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Copper Chaperone for SOD1 (CCS) Transcript in Whole Blood: A Potential Indicator of Copper Deficiency in Sheep (*Ovis Aries*)

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Abstract

Early copper deficiency detection is important for sheep, a sensitive animal to copper (Cu) nutrition. Diagnosis through blood copper, ceruloplasmin, and superoxide dismutase 1 (SOD1) enzymes is not ideal due to the multifactorial control of these parameters. The copper chaperone for superoxide dismutase (CCS) protein is reported to be a specific biomarker for Cu deficiency in cattle and mice but not in sheep. Theoretically, the CCS transcript might change in Cu-deficiency but there is no evidential proof. This study is thus designed to determine the CCS chaperone and SOD1 transcript expression in sheep fed with Cu-adequate and Cu-deficient diets. Twenty-four sheep (n = 24) were divided into 2 groups of 12 animals each and fed with either Cu-adequate or Cu-deficient diets. The onset of Cu-deficiency was determined by the skin and wool lesions, intake and absorption of Cu and the inorganic Cu- and ceruloplasmin status in blood. After the onset of deficiency, whole blood and erythrocytes were collected from all the sheep and liver samples from the selected (n=3 from each group) sheep for determination of CCS and SOD1 transcript expressions by qPCR. The results indicated the intake and absorption of copper coincided with the level feeding in sheep. The Cu-deficient diet reduced Cu ($p<0.01$) in the liver and blood, ceruloplasmin ($p<0.001$) in the blood, and up-regulated the CCS transcripts ($p<0.001$) in the liver, whole blood, and erythrocytes, respectively. However, the SOD1 transcripts were down regulated in liver ($p<0.001$), up-regulated in whole blood ($p<0.001$), and did not change in erythrocytes. The CCS transcripts were highest in whole blood compared to liver and erythrocytes in Cu-deficient sheep. Our study indicated that the whole blood CCS transcript up regulation might be a useful biomarker of Cu deficiency in sheep since the increase of transcript might not be related to the other factors.

Keywords: Sheep; Copper Biomarker; Copper Deficiency; CCS Transcript; SOD1 Transcript

Introduction

Copper (Cu) is an essential micro-mineral, an integral component of several enzymes involved in the formation of connective tissue, myelin, melanin pigment, and keratin; in iron metabolism, cellular respiration, protection against oxidative stress, catecholamine biosynthetic pathway, and also is a key trace element required for an effective immune response in humans and animals[1, 2]. A low Cu status thus contributes to the development of a wide range of hepatic, neurological, and other types of disorders [1- 3]. In addition, the excess of Cu is also known to cause toxicity in humans and animals [2, 4, 5]. The monogastric species tolerate the excess Cu much better than the ruminants bearing multi-compartment stomachs. Compared to other ruminants, sheep are more susceptible to Cu toxicity because of their less efficient Cu excretory mechanism [1, 6-9]. Cu-toxicity is widespread in sheep flocks mainly due to chronic exposure to Cu [10, 11] causing significant economic losses to the farmers due to the high mortality rates in the clinically affected animals [5, 10, 11]. Many factors can predispose the sheep to Cu- deficiency and poisoning which are considered significant illnesses affecting the sheep industry in many countries [6, 7, 11]. Evidence indicates that the incidence of this disease is increasing due to the adoption of more intensive sheep production methods and/or breed susceptibility to Cu-deficiency and toxication [12, 13]. In ruminants, Cu absorbed from the small intestine is transported through blood transcuprein and albumin and stored in the different organelles of the liver. In Cu overload, Cu is stored in the lysosomes in other species but not in sheep making this species susceptible to Cu toxicity [14]. This mechanism of susceptibility is not similar to the two human genetic disorders Menke's disease and Wilson disease which happens due to the inability to absorb and utilize Cu; and impaired excretion of Cu from the liver to the body, respectively [15].

