1 Title: Prompt rise in urinary ammonium excretion suffices to mitigate metabolic acidosis in

2 an experimental animal model of severe normovolemic hemodilution

3 Short title: Prompt rise in urinary ammonium excretion

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1 **<u>Summary</u>**

2 Recently, we have established a model of severe stepwise normovolemic hemodilution to a

3 hematocrit of 10% in rats employing three different colloidal volume replacement solutions

4 (Voluven, Volulyte and Gelafundin) that are routinely used in clinical practice at present. We

5 did not see severe dilutional acidosis as to be expected, but a decline in urinary pH. We here

6 looked on further mechanisms of renal acid excretion during normovolemic hemodilution.

7 Bicarbonate, which had been removed during normovolemic hemodilution, was calculated

8 with the help of the Henderson-Hasselbalch equation. The urinary amount of ammonium as

9 well as phosphate was determined in residual probes. The absolute amount of free protons in

10 urine was obtained from the pH of the respective samples. The amount of protons generated

11 during normovolemic hemodilution was approximately 0.6 mmol. During experimental time

12 (5.5 h), distinct urinary ammonium excretion occurred (Voluven 0.52 mmol, Volulyte 0.39

13 mmol and Gelafundin 0.77 mmol). Proton excretion via the phosphate buffer constituted 0.04

14 mmol in every experimental group. Excretion of free protons was in the range of 10^{-6} mmol.

15 The present data prove that the prompt rise in urinary ammonium excretion is also valid for

16 acute metabolic acidosis originating from severe normovolemic hemodilution.

17 **Key words**

18 acute metabolic acidosis, ammonium excretion, acid-base homeostasis, normovolemic

19 hemodilution, renal acid excretion

20

1 **<u>Introduction</u>**

2 Metabolic acidosis is defined as a condition in which plasma bicarbonate is reduced to a

3 degree that equals a base excess below -2 mEq/L (Astrup *et al.* 1960, Kraut and Madias

4 2012). Severe manifestation of this type of acid-base disturbance is associated with increased

5 morbidity and mortality, and therefore with poor clinical outcome (Gunnerson *et al.* 2006,

6 Kraut and Madias 2012). The miserable prognosis can be explained by the fact that acute

7 metabolic acidosis is known to have several deleterious effects, a decrease in cardiac

8 contractility and cardiac output, hypotension due to peripheral vasodilation, and decreased

9 binding of insulin and catecholamines, for instance (Oliva 1970). In Western countries, most

10 cases of acidosis with metabolic rather than respiratory origin are caused by lactic acidosis or

11 ketoacidosis (Kraut and Madias 2012). Nonetheless, in clinical situations that make the

12 administration of large volumes of saline fluids necessary, a metabolic acidosis might occur

13 due to dilutional effects. Dilutional acidosis by definition develops, if the buffer base

14 bicarbonate is diluted due to administration of volume replacement solutions that are devoid

15 of bicarbonate, whereas the buffer acid carbon dioxide is not (Waters *et al.* 1999). In the

16 clinical setting outlined above, colloidal volume replacement solutions are often used due to

17 their oncotic properties in contrast to pure crystalloid solutions. Concerning their

18 composition, colloidal solutions differ as regards the contained colloid, electrolytes and

19 possibly metabolizable anions.

20 Recently, we have established a model of severe stepwise normovolemic hemodilution to a

21 final hematocrit of $10 \pm 1\%$ in rats (Teloh *et al.* 2013). For hemodilution, we employed three

22 different approved volume replacement solutions (one per experimental group), which all

23 have been commonly used in clinical routine (Gelafundin, Voluven, Volulyte). Starting at a

24 value of -0.6 mEq/L, current base excess fell to a value of -3.5 mEq/L at the end of the

25 experiment in all groups, indeed indicating the existence of an acute metabolic acidosis, but to

1 a much lesser extent than expected. Despite the severe degree of hemodilution, we did not

2 detect any drop in plasma pH in none of the three experimental groups (Gelafundin, Voluven,

3 Volulyte), instead staying at a rather constant level. To find an explanation for this

4 unexpected finding and since the urinary pH decreased in all groups from an initial value

5 slightly above neutrality to approximately 6.3 in the observation phase, we decided to

6 measure phosphate and ammonium content in residual urine probes of this experiment and

7 compared these values quantitatively with the amount of bicarbonate removed during

8 stepwise severe normovolemic hemodilution.

