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# SUBNUCLEAR LIPID-CONTAINING VACUOLIZATION IN CASES OF KETOACIDOSIS - CORRELATION OF MORPHOLOGICAL FINDINGS AND KETONE BODY CONCENTRATIONS

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**Abstract:** In cases of different causes of ketoacidosis, vacuole changes in the epithelium of the proximal renal tubules have been observed. Meanwhile it has been proven that subnuclear vacuolization is not only found in alcoholics but also in diabetic coma and fatal hypothermia. In this work, concentrations of the ketone body beta-hydroxybutyrate were determined and a histological examination of the kidneys was performed (HE-, PAS-, Sudan-staining). In a collective of 30 cases with alcohol abuse, fatal hypothermia, diabetes or combinations and 55 control cases, a strong correlation of the measured ketone body concentrations with subnuclear lipid-containing vacuolization in kidneys could be demonstrated. If the cause of death could not be clarified, this finding could be the reason for specific ketone body determinations to clarify the cause of death.

**Keywords:** ketoacidosis, kidney, Sudan staining, vacuolization, ketone body, diabetes, alcohol abuse, hypothermia.

## INTRODUCTION

Ketone bodies are formed in catabolic metabolic situations. Ketoacidosis, a form of metabolic acidosis, is caused by elevated concentrations of ketone bodies in the blood. Lethal ketoacidosis is observed in chronic alcohol abuse, diabetes mellitus and is considered to be one of many factors contributing to fatal hypothermia [1-3]. The main parameter to determine ketoacidosis or ketogenic metabolic situations is the ketone body beta-hydroxybutyrate (BHB). Lethal thresholds are generally considered to be > 250µg/ml and physiological values <50µg/mL [4].

Besides the Armanni-Ebstein-cells, which are a characteristic finding in diabetic coma [5], the subnuclear lipid-containing vacuolization in epithelial cells of renal tubules must be distinguished [6]. These findings are not only present in metabolic disturbances due to diabetes but also in chronic alcohol abuse and hypothermia [7, 8]. Our institute already has been able to show that there seems to be a correlation between the measured concentrations of ketone bodies and lipid-containing vacuolic changes in renal tubuli [9].

Since deaths due to chronic alcohol abuse, metabolic disturbances in diabetes and hypothermia can be difficult to clarify because macroscopical findings in these cases are often not sufficient to determine a cause of death or even remain completely absent, the histological evidence of subnucelar lipid-containing vacuolization could be useful in the clarification of the cause of death. In this work, cases of alcohol abuse, diabetes, hypothermia as well as combinations were studied to further investigate a correlation of the level of ketone body concentrations with subnuclear lipid-containing vacuolization of the kidney. If the cause of death remains unclear, histological examination of the kidneys may contribute to clarification.

## MATERIAL AND METHODS

### *Samples*

The study included a prospective series (n=30) of alcoholics, patients suffering from diabetes and cases of hypothermia, combinations of them as well as controls. The control group (n=55) consisted of patients in whom the medicolegal autopsy revealed natural causes of death, especially cardial, or unnatural causes of death, like suicidal hanging or drowning.

In all cases the concentration of ketone body BHB was analyzed, mainly in vitreous humor. If not

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available, cerebrospinal fluid or femoral blood was used.

In each case, histological examinations of kidneys were carried out using HE-, Sudan-, and PAS-staining. The histological preparations were each meandered under the light microscope in 10 fields of view at 200x magnification. For each field of view, the structures stained with Sudan (renal tubuli) were related to the total number of structures. From the 10 visual fields, a percentage mean value was calculated for each preparation, which was used to determine the degree of Sudan-staining (Table 1, Fig. 1). Measured BHB-concentrations were then correlated with the degree of Sudan-stainability in the renal tissue.

