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Mannitol and the Combination of Mannitol and Gelatin Impair Whole Blood Coagulation and the Platelet Function *In Vitro*

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Abstract

Objective: Mannitol 20% and succinylated gelatin 4% are routinely used in neurosurgical procedures. The aim of this *in vitro* study was to explore the influence of both agents on blood coagulation and platelet function.

Methods: Blood from 21 healthy volunteers was obtained and then diluted so as to form five groups: (1) 7% dilution with mannitol; (2) 10% dilution with gelatin; (3) 17% dilution with isotonic balanced electrolyte solution; (4) 17% dilution with mannitol+gelatin; and (5) undiluted blood. The extrinsic thrombelastometry (EXTEM) and fibrin thrombelastometry (FIBTEM) tests were examined by rotational thrombelastometry via ROTEM®, and thrombocyte aggregometry with the aspirin inhibiting- (ASPI), adenosine diphosphate- (ADP), and thrombin-activating protein (TRAP) tests performed by Multiplate.

Results: In the EXTEM test clot formation time, the alpha angle, and maximum clot firmness were significantly reduced by mannitol and the combination of mannitol with gelatin. The platelet function tested in the ADP test was also significantly reduced with this combination.

Conclusion: In this *in vitro* study, clinically relevant dilutions of mannitol and gelatin showed a significant inhibition of whole blood coagulation and the platelet function, which could be detrimental in neurosurgical settings.

Keywords: Coagulation, gelatin, mannitol, platelet aggregometry, platelet function, thrombelastometry

Introduction

Osmotherapy is a standard treatment in patients undergoing elective cranial neurosurgery and with traumatic brain injury. However, there is still an ongoing debate on the best hyperosmolar infusion (1-3). The most popular infusions are probably mannitol or hypertonic saline (HS). Both infusions are able to provide a significant brain relaxation and a decrease in the intracranial pressure. The proposed mode of action of both infusions is the initiated fluid gradient out of the brain cells into the intravascular compartment with a consecutive reduction in intracranial hypertension and therefore an increase in cerebral perfusion. In addition to negative effects of mannitol on renal function, it may cause a secondary hypovolaemia due to the osmotic diuretic action (4). The recommended dose of mannitol is 0.25-1.0 g kg^{-1} body weight (5, 6). Previous *in vitro* studies showed dose-dependent negative effects of mannitol and HS on blood coagulation and clot formation (7-9). Lindroos et al. (8) investigated the *in vitro* effects of the combination of mannitol and hydroxyethyl starch (HES) 6% (130/0.4) on blood coagulation. They showed that the fibrinogen/fibrin axis of clot formation was significantly more inhibited by mannitol and HES 6% as compared to the combination of mannitol and Ringer acetate. However, the dilutions of the blood samples in their study were not all comparable to the clinical setting. For example, a 20% dilution with mannitol 15% as performed in the study would result in a dose of mannitol of 2.1 g kg^{-1} body weight, thus by far exceeding the recommended dose of 0.25-1.0 g kg^{-1}.

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Colloids are widely used in clinical practice and play a crucial role in the treatment of patients with hypovolaemic shock and intraoperative bleeding. Since the European Medical Agency is going to suspend the market authorization of HES infusions, alternative substances like albumin or gelatin are currently being used. Because of the higher costs of albumin in Germany, gelatin infusions are the preferred alternative in our university hospital. For all artificial colloids (dextran, HES, gelatin) as well as for albumin however, dose-dependent alterations of blood coagulation have been shown (10).

In the clinical setting in neurosurgical operating theaters, it is to expect that a combination of a colloid infusion and mannitol could be administered. This combination could potentially aggravate the individual negative effects on coagulation and lead to further harm in our patients due to extensive bleeding.

To the best of our knowledge, it is still unknown whether usual amounts of administered mannitol (1 g kg^{-1}) in combination with gelatin (5-10 mL kg^{-1}) lead to an impairment of whole blood coagulation and the platelet function. We therefore conducted this *in vitro* study to investigate the effects of clinically relevant dilutions of whole blood with mannitol and gelatin infusions on whole blood coagulation and the platelet function.

