EVALUATION OF THE AMPLITUDE OF CYTOGENETIC DAMAGE INDUCED BY TWO FOOD ADDITIVES IN WHEAT

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Abstract. Sulfites and metabisulfites, nitrates and nitrites are among the most utilized food additives. They can determine negative reactions in human body, but some of them showed genotoxic and carcinogenic action in various test-systems. In this study, we analyzed the amplitude of the cytogenetic changes induced by the exposure to different concentrations (0.1%, 0.5%, and 1.0%) of sodium nitrate (E251; SN) and sodium metabisulfite (E223; SMB) in wheat root tips meristems, by scoring the mitotic index and the frequency of division phases, the rates of ana-telophase chromosome aberrations, and of metaphase disturbances. Also, the influence on plantlet length was analyzed in young plantlets. The two chemicals increased the amount of chromosome aberrations, depending on concentration and time exposure.

INTRODUCTION

The continuous increase of the world's population requires the identification of new food resources, as well as the finding of effective methods to ensure their storage and the long-term maintaining of the quality or to optimize their nutritional quality, the flavour and the appearance. More than 3,000 food additives (preservatives, antioxidants, emulsifiers, stabilizers, coloring agents, sweeteners, taste and small improvers, thickeners, gelling agents, anti-caking agents etc.) are used in modern food processing and technology. About 75% of the Western diet is made up of processed foods, each person consuming an average of 3.6-4.5 kg of food additives per year (Zengin *et al.*, 2011). Although food preservatives are so widely present in our foods, the information about their genotoxic potential are inconclusive. Sometimes, the reported results are highly contradictory concerning the same tested substance. Numerous studies reported that certain food additives, especially antimicrobial agents, were genotoxic in different test systems (Meng and Zhang 1992; Rencuzogullari *et al.* 2001a, b; Gomurgen 2005; Arslan *et al.*, 2008; Carvalho *et al.*, 2011; Turkoglu, 2009, 2013; Yavuz-Kocaman *et al.*, 2008; Yilmaz *et al.*, 2012).

Sulfites and metabisulfites (E221-227), nitrates and nitrites (E249-252) are among the most utilized food additives. They can determine negative reactions in human body (anaphylactic shock, asthma, nausea etc.), but some of them are genotoxic and carcinogenic in various test systems (Sarikaya and Cakir, 2005; Kayraldiz and Topaktas, 2007; Zengin *et al.*, 2011). On the contrary, Nair and Elmore (2003) reviewed that all the sulfites including PMB and SMB were not teratogenic in mice, rats, hamsters, or rabbits and gave negative mutagenic effects in mutagenicity tests.

Sulfites and metabisulfites, with antioxidant, antibacterial, antifungal action, are added as preservatives in biscuits and chocolate (50 mg/kg), processed meat (450 mg/kg), alcoholic drinks, dried fruits (2,000 mg/l), fruit juices, as an antioxidant in wine (2,000 mg/l) etc. Chemically, sodium and potassium metabisulfites are very similar, so that they are interchangeable (Yavuz - Kocaman *et al.*, 2008). Admissible daily intake (ADI) dose of sulfites is up to 0.7 mg/kg body weight in equivalent sulfur dioxide (*http://www.food-info.net/uk/e/e223.htm*). Turkish Ministry of Agriculture (1997) suggested that SMB can be introduced into foods as antimicrobial substance to the maximum dose of 300 mg/L, and Yavuz - Kocaman *et al.* (2008) consider that sulfites can be used in food up to a maximum dose of 2000 mg/L (kg). In water, SMB is converted into sulphur dioxide and sodium bisulfite which causes deamination of cytosine in the nucleic acids, so leading to base substitutions and mutations in the genes (Chen and Shaw, 1994).

Because some studies have shown some connection between the risk of human gastrointestinal cancer and high levels of nitrates in vegetables, nitrate content in daily diet is of great interest. The addition of sodium nitrite is more controversial than that of sodium nitrate because of nitrosamine generation at high temperatures. ADI for NO_3 is up to 3.7 mg/kg body weight (Tamme *et al.*, 2006) or 5 mg/kg body weight (Schuddeboom, 1993). Despite the risk, it is not possible, however, to completely give up their addition in processed meats because these compounds act against highly pathogenic bacterium *Clostridium botulinum* (Loutsidou *et al.*, 2012). For some additives (boric acid, citric acid, and sodium metabisulfite), genotoxic effects were reported even at doses lower than those considered as safe (Rencuzogullari *et al.*, 2001).

