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Modulating effect of vitamin D3 on the mutagenicity and carcinogenicity of doxorubicin in *Drosophila melanogaster* and *in silico* studies

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ABSTRACT

Vitamin D3 (VD3) deficiency increases DNA damage, while supplementation may exert a pro-oxidant activity, prevent viral infections and formation of tumors. The aim of this study was to investigate the mutagenicity and carcinogenicity of VD3 alone or in combination with doxorubicin (DXR) using the Somatic Mutation and Recombination Test and the Epithelial Tumor Test, both in *Drosophila melanogaster*. For better understanding of the molecular interactions of VD3 and receptors, *in silico* analysis were performed with molecular docking associated with molecular dynamics. Findings revealed that VD3 alone did not increase the frequency of mutant spots, but reduced the frequency of mutant spots when co-administered with DXR. In addition, VD3 did not alter the recombinogenic effect of DXR in both ST and HB crosses. VD3 alone did not increase the total frequency of tumor, but significantly reduced the total frequency of tumor when co-administered with DXR. Molecular modeling and molecular dynamics between calcitriol and Ecdysone Receptor (EcR) showed a stable interaction, indicating the possibility of signal transduction between VD3 and EcR. In conclusion, under these experimental conditions, VD3 has modulatory effects on the mutagenicity and carcinogenicity induced by DXR in somatic cells of *D. melanogaster* and exhibited satisfactory interactions with the EcR.

1. Introduction

Cholecalciferol, also known as vitamin D3 (VD3), is a steroid hormone derived from cholesterol (secosteroid). VD3 is traditionally recognized as an important substance for maintaining serum calcium homeostasis and bone mineralization (Abdelghany et al., 2016). Calcium also exerts a reciprocal effect on the production of the pre-hormone calcidiol [25-hydroxyvitamin D - 25(OH)D_3] in the liver and calcitriol [1,25$(OH)_2D_3$] in the kidney, which is the biologically active form of vitamin D (VD) (Lucock et al., 2015; Jeon and Shin, 2018; Almaimani et al., 2019; El-Boshy et al., 2019). Woo and Eide (2010) report the need for a five to 30-min exposure to the midday sun, at least twice a week, for satisfactory VD3 synthesis.

In humans, VD3 is related to the development of therapies for autoimmune and chronic inflammatory diseases (Saul et al., 2019), besides to reducing the risk of viral infections (Gombart et al., 2020). Recent scientific studies have suggested that the adequate supplementation of VD may increase the resistance to the novel coronavirus (SARS-CoV-2), the causative agent of the Coronavirus Disease 2019 (COVID-19) (Wang et al., 2020). Therefore, VD supplementation might be a useful measure for reducing the risk of respiratory tract infections and for acting as one more therapeutic option for the treatment against this new virus (Grant et al., 2020; Zhang and Liu, 2020).

Prior studies supporting the role of VD in reducing risk of COVID-19

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indicate that the outbreak occurred in winter, when 25 (OH)D concentrations are low and, importantly, VD deficiency has been found to contribute to acute respiratory distress syndrome. In fact, VD levels have shown to be severely low in the aging population, especially in Spain, Italy and Switzerland, being the elderly the most vulnerable group of population for COVID-19. Therefore, it has been recommended that people at risk consider an intake of 10,000 IU/d of VD3 for a few weeks to rapidly raise 25 (OH)D levels (Grant et al., 2020; Ilie et al., 2020).

Mechanisms of signalling by VD3 are only possible through a highly specific VD nuclear receptor (VDR) (Grzesiak et al., 2019). Upon binding to calcitriol, the activated VDR recruits retinoid X receptor (RXR) and co-modulators for transcription of target genes, such as *cyp24a1* (Bunch et al., 2019). Thus, the [1,25$(OH)_2D_3$]-VDR complex is related to the control of gene expression, in addition to mediating pathologies, including breast cancer in humans (Huss et al., 2019).

According to previous studies, VDR expression is related to anti-tumor events in various tissues (Gharbaran et al., 2019; Shaker and Senousy, 2019; DeSantis et al., 2020). Supplementation with VD3 is necessary to activate the VDR pathway and thus prevent the formation of tumors, since, as reported in the literature, this vitamin has several anti-cancer mechanisms, such as: (i) induction of apoptosis, (ii) anti-proliferative effects, (iii) anti-inflammatory effects, (iv) stimulation of differentiation, (v) inhibition of angiogenesis and (vi) inhibition of invasion and metastasis (Fathi et al., 2019). Other researches, however, have reported that excessive VD3 supplementation may present potentially harmful effects on the body (Owens et al., 2017), including changes in the cell cycle regulatory pathways (Sakaki et al., 2014; Irving et al., 2015) and pro-oxidation (Koren et al., 2001; Halhali et al., 2010).

Among the different toxicological tests used to evaluate substances that can cause DNA damage and/or induce cancer, the wing Somatic Mutation and Recombination Test (SMART) and the Epithelial Tumor Test (ETT), both conducted in *Drosophila melanogaster*, have drawn considerable attention due to their sensitivity in assessing chemicals with mutagenic, recombinogenic and/or carcinogenic properties (Graf et al., 1984; Graf and van Schaik, 1992; Nepomuceno, 2015).

D. melanogaster has proven to be an excellent *in vivo* model organism for over a century. The major advantages of its use in research are the short life cycle, high offspring numbers and low costs for maintenance, being also an alternative model system to the use of vertebrates, since the fruit fly shares several basic biological, biochemical, neurological and physiological similarities with mammals. Furthermore, approximately 75% of genes related to human disease are conserved between humans and *Drosophila* (Pandey and Nichols, 2011; Abolaji et al., 2013; Koon and Chan, 2017).

The wing SMART allows to assess the potential of a chemical to induce loss of heterozygosity resulting from gene mutation, chromosomal rearrangement, chromosome breakage, or chromosome loss (Graf et al., 1984; De Andrade et al., 2003). Indeed, SMART has been successfully used to detect the mutagenic/recombinogenic as well as anti-mutagenic/antirecombinogenic properties of many chemical compounds (Orsolin et al., 2016; Oliveira et al., 2017, 2020; Naves et al., 2019).

The ETT allows to identify epithelial tumors induced by xenobiotic agents (Orsolin et al., 2012; Nepomuceno, 2015; Vasconcelos et al., 2017; Morais et al., 2017, 2018). The test uses a *D. melanogaster* strain containing the *wts* marker, which, when expressed in the wild type, acts as a tumor suppressor gene (Xu et al., 1995). The deletion of the wild-type gene *wts* and consequent expression of the mutant allele lead to the formation of highly invasive cell clones, hence resulting in the development of epithelial tumors in the body and appendages of adult flies (Nishiyama et al., 1999).

For conducting *in vivo* tests, it is essential to know whether the model organism has human orthologous receptors, which can be activated with the compound to be tested and, consequently, express a response in the model system. Therefore, *in silico* analyzes with molecular docking associated with molecular dynamics are recommended, in which, through computer simulation, it is possible to predict the best position and orientation of a ligand in another receptor molecule, an association that can be: (i) protein-peptide, (ii) protein-protein and (iii) protein-small molecule (Agrawal et al., 2019). In association with molecular docking, molecular dynamics (MD) is a tool that allows the simulation of the behavior of a molecular system; that is, MD leads to the understanding of ligand-receptor interactions with prediction of the intensity of stability and the consequent biological activity of this ligand-receptor system (Namba et al., 2008).

