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Hemostatic effect of a chitosan linear polymer (Celox®) in a severe femoral artery bleeding rat model under hypothermia or warfarin therapy

Hipotermi ve varfarin uygulanan şiddetli femoral arter kanamalı sıçan modelinde kitosan lineer polimer'in (Celox®) hemostatik etkinliği

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BACKGROUND
In this study, the hemostatic efficacy of Celox® in rats under hypothermia or warfarin treatment was investigated.

METHODS
A total of forty-eight Sprague-Dawley female rats weighing 200-350 g were used in the study. Six experimental study groups were designed, as follows: Group 1: Normothermia + compression; Group 2: normothermia + Celox®; Group 3: hypothermia + compression; Group 4: hypothermia + Celox®; Group 5: normothermia + warfarin + compression; and Group 6: normothermia + warfarin + Celox®.

RESULTS
Celox® provided effective hemorrhage control in all three tested groups. There was a statistically significant difference between compression and Celox® implementation in all groups in terms of hemostasis (p-values for the normothermia, hypothermia and warfarin groups were $p<0.05$, $p<0.01$ and $p<0.01$, respectively). Furthermore, the compression numbers were significantly lower in all of the groups that received Celox® than in those in which compression alone was applied (p-values for the normothermia, hypothermia and warfarin groups were $p<0.01$, $p<0.01$ and $p<0.001$, respectively).

CONCLUSION
Celox® provides effective hemorrhage control under conditions of normothermia, hypothermia and use of the oral anticoagulant agent warfarin.

Key Words: Bleeding control; Celox®; hypothermia; warfarin.

AMAÇ
Bu çalışmada hipotermi ve varfarin tedavisi altındaki sıçanlarda Celox®'un hemostatik etkinliği araştırıldı.

GEREÇ VE YÖNTEM
Çalışmada toplam 48 adet ortalama 200-350 gram ağırlığında Sprague-Dawley cinsi dişi sıçan kullanıldı. Her birinde 8 sıçan olan 6 grup oluşturuldu; 1. grup: normotermi + bası, 2. grup: normotermi + Celox®, 3. grup: hipotermi + bası, 4. grup: hipotermi + Celox®, 5. grup: normotermi + varfarin + bası, 6. grup: normotermi + varfarin + Celox®.

BULGULAR
Celox® uygulanan tüm gruplarda etkin kanama kontrolü sağlandı. Bası ve Celox® uygulanan tüm gruplar arasında hemostaz açısından istatistiksel olarak anlamlı farklılık saptandı (p değerleri normotermi, hipotermi ve varfarin grupları için sırasıyla; $p<0,05$, $p<0,01$ ve $p<0,01$). Benzer şekilde bası sayısı, Celox® uygulanan tüm gruplarda sadece bası uygulanan gruplardan anlamlı derecede daha azdı. (p değerleri normotermi, hipotermi ve varfarin grupları için sırasıyla; $p<0,01$, $p<0,01$ ve $p<0,001$).

SONUÇ
Celox®, sadece normotermide değil aynı zamanda hipotermide ve bir oral antikoagülan ajan olan varfarin kullanımında da etkin kanama kontrolü sağlamaktadır.

Anahtar Sözcükler: Kanama kontrolü; Celox®; hipotermi; varfarin.

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Uncontrolled hemorrhage after trauma is the leading cause of death among military personnel and the second leading cause of death among civilians worldwide.[1] Uncontrolled hemorrhage also has an important role in morbidity.[2] Better methods for providing hemostasis will improve survival and reduce the long-term effects of hemorrhage, and it is expected that one-third of the deaths can be prevented with these methods.[2] Consequently, numerous hemostatic agents have been produced in recent years, and significant improvements have been achieved in their use for traumatic hemorrhage.[2-7] These agents have become alternatives to classical methods of hemostasis like compression and tourniquet, especially for prehospital control of hemorrhage, because standard gauze field dressings and direct pressure are often inadequate in the control of hemorrhage.[8] However, the comparative evaluation of hemostatic agents in human clinical studies is very difficult, and animal studies are therefore needed.[9] Several studies have compared the effects of hemostatic agents, but the results of these studies are controversial.[2,4-7,9]

Most of the studies investigating the effectiveness of hemostatic products have employed normothermic conditions. However, hypothermia is a frequent problem encountered in trauma patients and is related to arduous hemorrhage control and increased mortality.[10-15] Thus, the effects of hemostatic agents should be evaluated under hypothermic conditions.