Therefore, we decided to assess in this work the genotoxic potential of two food additives – sodium metabisulfite and sodium nitrate – revealed by mitotic index, changes in frequency of mitotic stages, ana-telophase chromosome aberrations and metaphase disturbances, scored in root tips meristems of *Triticum aestivum* cv. Dropia. Also, the influence of wheat seed treatment with the two chemicals, depending on concentration and exposure time, on plantlet length was analyzed.

MATERIAL AND METHODS

Plant material. Seeds of *Triticum aestivum* cv Dropia were utilized. The two tested chemicals were sodium nitrate, $NaNO_3$ (MW = 84.9947 g/mol; E251; SN) and sodium metabisulfite, $Na_2S_2O_5$ (MW = 190.107 g/mol; E223; SMB), added as aqueous solutions at the concentrations of 0.1%, 0.5%, and 1.0%, for 6h and 12h. Controls were prepared in distilled water. Before treatments, the seeds were maintained overnight in distilled water. After treatment, the wheat seeds were placed on moistened filter paper, in covered glass Petri dishes, and incubated in the dark. The germinated seeds were then maintained at a photoperiod of 16h/8h, at room temperature. The length was measured in 7d old plantlets.

Preparation and analysis of slides. The newly emerged roots (10 – 20 mm in length) were fixed for 24h in alcohol:acetic acid (3:1, v/v), at room temperature, then they were washed, transferred to 70% ethyl alcohol, and stored at 4°C. After hydrolysis, the roots were stained in modified charbol fuchsin. Three rootlets/germinated seed were mounted on a slide and squashed in a drop of 45% acid acetic, under a cover-slip (squash technique). Five slides/variant (10 fields/slide) were analyzed using NOVEX Holland microscope, at 20 × objective lens. The photos were made using Nikon Eclipse 600 microscope, with Nikon Cool Pix 950 digital camera at 100 × oil immersion objective.

Cytogenetic parameters. Mitotic index (MI%), frequencies of division stages (expressed in prophase, metaphase, anaphase, and telophase indices – PI%, MeI%, AI%, and TI%), rates of ana-telophase chromosome aberrations (CA_{A-T}%) and of metaphase disturbances (M_{abn}%) were calculated according to the equations: MI%=TDC × 100/TC; PI% = prophase cells × 100/TDC; MeI%=metaphase cells × 100/TDC; AI%=anaphase cells × 100/TDC; TI%=telophase cells × 100/TDC, where TC=total analyzed cells (interphases + dividing cells), and TDC=total dividing cells. The rate of chromosome aberrations in ana-telophases and the rate of metaphase abnormalities were also evaluated in relation to the number of cells in mitosis: $CA_{A-T}\%=CA_{A-T} \times 100/TDC$; $M_{abn}\%=M_{abn} \times 100/TDC$.

Statistical analysis. The results were expressed as the mean values and standard error of the means ($\bar{x}$ ± SE). Statistical analysis was carried out according to Student's *t* test for each parameter in order to establish the differences between the treated variants and the controls. The differences were considered as significant at levels of $*p<0.05$, $**p<0.01$ and $***p<0.001$. The increase/decrease (–/+) rates were calculated based on the equation: *(1- x/y) × 100*, where y is the average value detected in the control and x is one of each treated samples. The Microsoft Office Excel 2003 software of Windows XP operating system was used to calculate and to graphically represent the statistical parameters.

RESULTS AND DISCUSSIONS

Influence on plantlet length. In 6h treatments, SN determined rises in growth of wheat plantlets as food additive concentration increased. Increase rates were 23.03%, 33.38%, and 45.74%, respectively, in the three tested concentrations, as compared to the control (Fig. 1).

