

# Assessment of genotoxic effects of a fungicide product and its active substances on human peripheral blood mononuclear cells

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## SUMMARY

The genotoxic potential of the plant protection product Signum<sup>®</sup> and its two active substances (pyraclostrobin and boscalid) was investigated by the comet assay (pH>13). Leukocytes isolated from whole blood were treated with different concentrations (0.1-25 µg/ml) of the fungicide for 2 h and 20 h. The Trypan Blue exclusion test showed higher cell viability (>84%) under all three concentrations and in the two treatment periods. The obtained results revealed that both Signum<sup>®</sup> and pyraclostrobin induced statistically significant DNA damage. In contrast, boscalid did not cause statistically significant DNA damage after 2 h exposure although it caused DNA damage at higher concentrations after a longer time exposure (20 h). It is deducible that pyraclostrobin and Signum<sup>®</sup> might be genotoxic. However, within the studied concentration ranges, none of the fungicides was found to be cytotoxic in the two treatment periods.

**Keywords:** genotoxicity; DNA damage; comet assay; fungicide; boscalid; pyraclostrobin

## INTRODUCTION

A pesticide formulation is described as a combination of active and inert ingredient(s) (EPA, 2006). Although „inert” refers to ingredients that are physically, chemically and biologically inactive, the EPA (1997) declared that an inert ingredient may possess biological activity of its

own. Thus, it may be toxic to humans. The combined effects of active substance(s) and inert ingredients are very difficult to predict. Therefore, the toxicity of such mixtures attracts great attention in the context of different health problems unrelated to evident causes. *In vitro* cell culture and animal models have shown that inert ingredients have the ability to increase the toxicity

of pesticides. For example, although the active substance glufosinate had no effect on blood pressure and did not change the heart rate of rats, a derived formulation caused the mentioned parameters to decrease (Koyama et al., 1997). Despite the active ingredients having no effect, the commercial formulation of glyphosate (Peixoto, 2005) and a formulation containing 2,4-D (2,4-dichlorophenoxyacetic acid) (Oakes & Pollak, 1999) may affect mitochondrial functions. As an active ingredient, glyphosate and its most common formulation, the herbicide Roundup, have been found to cause cytotoxicity and genotoxicity in a buccal epithelial cell line. Under all conditions, Roundup was more active than its active ingredient (glyphosate) (Koller et al., 2012). *In vitro* treatment of human peripheral blood mononuclear cells (PBMCs) with some pesticide formulations and active substances resulted in significantly higher values of induction (Bolognesi et al., 1997; Zeljezic et al., 2006).

Although active substances used so far have been approved as safe for human health, there are many diseases linked with pesticide exposure. This could indicate an inadequate assessment of the toxicological profile of both active substances and pesticide formulations (plant protection products). While only 7 out of 20 toxicological tests evaluate pesticide formulations, all other tests are carried out on active substances (Cox & Sorgan, 2006; European Commission, 2013a, 2013b). In addition, some significant aspects of human health, including cancer and genotoxic potential, are evaluated only in active substances (Cox & Sorgan, 2006). Specific test systems, including bacterial reverse mutation assay, *in vitro* mammalian cell assay and *in vivo* cytogenetic methods, are used on active substances. Pesticide formulations have apparently been inadequately assessed so far regarding their potential hazards. In addition, screening pesticide formulation(s) and active substances(s) by a different test system will enable us to see different aspects of potential hazards.

The comet assay is a preferred method to assess DNA damage and repair in individual cell populations (Dhawan & Anderson, 2009). The assay has been used to test the genotoxic potential of a broad range of chemicals (Bokan et al., 2013; de Barros et al., 2013; Guilherme et al., 2012; Syberg et al., 2015) and to determine the potential DNA damage level for human population (Benedetti et al., 2013; Costa et al., 2011; Kaur et al., 2011). Since the comet assay is not a routine test required for the registration of agrochemicals, the results of this test are new and reveal various aspects of the genotoxic potential of both the plant protection product (PPP) and active substances in pesticides.

