	5′-GGTGACGCAG-3′	70	OPB-07
PM7	5′-GGGTAACGCC-3′	70	OPA-09
PM8	5′-CCCGTCAGCA-3′	70	Not determined
PM9	5′-TCCGATGCTG-3′	60	OPS-07
PM10	5′-CTGCGCTGGA-3′	70	OPU-16
PM14	5′-GTTTCGCTCC-3′	60	OPB-01
PM15	5′-GTAGACCCAT-3′	50	Not determined
PM16	5′-AAGAGCCCGT-3′	60	Not determined
PM17	5′-AACGCGCAAC-3′	60	Not determined

PCR/RAPD test

To determine molecular genotoxicity of copper oxychloride in human lymphocytes with the RAPD method we used 10 oligonucleotide primers (Table 2) with variable GC base pair proportions, as described earlier by Atienzar and Jha (2006) with minor modifications.

We added peripheral blood from two healthy donors (one male and one female, both non-smokers, aged 21) to chromosome medium and incubated the culture at 37 °C for 72 h. The cultured cells were then treated with 3, 6 and 12 µg/mL concentrations of copper oxychloride solution 24 and 48 h. In addition to the negative and positive control cultures described above (untreated and EMS-treated, respectively), we used a second positive control culture treated with 2 µg/mL sodium azide (SA, Merck, Darmstadt, Germany, 106688). Ethyl methanesulfonate was used to cause frameshift mutation and SA base substitution (single-point mutation). At the end of the exposure period, the cultured cells were washed twice in sterile isotonic solution (0.9 % NaCl), and genomic DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol (http://www.roche-applied-science.com).

Thirty-five RAPD-PCR reactions were performed with a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) using the 10

oligonucleotide primers. PCR reaction mixtures (25 μL of total volume) consisted of the PCR buffer 1× [10 mM Tris–HCl, pH 8.8 and 50 mM KCl], 3 mM $MgCl_2$, 2.5 U/μL of Taq DNA polymerase (Fermantas, Vilnius, Lithuania, SB38), 1.25 μM of each dNTPs (Fermantas, RO191), 4 pmol of each primer, and 50 ng of the DNA sample. PCR conditions were used as follows: an initial denaturation at 95 °C for 5 min, was followed by 35 cycles at 95 °C for 1 min, at 34 °C for 1 min and at 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products were electrophoresed on 2 % agarose gel containing 0.5 μg/mL ethidium bromide and visualized under UV illumination. Fragment sizes were confirmed with DNA reference bands (DNA ladder, 100 bp) (Thermo Scientific, Waltham, MA, SM0321).

RAPD profiles and statistical analysis

All amplified bands in untreated control were scored as 0 and new and missing bands in treated groups were scored as 1. Bands in the treated groups that were the same as in control were scored as 0. By scoring the new and missing bands we were able to detect polymorphisms in RAPD profiles. The scores were then statistically analyzed using Student's *t* test. The genomic template stability (GTS) rate was calculated using the following formula: $GTS = (1 - y/z) \times 100$, where y is the total number of polymorphic bands in the treated groups (total number of new and missing bands) and z is the total number of bands in negative control, as described by Aras et al. (2011). Changes in RAPD profiles decreased the GTS rate, which indicated to genotoxic effects.

Results

Treatment with copper oxychloride significantly increased the percentage of CA and AC in human lymphocytes at all concentrations of all treatment periods when compared to negative control (Table 3). The effect was dose-dependent for CA in both 24 and 48-hour exposure ($r = 0.998$, $P = 0.042$ and $r = 0.997$, $P = 0.048$, respectively) and for AC in 24-hour exposure ($r = 0.999$, $P = 0.020$) (Fig. 1).

Copper oxychloride also significantly increased the MN frequency and the percentage of micronucleated binuclear cells (MNBN) at all concentrations of all treatment periods when compared to negative control (Table 4). However, the effect was not dose-dependent (Fig. 2).

