The effect of storage conditions on samples for the evaluation of copper status in blesbok (*Damaliscus pygargus phillipsi*)

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ABSTRACT

Investigaltions to determine the effect of sample storage on the concentration of copper in liver tissue and on the activity of erythrocyte superoxide dismutase were undertaken in preparation for a study of blesbok (*Damaliscus pygargus phillipsi*) that were suspected to be suffering from copper deficiency. Two liver samples were collected from each of 20 culled blesbok in a manner that simulated the collection of biopsies from the live animal. These samples were stored either in 10 % formalin or frozen at –20 °C until analysed 4½ months later. The effect of different methods of sample storage on superoxide dismutase activity was determined. Erythrocytes collected from 3 Jersey cows and 5 culled blesbok were washed and divided into 0.5 mℓ portions, stored at room temperature (~20 °C), in a refrigerator (4 °C), frozen at –20 °C in a freezer, and in liquid nitrogen (–200 °C). An analysis of superoxide dismutase activity was undertaken using a commercial assay kit at intervals of 2–4 days until the levels of activity had fallen significantly. The copper concentration in formalin-preserved liver samples was significantly lower than that measured in frozen liver tissue apparently as a result of leaching. The activity of superoxide dismutase in cattle blood was unchanged for 4 days at room temperature but fell appreciably after 2 days at 4 °C and –20 °C. Enzyme activity remained unchanged for 200 days in erythrocytes stored in liquid nitrogen. Superoxide dismutase activity levels in healthy blesbok were considerably lower than those measured in Jersey cows and remained unaffected for up to 6 days in samples stored at 4 °C and 20 °C. The level of activity fell significantly thereafter. Samples stored in liquid nitrogen were unchanged after 40 days.

Key words: blesbok, copper, formalin, frozen, liver, sample storage, superoxide dismutase.

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INTRODUCTION

A condition similar to 'swayback' was observed in blesbok (*Damaliscus pygargus phillipsi*) in the Karoo Nature Reserve, Graaff Reinet (32°18'S, 24°32'E). Affected animals developed signs of posterior paresis that became more severe when they bolted at the approach of a vehicle. An animal was shot and found to be suffering from chronic copper deficiency with typical lesions of myelopathy and an apparently low copper concentration in the liver at autopsy[12].

Copper plays an essential role in the biochemistry of living organisms. Although the amounts needed are minuscule, it is an essential component of many enzymes and biological catalysts. A deficiency in copper can cause a range of clinical diseases, such as 'swayback', synonymous with 'lamkruis' or enzootic ataxia in sheep and spontaneous fractures or falling disease of cattle[2,16]. The liver is the main storage organ of copper and the primary site of copper metabolism where it is incorporated into enzymes and proteins, stored or excreted[4]. Liver biopsies are commonly used in the evaluation of the copper status of animals. There are, however, only a limited number of studies that deal with the effect of different sample storage methods on mineral concentrations in the liver. Copper concentrations in formalin-fixed liver samples have been reported to be lower than those of the frozen samples[3]. Similarly, a significant difference between selenium values in formalin-fixed liver and fresh or frozen liver of swine has been reported[14]. In both of these reports the difference was suggested to have occurred as a result of leaching.

Copper, as part of superoxide dismutase (SOD), plays an important role in oxygen metabolism. Although oxygen is essential for life, highly damaging superoxide free radicals (O^-) are formed during metabolism from the univalent reduction of molecular oxygen[10]. In the body, free superoxide radicals are converted to hydrogen peroxide (H_2O_2) by superoxide dismutase according to the equation:

$$O^- + O^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad \text{(refs 8, 9)}$$

Fe (as part of catalase) then converts the H_2O_2 into H_2O and O_2.

