https://doi.org/10.48047/AFJBS.6.13.2024.5808-5830

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Dose-dependent effects of azathioprine administration on hemostatic processes, immune function, and antioxidant defense system in albino rats.

Heba Mohammed Abd El Latif [1*], Faten Roshdy Abdel Ghaffar[1], Asmaa El-Ashry [1], Hany Mohammed Ibrahim

[1] Zoology Department, Faculty of Science, Menoufia University, Shebin El-Kom (postal code: 32511), Egypt.

***Corresponding authors:**

*Heba M. Abd El Latif, Ph.D. Haematology & Physiology Unit, Zoology Department, Faculty of Science, Menoufia University, Shebin El-Kom, Egypt

Tel.: +02-0106-5721978, Fax: +02-048-2235689 E-mail: heba.abdellatif@science.menofia.edu.eg, ORCID : 0000-0002-9790-4158

Volume 6, Issue 13, Aug 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

doi: 10.48047/AFJBS.6.13.2024.5808-5830

Abstract

Azathioprine (AZA) is a widely used immunosuppressive drug to avoid transplant rejection and control autoimmune disorders. In this study, rats were split into four groups, with one designated as the control. They were given ascending graded doses of AZA (6.25, 12.5, and 25 mg/kg body weight) orally, once daily for a month. Our results demonstrated that AZA caused a dose- dependent reduction in platelet count, prolonged bleeding time, clotting time, prothrombin, and activated partial thromboplastin times with high significant effects observed in the group receiving 25 mg/kg body weight. Hemato-toxicity was also illustrated, with the highest impact seen in the moderate and high dose groups. AZA progressively cause a reduction in serum total immunoglobulin (Ig) concentration, quantitative hemolysis of SRBCs, hemagglutination titer, delayed type hypersensitivity, splenocyte proliferation rate, and the percentage of $CD3^+CD8^+$, $CD3^+CD4^+$, and $CD11b^+$ spleen cells in dose dependent way when compared to the control animals. Moreover, AZA ascending doses induced oxidative stress, renal and hepatic toxicity in a dose-dependent manner. This study is the first to highlight the examined ascending dose-dependent effects of AZA on rat hemostatic process, immune system, and organ functions. It emphasizes the importance of cautious monitoring and optimal dosing in clinical applications of AZA.

Keywords Azathioprine, coagulation, immune functions, blood counts, oxidative stress.

Cover letter

Dear Editor-in-Chief,

I am delighted to submit my research article titled "Dose-Dependent Effects of Azathioprine Administration on Hemostatic Processes, Immune Function, and Antioxidant Oxidant Defense System in Albino Rats" for consideration for publication in the African Journal of Biological Sciences.

This study was designed to comprehensively investigate the dose-dependent effects of azathioprine on rat hemostatic processes, immune function, and organ functions. Through administering graded ascending doses of azathioprine in male albino rats, we have discovered novel and significant insights into its impact on coagulation profile, immune markers, and oxidative stress.

Our findings demonstrate dose-dependent reductions in platelet count, prolonged clotting times, and hematotoxicity, with the most pronounced effects seen in higher doses. Moreover, Azathioprine progressively diminished immune responses and induced oxidative stress in a dose-dependent manner. These results highlight the importance of cautious monitoring and optimal dosing in clinical applications of Azathioprine.

We believe that our study's novelty lies in its comprehensive evaluation of Azathioprine's dose-dependent effects on multiple physiological aspects. By shedding light on these previously unexplored areas, our research contributes significantly to the understanding of Azathioprine's implications for immunosuppression and autoimmune disease treatment.

Thank you for considering our submission. We are eager to share our groundbreaking findings with the readers of your esteemed journal and the wider scientific community.

Sincerely,

Corresponding author

Heba M. Abd EL Latif

Introduction

Azathioprine (AZA) is an immunosuppressive medication that is widely used to prevent transplant rejection and treat autoimmune disorders. It was first introduced in the 1960s and has since become a cornerstone of immunosuppressive therapy (Patel et al., 2006; Broen and van Laar, 2020). AZA is a pro-drug that is quickly converted to its active metabolite, 6-mercaptopurine (6-MP), which in turn is converted into thioinosinic acid and incorporated into DNA and RNA, interfering with the proliferation of rapidly dividing cells, including immune cells, which helps to dampen the immune response (Fletcher and Maddocks, 1980).

The metabolism of AZA is complex process that involves multiple enzymes such as xanthine oxidase, thiopurine S-methyltransferase (TPMT), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Lennard, 2002). Activity of these enzymes can affect the efficacy and toxicity of AZA. In particular, low TPMT activity can lead to increased levels of toxic metabolites, while high TPMT activity can result in inadequate immunosuppression (Baker, 2003).

Previous reports revealed that the dosage of AZA can vary depending on the severity of the disease, the condition being treated, and the patient's individual response to the medication (Baker, 2003; Tiede et al., 2003; Meijer et al., 2017). In general, lower doses are typically used to treat autoimmune disorders (Johnson et al., 1995), while higher doses are used for organ transplantation (Bergan et al., 1998).

Despite its widespread use, AZA has several potential side effects, including bone marrow suppression (Connell et al., 1993), genotoxicity (El-elaimy et al., 2012), hepatotoxicity (Fraser et al., 2002), immunotoxicity, elevated infection risk (Matsumoto et al., 1990), and malignancy (Pedersen et al., 2014). Therefore, careful monitoring; ex: blood counts and liver function; is essential during therapy.

Overall, AZA can be a highly effective medication for patients with certain medical conditions. However, it is important for healthcare providers to carefully monitor patients for side effects and adjust dosage as needed to maximize the benefits and minimize the risks of treatment. Although many previous studies delt with the impact of using AZA, this research article is the first which comprehensively aims to explore the impact of three ascending doses of AZA (6.25, 12.5, and 25 mg/kg body weight) on coagulation indices, blood parameters, markers of immune

function, oxidative stress, as well as renal and hepatic functions in rats, in order to enhance patient outcomes and ensure safety while utilizing AZA as an immunosuppressive therapy.