Methods

After approval by the local ethics committee (Medical School Hannover 7051-2015), 22 healthy volunteers were enrolled into this prospective laboratory investigation. Written informed consent was obtained for blood sampling from all volunteers. All the participants were healthy, non-smoking, had a body mass index 20-25 and were 18-35 years old. None of them was allowed to take any medication, and female participants were only included 5 days before or after their menstrual period.

Approximately 40 mL of venous blood was drawn from the cubital vein. The tourniquet phase was kept as short as possible. The blood samples were stored in four citrated tubes (Sarstedt AG&Co., S-Monovette, 5 mL 9NC, Nuembrecht, Germany), and six tubes were filled with hirudin (Sarstedt AG&Co., S-Monovette, 2.7 mL r-Hirudin, Nuembrecht, Germany). The hirudin and the citrated blood samples were then diluted with mannitol 20% (MAN; Serumwerk Bernburg, Mannitol Infusionslösung 20% 250 mL, Bernburg/Saale, Germany), succinylated gelatin 4% (GEL; BBraun AG, Gelafundin 4% 500 mL, Melsungen, Germany), and isotonic balanced electrolyte infusion (ISO, BBraun AG, Sterofundin ISO, Melsungen, Germany) according to the dilution plan (Table 1). Whole blood coagulation analyses were performed with two thrombelastometry devices with a total amount of eight channels (ROTEM, Tem International GmbH, Munich, Germany). The ROTEM technique is commonly used and has been previously well described in detail (9, 11-13). The used tests were the EXTEM and the FIBTEM measurements, and they were carried out according to the manufacturer's manual. The EXTEM test measures the extrinsic clotting pathway induced by tissue factor. The determined parameters are the clotting time (EX-CT, start of the measurement until the onset of clotting), the clot formation time (EX-CFT, time from the onset of clotting until the clot firmness is 20 mm), the alpha angle in degree (EX-ALPHA, angle between the central line of the plot and the tangent of the curve at 2 mm of clot firmness), and the maximum clot firmness (EX-MCF, maximum amplitude [strength] of the clot). The FIBTEM test is similar to the EXTEM test, but the platelet effect on clotting is completely inhibited by cytochalasin D and therefore measures the fibrinogen/fibrin contribution to clotting. The only determined parameter in this test was the maximum clot firmness (FIB-MCF, maximum amplitude [strength] of the clot by fibrin).

The platelet function was analyzed by two 5-channel aggregometers (Multiplate, Roche Diagnostics, Grenzach-Wyhlen, Germany). This technology has been also described in detail before (14). The measurements were performed according to the manufacturer's manual. The prepared blood samples according to the dilution plan were diluted with normal saline to 50% dilution, and the measurement was carried out in single-use test cells. After incubation, the impedance change caused by the platelet aggregation on the test-cell surface was plotted against time. The area under the curve (AUC) expresses the aggregation response and is presented as so-called aggregation units. The following tests were performed: the thrombin-activating protein test (TRAP), the adenosine diphosphate activating test (ADP), and the arachidonic acid activating test (ASPI).

Table 1. Dilution plan

	Blood (mL)	Mannitol 20% (mL)	Gelatin 4% (mL)	Crystalloid (mL)
CON	2.5			
MAN (7% dilution)	2.325	0.175		
GEL (10% dilution)	2.25		0.25	
MAN+GEL (7%+10% dilution)	2.075	0.175	0.25	
ISO (17% dilution)	2.075			0.425

CON: control group without dilution; MAN: mannitol 20% group with 7% dilution; GEL: succinylated gelatin 4% group; GEL+MAN: mannitol 20% + gelatin 4% group; ISO: isotonic balanced electrolyte infusion with 17% dilution; mL: milliliters