In erythrocytes, the enzyme appears to be synthesised at the time of erythropoiesis and its half-life in blood correlates with the half-life of the erythrocyte[11]. Owing to the relatively long half-life of superoxide dismutase, the rate of decrease of this enzyme will be slower than other substances measured to evaluate copper status, such as plasma/liver copper or ceruloplasmin. Whereas ceruloplasmin activity decreases exponentially in the acute stages of copper insufficiency, decreases in erythrocyte superoxide dismutase activities are more linear[11]. Low erythrocytic superoxide dismutase values therefore have diagnostic significance, indicating a chronic or intense deficiency of available copper[15].

Little has been reported on the influence of time and temperature on the activity of superoxide dismutase in stored blood or tissue samples. The loss of enzyme activity in blood samples stored at room temperature was found to be variable. After 3 days human superoxide dismutase activity fell by 0–30 %[7]. Others report that no measurable loss of activity occurred for up to 10 days in heparinised human blood samples stored at 4 °C[1,13]. No significant changes in serum superoxide dismutase activity took place in samples that were kept for at least 2 months at –20 °C and the enzyme remained active in placental extracts kept at the same temperature for 8 years[17].

We report here on preliminary work undertaken to establish conditions for keeping blood and tissue samples that are to be transported over long distances to the laboratory in an optimal manner.

The 1st objective of was to determine the concentration of copper in liver samples stored at –20 °C or in formalin and compare the results. Second, the influence of the duration of storage at

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different temperatures on the activity of superoxide dismutase was determined.

MATERIALS AND METHODS

Liver samples

Whole livers were obtained from randomly selected blesbok in Willem Pretorius Game Reserve (28°16′S, 27°01′E), culled as part of population control measures (n = 20). Samples were collected using a 5-gauge biopsy needle without a stylet in a manner which simulated the technique described for collecting liver biopsies in sheep[5]. With the liver placed in an anatomically correct position, the biopsy needle was inserted into the dorsal right lobe in a cranio-ventromedial direction to a depth of 3 cm. The biopsy needle was rotated as it penetrated the tissue. Suction was applied to the needle using a 20-mℓ plastic syringe attached to the needle to draw the tissue into it. The collected biopsy tissue was expelled into a storage tube (Nunc, AEC Amersham, Johannesburg) and stored at –20 °C. A 2nd sample was collected in a similar manner at a site next to that of the 1st sample and placed into a rubber stoppered test tube containing 10 mℓ buffered formalin. Samples were stored for approximately 4½ months before analysis.

Analysis of hepatic copper concentration

Analysis of liver samples was performed by the Western Cape Regional Veterinary Laboratory, Stellenbosch.

The samples were placed onto clean watch glasses and dried in an oven at 90 °C overnight to remove all moisture. The samples were then weighed on an analytical balance (Mettler AE100) and transferred to clean, acid-washed digestion flasks containing 3 glass digestion beads. Ten millilitres of Acid Mix (1:5 70 % HNO_3:65 % $HClO_4$) (Merck) was added to each flask for biopsies weighing <0.40 g (15 mℓ for biopsies weighing >0.40 g). The flasks were allowed to stand for approximately 15 minutes and then gently heated to a maximum temperature of 250 °C until digestion was complete. The liver biopsies with a mass <0.40 g were diluted to 10 mℓ with deionised water, 20 mℓ was added when the mass of the samples was greater than 0.40 g. The copper levels were measured by flame atomic absorption spectrophotometer (GBC SDS-270 Avanta, GBC Scientific Equipment, Victoria, Australia).

Measurement of superoxide dismutase activity in blood

Blood was collected from 3 apparently healthy jersey cows kept at the Faculty of Veterinary Science, University of Pretoria, as part of a preliminary study into the use of the superoxide dismutase assay and to estimate the effect of different storage methods on enzyme activity over time.

Later a further 5 jugular blood samples were collected from blesbok culled in the Gariep Dam Game Reserve (30°38′S, 25°34′E) as part of population control measures.

Blood samples were collected into sodium heparin tubes (Becton Dickinson,USA) and placed into iced water in a cooler box, divided into 0.5-mℓ portions and prepared on site prior to being transported to the laboratory. The samples were centrifuged at 3000 rpm for 10 min. Thereafter the plasma was drawn off and the erythrocytes that remained were washed 3 times with a 0.9 % saline solution, centrifuging for 10 min at 3000 rpm after each wash.

