Original article

EFFECTS OF DRUG BETAMETHASONE ON HEMATOLOGICAL PARAMETERS OF *RATTUS NORVEGICUS* (BERKENHOUT 1769) *IN VIVO* CONDITIONS

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ABSTRACT

Glucocorticoids prevent or suppress the full inflammatory response to the infectious, immunological or physical agents by inhibiting early inflammatory events such as edema or cell exudation. The main effect of glucocorticoids on the inflammatory process is the inhibition or recruitment of neutrophils and monocytes. In this study, the effect of the glucocorticoid betamethasone on the hematological parameters of rats of the species *Rattus norvegicus* Berkenhout 1769 was examined *in vivo*. Betamethasone has been shown to affect the values of hematological parameters four hours after application and leads to a significant decrease in the prevalence of lymphocytes and monocytes, but also leads to a statistically significant increase in erythrocyte count, hemoglobin concentration and hematocrit values.

Keywords: Glucocorticoid, hematology, *in vivo* condition,

INTRODUCTION

Corticosteroids are a class of steroid hormones secreted by the adrenal cortex and are divided into mineralocorticoids and glucocorticoids [1]. In addition to affecting carbohydrate metabolism, glucocorticoids regulate other processes such as cell growth and development, metabolism, maintaining homeostasis and suppressing the immune (immune) response, which is why they are widely used as therapeutic agents against a large number of different diseases, including inflammation, autoimmune diseases, and cancer [2, 3]. Corticosteroids are metabolized in the liver by cytochrome P450 3A4 (CYP450) through enzymatic transformations that reduce their physiological activity and increase water solubility to increase their urinary excretion [4]. As a general rule, the metabolism of steroid hormones involves the sequential addition of oxygen and hydrogen atoms, followed by conjugation with water-soluble derivatives [5]. Double bond reduction exists in both hepatic and extrahepatic tissues of corticosteroid metabolism to give inactive compounds [5]. Corticosteroid metabolism occurs primarily in the liver. Certain liver diseases resulted in elevated free hormone due to decreased corticosteroid metabolism and decreased serum steroid-binding protein concentrations [4]. Betamethasone (16β-methyl-9α-fluoroprednisolone) is a long-acting synthetic fluorinated glucocorticoid with metabolic, immunosuppressive, anti-inflammatory and antiproliferative effects and is most commonly used for topical application in the treatment of dermatoses [3,6].

Cells that synthesize steroid hormones - testicular, ovarian and adrenal gland cells are endocrine cells morphologically and therefore physiologically adapted to produce and secrete steroid hormones. They have a particularly developed smooth endoplasmic reticulum, which contains enzymes necessary for the synthesis of cholesterol from acetate and other substrates, as well as for the transformation of pregnenolone produced in mitochondria into sex hormones. Pregnenolone is formed in the mitochondria by breaking the side chains of cholesterol in enzymatically catalyzed reactions. Steroid hormones, unlike some other hormones, are synthesized and secreted as needed, and their excretion does not require an exocytosis process [1]. The classical genomic actions of glucocorticoids are mediated by the glucocorticoid receptor (GR). In the absence of hormones, GR is mainly found in the cytoplasm of cells as part of a large multiprotein complex that includes chaperones (hsp90, hsp70, and p23) and immunophilins (tacrolimus-binding protein 51, FKB51, and tacrolimus-

binding protein 4, FKB52). The multiprotein complex maintains GR in a conformation conducive to high-affinity ligand binding. Ligand binding results in a conformational change in GR, resulting in dissociation of the multiprotein complex. This leads to structural reorganization of the GR and exposure of the two nuclear localization signals, and the ligand-GR complex is rapidly translocated to the nucleus through the nuclear pores. When it enters the nucleus, GR binds directly to (GRE) (a special domain of nuclear receptors called glucocorticoid response elements) and stimulates the expression of the target gene [2]. Glucocorticoid treatment leads to an increase in polymorphonuclear leukocytes in the blood as a result of an increased rate of bone marrow entry and a reduced rate of elimination from vascular compartments. In contrast, the number of lymphocytes, eosinophils, monocytes, and basophils decreases after glucocorticoid administration. A single dose of cortisol reduces the number of lymphocytes by 70% and monocytes by 90% four to six hours after treatment and persists for the next 24 hours. The number of cells then increases 24 to 72 h after treatment application [4]. Capillary and fibroblast proliferation, collagen deposition, and scarring are also inhibited by glucocorticoids [4].

