# Biphasic effects of ankaferd blood stopper on renal tubular apoptosis in the rat partial nephrectomy model representing distinct levels of hemorrhage

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## ABSTRACT

**الأهداف:** دراسة تأثير علاج أنكافارد لوقف نزيف الدم (ABS) على الموت الخلوي المبرمج لأنبوب الكلية وكذلك تأثيره على إنزيمات سينثاز أكسيد النيتريك البطاني (eNOS)، وسنثاز أكسيد النيتريك القابل للتحريض (iNOS)، والعامل التنشيطي للموت الخلوي بروتياز رقم1 (Apaf–1) وذلك في نفس الكلية بعد إجراء عملية استئصال جزئية للكلية لمجموعة من الجراذ.

**الطريقة:** لقد تم إجراء الدراسة في عام 2009م بمستشفى أنقرة للأبحاث والتدريب، معمل الحيوانات الإختباري، أنقرة، تركيا. لقد قمنا بتقسيم 24 جرذ من النوع ويستر إلى أربع مجموعات. أخضعنا المجموعة الأولى (ج1) إلى استئصال الكلية الجزئي (PN) مع مراقبة هيليوم الكلية كما يتم في عمليات الاستئصال التقليدية، في حين تم تطبيق عملية الاستئصال التقليدية على المجموعة الثانية (ج2) مع استخدام علاج (ABS). أما المجموعة الثالثة (ج3) فتم استخدام علاج (ABS) للجزء الوظيفي للكلية وللقناة الجامعة مع مراقبة هيليوم الكلية (لم يتم علاجها جراحياً)، وتم إجراء عملية استئصال جزئي للكلية مع استخدام علاج (ABS) مع المجموعة الرابعة (ج4) ولكن من دون مراقبة هيليوم الكلية. لقد تم اللجوء لمحلول علاج (ABS) بجرعة مقدارها (1سم مكعب) لوقف نزيف الأنسجة الكلوية أثناء الجراحة. توفيت كل الجراذ في الشهر الأول في حين تم تحديد أسباب الموت الخلوي لأنبوب الكلية.

**النتائج:** يتكون تركيب الخلايا التالفة في (ج1) من 20% (iNOS)، و20% (eNOS)، و10% (Apaf–1). أما (ج2) فكانت النسبة 10% من (iNOS)، و 20% من (eNOS)، و5% من (Apaf–1). ووصلت نسبة الخلايا التالفة في (ج3) إلى 40% من (iNOS)، و50% من (eNOS)، و30% من (Apaf–1)، وفي (ج4) كانت النسبة 5% من (iNOS)، و5% من (eNOS)، و3% من (Apaf). لم يكن هناك نقصاً واضحاً في عدد الخلايا التالفة في المجموعات ج2 وج3 وج4 والتي تم علاجها بـ (ABS). ظهرت أعلى نسبة للموت الخلوي في ج3 ورافق ذلك التهاباً حاداً، في حين ظهرت أقل نسبة للموت الخلوي في ج4 وهي المجموعة التي لا تعاني من إفقار الكلية. لقد كان لعلاج (ABS) تأثيراً مرحليا مزدوجاً على الموت الخلوي المبرمج وذلك منذ بداية التجربة.

**خاتمة:** يؤدي النزيف الحاد لأنبوب الكلية إلى إضعاف عمل علاج (ABS) في مجرى الدم وذلك بوجود جزيئات الموت الخلوي. وهذا يشير إلى أن علاج (ABS) يعمل كمساعد موضعي من أجل تحفيز استجابة الجسم.

**Objectives:** To investigate the effect of Ankaferd Blood Stopper (ABS), on renal tubular apoptosis and on expressions of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and apoptosis protease-activating factor-1 (Apaf-1) in the ipsilateral kidney after an experimentally formed partial nephrectomy in a rat model.

