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ORIGINAL ARTICLE

Changes in plasma alpha-1 acid glycoprotein following hemorrhagic trauma: Possible role in dose differences of ALM drug therapy in rat and pig resuscitation

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Abstract

Introduction: The binding of drugs to plasma proteins is an important consideration in drug development. We have reported that the dose of adenosine, lidocaine, and magnesium (ALM) fluid therapy for resuscitation from hemorrhagic shock is nearly 3-times higher for pigs than rats. Since lidocaine strongly binds to serum alpha-1-acid glycoprotein (AGP), the aim of the study was to investigate the effect of hemorrhagic shock on levels of AGP in rats and pigs.

Materials and Methods: Healthy adult male Sprague–Dawley rats and female cross-bred pigs ($n = 33$ each) underwent tail vein and peripheral ear vein blood sampling, respectively, to collect plasma for AGP measurements. Rats ($n = 17$) and pigs ($n = 16$) underwent surgical instrumentation and uncontrolled hemorrhage via liver resection, and were treated with 3% NaCl ± ALM IV bolus followed 60 min later by 4 h 0.9% NaCl ± ALM IV drip. Rats were monitored for 72 h with blood samples taken post-surgery, and at 5.25, 24, and 72 h. Pigs were monitored for 6 h with blood samples taken post-surgery, and at 60 min and 6 h. Plasma AGP was measured with rat- and pig-specific enzyme-linked immunosorbent assay kits.

Results: Baseline AGP levels in rats were 3.91 μg/mL and significantly 83-fold lower than in pigs (325 μg/mL). Surgical instrumentation was associated with ~10-fold increases in AGP in rats and a 21% fall in pigs. AGP levels remained elevated in rats after hemorrhage and resuscitation (28–29 μg/mL). In contrast, no significant differences in plasma AGP were found in ALM- or Saline-treated pigs over the monitoring period.

Conclusions: We conclude that the trauma of surgery alone was associated with significant increases in AGP in rats, compared to a contrasting decrease in pigs. Higher levels of plasma AGP in pigs prior to hemorrhagic shock is consistent with the higher ALM doses required to resuscitate pigs compared with rats.

KEYWORDS
ALM, alpha-1 acid glycoprotein, dosage, hemorrhage, serum proteins, trauma

Abbreviations: AGP, alpha-1 acid glycoprotein; ALM, adenosine, lidocaine, and magnesium; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assays; IL-1, interleukin-1; IL-6, interleukin-6; MAP, mean arterial pressure; ORM, orosomucoid; SPF, specific-pathogen free.

1 | INTRODUCTION

The binding of drugs to plasma proteins is an important consideration in drug development, pharmacokinetic profiling, and clinical use.[1–3] Serum albumin and alpha-1-acid glycoprotein (AGP) (orosomucoid [ORM]) are the two major plasma proteins, and globulins and lipoproteins are less abundant.[2–5] Although AGP represents 1%–3% of total plasma protein compared to albumin (50%–60%), it plays a major role in drug pharmacokinetics.[1,3,5] AGP is considered a high-affinity/low-capacity binder, whereas albumin is a low-affinity/high-capacity binding protein.[4,6,7] In addition to drug binding, AGP is a positive acute-phase protein that can change its level after surgery, infection, trauma, burns, or cancer,[4,8–14] which in turn can influence the bioavailability of a drug with clinical implications.[11] Like most acute-phase proteins, AGP can be induced by inflammatory cytokines, such as interleukins-1 (IL-1) and IL-6, and tumor necrosis factor-α, and corticosteroids.[4,10–12] However, AGP comprises 12 to 20 glycoforms with many other immunomodulatory properties, including controlling neutrophil function, cyclic adenosine monophosphate-dependent endothelial barrier functions, platelet migration, and anticoagulant properties.[4,14,15]