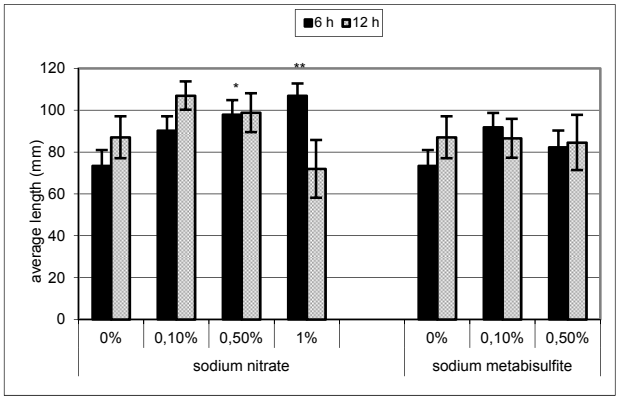

Fig. 1. Average values of length in 7-days old wheat plantlets, after seed exposure to different concentrations of SN and SMB. Vertical bars = standard errors of the mean. Asterisks = level of significance (* = p<0.5; ** = p<0.01)

In 12h exposure, the length of plantlets exposed to SN was also superior to the control at the concentrations of 0.1% and 0.5%, but this compound induced lowering of growth at the maximum tested concentration (decrease rate = 17.33%). SMB-1.0% determined a quasi total inhibition of the germination and growth. In short exposure (6h), after an increase rate of 25.17% in 0.1% SMB, at 0.5% concentration a decline occurred, although the values are still over the control. The plantlets issued from seed treated for 12h with this compound had heights smaller than the control. The results differ significantly from the control only at the highest concentrations of SN (0.5% and 1.0%) in 6h-treatment ($p<0.05$, respectively $p<0.01$). Teresa *et al.* (2003) also evidenced increases in length of *Vigna sinensis* plants under SMB impact, while the germination was negatively influenced by this compound. Pre-sowing for 24h of *Withania somnifera* seeds in SN stimulated the plant growth at 0.5%, but especially at 1.0%, where the plant height was with ~50% higher than the control value (Kattimani and Reddy, 2001).

Mitotic index and division phases. In both exposures, SN had stimulative effects on mitotic index at the concentrations of 0.1% and 0.5% (Fig. 2). The average values, although superior to the control, registered a decline starting from 0.5%, the length of the plantlets showing average values below the control at 1.0% (in 6h-treatment - 3.79±0.30%, as compared to 4.27±0.21% in control; in 12h-treatment - 3.22±0.23%, as compared to 4.46±0.08%, for control), the decrease rate being important in the longer treatment (27.81%) than in the short-term exposure (11.25%).

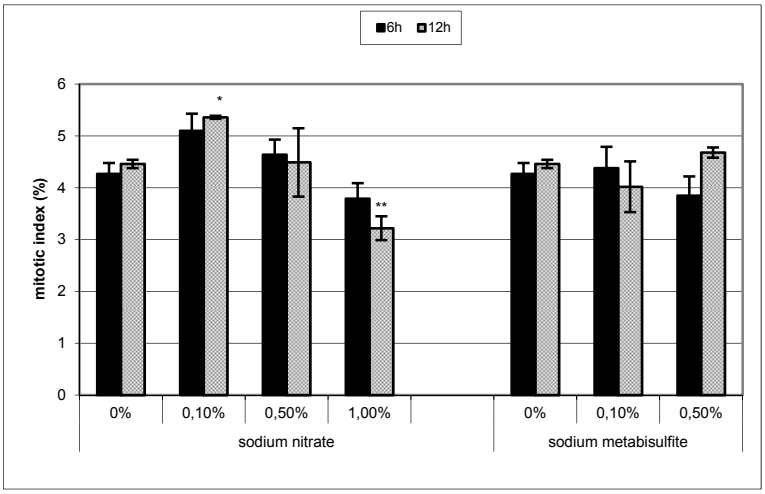

Fig. 2. Evolution of mitotic index in wheat after treatment with SN and SMB. Vertical bars = standard errors of the mean. Asterisks = level of significance (* = $p<0.05$; ** = $p<0.01$)

Loutsidou *et al.* (2012) evidenced that the short-term exposure (2h) of the protozoan *Tetrahymena pyriformis* to doses of SN equivalent to ADI resulted in significant increase in DNA content, fact indicating the stimulation of the mitosis and possibly an uncontrolled mitogenic proliferation, sometimes linked with chemical carcinogenesis. SMB was reported to decrease the mitotic index in different plants (Rencuzogullari *et al.*, 2001a; Kumar and Panneerselvam, 2007), but also in human lymphocytes (Rencuzogullari *et al.*, 2001b), while potassium nitrate and potassium metabisulfite inhibited mitosis in onion (Gomurgen, 2005). At

concentrations smaller than those tested in our experiment, but in longer exposures, SMB affected root growth and cell division in *Allium cepa* (Mishra and Yadav, 2011).

