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TITLE: Survival and Injury Outcome After TBI: Influence of Pre- and Post-Exposure to Caffeine

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14. ABSTRACT

Soldiers on active duty consume large amounts of caffeine, however little is known how chronic and acute caffeine use affects the outcome of traumatic brain injury (TBI). Data from our lab document that an acute dose of caffeine given after severe TBI in rats results in a dramatic reduction in mortality. The experiments presented herein provide a systematic analysis how acute versus chronic caffeine administered before or after TBI - modeled here in the rat lateral fluid percussion injury (FPI) model - affects survival and morbidity following TBI. We present data demonstrating that (i) chronic caffeine consumption is safe in regards to acute outcome parameters following TBI, (ii) default caffeine withdrawal after TBI is beneficial, whereas chronic caffeine consumption after TBI should be avoided, and (iii) a single bolus of caffeine after severe TBI can prevent lethal outcome, regardless of pre-injury caffeine consumption. We demonstrate that a wide dose range of caffeine is safe, with a moderate dose (equivalent to 2-3 cups of coffee for a human) was most effective.

15. SUBJECT TERMS
TBI, caffeine, morbidity, mortality

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INTRODUCTION

Soldiers on active duty consume large amounts of caffeine, however little is known how chronic and acute caffeine use affects the outcome of traumatic brain injury (TBI). Recent data in animals suggest a potential a link between caffeine intake and the outcome of TBI, which may have translational relevance with respect to caffeine consumption by military personnel and the treatment of TBI. We have previously demonstrated in rats that an acute dose of caffeine given after severe TBI dramatically reduces mortality, supporting a beneficial effect of caffeine in the treatment of TBI. Here, we use the rat lateral fluid percussion injury (FPI) model to systematically evaluate the therapeutic potential of acute vs. chronic caffeine treatment given either before or after TBI to improve the outcome of TBI. The deliverable of these studies is a caffeine-based therapeutic regimen to (i) reduce acute mortality, and (ii) avoid morbidity following TBI.

BODY

Detailed methods and referenced figures are in the Supporting Materials section of the report.

Animal numbers reported in Tables 1-3 reflect the animals used to generate the final data presented herein (a total of 349 rats). The original statement of work included 456 rats. The additional rats were used for FPI device calibration, met experimental exclusion criteria, or were out of the specified weight range.

Phase 1: Study the prophylactic effects of caffeine exposure prior to FPI

Task 1: Pre-treatment with acute and chronic caffeine

In our first publication resulting from this grant (Lusardi, et al., 2012), we demonstrated that a single bolus of caffeine can protect against lethal apnea associated with severe traumatic brain injury (TBI), modeled using fluid percussion injury (FPI). It is our hypothesis that the adenosine surge caused by severe TBI (Bell, et al., 1998, Nilsson, et al., 1990) acts at adenosine receptors in the respiratory centers within the brain stem, repressing the respiratory drive. Caffeine protection from apnea likely stems from non-specific antagonism of the A1 receptor. Before this finding can be translated to field (or pre-hospital) use, however, it is necessary to understand how daily caffeine consumption, might influence the therapeutic efficacy of caffeine. Here, we examined the influence of caffeine preconditioning on rats, comparing *(i)* a single bolus of caffeine 1 hour prior to FPI or 48 hours of caffeine withdrawal in chronically caffeinated rats or *(ii)* the influence of a single bolus of caffeine in caffeine naïve rats. While the focus of these studies was protection from acute mortality, we also included sham and moderate injuries in the experimental design to evaluate the potential influence of caffeine on injury severity and outcome across the injury spectrum.

Table 1 details the number of rats used in Task 1, their average weight at injury, and the FPI peak pressure. There were no significant differences in weight at injury except for the sham group; rats that were over the 400 g weight limit at injury were assigned to the sham group as excessive weight appears to be protective. Within each injury group, there were no differences in the injury severity (measured in atmospheres, atm), nor was there overlap in the injury parameters.

Table 1: Task 1 Experimental matrix and injury parameters (mean ± SD)

Pre-Injury		Sham			Moderate			Severe		
Chronic Caffeine[1]	**Acute**	**n**	**Weight (g)**	**FPI (atm)**	**n**	**Weight (g)**	**FPI (atm)**	**n**	**Weight (g)**	**FPI (atm)**
0.3 g/l	None	6	397 ± 23	0 ± 0	8	370±11	1.81±.09	20	366±18	2.80±.26
	Bolus[2]	6	396 ± 22	0 ± 0	7	366±17	1.81±.04	20	370±16	2.83±.23
	Withdraw[3]	6	394 ± 28	0 ± 0	6	366±10	1.75±.10	20	365±18	2.84±.24
0 g/l	None	6	384 ± 26	0 ± 0	6	367±20	1.79±.12	22	367±20	2.80±.24
	Bolus[2]	6	364 ± 24	0 ± 0	9	372±13	1.80±.08	20	368±14	2.88±.21

[1]Chronic Caffeine: administered *ad libitum* in drinking water for 3 weeks prior to FPI
[2]Bolus: 25 mg/kg, administered intra peritoneal 1 hour prior to FPI
[3]Withdraw: 48 hours caffeine withdrawal prior to FPI

Task 1: Results

The influence of pre-injury caffeine in caffeine-preconditioned rats

In the caffeine preconditioned rats, we observed acute mortality only after severe injury in all caffeine conditions. A single bolus of caffeine administered 1 hour prior to severe FPI did result in lower acute mortality compared to a saline bolus (not significant by logistic regression), while there was no effect of withdrawal on mortality (Fig. 1a). We used a non-parametric analysis to assess the duration of apnea following FPI, assigning a value of 300 seconds to the rats that had no evidence of respiration after FPI. While we found a significant increase in apnea duration with injury severity ($p<0.001$), we did not measure a significant effect of a pre-injury bolus of caffeine or caffeine withdrawal on apnea duration (Fig. 1b). Similarly, we found a significant effect of injury severity on righting time (ANOVA, $p<0.001$), but no influence of a single pre-injury bolus of caffeine or caffeine withdrawal (Fig. 1c).

24 hours after FPI, we measured motor function by neuroscore. As expected following an injury centered on the left-hemisphere, we found more impairment in the right limbs than the left (Fig. 1d & e). There was a significant effect of injury severity in both the left and right limbs ($p<0.005$), but no significant effect of a bolus of caffeine or caffeine withdrawal. We also used rotarod performance to assess motor function at both 1 day and 7 days after FPI in comparison to pre-injury performance, assessing results by repeated measures ANOVA (Fig. 1f). In the sham and moderately injured rats, we found no influence of either time after injury or pre-injury caffeine on outcome. After severe FPI, however, we found a significant decrease in performance at 24 hours after FPI, with a partial recovery at 7 days ($p<0.0001$). We still found no significant effect of pre-injury caffeine consumption on the outcome.

The influence of pre-injury caffeine in caffeine-naive rats

In caffeine naive rats, we observed acute mortality only after severe injury with or without a pre-injury caffeine bolus. A single caffeine bolus prior to injury resulted in a lower (but not significantly lower) mortality rate (Fig. 2a). Apnea duration (Fig. 2b) and righting time (Fig. 2c) both increased with injury severity ($p<0.001$ in both cases), but a caffeine bolus had no influence on either one.

Neuroscore measures at 24 hours after FPI showed a significant effect of injury severity in both hemispheres ($p < 0.02$, Figs 2d & e), but again, no significant effect of pre-injury bolus. In rotarod tests at 1 and 7 days post FPI showed no effect of sham or moderate injury, but a significant deficit in after severe injury ($p<0.001$). Again, there was no significant effect of pre-injury caffeine bolus (Fig. 2f).

Task 1: Conclusions

These results demonstrate that pre-injury caffeine paradigms do not significantly affect mortality or morbidity after FPI. These results vary slightly from those presented in our Annual Report (10/15/2011) as we added additional rats to the severe injury group to improve the power of our results. Overall, results in the caffeine naïve rats (Fig. 2) were similar to those measured in the caffeine preconditioned rats (Fig. 1). In a separate analysis, we re-examined the data to consider any interaction between caffeine naïve vs. preconditioned and caffeine vs. saline bolus prior to injury. In this analysis, we do find a near-significant protective effect of pre-injury caffeine (Fig. 3, † $p=0.06$); similarly, there is a trend toward a protective effect of pre-injury caffeine on post-injury measures, as previously reported.

These results complement existing literature demonstrating the deleterious effects of pre-injury caffeine at very high doses (Al Moutaery, et al., 2003); our caffeine dose of 25 mg/kg is consistent with recommendations in *Caffeine for the Sustainment of Mental Task Performance: Formulations for Military Operations*. When we increase the post-injury caffeine bolus to 50 mg/kg (Fig. 3), we also find diminished recovery. In addition, Al Moutaery et al used lighter female Wistar rats. In our experience, rat weight has a significant effect on injury severity, with heavier rats more tolerant of injury. In mice, chronic caffeine has been shown to have a largely protective effect, with little influence of an acute bolus prior to injury (Li, et al., 2008). In this case, while there are likely model and species specific effects, differences may stem from the outcome measures examined in surviving mice. Clinically, elevated CSF caffeine at the time of TBI is associated with improved outcome 6 months after injury (Sachse, et al., 2008), suggesting that caffeine at the time of injury has long-term benefits that may not be readily apparent in the time scale examined in this study.

Overall, we have demonstrated that there is a minimal effect of pre-injury caffeine consumption on short-term outcome measures after FPI. However, the trends observed here suggest that additional environmental factors (eg, sleep disruption, nicotine use, nutrition) may enhance these trends to significance. Further, the nature of modeled injury results in a "clean" TBI, which is not typical of clinical injuries. As caffeine influences many physiologic systems (notably the cardiovascular system), the influence of caffeine at the time of injury should not be underestimated.

In summary, our data demonstrate that chronic caffeine consumption during active duty is safe in regards to acute outcome parameters following TBI.

Phase 2: Evaluate the therapeutic benefit of post-injury caffeine treatment

Task 2.1: Post-treatment with chronic caffeine

While we have demonstrated that an acute bolus of caffeine administered immediately after a severe FPI can protect against lethal apnea, there are no studies examining the chronic use of caffeine after TBI. Therefore, in this task, we examined the interaction of chronic caffeine consumption before and after FPI.

Experimental parameters for Task 2.1 are detailed in Table 2. There were no significant differences in weight at injury except for the sham group; rats that were over the 400 g weight limit at injury were assigned to the sham group as excessive weight appears to be protective. Within each injury group, there were no differences in the injury severity (measured in atmospheres, atm), nor was there overlap in the injury parameters.

Table 2: Experimental matrix and injury parameters (mean ± SD)

Caffeine[1]		**Sham**			**Moderate**			**Severe**		
Pre-Injury[2]	**Post-Injury[3]**	**n**	**Weight (g)**	**FPI (atm)**	**n**	**Weight (g)**	**FPI (atm)**	**n**	**Weight (g)**	**FPI (atm)**
0 g/l	N/A[4]	-	-	-	-	-	-	9	364 ± 15	3.25 ± .04
	0 g/l	4	381 ± 33	0 ± 0	6	361 ± 9	1.97 ± .07	4	375 ± 02	3.27 ± .05
	0.3 g/l	4	392 ± 13	0 ± 0	6	363 ± 19	1.96 ± .04	5	362 ± 09	3.25 ± .02
0.3 g/l	N/A[4]	-	-	-	-	-	-	5	371 ± 16	3.23 ± .04
	0 g/l	6	398 ± 12	0 ± 0	3	357 ± 26	1.87 ± .02	3	352 ± 20	3.21 ± .08
	0.3 g/l	5	379 ± 37	0 ± 0	3	358 ± 14	1.93 ± .12	6	356 ± 27	3.21 ± .04

[1]Chronic Caffeine: administered *ad libitum* in drinking water for 3 weeks
[2]Pre-injury caffeine exposure for 3 weeks
[3]Post-injury caffeine exposure from FPI until sacrifice
[4]N/A Indicates rats that died as a result of the FPI (Severe FPI only)

Task 2.1: Results

The influence of caffeine preconditioning on acute outcome measures

As in Task 1, only severe FPI was associated with acute mortality. While we did find a decrease in acute mortality associated with caffeine preconditioning (Fig. 4a), it was not significant. Again, apnea duration was associated with injury severity ($p < 0.001$), but not influenced by pre-injury caffeine consumption (Fig. 4b). Righting time was also correlated to injury severity ($p<0.001$), and with pre-injury caffeine consumption ($p<0.05$); post-hoc analysis confirms that that the effect of pre-injury caffeine in this study was limited to severe injury, where chronic caffeine consumption prior to FPI resulted in a significantly shorter righting time (Fig. 4c, ** $p<0.01$).

The influence of chronic caffeine consumption after injury

We examined neuroscore at 1 day after FPI, and found no deficits in either right or left limbs after sham injury. After moderate injury, there was no deficit in left limbs, but small (non-significant) deficits in right limbs, corresponding to the left hemisphere brain injury. Severe injury caused deficits in both left and right limbs (Fig. 5a & b), with deficits worse in right limbs. In the left limbs, there was a pronounced (but not significant) increase in deficits in the rats that received caffeine both before and after FPI (Fig. 5a). In the left limbs, there was a pronounced (but again, not significant) reduction in deficits measured by neuroscore in the rats that were in caffeine withdrawal at the time of assessment (chronic caffeine prior to FPI, standard drinking water after FPI, Fig. 5b).

