



ORIGINAL PAPER

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## Model development of hydroxyproline induced hyperoxaluria in young growing pigs

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### ABSTRACT

**Aim of the study.** In this study, we sought to create a model of reversible hyperoxaluria in pigs by feeding with hydroxyproline (HP).

**Materials and methods.** The experiment included 12 pigs divided into 2 groups (n = 6). The pigs were fed twice a day. At the beginning of the experiment, in the adaptation period, all pigs were given standard feed. In the next 7 days, an increasing amount of hydroxyproline (1–3% HP), was added to the feed. In next 14 days, 4% HP was administered in each pig meal. After 14 days of 4% HP diet, the pigs were randomly divided into 2 groups. For 6 pigs, 4% HP treatment had been continued for the next 14 days while the second group of pigs for the next 14 days received a standard HP free diet. 24h urine samples, blood and fecal samples were collected on particular days.

**Results.** The addition of HP to the diet increased urinary oxalate excretion. A characteristic increase was noted after 12 days of treatment with 4% HP. During the removal period, oxalate excretion decreased in the group without HP in diet, while in the group which continued with a 4% HP diet, oxalate excretion significantly increased. Gross examination of kidneys showed that in the group which had 4% HP diet for 4 weeks, kidneys were fibrotic with enlarged cavities, and had small visible urinary stones. In second group, kidneys were relatively normal looking with no visible stones.

**Conclusion.** Hyperoxaluria is reversible, if HP is removed 14 days after the start of 4% HP diet. Prolonged exposure up to 4 weeks causes pathologic changes in kidneys including crystals, sand and small stone formation.

**Keywords.** pig model, hyperoxaluria, kidney stones, hydroxyproline

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**Participation of co-authors:** A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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## Introduction

Kidney stones formation is a known problem worldwide affecting from 5 to 15% of the population. The development of stones has been shown to be a multifactorial process, caused by metabolic and environmental factors.<sup>1</sup> This disease mainly occurs in the 3<sup>rd</sup> to 5<sup>th</sup> decade of life. Men suffer three times more often than women. 15% of people who have experienced an episode of renal colic, will sustain another attack within 3 years, and 30–50% in the next 15 years.<sup>2</sup> Patients forming kidney stones are prone to recurrence even after surgical removal. Recurrence rates are close to 50%.<sup>3</sup> There are about 16 types of different minerals that form urinary tract stones. The most common form of stones that occurs in about 70% of cases consist of calcium oxalate.<sup>4</sup> Hyperoxaluria is a major risk factor in calcium oxalate stone disease. This disorder is characterized by an excessive production of oxalate. Oxalate is an end product of metabolism, which normally over 90% is excreted by healthy kidneys. Increased excretion of oxalate in urine may lead to oxalic acid combining with calcium and formation of small crystals and eventually kidney and bladder stones. The conditions of increased excretion of oxalate in urine are defined as a primary and secondary hyperoxaluria. Both are autosomal recessive diseases.

Primary hyperoxalurias (Type I and II) are genetic defects that cause faults in the metabolism of glyoxylate and oxalate. In Primary hyperoxaluria Type I (PHI) alike oxalate acid, glycolic acid is also excreted in excess amount. It's cause is reduced or completely inhibited activity of the liver specific, peroxisomal, pyridoxal phosphate – dependent enzyme alanine: glyoxylate amino transferase. The gene for this enzyme-AGXT is located on chromosome 2q37.3. The second type PH2 is caused by a deficiency of cytosolic enzyme, glyoxylate reductase. In this type oxalic acid and L-glyceric acid are excreted in excessive amounts. Location of the gene for this enzyme-GHPR is on chromosome 9p11. PH1 is the most common form of PH.<sup>5,6</sup>

The western type diet, which has an abundance of animal protein, has been implicated as an increased risk factor for the formation of kidney stones in humans.<sup>7</sup> In rats, oral intake of ethylene glycol or hydroxyproline (HP) increased urinary oxalate. Pigs fed with HP became hyperoxaluric, causing crystalluria, calcium oxalate plaques formation on renal papillae and stones.<sup>7,8</sup> It was also shown that in healthy humans, food enriched with gelatin, a food ingredient that contains HP, caused elevated excretion of urinary oxalate and glycolate.<sup>9</sup>

## Objectives

The purpose of this study was to establish a reversible model of hyperoxaluria in young growing pigs as a result of a diet enriched in hydroxyproline. Trans-4-hydroxyproline (HP) used in this study is a physiological pre-

cursor of oxalate, proline derivative and a component of collagen. The intention was to develop a model for imitation of human primary hyperoxaluria disease.