The neurodegenerative disease 'Scrapie' in sheep is known to be related to Cu deficiency [16]. Assessing the blood Cu levels and the Ceruloplasmin protein that carries the Cu in bound form aids in the diagnosis of Cu deficiency [17]. Ceruloplasmin a 132 kDa protein consisting of 1046 amino acids, is a member of the copper oxidase family enzyme, primarily synthesized in the liver parenchymal cells, although small amount is also produced by the immune cells, including macrophages and lymphocytes [18]. The polypeptide chain of ceruloplasmin is first synthesized (apo-enzyme) then copper atoms are added from an intracellular ATPase [19]. Copper availability is not necessary for ceruloplasmin translation, but the binding of Cu is essential for the adequate folding of ceruloplasmin protein, normal attachment of the side-chain oligosaccharides, and the function. The apo-ceruloplasmin is structurally less stable without Cu, as most of them undergo intracellular degradation and the small portion that reaches the circulation gets degraded fast because of the short half-life of 5 to 6 h compared to the holoenzyme (ceruloplasmin plus copper), which has a half-life of a few days [20]. The ceruloplasmin is a catalyst for redox reactions in plasma. It oxidizes iron from ferrous (2+) to ferric iron (3+) to assist iron binding to transferrin. In hereditary ceruloplasmin deficiency, neurological disorders occur due to the problem of iron transport in the brain. It is also thought to be involved in controlling membrane lipid oxidation. In the presence of superoxide, ceruloplasmin promotes low-density lipoprotein (LDL) oxidation *in vitro* and is co-localized with oxidized LDL in atherosclerotic lesions [21]. More than 90 % of the copper is exported from the liver to the plasma in the form of ceruloplasmin however, the ceruloplasmin has a limited role in copper transport from the peripheral tissues. Albumin and Transcuprein appear to be the other major copper transport proteins, especially immediately after post-gastrointestinal absorption [22]. The copper-albumin complex is carried to the liver via the portal system in circulation. In humans, the low ceruloplasmin content in blood samples indicates pathological conditions such as Wilson disease, Menke disease, copper deficiency, aceruloplasminemia, or states of low protein energy intake (e.g., malnutrition.) [23][24]. Whereas the high blood ceruloplasmin contents may be related to pregnancy, oral contraceptive use, copper toxicity/zinc deficiency, lymphoma, lung cancer, acute and chronic inflammation, rheumatoid arthritis, angina, Alzheimer's disease, schizophrenia, and obsessive-compulsive disorder [25-27].

The problem with the blood inorganic Cu assay is that the levels do not change even if the animal suffers from deficiency due to the homeostasis mechanism, and the Ceruloplasmin being the acute phase protein, changes during inflammation and infection [28]. Nevertheless, these assays can diagnose the very severe deficiency of Cu. To overcome this problem of diagnosing ani-

mals that are actually deficient but are not reflected by the existing diagnosis methods, the ceruloplasmin: Cu ratio [29] and non-ceruloplasmin copper (NCPC) tests [30] have been used. However, none of the tests are found robust for accurate detection of subclinical cases therefore is not useful for field application. The Cu/Zn-dependent superoxide dismutase 1 (SOD1) is another indicator used as a marker of Cu deficiency by many. The reason is that SOD1 contains two covalently bound Cu^{++} that give structural stability and are essential for the activity of this enzyme. However, the level of SOD1 changes under oxidative and other stresses [31] therefore cannot be considered as a reliable marker of Cu deficiency either. On the other hand, it has been discovered that the Cu insertion into SOD1 is dependent on the copper chaperone for SOD1 (CCS) protein. The CCS protein increases linearly in the liver and erythrocytes with the decrease in dietary Cu levels and is reported to be a useful biomarker for Cu deficiency in rats and cattle [32, 33].

The CCS protein expression is mostly determined by Western blot analysis [33, 34]. The CCS transcript is determined by PCR only in humans [35, 36]. Keeping the above in mind this experiment was designed to assess the CCS and SOD1 mRNA in the whole blood, erythrocytes, and liver tissues of sheep fed with adequate and deficient copper diets for an implication of using this as a potential biomarker of Cu deficiency.

Materials and Methods

Animals, Housing and Diets

The experiment was conducted at the Experimental Livestock unit of ICAR-National Institute of Animal Nutrition and Physiology (ICAR-NIANP). All care, handling, and sampling procedures of sheep used in the study were approved by the ICAR-ICAR-NIANP Animal Ethics Committee, Bangalore, and Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA), Ministry of Environment and Climate Change, Government of India, New Delhi with prior to intimation of the experiment. Twenty-four sheep (n = 24) used for this study had an average body weight of 15 ±1.23 kg. The experimental sheep were randomly divided into two groups of 12 animals in each and fed with either a Cu-adequate or a Cu-deficient diet. A semi-purified basal diet containing ragi straw and concentrate mixture with 4.0 mg of Cu per kg dry matter was fed to all the animals in an individual small pen. The experimental sheep were fed a basal diet consisting of ragi straw (*Eleucine coracana*) and a concentrate mixture for 240 days. The concentrate mixture (semi-purified) was prepared using casein as the protein source and corn and corn starch as an energy source to supplement the deficient level of Cu in sheep. Ingredients composition of the basal diet is given in Table 1. The Ragi straw and concentrate mixture used as basal diet contained 3.8% and 17.0% crude protein, respectively by analysis, and had a total of 9.74% crude protein on calculation. The formulated Cu-deficient basal diet contained 3.6 mg of Cu/kg dry feed on chemical analysis. In the Cu-adequate group feed the Cu content was 9.7 mg/kg DM as per the analysis as compared to the actual requirement of Cu 10 mg/kg dry feed in sheep (ICAR 2012) [37]. The basal diet was formulated to meet all other nutrient requirements except the Cu for sheep. The Cu contained in the basal diet was approximately one-third of the normal dietary requirement of Cu for sheep. Sheep were fed individually once daily at 09:00 h. The required quantity of Cu (10 ppm from feed-grade inorganic copper sulfate) was supplemented to Cu-adequate sheep individually daily by mixing with concentrate mixture just before feeding. All the animals had free access to water all the time. Feed intake and body weight of the experimental sheep were recorded at 15-day intervals to adjust the feed offered to animals as per their body weight.

