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Original Article

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Neutrophils in acidotic haemodialysed patients have lower intracellular pH and inflamed state

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Abstract

Background. The effect of intracellular pH (pHi) on neutrophils has not been clearly defined.

Methods. We used pre-dialysis neutrophils from three groups of haemodialysis (HD) patients having different levels of pre-dialysis plasma bicarbonate concentrations (P_{HCO3}) and pH values (pre-dialysis P_{HCO3} of groups A, B and C were consistently $\leq$21, 21–26 and $\geq$26 mmol/l [mEq/l], respectively) and neutrophils from age- and sex-matched healthy controls to determine pHi, apoptosis, phagocytosis and oxidative burst reactions *in vivo*. We also studied, in group A, the effect of metabolic acidosis correction on neutrophil function. Furthermore, we investigated the effect of intracellular acidification on neutrophil functioning *in vitro*.

Results. Neutrophils from the HD patients in group A exhibited significantly lower pHi than those in groups B and C. In addition, group A neutrophils had significantly delayed apoptosis, enhanced phagocytosis and increased oxidative burst reactions compared with those in groups B and C. These alterations in neutrophil function in group A were reduced by correcting metabolic acidosis over a period of 1 month. Moreover, our *in vitro* studies demonstrated that the pHi of neutrophils is positively correlated with apoptosis and inversely correlated with phagocytosis and oxidative burst reactions.

Conclusion. HD patients having low P_{HCO3} exhibited low neutrophil pHi. This intracellular acidification may contribute to the delayed apoptosis, enhanced phagocytosis and increased oxidative burst reactions observed in these neutrophils compared with neutrophils having normal or higher pHi.

Keywords: apoptosis; haemodialysis; intracellular pH; neutrophil; phagocytosis

Introduction

Low pre-dialysis plasma bicarbonate (HCO_3^-) concentrations (P_{HCO3}) are still commonly found in a number of haemodialysis (HD) patients, and even in subjects that are apparently well dialysed on standard bicarbonate dialysis (35 mmol/l) (mEq/l) [1,2]. Here, we studied uraemic patients on maintenance haemodialysis that had been divided into three groups of patients by three consecutive monthly pre-dialysis P_{HCO3} ($\leq$21, 21–26, $\geq$26 mmol/l) [3]. These three groups of patients differed in terms of anthropometry, nutritional and biochemical markers, suggesting that they may also differ in physiological and/or patho-physiological status. Whether they exhibited any behavioural differences remains unknown.

Although uraemic acidosis is mild, long-term metabolic acidosis of this type is associated with many adverse effects, including negative nitrogen balance [4], increased protein decomposition [5], fatigue, bone lesion [6], hypercalciuria [7], enhancement of parathyroid hormone secretion [8], hyperphosphataemia, reduced effect of 1α-hydroxylase [9], impaired cardiovascular function (depression of myocardial contractility, sensitization to arrhythmia [10], increased pulmonary vascular resistance [11]), hormonal disturbances [12], insulin resistance [13], gluconeogenesis cycle disorders [14] and altered triglyceride

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metabolism [15]. However, little is known about the effect of uraemic acidosis on immune regulation in HD patients.

Neutrophils are relatively short-lived and they constitutively undergo apoptosis. Apoptotic neutrophils are phagocytosed by macrophages without release of pro-inflammatory mediators, leading to limitation of tissue injury and resolution of the inflammatory process. A suppressed neutrophil apoptosis results in prolongation of its life span and may be beneficial for the host defense against systemic bacterial invasion. However, prolonged survival of activated neutrophils could cause the uncontrolled release of cytotoxic metabolites and pro-inflammatory substances, and lead to amplified systemic inflammation, tissue injury and organ failure. Thus, a balance between neutrophil apoptosis and phagocytosis is considered to be important for HD patients, who are generally considered to be in an inflamed state.

Surprisingly, few investigators have studied the effects of extracellular pH (pHe), intracellular pH (pHi) or both on neutrophil function in HD patients. The present study was designed to evaluate *in vivo* neutrophil pHi and function, which included apoptosis, phagocytosis and oxidative burst reactions in three groups of HD patients with different pre-dialysis P_{HCO3} and pH values. We additionally investigated the *in vitro* effects of altered pHe and pHi (within the acidic pH range) on the regulation of neutrophil function. Finally, we studied the effect of correction of metabolic acidosis on neutrophil function in acidic-HD patients.

Subjects and methods

In vitro *study design*

This study consisted of a multi-centred (3 HD centres), comparison of three groups of HD patients (group A had a pre-dialysis P_{HCO3} that was consistently $\leq$21 mmol/l and pH $\leq$7.38; group B had a pre-dialysis P_{HCO3} that was consistently between 21 and 26 mmol/l and pH 7.38–7.42; and group C had a pre-dialysis P_{HCO3} that was consistently $\geq$26 mmol/l and pH $\geq$7.42.) with a group of age-, sex-matched healthy subjects (group CN) in order to study neutrophil pHi and function, including apoptosis, phagocytosis and oxidative burst reactions. To evaluate the effect of metabolic acidosis on neutrophils, we investigated neutrophil functions in acidic HD patients (group A) after restoring their pre-dialysis P_{HCO3} to 23–26 mmol/l over a period of 1 month (group A^+). To achieve this goal, the dialysate HCO_3^- concentration was increased from 35 mmol/l to 38–40 mmol/l. Blood was drawn from the arterial side of the arteriovenous (AV) fistula at the start of dialysis on a mid-week day to avoid different pre-dialysis P_{HCO3} levels on different weekdays. During blood sampling, the patient was at rest and there was no hand motion. Arterial blood gas was measured at 4°C from a whole blood sample taken anaerobically. The samples were analysed by an ABL 510 (Radiometer, Copenhagen, Denmark) within 5 min to avoid a decrease in P_{HCO3} due to delayed measurement. All measurements, except when mentioned otherwise, were performed within 1 h after the samples were taken. In addition, all measurements were assayed on at least three separate dialytic sessions for each patient and means of these data are presented.