A power analysis was performed based on the data of previously published studies (8, 9). Assuming an alpha error of 0.05 with a power of 0.85, we calculated a minimum sample size of 18 to show a significant reduction in FIB-MCF, induced by a 17% dilution of the blood sample. Based on this calculation, we included 22 volunteers. A statistical analysis was performed using the SPSS 22 (IBM Deutschland, Ehningen, Germany). All variables were tested for normal distribution (Shapiro-Wilks test). Normally distributed variables are expressed as the mean±standard deviation (SD). The median and interquartile range were used for variables for which a normal distribution could not be determined. Intergroup differences were determined by the Friedman test. Post-hoc testing was performed with the Wilcoxon test, and the Bonferroni correction for repeated measures was applied. Dichotomous variables were expressed as numbers and percentages, and differences were calculated using the chi-squared test. A p-value <0.05 in all tests was considered to be statistically significant.

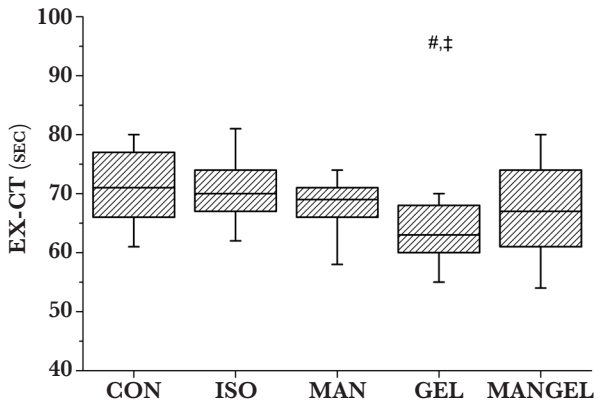

Figure 1. Clotting Time with ROTEM-EXTEM Test. Tested variable is the clotting time (EX-CT). Comparison of an undiluted blood sample (CON), 17% dilution with balanced isotonic electrolyte infusion (ISO), 7% dilution with mannitol (MAN), 10% dilution with succinylated gelatin (GEL), and 17% dilution with 7% mannitol 20% plus 10% succinylated gelatin (MANGEL). Boxplots are the median and interquartile range, and whiskers the 10/90% percentiles. # indicates a difference (p<0.05) compared with CON; ‡ indicates a difference (p<0.05) compared with MAN.

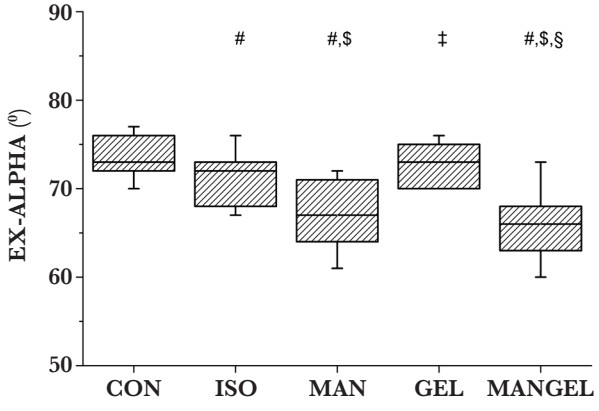

Figure 2. Alpha Angle with the ROTEM-EXTEM Test. Tested variable is the alpha angle (EX-ALPHA) in degrees. Comparison of an undiluted blood sample (CON), 17% dilution with balanced isotonic electrolyte infusion (ISO), 7% dilution with mannitol (MAN), 10% dilution with succinylated gelatin (GEL), and 17% dilution with 7% mannitol 20% plus 10% succinylated gelatin (MANGEL). Boxplots are the median and interquartile range, and whiskers the 10/90% percentiles. # indicates a difference (p<0.05) compared with CON; $ indicates a difference (p<0.05) compared with ISO; ‡ indicates a difference (p<0.05) compared with MAN; § indicates a difference (p<0.05) compared with GEL.

Results

Twenty-two volunteers participated in the study. One study subject had to be dropped out due to subsequent information on the use of non-steroidal anti-inflammatory drugs. Data of the 21 remaining probands were included for statistical analysis. Nine male and 13 female study subjects contributed to the study (not significant). The average age was 27.5±5.4 years (mean±SD). All measurements were successfully terminated.

