Brazilian Journal of Biological Sciences, 2017, v. 4, No. 8, p. 293-300.
ISSN 2358-2731
https://dx.doi.org/10.21472/bjbs.040807

Evaluation of mutagenic effects of pure hydroxyapatite doped with chromium (III) through the SMART Test in *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae)

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Abstract. Hydroxyapatite (HAP) is a bioceramic used in the medical and dental areas as a bone replacement factor due to its chemical similarity to the mineral phase of bones and teeth. Its use in implants stimulate the growth of bone tissue, showing no toxicity or rejection of the host tissue. Its nanostructured form has been shown to be a viable alternative for photoprotection when doped with metal ions, such as trivalent chromium (Cr^{+3}). Due to the reach of this form among the population, this work evaluated the mutagenic potential of pure nanostructured hydroxyapatite (HAP) and doped with trivalent chromium (Cr^{+3}) (HCrIII) by means of the Somatic Mutation and Recombination Test (SMART Test) on *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) wings. Larvae resulting from standard crosses (ST) and high metabolic bioactivation (HB), treated with PAH and with HCrIII at concentrations 16.66 mg/mL, 8.33 mg/mL, and 4.16 mg/mL. As positive and negative controls, urethane and dodecyl sulfate sodium (SDS) were used, respectively. The frequencies of the different categories of mutant spots observed in offspring of HAP (HAP) and HCrIII treated crosses were not significantly different from those observed in the negative control. These data show that pure and chromium-doped (Cr^{+3}) nanoestructured hydroxyapatite do not exhibit mutagenicity.

Keywords: Hydroxyapatite; Chromium; Somatic Mutation and Recombination Test; SMART Test; Mutagenicity.

Received
November 18, 2017

Accepted
December 21, 2017

Released
December 31, 2017

Open Access
Full Text Article

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Introduction

Ceramics materials mainly based on calcium phosphate salts have been used in various applications in healthcare as implant materials, tissue repair and regeneration. Its wide acceptance is due mainly to its high degree of biocompatibility, since they have no local or systemic toxicity, absence of inflammation or foreign body responses and apparent ability to bind to the host tissue, as well as having great chemical similarity with the materials normally found in the bone tissue (Dourado, 2006; Lacerda et al., 2009; Trommer et al., 2006; Hench, 2013). Among bioceramics with improved performance are bioglasses, alumina, beta-tricalcium phosphate and hydroxyapatite (HAP). This one attracts considerable interest for being the principal mineral phase found in bone tissues and it has high biocompatibility and bioactivity, in addition to have excellent adhesion not only to the cells, but also to soft and hard tissues (Chai and Ben-Nissan, 1999; Okada and Furuzono, 2010, Wang et al., 2010). Furthermore, hydroxyapatite has great importance in many industrial applications such as catalyst support, liquid chromatography, illumination of materials, transport of substances, chemical sensors, ion conductors and drug carriers (Wang et al., 2010).

The particle size of hydroxyapatite may be decisive for its use, and the crystals obtained from preparations can vary at the scale from millimeters to nanometers (Elliott, 1994). The nanostructured hydroxyapatite has optical properties which are maximized when it acts connected with a metal ion dopant, such as trivalent chromium (Cr^{+3}), so it shows great potential for application as sunscreen (Araújo et al., 2008).

The use of hydroxyapatite doped with chromium to formulate sunscreens is an important and innovative output to the cosmetics market. However, because it is a product that will interact directly with the human body, and also for being an innovative product, it is essential the conduction of several tests before the release of such material for commercialization, to prove that it does not harm the user's health especially taking into consideration that nanostructured materials have larger contact area with the skin (Araújo, 2006).

This fact may be beneficial, but can also bring risks given that when on nano-scale the materials have specific and distinct physicochemical properties, which are different from the properties of the same material at larger scales. Therefore, a product that does not bring health risks when it is at a macroscopic scale can become harmful when nanostructured (Emerich and Thanos, 2003; Sahoo and Labhasetwar, 2003; Nogueira, 2005).