In vitro *study design*

Neutrophils from each HD patient were assayed for neutrophil pHi and function, including apoptosis, phagocytosis and oxidative burst reactions, after incubating in different pH-adjusted (7.4, 6.5, 6.0, 5.5) culture media for 1 h. This created four groups of data (7.4, 6.5, 6.0, 5.5) for each patient. We chose bicarbonate-buffered RPMI 1640 supplemented with 1% FCS [FCS contained < 5 pg 100 ml of lipopolysaccaride (LPS) as certified by the manufacturer (HyClone, South Logan, UT, USA)], which has been previously adjusted to the desired pH values (7.4, 6.5, 6.0, 5.5). Aliquots of 10^6/ml were cultured at 37°C in humidified 5% CO_2 (for cells suspended in medium at pH 7.4) or 7% CO_2 (for cells suspended in medium at lower pH values) incubators to maintain the desired pH in media. The pH of the culture media did not significantly change during 1 h incubation (data not shown).

Selection of patients

After approval by the local medical ethics committee, 123 anuric patients undergoing maintenance HD (62 men and 61 women) gave informed consent and were entered the study. The mean age was 52.3 years (range, 40–74 years). Mean duration of dialysis was 39.6 months (range, 8–52 months). The underlying primary renal diseases were glomerulopathy ($n=58$), polycystic kidney disease ($n=10$), tubulointerstitial nephritis ($n=28$), nephrosclerosis ($n=18$) and unknown ($n=9$). In addition, all individuals had serum C-reactive protein (CRP) levels below 0.6 mg/dl and none of them had diabetes mellitus, clinical infection, heart failure, liver cirrhosis or keto- and lactic-acidosis. Further, none of the individuals were taking calcium channel blockers, oral or intravenous active vitamin D or iron. The HD patients in groups A, B and C were screened for inclusion criteria in order to have similar [serum ferritin (200–250 µg/l), transferin saturation (TSAT) (20–50%), haemoglobin (10–11 g/dl), intact parathyroid hormone (i-PTH) [150–300 ng/l], length of HD (36–48 months) and Kt/V (1.2–1.5) (markers for adequacy of HD efficiency)] during the experimental time period (monthly intervals for 3 months). All patient laboratory values presented in this study were within inclusion limits. As shown in Table 1, there were no statistically significant differences in serum ferritin, TSAT, haemoglobin, i-PTH, length of HD or Kt/V in HD patients among groups A, B and C. The amount of maintenance erythropoietin supplement depended on clinical indications.

Haemodialysis

HD was performed using a dialyser (Fresenius Polysulfone F8 HPS–F10 HPS) having a 1.8–2.4 m^2 polysulfone membrane and a dialysate flow rate of 500 ml/min. The dialysate fluid composition was sodium 140 mmol/l, potassium 2 mmol/l, calcium 1.75 mmol/l

Table 1. Subject characteristics in four study groups

Subject group	CN	≤21 (A)	21–26 (B)	≥26 (C)	(A^+)
No. of patient	29	35	52	36	35
pH		7.33 ± 0.004	7.40 ± 0.002	7.46 ± 0.006	7.41 ± 0.004
HCO_3^- (mmol/l)		19.8 ± 0.2	24.2 ± 0.5	27.6 ± 0.3	24.1 ± 0.3
Sex (M/F)	14/15	17/18	26/26	18/18	17/18
Age (years)	52.5 ± 1.4	52.6 ± 1.6	53.2 ± 1.8	52.4 ± 1.4	52.6 ± 1.6
PHi	7.25 ± 0.08[a]	7.09 ± 0.05[e,f,g]	7.20 ± 0.06[f]	7.28 ± 0.08	7.21 ± 0.04
Length of HD (months)		39.2 ± 0.6	40.3 ± 0.8	40.8 ± 0.5	39.2 ± 0.6
Kt/V		1.35 ± 0.06	1.36 ± 0.04	1.35 ± 0.06	1.36 ± 0.06
Haemoglobin (g/dl)	14.3 ± 0.8[a,b,c,d]	10.6 ± 0.4	10.2 ± 0.5	10.5 ± 0.4	10.5 ± 0.4
Biochemical markers					
K^+ (mmol/l)	4.3 ± 0.2[a]	5.2 ± 0.2[e,f,g]	4.6 ± 0.3[h]	4.2 ± 0.2[i]	4.5 ± 0.2
Phosphorus (mmol/l)	1.28 ± 0.21[a,b,c,d]	2.31 ± 0.15	2.11 ± 0.14	1.82 ± 0.22	2.31 ± 0.22
TSAT	0.37 ± 0.11	0.35 ± 0.08	0.37 ± 0.07	0.36 ± 0.05	0.35 ± 0.05
Ferritin (μg/l)	105.6 ± 5.9[a,b,c,d]	215.4 ± 3.6	218.7 ± 4.2	216.2 ± 2.5	214.2 ± 2.4
Albumin (g/l)	44.3 ± 1.5[a,b,c,d]	40.3 ± 1.2	40.8 ± 1.4	40.2 ± 1.4	40.3 ± 1.4
I-PTH (ng/l)	42.5 ± 1.4[a,b,c,d]	132.5 ± 9.4	142.5 ± 10.5	135.7 ± 11.2	132.7 ± 10.1
IL-6 (ng/l)	1.4 ± 0.2[a,b,c,d]	5.9 ± 0.4	6.1 ± 0.3	6.1 ± 0.2	6.0 ± 0.3

Data presented as mean ± SEM. One-way ANOVA followed by Scheffe''s test for multiple comparisons was used to compare five study groups. To convert haemoglobin in g/dl to g/l, multiply by 10; phosphorus in mg/dl to mmol/l, multiply by 0.3229; albumin in g/dl to g/l, multiply by 10.

TSAT, transferin saturation; IL-6, interleukin-6; I-PTH, intact parathyroid hormone.

[a]CN *vs* A, $P < 0.05$; [b]CN *vs* B, $P < 0.05$; [c]CN *vs* C, $P < 0.05$; [d]CN *vs* A^+, $P < 0.05$; [e]A *vs* B, $P < 0.05$; [f]A *vs* C, $P < 0.05$; [g]A *vs* A^+, $P < 0.05$; [h]B *vs* C, $P < 0.05$; [i]C *vs* A^+, $P < 0.05$.