**Methods.** The study was performed in 2009 at the Ankara Training and Research Hospital, Animal Laboratory Center, Ankara, Turkey. We divided 24 Wistar rats into the following 4 groups. Group I (GI) - partial nephrectomy (PN) with hilar control as the conventional technique, Group II (GII) – the conventional technique with ABS, Group III (GIII) - received ABS application to the renal parenchyma and collecting duct with hilar control (non-sutured group). Group IV (GIV) - PN and ABS were performed without hilar control. The ABS solution (1 cc) was applied during the surgery to stop bleeding from resected renal tissue. At first month, all rats were sacrificed. Renal tubular apoptosis was investigated.

**Results:** The mean percentage of apoptotic cell counts in GI were 20% iNOS, 20% eNOS, and 10% Apaf-1. In GII they were 10% iNOS, 20% eNOS, 5% Apaf-1, in GIII they were 40% iNOS, 50% eNOS, 30% Apaf-1, and in GIV they were 5% iNOS, 5% eNOS, and 3% Apaf-1. There was no significant decrease in apoptotic cells in GII, GIII, and GIV, to which we applied ABS. The highest percentage of apoptosis was shown in GIII accompanied by significant inflammation. The lowest percentage was determined in GIV, the non-warm ischemia group. The ABS has a dual biphasic de novo effects on apoptosis.

**Conclusion:** The challenge of severe hemorrhage in the renal tubular cellular micro-environment causes ABS-induced down-regulations in the expressions of apoptotic molecules, indicating that ABS may act as a topical biological response modifier.

***Saudi Med J 2010; Vol. 31 (8): 864-868***

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*Received 10th April 2010. Accepted 20th June 2010.*

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Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum, and Urtica dioica are major ingredients of Ankaferd Blood Stopper (ABS), which is a medicinal plant extract. It has historically been used as a hemostatic agent in Turkish folkloric medicine among other traditional methods.[1] The basic mechanism of action for ABS is the formation of an encapsulated protein network that provides focal attachment points for very rapid (<1 second) vital erythrocyte aggregation.[1] Previous proof of erythrocyte aggregation at the tissue level was shown in a rat partial nephrectomy model.[1] Ankaferd-induced protein network formation with blood cells, particularly erythrocytes, covers the primary and secondary hemostatic system without disturbing individual coagulation factors.[2-5] The in vitro anti-infective and anti-neoplastic actions of Ankaferd have also been demonstrated.[6-8] The molecular components of the Ankaferd-induced hemostatic network include the vascular endothelium, proteins, and blood cells.[2] Partial nephrectomy is a conventional approach for the management of small renal masses. Bleeding and ischemic renal damage due to the warm ischemia period are the most important complications following the surgery.[9] The association between surgical damage to renal tubular cells, bleeding, and wound healing, in relation to Ankaferd-induced hemostatic effects, are 3 main courses that shall be investigatedin this study. The hemostatic effect of Ankaferd on renal tissue in a partial nephrectomy (PN) model was extensively investigated.[1] In our previous study, we observed significant glomerular necrosis and calcification due to conventional PN, indicating the renal tissue damage histopathologically.[1] Accordingly, the aim of this study is to assess ultrastructural changes in renal tubular cells (namely, the main part of the collecting duct system of the kidney) following PN. Apoptosis is a pathobiological process that is responsible from the programmed cell death, indicating tissue damage.[10] We will also focus on the effect of Ankaferd on renal tubular apoptosis. For this aim, we have incorporated unique markers of apoptosis into our previously described PN model testing the Ankaferd hemostatic effect.[1] Nitric-oxide synthase (NOS), the enzyme responsible for the production of NO, has 3 major isoforms, neuronal, endothelial, and inducible NOS. Although endothelial NOS (eNOS) is calcium ion (Ca2+) dependent and is expressed in many tissues, including the testes, inducible NOS (iNOS) is Ca2+ independent and is induced in tissues after exposure to inflammatory cytokines or ischemia.[11] The increasing level of NOS expression leads to excessive NO production, and NO at high concentrations may cause cell death.[11] We, therefore, investigated the effect of Ankaferd, a novel hemostatic agent, on renal tubular apoptosis, and also on expressions of eNOS, iNOS, and apoptosis protease-activating factor-1 (Apaf-1) in the ipsilateral kidney after an experimentally formed PN in a rat model.