Materials and Methods

Chemicals

In the study, medical grade tablets of AZA Mylan (50 mg/tablet) from DEPHARM LILLE SAS company in France were utilized. Concanavalin A (Con A) and RPMI 1640 medium was purchased from Sigma-Aldrich in St. Louis, MO and supplemented with ten percent fetal calf serum, streptomycin (100 µg/ml) & penicillin (100 U/ml) (Sigma). Con A from *Canavalia ensiformis*, with a stock solution concentration (5 mg/ml) in RPMI 1640 medium, was prepared for further use. Anti-mouse monoclonal anti-mouse antibodies labeled with Fluorescein isothiocyanate (FITC) for CD3 (clone: 17A2), Allophycocyanin (APC) for CD4 (clone: RM4-5), Phycoerythrin-cyanine 5 (PE.Cy5) for CD8 (clone: 53-6.7), and APC for CD11b (clone: M1/70) were purchased from BD Bioscience Company (USA). All other chemicals and reagents used in the study were of the utmost purity available.

Animals

Twenty male rats (*Rattus norvegicus*), weighing (200-250g), were bought from the Vaccination and Serology CO. (Egypt). In order to collect the complement for QHS assay female guinea pigs, (220±20g) was utilized. The rats were acclimatized to standard laboratory conditions for two-week and then the experiment began. The animals were kept in standard housing conditions with a twelve-hour dark/light cycle & full freedom of access to water and food.

Experimental design

All rats were split into four groups (n=5): Negative control group received orally 0.5 ml of the vehicle, only distilled water, AZA high dose group, received orally 0.5 ml of AZA dissolved in distilled water at 25 mg/kg b.w. according to El-Ashmawy et al. (2010), AZA moderate dose group, received orally 0.5 ml of AZA dissolved in distilled water at 12.5 mg/kg b.w., and AZA low dose group, received orally 0.5 ml of AZA dissolved in distilled water at 6.25 mg/kg b.w. AZA dosages were calculated based on the body weight of the rats, and the drug was administered orally once a day for thirty consecutive days. Alsever's solution was used to collect sheep red

blood cells (SRBCs), and then SRBCs were washed three times with sterile saline and be ready for use (Ibrahim, 2014). Moreover, the rats were immunized intraperitoneally with 1 $\times 10^8$ SRBC in saline on day 22 of the treatment.

Blood and tissue sampling

By the end of day 30, the animals were fasted over-night, anesthetized using isoflurane, and dissected. From the hepatic portal vein, the blood samples were collected, divided into 3 tubes, one of them mixed with sodium citrate, the second was mixed with EDTA while the third one was permitted to clot. Centrifugation was used to separate serum samples for 15 min. at 3000 rpm. The aliquot serum was kept in deep freezer at -80 °C. The collected spleens were washed in sterile saline and used for preparation of single cell suspension according to Ibrahim et al. (2010).

Evaluation of hematological parameters

According to Song et al. (2012), bleeding time was estimated using a cutting-tail rat model. According to Ibu and Adeniyi (1989), Clotting Time was performed using Ivy's approach. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured in citrated plasma using the BioMed-Liquicellin-E kit (BioMed Diagnostics, Germany) and Phosphoplastin RL prothrombin time reagent (R2 Diagnostics, UK), respectively. Blood picture was also performed (Dacie and Lewis, 1991).

Evaluation of the humoral immune response

As described by Hassouna et al. (2015), Total serums Igs were determined using "zinc sulfate turbidity test" where the final product was measured at a wavelength of 545 nm. Hemagglutination (HA) titer was measured using serum aliquots from immunized rats with SRBCs according to Bin-Hafeez et al. (2003). The highest hemagglutination dilution was used as antibody titer. Spleen cell suspension (one million cells per ml) was prepared in saline and incubated with 1 ml of SRBC (0.2%) and 1 ml of the serum that collected from guinea pig (10%) for QHS assay according to Hassouna et al. (2015).

Evaluation of the cellular immune response

The reaction of delayed type hypersensitivity (DTH) was determined according to Bin-Hafeez et al. (2003). Immunized animals had their right hind foot pad challenged with 1×10^8 SRBC

on day 29, while the contra-lateral paw was challenged with saline. After 24 h, the difference in thickness between the right and left hind paws served as an indicator of the DTH reaction. The proliferative response of splenocytes to the mitogen Con A was examined using a micro tissue culture system, based on the procedure described by Ibrahim et al. (2010). In this assay, spleen cells (5×10^5) were suspended in prepared RPMI 1640 medium and cultured with Con A for 24 h. After incubation with Cell Counting Kit-8 (Sigma, USA) reagent for three hours at 37 °C in 5% CO_2, the optical density was detected using a Seac Radim Company (Italy) microplate reader at 450 nm. The detection of splenic $CD3^+CD8^+$, $CD3^+CD4^+$, and $CD11b^+$ cells expression was measured using a flow cytometer (BD Accuri C6, San Jose, CA, USA) and anti-mouse monoclonal antibodies in accordance with the manufacturing instructions (Morsi et al., 2023).

Biochemical evaluation

Collected serum was used for oxidative stress evaluation through measuring the level of malondialdehyde (MDA; Elabscience, E-BC-K025-M, USA), reduced glutathione (GSH; Elabscience, E-BC-K030-M, USA), superoxide dismutase (SOD; Biodiagnostic, SD 2521, Egypt), and catalase (CAT; Biodiagnostic, CA 2517, Egypt) using colorimetric assay kits according to the manufacturer's instructions. Moreover, serum concentrations of aspartate aminotransferase (AST; Linear Chemicals, REF 1109000, Spain), alanine aminotransferase (ALT; Linear Chemicals, REF 1105000, Spain), creatinine (SPINREACT, Ref: MD1001111, Spain), and urea (SPINREACT, Ref: 1001333, Spain) were measured using colorimetric assay kits according to the manufacturer's instructions.

Ethics statement

The current research was conducted after getting approved by Menoufia University Institutional Animal Care and Use Committee (MUFS/S/PH/2/22).

Statistical analysis

Using SPSS program Version 22 (IBM Corp., Armonk, NY USA), for analyzing the data using one-way ANOVA followed by LSD multiple comparisons test and was recorded as the mean ± standard deviation (SD). Results were considered statistically significant when $P < 0.05$.

