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Section **animal welfare**

A pilot study to develop a screening test for brachycephalic dogs

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Aim of the study: to establish a feasible blood-borne test for screening examinations and objective assessment of the severity of BOAS

Material and methods
Blood samples of 42 healthy dogs belonging to 18 brachycephalic and 24 non-brachycephalic dogs with no clinical symptoms have been collected. Laboratory baseline data, i.e., chemogramm, hematology with white blood cell differential and reticulocyte counts, Coombs test, basal cortisol as well as CRP detection were performed. For Part A of the study, the adjustment of RBC analysis methodologies known from other species for canine cells was made. Redox state markers (reduced (GSH) and oxidised glutathione (GSSG) and total reduced thiol detection), markers of red blood cell age and damage (deamidation of band 4.1 and membrane loss), markers showing the changes in rheological properties as well as plasma nitrite/nitrate levels as markers of NO production were assessed in blood samples (6ml each) of 20 dogs. We also measured Hb state (oxygenation, oxidation, carboxylation), glucose and lactate levels in plasma. In Part B the impact of sample transportation for 6 or 24 hours (room temperature, shaking) on the parameters of interest was investigated in blood samples of 22 additional dogs.
In more detail, the following variables have been investigated:

- Intracellular reduced total protein thiols (monobromobimane, flow cytometry) and non-protein thiols (GSH and GSSG, Ellmann's reagent) were measured - Nonprotein thiols [39], along with hemoglobin O_2 saturation (GEM® OPL™ analyser)
- NO_2- and NO_3- levels in plasma were determined using chemiluminescence assay [40, 41]
- Reticulocyte counts were detected as RNA+ cells by flow cytometry,
- RBC shape and membrane abundance were determined using forward and side scatter, and band 3 abundance was assessed determined by flow cytometry using eosin 5-maleimide (EMA) staining,
- Cell projected area and morphological features (microscopy) [46] were assessed
- Band 4.1a:b ratio [39] was determined as a marker of red blood cell age
- Finally, markers showing the changes in rheological properties, i.e., hydration, deformability and osmotic fragility were assessed using ektacytometry and in a swelling test using flow cytometry [46, 47]

Statistical approach
Data were tested for normal distribution (SigmaPlot v14) and further evaluated using rank sum test or unpaired t-test for the brachycephalic vs control group. Repeated measures of values in transportation study were analysed using Anova on ranks for time-dependence.

Results and significance

Despite the small numbers of cases included in this pilot study, RBC analysis to assess parameters associated with stress, intermittent hypoxia and red blood cell turnover adjusted for canine cells revealed significant differences between brachy- and non-brachycephalic dogs for some of the red blood cell indices, rheological properties, redox state markers as well as of some markers of metabolism. This is especially remarkable, since this study mainly was to refine the methodological approach, i.e. to adapt and validate the methodological approaches for assessment of hypoxia-dependent marker in canine blood.

In the brachycephalic dogs compared to the non-brachycephalic group mean corpuscular haemoglobin content, mean corpuscular haemoglobin and plasma protein (P=0.013, P=0.052 and P=0.036, respectively) were increased and EMA staining was decreased, reflecting band 3 protein and membrane surface loss. Two parameters reflecting the cell size variability and/or shape, i.e., Red Blood Cell Distribution width and flow cytometric forward light scatter standard deviation, were higher in the brachycephalic group (P= 0.000017 and P=0.004, respectively). Increase in RDW and forward scatter variance were earlier on reported as nonspecific predictors of stress or pathology for humans. Deformability and osmotic fragility determined by osmoscan (LORRCA) did not differ significantly between the control and brachycephalic group.

Signs of stress erythropoiesis, i.e. minor increase in reticulocyte count (P=0.076) as well as retarded reticulocyte maturation determined by RNA staining intensity (P=0.036), were found in the brachycephalic group, with no concurrent signs of increased RBC turnover (band 4.1 deamidation).

Differences between both groups were also found for metabolic markers (serum lactate and RBC lactate production, cholesterol, triglycerides, lipemic index) and redox state (intracellular reduced glutathione) markers. RBCs of brachycephalic dogs were lower in intracellular GSH (P=0.0046, not measured before in any study) when measured immediately after blood sampling. GSH levels in RBCs of brachycephalic dogs recovered *ex vivo* within 24 h (transportation study) reaching the values we have measured in control dogs. The observed recovery of redox balance suggests that the systemic oxidative load on RBC was higher in brachycephalic dogs and the cells restored their redox state as soon extracellular oxidant flow was interrupted. These findings are consistent with the increased levels in pro-inflammatory cytokines reported for the brachycephalic dogs before ([1,2]). Plasma NO was upregulated (supporting [2,3]) providing vasodilatation, and lactate levels and production were slightly increased in brachycephalic dogs relying more on anaerobic glycolysis, while glucose levels and consumption were similar in both groups.

Finally, the serum Cortisol level was increased in brachycephalic dogs (<0.001), indicating stress and increased metabolic demand, while some other markers of metabolism determined for the general health check, i.e., cholesterol and triglycerides (P=0.002 and P=0.004, respectively) were decreased in brachycephalic dogs.

Results of Part B of the study show that some of the parameters differing between brachy- and non-brachycephalic are not stable over time without further stabilization. Fixation of cells will now be performed in a second step to stabilize RBC shapes and prevent membrane loss during storage or transportation.

Outlook

We hope that these promising results will enable us to get funding support for more extended clinical studies, in which red blood cell indices, reticulocyte counts, plasma ions and proteins, metabolic parameters, cortisol, inflammation markers as well as flow cytometry (forward and side scatter, EMA test, MBBR), lactate, NO, and glutathione measurements will be tested for association with severity of airway obstructive syndrome assessed by whole-body barometric plethysmography. Parameters with high diagnostic power will be suggested for use by the FSVO to prevent breeding as well as exhibition of BOAS affected dogs (“Verordnung des BLV über den Tierschutz beim Züchten Art. 5 Abs. 2-5“). E.g.,- restricting breeding to dogs with blood parameters better than the population mean will improve the health of brachycephalic breeds over time. Furthermore, these parameters might be used for diagnostic testing in the future to identify potential candidates for surgical intervention early in the progress of the disease and to objectively weigh outcome of the various repair techniques to manage this syndrome (Evaluation of different approaches for multilevel surgery, different techniques for palate shortening and thinning (folded flap versus H-plasty versus standard staphylectomy). Both aspects- improving the phenotype of affected breeds by selected breeding over time as well as optimizing treatment of affected dogs are mandatory to decrease the suffering of affected dogs in the future.

Publications, posters and presentations

Publication in preparation

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