9 **Methods**

10 We took residual urine probes from our previous experiments (Teloh *et al.* 2013). In those

11 experiments, stepwise normovolemic hemodilution in male Wistar rats (strain WIS/WU, body

12 weight 410 – 470 g, estimated blood volume of 25 - 29 ml according to the equation of Lee

13 and Blaufox (Lee and Blaufox 1985)) until a final hematocrit of $10 \pm 1\%$ using one of the

14 colloidal volume replacement solutions Voluven, Volulyte or Gelafundin was performed. For

15 blood withdrawal and volume replacement, one catheter was placed within the right femoral

16 artery and the right femoral vein each. Per step of dilution, 3 ml of blood were exchanged,

17 followed by a pause interval of 15 min. For urine collection, a catheter was inserted into the

18 bladder and the urine collected hereby pooled for the respective phase of the experiment

19 (initial, phase of dilution and observation phase).

20 **Measurements**

21 For ammonium quantification, capillary electrophoresis was used. For this purpose, a *fused*

22 *silica* capillary was employed with an effective length of 50 cm, an I.D. of 75 µm and an O.D.

23 of 375 µm. Samples of initial urine were diluted with ultrapure water 1:50, whereas samples

24 of the phase of dilution as well as observation phase were diluted 1:100. Analysis was

25 performed using a cation analysis kit (ABSciex, Fullerton, USA). Per analysis, the volume

1   was applied using pressure injection. The subsequent separation proceeded using a voltage of

2   30 kV and normal polarity of the capillary. Indirect detection was performed employing a

3   photo diode array at a wavelength of 200 nm.

4   Quantification of phosphate in urine samples was realized with the help of a colorimetric test

5   at a wavelength of 340 nm (Daly and Ertingshausen 1972). Here, inorganic phosphate reacts

6   with ammonium molybdate to form an unreduced phosphomolybdate complex. The obtained

7   results had already been corrected for background absorption by individual blank

8   measurements that were automatically subtracted from the sample's absorbance by the

9   analyzer.

10   **Calculations**

11   Bicarbonate having been removed during every single step of normovolemic hemodilution

12   was calculated with the help of the Henderson-Hasselbalch equation on the basis of measured

13   pH and carbon dioxide partial pressure at the respective point of time (Teloh *et al.* 2013). It

14   was assumed, that during the pause interval with a length of 15 min after every step of

15   dilution, a new equilibrium had adjusted.

16   The absolute amount of free protons in urine, which is responsible for its acidification, was

17   obtained from the pH of the respective sample (Teloh *et al.* 2013). Absolute amounts of

18   ammonium as well as phosphate were calculated taking into account the volumes of the

19   respective urine samples.

20   **Statistical Analysis**

21   Experiments were performed with six animals per group. The data are expressed as mean

22   values ± standard error of the mean (SEM). Comparisons within a single group were

23   performed using repeated measurement two-way independent analysis of variance (ANOVA)

24   followed by a Dunnett post hoc analysis. Comparisons among multiple groups were

1   performed during two-way independent ANOVA followed by a Tukey post hoc analysis. A *P*

2   value < 0.05 was considered significant.

3   **Results**

4   The absolute amount of ammonium in initial urine varied between 0.012 ± 0.003 mmol

5   (Volulyte group) and 0.021 ± 0.005 mmol (Gelafundin group) (Figure 1). During the phase of

6   dilution, the absolute amount of ammonium increased to values of 0.194 ± 0.022 mmol,

7   0.296 ± 0.024 mmol for the Volulyte and Voluven group, but to 0.455 ± 0.051 mmol for the

8   Gelafundin group. In the observation phase, the values amounted to 0.201 ± 0.030 mmol,

9   0.160 ± 0.044 mmol and 0.425 ± 0.026 mmol, respectively.

10   The absolute phosphate amount in initial urine was 0.0004 ± 0.0002 mmol (Voluven group)

11   and 0.0010 ± 0.0004 mmol (Gelafundin group) (Figure 2). The urinary amount increased to

12   0.038 ± 0.002 mmol, 0.015 ± 0.007 mmol   and 0.032 ± 0.012 mmol   (Voluven,   Volulyte,

13   Gelafundin) in the phase of dilution and was 0.012 ± 0.003 mmol, 0.022 ± 0.014 mmol and

14   0.011 ± 0.005 mmol, respectively, in the observation phase.

