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Research Article

The Effects of 4-Vinylcyclohexene Diepoxide on Gene Expression in Dog Testes

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ABSTRACT

In this study, the effects of 4-Vinylcyclohexene diepoxide (VCD) on the testicular gene expression in dogs were investigated. Eighteen male dogs were used as material and were randomly allocated to 3 groups. The first group (n=6) was administered sesame oil by intraperitoneal (i.p.) route once a day for 8 days, and this group was served as a control. The second group (n=6) received 80 mg/kg VCD and the third (n=6) group was administered VCD (i.p.) at a dose of 320 mg/kg once daily for 8 days. The testes were removed on following day the last VCD administration. The testes were immediately placed in the RNA stabilization solution and stored at -20°C. Later mRNAs were obtained from tissue samples. After RNA isolation, the cDNAs were synthesized. Finally, MasterMix was prepared and gene expression levels were examined at the real-time PCR. The expression of B cell lymphoma 2 (Bcl-2), tumor suppressor protein (tp53), Bax, CASP2, CASP3, CASP8, CASP9 and β-actin were investigated in the all groups. Cross-group comparisons were made by using student t test. The tp53 gene expression in the second group was found to be higher compared to the control group. It was determined that the expression of Bcl-2 was reduced in the testes of animals treated with high-dose VCD. CASP-3 was found to be more expressed and CASP-9 was down-regulated in the third group. In addition, CASP-8 gene expression was significantly reduced in both treatment groups. As a result, it can be concluded that expression changes in the tp53, Bcl-2, CASP-3, CASP-8 and CASP-9 genes in the testes of male dogs may cause apoptotic changes with the VCD application.

Key words: 4-Vinylcyclohexene diepoxide, Apoptosis, Dog, Testis

INTRODUCTION

Free-roaming domestic dog population is more than 700 million in the world (Massei and Miller 2013). It is necessary to limit the breeding of these animals because of some serious problems, such as giving birth to many puppies, carrying important zoonotic diseases, exhibiting aggressive behaviors, and attacking humans and other animals. For these reasons, different methods have been tried to keep the number of stray dogs under control. Surgical castration is usually performed for permanent sterilization (Kutzler and Wood, 2006). However, complication after surgery is an important problem (Hamilton *et al*., 2014). Therefore, applications with long-lasting and minimal side-effects are becoming important (Wiebe and Howard, 2009; Goericke-Pesch, 2010; Aydın and Abay, 2013). Today, hormone use, immuno-contraceptive vaccines and chemical agents are among the most commonly used sterilization methods in male animals (Kutzler and Wood, 2006; Jana and Samanta, 2007; Massei and Miller, 2013).

The chemical sterilization process is carried out using various substances such as cadmium chloride, chlorhexidine, danazol and zinc, which cause sclerosing or toxic effects (Dixit, 1977; Jana and Samanta, 2007; Kutzler and Wood, 2006; Mogheiseh *et al*., 2017).

4-Vinylcyclohexene diepoxide (VCD), which is a candidate for entry into these agents, has come to the forefront with recent studies. VCD is a metabolite of 4-Vinylcyclohexene (VCH). VCH is a chemical that is produced in the production of materials such as rubber, plastics and pesticides. VCH is converted into epoxide derivatives by metabolism in the body (Bhattacharya and Keating, 2011). VCD selectively shows toxic effects on ovaries (Van kempen *et al*., 2011). Studies investigating the effect of VCD on male animals are also increasing. However, this substance does not have the same effect on the all species. In a study on mice and rats, ovaries and testes were affected in mice, but not in rats (Chhabra 1990a). But, recent studies were performed on rats and the effects of VCD on the testes were investigated (Adedera *et al*., 2016; Abolaji *et al*., 2016). However, there is no study investigating the effect of VCD on gene expression on dog testes. Therefore, the aim of this study was to investigate the effects of VCD on gene expression in testes.

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MATERIALS AND METHODS

The material of the study consisted of 18 male dogs (1-2 years old and body weight of 12-23 kg). VCD (purity>96%) was obtained from Sigma Aldrich Chemical and sesame oil from Acros Organics. The primers were taken from Qiagen. Before initiating the study; permissions were taken from the Gumushane University, Animal Experiments Local Ethical Board (dated 04.05.2016 and 2016-02 approval number).