We measured rotarod performance at 1 and 7 days after injury. Within each caffeine paradigm, we found a graded effect of injury level. At 1 day after severe injury, we measured a significant reduction in performance for both caffeine naïve and preconditioned rats, regardless of their post-injury caffeine consumption. While all groups demonstrated recovery of rotarod performance at 7 days after FPI, caffeine after injury inhibited recovery (Fig. 5c & d, * $p < 0.05$).

Task 2.1: Conclusions

These results support the observations in Task 1 that there is little effect of pre-injury caffeine consumption on injury outcome at the time scale examined herein. Further, they demonstrate that, after severe injury in particular, continued chronic caffeine consumption after the injury impairs recovery of motor function.

There is little evidence-based guidelines for the use of caffeine after TBI. Anecdotal reports suggest that caffeine consumption after injury may promote memory, attention, and task performance, or it may cause irritability,

insomnia, and harmful prescription drug interactions. As with all matters related to TBI, the heterogeneous nature of the injury itself may contribute to the confusion. The results presented here suggest that chronic caffeine after injury, particularly in caffeine preconditioned individuals, restricts recovery of motor function after injury (Fig. 5d).

Further studies examining the long-term effects of caffeine consumption after injury would be useful. For example, understanding the interaction between caffeine and commonly prescribed drugs to treat pain, commonly comorbid with TBI, may shed light on the difficulties in pain management in this population.

In summary, default caffeine withdrawal after TBI is beneficial, whereas chronic caffeine consumption after a TBI should be avoided.

Task 2.2: Post-treatment with acute caffeine

In this task, we examine the parameters governing the use of a single bolus of caffeine after severe injury to protect against acute, apnea associated mortality. As we found no significant differences between caffeine naïve and preconditioned rats in Tasks 1 and 2.1, and the prevalence of caffeine consumption in both the military and civilian environments, all rats in Task 2.2 are caffeine preconditioned. Note that prior to beginning these studies, we performed FPI calibration studies to determine the injury severity necessary to achieve 40-50% mortality in caffeine naïve rats. We address three questions in this experiment: *(i)* is caffeine rescue dose dependent, *(ii)* does the time of bolus affect the efficacy of the bolus, and *(iii)* are there negative effects of an unnecessary caffeine bolus after mild or moderate injury?

The experimental matrix for Task 2.2 is outlined in Task 2.2. Animals with weight in excess of 400 g were assigned to the sham injury group; weight distributions in the mild, moderate, and severe groups were not significantly different from one another. FPI severity (measured in atm) were not significantly different within a severity group, but were significantly different from one another.

Table 3: Task 2.2 Experimental Matrix and injury parameters (mean ± SD)[1].

Caffeine Bolus[2]		Sham[3]			Mild			Moderate			Severe		
Time (sec)	Dose (mg/kg)	n	Weight (g)	FPI (atm)	n	Weight (g)	FPI (atm)	n	Weight (g)	FPI (atm)	n	Weight (g)	FPI (atm)
10	0	8	400 ± 12	0 ± 0	8	373 ± 17	0.60 ± .03	8	373 ± 9	1.90 ± .05	12	371 ± 16	3.01 ± .07
	12.5	-	-	-	-	-	-	-	-	-	12	380 ± 12	2.98 ± .05
	25	6	404 ± 7	0 ± 0	8	378 ± 15	0.62 ± .03	8	373 ± 16	1.90 ± .08	16	368 ± 11	2.98 ± .04
	50	-	-	-	-	-	-	-	-	-	12	375 ± 14	3.03 ± .05
90	25	-	-	-	-	-	-	-	-	-	14	378 ± 12	3.01 ± .07

[1] All rats in this study were administered caffeine in their drinking water (0.3 g/l) for 3 weeks prior to FPI
[2] Bolus delivered IP in 1 ml/kg saline
[3] Heavy rats were assigned to the Sham FPI group

Task 2.2: Results

Caffeine rescue after severe FPI is dose dependent

We examined the acute mortality after a post-FPI caffeine dose of 12.5, 25, or 50 mg/kg in comparison to a vehicle (saline) injection. We found that protection against acute mortality increases with caffeine dose up to 25 mg/kg, but higher doses have no protective effect (Fig. 6a), with maximal protection at 25 mg/kg (* $p<0.05$). Apnea duration varied similarly, with the shortest duration after the 25 mg/kg dose (Fig. 6b, not significant). Righting time was consistent up to and including the 25 mg/kg bolus group, but increased significantly in the 50 mg/kg group (Fig. 6c).

We examined motor function using neuroscore at 1 day after injury (Fig. 6d), and found a significant effect of caffeine dose ($p < 0.01$) and greater impairment on the right side, consistent with a left brain injury ($p<0.001$ by two-way ANOVA). Post hoc analysis showed a significant effect between the left and right limbs only after the 50 mg/kg dose (** $p<0.01$). We examined rotarod performance at 1 and 7 days after injury (Fig. 6e), and found significant effect of time after injury ($p<0.001$), but no main effect of dose.

These results demonstrate conclusively that 25 mg/kg in rats is the optimal dose after severe FPI to protect against acute mortality.

Caffeine rescue after severe FPI is time sensitive

Using the 25 mg/kg dose of caffeine demonstrated to provide maximum protection against acute mortality, we examined the effects of delayed caffeine treatment. In addition to the 10 second time point, we examined a 90 second time point, which is within the time frame of survivable apnea. We found that mortality was higher with delayed administration, but not significantly (Fig. 7a). Apnea was longer with increased delay, though not significantly (Fig. 7b). Righting time also increased with delayed administration (Fig. 7c, * $p<0.05$).

Neuroscore assessment at 1 day after FPI demonstrated a significantly worse impairment in the right limbs as compared with the left, again consistent with a left hemisphere injury ($p < 0.01$, Fig. 7d). Post hoc tests show a significant difference in the 90 second delay group (Fig. 8d, * $p< 0.05$). Rotarod assessments showed no significant difference in impairment or recovery based on the delay to caffeine bolus (Fig. 7e).

These results demonstrate that a caffeine bolus is most effective when delivered as soon as possible after severe injury. Delayed caffeine delivery does not worsen survival compared with no treatment, and in rats that survive, the delayed bolus does not exacerbate injury.

An acute bolus of caffeine is not detrimental after mild or moderate injury

As demonstrated above, the therapeutic effect of caffeine is strongest when administered as soon as possible following injury. One of our goals for the transition of caffeine to field use is to have a therapy that does not require specialized knowledge or assessment to determine treatment appropriateness. Therefore, we evaluate here the use of caffeine after sham, mild, and moderate injury, which do not cause acute mortality, to determine whether unnecessary caffeine administration could cause harm.

We only observed apnea after moderate and severe injury, but did not measure any significant influence of caffeine bolus on apnea. Similarly, righting time scaled with injury ($p < 0.0001$, Fig. 8a), but there was no influence of caffeine bolus.

Neuroscore was not sensitive enough to measure significant changes at 1 day after sham, mild, or moderate injury. Rotarod results (Fig. 8b) demonstrated a slight reduction in rotarod performance after a caffeine bolus, compared to a saline bolus. After sham (b.*i*), mild (b.*ii*), or moderate (b.*iii*) injury, the deficits were not significant; after severe injury (Fig. 9b.*iv*), repeated measures ANOVA evaluation revealed a nearly significant effect of caffeine ($p = 0.051$), with post hoc analysis at 7 days demonstrating a significantly reduced recovery of rotarod function (* $p<0.05$).

Task 2.2: Conclusions

These results add further support to the use of a single bolus of caffeine to prevent lethal apnea after severe TBI. Further, they demonstrate a range of efficacy for a single bolus of caffeine that suggest that these findings can be translated to field use with broad success. Finally, our findings that a single bolus of caffeine does not significantly impair recovery in cases when apnea is not present establish that caffeine could be utilized in a field setting by personnel with minimal training, stabilizing a TBI victim for transport and thus saving a life.

The recognition that pre-hospital apnea associated mortality is a significant concern after TBI was the impetus behind the OPALS Major Trauma Study (Stiell, et al., 2008), which was designed to assess the standardized use of pre-hospital intubation and resuscitation support on pre-hospital mortality. Unfortunately, the result from this study has been consistently poor (Davis, 2008, Stiver and Manley, 2008). The use of caffeine to restore respiration after TBI has the advantages of simplicity and directness. There is little risk of exacerbating neck or spinal damage with an intraperitoneal injection of caffeine, unlike intubation which can be particularly difficult in a field situation. A bolus of caffeine likely acts in the central nervous system, blocking the inhibitory adenosine input to respiratory neurons, restoring breathing.

In summary, a single bolus of caffeine can prevent lethal outcome after severe TBI. Administration should occur as early as possible. While a wide dose range of caffeine was found to be safe, a moderate dose of caffeine – compared to the human consumption of 2-3 cups of coffee – was shown to be most effective.

Histological Results

We examined post-mortem brains to determine whether a post-injury caffeine bolus could exacerbate histological damage. We found no increased in brain atrophy in the caffeine treated group (Fig. 9), further supporting a protective role for caffeine after TBI. In addition, we had planned to use immunohistochemistry to evaluate changes in adenosine A_1 and A_{2A} receptor distribution in response to chronic caffeine consumption and the various trauma conditions. Unfortunately, as we evaluated the commercially available A_1 and A_{2A} antibodies in our $A_1^{-/-}$ and $A_{2A}^{-/-}$ mice, we have found that both of these antibodies have significant non-specific staining patterns. Brain tissue from each rat in this study has been preserved for future histologic evaluation.

KEY RESEARCH ACCOMPLISHMENTS

- Caffeine bolus after TBI significantly reduces lethal outcome
- Caffeine bolus after TBI has a wide effective dose range
- Caffeine bolus pre-TBI decreases mortality and morbidity
- Chronic caffeine pre-TBI is safe and does not negatively impact TBI outcome
- Chronic caffeine post-TBI impairs recovery

REPORTABLE OUTCOMES

Published Manuscripts (full text in appendix)

- Lusardi, T. A., Lytle, N. K., Szybala, C., and Boison, D., 2012. Caffeine prevents acute mortality after TBI in rats without increased morbidity. Exp Neurol 234, 161-168. Pubmed: 22226594

Abstracts (full text in appendix)

- Lytle, N. K., Boison, D., Lusardi, T. A. Acute mortality following severe traumatic brain injury is rescued by caffeine. Society for Neuroscience, 42nd Annual Meeting, New Orleans, LA October 13-17, 2012.
- Lusardi, T. A., Lytle, N. K., Boison, D. Effects of caffeine withdrawal after traumatic brain injury. Society for Neuroscience, 42nd Annual Meeting, New Orleans, LA October 13-17, 2012.

Presentations

- "Survival and Injury Outcome after TBI: Influence of Pre- and Post-Exposure to Caffeine", TATRC Warfighter Neuro System Dysfunction, 13 September 2011.
- "Can coffee save your life?" 3rd Meeting of the Brazilian Purine Club, Ouro Preto, MG, Brazil, September 22, 2012
- "Caffeine & TBI: preventing prehospital mortality." Department of Anesthesia and Perioperative Medicine, Oregon Health Sciences University. *Scheduled: 10/31/2012*

Funding applied for based on work supported by this award

- *Pending*: "Survival and Injury Outcome after TBI, Phase III: Influence of Sleep Disruption and Caffeine Consumption." TATRC Proposal #12325001
- *Not Funded:* "Caffeine-Based Resuscitation after Traumatic Brain Injury and Hemorrhagic Shock in Pre-Caffeinated and sleep Disrupted Populations." PHTBI-ANRA 2011 Proposal PT110667.

Personnel receiving pay from this research effort

- Detlev Boison, PhD
- Theresa A. Lusardi, PhD
- Nikki K. Lytle, BA

CONCLUSION

This grant has provided evidence that caffeine consumption during active duty is safe in terms of acute outcome parameters after TBI. Of note, caffeine withdrawal after severe TBI is beneficial, and daily caffeine administration should be avoided in the early time frame after TBI. Most importantly is the demonstration that an acute caffeine bolus delivered intraperitoneally *after* TBI significantly reduced acute apnea associated mortality. As apnea is a major cause of pre-hospital mortality, treatments that can be easily applied at the scene of injury are desperately needed. We further demonstrated that a wide-range of doses are effective for the restoration of respiration after injury, and that a single bolus of caffeine given when apnea is not apparent does not have negative consequences. Together, these results suggest that a caffeine bolus can be successfully administered in the field by an individual with minimal specialized medical training, and support the development of a "caffeine pen" that could be carried as a part of a personal emergency medical kit for administration to an injured individual.

In the process of these experiments, we found an optimal dose of caffeine in rodents (25 mg/kg, on the order of 2-3 cups of coffee for humans); doses above this level were ineffective for preventing mortality. Future studies should also include a longer survival time with a larger variety of outcome measures to include cognitive and psychosocial outcomes. In these studies reported here, we found several outcomes that were "nearly" significant, suggesting that these studies were underpowered and that power analyses must be revisited for further studies.

Additional studies to examine a wider range of environmental factors on outcome both before and after TBI. Environmental factors (caffeine, nicotine, alcohol, nutrition, stress) all likely contribute to resilience (or susceptibility) at all injury severities. After injury, we find that continued caffeine consumption in the early time frame is detrimental; studies to determine "return to caffeine" recommendations would fill the current evidence gap regarding post-trauma caffeine use. Studies targeted to systematically address environmental factors will provide additional understanding of the variety of individual susceptibility to similar injuries and will provide guidance for individualized treatment following injury.