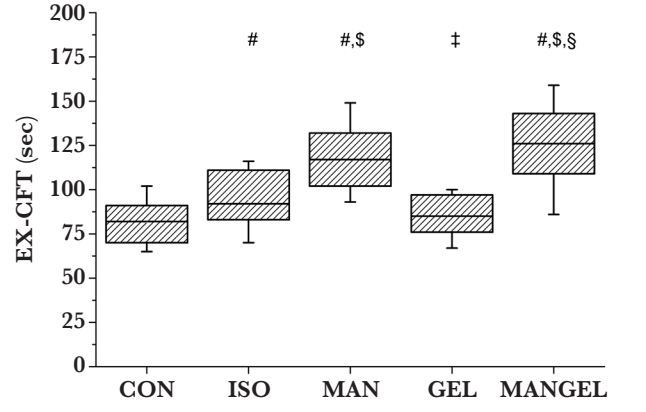

Figure 3. Clot Formation Time with The ROTEM-EXTEM test. Tested variable is the clot formation time (EX-CFT) in sec. Comparison of an undiluted blood sample (CON), 17% dilution with balanced isotonic electrolyte infusion (ISO), 7% dilution with mannitol (MAN), 10% dilution with succinylated gelatin (GEL) and 17% dilution with 7% mannitol 20% plus 10% succinylated gelatin (MANGEL). Boxplots are the median and interquartile range, and whiskers the 10/90% percentiles. # indicates a difference (p<0.05) compared with CON; $ indicates a difference (p<0.05) compared with ISO; ‡ indicates a difference (p<0.05) compared with MAN; § indicates a difference (p<0.05) compared with GEL.

Thrombelastometry

In the EX-CT test, only the GEL group 63 sec (60-68) showed a significant difference when compared to the CON group 71 sec (65-77), as well as with the MAN group 69 sec (64-71) (Figure 1). The alpha angle in the EX-ALPHA test was significantly reduced in all groups except for the GEL group (Figure 2). The mannitol and gelatin (MANGEL) group had the biggest difference with 66° (63-68) vs. 73° (71-76) in the CON group. Looking at the clot formation time in the EX-CFT test (Figure 3), we found similar results. Except for the GEL group, all groups showed a significantly prolonged clot formation time as compared to the CON group with 81.5 sec (70-90). The strongest effects were detected in the MAN and the MANGEL group with 116.5 sec (98-139) and 126 sec (109-150), respectively. Both groups were also significantly higher than the ISO group with 90.5 sec (75-111). The maximum clot firmness in the EX-MCF test showed only minimal changes (Figure 4). However, the ISO 58 mm (56-63), MAN 59 mm (56-61.5), and MANGEL group 57 mm (56-62) were significantly lower in their maximum clot amplitude as compared to the CON group with 64 mm (61-66.5). The maximum clot firmness of fibrinogen/fibrin axis (FIB-MCF) showed minor but significant changes in test groups, as compared to the CON 15 mm (12.5-20) group (Figure 5). The strongest effect was seen in the MANGEL group with 9.5 mm (8-11).

Thrombocyte aggregometry

We next investigated the platelet function by aggregometry. In the ADP test (Table 2), all groups except for the MAN group showed a significant decrease in AUC when compared with the CON group. The most pronounced effects were in the MANGEL, GEL, and ISO groups. We found no significant differences between the CON group as compared to the other groups in the ASPI test (Table 2). However, the MAN

