JOURNAL OF
Veterinary Emergency
AND Critical Care

CO-oximetry measurements and antioxidant effects of ascorbic acid and methylene blue in equine methaemoglobinaemic blood

Journal:	*Journal of Veterinary Emergency and Critical Care*
Manuscript ID	JVECC-19-02-0008.R2
Wiley - Manuscript type:	Original Study
Keyword:	CO-oximeter, methaemoglobin, horse, methylene blue, ascorbic acid

Summary

Objectives: The purpose of this study was to determine the effects of time after sampling on CO-oximetry measurements of equine blood samples and the effects of adding ascorbic acid (AscAc) and methylene blue (MetBlue) to samples with *in vitro* induced methaemoglobinaemia.

Design: Experimental study.

Setting: University teaching hospital.

Animals: Thirty healthy adult horses assigned to 5 different groups.

Interventions: Repeated CO-oximetry determinations were performed on venous (n=6) and arterial blood samples (n=7) stored at 0°C (ice water) for 48h. Methaemoglobinaemia was induced *in vitro* in 17 additional blood samples. Six were used as untreated controls, six had AscAc and five had MetBlue added. Total haemoglobin, oxyhaemoglobin (O_2Hb), carboxyhaemoglobin, methaemoglobin (MetHb) and oxygen saturation of haemoglobin were measured. Samples were kept in 1ml airtight plastic syringes.

Measurements and main results: Oxyhaemoglobin and SO_2 increased from 69.8±10.2% and 90±3% to 82.8±7.9% and 99±3% respectively after 8h in venous blood (mean±SD, $p<0.001$). There was an effect of treatment ($p=0.032$) and of time (interaction $p=0.003$) on MetHb% in methaemoglobinaemic samples. The difference in absolute MetHb% from time 0 were as follows: 7.0% [IQR:-1.2, 20.0], -0.2% [IQR:-2.0, 1.5] and -4.4% [IQR:-7.0, -1.8] at 48h in Control, AscAc and MetBlue groups respectively ($p<0.05$). There was no effect of time on MetHb% in the AscAc group (23% [IQR:9.2, 61.8] at time 0 to 23.2% [IQR:7.0, 63.6] after 48h).

Conclusions: Storage of blood in ice water to determine HbO_2 and SO_2 using a CO-oximeter should not exceed 4h. Measurement of MetHb% could be delayed by up to 48h if AscAc is added to the sample. Methylene blue significantly decreased MetHb% over time. The limitations of this study include the fact that the antioxidant effects of AscAc and MetBlue were evaluated *in vitro* and not *in vivo*. Further studies are needed to evaluate different storage temperatures and syringe types.

Abbreviations

AscAc	Ascorbic acid
COHb	Carboxyhaemoglobin
Hb	Haemoglobin
MetBlue	Methylene blue
MetHb	Methaemoglobin
O_2Hb	Oxyhaemoglobin
SO_2	percentage oxygen saturation of haemoglobin
TotHb	Total haemoglobin

INTRODUCTION

CO-oximetry is the gold standard method of measurement of oxygen saturation of haemoglobin and provides the spectrophotometric measurement of total haemoglobin as well as the percentages of the main haemoglobin derivatives, which include oxyhaemoglobin (O_2Hb), deoxyhaemoglobin, carboxyhaemoglobin (COHb) and methaemoglobin (MetHb).[1] CO-oximetry is mainly used in the critical care setting and is indicated in patients in which hypoxaemia does not improve with oxygen therapy, when there is a history consistent with toxin exposure or when the clinician suspects dyshaemoglobinaemias (i.e.: methaemoglobinaemia or carboxyhaemoglobinaemia).[1]

Measurement of oxygen status and haemoglobin (Hb) species is problematic, as oxygenation parameters as well as percentages of different Hb species change after the blood has been sampled. Rapid changes in oxygenation parameters are reported in human and animals after blood samples have been drawn from these patients.[2-5] Most studies in animals on the effects of time of storage on blood oxygenation parameters have used standard blood gas machines. These calculate oxygen haemoglobin saturation (SO_2) based on the PO_2 instead of measuring SO_2 or O_2Hb directly. CO-oximetry measures O_2Hb percentage and total Hb allowing for an accurate determination of the SO_2. It has been shown that CO-oximetry provides more precise SO_2 and O_2Hb measurements compared to standard blood gas machines.[6] Although the effects of exercise on SO_2 or O_2Hb as measured by CO-oximetry have been determined on equine whole blood stored at 0°C in (ice

water),[7] the effect of time of storage on equine blood CO-oximetry values has not been previously described.