Many patients today use anticoagulant drugs for thrombotic diseases, mainly heparin, low-molecular-weight heparin, pentasaccharide, and warfarin. The effects of hemostatic agents on trauma patients receiving this kind of therapy have been examined; however, these studies were generally performed with heparin, thus necessitating the evaluation of warfarin.

In the present study, Celox®, a hemostatic agent containing chitosan that is approved by the FDA (Food and Drug Administration) for external use, was evaluated in rats with severe femoral artery bleeding under hypothermia or warfarin therapy.

MATERIALS AND METHODS

Forty-eight Sprague-Dawley female rats weighing 200-350 g, obtained from Uludag University Faculty of Medicine Laboratory Animals Growing Application and Research Center, were used in the study. The study was supported by the Uludag University Research Fund and was approved by the Uludag University Animal Experiments Ethics Committee.

The rats were anesthetized with 3% isoflurane after being fasted for 12 hours, and after tracheostomy, they were maintained under anesthesia with 2% isoflurane with 21% oxygen support, permitting spontaneous respiration via the anesthesia system during the procedure (SurgiVet, Inc., Veterinary Anesthesia and Monitoring Equipment, Multi-Station Lab Research Anesthesia System).

Rat temperatures were recorded by rectal probe (Biopac Systems, Inc., SSGL Fast Temp. SN6053859), and were maintained at 37±0.5 °C in the normothermia group by heater pads and at 32±0.5 °C in the hypothermia group by the application of alcohol to the skin under an electric fan.

The left femoral artery was cannulated to monitor blood pressure and heart rhythm and to perform blood sampling. The catheter was connected to a volumetric pressure transducer (Biopac Data Acquisition Unit MP30), and blood pressure and heart rate were recorded continuously. The right femoral artery was perforated with a 24-gauge needle, which was then removed to allow free bleeding for 30 seconds (sec). Standard compression or compression + Celox® was then applied according to the respective groups. All bleeding and pressure procedures were performed by the same person. The rats were divided into six groups of eight rats each to evaluate the hemostatic effect of Celox®.

Normothermia + compression group: In this group, the core temperature of the rats was maintained at 37±0.5 °C, and after 30 sec of bleeding from the femoral artery, compression was provided by 100 g of standard weight for 30 sec. Hemostasis was subsequently evaluated, and the test was terminated if the bleeding stopped. If the bleeding continued, compression was applied for an additional 30 sec and bleeding was again assessed for hemostasis. If hemostasis was not achieved after the second compression, a final 30 sec of compression was applied. The test was regarded as a failure if bleeding continued following the third compression.

Normothermia + Celox® group: The core temperature of the rats was maintained at 37±0.5 °C. After 30 sec of bleeding from the femoral artery, Celox® (1 g/kg) was applied to the bleeding area, and compression was provided by 100 g of standard weight for 30 sec. Subsequently, hemostasis was evaluated and second and third compressions were applied if necessary. If hemostasis was not achieved after 90 sec of treatment, uncontrolled hemorrhage was concluded.

Hypothermia + compression and hypothermia + Celox® groups: In these groups, the core temperature of the rats was maintained at 32±0.5 °C, and bleeding, compression, Celox® administration, and the test evaluation were performed as described for the normothermia groups.

Normothermia + warfarin + compression and normothermia + warfarin + Celox® groups: Warfa-

rin (0.06 mg/kg) was administered to the rats by oral gavage for three days, and the experiments were performed 30 minutes (min) after the final dose. In these groups, the core temperature of the rats was maintained at 37±0.5 °C, and bleeding, compression, Celox® administration, and the test evaluation were performed as described for the other normothermia groups.