In *Calendula officinalis*, significant decreases in division intensity and high frequencies of chromosome aberrations were observed after treatment with metabisulfites (Marc and Capraru, 2008). Onyemaobi *et al.* (2012) evidenced an inverse correlation between concentration and exposure to metabisulfite and the evolution of root length and mitotic index in onion, at concentrations ranging from 0.025 M to 0.250 M.

As regards the frequency of mitotic stages (Fig. 3), the treatment with SN – 6h progressively declined prophase number with increasing concentration, but determined the increase of amount of anaphases + telophases (at 0.5%, AI% + TI% was 57.15%, comparatively with 41.97% in control) and of metaphases, at the concentrations of 0.1% and 0.5%. At the exposure of 12h, the PI% was slightly superior to control at 0.1% and 1.0%, and MeI% significantly surpassed the control at the concentration of 0.5% (30.34±3.24%, as compared to 24.14±1.20%, in control). The high values of MI% at 0.1% SN come from the high number of metaphases (6h) or anaphases (12h).

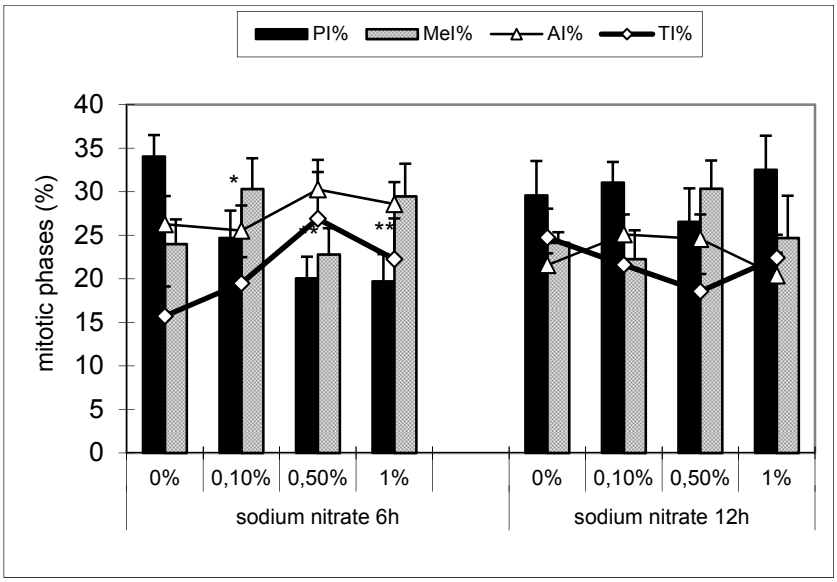

Figure 3. Evolution of the frequency of division stages in treatment with SN. Vertical bars = standard errors of the means. Asterisks = level of significance: * $p<0.05$; ** $p<0.01$.

In SMB 6h–treated (Fig. 4), PI% markedly decreased (with ~ 45%), but AI% + TI% increased (mainly based on telophases). The longer exposure to 0.1% diminished the frequency of prophases and metaphases, and increased ana-telophase proportion. The differences are significant ($p<0.01$) for PI% in 6h treatments, for both SN and SMB. In literature, the food additives are reported to cause changes in the frequencies of cell stages, but the results are different, depending on class of food additive, concentration, exposure time, species etc. (Rencuzogullari *et al.* 2001a; Gomurgen 2005; Turkoglu, 2009).

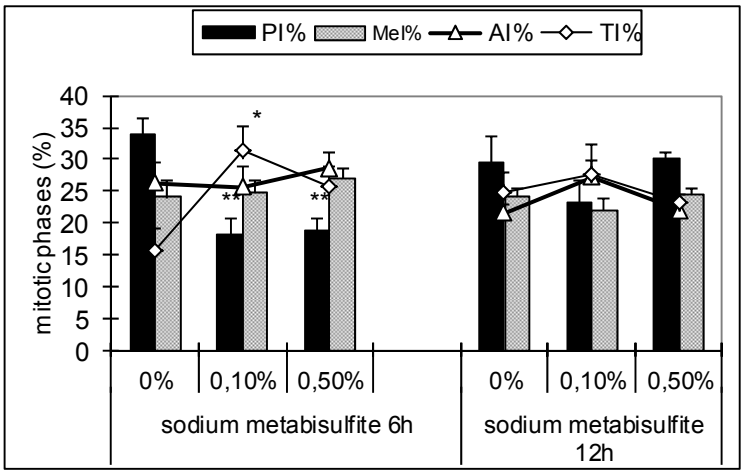

Fig. 4. Frequency of division stages in treatment with SMB. Vertical bars = standard errors of the means. Asterisks = level of significance: * $p<0.05$; ** $p<0.01$)

The frequency of chromosome aberrations. The amount of ana-telophase aberrations registered an ascendant trend in 6h treatment with SN (increase rate is 40.15% for 0.1%, and 50.27% at 0.5%) (Fig. 5).

