

Amylase and Lipase Detection in Hemorrhaged Animals Treated with HBOC-201

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Abstract: HBOC-201 may alter lipase and amylase detection on chemistry analyzers using optical methods and affect pancreatic function after trauma. Amylase and lipase measurements were correlated against HBOC-201 to evaluate interference on samples spiked with 0–6g/dL HBOC-201. The detection threshold was 2.5g/dL or none when measured, respectively, on Vitros 250 or Advia 1650 instruments. Amylase and lipase from blood samples collected from 55% EBV hemorrhaged Yucatan min-pigs showed peaks around 24–48 hours. Amylase increase was not significant between treatments but lipase was higher in HBOC-201-treated animals. Animals particularly affected by the injury had elevated enzymes after hemorrhagic shock, without significant clinical consequences.

Keywords: interference, HBOC, trauma, hemorrhage, pancreas, clinical chemistry

INTRODUCTION

Amylase and lipase are two pancreatic enzymes that digest complex and high molecular weight carbohydrates and lipids, respectively. These enzymes are particularly active within the digestive system where they are primarily secreted by pancreas, and also parotid salivary glands for amylase. Nonetheless, they can also be found in other organs such as the liver and participate to other regulatory mechanisms [1–3]. Studies show that inflammatory processes can contribute to abnormally high serum amylase (hyperamylasemia) and lipase (hyperlipasemia) [4]. Consistent with this explanation, the pancreas also experiences stress in the event of trauma and hemorrhagic shock, with elevated amounts of amylase and lipase released into the bloodstream [5].

HBOC-201 (Hemoglobin Based Oxygen Carrier) (Hemopure, Biopure Corporation¹, Cambridge, MA) is a bovine polymerized hemoglobin that has been proposed to treat hemorrhagic shock as a resuscitation fluid in emergency situations, when whole blood transfusion is not

immediately available. HBOC-201 is able to deliver oxygen to tissues as well as to restore blood volume whereas current emergency resuscitation fluids (e.g. Hextend (HEX) or Lactated Ringer's) restore only volume and blood pressure. It has been shown that HBOC-201 improved survival in various animal hemorrhage models in surgical pre-clinical trials [6–8]. By improving microcirculation HBOC could show benefit on organs such as the pancreas but, based on Diaspirin cross-linked hemoglobin (DCLHb) reports, lipase and amylase serum levels could possibly be affected and pancreatic function be disturbed [9].

HBOC-201, as a purified acellular agent, will be present in serum or plasma of blood samples collected from animals treated with HBOC-201 and will cause samples to be identified as hemolysed [10]. Routinely, clinical samples are tested for pancreatic enzyme levels using standard diagnostics that may be limited by the presence of hemoglobin, particularly for laboratory assays that use optical methods to quantify parameters if the range of wavelengths utilized are close to that of HBOC-201 [10]. When HBOC-201 exceeds a certain concentration,

¹Biopure Corp is now OPK and it is located in Cambridge, MA.

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this interference may invalidate the results. The degree of interference depends on the individual assay as well as the testing method [10–12]. In addition, Ma et al. [13] reported that measurements of amylase and lipase performed on the Hitachi 747 and Vitros 750 were differently affected by HBOC. Thus establishment of threshold above which the substance exerts undesirable interference also depends upon the instrument used and therefore needs to be determined for each specific testing device.

Experiments presented here were designed first to assess the effects of increased HBOC-201 concentration *in vitro* on amylase and lipase measurements, and second to address changes in the *in vivo* levels of these enzymes from animals that underwent a 55% controlled hemorrhage and resuscitation with HBOC-201.

