NOTE *Internal Medicine*

Evaluation of Neutrophil Function during Hemodialysis Treatment in Healthy Dogs under Anesthesia with Sevoflurane

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(Received 1 November 2013/Accepted 24 July 2014/Published online in J-STAGE 21 August 2014)

ABSTRACT. This study evaluated the number and function of neutrophils during 3 hr of hemodialysis in healthy dogs under anesthesia. Isolated neutrophils were used to assess neutrophil adhesion, phagocytosis and the oxidative burst. At 0.5 and 3 hr after the start of hemodialysis treatment, there was a decrease in neutrophil number. The phagocytic ability of neutrophils was decreased 3 hr after the start of hemodialysis. In conclusion, this study demonstrated that hemodialysis reduces the number and phagocytic ability of neutrophils during treatment. However, these changes recover within 24 hr of hemodialysis.

KEY WORDS: canine, hemodialysis, immune function, neutrophil function

doi: 10.1292/jvms.13-0541; *J. Vet. Med. Sci.* 76(11): 1539–1543, 2014

Intermittent hemodialysis (HD) is a useful treatment that improves the outcome in dogs and cats with kidney injuries that do not respond to medical management. Indications for intermittent HD include drug or toxin ingestion, acute or acute-on-chronic kidney injury and chronic kidney disease [4].

During HD, there is an early, transient decrease in the number of polymorphonuclear cells [19]. In dogs, this transient granulocytopenia occurs shortly after the beginning of HD [27]. The neutrophils that disappear from the circulation during the development of dialysis neutropenia are sequestered in the pulmonary capillaries, but the sequestration mechanism remains controversial. The most widely held theory is that activated neutrophils adhere to the endothelial walls of the pulmonary capillaries, the first vascular surface with which they come into contact after leaving the dialyzer [16].

Neutrophils, the major cellular component of the innate immune system, are pivotal in the early response to inflammation and infection. Clinical correlates of depressed neutrophil function are associated with an increased risk of infection and higher infection mortality rates in patients undergoing HD [3]. In humans, this is especially true for patients undergoing HD for end-stage renal disease, in whom infection is one of the leading causes of morbidity and mortality. Therefore, the neutrophil function of patients undergoing HD is important. However, studies of neutrophil function in HD patients have produced conflicting results [6, 11, 21, 28]. Work in this area has addressed the effects of uremia [15] and acute intradialytic blood-membrane interactions [17] on neutrophil function. No study has identified the determinants of neutrophil function in dogs during HD. In particular, the specific relationship between neutrophil function and the dialyzer membrane is not clear. Data are needed to evaluate the effects of HD on neutrophil function in dogs. Therefore, this study evaluated the effects of HD on neutrophil function in clinically healthy dogs.

This study was performed with 4 adult beagle dogs weighing 9.5 to 12 kg that were maintained for experimental purposes. All dogs were housed individually in cages and fed commercial dry food once per day at 5:00 p.m.; all had free access to water. The study was conducted in accordance with the guidelines of the Animal Experiment Committee of Iwate University, Japan.

Each dog was pretreated with a subcutaneous injection of atropine sulfate (0.04 mg/kg body weight). Induction and intubation were achieved with the intravenous administration of propofol (Rapinovet, Takeda Schering-Plough, Tokyo, Japan)(6 mg/kg to effect), and anesthesia was maintained with sevoflurane (Sevoflo, Dainippon-Sumitomo Pharama, Osaka, Japan) (vaporizer setting 1.5% in oxygen). A 20-cm double-lumen transcutaneous silicone HD catheter (UB-1220-WHAY, Unitika, Osaka, Japan) was placed in the right jugular vein via a cutaneous cut-down and venotomy and positioned so that the tip of the catheter extended into the right atrium. To prevent thrombus formation within the catheter, the lumen of each catheter was heparin-locked with a flush solution containing 1,000 U/m*l* heparin sulfate.

The physical findings, body weight and rectal temperature were recorded for each dog before each HD session. Dialysis was performed with a dialysis delivery system (NCU-A, Leaf International, Osaka, Japan) and a 0.3-m^2 hemofilter (FB-

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Table 1. Blood urea nitrogen (BUN) before and after HD and the urea reduction ratio

	PRE (mg/d*l*)	180 min (mg/d*l*)	Reduction rate (%)
BUN	13.7 ± 1.3	10.6 ± 1.6[a)]	21.9 ± 1.4

a) Data shown are means ± SEM. An asterisk indicates a significant difference compared with the pretreatment value (P<0.05).