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APPENDICES

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Caffeine prevents acute mortality after TBI in rats without increased morbidity

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ABSTRACT

Severe traumatic brain injury (TBI) is associated with a high incidence of acute mortality followed by chronic alteration of homeostatic network activity that includes the emergence of posttraumatic seizures. We hypothesized that acute and chronic outcome after severe TBI critically depends on disrupted bioenergetic network homeostasis, which is governed by the availability of the brain's endogenous neuroprotectant adenosine. We used a rat lateral fluid percussion injury (FPI) model of severe TBI with an acute mortality rate of 46.7%. A subset of rats was treated with 25 mg/kg caffeine intraperitoneally within 1 min of the injury. We assessed neuromotor function at 24 h and 4 weeks, and video-EEG activity and histology at 4 weeks following injury. We first demonstrate that acute mortality is related to prolonged apnea and that a single acute injection of the adenosine receptor antagonist caffeine can completely prevent TBI-induced mortality when given immediately following the TBI. Second, we demonstrate that neuromotor function is not affected by caffeine treatment at either 24 h or 4 weeks following injury. Third, we demonstrate development of epileptiform EEG bursts as early as 4 weeks post-injury that are significantly reduced in duration in the rats that received caffeine. Our data demonstrate that acute treatment with caffeine can prevent lethal apnea following fluid percussion injury, with no negative influence on motor function or histological outcome. Further, we show epileptiform bursting is reduced after caffeine treatment, suggesting a potential role in the modulation of epilepsy development after severe injury.

Introduction

Severe traumatic brain injury (TBI) constitutes a major cause of mortality and morbidity, and the incidence of TBI is on the rise. Both acute mortality and chronic consequences of TBI such as posttraumatic epilepsy (PTE) constitute major unmet medical problems. One of the first acute consequences of TBI is a surge of the neuromodulator adenosine (Clark et al., 1997). Apnea, caused by central respiratory depression, and resulting hypoxia is a major cause of acute mortality after TBI; indeed, prolonged apnea after TBI in humans is associated with a mortality rate of 50% (The Brain Trauma Foundation and The American Association of Neurological Surgeons, 2000).

The rostral end of the brain stem, the medulla oblongata, plays an important role in respiration control (Roth and Roehrs, 2000). Within this respiratory center, the inhibitory neuromodulator adenosine acts at adenosine receptors to regulate respiratory functions (Runold et al., 1989). It has been demonstrated that overstimulation of both adenosine A_1 and A_{2A} receptors can cause the suppression of vital respiratory and cardiovascular functions (Barraco et al., 1990; McCarley 2007; Tseng et al., 1988). Under physiological conditions, adenosine concentrations are kept within the nanomolar affinity range for its receptors (Fredholm et al., 2005). However, under conditions of extreme metabolic stress, as occurs during TBI, a surge of micromolar levels of adenosine results (Clark et al., 1997). In rodents, this increase has been demonstrated as early as 10 min after injury (Bell et al., 1998; Headrick et al., 1994; Nilsson et al., 1990). It was further demonstrated that a seizure-induced surge in adenosine coupled to a pharmacologically induced deficiency in metabolic adenosine clearance triggered lethal apnea in mice (Shen et al., 2010). Likewise, deficient adenosine clearance induced by genetic disruption of adenosine kinase in mice led to intermittent periods of apnea and perinatal mortality (Boison et al., 2002). These findings suggest that increased brain levels of adenosine can be a likely cause for lethal apnea. In non-toxic concentrations, the methylxanthines caffeine and theophylline are non-selective antagonists of the adenosine receptors (Fredholm 2007; Fredholm et al., 1999). Clinically, apnea of prematurity can be treated effectively with caffeine (Schmidt et al., 2007); likewise, inhibition of adenosine receptors with theophylline was shown to restore spontaneous respiration after spinal cord injury (Nantwi, 2009). Thus, methylxanthines can be used experimentally and therapeutically to influence adenosine-based modulation of respiratory function.

In addition to the risk of acute mortality, severe TBI is a risk factor for the development of PTE; the complexity of TBI and the long latency to clinically evident spontaneous seizures has made the PTE etiology difficult to assess. Gene profiling studies suggest that changes in neuronal plasticity, cell death, proliferation, and inflammatory or immune responses all contribute to the development of PTE (Pitkanen

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and Lukasiuk, 2009, 2011). Further, changes in homeostatic functions exerted by astrocytes (Stewart et al., 2010) contribute to the development of PTE, and brief focal, recurrent and spontaneous epileptiform electrocorticography events constitute an early event in the pathogenesis of PTE (D'Ambrosio et al., 2009). As a homeostatic bioenergetic network regulator, adenosine modulates immune functions including inflammatory processes and cytokine release in the brain (Hasko et al., 2005), in addition to the regulation of homeostatic functions of astrocytes (Boison, 2008a, 2008b). Clinical studies demonstrate elevated CSF adenosine with micromolar spikes for up to 18 h following TBI (Bell et al., 2001), raising the possibility that the post-injury adenosine surge persists long enough to trigger network alterations that may contribute to the development of a posttraumatic epileptic phenotype.

We therefore hypothesized that both acute mortality and post-traumatic chronic consequences might be influenced by an acute surge in adenosine. If true, blockade of adenosine by the non-selective adenosine receptor antagonist caffeine should protect against both acute as well as chronic consequences of TBI. We modeled severe TBI in the rat by lateral fluid percussion injury, which has been shown to cause acute lethal apnea and to trigger PTE (Kharatishvili et al., 2006). We demonstrate that treatment with caffeine prevents lethal apnea and reduces epileptiform EEG activity without negative impact on neuromuscular behavior or histological outcome.

Materials and methods

Animals

Procedures were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care according to protocols approved by the Legacy Institutional Animal Care and Use Committee, the USAMRMC Animal Care & Use Review Office, and guidelines from the National Institute of Health.

Lateral fluid percussion injury (FPI)

Male Sprague–Dawley rats (352.2 ± 2.7 g at FPI, n = 42, Charles River, Wilmington, MA) were anesthetized with isoflurane (2% isoflurane at 2 ml/min in 2:1 N_2O:O_2), then affixed into a stereotactic frame. A 5 mm trephine hole was drilled centered at bregma −4.5 mm anterior–posterior and +2.8 mm medial-lateral (Kharatishvili et al., 2006). TBI was produced by a fluid-percussion device (Custom Design and Fabrication, Richmond, VA). A 21–23 ms fluid pulse with peak pressure of 1.97 ± 0.02 ATM was applied to the exposed dura, measured by an external pressure transducer, digitized by a PowerLab A/D converter (ADInstruments, Colorado Springs, CO), then recorded using Scope (ADInstruments). There were no significant differences in weight and pressure distributions for the severe injury group by post-injury caffeine and mortality, as presented in Table 1. Following FPI, rats were placed in dorsal recumbency for continuous observation of respiratory activity. The duration of apnea was measured from the time from FPI until the first acute inspiratory effort, or "gasp." Monitoring continued until regular spontaneous ventilation was apparent, or until the complete cessation of inspiratory effort for at least 5 min. Sham-injured controls were generated using identical manipulations without impact.

Table 1
FPI parameters.

FPI	Caffeine (mg/kg)	Survival	Weight (g)	FPI pressure (atm)
Severe	0	Died (n = 7)	352 ± 9	1.96 ± 0.04
Severe	0	Survived (n = 8)	345 ± 4	1.98 ± 0.04
Severe	25	Died (n = 0)	NA*	NA*
Severe	25	Survived (n = 8)	356 ± 4	1.96 ± 0.03
Sham	0	Survived (n = 9)	358 ± 5	0
Sham	25	Survived (n = 10)	349 ± 7	0

*None of the severe injury group died after receiving caffeine.

Caffeine

The caffeine dose (Sigma-Aldrich, St. Louis, MO, 25 mg/kg, i.p.) was selected based on its pharmacokinetic profile in rats compared with humans. Considering metabolic body weight [body weight raised to the 0.75 power] this is equivalent to about 700 mg of caffeine in an 85 kg adult human, well below the lethal dose of approximately 10 g in humans (Fredholm et al., 1999).

Neuroscore

Motor function was assessed prior to and 24 h after FPI by an individual blinded to the experimental condition. Rats were scored from 0 to 4 for their left and right side performance on contraflexion (forelimb reaching), hindlimb flexion, and lateral pulsion. Scores range from 0 to 4, indicating no response to normal performance respectively, for a total of 24 possible points (McIntosh et al., 1989).

Open field testing

Locomotor activity was assessed in the open field (Yee et al., 2007). Tests were performed 24 h and 4 weeks after FPI in squads of 4 animals. Each rat was placed in the center of a 50 × 50 cm open field and videotaped for 50 min. Total distance traveled was measured using EthoVisionXT (Noldus Information Technology, Wageningen, The Netherlands). Distance was assessed for each animal in 5 min bins for analysis.

Electrode implantation

Three weeks after FPI, rats were anesthetized with isoflurane (as for FPI), then affixed into a stereotactic frame. Twisted-pair electrodes (PlasticsOne, Roanoke, VA, USA) were inserted into the brain with respect to bregma: −5.0 mm anterior–posterior, +4.0 mm medial–lateral, and −7.5 mm dorso-ventral. Stainless steel reference screw electrodes (PlasticsOne) were implanted over the cerebellum. Electrodes were inserted into a multi-channel electrode pedestal (PlasticsOne), and secured using dental cement (CO-Oral-Ite Dental Mft Co, Diamond Springs, CA). An additional 10 naïve adult male Sprague Dawley rats (Charles River) were included in the video-EEG analysis.

Video-EEG recording

Four weeks after FPI, rats were connected to an amplifier (Grass Technologies, West Warwick, RI) for EEG monitoring. EEG signals were digitized (PowerLab, AD Instruments, Colorado Springs, CO) and recorded to a personal computer (Dell, Round Rock, TX). Video was acquired using an IR sensitive video camera (Noldus). Animals were subjected to 24 h of continuous video-EEG monitoring. Epileptiform EEG-bursts were defined as high amplitude, periodic spike waveforms with greater than 5 s duration (Fig. 3). EEG activities associated with grooming, eating, or drinking were excluded from analysis.

Histology

Following video-EEG, rats were deeply anesthetized with isoflurane, then transcardially perfused with 0.9% saline and 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde, sunk in sucrose, frozen, and sectioned by cryostat. Nissl staining at Bregma −3–4 mm was used to calculate cortical thinning and lateral

ventricular enlargement. Sections were scanned (HP 8350, Hewlett Packard), then quantified using ImageJ (NIH, Bethesda, MD).

Statistics

All statistics were performed using StatView (SAS Institute, Cary, NC). The distributions of weight, injury pressure, and apnea for each group were compared by *t*-test, and were not different among groups (Table 1). Correlation between caffeine treatment and mortality was assessed using logistic regression followed by a chi-squared test. Effects of caffeine and injury on Neuroscore were assessed by two-way ANOVA. Effects of caffeine and injury on Open Field performance were assessed by repeated measures ANOVA. Incidence of seizure activity was analyzed by logistic regression and the effect of caffeine treatment on burst duration by *t*-test. Results are presented as mean ± SEM.

Results

Acute mortality following severe TBI can be prevented by a single acute dose of caffeine

Clinically, acute and prolonged lethal apnea is a pathological hallmark of severe TBI (The Brain Trauma Foundation and The American Association of Neurological Surgeons, 2000). To assess whether the duration of apnea correlates with lethal outcome, we induced severe TBI in rats by lateral fluid percussion injury (FPI). 15 rats received severe FPI, resulting in prolonged apnea and a mortality rate of 47%. A total of 8 rats survived the procedure, whereas 7 animals died within 10 min. Apnea was significantly longer in animals that died after severe FPI (Fig. 1A) compared to the apnea duration in survivors. Body weight and FPI pressure distribution were consistent between groups (Table 1). Thus, prolonged apnea correlates with lethal outcome.

Fig. 1. Caffeine treatment prevents acute mortality after severe lateral fluid percussion injury. (A) Acute mortality is associated with longer apnea duration (188 ± 40 s) after severe FPI than for survivors (61 ± 11 s). (B) Treatment with a single IP bolus of caffeine within 1 min after FPI reduces apnea duration to 53 ± 9 s and prevents acute mortality. * $p<0.01$.

To investigate whether lethal apnea is due to an adenosine-related mechanism and thereby preventable, we treated a second group of 8 rats within 1 min after FPI with a single acute dose of caffeine (25 mg/kg, intraperitoneal). This treatment completely prevented the extended apnea seen in untreated non-survivors, resulting in apnea durations comparable to untreated survivors. Most importantly, caffeine completely prevented apnea-related acute mortality (Fig. 1B). There were no statistical differences in the weight and FPI pressure distributions in any of the injury groups (Table 1). Thus, lethal apnea can be prevented by caffeine treatment after injury.