**Methods.** The study was performed in 2009 at the Ankara Training and Research Hospital, Animal Laboratory Center, Ankara, Turkey. Twenty-four Wistar rats were divided into 4 groups and underwent right lower pole PN. The PN techniques of the groups were determined as: Group I (GI) underwent right PN with hilar vascular control - intracorporeal suturing of renal parenchyma and collecting duct as conventional PN. Group II (GII) underwent conventional PN with application of ABS. Group III (GIII) received ABS application to the renal parenchyma and collecting duct with hilar control (non-sutured group). Group IV (GIV) underwent PN and application of ABS without hilar control (non-sutured group). Warm ischemia time (WIT) is used for the duration of the hilar clamping period. Warm ischemia during the PN was applied to GI, GII, and GIII, but not to GIV. The ABS solution (1 cc) was separately used for GII, GIII, and GIV to achieve hemostasis as a standard technique. The ABS was commonly applied during renal hilar control. The application of the drug was performed in a compressive fashion using a pad. After one month, the rats were sacrificed and right nephrectomy was performed to evaluate histopathologic results. The evaluations of specimens were performed following the surgery. Histological sections were evaluated in a blind fashion by 2 pathologists by light microscopy. Renal tubular apoptosis was investigated by the expression of eNOS, iNOS, and Apaf-1 in the ipsilateral kidney after an experimentally formed PN in a rat model. The pathologists counted the apoptotic cells manually. A midline incision was made in the abdomen after sterile prep and draping following the administration of a single dose of prophylactic broad-spectrum antibiotics. The right kidney was completely mobilized. The right renal artery and vein (hilar control) were then occluded with Rommel vascular clamp, and the lower 1/3 of the right kidney was resected in guillotine fashion with a single stroke of an amputating knife. The surgeon with assistant immediately began one of the 4 randomly determined, reparative procedures. After the procedure, sponges were used to collect the clothes and blood. The main evaluated parameter was the renal tubular apoptotic cell index in groups of warm ischemia; (+) and (-). However, each group was compared with the conventional PN group (GI) regarding the apoptotic cell differentiation.

**Results.** All specimens were histopathologically evaluated on the date of sacrifice. The mean percentage

of apoptotic cell counts in histopathologic view (100x10) in GI was 20% iNOS, 20% eNOS, and 10% Apaf-1 (**Figure 1**). The percentages for GII were 10% iNOS, 20% eNOS, 5% Apaf-1 (**Figure 2**), for GIII were 40% iNOS, 50% eNOS, 30% Apaf-1 (**Figure 3**), for GIV were 5% iNOS, 5% eNOS, and 3% Apaf-1 (Figure 4). In GII and GIII in which warm ischemia was applied, increased apoptosis was demonstrated while in the non-warm ischemia group, decreased renal tubular apoptosis was demonstrated in comparison with the control group. Among the GII, GIII, GIV 'Ankaferd-applied' groups, only the GIV non-ischemia group was associated with an increased level of bleeding, the less apoptosis was detected.

**Discussion.** In this study, we observed that Ankaferd has dual diverse dynamic actions on renal tubular apoptosis based on the level of hemorrhage. When the bleeding was associated with the surgery of PN it is mild or moderate, and ABS can initially increase renal tubular apoptosis. On the contrary, during the increased amount of massive bleeding from the kidney tissue, Ankaferd decreases apoptosis in renal tubular cells (**Figure 4**). These results indicate that Ankaferd modulates the cellular apoptotic responses to hemorrhagic stress as well as its hemostatic hemodynamic activity. The paradoxical biologic actions of ABS on apoptosis in renal tubular cells seem to be related to its vasodynamic actions. The initial vascular dynamic response to ABS is vasoconstriction, while the late effect is vasodilatation, quite the reverse.[12] There is a close relationship between apoptotic cascades, particularly p53, and vasospasm during tissue hemorrhage.[13] Ankaferd can increase the activity of pro-apoptotic p53 and anti-apoptotic YY1 as demonstrated in human vascular endothelial cells.[13] Therefore, enhanced renal tubular apoptosis could be related to initial vasospasm and increased p53 activity due to ABS. Immediate enhanced expression of pro-hemostatic apoptotic molecules and down-regulated anti-coagulant proteins upon the exposure of ABS are compatible with the sudden anti-hemorrhagic efficacy of Ankaferd previously tested in experimental,[3,4,15] and clinical backgrounds.[16-19] However, when the amount of bleeding reaches massive levels threatening tissue vitality, the vasodilator actions of ABS12 and promotion