During adenosine, lidocaine, and Mg^{2+} (ALM) preclinical drug development for resuscitation following hemorrhagic shock, we found that rats required one-third of the dose per kg to raise mean arterial pressure (MAP) from ~30 to 60 mmHg compared to pigs.[16–19] The difference suggests that one (or more) of the actives in ALM has less bioavailability in pigs than in rats. Under physiological conditions, adenosine has a high affinity and selectivity for adenosine receptors and is not likely to be a strong binder to serum albumin, and less so to AGP.[20] Wzorek et al. recently concluded that: "the affinity values of adenosine and its analogs to serum albumin are in the lower range limit of typical binding constants".[20] Similarly, magnesium weakly binds to albumin,[21] and probably is not a major contributor to ALM dosage differences. In addition, serum albumin levels in rats and pigs are similar (2.5–3.5 g/100 mL).[22,23] Lidocaine, on the other hand, is known to tightly bind to AGP (up to 90%) with a binding constant of $1.1–1.7 \times 10^5 M^{-1}$ at 37°C and pH 7.4,[8,24] and only weakly to albumin.[9,25,26] Furthermore, baseline AGP levels have been reported to be up to 100 times higher in pigs than in rats or humans.[5,7,27] Compared with serum albumin, very little is known on the levels of AGP in rats and pigs following hemorrhage. Thus, the aim of our study is to examine changes in AGP levels in rats and pigs before and following hemorrhagic shock. Our hypothesis is that hemorrhagic shock may lead to higher plasma levels of AGP in pigs compared with rats, and possibly linked to the higher ALM dose per kg in pigs.

2 | MATERIALS AND METHODS

2.1 | Ethics

The study was approved by the James Cook University (JCU) Animal Ethics Committee (A2296), the Danish National Committee on Animal Research Ethics (2016-15-0201-00999), and the US Army Animal Care and Review Use Office (ACURO; SO150053). This study conforms to the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition, 2013, and the Danish Animal Welfare Act 2013, and complies with institutional and ACURO guidelines.

2.2 | Rat model of uncontrolled hemorrhage

Conventional adult male Sprague–Dawley rats (413 [SD = 34] g; $n = 50$) were bred and housed in a 14–10 h dark–light cycle in a temperature- and humidity-controlled non-specific-pathogen free (SPF) facility, with free access to food and water *ad libitum*. Healthy rats ($n = 33$) underwent blood sampling via the tail vein for storage of plasma for quantification of baseline AGP. The surgical procedure and model of uncontrolled hemorrhage induced by liver resection have previously been described.[28,29] Briefly, animals were anesthetized with isoflurane 1.5–5% (in 100% O_2), with animals breathing spontaneously. Sterile chronic catheters (Access Technologies) were implanted in the left femoral artery and vein for fluid infusion, blood sampling, and blood pressure measurement, respectively.[28] A 0.6 mL arterial blood sample was taken after catheterization, and plasma stored for quantification of post-surgery AGP. To induce uncontrolled hemorrhage, a 3 cm transverse laparotomy was performed to expose the abdominal cavity, and 50% of the left lateral and medial liver lobes were resected with sharp dissection and allowed to bleed freely into the peritoneal cavity.[17] Sham animals underwent surgical catheterization and laparotomy without liver resection and bleeding. Fifteen minutes after liver injury, animals received a 0.7 mL/kg IV bolus via the femoral vein catheter of: (1) 3% NaCl (Sham and Saline group), or (2) 3% NaCl ALM (ALM group). ALM comprised 1 mM adenosine (0.19 mg/kg), 3 mM lidocaine (0.61 mg/kg), and 2.5 mM $MgSO_4$ (0.21 mg/kg).[28] After 60 min bolus resuscitation, animals were recovered from anesthesia and received a 4 h drip infusion of 0.5 mL/kg/h 0.9% NaCl (Sham and Saline group) or 0.9% NaCl ALM (ALM group; 0.25 mg/kg/h adenosine, 0.5 mg/kg/h lidocaine, and 0.25 mg/kg/h $MgSO_4$.[28] Animals received 0.05 mg/kg s.c. Temgesic™ (buprenorphine-HCl) 10 min prior to recovery from anesthesia for post-operative analgesia, with repeat doses at 6–12 h intervals. Rats were monitored for up to 72 h, with additional blood sampling at 5.25 h (end of drip resuscitation) and 24 h, for AGP measurements.