Copper oxychloride decreased the MI at only the highest concentration (12 μg/mL) and decreased the NDI at the two highest concentrations (6 and 12 μg/mL)

Table 3 Structural chromosome aberrations and percentage of abnormal cells in cultured human lymphocytes treated with copper oxychloride

Test substances[a]	Treat. time (h)	Concent. (μg/mL)	Abnormalities[+]			Structural CA % mean ± SE[b]	Abnormal cell % mean ± SE
			B′	B″	P		
Control	–	–	10	6		3.20 ± 0.48	3.20 ± 0.48
EMS	24	0.22	125	29	4	61.60 ± 4.26	40.39 ± 5.11
CuOCl	24	3	40	7	1	9.40 ± 1.28*	9.00 ± 1.14*
CuOCl	24	6	52	13	3	13.00 ± 1.09**	11.80 ± 0.58**
CuOCl	24	12	99	14	3	22.60 ± 0.81**	18.20 ± 1.39**
EMS	48	0.22	216	120		96.00 ± 9.71	52.20 ± 4.90
CuOCl	48	3	61	14	1	15.00 ± 1.26**	13.60 ± 0.81**
CuOCl	48	6	74	19	2	18.60 ± 1.20**	14.60 ± 1.12**
CuOCl	48	12	123	20	1	28.60 ± 2.87**	20.60 ± 1.63**

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

[+] B′: Chromatit type; B″: Chromozome type; P: Polyploidy

[a] CuOCl: copper oxychloride; EMS: ethyl methanesulfonate, served as positive control

[b] Significant compared to control, SE – standard error

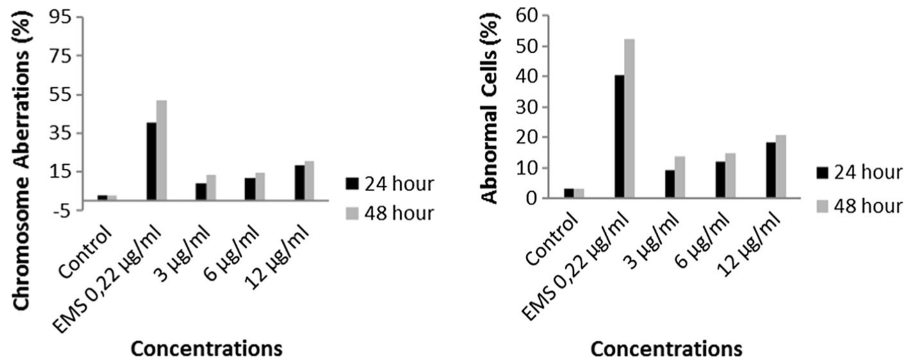

Fig. 1 The percentage of chromosome aberrations and abnormal cells in cultured human lymphocytes treated with CuOCl for 24 and 48 h

Table 4 The frequency of micronucleus (MN) and micronucleated binuclear (MNBN) cells in cultured human lymphocytes treated with copper oxychloride

Test substances[a]	Treatment time (h)	Concentrations (µg/mL)	MN % mean ± SE[b]	MNBN % mean ± SE
Control	–	–	0.64 ± 0.03	0.63 ± 0.02
EMS	24	0.22	2.43 ± 0.47	2.03 ± 0.34
CuOCl	24	3	1.63 ± 0.12***	1.40 ± 0.09***
CuOCl	24	6	2.00 ± 0.13**	1.72 ± 0.15**
CuOCl	24	12	1.99 ± 0.25**	1.77 ± 0.25*
EMS	48	0.22	4.23 ± 0.31	3.43 ± 0.16
CuOCl	48	3	1.39 ± 0.19*	1.28 ± 0.17*
CuOCl	48	6	1.98 ± 0.30*	1.83 ± 0.29*
CuOCl	48	12	1.97 ± 0.29*	1.81 ± 0.25**

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

[a] CuOCl: copper oxychloride; EMS: ethyl methanesulfonate, served as positive control