The dogs were randomly divided into 3 groups. Each group consisted of 6 dogs. Later, the VCD with the sesame oil were intraperitoneally given to treatment groups. The sesame oil was only given to the control group. All groups were administered at the doses and times indicated below.

Group 1 (n=6): Sesame oil (2 ml) was given intraperitoneally for 8 days. Group 2 (n=6): VCD (80 mg/kg) was given intraperitoneally for 8 days. Group 3 (n=6): VCD (320 mg/kg) was given intraperitoneally for 8 days.

These doses and application periods were determined based on previous studies (Devine *et al*., 2001; Hooser *et al*., 1995; Mayer *et al*., 2002; Mayer *et al*., 2004; Sahambi *et al*., 2008). VCD were given to the median line of the animal under sedation. One day after the last application, dogs were castrated and its testes were taken. Testes were placed in RNA stabilization solution (RNAlater, Ambion, USA) and maintained at -20°C for genetic analysis. Storage of tissue samples, PCR and mRNA synthesis were performed at Gumushane University Center Laboratory.

mRNA Extraction: A sample of 30 mg was taken from the dissected testis tissue at + 4°C and transferred to ependorf tubes. 600 µl RLT buffer + beta-mercaptoethanol was added to the tissue. The sample mixed with the buffer was kept in the homogenizer for 2 minutes and then subjected to dispersion and homogenization. The lysate was centrifuged at maximum speed for 3 minutes and the supernatant was taken for the next step. It was placed to another tube. 600 µl of ethanol (70%) was added to the lysate and pipetted. Lysate was added to the gDNA elimination columns in the RneasyPlus Mini Kit (Qiagen, Hilden, Germany) and centrifuged at 10000 rpm for 30 seconds. The bottomed fluid was taken and poured into the spine column, and later centrifuged at 8000xg for 15 seconds. After the liquid in the bottom was poured, the RNA in the upper filter has been collected. The collected RNA was taken to washing. For this, 700 µl RW1 was added to the spine column and centrifuged at 8000xg for 15 seconds. The liquid in the bottom was poured. Then, 500 µl RPE was added and centrifuged at 8000 x g for 15 seconds. 500 µl RPE was again added and centrifuged at 8000 x g for 2 minutes. The liquid in the bottom was poured; the residue was centrifuged at 21100x g at maximum speed. The spine column was placed in the elution tube and 30 µl of elution buffer was added to it and waited for a minute. Then, it was centrifuged at 8000 x g for 1 minute. Thus, the RNA passed into the bottom collection tube, and the spine column was discarded.

cDNA Synthesis: Total RNA quantities were calculated using a spectrophotometer (Qiaxpert, Qiagen, Germany) according to the ratio 260/280 nm and the results were found to be ng/µl. After RNA measurement, sample concentrations were prepared for cDNA stage, equaling 100 ng at 1 µl. Then, cDNA was synthesized using RT2 First Strand Kit Qiagen, Germany. The procedure was realized by following the steps of the kit manufacturer's manual.

To prepare each sample, 6,5 µl RNase-free water, 2 µl 5x gDNA elimination buffer and 1,5 µl templete RNA were added to PCR tubes and thoroughly mixed by pipetting. The tubes prepared for each sample were placed in a thermal cycler and incubated at 42°C for 5 minutes. At this stage, the genomic DNA contamination was removed from RNA samples.

For each sample, a master mix was prepared using 4 µl 5x Buffer BC3, 1 µl Control P2, 2 µl RE3 Reverse Transcriptase Mix, and 2 µl RNase-free water. 10 µl of the master mix was added to each gDNA cleaned sample and pipetted. The prepared samples were subjected to PCR thermal cycler for cDNA reaction. The cDNAs were synthesized by incubating for 15 minutes at 42°C and 5 minutes at 95°C. RNase-free water (91 µl) was added to the tubes containing the cDNA and mixed.