MATERIAL AND METHODS

Assay Calibration

Visible spectrum (480 – 680 nm) of HBOC-201 was determined using a spectrophotometer (Cary 100-UV-VIS, Varian, Palo Alto, CA) to identify the peaks of absorption. Plasma hemoglobin concentration in sample was measured on a blood gas analyzer (ABL-700, Radiometer, Denmark). A Vitros 250 device (Ortho-Clinical Diagnostics, Johnson and Johnson, Rochester, NY) was the instrument selected in our facility to measure chemistry parameters; it uses dry chemistry methodologies (120 2670 and 166 8409 cartridges, respectively, for amylase and lipase). An Advia 1650 device (Bayer/Siemens HealthCare, Deerfield, IL) using wet chemistry methodologies was used as a second device of choice. The influence of HBOC-201 concentration on amylase and lipase determination was examined on swine samples (freshly collected at the start of the surgical experiment). The samples were spiked with a calibrated standard HBOC-201 solution (12 g/dL) and bovine serum albumin (BSA) as a diluent to achieve a final concentration of HBOC ranging from 0 to 6 g/dL. A linear regression was performed on the data and the threshold above which interference was considered detrimental was determined at 20% above the baseline [10]. Because blood is either collected with or without heparin, plasma and serum samples were also compared. Then, using the Vitros 250 calibrator as a known source of amylase and lipase for preparing the samples, interference was tested with increasing amylase levels (from 400 U/L to 1200 U/L) and lipase levels (150 U/L to 1800 U/L) at a fixed HBOC-201 concentration of 1.25 g/dL, a concentration below interference.

In Vivo Experiments

The experiments were conducted according to the principles set forth in the "Guide for the Care and Use of

Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The study was approved by the WRAIR Institutional Animal Care and Use Committee (IACUC) and all procedures were performed in an animal facility approved by the Association for Assessment and Accreditation for Laboratory Animal Care International (AAALAC).

Animal Surgery and Sample Collection: Simulation of vascular injury was addressed using 55% estimated blood volume (EBV) controlled hemorrhage in a swine model. Blood withdrawal was performed via catheter withdrawal on anesthetized Yucatan mini-pigs (~32kg). Surgical procedures and findings from these studies have been extensively reported previously [6,14,15]. Briefly, the animals were randomly allocated to different treatments groups: no resuscitation (None) (n = 4), HBOC-201 (n = 14) and Hextend (HEX, 6% hydroxy-ethyl starch, Abbott Laboratories, Abbott Park, IL) (n = 14) resuscitation. Following hemorrhage, fluid resuscitation to prevent hemorrhagic shock took place with five (5) infusions during the *pre-hospital* phase from 15 minutes post injury to 3 hours. Each animal received appropriate infusion for blood pressure less than 40 mmHg. After the pre-hospital phase, the animals entered a *hospital arrival* phase and were eligible to blood transfusion. The animals received whole blood transfusions and/or saline, depending on their hemoglobin concentration (hemoglobin < 7 g/dL) and saline for blood pressure less than 40 mmHg. To reflect delayed access to full medical care in a combat situation, the animals were divided into two delay groups for this phase: 4 hours hospital arrival (55-4 group), and 24 hours hospital arrival (55-24 group). Thus in the 24 hour delay group animals received no blood or saline for 24 hours as in extreme combat situations. Blood samples were collected in vacutainer (BD, Franklin Lakes, NJ) without anticoagulant or with heparin at five time points: time 0, 4 hours, 24 hours, 48 hours, and 72 hours. All samples were centrifuged at 3400 rpm for 20 minutes at 4°C and supernatant was processed immediately or stored frozen at -80°C for laboratory analysis. Fibrinogen was also measured in this study using a Sta compact instrument (Diagnostica Stago, Parsippany, NJ) and thromboelastography using a TEG-5000 (Haemoscope, Niles, IL).

In vivo Amylase and Lipase Measurement: *In vivo* samples from the 55% EBV controlled hemorrhagic shock study were aliquoted and assayed for amylase and lipase. Plasma HBOC-201 concentration was determined using a blood gas analyzer (Radiometer, Copenhagen). Amylase samples with HBOC-201 concentrations above the threshold (as determined in the previous calibration section) were diluted to bring the HBOC-201 concentration at or below the required threshold level. Amylase and lipase data were compared across treatment groups.

Statistics

All samples were run in triplicate. Results, data, and figures are presented as mean $\pm$ standard deviation (SD) unless otherwise stated. Analysis of variance (ANOVA) and Student's t-test were used for direct comparison of normally distributed samples. For multiple variables and for data collected over time, results were analyzed using the mixed statistical model for global inspection of continuous measurements. P value 0.05 or less was considered significant.

