

The effects of prolonged azathioprine administration on blood cells, lymphocytes and immunoglobulins of Iranian mixed-breed dogs

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Abstract:

BACKGROUND: Azathioprine is an immunosuppressive agent that is used in a variety of dermatologic, digestive and hematologic disorders in both humans and small animals. **OBJECTIVES:** The effects of long term Azathioprine administration on complete blood count, rate of CD4+ and CD8+ lymphocytes, serum immunoglobulins and protein concentrations of mixed-breed dogs were evaluated in this study. **METHODS:** 24 healthy mixed-breed dogs were divided randomly into two equal control and treatment groups. Dogs in the treatment group received the therapeutic dose of Azathioprine for four months, while the dogs in the control group did not receive this drug. Peripheral blood samples were taken from both groups before and after the trial to check CBC, CD4+ and CD8+ lymphocytes and the concentrations of total protein, albumin, serum IgM and IgG. **RESULTS:** There was significant decrease in the levels of WBC, RBC, hematocrit and CD4 lymphocyte and double positive CD4/CD8 rates (all p values < 0.001), concentrations of total protein, albumin, serum IgG and IgM (P values: 0.014, 0.001, 0.007 and 0.041 respectively) in treatment group after the trial. **CONCLUSIONS:** Myelotoxicity induced by Azathioprine could be the probable cause of decrements in the rate of WBC and RBC. Decrease in the rate of dpCD4/CD8 might be due to decrement in dpCD4/CD8 progenitor cells and/or decrease in the activation rate of single positive T cells as the result of pharmacological effect of Azathioprine. Disrupted synthesis processes, from genes to proteins through Azathioprine might be the cause of decreases in the level of serum gamma globulins and protein.

Introduction

Azathioprine is an immunosuppressive drug used to treat immune-mediated dermatologic, digestive and hematologic disorders, to prevent rejections of transplants and to treat some types of leukemia in humans and dogs (Lennard et al 1987; Rinkardt and Kruth 1996; Sebbag et al 2000; Snow and Gibson 1995; Viviano 2013 and White et al 2000). Azathioprine is a purine analogue and 6-mercaptopurine (6-MP) prodrug (Aarbakke et al 1997 and Elion 1993). Azathioprine is rapidly absorbed following oral administration and then metabolized to 6-MP by a variety of enzymes (Lennard 1998) and only about 2 percent of Azathioprine is excreted unchanged in the urine (Stern et al 2005). It undergoes non enzymatic degradation due to reactions with sulfhydryl compounds in the liver, erythrocytes and body tissues and is converted into 6-MP. Then 6-MP is metabolized by the variety of enzymes. Enzyme Hypoxanthine- Guanine Phosphoribosyl Transferase (HGPRT) converts 6-MP into 6-Thioinosine-5'-Monophosphate (TIMP). Other enzymes that are responsible for inactivating Azathioprine are Thiopurine Methyl Transferase (TPMT), Aldehyde Oxidase (AO) and Xanthine Oxidase (XO). Thiopurine Methyl Transferase (TPMT) results in TIMP methylation and MeTIMP, an inactive metabolite formation. Xanthine Oxidase (XO) oxidizes Azathioprine forming inactive 6-thiouric acid metabolite and Aldehyde Oxidase (AO) causes Azathioprine dehydroxylation forming inactive Hydroxylated 6-Thioguanine Nucleotide (Hydroxylated 6-TGN) metabolite.

Following the conversion of 6-MP to TIMP, TIMP is converted into 6-Thioguanosine-5'-monophosphate (TGMP) and

then TGMP is converted to a 6-TGN metabolite, Deoxy-6-thioguanosine-5'-phosphate (dGS), due to effects of some kinases and reductases. dGS disrupts DNA/ RNA replication, transcription and translation processes as a false base resulting in cell cycle arrest and apoptosis (Stern et al 2005).

Xanthine oxidase (XO) and aldehyde oxidase (AO) enzymatic competition causes only 16% of Azathioprine dose converted into 6-MP. However, TPMT enzyme plays important roles in systemic distribution of 6-MP levels by metabolizing them into 6-Methylmercaptopurine (6-MMP) and MeTIMP. The most important cells affected by Azathioprine are the lymphocytes since these cells do not have Salvage Purine pathways in their metabolisms (Viviano 2013) and these abnormal purine metabolites are constantly used in DNA and RNA synthesis in lymphocytes (Maltzman and Koretzy 2003).

Researchers are still studying the effects of Azathioprine on dog's immune system changes. To the best of our knowledge, there is no published document regarding long term in vivo effects of Azathioprine administration on dogs, based on searching along databases up to now. The aim of this study was to evaluate the effects of long term administration of Azathioprine in therapeutic dose, which is recommended for treatment of Canine Inflammatory Bowel Disease (Papich 2016 and Plumb 2007), on mixed-breed dogs and assessment of hematologic, CD4+ and CD8+ lymphocyte percentages and the level of serum gamma globulins changes after this treatment period.

Materials and Methods

In this study, all institutional and national

guidelines for the care and use of animals were followed. Twenty-four mixed breed dogs, 12 males and 12 females, with age range of 1-2 years (mean \pm SD: 17.16 \pm 3.80 month), and average weight of 15.26 \pm 2.93 kg (mean \pm SD), were randomly divided into 2 equal groups as control and treatment. Before the trial, for health evaluation of dogs, all of them were clinically and paraclinically assessed by clinical examination, radiographic assessment of thoracic cavity and laboratory evaluation of CBC and blood biochemistry profile including: BUN, Creatinine, Alkaline Phosphatase, Alanine Aminotransferase, Aspartate Aminotransferase, serum total protein and albumin. After attaining assurance of the physical health of all dogs, each one received anti-parasitic drugs for external and internal parasitism as prophylaxis (Ivermectin, 0.4 mg/kg, SC and Praziquantel forte, 1 tablet for each 10 kg body weight, PO).