Given that VD3 has already been associated with events that result in modulation of genetic instability (Elhusseini et al., 2018; Fagundes et al., 2019), the aim of the present study was to evaluate the mutagenicity and carcinogenicity of VD3 when administered alone or its anti-mutagenicity and anticarcinogenicity when administered simultaneously with DXR, through SMART and ETT. Additionally, we performed *in silico* analysis with molecular docking and simulation of molecular dynamics between VD3 and receptors VDR and EcR.

2. Material and methods

2.1. Chemical agents

Vitamin D3 (VD3) (CAS 67-97-0) was obtained from Gemini Indústria de Insumos Farmacêuticos Ltda., Anápolis (GO), Brazil (Fig. 1A). Doxorubicin (DXR) (CAS 25316-40-9), commercially known as Adriblastina® RD, was manufactured and packaged by Activis Italy Sp - Nerviano (Milan, Italy) and imported by Pfizer Laboratório Ltda., São Paulo, Brazil (Fig. 1B).

2.2. Drosophila stocks

Three *D. melanogaster* strains were used to investigate the mutagenicity and recombinogenicity of VD3 when administered alone or its antimutagenicity and antirecombinogenicity when administered simultaneously with DXR: [1] multiple wing hairs (*mwh/mwh; y; mwh jv*, 3 (3–0.3)); [2] flare-3 (flr^3/*In*(3LR)TM3, *ri* p^p *sep l (3)89Aa* bx^{34e} and Bd^S); and [3] ORR; flare-3 (*ORR/ORR*; flr^3/*In*(3LR) TM3, *ri* p^p *sep l (3) 89Aa* bx^{34e} and Bd^S). Two *Drosophila* strains were used to investigate the carcinogenicity of VD3 when administered alone or its anti-carcinogenicity when administered simultaneously with DXR: [1] multiple wing hairs (*mwh/mwh; y; mwh jv*, 3 (3–0.3)) and [2] "warts" (*wts*/TM3; ST [1] *in* [1] *kni* [*ri*-1] *wts* [3–17]/TM3, Sb^1). These strains were kept in glass vials filled with a maintenance medium (i.e., agar-agar, banana, yeast, methylparaben, water and penicillin/streptomycin) in a Bio-Oxygen Demand-type (B.O.D.) chamber (Model: SL224, SOLAB – Equipamentos para Laboratórios Ltda., São Paulo, SP, Brazil) at 25 ± 1 °C, under 12 h light/12 h dark cycles of photoperiod.

2.3. Somatic Mutation and Recombination Test (SMART)

2.3.1. Crosses and treatments

Two crosses were carried out to produce the experimental larval progeny: (1) Standard (ST) cross: *mwh/mwh* males crossed with flare-3 virgin females (Graf et al., 1984, 1989); (2) High bioactivation (HB) cross: *mwh/mwh* males crossed with ORR; flare-3 virgin females (Graf and van Schaik, 1992). These crosses yielded two types of offspring: marked trans-heterozygous (MH) (*mwh* +/+ flr^3) flies with phenotypically wild-type wings and balanced heterozygous (BH) (*mwh*+/+TM3, Bd^S) flies with phenotypically serrated wings. These offspring are phenotypically distinct due to the marker TM3, Bd^S.

Eggs were collected over 8 h in vials containing a solid agar base (4% agar in water) and a layer of yeast (*Saccharomyces cerevisiae*) supplemented with sucrose. After 72 ± 4 h, third instar larvae were washed up with tap water and collected using a fine mesh sieve. We performed a pilot study to test the VD3 toxicity in the SMART. To calculate the survival rates upon exposure, larvae were counted before the distribution

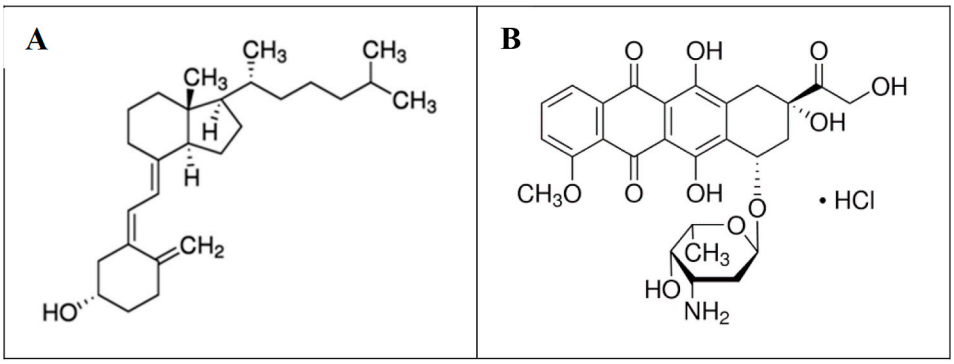

Fig. 1. Structural formula of (**A**) Vitamin D3; (**B**) Doxorubicin.

into glass tubes containing an alternative culture medium, prepared with instant potato puree Yoki® Alimentos S.A (Spanó et al., 2001). and different concentrations of VD3 alone (12.5; 25.0; 50.0 and 100.0 mM) dissolved in a mixture of 1% Tween 80 (Labsynth Produtos para Laboratório Ltda., Diadema, Brazil) and 3% ethanol (Neon Comercial Ltda., São Paulo, Brazil) or in association with DXR (0.4 mM) (for co-treatments). The survival tests were performed only once, without replicates. The hatched flies were counted and stored in 70% ethanol. Chi-squared test was used for statistical comparisons of the survival rate ratios for independent samples (De Rezende et al., 2013).

Therefore, for SMART the VD3 concentrations were based on survival assays in *D. melanogaster* (Fig. 2). Treatments were done in two independent experiments, with two replicates, considering four concentrations of VD3 alone (12.5; 25.0; 50.0 and 100.0 mM) and three concentrations of VD3 (12.5; 25.0 and 50.0 mM) in association with DXR (0.4 mM) (for co-treatments). Three controls were included: (1) negative control (ultrapure water); (2) solvent control (1% Tween 80 + 3% ethanol) and (3) positive control (doxorubicin - 0.4 mM DXR).

2.3.2. Analysis of flies

Emerging adult flies from the different treatments were collected and fixed in 70% ethanol. The wings were removed from the flies, soaked in Faure's solution (30 g of gum arabic, 20 mL of glycerol, 1.5 g of chloral hydrate and 5.0 mL of distilled water) and arranged on a dry slide. The slides were dried for 1 h on a hot plate (40 °C). Then, the slides were coverslipped and dried at room temperature.

Wings were examined on a microscope (Nikon Eclipse E200, 400 X) to record the number and types of spots (single or twin) as well as their size and position along the wing. Approximately 24,400 cells per wing were analyzed.