2.3 | Pig model of uncontrolled hemorrhage

Female adult crossbred Landrace/Yorkshire pigs ($n = 49$; 70.5 ± 9.0 kg) were fasted overnight but allowed free access to water. Healthy pigs ($n = 33$) were sedated with intramuscular midazolam (0.625 mg/kg), ketamine (6.25 mg/kg), and atropine (0.5 mg), for blood sampling via a peripheral intravenous ear catheter and storage of plasma for quantification of baseline AGP. The surgical procedure and model of uncontrolled hemorrhage induced by laparoscopic liver resection

has previously been described. Briefly, pigs were orally intubated and volume-controlled ventilated, with anesthesia maintained with continuous infusion of propofol (3 mg/kg/h) and fentanyl (15 μg/kg/h). Surgical preparations included creation of three burr holes through the skull for neuromonitoring probes, placement of vascular sheaths and catheters into the right carotid artery and external jugular vein for hemodynamic and cardiac function measurements and blood sampling, and insertion of three 12 mm laparoscopic ports in the abdomen. An arterial blood sample was taken after a 60 min stabilization period, for post-surgery AGP measurement. For uncontrolled hemorrhage, the left lateral lobe of the liver was isolated via the laparoscopic ports, and transected 5 cm from the tip. Bleeding was allowed to continue for 20 min, after which the medial-lateral lobe was resected for a further 10 min bleeding. After 30 min hemorrhage, pigs received an IV bolus of (1) 4 mL/kg 3% NaCl (Saline group), or (2) 4 mL/kg 3% NaCl ALM (ALM group). The bolus dosing (0.5 mM adenosine [0.54 mg/kg], 1.5 mM lidocaine [1.74 mg/kg], and 1.25 mM $MgSO_4$ [0.6 mg/kg]) was based on our previous pressure-controlled hemorrhagic shock and endotoxemia studies as the minimum dosing for shock resuscitation.[16,30] After 60 min bolus resuscitation, an arterial blood sample was taken for AGP measurement, and pigs received a 4-h resuscitation infusion of 3 mL/kg/h 0.9% NaCl ± ALM (7.2 mg/kg/h adenosine, 14.4 mg/kg/h lidocaine, 8.04 mg/kg/h $MgSO_4$). After drip resuscitation, pigs received an autologous blood transfusion of 450 mL and were monitored under anesthesia for a further 60 min before final blood sampling and euthanasia.

2.4 | Alpha-1-acid glycoprotein analysis

Rat ORM enzyme-linked immunosorbent assay (ELISA) Kit (MBS007612, Lot: 05/2020, MyBiosource) and Pig AGP 1 (ORM1) ELISA Kit (MBS9718408, Lot: ATTMA0601, MyBiosource) were used to measure plasma levels of AGP on POLARstar Omega microplate reader (BMG Labtech), according to manufacturer's instructions. All samples were measured in duplicate as per the literature standard. Detection ranges for rat and pig kits were 3.12–100 ng/mL and 15–240 μg/mL, respectively. Assay sensitivities (minimum detectable concentration), intra- and inter-assay precision (%CV) were 1.0 ng/mL, <10%, and <15% for rat ORM ELISA, and <1 μg/mL, <9%, and <11% for pig ORM1 ELISA. Total plasma protein was determined using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific).

2.5 | Statistical analysis

SPSS Statistical Package 25 (IBM) was used for statistical analysis. Data are presented as mean (standard deviation). Prism 9 4-parameter-logistic curve fitting was applied to ELISA data (GraphPad). Between species differences were analyzed using a one-way analysis of variance (ANOVA). Longitudinal AGP data were assessed with General Linear Model Repeated Measures ANOVA. Statistical significance was defined as $p < .05$.