In the beginning of trial, 15 ml jugular venous blood samples were taken from all dogs. The sample was divided into three equal parts as one for complete blood count (CBC) in EDTA tube, one for CD4 and CD8 lymphocyte flow cytometry in heparinized tubes and one part to measure total protein, albumin and serum immunoglobulins in clotting tubes.

Then dogs of the treatment group received the therapeutic dose of Azathioprine that is indicated for treatment of Canine Inflammatory Bowel Disease (as a model for long term consumption of this drug) as 2 mg/kg, PO, once a day for 4 weeks, then 2 mg/kg, PO, every 48 h for 4 weeks and finally 1 mg/kg, PO, every 48 h for 8 weeks; while the dogs of the control group have not received this drug for a four month trial period. To prevent probable opportunistic infections,

all dogs in both groups received Ivermectin (0.4 mg/kg, SC, monthly), and Cefixime (10 mg/kg, PO, q12h) and Coamoxyclave (15 mg/kg, PO, q12h) from the second month to the end of the trial.

Doses of all drugs including: Azathioprine, Ivermectin, Cefixime and Coamoxyclave were determined based on that documented in Saunder's Handbook of Veterinary Drugs (Papich 2016) and Plumb's Veterinary Drug Handbook (Plumb 2007).

In the whole of the trial, each dog was housed in separated individual cage with approximate dimensions of 2 \times 2 \times 1.5 m (length, width and height respectively) with ambient temperature of 20 to 25 degrees centigrade in the ward of Small Animal Hospital of School of Veterinary Medicine of Tehran University.

The diet of all dogs was the same during the whole period of the study and they were fed with Nutripet[®] dry food (Behindast Co, Iran) as ad libitum water and food status. The amount of daily required feed for each dog was calculated based on amount of energy of dry food (kcal/100g) and total daily energy requirement as the following formula (Bermingham et al 2014):

Resting Energy Requirement (RER) = 70 \times Body Weight^{0.75}

Total Energy Requirement (TER) = 1.5 \times RER

At the end of trial, blood samples were taken from all dogs (15 ml from jugular vein) and equally divided into three tubes as the method in the initiation of the trial, to evaluate CBC, Flow cytometry of CD4 and CD8 lymphocytes and serum total protein, albumin and serum immunoglobulins (IgG and IgM) concentrations.

CBC: To count and differentiate blood cells, 5 ml of each dog's jugular vein blood

sample in EDTA tube was sent to the Laboratory of the Small Animal Hospital of School of Veterinary Medicine of Tehran University. The RBC and WBC count were measured with Automated Hematology Analyzer (Celltac α , MEK-6450, NIHON KOHDEN $^{\text{®}}$, Japan) and air dried blood smear stained with Giemsa's solution (Merck KGaA, Germany), evaluated microscopically (1000x magnification) for determination of different blood WBC percentages.

CD4+ and CD8+ lymphocytes flow cytometry: For flow cytometric assessment of CD4+ and CD8+ peripheral blood lymphocytes, 5 ml heparinized jugular vein blood sample from each dog was sent to Dr. Rastegar Laboratory of School of Veterinary Medicine of Tehran University.

To do so, Rat anti-dog CD4: FITC/CD8: RPE kit (AbD Serotec, Bio-Rad Co, USA) was used together with its isotype control, Rat IgG2a: FITC/ Rat IgG1: RPE (AbD Serotec, Bio-Rad Co, USA).

To perform the flow cytometry assay, 100 μ l heparinized jugular vein whole blood was prepared. Then 10 μ l of reconstituted (1 ml distilled water in each lyophilized cocktail) Rat anti-dog CD4: FITC/CD8: RPE and for isotype control, 10 μ l of reconstituted (0.5 ml distilled water in each lyophilized cocktail) was added to each 100 μ l heparinized whole blood sample. Then the antibody- whole blood mixture was properly mixed together and incubated for 30 min in room temperature. After that, 2-3 ml of 0.1x ammonium chloride based RBC lysis buffer for flow cytometry (Erythrolyse BUF04, AbD Serotec, Bio- Rad Co, USA) was added to each tube and incubated for 10 min at room temperature. Then each tube was centrifuged for 5 min in 400 \times g at 18-20 $^{\circ}$ C and its supernatant was discarded. There-

after each sample was washed with 2 ml of PBS/BSA and was centrifuged for 5 min in 400 \times g at 18-20 $^{\circ}$ C and its supernatant was discarded. Then the pellet cells were re-suspended in 0.2 ml PBS/BSA and finally each sample was analyzed with ParTECpas Flow Cytometry Machine (PARTEC, GmbH, Munster, Germany) and Flowmax $^{\text{®}}$ software. 10000 cells were counted in each run and primary gating of lymphocyte population was performed based on forward and side scattering light properties in linear scaling dot-plot (Fig. 1-A). Quadrant frame positioning was determined by means of isotype control analysis and ascertainment of negative cells population (Fig. 1-B). Finally, on lymphocyte gated population, the percentages of CD4+ and CD8+ lymphocytes were determined by means of analysis of traced events on FL1 (CD4: FITC) versus FL2 (CD8: RPE) channels in logarithmic scaling on dot-plot.