A digestibility trial of 5 days duration was conducted following the feeding trial on the same animals to assess the gut absorption of copper. The animals received the same feed mixtures that they had been receiving since 8 months of the feeding trial. All sheep were fed at 08:00 h and had ad libitum access to fresh water. The feed intake during the digestion trial was calculated before morning feeding by weighing the amount of feed offered and refused, respectively. Each day, the total faeces collected during the previous 24 h and faecal samples were collected using plastic bags that were fixed on the buttocks of each sheep. The

animals were adapted to the harnessing of faecal bags for three days followed by a faecal collection for five consecutive days. About 15% of the sub-sample was taken daily from the faeces of an individual animal, composited in a container (airtight plastics), and stored at -20°C till the end of the collection period. The representative faecal samples from each sheep were spread out in drying trays and placed in a drying oven maintained at a temperature of 90°C to 95°C for determining the moisture content in faeces. When dry, the faeces from each animal were placed in separate tightly covered cans. The dried samples of feed and faeces were ground in a hammer mill, thoroughly mixed, kept in polythene container at room temperature, and sampled for chemical analyses.

Sampling Procedures

Jugular vein blood samples were collected at monthly intervals to monitor the Cu status by determining the plasma Cu, ceruloplasmin (CP), Cu: CP, non-ceruloplasmin Cu (NCPC), and hemoglobin. Ten milliliters of whole blood was collected in the heparinized tube and kept on ice until centrifugation. Samples were centrifuged (Eppendorf, Germany) at 1811xg for 10 min at 4°C. Plasma was separated and stored in a storage vial at – 20°C until analyzed for biochemical indices of Cu and plasma Cu level. Biochemical indicators of Cu in blood plasma were monitored at monthly intervals till the sheep under the Cu-deficient group showed a deficiency of Cu status. Once the deficient group sheep showed the changes in biochemical indicators (plasma Cu/ceruloplasmin/Cu:CP/NCPC and Hemoglobin) and the clinical symptoms of anaemia about 10 mL jugular vein blood samples were collected for determining the conventional indicators of Cu status and for total RNA isolation from whole blood and erythrocytes. The whole blood samples of sheep fed with Cu-adequate (n =12) and Cu-deficient (n=12) diets were collected into 15 ml conical bottom tubes containing 200 µL of 40 % (w/v) EDTA solution (anticoagulant) and carried to the laboratory at ice-cold temperature. An aliquot of 250 µl of whole blood was taken and mixed with 750 µl of Tri-Reagent BD (Cat # T3809, Sigma Aldrich, St. Louis, MO, USA) in 2 mL microcentrifuge tubes and stored at – 80°C until use. The remaining blood was centrifuged (Eppendorf, Germany) at 453 x g in a swing-out rotor at 4°C for 15 min. The plasma and the buffy coat were removed carefully and 250 µl of red blood cells were taken from the bottom of the tube into 750 µl of Tri-Reagent BD, mixed well, and stored at – 80°C until use.

Three sheep from Cu-deficient and adequate groups were slaughtered in the local abattoir following the standard practice. The liver tissue samples were collected aseptically immediately after slaughter at ice-cold temperatures. For Cu-analysis, part of the tissue samples were kept at – 80°C until use. Whereas for RNA isolation a small piece of tissue from each animal was kept in pre-chilled RNA later solution (Cat # 76104, Qiagen, Germany) and incubated at 4°C overnight before storing at – 80°C until use.

Analytical Procedures

Haemoglobin in sheep blood was determined by using Drabkin's reagent (Sigma-Aldrich, USA) method. Plasma ceruloplasmin level in sheep was analyzed by its p-Phenylenediamine oxidase activity (Sunderman and Nomoto 1970) [38] and plasma superoxide dismutase (SOD) was estimated by the pyrogallol method (Marklund and Marklund 1974) [39]. One unit of SOD was described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 1.0 ml of assay mixture. Non-ceruloplasmin copper (NCPC), and Cu: Cp were calculated as described by Twomey et al. (2005) [30]. The NCPC was derived from the equation: NCPC (µg/dl) = plasma Cu (µg/dl) - 3 x CP (mg/dl) whereas Cu: CP was determined using the equation: Cu: CP ratio = plasma Cu (µmol/L*0.132/CP g/L). The plasma and liver tissue samples were prepared for Cu analysis as described by Pal et al. (2010) [40]. Diets were analyzed for Crude Protein and Cu as per the procedure of AOAC (1990) [41]. Feed samples were dried in a hot air oven at 70°C overnight (Culture Instruments, Bangalore, India) and then dry-ashed for mineral analysis in a muffle furnace (CEM Corporation, USA) at 600°C for 6 h (AOAC 1990) [41]. The copper was analyzed by ICP-OES (Perkin Elmer Optima 8000, Singapore) at 327.393 nm wavelength in plasma axial view at 1300W RF incident power using a crow-flow nebulizer. The Cu concentration was determined using a minimum of duplicate measurements with an in-

tra-assay CV of ≤ 10%. Corrections were made for baseline drift (e.g., a slight change in baseline absorbance) every 10 samples. The accuracy of the ICP method was evaluated by analyzing a laboratory-certified material (ICAR-NIANP, Corn grain) and NIST-certified standards obtained from Perkin Elmer, Bangalore.