(7.01 mg/dl), HCO_3^- 35 mmol/l, acetate 4 mmol/l and glucose 5.5 mmol/l (99.1 mg/dl). Treatment duration was 4 h and blood flow rates were around 200–250 ml/min. Anticoagulation was achieved using a loading dose followed by constant infusion of heparin. Net fluid removal was set on an individual basis according to the clinical needs of each patient.

Biochemical analysis

Albumin was measured with the Beckman Synchron CX9 Delta Chemistry Analyzer (GMI, Minnesota, USA) according to the manufacturer's instructions. Serum iron and total iron binding capacity were determined using spectrophotometry and detected by Integra 700/Roche Diagnostics (Basel, Switzerland). Ferritin was measured with a polyclonal goat anti-ferritin antibody labelled with acridinium ester and detected by ACS:180 (R) SE Automated CL System-Centaur (Bayer, Tarrytown, NY, USA). Intact-PTH was determined with biotinylated polyclonal goat anti-human PTH antibody compounded with acridinium ester-labelled polyclonal goat anti-human PTH antibody in phosphate buffer with goat IgG, and detected by the ADVIA Centaur Immunoassay system (Bayer, Tarrytown, NY, USA). Plasma CRP concentration was determined by rate nephelometry (Beckman Instruments, Inc., Galway, Ireland). Plasma IL-6 concentration was measured by ELISA (R&D Systems Inc. Minneapolis, MN, USA).

Neutrophil isolation and preparation

Heparinized blood was obtained from the AV fistula or graft in HD patients during pre-dialysis in groups A, B and C, and from age- and sex-matched healthy individuals who had taken no medication for at least 10 days before sampling. Neutrophils were isolated by sequential sedimentation in 6% of dextran in 0.9% sodium chloride for 40 min at 22°C. Centrifugation was in Ficoll-Paque at 1500 rpm for 30 min to pellet granulocytes and remaining erythrocytes. The resuspended pellet was centrifuged over an 81% isotonic Percoll gradient at 1750 rpm for 15 min to pellet erythrocytes. The diffused layer at the interface contained neutrophils which were harvested, washed, resuspended in a medium and counted. Cell viability was >98%, as determined by trypan blue exclusion. The preparation was routinely comprised of >95% neutrophils. Eosinophils were the principal contaminants as determined by Rapi-diff II staining (Diagnostic Developments, Lancashire, UK) of the cytocentrifuged samples.

Measurement of pHi

Neutrophils were isolated from the different groups as described earlier. Measurement of pHi was performed using carboxy-SNARF-1-AM as previously described [16]. Neutrophils [5×10^6/ml in indicated medium, previously pH adjusted for the *in vivo* studies, pH was adjusted to match the plasma pH in each study subject.] were loaded with 10 μM carboxy-SNARF-1-AM during 1 h at 37°C, washed in phosphate buffer solution (PBS), and resuspended in the same buffer at 5×10^6 in 100 μl. Assays were performed by flow cytometry, with excitation at 488 nm and emission analysis at FL2 and FL3. Ten thousand events were collected. The pHi was estimated from the ratio of emission intensities at the two wavelengths and was standardized by comparison with the fluorescence intensity ratios of cells whose pHi values were fixed by incubation with nigericin (10 μM) in high-potassium buffers, as previously described [16].

Neutrophil viability, apoptosis and necrosis

Flow cytometry. Cell apoptosis was measured by annexin V staining. Neutrophils were isolated from different conditions as described earlier and were washed in PBS, and density was adjusted to 1×10^6 ml [17]. Subsequently, 100 µl of the solution (1×10^5 cells) was transferred to a 5 ml culture tube. The 5 µl of annexin V-FITC (Pharmingen, San Diego, CA, USA) and 2 µl of propidium iodide (PI) were added and the cells were incubated for 15 min at room temperature in the dark and 400 µl of binding annexin V-buffer was added to each tube. The following controls were used: unstained cells, cells stained with Annexin V-FITC (no PI) and cells stained with PI (no Annexin V-FITC). The degree of apoptosis was assessed by flow cytometry (FACScal-Systems, San Diego, CA, USA) within 1 h. Cells staining positive for PI were considered dead cells (necrosis or late apoptosis), cells staining positive only for annexin V were considered apoptotic and cells negative for both were considered viable.

DNA fragmentation assay. Neutrophils were isolated from different conditions as described earlier and were lysed, and genomic DNA was extracted according to the protocol for apoptotic DNA laddering kit (R&D Systems). Samples (2 µg of DNA/lane) were analysed by gel electrophoresis (1.8% agarose) and ethidium bromide staining. The gel was visually examined under 305 nm of UV illumination.

Measurement of caspase-3 activity. Caspase-3 activity was measured with a colorimetric assay kit (Sigma). Neutrophils were isolated from different conditions as described earlier. After washing with PBS, neutrophils were lysed in 60 µl lysis buffer [50 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, pH 7.4, 5 mM 3-[(3-cholamindopropyl) dimethlyammonio]-1-propanesulfonate and 5 mM dithiothreitol], disrupted on ice by sonication and centrifuged ($17\,400 \times g$ for 10 min). The supernatants (10 µl; 5×10^5 cell equivalents, containing ~20 µg protein) were incubated with a 2 mM acetyl-Asp-Glu-Val-Asp-p-nitroanilide substrate with or without 200 µM acetyl-Asp-Glu-Val-Asp-al, a specific caspase-3 inhibitor, at 37°C for 6 h in a total volume of 100 µl assay buffer. Caspase-3 activity was measured at 405 nm in a Model 3550-UV microplate reader (Bio-Rad), and expressed as nmol of *p*-nitroanilide liberated/10^6 cells/h [18]. The protein contents were determined with a BCA protein assay kit (Pierce).