Signum<sup>®</sup> is a fungicide (PPP), and its active substances are pyraclostrobin and boscalid. Their genotoxic potentials have been assessed, using the cytokinesis-block micronucleus (CBMN) cytome assay, by our research group (Çayır et al., 2014). Since the comet assay measures different DNA damages, compared to the CBMN assay, we aimed to investigate the genotoxic potential of the PPP Signum<sup>®</sup> and its active substances pyraclostrobin and boscalid individually on isolated human PBMCs using the alkaline comet assay (pH>13).

## MATERIALS AND METHODS

Signum<sup>®</sup>, composed of 26.7% boscalid and 6.7% pyraclostrobin, was purchased as a commercial formulation (99.9% purity, Sigma, Germany) from an agrochemical sales outlet in Canakkale, Turkey. All chemicals were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany). In the study, blood samples were taken from two healthy voluntary donors. In each experiment, we used 100 µM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, Merck, Germany) as a positive control, and solvent (<1% DMSO) as a negative control. The ethical permission of the research was granted from the Medicine Faculty at Canakkale Onsekiz Mart University, Turkey.

### Comet assay

In the present study, the alkaline comet assay was carried out with minor modification, according to Dhawan's method (Dhawan et al., 2003). Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque from whole blood of two volunteers. The cells were exposed to Signum<sup>®</sup>, boscalid and pyraclostrobin (0.1-25 µg/ml) for 2 h and 20 h. At the end of incubation, all cultures were washed three times with cold phosphate buffered saline (PBS, Sigma, Germany). The cells were mixed with low melting agarose (Invitrogen, USA) and the mixture was dropped on coated slides with normal melting agarose (Invitrogen, USA). Lysing and electrophoresis steps were carried out at +4°C in the dark. At the lysing stage, the slides were immersed in cold lysing solution for one hour. After the lysing step, the slides were placed in cold electrophoresis solution (pH>13) for 20 minutes, and then electrophoresis was performed at 300 mA and 25 V for 20 minutes. Finally, the slides were kept in a neutralized solution (pH=7.5) for 15 minutes. The slides were dyed with ethidium bromide (20 µg/ml) (AppliChem, Germany). Calculation of the arbitrary

unit (AU) for each slide and microscopic evaluation of the slides were conducted according to Collins (2004).

### Cell viability

We applied the trypan blue assay to measure cell viability. After exposure, the cells (10  $\mu$ l), which were treated and then washed, were incubated by trypan blue (10  $\mu$ l, Gibco, USA) for two minutes. Five hundred cells per each slide were counted to measure viability under a light microscope (Primo Star, Zeiss) using 40X magnification. The stained blue cells were counted as dead. We calculated cell viability % by using the number of dead and alive cells.

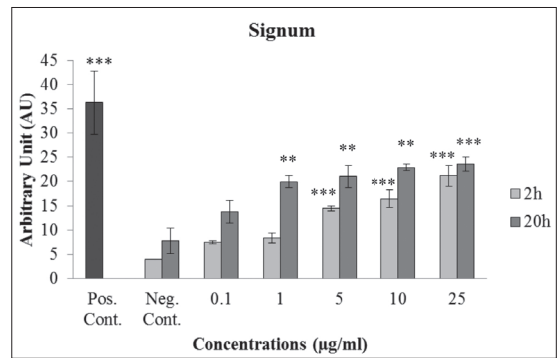
### Statistical analysis

DNA damage induced by different concentrations of the fungicides and control values were compared using the Kruskal-Wallis and Dunn's multiple comparison tests. A linear regression analysis was performed to show the dose dependence formation of the genetic end-point. These tests were performed using the GraphPad Prism 5 software for Windows. For all analyses,  $p < 0.05$  value was accepted as the significant level.