Caffeine rescue does not impact neuromuscular performance after severe FPI

To rule out the possibility that caffeine rescue aggravates neurological deficits, we performed tests of basic neurological and motor functions 24 h and 4 weeks after FPI. Neuroscore assessment of motor skills showed significant impairment in severely injured survivors in both groups compared to sham-injured animals, with a trend

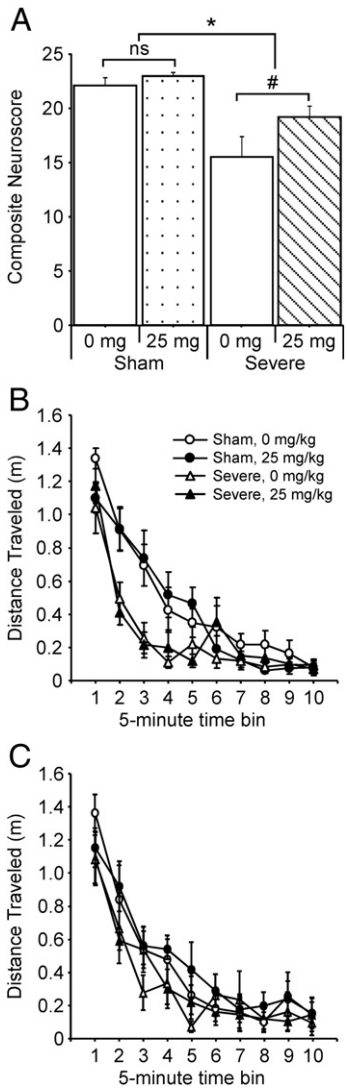

Fig. 2. Caffeine rescue does not impair behavioral outcome parameters. (A) 24 h after FPI, gross motor function was assessed by composite neuroscore on a scale of 0 (no function) to 24 (normal function; see methods). Neuroscore is significantly reduced in severely injured rats (*$p<0.001$), with a trend toward improved function in the caffeine treated rats (# $p<0.055$). (B) Severe injury results in reduced locomotion in the open field when compared with sham injury ($p<0.01$), with no influence of caffeine treatment. (C) Four weeks after injury, there is no effect of FPI or caffeine treatment on open field performance.

towards improvement in the rats receiving caffeine (Fig. 2A). Spontaneous locomotor activity in the open field showed a significant reduction in distance traveled by the severely injured rats (Fig. 2B), but no significant effect of caffeine, and no interaction between injury and caffeine. At 4 weeks after FPI, there was caffeine-independent recovery of open field activity in the severe injury group (Fig. 2C). Thus, a single acute dose of caffeine given after the FPI did not negatively impact neuromotor behavior when assessed either 24 h or 4 weeks after brain injury.

Epileptiform EEG-bursts constitute an early consequence of severe TBI and can be reduced in duration by post-injury caffeine

Severe TBI is associated with the development of posttraumatic epilepsy (PTE) (Frey, 2003; Lowenstein, 2009). Here we used an established model of PTE, which induces spontaneous hippocampal seizures after a latency of 2–12 months (Kharatishvili et al., 2006). To understand the early consequences of FPI for neuronal excitability in the hippocampus, we acquired simultaneous video and EEG recordings for 24 h in each animal at four weeks after FPI. Video analysis revealed no evidence of clinical seizures. However, evaluation of the EEGs revealed frequent epileptiform bursting in 7 of the 16 severely injured animals, 1/10 sham injured animals, and 0/10 naïve animals. Bursting was distinguished by amplitude, periodicity, spike waveform, and duration greater than 5 s (Fig. 3A), and increased EEG-power (Fig. 3C) as compared to quiescent periods (Fig. 3B). EEG activity associated with grooming, eating, or drinking were excluded from the analysis. While the number of bursts was not significantly affected by the single acute caffeine treatment after the injury,

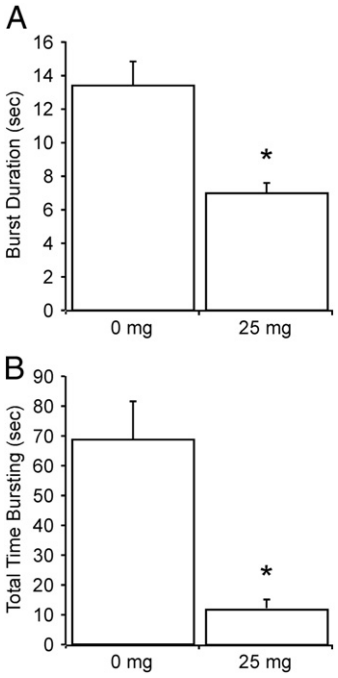

Fig. 4. Caffeine treatment reduces the incidence of epileptiform bursting after severe FPI. (A) Burst duration was significantly reduced from 13.35 ± 1.4 s in the non-caffeinated rats to 6.94 ± 0.5 s in the rats that received an acute caffeine bolus. (B) The total time spent bursting over a 24 hour period was significantly reduced from 68.53 ± 12.78 s in the non-caffeinated rats to 11.88 ± 3.0 s in the acute caffeine group . (*$p<0.05$).

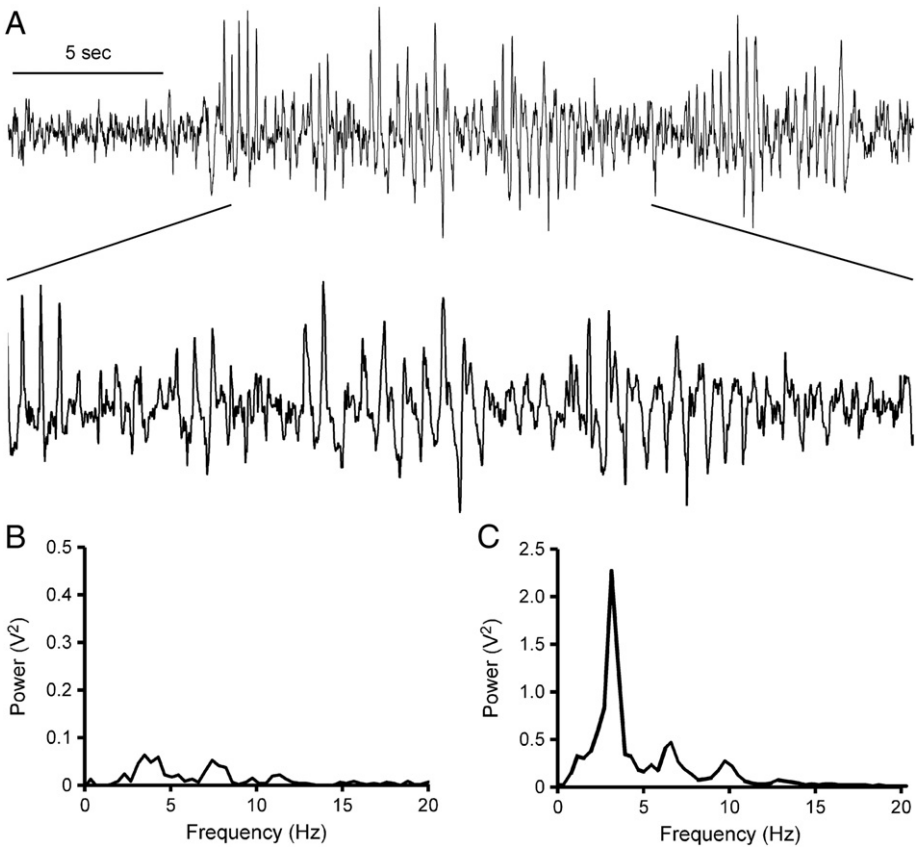

Fig. 3. Epileptiform bursting measured in the injured hippocampus. (A) Bursts associated with eating, drinking, or grooming, or shorter than 5 s were excluded from analysis. Bursts were characterized by increased amplitude (A) and power (B, C) as compared to quiescent periods.

the burst duration was significantly reduced in the caffeine-treated rats as compared to the non-caffeinated rats (Fig. 4A). In addition, the total time spent bursting was significantly reduced in the acute caffeine group (Fig. 4B). These data demonstrate *(i)* that epileptiform EEG-bursts constitute an early pathological consequence of TBI, and *(ii)* that a single acute bolus of caffeine administered immediately after the injury can reduce the duration of those bursts, suggesting that the acute injury-associated adenosine surge is mechanistically linked to the development of PTE.

A caffeine bolus after severe FPI does not worsen histological damage

As lethal apnea is associated with severe injury, we were concerned that caffeine rescue might be associated with more severe brain damage that was not evident in the behavioral tests presented here. We assessed thinning in the injured cortex with respect to the contralateral cortex, and found a significant effect of severe injury on cortical thickness ($p<0.05$), but no effect of (or interaction with) caffeine (Fig. 5A). We also assessed lateral ventricle enlargement at the same location, again comparing the area of the ventricle in the injured hemisphere to that of the contralateral hemisphere. Again, we found a significant effect of severe FPI that was independent of caffeine (Fig. 5B). Sample sections used for quantification are shown for each experimental condition (Fig. 5C–F). These results provide additional evidence that caffeine rescue after severe FPI does not result in a more severe injury phenotype.

Discussion

Here we addressed an adenosine-based mechanism for mortality and morbidity following severe TBI by testing the hypotheses that an acute trauma-induced surge in adenosine (Clark et al., 1997) influences acute and chronic outcomes after TBI, and that transient blockade of adenosine with the non-selective adenosine receptor antagonist caffeine would ameliorate acute and chronic consequences of severe TBI. We provide evidence that in severe TBI induced by a lateral fluid percussion, (i) lethal outcome correlates with apnea duration, (ii) lethal apnea can be prevented by antagonizing the effects of adenosine with a single acute dose of caffeine, (iii) caffeine rescue does not worsen neurological outcome, (iv) EEG bursts occur within 4 weeks after severe TBI, and (v) EEG-bursts can be ameliorated by a single dose of caffeine, suggesting a role of adenosine in their

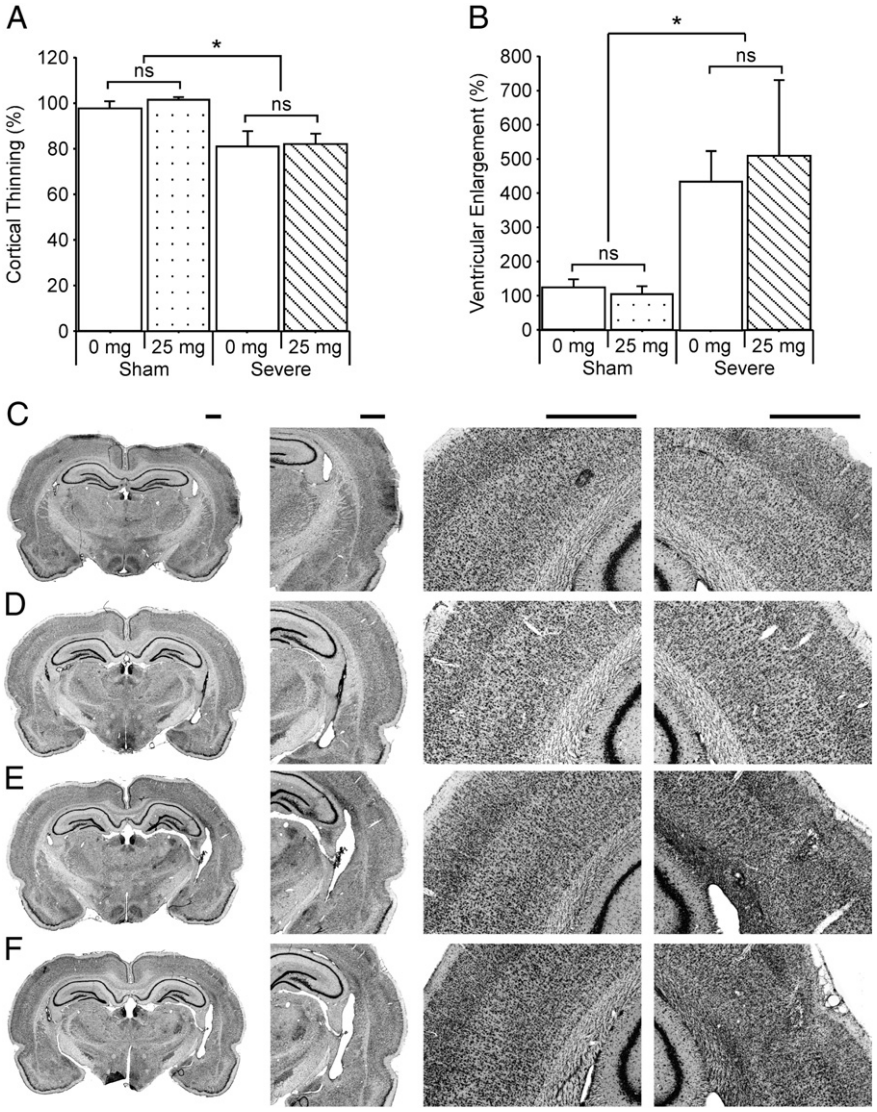

Fig. 5. Severe FPI causes (A) cortical thinning and (B) ventricular enlargement, though neither measure is influenced by caffeine treatment (*$p<0.05$). Sample Nissl stained sections from Bregma −3.3 mm: (C) Sham, (D) Sham + 25 mg/kg caffeine, (E) Severe TBI, and (F) Severe TBI + 25 mg/kg caffeine used for quantification. The first image contains the whole image used for quantification, with the uninjured hemisphere on the left, and the injured hemisphere on the right. The next column is magnified to illustrate ventricular enlargement. The third and fourth images in each row depict the uninjured and injured cortices. Scale bars at the top of each column 1 mm.

pathogenesis. These findings are of direct therapeutic significance and suggest a novel and unexpected therapeutic potential for caffeine.