3 | RESULTS

Baseline AGP values for rats and pigs were 3.91 (1.03) and 325 (299) μg/mL, respectively ($p < .001$; Table 1). In rats prior to traumatic hemorrhage, AGP significantly increased 10-fold ~40 μg/mL ($p < .001$; Figure 1A). This increase was due to surgical catheterization procedures in all groups. In Sham rats (no hemorrhage), AGP remained elevated over the next 5 h post-surgery, then decreased by 30% to 28 μg/mL at 24 h, where it remained for the next 2 days (Figure 1A). In contrast, AGP levels in Saline and ALM-treated animals immediately both fell by 27% after hemorrhage over 5.25 h, then stabilized at concentrations of 28–29 μg/mL AGP (Figure 1A).

In direct contrast, in the pig, AGP values started high (325 μg/mL) and *fell* 21% after surgery in both groups ($p > .05$). Over the next 60 min, following hemorrhage and fluid resuscitation, AGP levels decreased further to 73% of baseline, and continued to decline over the next 5 h to 64% of baseline in ALM-treated (207 μg/mL) but not in the Saline group (242 μg/mL; Figure 1B). No significant differences in plasma AGP were found between Saline controls and ALM-treated pigs over the pre- or post-surgical monitoring period (Figure 1B).

TABLE 1 Summary of baseline plasma levels of AGP in healthy rats and pigs.

Animal	AGP (μg/mL)	Literature
Sprague Dawley rat	3.91 ± 1.03[a] (1.76–5.67)	• Varies with age and sex • 50–320 μg/mL[b7,31]
Yorkshireandrace/Duroc pig	325 ± 299* (162–1886)	• Highly variable dependent on age, strain and SPF status • 300–2500 μg/mL in conventional cross-bred adult pigs[7]

Abbreviations: AGP, α1-acid glycoprotein; SPF, specific-pathogen free.

Note: Data represent mean ± SD (range).

*$p < .001$ compared to rats. Effect size 0.4 (95% CI: 0.2–0.5).

[a]Determined using highly sensitive (0.001 μg/mL) quantitative sandwich ELISA.

[b]Determined using seromucoid assay or spectrofluorometric method.

