J Vector Borne Dis 50, June 2013, pp. 133–136

The role of heparan sulphate in pathogenesis of Crimean-Congo hemorrhagic fever disease

Fatma Mutlu Kukul Guven[1], Hüseyin Aydin[2], Ali Kaya[3], Aynur Engin[4], V. Kenan Celik[2], Ilhan Korkmaz[1], Bahri Atli[5] & Dilara Icagasioglu[3]

[1]*Department of Emergency Medicine;* [2]*Department of Biochemistry;* [3]*Department of Pediatrics;* [4]*Department of Infectious Disease and Clinical Microbiology Medicine, Cumhuriyet University School of Medicine, Swas;* [5]*Emergency Service, Karabuk State Hospital, Karabuk, Turkey*

ABSTRACT

Background & objectives: Crimean-Congo hemorrhagic fever (CCHF) is a viral infection typically transmitted by tick bite. This study is to define the level of heparan sulphate (HS) in serum/urine since HS may play a role in the pathogenesis of hemorrhagic events in the patients with CCHF.

Methods: In this study, the patient group consisted of 79 cases with a positive diagnosis of CCHF according to PCR/ELISA outcome among the patients referred to Cumhuriyet University, School of Medicine in 2010. A total of 81 volunteers who had not any viral or metabolic disease were enrolled as the control group. The blood samples were centrifuged, and the serum and urine samples obtained were stored at –80°C until they were studied. Then, these samples were simultaneously dissolved, and HS level was spectrophotometrically measured using glycosaminoglycans specific 1–9, dimethyl-methylene blue (DMMB) stain.

Results: A statistically significant increase in the HS_{serum} values was found both in the individuals under and above 16 yr old in the patient groups compared to the controls ($p < 0.05$). Also there was a statistically significant increase in the urine levels of HS in the cases >16 yr old compared to the controls ($p < 0.05$).

Interpretations & conclusion: Increase of the serum/urine levels of HS was though to be due to vascular endothelium damage and to liver injury as well as vascular endothelium damage in the patients who died. Further, comprehensive studies are needed to demonstrate whether the serum/urine levels of HS are correlated to liver and vascular endothelium damage and prognosis of the disease.

Key words Crimean-Congo hemorrhagic fever; hemorrhage; heparan sulphate; viral infections

INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) is an infectious disease caused by Nairovirus genus from Bunyaviridae family. It begins with fever, severe headache, weakness, nausea, vomiting and progresses with bleeding in different parts of the body. The bleeding findings emerge as subcutaneous bleeding, nasal bleeding, gingival bleeding and visceral bleeding[1].

CCHF virus is known to be infected by mononuclear phagocytic cells, liver cells and endothelial cells; however, its pathogenesis is not fully clarified[2–4]. The disease courses subclinically in some cases, while may result in mortality in other cases with developing shock and disseminated intravascular coagulopathy (DIC). Although genetic strains similar to CCHF have been detected in Turkey[5], the cause of the clinical differences between the cases is not known.

Heparan sulphate (HS) is a proteoglycan synthesized in the endothelial cells and found in the extracellular part of the plasma membrane. HS interacts with growth factors such as sulphate basic fibroblast growth factor (bFGF), collagen, laminin and fibronectin better supports the integrity of the vessels[6]. HS is considered as the initial receptor for numerous viruses. A number of enveloped viruses bind to HS, while few number of the bound non-enveloped viruses is limited[7]. In this study, serum/urine levels of HS in the infected patients with CCHF that is an enveloped virus were studied and its impacts on the hemorrhagic events were investigated.