As previously indicated, physiological parameters of mean blood pressure, heart rate and rectal temperature were recorded continuously during the tests. Blood samples were obtained to analyze the hemoglobin levels, platelet counts, red blood cell counts, prothrombin time, and the International Normalized Ratio (INR) values before bleeding (0 sec) and at the end of the test. All animals were euthanized by high-dose isoflurane inhalation at the end of the experiment.

Statistical Analysis

Statistical analysis was performed using SPSS version 13.0 for Windows. The Mann-Whitney U test was used to compare two independent groups, the Kruskal-Wallis test to compare more than two independent groups and the Wilcoxon test to compare two dependent groups. Pearson's and Fisher's chi-square tests were used to compare the distributions of categorical variables. Data are expressed as the mean ± SD and p-values less than 0.05 were regarded as statistically significant.

RESULTS

The mean blood pressure and heart rate of the rats were recorded before (0 sec) and 30, 60 and 90 sec after hemorrhage, and the difference between before and after bleeding represented the percent change. There were no statistically significant differences between the groups with respect to changes in the mean blood pressure and heart rate (Tables 1 and 2).

No statistically significant differences were observed between the compression and Celox® groups with regard to hemoglobin levels, red blood cell counts, platelet counts, prothrombin time, and INR evaluated before and after bleeding. In the warfarin groups, prothrombin time and INR were very high but could not be accurately determined in the laboratory, thus precluding a statistical analysis of these groups.

Hemostasis was achieved in 4 of 8 rats in the normothermia + compression group (50%), whereas it was provided in all rats in the normothermia + Celox® group (100%). In the hypothermia + compression group, hemostasis was observed in 6 of 8 rats, whereas it was 100% successful in the hypothermia + Celox® group. In the groups that received warfarin therapy, hemorrhage control was achieved in only 2 of 8 rats in the compression group; however, it was successful in all rats in the normothermia + warfarin + Celox® group (Fig. 1). There was a statistically significant difference

Table 1. Blood pressure changes (%) in all groups

	Blood pressure change (%)		
	30 sec	60 sec	90 sec
Normothermia + compression	73.9±0.2	73.7±0.2	74.2±0.2
Normothermia + Celox®	62.9±0.1	61.6±0.1	52.7±0.1
Hypothermia + compression	46.7±0.3	52.6±0.3	53.5±0.3
Hypothermia + Celox®	39.4±0.9	42.5±0.1	42.5±0.1
Warfarin + compression	49.4±0.1	50.4±0.1	53.5±0.3
Warfarin + Celox®	43.3±0.1	53.3±0.1	55.9±0.2

Blood pressure was recorded and allowed to stabilize for 30 min prior to bleeding. Blood pressure changes (%) from baseline levels were determined by comparison of the levels at 30, 60, and 90 sec of bleeding. No significant differences were observed between the groups (p>0.05).

Table 2. Heart rate changes (%) in all groups

	Heart rate changes (%)		
	30 sec	60 sec	90 sec
Normothermia + compression	20.9±0.4	24.2±0.6	25.5±0.7
Normothermia + Celox®	29.2±0.1	26.8±0.9	29.3±0.2
Hypothermia + compression	26.7±0.2	29.6±0.2	36.7±0.2
Hypothermia + Celox®	19.4±0.3	26.5±0.3	23.1±0.3
Warfarin + compression	9.0±0.1	8.9±0.1	8.9±0.2
Warfarin + Celox®	16.8±0.2	12.3±0.1	9.1±0.9

Heart rate was recorded and allowed to stabilize for 30 min prior to bleeding. Heart rate changes (%) from baseline levels were determined by comparison of the levels at 30, 60, and 90 sec of bleeding. No significant differences were observed between the groups (p>0.05).