Results

Impact of AZA different doses on coagulation profile and blood components

Impact of different AZA doses on coagulation profile and complete blood counts are illustrated in Table 1. There were statistically significant changes between control measurements and measurements after administration of AZA. Exposure of albino rats to the examined doses of AZA resulted in marked decline in thrombocyte count, that was highly significant ($P < 0.01$) with the high dose, along with retarded blood bleeding and clotting time in dose dependent manner. The effect on bleeding time was highly significant ($P < 0.01$) with high dose, while the effect on clotting time was highly significant ($P < 0.01$) with moderate and high doses. In the same line, dose dependent prolongation was demonstrated on PT after exposure to AZA that was significant ($P < 0.05$) at moderate dose, and highly significant ($P < 0.01$) at high dose. AZA resulted in mild aPTT prolongation that was not significant ($P > 0.05$).

Hematological investigations revealed that exposure to varying ascending doses of AZA led to a dose-dependent decline in RBC count, hemoglobin content, and hematocrit value, indicating the presence of anemia. The decrease was statistically significant ($P < 0.01$) at both moderate and high doses of AZA. Moreover, when different ascending doses of AZA were administered, a highly significant ($P < 0.01$) decrease in total leukocyte count and lymphocyte subpopulation was detected, accompanied by a significant ($P < 0.05$) increase in granulocytes in low dose and highly significant ($P < 0.01$) with moderate and high doses, was found in a dose-dependent way. However, the impact of AZA on monocytes did not reach statistical significance ($P > 0.05$).

Table 1. Impact of azathioprine dosages on coagulation profile and blood components

	Control	**AZA low dose 6.25 mg/kg**	**AZA moderate dose 12.5 mg/kg**	**AZA high dose 25 mg/kg**
Platelets (10^3/mm^3)	319.25±40.06	287.42±30	275.86±59.48	191.85±21.08**#$
Bleeding time(second)	98.00±5.88	100.75±13.37	108.75±15.47	175.25±4.99**#$
Clotting time (second)	140.25±6.29	151.66±6.23	168±7.39**#	230.25±11.61**#$
PT (second)	17.00±3.16	20.50±3.31	24±2.82*	33±4.76**#$
aPTT (second)	26.25±1.70	25.50±3.31	27.50±3.69	28±1.41
RBCs (10^6/mm^3)	5.21±0.54	5.20±0.36	3.73±0.27**#	3.31±0.26**#
Hemoglobin (g/dL)	14.8±1.10	14.67±0.49	10.92±0.61**#	10.22±1.06**#
Hematocrit (%)	47.50±3.18	44.23±1.86	36.03±0.89**#	34.23±2.17**#
WBCs (10^3/mm^3)	7.81±0.82	4.24±0.55**	3.26±0.52**#	3.18±0.22**#
Granulocytes (%)	51.25±2.50	56±2.16*	59.75±1.89**#	64.50±2.64**#$
Lymphocytes (%)	42.75±2.21	37±2.94**	34.25±2.62**	29.50±2.08**#$
Monocytes (%)	6.00±0.81	7±0.81	6±0.81	6±1.41

Results are expressed as "mean ± SD", n = 5/group. * $P < 0.05$, ** $P < 0.01$ denote significant change compared to control rats; $^{\#}$ $P < 0.05$ denote significant difference compared to AZA low dose (6.25 mg/kg); $^{\$}$ $P < 0.05$ denote significant change compared to AZA moderate dose (12.5 mg/kg). AZA: azathioprine, PT: prothrombin time, aPTT: activated partial thromboplastin time, RBCs: red blood cells, WBCs: white blood cells.

Impact of AZA different doses on humoral and cellular immunity

The impact of AZA on humoral and cellular immunity has been observed and documented in table (2) and Figure (1) and (2). The administration of varying doses of AZA showed a significant ($P < 0.05$) reduction in the concentration of serum total Igs, the antibody titer against SRBCs and the percentage of hemolyzed SRBCs in the QHS assay compared to control group. This dose-dependent effect was particularly significant ($P < 0.05$) at lower AZA doses and highly significant ($P < 0.01$) at both moderate and high doses of AZA. Furthermore, the impact on the QHS assay was highly significant ($P < 0.01$) across all three tested AZA doses. Similarly, the different doses of AZA resulted in a highly significant ($P < 0.01$) decrease in the DTH reaction and the percentage of splenocyte proliferation in dose dependent way when compared with control rats table (2).

The administration of different ascending doses of AZA resulted in a gradual reduction in the percentage of $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD11b^+$ splenocytes. AZA impact on $CD3^+CD4^+$ and $CD3^+CD8^+$ was found to be highly significant ($P < 0.01$) across all three doses, except for the effect of the low dose on $CD3^+CD8^+$, which was only significant ($P < 0.05$). Additionally, the effect on $CD11b^+$ reached significance ($P < 0.05$) with the moderate dose and showed high significance ($P < 0.01$) with the high dose of AZA when compared to control values (Figure 1 & 2).

Table 2. Impact of azathioprine dosages on humoral and cellular immune responses

	Control	**AZA low dose 6.25 mg/kg**	**AZA moderate dose 12.5 mg/kg**	**AZA high dose 25 mg/kg**
Total immunoglobulin concentration (g/L)	5.96±0.43	4.69±0.93*	4.15±0.51**	3.82±0.42**
Hemagglutination titer (Log ^{2} titer)	6.66±0.47	5.75±0.50*	5.33±0.47**	4.75±0.50**#
Quantitative hemolysis of SRBCs (%)	77.46±7.86	56.69±4.84**	41.79±6.58**#	24.18±0.81**#$
DTH (mm)	1.27±.25	0.75±0.10**	0.41±0.09**#	0.12±0.04**#$
Splenocytes' proliferation (%)	100.00±9.62	77.74±4.19**	36.69±4.93**#	25.09±3.74**#$

Results are expressed as mean ± SD, n = 5/group. * $P < 0.05$, ** $P < 0.01$ denote significant change compared to control group; $^{\#}$ $P < 0.05$ denote significant change compared to AZA low dose (6.25 mg/kg); $^{\$}$ $P < 0.05$ denote significant change compared to AZA moderate dose (12.5 mg/kg). AZA: azathioprine, SRBCs: sheep red blood cells, DTH: delayed type hypersensitivity.

