Saudi Journal of Biological Sciences 27 (2020) 3157–3160

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

The effect of different sample collection methods on rabbit blood parameters

Martin Massányi [a,*,1], Ladislav Kohút [b], María-José Argente [c], Marko Halo [a], Anton Kováčik [d], Eva Kováčiková [e], Ľubomír Ondruška [f], Grzegorz Formicki [g], Peter Massányi [d]

[a] Department of Animal Husbandry, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture, Nitra, Slovak Republic
[b] Department of Small Animal Science, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture, Nitra, Slovak Republic
[c] Departamento de Tecnología Agroalimentaria, Universidad Miguel Hernández de Elche, Orihuela, Spain
[d] Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Nitra, Slovak Republic
[e] Research Centre AgroBioTech, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic
[f] Institute of Farm Animals, Animal Production Research Centre Nitra, Luzianky, Slovak Republic
[g] Department of Animal Physiology, Institute of Biology, Faculty of Exact and Natural Sciences, Pedagogical University of Cracow, Cracow, Poland

ARTICLE INFO

Article history:
Received 5 April 2020
Revised 11 June 2020
Accepted 13 July 2020
Available online 17 July 2020

Keywords:
Rabbit
Blood collection
Haematology
Cortisol
Mineral profile

ABSTRACT

Nowadays great deal of research is physiological field is conducted on experimental animals and there is a lot of criticism from the wide public on methods used. Therefore, recently there is a lot of effort focused on the welfare of the animals. Main aim of this study is to determine the effect of experimental sample collection method on the selected parameters of stress. In the experiment two sample collections of rabbit blood from marginal ear vein were realized – first using standard method with one person fixing the animal and other collecting the blood using gently fixating the animal. In the second groups experimental method of inserting the experimental animal into a sack and further collection in dark was realized. During the experiment the levels of cortisol – main stress indicator in organism and other health parameters of animals including mineral profile and haematological parameters were observed. Our results show no significant changes in levels of cortisol but also a decreasing tendency in the sample from the second (dark) collection. Haematological parameters were generally in the reference values and any significant changes except levels of lymphocytes and percent of lymphocytes which shown significant increase in the second collection period were found. Also the levels of mean corpuscular haemoglobin and percent of neutrophils unveiled a significant decrease in values. Values of mineral profile parameters have indicated no significant changes except the levels of phosphorus. Based on the result we can state that the experimental sample collection has no effect on blood parameters of the animals but we spectated a statistically insignificant decrease in the levels of cortisol which can suggest that the dark collection is possibly less stressful to the animals.

1. Introduction

Generally, there are many types of experimental animals which frequently undergo different sample collections. For the welfare of the animals it is important to update or change the methods to lower the amount of stress as much as possible for the sake of animals well-being as well as it can cause changes in the results of measured parameters. Collection of blood from laboratory animals is necessary for a wide range of scientific research and there are number of available methods that are efficient (Parasuraman et al., 2010).

Rabbit ear artery and vein are the most common locations used to obtain a blood sample in the practice. The rabbit may be gently restrained in dorsal or lateral recumbency. Fractious animals may

* Corresponding author at: Department of Animal Husbandry, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovak Republic.
E-mail address: martinmassanyi@yahoo.com (M. Massányi).
[1] Permanent address: Panská dolina 70, 949 01 Nitra, Slovak Republic.
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2020.07.016
1319-562X/

need to be anesthetized or lightly sedated (Jeffrey, 2008). Since few information are reported on testing of different sample collecting techniques, we decided to test a different method of blood collection to describe possible differences in levels of stress. Stress is a sum of non-specific regulation reactions which engage in the case of possible danger to internal homeostasis. Stress reaction is activated by certain parts of nervous and endocrine system. Stress causes mobilization of nutrients and its transport to the tissues with priority supply – result being adjustment of fluctuating values of internal organism environment (Kováčik et al., 2015).