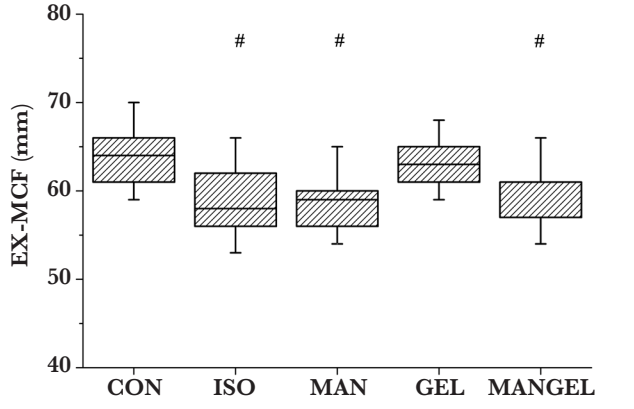

Figure 4. Maximum Clot Firmness with the ROTEM-EXTEM test. Tested variable is the maximum clot firmness (EX-MCF). Comparison of an undiluted blood sample (CON), 17% dilution with balanced isotonic electrolyte infusion (ISO), 7% dilution with mannitol (MAN), 10% dilution with succinylated gelatin (GEL), and 17% dilution with 7% mannitol 20% plus 10% succinylated gelatin (MANGEL). Boxplots are the median and interquartile range, and whiskers the 10/90% percentiles. # indicates a difference (p<0.05) compared with CON.

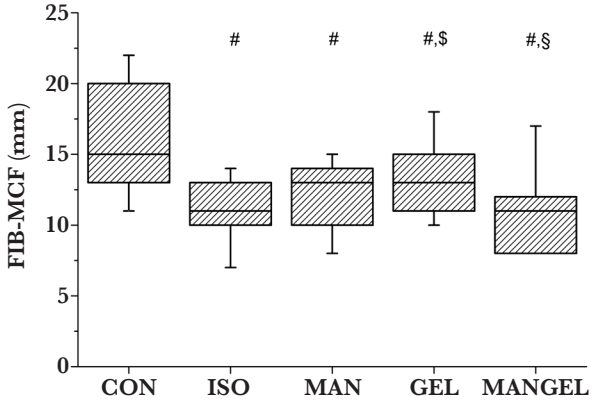

Figure 5. Maximum Clot Firmness with the ROTEM-FIBTEM Test. Tested variable is the maximum clot firmness (FIB-MCF). Comparison of undiluted blood sample (CON), 17% dilution with balanced isotonic electrolyte infusion (ISO), 7% dilution with mannitol (MAN), 10% dilution with succinylated gelatin (GEL) and 17% dilution with 7% mannitol 20% plus 10% succinylated gelatin (MANGEL). Boxplots are the median and interquartile range, whiskers the 10/90% percentiles. # indicates a difference (p<0.05) compared with CON; $ indicates a difference (p<0.05) compared with ISO; § indicates a difference (p<0.05) compared with GEL.

Table 2. Platelet function analyzed by thrombocyte aggregometry (multiplate)

	CON	ISO	MAN	GEL	MANGEL
ADP (AUC)	92 (74.5-100.5)	62 (53-89)#	75 (58.5-106)$	69 (55-86.5)#	61 (47.5-81.5)#
ASPI (AUC)	89 (78.5-107)	86 (72.5-98.5)	99 (86.5-113)$	82 (65.5-100)‡	75 (62.5-96)‡
TRAP (AUC)	106 (90-131)	97 (77-107)#	108 (90.5-119)	92 (77.5-110.5)#,$	95 (83.108)#,§

Platelet function analyzed by thrombocyte aggregometry. Tested variables: adenosine diphosphate test (ADP); arachidonic acid test (ASPI); and thrombin activated test (TRAP). All tests displayed in aggregatory units*10 per minute (AUC). Comparison of undiluted blood sample (CON), 17% dilution with balanced isotonic electrolyte infusion (ISO), 7% dilution with mannitol (MAN), 10% dilution with succinylated gelatin (GEL), and 17% dilution with 7% mannitol 20% plus 10% succinylated gelatin (MANGEL). # indicates a difference (p<0.05) compared with CON; $ indicates a difference (p<0.05) compared with ISO; ‡ indicates a difference (p<0.05) compared with MAN; § indicates a difference (p<0.05) compared with GEL.

group showed significantly higher values as compared to the ISO, GEL, and MANGEL groups. We finally performed the TRAP test and found significant but minor decreases for the ISO, GEL, and MANGEL groups, as compared with the CON group.

Discussion

Mannitol and gelatin are standard components used in the treatment of neurosurgical patients. We therefore conducted this study to investigate the effects of gelatin in a combination with mannitol, which in this study significantly impaired whole blood coagulation and platelet function.