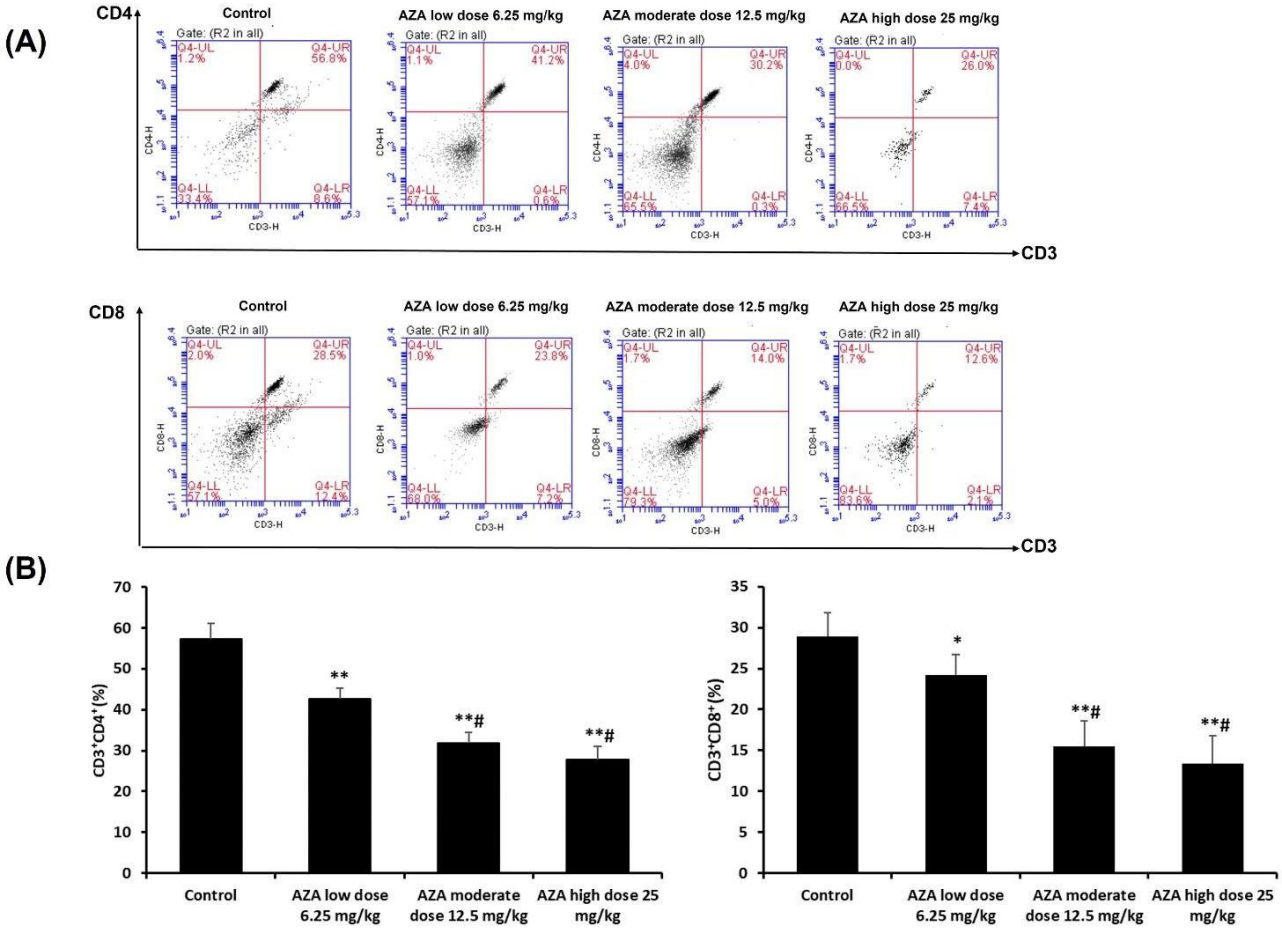

Figure 1. Effects of ascending doses of AZA on spleen T-cell subtyping. $CD3^+CD4^+$ and $CD3^+CD8^+$ spleen cells were gated by flow cytometry based on their staining patterns. (A) A dot plot of one representative experiment out of 5 independent trails is shown. (B) Pooled data from 5 experiments are expressed as the mean percentage of T-cells ± SD. Statistical change was calculated with ANOVA test and follow-up test (LSD). * $P < 0.05$, ** $P < 0.01$ indicate significant difference compared to control group; # $P < 0.05$ indicate significant difference compared to AZA low dose (6.25 mg/kg).