RESULTS

HBOC Interference

The peak absorption of HBOC-201 varied depending on the oxygen binding level of the molecule. It was identified at 542, 577 nm (predominantly for the oxygenated form with bound oxygen in the heme group) and 632 nm (higher in the deoxygenated form without the bound oxygen) and with methemoglobin due to oxidation to the ferric state). The HBOC spectrum was similar to human hemoglobin (Figure 1). Vitros 250 instrument using a dye that absorbed at 540 nm for biochemical detection of amylase and lipase, thus, overlapping with HBOC-201, caused positive interference of the testing conditions. Advia 1650 that computes dual wavelength at specifically 545 nm and 694 nm to obtain activity, showed no interference for amylase and lipase.

Average level for lipase and amylase in freshly collected swine samples determined on both instruments were 10 ± 3 and 396 ± 130 U/L, respectively. The results on Vitros for samples spiked with increasing concentration

of HBOC-201 are illustrated in Figure 2 as normalized results. Amylase level determined on both serum and plasma varied positively with increasing concentration of HBOC-201. Spiked samples were found to be correlated exponentially up to the maximum HBOC-201 concentration tested (i.e. 6 g/dl) and yielded similar values regardless of the sample collection: $\text{Amyl} (\%) = 100^{0.0899} * \text{HBOC-201 concentration}$; $r = 0.993$ for serum and $\text{Amyl} (\%) = 100^{0.0858} * \text{HBOC-201 concentration}$; $r = 0.989$ for plasma (no significant difference). The vertical solid line in Figure 2 denotes the HBOC-201 concentration value at which amylase readings exceeded 20% positive error on Vitros 250. We found that 2.5 g/dL HBOC-201 imposed an interference threshold. Thus, amylase results for HBOC-201 < 2.5 g/dL were considered acceptable without correction and linear regression for HBOC-201 > 2.5 g/dL was acceptable ($\text{Amyl} (\%) = 100 + 7.63 * \text{HBOC-201}$; $r = 0.998$). When read on Vitros 250 adjustments could be performed either by the linear regression or by diluting the samples to reach levels of HBOC at or lower than 2.5 g/dL. Spiked specimens were tested with increased enzyme concentration and fixed HBOC-201 level at 1.25 g/dL (below the interference threshold). It was found that HBOC-201 did not interfere in assays with amylase levels below 400 U/L. However, departure from linearity occurred for higher concentrations (Figure 3a). No lipase readings could be obtained at any HBOC concentration by dry chemistry, making lipase not measurable with the Vitros 250 instrument. Because of these findings samples were measured on Advia 1650. We found that lipase measurements were unaffected up to the maximum concentration of HBOC-201 used (6g/dl). Lipase results showed complete linearity on the Advia 1650 for all lipase concentration ranges tested with 1.25 g/dL HBOC-201 (Figure 3b).

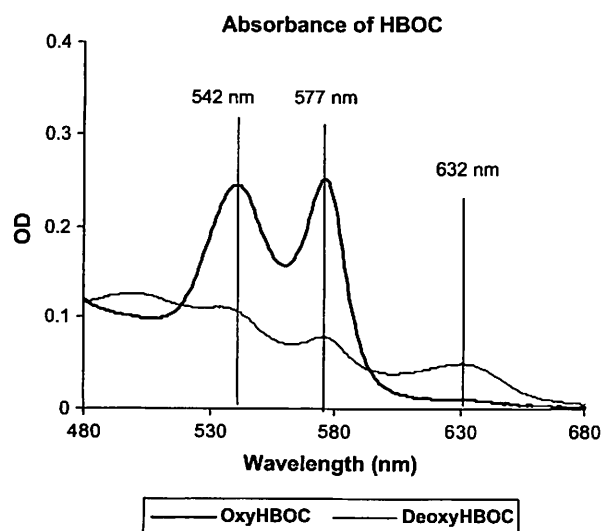


Figure 1. Absorption spectrum for HBOC-201 in the oxygenated state (dark line) with high absorption at 542 and 577 nm or deoxygenated state (dotted line) with high absorption at 632 nm.

