Characterization of the cortisol response to traumatic hemorrhage and intra-abdominal contamination models in Cynomologus Macaques

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ABSTRACT

Introduction: Trauma, hemorrhage, and peritonitis have widely varying impacts on endocrine response in the injured patient. We sought to examine cortisol response in established non-human primate models of traumatic hemorrhage and intra-abdominal contamination.

Methods: Cynomologus Macaques were separated into two experimental groups, the polytrauma and hemorrhage model, involving a laparoscopic liver resection with uncontrolled hemorrhage, cecal perforation, and soft tissue excision; and the traumatic hemorrhage model, involving only liver resection and uncontrolled hemorrhage. Cortisol levels were measured pre-operatively, at the time of injury, and at regular intervals until post-operative day 1.

Results: Cortisol levels increased 600% from the pre-operative value in the polytrauma and hemorrhage model, with minimal changes (20%) in the hemorrhage only model.

Conclusion: Cortisol levels increase dramatically in response to polytrauma and intra-abdominal contamination as compared to hemorrhage only. The lack of response in the hemorrhage only group may be due to relative adrenal insufficiency caused by the shock state or lack of enticing stimuli from fecal peritonitis.

1. Introduction

In the acute trauma setting, hormones elicit multiple effects on the body. These effects may serve to strengthen the host response to trauma, or lead to a maladaptive immune response that worsens survival. Cortisol modifies the immune response, inflammatory cascade, metabolism, and healing in response to stressors, such as physical trauma and infection.

Cortisol increases metabolic activity by stimulating gluconeogenesis, raising blood glucose levels (Cidlow et al., 2016). Additionally, cortisol has anti-inflammatory effects by directly inhibiting pro-inflammatory mediators, such as IL-12, IFN-γ, and IFN/TFN-α (Cidlow et al., 2016). Further inhibition of cytokine production from lymphocytes occurs secondary to the action of cortisol on NF-κB (McKay and Cidlow, 1999). Prolonged elevated levels of cortisol can decrease wound healing as well osteogenesis and chondrogenesis (Khani and TAYEK, 2001; Chyun et al., 1984; Simmons et al., 1984). After trauma, cortisol has been shown to be elevated when measured at the point of injury, though severely injured patients have shown signs of early cortisol suppression (Hetz et al., 1996; Foster et al., 2020). When measured past the initial hours after injury, cortisol values have varied considerably (Agha et al., 2004; Foster et al., 2020). Additional variability in cortisol levels has occurred with different traumatic stimuli, such as lower cortisol levels seen in patients with TBI versus other forms of trauma (Pfefferle et al., 2018).

While the effects of cortisol have been described in numerous models of animal stress, there is a paucity of data regarding initial 24-h cortisol levels in traumatic models, especially in non-human primates (Pfefferle et al., 2018; Hamel et al., 2017). Our group recently published a work describing corticosterone (a cortisol analogue in rodents) in a rat traumatic injury model. In this model, the most severely injured animals had the highest peak concentrations of corticosterone, and reached these peaks in the shortest amount of time after trauma (Qin et al., 2020). Another rat model measuring adrenal response (via corticosterone secretion) in hypotensive hemorrhagic shock showed initially elevated corticosterone levels, with subsequent decrease over the course of continued hypotension. These rats showed evidence of adrenal infarction on pathology, leading investigators to postulate that the drop in
Animals were quarantined upon arrival to the research facility for males weighing an average of 7.3 kg (Worldwide Primates, Miami, FL). primate polytrauma and intra-abdominal contamination model (herein increased cortisol levels at presentation as compared to survivors (De Bograd et al., 2015; Bradley et al., 2017). Our research group has used cortisol values. The complete methods, pre-surgical treatment, and hemorrhage-only model (herein referred to as, referred to as
showing that non-survivors from septic shock had significantly representing the transition in protocols from the THM to the PHM).

Several human studies have looked at cortisol levels in traumatically injured patients, specifically those with severe hemorrhage. A study of cortisol levels in acutely hemorrhaging patients found lower levels of cortisol obtained on arrival to care were associated with significantly higher rates of mortality (Stein et al., 2013). Another study of hypotensive patients from traumatic hemorrhage revealed that 93% of patients studied had relative adrenal insufficiency (as demonstrated by serum cortisol < 25 mcg/dL) during their first 24 h of admission (Rushing et al., 2006a). Sepsis, especially septic shock, seemingly has the opposite effect on cortisol, as demonstrated by a recent study showing that non-survivors from septic shock had significantly increased cortisol levels at presentation as compared to survivors (De Castro et al., 2019).