15   **Discussion**

16   During stepwise normovolemic hemodilution, bicarbonate is removed as a result of blood

17   withdrawal and replacement of blood by non-bicarbonate-containing fluids. In the present

18   experiments, the amount of bicarbonate removed that way approximated 0.6 mmol (mean of

19   the three experimental groups, Table 1). Since every molecule bicarbonate removed left

20   behind one proton, the deficit of bicarbonate equaled the surplus of protons and thus, about

21   0.6 mmol protons had been generated during the phase of hemodilution.

22   Generally, three different renal mechanisms exist to excrete or neutralize excess protons

23   (Adam 1981, Rector 1973). First, excretion of free protons into urine. Since, however, the pH

24   of urine is restricted between 4.5 and 7.5, only small amounts of protons can be excreted this

1   way (Lim 2007, Rector 1973, Rose and Post 2001). In the present experiments, during

2   hemodilution and the subsequent observation time, urinary pH decreased from approximately

3   7.3 to 6.3 (Teloh *et al.* 2013), indicating an additionally excreted amount of free protons of

4   about $5 \cdot 10^{-6}$ mmol. Hence, the excretion of free protons is quantitatively negligible as

5   compared to the amount of 0.6 mmol protons formed during normovolemic hemodilution.

6   Second, removal of excess protons with the help of the phosphate buffer system. Attributable

7   to the acidification of urine, HPO_4^{2-} being present in the urine is further protonated to yield

8    $H_2PO_4^-$. However, this buffer system is barely gradable due to limited available phosphate

9   (Lim 2007, Pitts 1973, Rose and Post 2001). In our experiments, compared to the initial value,

10   during and following hemodilution, the absolute amount of phosphate excreted is increased

11   (Figure 2), but these values are still around the reference of daily phosphate excretion in rats

12   that amounts to approximately 0.35 mmol, i.e. 0.04 mmol within 3 hours (Waynforth and

13   Flecknell 1992). In total, excretion of protons via further phosphate protonation and increase

14   in phosphate excretion may amount to approximately 0.03 mmol. Thus, as compared to a

15   value in the order of 0.6 mmol protons formed, this mechanism also does not constitute an

16   adequate machinery to excrete the surplus of protons being generated during hemodilution.

17   Third, enhanced urinary ammonium excretion. Ammonium release from glutamine and

18   hereinafter glutamate takes place in the kidney and is mediated by two enzymes, namely

19   glutaminase I and glutamate dehydrogenase (Damian and Pitts 1970, Fahien and Strmecki

20   1969, Goldstein and Schooler 1967). In acidotic states, urinary ammonium excretion rises

21   resulting from an interplay between renal glutamine degradation with a concomitant fall in

22   glutamine concentration and increased ammonium excretion due to urinary acidification. In

23   the liver, re-synthesis of glutamine occurs for disposal of waste nitrogen at the expense of

24   ureagenesis. Since nitrogen excretion via ammonium does not utilize any bicarbonate, but

25   nitrogen excretion via urea does, the saved bicarbonate can neutralize excess protons.

1 Compared with the initial value, the absolute amount of ammonium in urine increased in all

2 groups during experimental time (Figure 1). According to the literature (Oliver and Bourke

3 1975), and also reference values of other in-house animals (unpublished data), animals are

4 supposed to excrete approximately 0.42 ± 0.01 mmol ammonium within 24 hours under

5 physiological conditions. For the duration of the phase of dilution (3 hours) and the

6 subsequent observation phase (2.5 hours), this amounts to 0.05 mmol and 0.04 mmol,

7 respectively. Therefore, the amount of ammonium excreted in the respective experimental

8 phases (0.32 mmol and 0.26 mmol, respectively, on average) is distinctly elevated as opposed

9 to the aforementioned physiological baseline conditions. The sum of excreted ammonium

10 during the phase of dilution and observation phase in each group was close to or even above

11 0.6 mmol. These values clearly show that the vast majority of protons produced during

12 normovolemic hemodilution was compensated by urinary ammonium excretion. However,

13 animals having received Gelafundin as volume replacement excreted in sum (phase of