2.3.3. Statistical analysis

The wings of 40 flies from each treated series were scored, including controls. The data were evaluated according to the multiple-decision procedure of Frei and Würgler (1988, 1995), resulting in three different diagnoses: negative, positive or inconclusive. The frequency of each type of spot (small single, large single or twin) and the total frequency of spots per fly for each treatment were compared pair-wise, i.e., solvent control vs. VD3 alone; and positive control (DXR) alone vs. DXR plus VD3, following recommendations of Kastenbaum and Bowman (1970) with $p = 0.05$. All inconclusive and weak results were analyzed with the non-parametric *U* test of Mann, Whitney and Wilcoxon (a = b = 0.05, one sided) to exclude false positives (Frei and Würgler, 1995).

Modulating effects of VD3 on the mutagenicity and recombinogenicity of DXR were quantified by comparing the two genotypes (*mwh/flr*^{3}and *mwh*/TM3) and by applying the formulas: Recombination (R) = 1 - [(control corrected n/negative control in BH flies)/(control corrected n/negative control in MH flies)] x 100; Mutation (M) = 100 – R (Frei and Würgler, 1996).

Based on the control-corrected spot frequencies per 10^5 cells, the percentage of VD3 inhibition was calculated as: [DXE alone – (DXR + VD3)/DXR alone] x 100 (Abraham, 1994).

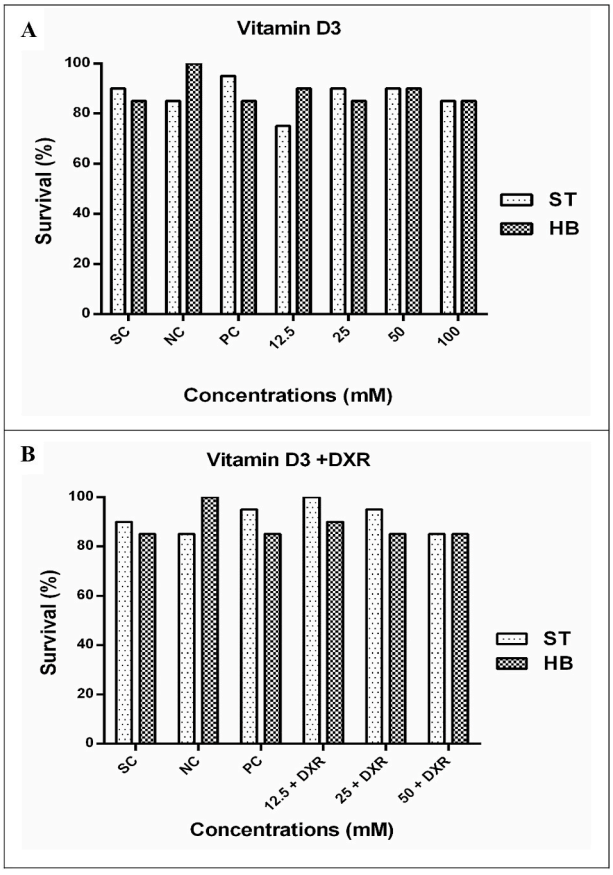

Fig. 2. Survival rates (%) of individuals from ST and HB crosses upon exposure to different concentrations of (**A**) VD3 (Vitamin D3 - mM) alone; (**B**) VD3 in combination with DXR (Doxorubicin - 0.4 mM). SC: Solvent control (1% tween 80 and 3% ethanol); NC: Negative control (ultrapure water); PC: Positive control (DXR - 0.4 mM). Data are representative of survival tests performed only once, without replica. Statistical comparisons were made by using Chi-square test for ratios for independent samples ($p > 0.05$).

2.4. Epithelial Tumor Test (ETT)

2.4.1. Cross and treatments

One cross was carried out to produce the experimental larval progeny: *mwh/mwh* males were crossed with *wts,*TM3, *Sb*^{1} virgin females (Nepomuceno, 2015). The wts strain was supplied by the Bloomington *Drosophila* Stock Center of the University of Indiana (USA), registered

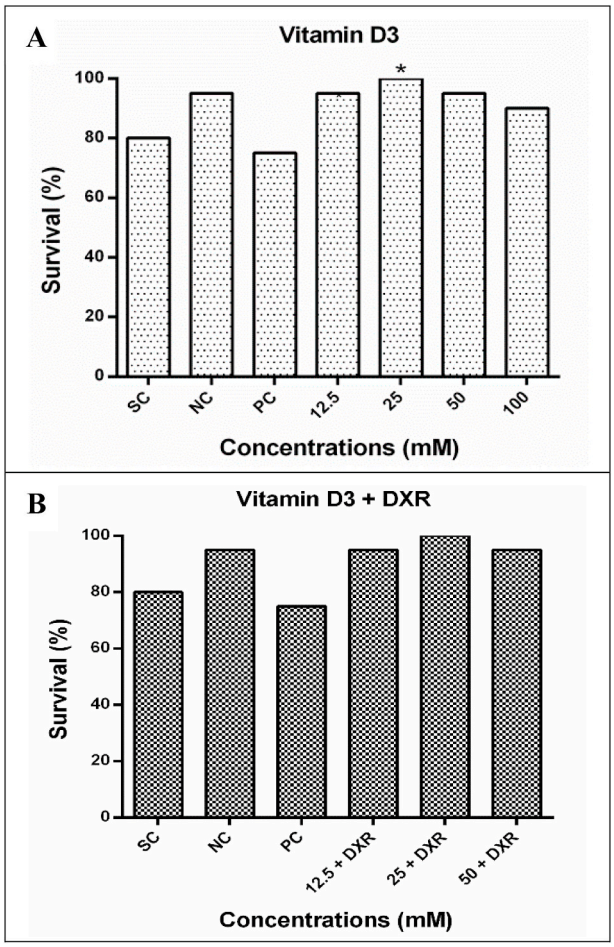

Fig. 3. Survival rates (%) of individuals from ETT upon exposure to different concentrations of (**A**) VD3 (Vitamin D3 - mM) alone; (**B**) VD3 in combination with DXR (Doxorubicin - 0.4 mM). SC: Solvent control (1% tween 80 and 3% ethanol); NC: Negative control (ultrapure water); PC: Positive control (DXR - 0.4 mM). Data are representative of survival tests performed only once, without replica. Statistical comparisons were made by using Chi-square test for ratios for independent samples ($p > 0.05$).

under the number Bloomington/7052.

Eggs were collected over 8 h in vials containing a solid agar base (4% agar in water) and a layer of yeast (*Saccharomyces cerevisiae*) supplemented with sucrose. After 72 ± 4 h, third instar larvae were washed with tap water and collected using a fine mesh sieve.

We performed a pilot study to test the VD3 toxicity in the ETT. In order to calculate the survival rates after exposure, larvae were counted before distribution in glass tubes containing an alternative culture medium rehydrated with the same concentrations of VD3 alone or in association with DXR, as described previously in the item 2.3.1 of SMART. These survival tests were performed only once. Chi-squared test was performed for statistical comparisons of the survival rate ratios for independent samples (De Rezende et al., 2013).

Therefore, for ETT the VD3 concentrations were based on survival assays in *D. melanogaster* (Fig. 3). Treatments were done in two independent experiments, with two replicates, with four concentrations of VD3 alone (12.5; 25.0; 50.0 and 100.0 mM) and three concentrations of VD3 (12.5; 25.0 and 50.0 mM) in association with DXR (0.4 mM) (for co-treatments). Three controls were included: (1) negative control (ultrapure water); (2) solvent control (1% Tween + 3% ethanol) and (3) positive control (doxorubicin - 0.4 mM DXR).

