

Concentrations of Free (Ionized) and Total Calcium and Magnesium in Healthy Captive Asian Elephants (*Elephas maximus*) and Effects of Sample Type and pH on measured Free Calcium and Magnesium Concentrations

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ABSTRACT

The Asian elephant (*Elephas maximus*) is an endangered species, with an overall low reproduction rate in captivity, and a long, 22-month gestation, mostly with a single calve. Calcium and magnesium are important for the normal progression of gestation and parturition. This study measured blood total and ionized calcium (tCa and iCa, respectively) and total and ionized magnesium (tMg and iMg, respectively) in four healthy, captive Asian elephant cows in the Tisch Family Zoological Gardens, Jerusalem, every alternative month, over a 1-year period, to establish their reference intervals and examine sample pH and sample type effects on measured iCa and iMg concentrations. iCa and iMg were measured using an ion-selective electrode electrolyte analyzer. Calcium and magnesium levels in diet samples were measured. The iCa:tCa and iMg:tMg ratios were 0.44 and 0.73, respectively. Mean iMg concentrations in whole-blood, heparinized plasma and serum were 0.58, 0.65 and 0.66 mmol/L, respectively. iCa and iMg concentrations in the three sample types were highly correlated, with no sample type effect on measured iCa concentration, but significant effect on iMg concentration, with significantly lower whole-blood levels vs. serum and plasma. Serum albumin and both tCa and tMg concentrations positively correlated. Sample pH had no effect on measured iCa or iMg levels. This study is the first to measure iMg in Asian elephants, and assess the effects of sample type and pH on the results. It was concluded that different iMg reference intervals should be established for each sample type.

Keywords: Asian Elephant; Ionized Magnesium; Serum; Plasma; Whole Blood.

INTRODUCTION

The Asian elephant (*Elephas maximus*) is an endangered species, with estimated worldwide population size in 2003 of 41,400 to 52,000 elephants, including 15,000 domesticated elephants in Asia, and 1,000 in zoos worldwide (1). In Israel, there are 12 captive Asian elephants, and those held in the Tisch Family Zoological Gardens Jerusalem (TFZGJ; formerly the Jerusalem Biblical Zoo) are the only trained elephants in Israel, and therefore, the only ones from which routine blood samples can be obtained. Being an endangered species, with a long gestation period (22 months), mostly

with a single calf, successful gestation and parturition are essential. Zoos worldwide encounter difficulties in mating elephants, resulting in low overall reproduction rates (2). The aggressive (musth) period of elephant bulls makes their handling extremely dangerous for the staff, limiting the number of adult elephant bulls in zoos (2-4). Other reasons for low reproduction rates include lack of breeding programs, old, non-cyclic females, high infant mortality rate and diseases (e.g., endotheliotropic elephant herpes virus infection, a common cause of fetal death and stillbirth in captive Asian elephants), which even in the relatively uncommon

events of successful mating, both gestation and parturition are frequently complicated (5, 6). Thus, understanding the pathogenesis of dystocia in the elephant is important in its prevention.

Calcium and magnesium are important divalent cations, with significant roles in the normal progression of gestation and parturition (8, 9). Total serum magnesium (tMg) and calcium (tCa) are distributed among three major fractions: protein-bound, complexed with small anions (e.g., phosphates, bicarbonate and lactate) and free-ionized (iMg and iCa, respectively), which are the active forms (10-12). Magnesium is mostly an intracellular cation, less tightly regulated compared to calcium, with a wider physiological range. Its roles include monitoring of calcium pumps, ATPases, neural and cardiac muscle function, and as a cofactor in several enzymatic reactions (12-14). It also has important roles in fetal development (15). During normal pregnancy in women, serum magnesium levels decline, while severe hypomagnesaemia is associated with abortion, and dietary magnesium deficiency plays an important role in the pathogenesis of eclampsia and hypertension (15, 16). Hypomagnesaemia in ruminants and horses might lead to recumbency, ataxia and other neurological signs (10). Total magnesium (tMg) concentrations in Asian elephants were previously reported (8), but their normal iMg levels are unknown.