Total protein, albumin and globulin measurement: Serum total protein and albumin concentrations were measured by spectrophotometry kit (using Teb Gostaran Hayyan Iranian Co. Iran) with Automated Biochemistry Analyzer (Selectra ProM, ELITech Group) in Laboratory of Small Animal Hospital of School of Veterinary Medicine of Tehran University. The levels of serum globulins were calculated according to the difference between the concentrations of albumin and total protein.

Serum IgG and IgM measurement in dogs: Dog's serum total IgG and IgM were measured by Quantitative Sandwich ELISA assay using Abcam $^{\text{®}}$ Eliza Kits (canine IgG ELISA kit ab193768, canine IgM ELISA kit ab157702, Abcam, UK), in Dr. Rastegar Laboratory of School of Veterinary Medicine of Tehran University.

Table 1. Changes of the numbers of blood cells in the control and treatment groups, before and after the trial in Mean \pm SD. A P value less than 0.05 is considered statistically significant. In the treatment group, the levels of WBC, RBC and Plt were significantly decreased after 4 months receiving Azathioprine. WBC: white blood cells, RBC: Red Blood Cells, Plt: Platelets.

	WBC (cell/ μ l)	RBC (cell/ μ l)	Plt (cell/ μ l)	
Control Group	Before	7666.67 \pm 3727.19	6241666.67 \pm 479504.16	423333.33 \pm 85634.88
	After	9244.67 \pm 1920.29	6091666.67 \pm 519542.34	420833.33 \pm 80165.55
	P Value	0.194	0.154	0.950
Treatment Group	Before	9912.50 \pm 1911.11	6375000.00 \pm 771215.10	380000.00 \pm 93127.48
	After	2679.17 \pm 2989.10	2708333.33 \pm 1098311.38	135250.00 \pm 61224.07
	P Value	0.001	0.001	0.001

Table 2. Changes of different WBC types numbers in the control and treatment groups, before and after the trial in Mean \pm SD. A P value less than 0.05 is considered statistically significant. In the treatment group, after 4 months receiving Azathioprine, the levels of neutrophil, lymphocytes and monocytes were significantly decreased. Neut: Neutrophil, Lymph: lymphocyte, Band Neut: Band Neutrophil, Mon: Monocyte, Eos: Eosinophil.

		Neut. (cell/ μ l)	Lymph. (cell/ μ l)	Band Neut. (cell/ μ l)	Mon. (cell/ μ l)	Eos. (cell/ μ l)
Control Group	Before	5593.25 \pm 2173.35	2878.83 \pm 1191.94	20.25 \pm 47.50	50.92 \pm 78.24	38.83 \pm 78.76
	After	6256.50 \pm 1907.13	2780.58 \pm 823.01	16.75 \pm 40.72	103.83 \pm 93.08	86.50 \pm 94.26
	P Value	0.437	0.821	0.593	0.156	0.093
Treatment Group	Before	6360.92 \pm 1586.13	3368.17 \pm 978.47	26.58 \pm 6468	79.83 \pm 86.42	77.00 \pm 109.34
	After	1689.50 \pm 1802.60	948.80 \pm 1208.01	2.86 \pm 6.83	15.80 \pm 25.71	22.25 \pm 46.28
	P Value	<0.001	<0.001	0.258	0.019	0.161

Table 3. Changes of RBC's factors in the control and treatment groups, before and after the trial in Mean \pm SD. A P value less than 0.05 is considered statistically significant. In the treatment group, after 4 months receiving Azathioprine, the levels of Hct and Hb were significantly decreased. Hct: Hematocrit, Hb: Hemoglobin.

		Hct (%)	Hb (g/dl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Control Group	Before	44.00 \pm 3.52	14.17 \pm 1.15	70.50 \pm 3.90	23.37 \pm 1.52	33.50 \pm 1.09
	After	42.83 \pm 3.86	14.28 \pm 1.10	70.42 \pm 4.44	23.37 \pm 1.52	33.50 \pm 0.90
	P Value	0.095	0.143	0.777	1	1
Treatment Group	Before	44.17 \pm 4.59	14.76 \pm 1.30	69.42 \pm 2.43	22.84 \pm 1.56	33.58 \pm 1.08
	After	18.91 \pm 8.12	6.29 \pm 2.67	69.42 \pm 2.27	22.47 \pm 1.41	33.50 \pm 2.54
	P Value	0.001	0.001	1	0.118	0.811

To do so, 5 ml of jugular vein blood sample from each dog, was collected into clotting tube. After clot formation, samples were centrifuged at 2000 \times g for 10 min at room temperature and their serums were separated. Then all reagents, working standard and samples were prepared as were directed in assay procedure of each kit.

For IgG measurement, 50 μ l of pre-diluted serum samples (1/1 \times 10⁶) and different concentrations of serially diluted stan-

dard solutions (with concentrations of: 20, 10, 5, 2.5, 1.25, 0.63, 0.31 and 0 ng/ml) were added into the appropriate ELISA kit's wells. Then 50 μ l of antibody cocktail was added to each well and the wells were incubated for 1 h at room temperature on plate shaker (400 rpm). After that, the wells were washed with wash buffer (3 times). Then 100 μ l TMB - Substrate was added to each well and the wells were incubated 30 min in the dark in plate shaker (400 rpm).

Table 4. Percentages and intensities of blood cells changes in comparison to their reference intervals in the treatment group, after the trial. The total numbers of treatment group's members were 12 dogs. Sev: severe, Mod: Moderate.