Methaemoglobin concentrations also vary after sampling due to the instability of MetHb.[8] The efficacy of ascorbic acid (AscAc) and methylene blue (MetBlue) for the treatment of horses with methaemoglobinaemia has been questioned. [9] In addition it has been shown that in contrast to other species, equine MetHb is more sensitive to oxidation[10] and has a decreased reduction rate by MetBlue using equine blood samples. [11] In a previous *in vitro* study using equine washed red blood cells, MetBlue did not have an effect on MetHb%.[11] However the antioxidant effects of AscAc and MetBlue on equine whole blood and the effects of time after sampling on samples to which these compounds were added is unknown.

Objectives

The purpose of this study was to determine the effects of time after sampling on CO-oximetry measurements of equine blood samples and the effects of adding AscAc and MetBlue to samples with *in vitro* induced methaemoglobinaemia. We hypothesized that 1) there would be an effect of a prolonged delay-in-time from sampling to determination of CO-oximetry measurements in arterial and venous blood from healthy horses and that 2) there would be an antioxidant effect of methylene-blue and ascorbic-acid on equine blood samples with *in vitro* induced methaemoglobinaemia. A tertiary hypothesis was that 3) there would be an effect of a prolonged delay-in-time from sampling to determination of methaemoglobin in methaemoglobinaemic blood samples.

MATERIALS AND METHODS

Animals

Venous blood was collected from the jugular vein of 23 healthy horses, and arterial blood samples were collected from the transverse facial artery in another seven horses (4-25 years of age and mean age of 11.6, 17 males and 13 females). The effect of time after sampling on CO-oximetry measurements was evaluated in the arterial blood from seven and venous blood from six healthy horses respectively. Venous blood from the remaining 17 horses was used to determine the effects of delay-in-time from sampling to determination of MetHb in methaemoglobinaemic equine venous blood (6 horses) and to evaluate the antioxidant effects of AscAc (6 horses) and MetBlue (5 horses) on whole venous blood with *in vitro* induced methaemoglobinaemia. Animals were randomly allocated to the 3 different groups. Sample size was calculated following a resource equation approach, using a design specific for group comparison, one between and one within factor, repeated-measures ANOVA, as previously described.[13]

Quality controls for the CO-oximeter included an initial calibration with all liquid controls (RNA CO-Oximeter controls[a]). Weekly testing of one different level of liquid control was performed thereafter. In addition, daily optical quality controls were performed during the whole testing period.

Procedures

Effects of time after sampling on percentage O_2Hb, COHb, MetHb and SO_2 as measured by CO-oximetry (Avoximeter 4000[b]) were evaluated in arterial and venous equine blood samples. Aliquots of 1 ml of blood were stored in air tight

sealed plastic syringes placed in ice water and CO-oximetry measurements repeated at 0, 1, 2, 4, 8 and 24 hours after sampling. Blood samples were drawn in 1ml plastic syringes (ENFA 1 ml. Tub. 3C-Luer cen.[c]) through 22G 2.5cm (SOL-M™ hypodermic needle[d]) needles previously washed with liquid heparin (5000 IU/ml) left to fill the dead space. A rubber cup was immediately placed at the end of the needle to create an airtight environment. Syringes were filled to total capacity and then inverted (at least 20 times) and rolled during 60 seconds after sampling. To determine the effects of delay-in-time from sampling to determination of MetHb percentage (MetHb%) and to evaluate the antioxidant effects of MetBlue and AscAc on methaemoglobinaemic equine blood samples, methaemoglobinaemia was induced *in vitro* as described elsewhere.[14] Briefly, a 0.1M stock solution of sodium nitrite was prepared. A variable percentage of MetHb was induced *in vitro* by adding equal amounts (0.1 ml) of sodium nitrate solution using a 2-fold serial dilution factor up to a 64-fold total dilution of the original stock solution in 1 ml of blood. Venous blood was incubated with sodium nitrate for 20 minutes at room temperature. Serial CO-oximetry measurements were performed at 0, 8, 24 and 48h after incubation for the 3 groups namely: {1} Control group (Control), {2} a group with added AscAc and {3} a group with added MetBlue.