[b] Significant compared to control

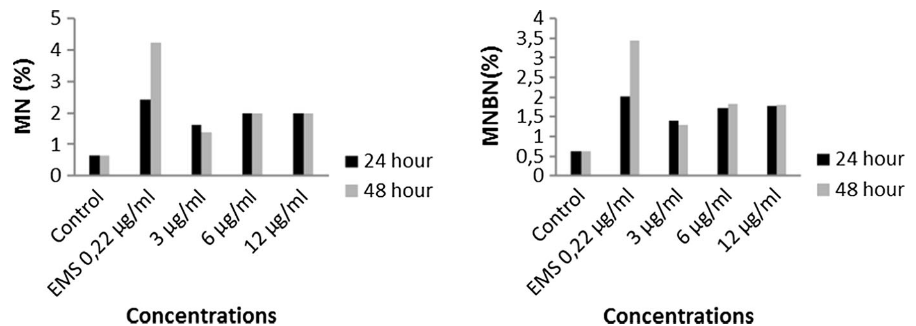

Fig. 2 The percentage of micronucleus (MN) and micronucleated binuclear (MNBN) cells in cultured human lymphocytes treated with CuOCl for 24 and 48 h

in both exposure periods (Table 5). Again, the effect was not dose-dependent for either index (Fig. 3).

The significant increase in total polymorphic bands, observed for all concentrations and treatment periods, was also not dose-dependent (Table 6). The GTS rate also significantly increased in all concentrations and treatment periods but not in a dose-dependent manner (Table 7; Fig. 4).

Table 5 The mitotic index (MI) and nuclear division index (NDI) in cultured human lymphocytes treated with copper oxychloride

Test substances[a]	Tratment time (h)	Concentrations (μg/mL)	MI mean ± SE[b]	NDI mean ± SE
Control	–	–	6.66 ± 1.13	1.53 ± 0.02
EMS	24	0.22	1.83 ± 0.24	1.05 ± 0.01
CuOCl	24	3	5.12 ± 1.35	1.11 ± 0.01
CuOCl	24	6	4.05 ± 1.27	1.07 ± 0.01**
CuOCl	24	12	3.73 ± 0.99*	1.07 ± 0.01*
EMS	48	0.22	0.91 ± 0.35	1.04 ± 0.01
CuOCl	48	3	5.40 ± 1.46	1.16 ± 0.02
CuOCl	48	6	5.13 ± 1.37	1.09 ± 0.01*
CuOCl	48	12	4.06 ± 0.72*	1.07 ± 0.01**

* $P \leq 0.05$; ** $P \leq 0.01$

[a] CuOCl: copper oxychloride; EMS: ethyl methanesulfonate, served as positive control

[b] Significant compared to control

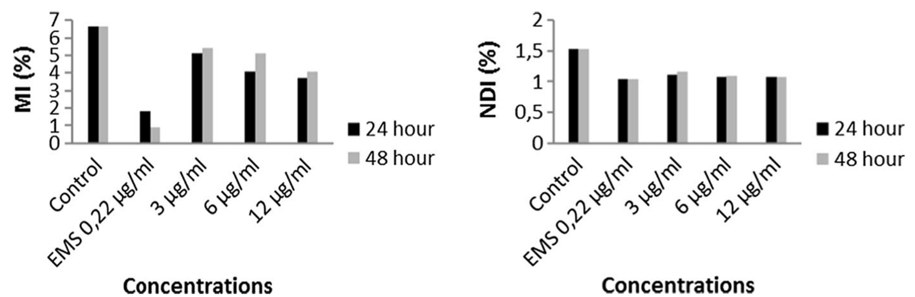

Fig. 3 The MI and NDI in cultured human lymphocytes treated with CuOCl for 24 and 48 h

Table 6 The RAPD bands profile in female and male DNA in human cells exposed to copper oxychloride