Neutrophil phagocytosis. Bacteria phagocytosis capacity was assayed by means of a commercially available kit (PHAGOTEST; ORPEGEN Pharma, Heidelberg, FRG). For the *in vitro* study, neutrophils were isolated as described earlier and resuspended in 50% RPMI 1640, 50% autologous plasma in v/v (previously pH adjusted), then the opsonized FITC-labelled *Escherichia coli* were added and were incubated for 30 min in a humidified 5% or 7% CO_2 incubators. For the *in vivo* study, heparinized whole blood was used for assay. The following procedure was performed following the manufacturer's instructions.

Oxidative burst reactions

Superoxide assay. The generation of superoxide (SO) anion was measured as the SO dismutase-inhibitable reduction of cytochrome-c by measuring absorbance at 550 nm as described by Babior *et al*. [19]. Neutrophils (10^6) were isolated as described above and were incubated in RPMI 1640 [previously pH adjusted (for the *in vivo* studies, pH was adjusted to match the plasma pH in each study subject)], with ferricytochrome-c (BDH Chemical, Toronto, Canada). Superoxide release was assayed with or without using phorbol myristate acetate (PMA; Sigma; final concentration, 0.5 µg/ml) as the stimulus. The reaction mixture was incubated at 37°C for 1 h in humidified 5% (for *in vivo* study) or 7% CO_2 incubators and then centrifuged for 5 min at $800 \times g$ at 4°C. Absorbance of the supernatant at 550 nm was read on a Beckman DU-8 spectrophotometer. The blank contained all the components listed earlier along with 60 µg of SO dismutase (Sigma) per millilitre to correct for ferricytochrome-c reduction by agents other than SO. The amount of reduced cytochrome-c present was calculated by using an extinction coefficient of 21.1/mM/cm at 550 nm. Results were expressed as nanomoles of SO/10^6 cells/1 h. Studies with PMA contained 60 mM ferricytochrome-c. At each of these concentrations, the total amount of ferricytochrome-c available for reduction, as determined by sodium dithionite, exceeded the amount reduced by PMA stimuli.

Hydrogen peroxide assay. Hydrogen peroxide release (H_2O_2) was measured by the oxidation of phenol red as described by Pick and Keisari [20]. Briefly, the reaction mixture (1.0 ml) consisted of RPMI 1640 [previously pH adjusted (for the *in vivo* studies, pH was adjusted to match the plasma pH in each study subject)] with 2×10^6 neutrophils, 0.28 mM phenol red (Sigma), 8.5 U of horseradish peroxidase (Worthington Diagnostics, Freehold, NJ), with or without PMA as the stimulus. After a 1 h incubation at 37°C in humidified 5% (was selected for *in vivo* study) or 7% CO_2 incubators, 0.010 ml of 1M NaOH was added and the tubes were centrifuged at $800 \times g$ for 5 min at 4°C. Absorbance of the supernatant at 610 nm was determined spectrophotometrically. Results were expressed as nanomoles of H_2O_2 produced per 10^6 neutrophils per 1 h.

Statistical analyses. All results are expressed as means ± SEM. Differences among groups were analysed by one-way analysis of variance (ANOVA) followed by Scheffe′'s test for multiple comparisons. Pearson's correlation coefficient was used to determine the relationship between the neutrophil pHi and functions. A P-value of < 0.05 was considered statistically significant.

Results

Neutrophil apoptosis in patients and healthy individuals

In the initial experiments, flow cytometry using FITC-conjugated annexin V revealed that the neutrophils of the HD patients in groups A (A), B (B) and C (C) underwent rapid apoptosis. As shown in the A, B and C subparts of Figure 1A, 18–25% of the neutrophils were in the early stage of apoptosis with