## RESULTS

DNA damage induced by the positive control showed a statistically significant difference compared to the negative control throughout all stages of the experiment. In the present study, we eliminated cytotoxicity as a confounder factor since it causes a misleading result in the comet assay (Hartmann et al., 2003). Therefore, the trypan blue exclusion test was performed using the treated cells. The results revealed that Signum<sup>®</sup>, boscalid and pyraclostrobin did not cause higher cell death within the tested concentration range. Cell viability at the end of both treatment periods for all three fungicides was found to be  $>84\%$ .

The results of the comet assay performed on the PBMCs treated with Signum<sup>®</sup> for 2 h and 20 h are presented in Figure 1. In general, it was observed that all treatments resulted in an increase in DNA damage for both exposure periods. According to the obtained data, 5, 10, and 25  $\mu$ g/ml concentrations of Signum<sup>®</sup> exerted statistically significant DNA damage, compared to the negative control for 2 h treatment period. In the case of 20 h period, statistically significant DNA damage occurred in the 1–25  $\mu$ g/ml dose range. It infers that longer time exposure increased DNA damage.

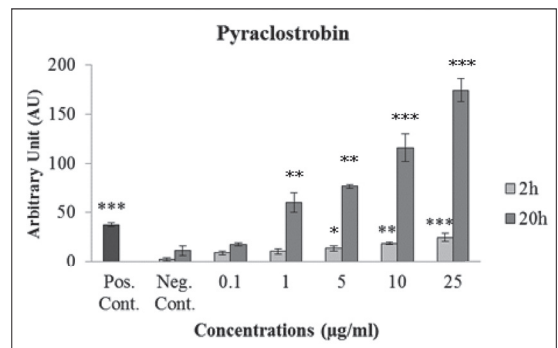


**Figure 1.** Effect of Signum on DNA damage after 2 h and 20 h exposure (Pos. Con. = Positive Control  $\times$  10; Neg. Cont. = Negative Control)

\*\* $P < 0.01$

\*\*\* $P < 0.001$

A clear increase in DNA damage induced by pyraclostrobin was observed for both treatment periods (Figure 2). The DNA damage obtained at the highest concentration elevated DNA damage by 18.6-fold when compared to the negative control. Statistically significant DNA damage was found at the concentrations of 5, 10, and 25  $\mu$ g/ml for the 2 h treatment. DNA damage increased linearly in a dose-dependent manner ( $r^2 = 0.79$ ,  $p = 0.01$ ). More DNA damage was observed after treatment over the longer 20 h period, statistically significant at the concentrations of 1–25  $\mu$ g/ml of pyraclostrobin. The DNA damage induced by pyraclostrobin increased linearly with the concentration increment over 20 h ( $r^2 = 0.87$ ,  $p = 0.004$ ).



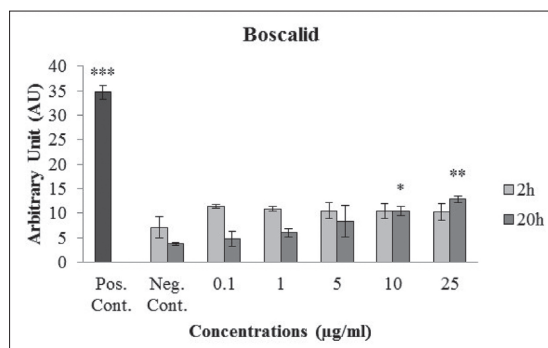
**Figure 2.** Effect of pyraclostrobin on DNA damage after 2 h and 20 h exposure (Pos. Con.=Positive Control  $\times$  10; Neg. Cont.= Negative Control)

\* $P < 0.05$

\*\* $P < 0.01$

\*\*\* $P < 0.001$

In contrast to Signum® and pyraclostrobin, the PBMCs treated with boscalid showed different responses in both treatment periods. During the 2 h exposure, DNA damage did not occur with an increase in boscalid concentration and no statistically significant response was obtained. On the other hand, the two higher concentrations of boscalid induced significant DNA damage when compared to the negative control (10-25 µg/ml) (Figure 3). A linear regression analysis showed that DNA damage induced by boscalid led to a dose-dependent increase over the 20 h period ( $r^2=0.83$ ,  $p=0.007$ ).