Quantification of CCS and SOD1 Gene Expression by Qpcr Assay

Isolation of Total RNA From Whole Blood, Erythrocytes, and Liver Samples

Total RNA from the whole blood was isolated using the Tri Reagent BD reagent and protocol. However, a combined Tri-reagent BD and RNeasy Micro kit (Cat # 74004, Qiagen, Germany) protocols were used for isolation of the total RNA from erythrocytes (RBC). In the combined protocol the aqueous phase from the chloroform step of the Tri-reagent BD protocol was mixed with 1.25 volumes of absolute ethanol and passed through the RNeasy micro spin column by a brief centrifugation at 19,357 x g (Rota 4R-V/Fm $^{(R)}$, Plastocraft, Mumbai, India) for 15 sec. The bound nucleic acids in the column matrix were washed once with 700 µl RW-1 buffer, then twice with 500 µl of wash buffer II (RPE), and finally one more time with 80% ethanol. All the flow through were discarded and the empty column after the last spin was centrifuged at 19,357x g for 5 min keeping the lids open. Finally, the column was transferred to a fresh 1.5mL nuclease-free collection tube, and total RNA was eluted with pre-warmed 30 µl nuclease-free water.

The total RNA from about 60 mg liver tissues was isolated using an RNeasy mini kit (Cat # 74104, Qiagen, Germany) following the manufacturer's protocol. In brief, the RNA latter stored tissues were weighed and homogenized in 675 µL RLT buffer using Tissue Ruptor (Cat # 9001271, Qiagen, Germany) and disposable probes (Cat #990890) in 3 pulses of 15 – 20 s each and passed through the genomic DNA removal column. The flow-through was treated with an equal volume of 70 % ethanol, loaded into the binding column, and centrifuged at 19,357 x g for 15 s. The unbound materials from the column were washed once with 700 µL of RW1 buffer by spinning at 19,357 x g for 15 s. The contaminating DNA from the column if any was removed by in--column digestion of RNase-free DNase (Cat#.79254, Qiagen, Germany). To accomplish DNA digestion 10 µL (30 Units/column) of RNase-free DNase was mixed with 70 µL of reaction buffer (RDD) before adding to the column and incubated at room temperature for 15 min. The column was then washed once with 700 µL of RW1 buffer and twice with 500 µL of wash buffer II (RPE) by a brief spin at 19,357 x g for 15 sec every time. The empty spin column was centrifuged at 19,357 x g for 1 min to dry off the traces of water and then transferred to a fresh nuclease-free tube. The total RNA was eluted with pre-warmed 30 µl nuclease-free water. Total RNA samples isolated from the whole blood, red blood cells, and liver samples were quantified at 280 and 260 nm absorbance using Nanodrop Spectrophotometer 2000C (Thermo Scientific, USA). The quality of total RNA was tested by separating it in 1% agarose gel by electrophoresis and ethidium bromide staining.

The first strand cDNA was synthesized from total RNA by reverse transcription using Revert Aid H minus First Strand cDNA synthesis kit (Cat # K1621, Thermo Scientific, USA) according to the manufacturer's protocol. The qPCR primers for the test genes CCS and SOD1, endogenous control gene GAPDH, and white blood cell (WBC) marker genes CD45(Table 2) were designed using Primer 3 web tool Version 4.0.0 [42] and synthesized from Xcelris Genomic Pvt Ltd, India. The presence of CD45 in the samples was tested to understand the presence of WBC in the samples. The PCR efficiency was determined for all the genes by testing amplification of in 1:5 serially diluted cDNA in duplicate samples. The melting curves were checked for all the tested samples to ensure consistent amplification of a single PCR product.

The relative expression of the CCS and the SOD1 genes compared to the endogenous control gene GAPDH was determined in about 10 ng total RNA equivalent cDNA template in the whole blood, red blood cells, and liver samples. All the assays were performed in triplicates along with negative reverse transcription controls (similarly diluted RNA sample used as a template) and no template controls as described earlier. The captured data was quality checked for amplification and melt curve analysis using Light Cycler® 96 software (Version 1.1.0.1320, Roche Diagnostics International Ltd, Switzer land). A comparison of means

was performed by taking the ΔCt values of each gene in different biological replicates of adequate and deficient groups. The Ct values were exported into the MS Excel spreadsheet for further analysis. The relative quantity (ΔCt) of the test genes (CCS and SOD1) in each sample was calculated by subtracting the average Ct of the test genes and the average Ct of the endogenous control gene (GAPDH). The fold changes in sheep fed with Cu-deficient diet relative to Cu-adequate diet-fed sheep were calculated using the formula, $E^{-\Delta\Delta Cq}$ [43], where E = PCR efficiency of the gene of interest and ΔΔCq = (mean ΔCq of deficient – mean ΔCq of adequate).

