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In Vivo Genotoxicity Testing Of Vitamin C And Naproxen Sodium Using Plant Bioassay

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Abstract

Vitamin C (ascorbic acid) is a water-soluble vitamin and essential for collagen, carnitine and neurotransmitter biosynthesis. Naproxen sodium is propionic acid derivative and anti-inflammatory non-steroid agent. The aim of the study was to assess genotoxicity of selected medicaments and their possible effects on genetic material using *Allium* bioassay. The treatment of onion bulbs with fresh solutions of Naproxen Sodium (Nalgesin S®) and Vitamin C was performed using selected concentrations (550, 825 µg/ml and 250, 500, 1000 µg/ml respectively) in 72 hours time period. Control group was also set up.The microscopic parameters (mitotic index and chromosomal aberrations) of *Allium* root tips as well as the frequency of aberrant mitotic phases were analyzed. Both medicaments (vitamin C and Naproxen Sodium) caused increased frequency of abnormal mitosis when compared to control group.

1. INTRODUCTION

Plant bioassays are well-established test systems used for screening and monitoring of chemical substances with mutagenic and carcinogenic potential. Using plant bioassays for testing and monitoring have several advantages such as: they are easy to handle, inexpensive and in many cases more sensitive that other available systems (Maluszynska *et al.* 2005). The *A. cepa* bioassay is important since it is an excellent model *in vivo*, where the roots grow in direct contact with the substance of interest (Pastori *et al*. 2013). Correlation studies regarding the sensitivity of the *A. cepa* bioassay and other systems are important for the evaluation of the environmental risk and the obtained data can be extrapolated to other organisms, including humans (Leme *et al.* 2009, Pastori *et al*. 2013).

The present study was designed to examine the effect of Vitamin C (ascorbic acid) and Naproxen Sodium (Nalgesin S®) on cell divisions in the root meristems of *Allium cepa,* to reveal the genotoxic effect and chromosomal abnormalities induced by selected medicaments.

2. MATERIALS AND METHODS

We have chosen to perform cytogenetic analyses in the species *Allium cepa, L.* because it has been considered as encouraging higher plant for the assessment of chromosomal damages and disorders in mitosis, because of low chromosomal number (2n=16), large chromosomes, understanding the duration of the cell cycle and its response in the existence of many known mutagenic agents and apical meristems containing cells in division (Fiskesjö, 1985, Sarhan, 2010, Tănasie, 2012).

- **Vitamin C (ascorbic acid)**

Vitamin C or L-ascorbic acid ($C_6H_8O_6$) is important micronutrient and chemically is 2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol (Figure 1). It is one of the most common antioxidants in fruits and vegetables and majority of the plants and animals can synthesize ascorbic acid from D-glucose or D-galactose in liver. However, fruit eating bats, guinea pigs, apes and humans can not synthesize ascorbic acid due to the absence of enzyme L-gulonolactone oxidase. (Luo *et al.* 2014;

Naidu, 2003). The major dietary forms of vitamin C are L-ascorbic acid and dehydroascorbic acid.

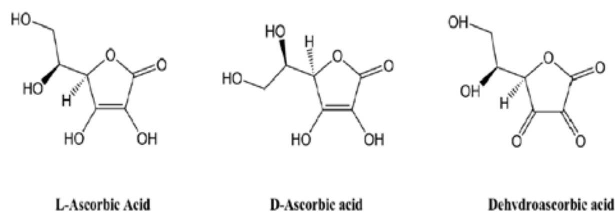

Figure 1. Forms of Vitamin C (ascorbic acid) (Source: Carr *et al.* 2013).

Ascorbyl palmitate is used in commercial antioxidans preparations because of its grater lipid solubility. Other commercial forms of vitamin C are soluble in water. Ascorbic acid is labile molecule whose stability is influenced by temperature, pH, oxygen levels or presence of transition metals. (Luo *et al*. 2014, Mazid *et al*. 2011).

Vitamin C (ascorbic acid) has many physiological functions that are largely dependent on its oxido-reduction properties. L-ascorbic acid is a co-factor for monooxigenase and hydroxylase enzymes involved in the synthesis of collagen, carnitine and neurotransmitters (Naidu, 2003). Vitamin C plays an important role in the maintenance of collagen which constitutes about one third of the total body proteins. Also, vitamin C is essential for the synthesis of muscle carnitine that is required for transport and transfer of fatty acids into mitochondria where they can be used for energy production. Further, ascorbic acid serves as co-factor for the dopamine-β- hydroxylase enzyme, important for conversion of neurotransmitter dopamine to norepinephrine (Naidu, 2003). Ascorbic acid is necessary for the transformation of cholesterol to bile acids, whose deficiency leads to accumulation of cholesterol in liver. Vitamin C enhances the availability and absorption of iron from non-heme iron substrates. (Oguntibeju, 2008, Naidu, 2003).

