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Investigation of Immunological Differences in Mecsina Hemostopper©, Ankaferd Blood Stopper® and Tranexamic Acid Used as Haemostatic Agents with Cell Culture Study

Hemostatik Ajan Olarak Kullanılan Mecsina Hemostopper©, Ankaferd Blood Stopper® ve Traneksamik Asidin İmmunolojik Farklılıklarının Hücre Kültürü Çalışması ile Araştırılması

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Öz

Amaç:Tıp ve diş hekimliğinin birçok uygulamasında, tedavi sırasında veya sonrasında görülen beklenmedik kanamalar, yapılan işlemin büyüklüğünden bağımsız olarak gelişebilecek bir komplikasyon olarak değerlendirilir. Kanamanın kontrol altına alınmasını kolaylaştırmak amacıyla cerrahi işlem öncesinde ve/veya sonrasında kullanılabilecek birçok biyomalzeme üretilmiştir. Bu çalışmada kanama durdurucu ajan olarak kullanılan Ankaferd Blood Stopper® (ABS), Traneksamik Asit (TA) ve yeni bir kanama durdurucu olan Mecsina Hemostopper®'ın(MH) immünolojik etkinlikleri ve etkilerinin karşılaştırılması amaçlanmıştır. **Yöntem:** Çalışmada pıhtılaşma ve fibrin oluşumu üzerinde en çok çalışma yapılan ölümsüz hücre içeriğine sahip insan umblikal kord hücreleri (HUVEC, Human Umbilical Vein Endothelial Cell) kullanılmıştır. Her flaska her bir ilaç için 5000 hücre olacak şekilde 5 gruba (ABS grubu, TA grubu, MH grubu, distile su uygulanan negatif grup ve herhangi bir uygulama yapılmayan kontrol grubu) hücreler dağıtıldı. **Bulgular:** Elde edilen verilere göre 1:2 ve 1:10' luk konsantrasyonlarda; MH uygulaması yapılan hücrelerde Tümör Nekrozis Faktör Alfa (TNF-α) seviyeleri diğer ilaç gruplarına göre daha düşüktür ($p<0,05$). İnterlökin 1 Beta (IL-1β) ve İnterlökin- 6 (IL-6) seviyelerinde ise hem 1:2 hem de 1:10' luk konsantrasyonlarda tüm ilaç uygulamaları sonucunda kontrol grubuna kıyasla anlamlı derecede artış olduğu gözlenmiştir ($p<0,05$). TNF-α seviyelerinde ise 1:2 konsantrasyonda tüm ilaç uygulamalarında kontrole göre artış ($p<0,05$) gözlenmişken, 1:10 konsantrasyonda MH uygulamasında anlamlı bir değişiklik görülmemiş ($p>0,05$) ancak TA ve ABS uygulamalarında ise anlamlı derecede artış olduğu belirlenmiştir ($p<0,05$). **Sonuç:** Sonuç olarak, HUVEC hücre gruplarında farklı konsantrasyonlarda uygulanan farklı anti hemorajik ajanların hücre içi sitokin seviyelerinde önemli ölçüde bir artış meydana getirdiği görülmüştür. Sonuçlar göz önüne alındığında MH uygulamasının ABS (ve özellikle TA uygulamalarına kıyasla daha etkili bir anti hemorajik ajan olduğunu söyleyebilmekteyiz.

Anahtar Kelimeler: HUVEC, Interleukin-6, Interleukin-1 βeta, Tumor Necrosis Factor Alpha, Mecsina

Abstract

Objective: Hemorrhagic complications may develop in many branches of medicine and dentistry after or during the treatment independently of the ex-

tent of the procedure performed. Various biomaterials have been used in the medical and dental practice procedures before and after surgical procedures. The aim of this study was to compare the immunological efficacies and effects of Ankaferd Blood Stopper® (ABS), Tranexamic acid (TA) used as anti-hemorrhagic agents and Mecsina Hemostopper©(MH), a new anti-hemorrhagic agent. **Method:** The immortalized Human Umbilical Vein Endothelial Cell (HUVEC) cell lines, which are human umbilical cord cells and used in many studies on coagulation and formation of fibrin, were commercially purchased for the study. The cells, 5000 cells per flask for each drug, were distributed into the 5 groups (ABS Group, TA group, MH group, distilled water-administered negative group and control without any administration). **Results:** According to the data we obtained, Tumor Necrosis Factor Alpha (TNF-α) levels were found to be lower in the cells, to which MH was administered at concentrations of 1: 2 and 1:10, than other drug groups (p<0,05). There was a significant increase in Interleukin 1β (IL-1β) and Interleukin 6 (IL6) levels in all drug administrations at both concentrations of 1: 2 and 1: 10 compared to the control group (p <0,05). While there was no significant increase in TNF-α levels in all drug administrations at a concentration of 1: 2 (p <0,05), there was no significant correlation in MH administration at a concentration of 1:10 (p> 0,05), but a significant increase was found in TA and ABS administrations (p <0.05). **Conclusion:** In conclusion, it has been observed that different anti-hemorrhagic agents administered at different concentrations in HUVEC cell groups produced a significant increase in intracellular cytokine levels. Considering the results, we can say that MH administration is a more effective anti-hemorrhagic agent than administrations of ABS and especially TA.