Calcium, mostly an extracellular cation, is kept within a narrow physiological range and has important roles in muscle contractility, skeletal structure and neural function. It is an intracellular messenger and cofactor in numerous enzymatic and coagulation reactions (17). Hypocalcaemia leads to post parturient paresis in lactating dairy cows, to flaccid paralysis in pregnant ewes, and is one of the major disorders leading to dystocia in ruminants (10). Pregnant mares and women have lower iCa than non-pregnant counterparts, probably due to the growing fetus calcium demands (10, 13, 17, 18). It is suggested, but not confirmed, that captive Asian elephants have low calcium levels, which might play a role in the pathogenesis of dystocia, by causing initial progression failure of the second stage of labor (19). Several, partially documented reports of dystocia in captive Asian elephants worldwide are available, some of which responded to intravenous calcium therapy (8).

Nevertheless, information of calcium metabolism in Asian elephants is limited. The mean absorption rate of dietary calcium is 60%, irrespective of its dietary level. The digestive physiology of elephants resembles that of horses in

the facts that dietary crude fiber concentration influences dry matter digestibility, the presence of pelleted feeds influences calcium absorption, and concentrate feeds lead to increased fecal volatile fatty acids. In both species, being hindgut fermenters, intestinal calcium absorption is higher compared to ruminants and in both, excess calcium is excreted in the urine (6, 8, 20). Unlike ruminants, they are almost completely dependent on calcium absorption rather than on its skeletal mobilization (6). A clinical study, performed in Asian elephants in the Rotterdam Zoo, showed that following a high calcium diet, serum tCa levels increased, while its urinary excretion remained unchanged, suggesting prior sub-clinical hypocalcaemia. The authors concluded that captive Asian elephants should be fed a calcium-rich diet, especially around parturition. That study has suggested that the reference interval (RI) concentrations of plasma tCa and iCa in captive Asian elephants should be around 3.6 mmol/L, and 1.25 mmol/L, respectively (8, 21). Another study investigated the effects of dietary calcium or cholecalciferol supplementation on serum calcium concentration in elephants. It was concluded, that in Western Europe, captive Asian elephants possibly suffer from summer-associated subclinical hypocalcemia, and that the advisable dietary calcium and cholecalciferol levels should be higher than the currently used guidelines (22). According to the International Species Information System (ISIS), mean serum tCa and tMg concentrations in captive Asian elephants are 2.64 mmol/L (10.6 mg/dL) and 0.893 mmol/L (2.17 mg/dL), respectively, based on measurements in 216 and 105 healthy Asian elephants, respectively (23). The RIs or mean concentrations of iMg are not available.

During parturition, calcium levels should be closely monitored, and if serum tCa decreases <2.5 mmol/L, or if iCa falls < 1.2 mmol/L, IV or oral calcium should be administered (6). The uterine effect of such calcium therapy should be confirmed by rectal palpation (i.e., increase in uterine contractility is considered a desired response) and serum calcium measurement (24). Due to the limited data on the physiological and periparturient serum calcium and magnesium levels in wild Asian elephants, it is currently unclear whether the serum levels measured in captive elephants are a dominant factor in the pathogenesis of dystocia. It is of great importance to establish RIs for both cations, and to routinely monitor zoo elephants, especially cows, before and during parturition, and correct abnormalities in order to improve their reproduction rate.

The Asian elephant's nutrition in the wild is based on plants and trees, while in captivity it mainly includes hay and pellets, with major differences in elephant diets between zoos (25). No commercial food designed for elephants is available, except for mineral additives; therefore their nutrition in captivity, especially regarding calcium and magnesium levels, might be incomplete and imbalanced, especially during stressful periods, such as gestation, parturition and early lactation. Calcium absorption is significantly decreased by pelleted feed addition, in both equids and elephants (26). Most zoos apply the equine nutritional requirements for elephants. However, experiments have shown that elephants have much higher gastrointestinal passage rates compared to horses, thus leading to a lower absolute digestibility coefficient (20). According to the Nutrient Requirements Council (NRC) guidelines for horses, currently serving as elephant nutrition guidelines, the minimum calcium feed recommended concentrations for maintenance and late pregnancy, respectively, are 0.3% and 0.5%, and the minimum one for magnesium is 0.1% (26, 27).