Briefly, for the AscAc group, 1 ml of blood with *in vitro* induced methaemoglobinaemia was exposed to 0.1 ml (0.03 uM) of a freshly prepared solution of AscAc. For the MetBlue group, 1 ml of blood with *in vitro* induced methaemoglobinaemia was exposed to 0.1 ml (10 uM) of a freshly prepared MetBlue solution. Control group samples were composed of methaemoglobinaemic samples to which 0.1 ml of normal saline solution was added. All samples were

stored in airtight sealed plastic syringes placed in ice water and kept until the parameters were evaluated by CO-oximetry.

Data analysis

The data were analysed using Sigmastat® 3.0.1[e] using repeated measures analysis of variance (ANOVA) as assumptions of normal data distribution using a Kolmogorov-Smirnov test with Lilliefor´s correction were met. Methaemoglobin was logarithmically transformed to meet these criteria and converted back to original units. All data are presented as mean±SD except for MetHb% that is presented as median and interquartile range 25-75 [IQR]. The reduction of MetHb over time for the 3 different groups is expressed as the absolute reduction rate (%) from time 0 at 8, 24 and 48h. Multiple comparisons within treatment and time were performed by the Holm-Sidak method. Differences were considered statistically significant when $p \leq 0.05$.

RESULTS

Oxygenation status

There was a significant increase in O_2Hb and SO_2 over time from 69.8±10.2% and 90±3% to 82.8±7.9% and 99±3% respectively at 8h (mean±SD, $p<0.001$) in venous blood samples stored at 0°C (ice water). There were no statistically significant changes observed in arterial blood samples for any of the CO-oximetry measurements (Table 1).

Antioxidant effects of ascorbic acid and methylene-blue on MetHb%

Methaemoglobinaemic samples from each group had variable percentages of MetHb ranging from 4 to 73% at time 0 (Table 2). There was a significant effect of treatment (p=0.032) and of time (interaction p=0.003) on MetHb%. There was a significant increase in absolute MetHb% in samples from the control group at 24 and 48 hours (7.0% [IQR:-1.2, 20.0]) (p<0.001) (Figure 1A). There was no significant effect of time on MetHb% in the AscAc group at any point in time (23% [IQR:9.2, 61.8] at time 0 to 23.2% [IQR:7.0, 63.6] after 48h) (Figure 1B). There was an effect of time on absolute MetHb% in the MetBlue group at 48h (17.2% [IQR:9.8, 32.8] at time 0h to 7.6% [IQR:3.5, 28.4] after 48h; p=0.003) (Fig 1C). In samples with added AscAc and MetBlue, changes in MetHb% were as follows: -0.25% [IQR:-2.0, 1.5] and -4.4% [IQR:-7.0, -1.8] at 48h for the AscAc and MetBlue groups respectively (p<0.039) (Fig 2).

DISCUSSION

Our results revealed that O_2Hb and SO_2 increased significantly beyond 4 hours of storage in airtight plastic syringes placed ice water. Addition of AscAc to methaemoglobinaemic samples had a negligible antioxidant effect but was very effective in preventing further oxidation of MetHb during the 48 hours study period. Methylene blue resulted in a significant reduction on MetHb concentration over time.