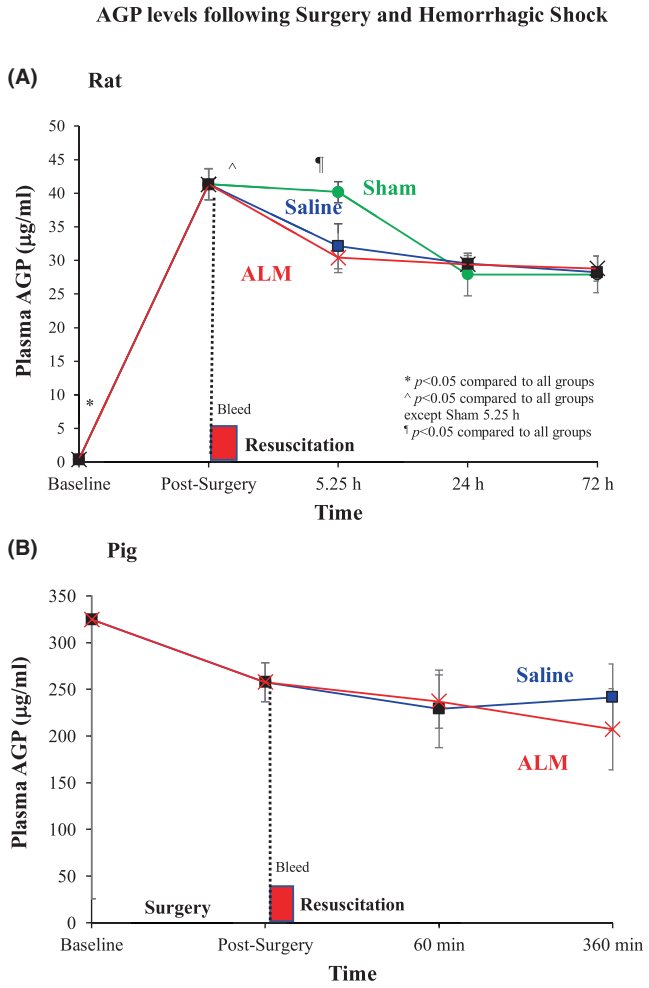

FIGURE 1 Plasma α1-acid glycoprotein concentrations pre- and post-hemorrhage in healthy rats (A) and pigs (B). Values represent mean ± SD. For rats, $n = 33$ for baseline; $n = 13$ for post-surgery; $n = 7$ for Sham 5.25 h; $n = 5$ for Saline 5.25 h, ALM 5.25 h, and Sham 72 h; $n = 4$ for Sham 24 h, Saline 24 h and ALM 72 h; $n = 3$ for ALM 24 h; and $n = 1$ for Saline 72 h. For pigs, $n = 33$ for baseline; $n = 16$ for post-surgery; $n = 8$ for Saline and ALM groups. Overall between groups significance for rats (A) is $p < .001$. * $p < .05$ compared to all groups; ^ $p < .05$ compared to all groups except Sham 5.25 h; ¶ $p < .05$ compared to all groups except post-surgery. Overall between groups significant for pigs (B) is $p = .533$. AGP, α1-acid glycoprotein; ALM, adenosine, lidocaine, and magnesium.

4 | DISCUSSION

Alpha-1 acid glycoprotein (AGP) is a significant high-affinity/low-capacity drug binding and acute-phase protein present in animals and humans.[3,5] With respect to changes in AGP in rats and pigs after hemorrhagic shock, we report the following: (1) mean baseline plasma AGP concentration is 83-fold higher in pigs than in rats; (2) surgical procedures alone were associated with a ~10-fold increase in AGP in rats and a contrasting 21% decrease in pigs; and (3) after hemorrhage and resuscitation, AGP levels in rats decreased 26% over 5 h with little change over the next 3 days, while AGP values in pigs decreased ~8% over 60 min and less than 10% decrease over the next 6 h. We will now discuss these findings, and the possible clinical relevance to higher ALM-doses required in pigs to resuscitate following hemorrhagic shock.

4.1 | Baseline levels of plasma AGP in rats and pigs

Baseline AGP levels in adult Sprague–Dawley male rats were 3.91 µg/mL (Table 1). This is lower than those of Boyle et al. who reported values of 40 to 70 µg/mL in adult male Sprague–Dawley rats.[5] The reasons for the differences are unclear. Both studies used isoflurane anesthesia for tail vein blood sampling, however, they differed in breeding status. Our study employed conventionally bred and housed rats, whereas Boyle et al. used SPF rats.[5] SPF rats have different gut microbiomes, which is known to alter inflammatory, coagulation, and immunological status, and, therefore, possibly AGP levels.[32–34] With respect to 5-months crossbred female pigs, we measured AGP values that were 83-fold higher (325 µg/mL) than in rats (Table 1). Pigs are atypical among mammals, including humans, by having extremely high-plasma AGP values.[7,27] Our basal values agree with published levels of Itoh and colleagues of 338 (79) µg/mL (5–10 months),[35] and with those of Clapperton et al. who reported 388 µg/mL in conventional (non-SPF) pigs.[36] Interestingly, the Clapperton et al. found that AGP in SPF pigs increased nearly 2-fold to 744.8 µg/mL,[36] presumably due to differences in the gut microbiome.

From a literature survey, we acknowledge there is wide variability of resting AGP levels in rats, pigs, and humans.[4,5,8–13,31] Notwithstanding interspecies and strain differences, serum AGP of any one species can change rapidly in response to changing physiological status such as animal handling, housing, stress, age, health status, surgical preparation, activity level, and the presence of inflammation, infection, or injury. The specificity of the analytical AGP method may also contribute to differences where older studies used multi-step electrophoretic immunodiffusion procedures,[8] while more recent studies use highly specific ELISA.[2,3,27] In summary, when comparing AGP values in the serum of animals and humans, and before standard reference values can be established, it is important to understand the procedural methods and health status of the animal prior to measurement.