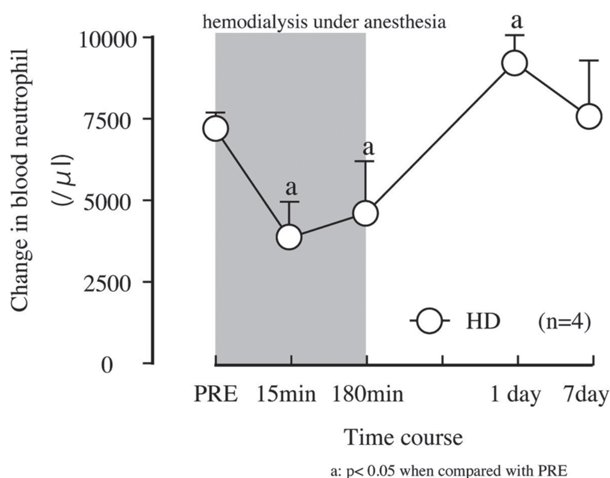

Fig. 1. The neutrophil count in the peripheral blood of dogs treated with HD (n=4). Data are means ± SD. An asterisk indicates a significant difference compared with the pretreatment value (P<0.05).

30U GA, Nipro, Osaka, Japan). The total priming volume of the circuit and hemofilter was 33 m*l*. The circuit and hemofilter were rinsed with 500 m*l* of 0.9% sodium chloride. The maximum extracorporeal blood flow rate was 2 m*l* (kg·min), and the dialysate flow rate was 200 m*l*/hr. The initial blood flow rate was adjusted to half of the maximum blood flow rate and increased gradually to the maximum rate during the first 10 min. The HD protocol was as follows: blood flow, 200 m*l*/min; dialysate flow, 500 m*l*/min; duration, 3 hr; and heparin sulfate at 100 U/kg prime for anticoagulation, administered at 700–1,200 U/hr and adjusted to maintain the activated clotting time between 150 and 200 sec. A bicarbonate-based dialysate was used (Carbostar, Ajinomoto Pharmaceuticals, Tokyo, Japan); the solution contained 140 mEq/*l* sodium, 2.0 mEq/*l* potassium, 111 mEq/*l* chloride, 3.0 mEq/*l* calcium, 1.0 mEq/*l* magnesium, 35 mEq/*l* bicarbonate, 2.0 mEq/*l* citrate and 1,500 mg/*l* glucose.

The urea reduction ratio, a measure of the efficacy of urea removal, uses the blood urea nitrogen (BUN) concentration and was calculated as 1−postdialysis BUN / predialysis BUN (Table 1). After 3 hr of dialysis, the serum BUN decreased significantly from 13.7 ± 1.3 (range 12.4–15.5) to 10.6 ± 1.6 (range 8.3–12.1) mg/d*l*, and the urea reduction ratio was 0.21 ± 0.14 (range 0.09–0.39).

The dogs were positioned in the right lateral recumbent position during the experiments, and 10-m*l* blood samples were obtained from the cervical vein. Cell counts, CD11b and CD18 expression, phagocytic activity and the oxidative burst of neutrophils were measured as described below.

Neutrophils were isolated from 10 m*l* of blood using dextran sedimentation and Ficoll solution (specific gravity 1.077; Daiichi-Sankyo, Tokyo, Japan) for density-gradient separation, as reported elsewhere [25]. The viability of the isolated polymorphonuclear leukocytes was determined by 0.2% Trypan blue staining (>95%).

The production of superoxide was measured by chemiluminescence with luminol, as described previously [25]. The chemiluminescence was measured with a luminometer (Luminescencer-PSN, ATTO, Tokyo, Japan) at 2-sec intervals for 30 min at 37°C.

Neutrophil phagocytosis of fluorescent microspheres was measured using the following flow cytometry technique [25]. Neutrophils and non-opsonized microspheres were incubated for 30 min at 37°C, and then, phosphate-buffered saline (PBS) with 3 mM EDTA-2Na was added. The cells were resuspended in 0.5% paraformaldehyde in PBS. The phagocytic activity was expressed as the percentage of the total neutrophil population ingesting fluorescent microspheres. The microspheres in neutrophils were analyzed by flow cytometry (Guava EasyCyte Mini, Guava Technologies, Chicago, IL, U.S.A.).

After staining with FITC-labeled anti-dog CD11b and CD18 monoclonal antibody (Beckman Coulter, Brea, CA, U.S.A.), the cells were resuspended in 0.5% paraformaldehyde in PBS. Cells were also labeled with FITC-labeled anti-mouse IgG antibody (Beckman Coulter, Brea, CA, U.S.A.) to serve as negative controls. The analysis gate for neutrophils was expressed as the mean fluorescence intensity (MFI) on a log-scale analyzing 10,000 cells per sample as follows: mean fluorescence intensity = (geometric mean of target antibody − geometric mean of negative control) / (geometric mean of negative control).