CO-oximetry is the gold standard for the determination of oxygenation status in patients. However, CO-oximetry is not widely available hence the need to store

blood for later determination at reference laboratories. Storage of blood samples in ice water is a practical and standard way to keep samples refrigerated until arrival to the laboratory. Previous work performed in healthy dogs has shown that CO-oximetry measurements in arterial blood samples were stable for up to 4h in plastic syringes placed in ice water[12] and study measurements were performed at 2, 4 and 48h after sampling. The frequency of sampling in the present study was adapted to determine if parameters would remain stable at 1, 2, 4, 8 and 24h. These collection times were chosen to determine if testing could be delayed beyond 4h in horses. In addition to arterial blood, venous samples were also included in order to evaluate whether samples with lower O_2Hb and SO_2 levels would be affected by storage conditions. Air was removed from all samples and a rubber cap was placed at the end of the needle to create an airtight environment. In the present study SO_2 and O_2Hb values in arterial samples remained unchanged throughout the study period. This may be explained by the high SO_2 and O_2Hb percentages found in normal arterial blood samples, thus these parameters remained stable (Table 1).

In the present study, venous blood SO_2 and O_2Hb had a 9 and 10% absolute increase respectively at 8h of storage in ice water (Table 1). The results of the present study are in agreement with those obtained by Rezende in dogs. In their study oxygenation status variables remained stable for up to 4h post-sampling.[10] However, these results were only observed on samples with lower oxygen saturation of haemoglobin (venous blood), while no changes were seen on samples with very high oxygen saturation of haemoglobin (arterial samples). It is likely that due to a higher oxygen saturation of haemoglobin, a modest increase in partial pressures of oxygen within the sample did not affect the percentage of

oxyhaemoglobin. Increasing the sampling frequency confirmed that blood samples stored under these conditions suffered spurious increases in SO_2 and O_2Hb after 4h of storage.

Previous studies evaluating the effects of time and conditions of storage performed in horses and other animal species evaluated PaO_2 and the calculated SO_2 using standard blood gas machines.[2-5] Partial pressure of oxygen accounts for less than 3% of the oxygen content in blood, which can be a deficient reflection of the remaining 97% total oxygen present in the blood. In case of dyshaemoglobinaemias PaO_2 and PvO_2 and a calculated SO_2 as measured by standard blood gas machines may remain within normal limits, while O_2Hb and the real SO_2 could be in fact decreased. Previous studies have described the effect of time and syringe type (glass versus plastic) on PaO2 and PaCO2 measurements as well as the calculated SO_2 in blood samples from horses. [2-5] In the present study we used plastic syringes. The effects of time of storage on HbO_2 and directly measured SO_2 have not been previously described in horses.

The *in vitro* experiments in the present study demonstrated a significant increase in the concentration of MetHb over time in the control group. This is in agreement with previous observations in humans in which MetHb oxidation continued over time when whole blood methaemoglobinaemic samples were stored at room temperature or at -30°C.[15] This could be caused by a process known as autoxidation of MetHb.[16] It has been shown that oxidation of Hb into MetHb occurs by different mechanisms, direct oxidation and autocatalytic oxidation.[17] However, only control samples with MetHb >20% in our study had an increase over time,

whereas control samples with lower MetHb% would subjectively experience a decrease over time. Perhaps, this effect was related to homeostatic mechanisms of soluble and enzymatic antioxidant mechanisms that may be overwhelmed when a given MetHb% is surpassed.

The MetBlue group showed a significantly decreased MetHb concentration when compared to the control group at 48 hours. This is in contrast to a previous study in which no effect of MetBlue on equine MetHb% was observed.[11] In the study by Medeiros et al. CO-oximetry measurements were evaluated up to 24h, while in the present study CO-oximetry measurements were evaluated for a longer period of time (48h). For the present study whole blood was used instead of washed red blood cells placed in a medium containing glucose and methylene blue as described by Medeiros.[11] Methylene blue is a pro-oxidant that needs to be first reduced to leukomethylene blue in order to reduce methaemoglobin.[18] It is likely that some degree of oxidation due to methylene blue occurred at a similar rate than red blood cell reduction causing concentrations to remain levelled throughout Medeiros' experiment. Although a decrease in MetHb was observed in this study, the magnitude of the effect appeared to be less pronounced compared to the reducing effect of MetBlue in cases of acquired methaemoglobinaemia in human subjects.[19] However, the effect was relevant since control samples had a large increase in absolute percentages of MetHb while MetHb% in AscAc and MetBlue treated samples either stabilized or decreased.