Table 3. Evaluation of the number of compressions needed for hemostasis after severe femoral artery bleeding in normothermia, hypothermia and warfarin groups with compression and Celox® implementation

Groups	Compression			Celox®		
	1st compression	2nd compression	3rd compression	1st compression	2nd compression	3rd compression
Normothermia	–	–	4	7	1	–*
Hypothermia	–	–	6	4	3	1*
Warfarin	–	–	2	6	2	–**

The differences between compression and Celox® were statistically significant for compression time in terms of hemostasis (*p<0.01, **p<0.001).

between compression and Celox® implementation in all groups in terms of hemostasis (p-values for the normothermia, hypothermia and warfarin groups were p<0.05, p<0.01 and p<0.01, respectively).

Assessment of the number of compressions in rats required to achieve hemostasis revealed hemorrhage control with the third compression in 4 rats in the normothermic + compression group, whereas it was achieved after the first compression in 7 of 8 rats and after the second compression in 1 rat in the normothermia + Celox® group. In the hypothermia + compression group, hemostasis was successful in 6 of 8 rats and in all rats after the third compression. However, when hypothermic rats were treated with Celox®, hemostasis was possible in 4 rats after the first, in 3 rats after the second and in 1 rat after the third compression. Hemostasis was achieved in 2 rats in the group that received warfarin therapy + compression after the third compression and in 6 and 2 rats in the normothermia + warfarin therapy + Celox® group after the first and second compression, respectively (Table 3). The compression numbers were significantly lower in all of the groups that received Celox® (p-values for the normothermia, hypothermia and warfarin groups were p<0.01, p<0.01 and p<0.001, respectively).

DISCUSSION

The early recognition and treatment of bleeding is a critical process and currently remains a serious clinical issue. At present, local hemostatic agents are added to conventional methods such as manual compression, ligation and tourniquet application for the control of bleeding.[16,17] It is estimated that hemostatic agents are particularly useful in comparison to conventional methods in cases of hemorrhage in difficult anatomic regions such as the axillary and femoral areas.

In several studies,[18-21] Celox® was found to be 100% effective in rats with severe femoral artery bleeding under normothermic conditions. In a study reported by Ersoy et al.,[1] the time required for hemostasis was found to be significantly shorter with the implementation of TraumaDEX® (microporous polysaccharide hemisphere) in rats with uncontrolled hemorrhage. In the present study, the use of Celox® provided hemostasis control in 100% of the animals, and compression numbers were significantly lower than in the groups in which compression alone was applied.

In a comparative study, Kozen et al.[19] demonstrated the superiority of Celox® for survival as compared to HemCon®, QuikClot® and standard compression technique in a porcine model of inguinal injury. In the present study, the animals were not resuscitated and were euthanized at the end of the study; therefore, despite the achievement of hemostasis with Celox® in all rats, survival could not be evaluated.

Devlin et al.,[18] using a porcine model study, demonstrated that local hemostatic agents (ChitoFlex®, OuikClot®, Celox®) were not superior to standard gauze. They emphasized that these agents could be effective for venous or mixed hemorrhage but were not suitable for high pressure artery bleeding. In the present study, Celox® was significantly effective for the treatment of severe femoral artery bleeding in a rat model. The only difference between these two studies was the animal model used for the analysis. In addition

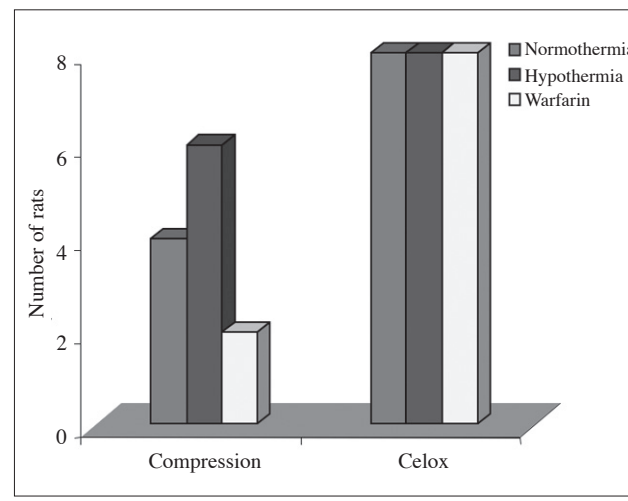

Fig. 1. Evaluation of hemostasis after severe femoral artery bleeding in the normothermia, hypothermia and warfarin groups with compression and Celox® implementation. *There was a statistically significant difference in successful hemostasis in favor of the Celox® group when compared to the compression group (*p<0.05, **p<0.01).