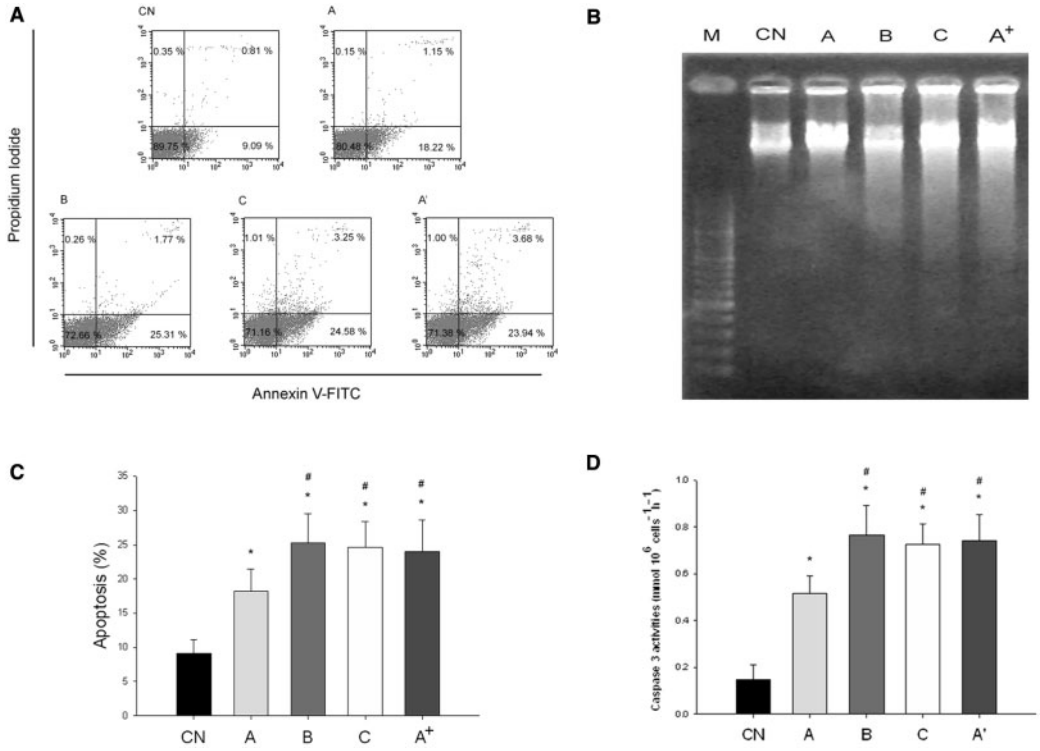

Fig. 1. Neutrophil apoptosis was assessed by PI and annexin V-FITC staining in groups CN (CN; n=29), A (A; $n = 35$), B (B; $n = 52$), C (C; $n = 36$) and A^+(A^+; $n = 35$). (**A**) Representative results from flow cytometric analysis of binding of annexin V-FITC and PI in neutrophils from the five experimental groups. The relative distribution of cells manifesting viable (annexin$^-$ PI$^-$), early (annexin$^+$ PI$^-$), and late (annexin$^+$ PI$^+$) apoptosis is shown as percentages. (**B**) Agarose gel electrophoresis of internucleosomal DNA fragmentation in neutrophils. The molecular markers are indicated to the left (M). Five experimental groups are indicated as CN, A, B, C and A^+. (**C**) Results from statistical analysis showing the proportions of apoptotic cells (mean ± SEM) in the five experimental groups. $^*P < 0.01$ compared with CN, $^{\#}P < 0.01$ compared with A. (**D**) Statistical results showed the caspase-3 activity (mean ± SEM) and expressed as nmol of p-nitroanilide liberated/10^6 cells/h in five experimental groups. $^*P < 0.01$ compared with CN, $^{\#}P < 0.01$ compared with A.

preserved membrane integrity and 1–4% were positive for both annexin V and PI. The neutrophils of healthy individuals (group CN) revealed a much lower degree of apoptosis. As shown in the CN subparts of Figure 1A, approximately 9% of the neutrophils underwent apoptosis and <1% were positive for both annexin V and PI. As shown in Figure 1B–D, similar results were obtained in complementary experiments that were performed to assay DNA fragmentation. Flow cytometry and examination of caspase-3 activity revealed that neutrophil apoptosis was significantly increased in groups A, B and C compared with group CN. However, neutrophil apoptosis was significantly lower in group A than in groups B and C, and it did not differ between groups B and C. Furthermore, as shown in Figure 1A–D, we found that the lower neutrophil apoptosis in group A was increased after we restored the pHi by increasing the delivered bicarbonate dose (group A^+). In accordance, we found that neutrophil apoptosis was significantly increased in the HD patients compared with the healthy individuals. In addition, neutrophil apoptosis in group A (that had lower P_{HCO3} and pHi values) was delayed compared with that in groups B and C. Moreover, the pHi-associated decrease in neutrophil apoptosis could be reversed by increasing the bicarbonate dose.

Neutrophil phagocytosis in the patients and healthy individuals

Uptake of FITC-labelled bacteria by neutrophils in whole-blood samples was tested using a gram-negative species. Flow cytometric analysis revealed that neutrophil-opsonized phagocytosis occurred in groups A (A), B (B), C (C) and A^+ (A^+). As shown in the CN, B and C subparts of Figure 2A, 53–57% of the neutrophils phagocytosed FITC-labelled bacteria, while approximately 67% of the neutrophils from group A patients showed phagocytosis. As shown in Figure 2B, neutrophil phagocytosis was significantly increased in group A compared with groups CN, B and C. However, there was no significant difference in neutrophil-opsonized phagocytosis among groups B, C and CN. Furthermore, phagocytosis by neutrophils in group A^+ was reduced. Similarly, neutrophil-opsonized phagocytosis was increased in HD patients having lower P_{HCO3} and