Washed erythrocytes were stored at different temperatures, namely at room temperature (~22 °C), in a refrigerator (4 °C) or freezer (–20 °C) and in liquid nitrogen (–200 °C). Blesbok erythrocyte samples were placed in iced water, taken to the laboratory at the Faculty of Veterinary Science and then stored in a fridge (4 °C) and freezer (–20 °C). Washed erythrocyte samples which were assigned for storage at –200°C were placed liquid nitrogen at the reserve.

Superoxide dismutase assay

A commercial assay kit (RANSOD Cat. No. SD 125, Randox Laboratories, Crumlin, UK) was used to measure superoxide dismutase activity. The method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity is measured by the degree of inhibition of this reaction.

In the laboratory, the samples, if frozen, were defrosted at room temperature. Samples were made up to 2.0 mℓ with re-distilled water, mixed and left to stand at 4°C for 15 minutes. The lysate was further diluted with 0.01 mmol/ℓ phosphate buffer (pH 7.0) to a dilution of 1:100 and added to the mixed substrate. Xanthine oxidase was then added to start the reaction. Absorbance was read using a spectrophotometer (Beckman DU 650, USA). The wavelength was set at 505 nm and the temperature at 37 °C. The initial absorbance was read 30 seconds after the addition of xanthine oxidase. The final absorbance was read 3 minutes after the 1st reading. A change in absorbance per minute was calculated and the percentage inhibition determined by comparing this reaction to the uninhibited one (xanthine oxidase added to the mixed substrate). A standard curve was used to determine the superoxide dismutase activity (U/mℓ) from the percentage inhibition.

Each sample was analysed 3 times and the average calculated. Measurements not within a 30–60 % inhibition range were discarded and a different dilution made so that the percentage inhibition would fall between 30 and 60 %. Precision was controlled by using a RANSOD control (Cat. No. SD 126, Randox Laboratories, Crumlin, UK).

RESULTS

Effect of storage on copper levels in blesbok liver samples

The mean liver copper concentration in formalin (2094.6 mmol/kg, SD = 927.4, n = 19) was significantly lower than the mean copper concentration measured in frozen livers (2274.4 mmol/kg, SD = 935.0, n = 19) (paired t-test: t = –4.13, df = 18, P < 0.001). One sample bottle was damaged in transit and the liver tissue was not analysed.

The effect of the duration of storage at different temperatures on the activity of superoxide dismutase

The effect of the duration of storage and the temperature on the activity of superoxide dismutase in blood collected from Jersey cows is represented graphically in Fig. 1. The activity of superoxide dismutase appears to have been maintained for approximately 4 days at room temperature but only for 2 days at 4 °C. Insufficient data are available from samples kept in the freezer (–20 °C) to be able to assess the daily rate of decline of enzyme activity. It is clear that a considerable loss of activity had occurred after 7 days. The activity of the enzyme remained stable for at least 6 months in liquid nitrogen.

The high and variable ambient temperatures experienced in collecting blood samples from culled blesbok at the Gariep Dam game reserve precluded the storage of samples at room temperature. Samples from these animals were kept in iced water until they reached the laboratory 12 hours later and were then placed into either a refrigerator or freezer. The superoxide dismutase remained unchanged to 6 days but fell rapidly thereafter.

The activity of superoxide dismutase in blesbok blood was maintained for the duration of the study period (40 days) when the samples were stored in liquid nitrogen (Fig. 2).