Ascorbic acid concentrations in this study were of similar molar concentration described to be effective in reducing MetHb in methaemoglobinaemic samples in an *in vitro* study in ruminants.[20] In Atyabi's experiment, blood was pretreated with different vitamins at varying concentrations adding the AscAc before induction of methaemoglobinaemia. Higher concentrations of AscAc resulted in a smaller antioxidant effect when compared to a medium-low concentration.[20] In this study AscAc was added after incubation with sodium nitrate.

The main limitations of this study are that only one type of syringe and one method of cooling of the blood samples were used. Further studies are needed to evaluate the effects of different storage temperatures and types of syringes on CO-oximetry parameters in horses. Another limitation is that the temperature of the water may have fluctuated depending on the volume of ice and water components, however, this parameter was not recorded. As the antioxidant effects of AscAc and MetBlue were evaluated *in vitro*, it is not known if these results may be extrapolated for use in clinical cases.

In conclusion, whole blood can be stored in regular airtight plastic syringes for up to 4 hours before measurement of HbO_2 and SO_2 by CO-oximetry. Methaemoglobin may continue to increase after sampling in equine whole blood. The addition of freshly prepared AscAc solution may prevent this spurious pre-analytical increase in MetHb. Methylene blue reduces MetHb *in vitro* on equine whole blood and it should be further investigated in horses using *in vivo* models.

Manufacturers' addresses

[a] RNA medical, Devens, MA, USA

[b] ITC international Technydine Corporation, Edison, NJ, USA

[c] Sol-Millenium Medical Inc., Lawrenceville, GA, USA

[d]Jiangsu Kanghua Medical Equipment Co. Ltd., Jiansu, P.R. China

[e] SYSTAT Software Inc., San José, CA, USA

279 Table 1: Mean (±SD) percentage oxyhaemoglobin (O_2Hb) and oxygen saturation
280 (SO_2) in arterial blood samples at 0, 1, 2, 4, 8 and 24h and venous blood samples
281 at 0, 1, 2, 4 and 8h as well as, magnitude of change (Δ) of O_2Hb and SO_2 from time
282 0h for both types of samples (calculated as median of the differences). Results are
283 expressed as mean [SD], Repeated measures ANOVA, n=7 (arterial group), n=6
284 (venous group). There was a significant effect of time at 8h * $p<0.01$, as compared
285 to 0h for the venous blood group.

	T0	T1	T2	T4	T8	T24
Arterial O_2Hb	99.6 (±0.8)	99.1 (±1.9)	99.3 (±1.5)	99.3 (±1.5)	99.7 (±0.3)	99.7 (±0.4)
Arterial SO_2	99.6 (±0.6)	99.2 (±1.8)	99.3 (±1.5)	99.4 (±1.2)	99.8 (±0.1)	99.8 (±0.2)
Venous O_2Hb	69.8(10.2)	73.5(8.6)	75.5(7.5)	78.3(7.5)	82.7(7.8)*	---
Venous SO_2	70.1(10.3)	73.4(8.9)	75.3(7.8)	78.1(7.6)	82.4(8.0)*	---
		ΔT0-T1	ΔT0-T2	ΔT0-T4	ΔT0-T8	ΔT0-T24
Arterial O_2Hb	---	-0.4 (±1.0)	-0.3 (±0.7)	-0.3 (±0.6)	0.2 (±0.5)	0.2 (±0.5)
Arterial SO_2	---	-0.5 (±1.1)	-0.3 (±0.9)	-0.2 (±0.6)	0.2 (±0.5)	0.2 (±0.4)
Venous O_2Hb	---	3.7(4.8)	5.7(4.3)	8.5(5.7)	12.9(7.5)*	---
Venous SO_2	---	3.3(4.7)	5.3(4.2)	8.1(5.7)	12.3(7.5)*	---

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Table 2. Median [IQR] methaemoglobin percentage (MetHb%) in venous blood samples of the control, ascorbic acid (AscAc), and methylene blue (MetBlue) groups with variable *in vitro* induced methaemoglobinaemia at 0, 8, 24 and 48h, as well as, magnitude of change (Δ) of MetHb% from time 0h (calculated as median of the differences). ~~Results are expressed as median [IQR], Repeated measures ANOVA, n=6 (control and AscAc groups) n=5 (MetBlue group).~~ There was a significant effect of time and treatment at 24h and 48h *p<0.01, when compared to 0h for the control group, and §p<0.01 when compared to 0h for the MetBlue group.