Lightcycler Mastermix preparation and loading

Solutions of RT2 Primer Assay Primer (Qiagen, Germany) and master mix RT2 SyberGreen MasterMix (Qiagen, Germany) were melted and spun, and then their caps were opened. Mixture was prepared by adding 12,5 µl RT2 SyberGreen Master Mix, 1 µl RT2 Primer Assay and 6,5 µl H_2O. The total volume was 20 µl.

Finally, 5 µl cDNA was added. Thus, the reaction volume was 25 µl. The sealing foil was pulled onto the plate. The plate was centrifuged 15 seconds at 3750 rpm in the plate centrifuge. The plate was then placed in the Roche Lightcycler 480, the device was programmed and run according to the protocol. 10 min at 95°C Hold, 15 seconds at 94°C, 30 seconds at 60°C, Annealing - 40 cycles.

Quantification of gene expression as semi-quantitative

RT2 qPCR Primer Assay, Qiagen kits for SYBR® Green-based real-time PCR kits were used to quantitatively measure mRNA encoding B-cell lymphoma 2 (Bcl-2), tumor suppressor protein (tp53), Bax, Caspase-2 (CASP2), CASP3, CASP8, CASP9 and β-actin proteins in dogs. Quantification of the amplified cDNAs of the target and reference gene (β-actin) was carried out using the relative quantification method. First, the C_T values were normalized to the reference gene (β-actin). The data analysis portal calculated fold change/regulation using the Delta Delta C_T method. Delta C_T was calculated between the target gene and an average of housekeeping genes, followed by Delta Delta C_T calculations [Delta C_T (treatment) - Delta C_T (control)]. Fold Change was then calculated using 2^(- Delta Delta C_T) formula. Fold-Change (2^(- Delta Delta C_T)) is the normalized gene expression (2^(- Delta C_T)) in the Test Sample divided the normalized gene expression (2^(- Delta C_T)) in the Control Sample. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-

change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change (Dikbas *et al*., 2017).

Statistical analysis

The C_T values were entered in the excel file according to the table format. This table was uploaded to the gene globe data analysis center (Qiagen, http://www.qiagen.com/geneglobe). Student's t test was used for comparison of the available data (Dikbas *et al*., 2017). Statistical significance was assumed at P>0.05.

RESULTS

Compared to the gene expression in the testes of the animals in the control group, there was a significant decrease in CASP-8 expression in the treatment groups (P<0.05). In the VCD-treated groups, the expression levels of other apoptosis genes were different depending on the dose (Figure 1, Table 1-2).

Apoptosis-stimulating tp53 gene expression was increased in the testes of the animals in the treatment groups. This increase was significant at 80 mg/kg VCD (P<0.05). However, apoptosis-inhibiting Bcl-2 gene expression was down regulated in testes of animals treated with 320 mg VCD compared to control (P<0.05). In the low-dose VCD group, Bcl-2 expression was higher than the control group (P>0.05).

When Bax gene expression is examined, there is an insignificant decrease in the treatment groups. This reduction was found to be greater at 80 mg/kg VCD (P>0.05). When we look at the genes that induce apoptosis from the caspase gene family, CASP-2 appears to be more expressed in the high dose group (P>0.05). CASP-9 decreased expression in the treatment groups compared to the control, and this decrease was significant in high dose VCD (P<0.05). CASP-3 gene expression was significantly higher in dogs treated with 320 mg VCD than control group (P<0.05).

Table 1: Fold changes from genes between the control group and low dose VCD treated in testes of dogs.

Genes	2^(- Delta Ct)		P value	Fold change	Comments
	Group 1	Group 2		Group 1/2	
CASP8	0,000361	0,000084	0,000138*	0,2333	A
TP53	0,002923	0,005786	0,001004*	1,9793	
BCL2	0,058924	0,086669	0,059599	1,4709	
ACTB	1	1	0	1	
BAX	0,009442	0,005983	0,171431	0,6336	
CASP3	0,005962	0,004905	0,42471	0,8226	
CASP2	0,006968	0,006592	0,825779	0,9461	
CASP9	0,013094	0,010871	0,457262	0,8303	

A: This gene's average threshold cycle is relatively high (>30) in either the control or the test sample, and is reasonably low in the other sample (<30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result. This fold-change result may also have greater variations if P value >0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.*The p values are calculated based on a Student t-test of the replicate 2^(- Delta C_T) values for each gene in the control group and treatment groups, and P values less than 0.05 are indicated as follows: *<0.05.