Ionized Mg and Ca (iCa and iMg, respectively) can be measured in whole blood, serum or heparinized plasma, using ion selective electrode analyzers. Their concentrations decrease with increase in sample pH, *in vivo* or *in vitro*, and vice versa (28-31). Therefore, anaerobic blood sample handling, using vacuum-sealed tubes is recommended, to prevent gas-exchange (16, 18).

MATERIALS AND METHODS

Animals and diet

The study included four Asian elephant cows (aged 21 to 28 years) held at the same facility and fed the same diet, in the TFZGJ, the only zoo in Israel that holds trained elephants, from which blood samples were obtained every alternative month over one year. At the beginning of this study, one cow was in mid-gestation, and was expected to give birth during the study period. Another, had been lactating over the preceding 3.5 years, and the other two had never been pregnant. Diet samples were obtained during the trial period for the measurement of calcium and magnesium concentrations.

Each cow was fed 3 meals daily consisting of wheat hay (35 kg), dairy cow pellets, (5 kg; 16% protein), pruned branches, mainly carobs (*Ceratonia siliqua*) (10-15 kg), vegetables (20 kg) and one tablet (138 g) of equine vitamin and mineral supplement (Salvana Tiernahrung GmbH, Klein

Offenseth-Sparrieshoop, Germany) containing 9.5% calcium and 1.5% magnesium. The wheat hay contained 0.58% calcium and 0.1% magnesium in dry matter. The pellets contained 1.3% calcium and 0.24% magnesium in dry matter. The total daily calcium and magnesium intake, excluding the pruned branches and vegetables, were 280 g (0.7%) and 47 g (0.12%), respectively. The diet analysis was carried out by the Forage and Feed Laboratory, E.H. Smoler Consulting and Research for Agricultural Science Ltd., Yoav Business Center, Re'e'm Junction, Israel.

Blood samples and laboratory methods

Blood was obtained from the saphenous or cephalic veins. In order to minimize gas exchange and resultant *in vitro* sample pH changes, samples were collected using vacuum-sealed tubes (Vacutainer, BD Diagnostics and Preanalytical Systems, Franklin Lakes, NJ, USA), and were analyzed within four hours from collection. Blood from each elephant was collected in three sealed lithium-heparin tubes and two plain serum tubes with separators. The heparin tubes were used for whole-blood and plasma iCa and iMg measurements. One was centrifuged within 30 minutes from collection, and the plasma was immediately transferred to sealed Eppendorf tubes, filled to their full capacity to prevent gas exchange, and analyzed within four hours from collection. The plain tubes were allowed to clot, centrifuged, and harvested serum was handled as described above. One plain separator tube was used for tCa and tMg concentration measurement, and the other was used for iCa and iMg measurement. All tubes were kept chilled on ice pending analysis.

Electrolytes were measured using an ion selective electrode (ISE) electrolyte analyzer (Nova 8, Nova Biomedical, Waltham, MA, USA; at 37°C). Serum biochemistry analysis, including tCa and tMg, phosphorus and albumin, was performed by wet chemistry autoanalyzers (Vitros 5,1 FS, Ortho Clinical Diagnostics, NJ; Cobas Integra 400 Plus, Roche, Mannheim, Germany; at 37°C).

Statistical analysis

The distribution pattern of continuous variables was assessed using the Kolmogorov-Smirnov test. Pearson's correlation coefficient was used to evaluate the correlation between two continuous variables (e.g., pH and iCa). Intra-class correlation (both definitions of consistency and absolute agreement) was calculated in order to evaluate the association between

Table 1: Concentrations of ionized calcium and magnesium in whole-blood, plasma and serum, total serum calcium and magnesium in four captive Asian elephant cows in the Jerusalem Biblical Zoo over one year

Sample	Ionized calcium (mmol/L)			tCa (mmol/L)		Reported ^a tCa (mmol/L)	Ionized magnesium (mmol/L)			tMg Serum	Reported tMg ^a (mmol/L)
	Whole blood	Plasma	Serum	Serum	Serum		Whole blood	Plasma	Serum		
Mean	1.12	1.11	1.16	2.70	0.44	2.65	0.58	0.65	0.66	0.91	0.85
SD	0.18	0.23	0.20	0.39	0.12	0.2	0.10	0.09	0.08	0.12	0.2
Minimum	0.65	0.45	0.68	1.49	0.4	NA	0.31	0.47	0.46	0.54	NA
Maximum	1.34	1.38	1.39	3.17	0.56	NA	0.72	0.82	0.81	1.1	NA

tCa = total calcium; iCa = ionized calcium; tMg = total magnesium; iMg = ionized magnesium; a = ISIS physiological reference values (23); NA = not available.