to these studies, Kheirabadi et al.[20] showed the positive effect of several new hemostatic agents including Celox® on artery bleeding. In contrast to Devlin's study, Gustafson et al.[21] reported that local hemostatic agents containing chitosan were found to be 100% effective in a porcine model of femoral artery bleeding, consistent with the present results. Gustafson et al. noted that the use of different techniques and animal models could affect study outcomes, potentially explaining the different results obtained in our study and that reported by Devlin et al.

In a porcine model study of mortal inguinal injury reported by Alam et al.,[22] QuikClot® (mineral zeolite) decreased blood loss and resulted in 100% survival; however, the mortality was found to be 83% in the compression group. Concordantly, Pusateri et al.[23] and Baker et al.[24] reported the superiority of QuikClot® as compared to other homeostatic agents. Although blood loss was not evaluated in the present study, 100% hemostasis was achieved with Celox®. In contrast, compression provided successful hemostasis in only 50% of the normothermic, 75% of the hypothermic and 25% of the warfarin-treated groups. In considering the results of these studies, it is important to note that QuikClot® causes a local increase of heat via an exothermic reaction and causes minimal tissue damage.[4]

Hypothermia due to environmental losses and after surgery is related with uncontrolled hemorrhage and mortality in trauma patients.[10-15,25] Several studies[26-28] have demonstrated increased survival under conditions of hypothermia with controlled hemorrhage; however, another study[29] reported a higher mortality with hypothermia. In the present study, controlled, moderate hypothermia (32±0.5 °C) was assessed in one group of rats. Successful hemostasis was observed in six of eight rats in the hypothermia group and in four of eight rats in the normothermia group, consistent with previous studies. Therefore, we can conclude that Celox® has a favorable effect during hypothermia. In addition, we found that Celox® had similar effects in the hypothermia and normothermia groups.

Several studies have assessed the effects of local hemostatic agents after trauma on patients undergoing anticoagulant therapy, and most of these studies have employed heparin, a parenteral anticoagulant agent.[30,31] Klokkevold et al.[30] demonstrated the efficacy of chitosan in a heparinized rabbit model, and Tuthill et al.[31] showed significantly decreased blood loss through the use of a fibrin sealant hemostatic agent in heparinized rats undergoing heminephrectomy. Schwaitzberg et al.[32] asserted that local hemostatic agents containing poly-N-acetylglucosamine are effective even in congenital or acquired diseases of coagulopathy in a study performed in dogs with hemophilia and in heparinized pigs. In the present study, we investigated the efficacy of Celox® with warfarin treatment. As expected, hemostasis was not achieved with compression in six of eight rats in the warfarin treatment group. In contrast, hemostasis was successfully provided by Celox® in all rats, demonstrating that warfarin therapy did not influence the effect of Celox®. The efficacy of Celox® even under warfarin therapy, a condition presenting a deficit of coagulation factors, is consistent with previous studies[33-35] suggesting that the capacity of Celox® to achieve hemostasis is independent of the effects of coagulation factors and platelets.

As a limitation of the study, animal studies are not universally accepted to reflect the effect of hemostatic agents on wounds in humans. In addition, small animal models likely do not reflect the effect of hemostasis in the context of high-pressure bleeding. Although the present study was performed to evaluate the hemostatic efficiency of Celox®, the effect of Celox® on blood loss and mortality was not determined. In addition, the results of the present study were not blinded, as the implementation of compression and hemostatic agents and the evaluation of hemostasis were performed by the same researcher.

In conclusion, Celox® provides hemostasis not only under normothermic conditions but also under conditions of hypothermia and warfarin therapy. However, further clinical investigations are needed to validate the results of these animal studies.

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