MATERIAL & METHODS

In this study, serum samples are taken from the patients referred to the Cumhuriyet University School of Medicine, Department of Emergency Medicine, Department of Infectious Diseases, Department of Clinical Microbiology, and the Department of Pediatrics with a tick bite history/pre-diagnosis of CCHF in 2010. Of these samples, 79 cases with a positive diagnosis of CCHF according to the PCR/ELISA results from Refik Saydam Institute of Public Health were selected for the study. Age

and gender matched, 81 volunteers who had not any viral or metabolic disease were enrolled as the control group. The serum samples of both the groups were centrifuged at 4000 rpm for 5 min using Hettich Universal 30 centrifuge device. The serum and urine samples were stored at –80°C until the study was carried out. All the samples were simultaneously dissolved, and HS level was spectrophotometrically measured using glycosaminoglycans specific 1–9, dimethyl-methylene blue stain and standard[8].

This study was conducted by the approval of Cumhuriyet University School of Medicine Ethics Committee. Informed consent was obtained from the patients and healthy volunteers.

Statistical analysis

Data obtained from the study were entered to the SPSS 15.0 software. Data were analysed using chi-square, *t*-test for two independent variables; and Pearson's correlation test and receiver operating characteristic (ROC) analysis. Appropriate values of $p < 0.05$ were considered statistically significant. All the variables were expressed as mean ± standard deviation.

RESULTS

A total of 79 CCHF patients and 81 volunteer individuals were enrolled to this study. The differences between the controls and patient groups in terms of age and gender were not found statistically significant ($p > 0.05$). Demographic features of the controls and patient groups are given in Table 1.

A statistically significant increase was observed in the levels of HS_{serum} in the persons under and >16 yr-old both in the patients according to control groups ($p < 0.05$) (Table 2). Urine levels of HS could not be evaluated in those <16 yr-old since the samples could not be collected. However, levels of HS_{urine} were found statistically significant higher in those >16 yr-old than in the controls ($p < 0.05$) (Table 2).

CCHF patients under the age of 16 yr could not be included for Swanepoel criteria and ROC analysis due to information of the patients could not be attained. According to Swanepoel *et al*[9] criteria, 26 patients were defined as severe. These patients had hemorrhagic events. The types of hemorrhagic events were mostly epistaxis, vaginal bleeding and gastrointestinal bleeding, gingival bleeding, petechiae, and hematomas in different parts of the body. ROC analysis was performed for calculating the cut-off value in patients with bleeding for HS_{serum}. The size of the area under the curve was found to be 0.662 ($p = 0.047$, $p < 0.05$). This area of 95% confidence limits was found to be 0.514 to 0.813. Accordingly, in patients with bleeding, the cut-off value of HS_{serum} was obtained as 13.25. The sensitivity value for this point was found as 0.769, the specificity value 0.526. ROC analysis related to this subject in shown is Fig. 1.

Table 1. Demographic characteristics of the patients

	Age (yr)	Male (%)	Female (%)
<16 yr			
Control (n=30)	10.20 ± 4.44	23 (76.7)	7 (23.3)
Patient (n=28)	10.14 ± 4.03	22 (78.6)	6 (21.4)
>16 yr			
Control (n=51)	47.22 ± 15.63	29 (56.9)	22 (43.1)
Patient (n=51)	50.11 ± 16.55	28 (54.9)	23 (45.1)

Table 2. Levels of heparan sulphate in the patient and control group

Measurements	Control	Patient	*P*
<16 yr	n = 30	n = 28	
HS_{serum} (mg/mL)	10.30 ± 2.72	20.34 ± 7.57	0
>16 yr	n = 51	n = 51	
HS_{serum}(mg/mL)	12.48 ± 3.24	18.55 ± 8.07	0
HS_{urine}(mg/mL)	3.72 ± 1.08	8.69 ± 3.49	0

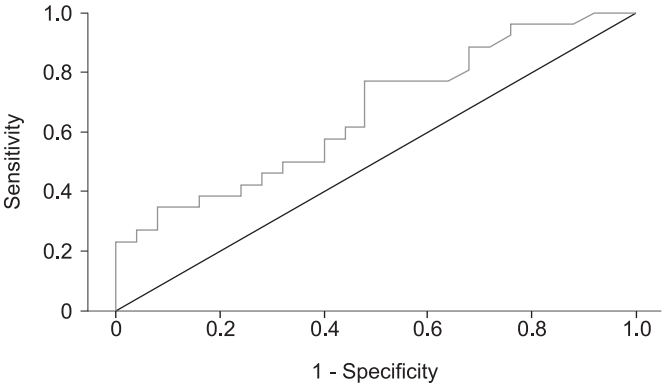

Fig. 1: ROC analysis for cut-off value of HS_{serum} in patients with bleeding.