2.4.2. Analysis of flies

Emerging flies with long and thin hairs were analyzed because they were carriers of the *wts* gene and lacked the chromosome balancer (TM3, Sb^1). Individuals were transferred to concave slides containing glycerol and then examined on a stereoscopic microscope (Bel® Photonics) for visualization and tumour counting. The presence of tumors was evaluated and recorded on a standard spreadsheet.

2.4.3. Statistical analysis

Statistical differences between tumor frequencies in the experimental (at the concentrations tested) and control groups were calculated using the non-parametric Mann-Whitney *U* test at a significance level $\alpha = 0.05$.

2.5. In silico analysis

2.5.1. Molecular modeling and docking

The Ecdysone Receptor Protein (ECR) of *D. melanogaster* was modeled using homology modeling by the program Modeller (Webb and Sali, 2017). The human Vitamin D3 Receptor (VDR) protein was used as template (PDBid: 3B0T). During the modeling, 1000 structures were generated. Among all, the best quality structure was selected after evaluation by Dope (Shen and Sali, 2006), Verify3D (Eisenberg et al., 1997), Ramachandran (Ramachandran et al., 1963) and ERRAT (Colovos and Yeates, 1993) programs.

Both VDR and ECR were subjected to docking simulation studies with calcitriol (active vitamin D) through the program GOLD (Jones et al., 1997) using the parameters predefined by the program, except for the flexibility of the ligand, which was defined as 200%. Each docking was performed 50 times and the best docking positions were assessed based on a ranking of the ChemPLP scoring function. Then, a 2D plot of the protein-ligand interactions was performed.

2.5.2. Molecular dynamics simulation

The best calcitriol poses for VDR and ECR from molecular docking were submitted to a receptor-ligand molecular dynamics (MD) simulation using GROMACS (Abraham et al., 2015). The ligand topology parameters were generated by SwissParam (Zoete et al., 2011) using the CHARMM force field.

The protein-ligand complex MD were performed on GROMACS (Abraham et al., 2015) using TIP3P as water model. The unit cell was defined as triclinic shape and water and ions were added. After energy minimization, an equilibrium phase was carried out using NPT and NVT conditions. The production phase was conducted by a 20 ns. The trajectories were analyzed by means of protein root mean square deviation (RMSD), H bond number and binding energy between protein and ligand.

3. Results

3.1. Somatic Mutation and Recombination Test - SMART

The wing SMART of *D. melanogaster* was performed to assess the mutagenic and recombinogenic potential of VD3 and its possible modulating effects on DNA damage induced by doxorubicin (DXR).

The different concentrations of VD3 used alone or in combination with DXR were selected based on survival assays with *Drosophila*. The survival rates (%) of individuals from ST and HB crosses are depicted in Fig. 2. Therefore, these survival data validated the use of four concentrations (12.5; 25.0; 50.0 and 100.0 mM) of VD3 alone, and three concentrations of VD3 (12.5; 25.0 and 50.0 mM) in combination with DXR, which were tested in two independent experiments. The data were pooled after verifying that there were no significant differences between repetitions.

The results for the MH and BH descendants, derived from the Standard Cross (ST), treated with different concentrations of VD3 alone or in

Table 1
Summary of results obtained with the *Drosophila melanogaster* wing Somatic Mutation and Recombination Test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the standard (ST) cross after chronic treatment of larvae with different concentrations of vitamin D3 (VD3 - mM), ultrapure water (negative control), solvent control and doxorubicin 0.4 mM (DXR - positive control).

Genotypes and Treatments (mM)	Number of flies	Spots per fly (number of spots); statistical diagnoses[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per division[d]		Recombination (%)	Inhibition[e] (%)
		Small single spots (1–2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
mwh/flr³										
Negative control	40	0.95 (38)	0.03 (1)	0.00 (0)	0.98 (39)	37	1.90			
Solvent control	40	0.58 (23) -	0.18 (7) +	0.03 (1) i	0.78 (31) -	28	1.43	−0.47		
VD3 12.5	40	0.85 (34) i	0.05 (2) -	0.05 (2) i	0.95 (38) -	37	1.90	0.47		
VD3 25.0	40	0.80 (32) i	0.10 (4) -	0.00 (0) i	0.90 (36) -	36	1.84	0.41		
VD3 50 .0	40	0.60 (24) -	0.05 (2) -	0.00 (0) i	0.65 (26) -	26	1.33	−0.10		
VD3 100.0	40	0.58 (23) -	0.05 (2) -	0.03 (1) i	0.65 (26) -	25	1.28	−0.15		
DXR 0.4	40	6.20 (248) +	8.60 (344) +	6.95 (278) +	21.75 (870) +	824	42.21	40.78	96.62	
VD3 12.5 + DXR 0.4	40	5.80 (232)	4.28 (171) *	4.20 (168) *	14.28 (571) *	555	28.43	27.00	99.26	33.79
VD3 25.0 + DXR 0.4	40	2.70 (108) *	2.88 (115) *	2.55 (102) *	8.13 (325) *	310	15.88	14.45	97.85	64.57
VD3 50.0 + DXR 0.4	40	2.93 (117) *	1.80 (72) *	1.45 (58) *	6.18 (247) *	235	12.04	10.61	94.15	73.98
***mwh*/TM3**										
Negative control	40	0.58 (23)	0.03 (1)	f	0.60 (24)	24	1.23			
DXR 0.4	40	1.03 (41) +	0.25 (10) +		1.28 (51) +	51	2.61	1.38		
VD3 12.5 + DXR 0.4	40	0.40 (16) *	0.10 (4)		0.50 (20) *	20	1.02	−0.20		
VD3 25.0 + DXR 0.4	40	0.33 (13) *	0.13 (5)		0.45 (18) *	18	0.92	−0.31		
VD3 50.0 + DXR 0.4	40	0.25 (10) *	0.05 (2) *		0.30 (12) *	12	0.61	−0.62		

Marker-trans-heterozygous flies (*mwh/flr³*) and balancer-heterozygous flies (*mwh*/TM3) were evaluated.
[f] Balancer chromosome TM3 does not carry the *flr³* mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.
[a] Statistical diagnose according to Frei and Würgler (1988, 1995). *U* test, two sided; probability levels: -, negative; +, positive; i, inconclusive; $p < 0.05$ DXR vs. negative control; VD3 vs. solvent control; *, positive; $p \leq 0.05$ VD3 + DXR vs. DXR (0.4 mM) only.
[b] Including rare *flr³* single spots.
[c] Considering mwh clones from mwh single and twin spots.
[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).
[e] Calculated as {[DXR alone – DXR + VD3]/DXR} X 100, according to Abraham (1994).

combination with DXR are shown in Table 1. In the MH individuals, VD3 alone did not exhibit any mutagenicity at the doses used. DXR treatment, as expected, induced positive results for all classes of spots (small single, large single and twin spots) when compared to the negative control ($p < 0.05$). The simultaneous administration of VD3 (12.5; 25.0 or 50.0 mM) with DXR (0.4 mM) inhibited significantly ($p < 0.05$) the number of DXR-induced mutant spots (33.79, 64.57 and 73.98%, respectively) in comparison to DXR alone. Due to the significant reduction observed in flies simultaneously treated with VD3 plus DXR, the wings of the BH descendants resulting from these treatments were also scored. Based on the clone induction frequency per 10^5 cells, we compared the number of observed spots in the MH and BH individuals and quantified the contribution (%) of mutation and recombination to the total number of observed spots (Frei and Würgler, 1996). The observed frequency of recombination was higher than 94% for all treatments.