- **Naproxen Sodium (Nalgesin S®)**

Naproxen (NpSd) is chemically *(S)-6-methoxy-α-methyl-2-naphthaleneacetic acid* as sodium salt (Rao *et al*., 2013) shown at Figure 2. The molecule has anti-inflammatory, analgesic and antipyretic properties (Zuberi *et al.* 2014). The drug is commonly used for reduction of moderate to severe pain, in treatment of rheumatic or musculoskeletal disorders (Rao *et al.,* 2013, Zuberi *et al*. 2014, Redasani *et al.* 2013). It works as cyclo-oxygenase inhibitor (inhibitor of COX-1 and COX-2 enzymes) with effect in decrease of prostaglandin synthesis (Hawkey, 2001).

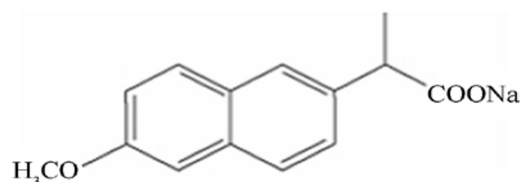

Figure 2. Naproxen Sodium (Source: Zuberi *et al*. 2014).

3. METHODS

The *Allium* bioassay was performed following Fiskesjö protocol, with some modifications (Fiskesjö, 1985, Fiskesjö 1993, Fiskesjö 1997). Healthy onion bulbs not treated with pesticides and obtained from commercial sources were used for each treatment group. Bulbs were left to germinate in transparent glass tubes filled with common tap water until roots reached 2-3cm in length. Freshly emerged roots were treated by fresh solutions of synthetic vitamin C (ascorbic acid, ZADA Pharmaceuticals) and naproxen sodium (Nalgesin S®, Krka-Pharma) in 72 hours time period (Jangala, M. et al. 2012). We have used five different solution concentrations: 250, 500 and 1000μg/ml for vitamin C, and 250, 825μg/ml for nalgesin S. Control group was set up using distilled water. Five onion bulbs were used for each treatment group. The total of 4000 cells were observed for each treatment and control group. Subsequently, treated roots were fixed using Farmer's fixative (absolute ethanol and glacial acetic acid, 3:1, v/v) for 24 hours. After fixation process, microscopic preparations were made. For each treatment, four slides were prepared using 4-5 root tips hydrolyzed in 1N HCl for 5 minutes and washed in distilled water. The fragmented meristemic tissue was stained with 2% acetic orcein (Guerra et al., 2002).

Mitotic index (MI) and chromosomal aberrations (CA) were analysed. MI was calculated as the ratio between number of mitotic cells and the total number of cells scored and expressed as percentage (MI %). Chi-square test is performed as well, using BioStat 2009 software ver. 5.8.0.0. and Microsoft Office Excel 2007.

4. RESULTS

Treatment with synthetic vitamin C (ascorbic acid) resulted in increase in the mitotic activity (in all treatments – 250 μg, 500 μg, 1000 μg/ml) and observed increase was statistically significant in all treatments (χ^2 test, $p< 0.05$), as shown in Table 1.

Regarding the number of individual mitotic phases, we observed that the frequency of prophases was increased in all treatments (250 μg, 500 μg, 1000 μg/ml) compared to control

group. All observed increments were statistically significant ($p < 0.05$). The number of metaphases was increased in 500 µg/ml (statistically significant $p < 0.05$) and 1000 µg/ml treatments, but slightly decreased in 250 µg/ml treatment. The number of anaphases and telophases was decreased comparing to control group and decrease was statistically significant ($p < 0.05$), except for number of telophases in 1000 µg/ml treatment where the number was equal to one observed in control group (Table 2).

Table 1.	**Changes in mitotic activity of *A. cepa* root tip cells treated with *vitamin C* compared to control group**			
Treatment length (in hours)	***Control***	***250 µg/ml***	***500 µg/ml***	***1000 µg/ml***
72	**9.73**	14.13*	16.33*	14.1*
Legend: Statistically significant at p<0.05 compared to untreated control*				

Table 2.	**Changes in frequency of individual phases in *A. cepa* root tip cells in comparison to control group**			
Frequency (%)	***Control***	***250 µg/ml***	***500 µg/ml***	***1000 µg/ml***
Prophase	**5.33**	10.98*	11.23*	10.13*
Metaphase	**1.98**	1.93	4.18*	2.38
Anaphase	**1.40**	0.63*	0.38*	0.58*
Telophase	**1.03**	0.60*	0.55*	1.03
Legend: Statistically significant at p<0.05 compared to untreated control*				

In this study, the most common chromosomal aberrations (CAs) found were: abnormal kinetics, chromosome laggards, anaphase bridges, unequal spiralisation (Figure 3). Chromosomal aberrations were observed in each treatment comparing to control and most of the observed irregularities were related to abnormal chromosomal kinetics.