variable measurements in plasma, serum, and whole blood. Assessment of differences between analyte concentrations in the different sample types was carried out using repeated measures ANOVA, applied twice, with, and without elephant effect. The Greenhouse-Geisser test was applied to test the effect within subjects. If differences between sample types were statistically significant in the ANOVA, Student's paired *t*-tests, with Bonferroni's correction of the significance level were used to compare sample type pairs at each time-point. All tests applied were two-tailed, and a *P* value ≤ 0.05 was considered statistically significant.

RESULTS

All measured analytes and calculated variables were normally distributed. The mean iCa, tCa, iMg and tMg concentrations, the calculated iCa:tCa and iMg:tMg in whole-blood, plasma and serum in the four elephant cows over the study period are presented in Table 1. The mean sample pH of whole-blood, plasma and serum over the study period were 7.38 (SD 0.05; range 7.29-7.51), 7.49 (SD 0.03; range 7.43-7.57) and 7.51 (SD 0.04; range 7.45-7.62), respectively. Mean serum albumin, total protein (TP) and phosphorus concentrations over the study period were 3.04 (SD 0.4) g/dL, 8.1 (SD 0.52) g/dL and 4.41 (SD 0.83) mg/dL, respectively.

The intra-class correlation coefficients using consistency definition (assuming absence of interaction, because it was not estimable otherwise) for iMg, iCa and pH in whole blood, serum and plasma samples were $r=0.891$, $r=0.790$ and $r=0.632$, respectively. The respective intra-class correlation coefficients using absolute agreement definition (assuming absence of interaction, because it is not estimable otherwise) were $r=0.815$, $r=0.791$, and $r=0.230$, respectively.

Whole blood and plasma iMg were strongly correlated

($r=0.826$, $P<0.005$; $n=20$), with a weaker correlation between whole blood and serum iMg ($r=0.690$, $P=0.001$; $n=20$) and between plasma and serum iMg ($r=0.678$, $P=0.001$; $n=20$). For iCa, the strongest correlation was between plasma and serum iCa ($r=0.808$, $P<0.005$; $n=21$), followed by that between whole blood and serum iCa ($r=0.454$, $P=0.039$; $n=21$) and between whole blood and plasma iCa ($r=0.395$, $P=0.077$; $n=21$). There were significant moderate correlations between whole blood and plasma pH ($r=0.584$, $P=0.005$; $n=21$) and between plasma and serum pH ($r=0.584$, $P=0.005$; $n=21$). There was no significant correlation between blood and serum pH.

There were significant strong correlations between albumin and tCa ($r=0.877$, $P<0.001$) and between albumin and tMg ($r=0.869$, $P<0.001$) (Figure 1) and a moderate significant correlation between albumin and phosphorus ($r=0.536$, $P=0.012$). There were no other significant correlations between measured analytes.

When analyzing the sample type and elephant effects on the results using the Greenhouse-Geisser's test, there was a significant sample type effect on pH ($P<0.001$) and on iMg concentration ($P=0.00001$), but no significant sample type effect on iCa ($P=0.367$). There were no elephant effects on pH or iMg and iCa concentrations ($P=0.413$, $P=0.840$ and $P=0.675$, respectively). When examining the elephant effect on the concentrations of total protein, albumin and phosphorus, the only significant difference was in mean serum total protein concentration ($P=0.001$).

Based on paired Student's *t*-test, significant differences were found between whole-blood and heparinized plasma pH ($P<0.001$) and between whole-blood and serum pH ($P<0.001$), with no significant difference between plasma and serum pH ($P=0.038$). Sample pH was consistently lower in whole-blood compared to plasma and serum.