In the present study, we aimed to mimic a clinically relevant situation with both agents, that is, the used dilutions of both substances are likely to be close to the plasma concentrations found in patients. The dilution plan in our study was defined assuming that normal healthy people have a total blood volume of 70 mL kg^{-1} body weight. A recommended amount of mannitol during neurosurgical procedures is 1 g kg^{-1} mannitol (5, 6). By using a 20% MAN solution, this would be 5 mL kg^{-1} that would cause a 6.7% dilution of the blood in a patient weighing 70 kg. GEL is used as a plasma expander in surgical procedures, and the initially used dose of 5-10 mL kg^{-1} would cause a dilution of 6.7%-13.4%. Because infusions are usually in the form of bottles or bags, a patient weighing 70 kg would receive 500 ml of GEL (7.14 mL kg^{-1}). This dose would lead to a blood dilution of 9.3%. To keep it simple, we used a 7% dilution for MAN and a 10% dilution for GEL, when administered separately, and 17% for the combination of both. We also used a 17% dilution with ISO to rule out a dilution effect.

Effects of MAN and GEL on thrombelastometry

Gelatin infusions have previously been demonstrated to have an influence on haemostasis. Osthaus et al. (15) tested a bolus of 10 mL kg^{-1} of gelatin in children under 12 years of age and found a significant decrease in EX-CFT and EX-MCF, but not FIB-MCF and EX-CT, when compared to undiluted blood. In an animal study, a dose-dependent decrease of EX-MCF with the use of gelatin in a haemorrhagic shock model was found (16). However, this effect was only significant with dilutions from 20 mL kg^{-1} or higher. Similar effects were found by Mardel et al. (17). They were able to point out that in their *in vitro* setting with dilutions of 15% or higher, the maximum clot amplitude was reduced by gelatin in a dose-dependent manner. Similar effects have also been reported by other authors (10, 18, 19). As our data on EX-MCF and EX-CFT did not reveal any effects by GEL as compared to baseline, there seems to be a discrepancy between our data and previously published reports. However, as our dilutions with GEL were lower and most probably closer to the plasma levels of GEL in patients, we believe that our data are more valid.

Looking at mannitol, we found that our 7% dilution resulted in a significant deterioration of EX-ALPHA, EX-MCF, EX-CFT, and FIB-MCF. However, all variables stayed within the normal range (20). These findings are in line with other *in vitro* investigations that used considerably higher concentrations of mannitol (10% or more) (7-9). Ali et al. (21) used the same mannitol dilution (7%) like in our study, and indeed, they found very similar effects with a decrease in FIB-MCF and a prolongation of EX-CFT.

The combination of MANGEL showed the most pronounced effects. In this group, we had a dilution of 17%, and when compared with the ISO group (with 17% dilution as well), we could determine significant effects on EX-CFT and EX-ALPHA. Although not significant, the EX-CT and the FIB-MCF were also slightly reduced when compared to the ISO group. These data seem to indicate that mannitol and gelatin induce additional effects on haemostasis, except for dilution.

Yozova et al. (4) conducted an *in vivo* study in 15 dogs. They compared 5 mL kg^{-1} mannitol 20% with 4 mL kg^{-1} HS (7.2%) and found no significant changes in EX-CT, EX-CFT, EX-ANGLE, and EX-MCF. Only the FIB-MCF test was significantly reduced, but still within the normal range. Similar results were found by Adamik et al. (22). In an *in vitro* setting with healthy dogs, they tested two dilutions of mannitol (12.5% and 6.25%) and found significant effects on EX-CT, EX-CFT, but not on EX-MCF and FIB-MCF for both dilutions. However, only the results for the 12.5% dilution were outside the normal range. In a clinical study that included 40 elective craniotomy patients, the effects of mannitol 20% and HS (3%) on whole blood coagulation were investigated (23). Interestingly, the authors did not detect any significant differences for EX-CT, EX-CFT, EX-ANGLE, EX-MCF, or FIB-MCF. These findings are contrary to all previously published *in vitro* findings (7-9, 21), as well as to our results. The authors concluded that *in vitro* studies are limited and probably not able to simulate the complex *in vivo* pathways of blood coagulation. Another clinical study that included patients with traumatic brain injury also failed to detect any significant effect of mannitol and HS blood coagulation (24). There is, however, a potential reason for this discrepancy. Abrahams et al. (25) were able to show that blood coagulation increased over the course of the operation/anaesthesia in elective craniotomy patients. The most pronounced effect was found between intubation and skin incision, but it increased further during the surgery. Thus, although the mechanisms for this effect remain elusive, an improvement in coagulation occurring during invasive procedures obviously does not occur i*n vitro* and might explain why the effects of mannitol and/or gelatin found *in vitro* are not evident *in vivo*.

