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Hepatic Hepcidin Gene Expression in Dogs with a Congenital Portosystemic Shunt

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Background: Microcytic anemia is common in dogs with a congenital portosystemic shunt (cPSS) and typically resolves after surgical attenuation of the anomalous vessel. However, the pathophysiology of the microcytic anemia remains poorly understood. Hepcidin has been a key role in controlling iron transport in both humans and animals and in mediating anemia of inflammatory disease in humans. The role of hepcidin in the development of microcytic anemia in dogs with a cPSS has not been examined.

Hypothesis: To determine whether hepatic hepcidin mRNA expression decreases, while red blood cell count (RBC) and mean corpuscular volume (MCV) increase in dogs after surgical attenuation of a cPSS.

Animals: Eighteen client-owned dogs with confirmed cPSS undergoing surgical attenuation.

Method: Prospective study. Red blood cell count (RBC) and mean corpuscular volume (MCV), together with hepatic gene expression of hepcidin, were measured in dogs before and after partial attenuation of a cPSS.

Results: There was a significant increase in both RBC (median pre 6.17×10^{12}/L, median post 7.08×10^{12}/L, $P < .001$) and MCV (median pre 61.5fl, median post 65.5fl, $P = .006$) after partial surgical attenuation of the cPSS. Despite the increase in both measured red blood cell parameters, hepatic gene expression of hepcidin remained unchanged.

Conclusions and Clinical Importance: This study found no evidence that dysregulated production of hepcidin was associated with anemia in dogs with a cPSS.

Key words: Anemia; Hepcidin; Iron; Portosystemic shunt; qPCR.

Anemia is commonly reported in dogs with a congenital portosystemic shunt (cPSS) and has been associated with abnormalities in iron metabolism.[1] However, the precise pathogenesis of cPSS-associated anemia remains unclear. Anemia has been documented in both spontaneous[1,2] and experimentally induced PSS[3] and is often microcytic, which has prompted investigations into iron status of dogs with a cPSS. Previous studies have revealed a high incidence of hypoferremia in dogs with a cPSS, ranging from 56 to 70%.[1,2] Hypoferremia does not appear to be associated with absolute iron deficiency, as evaluation of other markers of iron status do not support this conclusion. For example, total iron binding capacity (TIBC) has been found to be normal or reduced and transferrin saturation is highly variable in dogs with cPSS, whereas more reliable markers of nonheme iron stores (serum ferritin concentration and hepatic stainable iron) are normal or increased in many individuals.[1,2,4] In addition, liver iron content (analyzed quantitatively) doubled in dogs after experimentally induced PSS.[3] Iron parameters have been shown to return to normal after partial shunt attenuation, supporting a causal relationship between abnormalities and cPSS.[1] It is therefore suspected that hypoferremia in dogs with cPSS is caused by abnormal iron sequestration or transport rather than true iron deficiency.[1,5] In summary, although these studies have indicated that iron metabolism is dysregulated in dogs with a cPSS, they have not clearly defined the pathogenesis of microcytic anemia in this disease.

Abbreviations:

cPPS	congenital portosystemic shunt
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
qPCR	quantitative polymerase chain reaction
RBC	red blood cell count
TIBC	total iron binding capacity

Hepcidin, a hormone synthesized predominantly by hepatocytes, controls iron transport by binding and inhibiting (via degradation) the iron-export protein ferroportin.[6] Hepcidin expression is increased in both anemia of chronic disease and iron overload, and decreased with regenerative anemia/erythropoiesis, hypoxia, some forms of hereditary hemochromatosis, and experimentally induced iron deficiency.[6] Increased hepcidin expression, induced by sustained up-regulation of IL-6 during chronic inflammation, has been proposed as the main mediator of anemia of chronic disease because of iron-restricted erythropoiesis.[7,8] This observation is particularly relevant to dogs with a cPSS because IL-6 concentrations are increased in dogs with

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a cPSS compared to healthy control dogs (Kilpatrick S, Gow AG, Foale RD, Tappin SW, Carruthers H, Reed N, Yool DA, Woods S, Marques AI, Jalan R, Mellanby RJ, personal communication). Therefore, IL-6-induced up-regulation of hepcidin is a plausible explanation for the frequent occurrence of microcytic anemia which is commonly observed in dogs with a cPSS.