Cortisol is a hormone from the group of glucocorticoids and is produced by the adrenal cortex. It increases the level of glucose in blood as it stimulates degradation of muscle proteins to amino acids captured by liver from the bloodstream (Poráčová et al., 2015) and supports metabolism of fatty acids more than metabolism of saccharides and conserves glucose for the brain – secures complex regulation of energy metabolism (Longenbaker, 2011). Cortisol is also a potent anti-inflammatory hormone and its dysfunction is likely to result in widespread inflammation following the reactivation of acute pro-inflammatory stress response (Hannibal and Bishop, 2014). Glucocorticoid steroids inhibit fibroblast growth and specific cell products output (i.e. collagen and mucopolysaccharide) *in vivo* (Pratt, 1978). ACTH is responsible for vital role in regulating the adrenal steroid synthesis and secretion and also exerts profound trophic effect on the adrenal gland (Rhodes, 2017).

Stressors are a potential threat to the organism, whereas the stress response is integral to adapting to the stressor. Stressors both physical and psychological are pervasive and challenge the well-being of living organism (Shors and Horvath, 2001). Stressors are load factors affecting the organism from the environment.

Physiological research in animal breeding is the first step to improve welfare and possibly intensification of experimental processes in certain population. The aim of the study was to observe the difference between two different blood sampling methods *in vivo* in two separate sample collection times. The target was based on different animal fixation with expectation of different levels of stress (cortisol) and some other blood parameters.

2. Material and methods

All experimental procedures and management of animals were conducted in accordance with European Community guidelines m. 86/609/EEC regarding the protection of animals for experimental purpose. Rabbits (n = 10) selected for the experiment were focused on their welfare and possibly related less invasive or less stressful collection method for animal. Experiment had duration of 2 weeks with one blood sample collection each week on the same day and same time of day. Experimental animals were provided at National Agricultural and Food Centre, Nitra (Slovak Republic). All rabbits were in the age of 18 months. Over the duration of the experiment, animals were placed in separate cages that were equipped with a feeder and automatic watering system. Environmental condition in the rabbitry was 16 h of light and 8 h of dark per day (maximal intensity 80 lx), air temperature 20–24 °C and 65% humidity.

2.1. Blood sampling and analysis

Samples were collected from all animals once a week – first week using common blood sampling technique (light/open collection) with one person fixating the animal and other collecting the blood from the ear marginal vein. On the second week animals were placed into a bag (dark collection) with only the ear standing out. The blood was collected into two tubes. Samples for biochemical analysis were placed into tubes without additive, and tubes containing EDTA as an anticoagulant, were used for the haematological analysis, mixed and placed into thermobox. After finishing all blood sample collection, the samples were transported into laboratory where haematological parameters were measured and later after centrifugation of the samples, blood plasma and serum was stored in a deep freezer (−80 °C).

2.2. Analysis of blood parameters

Hematologic parameters were measured using Abacus Vet5 (Diatron MI Ltd., Budapest, Hungary) – a fully automatic haematological analyser which operational principle is based on differences of resistances of individual blood elements spurred thru soft microcapillaries. Following haematological parameters were analyzed: total count of leukocytes (WBC, 10^9/l); total count of lymphocytes (LYM, 10^9/l); percentage of lymphocytes (LYM, %); total count of monocytes (MON, 10^9/l); percentage of monocytes (MON, %); total count of neutrophils (NEU, 10^9/l), percentage of neutrophils (NEU, %); total count of erythrocytes (RBC, 10^{12}/l); haemoglobin (HGB, g/l); haematocrit (HCT, %); average volume of erythrocytes (MCV, fl); mean corpuscular haemoglobin (MCH, pg); mean corpuscular haemoglobin concentration (MCHC, g/l); red cell distribution width (RDWc, %); total count of platelets (PLT, 10^9/l)); percentage of platelets (PCT, %); mean platelet volume (MPV, fl) and platelet distribution width (PDWc, %) (Massányi et al., 2014).

After both sample collections further analysis of cortisol levels and mineral profile parameters were completed. For analysis of cortisol DIALAB Elisa test kit (DIALAB Pruduktion and Vertrieb von chemish – technischen Produkten und Laborinstrumenten Gesellschaft m. b. H., Wiener Neudorf, Austria) was used. Mineral profile analysis was measured using commercial DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) on the Randox RX Monza analyser (Crumlin, United Kingdom) (Kováčik et al., 2017; Kovacik et al., 2019).