There were significant iMg concentration differences be-

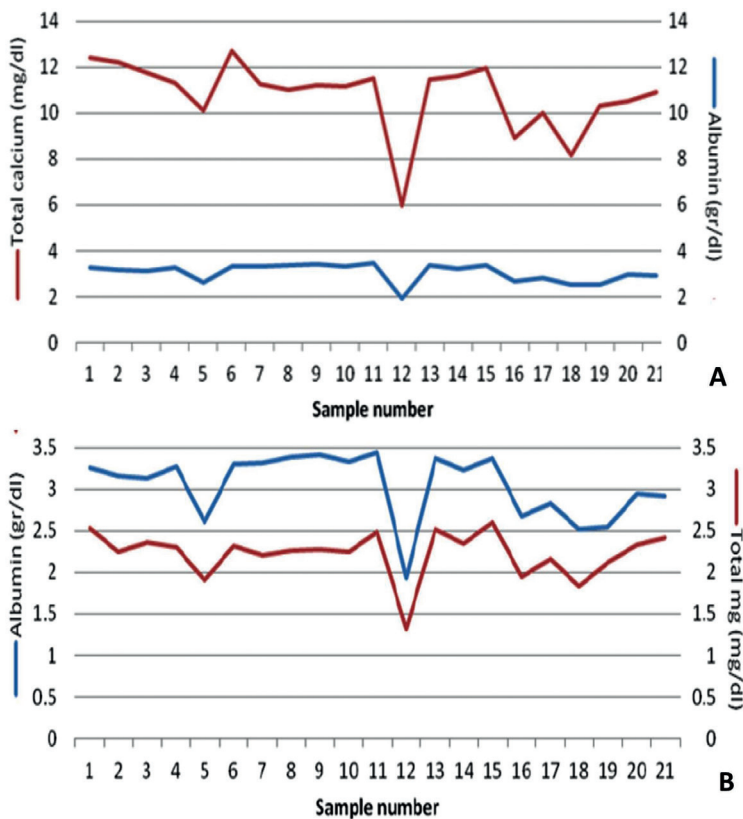


Figure 1: Changes in mean total calcium (A) and total magnesium (B) vs. serum albumin over the study period in four captive Asian elephants in the Jerusalem Biblical Zoo. Note the parallel changes in both total calcium and total magnesium with changes in serum albumin. There were significant ($P < 0.001$), positive strong correlations between changes in total calcium and total magnesium vs. serum albumin ($r = 0.877$ and $r = 0.869$, respectively).

tween whole-blood and heparinized plasma ($P < 0.0001$) and between whole-blood and serum ($P < 0.0001$), with no significant difference between plasma and serum iMg ($P = 0.69$). iMg concentrations were consistently lower in whole-blood compared to heparinized plasma or serum. There were no significant differences between iCa concentrations in different sample types.

There was a significant moderate correlation between whole blood iCa and iMg ($r = 0.699$, $P = 0.001$), and no correlations between whole blood pH and iMg or iCa. There were significant moderate correlations between plasma iCa and iMg ($r = 0.575$, $P = 0.006$), and between serum iCa and iMg ($r = 0.567$, $P = 0.007$), and a weak one between plasma pH and iMg ($r = -0.459$, $P = 0.036$). There was no correlation between plasma pH and iCa, and between serum pH and iCa or iMg.

The pregnant elephant cow included in the present study had started labor five months from the beginning of the

study. It did not proceed beyond the second stage of labor, while the calf was still within the birth canal and did not respond to oral and intravenous calcium treatment (Table 2). A vaginal vestibulotomy was performed, and attempts were made to pull the calf out, but these were unsuccessful, as it was too large. This was followed by a fetotomy procedure, a procedure performed only twice before in elephants, and was successful only once (19). Only one third of the calf was evacuated, while the rest remained in the birth canal. Three days later, despite intensive supportive care, the elephant suddenly died. Necropsy was not conducted, so the cause of death was undetermined. Blood samples obtained during parturition were analyzed only for total calcium (Table 2) due to the urgency of events, and were not included in the analyses.

DISCUSSION

This study followed over a period of one year iCa, iMg concentrations and pH in three different sample types every alternative month in four captive, healthy adult Asian elephant cows in different physiological states. To the best of our knowledge, this is the first study to report iMg concentrations in elephants in general, and particularly in Asian elephants. Because these results are novel, a comparison with previously published data regarding iMg could not be performed. These results may serve as reference intervals for future studies, although, these results should be applied cautiously, due to the limited number of elephants included herein. Additionally, the present study includes novel findings regarding the influence of the sample type (e.g., whole-blood, heparinized plasma and serum) on iCa and iMg measurements, and their associations of sample pH in Asian elephants.