## Materials and methods

All experimental procedures were approved by the University of Lund Ethics Review Committee on Animal Experiments.

## Animals and housing

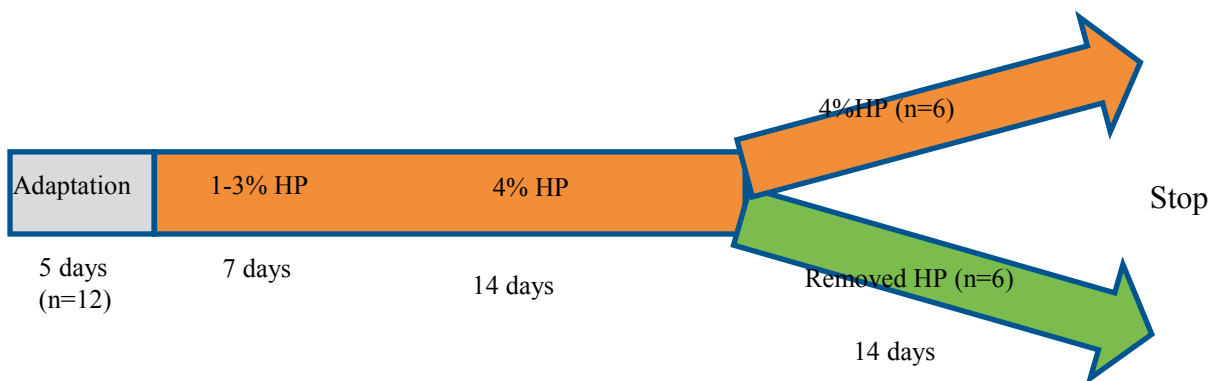
The experiment was carried out at the research farm of the Department of Agricultural Biosystems and Technology, Swedish University of Agricultural Sciences. Batches of 12 pigs (Large White × Landrace, males) that were 6–12 weeks of age and weight  $12.1 \pm 1.2$  kg at the beginning of experiment, were used in the experiment. The pigs were housed in identical, individual “home - design” metabolic cages. All cages were equipped with perforated flooring, heating lamps and a drinking nipple. For the first 5 days animals were trained and acclimated to metabolic cages.

## Model design

All the pigs were fed twice daily (2% body mass per meal) in the morning and in the evening with standard cereal based pellet feed. The adaptation and experimental periods lasted for 40 days. After 5 days of adaptation period, pigs were challenged with hydroxyproline (HP). HP was administered twice a day to pigs as a mixture with feed. HP concentration in the diet was increased during the 7 days of the experiment from 1 to 3% HP. After adjustment, in the next 14 days, 4% of HP was administered in each pigs meal. After 14 days of the 4% HP diet, pigs were divided into 2 groups. For 6 pigs, 4% HP treatment was continued for the next 14 days. This means that this selected group of animals obtained, twice a day, 4% HP during 28 days. The second group of pigs for next 14 days got a standard HP free diet. This means that this group of pigs was kept on 4% HP diet only for 14 days. 24 h drinking water intake was measured during the study. At the end of the study pigs were scarified and internal organs including kidneys, liver and urine bladder were taken for further studies. A general study design and model of hydroxyproline induced hyperoxaluria development in pigs is shown on Figure 1.