Treatment with naproxen sodium (Nalgesin S®) resulted in decrease in the mitotic activity (550µg/ml treatment). The decrease was statistically significant (χ^2 test, $p < 0.05$). But, the higher concentration of naproxen sodium (825 µg/ml) led to slightly increased mitotic activity compared to control group, but with no statistical significance ($p < 0.05$) as shown in **Table 3.**

The number of individual mitotic phases in both treatments (550 µg, 825µg/ml) was decreased compared to control group, except in case of prophases in treatment with 825 µg/ml where the number of prophases was increased, with statistical significance ($p < 0.05$). Regarding decrease in number of metaphases, anaphases and telophases compared to control group, the decrease in case of metaphases and anaphases was statistically significant ($p < 0.05$) and regarding telophases was not (**Table 4**).

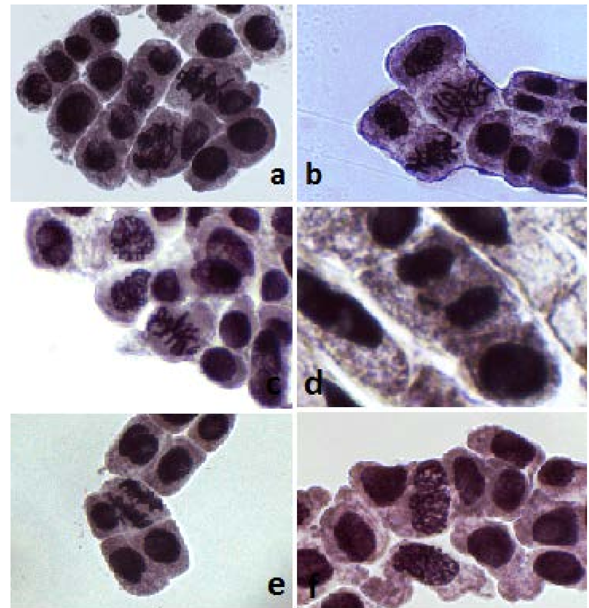

Figure 3. Photomicrographs of abnormal cell divisions induced by vitamin C (ascorbic acid) in root meristem cells of *A. cepa*: a) irregular kinetics and anaphase bridges; b) abnormal kinetics c) and d) lagging chromosome; e) anaphase with vagrant chromosomes; f) unequal spiralisation.

Table 3.	**Changes in mitotic activity of *A. cepa* root tip cells treated with *Naproxen Sodium* compared to control group**		
Treatment length (in hours)	***Control***	***550 µg/ml***	***825 µg/ml***
72	12.1	9.65*	12.68
Legend: Statistically significant at p<0.05 compared to untreated control*			

Table 4.	**Changes in frequency of individual phases in *A. cepa* root tip cells in comparison to control group**		
Frequency (%)	***Control***	***550 µg/ml***	***825 µg/ml***
Prophase	6.63	5.45*	9.13*
Metaphase	2.93	2.3*	1.73*
Anaphase	1.43	0.8*	0.73*
Telophase	1.13	1.1	1.1
Legend: Statistically significant at p<0.05 compared to untreated control*			

In relation to cellular abnormalities, chromosomal aberrations were observed in each treatment comparing to control. Observed CAs comprised: chromosomal agglutination,

vacuolization, chromosomal lagging and stickiness, anaphase bridges, unequal spiralisation, scarce occurrence of micronuclei (MNi). Also, changes in cell morphology are observed in form of elongated nuclei and cytoplasm changes (Figure 4).