Statistical Analysis

Statistical analysis of variables for the experiment was performed by ANOVA for a completely randomized design using SPSS software. The model statement contained the fixed effect of dietary Cu status (Cu-adequate vs. Cu-deficient) and the random effect of animals. Plasma Cu, hemoglobin, ceruloplasmin, Cu/Zn-SOD, NCPC, Cu: CP ratio, and liver Cu were analyzed using ANOVA to determine the effectiveness of each assay in diagnosing Cu deficiency. The difference in mean fold of gene (mRNA) expression was considered significant when $p \leq 0.05$ by independent samples t-test (SPSS version 18.0).

Data on feed intake, plane of nutrition, and Cu absorption parameters were calculated using a t-test model on SPSS software (version 18). Statistical significance was set at $p < 0.05$

Results

Copper Status

Although the body weights in the Cu-deficient diet fed reduced by approximately 10 -12% than Cu-adequate diet sheep the difference was not significant (($p=0.278$). Similarly, the feed intake was also not affected by the dietary Cu levels (Table 3). The Cu intake was lower ($p<0.0001$) in Cu-deficient than Cu-adequate diet-fed sheep (Table 3). The Cu-deficient sheep showed lowered ($p < 0.01$) Cu absorption from gastro gastrointestinal tract.

All the biochemical indices of Cu were significantly lowered in sheep fed with a Cu-deficient diet (Table 4). Inorganic Cu in sheep plasma decreased ($p<0.05$) from the starting level of 1.20±0.07 mg/L to 0.49 ±0.04 mg/L by 240 days of feeding were due to deficient Cu diet whereas the normal values of 1.20 ±0.07 mg/L in the latter group was due to the adequate Cu diet. The differences in plasma Cu, ceruloplasmin, and non-ceruloplasmin copper concentrations between Cu-deficient and Cu-adequate sheep were extreme ($p < 0.0001$; 1.20 vs. 0.58 mg of Cu/L of plasma; 135.60 vs. 88.96 mg of CP/L of plasma and 79.60 vs. 25.27 μg of NCPC/dl of plasma, respectively). The sheep in the Cu-deficient group showed severe debilitation although appetite remained normal. Most of the animals in this group showed clinical signs like paleness of mucosa (anemia), stunted growth, and signs of scabby skin appearing around the mouth and eyes (*Oculofacial achromortichia*). Although diarrhoea occurred in some of the animals, persistent diarrhoea was not observed. Two sheep of the Cu-deficient diet group died and found liver Cu concentrations of <15 μg/g (12.35 and 14.23 μg/g). The wool texture also changed in sheep as compared to the Cu-adequate group. Liver Cu concentration was significantly ($p< 0.01$) lower in Cu-deficient sheep as compared to Cu-adequate sheep. These biochemical indices, clinical symptoms, and liver Cu concentrations confirmed the Cu deficiency in sheep fed a Cu-deficient diet, and further gene expression profiles in Cu-adequate and Cu-deficient sheep were carried out accordingly.

Changes in the Relative Expression of CCS, SOD1 Genes in Sheep Fed With Cu-Adequate and Cu-Deficient Diets

Total RNA isolated from whole blood, red blood cells, and liver tissue had intact 28 S and 18 S ribosomal RNA bands as detected by 1% denaturing agarose gel electrophoresis separation and ethidium bromide staining (Figure 1).

In liver samples, as the liver Cu concentrations decreased the mRNA expression of the SOD1 gene was down-regulated (-) 0.59 folds ($p<0.05$) but the CCS expression was up-regulated 0.67 folds ($p<0.01$) in Cu-deficient sheep. In erythrocytes (RBC) the SOD1 expression did not change however, the CCS expression was up-regulated 2.3 folds ($p<0.001$) in deficient as compared to the adequate sheep. In whole blood, both the SOD1 and CCS were up-regulated 3.34 folds and 6.96 folds ($p<0.001$), respectively in sheep fed with Cu-deficient as compared to the Cu-adequate diets (Figure 2). The CD45 amplification was observed in whole blood samples but not in separated erythrocyte samples (data not shown)

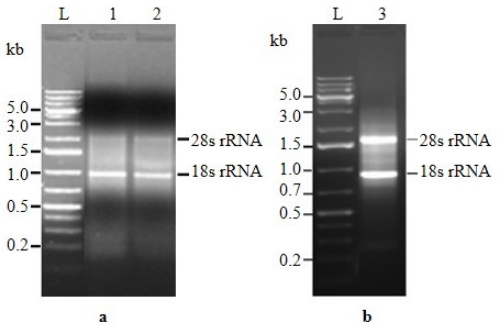


Figure 1: Quality of total RNA: location of 28s and 18s ribosomal RNA (rRNA) bands shown in 500ng total RNA isolated from whole blood (lane 1), red blood cells (lane 2) and liver tissue (lane 3) on the image of ethidium bromide stained 1% denaturing Agarose gel. The bands of GeneRuler™ 1Kb plus DNA Ladder (L) are shown in each gel.