	T0	T8	T24	T48
Control	21.9 [11.0, 29.2]	25.5 [11.8, 42.3]	27.6 [11.7, 46.8]*	28.9 [9.1, 52.4]*
AscAc	23.1 [9.2, 61.8]	24.8 [10.3, 62.5]	24.1 [8.6, 62.2]	23.2 [7.0, 63.6]
MetBlue	17.2 [9.8, 32.8]	16.5 [7.3, 35.1]	12.8 [3.7, 31.9]§	7.6 [3.5, 28.4]§

		ΔT0-T8	ΔT0-T24	ΔT0-T48
Control	--	3.3 [0.7, 10.0]	5.7 [0.7, 14.4]*	7.0 [-1.2, 20.0]*
AscAc	--	0.7 [0.25, 1.2]	-0.5 [-0.7, 0.5]	-0.2 [-2.0, 1.5]
MetBlue	--	-0.9 [-1.4, -0.7]	-2.7 [-4.4, -1.2]§	-4.4 [-7.0, -1.8]§

AscAc, ascorbic acid; MetBlue, methylene blue.

Figure legends:

Figure 1. Median [IQR] (solid line [error bars]) and individual horses' (dashed lines) methaemoglobin % in venous blood samples with in vitro induced methaemoglobinaemia from (A) the control group, (B) the ascorbic acid (AscAc) group, and (C) the methylene blue (MetBlue) groups. ~~The interquartile range is depicted by the error bars arising from the solid lines (median methaemoglobin concentration) at each time point. The methaemoglobin concentrations in (A) the control group increased over time while the (B) AscAc group showed virtually no changes during the study period. The methaemoglobin concentration of (C) the MetBlue group showed a decrease over time.~~ The median (solid line) of figure C obscures one individual horses' methaemoglobin % as both (median and horse) have the same values. Asterisks and § signs identify statistically significant changes from baseline at specific times. Repeated measures ANOVA, ~~n=6 (control and AscAc groups) n=5 (MetBlue group),~~ * 24h and 48h statistically different (p=0.001), ~~when compared to~~from 0h for the control group, § 24h and 48h statistically different (p=0.003) ~~when compared to~~from 0h for the MetBlue group.

Figure 2. Median [IQR] of change (Δ) in methaemoglobin % from time 0h in venous blood samples with *in vitro* induced methaemoglobinaemia of the control (dashed line with circles), ascorbic acid (solid line with rectangles) and methylene blue (dashed line and dots with triangles) groups.

Supporting information

~~Figure 3. Individual sample's magnitude of change (Δ) in methaemoglobin % (MetHb%) from time 0h, in venous blood samples with *in vitro* induced methaemoglobinaemia of the control, ascorbic acid (AscAc) and methylene blue (MetBlue) groups respectively.~~