The results of the HB cross are summarized in Table 2. The findings obtained with the MH individuals treated with VD3 alone were negative at all tested concentrations when compared to the solvent control. DXR statistically increased ($p < 0.05$) all categories of spots when compared to the negative control. The recombinogenic activity was the major response to DXR-induced DNA damage (98.94%). When administered with DXR, all concentrations of VD3 (12.5; 25.0 or 50.0 mM) were found to significantly decrease the number of spots ($p < 0.05$) induced by DXR. The inhibition rate was, respectively, 45.25; 49.52 and 50.27%. By comparing the number of observed spots in the MH and BH individuals, we found that the induced spots were mainly due to recombination (respectively 95.13; 98.10 and 97.44%).

3.2. Epithelial Tumor Test - ETT

The ETT of *D. melanogaster* was performed to assess the carcinogenic potential of VD3 or its anticarcinogenic potential when associated to doxorubicin (DXR). The different concentrations of VD3 used alone or in combination with DXR were selected based on survival assays with *Drosophila*. The survival rates (%) are depicted in Fig. 3.

Table 3 shows the frequency of tumors found in the different segments of the body of *D. melanogaster* treated with different concentrations of VD3 alone or in combination with DXR.

Table 2
Summary of results obtained with the *Drosophila melanogaster* wing Somatic Mutation and Recombination Test (SMART) in the marker-heterozygous (MH) and balancer-heterozygous (BH) progeny of the high bioactivation (HB) cross after chronic treatment of larvae with different concentrations of vitamin D3 (VD3 - mM), ultrapure water (negative control), solvent control and doxorubicin 0.4 mM (DXR - positive control).

Genotypes and Treatments (mM)	Number of flies	Spots per fly (number of spots); statistical diagnoses[a]				Spots with *mwh* clone[c]	Frequency of clone formation/10^5 cells per division[d]		Recombination (%)	Inhibition[e] (%)
		Small single spots (1–2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
mwh/flr³										
Negative control	40	1.10 (44)	0.15 (6)	0.08 (3)	1.33 (53)	49	2.51			
Solvent control	40	1.15 (46) i	0.08 (3) i	0.13 (5) i	1.35 (54) -	51	2.61	0.10		
VD3 12.5	40	1.25 (50) -	0.15 (6) i	0.08 (3) i	1.48 (59) -	58	2.97	0.36		
VD3 25.0	40	1.10 (44) -	0.18 (7) i	0.05 (2) -	1.33 (53) -	53	2.72	0.10		
VD3 50 .0	40	0.93 (37) -	0.08 (3) i	0.03 (1) -	1.03 (41) -	41	2.10	−0.51		
VD3 100.0	40	0.75 (30) -	0.15 (6) i	0.10 (4) i	1.00 (40) -	40	2.05	−0.56		
DXR 0.4	40	6.85 (274) +	10.20 (408) +	8.95 (358) +	26.00 (1040) +	988	50.61	48.00	98.94	
VD3 12.5 + DXR 0.4	40	5.40 (216) f+	4.73 (189) +	4.60 (184) +	14.73 (589) f+	564	28.89	26.28	95.13	45.25
VD3 25.0 + DXR 0.4	40	4.25 (170) f+	4.80 (192) +	4.65 (186) +	13.70 (548) +	524	26.84	24.23	98.10	49.52
VD3 50.0 + DXR 0.4	40	3.73 (149) +	4.90 (196) +	4.88 (195) +	13.50 (540) +	517	26.49	23.87	97.44	50.27
***mwh*/TM3**										
Negative control	40	0.95 (38)	0.15 (6)	f	1.10 (44)	44	2.25			
DXR 0.4	40	0.90 (36) -	0.45 (18) +		1.35 (54) -	54	2.77	0.51		
VD3 12.5 + DXR 0.4	40	1.48 (59) +	0.25 (10) i		1.73 (69) -	69	3.53	1.28		
VD3 25.0 + DXR 0.4	40	1.00 (40) -	0.33 (13) i		1.33 (53) -	53	2.72	0.46		
VD3 50.0 + DXR 0.4	40	1.18 (47) -	0.23 (9) i		1.40 (56) -	56	2.87	0.61		

Marker-trans-heterozygous flies (*mwh/flr³*) and balancer-heterozygous flies (*mwh*/TM3) were evaluated.
[f] Balancer chromosome TM3 does not carry the *flr³* mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.
[a] Statistical diagnose according to Frei and Würgler (1988, 1995). *U* test, two sided; probability levels: , negative; +, positive; i, inconclusive; $p < 0.05$ DXR vs. negative control; VD3 vs. solvent control; *, positive; $p \leq 0.05$ VD3 + DXR vs. DXR (0.4 mM) only.
[b] Including rare *flr³* single spots.
[c] Considering mwh clones from mwh single and twin spots.
[d] Frequency of clone formation: clones/flies/48,800 cells (without size correction).
[e] Calculated as {[DXR alone – DXR + VD3]/DXR} X 100, according to Abraham (1994).

Table 3
Summary of results obtained with the *Drosophila melanogaster* Epithelial Tumor Test (ETT) after chronic treatment of larvae with different concentrations of vitamin D3 (VD3 - mM), ultrapure water (negative control), solvent control and doxorubicin 0.4 mM (DXR - positive control).

Treatment mM	Number of flies	Frequency of tumors analyzed (total of tumors)						Frequency of tumor	Reduction (%)
		Eyes	Head	Wings	Body	Legs	Halters		
Negative control	200	0.000 (00)	0.085 (17)	0.040 (08)	0.110 (22)	0.025 (05)	0.000 (00)	0.260 (52)	
Solvent control	200	0.000 (00)	0.070 (14)	0.055 (11)	0.150 (30)	0.055 (11)	0.000 (00)	0.330 (66)	
Positive control	200	0.045 (09)	0.130 (26)	1.390 (278)	0.770 (154)	0.570 (114)	0.085 (17)	2.990 (598)*	
VD3 12.5	200	0.000 (00)	0.050 (10)	0.030 (06)	0.080 (16)	0.045 (09)	0.000 (00)	0.205 (41)	
VD3 25.0	200	0.005 (01)	0.085 (17)	0.055 (11)	0.110 (22)	0.030 (06)	0.005 (01)	0.290 (58)	
VD3 50.0	200	0.000 (00)	0.050 (10)	0.030 (06)	0.130 (26)	0.010 (02)	0.015 (03)	0.235 (47)	
VD3 100.0	200	0.000 (00)	0.085 (17)	0.050 (10)	0.190 (38)	0.080 (18)	0.000 (00)	0.405 (81)	
VD3 12.5 + DXR 0.4	200	0.015 (03)	0.055 (11)	0.305 (61)	0.435 (87)	0.210 (42)	0.045 (09)	1.065 (213)**	64.4
VD3 25.0 + DXR 0.4	200	0.030 (06)	0.050 (10)	0.210 (42)	0.425 (85)	0.145 (29)	0.015 (03)	0.875 (175)**	70.7
VD3 50.0 + DXR 0.4	200	0.010 (02)	0.035 (07)	0.140 (28)	0.290 (58)	0.140 (28)	0.005 (01)	0.620 (124)**	79.3

Statistical diagnosis according to the Mann and Whitney Test. *, Different from the negative control. Level of significance $p \leq 0.05$. **, Different from the positive control. Level of significance $p \leq 0.05$.