Abnormality	Thrombocytopenia (PLT<15×10 ³ cell/μl)				Anemia (Hct<37)			Leukopenia (WBC<5000 cell/μl)		
	Severe (<50×10 ³ cell/μl)	Mod. (50- 100×10 ³ cell/μl)	Mild (100- 150×10 ³ cell/μl)	Very Sev. (<13)	Severe (13-19)	Mod. (19-29)	Mild (30-37)	Severe (<1700 cell/μl)	Mod. (1700- 3500 cell/μl)	Mild (3500- 5000 cell/μl)
Total Percentage (%)	58.3				100			75		
Relative Percentage (%)	14	43	43	17	33	42	8	77	23	-
Total Percentage (%)	8.3	25	25	17	33	42	8	58	17	-

Table 5. Flow cytometric results of percentages of CD4+ and CD8+ blood lymphocytes and their changes before and after the trial in Mean ± SD. A P value less than 0.05 is considered statistically significant. In the treatment group, after 4 months receiving Azathioprine, the percentages of CD4+, dp-CD4/CD8, dn-CD4/CD8 and CD4+/CD8+ lymphocytes were significantly decreased. dp-CD4/CD8: double positive CD4/CD8 lymphocytes, dn-CD4/CD8: double negative CD4/CD8 lymphocytes, CD4+/CD8+: ratio of CD4+ lymphocytes to CD8+ lymphocytes.

		CD4+ (%)	CD8+ (%)	dp-CD4/CD8	dn-CD4/CD8	CD4+/CD8+
Control Group	Before	43.56±3.31	19.50±2.20	2.16±0.29	34.80±5.29	2.25±0.17
	After	43.17±3.14	20.19±2.08	2.13±0.31	34.51±4.85	2.16±0.17
	P Value	0.583	0.247	0.780	0.820	0.111
Treatment Group	Before	44.27±3.70	19.43±1.70	2.19±0.35	34.11±5.05	2.27±0.16
	After	34.71±2.56	20.29±2.61	0.52±0.23	44.47±3.56	1.74±0.27
	P Value	0.001	0.321	0.001	0.001	0.001

Table 6. Results of changes of serum proteins and immunoglobulins concentrations in the control and treatment groups, before and after the trial in Mean ± SD. A P value less than 0.05 is considered statistically significant. In the treatment group, after 4 months receiving Azathioprine, the levels of concentration of serum total protein, serum albumin, IgG and IgM were significantly decreased.

		Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	IgG (g/dl)	IgM (g/dl)
Control Group	Before	6.57±0.53	3.31±0.46	3.27±0.25	1.29±0.28	0.16±0.06
	After	6.77±0.46	3.62±0.39	3.14±0.28	1.25±0.25	0.14±0.07
	P Value	0.228	0.081	0.297	0.954	0.271
Treatment Group	Before	6.52±0.53	3.47±0.45	3.06±0.24	1.16±0.29	0.18±0.06
	After	5.97±0.72	2.76±0.21	3.22±0.77	0.81±0.18	0.14±0.18
	P Value	0.014	0.001	0.520	0.007	0.041

Thereafter, 100 μl of stop solution was added to each well and then the optical density (OD) of samples and serially diluted solutions of standard were measured in 450 nm wavelength using ELISA Reader (BioTek®, ELx800 Absorbance Reader).

For IgM measurement, 100 μl of diluted serum samples (1/1×10⁴) and serially diluted solutions of standard (with concentrations

of: 800, 400, 200, 100, 50, 25 and 0 ng/ml) were added to each well. After 30 min incubation at room temperature, each well was washed with wash buffer (3 times). Then 100 μl of Enzyme- Antibody conjugate was added to each well and the wells were incubated 30 min in dark at room temperature. After that, wells were washed again (3 times with wash buffer). Thereafter, 100 μl TMB

– Substrate was added into each well and was incubated for 10 min at room temperature. After 10 min, 100 µl of stop solution was added to each well and then the OD of each well was measured in 450 nm wavelength with ELISA Reader.

After OD recording, the standard curve of serially diluted standard solutions was prepared (for both IgG and IgM in separated curves). Then the recorded OD values of each pre-diluted canine serum sample were put into standard curve and immunoglobulin concentrations of them were submitted. Finally, the achieved values of immunoglobulins of each diluted sample were multiplied by their dilution factors to find out the immunoglobulins concentrations in each serum sample in g/dl unit.

Statistical analysis: The results were analyzed using SPSS Software V.16 (SPSS-Chicago, IL, USA). At the beginning, the normality of data was examined using Kolmogorov-Smirnov test. Then, Paired Sample-T Test was used to analyze the normal distributed possibilities and Wilcoxon Signed-Ranks test was used for those with non-normal distributed possibilities (numbers of Band neutrophils, Eosinophils, MCHC and values of IgM concentrations). The results were recorded as Mean ± Standard Deviation (Mean ± SD) and a P values less than 0.05 were considered as statistically significant.

Results

The results of blood cell count: The results of the statistical test show that the number of white blood cells (WBC), red blood cells (RBC) and the levels of hematocrit and hemoglobin are significantly decreased after the trial in the treatment group

(Tables 1, 2 and 3). Also, comparison of hematologic results with their reference intervals (reference intervals submitted in Schalm's veterinary hematology, sixth edition (Weiss and Wardrop 2010)) shows that, in control group (before and after trial and in treatment group (before trial) all of the hematologic values were within their reference intervals range. But in the treatment group after the trial, 58.3, 100 and 75% of the dogs suffered from thrombocytopenia, anemia and leukopenia, respectively (Table 4). The results show concurrent thrombocytopenia in 66.6% of leukopenic individuals and concurrent lymphopenia in 88.9% of neutropenic ones in treatment group after the trial.