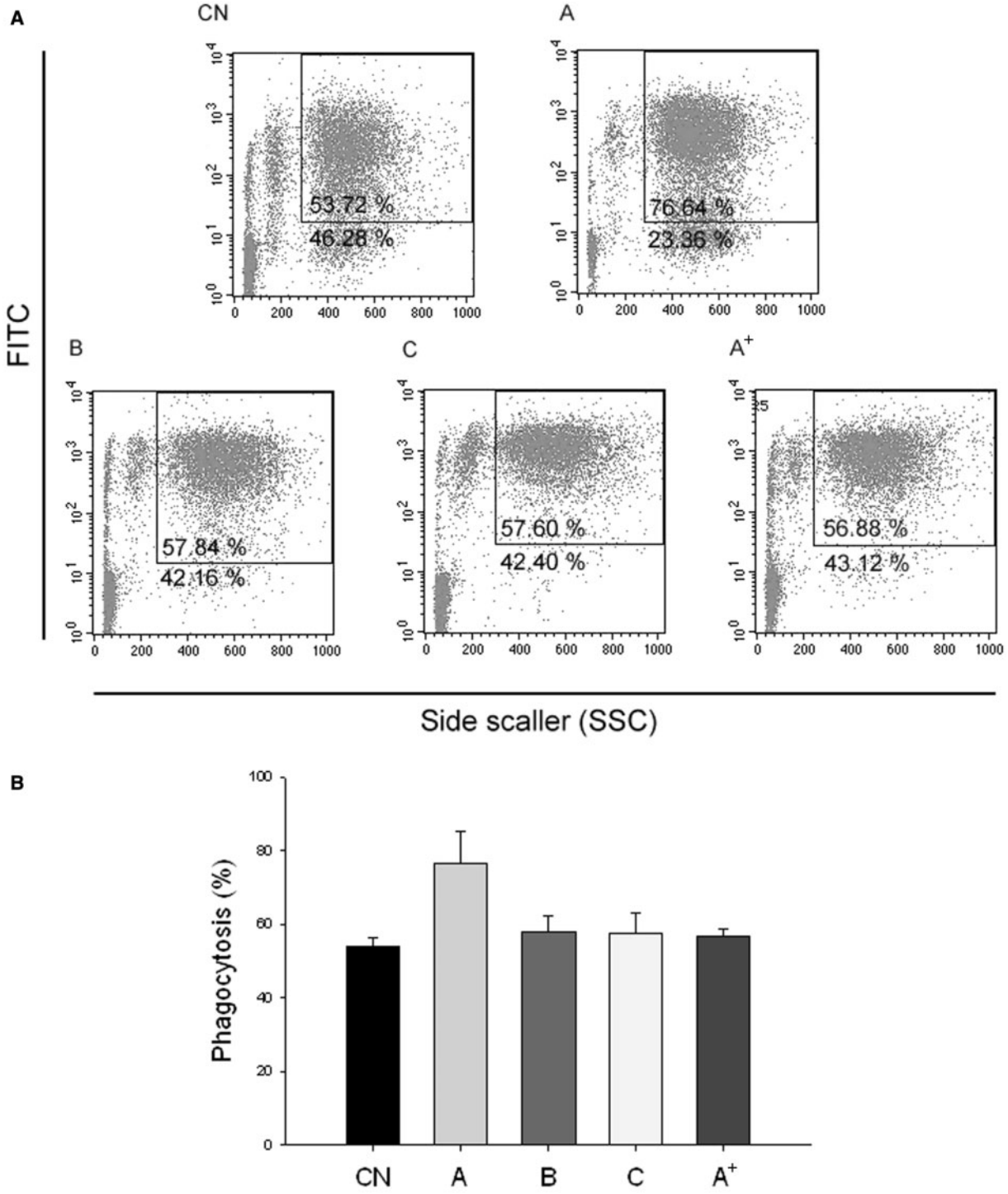

Fig. 2. Neutrophil phagocytosis was assessed by uptake of FITC-labelled *E. coli* and DNA staining in groups CN (CN; $n = 29$), A (A; $n = 35$), B (B; $n = 52$), C (C; $n = 36$) and A^+(A^+; $n = 35$). (**A**) Representative results showing flow cytometric analysis of uptake of FITC-labelled *E.Coli* and DNA staining in neutrophils from the five experimental groups. The relative distribution of cells manifesting uptake of FITC-labelled *E. coli* is shown as percentages. (**B**) Results from statistical analysis showing the proportions of neutrophils that had phagocyted FITC-labelled *E. coli* (mean ± SEM) in the five experimental groups. $^*P < 0.05$ compared with A.

pHi values compared with patients having normal or higher P_{HCO3} and pHi values. Again, this increase in neutrophil function observed in group A could be reduced by increasing the administered bicarbonate dose.

Neutrophil-mediated oxidative burst reactions in the patients and healthy individuals

Oxidative burst reactions were estimated by SO and H_2O_2 production by neutrophils with or without PMA

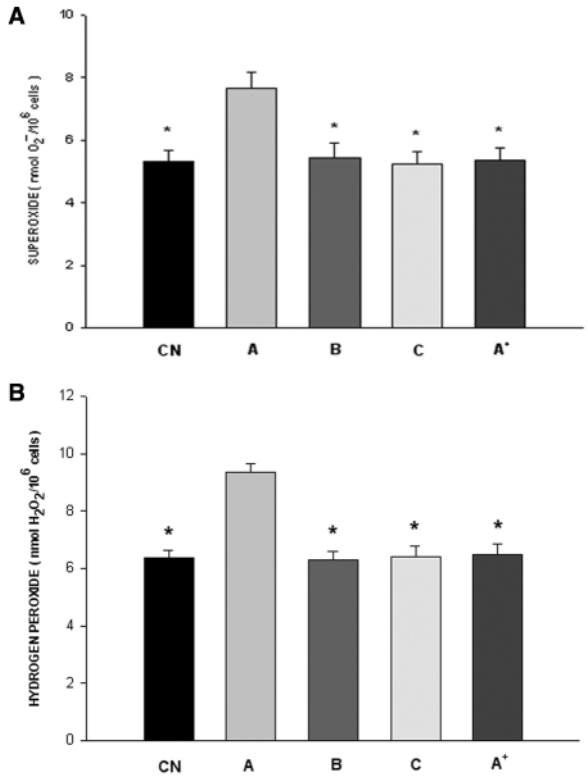

Fig. 3. Findings from statistical analysis showing (mean ± SEM) the production of superoxide (SO) (**A**) and of hydrogen peroxide (H_2O_2) (**B**) in uraemic neutrophils from groups CN (CN; $n = 29$), A (A; $n = 35$), B (B; $n = 52$), C (C; $n = 36$) and A^+ (A^+; $n = 35$). $^*P < 0.05$ compared with A.

as a stimulus. There was no significant difference in baseline (resting) neutrophil SO or H_2O_2 production among the groups CN, A, B, C and A^+ (data not shown). As shown in Figure 3A and B, PMA-stimulated SO and H_2O_2 production by the uraemic neutrophils from group A patients increased significantly compared with that of neutrophils from the individuals in groups CN, B and C. However, there was no significant difference in neutrophil SO and H_2O_2 production among groups CN, B, C and A^+. Further, the incidence of neutrophil oxidative burst reactions in HD patients with normal pHi values was significantly lower in neutrophils from HD patients with lower pHi values, however, it was similar to that in neutrophils from healthy individuals. Furthermore, correction of metabolic acidosis restored the oxidative burst reactions in uraemic neutrophils.

Effect of pHi on apoptosis and phagocytosis in uraemic neutrophils

To explore the role of pHi in the regulation of uraemic neutrophil function, neutrophils from the HD patients were incubated for 1 h in culture media having various pH values (7.4, 6.5, 6.0, 5.5), and were assayed for pHi as well as functions including apoptosis, phagocytosis and oxidative burst reactions.

Table 2. Uraemic neutrophil intracellular pH after 1 h incubation at various medium pH values

Medium pH	Intracellular pH
7.4	7.2 ± 0.2
6.5	6.3 ± 0.3*
6.0	6.0 ± 0.2$^{\#}$
5.5	5.8 ± 0.3$^{\#}$

Neutrophils loaded with carboxy-SNARF-1-AM were suspended at 10^6/ml in the pH-adjusted medium and pHi was determined after 1 h incubation. Shown are the means ± SEM of 21, each was in duplicate, experiments.
$^*P < 0.05$ *vs* similar medium at pH 7.4; $^{\#}P < 0.001$ *vs* similar medium at pH 7.4.

The pHi values of uraemic neutrophils after incubation for 1 h at various external pH levels are shown in Table 2. The pHi varied significantly with the external pH. As shown in Figure 4A and B, pHi was positively correlated with neutrophil apoptosis (A), whereas it was inversely correlated with phagocytosis (B). Accordingly, intracellular acidification of uraemic neutrophil delayed apoptosis but enhanced phagocytosis.