Voluntarily or not, during its existence, living organisms are exposed to mutagens agents that can cause changes in the DNA sequence. Many of these changes become harmful, because they interfere in vital processes to the cells, such as DNA replication and gene transcription. These changes can result in mutations that trigger cancerous processes, which can lead eventually to cell death. So, it is important to evaluate the genotoxic and mutagenic potential of several substances, especially the new products to minimize undesirable effects on living organisms (Costa and Menck, 2000).

For this aim, the Somatic Mutation and Recombination Test (SMART Test) in *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) can be used as a fast, safe and reliable alternative. Through the SMART it is possible to detect point mutations, chromosomal breaks and losses and also the presence of somatic mutations when analyzed their markers, namely, through analysis of the mutant hair (Graf et al., 1984; Frei and Würgler, 1995).

Considering these facts, the present study aimed to evaluate, by means of the SMART Test in *Drosophila melanogaster* (fast, safe and reliable test), the possible mutagenic effects of hydroxyapatite pure and doped with Cr^{+3}.

Material and methods

Three different lineages of *D. melanogaster* were used for the experiment, multiple wing hair (mwh), flare-3(flr³) and Oregon R, flare³ (ORR; flr³). These lineages were kept in an oven at controlled temperature of 25°C ± 1°C in glass bottles of 250 mL standard culture medium the basis of distilled water (820 mL), biologic yeast (25 g), agar (11 g), banana (156 g) and Nipagin (1 g).

For treatment effect two types of crossings were used, the pattern - Standard Cross (ST), which characterizes the direct action of the compound investigated, and the high metabolic bioactivation - High Bioactivation Cross (HB), characterized by indirect action (Graf et al, 1984). At the Standard Cross (ST), virgin females flr³ (*flr³/In(3LR)TM3, ri p^{p} sep l(3)89Aa bx^{34e} and Bd^{S}*) were crossed with males of mwh lineage with genetic constitution *mwh/mwh* (Graf et al, 1984).

At the crossing HB, virgin females of the lineage ORR/flr³ (*ORR/ORR: flr³/In(3LR)TM3, ri p^{p} sep l(3)89Aa bx^{34e} and Bd^{S}* were used with *mwh/mwh* male. The ORR/*flr³* females carry chromosomes 1 and 2 from the lineage Oregon R (R), DDT-resistant (Dapkus and Merrell, 1977). These genes are responsible for high levels of metabolic enzymes of the Cytochrome P450 (CYP6A2) type (Graf and Schaik, 1992). From both crosses two kinds of larvae were originated: the trans-marked heterozygous (MH) which have regular/smooth edges of wings and the balanced larvae heterozygous (BH) for chromosome TM3, which have irregular/serrated edge of wings, which were exposed to the tested substances.

Samples of nanostructured hydroxyapatite pure and doped with Cr^{+3} at the concentration of 1% mol were used for treatment. Sodium dodecyl sulphate (SDS) at 3%, which in this experiment was used as a solvent because it proved to be more efficient than distilled water at the dispersion of hydroxyapatite's molecules, was used as the negative control and urethane (URE) 0.891 mg/mL as the positive control. For each treatment three concentrations were determined and for each concentration three repetitions were determined. The concentrations that were determined for both treatment are 16.66 mg/mL, 8.33 mg/mL, and 4.16 mg/mL.

For each treatment 80 *flr^{3}* virgin females were used (standard cross - ST) and 80 ORR, flr³ virgin females (crossing with high bioactivation - HB) with 40 *mwh* males in separated bottles per crossing, containing standard culture medium for two days. After this period the parental flies were transferred to other containers of about 400 mL, which contained oviposition medium-based agar, biological yeast and sugar, all cooked in a water bath at a temperature of 38 ºC ± 2 ºC, where they remained for 8 h. Afterwards the adults were discarded. 72 h ± 4 h after the beginning of oviposition, the larvae of the third stage were collected by flotation in flowing water at 25 °C through a metal strainer with fine mesh (Graf et al, 1984).