The purpose of this study is to compare the effect of a non-human primate polytrauma and intra-abdominal contamination model (herein referred to as “Polytrauma and Hemorrhage” or PHM) versus a hemorrhage-only model (herein referred to as, “Traumatic Hemorrhage” or THM) on cortisol levels obtained over 24 h. Additionally, we sought to compare the changes in cortisol levels to the inflammatory profiles of various cytokines drawn from these two models. Our hypothesis is that the animals who undergo PHM will have a significantly higher cortisol and cytokine response as compared to the animals that undergo THM.

2. Methods

Cynomolgus Macaques (Macaca fascicularis) Non-Human Primates (NHPs) were utilized as experimental models. The animals were adult males weighing an average of 7.3 kg (Worldwide Primates, Miami, FL). The study protocol (19-OUMD-14LS) was reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee in compliance with all applicable Federal regulations governing animal protection in research. Animals were quarantined upon arrival to the research facility for at least 45 days. Animals had free access to food and water until 12 h prior to surgery. Serum and plasma samples were collected for each NHP 1–2 weeks prior (t = Pre-operative) to surgical intervention for baseline cortisol values. The complete methods, pre-surgical treatment, and surgical protocol has been detailed previously (Vicente et al., 2018; Bograd et al., 2015; Bradley et al., 2017). Our research group has used these models (THM and PHM) to test various physiologic, laboratory, inflammatory, and endocrine trends in traumatic injury. While the samples were drawn from historical animals of the THM and PHM, the samples were drawn from appropriately contemporaneous protocols (all samples were obtained from sequential animals from 2012 to 2013, representing the transition in protocols from the THM to the PHM. There were no protocol changes, within each protocol, or modifications during this time period.

2.1. Traumatic hemorrhage model (THM) and polytrauma and hemorrhage model (PHM)

NHPs in the THM experimental arm underwent a 60% left-lobe liver resection under laparoscopic visualization without control of the resulting hemorrhage. This injury designated time zero (t = 0 min) in both groups. NHPs in the PHM experimental arm underwent a laparoscopic 4 cm anti-mesenteric cecal injury to induce feculent peritonitis, followed by the same 60% left-lobe liver resection as the traumatic hemorrhage model. Additionally, the PHM group underwent a standard sized (18.85 cm²) right flank soft tissue excision down to the level of the muscular fascia. Laparoscopic ports were removed and temporarily closed. Serum and plasma samples were collected intra-operatively during the uncontrolled hemorrhage and resuscitation (t = 0, 60, 120, 180, 240), see Fig. 1.

2.2. Resuscitation and recovery

At t = 15 min resuscitation was initiated with normal saline administration (20 mL/kg) for both THM and PHM groups. All vitals were continuously monitored and no further interventions occurred until 2 h post injury (t = 120) to simulate a prolonged field-care scenario. At t = 120, both THM and PHM underwent surgical exploration with midline laparotomy to expose the abdominal cavity, repair the hepatic and celiac injuries; and removal of the hematoma and/or fecal contamination. Hemostasis at the liver bed was achieved via suture ligation, cautery, and Surgicel (Ethicon, Somerville, NJ). The peritoneal cavity was irrigated with normal saline to remove all fecal matter. The organs were carefully inspected prior to closure. Both THM and PHM NHP abdomens were closed at t = 240 min when the final intra-operative plasma and serum sample was retrieved. Appropriate monitoring and additional resuscitation efforts were conducted as detailed in the protocol and previous publications of this study design, see Table 1 (Vicente et al., 2018; Bograd et al., 2015; Bradley et al., 2017). NHPs were survived up to two weeks post-operatively and monitored by trained personnel and veterinary staff. An additional serum and plasma extraction was collected 24 h after the last intra-operative collection on post-operative day one (POD 1).

2.3. Data collection and enzyme-linked immunosorbent assay methods

The serum and plasma samples were centrifuged and aliquoted prior to being placed in storage at −80 °C. All samples were stored in small aliquots, in the same freezer. All samples were analyzed contemporaneously, during a one month period in 2018. Due to project requirements, plasma samples were ultimately used to analyze the PHM, and serum samples were utilized for the THM. Serum and plasma samples were analyzed for cortisol elevation utilizing the Cortisol ELISA Kit (Cayman Chemical Item # 500,360) protocol, as follows.

Samples were acidified to pH 1–2 using 3 M HCl and verified with paper strip indicators. They were then extracted by the addition of methylene chloride to allow separation of the solvent and sample. Solvent layer was removed and the sample was evaporated using nitrogen gas and heat. Each sample was then reconstituted with 1 × ELISA buffer and stored at 4 °C prior to cortisol assay.