Table 2: Fold changes from genes between the control group and high dose VCD treated in testes of dogs.

Genes	2^(- Delta Ct)		P value	Fold change	Comments
	Group 1	Group 3		Group 1/3	
CASP8	0,000361	0,000113	0,000277*	0,3132	A
TP53	0,002923	0,003979	0,149853	1,3613	
BCL2	0,058924	0,032017	0,00631*	0,5434	
ACTB	1	1	0	1	
BAX	0,009442	0,008032	0,334543	0,8507	
CASP3	0,005962	0,008116	0,006014*	1,3613	
CASP2	0,006968	0,008163	0,352632	1,1715	
CASP9	0,013094	0,00775	0,000224*	0,5919	

A: This gene's average threshold cycle is relatively high (>30) in either the control or the test sample, and is reasonably low in the other sample (<30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result. This fold-change result may also have greater variations if P value >0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.*The p values are calculated based on a Student t-test of the replicate 2^(- Delta C_T) values for each gene in the control group and treatment groups, and P values less than 0.05 are indicated as follows: *<0.05.

DISCUSSION

VCH formed by the catalytic dimerization of 1,3-butadiene is an industrial chemical. VCD manufactured by epoxidation of VCH (IARC 1994; Dhillon and Von Burg 1996, Van Kempen *et al*., 2011). There are only a few studies investigating the effects of VCD on testes (Hooser *et al*., 1995; Chhabra *et al*., 1990a; Chhabra *et al*., 1990b). These studies were also conducted to investigate the toxicity of VCD. In the present study, it was determined that VCD causes a decrease in apoptosis-inducing Caspase-8 gene expression.

Apoptosis is known as programmed cell death and is the mode of death in which the cell destroys itself (Jacobson *et al*., 1995). Thymus retraction and uterine shrinkage in menopause can be given as an example (Başaran, 1999). Another example is the destruction of a large number of B and T cells, which are produced by the immune system every day in large numbers (Ganea, 1996). In the case of apoptosis failure, it is known that problems such as cancer and autoimmune diseases arise (Fadeel *et al*., 1999; Renehan *et al*., 2001; Elmore, 2007). During the apoptosis, the intracellular elements do not dissipate in the intercellular space. Since there are no proteolytic enzymes or toxic oxygen release, no inflammatory response occurs and damage to neighboring cells is prevented. Apoptosis can be triggered by internal and external signals and initiated by different signal pathways. Extrinsic apoptosis is initiated via the receptor in the cell membrane. These receptors include members of the tumor necrosis factor (TNF) receptor superfamily (Elmore, 2007). The effects of the proapoptotic members of the Bcl family on external mitochondrial membrane permeability, which trigger caspase activation in intrinsic apoptosis, are important. Proapoptotic molecules initiate apoptosis through mitochondria (Fulda *et al*., 2006). In our study, it was determined that Bcl-2 gene expression

increased in testes of animals given low dose VCD. On the contrary, high doses of VCD were reduced. This may be the answer of the testes to block apoptosis. Because the testes increased the expression of Bcl-2 while trying to tolerate low-dose VCD, and at higher doses, this mechanism may have been overrun because the damage was excessive.