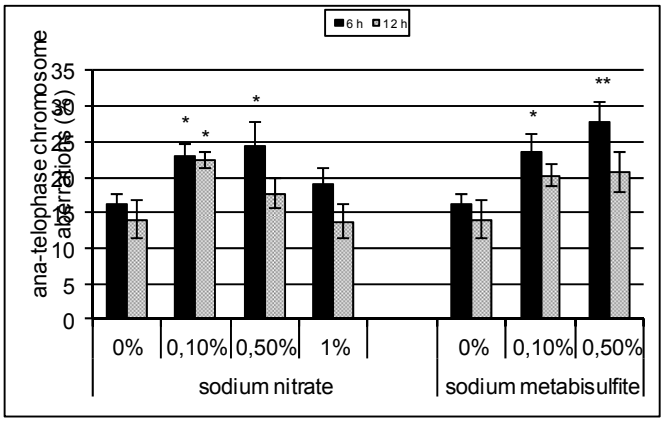

Fig. 5. Rate of ana-telophase aberrations after the treatment with SN and SMB. Vertical bars = standard errors of the means. Asterisks = level of significance: * $p<0.05$; ** $p<0.01$)

A decline is visible starting with 1%, but the aberration percentage (19.03%) still maintains over the control (16.31%). In 12h exposure, after an increase of ~ 1,5× at 0.1%, the aberration rate is smaller, so that at 1% it is lower than control (decrease rate=2.2%) SMB induced high percentages of ana-telophase aberrations in both exposures, but mainly in 6h-treatment (at 0.5%, the increase rate was 70.63%). The differences are significant ($p<0.05$; $p<0.01$) at 0.1% and 0.5% for both additives. The bridges (unique and multiple) (Fig 6c,d,e) are the most numerous aberrations. Some authors confirmed their presence in different plant systems after treatment with sodium and potassium sulfites, metabisulfites, and nitrates (Njagi and Gopalan, 1982;

Gomurgen, 2005), but in other studies they have not been evidenced (Kumar and Panneerselvam, 2007). In SN-treated variants laggiards (Fig. 6j,k), complex aberrations (Fig 6 g,h,i), and multipolarity were observed, but in smaller proportion (Fig. 6l). The most numerous complex aberrations occurred in 0.1% SN (6h – 2.99±1.22 %; 12h – 3.60±1.59%), while the highest proportion of laggards was noted in 0.5% - 6h (3.69±1.42). SMB 0.5% - 6h determined the formation of 2.46±1.93% lagging chromosomes, and the most numerous complex aberrations occurred in SMB 0.1% - 6h (3.66±1.92%). Gomurgen *et al.* (2005) reported the presence of lagging chromosomes in onion after the treatment with potassium metabisulfite and potassium nitrate. The presence of this kind of aberrations indicates an alteration of mitotic spindle, at structural or functional level.

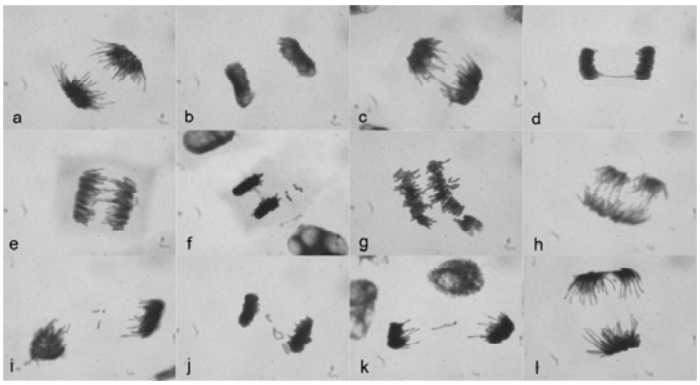

Fig. 6. Ana-telophase aberrations. a. normal anaphase (SMB 0.1%-12h); b. normal telophase (SN 0.1% - 12h); c. one anaphase bridge (SN 0.1%-6h); d. one telophase bridge (SN 0.1%-12h); e. multiple anaphase bridges (SN 0.1%-6h); f. complex aberration (telophase with bridge + vagrants) (SN 0.1%-6h); g. complex aberration (anaphase with bridges + expulsed chromosomes) (SN 0.1%-12h); h. complex aberration (multipolar anaphase + bridges) (SMB 0.5%-6h); i. complex aberration (A-T with laggards + fragment) (SN 0.5%-6h); j. laggards in telophase (SN 0.1%-12h); k. laggards in ana-telophase (SMB 0.1%-12h); l. tripolar anaphase (SMB 0.1%-12h).