Keywords: HUVEC, Interleukin-6, Interleukin-1 βeta, Tumor Necrosis Factor Alpha, Mecsina

Introductıon

As a result of all surgical procedures, different levels of hemorrhage occur in the operation area. The maintenance of homeostasis pre- and post-operatively is one of the most important surgical requirements for a good and effective surgical operation.[1] Besides mechanical techniques, the use of anti-hemorrhagic agents is vital for bleeding control, especially in patients with bleeding problems.[2] Ankaferd Blood Stopper (ABS) is a hemostatic agent consisting of 5 different plant extracts. It is known to be an effective anti-hemorrhagic agent routinely used during surgical operations. Each of these plants, composing the content of ABS, has different effects on blood cells, endothelium, angiogenesis, cell proliferation and cellular mediators.[3] Tranexamic acid (TA) is an antifibrinolytic agent that prevents the transformation of plasminogen to plasmin and is also used in patients with postoperative long-term bleeding after surgery.[4] TA competitively inhibits the interaction between lysine residues on the fibrin surface and the heavy chain of plasminogen and plasmid, blocking the lysine-binding portion on the plasminogen molecule. It reduces other plasma proteins, including fibrin clot, fibrinogen, FV and FVIII.[5] In the use of TA does not increase the risk of mortality, myocardial infarction, stroke, deep venous thrombosis, pulmonary embolism and renal dysfunction, in addition to that, causes a significant reduction in the length of hospital stay.[6] Anti-hemorrhagic agents may prevent hemorrhage by using different mechanisms of action. Mecsina Hemostopper (MH) is made from herbal agents such as Glycyrrhiza glabra extract, Alpinia officinarum, Thymus serpyllum, Syzygium aromaticum, Hypericum perforatum, Vitis vinifera, Urtica angustifolia, Mentha arvensis. MH provides a vital erythrocyte aggregation by forming a protein network in the environment. It has been proved in electron microscope experiments that it forms a protein network by binding especially to fibrinogen, and the erythrocytes are arranged in the formation in this network. By this means, its effect on hemostasis could be observed. In this study, we aimed to compare the immunological and effects of ABS, TA routinely used as hemostatic agents, and MH, a new hemostatic agent.

Materıals and Method

Cell Culture

In this study, the immortalized HUVEC (C0155 , Invitrogen, Carlsbad, CA, USA) cell lines, which are human umbilical cord cells and used in many studies on coagulation and formation of fibrin, were commercially purchased. They were incubated and grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12) medium containing 10% fetal bovine serum (12483012, Invitrogen, Carlsbad, CA, USA) at 37 ° C in 5% carbon dioxide atmosphere with 95% humidity. The cells grown in 25 cm2 flasks were removed from

the flask surface with 0.05% trypsin/EDTA (25200056, Invitrogen, Carlsbad, CA, USA) solution when they covered the cell culture plates. During passaging, the cells were transferred so as to be 1: 2 cell in the new passage. The culture medium was changed every two days after the passaging. It was then stored as stock by applying a cryopreservation protocol with Dimethylsulfoxide (DMSO, sc-358801 Santa Cruz Biotechnology, California, USA). After dying with Sigma branded Trypan Blue (0.05%) dye, the amount of living/dead cells was determined by Celeromics (Grenoble, France) branded cell counting device. After reaching a sufficient number of cells, the cells, 5000 cells per flask for each drug, were distributed into 5 groups (ABS, TA, MH, distilled water, distilled water-administered negative control without any administration).

Enzyme-Linked Immunosorbent Assay (ELISA) Study

The control cells and HUVEC cells, to which 1:2 and 1:10 drug doses were administered, were planted on 96-well plate so as to be 10.000 cells per plate. After 24 hours of drug administration, the cell extract was obtained.

Protocol for Cell Extract Preparation

In the study, the protocol was carried out using the ProtinEx total protein extraction solution kit (South Korea, Geneall) to measure the antibody levels in both monolayer and supernatants. Supernatants were used for ELISA plates after being measured with NanoDrop spectrophotometer. (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA)

In the ELISA study,) IL-1β (28 KHC0011, Invitrogen, Carlsbad, CA, USA), IL-6 (27KHC0062, Invitrogen, Carlsbad, CA, USA)) and TNF-α (29 KHC3012, Invitrogen, Carlsbad, CA, USA) kits were used. After the standards of the study were prepared, pipetting was performed, following the steps in the manuals of the kits. 2 hours later, the color change occurred was measured by ELISA reader with 450 nm filter. After creating a linear curve according to the standard concentrations and optical density (OD) values, the OD values of the samples were written in their place in the equilibrium on the resulting graph and their concentrations were calculated.