The anti-inflammatory mechanism of glucocorticoids, which is not fully elucidated, is of great therapeutic importance and is the subject of intensive scientific research [4]. Glucocorticoids reduce the ability of neutrophils to bind to capillary endothelial cells by a double mechanism. They block the normal increase in the expression of endothelial adhesion molecules (ELAM-1) and intracellular adhesion molecules (ICAM1) and induce lipocortin, a phospholipase A2 inhibitor protein (PLA2). As the PLA2 enzyme is involved in prostaglandin synthesis, glucocorticoids ultimately reduce the synthesis and release of prostaglandin mediators of cell adhesion. The ability of GR to repress the activity of NF-κB and AP-1, as well as other key immunomodulatory transcription factors, is the main focus of research on the mechanisms underlying the anti-inflammatory effects of glucocorticoids [7]. During T cell development, immature thymocytes progress from double-negative to double-positive cells (CD4 + CD8 +) that are subject to positive selection (only thymocytes that bind MHC in complex with their own antigen survive) and negative selection (against cells that interact too strongly with their own antigens) to mature either CD4 + or CD8 + cells [7]. Double-positive cells, which make up the majority of the thymocyte population, are extremely sensitive to glucocorticoid-induced apoptosis and at physiological glucocorticoid levels [8, 9]. The glucocorticoid receptor (GR) in myeloid cells, but not in T cells, is required for glucocorticoid suppression of contact allergy (T cell-dependent delayed type of hypersensitivity response that exists, e.g. in response to metals or poison ivy) [10] The immunosuppressive effects of GR are likely the result of a number of mechanisms that are dependent on cell type and stimulus type [7]. In addition to affecting immune cell function, glucocorticoids also alter progenitor cell differentiation programs [11].

Thus, chronic stress states (in which the production of endogenous glucocorticoids is high) or glucocorticoid pharmacotherapy can alter the differentiation of immune system cells and possibly shape the immune response as it develops [12].

MATERIALS AND METHODS

Animals: The research was conducted on laboratory rats of the species *Rattus norvegicus* (Wistar strain) Berkenhout, 1769, bred in the vivarium of the Department of Biochemistry and Physiology, Department of Biology, University of Sarajevo, BiH. The laboratory rat, *Rattus norvegicus*, belongs to the order Rodentia of the family Muridae. They are characterized by short fur and a long bare tail, rounded erect ears, a pointed snout with long vibrations and five toes on each foot. Laboratory rats of the Wistar strain are albino due to a mutation in the tyrosinase gene, have red eyes and have poor eyesight. They can reach a length of up to 400 mm and a weight of 140 to 500 g.

Experimental data and methods:

The study examined the effect of (different) concentrations of betamethasone (pure active substance), as well as betamethasone itself, on the hematological parameters of 10 individuals of *Rattus norvegicus* divided into two groups. The first group of five was administered intraperitoneally (i.p.) betamethasone at a concentration of 0.2 mg/kg body weight, while the second group of five individuals was administered intraperitoneally betamethasone at a concentration of 0.4 mg/kg body weight. In both groups, the rats were three months old, all individuals were bred in the same environmental conditions, and at the very beginning of the study, the individuals were given body weight to administer the correct dose of betamethasone.