Micronuclei were observed in all division phases (Fig. 7 b,d), as well in interphases (Fig. 8a,c), after the treatment with SN and SMB. Micronucleus formation implies loss of genetic material and reflects the genotoxicity of the tested agents. They were recorded by many investigators following treatment with different food additives both in plants (Gomurgen, 2005; Turkoglu, 2013) and in animal and human systems (Meng and Zhang, 1992; Rencuzogullari *et al.*, 2001b).

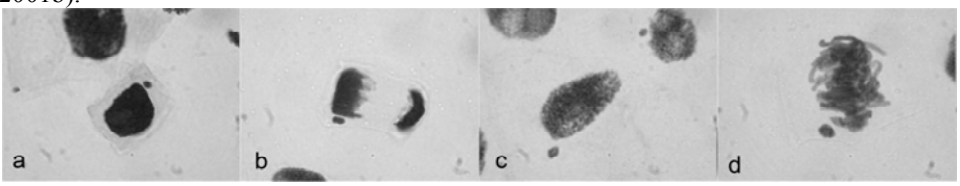

Fig. 7. Micronuclei. a. interphase micronucleus (SN 0.5% - 6h); b. ana-telophase micronucleus (SN 0.1% - 12h); c. interphase micronuclei (SMB 0.5% - 12h); d. metaphase micronucleus (SN 0.1% - 12h)

Presence of stickiness, c-mitoses, laggards, bridges, micronuclei, fragments after SMB were also reported in other plant systems (C*alendula officinalis* L., *Vicia faba, Allium cepa*) (Rencuzogullari *et al.*, 2001; Gomurgen 2005; Marc si Capraru, 2008; Mishra si Yadav, 2011).

Increases in metaphase disturbances (Fig. 8) were evidenced in 0.5% - 6h and 1.0% - 6h SN, but the frequencies were higher in 12h exposure, at 0.1% and 1.0%. SMB induced higher amounts of metaphase abnormalities than SN at 0.1% - 6h, 0.1% - 12h, and 0.5% - 12h

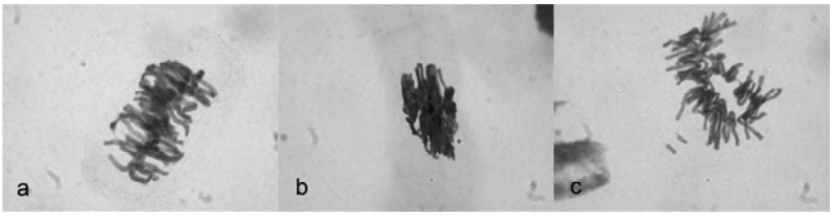

Fig. 8. Normal and abnormal metaphases induced by food additives. a. normal metaphase (SN 0.5% - 6h); b. sticky metaphase (SN 0.1% - 6h); c. metaphase with vagrants (SN 0.1% - 12h)

The most numerous were those metaphases showing chromosomes or fragments expulsed from equatorial plate (Fig. 9c). Sticky metaphases were found in low proportion (Fig. 9b), and the presence of c-metaphases was sporadic in SMB 0.5% - 12h (0.64%). The variant SMB 0.1% - 6h showed the highest percentage of abnormal metaphases (5.05%, as compared to 0.49% in control), 3.53% from these being sticky metaphases, and 1.52% being figures with expulsed chromosomes.

CONCLUSIONS

This study provides additional information about the toxicogenomic profile of SN and SMB, two chemicals commonly used as food additives. The results indicate a high amplitude of the changes induced in the genetic material, changes generated by both clastogenic and aneugenic mechanisms. This fact recommends the maintaining of a permanent alert on the genotoxic potential of these two additives (already utilized on large scale in food processing), with the reconsideration of their excessive addition and the identification of safer alternatives in terms of consumer health.

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