14 dilution and observation phase) approximately twice as much ammonium as animals of the

15 Volulyte group, being the group that did excrete fewest of all (Gelafundin 0.88 mmol,

16 Voluven 0.46 mmol and Volulyte 0.40 mmol) suggesting a possible correlation of ammonium

17 excretion with the colloid's chemical structure. Gelafundin comprises succinylated gelatin

18 whose terminal carboxyl groups are deprotonated at physiological pH, hence, carrying a

19 negative charge. Due to the fact that succinylated gelatin is rapidly excreted via the urine

20 (Teloh *et al.* 2013), it could have favored the excretion of cations like ammonium for

21 electrochemical reasons. In contrast, hydroxyethyl starch, being the colloidal component in

22 Voluven and Volulyte, is uncharged at physiological pH. Based on the increased ammonium

23 excretion in the Gelafundin group, one might even speculate that infused succinylated gelatin

24 may lead to a disproportional ammonium excretion favoring alkalization. In addition to the

25 colloid, the carrier solution's composition appears to be also of importance as regards the

26 ability to excrete ammonium. Volulyte is the only solution of the three employed here whose

1   carrier solution is plasma-adapted and therefore contains a metabolic anion in terms of

2   acetate. Based on the theory of metabolizable anions (Zander 2009), metabolic acidosis

3   should be antagonized by producing bicarbonate during their metabolization. Taking into

4   account absolute values of ammonium excretion (Figure 1), our data of the Volulyte group are

5   in line with this theory. In sum (phase of dilution plus observation phase), urine contained

6   only 0.40 mmol ammonium (in contrast to 0.46 mmol and 0.88 mmol of the Voluven and

7   Gelafundin group, respectively) but base excess values were simultaneously closest to

8   neutrality of the three groups (Teloh *et al.* 2013), indicating, that the remaining surplus of

9   protons is neutralized with the aid of bicarbonate originating from acetate metabolism.

10   In the past, a lot of experimental animal studies were conducted examining ammonium

11   excretion as a response to acute metabolic acidosis (Bishop *et al.* 2010, Goldstein and

12   Schooler 1967, Lee *et al.* 2009, Leonard and Orloff 1955, Oliver and Bourke 1975, Parry and

13   Brosnan 1978, Veverbrants *et al.* 1977), showing, that urinary ammonia excretion is the

14   primary component of the increase in net acid excretion (Bishop *et al.* 2010, Hamm and

15   Simon 1987, Weiner and Verlander 2013). All of these studies more or less uniformly showed

16   that metabolic acidosis enhanced urinary ammonium excretion within a few hours after

17   acidosis induction. However, metabolic acidosis was always induced by administration of

18   either hydrochloric acid or ammonium chloride. Therefore, these models were highly artificial

19   compared to patients developing acute metabolic acidosis. In contrast, in the present study,

20   normovolemic hemodilution was the employed model to establish an acidosis of dilutional

21   origin which regularly develops in the clinical setting, especially in patients undergoing

22   cardiac surgery with the need of an extracorporeal circulation (Teloh *et al.* 2015). Thus, we

23   here confirmed that urinary ammonium excretion is also the primary component of net acid

24   excretion in the context of normovolemic hemodilution and thus suffices to mitigate

25   metabolic acidosis in terms of a compensatory mechanism. Additionally, differences in the

26   need for urinary ammonium excretion became obvious due to the presence or absence of

1   metabolizable   anions   in   the   colloidal   volume   replacement   solution.

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9

1   **Tables**

2   <u>Table 1</u>

3   **Calculation of excess protons generated during the phase of severe stepwise**

4   **normovolemic hemodilution with Voluven, Volulyte and Gelafundin whereby the**

5   **exchanged volume of the last of nine steps of dilution was kept variable to reliably reach**

6   **the final hematocrit of 10%.**

| Volume replacement solution used for hemodilution	Current HCO_3^- [mmol]	Exchanged volume during dilution [ml]	Excess H^+ [mmol]
Voluven	23	26	0.60 ± 0.02
Volulyte	26	26	0.68 ± 0.02
Gelafundin	23	25	0.58 ± 0.04

7   *Note: Calculations were made based on the values of six individual animals per group at*