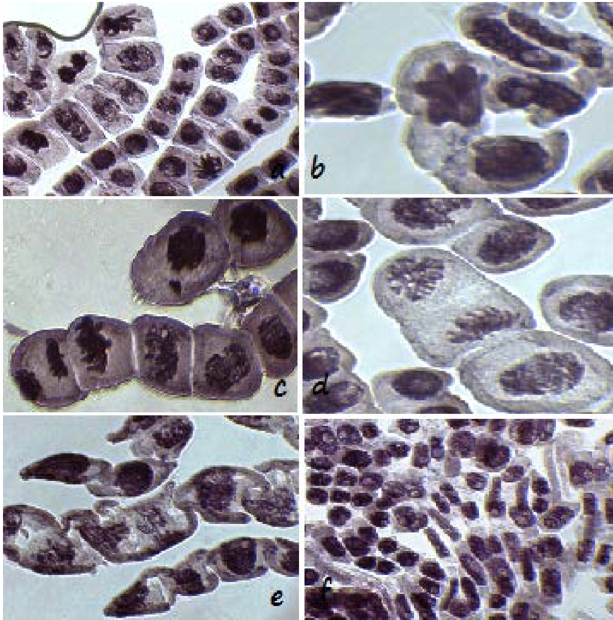

Figure 4. Photomicrographs of abnormal cell divisions induced by Naproxen Sodium in root meristem cells of *A. cepa*: a) irregular kinetics and chromosomal agglutination; b) agglutination; c) micronucleus in interphase and clumped chromosomes in metaphase d) unequal spiralisation; e) and f) changes in cell morphology

4. DISCUSSION

As shown in the previous classical studies of Fiskesjö (1985, 1993, 1997) and others (Hoshina *et al.* 2009, Leme *et al*. 2008) *Allium cepa* bioassay is low-cost, simple, effective and reproducible model for genotoxicity and cytotoxicity evaluation of chemical and mixture substances (Iwalokun *et al*. 2011). The reason for good genotoxic assay performance of *Allium cepa* can be attributed to large chromosomes and low chromosomal number of the species (2n=16) and the ability to interpolate outcomes of the assay with those in mammalian cells, consequently with those in human cells as well. (Fiskesjö, 1985, Iwalokun *et al*. 2011, Tadesco *et al*. 2012).

Allium root tip meristem cells were treated with freshly made synthetic vitamin C solutions (250, 500 and 1000 μg/ml) in 72 hours time period. The treatment of meristem cells showed increased mitotic activity in all used vitamin C concentrations, with statistical significance ($p<0.05$). The increased values of MI comparing to control may be explained by its profound influence (together with vitamins A, β-carotene, E) on cell growth and differentiation, in accordance with its anti-oxidant properties (Walingo, 2005). According to the study performed in cultures of human lymphocytes treated with Vitamin C with the same concentrations, the MI was reduced (Nefic, 2008). The mitotic abnormalities observed in our study were mostly in form of disturbances in chromosomal kinetics. The chromosomal abnormalities comprised chromosome stickiness, anaphase bridges, chromosome laggards and unequal spiralisation. Other studies (Nefic, 2008) showed that Vitamin C induce similar CAs in treatments with the same concentrations (250, 500, 1000 μg/ml). Although many studies have demonstrated the antimutagenic activity of ascorbic acid (Aly, *et al.* 2002, Assayed *et al.* 2010) in some cases, it can have co-mutagenic effects (Kaya et al. 2002, Konapacka *et al.* 1998). It is considered that Vitamin C protects cells from oxidative DNA damage and has antimutagenic effect (Luo *et al.* 2014), but its effect may be exhert as pro-oxidant and generate changes in DNA depending on dosage (Halliwell, 2001). The data on Vitamin C and DNA damage are conflicting and inconsistent (Naidu, 2003). This study can contribute to further understanding of genotoxic potential of vitamins, in this case of Vitamin C.

In treatment with Naproxen Sodium (Nalgesin S®) with 550 μg and 825μg solution concentrations, the value of MI was decreased in 550 μg treatment with statistical significance ($p<0.05$); MI value was slightly increased in 825 μg treatment without statistical significance. The data regarding evaluation of Naproxen Sodium on cell proliferation were rather limited. In available study, Naproxen had concentration-dependent inhibitory effect on cell proliferation in cancer (osteosarcoma) cells (Correia *et al*. 2014).

Regarding the number of individual mitotic phases, we observed that the frequency of all phases (prophase, metaphase, anaphase, telophase) was decreased except the frequency of prophases in 825 μg treatment where it was increased with statistical significance ($p<0.05$). Decrease in number of telophases had no statistical significance.

The mitotic abnormalities observed in this study comprised disturbances in chromosomal kinetics. The chromosomal abnormalities observed were in form of chromosome stickiness, increased agglutination, anaphase bridges and unequal spiralisation. In case of observed numerical abnormalities (NAs) the scarce presence of cells with micronuclei (MNi) was observed. Since available reports about genotoxicity assessments of Naproxen Sodium in literature sources are rather scarce, there was limited information on the potential mutagenic effects of this drug on

cells. In the study of Correia *et al.*, DNA damage in used cells (osteosarcoma cells) was reported (Correia *et al*. 2014).

5. CONCLUSION

The results of this study indicate a need for further *in vitro* and *in vivo* studies of genotoxic potential of selected substances, as well as using different test systems besides *Allium* assay. The data obtained will add more information regarding safe use of these substances.

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