The acute administration of caffeine before an injury in caffeine-naïve subjects is generally believed to promote injury and to have pro-convulsant effects (Boison, 2010); pretreatment of rats with caffeine caused significant, dose-dependent mortality after a cortical contusion injury (Al Moutaery et al., 2003). While acute adenosine receptor blockade prior to TBI can promote injury, post-injury treatment with caffeine, as demonstrated here for the first time, can completely prevent trauma-associated lethal apnea, however without aggravating behavioral outcome parameters. These findings *(i)* support our hypothesis that lethal apnea following a severe TBI is causally linked to a surge of adenosine triggered by the injury and *(ii)* demonstrate beneficial effects of post-injury caffeine on morbidity.

Injuries to the brain in general, such as TBI, stroke, or excessive seizures, are known to result in a surge of adenosine (Clark et al., 1997; During and Spencer, 1992; Gouder et al., 2004; Pignataro et al., 2008). In further support of our hypothesis, lethal apnea and post-ictal brain shutdown in sudden, unexplained death in epilepsy (SUDEP) (Hirsch, 2010) has been attributed to excessive levels of adenosine due to deficiencies in the metabolic clearance of adenosine (Shen et al., 2010). In those studies, an acute dose of 40 mg/kg caffeine was shown to extend the life-span of mice following excessive seizures, suggesting a mechanistic relationship between SUDEP and lethal post-ictal apnea. To make therapeutic use of post-injury caffeine, it is important to rule out that caffeine, while preventing mortality, might negatively impact neurological outcome. In studies of gross neurological and motor functions we show that the animals that received caffeine show a slight but non-significant improvement compared to non-caffeinated controls, suggesting that there are no overt deleterious effects of the acute caffeine treatment.

Severe TBI is associated with an increased risk of PTE (Lowenstein, 2009). Studying the mechanisms of PTE is complicated by its long latency, often months or years after the traumatic event. Early electrophysiological studies using acute hippocampal slices demonstrate increased excitability at 1 week (Santhakumar et al., 2000) and as late as 15 weeks (Golarai et al., 2001) after brain injury. More recently, the progression to spontaneous seizures has been demonstrated in vivo after FPI (D'Ambrosio et al., 2004), with 92% of rats demonstrating electrographic seizure activity at 8 weeks, progressing to generalized seizures in 50% of the survivors by 1 year after injury (Kharatishvili et al., 2006). The incidence, frequency, and latency of these seizures demonstrate the face validity of the FPI model for the study of PTE, yet the logistics of the model make it difficult to efficiently propose and test hypotheses. We examined hippocampal electrographic activity at 4 weeks following FPI, and have found epileptiform bursts in the severe injury group, consistent with other studies (D'Ambrosio et al., 2009). Remarkably, a single acute dose of caffeine given immediately after the FPI was linked to a significant reduction in burst duration 4 weeks after the injury. This finding indicates potential disease-modifying consequences of early intervention with adenosine signaling at an early time point following FPI. More work is certainly needed to investigate the mechanistic relationship between an early surge of adenosine (blocked here at least partly with caffeine) and subsequent epileptogenesis. While it is not clear from our studies whether these bursts would develop into spontaneous generalized seizures, they provide evidence of early epileptiform activity and constitute a rational early target for evaluating interventions to modify the long-term outcome after TBI.

Caffeine, a non-selective antagonist of adenosine receptors at doses normally reached during human caffeine consumption, is the most widely used psychoactive substance, with a well-understood pharmacodynamic and pharmacokinetic profile (Fredholm et al., 1999). By antagonizing the function of adenosine, which acts as an endogenous anticonvulsant and neuroprotectant of the brain (Dragunow, 1986; Dragunow and Faull, 1988; Dunwiddie, 1980; Ribeiro, 2005; Ribeiro et al., 2003), the acute use of caffeine is generally thought to aggravate neuronal injury and to promote epileptic seizures (Boison, 2010). Using a model of closed head injury in female rats, the high mortality associated with pre-injury caffeine was delayed beyond the acute period evaluated in our study (Al Moutaery et al., 2003). It is important to point out that in the present study we specifically assessed the immediate acute apnea-related mortality that has not been assessed in previous studies. This distinction might be model-dependent, since not all models of TBI recreate the immediate apnea-related mortality studied here.

In contrast, the chronic use of caffeine is generally thought to be neuroprotective, at least in part by effect inversion and adenosine receptor desensitization, at least under certain dosages (Fredholm, 1997; Jacobson et al., 1996). In line with the neuroprotective effects of chronic caffeine, 3 weeks of caffeine administration to mice provided profound neuroprotection following a cortical contusion injury, whereas an acute dose of caffeine in the same model was without effect (Li et al., 2008). The detrimental effects of acute caffeine are likely based on the blockade of A_1Rs. This notion is supported by findings that TBI in A_1R knockout mice led to lethal status epilepticus (SE) (Kochanek et al., 2006). Likewise, A_1R knockout mice subjected to an excitotoxin succumbed to lethal SE (Fedele et al., 2006). In human populations the use of an A_1R antagonist for the treatment of acute heart failure with renal impairment was associated with seizures as one of the observed side effects (Cotter et al., 2008). Our current findings demonstrate for the first time that a single acute dose of caffeine, when given immediately *after* the injury prevents lethal outcome and are in apparent contrast to previous data discussed above.

The time-point of acute caffeine administration may contribute to the observed differences in pre- and post-injury caffeine administration. In vitro, stretch injury of neurons limits the effect of caffeine on calcium-induced calcium release (Weber et al., 2002), suggesting that pre-injury caffeine in a caffeine-naïve neuron is additive with injury, but post-injury caffeine is not. In vivo studies examining the effect of a single bolus of caffeine on outcome after TBI considered a single time point, 30 min, prior to injury, sufficient for the caffeine to be well distributed throughout the brain, yet the relatively short half-life of caffeine (0.8 h) suggests that some clearance has occurred prior to injury (Bonati et al., 1984). In addition, caffeine when given prior to the injury is likely to affect the entire brain, whereas caffeine perfusion into the injured brain might be compromised in the most severely affected brain areas. This could be a likely explanation why the acute dose of caffeine after the injury did not worsen morbidity. However, full penetration of caffeine into brainstem is likely, since this region is not directly affected by the lateral fluid percussion injury. In our experiments, we found that post-injury caffeine treatment rapidly restored spontaneous breathing; the reduced hypoxia as a result of limiting apnea may surpass any consequent negative effects of continued A_1R blockade. The relatively low affinity and rapid clearance of caffeine also may serve to limit the detrimental effects of A_1R blockade demonstrated in A_1R knockout mice (Kochanek et al., 2006). Based solely on its actions as an adenosine receptor antagonist, we would predict that caffeine delivered to caffeine-naïve subjects immediately prior to TBI might act similarly to post-injury administration to prevent lethal apnea. Of more significant clinical importance, the influence of chronic caffeine consumption on A_1R and A_{2A}R expression (Svenningsson et al., 1999) indicates that we must consider the protective effects of a single bolus of caffeine in both caffeine naïve and chronically caffeinated subjects.

The fact that caffeine is rapidly absorbed in the gut, readily crosses the blood–brain barrier, and is a relatively weak, non-selective antagonist may partly account for its post-injury efficacy without negative consequences. Adenosine has evolved to maintain homeostasis across organ systems and within the brain across neurotransmitters (Boison, 2008a, 2008b; Boison et al., 2011; Fredholm, 2007); to maintain stability, there are likely many as yet unrecognized compensatory

mechanisms activated in response to typical environmental challenges. The most successful therapeutics may be those that blunt the acute effects of a traumatic event, and then allow endogenous compensatory mechanisms to fully function. Caffeine is a widely consumed psychoactive substance (Barone and Roberts, 1996), and chronic consumption is known to affect adenosine receptor expression (Svenningsson et al., 1999). Sleep disruption, another common fact of modern life and likely contributor to risk of TBI, may further alter adenosine receptor expression (Basheer et al., 2004; Elmenhorst et al., 2009). While chronic caffeine consumption is associated with favorable outcome after TBI (Sachse et al., 2008), further studies to examine the combined effects of chronic pre-injury caffeine treatment with a protective post-injury caffeine treatment are essential to support the translation of the current findings to broader therapeutic use.

Our results are the first to demonstrate that caffeine limits apnea duration and prevents mortality when administered rapidly following severe TBI, without negative consequences. As apnea is a major cause of pre-hospital mortality, this finding presents a major therapeutic opportunity for first responders in both civilian and military environments. We also demonstrate that a single post-injury dose of caffeine can have long-lasting effects on electrographic burst durations, indicating that caffeine might beneficially influence processes involved in posttraumatic epileptogenesis, an interesting observation that warrants further experimentation. In conclusion, our studies show that, at a safe dose and without adverse neurological outcome, caffeine has the potential to prevent lethal apnea following TBI, and may reduce brain excitability long-term. Since caffeine is a well characterized and widely consumed drug, our findings present a translatable strategy to reduce acute lethal outcome after severe TBI.

Acknowledgments

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NEUROSCIENCE 2012

Presentation Abstract

Program#/Poster#: 555.28/P20

Presentation Title: Acute mortality following severe traumatic brain injury is rescued by caffeine

Location: Hall F-J

Presentation time: Tuesday, Oct 16, 2012, 11:00 AM -12:00 PM

Authors: **N. LYTLE**, T. LUSARDI, *D. BOISON;
Legacy Res. Inst., PORTLAND, OR

Abstract: Every year, approximately 1.4 million Americans suffer from a traumatic brain injury (TBI). However, few pre-hospital treatment strategies are available. Acute mortality from severe TBI may be mediated via suppression of respiratory centers in the brainstem caused by an injury-induced surge of adenosine. Caffeine is a fast-acting adenosine receptor antagonist that is FDA approved and commonly used in many forms. In addition to caffeinated teas, sodas, and pills, more than 50% of Americans consume coffee daily. Since chronic caffeine consumption modulates adenosine receptor expression, we evaluated the effects of caffeine on TBI outcome. We used a chronic caffeine exposure model in rats equivalent to the human consumption of 2-3 cups of coffee per day (300mg/day). Severe TBI, modeled by lateral fluid percussion injury, was defined as having a mortality rate of 36-50% in caffeine naïve animals (n=12-18). Prior to injury, adult male Sprague Dawley rats received either standard drinking water or drinking water with 0.3g/l caffeine for 3 weeks. Additionally, rats received a bolus of caffeine or saline (i.p.) pre- or post-TBI and were supplied with caffeine-free drinking water following injury. We demonstrate that mortality was reduced in all experimental groups that received a caffeine bolus compared to groups that received saline. However, mortality reduction was dependent on the pre-injury caffeination state, the time of caffeine bolus delivery, and the bolus dose. A 25mg/kg caffeine bolus delivered 1 hour before severe TBI reduced mortality from 36% to 20% in caffeine-naïve rats and from 35% to 15% in chronically caffeinated rats. A bolus of 12mg/kg, 25mg/kg, or 50mg/kg caffeine delivered 10 seconds after injury reduced mortality in chronically caffeinated rats from 42% to 17%, 6%, and 33%, respectively. Finally, a 25mg/kg caffeine bolus delivered 90 seconds after injury reduced mortality from 42% to 27%. Next we assessed gross motor deficits in surviving animals by performing angleboard and neuroscore 24 hours post-TBI, and rotarod 24 hours and 7 days post-TBI. In all groups that received a caffeine bolus, injury-

induced motor deficits were equivalent to groups that received a saline bolus. Overall, our results demonstrate that a caffeine bolus delivered immediately after TBI can prevent acute mortality without exacerbating motor deficits.

Disclosures: **N. Lytle:** None. **T. Lusardi:** None. **D. Boison:** None.

Keyword(s): TRAUMATIC BRAIN INJURY

CAFFEINE

Support: USAMRMC Grant 09051005

[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

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NEUROSCIENCE 2012

Presentation Abstract

Program#/Poster#: 555.26/P18

Presentation Title: Effects of caffeine withdrawal after traumatic brain injury

Location: Hall F-J

Presentation time: Tuesday, Oct 16, 2012, 9:00 AM -10:00 AM

Authors: ***T. A. LUSARDI**, N. K. LYTLE, D. BOISON;
RS Dow Neurobio. Labs, Legacy Res. Inst., PORTLAND, OR

Abstract: Caffeine is widely consumed, with ~50% of Americans drinking coffee daily, suggesting that prior caffeine consumption is likely at the time of a traumatic brain injury (TBI). Caffeine consumption has complex neurologic effects. Caffeine acts rapidly as a non-specific adenosine receptor antagonist, and chronic caffeine consumption modulates adenosine receptor expression. Adenosine receptors at synapses are generally inhibitory, while those at astrocytic endfeet trigger vasodilation. Thus, adenosine receptors maintain local energetic homeostasis in the brain, which may be altered by caffeine consumption. While negative consequences of caffeine withdrawal such as headache and fatigue are well known, it is not yet clear how caffeine withdrawal influences outcome after TBI. We used the lateral fluid percussion injury (FPI) model to examine the role of caffeine withdrawal after TBI. A subset of rats was preconditioned with caffeine (0.3 g/l) for 3 weeks prior to FPI. After FPI, rats were randomly assigned to receive either chronic caffeine or plain drinking water. We assessed motor function using the rotarod and found that chronic caffeine consumption after FPI impairs recovery of rotarod performance between 24 hours and 7 days after injury, regardless of pre-injury caffeine consumption. We examined pain threshold using the tail flick assay at 7 days after FPI. In caffeine naïve rats, FPI shortened the latency to tail flick, suggesting a lower pain tolerance after injury. Chronic caffeine consumption after FPI had little effect on tail flick latency, despite impaired rotarod activity. Surprisingly, in sham operated rats, we found a significant decrease in the latency to tail flick in the caffeine withdrawal group. After FPI, however, caffeine mediated differences in tail flick latency disappeared with increasing injury severity. These results suggest that caffeine consumption after TBI has negative effects on motor performance, but caffeine withdrawal may reduce pain tolerance.
This work was supported by TATRC (W81XWH-10-1-0757).