2.3. Statistical analysis

Statistical analysis were conducted using GraphPad Prism 6.1 (6.1 version for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com) to elaborate obtained experimental data. Values were compared using *t*-test and significance differences between groups were set to $P < 0.05$ and column statistics were calculated. Results are presented as mean ± S.D.

3. Results

While observing haematological parameters any significant changes in levels of total count of leukocytes, total count of monocytes, percent of monocytes, total count of neutrophils, total count of erythrocytes, haemoglobin, haematocrit, average volume of erythrocytes, mean corpuscular haemoglobin concentration, red cell distribution width, total count of platelets, percent of platelets, mean platelet volume, platelet distribution width were detected. On the other hand, a significant increase in total count of lymphocytes and percent of lymphocytes was found – level of both parameters was significantly higher in the samples collected in the second (experimental/dark) group. Likewise, also a decrease of percentage of neutrophils in the dark collection time compared to the light collection was observed. The level of mean corpuscular haemoglobin has significantly decreased in the samples from dark collection (Table 1).

Any significant change in the levels of cortisol between the two different collection methods though was observed, but a decreasing tendency in the dark collection method was found suggesting lower stress of animals during the sample collection (Table 2).

Table 1
Haematological parameters (results are presented as mean ± S.D.)

Parameter	Light collection	Dark collection	p – value
WBC	8.453 ± 2.924	8.224 ± 2.776	ns
LYM	**2.162 ± 1.423**	**3.496 ± 0.996**	*
MON	0.459 ± 0.252	0.452 ± 0.154	ns
NEU	5.832 ± 2.401	4.276 ± 1.901	ns
LYM%	**28.85 ± 19.11**	**43.84 ± 8.63**	*
MON%	5.53 ± 2.33	5.50 ± 1.45	ns
NEU%	**65.62 ± 18.31**	**50.66 ± 9.40**	*
RBC	5.475 ± 0.359	5.780 ± 0.236	ns
HGB	11.65 ± 1.08	11.67 ± 0.75	ns
HCT	39.24 ± 4.84	40.09 ± 2.76	ns
MCV	69.70 ± 4.86	67.30 ± 3.47	ns
MCH	**20.71 ± 0.99**	**19.62 ± 1.26**	*
MCHC	29.81 ± 1.96	29.17 ± 1.79	ns
RDWc	16.44 ± 1.56	16.70 ± 0.85	ns
PLT	165.4 ± 91.30	197.9 ± 73.36	ns
PCT	0.101 ± 0.058	0.114 ± 0.044	ns
MPV	6.06 ± 0.29	5.83 ± 0.30	ns
PDWc	29.19 ± 1.35	28.28 ± 1.70	ns

Abbreviations: total count of leukocytes (WBC, 10^9/l); total count of lymphocytes (LYM, 10^9/l); total count of monocytes (MON, 10^9/l); total count of neutrophils (NEU, 10^9/l); percentage of lymphocytes (LYM, %); percentage of monocytes (MON, %); percentage of neutrophils (NEU, %); total count of erythrocytes (RBC, 10^{12}/l); haemoglobin (HGB, g/l); haematocrit (HCT, %); average volume of erythrocytes (MCV, fl); mean corpuscular haemoglobin (MCH, pg); mean corpuscular haemoglobin concentration (MCHC, g/l); red cell distribution width (RDWc, %); total count of platelets (PLT, 10^9/l)); percentage of platelets (PCT, %); mean platelet volume (MPV, fl) and platelet distribution width (PDWc, %).

Table 2
Cortisol blood levels (results are presented as mean ± S.D.)

Parameter	Light collection	Dark collection	p – value
Cortisol (ng/ml)	0.490 ± 0.169	0.426 ± 0.174	ns

Table 3
Mineral profile parameters (results are presented as mean ± S.D.)

Parameter	Light collection	Dark collection	p – value
Chlorides (mmol/l)	102.90 ± 3.63	104.80 ± 2.55	ns
Phosphorus (mmol/l)	**1.87 ± 1.55**	**3.83 ± 2.05**	*

Any significant changes in levels of chlorides over the duration of the experiment were found, whereas a significant increase in phosphorus level in the second sample collection method was found (Table 3).