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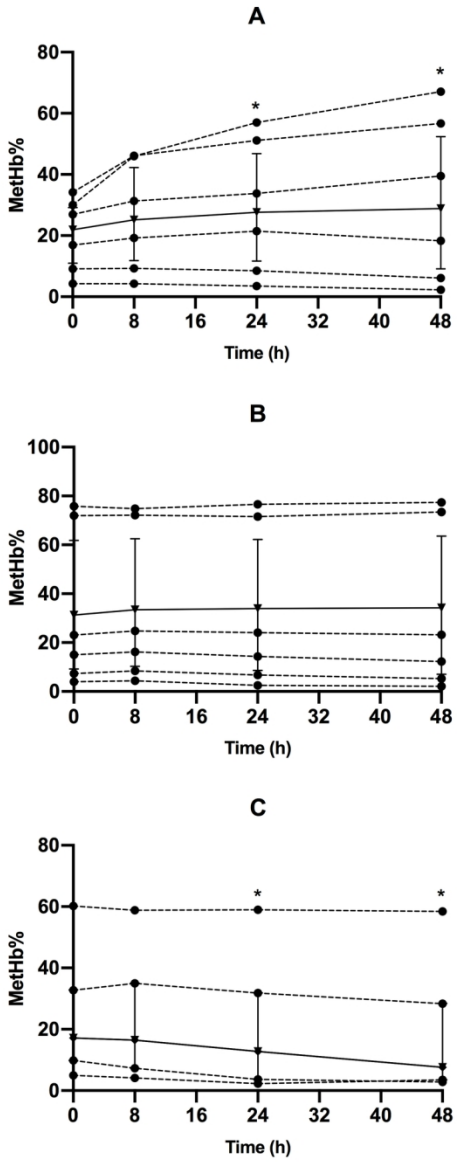

Figure 1. Median [IQR] (solid line) and individual horses' (dashed lines) methaemoglobin % in venous blood samples with in vitro induced methaemoglobinaemia from (A) the control group, (B) the ascorbic acid (AscAc) group, and (C) the methylene blue (MetBlue) groups. The interquartile range is depicted by the error bars arising from the solid lines (median methaemoglobin concentration) at each time point. The methaemoglobin concentrations in (A) the control group increased over time while the (B) AscAc group showed virtually no changes during the study period. The methaemoglobin concentration of (C) the MetBlue group showed a decrease over time. The median (solid line) of figure C obscures one individual horses' methaemoglobin % as both (median and horse) have the same values. Asterisks and § signs identify statistically significant changes from baseline at specific times. Repeated measures ANOVA, n=6 (control and AscAc groups) n=5 (MetBlue group), * 24h and 48h statistically different (p=0.001), when compared to 0h for the control group, § 24h and 48h statistically different p=0.003 when compared to 0h for the MetBlue group.

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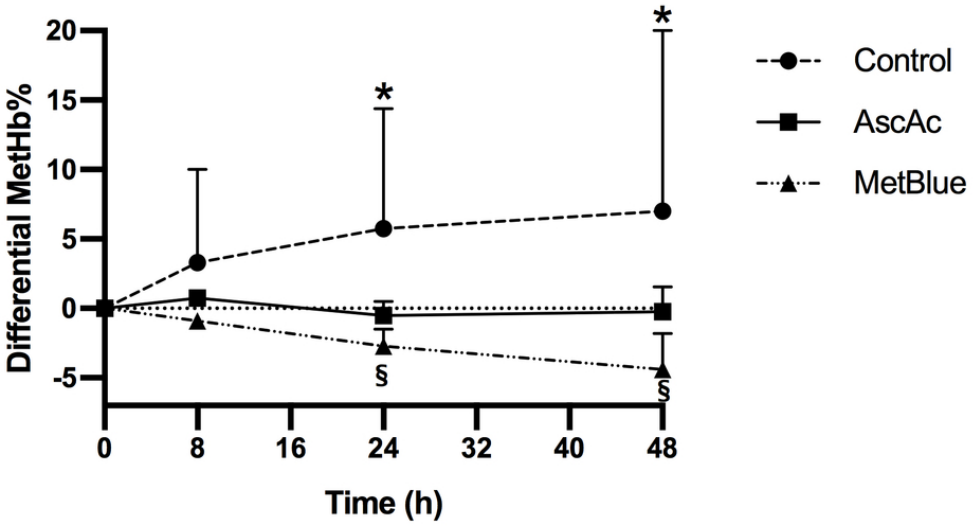

Figure 2. Median [IQR] of change (Δ) in methaemoglobin % from time 0h in venous blood samples with in vitro induced methaemoglobinaemia of the control (dashed line with circles), ascorbic acid (solid line with rectangles) and methylene blue (dashed line and dots with triangles) groups.

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