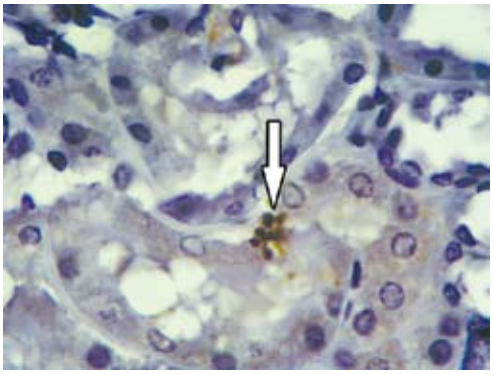

**Figure 1 -** The immunohistochemical appearance of renal apoptotic cells-control group (apoptosis protease-activating factor-1 100x10).

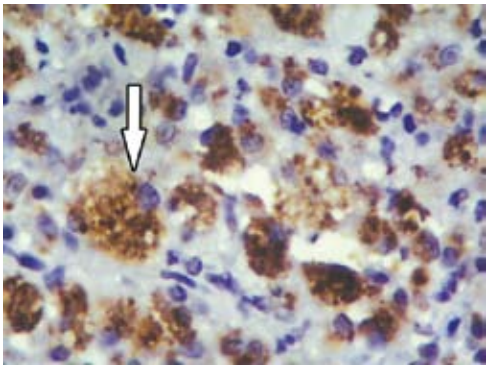

**Figure 3 -** The immunohistochemical appearance of renal apoptotic cells-Group III (apoptosis protease-activating factor-1, 100x10).

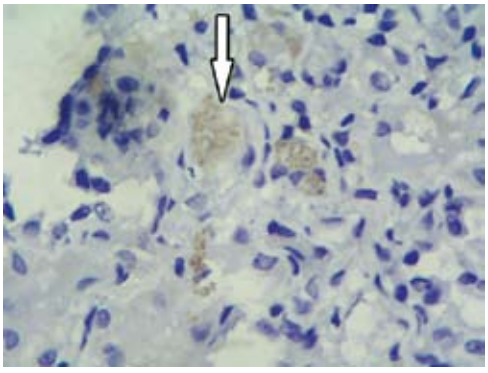

**Figure 2 -** The immunohistochemical appearance of renal apoptotic cells-Group II (endothelial nitric oxide synthase, 100x10).

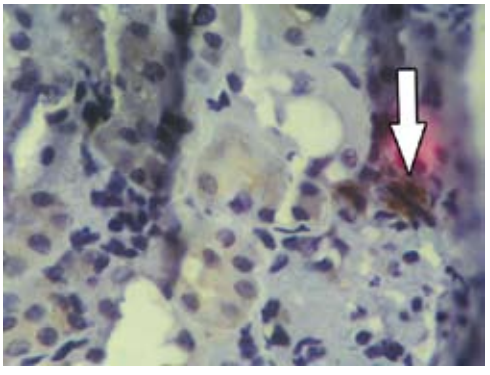

**Figure 4 -** The immunohistochemical appearance of renal apoptotic cells-Group IV (apoptosis protease-activating factor-1 100x10).