These collected larvae were placed in smaller containers (approximately 100 per container) containing 1.5 g of synthetic medium (yoki ® mashed potatoes) and 5 mL of the solution to be tested - treatment. This procedure was the same for both controls as negative to positive. The larvae remained under these conditions for about 48 h (chronic treatment), until reaching the emergence of the larvae. All adults derived from the treatments were maintained in 70% ethanol until the assembly of blades. For this step, the wings were extracted in stereomicroscope and mounted between blade and coverslip with Faure solution (30 g of arabic gum, 20 mL of glycerol, 50 g of chloral hydrate and 50 mL of water) and analyzed for the occurrence of different types of mutant spots in a light optical microscope, at increased 400x. Statistical analysis was performed according to the chi-square (X2) proposed by Frei and Würgler (1988), for two-tailed proportions significance level: $\alpha = \beta = 0.05$, in order to compare the frequencies obtained from the groups which were treated with the negative control frequency.

Results and discussion

Hydroxyapatite (HAP) has been used successfully for a long time in the medical implant and bone tissue repair, as a biocompatible and bioactive material cytologically. But in recent years, it has attracted attention as an excellent alternative for environmental control, as biosensor and intelligent drugs vehicle (Araújo et al, 2008). In its most recent use, nanostructured hydroxyapatite doped with chromium (Cr^{+3}) has been studied as a viable alternative for the production of sunscreen. In this study samples of pure nanostructured hydroxyapatite and nanostructured hydroxyapatite doped with Cr^3 + 1% mol at different concentrations (16.66 mg/mL, 8.33 mg/mL, and 4.16 mg/mL) were tested through the SMART Test, and the respective controls: positive (urethane 0.891 mg/mL) and negative (sodium dodecyl sulphate - 3% SDS). The controls, positive and negative, were simultaneously tested in each experiment and the results obtained in the isolated treatments were compared to negative control.

Mutagenic analysis of pure hydroxyapatite (HAP)

Table 1 shows the results of the frequencies of mutant spots observed in the MH descendants for both crossings (ST and HB), treated with different concentrations of pure Hydroxyapatite (HA) and their respective controls. For this treatment, the frequency of mutant spots – the small simple ones, the large simple ones and the twins, as well as the total number of spots observed were not statistically significant ($\alpha \leq 0.05$) when compared to their respective negative controls for the two crossings, both ST as HB.

Table 1. Frequency of mutant spots observed in the MH descendants wings of *D. melanogaster* from ST and HB, after chronic treatment (~48h) with different concentrations (16.66 mg/mL, 8.33 mg/mL, 4.16 mg/mL) of pure nanostructured hydroxyapatite and its respective controls: negative (3% SDS) and positive (URE 0.891 mg/mL).

Treatments Concentrations (mg/mL)	N.° of flies (*N*)	Spots per individual (n° of spots) statistical diagnostic[a]										Total of Spots mwh[c]
		Small Simple Spots (1-2 cells)[b] *m* = 2			Large Simple Spots (>2 cells)[b] *m* = 5			Twin spots *m* = 5			Total of Spots *m* = 2			
ST Crossing *mwh/flr*^{3}														(*n*)
Negative Control	50	0,48	(24)		0,06	(03)		0,02	(01)		0,56	(28)		25
HAP [1]	50	0,40	(20)	-	0,02	(01)	i	0,00	(00)	i	0,42	(21)	-	18
HAP [2]	50	0,24	(12)	-	0,00	(00)	-	0,00	(00)	i	0,24	(12)	-	12
HAP [3]	50	0,30	(15)	-	0,02	(01)	i	0,00	(00)	i	0,32	(16)	-	15
URE (0,891 mg/mL)	40	2,85	(114)	+	0,20	(08)	i	0,00	(00)	i	3,05	(122)	+	107
HB Crossing *mwh/flr*^{3}														
Negative Control	40	0,48	(19)		0,10	(04)		0,00	(00)		0,58	(23)		17
HAP [1]	49	0,47	(23)	-	0,08	(04)	i	0,00	(00)	i	0,55	(27)	-	24
HAP [2]	50	0,36	(18)	-	0,02	(01)	-	0,00	(00)	i	0,38	(19)	-	18
HAP [3]	50	0,42	(21)	-	0,02	(01)	-	0,00	(00)	i	0,44	(22)	-	21
URE(0,891mg/mL)	40	3,83	(153)	+	0,75	(30)	+	0,10	(04)	i	4,68	(187)	+	149