Disclosures: **T.A. Lusardi:** None. **N.K. Lytle:** None. **D. Boison:** None.

Keyword(s): CAFFEINE

TRAUMATIC BRAIN INJURY

PAIN

Support: TATRC (W81XWH-10-1-0757)

[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

SUPPORTING DATA

Methods

Animals Procedures were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care according to protocols approved by the Legacy Institutional Animal Care and Use Committee, the USAMRMC Animal Care & Use Review Office, and guidelines from the National Institute of Health.

Caffeine Rats exposed to chronic caffeination (Sigma-Aldrich, St. Louis, MO) prior to FPI received a dose of 0.3g/l of drinking water changed every other day for 3 weeks. Chronically caffeinated rats with an acute withdrawal period were provided standard water for 36 hours prior to FPI. A subset of rats received a caffeine bolus of 0, 12.5, 25, or 50 mg/kg in sterile saline, i.p. at time points indicated in the results.

Lateral Fluid Percussion Injury (FPI) Male Sprague–Dawley rats (Charles River, Wilmington, MA) were anesthetized with isoflurane (2% isoflurane at 2ml/min in 2:1 N_2O:O_2), then affixed into a stereotactic frame. A 5mm trephine hole was drilled centered at bregma – 4.5mm anterior-posterior and + 2.8mm medial-lateral (Lusardi, et al., 2012). TBI was produced by a fluid-percussion device (Custom Design and Fabrication, Richmond, VA). A 21–23 ms fluid pulse was applied to the exposed dura, measured by an external pressure transducer, digitized by a PowerLab A/D converter (ADInstruments, Colorado Springs, CO), then recorded using Scope (ADInstruments). For each of the three studies, FPI severity was calibrated using caffeine naïve rats to determine the peak pressure resulting in 40-50% mortality, considered "severe" injury (Tables 1, 2, and 3). Mild and moderate injuries were scaled accordingly. Following FPI, rats were placed in dorsal recumbency for continuous observation of respiratory activity. The duration of apnea was measured from the time from FPI until the first acute inspiratory effort, or "gasp." Monitoring continued until regular spontaneous ventilation was apparent, or until the complete cessation of inspiratory effort for at least 5 minutes. Sham-injured controls were generated using identical manipulations without impact.

Motor Function Assessment Rats were assessed for motor performance once per week for 3 weeks prior to FPI and following FPI at 24 hours and 7 days by an individual blinded to the experimental condition. Neuroscores were determined for their left and right side on contraflexion (forelimb reaching), hindlimb flexion, and lateral pulsion. Scores range from 0-4, indicating no response to normal performance respectively, for a total of 24 possible points (McIntosh, et al., 1989). Sensorimotor function was assessed using an accelerating rotarod. Rats were placed on the rotarod rotating at a constant rate of 2 rpm. Acceleration began at time 0 and reached a top speed of 30 rpm at 96 seconds. The time for each trial was recorded at the point that the rat fell off the rod or rotated with the rod for 2 revolutions. The rotarod test consisted of 3 trials, each a maximum of 120 seconds and a rest period of 2 minutes between trials.

Statistics All statistics were performed using StatView (SAS Institute, Cary, NC) or Prism 5 (GraphPad, San Diego, CA). The distributions of weight, injury pressure, and apnea for each group were compared by ANOVA (StatView), and were not different among groups (Tables 1-3). Correlation between caffeine treatment and mortality was assessed using logistic regression test (StatView). The influence of treatment on apnea duration and righting time was assessed using a non-parametric Kruskal-Wallace test (Prism); rats with total apnea were assigned an apnea duration of 300 seconds, and those that did not recover from anesthesia were assigned a righting time slightly longer than the longest value measured in that group. All behavior results (Neuroscore and Rotarod) were assessed only for those rats that survived the procedure, and were thus evaluated using ANOVA. Results are presented as mean ± SEM.

Figures

Following pages.

Figure 1: Pre-injury Caffeine in Caffeine Preconditioned Rats

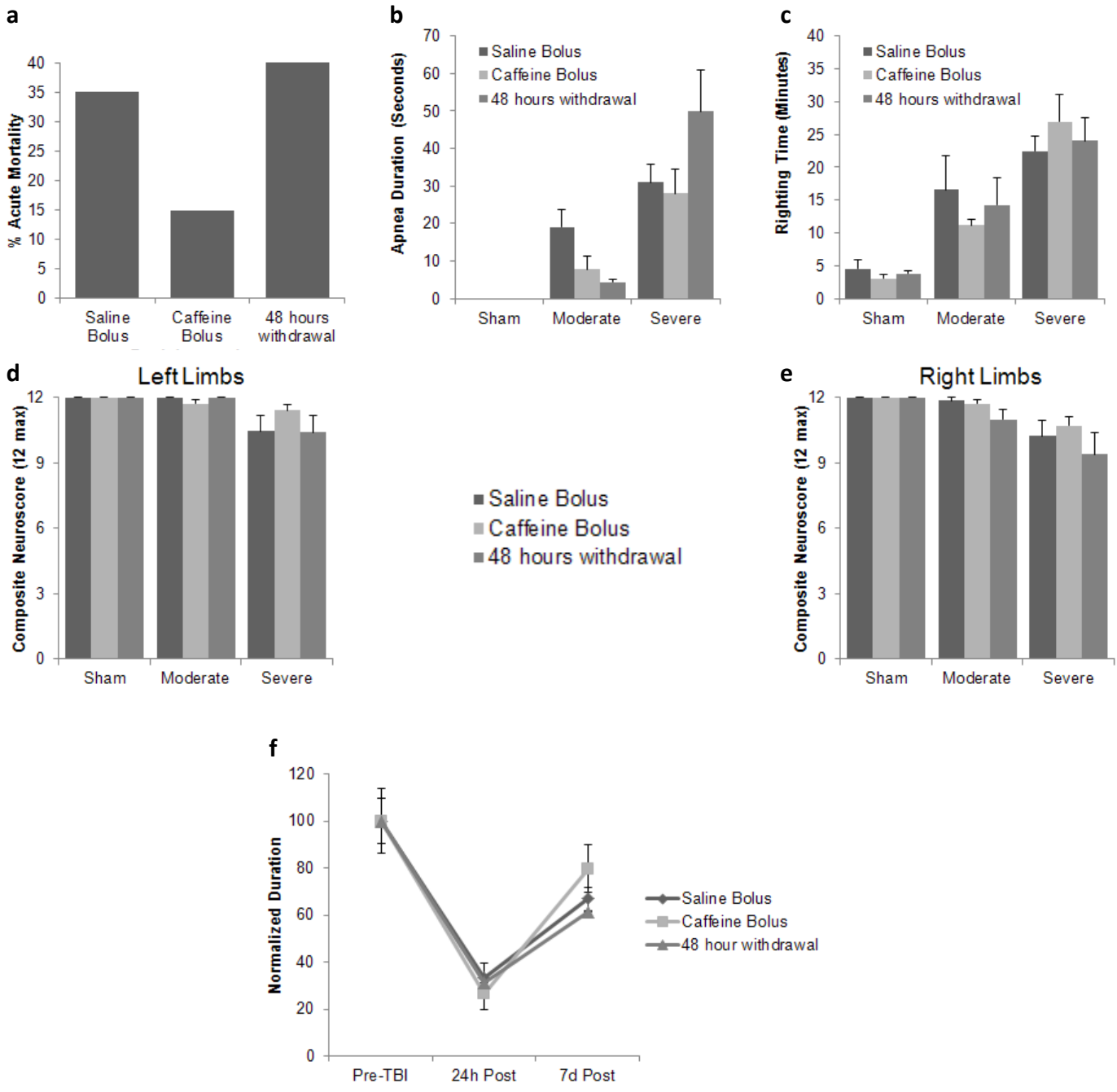

Figure 1: We examined the influence of a single bolus of caffeine (25 mg/kg, *IP*) before FPI in caffeine preconditioned rats. **(a)** A bolus of caffeine prior to severe FPI was associated with lower mortality rate. **(b)** Caffeine regimen prior to FPI did not have a significant influence on apnea duration. **(c)** Righting time was not significantly influenced by pre-injury caffeine regimen. Composite neuroscore was lower in the right limbs **(e)** than the left **(d)**, but was not influenced by the pre-injury regimen. **(f)** Rotarod performance at 1 and 7 days after severe injury was not influenced by pre-injury caffeine.

Figure 2: Pre-injury Caffeine in Caffeine Naïve Rats

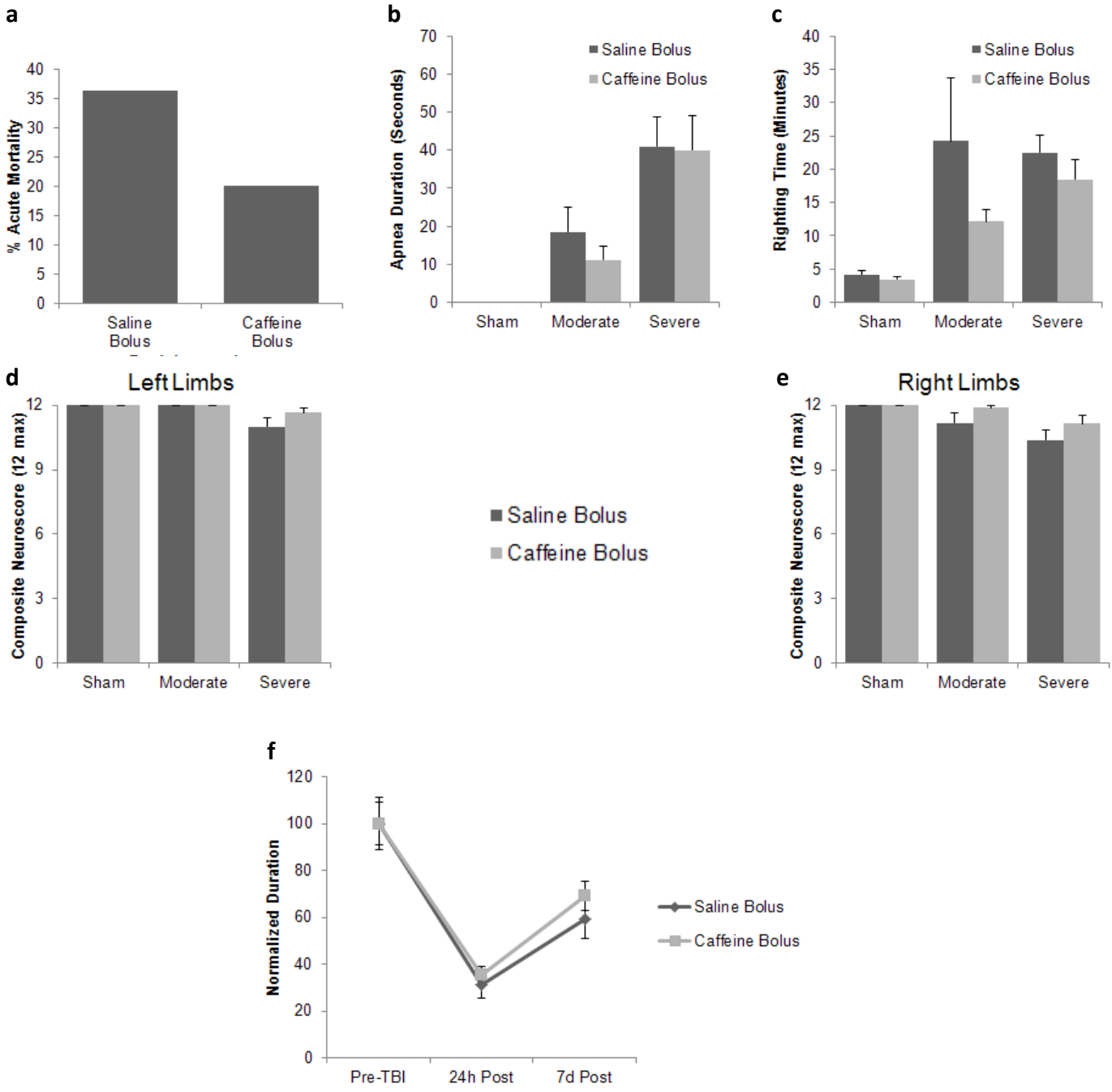

Figure 2: We examined the influence of a single bolus of caffeine (25 mg/kg, *IP*) before FPI in caffeine naïve rats. **(a)** A bolus of caffeine prior to severe FPI was associated with lower mortality rate. **(b)** A caffeine bolus prior to FPI did not have a significant influence on apnea duration. **(c)** Righting time was not significantly influenced by a pre-injury caffeine bolus. Composite neuroscore was not influenced by the pre-injury regimen in the left **(d)** or right **(e)** limbs. **(f)** Rotarod performance at 1 and 7 days after severe injury was not influenced by pre-injury caffeine bolus.