of molecules associated with resistance to apoptotic stimuli such as YY114 could take place. The process of apoptosis is located at the critical crossroads of important pathobiological events, including bleeding, trauma, and sepsis.[20-22] The fate of living tissue cells are determined by apoptotic responses during those catastrophic alterations.[23-25] Several pharmacological agents and resuscitation attempts can modulate apoptosis to help tissue healing associated with those difficult pathologies such as trauma and sepsis.[26-28] Based on the results of this present study, ABS could also affect the apoptotic responses and may help tissue healing as well as its anti-hemorrhage effects. Preliminary observations disclosed that ABS may have a "wound healing" function in different clinical states, such as infected dental areas, rectal ulcers, penile fracture or neoplastic lesions.[18,29,30] Ankaferd, besides its hemostatic activity, may also inhibit the growth of bacteria.[6,7] The anti-infectious activity of Ankaferd represents an advantage over its current clinical use, since it inhibits the growth of bacteria in the area used mainly for its hemostatic activity, such as traumatic infected wounds.[6] The antimicrobial activity of Ankaferd was tested against many pathogens. The isolates included *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter spp., Stenotrophomonas maltophilia, Methicillin-resistant Staphylococcus aureus*, methicillin resistant coagulase negative Staphylococcus, vancomycin susceptible Enterococcus and VRE. Antibacterial activities of Ankaferd against several gram positive and gram negative food and human pathogens, were also reported.[6,7] The mechanism of action regarding the anti-infective effects of ABS is currently unknown. Therefore, the inter-relationships between bacterial death and our findings on ABS-effects on apoptosis should be further researched. There are distinct important molecular components of the Ankaferd-induced hemostatic network. Vital erythroid aggregation takes place with the spectrin ankrin and actin proteins on the membrane of red blood cells. Essential erythroid proteins (Ankrin recurrent and FYVE bundle containing protein 1, Spectrin alpha, Actin-depolimerisation factor, Actin-depolimerizing factor, LIM bundle and actine binding subunit 1 isoform a, LIM bundle and actine binding subunit 1 isoform b, NADP-dependent malic enzyme, NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex, mitochondrial NADP (+) dependent malic enzyme 3, Ribulose bisphosphatecarbocsilase large chain, maturase K) and the required ATP bioenergy (ATP synthase, ATP synthase beta subunit, ATP synthase alpha subunit, ATP-binding protein C12, TP synthase H+ transporter protein, ADF, Alpha-1,2-glycosyltransferase ALG10-A) are included in the protein library of Ankaferd. Ankaferd also upregulates GATA/FOG transcription systems affecting erythroid functions and urotensin II.[31,32] Thus, the unique protein content of Ankaferd and its effects on apoptosis should also be investigated as a possible cause-and-effect relationship in further upcoming studies. In this study, we preferred to show the apoptotic activity using 3 main markers, namely, Apaf-1, eNOS, and iNOS, which were available at our Molecular Laboratory of Pathology Department, and to focus on the morphologic evidence demonstrating the apoptotic activity. It was also a cost-effective choice to avoid using extra markers (such as annex in Van and so forth) to evaluate the apoptosis. The restrictive factor of this study could be that the expression of eNOS, iNOS, and Apaf-1 were not detected on a molecular biology level (mRNA and protein expression) due to technical reasons. However, the limited number of rats in the groups are the limitations of this study. The rat PN model is a similar operation to PN in humans. The steps were the same, however, the small kidney size and related lower amount of bleeding from the resected area are restrictive factors. It is exact that these findings have been related with the apoptotic effects of ABS demonstrated in the literature previously[13,14] so, not restricted but resistant factor of this study could be mentioned to research a novel agent regarding the different effect of apoptosis, which was the different from the hemostasis. We believe that the effect of ABS has a similar histopathologic effects in bigger kidneys such as the porcine or calf kidney.

In conclusion, ABS has dual biphasic de novo effects on apoptosis. The challenge of severe hemorrhage in the renal tubular cellular micro-environment causes ABS-induced down-regulation in the expressions of apoptotic molecules, indicating that Ankaferd may act as a topical biological response modifier. Since ABS is currently being developed in basic and clinical grounds, those novel observations should prompt further research focusing on the pleiotropic effects of this unique hemostatic agent.

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