The results of peripheral blood CD4+ and CD8+ lymphocytes flow cytometry: Peripheral blood lymphocytes in the treatment and control groups before and after the trial are shown in Table 5. The results of statistical analysis show that the percentages of all blood lymphocyte subgroups except CD8+ lymphocytes were significantly different after the trial in a way that the percentages of CD4+ and double positive CD4/CD8 cells are decreased and the percentage of double negative cells are increased after Azathioprine administration (Fig. 2).

Measuring serum protein and immunoglobulins concentrations: The average and standard deviation of serum immunoglobulins and protein concentrations of the treatment and the control groups before and after the trial are shown in Table 6. The results of statistical analysis show significant decreases in total protein concentration, albumin, albumin to globulin ratio, and IgG and IgM concentrations of the treatment group after the trial compared with the results of this group before the trial. Com-

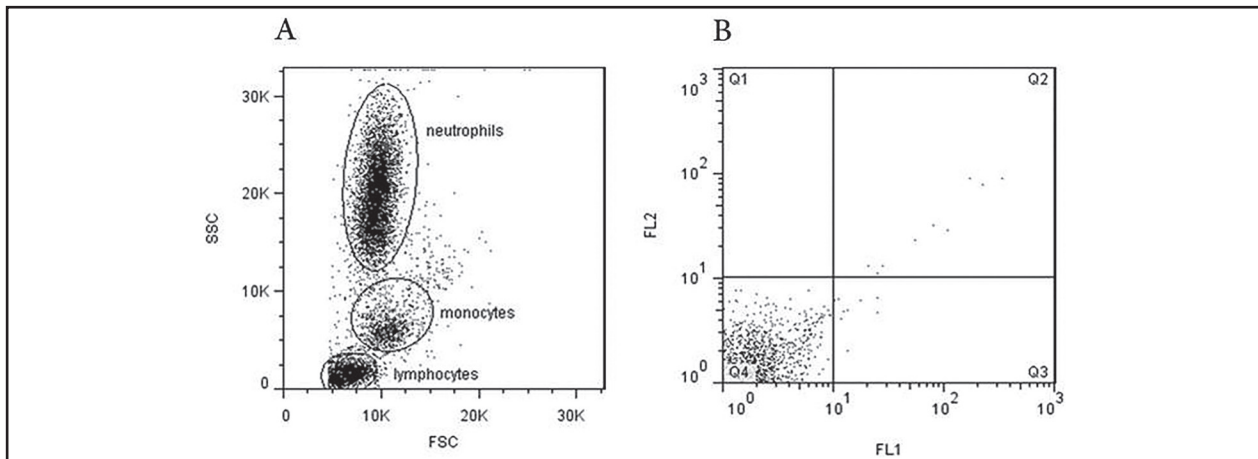


Figure 1- A: Forward versus side scattering properties of canine erythrolyzed whole blood in linear scaling dot- plot. Neutrophils, Monocytes and lymphocytes populations are gated based on forward (their size) and side (their granularity) scattering properties. Events for lysed RBC, debris and platelets are electronically excluded (with proper flow cytometer’s threshold setting) during flow cytometer analysis. FSC: Forward Scattering, SSC: Side Scattering. B: Flow cytometric isotype control analysis (Rat IgG2a: FITC/ Rat IgG1: RPE) on Canine erythrolyzed whole blood and proper quadrant positioning for determination of negative population in dot- plot logarithmic scaling. FL1: Rat IgG2a: FITC, FL2: Rat IgG1: RPE. Q1: Area of CD8+ lymphocyte population, Q2: Area of Double Positive CD4/CD8, Q3: Area of CD4+ lymphocyte population and Q4: Area of Double Negative CD4/CD8 lymphocyte population.

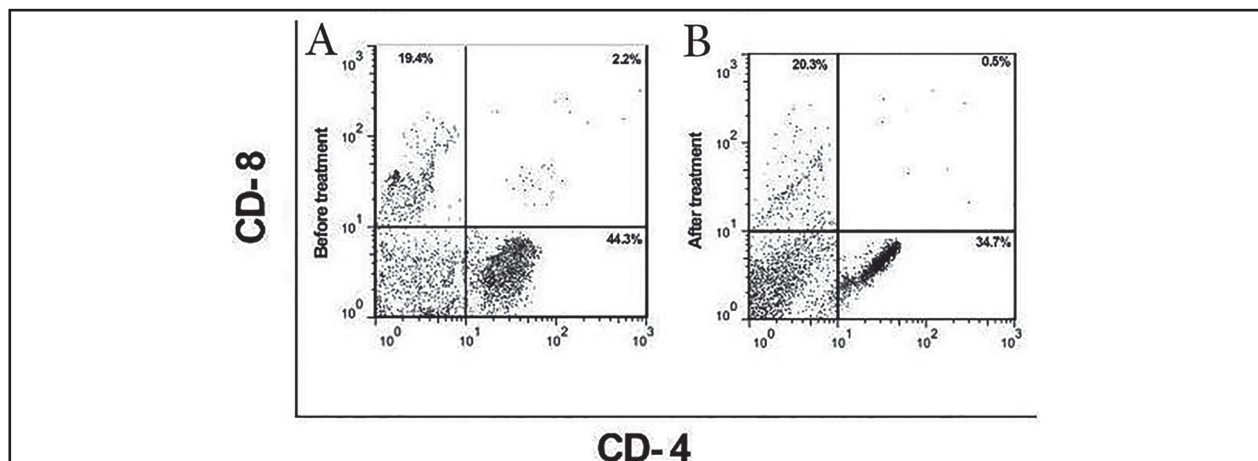


Figure 2. Representation of flow cytometric dot-plot percentages of CD4+, CD8+, double positive CD4/CD8 and double negative CD4/CD8 lymphocytes in lymphocyte gated population of canine erythrolyzed whole blood sample, A: before the trial and B: after the trial in one of the members of treatment group, using Rat Antidog CD4: FITC/CD8: RPE antibody cocktail.

parison of serum concentrations of IgG and IgM in members of treatment and controls groups, before and after the trial, with their reference intervals (Day 2010) shows that serum concentrations of IgG and IgM in control groups before and after the trial and in treatment group before the trial were between reference range, but in spite of significant decrease in their values in treatment group after the trial, only in 8.8% and 30%

of individuals, concentrations of serum IgG and IgM were below reference intervals respectively.