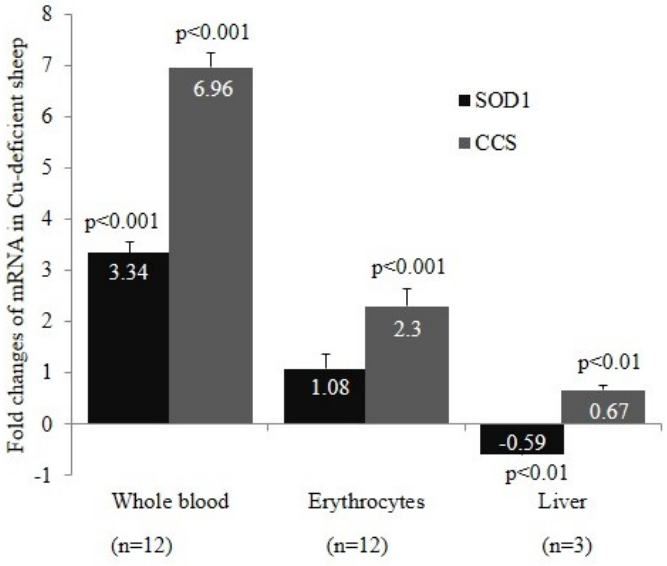


Figure 2: Expression of SOD1and CCS mRNA: in liver tissues (n = 3), whole blood (n = 12), and red blood cells (n = 12) samples of Cu-deficient and Cu-adequate group sheep. The relative mRNA expression was determined by establishing SYBR green-based quantitative PCR assay relative to the expression of glyceraldehydes 3 phosphate dehydrogenase (GAPDH) endogenous control gene. The magnitudes of up and down regulations are calculated by a standard formula [43].

Discussion

Copper is an essential trace mineral for all eukaryotes and most prokaryotes. The role of this mineral is described in a variety of physiological functions related to growth, reproduction, and immune competence. In sheep, copper nutrition is very important

for their sensitivity to deficiency and overfeeding toxicity. We report here the experimental induction of copper deficiency in sheep and its consequences on health. Also we reported the upregulation of CCS transcript in the RBC and whole blood. To our knowledge, this is the first report that demonstrated increased expression of CCS mRNA in whole blood that coincided with the Cu deficiency in ovine species and can be explored further for the possibility of using it as biological marker of Cu deficiency. Identification of this biological indicator might go a long way for better management of sheep.

Copper (Cu) deficiency is a problem in ruminants in many parts of the world due to low dietary Cu intake or when surplus Cu antagonists, such as molybdenum (Mo), sulfur (S), or iron (Fe) are fed through diet [44]. Dietary Cu antagonists present at high concentrations in soils, feedstuffs, and even water can greatly reduce the Cu status in ruminants [45, 46]. Deficiency of Cu causes neurodegenerative diseases such as 'Scrapie' in sheep [16]. Although Cu-toxicity is common in sheep, is rare in cattle and goats. It is indicated that the incidence of Cu deficiency and Cu toxicosis in sheep varies among the breeds [47] whereas, differences in Cu metabolism between cattle breeds are not as dramatic as those reported in sheep [48]. Copper is essential for Cu/Zn-SOD activity [25, 26]. In the cells where the concentration of free copper is extremely low, copper chaperones (CCS) facilitate the insertion of copper into specific target proteins. The CCS is identified as the chaperone essential for the incorporation of copper into SOD1 [51]. The CCS protein changes consistently in experimentally induced and recovered copper deficiency models [32-34]. In ruminants, a few studies have approached the idea that CCS may be a copper deficiency biomarker [52, 53]. Most studies in animals and hepatic cell lines have estimated CCS protein [32- 34], while studies in humans assessed the CCS transcripts [35, 36].

No change in feed intake in the two experimental group sheep indicated the Cu-deficient diet did not affect the consumption of feed. Since the intake of straw was similar, the variation in Cu-intake in the adequate group arose due to the concentrate feed that had additional Cu contents. Thus total Cu intake and the absorbed Cu were higher ($p<0.0001$) in Cu-adequate than in Cu-deficient group sheep. Interestingly, since we fed the animals with the copper-deficient diet in a controlled experimental condition for an extended period we could observe the typical signs of copper deficiency symptoms. Consequently, the deficient diet could lower the inorganic blood copper level including the ceruloplasmin and the nonceruloplasmin copper contents.