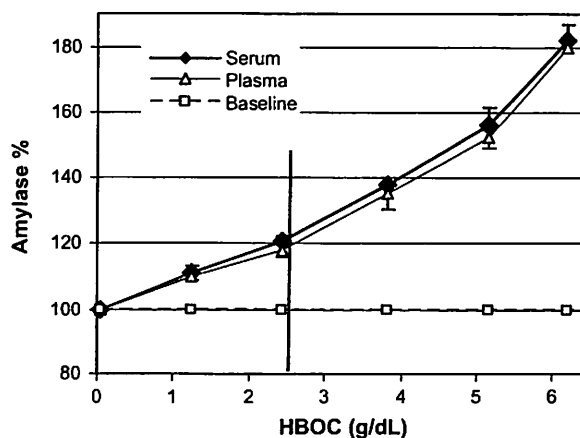


Figure 2. Amylase measurement on Vitros 250 in the presence of increasing concentration of HBOC-201. Vertical bar indicates the maximum concentration HBOC-201 for 20% amylase readings above baseline. Regression Equations: $\text{Amyl} (\%) = 100^{0.0899} * \text{HBOC-201 concentration}$ ($r = 0.993$) for serum; $\text{Amyl} (\%) = 100^{0.0858} * \text{HBOC-201 concentration}$ ($r = 0.989$) for plasma.

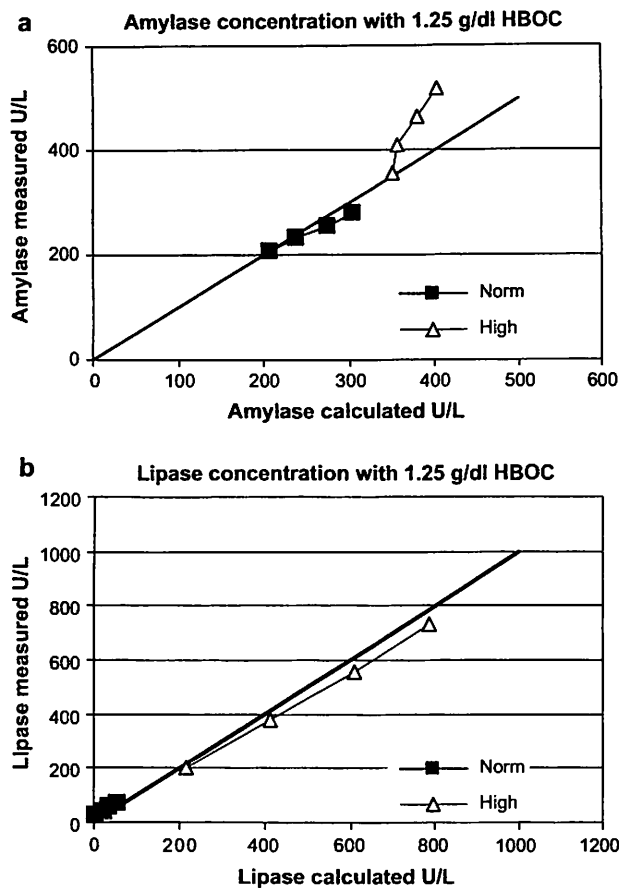


Figure 3. Increasing concentration of amylase (a) and lipase (b) with a HBOC-201 concentration fixed at 1.25 g/dl. Normal level (norm, black square) and high level (high, empty triangle) were tested for amylase (366 U/L – 1138 U/L) on Vitros 250 and for lipase (166 U/L – 1821 U/L) on ADVIA. The line represents regression.

HBOC-201 Detection in In Vivo Swine Samples

Plasma HBOC in both groups was similar and Figure 4 illustrates the level of HBOC-201 in the supernatant of swine samples from both the 55-4 and 55-24 HBOC groups during the resuscitation phase after hemorrhagic shock. HBOC-201 concentration peaked at 4 hours but the maximum level varied among individual animals due to their hospital fluid requirement. HBOC-201 levels decreased thereafter due to metabolic degradation; HBOC-201 was found to have a half life of ~20 h.