In living patients, between ALT, AST and HS_{serum} negative way, a correlation coefficient was found. This correlation coefficient is statistically insignificant and very small. In the patients who died, between HS_{serum} with international normalised ratio (INR), ALT, AST, ALP and LDH was found with a statistically insignificant positive correlation. Between ALT, AST and LDH moderate correlations were found. The reason why the correlation insignificant can be explained by the low number of subjects. The comparison of level of HS with liver enzymes and INR in the group of patients and controls is shown in Fig. 2.

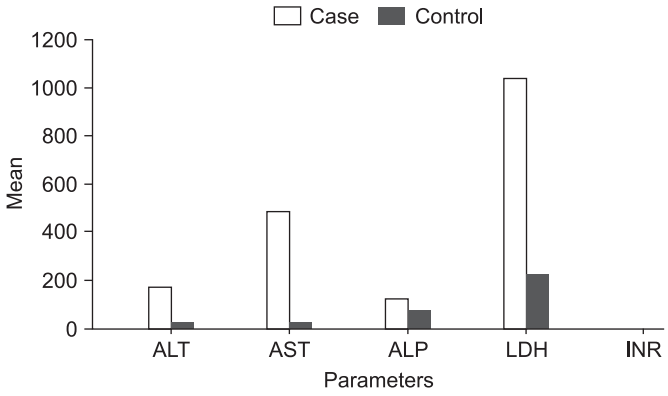

Fig. 2: The comparison of level of heparan sulphate with liver enzymes and INR in the group of patient and control.

DISCUSSION

In this study, a statistically significant increase defined in the serum/urine levels of HS in the patients with CCHF compared to the control group was found. HS is widely found on the cell surfaces and extracellular matrix of the mammals. This contributes to many biological processes such as blood coagulation, viral infections, embryonic development, wound healing and suppression of tumor growth [10–15]. HS causes tumor metastasis in the organs such as liver, kidney and spleen by interaction with cancer cells, platelets and endothelial cells[16, 17].

HS binds to many ligands and microorganisms such as growth factors, cytokines and chemokines. Binding to HS on the cell surface have a critical importance for many viruses to enter the cell[10]. HS synthesized in the endothelial cells is found in the extracellular part of the plasma membrane and helps to preserve the integrity of the vessels. It is one of the most common glycosaminoglycans found in the vascular endothelium. It is normally found in the blood with a very low amount. HS has an anticoagulant effect like heparin, by inhibiting antithrombin-III (AT III)[7, 12, 18, 19]. Endogenous HS stimulates heparin AT III, which is similar to itself in structure to inhibit the occurrence of thrombin[20]. Therefore, increase of the serum levels of HS associted with various factors leads to bleeding.

CCHF is a zoonotic character infection which affects many organs and systems, characterized by bleeding and liver function abnormalities and takes place among the viral hemorrhagic fever syndromes[21].The pathogenesis of viral hemorrhagic fevers shows similarity[22, 23]. Thus, understanding of the pathogenesis is important in planning of the treatment. Although pathophysiology may differ for each factor of the viral hemorrhagic fever, major common features such as microvascular disease and disruption of hemostasis come to the forefront[23, 24].

Capillary endothelium is directly or indirectly targeted in CCHF disease as with all viral hemorrhagic fevers. CCHF virus mainly activates mononuclear cells and leads to release of various cytokines and chemokines. These chemokines indirectly target the endothelium. Furthermore, the destruction occurs also as a result of the direct infection of endothelium. Detection of the tubuloreticular bodies related to the virus in the endothelial cells suggests dysfunctions developed in the capillary vessels lead to clinical and pathological changes during the disease. Furthermore, increase of the capillary permeability and coagulation dysfunctions cause a tendency to bleed[21, 25, 26].