4.2 | Effect of hemorrhagic trauma on plasma AGP levels

A standout finding of the present study was the opposing effects of surgical preparation and procedures on plasma AGP levels in rats and pigs prior to hemorrhage (Figure 1A,B). Serum AGP levels in rat significantly increased ~10-fold, whereas levels in pigs decreased by 21% (Figure 1A,B). To our knowledge, this has not been reported before following surgical trauma, although AGP levels in pigs have been shown to display negative acute-phase protein behavior following infection and sepsis.[4,7,27]

Although the mechanisms are unknown, a possible explanation for rat-pig differences is that serum AGP in pigs has different surface motifs with different affinities to immune and inflammatory mediators, damage-associated molecular patterns, and/or pathogen-associated molecular patterns that are released into the circulation after trauma and infection.[37] Moreover, the extremely high-serum AGP levels in pigs may reflect an evolutionary adaptation to the constant immunomodulatory stress from the squalor conditions in which they live, and any further increases from trauma or infection are of little or no benefit.

Following the hemorrhagic shock, our study further showed that AGP levels decreased only slightly in rats (26%) and pigs (~8%) relative to post-surgical values (Figure 1A,B). High values in both species indicate that the regulation of *AGP* mRNA expression in the liver continued to maintain the steady state, albeit slightly decreasing, that is consistent with an inflammatory, post-hemorrhagic shock state with widespread ischemia.[16–19] The other interesting finding of our study was that Sham rats had higher AGP levels in the first 5.25 h post-surgery than bleeding rats, indicating that the initial 26% fall in the latter was due to hemorrhage and/or fluid resuscitation. This was not the case between 24 and 72 h because Sham AGP converged at 24 h (Figure 1A). Shams only received surgical procedures (catheterization and laparotomy) with no bleed or further trauma, which again demonstrates the major effect of the trauma of surgery on levels of serum AGP. We did not measure AGP in Sham pigs to compare the effect of surgery in this species.

4.3 | Resuscitation and possible clinical significance

Given the extensive literature on the binding of lidocaine to AGP, the differences between rats and pigs in AGP in this study are consistent with the higher ALM doses required in pigs to raise MAP following hemorrhagic shock. Since the 1970s, plasma lidocaine binding has been recognized to be almost entirely due to changes in AGP.[8,9,24,25] Bailey and Briggs have further shown that increases in the AGP concentration decreases the unbound (free) or "active" concentration of lidocaine.[38] Thus, the binding ratio (bound concentration/free concentration) correlates strongly with AGP concentration in animal models and trauma patients.[8] This data has important clinical significance when understanding the dosing and physiological function of lidocaine in the clinical setting, and helps explain the higher ALM doses required to resuscitate pigs compared with rats. It is likely that humans would require similar dosing to rats, given the high-baseline levels and unique differences demonstrated in pigs. These findings also have important implications for the development of other basic and neutral drugs which preferentially bind AGP. Despite a vast literature on the subject, the physiological functions of the 12 to 20 different glycoforms of AGP,[14] from their role in the immune response to drug binding, still remain to be elucidated.[13,27,39]

5 | CONCLUSIONS

We conclude that the trauma of surgery alone was associated with ~10-fold increases in plasma AGP in the rat. In contrast, plasma AGP decreased by 21% in pigs. These contrasting effects may relate to the 83-fold higher levels of AGP circulating in normal states compared to rats. Significantly higher baseline levels of AGP in pigs is consistent with the higher ALM doses required to resuscitate pigs from hemorrhagic shock. The underlying mechanisms for AGP as a negative acute-phase protein in pigs following trauma requires further investigation.

AUTHOR CONTRIBUTIONS

Geoffrey P. Dobson, Jodie L. Morris, and Hayley L. Letson contributed equally to the design, literature analysis, implementation, data collection, and writing of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Geoffrey Dobson is the sole inventor of the ALM concept for cardioplegia, organ preservation, surgery, infection, sepsis, and trauma.

DATA AVAILABILITY STATEMENT

The data can be made available upon request.

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