4. Discussion

In study conducted by Voight et al. (2004), a minimally invasive blood sampling method was used to collect blood from domestic rabbits using blood-sucking bugs (Reduviidae, Heteroptera). The results showed that the concentration of progesterone, testosterone and hydrocortisone in blood ingested by bugs did not deviate significantly from concentrations in blood obtained with conventional methods from the same animals. Similarly, in this study we likewise found no significant differences between stress hormone levels in the standard and alternative methods used in our experiment.

Self-stimulation status of the brain lateral hypothalamus was utilized as a model for positive reinforcement. Basal concentration level in intact rabbits, determined by means of radioimmunoassay was equal to 5.31 ± 0.87 μg% over the period of 10 to 2 h p.m. Using the method of the ear marginal vein dissection shown an increase of blood cortisol by 19.4% 30 min after the sample collection. A significant rise of hormone release into blood was spectated between five and fifteen minutes following the self-stimulation test. It was also observed that the levels of cortisol remained higher than normal for at least 1 h after the self-stimulation experiment (Morla et al., 1984). In our experiment we observed the cortisol concentration of cortisol levels – 0.490 ± 0.169 (ng/ml) in the light collection and 0.426 ± 0.174 (ng/ml) in dark collection.

Szeto et al. (2004), had an important finding that rabbits maintained in a controlled laboratory colony secrete both corticosterone and cortisol in a circadian rhythm which peaks in the afternoon and reaches nethermost value at 06:00. Levels of cortisol peaked at 12:00. In our experiment we gathered samples on the same day of the week and in the same time which should eliminate the effect of different cortisol levels depending on day-cycle.

In various literature also hypothalamus–pituitaryadrenal axis responses are swayed by the subordination of stress, fear and defeat (Raab et al., 1986; Blanchard et al., 1993; Sapolsky 1995; Koob and Heinrichs, 1999).

Kalaba (2012) study aimed to spectate the physiological response and stress indicators of California rabbits under intensive conditions in Egypt found out that cortisol levels were highest in group with 16 rabbits and stated that 4 rabbits per cage is acceptable threshold of animals per cage without adverse effect on welfare of rabbits. In our experiment the animals were held in cages separately.

Moore et al. (2015) observed reference ranges of similar haematological parameters in the New Zealand white rabbits. Our values were mostly in the range of reference ranges except total count of platelets, percent of neutrophils which were lower in samples from both our sample collections and average volume of erythrocytes which was slightly higher.

Özkan et al. (2012) observed normal values of haematological and biochemical parameters in serum and urine of New Zealand white rabbits. The normal range of chlorides is 102.2–116.9 mmol/l which is in consent with our findings (Simek et al., 2017). Reportedly the normal results of phosphorus are 1.09–1.68 mmol/l which is slightly lower than our measure values. As phosphorus is present inside cells, concentration of blood phosphate is easily increased by hemolysis (e.g., spontaneous or sampling problems). Increased levels of phosphorus in "dark collection" can be caused by soft tissue trauma. The release of phosphate from the large intracellular pool as a result of cell injury was reported (Edwards, 2004). On the other hand, hypophosphatemia is not rare, but its clinical significance is unknown and is usually related to dietary deficiencies or reduced intestinal absorption (Melillo, 2007).

In this study, a significant difference in the count of lymphocytes and neutrophils was observed. In another study where animals were tested after surgeries results indicated that major surgery induces a redistribution of lymphocytes from peripheral blood to lymphatic tissue. It is suggested that the endocrine stress response may be of major importance (Toft et al., 1993). The availability of biological markers that objectively quantify stress is a highly relevant issue. However, experimental evidence suggests that most physiological changes elicited by stressors do not reflect their intensity and are not useful for this purpose. Plasma levels of some hormones and elements have been found to reflect, at least under certain conditions, the intensity of stressors in animals and probably in humans (Armario et al., 2010).

5. Conclusion

The target of this study was to observe the effect of different (experimental) blood sample collection methods with special focus

on haematological and biochemical parameters to indicate stress during sample collection using light and dark methods and compare results.