The mean serum tCa concentration measured presently (2.7 ± 0.37 mmol/L) is similar to findings in 216 healthy Asian elephants (2.64 ± 0.22 mmol/L) (23), while mean plasma iCa concentration herein (1.11 ± 0.23 mmol/L) is somewhat higher compared to previously published data (0.93 ± 0.11 mmol/L) (8, 22, 32). This difference appears clinically insignificant. Nevertheless, both iCa concentration herein and in the previously published study are lower than its recommended target concentration of 1.25 mmol/L

Table 2: Serum total calcium concentration, treatment and events in an Asian Elephant cow with dystocia at the Jerusalem Biblical Zoo

Date*	Serum calcium (mmol/L) [mg/dL]	Calcium treatment and comments
14.7.09	2.4 mmol/L [9.6 mg/dL]	4 Salvana horse pills in the food
16.7.09	2.5 mmol/L [10 mg/dL]	4 Salvana horse pills in the food
19.7.09	2.4 mmol/L [9.6 mg/dL]	4 Salvana horse pills in the food
20.7.09	2.6 mmol/L [10.4 mg/dL]	4 Salvana horse pills in the food; Ca-borogluconate (2L, 23% solution) PO. Serum progesterone concentration decreased.
21.7/09	2.6 mmol/L [10.4 mg/dL]	4 Salvana horse pills in the food; Ca-borogluconate (2L, 23% solution) PO
22.7.09	2.7 mmol/L [10.8 mg/dL]	4 Salvana horse pills in the food; Ca-borogluconate (2L, 23% solution) PO
23.7.09	2.6 mmol/L [10.4 mg/dL]	4 Salvana horse pills in the food; Ca-borogluconate (2L, 23% solution) PO
24.7.09		4 Salvana horse pills in the food; Ca-borogluconate (2L, 23% solution) PO
25.7.09 (16:00)	2.63 mmol/L [10.5 mg/dL]	4 Salvana horse pills in the food; Ca-borogluconate (5L, 23% solution) PO
26.7.09 (05:20)	2.42 mmol/L [9.7 mg/dL]	At 06:00: Ca-borogluconate IV (800 mL; 23% solution) At 12:30: Ca-borogluconate (2L, 23% solution) PO; 4 Salvana horse pills in the food
26.7.09 (22:30)	2.28 mmol/L [9.1 mg/dL]	At 23:00: Ca-borogluconate IV (400 mL; 23% solution)
27.7.09		At 07:10: Ca-borogluconate IV (400 mL; 23% solution)
28.7.09 (14:30)	2.25 mmol/L [9.0 mg/dL]	Vestibulotomy**

* when the time is mentioned, it refers to the time in which the blood sample was obtained;

** no further measurements of serum calcium were made after the vestibulotomy.

in Asian elephants, fed a calcium-rich diet (8). It has been recommended that when iCa level decrease to <1.2 mmol/L during parturition, IV calcium should be administered in order to maintain these iCa levels, a calcium-rich diet should be fed at all times, particularly around parturition. Avoiding pellets in the diet might improve calcium absorption in elephants (8, 26). Since most zoos (including the TFZGJ) include pellets in elephant diets, and since blood calcium concentrations recorded herein and previously are below its target concentration, possibly, such elephant diets in zoos fail to meet physiologic calcium demands. A summer-associated hypocalcemia has been suggested in captive Asian elephants in Western Europe as well (22), and may play a role in the high incidence of dystocia among captive Asian elephants. If so, increasing dietary calcium content in captive Asian elephant diets should be considered to maintain health and prevent the potential effects of subclinical hypocalcemia on reproduction. iCa was unfortunately not measured in the elephant cow that had dystocia during this study. However, total serum calcium concentration range, measured during parturition in this cow, was 2.275 to 2.7 mmol/L, despite oral and intravenous calcium administration throughout parturition (Table 2). Given a calculated average iCa:tCa ratio of 0.44, an iCa concentration range of 1.001 to 1.188 mmol/L during parturition may be assumed, which is below the desired concentration of 1.25 mmol/L (8). This supports

the presence of hypocalcemia in this cow during dystocia, but cannot prove a direct cause and effect between this hypocalcemia and dystocia. Therefore, the present study, as in previous ones (8, 22), did not conclusively prove a direct association between low blood Ca concentrations and dystocia in elephants.