The hypothesis of this prospective study was that hepatic hepcidin mRNA expression would decrease, while red blood cell count (RBC) and mean corpuscular volume (MCV) would increase in dogs with a cPSS after partial surgical attenuation of the anomalous vessel. This hypothesis was examined by measuring RBC and MCV, together with hepatic gene expression, before and after partial surgical attenuation of the anomalous vessel.

Dogs undergoing surgery for cPSS were considered for inclusion into the study. Dogs were treated by partial or complete suture attenuation determined by subjective and objective intraoperative assessments of portal hypertension. Dogs treated with partial attenuation had a follow-up surgery approximately 3 months later for complete attenuation. Dogs that had partial attenuation and follow-up surgery were eligible for inclusion in the study.

Blood samples were collected before each surgery by jugular venipuncture for hematology which included red blood cell count and mean corpuscular volume assessment. A single liver biopsy specimen was collected during the initial and follow-up shunt surgery for routine histopathology in formalin and for qPCR in RNAlater. RNAlater samples were stored at −80°C.

Hematocrit, erythrocyte number (RBC), and mean corpuscular volume (MCV) were determined in EDTA anticoagulated blood with an automated hematology analyzer (impedence counter and laser flow cytometer).[a]

RNA was extracted from approximately 20–30 mg of each hepatic biopsy sample with a Mammalian Total RNA Miniprep Kit.[b] The tissue was homogenized in 500-μl Lysis Solution with a Mixer Mill.[c] An in-solution DNase digestion was performed with the Ambion TURBO DNA-*free* Kit[d] to remove any contaminating DNA. RNA quality and quantity was assessed by microfluidic capillary electrophoresis with a Bioanalyzer.[e] Two separate cDNA were synthesized from each RNA sample using a mixture of random hexamer and oligo $(dT)_{15}$ primers and reverse transcriptase enzyme.[f] Where possible the amount of RNA template for cDNA synthesis was standardized at 1 μg. The cDNA was diluted to a final volume of 100 μL with nuclease-free water and was stored at −20°C before further use.

Quantitative polymerase chain reaction (qPCR) was used to measure the relative expression of hepcidin mRNA in hepatic tissue from cPSS dogs at first and second surgery. Previously published canine gene specific primers for hepcidin[6] and 4 liver-specific reference genes[9], hydroxymethyl-bilane synthase (HMBS), ribosomal protein L13a (RPL13A), ribosomal protein L32 (RPL32), and ribosomal protein S18 (RPS18), were used (Table 1).

For quantification, each liver sample had 2 cDNA samples analyzed in duplicate. Reactions were carried out in 25-μL volumes with a Real-Time PCR Detection System thermocycler.[g] Each reaction consisted of 1-μL cDNA as the template with Immobuffer (1× concentration), Hi-Spec Additive (1× concentration), dNTP (final concentration 1 mM), magnesium chloride (final concentration 2.5 mM for genes of interest, 4.5 mM for reference genes), 1 unit Immolase DNA polymerase,[h] and EvaGreen dye[f] (0.06× diluted 1:4 with nuclease free water). Samples were incubated at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 10 seconds. A primer-dimer melting temperature of 80°C for 1 second was programmed before fluorescence readings were taken at the end of each cycle. A melting curve analysis from 65 to 95°C with a plate read every 0.5°C was performed at the end of 40 cycles. Initial qPCR analysis was performed,[i] followed by analysis of raw real-time data.[j] Relative gene expression was quantified as previously described.[10] Quantification cycle (Cq) values were corrected using the calculated efficiencies for each primer set. Normalization of each sample Cq for hepcidin was performed relative to the geometric normalization of the 4 reference genes. The relative expression of hepcidin mRNA in each cDNA sample was calculated using the normalized Cq of each sample relative to the average Cq of all of the samples.

Statistical analysis was performed by a statistical software package.[k] Median and range were reported, and data compared with the Wilcoxon Signed Rank test. Significance was set at the 5% level ($P = .05$).

Table 1. Primers used in quantitative polymerase chain reaction.

