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Evidence that the radioprotector effect of ascorbic acid depends on the radiation dose rate

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ABSTRACT

Many studies have revealed that ascorbic acid (Aa) acts as a powerful inhibitor of genetic damage. The objetive of the present study was to evaluate the radioprotector effect of Aa at two diferent radiation dose rates. The somatic mutation and recombination test in *Drosophila melanogaster* was used. 48 h larvae were treated for 24 h with 25, 50 and 100 mM of Aa. After pretreatment, larvae were irradiated with 20 Gy of gamma rays administered at 36 or 960 Gy/h. Toxicity, development rate and frequency of mutant spots were recorded. Results provide evidence of a radioprotective effect for all tested concentrations of Aa only when 20 Gy were delivered at 36 Gy/h and only with 25 mM using the 960 Gy/h. To consider the use of Aa as radioprotector or therapeutic agent, it is necessary to know its potential under different situations to avoid unwanted injuries.

1. Introduction

The harmful effects of ionizing radiation in biologicalsystems are produced essentially through direct deposition of energy into crucial molecules (direct effect), or through the intracellular generation of reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide, hydroxyl radicals, peroxide radicals, and other free radicals (Azzam et al., 2012). The most common reactive ROS include superoxide anion (O^2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). At low levels, ROS show beneficial effects: they participate in cellular response and immunity (Selim et al., 2016), but its excess provokes different effects including lipid peroxidation, removal of thiol groups from cellular and membrane proteins, strand breaks and base alterations leading to DNA damage (Shukla et al., 2010). Free radicals produce single and double DNA strand breaks, which lead to mutations. Nowadays, it is well known that DNA damage plays a significant role in the development of atherosclerosis and other degenerative diseases including some kind of autism, Alzheimer, Parkinson and cancer (Jones, 2010; Mirończuk-Chodakowska et al., 2018; Sanz, 2016). The risk of injury from radiation is a function of the doses received; the type of radiation (low or high linear energy transfer), the type of cell and dose rate, among others (Kelsey et al., 2014). Dose rate is defined as the radiation dose absorbed per unit of time (Ślosarek et al., 2014).

Nowadays, the extensive use of radiation for medical treatment, diagnosis; its use in the energy sector, industry, nuclear accidents, nuclear terrorism and in some other activities such as outer space or air traveling, has increased the necessity to identify, develop, and validate potential strategies to protect normal tissues from the harmful effects of ionizing radiation (Singh and Krishnan, 2015). Pharmacological intervention could be the most prudent strategy, given that compounds, especially those of natural origin that can act as free radical scavengers and antioxidants, are able to reduce or mitigate the deleterious effects of ionizing radiation (Maurya et al., 2006).

Radioprotective agents are chemical compounds that reduce the effects of radiation in healthy tissues and have been used to reduce morbidity or mortality produced by ionizing radiation (Greenberger, 2009), they also have a practical use in clinical radiotherapy because normal tissues should be protected against radiation injury while cancerous tissues are exposed to higher doses of radiation to obtain better results. A large number of compounds showed good radioprotection in *in vitro* studies, but most of them failed *in vivo* application due to acute toxicity and side effects (Weiss and Landauer, 2003). There have been many attempts to find an ideal radioprotector that can preferentially protect normal tissues from radiation damage without affecting the sensitivity of tumor cells (Bump and Malaker, 1997). The chemicals that can scavenge free radicals reduce the occurrence of DNA strand breaks. Thus they can prevent the formation of free radicals or destroy free radicals by reacting with them, thereby inhibiting their reaction with biomolecules. Since free radicals are short-lived, it is necessary for such radioprotective molecules to be present in the cellular media at the

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time of radiation exposure.

To keep the damage caused by ROS from occurring, eukaryotic cells possess antioxidant systems, such as enzymes that prevent or limit the free radical production: superoxide dismutase, catalase, and glutathione peroxidase. These enzymes provide important protection against radiation exposure (Pisoschi and Pop, 2015). Besides the enzymes, the cell can prevent the free radical damage by means of dietary antioxidants, including vitamins A, C, and E, polyphenols, anthocyanins, flavonoids and isothiocyanates (Weiss and Landauer, 2003; Meyers et al., 2008).

Vitamin C is the reduced form of ascorbic acid (Aa), it is a water-soluble ketolactone with two ionizable hydroxyl groups that readily undergoes two consecutive one-electron oxidations to form the poorly reactive ascorbate radical and the dehydroascorbic acid (Du et al., 2012). Administration of Aa before gamma irradiation prevents chromosomal damage in bone marrow cells and radiation-induced lethality (Konopacka et al., 1998). The protective properties of this compound are due mainly to its scavenging activity of reactive oxygen species before they reach macromolecules, protecting lipid membranes and proteins from oxidative damage (Berger et al., 1997). It has been reported that Aa can prevent the adverse effects of whole body irradiation through increasing the antioxidant defense systems in the liver and kidney of irradiated animals (Mortazavi et al., 2015). Pretreatment with Aa inhibits lipid peroxidation, protects mice against mortality and sickness induced by irradiation, and produces an improvement of wound healing after exposure to whole body gamma radiation (Prasad et al., 2002).