All data are expressed as means ± standard deviation. Intergroup comparisons were performed using one-way analysis of variance (ANOVA) for repeated measures with the Bonferroni post hoc test. A P-value<0.05 was considered to indicate statistical significance.

Figure 1 is representative of the change in the neutrophil count during the study. It decreased rapidly after the start of dialysis before rebounding, consistent with previous reports [19, 27]. The neutrophil count was decreased significantly at 15 min (P<0.05 vs. PRE) and 180 min (P<0.01 vs. PRE) compared with the baseline value and then increased 1 day after HD. Figure 2A and 2B shows the changes in CD11b and CD18 expression on neutrophils during the experiment. No differences in either CD11b or CD18 were found during the study. Figure 2C shows the changes in the phagocytic activity of the neutrophils during the study. The percentage of microbead-engulfing neutrophils was decreased significantly at 180 min compared with the baseline and then increased 1 day after HD. The phagocytic activity recovered by day 7. Figure 2D shows the changes in the neutrophil oxidative burst during the experiment. This oxidative burst was evaluated by a chemiluminescent method during serum-opsonized zymosan stimulation. There were no significant

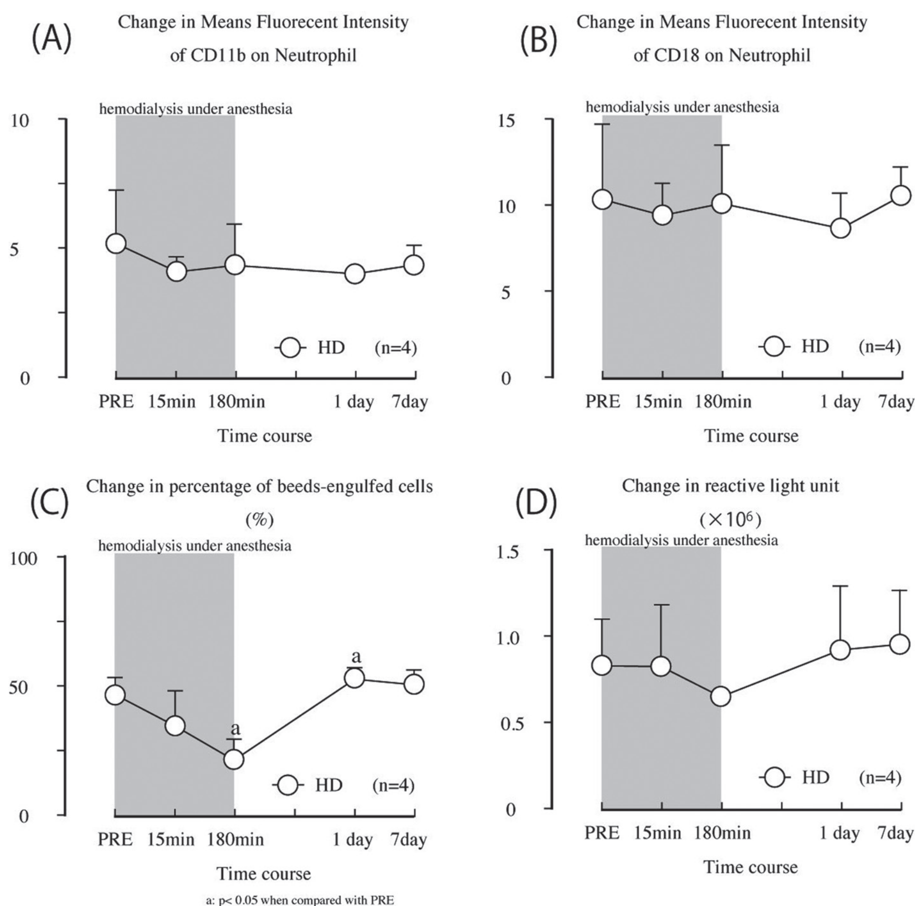

Fig. 2. Flow cytometric measurement of (A) CD11b and (B) CD18 expression in neutrophils of dogs treated with HD and (C) the phagocytic capacity of neutrophils in dogs treated with HD. (D) Chemiluminescence as an index of the oxidative burst capacity of neutrophils of dogs treated with HD. Isolated neutrophils were used for this analysis. Data are presented as means ± SD. An asterisk indicates a significant difference compared with the pretreatment value (P<0.05).

changes during the study.