A total of six males and four females were used in the experiment. A heart puncture was performed four hours after betamethasone administration. During the puncture of the heart, syringes with EDTA (ethylenediaminetetraacetic acid) were used, from which the blood was transferred to a test tube, also with EDTA, which was used as an anticoagulant. Analyzes were performed immediately after the heart puncture was completed. Leukocyte count (WBC) determination was performed by a standard method in a hemocytometer using Türk's reagent. Differential blood count was determined microscopically by examination of peripheral blood smears stained by the Pappenheim method [13]. Determination of erythrocyte count (RBC) was performed by a standard method in a hemocytometer using Hayem reagent. Hemoglobin concentration was determined spectrophotometrically using Drabkin's reagent. The method is based on the oxidation of hemoglobin and its derivatives other than sulfhemoglobin in the presence of potassium ferricyanide to methaemoglobin which reacts with potassium cyanide to the stable compound cyanomethaemoglobin, which has a maximum absorption at 540 nm [13]. To determine the hematocrit, we used the microhematocrit method, centrifuging the blood for five minutes at 16,000 rpm. Hematocrit (Hct) is the ratio of blood elements to plasma obtained after centrifugation. Hematological indices were the mean erythrocyte volume (MCV), the average amount of hemoglobin in one erythrocyte (MCH) and the average hemoglobin concentration in erythrocytes (MCHC) calculated mathemati-

cally based on the values of the total number of erythrocytes, hematocrit and hemoglobin.

Statistical methods

Analysis and processing of results were done by the method of descriptive and analytical statistics using Microsoft Excel 2013, PAST (Paleontological Statistics) 3.25 and Minitab 19. As mathematical and statistical indicators were used: arithmetic mean (mean), standard deviation, minimum and maximum value, mode, variance, coefficient of variation, median, skewness, kurtosis, upper control line, lower control line, t-test, analysis of variance, Kolmogorov-Smirnov and Shapiro-Wilk test.

RESULTS

The specimens of both experimental groups of rats of the species *Rattus norvegicus* Berkenhout, 1769, were determined sex and body weight from morphometric parameters and from hematological parameters, total leukocyte count, differential blood count, erythrocyte count, hemoglobin concentration, hematocrit and hematological indices: MCV, MCH and MCHC. After collecting the samples and their analysis, the results were obtained, which were statistically processed and presented in a table. Descriptive statistics for body weight are shown in Table 1.

Table 1. Body weight of the control and experimental group of rats *Rattus norvegicus*

	Mass of individuals of the first experimental group (g)	Mass of individuals of the second experimental group (g)
Number of individuals (N)	5	5
Average value	207.40	179.20
Standard deviation	52.24	49.29
Minimum value	148.00	120.00
Maximum value	280.00	250.00
Mod	-	-
Skewness	0.49	0.47
Courtesy	-0.91	0.08
Coefficient of variation	25.19	27.50

In Table 1, we see that in the individual of the first group, a positive symmetric dispersion of data (skewness) in relation to the ordinate was determined, as well as a dispersion of data per abscissa (kurtosis) which was -0.91, i.e. platicurtic distribution of data. The mean body weight of the individuals inthe second group was 179.20 ± 49.29 g, with a relatively wide range of individual variation from 120.00 g to 250.00 g and a coefficient of variation of 27.50%. There was no recurring value in either group. From the above, we notice that the mean value of body weight was higher in individuals of the first group compared to individuals in the second group, while the coefficient of variation was lower in the first group compared to the second. The results of the examined hematological parameters are given in Table 2.

Table 2. Results of hematological parameters of the first and second experimental groups of rats *Rattus norvegicus*

Hematologically parameters	[Betamethasone] 0.2 mg/kg First group	[Betamethasone] 0.4 mg/kg Second groupa	t test p	ANOVA p	Kolmogorov-Smirnov test p	Shapiro-Wilk test p
Leukocytes (x 109/L of blood)	4.93 ±0.25	6.51±0.23	0.00	0.00	0.01	0.28
Lymphocytes (%)	64.00±2.83	51.00±2.55	0.00	0.00	0.01	0.06
Segmented neutrophils (%)	25.80±1.92	36.20±1.48	0.00	0.00	0.01	0.73
Unsegmented neutrophils (%)	3.40±0.89	8.60±1.82	0.00	0.00	0.01	0.45
Eosinophils (%)	0.60±0.55	1.20±0.84	0.22	0.22	0.55	0.37
Monocytes (%)	6.20±1.10	3.00±1.58	0.01	0.01	0.08	0.58
Erythrocytes (x 1012/L of blood)	6.10±0.33	7.28±0.27	0.00	0.00	0.01	0.45