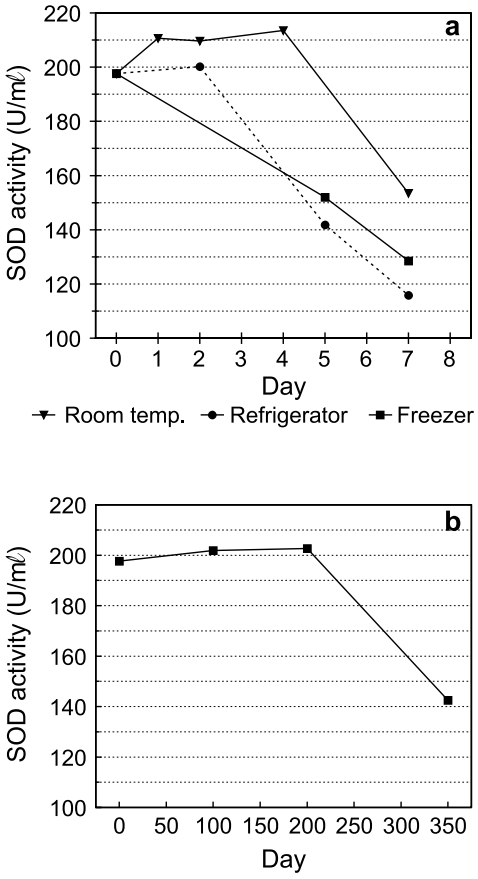

Fig. 1: a: **Mean bovine erythrocyte superoxide dismutase activity in samples kept at room temperature, in a refrigerator and freezer** (n = 3); b: **mean bovine erythrocyte superoxide dismutase activity in samples kept in liquid nitrogen** (n = 3).

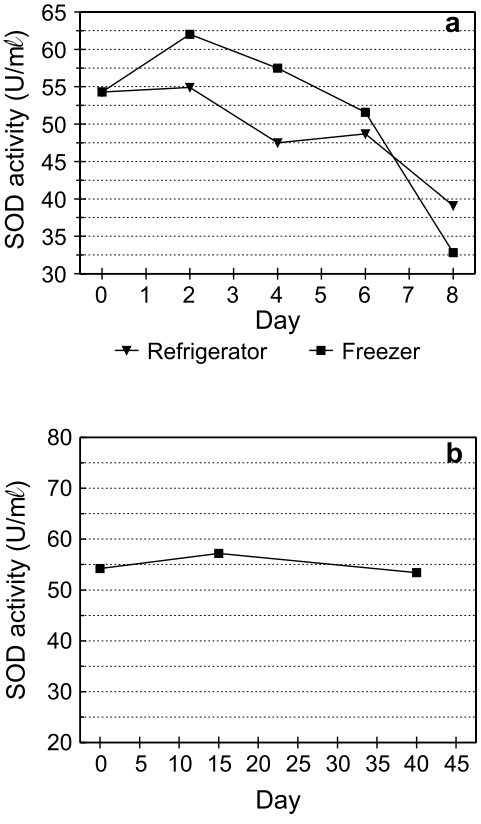

Fig. 2: a: **Mean bovine erythrocyte superoxide dismutase activity in blesbok blood kept in a refrigerator and freezer** (n = 3)**;** b: **mean bovine erythrocyte superoxide dismutase activity in blesbok blood kept in liquid nitrogen** (n = 3)**.**

DISCUSSION

Storage of liver biopsies

The copper concentration in formalin-fixed liver samples was significantly lower than in frozen liver tissue. This probably occurred as a result of leaching[3,14]. As the degree of leaching over time is likely to be variable, samples should be frozen if the liver copper concentration is to be measured.

Superoxide dismutase activity

The duration of storage and the temperature at which this takes place was found to have an important effect on the activity of erythrocyte superoxide dismutase. Though the more prolonged maintenance of enzyme activity found at room temperature appears anomalous, similar findings in other enzymes have been reported[6]. The differences in superoxide dismutase activity in the blood of the jersey cows and the blesbok are apparently the result of differences commonly seen between species[6]. The culled blesbok were in good health.

From these data it is recommended that bovine and blesbok blood samples be kept cool and analysed within 6 days for an accurate measurement of superoxide dismutase activity. Storage of samples in liquid nitrogen is the only method of maintaining the activity of superoxide dismutase for prolonged periods.

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