Along with plasma Cu, the determination of free Cu (non-ceruloplasmin-copper, NCPC) provided a more accurate measurement for screening, diagnosis, and monitoring of Cu deficiency. The lower NCPC fraction in the sheep fed with Cu-deficient than Cu-adequate diet suggested this could be a marker of Cu status as indicated by others [29]. The quantity of NCPC was 68% less in plasma of Cu-deficient than that in Cu-adequate sheep (0.25 mg/L and 0.79 mg/L, respectively). In general, the Cu deficiency impairs the effective absorption and utilization of dietary iron in animals [30]. This might have resulted in lowering ($p<0.001$) the level of hemoglobin (Hb) and consequent development of anemia in sheep fed with Cu-deficient as compared with Cu-adequate diet in our study. It has been observed that the Cu deficiency leads to the degradation of Hb by an increase in superoxide anion [55]. The supporting evidence of lower SOD activity in the Cu-deficient sheep indicated there could be an increase of superoxide ions in these groups of animals. As Cu is a catalytic cofactor for metalloenzymes like Cu/Zn SOD and ceruloplasmin (CP), decreased (~30%) activities in sheep fed Cu deficient diet compared to Cu-adequate sheep were due to deficient-diet. Low Cu/Zn-SOD and CP activity in Cu-deficient animals was reported earlier [56, 57]. Ceruloplasmin, although is termed as the acute phase protein, it seems to be a useful indicator of Cu nutrition status in cattle and sheep as CP and plasma Cu concentration decreased with nutritional Cu depletion [17] provided animals are not suffering from any inflammation and other disease conditions. Hence, in the present study, the copper deficiency was confirmed by the decreased plasma copper levels (<0.6mg/L) along with a reduction of other biochemical indicators, clinical signs, and liver Cu-content in sacrificed representative sheep fed with a Cu-deficient diet.

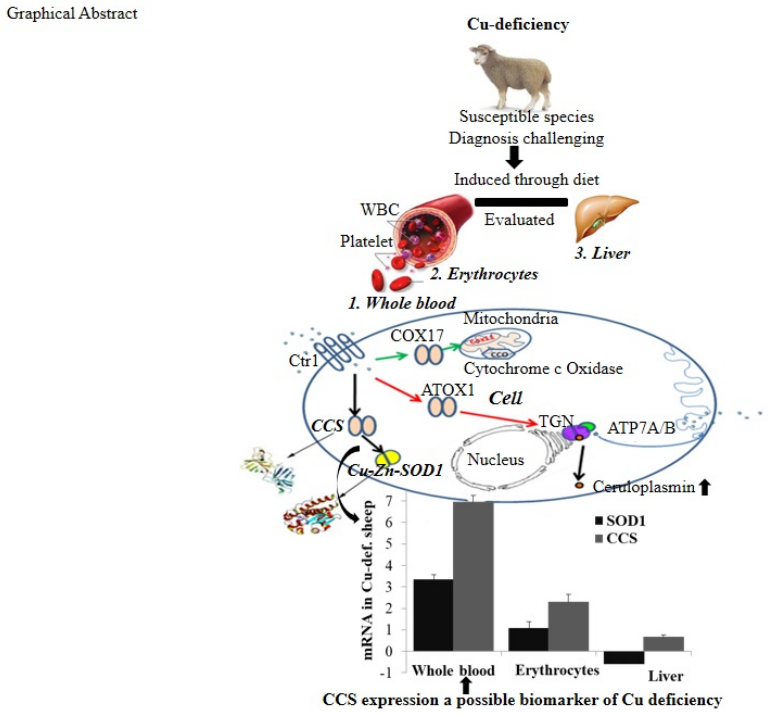
Copper deficiency in animals is normally diagnosed either by blood analysis (low copper in case of deficiency) or by nutritional analysis (to detect if the diet is low in copper). In addition for most of the experimental *in vivo* Cu-related animal model