In this study, high serum levels of HS in the patients with CCHF support efficiency of HS in the pathophysiology of these events and accordingly disruption of the vascular integrity. The levels of enzymes released by liver and muscles (AST, ALT, LDH) remain higher in the patients with a severe course of the disease[1].

Major markers of a poor prognosis of the disease resulting in mortality compared to the other patients may include a deep leukopenia and thrombocytopenia, high levels of alanine aminotransferase and aspartate aminotransferase and prolongation of the partial thromboplastin time[4, 27–29]. There was a strong same directional correlation between the serum levels of HS and the levels of ALP, ALT, AST, LDH and INR in the patients who died from CCHF, although this was not found statistically significant since the number of the case was insufficient.

In conclusion, we believe HS values found high in CCHF disease will clarify pathophysiology of the hemorrhagic findings. Further, comprehensive studies are needed on this subject.

ACKNOWLEDGEMENTS

The authors thank Refik Saydam, Hygiene Center of Ankara, Turkey for testing the serum samples and to colleagues from the Turkish Ministry of Health, Turkey for their contributions.

Conflict of interest: No conflict of interest to declare.

REFERENCES

1. Ergönül O. Kirim Kongo Kanamali Atesi. Klinik Gelisim 2010; 23: 3 Salgin Hastaliklar 14–27. Available from: http://www.klinikgelisim.org.tr/kg_23_3/4.pdf (In Turkish).
2. Burt FJ, Swanepoel R, Shieh WJ, Smith JF, Leman PA, Greer PW, *et al*. Immunohistochemical and *in situ* localization of Crimean-Congo hemorrhagic fever (CCHF) virus in human tissues and implications for CCHF pathogenesis. *Arch Pathol Lab*