Amylase Results

Amylase and lipase levels were measured in animals from five pre-hospital treatment groups: none (not resuscitated) or resuscitated with HBOC-201 or HEX in both the 55-4 and 55-24 hour delay hemorrhaged categories (Figure 5). Because of the small number of animals due

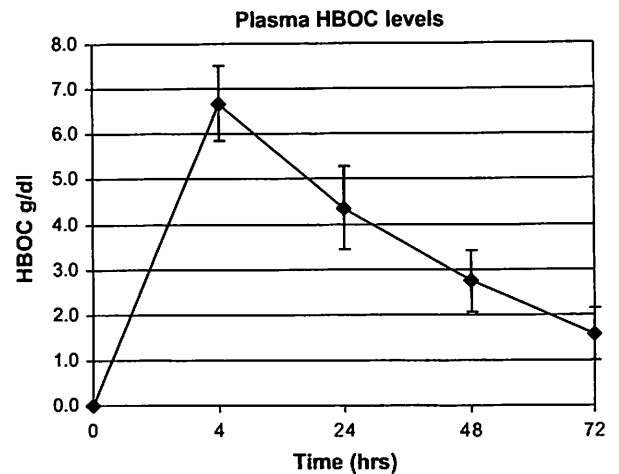


Figure 4. HBOC-201 concentration in swine samples during the course of resuscitation. Both data from the 55-4 and 55-24 groups were combined.

to early death and the absence of difference in the results, data were pooled for the 55-4 and 55-24 groups in the None group (Figure 5a). Amylase patterns for None increased to a maximal level at 24 h and then decreased towards baseline. In contrast in all resuscitated animals amylase decreased slightly at 4 hours, likely due to hemodilution and increased thereafter. In HEX groups, amylase levels peaked at 24 hours in comparison with animals treated with HBOC-201 where amylase peaked between 24 and 48 hours and returned toward baseline at 72 hours to comparable levels as in the None group. Is it noteworthy that the peak in the 55-24 group was higher than in the 55-4 group ($p = 0.041$). Similarly with HBOC-201, 55-4 and 55-24 groups were different after 4h. At 24 h amylase levels for the animals in the 55-24 were significantly higher than in the 55-4 treatment group (701 ± 199 vs 394 ± 134 , respectively; $p < 0.05$) and at 48 h (948 ± 393 vs 655 ± 295 , respectively). Nonetheless, the data showed large animal heterogeneity in the 55-4 and 55-24 groups (Table 1). There were more occurrences of elevated amylase from baseline (> 800 U/L) in the 55-24 group compared to the 55-4 group (9/19 vs 3/16, respectively) for HEX or HBOC treatment combined, and there were more occurrences of elevated amylase after HBOC treatment than HEX (10/30 vs 2/7, respectively).

Lipase Results

Animals in the None and HEX treatment groups showed no noticeable increase pattern in lipase levels over time (Figure 5b) and lipase level for animals in the HEX group was lower than with the HBOC-201 treatment groups. There was no difference between the HEX 55-4 and HEX 55-24 groups. Lipase showed the same pattern as amylase in the HBOC groups, peaking between 24 and

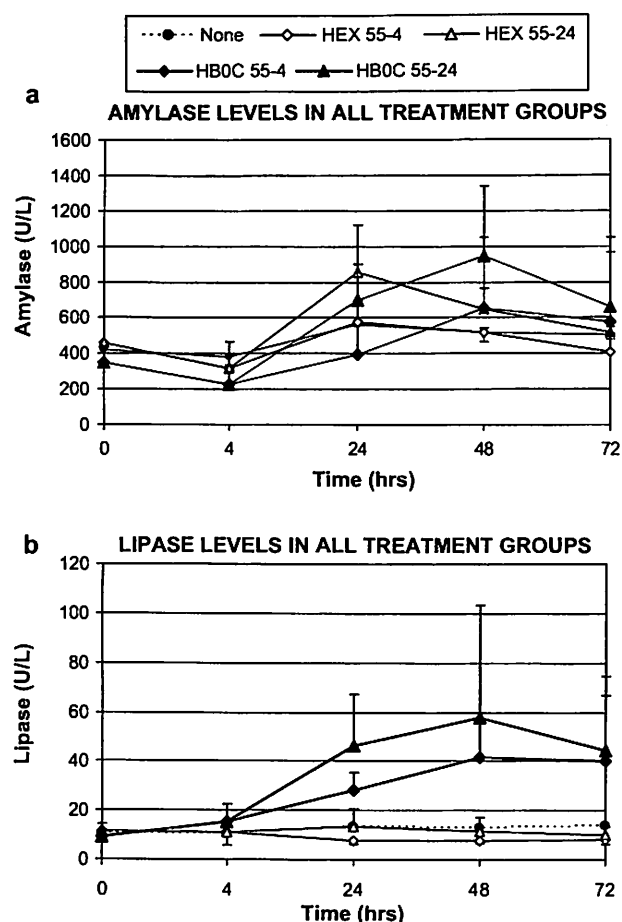


Figure 5. Amylase (a) and lipase (b) level for all treatment groups (Mean $\pm$ SD) for HBOC-201, (—) and HEX (---) treated animals in 55% - 4 h delay ($\diamond$) and 55% 24 h delay (Δ) groups.