***GC-MS analyses***

Beta-hydroxybuyrate (BHB) concentrations were determined by means of a gas chromatographic mass spectrometric (GC-MS) method. The method was validated according to a forensic guideline of the Society of Toxicological and Forensic Chemistry (GTFCh) [11]. Water was used as the matrix during method validation. The linear range was established between 10 µg/mL and 500 µg/mL. Limit of detection (LOD) and limit of quantification (LOQ) for the detection of BHB were 1.4 µg/mL and 3.3 µg/mL, respectively. Accuracy and precision (at concentrations of 32 µg/mL, 60 µg/mL and 325 µg/mL) were in accordance with the acceptance criteria of the guideline (<15% bias and precision). During analyses of vitreous humor, cerebrospinal fluid or femoral blood samples, negative controls (water) as well as quality control samples prepared in water were carried along each analysis sequence.

*Chemicals*

(±)-ß-Hydroxybutyrate-d4 (sodium salt, BHB-d4) was purchased from Cayman Chemicals (Ann Arbor, MI, United States), sodium 3-Hydroxybutyrate from LGC (Teddington, United Kingdom), methanol from Honeywell Riedel-de-Haën™ (Seelze, Germany), Silyl-991 (N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1 % trimethylchlorosilane (TMCS)) from Macherey-Nagel (Düren, Germany).

*Sample preparation for GC-MS analyses*

Twenty µL of the matrix sample (vitreous humor, cerebrospinal fluid or femoral blood) were fortified with 10 µL of internal standard solution containing 125 µg/mL BHB-d4. Protein precipitation was performed by addition of 100 µL of methanol, subsequent vortexing and centrifugation for 10 min. Before derivatization, the methanolic supernatant was evaporated to dryness on a rotary evaporator at 60°C. Derivatization was done by redissolving the residue in 50 µL BSTFA (with 1% TMCS) and 450 µL isooctane and subsequent incubation at 80°C for 10 min. 2 µL of the derivatized extract were subsequently injected splitless and analyzed by GC-MS.

*GC-MS analysis*

Gas chromatographic separation was achieved using an Agilent 6890N gas chromatography device (Agilent Technologies, Santa Clara, US). For chromatographic separation, a HP-5ms (5%-Phenyl-methylpolysiloxane) GC-column (Agilent Technologies, Santa Clara, US) was used. Helium was

**Table 1.** Graduation of Sudan-staining (according to [7])

| Distribution (%) | Graduation | Staining |
|---|---|---|
| 0 | 0 | none |
| > 0-29,99 % | 1 | weak |
| 30-59-99 % | 2 | moderate |
| 60-100% | 3 | intensive |