The total frequency of tumors observed in the heterozygote descendants of *D. melanogaster* treated with different concentrations of VD3 (12.5; 25.0; 50.0 or 100.0 mM) was not statistically significant ($p >$ 0.05) when compared to the frequencies observed in the solvent control. Thus, the results reported no carcinogenic potential of VD3 in the concentrations used, in *D. melanogaster*. The frequency of tumors observed

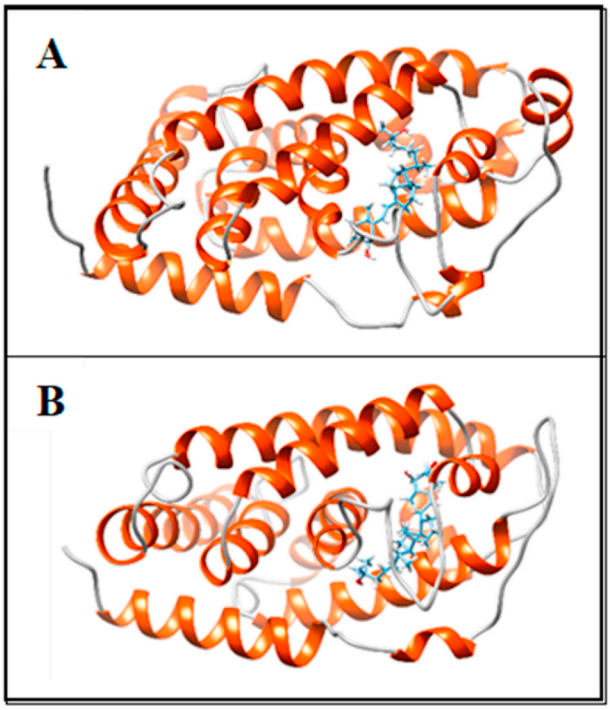

Fig. 4. Molecular modeling and docking between: (**A**) calcitriol (active vitamin D) and its VDR receptor; (**B**) calcitriol and the ecdysone receptor.

in those treated with DXR (0.4 mM) was statistically significant ($p < 0.05$) when compared to the frequencies in the negative control. On the other hand, the frequencies of tumors observed in the fruit flies treated with VD3 (12.5; 25.0 or 50.0 mM) in combination with DXR were significantly different ($p < 0.05$) from the frequencies observed in those treated with DXR alone. Table 3 also demonstrates that the reduction occurs in the number of tumors, in a dose dependent manner. Therefore, these results revealed that VD3 has modulatory effects on the carcinogenicity induced by DXR.

3.3. *In silico* analysis

Fig. 4 indicates the result of molecular modeling and docking between calcitriol (active vitamin D) and its VDR receptor (Fig. 4A) and also between calcitriol and the ecdysone receptor (EcR) (Fig. 4B). Accordingly, the calcitriol in the EcR protein occupies the binding site in an inverted form compared to the VDR protein.

Fig. 5 illustrates a 2D plot of the ligand interactions with receptor proteins, in this case, between calcitriol and VDR (Fig. 5A) and also between calcitriol and EcR (Fig. 5B). In this plot, it is possible to visualize, in pink, the hydrophobic interactions; in green, hydrogen bonds and in red, unfavorable interactions. Despite the unfavorable interaction detected in the interaction between calcitriol and EcR, the diagrams evidence the presence of hydrogen bonds and hydrophobic interactions that are essential for stabilization of calcitriol in the binding site.

Fig. 6 shows the simulation of the molecular dynamics between calcitriol and VDR and EcR receptor proteins. Fig. 6A reveals the stability of the proteins, being noticeable that, for both, there is not much variation, mainly in the second half of the simulation, thus indicating that the proteins are in equilibrium with the system. Fig. 6B exhibits the hydrogen bonds made by calcitriol and proteins, with a result considered satisfactory, because during the 20 ns of simulation these bonds increased, demonstrating higher stability between calcitriol and receptors. Fig. 6C reports the measurement of the binding energy between the ligand and the protein; in both cases, there was a downward trend, highlighting an increase in the stability of both protein-ligand complexes.

In silico analysis indicate that, as with VDR, the calcitriol and EcR binding, although being in an inverted position when compared to the first one, revealed strong evidence of its stability with protein, as verified by docking and MD analysis. These considerations, in accordance with experimental data, confirm the possibility, in *D. melanogaster*, of signal transduction between the active form of VD3 (calcitriol) and the ecdysone receptor, enabling the occurrence of the different events attributed to this receptor at distinct stages of the life cycle in this model organism.

4. Discussion

Our results demonstrated that vitamin D3 (VD3) is not mutagenic neither carcinogenic and displays antimutagenic and anticarcinogenic effects when co-administered with doxorubicin (DXR) on *Drosophila* through the Somatic Mutation and Recombination Test (SMART) and the Epithelial Tumor Test (ETT). Molecular modeling and molecular dynamics between VD3 and ecdysone receptor (EcR) showed a stable interaction, indicating the possibility of signal transduction between VD3 and EcR.

In order to carry out this work, VD3 was diluted in Tween 80 and ethanol in ultrapure water. Tween 80, also known as polysorbate 80, is a ubiquitously used solubilizing agent with hydrophilic characteristic, which enables the formation of hydrogen bonds with the water molecule. Besides that, the presence of an extensive carbon chain ($C_{64}H_{124}O_{26}$) allows solubility in nonpolar compounds. The emulsifying property of Tween 80 enables a solution between VD3 (nonpolar) and water (polar) (Feng et al., 2006; Perazzo et al., 2012). Thus, the findings observed in fruit flies treated with VD3 in both tests (SMART and ETT) were compared with the results obtained in the solvent control (1% Tween 80 and 3% ethanol in water). On the other hand, DXR was dissolved in ultrapure water. The results observed in flies treated with DXR in both tests (SMART and ETT) were compared with the negative control (ultrapure water).

In the SMART, VD3 alone or in association with DXR was tested in two independent experiments with two replicates. The data were pooled after verifying that there were no significant differences between repetitions.

The results observed with VD3 alone, in both crosses (ST and HB) of the SMART, were rather similar. VD3 itself did not show genotoxicity at the doses used. Prior studies found in the literature reported the lack of mutagenicity of VD3 in different organisms and in different test systems. Remarkably, SMD-502, a VD3 analog, was not mutagenic in skin and liver of *gpt* delta transgenic mice and in GDL1 cells (Takeiri et al., 2012). Moreover, there was no overall relationship between 25(OH)D and DNA damage in human lymphocytes (Nair-Shalliker et al., 2012); and VD3 supplementation reduced oxidative stress and DNA damage in patients with type 2 diabetes (Fagundes et al., 2019). In turn, no association between VD and oxidation-induced DNA damage was observed in peripheral lymphocytes of young (18–26 years) adults, but VD deficiency was highly prevalent in the young adults studied, and the authors could not rule out an ameliorative effect of correction of VD deficit on DNA damage (Wang et al., 2016).