**Figure 3.** Effect of boscalid on DNA damage after 2 h and 20 h exposure (Pos. Cont.=Positive Control  $\times 10$ ; Neg. Cont.= Negative Control)

\* $P < 0.05$

\*\* $P < 0.01$

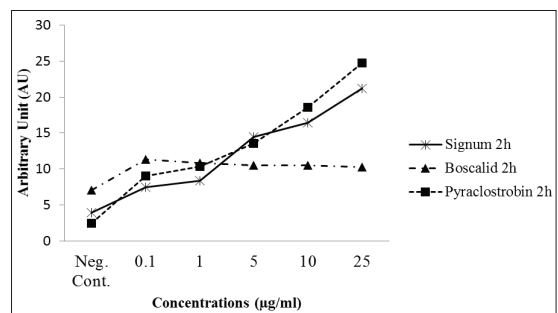
## DISCUSSION

In the study, the genotoxic effects of Signum®, pyraclostrobin and boscalid were investigated by using the comet assay. The obtained results showed that DNA damage was observed in PBMCs as a response to pyraclostrobin and Signum® treatments for 2 h. Furthermore, a clear genotoxic effect was observed when PBMCs were treated with pyraclostrobin and Signum® for 20 h. While DNA damage has not been correlated with cancer risk in individuals, it is generally assumed that elevated levels of DNA damage are linked with human health issues (Dhawan & Anderson, 2009). The alkaline version of the comet assay could assess single strand breaks (SSB), which have been associated with incomplete excision repair sites and double strand breaks (DSB), as well as alkali labile sites (ALS) (Dhawan & Anderson, 2009). In the present study, Signum® and pyraclostrobin showed a potential to induce SSB, DSB, and/or ALS. Furthermore, the genotoxic potential of the

two fungicides increased in proportion to the increment of the exposure time.

While working and/or spraying, workers are exposed to PPPs composed of active substance(s) and inert ingredient(s). Thus, it is crucial to evaluate the genotoxicity and cytotoxicity of active substances, as well as their PPPs. Many studies have investigated the genotoxicity and cytotoxicity of pesticides by using different methods (Koller et al., 2012; Moretti et al., 2002; Ribas et al., 1997; Santovito et al., 2011; Underger & Basaran, 2005; Villarini et al., 1998). The genotoxicity of Signum®, evaluated by the cytokinesis-block micronucleus (CBMN) assay on human peripheral blood lymphocytes, showed that some concentrations of Signum® increased micronucleus frequencies when compared with control (Çayır et al., 2014). In the present study, after both treatment periods, Signum® induced statistically significant DNA damage, compared to control values. It could be inferred that Signum® might have a potential to exert genotoxicity on dividing and non-dividing cells.

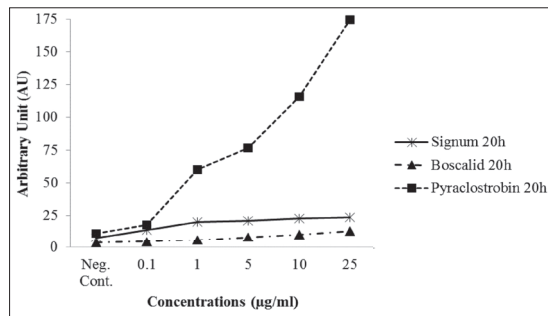
Figure 4 shows the effects of pyraclostrobin and boscalid and their PPP Signum® on human PBMCs after 2 h treatment. DNA damage caused by each fungicide is more or less similar at doses below 5 µg/ml in the 2 h treatment. On the other hand, at doses higher than 5 µg/ml, boscalid did not alter DNA damage. A linear increase in DNA damage was observed at corresponding doses of Signum® and pyraclostrobin. Pyraclostrobin evidently induced higher levels of DNA damage when compared with Signum® and boscalid at higher doses after 2 h treatment.