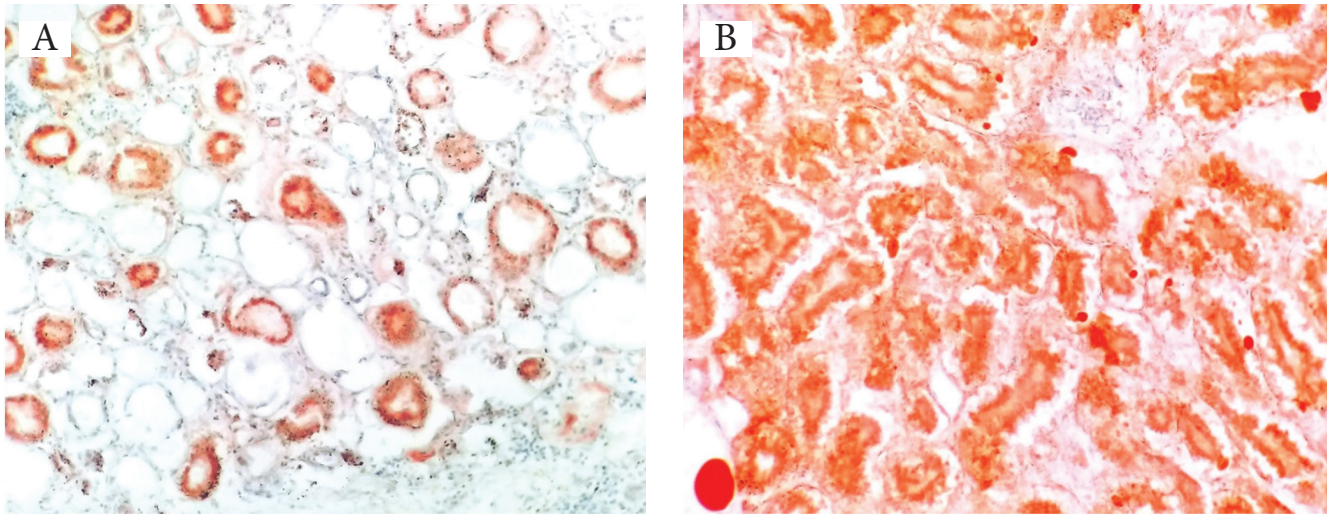


**Figure 1.** Degree of Sudan - staining: A) Grade 2, B) Grade 3.

**Table 1.** Study group

| No. | Age | Sex | Cause of death | Prehistory | BHB (µg/mL) | Sudan stain Grade |
|---|---|---|---|---|---|---|
| **1** | 36 | M | Combined drug intoxication | A | 17.81 | 1 |
| **2** | 31 | M | Diabetic ketoacidosis | DM | 1333.94 | 3 |
| **3** | 59 | M | Intoxication | A | 17.57 | 0 |
| **4** | 33 | M | Heroin intoxication | A + DM | 31.81 | 1 |
| **5** | 33 | M | Combined drug intoxication | A | 20.19 | 0 |
| **6** | 46 | M | Combined drug intoxication | A | 13.7 | 0 |
| **7** | 66 | F | Hypothermia | A | 229.67 | 1 |
| **8** | 49 | M | Hypothermia | A | 1428.33 | 3 |
| **9** | 53 | M | Intoxication | A | 872.33 | 3 |
| **10** | 70 | F | Hypothermia | | 255.14 | 1 |
| **11** | 81 | M | Hypothermia | | 257.06 | 1 |
| **12** | 88 | M | Hypothermia | | 157.06 | 0 |
| **13** | 21 | F | Intoxication | A* | 34.96 | 0 |
| **14** | 40 | F | Combination of previous illness and alcohol abuse | A | 32.01 | 1 |
| **15** | 46 | M | Combined drug intoxication | A | 26.63 | 0 |
| **16** | 62 | M | Intoxication | A | 78.5 | 3 |
| **17** | 43 | M | Hypothermia | | 16.4 | 0 |
| **18** | 36 | F | Hypothermia | DM | 1699.53 | 3 |
| **19** | 57 | M | Metabolic imbalance | A + DM | 120.89 | 2 |
| **20** | 51 | M | Hypothermia | A | 48.4 | 2 |
| **21** | 53 | M | Metabolic imbalance | DM | 21 | 0 |
| **22** | 33 | F | Hypothermia | DM | 2415.83 | 3 |
| **23** | 23 | M | Hypothermia | A | 305.83 | 3 |
| **24** | 73 | F | Hypothermia | A* | 123.73 | 2 |
| **25** | 57 | M | Hypothermia | DM | 1044 | 1 |
| **26** | 77 | F | Hypothermia | A | 73.87 | 3 |
| **27** | 62 | M | Hypothermia | A | 1571.57 | 3 |
| **28** | 27 | F | Alcohol intoxication | A | 47.56 | 0 |
| **29** | 76 | M | Hypothermia | | 271.46 | 1 |
| **30** | 70 | M | Hypothermia | | 442.21 | 2 |

F: female, M: male, A: chronic alcohol abuse, DM: suffered from diabetes mellitus. * = Chronic alcohol abuse not explicitly mentioned in prehistory, but empty liquor bottles found next to the body.

used as carrier gas (flow rate of 1 mL/min). Injector was hold at constant temperature of 250°C. The column was heated with the following temperature program: temperature was hold at 80°C for 1 min, increased up to 120°C with a heating-up rate of 10°C/min and hold for 1 min at 120°C, raised up to 160°C with a heating-up rate of 30°C/min and further increased up to 300°C with a heating-up rate of 85°C/min, hold for 3 min at 300°C and rapidly decreased back to 80°C after the run. An Agilent 5973N single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, US) was used for the detection of BHB and BHB-d4 using electron-impact-ionization and single-ion-monitoring (SIM) detection mode. For TMS-derivative of BHB the ion with a mass to charge ratio (m/z) of 233 was used as target and ions with m/z 191 and 234 were used as qualifiers. For TMS-derivate of BHB-d4 the ion with m/z 237 was used as target (additional ions for identification: m/z 195 and 238). The detector was hold at constant temperature of 210°C. Total runtime was 12 minutes.