Discussion

To the best of our knowledge, there is no published document regarding long term in vivo effects of Azathioprine administration on dogs up to now. In this study effects of long term administration of Azathioprine in

therapeutic dose, which is recommended for treatment of Canine Inflammatory Bowel Disease on mixed-breed dogs were evaluated and hematologic, CD4 and CD8 lymphocyte percentages and the level of serum gamma globulins changes after four months of Azathioprine treatment were assessed.

Blood cells counting and differentiating results of this study demonstrate occurrence of myelosuppression with different severity in dogs of treatment group after the trial.

Rinkardt et al studied the effects of Azathioprine on dogs in a 14-day period in 1999 and stated that there were no changes in complete blood counts of the dogs that received Azathioprine (Rinkardt et al 1999). This was probably due to the short treatment period. The studies indicated that the therapeutic effects of Azathioprine began about 2 to 5 weeks after the start of IMHA treatment in humans and dogs (Al-Ghazlat 2009 and Fox 2013).

Campbell et al observed in a study on Azathioprine effects on the human patients with inflammatory bowel disease (IBD) that administering 2-5 mg/kg/day dose of Azathioprine for a year caused significant lymphopenia in the treatment group. Although the neutrophil counts were decreased in this group, the difference was not significant compared to the group who suffered from IBD but did not receive Azathioprine. There was also no significant difference between the platelet counts of the group that received Azathioprine and the group which did not (Campbell et al 1976).

Several clinical reports about bone marrow suppression, though in a few percentages of dogs and humans administered standard dose of Azathioprine, were published (Andersen et al 1998; Lennard and Lilleyman 1996; Lennard et al 1987; Snow

and Gibson 1995; Stolk et al 1998). A wide range of disorders in counting blood cells such as leukopenia, neutropenia, thrombocytopenia, anemia and pancytopenia, with the most occurrence of lymphopenia were observed in these reports. In this study anemia and in the second place leukopenia were the most hematologic changes in dogs which received Azathioprine.

One of the enzymes involved in Azathioprine metabolism is TPMT enzyme. The average TPMT enzyme activities are 37.1, 2.8 and 35.7 nmol/bHb/h in dogs, cats and humans, respectively (Foster et al 2000; Tavadia et al 2000). The studies indicated that the activity of this enzyme is high in 88.6 %, moderate in 11.1% and low in 0.3 % of human population. In people with low activity level of this enzyme, Azathioprine metabolism caused the production of 6-MMP inactive metabolites to decrease and the production of 6-TGN active metabolites to increase. These people would suffer from toxicity and acute bone marrow suppression (Rinkardt and Kruth 1996; Rodriguez et al 2004). Human studies indicated that measuring TPMT enzyme activity in RBCs could be considered as a predictive factor for thiopurine toxicity occurrence in those administering Azathioprine (Andersen et al 1998; Klemetsdal et al 1993; Lennard et al 1987; Snow and Gibson 1995; Stolk et al 1998). Nowadays, for minimizing Azathioprine induced toxicity, many physicians believe that TPMT enzyme activity should be evaluated phenotypically (measuring this enzyme in tissues like RBC) and/or genotypically (analyzing TPMT encoding genes) before treating with Azathioprine (Sebbag et al 2000; Stolk et al 1998; Tavadia et al 2000). On the other hand, Azathioprine dosage could be calculated based on TPMT en-

zyme activity level (Lennard and Lilleyman 1996; Lennard 1998).

Recent studies indicated that the variety and distribution of TPMT enzyme in dogs are similar to humans (Foster et al 2000; White et al 2000). Rodriguez et al in a study on 300 dogs in 2004 found out that TPMT enzyme activity is moderate in 58% and high in 42% of the studied dogs and none of them showed low enzyme activity (Rodriguez et al 2004). All studies on dogs have not indicated any low levels of TPMT activity (Rodriguez et al 2004; Stolk et al 1988; White et al 1984; White et al 2000). However, in another study dogs with moderate TPMT activity had significantly lower neutrophil counts after 4-week Azathioprine treatment and indicated that mild to moderate bone marrow suppression (severe neutropenia, leukopenia and thrombocytopenia) could occur in dogs with moderate TPMT activity (Rodriguez et al 2004). One possible explanation for these findings is that the threshold of TPMT activity at which dogs will experience myelotoxicity when receiving Azathioprine differs from the threshold in humans. Other possible causes include the existence of some mechanisms, other than TPMT enzyme, involved in Azathioprine myelotoxicity in dogs such as low activity levels of inosine triphosphate pyrophosphohydrolase (ITPP) (Fraser et al 1975; Lennard 2002) and xanthine oxidase (XO) or increasing concentration of methylated ribonucleotides (Dubinsky et al 2001) and/or idiosyncratic reactions (Giger et al 1985; Trepanier et al 2003).