Figure 3: A pre-injury caffeine bolus reduces mortality

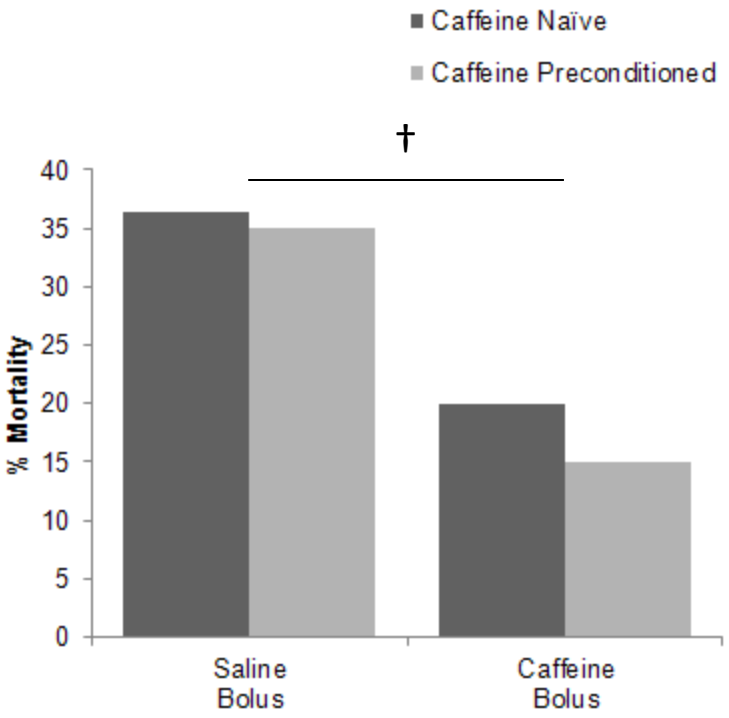

Figure 3: Using an alternate analysis, we re-evaluated the interaction between chronic caffeine pre-injury and an acute bolus of caffeine pre-injury. In this analysis, chronic caffeine consumption prior to injury has no influence on acute mortality. However, an acute bolus of caffeine administered 1 hour prior to injury did result in a reduced mortality († $p < 0.06$).

Figure 4: Chronic caffeine before FPI has a minimal effect on acute measures

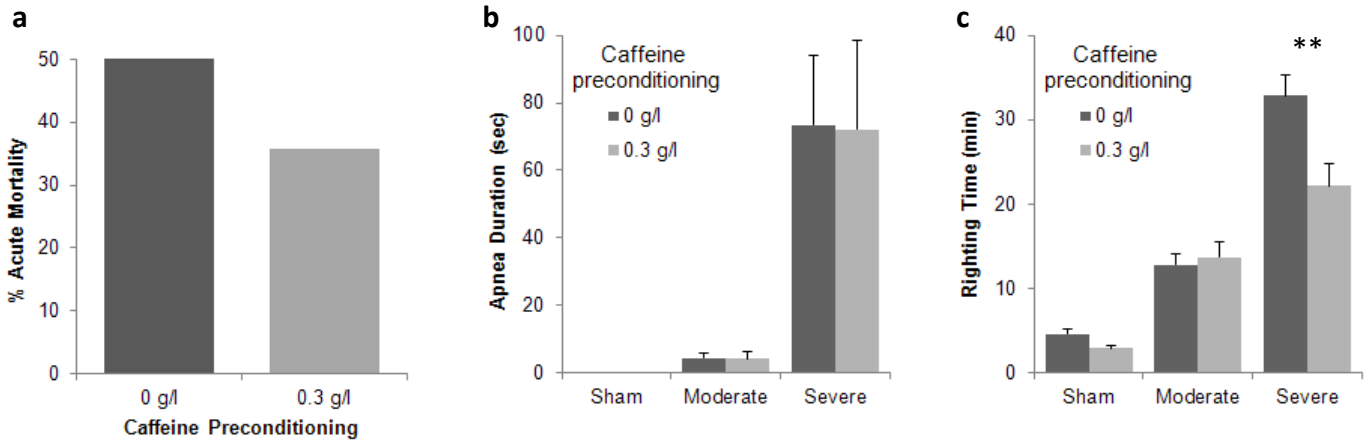

Figure 4: We examined the effect of chronic caffeine (0.3 g/l , *ad lib*) for 3 weeks before FPI. **(a)** Chronic caffeine consumption prior to severe FPI was not associated with a statistically significant lower mortality rate. **(b)** Chronic caffeine consumption prior to FPI did not have any influence on apnea duration. **(c)** Righting time was significantly influenced by chronic caffeine consumption after severe FPI (** $p < 0.01$).

Figure 5: Chronic caffeine after Severe FPI has detrimental effects

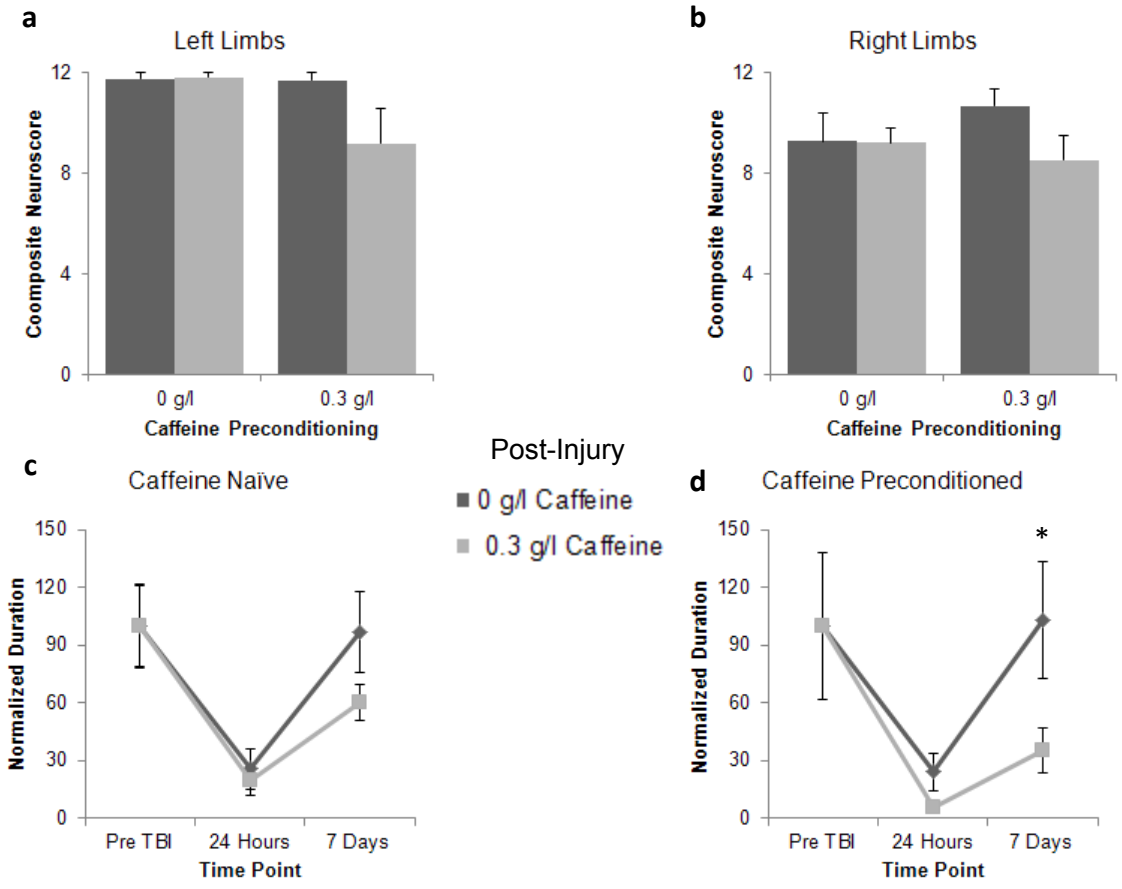

Figure 5: We examined the influence of chronic caffeine consumption (0.3 g/l , *ad lib*) for 1 week after severe FPI. Composite neuroscore at 1 day after severe FPI demonstrated worse deficits in the right limbs **(b)** compared to the left limbs **(a)**. Rotarod tests in caffeine naïve **(c) and caffeine preconditioned (d) rats demonstrate that in both cases, recovery of rotarod function at 7 days after injury was lower in the rats that received chronic caffeine after injury.** (* $p < 0.05$).

Figure 6: Caffeine rescue after severe FPI is dose dependent

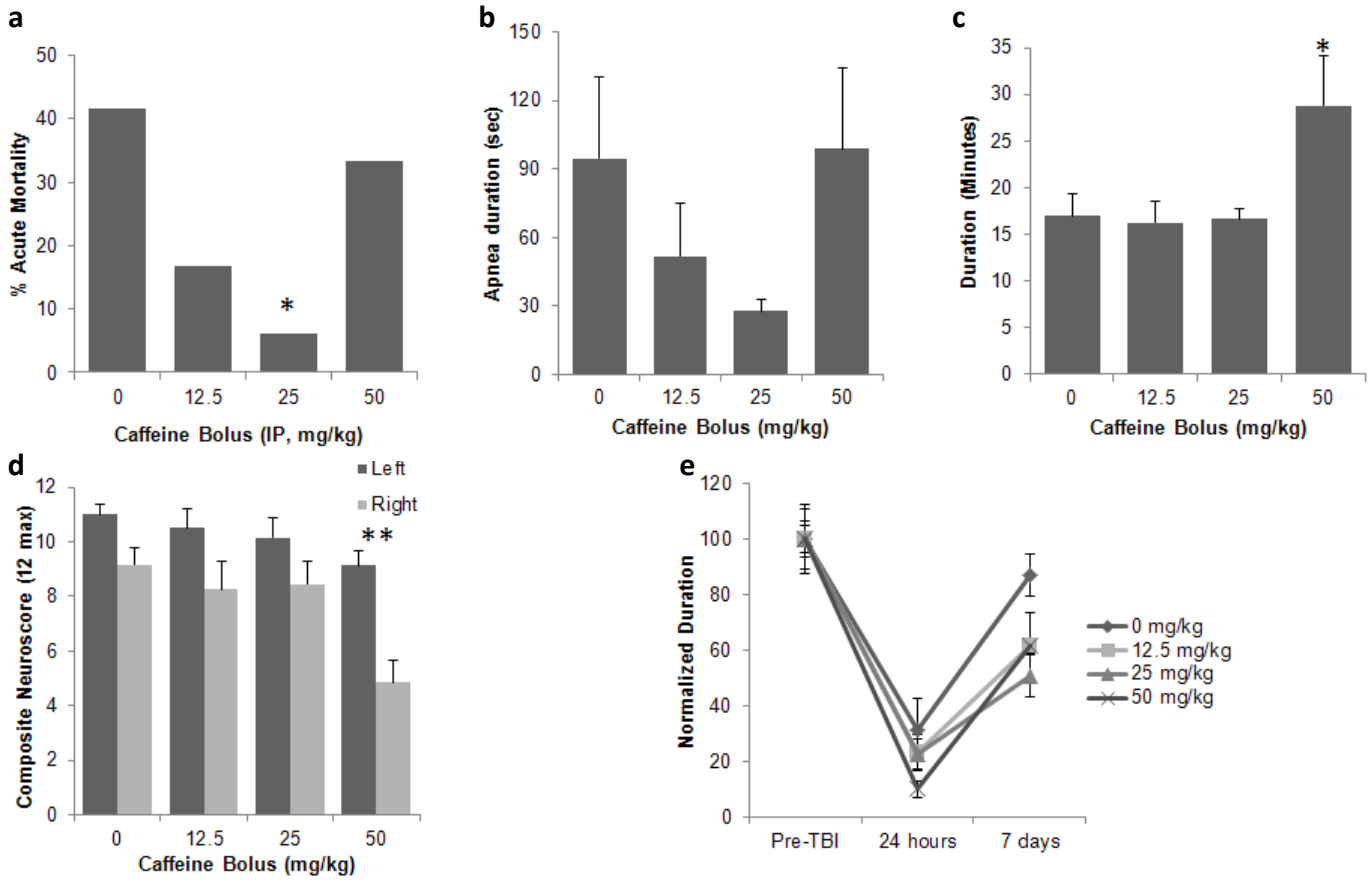

Figure 6: (a) Caffeine rescue after severe FPI is dose dependent (* $p < 0.05$). **(b)** Apnea duration followed similar trends as mortality, but was not significantly affected by the caffeine dose. **(c)** In surviving rats, righting time was significantly longer after a 50 mg/kg dose of caffeine (*$p < 0.05$). **(d)** Changes in composite neuroscore was assessed by ANOVA (caffeine dose x hemisphere), showing a significant effect of the caffeine dose (**$p < 0.01$, post hoc significant at 50 mg/kg), with worse deficits in the right limbs ($p < 0.001$). **(e)** Rotarod performance was assessed 1 day and 7 days after FPI. Repeated measures ANOVA demonstrated a significant effect of time after injury ($p < 0.0001$), but no primary effect of dose; post-hoc tests do suggest lower performance after 25 mg/kg at 7 days ($p < 0.05$).