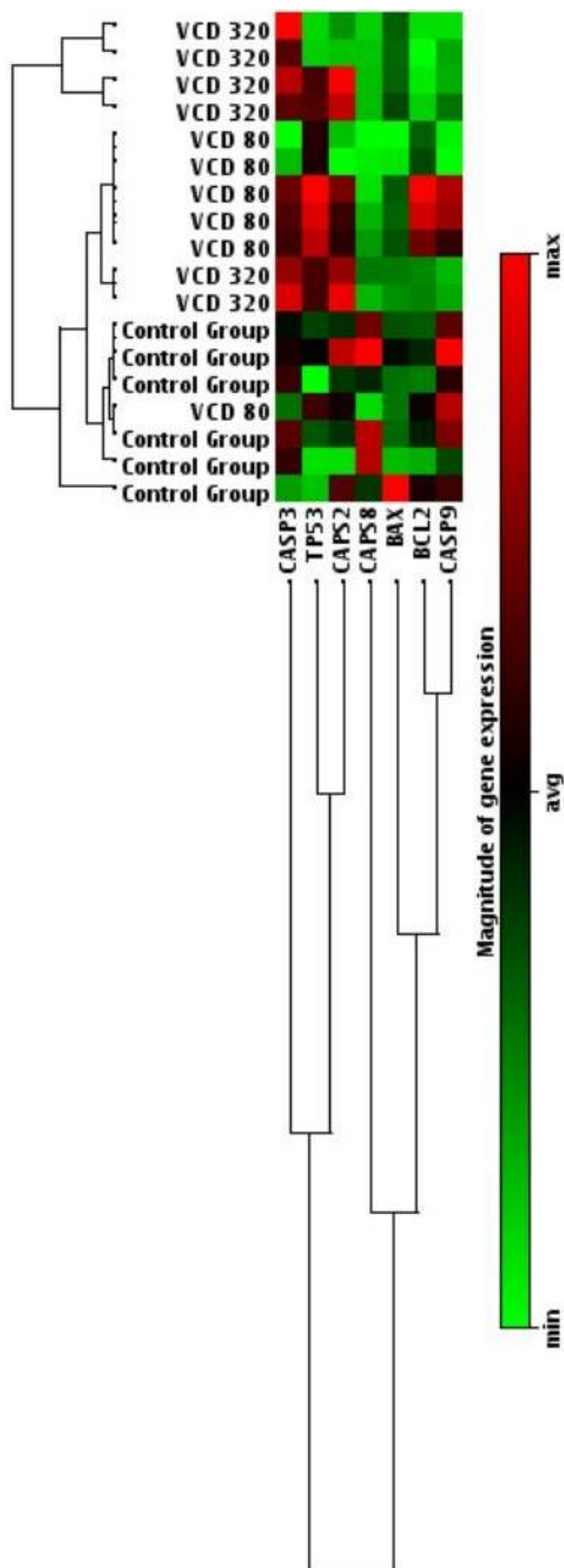

Fig. 1: The dendogram of seven genes hierarchical clustering analysis.

When apoptosis is induced by intrinsic and extrinsic signals, caspase is activated in the cell and the path leading to cell death begins. Caspases, which are the main regulators of the apoptotic cell death pathway, work by proteolytically cleaving more than 400 substrates (Zhang *et al*., 2012; Vurusaner *et al*., 2012). Caspases are biologically studied in two different groups in the apoptotic process. The first is the apoptosis-inducing caspases (caspase-2, 8, 9, 10), which are caspases responsible for the initiation of the caspase cascade by converting various intracellular or extracellular signals into proteolytic activity. Initiator caspases (caspase-8) that are activating with extracellular signals, trigger the caspase cascade by initiating the receptor-mediated (extrinsic) pathway. Initiator caspases activated by intracellular signals (caspase-9) initiate the mitochondria-mediated (intrinsic) pathway to trigger the caspase cascade (Zhang *et al*., 2009; Petersen *et al*., 2011). In the present study, it can be said that the down regulation of caspase-8 and 9 is down-regulated at the level of apoptosis, that is, the formation of apoptosis is reduced through this gene. Decreasing apoptosis through this gene can indicate that the agent used is harmful to the cell and that this is true for both doses we apply.

The second group of caspases are apoptosis-executing (caspase-3, 6, 7), which proteolytically cleave specific polypeptide targets within the cell (Zhang *et al*., 2009; Petersen *et al*., 2011). In this study, it was determined that high dose VCD increased both CASP-2 and CASP-3 gene expression. This result shows that VCD application stimulates both intrinsic and extrinsic pathways of apoptosis.

In conclusion, this study showed that VCD causes male dogs to cause a significant apoptosis in testes. The fact that caspase-8 is down-regulated at the gene level shows that the chemical used damages the testicular tissue. However, the effects of this chemical on other organs and tissues must be demonstrated with future studies. It is thought that our work will give light to similar investigations to be made. It will be useful for those who want to research on this topic in the future to consider this issue.

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