Effect of pHi on oxidative burst reactions in uraemic neutrophils

Figure 4C and D illustrate the effects of pHi on SO and H_2O_2 production in neutrophils when PMA was used as a stimulus. The production of SO and H_2O_2 in neutrophils incubated in medium with a low pH increased significantly compared with that of neutrophils incubated in medium with a high pH. In addition, SO and H_2O_2 production in uraemic neutrophils was inversely correlated with pHi.

Discussion

To the best of our knowledge, this is the first study to show that uraemic patients undergoing maintenance haemodialysis can be classified into three categories of P_{HCO3} and pH status and that these categories differ in terms of neutrophil function and pHi. The neutrophils from patients with low P_{HCO3} and pH values exhibited low pHi values. Further, the low pHi in this group may contribute to the delayed apoptosis, enhanced phagocytosis and increased oxidative burst reactions as compared with these processes in neutrophils having normal or higher pHi values. In addition, restoration of pre-dialysis P_{HCO3} and pH values in acidotic HD patients (group A) counteracted the alterations in neutrophil function and pHi. *In vitro* studies demonstrated that neutrophil pHi was positively correlated with apoptosis, but correlated inversely with phagocytosis and oxidative burst reactions. Finally, this is the first *in vivo* study to suggest that P_{HCO3} influences the regulation of pHi in circulating cells.

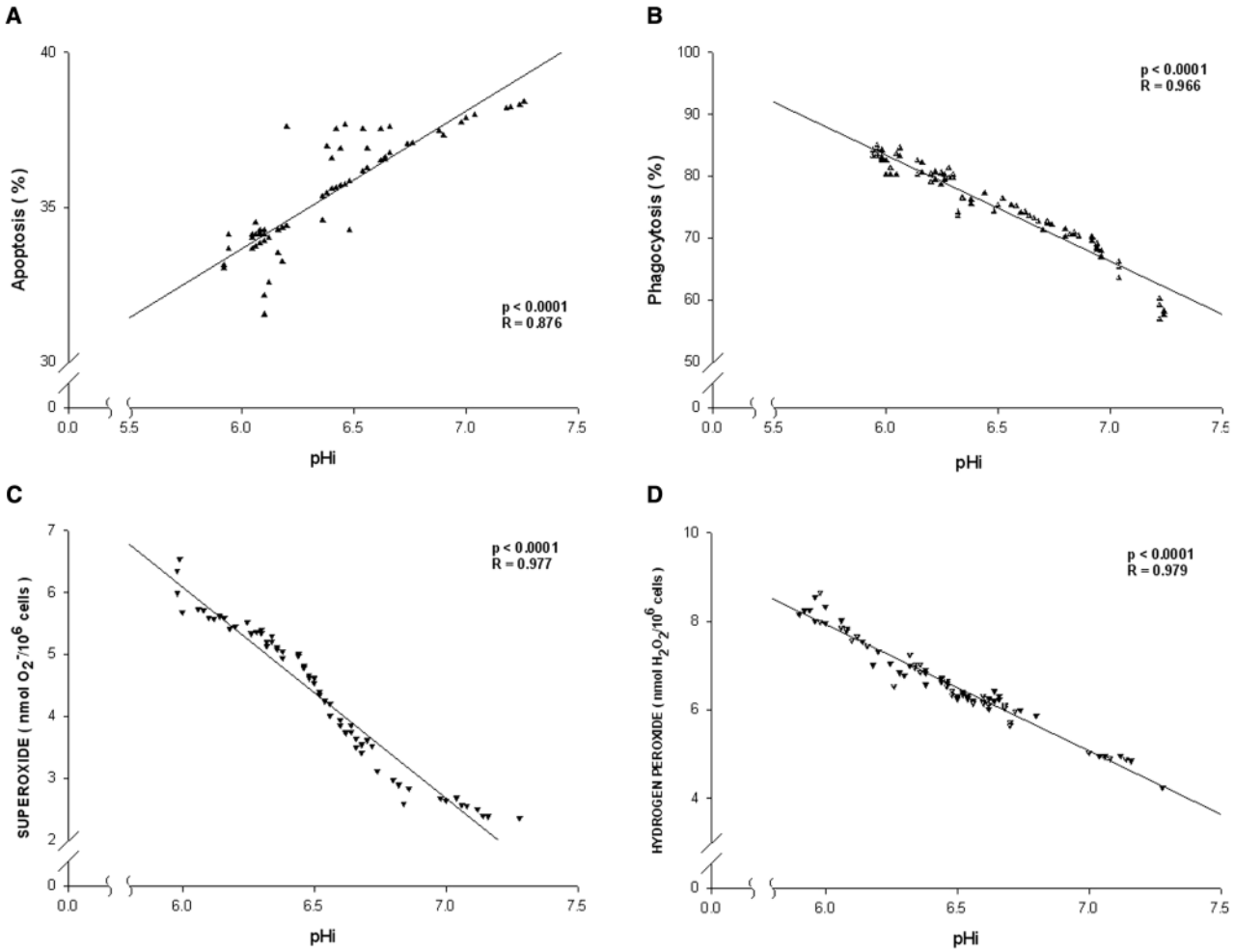

Fig. 4. Correlations between neutrophil pHi and apoptosis (**A**) phagocytosis (**B**) SO, (**C**) H_2O_2 and (**D**) production from HD patients. Neutrophils (10^6 cells/ml) were incubated in RPMI 1640 at different pH (7.4, 6.5, 6.0, 5.5) for 1 h. After incubation, uraemic neutrophil pHi, apoptosis, opsonized phagocytosis, SO and H_2O_2 production were assayed as described in subjects and methods. For each experimental set-up $n = 21$.