Med 1997; *121*(8): 839–46.
3. Whitehouse CA. Crimean-Congo hemorrhagic fever. *Antiviral Res* 2004; *64:* 145–60.
4. Ergonul O. Crimean-Congo haemorrhagic fever. *Lancet Infect Dis* 2006; *6:* 203–14.
5. Midilli K, Gargili A, Ergonul O, Sengoz G, Ozturk R, Barkar M, *et al*. Imported Crimean-Congo hemorrhagic fever cases in Istanbul. *BMC Infect Dis* 2007; *7:* 54.
6. Han J, Mandal AK, Hiebert LM. Endothelial cell injury by high glucose and heparanase is prevented by insulin, heparin and basic fibroblast growth factor. *Cardiovasc Diabetol* 2005; *4*(1): 12.
7. Escribano-Romero E, Jimenez-Clavero MA, Gomes P, García-Ranea JA, Ley V. Heparan sulphate mediates swine vesicular disease virus attachment to the host cell. *J Gen Virol* 2004; *85*(3): 653–63.
8. De Jong JG, Wevers RA, Laarakkers C, Poorthuis BJ. Dimethylmethylene blue-based spectrophotometry of glycosaminoglycans in untreated urine: A rapid screening procedure for mucopolysaccharidoses. *Clin Chem J* 1989; *35*(7): 1472–7.
9. Swanepoel R, Gill DE, Shepherd AJ, Leman PA, Mynhardt JH, Harvey S. The clinical pathology of Crimean-Congo hemorrhagic fever. *Rev Infect Dis* 1989; *11* (Suppl 4): S794–S800.
10. Liu J, Thorp SC. Cell surface heparan sulfate and its roles in assisting viral infections. *Med Res Rev* 2002; *22:* 1–25.
11. Rosenberg RD, Shworak NW, Liu J, Schwartz JJ, Zhang L. Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? *J Clin Invest* 1997; *100* (Suppl ii): 67–75.
12. Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, *et al*. Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999; *68:* 729–77.
13. Esko JD, Lindahl U. Molecular diversity of heparan sulfate. *J Clin Invest* 2001; *108:* 169–73.
14. Alexander CM, Reichsman F, Hinkes MT, Lincecum J, Becker KA, Cumberledge S, *et al*. Syndecan-1 is required for Wnt-1-induced mammary tumorigenesis in mice. *Nat Genet* 2000; *25*(3): 329–32.
15. Shukla D, Spear PG. Herpes viruses and heparan sulfate: An intimate relationship in aid of viral entry. *J Clin Invest* 2001; *108*(4): 503–10.
16. Varki NM, Varki A. Heparin inhibition of selectin-mediated interactions during the hematogenous phase of carcinoma metastasis: Rationale for clinical studies in humans. *Semin Thromb Hemost* 2002; *28*(1): 53–66.
17. Smorenburg SM, Van Noorden CJ. The complex effects of heparins on cancer progression and metastasis in experimental studies. *Pharmacol Rev* 2001; *53*(1): 93–105.
18. Capila I, Linhardt RJ. Heparin-protein interactions. *Angew Chem Int Ed Engl* 2002; *41*(3): 391–412.
19. Damus PS, Hicks M, Rosenberg RD. Anticoagulant action of heparin. *Nature* 1973; *246*(5432): 355–7.
20. Griffin JH. Control of coagulation reactions. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U, editors. *Williams Hematology*, XI edn. New York, USA: MCGraw-Hill 2001; p. 1435–51.
21. Bakir M, Elaldi N, Kirim-Kongo, Hemorajik Atesi, Ankem Dergisi. Available from: *http://www.ankemdernegi.org.tr/ANKEMJOURNALPDF/ANKEM_20_Ek2_227_231.pdf* (in Turkish).
22. Bausch DG, Ksiazek TG. Viral hemorrhagic fevers including hantavirus pulmonary syndrome in the Americas. *Clin Lab Med* 2002; *22:* 981–1020.
23. Bray M. Comparative pathogenesis of Crimean-Congo hemorrhagic fever and ebola hemorrhagic fever. In: Ergonul O, Whitehouse CA editors. *Crimean-Congo hemorrhagic fever: A global perspective*. Dordrecht (NL) Springer 2007; p: 221–31.
24. Peters CJ, Zaki SR. Overview of viral hemorrhagic fevers. In: Guerrant RL, Walker DH, Weller PF, editors. *Tropical infectious diseases: Principles, pathogens, and practice*. Philadelphia, PA: Churchill Livingstone 2006; p. 726–33.
25. Uludag Üniversitesi Veterinerlik Fakültesi. Kirim Kongo hemorrhagic fever: Viral etiyoloji. Available from: *www.veteriner.uludag.edu.tr- Erisim* 12.07.2006. (in Turkish).
26. Türk Enfeksiyon. Web Sitesi. Kirim Kongo Kanamali Atesi. Available from: *http://www.infeksiyon.org, Erisim* (Accessed on July 12, 2006).
27. Ergonul O, Celikbas A, Dokuzoguz B, Eren S, Baykam N, Esener H. Characteristics of patients with Crimean-Congo hemorrhagic fever in a recent outbreak in Turkey and impact of oral ribavirin therapy. *Clin Infect Dis* 2004; *39:* 284–7.
28. Çevik M. Kirim-Kongo Kanamali Atesi: Klinik özellikleri. *Klimik Derg* 2004; *17:* 59–61. Available from: *http://www.klimikdergisi.org/sayilar/49/59-61.pdf* (in Turkish).
29. Ergonul O, Celikbas A, Baykam N, Eren S, Dokuzoguz. B Analysis of risk-factors among patients with Crimean-Congo haemorrhagic fever virus infection: Severity criteria revisited. *Clin Microbiol Infect* 2006; *12:* 551–4.

Correspondence to: F. Mutlu Kukul Guven, Department of Emergency Medicine, Cumhuriyet University School of Medicine, 58140 Sivas, Turkey.
E-mail: mutlukukul@gmail.com

Received: 1 August 2012 *Accepted in revised form:* 3 March 2013