HAP = Pure Hydroxyapatite ; URE = Urethane; HAP [1] = 16.66 mg/mL, HAP [2] = 8.33 mg/mL, HAP [3] = 4.16 mg/mL. [a]Statistical diagnoses according to Frei and Würgler (1988): +, positive; -, negative; i, inconclusive. *m*, multiplication factor for the evaluation of the significantly negative results. Significance levels $\alpha = \beta = 0,05$. [b]Including rare simple spots *flr*^{3}. Considering mwh clones for mwh simple spots and for twin spots.

Chronic treatment of MH individuals with HA dissolved in 3% SDS resulted in a predominant distribution of simple and small spots for both crossings (ST and HB), indicating that the mutations observed in the cells of the wings' imaginal discs occurred at a late larval stage of the development of *D. melanogaster*, passing through one or two cycles of cell division in maximum once the larvae are exposed to the substances for 5 or 6 mitotic cycles, which correspond to 95% of all cell divisions that occur from the developing embryo to the beginning of the pupal stage (Frei et al., 1992). The large spots are formed by mutations, which occurred in the initial development stages passing through several cycles of cell division and, for this reason, it shows a higher number of cells per spot. Thus, in experiments with substances that have immediate genotoxic action, it is expected to find a prevalence of large spots, differently from what happened in this study.

The negative results found in MH descendants from the high bioactivation crossing (HB) permit the inference that HA under the experimental conditions used is not a pro-mutagenic agent, or with indirect action, dependent on metabolization, and that cytochrome P450 enzymes do not influence the genotoxic potential of this substance. Hydroxyapatite is widely used for medical application because of its high biocompatibility and low toxicity, even when it is in nanometric structure (Okada and Furuzono, 2010; Tomoda et al., 2010; Kumar et al., 2010; Wang et al., 2010a). Gouveia (2008) demonstrated through the CellTiter96® AQueous test that nanostructured hydroxyapatite is not cytotoxic. However, Motskin et al. (2009), testing hydroxyapatite nanopowders, they concluded that when nanostructured it assumes different physicochemical characteristics, but only becomes cytotoxic at higher concentrations. But there were no reports in the literature about tests for mutagenicity of nanostructured hydroxyapatite that could be compared to the results found in this study.

Mutagenic analysis of hydroxyapatite doped with trivalent chromium (HCrIII)

Table 2 shows the results of the frequencies of mutant spots in MH descendants for both crossings (ST and HB), treated with different concentrations of hydroxyapatite doped with trivalent chromium (HCrIII) and their respective controls. The frequency of total mutant spots observed on treatment with different concentrations of HCrIII were not statistically significant when compared to the frequencies of mutant spots found in their respective negative controls for the two crossings (ST and HB).

For the ST crossing, the frequency of large and simple spots was not statistically significant for any of the three concentrations when compared to negative control. The total of mutant spots showed no significant difference in any of the three concentrations when compared to the negative control, which indicates lack of genotoxicity of HCrIII. For MH descendants treated with HCrIII from the HB crossing, the results were similar to those from the ST crossing, because the frequency of simple spots as well as the total of mutant spots was not statistically significant for any of the three concentrations when compared to its negative control. For the descendants of the two crossings (ST and HB), HCrIII, although it has not showed statistically different results from the negative control, showed a higher frequency of small and simple spots, similar to the result of HAP. This indicates that mutations in the cells occured at a late stage in embryonic development of the larvae of *D. melanogaster*.

Under the experimental conditions used, HCrIII did not behave as a pro-mutagenic or with indirect action, since the MH descendants from HB crossing did not show statistically different results from negative control in any of the three concentrations that was analyzed, so it is not dependant on metabolization by cytochrome P450 enzymes.

Table 2. Frequency of mutant spots observed in MH descendants wings of *D. melanogaster* from ST and HB, after chronic treatment (~48h) with different concentrations (16.66 mg/mL, 8.33 mg/mL, and 4.16 mg/mL) of nanostructured hydroxyapatite doped with Cr3+ and its respective controls: negative (3% SDS) and positive (URE 0.891 mg/mL).