Test substances[a]	Treat. time (h)	Concent. (μg/mL)	Mean number of polymorphic bands		Total polymorphic bands, mean ± SE
			Female DNA ± SE[b]	Male DNA ± SE	
Control	–	–	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
EMS	24	0.22	0.216 ± 0.036	0.190 ± 0.035	0.201 ± 0.025
SA	24	2	0.168 ± 0.033	0.079 ± 0.024	0.123 ± 0.020
CuOCl	24	3	0.136 ± 0.030***	0.095 ± 0.026***	0.115 ± 0.020***
CuOCl	24	6	0.120 ± 0.029***	0.055 ± 0.020***	0.087 ± 0.017***
CuOCl	24	12	0.160 ± 0.032***	0.055 ± 0.021***	0.107 ± 0.019***
EMS	48	0.22	0.200 ± 0.035	0.158 ± 0.032	0.179 ± 0.024
SA	48	2	0.152 ± 0.032	0.134 ± 0.030	0.143 ± 0.023
CuOCl	48	3	0.144 ± 0.031***	0.142 ± 0.031***	0.143 ± 0.021***
CuOCl	48	6	0.168 ± 0.033***	0.190 ± 0.035***	0.179 ± 0.024***
CuOCl	48	12	0.136 ± 0.030***	0.142 ± 0.031***	0.139 ± 0.021***

*** $P \leq 0.001$

[a] CuOCl: copper oxychloride; EMS: ethyl methanesulfonate, served as positive control; SA: sodium azide, 2nd positive control

[b] Significant compared to control

Discussion

As our study was the first to investigate the genotoxic and cytotoxic effects of copper oxychloride in human lymphocytes, we were not able to compare our findings with similar studies. However, Pirtskhelani et al. (2008) reported a strong genotoxic effect of copper oxychloride in mice and Shivanandappa et al.

Table 7 Genomic template stability rate of the male and female DNA in human cells exposed to copper oxychloride

Test substances[a]	Treatment time (h)	Concentrations (µg/mL)	Genomic template stability mean ± SE[b]
Control	–	–	100.00 ± 0.00
EMS	24	0.22	77.00 ± 2.52
SA	24	2	85.90 ± 2.14
CuOCl	24	3	85.95 ± 2.67***
CuOCl	24	6	88.20 ± 2.91***
CuOCl	24	12	90.42 ± 2.46***
EMS	48	0.22	81.15 ± 2.92
SA	48	2	81.65 ± 3.32
CuOCl	48	3	83.47 ± 2.55***
CuOCl	48	6	79.63 ± 3.84***
CuOCl	48	12	82.40 ± 2.94***

*** $P \leq 0.001$

[a] CuOCl: copper oxychloride; EMS: ethyl methanesulfonate, served as positive control; SA: sodium azide, 2nd positive control

[b] Significant compared to control

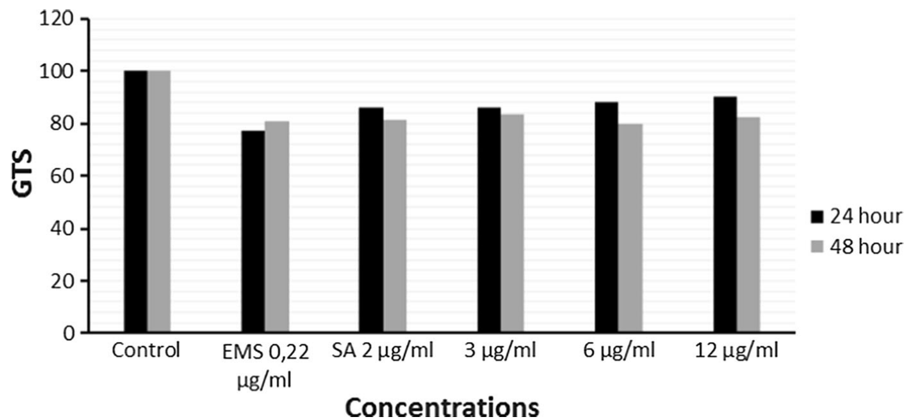

Fig. 4 The GTS rate of the male and female DNA in cultured human lymphocytes treated with CuOCl for 24 and 48 h

(1983) reported testicular atrophy in *Gallus domesticus* fed with acute doses of copper oxychloride. In addition, copper oxychloride caused infertility in some simple animals by decreasing the number of oocytes (Helling et al. 2000; Snyman et al. 2004, 2009). As for studies in humans, several have confirmed the genotoxic and mutagenic effects of copper oxide nanoparticles, which are used in a variety of industries (Ahamed et al. 2010; Wang et al. 2012; Alarifi et al. 2013; Di Bucchianico et al., 2013; Akhtar et al. 2013).