For haematological parameters of rabbit blood, no significant changes between the sample collections except significant increase of lymphocytes total count, percentage of lymphocytes and significant decrease of mean corpuscular haemoglobin and percent of neutrophils were found. Results indicate that the animals were healthy during the entire duration of the experiment. Observing the levels of cortisol, a slight non-significant change between light and dark collection was noticed, showing better tendency in the samples from dark collection. For mineral profile any significant changes in levels of chlorides were found, but a significant increase of phosphorus levels in the dark collection was observed. We can point out that our experimental sample collection method had no adverse effect on health parameters of the animals over the entire duration of the experiment. Cortisol levels had a decreasing tendency which can suggest that the animals could have been possibly less stressed even though our results were not statistically significant.

Sources of financing

This work was supported by projects VEGA 1/0539/18, VEGA1/0392/20, APVV-16-0289 and KEGA 010/SPU-4/2018.

Declaration of Conflict of Interest

Authors have no conflict of interest to declare.

References

Armario, A., Labad, J., Nadal, R., 2010. Focusing attention on biological markers of acute stressor intensity: Empirical evidence and limitations. Neurosci. Biobehav. Rev. 111, 95–103. https://doi.org/10.1016/j.neubiorev.2020.01.013.

Blanchard, D.C., Sakai, R.R., McEwen, B.S., Weiss, S.M., Blan-Chard, R.J., 1993. Subordination stress: Behavioral, brain, and neuroendocrine correlates. Behav. Brain Res. 58, 113–121. https://doi.org/10.1016/0166-4328(93)90096-9.

Edwards, R.M., 2004. Hyperphosphatemia. In: Martini, L. (Ed): Encyclopedia of Endocrine Diseases, Elsevier, 567–571. https://doi.org/10.1016/B0-12-475570-4/00662-4.

Hannibal, E.K., Bishop, D.M., 2014. Chronic stress, cortisol dysfunction, and pain: A psychoneuroendocrine rationale for stress management in pain rehabilitation. Phys. Ther. 94, 1816–1825. https://doi.org10.2522/ptj.20130597.

Jeffrey, R.J., 2008. Rabbit diagnostic testing. J. Exotic Pet Med. 17, 4–15. https://doi.org/10.1053/j.jepm.2007.12.003.

Kalaba, Z.M., 2012. Physiological response and stress indicators of California rabbits under intensive conditions in Egypt. Asian J. Poultry Sci. 6, 65–78. https://doi.org/10.3923/ajpsaj.2012.65.78.

Koob, G.F., Heinrichs, S.C., 1999. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. Brain Res. 848, 141–152. https://doi.org/10.1016/S0006-8993(99)01991-5.

Kováčik, A., Tvrdá, E., Fulopová, D., Čupka, P., Kováčiková, E., Zbyňovská, K., Massányi, P., 2017. In vitro assessment of gentamicin cytotoxicity on the selected mammalian cell line (Vero cells). Adv. Res. Life Sci. 1, 111–116. https://doi.org/10.1515/arls-2017-0018.

Kovacik, A., Tvrda, E., Miskeje, M., Arvay, J., Tomka, M., Zbynovska, K., Andreji, J., Hleba, L., Kovacikova, E., Fik, M., Cupka, P., Nahacky, J., Massanyi, P., 2019. Trace metals in the freshwater fish Cyprinus carpio: effect to serum biochemistry and oxidative status markers. Biol. Trace Elem. Res. 188, 494–507. https://doi.org/10.1007/s12011-018-1415-x.

Kováčik, J. et al., 2015. Animal Physiology – Fyziológia živočíchov (in Slovak). Slovak University of Agriculture, Nitra.

Longenbaker, S.N., 2011. Madeŕs Understanding Human Anatomy and Physiology. McGraw-Hill, New York.

Massányi, P., Stawarz, R., Halo, M., Formicki, G., Lukáč, N., Čupka, P., Schwarcz, P., Kováčik, A., Tušimová, E., Kováčik, J., 2014. Blood concentration of copper, cadmium, zinc and lead in horses and its relation to hematological and biochemical parameters. J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng. 49, 973–979. https://doi.org/10.1080/10934529.2014.894322.