48 hours and being higher in the 55-24 group compared to the 55-4 group. Lipase levels were significantly higher with HBOC-201 treatment than with HEX treatment ($p < 0.01$). A relatively high degree of heterogeneity in lipase levels was also visible in both 55-4 and 55-24 groups in a comparable fashion as for amylase (Table 1). There were more occurrence of elevated lipase from baseline (> 40 U/L) in the 55-24 HEX group compared to the 55-4 HEX group (4/21 vs none, respectively) and this was comparable in the HBOC group (12/24 vs 6/18, respectively, in 55-24 and 55-4). There were more occurrence of elevated

lipase after HBOC treatment than HEX (18/42 vs 4/21, respectively). Also, animals that had high lipase level in the HBOC-201 groups were the same animals that had abnormally elevated amylase at 24 h or 48 h. In contrast to amylase lipase in treated animals did not return to baseline as readily as amylase.

To account for the heterogeneity in pancreatic enzymes, data from the coagulation panel was analyzed. There was an increase in the reaction time (TEG-R) to form a clot and in the elevation of plasmatic fibrinogen after 24 h in animals that had high enzymes. There was a trend of higher fibrinogen (linear regression) with high amylase or lipase. Lipase showed a stronger correlation than amylase ($r = 0.56$, $n = 54$; $p < 0.001$ vs $r = 0.39$, $n = 54$; $p < 0.01$, respectively, for lipase and amylase).

DISCUSSION

The interference threshold for amylase was 2.5 g/dl and compared well with what has been reported in literature for the Vitros 250 or similar instruments [12,13, 16]. We found that lipase could not be measured with dry chemistry due to equipmental limitations and that the Vitros 250 device was not a preferred instrument for routine determination of lipase in the presence of HBOC-201. The exact reason for it was unclear and may be related to protein precipitation, thus methodologies using dual wavelength (Advia device) were considered and readings were possible with wet chemistry without interference up to 6 g/dL [12]. The upper limit of HBOC-201 set in this study corresponded to the maximum plasma hemoglobin concentration reached at 4 hours in the blood samples of hemorrhaged swine (Figure 4); thereafter the levels decreased due, in part, to HBOC-201 degradation and hemodilution [6,15]. *In vivo*, secretion of lipase and amylase up to 4 h was low (not different than baseline) but levels rose after 4 h and peaked around 24 h. The fact that plasmatic HBOC was at its highest concentration at 4 h and decreased rapidly thereafter indicated that the presence of HBOC-201 in the present study would minimally affect assay readings as the experiment progresses. The increase of enzyme together with residual HBOC-201 brings the maximum likelihood of interference around 24 h post

Table 1. Number of animals with elevated amylase and lipase in each treatment group. Elevated enzyme level was defined as three-fold that from baseline (amylase > 800 U/L and lipase > 40 U/L).

Groups	Number of animals with elevated pancreatic enzymes				
	Amylaseat 24h	Amylaseat 48h	Lipaseat 24h	Lipaseat 48h	Lipaseat 72h
HEX 55-4					
HEX 55-24	2/7		1/7	2/7	1/7
HBOC 55-4	1/8	2/8	6/8	4/8	2/8
HBOC 55-24	3/6	4/6	3/6	2/6	1/6

treatments. Therefore, to correct for potential erroneous readings of amylase on the Vitros 250 instrument, a sample dilution was the preferred choice and this method was implemented to test the *in vivo* samples. These samples were diluted by a factor of two or more in order to reduce the HBOC-201 level while keeping amylase levels within the linear range of detection by the Vitros 250. It should be noted that interference for other analytes such as liver enzymes was also observed with other HBOCs such as the Hemolink product, O-raffinose crossed-linked hemoglobin [18].