Hemoglobin (g/dL)	121.58±6.65	145.71±4.98	0.00	0.00	0.01	0.62
Hematocrit (L/L)	0.33±0.02	0.40±0.02	0.00	0.00	0.01	0.65
MCV (fL)	54.77±0.38	54.97±0.29	0.38	0.37	0.80	0.72
MCH (pg)	19.93±0.10	20.03±0.10	0.18	0.16	0.21	0.63
MCHC (g/dL)	363.94±2.71	364.34±2.14	0.80	0.80	1.00	0.30

The mean value of the total leukocyte count of the first experimental group of rats was $4.93 \pm 0.25 \times 10^9$/L of blood, with a range of variation from 4.65 to 5.20×10^9/L of blood. The coefficient of variation was 5.10%. There were no repetitive values.

Negative symmetric dispersion of data by ordinate (skewness) and negative dispersion by abscissa (kurtosis), i.e. platycurtic distribution of data, were observed. The mean value of the total number of leukocytes in the second experimental group of rats was $6.51 \pm 0.23 \times 10^9$/L of blood, with a range of variation from 6.30 to 6.85×10^9/L of blood.

The coefficient of variation was 3.54%.

The value of the total number of recurrent leukocytes was 6.30×10^9/L of blood. When we observe the average value of the number of erythrocytes in the first experimental group of rats, it is $6.10 \pm 0.33 \times 10^{12}$/L of blood with a range of variation from 5.79 to 6.63×10^{12}/L of blood. The coefficient of variation was 5.49%. There were no repetitive values. Positive asymmetric dispersion of data by ordinate (skewness) and positive platycuric dispersion by abscissa (kurtosis) were observed. The mean erythrocyte count of the second experimental group of rats was $7.28 \pm 0.27 \times 10^{12}$/L blood with a range of 6.96 to 7.60×10^{12}/L blood. In rats of the first group, a positive moderately asymmetric dispersion of data (skewness) in relation to the ordinate and a positive platycurtic dispersion of data per abscissa (kurtosis) were determined for the value of hemoglobin. The mean hemoglobin concentration of rats in the second experimental group was 145.71 ± 4.98 g/dL with a range of 139.48 to 152.20 g/dL. The mean hematocrit of rats of the first group was 0.33 ± 0.02 L/L with a range of individual variation from 0.32 to 0.36 L/L and a coefficient of variation of 5.01%. The repeated value was 0.32 L/L. The mean hematocrit value of rats in the second experimental group was 0.40 ± 0.02 L/L with a

range of variation from 0.38 to 0.42%. The coefficient of variation was 3.95%. There were no repetitive values. Analyzing the results from Table 2, we see that given the clear differences in the total number of leukocytes, lymphocytes, segmented and non-segmented neutrophils, erythrocytes, hemoglobin and hematocrit concentration, between the first and second experimental second, t-test and ANOVA indicate statistically significant values ($p <0.05$). T-test and ANOVA do not indicate significant differences when comparing the presence of eosinophils, monocytes and hematological indices in individuals of the first and second experimental groups ($p> 0.05$).

The Kolmogorov-Smirnov and Shapiro-Wilk tests indicate a normal distribution of eosinophils. The presence of lymphocytes is significantly lower in the second experimental group compared to the first. The Kolmogorov-Smirnov lymphocyte test rejected the normality of data distribution, while the Shapiro-Wilk test accepted the normality. We notice that the mean values of hematocrit, hemoglobin, erythrocytes, eosinophils, non-segmented neutrophils, segmented neutrophils, leukocytes, and lymphocytes are higher in individuals of the second experimental group to which a higher concentration of betamethasone was applied and that the coefficient of variation is lower in individuals in the same group first experimental groups. The value of the percentage of monocytes was higher in the individuals of the first experimental group to which a lower concentration of betamethasone was applied, as well as a much lower coefficient of variation compared to the individuals of the second group. Also, in Table 2, we see that the values of hematological indices (MCV, MCH and MCHC) are approximately the same in individuals of the first and second groups. Differences in the percentage of leukocyte forms between the first and second experimental groups are also presented in Graph 1.