**Figure 4.** Effects of Signum, pyraclostrobin and boscalid on DNA damage after 2 h exposure

In order to compare the genotoxicity of the PPP and its active substances, the observed DNA damage was summarized in Figure 5. It shows that pyraclostrobin was clearly the most genotoxic. In addition, at higher

fungicide concentrations, the DNA damage caused by pyraclostrobin was 7-8 times higher than those observed for Signum® and boscalid. The DNA damage induced by Signum® and boscalid was similar, but Signum® induced slightly more DNA damage than boscalid. It can be inferred from these results that pyraclostrobin may enhance the genotoxic potential of Signum®.



**Figure 5.** Effects of Signum, pyraclostrobin and boscalid on DNA damage after 20 h exposure

Pyraclostrobin has been tested for its genotoxic and mutagenic potential in different organisms. The results of those studies have revealed no genotoxic effect of pyraclostrobin. By contrast, pyraclostrobin induced micronucleus formation in human peripheral blood lymphocytes (Çayır et al., 2014). Similar findings were obtained by Bony et al. (2010) for azoxystrobin, which belongs in the same chemical group as pyraclostrobin (strobilurin). Azoxystrobin increased micronucleus frequency in erythrocytes and increased DNA damage assessed by the comet assay (Bony et al., 2010). Azoxystrobin (0.5–1 µg/l) induced DNA damage determined by the comet assay when compared with unexposed erythrocytes of *Phoxinus phoxinus* (Bony et al., 2008). Our results are consistent with previous findings.

Boscalid belongs to the carboxyanilides group (Hauke et al., 2004). The genotoxic and mutagenic potential of boscalid has been investigated in a range of *in vitro* and *in vivo* test systems. Those studies showed no genotoxicity or mutagenicity of boscalid in different cell types (EPA, 2003). In contrast, a study performed on human peripheral blood lymphocytes showed a possible genotoxic potential of boscalid (Çayır et al., 2014). In the present study, there is no clear DNA damage after 2 h exposure to boscalid. However, after 20 h exposure, higher concentrations of boscalid were found to induce DNA damage compared with control values. Comet assay may be suggested as the more sensitive test than those

used in previous studies on the evolution of genotoxicity of boscalid (European Commission, 2009).

In conclusion, pesticides pass several test systems for their safe usage. However, the increased DNA damage to PBMCs indicates the importance of reassessing the genetic hazard of both active substances and the PPP. These results suggest that personal protective equipment is necessary during application of the pesticide Signum®.

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## Ispitivanje genotoksičnih efekata fungicida i njegovih aktivnih supstanci na mononuklearne ćelije ljudske krvi

### REZIME

Ispitivani su genotoksični efekti fungicida Signum® i njegove dve aktivne supstance (piraklostrobin i boskalid) primenom komet testa (pH>13). Leukociti izolovani iz krvi tretirani su različitim koncentracijama (0.1-25 µg/ml) fungicida tokom 2 h i 20 h. Test sa tripansko plavim obojenjem pokazao je veću ćelijsku vijabilnost (>84%) kod sve tri koncentracije i u oba intervala primene. Rezultati su pokazali da Signum® i piraklostrobin dovode do statistički značajnog oštećenja. Nasuprot tome, boskalid nije doveo do statistički značajnog oštećenja DNK nakon 2 h izlaganja, iako je uzrokovao DNK oštećenja pri višim koncentracijama tokom dužeg izlaganja (20 h). Može se zaključiti da piraklostrobin i Signum® mogu imati genotoksično delovanje. Ipak, u okviru ispitivanog opsega koncentracija, nijedan od ispitivanih fungicida nije pokazao citotoksično delovanje tokom dva intervala primene.

**Ključne reči:** genotoksičnost; DNK oštećenje; komet test; fungicidi; boskalid; piraklostrobin