Melillo, A., 2007. Rabbit clinical pathology. J. Exotic Pet Med. 16, 135–145. https://doi.org/10.1053/j.jepm.2007.06.002.

Moore, D.M., Zimmerman, K., Smith, S.A., 2015. Haematological assessment in pet rabbits: Blood sample collection and blood cell identification. Clin. Lab. Med. 35, 617–627. https://doi.org/10.1016/j.cll.2015.05.010.

Morla, L., Ramirez, R., Belyi, V.P., Zavadskii, P.S., 1984. Changes in the level of cortisol in the blood of rabbits during self-stimulation. Probl. Endrokrinol. (Mosk) 30, 70–75. PMID: 6473320.

Özkan, C., Kaya, A., Akgül, Y., 2012. Normal values of haematological and some biochemical parameters in serum and urine of New Zealand White rabbits. World Rabbit Sci. 20, 253–259. https://doi.org/10.4995/wrs.2012.1229.

Parasuraman, S., Raveeendran, R., Kesavan, R., 2010. Blood sample collection in small laboratory animals. J. Pharmacol. Pharmacother. 1, 87–93. https://doi.org/10.4103/0976-500X.72350.

Poráčová, J. et al., 2015. Ecophysiology of animals and men – Ekofyziológia živočíchov a človeka (in Slovak). University of Prešov, Prešov.

Pratt, B.W., 1978. The mechanism of glucocorticoid effects in fibroblasts. J. Invest. Dermatol. 71, 24–35. https://doi.org/10.1111/1523-1747.ep12543774.

Raab, A., Dantzer, R., Michaud, B., Mormede, P., Taghzouti, K., Simon, H., Lemoal, M., 1986. Behavioural, physiological, and immunological consequences of social status and aggression in chronically coexisting resident-intruder dyads of male rats. Physiol. Behav. 36, 223–228. https://doi.org/10.1016/0031-9384(86)90007-7.

Rhodes, M.E., 2017. Adrenocorticotropic Hormone. Stress: Neuroendocrinology and Neurobiology. Handbook of Stress, 2, University of Melbourne, Australia, Elsevier. https://doi.org/10.1016/B978-0-12-802175-0.01001-9.

Sapolsky, R.M., 1995. Social subordinance as a marker of hypercortisolism. Ann. NY Acad. Sci. 771, 626–639. https://doi.org/10.1111/j.1749-6632.1995.tb44715.x.

Shors ,T.J., Horvath, B., 2001. Neural Basis of Stress. International Encyclopedia of the Social and Behavioral Sciences, first ed., Elsevier.

Simek, V., Zapletal, D., Strakova, E., Pavlik, A., Suchy, P., 2017. Physiological values of some blood indicators in selected dwarf rabbit breeds. World Rabbit Sci. 25, 27–36, https://doi.org/10.4995/wrs.2017.4110.

Szeto, A., Gonzales, J.A., Spitzer, S.B., Levine, J.E., Zaias, J., Saab, P.G., Schneiderman, N., McCabe, P.M., 2004. Circulating levels of glucocorticoid hormones in WHHL and NZW rabbits: Circadian cycle and response to repeated social encounter. Psychoneuroendocrinology. 29, 861–866. https://doi.org/10.1016/S0306-4530(03)00153-7.

Toft, P., Svendsen, P., Tønnesen, E., Rasmussen, J.W., Christtensen, N.J., 1993. Redistribution of lymphocytes after major surgical stress. Acta Anaesthesiol Scand. 37, 245–249. https://doi.org/10.1111/j.1399-6576.1993.tb03708.x.

Voight, C.C., Faßender, M., Dehnhard, M., Wibbelt, G., Jewgenow, K., Hofer, H., Schaub, A.G., 2004. Validation of a minimally invasive blood-sampling technique for the analysis of hormones in domestic rabbits, Oryctolagus cuniculus (Lagomorpha). Gen. Comp. Endocrinol. 135, 100–107. https://doi.org/10.1016/j.ygcen.2003.08.005.