8   *three different points of time. Therefore, calculations using mean values above may differ.*
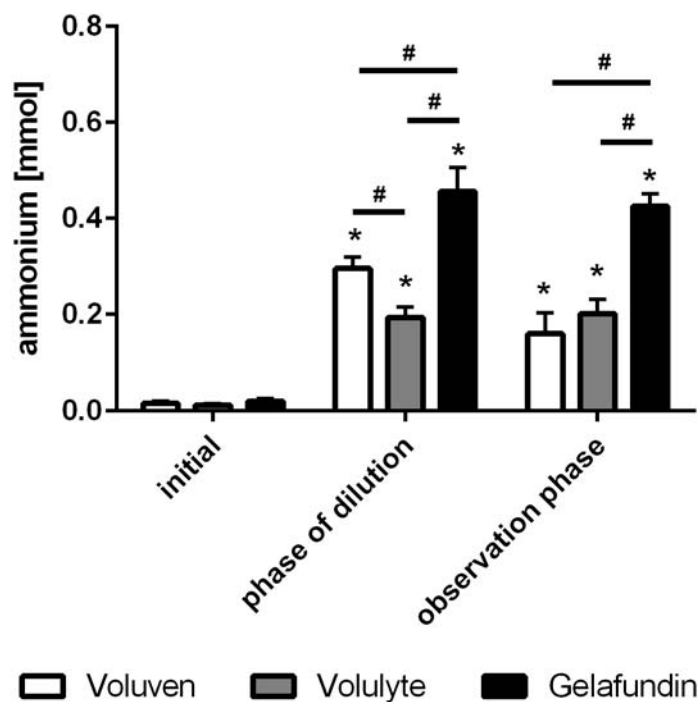
Figure 1. Ammonia excretion during and subsequent to severe normovolemic hemodilution with Voluven, Volulyte and Gelafundin. After insertion of the urinary catheter, the initial urine was collected. Collection was continued during the phase of hemodilution as well as during the observation phase. Experimental groups differ from each other in the colloidal volume replacement solution employed for normovolemic hemodilution. * $p < 0.05$ compared with the initial value. # $p < 0.05$. Sample size for every group: n=3 at initial due to the small urine volume; n=6 at phase of dilution; n=4 at observation phase due to 2 missing samples
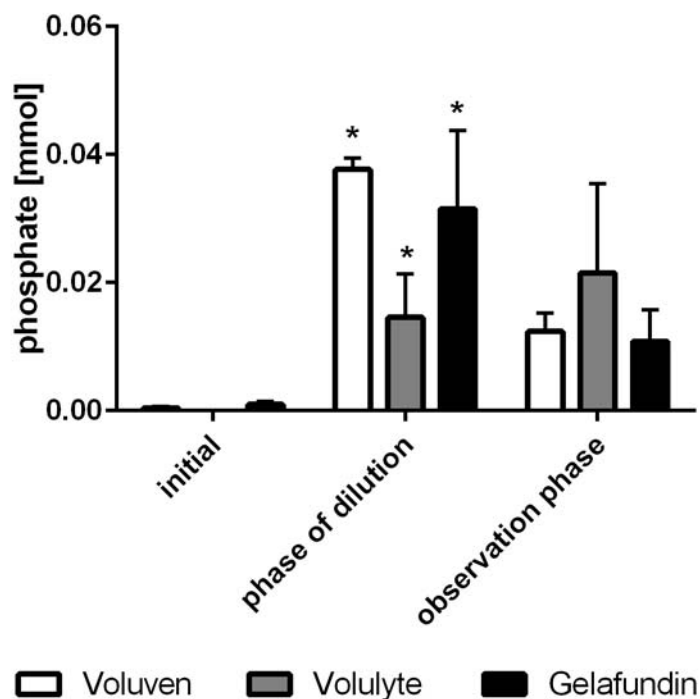
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2 **Figure 2. Phosphate excretion during and subsequent to severe normovolemic**

3 **hemodilution with Voluven, Volulyte and Gelafundin.** After insertion of the urinary

4 catheter, the initial urine was collected. Collection was continued during the phase of

5 hemodilution as well as during the observation phase. Experimental groups differ from each

6 other in the colloidal volume replacement solution employed for normovolemic hemodilution.

7 * $p < 0.05$ compared with the initial value. Sample size for every group: n=3 at initial due to

8 the small urine volume; n=6 at phase of dilution; n=4 at observation phase due to 2 missing

9 samples