In our study anemia and in the second place leukopenia were the most established hematologic changes. Studies on myelotoxicity effect of Azathioprine in humans showed that the most prevalent hematolog-

ic changes were Leukopenia, Anemia and thrombocytopenia respectively. Considering lack of salvage pathway for purine biosynthesis in lymphocytes and greater risk for leukopenia due to Azathioprine, from the aspect of the author of this article, this deference remains unexplained. Perhaps, the study of cumulative concentrations of Azathioprine and its metabolites in different canine bone marrow cell lineage and/or assessment of Azathioprine-related changes in hematopoietic and growth factors could explain these differences.

Studies on black and white races of humans in different geographical regions indicated high variety in TPMT enzyme activity in the populations (Gisbert et al 2007; Jang et al 1996; Lennard et al 1996; McLeod et al 1994). Kidd et al indicated that dog breeds including Giant Schnauzer and Alaskan Malamute had the most and least TPMT enzyme activity among the studied breeds of dogs, respectively (Kidd et al 2004).

In the current study, specific activities of TPMT, XO and ITTP enzymes were not measured. According to the high occurrence rate of blood cell changes in dogs treated with Azathioprine in this study, it was suggested to perform a more distributed study with various breeds of dogs including the mixed-breeds in Iran to measure and determine the activities of these enzymes. Also, it is necessary to evaluate and study the correlation between TPMT enzyme activities in main Azathioprine metabolizing tissues such as the liver with the activities of this enzyme in RBCs.

In the current study, significant decrements of CD4+ and Double Positive CD4+/CD8+ (dp-CD4/CD8) lymphocytes were observed in the treatment group after a 4-month Azathioprine administration peri-

od. In a study on the effects of administering Azathioprine for a short period, 14 days, in dogs, no changes in CD4+ and CD8+ lymphocytes were recorded (Rinkardt et al 1999). This was probably due to short administration period of Azathioprine since studies indicated that the shortest period needed to induce therapeutic effects of Azathioprine is two to five weeks (Al-Ghazlat 2009 and Fox 2013).

It could be concluded based on the results of the current study and revision of the previous studies that the reason of significant CD4+ lymphocyte decrement might be leukopenia occurrence followed by CD4+ and CD8+ lymphocyte decrements compared with the control group and/or decreasing the division and proliferation of T lymphocytes caused by Azathioprine administration in the treatment group.

In this study, there is significant decrease in percentages of dp-CD4/CD8 lymphocytes in treatment group after the trial.

Immature T cells were dp-CD4/CD8 lymphocyte. During the maturity process, these cells changed into single positive cells. Hence, it has been long thought that circulating mature T lymphocytes were CD4+ and/or CD8+ (Bukowska-Straková et al 2006). Zuckermann et al was indicated in 1992 that there are mature T lymphocytes in peripheral blood of human, primate, swine, rats, mouse and chickens that are simultaneously dp-CD4/CD8 (Zuckermann et al 1992). This cell population was also found in the dogs (Alexander- Pires et al 2010; Otani et al 2008). The rate of this cell population was 2.5% in dogs (Blue et al 1985; Nascimbeni et al 2004), 2-9% in humans (Bismarck et al 2012) and about 60% in swine (Zuckermann 1999). It seems the rates of these cells are positively correlated with age (Bismarck

et al 2012; Zuckermann 1999; Zuckermann and Husmann 1996) while the gender might not effect on dp-CD4/CD8 rate (Bismarck et al 2012). Since dp-CD4/CD8 cells lack CD1 (surface marker of immature T cells) in humans, the population is considered mature (Zuckermann 1999). In dogs, these cells are in fact mature TCR $\alpha\beta$ T cells and are divided into three groups as CD4bright CD8 α bright, CD4dim CD8 α bright and CD4bright CD8 α dim (Bismarck et al 2014).

The progenitor cells of dp-CD4/CD8 population are CD4 single positive T cells, single positive CD4 and single positive CD8 (with higher potency of CD8 Single Positive) and single positive CD4 and single positive CD8 (with higher potency of CD4 SPs) in swine, human and dogs, respectively (Bismarck et al 2014; Bismarck et al 2012; Saalmüller et al 2002).

In human and swine, the activation of T cells is directly correlated with becoming double positive (Saalmüller et al 2002; Sullivan et al 2001). In vitro developed canine dp-CD4/CD8 express high rate of CD25 in dog (Bismarck et al 2014). It is necessary to mention that CD25 is the alpha chain of interleukin 2 receptors that is expressed on activated T cells. In some clinical cases such as thymoma, allergy, transplantation (Bukowska-Straková et al 2006) and autoimmune diseases in humans, the rate of dp-CD4/CD8 has been increased (Alexander-Pires et al 2010; Bismarck et al 2014; Parel and Chizzolini 2004). Also, PBMC stimulation in dogs with parapox ovis, distemper virus, *Staphylococcus aureus* enterotoxin B, Concavalin A, IL-2 and Anti CD3 antibody resulted in increasing the population of this type of cells (Bismarck et al 2014; Fraser et al 1975 and Schütze et al 2009). The studies indicated that canine dp CD4+/CD8+ T

cells probably belonged to memory/effector T cells (Bismarck et al 2014).

In this study, the decrement of dp-CD4+/CD8+ cell population might be due to decreasing population of CD4+ and CD8+ cells as the progenitors of dp-CD4+/CD8+ and/or decreasing the level of single positive T cell antigenic activation as a result of administering Azathioprine.

In our study, the concentrations of albumin and IgG and IgM globulins were significantly decreased after administering Azathioprine for four months in the treatment group.