The use of pesticides containing heavy metals such as copper may lead to their transfer to humans through the food chain (Chen et al. 1997; Masaka and Muunganirwa 2007; La Pera et al. 2008; Pose et al. 2009). Waheed et al. (2013) reported that high accumulation of Cu in various organs of herbivorous and carnivorous edible fish may have increased the risk of metal toxicity in humans. Our tested formulation contained other heavy metals such as cadmium, iron, and lead, but in such trace amounts (0.1, 0.01 and 10 ppm, respectively) that they could not affect our findings.

In our study, all copper oxychloride concentrations increased the frequency of chromatid and chromosome breaks and the number polymorphisms and decreased cell proliferation. The resulting DNA damage most probably inhibited DNA replication and cell proliferation (Vock et al. 1998; Kirkland and Muller 2000). On the other hand, the high frequency of chromosome aberrations may have triggered mitotic selection and pressured cell proliferation (Madle et al. 1993; Vock et al. 1998; Galloway et al. 1998; Hillard et al. 1998; Armstrong et al. 1992; Kirkland and Muller 2000; Kocaman et al. 2014). The cell death

might have arisen from the oxidative stress and the high genotoxic effects that caused to apoptosis and necrosis which is the main mechanism of the cytotoxicity (Bakkali et al. 2008; Kocaman et al. 2011).

Copper (Cu) is essential for many biological systems as a cofactor of enzymes including cytochrome C oxidase, tyrosinase, dopamine-β-hydroxylase, superoxide dismutase (Suzuki et al. 2002; El-Gendy et al. 2009) and it has a very important role in hemoglobin synthesis involved in Fe metabolisms (WHO 1974). The estimated daily requirement of copper is 2 mg for adults because the copper intoxication may cause to hemolytic anemia (WHO 1974). On the contrary, the exceeding intake amount of Cu accumulated in liver and other organs and caused several diseases in humans such as chronic renal and liver disfunctions (Gunay et al. 2006; Hloch and Charvát 2012). The same results were reported for animals (Hurwitz et al. 2014; Ciji and Bijoy Nandan 2014). Woimant and Trocello (2014) divided the Cu disorders into two classes, in which one of them was genetically and related to inherited Cu transport disorders. The other ones are Cu disorders, which are associated with Cu deficiency or exceed. The mutant ATP7B gene caused Cu accumulation in tissue resulting in clinical disorders known as Wilson disease (Grazyna et al. 2014). The high concentrations of Cu led to oxidative stress and toxicity in all living beings (Gupta et al. 1996; Giller et al. 1998; Srivastava et al. 2006; Ciji and Bijoy Nandan 2014).

Even though our study on copper oxychloride and copper oxide nanoparticles demonstrate genotoxic and cytotoxic effects, copper oxychloride is still widely used as a fungicide. About twenty thousand metric tons of copper oxychloride were used in Turkey alone in 2013 (https://www.zauba.com). The most serious risk is that the copper oxychloride is still not classified as a mutagen or potential carcinogen for humans. Our short-term in vitro exposure has clearly shown the genotoxic effect of copper oxychloride, but for a better understanding of its genotoxicity long-term carcinogenic studies and in vivo animal genotoxicity studies are needed.

Conclusion

Our in vitro study has confirmed that copper oxychloride is genotoxic and cytotoxic to human lymphocytes. Therefore, it represents a certain risk for the environment and humans, and future studies should investigate that risk, especially via the food chain. In the meanwhile, caution is advised when using copper oxychloride as a fungicide for the sake of our and the next generations' health.

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Compliance with ethical standards

Conflict of interest The authors report no declaration of interest.

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