The serum iCa:tCa ratio in this study (0.44) is higher compared to previous findings (0.32) (8), despite the higher iCa concentration in the present study, and obviously higher results compared to the tCa concentration in the previous one. Since both ratios are lower than the ratio reported in horses (0.52) (18), this questions whether the current nutrient calcium demands for elephants, that are based on recommendations in equines, are adequate for elephants, and whether such species differences warrant devising elephant-specific dietary values. If the requirement in horses is to be applied in elephants, it seems that iCa concentration, measured herein and previously (8), are below optimal values.

Our results show significant strong correlations between iCa concentration among all three sample types, for both consistency and absolute agreement, with no significant sample type effect on measured iCa, suggesting that practically, any sample type of the three can be used to reliably measure iCa, accurately and interchangeably. These findings are not consistent with previous results which showed higher iCa concentrations in serum (20 samples; three elephants)

compared to heparinized plasma (25 samples; three elephants) (8). In horses, iCa concentration was also higher in serum compared to heparinized plasma and whole-blood, attributed to significantly higher sample pH in heparinized plasma compared to serum (18). The reasons for this discrepancy are unclear, and it might have resulted from sample pH differences between this study and the previous ones.

The present study's mean serum tMg concentration is similar to previous findings in 105 healthy Asian elephants (0.893 ± 0.08 mmol/L) (23). In the present study, the correlations between iMg concentrations in the three sample types were strong and significant, and stronger than those concerning iCa concentration. The iMg concentration range herein is narrower than that of iCa. However, in contrast with iCa, significant sample type effects were noted on iMg concentrations as demonstrated by pair-wise analyses, and these were consistently lower in heparinized whole-blood, in agreement with previous findings reported in horses (18). There are several potential explanations for these results. First, whole-blood and plasma samples were obtained with heparin as the anticoagulant, which binds Mg ions, thereby lowering their free fraction (33, 34). Second, erythrocytes and platelets, present in whole blood, might interfere with iMg analysis, through their influence on the electric potential of the ISE analyzer (33, 34). Third, platelets contain high intracellular magnesium, which is released during clot formation and ample centrifugation in serum harvesting. These findings are not consistent with those reported in humans, in which no sample type effect on measured iMg concentrations was noted (35). It is unknown if platelet magnesium levels in humans are lower compared to those in horses and elephants, thus warranting further studies. Nevertheless, the iMg concentration differences recorded, albeit statistically significant, are probably clinically insignificant.

The pH levels were significantly and consistently correlated, albeit only moderately, among the three sample types. The sample pH was consistently lower in whole-blood samples, similar to iMg and iCa concentrations, while pH was highest in serum samples. This is surprising, because previous studies have shown that a rise in pH leads to decreased iMg and iCa concentrations, and vice versa, since in alkaline solution calcium and magnesium ion binding to plasma protein increases, thereby lowering their free (ionized) form concentration (28-31). The higher pH levels in serum samples, compared to whole blood and plasma likely resulted

from some CO₂ evaporation during harvesting the serum, despite the strict protocol used to minimize it, which may have resulted in an increase in sample pH (36).

A previous study has suggested a theory to explain the lower whole-blood pH levels compared to serum and heparinized samples pH. According to this theory, in whole-blood samples, a fraction of CO₂ is associated with erythrocytes, and therefore evenly distributes within the collection tube similarly to physiological conditions. In contrast, when whole-blood is centrifuged, erythrocytes are packed, and CO₂ is trapped with the erythrocytes, and thus the supernatant plasma (or serum) CO₂ level is relatively lower (18). In addition, *in vitro* metabolism of glucose by leukocytes and platelets in whole-blood sample might have contributed to the formation of CO₂, thereby lowering the sample pH (35). Another possible explanation is post sampling *in vitro* changes, mainly CO₂ evaporation (from plasma and serum samples) during separation, despite the precautions taken to avoid such changes. Possibly, some evaporation is inevitable (36).