Results

In this study, the immunological effects of MH, ABS and TA at concentrations of 1: 2 and 1:10 on intracellular cytokines in cell extracts obtained from commercially purchased immortalized HUVEC cell lines were analyzed (Table 1). The inhibition of the drugs at these concentrations was compared with the control group.

According to the results obtained, at a concentration of 1:2, there was respectively a 3%, 28%, 14.25% increase with MH (Figure 1), 10.7%, 54%, 8% increase with ABS (Figure 2) and 9.5%, 15%, 19% increase with TA (Figure 3) in TNF-α, IL-6, and IL-1β levels. At a concentration of 1:10, there was respectively a

Table 1 Cytokine levels of different anti-hemorrhagic agents in the HUVEC cells

HUVEC cell line (1st day 24th hour)							
Cytokine	Mecsina Hemostopper		Ankaferd Blood Stopper		Tranexamic Acid		Statistical significance (p)
	1/2 dose administration	1/10 dose administration	1/2 dose administration	1/10 dose administration	1/2 dose administration	1/10 dose administration	
Tnf-a	3	-0.8	10.7	22.1	9.5	5.2	p<0,05
IL-6	28	65	54	90	15	106	p<0,05
IL-1b	14.3	15.4	8	20	19	22	p<0,05

0.8%, 65%, 15.4% increase with MH, 22.1%, 90%, 20% increase with ABS and 5.2%, 106%, 22% increase with TA in IL-6 and IL-1β levels. While there was 0,8% decrease in TNF-α levels with MH, there was a 22.1% increase with ABS and 5.2% increase with TA.

In our results, there was a significant increase in IL-1β and IL-6 levels compared to the control group with all drug administrations at both 1:2 and 1:10 concentrations ($p<0.05$). While there was a significant increase in TNF-α levels with all drug administrations at a concentration of 1:2 ($p<0.05$), there was no significant correlation with MH administration at a concentration of 1:10 ($p>0,05$), but a significant increase was found with TA and ABS administrations ($p<0,05$).

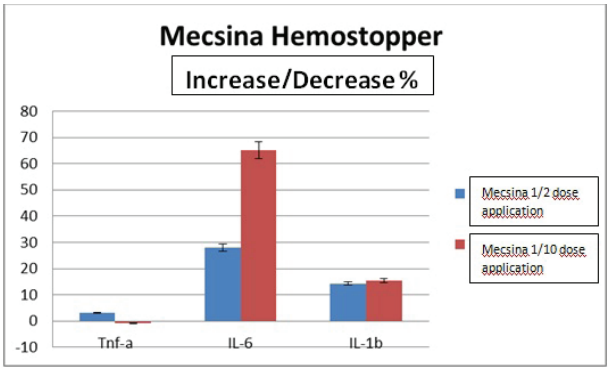

Figure 1. Demonstration of the increase or decrease rates (in percentage) of the cytokines in HUVEC cells to which Mecsina Hemostopper was administered at different doses

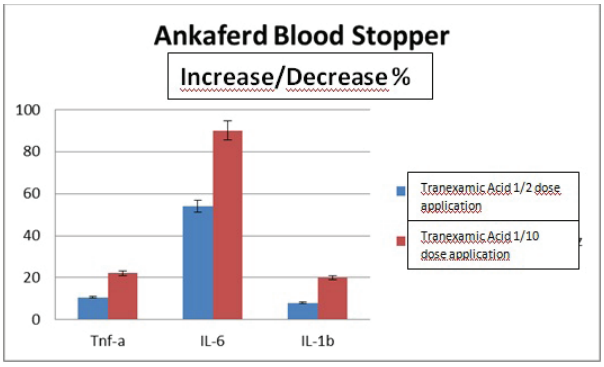

Figure 2. Demonstration of the increase or decrease rates (in percentage) of the cytokines in HUVEC cells to which Ankaferd Bloodstopper was administered at different doses