Swine baseline levels for amylase were comparable to the human range and lipase tended to be slightly lower, probably because of differences related to lipid metabolism. Increase of these levels by 5- to 10-fold would be indicative of pancreatitis [4,5]. In the presence of HBOC-201 the amylase elevation was more noticeable in the 55-24 delay group and was extended to 48 h. Despite the fact that the samples were diluted in order to reduce HBOC concentration, the persistently higher level of amylase with time, also observed in HEX treated animals, indicates some pathology rather than interference. In contrast lipase level did not increase after HEX treatment but was significantly higher with HBOC-201. Of note, only 4 / 14 animals showed high amylase and lipase that were 5 to 10 times greater than baseline or remaining high at 48 h and would qualify for pancreatitis whereas the other 10 animals showed no increase. Sole direct effect of HBOC-201 may be ruled out because plasmatic HBOC-201 concentration was no greater in these animals than others at any time point of the experimental course and because 2/7 HEX treated animals had also elevated enzymes. This suggests that animals more susceptible to injury responded more acutely and exhibited a higher rate of metabolic disturbance. Furthermore, elevated lipase occurred more readily in animals treated with HBOC-201 and increased past 48h. This response may be attributed to HBOC-201 but it cannot be firmly concluded that HBOC-201 only caused pancreas damage per se. It was shown that HBOC-201 altered hepatic function [6] and this can slow down the secretion of hepatic lipase, for example. Interestingly, no HBOC-201- resuscitated animals received blood transfusion immediately after hospital arrival as they had relatively high total hemoglobin levels compared to the HEX-resuscitated animals that required transfusion and this could be a confounding factor [14,15]. Increase in lipase was particularly obvious in the long delay group (55-24) where blood or saline transfusion was delayed. Indeed, at 24 h when the HEX treated animals had received hospital care the level decreases; but in the case of HBOC treatment the animals skipped the blood transfusion causing increased enzymes for additional 24 h. Therefore, HBOC-201 because of the treatment consequence (preferentially observed for amylase) or the direct effect of the product (preferentially observed for lipase) seems to contribute to pancreatic

enzymes elevation, but without clinical significance as the enzymatic level dropped at 72 h and the animals survived the 3 days experiments.

Elevation of amylase have also being reported in stress and trauma situations and was consistent with patients in critical conditions [5,19,20]. This 55% EBV hemorrhage battlefield model induced a severe ischemic injury leading to shock [14,15]. Thus the reported elevated enzyme levels around 24 hours (similar to an acute phase reactant for lipase in particular) may not reflect solely pancreatic damage but could be indicative of organ damage [1-3,21,22]. To support this idea, other *in vitro* laboratory variables were analyzed. Lactate level was found within normal range for this injury model and the same in all animals; however, we found higher value of fibrinogen in these animals. Fibrinogen, an acute phase reactant indicative of stress is related to hemorrhagic shock in our model [6,15]. Although under similar stress, HEX resuscitated animals had less elevated fibrinogen at 4 or 24 h as blood transfusion reduced plasma concentration of analytes. The absence of eligibility to fluid exacerbated the stress in the 55-24 HBOC-201 delay group, explaining the higher incidence of elevated enzymes. Lipase was the enzyme that showed the most sensitivity as indicated by a higher correlation with fibrinogen and higher fold in increase. However, it was not of clinical significance and the direct effect on the pancreas is still debatable. The literature indicates that polymerized HBOC's compared to DCLHb improved pancreatic function and reduced tissue damage in cases of severe pancreatitis [9,23-25] and, in addition, HBOC-201 was shown to improve pancreatic microcirculation [25,21]. Our results tend to point out that elevation of pancreatic enzymes may be related to individual animal sensitivities in response to shock and it is exacerbated by HBOC-201.

CONCLUSIONS

Amylase measurements on the Vitros 250 are strongly dependent on the level of HBOC-201 present. Accepting results with a 20% error above the expected value on this instrument brings the threshold of interference of HBOC-201 concentrations to around 2.5 g/dL and measurements can be considered accurate on the Vitros 250 for HBOC below this threshold. Clinically, compared to HEX treatment, HBOC-201 did seem to elevate the level of amylase or lipase particularly in animals more susceptible to injury, stress or hemorrhagic shock in this study. However, this transient elevation could be also reduced by blood transfusions and did not seem to bear long-term clinical relevance on pancreatic function.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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