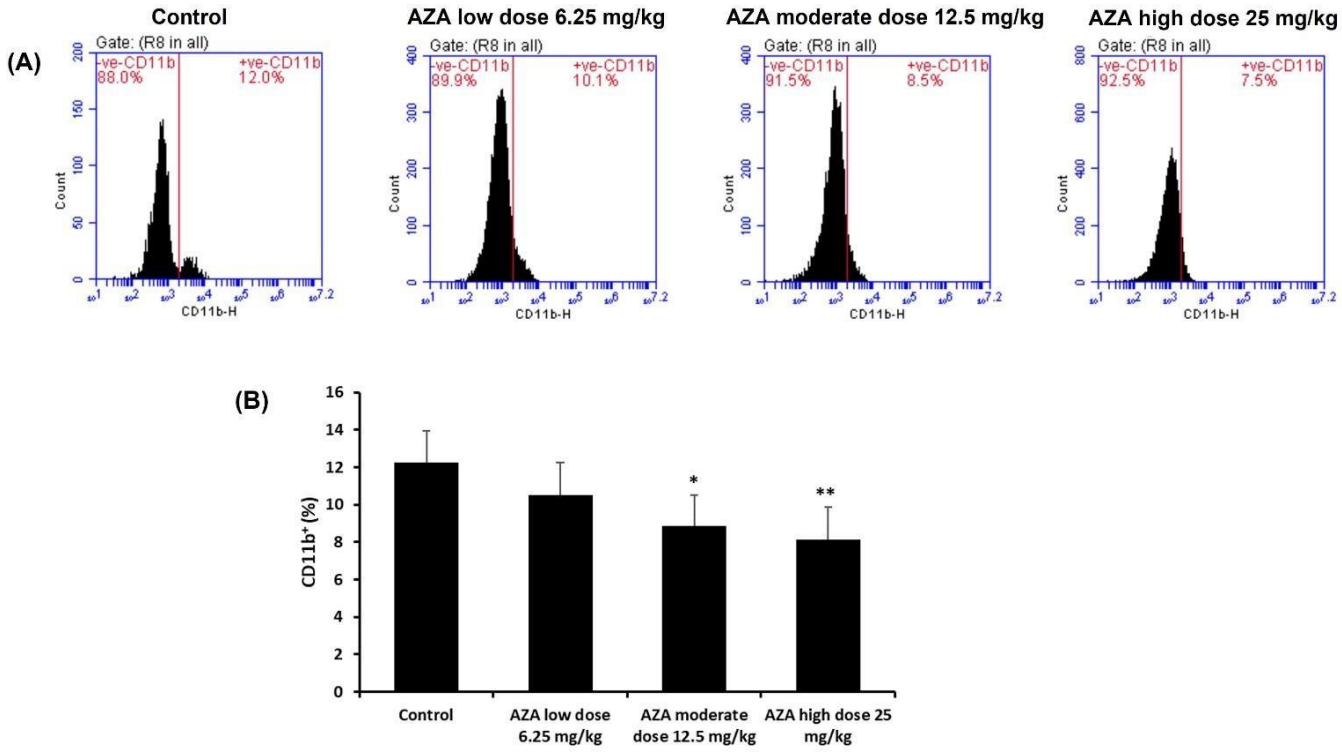

Figure 2. Effects of ascending doses of AZA on spleen $CD11b^+$ cells were gated by flow cytometry. (A) A dot plot of one representative experiment out of 5 independent trails is shown. (B) Pooled data from 5 experiments are expressed as the mean percentage of $CD11b^+$ cells ± SD. Statistical change was calculated with ANOVA test and follow-up test (LSD). * $P < 0.05$, ** $P < 0.01$ indicate significant difference compared to control group.

Impact of AZA different doses on antioxidant defense system

The administration of ascending doses of AZA resulted in a significant dose-dependent decrease in SOD, CAT, and GSH levels ($P < 0.01$) accompanied by a highly significant ($P < 0.01$) increase in MDA levels when compared to the control group (Table 3).

Table 3. Impact of azathioprine dosages on antioxidant defense system

	Control	**AZA low dose 6.25 mg/kg**	**AZA moderate dose 12.5 mg/kg**	**AZA high dose 25 mg/kg**
SOD(U/ml)	84.40±10.24	39.66±2.62**	32.53±3.67**	27.73±5.55**#
CAT (U/ml)	17.57±0.62	13.83±0.62**	11.78±0.66**#	9.70±0.32**#$
GSH (mmol/l)	5.15±0.17	2.67±0.52**	2.37±0.35**	2.32±0.32**
MDA (nmol/ml)	827.66±5.79	1018.33±99.0**	1107.33±125.46**	1124.66±7.36**

Results are expressed as mean ± SD, n = 5/group. ** $P < 0.01$ denote significant change compared to control group; # $P < 0.05$ denote significant change compared to AZA low dose (6.25 mg/kg); $ $P < 0.05$ denote significant change compared to AZA moderate dose (12.5 mg/kg). AZA: azathioprine, CAT: catalase, GSH: reduced glutathione, SOD: superoxide dismutase, MDA: malondialdehyde.

Impact of AZA different doses on liver and kidney functions

After treatment with both moderate and high doses of AZA, there was a gradual significant ($P < 0.01$) increase in ALT, AST, and creatinine levels compared to the control group. Furthermore, the low dose of AZA caused a highly significant ($P < 0.01$) increase in AST concentration. Only high dose of AZA induced a highly significant ($P < 0.01$) rise in urea levels compared to the control group. Interestingly, the negative observed impacts of AZA on liver and kidney functions were recorded in a dose dependent way (Table 4).

Table 4. Impact of azathioprine dosages on liver and kidney functions

	Control	AZA low dose 6.25 mg/kg	AZA moderate dose 12.5 mg/kg	AZA high dose 25 mg/kg
ALT(u/l)	24±4.54	28.32±5.24	42±3.74**#	51±4.89**#$
AST(u/l)	67±4.54	82.25±1.70**	92.32±3.39**#	103.32±6.12**#$
Urea (mg/dL)	19±1.63	20±0.81	20.5±2.85	26.5±1.22**#$
Creatinine (mg/dL)	0.81±0.09	0.84±0.03	1.01±0.01**#	1.85±0.06**#$

Results are expressed as mean ± SD, n = 5/group. ** $P < 0.01$ indicate significant difference compared to control group; # $P < 0.05$ indicate significant difference compared to AZA low dose (6.25 mg/kg); $ $P < 0.05$ indicate significant difference compared to AZA moderate dose (12.5 mg/kg). AZA: azathioprine, ALT: alanine aminotransferase, AST: aspartate aminotransferase.

Discussion

As far as our knowledge extends, no published document exists to date regarding the sub-chronic *in vivo* effects of AZA administration on blood coagulation indices in male albino rats for thirty days. This study represents the first comprehensive examination of the collective effects of sub-chronic administration of various ascending AZA doses on coagulation profile, blood counts, immune function parameters, oxidative stress markers, as well as hepatic and renal function tests in rats.

Clotting time serves as a qualitative measurement reflecting the factors involved in the intrinsic pathway. Consequently, any irregularities in the intrinsic pathway factors can impact clotting time (Tanko et al., 2012). Meanwhile, bleeding time evaluates both vascular and platelet responses in achieving hemostasis (Lind, 1984; Raaof et al., 2013). Furthermore, PT and aPTT provide insights into the functionality of the common coagulation pathway, with PT specifically measuring the extrinsic pathway and aPTT measuring the intrinsic pathway (Condrey et al., 2020).