Gene	Primer Sequences	PCR Amplicon Length (Base Pairs)	Genbank Accession Number	Primer Sequence Reference
Hepcidin	Forward: GGCCAGTGTCTCAGTCCTTC Reverse: GTTTTACAGCAGCCACAGCA	168	AY590589	Fry et al[6]
HMBS	Forward: TCACCATCGGAGCCATCT Reverse: GTTCCCACCACGCTCTTCT	112	XM546491	Peters et al[9]
RPL13A	Forward: GCCGGAAGGTTGTAGTCGT Reverse: GGAGGAAGGCCAGGTAATTC	87	AJ388525	Peters et al[9]
RPL32	Forward: TGGTTACAGGAGCAACAAGAAA Reverse: GCACATCAGCAGCACTTCA	100	XM848016	Peters et al[9]
RPS18	Forward: TGCTCATGTGGTATTGAGGAA Reverse: TCTTATACTGGCGTGGATTCTG	116	XM532106	Peters et al[9]

This study was approved by the Royal Veterinary College Ethics Committee and written informed consent was gained before recruitment of all cases.

Results

Eighteen dogs with a cPSS met the inclusion criteria. A variety of ages (median age 154 days, range 96–1769), breeds, and sex were represented (Table S1).

There was a significant increase in RBC count after partial attenuation of the shunting vessel (median pre 6.17×10^{12}/L [range 4.31–7.58], median post 7.08×10^{12}/L [range 5.49–8.54], $P < .001$). There was also a significant increase in MCV after partial shunt attenuation (median pre 61.5fl [range 51.4–67.7], median post 65.5fl [range 54.9–72.3], $P = .006$). These changes were in keeping with findings of previous studies.[1]

The median relative mRNA expression of hepcidin at first surgery was 2.51 (0.04–15.01) and at second surgery the median relative mRNA expression of hepcidin was 2.25 (0.01–8.87). This difference was not statistically significant.

The central finding of this study was that hepatic hepcidin mRNA expression showed no significant change after partial surgical ligation of the cPSS. The lack of change in hepatic gene expression of hepcidin cannot be explained by a lack of change in the RBC or MCV as both parameters significantly increased after partial attenuation of the shunting vessel. We were therefore unable to demonstrate a role for altered hepatic hepcidin mRNA expression in the pathogenesis of microcytic anemia in dogs with cPSS. However, future studies with more dogs may have a greater power to detect a difference in hepcidin expression postsurgery. It would have been useful to compare hepatic hepcidin mRNA expression in cPSS dogs (before attenuation) with age-matched control dogs (without hepatic disease) to further support the conclusion that hepcidin expression is unaltered in dogs with a cPSS. However, such controls were not available to us because of ethical and logistical limitations beyond our control.

We have also not ruled out the possibility that hepatic hepcidin up-regulation is present at the level of protein translation rather than mRNA expression, or even that extrahepatic sites of hepcidin expression exist and become more important in dogs with hepatic dysfunction. On the other hand, nonhepcidin mediators (including other acute phase proteins/cytokines implicated in the pathophysiology of anemia of inflammatory disease in dogs) could be involved and may warrant further investigation.

Footnotes

[a] Abbott Cell-Dyn 3500, Abbott Laboratories, Abbott Park, IL
[b] GenElute, Sigma-Aldrich Company Ltd, Dorset, UK
[c] MM 300, Retsch, Leeds, UK
[d] Ambion, Life Technologies Ltd, Paisley, UK
[e] Agilent 2100, Agilent Technologies, Cheshire, UK
[f] IMProm-II, Promega, Southampton, UK
[g] Bio-Rad CFX96, Bio-Rad Laboratories Ltd, Hertfordshire, UK
[h] Bioline, London, UK
[i] Biotium Inc, Hayward, CA
[j] GenEx professional version 4.4.2 software (Multid Analyses, Goteborg, Sweden)
[k] PASW Statistics 18.0.0; Education SPSS (UK) Limited IBM, Woking, UK

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Conflict of Interest: None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Individual data (signalment, time between surgeries, haematological and qPCR data pre and post partial shunt attenuation surgery).