studies, the CCS and SOD1 protein expression in liver tissues are determined. The problem with protein is that it undergoes degradation very easily even in low-temperature storage. In addition, the problem with SOD1 protein is that its level varies with the increase in the stress level of the animals. Nevertheless, the liver is the best tissue for analysis to understand the Cu status being the main organ of metabolism, storage, and release thus, assessed as a means of diagnosis in sheep in many countries. However, collecting liver tissue by sacrifice or biopsies from the live animals under field and experimental conditions only for copper deficiency assessment is time-consuming, technically more demanding, not easy, and not feasible in the farmer's field. This led us to attempt an alternative sample and parameter for analysis that might reflect the copper status of the animals. We thought the investigations of the mRNA expression of CCS and SOD1 gene in whole blood, erythrocytes, and liver samples by qPCR could be useful. Erythrocyte is used as the sample for this study as they also have been found to harbour different gene transcripts encoding many proteins that play critical roles in the differentiation and functions of mature erythrocytes [58, 59]. The absence of leucocytes in the erythrocyte samples was confirmed by checking the expression of the white blood cell marker gene CD45. It has been opined that the erythrocyte transcripts may simply be passengers, remnants of transcription that are stabilized by particular folding structures and RNA-binding proteins such as AGO2 [60]. We argue that the presence of intact 28 S and 18 S ribosomal RNA in erythrocytes (Figure 1) was an indication of active protein synthetic machinery. Indeed evidences from human indicated that RBCs (erythrocytes) contains genes encoding initiation, activation, and regulation of transcription and translation (for instance RNA polymerases I, II, III, zinc/PHD finger- DNA-binding proteins, cysteinyl, lysyl-tRNA synthetase), important RNA-stabilising factor - poly(A) binding protein, anti-apoptotic proteins (for instance beclin 1, reticulon 4, BCL2, IAP) together with genes for RNA degradation (for example ribonuclease T2) as well as genes encoding typical apoptotic proteins such as cyclooxygenase, apoptotic protease activating factor, caspase 8 [58]. The intact protein synthesis capability and posttranslational modifications probably help to get the desired proteins on demand for the survival and limited functions these cells perform during their short life span of 120 days. The presence of SOD1 and CCS transcripts observed in circulating erythrocytes of sheep is the first report so far known to us in literature. The changing SOD1 enzyme activity coinciding with the deficiency of Cu was reported earlier [40]. This study further confirmed that SOD1 mRNA expression changes in copper deficiency status in sheep liver (decreased, $p<0.01$) and whole blood (increased, $p<0.01$) which were fed with a Cu-deficient diet. The up-regulation ($p<0.01$) of CCS transcripts in the liver, whole blood, and erythrocytes due to the changes in dietary level of copper in sheep is also reported for the first time. Interestingly, we found that the expression of CCS in whole blood was much higher than in erythrocytes and liver in sheep. The human RBC (erythrocyte) contains less number of transcripts compared to the nucleated cells [59]. Since the whole blood contains the nucleated leucocytes the higher level of CCS transcript might be the outcome of that. However, why the liver tissue contained a lesser quantity of CCS transcript is not clear from this study. These probably need some more data to substantiate. Whatever could be the reason for their higher abundance, these findings prompted us to believe that the whole blood could be more convenient and attractive for field sampling due to the ease of collection and fewer processing steps as compared to the erythrocytes and liver samples. The advantage of the whole blood as the sample is that it can be collected easily without processing, the RNA isolation/stabilizing solution can be added just at the point of collection and transported to the laboratory at 4°C for isolation of total RNA subsequently followed by cDNA synthesis and gene quantification. The up-regulation ($p<0.01$) of CCS as observed in the Cu-deficient liver tissues of sheep in our study was also reported in rodents [61, 62]. The sheep that received Cu-deficient than Cu-adequate diets had down-regulation ($p<0.01$) of SOD1 transcript in liver tissue and up-regulation in erythrocytes and whole blood probably indicating differential regulation of the same gene expression in different tissues. The SOD1 is an important enzyme that protects the cells from oxidative damage by converting the superoxide anion to H_2O_2 and oxygen. The down-regulation of the SOD1 gene due to low cellular Cu concentrations might affect the translation of this enzyme in addition to the known problem of copper binding to the important structural component of this SOD1 enzyme it might result. Interestingly, the down-regulation of SOD1 transcript in the liver coincided with the lower SOD1 activity in the plasma of Cu-deficient sheep in the present study. The rats fed with Cu-deficient diets demonstrated decreased SOD1 activity due to Cu deficiency in the liver [63]. Copper atoms get incorporated into periplasmic and inner membrane cuproproteins like SOD1. However, how the lower cellular concentration of Cu resulted in lower ex-

pression of this gene in Cu-deficient sheep is not known from this study. A low intake of Cu (3.6 mg/kg) in sheep resulted in an increase in CCS mRNA expression in the whole blood and liver tissue, respectively, indicating that Cu plays an important role in the transcription of the CCS gene that might cause in the changed level of this protein. Higher expression of CCS protein is reported in earlier studies in rats and cattle [32- 34, 53]. This study indicated that Cu deficiency in the diet leads to higher expression of CCS. However, the mechanism by which the dietary Cu-restriction affects the CCS expression needs an elaborative study involving the regulatory sequence, their binding proteins, and transcription factors responsible for this gene expression in sheep. In addition, studies should also be undertaken to evaluate the transcriptional and post-translational regulatory mechanism of this protein in response to Cu status.

Conclusions

In summary, the dietary deficiency of copper reduced the intake, absorption, and Cu levels in the liver and blood, and ceruloplasmin in the blood. It up-regulated the CCS transcripts in the liver, whole blood, and erythrocytes, respectively. The up-regulation of CCS transcripts was maximum in the whole blood compared to the liver and erythrocyte samples in Cu-deficient sheep. Thus we conclude that the determination of CCS expression in the whole blood samples might be a useful biomarker of Cu deficiency in sheep as it is specific for the condition and one can use the advantage of ease of collection and analysis.



Graphical Abstract

Credit Author Statement

DT Pal: Original draft manuscript writing, conceptualization, planning and coordination, investigation and research

Jyotirmoy Ghosh: investigation, planning, coordination, data analysis, results interpretation, manuscript writing, and final editing.

BD Punith: Bench work, research and investigation, data analysis.

Conflict of Interest

Authors declare no conflicts of interest

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