By considering clotting time, bleeding time, platelet count, PT, and aPTT, we can comprehensively assess the integrity of the coagulation cascade and its response to AZA interventions. This holistic approach enhances our understanding of platelet function and coagulation dynamics in relation to the studied interventions. In the current study, the observed bleeding time, clotting time, and PT in AZA treated rats were consistently longer compared to the normal control rats. Notably, the most significant prolongation effects were observed in the AZA group treated with a dosage of 25 mg/kg body weight. These findings align with the general consensus that thrombocytopenia, as evidenced by reduced platelet count, contributes to a state of hypocoagulability in rats (Bashawri and Ahmed, 2007), which is consistent with the results obtained in our study. Thomason et al. (2018) conducted a previous study on dogs to examine the effects of five immunosuppressive drugs, including AZA, on the canine hemostatic system. The study found no significant difference in any hemostasis tests specifically related to AZA. However, it is important to note that the study only lasted for 7 days, while most clinical patients receiving immunosuppressive drugs require long-term treatment. Therefore, caution should be exercised when applying these findings to extended use of immunosuppressive drugs in clinical practice.

In addition to the detected thrombocytopenia our study revealed a dose dependent anemia with decreased RBCs count, Hb content, and Ht value, along with leucopenia with decreased differential relative lymphocytic count and relative increase in granulocytic count. These findings are correlated with those obtained from other previous studies (El-Ashmawy et al., 2010; Ahmed et al., 2014; Tochitani et al., 2022). Tochitani et al. (2022) reported the myelosuppressive effect of AZA through the decreased cellularity in the bone marrow that was reported previously via other researchers (Molyneux et al., 2008; Ahmed et al., 2014; Hassankhani et al., 2017). Ahmed et al. (2014) mentioned that AZA induced anemia may be due to the DNA synthesis inhibition in bone marrow precursors leaving both RNA & protein synthesis intact. Another investigation reported that the decreased numbers lymphocyte and monocyte counts were behind reduction in WBCs count after AZA treatment (Dewaal et al., 1995).

Previous studies have explored the negative effects of AZA, an immunosuppressive drug, on the immune system (Chang et al., 1983; Kabat-Koperska et al., 2016; El-sherbiny et al., 2021). However, our research aimed to expand this knowledge by specifically examining the immuno-toxic effects of AZA using different ascending doses. By investigating various doses, we aimed to understand how the drug's impact on the immune system varies depending on AZA administered amount. This information is crucial for healthcare professionals and researchers involved in prescribing and monitoring AZA, as it can improve patient care and safety. Our findings revealed that AZA inhibited both humoral and cellular immunity in rats, with dose-dependent effects. We observed reductions in serum total immunoglobulin levels, hemagglutination titers, and QHS, as well as suppressed DTH response, decreased proliferation of splenocytes when exposed to Con-A, and declines in $CD3^{+}C$, $D8^{+}CD3^{+}CD4^{+}$, and $CD11b^{+}$ spleen cells which might be rendered to bone marrow suppression and DNA interfering effects of AZA (Ahmed et al., 2014; Tochitani et al., 2022).

In a recent study conducted by El-sherbiny et al. (2021), it was observed that the administration of 5 mg AZA for 28 days led to a significant decrease in serum proteins and albumin. This decrease had an impact on the synthesis of Igs, as these proteins play a crucial role in their production. Hassankhani et al. (2017), have attributed these findings to the effects of AZA metabolites on processes such as DNA replication, translation, transcription, and ultimately protein synthesis, which are involved in the synthesis of these proteins. Disruptions in the synthesis processes from genes to proteins caused by AZA may explain the reduction in serum gamma globulins and protein levels (Hassankhani et al., 2017). Furthermore, it has been recorded that AZA administration results in a dose-dependent reduction in the total number of splenocytes and their functional activities, which aligns with our own findings (Matsumoto et al., 1990). Hassankhani et al. (2017), documented a significant decrease in $CD4^{+}$ and $CD8^{+}$ lymphocytes and suggested that this reduction may be attributed to the occurrence of leukopenia and the decreased division and proliferation ability of T lymphocytes induced by AZA administration, which is consistent with our study's results.

Our study revealed a clear presence of oxidative stress in the AZA groups compared to the control group. As the AZA dose increased, we observed a gradual decrease in the levels of important antioxidant markers including CAT, SOD, and GSH, associated with an increase in MDA levels. This decline in antioxidant production may be attributed to an increase in oxygen metabolites, which in turn suppresses the activity of the body's antioxidant defense-system (El-Beshbishy et al., 2010). Furthermore, we conducted a biochemical evaluation to assess the impact of AZA administration on liver and kidney functions. The results clearly indicated the development of hepatic and renal toxicities induced by the ascending doses of AZA. Specifically, we observed a significant increase in serum levels of AST, ALT, creatinine, and urea, particularly with the 12.5 mg and 25 mg doses of AZA. These findings align with previous research conducted by El-Ashmawy et al. (2010), which documented histopathological changes in the liver and a reduction in the antioxidant defense system following the administration of 25 mg of AZA. Similarly, another study conducted in 2016 revealed that a dose of 25 mg of AZA induced renal damage, evident from notable changes in the histological structure of the kidneys, significant increases in serum creatinine, urea, and MDA content in the tissue of the kidney (El-Ashmawy et al., 2016). These findings are consistent with our own results and can be explained by the metabolic effects of AZA on hepatic and renal tissues, leading to the generation of MDA, depletion of GSH, decreased ATP levels, mitochondrial injury, and cell death (Lee and Farrell 2001; Alghasham and Raza, 2011). These mechanisms ultimately contribute to increased levels of ALT, AST, urea, and creatinine, as demonstrated in our investigation.

Conclusion

Overall, our study demonstrates the obvious dose-dependent effects of AZA on hemostatic process, blood counts, immune responses, oxidative stress, and liver and kidney function. These findings highlight the potential risks and side effects associated with AZA administration, especially with 12.5 and 25 mg doses and emphasize the need for careful monitoring and dose optimization in clinical settings.

Acknowledgment

The authors gratefully acknowledge Prof. Dr. Ibrahim A. Elelaimy from Menoufia University for generously sharing valuable scientific comments that enhanced the quality of this manuscript.

Funding

Not applicable.

Conflict of interest

There is no conflict of interests regarding the publication of this article.