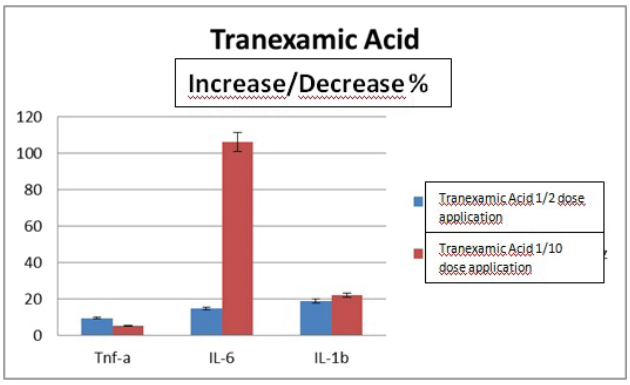

Figure 3. Demonstration of the increase or decrease rates (in percentage) of the cytokines in HUVEC cells to which Tranexamic Acid was administered at different doses

Discussion

Although a lot of active substances providing coagulation are recently available, the molecular mechanisms of some of them have not yet been fully clarified. ABS and MH, which are a completely herbal product, provide coagulation by taking haemostatic effect with erythrocyte aggregation in protein network environment independently of the classical coagulation cascade system.[3] The most defined mechanism of action for TA is that it is an anti-fibrinolytic.[7] TA is a lysine analog that competitively inhibits the plasma activation of plasminogen, thus preventing clot lysis.[8] It has reported in a study that TA had an anti-inflammatory effect in cardiopulmonary bypass.[9] As a result of experiments conducted with the HUVEC cell model, it may be possible to use anti-hemorrhagic agents with herbal active substances more actively and effectively in clinical practice, considering the fact that the investigation of genetic factors and intracellular activities is becoming more and more important. By determining whether these hemostatic agents are effective on procoagulants, anticoagulants and pro-fibrinolytic proteins, regulations in active substance doses and administration times, and improvements in potential bleeding risks may occur with the use of these agents at the most effective level possible. Within this scope, in our study, it was tried to investigate the effects of ABS, MH and TA hemostatic agents at different concentrations on intracellular cytokine levels in the HUVEC cell line and to compare them with each other.
In a study conducted on the transcription factors and

erythrocyte protein profile of ABS in HUVEC endothelium, it has been observed that ABS stopped bleeding effectively because of forming a rapid complex between the cells.[10] It has been also reported in the same study that ABS administration at low doses was not only effective in the outer part of the cells, but also very effective on endothelial cells inside the cell. It has been reported that oral systemic ABS was hematologically and biochemically reliable in rabbits in the short term.[11]According to our study results, less inflammatory cytokine release was observed in cells to which ABS was administered at low concentrations. This seems to be consistent with the previous studies. Moreover, while MH at low concentrations produced less inflammatory cytokines than both ABS and TA, interestingly, MH at higher concentrations caused minimum inflammation.

In a study investigating the effect of aprotinin and TA on surgical bleeding, aprotinin has been reported to be more effective than TA, and antifibrinolytics have been found to be clinically more advantageous.[12] It has been reported that the complication incidence was higher after the use of TA.[13] In studies evaluating plasma cytokine concentrations, the antiinflammatory characteristics of TA have been observed, and the cytokines have been indicated to play an important role in the inflammatory response after surgical procedure.[9] In a study, TA has been indicated to cause more regulation of proinflammatory and antiinflammatory genes. It has been stated in the same study that TA suppressed the TNF-α and IL-1β release by altering gene expression only in multiple organ failure patients involving down-regulation of IL-12α and IL-17β.[14] In our study, TNF-α cytokine levels were observed to be lower in the cells, to which TA was administered at lower or higher concentrations, than the cells to which ABS was administered. Conversely, in the case of MH administration, we noted that TNF-α levels were considerably lower than both TA and ABS.

In a study, the down- regulation of inhibin-beta that is released together with TNF-α has been noted at the onset of sepsis before IL-6 of TA administration.[15] In parallel with this, as in our results, IL-6 has been found to be higher than TNF-α levels in the cells to which ABS and TA and most significantly MH were administered. TNF-α and IL-1β are known as potent inflammatory cytokines that are up-regulated during inflammation, and play an important role in improving wound healing.[16] In a study, in which ABS was used in fracture healing, while TNF-α and IL-1β values were increased on the 7th day after the fracture compared to the control, the levels of both cytokines were decreased on the 21st and 45th days after fracture.[17] Similarly, in our study, TNF-α and IL-1β levels were significantly increased in the cell groups to which ABS and TA were administered, even at different concentrations, whereas IL-1B showed a significant increase in cell groups to which MH was administered, but there was no significant increase in TNF-α levels.

Conclusıon

In conclusion, it has been observed that different anti-hemorrhagic agents administered at different concentrations in the HUVEC cell groups produced a significant increase in intracellular cytokine levels. Considering the results, we can state that the MH administration is a more effective anti-hemorrhagic agent than ABS and especially TA administrations. The use of MH, a new generation of anti-hemorrhagic agent, may be appropriate to be able to produce less inflammatory effects in peroperatively and/or postoperatively, however we think that the importance of our statement in clinical practice will be increased by more advanced and more complex studies.

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