There were significant correlations between concentrations of iCa and iMg in the three different sample types, and mostly in whole-blood samples. In contrast, sample pH did not correlate with iCa or iMg concentrations in all three sample types. This may be explained by other more dominant factors, mentioned above, influencing the ions concentration (e.g., effects of heparin, platelets and erythrocytes). Conversely, significant correlations between sample pH and iCa concentration in serum and heparinized plasma were noted previously in Asian elephants (8, 31), with a much stronger correlation in serum samples compared to heparinized plasma samples ($r=0.819$ vs. $r=0.496$, respectively) (8). A significant, albeit weak negative correlation between sample pH and iCa concentration was noted in horses as well between sample pH and iMg levels (18). Our findings are in agreement with the latter.

Mean serum albumin and total protein and phosphorus concentrations in this study are similar to previous findings (32 ± 5 g/L, $n=206$; 81 ± 8 g/L, $n=209$ and 1.55 ± 0.39 mmol/L [4.8 ± 1.2 mg/dL]; $n=201$) (23). We did find a significant correlation between serum tCa and serum albumin, consistent with previous findings in horses (18), although in the present study the correlation is stronger ($r=0.877$ vs. $r=0.44$, respectively). This is probably because calcium strongly binds to serum proteins (about 45%), mainly albumin (11). In this study however, there was no correlation between serum tCa

and serum total protein, in contrast to a weak correlation described in horses (18). In this study there was also a significant correlation between serum tMg and albumin, in contrast to horses (18), likely due to strong magnesium binding to serum proteins (about 30%), mainly albumin (11). Surprisingly, there were no significant correlations between phosphorus and both tCa and tMg, although physiologically, phosphorus and calcium are closely regulated, and as dietary phosphorus influences intestinal calcium absorption, and serum phosphorus concentrations influence calcium resorption from bones and absorption from the intestine. Hyperphosphatemia can induce hypocalcaemia, and hypophosphatemia can induce hypercalcemia. Phosphorus also associates with magnesium; high dietary phosphorus inhibits magnesium absorption in the small intestine of monogastric animals (11).

There were no elephant effects on pH levels and iCa or iMg concentrations, supporting that iMg concentration differences resulted only from sample type differences and that the analytical method is not affected by individual animals. Although this study included only four elephants, these were in different physiological states (pregnancy, lactation and non-pregnancy), suggesting that the physiological status has no effect on measured pH, iCa and iMg.

The major limitation of this study is the small number of elephants included, that has been reduced further in the mid-study period, due to death of one elephant cow during parturition. However, this limitation is common worldwide in studies of captive elephants (8, 22, 32), and is inevitable, as in Israel, with only 12 Asian elephants in the country, of which only the four studied here were trained, allowing blood collection. Nevertheless, in order to partially overcome this limited number of elephants included, these were followed over a 12-months period. Thus, the total number of samples analyzed in the study is actually relatively high. Second, although most of the diet was unchanged during the study period, in some, dietary components changes were made over the study period, and this probably resulted in changes in their ingredients, precluding exact calculation of dietary calcium, magnesium and phosphorus at all times. Thus, potential changes in their dietary levels might have affected their blood concentrations, which could not be assessed in this study, thereby limiting our interpretation and recommendations. Third, the ISE electrolyte analyzer used in this study was designed for human blood; its performance in elephant blood has never been described or validated. Species differences

might have affected the results. Nevertheless, the fact that intra-assay correlations were high and significant, suggested that this factor is probably unimportant. Since this study is the first to examine the iMg levels in Asian elephants, our results could not be compared to an established reference interval or any other previous results. Lastly, during the unsuccessful parturition of the pregnant elephant, in the middle of the study period blood samples for iCa, iMg and pH were not obtained, due to the emergency nature of events, which would have been important to our understanding changes in these over this crucial period of dystocia.

In conclusion, this is the first study blood iMg and iMg:tMg ratio in elephants, and the first to compare the sample type and pH effects on iCa and iMg levels in elephants. Sample type affected iMg concentration, necessitating establishing different reference intervals for its concentration in whole-blood, plasma and serum. The sample type did not affect iCa levels, suggesting that a single reference interval could be applied for all three sample types. However, due to the small number of elephants and the fact that they were in different physiological states, these results should be applied cautiously. Additionally, this study showed no sample pH effect on iMg or iCa levels, in contrast with results in humans.

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