Plates were set up for 96-wells, including 2 blank wells (Blk), 2 non-specific binding (NSB) wells, 2 maximum binding (Bmax) wells, and 1 total activity (TA) well. Each standard (S1-S8) was run in duplicate as well as each of the plasma/serum samples. Additionally, samples were run at two dilutions in duplicate to ensure correct reference range within standards. Samples were initially diluted to either a 200 or 400 fold dilution, with repeat of 100 and 800 fold dilutions if the sample was out of range compared to the pre-set standards. This adjustment of dilution factors was made after the plates were read by the ELISA program. AChE tracer was added to each well excluding the TA and Blk wells. The cortisol monoclonal antibody was added to each well excluding the TA, NSB, and Blk wells. The plate was then covered with plastic film provided in the kit, covered in foil, and incubated over night at 4 °C.

Plate contents were removed by inverting the plate and tapping gently on a paper towel. The wells were then washed 5x with wash buffer and emptied prior to the addition of Ellman’s reagent for plate development. AChE tracer was added to the TA wells. The plate was allowed to develop for 90–120 min while covered on a shaker.

The plate was read at a wavelength between 405 and 420 nm. The quality of the plate was determined by assessing the value of the B0
wells, with the acceptable range between 0.3 and 1.5 A.U. (absorbance units with blank well value subtracted). The data was exported into an Excel (Microsoft) workbook provided by Cayman Chemical to convert the absorbance units into concentrations of cortisol.

The authors’ group previously published values of GCSF, IL-10, IL-6, IL-1ra, and MCP-1 in the THM and PHM models (Vicente et al., 2018). These values were included in the analysis to observe trends between the changes in cortisol levels as compared to the aforementioned cytokines.

2.4. Statistical analysis

Plasma samples obtained from the PHM were initially compared to previous serum samples from a PHM model per time point to determine if the difference between plasma and serum samples was significant. The lowest p-value of 0.36, it was determined that the levels obtained from serum vs. plasma were not significantly different and could be used for the analysis between PHM and THM cohorts. Each time point (t = PRE, 0, 60, 120,180, 240, POD1) was compared between groups. Serum and plasma samples were compared using a two tailed Students T-Test between the PHM (n = 7) and THM groups (n = 11). P values less than or equal to 0.05 were considered statistically significant. Statistical analysis was performed using Excel (Microsoft) and PRISM (GraphPad).

3. Results

3.1. Cortisol concentration in polytrauma and hemorrhage vs traumatic hemorrhage models

The PHM and THM groups demonstrated non-statistically distinguishable pre-operative cortisol values. At time 0 (the liver injury in both models, with the cecal injury occurring before the liver injury in the PHM), the cortisol levels began to diverge, with the levels in the PHM initially increasing by 358%. Cortisol levels continued to climb until post-operative day 1, when they reached the maximum increase of 612%. Cortisol levels remained close to baseline in the THM model, increasing by 21% at peak concentration at 120 min, then falling back to baseline levels. All differences between the cortisol concentrations after the pre-operative value were statistically significant, see Table 2 and Fig. 2.

3.2. Cortisol levels compared to GCSF, IL-10, IL-1ra, IL-6, and MCP-1

In the Polytrauma and Hemorrhage model, cortisol demonstrated a positive increase from pre-operative values between 358 and 612%. Multiple cytokines, such as MCP-1 and GCSF, demonstrated similar rates of change, with slightly lower values in the early time points and greater changes from baseline in the latter time points of the study. IL-6, IL-10, and IL-1ra demonstrated increases on the order of 100–10,000% of baseline values, with peak values occurring at similar times as seen in the Polytrauma and Hemorrhage model, see Table 3, Fig. 3.

In the Traumatic Hemorrhage model there was minimal to negative change in cortisol concentration (21% at maximum), with similar trends seen in MCP-1. The remainder of the cytokines increased on the order of 10,000 to 100,000% of preoperative values, see Table 3, Fig. 3.