## RESULTS

In the study group, in 9 cases no staining was found (grade 0): in 8 of these cases the concentration of BHB was <50 µg/mL, so that it can be assumed that no ketoacidosis or ketogenic metabolism was present, in one case a pathological but non-lethal BHB-concentration was found.

In 8 cases there was a weak stainability (grade 1), in 3 cases there was a physiological, in 5 cases a pathological BHB-concentration. All 3 cases with physiological concentrations were connected to alcohol abuse.

A moderate stainability was seen in 4 cases, whereby in 3 cases the BHB-concentration was above the normal value and in one case below.

In 9 cases of the group an intensive stainability could be detected, in 7 cases the BHB-concentration was above 250 µg/mL, i.e. in the range of lethal ketoacidosis. In 2 cases the value did not exceed 250 µg/mL, but was above the normal value. Overall, there was a clear

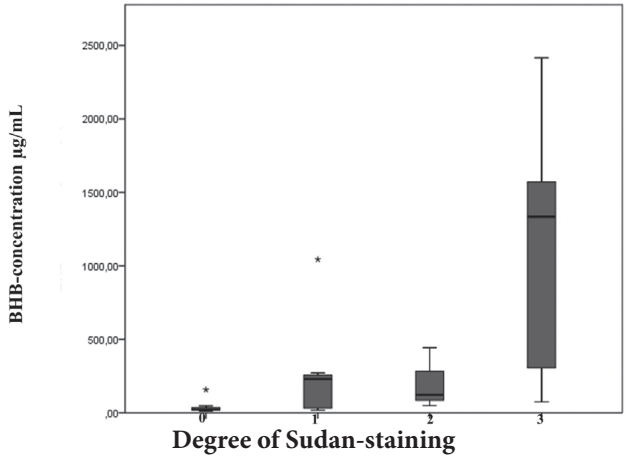


**Figure 2.** Distribution of BHB-concentrations in groups divided due to Sudan-stainibility. Bar shows median value with minimum and maximum values in each group (without outliers), * = outliers.

tendency in the study collective that the higher the BHB-concentrations measured, the more pronounced was the subnuclear, lipid-containing vacuolization of the renal tubules. Figure 2 shows Boxplots illustrating the distribution of BHB-concentrations in each group.

## DISCUSSION

The results show a close correlation between the ketone body concentration and the degree of subnuclear vacuolization. However, there was also one person in the study who, despite the presence of ketone bodies, did not show any stainability and, vice versa, there was one person who, despite low ketone bodies, showed moderate stainability. An exclusively histological examination would therefore not be reliable. In a case of a morphologically unexplainable cause of death, this finding could still account for a specific determination of ketone bodies to clarify the cause of death. Further studies could examine the cases in which the ketone body concentration does not correlate with the degree of Sudan-staining. Aspects (i.e. pre-existing conditions) might be found that have an influence either on the Sudan-staining or the ketone body concentration. This might allow an establishment of criteria that exclude certain cases from the histological examination and thus, providing a reliable method.

Another interesting approach is the correlation of the ketone body concentration with the respective triggers for ketoacidosis (alcohol, diabetes, hypothermia). Studies have already shown that deaths associated with diabetic metabolic conditions seem to have the highest ketone body concentrations [12]. Thus, conclusions might be drawn not only about an existing ketoacidosis as a cause of death but also about its underlying trigger.

**In conclusion,** the study showed that high BHB concentrations correlate with the degree of Sudan staining. However, there were few exceptions where this was not the case. Further studies with larger study collectives and more detailed investigation of these exceptions could clarify these cases and help establish the histological examination as a valid method to define ketoacidosis as the cause of death.

However, in cases of unclear deaths, histological examination of the kidneys in combination with the prehistory of the deceased is helpful to support the diagnosis of ketoacidosis.

### Conflict of interest

The authors declare that they have no conflict of interest.

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