Neutrophils are an integral component of the host defense against bacterial infections. In humans, there is substantial clinical evidence that patients undergoing chronic HD develop profound disturbances in neutrophil function. Impaired phagocytic capacity and bactericidal activity of these cells have been reported in patients undergoing HD [2]. Conversely, neutrophils can be stimulated by the artificial material that comprises the extracorporeal circulation in HD. Therefore, it is important to understand the effects of HD on neutrophil function in dogs. This study investigated the canine neutrophil response to HD.

The total number of neutrophils in peripheral blood decreased significantly during HD in this study. In humans, an early, transient decrease in the number of neutrophils during HD was first described by Kaplow and Goffinet [19].

Neutropenia is associated with the simultaneous upregulation of the integrin adhesion receptor CD11b [1]. According to current opinion, early HD-induced neutropenia occurs, because activated neutrophils are trapped in the lung vasculature [12], as demonstrated in postmortem studies [9]. Numerous human studies, both *in vitro* and *in vivo*, have shown that the binding of neutrophils to endothelial surfaces is mediated by upregulation of the adhesion molecule CD11b [5, 26]. The disappearance of neutrophils from the circulation during HD, probably caused by intrapulmonary sequestration, is accompanied by increased CD11b expression on neutrophils. The neutrophil numbers change similarly in dogs [27]. Nevertheless, we found no significant change in the expression of CD11b. A possible reason is that neutrophils with upregulated CD11b expression during HD might preferentially adhere to the pulmonary vasculature. Hence, the expression of CD11b on neutrophils obtained from the systemic circulation showed no changes.

In this study, the phagocyte function of neutrophils was suppressed during HD. Similarly, phagocyte function was suppressed during HD in human neutrophils [2]. It has been suggested that complement activation by membranes suppresses the phagocytic response both acutely and chronically. In humans, *in vitro* and *in vivo* data show that

this effect is dependent on complement activation via the alternative complement pathway [7, 8]. Even in our experiment, complement activation might have been stimulated, although it was not measured. However, the relationship between complement activation and the phagocytic function of neutrophils is controversial. It is necessary to evaluate the plasma complement components during HD in the dog.

Chemiluminescence assays that measure the production of reactive oxygen species (ROS) have been used widely as a sensitive assay for monitoring free radicals and reactive metabolites produced by enzymes, cells or organ systems [23]. Antioxidants affect the intensity of luminol-dependent chemiluminescence. The release of ROS by polymorphonuclear neutrophils is believed to play an important role in the neutrophil oxidative burst stimulated by HD treatment. However, we observed no significant differences in the chemiluminescence of neutrophils. Researchers have shown both the suppression and enhancement of neutrophil oxygen radical production during HD [10, 24]. Himmelfarb *et al.* [13, 14] found that fewer granulocyte oxygen species were produced in response to *Staphylococcus aureus* during HD with complement-activating membranes compared with non-complement-activating membranes. This was postulated to be due to the association between complement and oxygen species production.

In our study, HD was performed under general anesthesia with sevoflurane, which is known to influence neutrophils. In human research, inhalation anesthesia with sevoflurane induced oxidative stress, leading to the apoptosis of neutrophils [29]. In addition, sevoflurane inhalation caused decreased number *in vivo* [22] and neutrophil adhesion *in vitro* [20]. Therefore, the changes in neutrophil number and function identified in this study cannot be considered the result of HD treatment alone. However, in human, decreased number and function of neutrophil observed by HD treatment is well known [2, 8, 10]. The possibility of the HD effect to neutrophil cannot be denied. At least the result of current study is beneficial to HD treatment with general anesthesia.

Heparin is an essential anticoagulant in HD. Heparin suppresses phagocytosis and active oxygen production by neutrophils *in vitro* [18]. However, the heparin concentration used in that study (1,250–5,000 U/m*l*) was much higher than the amount used clinically and in this study (100 U/kg). The dose used in our study may have no effect on neutrophils.

In this study, with the exception of phagocytic function, no marked effect on neutrophil functions was found. Although various human studies have indicated that HD membranes alter neutrophil function [24] and that neutrophil function changes in the body during HD [2], there are only a few such studies of dogs. The HD treatment used in this study was similar to clinical HD. The neutrophil function in canines during and after clinical HD in veterinary medicine represents new knowledge.

This study did not investigate the cause of the change in the number and function of neutrophils during HD. Based on human research, complement plays a role in neutrophil function. Unfortunately, we could not find a report on complement in the dog during HD. A future study should examine complement to establish the cause of the change in neutrophil function during HD.

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