.References

Ahmed, W.M., Khalaf, A.A., Moselhy, W.A. and Safwat, G.M. (2014). Royal jelly attenuates azathioprine induced toxicity in rats. Environ Toxicol Pharmacol. 37(1):431-437. doi: 10.1016/j.etap.2013.12.010.

Alghasham, A.A. and Raza, M. (2011). Comparative protection by desferrioxamine against hepato- and nephro-toxicity induced by azathioprine. Int J Health Sci (Qassim). Jul 5(2 Suppl 1): 2– 3.

Baker, D.E. (2003). Pharmacogenomics of azathioprine and 6-mercaptopurine in gastroenterologic therapy. Rev Gastroenterol Disord.3(3):150-157.

Bashawri, L.A. and Ahmed, M.A. (2007). The approach to a patient with a bleeding disorder: for the primary care physician. J Family Community Med.14(2):53-58.

Bergan, S., Rugstad, H.E., Bentdal, O., Sødal, G., Hartmann, A., Leivestad, T. and Stokke, O. (1998). Monitored high-dose azathioprine treatment reduces acute rejection episodes after renal transplantation. Transplantation. 66(3):334-339. doi: 10.1097/00007890- 199808150-00010.

Bin-Hafeez, B., Haque, R., Parvez, S., Pandey, S., Sayeed, I. and Raisuddin, S. (2003). Immunomodulatory effects of fenugreek (*Trigonella foenum graecum* L.) extract in mice. Int Immunopharmacol. 3(2):257-265. doi: 10.1016/S1567-5769(02)00292-8.

Broen, J.C.A. and van Laar, J.M. (2020). Mycophenolate mofetil, azathioprine and tacrolimus: mechanisms in rheumatology. Nat Rev Rheumatol. 16(3):167-178. doi: 10.1038/s41584-020-0374-8.

Chang, K.Y., Cho, S.S. and Lee, W.J. (1983). Study on the inhibition of the immune response of the neonatal rat spleen by the azathioprine administered during pregnancy. Seoul Journal of Medicine. 24(4): 353-362.

Condrey, J.A., Flietstra, T., Nestor, K.M., Schlosser, E.L., Coleman-McCray, J.D., Genzer, S.C., Welch, S.R. and Spengler, J.R. (2020). Prothrombin time, activated partial thromboplastin time, and fibrinogen reference intervals for inbred strain 13/N Guinea pigs (*Cavia porcellus*) and validation of low volume sample analysis. Microorganisms. 8(8):1127. doi: 10.3390/microorganisms8081127.

Connell, W.R., Kamm, M.A., Ritchie, J.K. and Lennard-Jones, J.E. (1993). Bone marrow toxicity caused by azathioprine in inflammatory bowel disease: 27 years of experience. Gut. 34(8):1081-1085. doi: 10.1136/gut.34.8.1081.

Dacie, J.V. and Lewis, S.M .(1991): Practical haematology. Churchill Livingstone UK, 7th Ed.

Dewaal, E.J., Timmerman, H.H., Dortant, P.M., Kranjc, M.A. and Van Loveren, H. (1995). Investigation of a screening battery for immunotoxicity of pharmaceuticals within a 28-day oral toxicity study using azathioprine and cyclosporin A as model compounds. Regul Toxicol Pharmacol. 21: 327–338.

El-Ashmawy, I.M. and Bayad, A.E. (2016). Folic acid and grape seed extract prevent azathioprine-induced fetal malformations and renal toxicity in rats. Phytother, Res. 30(12):2027-2035. doi: 10.1002/ptr.5709.

El-Ashmawy, I.M., Gad, S.B. and Salama, M. (2010). Grape seed extract prevents azathioprine toxicity in rats. Phytother Res. 24(11):1710-1715. doi: 10.1002/ptr.3200.

El-Beshbishy, A.H., Tork, O.M., El-bab, M.F. and Autifi, M.A. (2010). Antioxidant and antiapoptotic effects of green tea polyphenols against azathioprine-induced liver injury in rats. Pathophysiology. 18(2):125-135 https://doi.org/10.1016/j.pathophys.2010.08.002.

Elelaimy, I., Elfiky, S., Hassan, A., Ibrahim, H. and Elsayad, R. (2012). Genotoxicity of anticancer drug azathioprine (Imuran): role of omega-3 (ω-3) oil as protective agent. JAPS. 2(4):14-23. doi: 10.7324/JAPS.2012.2404.

El-Sherbiny, E.M., Osman, H.F. and Taha, M.S. (2021). Effectiveness of *Echinacea purpurea* extract on immune deficiency induced by azathioprine in male albino rats. Bioscience Journal. 37: e37029. https://doi.org/10.14393/BJ-v37n0a2021-51270.

Fletcher, L. and Maddocks, J.L. (1980). Assay of thioinosinic acid, an active metabolite of azathioprine, in human lymphocytes. Br J Clin Pharmacol. 10(3):287-292. doi: 10.1111/j.1365-2125.1980.tb01757.x.

Fraser, A.G., Orchard, T.R. and Jewell, D.P. (2002). The efficacy of azathioprine for the treatment of inflammatory bowel disease: a 30 year review. Gut. 50(4):485-489. doi: 10.1136/gut.50.4.485.

Hassankhani, M., Aldavood, S.J., Khosravi, Sasani, F., Masoudifard, M., Ansari, F. and Taheri, M. (2017). The effects of prolonged azathioprine administration on blood cells, lymphocytes and immunoglobulins of Iranian mixed-breed dogs. Iran J Vet Med. 11(4):361-376. doi: 10.22059/ijvm.2017.225544.1004791.

Hassouna, I., Ibrahim, H., Abdel Gaffar, F., El-Elaimy, I. and Abd El Latif, H. (2015). Simultaneous administration of hesperidin or garlic oil modulates diazinon-induced hemato- and immunotoxicity in rats. Immunopharmacol Immunotoxicol. 37(5):442-449. doi: 10.3109/08923973.2015.1081932.

Ibrahim, H.M. (2014). Immunotoxicity of sub-chronic doses of diazinon in male albino Wister rats. Int J of Adv Res. **2**(2): 612-621.