Figure 7: Caffeine rescue after severe FPI is time sensitive

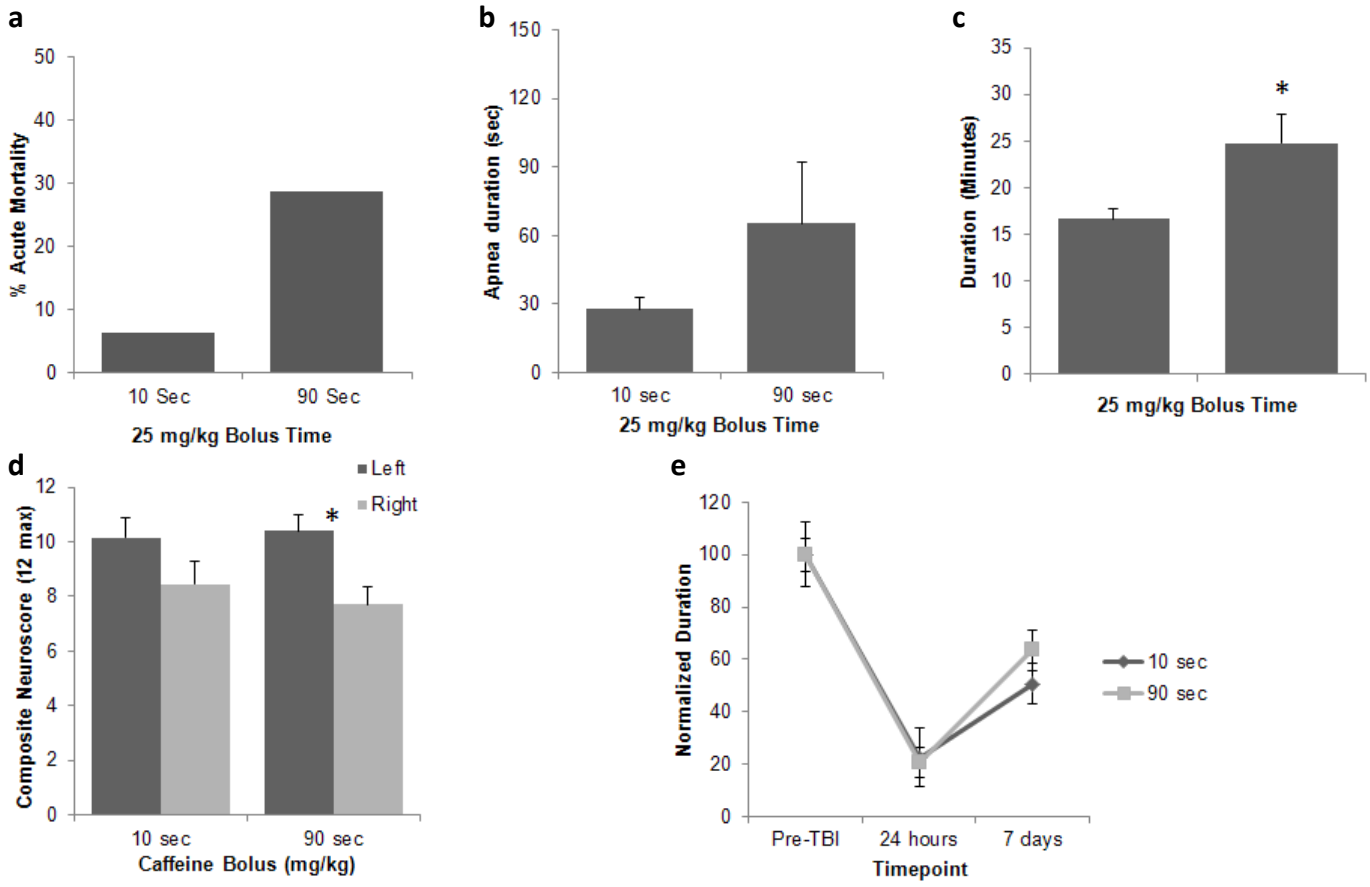

Figure 7: We examined the effect of the time of caffeine administration (25 mg/kg, IP) after severe injury. **(a)** Caffeine rescue was not significantly lower after delayed administration. Apnea duration **(b)** was not significantly longer with delayed caffeine administration, though righting time **(c)** was significantly longer with delayed administration (*$p<0.05$). **(d)** Changes in composite neuroscore was assessed by ANOVA (time of administration x left/right), showing no significant effect of the caffeine dose, but worse deficits in the right limbs (*$p < 0.05$). **(e)** Rotarod performance was assessed 1 day and 7 days after FPI. Repeated measures ANOVA demonstrated a significant effect of time after injury ($p < 0.01$), but no primary effect of administration time.

Figure 8: An acute bolus of caffeine is not detrimental after mild or moderate injury

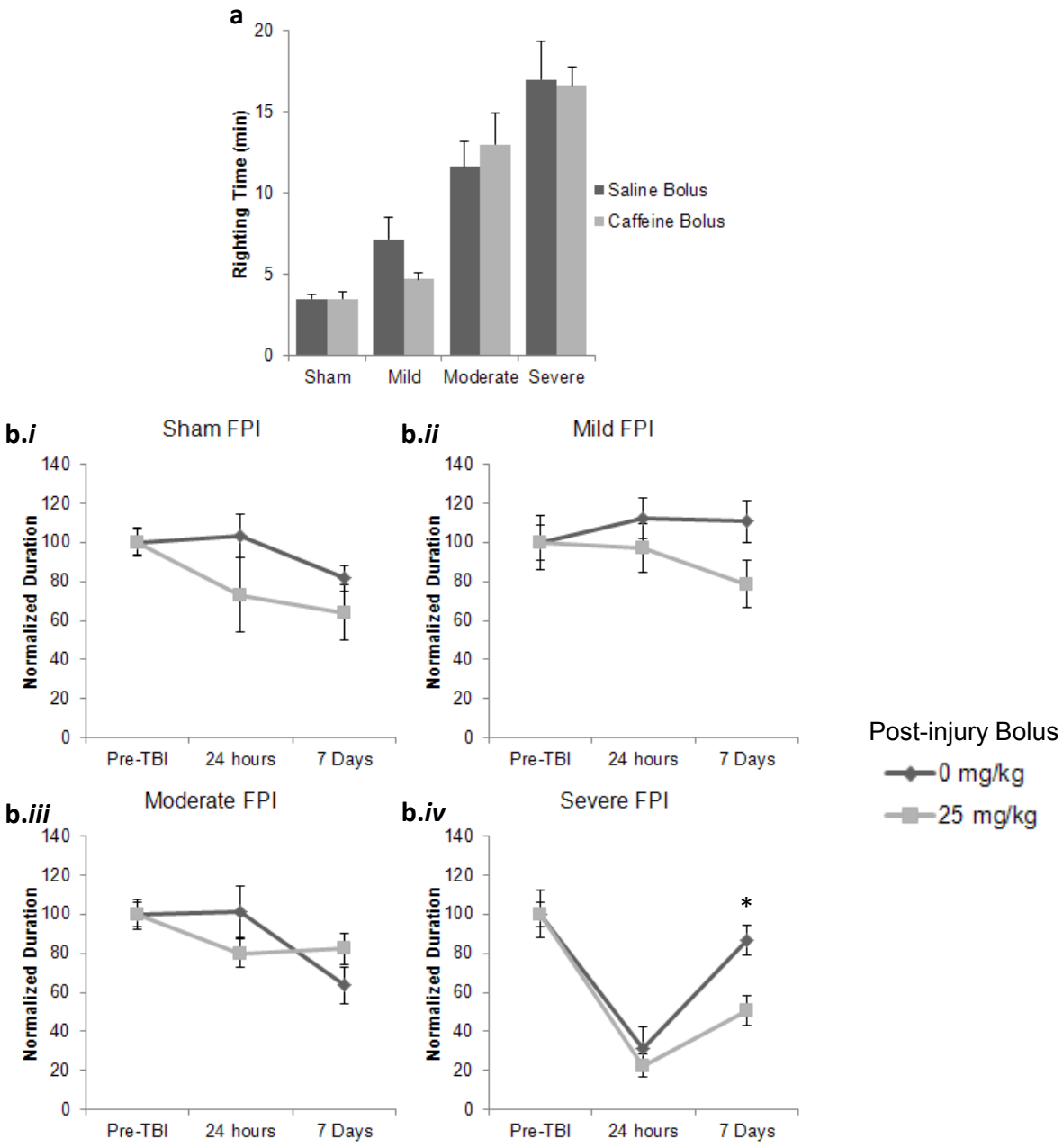

Figure 8: We examined the influence of a single bolus of caffeine (25 mg/kg, IP) on recovery after sham, mild, and moderate injury. **(a)** The influence of a single acute caffeine bolus after FPI was evaluated by two-way ANOVA (Injury x Caffeine). There was a significant main effect of injury severity ($p < 0.0001$), but no effect of caffeine dose on righting time. **(b)** Rotarod performance was assessed at 1 and 7 days after FPI. After sham **(b.*i*)** or mild **(b.*ii*)** injury, there was no significant effect of evaluation time or caffeine administration. After moderate injury **(b.*iii*)**, there was a significant effect of time after injury ($p < 0.01$), but no significant effect of caffeine. After severe injury **(b.*iv*)**, evaluation time point was significant ($p < 0.0001$), and caffeine dose was nearly significant ($p = 0.051$). Post hoc tests suggest that recovery at 7 days was impaired after caffeine treatment (* $p < 0.05$).

Figure 9: A single caffeine bolus after severe injury does not worsen brain atrophy

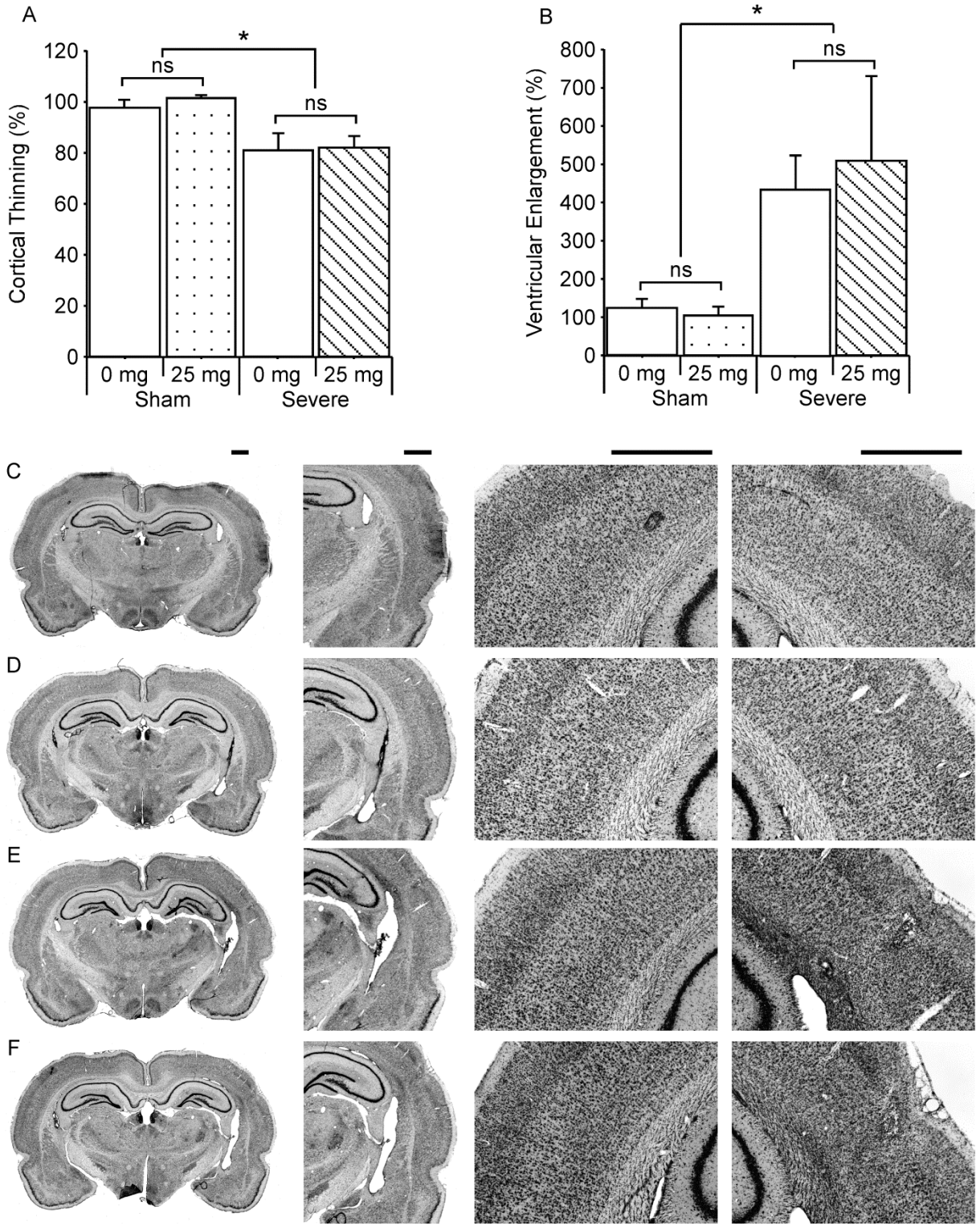

Figure 8: Severe FPI causes cortical thinning (A) and ventricular enlargement (B), though neither measure is influenced by caffeine treatment (* $p<0.05$). Sample Nissl stained sections Sham (C), Sham + 25 mg/kg caffeine (D), Severe TBI (E), and Severe TBI + 25 mg/kg caffeine (D) used for quantification.