		Spots per individual (nº of spots) statistical diagnostic[a]												
Treatments concentrations (mg/mL)	**Nº of flies (N)**	**Small simple spots**			**Large simple spots**			**Twin spots**			**Total spots**			**Total Spots mwh[c]**
		(1-2 cells)[b]			**(>2 cells)[b]**									
		m = 2			***m* = 5**			***m* = 5**			***m* = 2**			**(*n*)**
ST Crossing														
mwh/flr³														
Negative Control	50	0.48	(24)		0.06	(03)		0.02	(01)		0.56	(28)		25
HCrIII[1]	40	0.25	(10)	-	0.20	(08)	i	0.00	(00)	i	0.45	(18)	-	12
HCrIII[2]	48	0.21	(10)	-	0.00	(00)	-	0.00	(00)	i	0.21	(10)	-	10
HCrIII[3]	50	0.30	(15)	-	0.04	(02)	i	0.02	(01)	i	0.36	(18)	-	16
URE(0,891mg/mL)	40	2.85	(114)	+	0.20	(08)	i	0.00	(00)	i	3.05	(122)	+	107
HB Crossing *mwh/flr³*														
Negative Control	40	0.48	(19)		0.10	(04)		0.00	(00)		0.58	(23)		17
HCrIII[1]	38	0.32	(12)	-	0.05	(02)	i	0.00	(00)	i	0.37	(14)	-	12
HCrIII[2]	48	0.27	(13)	-	0.06	(03)	i	0.02	(01)	i	0.35	(17)	-	14
HCrIII[3]	40	0.25	(10)	-	0.00	(00)	-	0.03	(01)	i	0.28	(11)	-	11
URE (0,891 mg/mL)	40	3.83	(153)	+	0.75	(30)	+	0.10	(04)	i	4.68	(187)	+	149

HCrIII = Hydroxyapatite doped with Trivalent Chromium (Cr^3); URE = Urethane; HCrIII[1] = 16.66 mg/mL, HCrIII [2] = 8.33 mg/mL, HCrIII [3] = 4.16 mg/mL. [a]Statistical diagnoses according to Frei and Würgler (1988): +, positive; -, negative; i, inconclusive. m, multiplication factor for the evaluation of the significantly negative results. Significance levels $\alpha = \beta = 0{,}05$. [b]Including rare simple spots flr^3. [c]Considering mwh clones for mwh simple spots and for twin spots.

The trivalent chromium at high concentrations and long exposure is genotoxic (Çavaş and Ergene-Gözükara, 2005), although this compound has not shown genotoxicity in SMART (Graf and Singer, 1992).

The Cr^{+3} is an essential component for some biological activities, such as glucose metabolism and it does not show toxicity at low concentrations. However, Cr^{+3} compounds such as chromium chloride ($CrCl_3$) can be genotoxic *in vitro* leading to induction of micronuclei (MN) in human fibroblasts, according to study by Seoane and Dulout (2001). In a similar study with tannery workers exposed to Cr^{+3}, Meibian et al. (2008) demonstrated that this metal can cause DNA damage to human lymphocytes.

But, despite these studies, HCrIII showed no mutagenic activity under the experimental conditions used. Also, no work was found in the literature about HCrIII mutagenicity test.

Conclusions

In the experimental conditions used nanostructured hydroxyapatite both pure as doped with trivalent chromium (Cr^{+3}) at 1% mol, is not mutagenic nor with direct action nor with indirect action, dependent on metabolization. Moreover, hydroxyapatite doped with trivalent chromium showed similar results to those obtained from the analysis of mutagenicity of pure hydroxyapatite, so it is possible to conclude that, in this experiment, the presence of

chromium had no effect on its mutagenic potential. Nanostructured hydroxyapatite pure and doped with Cr^{+3}, based on experimental conditions used, showed results that support the safe use as raw material base for the manufacturing of products for human use, as sunscreen, for example.

Acknowledgments

We are grateful to the Brazilian funding agency CNPq and CAPES for financial support.

Conflicts of interest

Authors declare that they have no conflict of interests.

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