Uraemic neutrophil pHi and apoptosis

In vitro studies on neutrophils from uraemic patients have revealed that uraemic neutrophils undergo accelerated apoptosis [21]. However, little is known about the role that pHi plays in the regulation of uraemic neutrophil apoptosis. In this study, we observed that neutrophils from HD patients of group A, who have lower P_{HCO3} and pH, exhibited a lower pHi and delayed neutrophil apoptosis compared with those from patients in groups B and C. However, there was no significant difference in neutrophil apoptosis between groups B and C. In addition, and consistent with previous reports, neutrophils from the HD patients (groups A, B and C) underwent accelerated apoptosis compared with those from healthy individuals in group CN. Furthermore, in the *in vitro* study that was carried out in the acidic range of external pH, we demonstrated that pHi positively correlated with neutrophil apoptosis, although this correlation was not observed in the *in vivo* study. Our data suggest that intracellular acidification delayed uraemic neutrophil apoptosis. Nevertheless, further study is necessary to elucidate the relation between intracellular alkalification and uraemic neutrophil apoptosis.

During apoptosis, caspase are activated by cleavage of procaspases in a sequential manner. Caspase-3 is an effector caspase that plays a role in cell death induced by a variety of stimuli [22]. In this study, we observed that caspase-3 activity as well as the rate of uraemic neutrophil apoptosis was significantly higher in groups B and C than in groups A and CN. However, caspase-3 activity in uraemic neutrophils was not significantly correlated with apoptosis in the *in vivo* study (data not shown). Our data suggest that caspase-3 may be involved in the regulation of pHi-related neutrophil apoptosis in HD patients. Nevertheless, further study is necessary to elucidate the relations among caspase-3 activity, pHi and apoptosis in uraemic neutrophils.

Neutrophil pHi, phagocytosis and oxidative burst reactions

In previous work, there have been discrepancies in the effect of uraemia on neutrophil phagocytosis. A study from 1995 stated that, in general, the phagocyte activity is not impaired by uraemia [23]. This is inconsistent with earlier results reporting a reduced phagocytic activity in uraemic patients [24,25]. Recent studies suggested that neutrophil killing capability is reduced in HD patients, while the number of neutrophils that show phagocytic activity and produce reactive oxygen species (ROS) remains unchanged [26]. The mechanisms responsible for altered phagocytic activity of uraemic neutrophils are unclear and have been attributed to the time span from onset of dialysis [27], diabetes mellitus [28], iron stores [29], serum parathyroid hormone [30], dialyser bioincompatibility and reuse practice [31,32]. *In vivo* studies have demonstrated that pHi plays a role in the generation of SO radicals [33] as well as in chemotaxis [34] in normal neutrophils. However, the mechanisms by which pHi influences the regulation of uraemic neutrophil function have not been well established. Our present *in vivo* findings showed that uraemic neutrophils with low pHi values had enhanced phagocytosis and increased oxidative burst reactions in comparison to uraemic neutrophils having normal or high pHi values. In addition, our *in vitro* studies on uraemic neutrophils incubated in the acidic pH range demonstrated that pHi inversely correlated with phagocytosis and oxidative burst reactions. With regard to the generation of SO radicals, both our *in vivo* and *in vitro* data suggested that the production of ROS, including O_2^- and H_2O_2, showed increases under acidic conditions, resulting in the observed increase in cytotoxicity. These data are consistent with findings in normal neutrophils reported in previous studies. In contrast, Liberek and coworkers [35] suggested that a lowing of pHi in normal neutrophils was responsible for an observed inhibition of the respiratory burst reaction. On the other hand, our data do not necessarily contradict that of Trevani and co-workers [36] who used HCO_3^--free buffer solutions in their experiments. They also demonstrated that the changes in pHi depended on the presence of extracellular HCO_3^- and they observed only a slight change in neutrophil pHi and function when HCl was added to the HCO_3^--free medium. These findings suggest that HCO_3^- is necessary for the titration of extracellular H^+, which in turn permits inward CO_2 diffusion and subsequent intracellular acidification. In the present study, we observed that the HD patients in group A exhibited lower P_{HCO3} as well as reduced pHi values. Organic or inorganic acid in plasma, which cannot be efficiently removed by HD, can be titrated in the presence of HCO_3^- and this may result in intracellular acidification in uraemic neutrophils. Our data suggested that HD patients with low P_{HCO3} and pHi values, which indicate high acid production, may have radically altered neutrophil and/or immunological functions due to the low neutrophil pHi. Moreover, HCO_3^- may be one of the factors contributing to these discrepancies observed in neutrophil function. However, further study is necessary to explain these alterations.

Role of metabolic acidosis correction on uraemic neutrophil function

Pre-dialysis P_{HCO3} has been demonstrated to be negatively correlated with interdialytic weight gain and has been associated with β2-microglobulin accumulation [37]. In addition, moderate pre-dialysis acidosis ranging from 20.1 to 21.0 mEq/l (mmol/l) appears to be associated with better nutritional status and lower relative risk for mortality or hospitalization than is observed in patients with normal ranges of mid-week pre-dialysis serum bicarbonate concentration (approximately 24 mEq/l) or severe acidosis (<16 mEq/l) [38]. Correction of metabolic acidosis by increasing delivered bicarbonate dose has been shown to decrease catabolism [39] and improve nutrition status [40]. In this study, we observed that alterations in neutrophil function in acidotic HD patients could be restored by correcting metabolic acidosis over a period of 1 month. Our data, therefore, suggest that the pHi-associated activation of neutrophil function in HD patients can be reduced by restoring neutrophil pHi and pHe. However, further studies will be necessary to elucidate the effects that metabolic acidosis correction has on the innate immune system. Although most patients exhibit some degree of post-HD alkalaemia, we did not evaluate post-HD neutrophil function since other factors (pHi, dialyser, dialysate, etc.) may have contributed to the alterations in neutrophil function.

In summary, we demonstrated that HD patients having low P_{HCO3} and pH values also exhibited low pHi. This intracellular acidification may contribute to the delayed apoptosis, enhanced phagocytosis and increased oxidative burst reactions observed in these neutrophils compared with neutrophils having normal or high pHi. In addition, these alterations in neutrophil functions in acidotic HD patients were restored by correcting metabolic acidosis over a period of 1 month. We propose that HD patients having a higher acid production may have an inflamed innate immune state and that P_{HCO3} may play an important role in the regulation of pHi in neutrophils. Therefore, more attention should be given to the role of P_{HCO3} as well as pHi in the immune regulation of uraemic neutrophils.

Conflict of interest statement. None declared.

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