Rinkardt et al performed a short 2-week study on dogs and concluded that Azathioprine could not affect the concentrations of plasma immunoglobulins (Rinkardt et al 1999). This is probably due to the limited time of study.

Also, in another study on the effects of Azathioprine on immunoglobulin synthesis in humans for a long period, it was indicated that the synthesis rates of IgG and IgM were decreased 33.4 and 40%, respectively but the decreasing level of immunoglobulin concentration was not significant compared to the prior of the study (Levy et al 1972).

Wolf et al indicated that administering 6-thioguanine could suppress the immunoglobulins in rabbits (Wolff and Goodman 1963). Also, Mott et al studied the turnover of the immunoglobulins and showed that administering 6-MP in young rabbits could result in hypoglobulinemia (Mott et al 1968). The results of these studies are in agreement with the current study. The reason of gamma globulin decrement in these studies might be due to reduction of their synthesis. However, it seems the decrement is dose-dependent so that it only occurred in higher doses of Azathioprine (Levy et al

1972; Mott et al 1968; Wolff and Goodman 1963).

In this study in spite of significant decrease in concentration of IgG and IgM in treatment group, after the trial (Table 6), only in 8.8% and 30% of individual levels were below reference range respectively. This may be due to longer serum half-life of IgG comparing to IgM (Abbas et al 2015 and Bergeron et al 2014).

The decrement of serum proteins such as albumin and gamma globulins could be due to Azathioprine and its metabolites-related disruption in mechanisms such as DNA replication, transcription, translation and finally protein synthesis that are involved in synthesis of these proteins. Since occurrence of leukopenia is not required to suppress the synthesis of gamma globulins (Levy et al 1972), leukopenia might not be the definite cause of gamma globulin concentration's decrement in the current study.

Conclusion: It is concluded that Azathioprine can induce high rate occurrence of myelosuppression, decrease in CD4+, CD8+ and dp-CD4/CD8 peripheral blood lymphocytes percentages and decrement of serum IgG and IgM concentrations in Iranian mixed large breed dogs. Whereas some factors including: enzymes such as TPMT, ITPP, XO and breed differences, can possibly contribute in Azathioprine associated myelosuppression, it is suggested there is a correlation between these factors and Azathioprine associated myelotoxicity evaluated in more distributed and specified studies. Decrease in the rate of dpCD4/CD8 might be due to induced apoptosis in peripheral blood lymphocytes or decrement in dp-CD4/CD8 progenitor cells and/or decrease in the activation rate of single positive T cells as the result of pharmacological

effect of Azathioprine. Disrupted synthesis processes, from genes to proteins through Azathioprine might be the cause of decreases in the level of serum gamma globulins and protein.

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اثرات مصرف طولانی مدت آزاتیوپرین بروی سلول‌های خونی، لمفوسیت‌ها و ایمنوگلوبولین‌های سگ‌های نژاد مخلوط ایرانی

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چکیده

زمینه مطالعه: آزاتیوپرین یک داروی سرکوب‌ایمنی است که جهت سرکوب‌ایمنی در درمان طیف وسیعی از بیماری‌های پوستی، گوارشی و هماتولوژیک، در انسان و دام‌های کوچک مصرف می‌شود. هدف: هدف از این مطالعه بررسی اثرات تجویز طولانی مدت آزاتیوپرین بر روی تغییرات شمارش سلول‌های خونی (CBC)، درصد لمفوسیت‌های T و غلظت پروتئین‌ها و ایمنوگلوبولین‌های سرم در سگ‌های سالم نژاد مخلوط است. روش کار: بدین منظور تعداد ۲۴ قلاده سگ سالم نژاد مخلوط به صورت تصادفی به دو گروه شاهد و درمان (در هر گروه ۱۲ قلاده، ۶ قلاده نر و ۶ قلاده ماده) تقسیم شدند. سپس سگ‌های گروه درمان به مدت ۴ ماه آزاتیوپرین دریافت کردند، در حالیکه در اعضای گروه شاهد این دارو مصرف نگردید. قبل و بعد از مطالعه از تمامی سگ‌های گروه شاهد و درمان نمونه خون محیطی، جهت ارزیابی CBC، فلوسایتومتری لمفوسیت‌های CD۴+ و CD۸+، غلظت پروتئین تام، آلبومین، IgG و IgM سرم آنها، اخذ گردید. نتایج: نتایج آماری نشان دهنده کاهش معنی‌دار آماری در میزان WBC، RBC، هماتوکریت، هموگلوبین، درصد لمفوسیت‌های CD۴+ و CD۸+ (p value > ۰/۰۰۱) و غلظت پروتئین تام، آلبومین، IgG و IgM سرم (به ترتیب P value: ۰/۰۱۴، ۰/۰۰۱، ۰/۰۰۷ و ۰/۰۴۱) در سگ‌های گروه درمان، پس از مطالعه، است. نتیجه‌گیری نهایی: علل احتمالی این نتایج، سرکوب مغز استخوان، ایجاد آپوپتوز در لمفوسیت‌های خون محیطی، کاهش پیش‌سازهای سلول‌های CD۴+ double positive-CD۸ و یا کاهش فعال شدن single positive-T cells و کاهش سنتز پروتئین‌ها و گاماگلوبولین‌های سرم ناشی از ایجاد اختلال در فرایندهای تولید آنها (از ژن تا پروتئین)، می‌تواند باشد.

واژه‌های کلیدی: آزاتیوپرین، شمارش کامل سلول‌های خونی، سگ، گاماگلوبولین، لمفوسیت T

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