Effects of MAN and GEL on thrombocyte aggregometry
There is increasing evidence that the hyperosmolarity triggered by hypertonic solutions like mannitol or HS has an influence on the shape and aggregation properties of platelets (26). Possible mechanisms for this effect could be the influence of calcium homeostasis (27) or a change in the ligand expression on the surface of platelets (28).

Witt et al. (16) performed a study on pigs and were able to show a negative influence of gelatin on platelet function in the COL test with Multiplate. However, this effect was only detectable with dilutions that were 20% or higher. Independent on the dilution, no effects were observed in the ADP test or ASPI test. Another investigation by Winterhalter et al. (29) on cardiac surgery patients found no difference in the COL, ADP, or TRAP tests by the Multiplate analysis after an infusion of 500 mL of gelatin 4%. In our study population, we failed to determine significant mannitol-induced effects in the ASPI, TRAP, and ADP tests. These findings are contrary to the results by Adamik et al. (22). They found that both the 6.25% and 12.5% dilutions of blood samples with mannitol 20% displayed no changes in thrombocyte function according to the PFA-100 test (PFA clotting time). However, they did not use a Multiplate analyzer, and it is doubtful if these data are even comparable with our data.

In the GEL and especially the MANGEL groups, we found a significant reduction in AUC in the TRAP and ADP test. Furthermore, these recordings were in part under the normal range of 57 AUC. Thus, the combination of mannitol and gelatin seems to have an additive effect. As the 17% ISO dilution showed similar effects, however, we cannot exclude that these effects are due to simple dilution.

Our study has some limitations that have to be mentioned. We chose an *in vitro* setting, which will never be able to represent the complex pathways of coagulation. We included only young and healthy individuals, and our findings are therefore not fully applicable to a clinical setting in patients with comorbidities and those who underwent surgery. Significant effects found in our study were mostly within the normal range as they also were in most other studies (7-9).

Conclusion

In this *in vitro* study, we found the following major effects: (1) the EX-CFT and EX-ALPHA were mostly affected by MAN and the MANGEL combination; (2) The maximum clot firmness (EX-MCF) was slightly but significantly reduced in the ISO, MAN, and MANGEL groups; (3) the platelet function was reduced in the ISO, MAN, and MANGEL groups, according to the ADP test. Except for the result of the ADP test, all findings were within the normal range. Thus, one could question if our results are of clinical relevance. Importantly though, it is possible that in patients with marginal preoperative coagulopathy in neurosurgical procedures these changes could be the straw to tip the scale. Further larger studies on patients undergoing neurosurgery are needed to investigate the effects of mannitol in a combination with gelatin on blood coagulation and platelet function.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Medical School Hannover (7051-2015).

Informed Consent: Informed consent was obtained from all individual participants included in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – T.P., P.F.; Design – T.P., P.F.; Supervision – T.P., P.F.; Resources – T.P., P.F.; Materials – T.P., P.F.; Data Collection and/or Processing – E.K., J.H., H.E., D.S.; Analysis and/or Interpretation – T.P., H.E., D.S., P.F.; Literature Search – T.P., E.K., J.H., H.E., P.F.; Writing Manuscript – T.P., P.F.; Critical Review – T.P., E.K., J.H., H.E., D.S., P.F.

Conflict of Interest: The authors have no conflicts of interest to declare.

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