Graph 1. Percentage of individual forms of leukocytes of *Rattus norvegicus* rats of the first (0.2 mg/kg betamethasone) and second (0.4 mg/kg betamethasone) experimental groups.
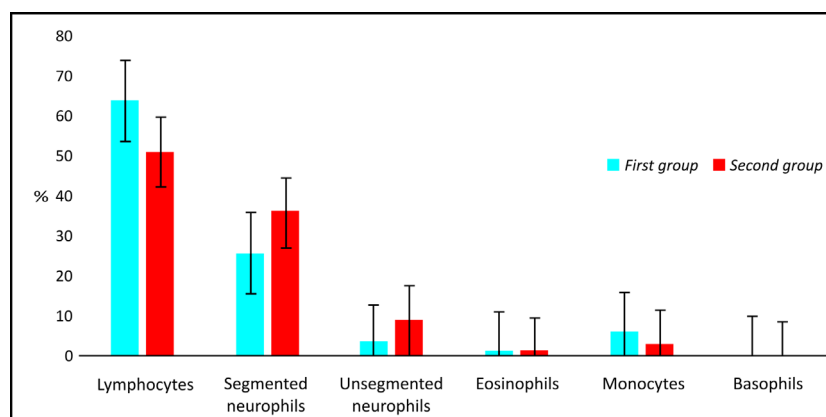
DISCUSSION

Betamethasone is, as previously written, a long-acting synthetic fluorinated glucocorticoid with metabolic, immunosuppressive, anti-inflammatory and antiproliferative effects [3, 6]. Like all other glucocorticoids, it has a systemic effect and is, therefore, a widely used therapeutic. Consequently, immunosuppressive, anti-inflammatory and antiproliferative effects will result, among other things, in a change in the total number of leukocytes as well as in an altered differential blood picture of the individual. Glucocorticoids, including betamethasone, also affect erythropoiesis and erythrophagocytosis, leading to an increase in the number of erythrocytes, hemoglobin and hematocrit [4]. All these effects are transient. The units of the first experimental group were administered betamethasone at a concentration of 0.0002 mg/g body weight, while the units of the second experimental group were administered betamethasone at a concentration of 0.0004 mg/g body weight. Comparing the hematological parameters of these two groups, a significant increase in the total number of leukocytes ($p = 0.00$) of individuals in the second experimental group was observed, probably as a result of an increase in the number of polymorphonuclear leukocytes as a result of increased bone marrow entry rate and decreased vascular compartment [4]. All this corresponds to previous research on this issue [14-17]. When it comes to differential blood counts, there is a significant increase in the representation of segmented ($p = 0.00$) and non-segmented neutrophils ($p = 0.00$) and a significant decrease in the representation of lymphocytes ($p = 0.00$) and monocytes ($p < 0.05$) in individuals of the second group. A higher concentration of betamethasone was administered compared to the subjects of the first experimental group four hours after application.

Betamethasone administration did not show significant changes in the presence of eosinophils and basophils ($p > 0.05$). The biological effects contributing to the increase in circulating polymorphonuclear leukocytes are multifactorial, with neutrophil demargination as the most important factor and delayed transmigration of polymorphonuclear leukocytes into tissues, delayed apoptosis and enhanced release of rod-shaped (unsegmented) neutrophils from the bone marrow into the circulation [18-23]. The decrease in the number of lymphocytes and monocytes is thought to be due to the redistribution of these cells. Additionally, certain lymphocytes undergo glucocorticoid-induced apoptosis, and T-lymphocytes are more sensitive to glucocorticoid-induced apoptosis than B-lymphocytes, and T cell subpopulations are differently sensitive to glucocorticoids [4]. Betamethasone therapy caused a transient increase in total leukocyte counts in pregnant women, and an increase in neutrophil count and a decrease in lymphocyte count was noted immediately two hours after the first injection [14]. Studies of the effects of betamethasone in pregnant women on white blood cells in preterm infants and other patients gave results equal to the results of our research [15-25]. Betamethasone, in all cases, caused a significant increase in the total number of leukocytes, seg-