4. Discussion

To our knowledge, our study is the first to evaluate the effect of traumatic hemorrhage and intra-abdominal contamination on cortisol response in non-human primate models. The use of non-human primates...
in this model allowed investigators to capture cortisol levels at the time of injury, which is not possible in human trauma. Additionally, these animal models allowed for nearly identical injuries to be created to a standardized fashion. The uniformity in hemodynamic, laboratory, and physiologic values between the two protocols is demonstrated in previous publications, which showed no difference between MAP, minimum pH, temperature, or maximum lactate, with the only significant difference between the PHM and THM being % of total blood loss (Vicente et al., 2018). This enabled our team to chart the initial response to traumatic hemorrhage and/or intra-abdominal contamination, in a reproducible model, before most human patients would present to care. Overall, the cortisol response was significantly increased in the Polytrauma and Hemorrhage Model (PHM) as compared to the Traumatic Hemorrhage Model (THM). Interestingly, this increase was apparent from the time zero (t = 0) measurement. Given the animals underwent the same liver resection and uncontrolled hemorrhage, the difference in the cortisol levels is likely a reflection of the effect of uncontained bowel spillage prior to the liver laceration in the PHM model, in addition to local inflammatory factors released during the soft tissue excision. While the 10 min interval between the cecal perforation and t = 0 liver laceration may seem like a short interval to develop a significant inflammatory response, we believe that the size of the defect (4 cm) and degree of frank fecal contamination likely explains this quick response. Clinically, many patients with perforated viscus injuries are able to pinpoint when their visceral abdominal pain transitioned to somatic pain at the time of perforation, leading us to speculate that a significant inflammatory response after perforation is likely very fast to develop. However, it is somewhat surprising that the trauma of a 60% left lobe liver resection and uncontained free hemorrhage into the peritoneum did not elicit a significant cortisol response in the THM group, as this represented a hemodynamically significant hemorrhage. Other models of hemorrhage were able to show a corticosterone response (a cortisol analogue in rats) in response to trauma and/or hemorrhage (Qin et al., 2020; Rushing et al., 2006b). In fact, cortisol concentration in this group (THM) decreased slightly from baseline at several separate time points. The suppression of the cortisol response to trauma has been shown in populations with adrenal insufficiency. Clinically low cortisol levels within the first 24-h after traumatic hemorrhage have been shown to predict increased morbidity and mortality (Stein et al., 2013; Rushing et al., 2006a). It has been postulated that in these instances of traumatic hemorrhage, if those patients with adrenal insufficiency could be identified in advance, they may benefit from supplemental corticosteroids (Stein et al., 2013; Rushing et al., 2006a, 2006b). While supplemental corticosteroids do not have a role in acute trauma, they still are in active use in critical care for various infectious, endocrine, and neurologic diseases of which hypotension may play a confounding factor (Annan et al., 2017). While our model doesn’t show a direct cause of adrenal insufficiency (such as adrenal necrosis secondary to hypotension), the low cortisol levels do point to some dysfunction that should be investigated in further studies.

Table 2
Cortisol concentrations (mcg/dL) pre and post-operatively in Polytrauma and Hemorrhage (PHM) and Traumatic Hemorrhage (THM).

| Test Group                        | Time Post-Operatively (minutes) | Cortisol Concentration (mcg/dL) |
|----------------------------------|---------------------------------|----------------------------------|
|                                  | 0                               | 60                               | 120                              | 180                              | 240                              | POD 1                             |
| Polytrauma and Hemorrhage (PHM)  | 3.691                           | 16.918                           | 16.652                           | 18.123                           | 18.460                           | 22.708                            | 26.276                            |
| Traumatic Hemorrhage (THM)       | 7.240                           | 5.132                            | 6.253                            | 8.337                            | 7.000                            | 8.132                             | 6.894                             |
| Student’s T-Test (P-Value)       | 0.139                           | < 0.001                          | 0.003                            | 0.007                            | 0.035                            | 0.006                             | 0.006                             |

Table 3
Concentrations of various cytokines in the Polytrauma and Hemorrhage Model.

| Cytokine   | Concentration (pg/mL) |
|------------|------------------------|
| GCSF       | 105.08                 |
| IL-10      | 148.27                 |
| IL-1ra     | 66.58                  |
| IL-6       | 37.34                  |
| MCP-1      | 841.79                 |

in this model allowed investigators to capture cortisol levels at the time of injury, which is not possible in human trauma. Additionally, these animal models allowed for nearly identical injuries to be created to a standardized fashion. The uniformity in hemodynamic, laboratory, and physiologic values between the two protocols is demonstrated in previous publications, which showed no difference between MAP, minimum pH, temperature, or maximum lactate, with the only significant difference between the PHM and THM being % of total blood loss (Vicente et al., 2018). This enabled our team to chart the initial response to traumatic hemorrhage and/or intra-abdominal contamination, in a reproducible model, before most human patients would present to care.
increasing cortisol levels, even in the face of adrenal insufficiency caused by hemorrhage, has major implications for the utility of following cortisol levels in traumatically injured patients. Clinicians measuring cortisol levels in a patient with traumatic hemorrhage could be falsely reassured by normal or elevated cortisol levels, while in fact it may be representative of another process in addition to hemorrhage, portending a worse injury pattern overall. In addition, perhaps there is a subset of these polytrauma patients that would benefit from supplemental cortisol as they would be considered to have adrenal insufficiency relative to similar polytrauma patients.