The positive control DXR, as expected, induced high frequencies of all types of mutant spots in both ST and HB crosses. Comparison of the frequencies of wing spots in the MH flies (*mwh/flr*^{3} genotype) and BH flies (*mwh*/TM3 genotype) from both ST an HB crosses indicated that induced recombination was the major response for the treatments with DXR alone. These findings were further supported by previous investigations with DXR in *Drosophila* wing SMART (Valadares et al., 2008; De Rezende et al., 2009; Orsolin et al., 2016; Silva-Oliveira et al., 2016; Oliveira et al., 2017).

DXR has different mechanisms that promote the onset of DNA damage, such as the binding and inhibition of the enzyme topoisomerase II, a DNA gyrase with high activity in proliferative cells (Kaiserová et al.,

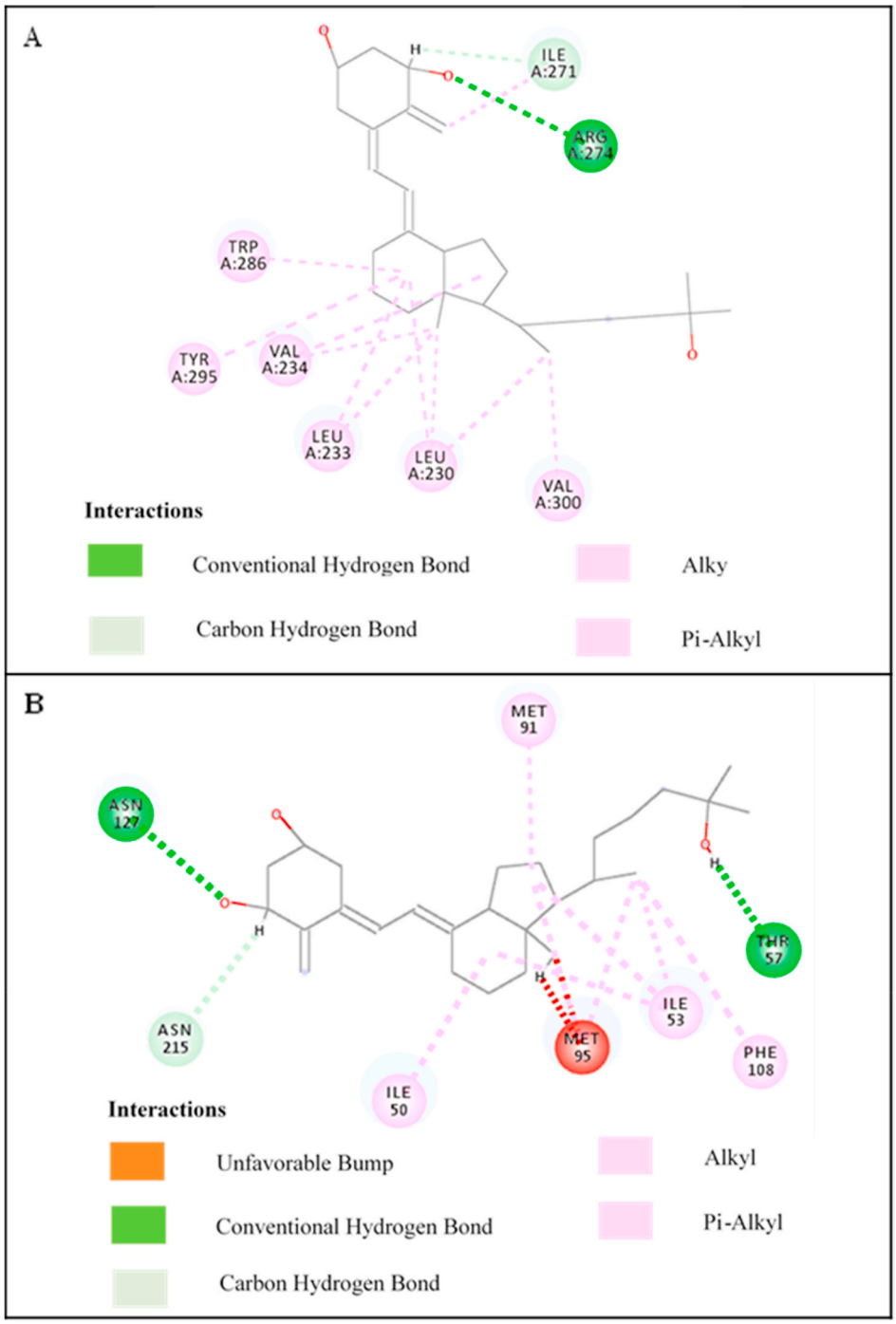

Fig. 5. 2D plotting of ligand-protein interactions: (**A**) Calcitriol and VDR receptor; (**B**) Calcitriol and the ecdysone receptor. Hydrophobic interactions (pink); hydrogen bonds (green) and unfavorable interactions (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2006; Marinello et al., 2018). Furthermore, the inhibition of the anti-cancer drug leads to genetic instability and causes reductive biotransformation of the quinone ring, yielding a semiquinone radical, which has a direct toxic effect or undergoes redox reactions (Ramji et al., 2003). DXR also contributes to reactive oxygen species (ROS) production, conferring secondary cytotoxicity (Gewirtz, 1999; Doroshow, 2019). In fact, Mokhtari et al. (2017) reported the pivotal role of VD3 in suppressing the NADPH oxidase enzyme complex, which acts in the formation of ROS.

The modulation of VD3 on DXR-induced mutant spots in *Drosophila* was also evaluated. Regardingly, VD3 was able to reduce the total frequency of mutant spots induced by DXR in all concentrations in both (ST and HB) crosses. In addition, VD3 was effective in reducing the mutagenic effect of DXR, but did not interfere on the recombinogenic effects of DXR. Ours findings reinforce the protective effects of VD3 on the damage generated by DXR directly and also after its metabolization.

Although previous studies have demonstrated that VD administration reportedly has lowered DNA damage in type 2 diabetic mice, and higher DNA damage was reported in mononuclear cells of severely asthmatic patients who were VD deficient (Wang et al., 2016), the current literature has shown controversial effects about the ability of VD3 to prevent or ameliorate oxidative stress biomarkers (Tagliaferri