Different factors modify the effect of Aa, the most studied are the concentration and the presence of transition metals (Halliwell, 1999), several experiments have shown that high concentrations of Aa increased DNA damage through the production of hydroxyl radicals from hydrogen peroxide by the Fenton reaction. Du et al. (2012) found that pharmacological doses of ascorbate induce cytotoxicity and oxidative stress in pancreatic cancer cells, but not in normal cells. Several studies have revealed that Aa, can also have prooxidant effects (Halliwell, 1996); in fact, the combination of transition metals (such as Fe and Cu) with ascorbate has long been used as an oxidizing system, and the combination of these two reagents also known as the “unfriend system” is used for the hydroxylation of alkanes, aromatics, and other oxidations (Odin, 1997; Halliwell, 1999; Cai et al., 2001; Mendes-da-Silva et al., 2014).

Although different experiments have been conducted to determine the radioprotective effect of ascorbic acid, results are not consistent so it is essential to explore under what circumstances antioxidants can decrease or increase the genetic damage caused by ionizing radiation. One of the factors modifying the effect of ionizing radiation is the dose rate which is very important in radiotherapy, that is why the main objective of the present work is to evaluate the radio protective effect of Aa using two different dose rates by means of the somatic mutation and recombination test (SMART) in the wing blade of *Drosophila*.

Since *Drosophila* is a holometabolous organism, toxicity can be assessed in the different developmental stages: embryo, larval, pupal, and adult. Various parameters such as: toxicity, development rate and genetic damage could be tested using the same individuals, under which circumstances antioxidants can protect from damage induced by ionizing radiation or increases it. From a radiobiological point of view, it is known that mitotic recombination is inducible by radiation and its significance in human oncogenesis is remarkable, the Drosophila SMART test offers the great advantage of strongly identifying this genetic point. Therefore, *D. melanogaster* could bring an essential contribution to the antimutagenic and radio protection field.

2. Material and methods

Biological material and pretreatment: Three-day-old virgin *mwh/mwh* females were mated to *flr*^{3}/TM3 *Ser* males in 250 ml flasks with regular food. Oviposition was restricted to 2 h to obtain homogeneous samples. Eggs were incubated in the culture room for 72 h to obtain second instar larvae that were separated from the culture medium by means of gradient density using a 20% sucrose solution. Larvae were treated for 24 h in empty bottles containing a disc of filter paper and 3.5 ml of 0, 25, 50 and 100 mM of Aa solution, using distilled water as solvent. Aa concentrations, treatment period as well as genetic damage-inducing dose were selected based on previous work in our laboratory (Olvera et al., 1995, 1997; Cruces et al., 2003, 2009).

Treatment with radiation: After Aa pretreatment, larvae for each concentration were divided in three groups: the first was irradiated with 20 Gy at low DR (LDR): 36 Gy/h in the Gamma-Cell 2000 irradiator, the second at high DR (HDR): 960 Gy/h in the Transelektro LGI-01 irradiator, and the third group was used as control. 1000 larvae were tested for each treatment in groups of 100 by homeopathic vial with 0.8 g of synthetic medium (Formula 4–24 Carolina Biological Supply Co.) and 2.5 ml of distilled water. All treated larvae were introduced into the culture room. After larvae completed their development, the number of emerged adults, males and females separately, were recorded daily.

Developmental rate and larvae-to-adult viability analysis: Larvae-to-adult developmental rate was determined by counting every day the number of emerged flies, until all had emerged, larvae-to-adult survival was measured as the ratio of the total number of emerged flies and toxicity was obtained by dividing total viable adults into the number of treated larvae. Once that data was obtained, survival curves were constructed. The slope (m) of the exponential phase of each curve was calculated for the development rate index and the emergence day for 50% of the individuals was determined by extrapolating the X axis. The Chi square test was used to establish differences between treatments.

Genotoxicity analysis: The wings of the subsequently emerged adults were mounted on slides following the standard procedure described by Graf et al. (1984). The wings were scored at 40x magnification for abnormal wing hair spots in small singles (1 or 2 cells), large (more than 3 cells) either *mwh* or *flr* and twin spots. Single *mwh* spots are inferred to arise from a separation between *mwh* and *flr*^{3}, from an interchange or from mutation/deletion at the *mwh*$^+$ locus; single *flr* spots occur from mutation/deletion at the *flr*$^+$ locus or following a double exchange; and twin spots from an interchange between *flr* and the centromere. The frequencies of each type of mutant clones per wing (s/p) were compared with the concurrent negative control.

Statistical analysis: The Chi-square test was used to identify statistical differences in larvae-to-adult survival between control and the remaining groups and to determine differences between genetic damage caused by different treatments.

3. Results

Developmental rate and larvae-viability analysis: Table 1 shows viability of second instar larvae treated with Aa 25, 50 or 100 mM, none of them decreased viability, however, in combination with 20 Gy at a HDR 50 and 100 mM they reduced the viability although no significant differences were found. Fig. 1 represents the development rate of the organisms treated with different concentrations of Aa and its treatment combined with 20 Gy gamma rays administered at two different dose rates. No differences were found between the slopes of the different treatments and the control groups.

Genotoxicity analysis: Table 2 shows the frequency of all kinds of spots (small, large, twin and total) produced by the treatment with the three Aa concentrations and in combination with 20 Gy of gamma rays administered at low or high dose ratios. None of the Aa concentrations modified the basal frequency of mutations, 20 Gy of gamma rays administered at 36 Gy/h, provoked an increase of 11.7 times in the total spots over the basal frequency. The lowest concentration of Aa combined with gamma rays reduced the frequencies of spots induced by 20 Gy: the small in 40%; the large in 20% and the total in 23%. The