Ibrahim, H.M., Xuan, X. and Nishikawa, Y. (2010). *Toxoplasma gondii* cyclophilin 18 regulates the proliferation and migration of murine macrophages and spleen cells. Clin Vaccine Immunol. 17(9):1322-1329. doi: 10.1128/CVI.00128-10.

Ibu., J.O and Adeniyi., K.O. (1989): A manual of practical physiology. Jos University Press, Jos, Nigeria.

Johnson, P.J., McFarlane, I.G. and Williams, R. (1995). Azathioprine for long-term maintenance of remission in autoimmune hepatitis. N Engl J Med. 333(15):958-963. doi: 10.1056/NEJM199510123331502.

Kabat-Koperska, J., Kolasa-Wołosiuk, A., Wojciuk, B., Wojciechowska-Koszko, I., Roszkowska, P., Krasnodębska-Szponder, B., Paczkowska, E., Safranow, K., Gołembiewska, E., Machaliński, B. and Ciechanowski, K. (2016). Changes in the immune system of female Wistar rats after exposure to immunosuppressive treatment during pregnancy. Scand J Immunol. 83(6):418-426. doi: 10.1111/sji.12434.

Lee, A.U. and Farrell, G.C. (2001). Mechanism of azathioprine-induced injury to hepatocytes: roles of glutathione depletion and mitochondrial injury. J Hepatol. 35: 756– 764.

Lennard, L. (2002). TPMT in the treatment of Crohn's disease with azathioprine. Gut. 51(2):143-146. doi: 10.1136/gut.51.2.143.

Lind, S.E. (1984). Prolonged bleeding time. Am J Med. 77(2):305-312. doi: 10.1016/0002-9343(84)90707-1.

Matsumoto, K., Sekita, K., Ochiai, T., Takagi, A., Takada, K., Furuya, T., Kurokawa, Y., Saito, Y., Teshima, R. and Suzuki, K., et al. (1990). Evaluation of immunotoxicity testings using azathioprine-treated rats: the International Collaborative Immunotoxicity Study (Azathioprine) Eisei Shikenjo Hokoku. (108):34-39. Japanese. PMID: 1364358.

Meijer, B., Wilhelm, A.J., Mulder, C.J.J., Bouma, G., van Bodegraven, A.A.and de Boer, N.K.H. (2017). Pharmacology of thiopurine therapy in inflammatory bowel disease and complete blood cell count outcomes: A 5-year database study. Ther Drug Monit. 39(4):399-405. doi: 10.1097/FTD.0000000000000414.

Molyneux, G., Gibson, F.M., Chen, C.M., Marway, H.K., McKeag, S., Mifsud, C.V., Pilling, A.M., Whayman, M.J. and Turton, J.A. (2008). The haemotoxicity of azathioprine in repeat dose studies in the female CD-1 mouse. Int J Exp Pathol. 89(2):138-158. doi: 10.1111/j.1365-2613.2008.00575.x.

Morsi, D.S., Barnawi, I.O., Ibrahim, H.M., El-Morsy, A.M., El Hassab, M.A. and Abd El Latif, H.M. (2023). Immunomodulatory, apoptotic and anti-proliferative potentials of sildenafil in Ehrlich ascites carcinoma murine model: *In vivo* and in silico insights. Int Immunopharmacol. 119:110135. doi: 10.1016/j.intimp.2023.110135.

Patel, A.A., Swerlick, R.A. and McCall, C.O. (2006). Azathioprine in dermatology: the past, the present, and the future. J Am Acad Dermatol. 55(3):369-389. doi: 10.1016/j.jaad.2005.07.059.

Pedersen, E.G., Pottegård, A., Hallas, J., Friis, S., Hansen, K., Jensen, P.E. and Gaist, D. (2014). Risk of non-melanoma skin cancer in myasthenia patients treated with azathioprine. Eur J Neurol. 21(3):454-458. doi: 10.1111/ene.12329.

Raaof, A., Al-Naqqash, Z.A., Jawad, A.M. and Muhsan, S.M. (2013). Evaluation of the activity of crude alkaloids extracts of *Zingiber officinale* Roscoe., *Thymus vulgaris* L. and *Acacia arabica* L. as coagulant agent in lab mice. Biomedicine and Biotechnology. 1(2): 11-16. doi: 10.12691/bb-1-2-3.

Simpson, M.A. and Gozzo, J.J. (1978). Spectrophotometric determination of lymphocyte mediated sheep red blood cell hemolysis *in vitro*. J Immunol Methods. 21(1-2):159-165. doi: 10.1016/0022-1759(78)90232-6.

Song, Q., Wang, S. and Zhao, W. (2012). Total steroidal alkaloids from *Veratrum patulum* L. inhibit platelet aggregation, thrombi formation and decrease bleeding time in rats. J Ethnopharmacol. 141(1):183-186. doi: 10.1016/j.jep.2012.02.017.

Tanko, Y., Eze, E.D., Jimoh, A., Yusuf, K., Mohammed, K.A., Balarabe, F. and Mohammed, A. (2012). Hemostatic effect of aqueous extract of mushroom (*Ganoderma lucidum*). European Journal of Experimental Biology.2(6): 2015–2018.

Thomason, J.M., Archer, T.M., Wills, R.W. and Mackin, A.J. Effects of immunosuppressive agents on the hemostatic system in normal dogs. J Vet Intern Med, 2018; 32:1325-1333. https://doi.org/10.1111/jvim.15132.

Tiede, I., Fritz, G., Strand, S., Poppe, D., Dvorsky, R., Strand, D., Lehr, H.A., Wirtz, S., Becker, C., Atreya, R., Mudter, J., Hildner, K., Bartsch, B., Holtmann, M., Blumberg, R., Walczak, H., Iven, H., Galle, P.R., Ahmadian, M.R. and Neurath, M.F. (2003). CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human $CD4^+$ T lymphocytes. J Clin Invest. 111(8):1133-1145. doi: 10.1172/JCI16432.

Tochitani, T., Sasaki, Y., Nishimura, N., Fujii, Y., Iwaisako, T., Umeya, N., Hashimoto, M., Inada, H., Chihara, K. and Miyawaki, I. (2022). Effects of microsampling on toxicity assessment of hematotoxic compounds in a general toxicity study in rats. J Toxicol Sci, 20