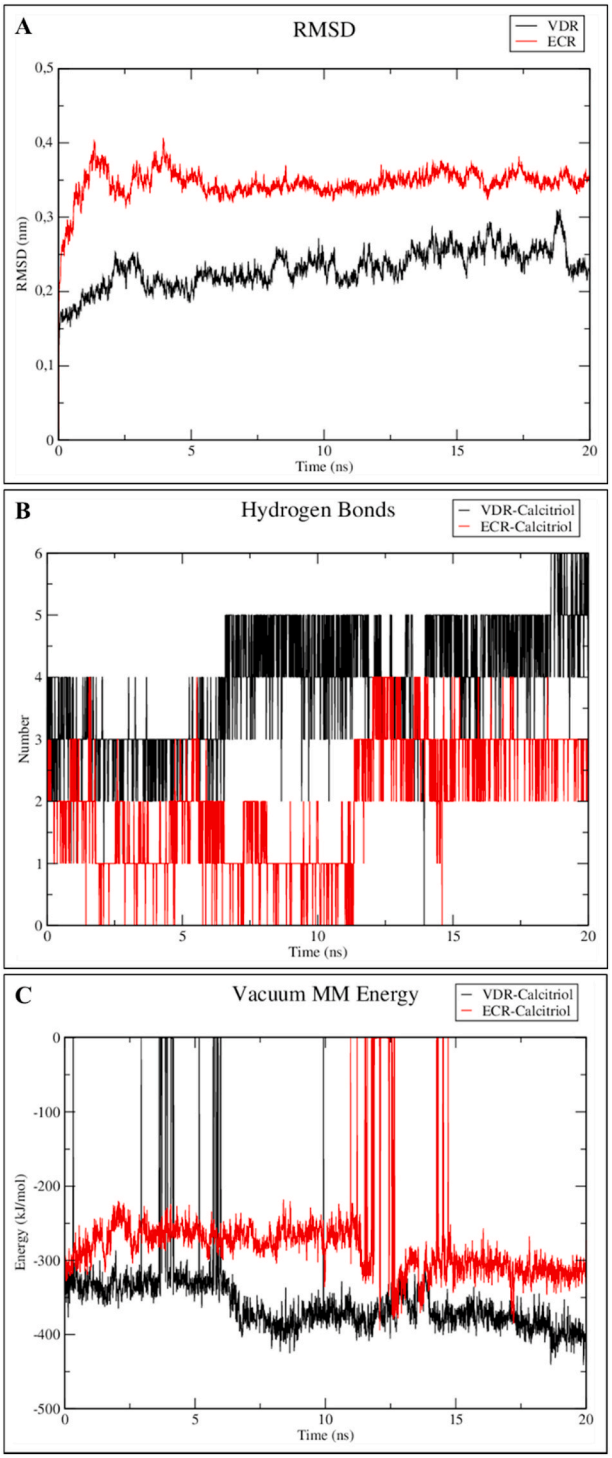

Fig. 6. Simulation of molecular dynamics between calcitriol and proteins. (**A**) Protein stability, VDR and EcR; (**B**) Hydrogen bonds between calcitriol and proteins; (**C**) Energy between binder and proteins.

et al., 2019). The protection against DXR-induced mutagenicity observed in the present research may be due to inhibition of free radicals and increased antioxidant status. Thus, further studies are needed to elucidate the antioxidant effect of VD supplementation.

In the ETT, VD3 alone or in association with DXR was tested in two independent experiments. The data were pooled after verifying that there were no significant differences between repetitions. The results observed with VD3 alone did not show carcinogenicity at the doses used, and the vitamin was able to significantly reduce the frequency of tumors induced by DXR in all concentrations analyzed. Pawlowska et al. (2016) described that the active form of VD3 removes DNA damage induced by ultraviolet (UV) radiation in precancerous cells via nucleotide excision repair (NER), which reduces the nitrosylation process of DNA repair enzymes.

Furthermore, prior studies demonstrated that VD3 may present oncoprotective properties through regulation of growth factor, cytokine synthesis and signaling, modulation of inflammation, cell proliferation and differentiation, angiogenesis, invasive and metastatic potential, apoptosis, miRNA expression regulation and modulation of the Hedgehog signaling pathway (Harris and Go, 2004; Merchan et al., 2017). A literature review offers an up-to-date analysis of VD and VDR roles in carcinogenesis (Merchan et al., 2017).

VD, as a prohormone, undergoes two-step metabolism in liver and kidney to produce a biologically active metabolite, calcitriol (1,25-dihidroxicholecalciferol [1,25$(OH)_2D_3$]), which binds to the VD receptor (VDR) for the regulation of expression of diverse genes (Jeon and Shin, 2018; Almaimani et al., 2019; El-Boshy et al., 2019).

Calcitriol is synthesized by a steroid precursor, cholecalciferol or pre-VD3, recognized as a molecule similar to the cell membrane antioxidants, which is bioactivated through two steps under the action of the *cyp24a1* gene, thereby resulting in the formation of calcidiol (25(OH)D_3) and calcitriol (Wheeler and Nijhout, 2003; Sakaki et al., 2014). The *cyp24a1* gene, found in humans, has an ortholog in *D. melanogaster*, the *cyp12b2* (NCBI, 2020).

In vertebrates, it has been described the existence of a nuclear VDR with the intrinsic ability to be activated by calcitriol. Apart from this active metabolite, VDR is heterodimerized by the retinoid X receptor (RXR) and its ultraspiracle homolog (USP), found in *D. melanogaster* (Yao et al., 1992). Dela Cruz et al. (2000) relate a remarkable activation of VDR when associated with USP, with no requirement that this receptor always be linked to the active metabolite of VD3. Laudet et al. (1992) mentioned a close evolutionary relationship of VDR and the Ecdysone Receptor (ECR) found in *D. melanogaster*, owing to the similarity in the ligand binding domain present in these two receptors. ECR has three isoforms that, when activated by USP and the steroid hormone 20-hydroxyecdysone (20E), induce cell proliferation during the larval period and maturation during the pupal period (Dela Cruz et al., 2000; Delanoue et al., 2010). Signaling by the ECR complex for cell proliferation occurs until the end of the larval period, due to the gradual increase of 20E and, upon reaching ecdysone peak, novel genes will be activated for the purpose of maturation during the pupal period (Delanoue et al., 2010; Tsao et al., 2016).

In the present study, for better understanding of the molecular interactions of VD3 and receptors, we performed *in silico* analysis with molecular docking and molecular dynamics, which allowed us to verify a stable interaction between calcitriol and EcR. Thus, when VD3 was tested at ETT, it was verified that the larval and pupal periods occurred in regular time, which can be explained by the favorable interaction between calcitriol and EcR. In this sense, we suggest that the VD3 active metabolite, at the end of the larval period, stopped inducing cell proliferation, as previously described by Sakaki et al. (2014), hence inhibiting the carcinogenesis process.

5. Conclusion

The results obtained in this study allow us to conclude that, under the experimental conditions, VD3 is not toxic, mutagenic neither carcinogenic and has modulatory effects on the mutagenicity and carcinogenicity induced by DXR in *D. melanogaster*. *In silico* analysis with molecular modeling and molecular dynamics between calcitriol and Ecdysone Receptor (EcR) showed a stable interaction, indicating the possibility of signal transduction between VD3 and EcR. In this context, based on literature data, our findings suggest that the modulatory effects of VD3 can be explained by its antioxidant and apoptotic properties.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mirley Alves Vasconcelos: Conceptualization, Data curation, Formal analysis, Writing - original draft. **Priscila Capelari Orsolin:** Writing - original draft. **Victor Constante Oliveira:** Writing - original draft. **Paula Marynella Alves Pereira Lima:** Writing - original draft. **Maria Paula Carvalho Naves:** Conceptualization, Data curation, Formal analysis, Writing - original draft. **Nilson Nicolau-Júnior:** Conceptualization, Data curation, Formal analysis, Writing - original draft. **Ana Maria Bonetti:** Writing - original draft. **Mário Antônio Spanó:** Writing - original draft.

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