mented and non-segmented neutrophils, and a significant decrease in the number of lymphocytes. Four hours after corticosteroid administration, for the purposes of one research work, there was a decrease in the number of lymphocytes and monocytes [26]. Monocyte production and release have been shown to be suppressed in mice injected with hydrocortisone, which may be somewhat related to the results of this study since hydrocortisone, as well as betamethasone, is a corticosteroid [27]. Circulating lamb monocytes of sheep that received corticosteroid injections before farrowing had reduced in vitro responses to endotoxin stimulation [28]. Corticosteroids cause decreased monocyte chemotaxis, migration, bactericidal activity, and phagocytosis [29]. On the other hand, corticosteroid therapy has led to an increase in the number of monocytes in pregnant female rats, which speaks in favor of the fact that the effect of corticosteroids on monocytes has not been sufficiently investigated and raises many questions [30]. There was a significant increase in erythrocyte count ($p = 0.00$), hemoglobin concentration ($p = 0.00$) and hematocrit ($p = 0.00$) in individuals of the second experimental group to whom a higher concentration of betamethasone was applied, which is consistent with most previous studies [14, 16]. Betamethasone had no significant effect on the values of hematological indices MCV, MCH, MCHC ($p > 0.05$). The increase (number) of erythrocytes is probably due to the fact that erythropoiesis in normal bone marrow is stimulated by corticosteroid therapy [31]. Glucocorticoids prolong the expression of genes that antagonize and slow down the induction of genes that trigger terminal erythroid differentiation [32]. Also, corticosteroids increase hemoglobin concentration and erythrocyte count, probably slowing erythrophagocytosis. This effect has been demonstrated by the occurrence of polycythemia in Cushing's disease and mild normochromic anemia in Addison's disease [4]. One study showed that steroid therapy is very effective in increasing hemoglobin concentration ($p = 0.015$) in hemolytic crises and leads to a 20% increase in hemoglobin concentration [33]. On the other hand, a study in pregnant women showed that betamethasone leads to a decrease in erythrocyte count and a decrease in hemoglobin concentration, and a study in pregnant and non-pregnant female rats showed that hydrocortisone also leads to a decrease in erythrocyte count and decreased hemoglobin in non-pregnant females, which is contrary to the results of our study [17, 30].

The application of betamethasone gave results similar to the results of most studies conducted on the hematological effects of betamethasone and other corticosteroids. The experimental group of rats to which betamethasone was administered at a higher concentration had a significantly higher total number of leukocytes, segmented and non-segmented neutrophils, reduced lymphocyte and monocyte count, increased erythrocyte count, higher hemoglobin concentration and higher hematocrit values compared to the first experimental group-administered betamethasone at a lower concentration.

CONCLUSION

Based on statistically processed data and analysis of the obtained results of hematological parameters, it can be concluded that betamethasone significantly affects the values of hematological parameters four hours after application. We also see that betamethasone leads to a statistically significant increase in erythrocyte count, hemoglobin concentration, and hematocrit. Higher drug concentrations significantly affect all examined hematological parameters. These changes were pronounced regardless of the sex of the individual, and it was observed that betamethasone also leads to a significant decrease in the representation of lymphocytes and monocytes in the tested individuals. Given the results obtained, we can conclude that supervision must be ensured during treatment with topical corticosteroids, as a distinction can be made between successful treatment and exacerbation of the disease.

ABBREVIATIONS:

P450 3A4 (CYP450) - cytochrome;

GR - glucocorticoid receptor;

FKB51 - tacrolimus-binding protein 51;

FKB52 - tacrolimus-binding protein 4;

ELAM-1 - endothelial adhesion molecules;

ICAM1 - intracellular adhesion molecules;

PLA2- phospholipase A2 inhibitor protein;

EDTA - ethylenediaminetetraacetic acid;

WBC - leukocyte;

RBC - erythrocyte;

MCV - hematological indices were the mean erythrocyte volume;

MCH - the average amount of hemoglobin in one erythrocyte;

MCHC - the average hemoglobin concentration in erythrocytes;

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