The studied cytokines all showed increased concentrations in the Polytrauma and Hemorrhage Model (PHM) as compared to the Traumatic Hemorrhage Model (THM). While there exists some response in the THM, on the order of 100–10,000% percent increases in certain cytokines, the PHM group showed significantly greater increases, with some cytokines reaching greater than 100,000% increases. Again, this is likely due to the additional fecal contamination and or soft tissue excision stimuli present in the PHM as compared to the THM. Interestingly, in the THM, while there was minimal cortisol response to injury, there was still a significant cytokine response. The fact that the THM model produced a significant cytokine response, and the lack of cortisol response, suggests that there may be physiologic cause for the lower than expected rise in cortisol, such as the relative adrenal insufficiency previously mentioned. While the peak levels were lower than in the PHM group, the peak cytokine levels in the two groups mostly occurred later in the study (@240 min and POD 1). This finding shows that there is still significant immune response generated by the THM as compared to the PHM, but it is not readily manifested in cortisol levels. The early increase in cytokine levels trend was slightly different in GCSF, which showed initial suppression in the Traumatic Hemorrhage group (the only cytokine to show initial negative response), although it eventually peaked at the same time as the other cytokines. This initial suppression in the Traumatic Hemorrhage model likely represents the natural response in pure hemorrhage, prioritizing non granulocyte hematopoiesis vs the granulocyte response needed for fighting the septic insult seen in the Polytrauma and Hemorrhage model.

4.1. Limitations and further studies

Our study has several limitations worth mentioning. Due to the experimental design and ethical goals of reducing the number of animals in the protocol, we did not have a separate control for animals undergoing only the soft tissue excision in the absence of the cecal perforation. This makes it difficult to definitively state which insult is causing the

Table 4

Concentrations of various hormones and cytokines in the Traumatic Hemorrhage Model (THM).

| Cytokine | Concentration (pg/mL) |
|----------|-----------------------|
| GCSF     | 6.31 5.84 26.55 110.10 167.02 |
| IL-10    | 43.85 193.68 588.25 201.50 77.11 |
| IL-1ra   | 16.63 88.63 299.91 2510.94 186.73 |
| IL-6     | 43.10 238.34 615.24 898.85 420.25 |
| MCP-1    | 736.56 1045.31 1399.38 1784.38 4229.17 |

Fig. 3. Percent change from pre-operative value of various hormones and cytokines in the Polytrauma and Hemorrhage Model (PHM).

Fig. 4. Percent change from pre-operative value of various hormones and cytokines in the Traumatic Hemorrhage Model (THM).
increased cortisol levels. However, for our intended study goals (emulating complex battlefield polytraumatic injury), the combination of a polytrauma and hemorrhage model seems to best match the injury patterns that we are hoping to model. Additionally, while we postulate adrenal insufficiency may play a role in the lack of cortisol response in hemorrhage only model, we did not test for any other HPA axis precursor hormones or adrenal pathology at the time of necropsy (due to the use of historical samples). We hope to incorporate a greater biochemical view of the entire HPA axis in trauma in our future development of these models. Our further goals with this project include extending the length of time the animals are kept in recovery to simulate prolonged ICU care and how this can modulate the initial cortisol response.

5. Conclusion

In summary, the authors present a novel study measuring cortisol levels in two models of hemorrhage and intra-abdominal contamination in non-human primates. Cortisol rapidly increases in the Polytrauma and Hemorrhage Model as compared to the traumatic hemorrhage model, likely due to the impact of additional stimuli from fecal peritonitis. The minimal change in cortisol in the Traumatic Hemorrhage model suggests that cortisol is being under produced relative to the trauma sustained by possible adrenal insufficiency caused by the hemorrhage, or cortisol is not a sensitive marker for endocrine response in pure hemorrhage. Cytokine levels, while mostly increasing in both groups, increased more in the Polytrauma and Hemorrhage model, likely due to the additional impact of fecal contamination and soft tissue excision in the PHM group. The elevation of cytokines in both groups confirms that a significant immune response was generated in both models, although cortisol did not seem to contribute to this response in the THM group.

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CRediT authorship contribution statement

Rex E. Atwood: Writing - original draft, Writing - review & editing. Dana M. Golden: Investigation, Data curation, Writing - review & editing. Stephen A. Kaba: Project administration, Writing - review & editing. Matthew J. Bradley: Conceptualization, Methodology, Resources, Supervision, Funding acquisition, Writing - review & editing.

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