## Sample collection and analysis

**Urine samples.** To monitor hyperoxaluria development, 24 h urine samples were collected at the following time points: basal (at the end of adaptation time), the last days on 1% HP and 3% HP, every 2–3 days before randomization, and every 2–3 days during the removal period. 24 hour urine samples were gathered to a pot containing 5–15 mL of



**Figure 1.** Model development of hydroxyproline induced hyperoxaluria in pigs

6 N HCl to acidify the samples and achieve pH~3 to preserve all oxalate soluble in the urine. Total volume of urine was measured and 3 ml samples were transferred to plastic tubes and stored for further analysis.

**Blood samples.** 5 ml blood samples were collected at the following time points: on the last day of adaptation to metabolic cages period, first day on 3% HP, every 3–5 days during treatment period. Samples were collected before feeding from the jugular vein. Collected blood samples were placed in vacutainer heparin tubes. After collection, tubes were centrifuged for 15 minutes at 3000 rpm and blood plasma was separated to new tubes. Two 0.5 mL samples were stored at  $-20^{\circ}\text{C}$  for further analysis.

**Fecal samples.** A small fraction of fecal sample was occasionally collected from pigs in the morning for oxalate estimation. Each sample was weighed and stored at  $-20^{\circ}\text{C}$  in plastic bags for further analysis.

#### Estimation of renal function by creatinine clearance measurement

Creatinine clearance is expressed as excretion rate ( $U_{cr} \times V$ ), where  $U_{cr}$  presents concentration of creatinine (mg/dL) in  $V$  (the 24h urine sample (mL/24h)), divided by plasma creatinine ( $P_{cr}$ ) (mg/dL). This is represented mathematically as:

$$C_{cr} = \frac{(U_{cr} \times V)}{(P_{cr} \times 24)} = \frac{mL}{h}$$

#### Collection of organs for histo-pathological analysis and gross examination

**Gross analysis.** At the end of the experiment, pigs were euthanized with sodium pentobarbiturate (20 mg/kg) and submitted to gross postmortem examination. Kidneys, liver and small and large intestine were gross examined. Kidneys and liver weight was recorded. Both right and left kidney were divided transversely longitudinally, exposing the corticomedullary surface and papillary tips. Gross appearance of the kidneys was recorded

and digital images were obtained. After the gross examination, specimens of the kidneys, fixed in 10% formalin, were taken for future histopathology analysis and Yasue specific staining.

**Histopathology.** Each kidney was cut in 12 serial sections at  $4 \mu\text{m}$  per kidney and stained with hematoxylin and eosin for routine histological examination, or by specific Yasue metal substitution histochemical method to detect the presence of calcium oxalate crystals in the renal tissue.

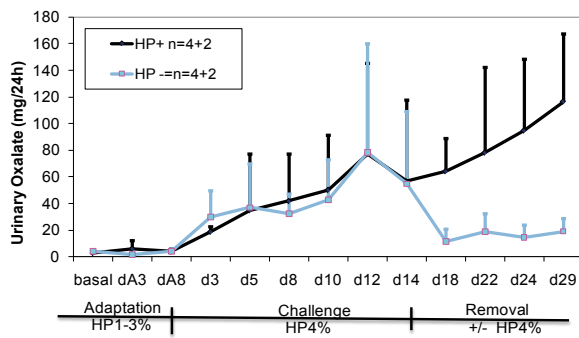
#### Statistical Analysis

Statistical analysis was performed on the data generated from this study using the unpaired two-tailed Student's t-test. Differences were considered significant if  $p \leq 0.05$ , all data are expressed as a mean  $\pm$  standard deviation ( $\pm$  SD).

#### Results

**Determination of the oxalates concentrations in urine**  
Hyperoxaluria development was monitored by measurement of changes in urinary oxalate excretion. Presence of oxalates in acidulated urine were determined by the Jaffe method with the help of a commercial oxalate kit from Trinity Biotech. Amount of oxalates was expressed in mg per twenty four hours.

A slow increase of urinary oxalate from basal concentration  $3.83 \pm 3.0$  mg/24h was demonstrated during the HP challenge period. A significant increase was noted after 12 days of treatment with 4% HP when urinary oxalate levels reached  $78.15 \pm 94.53$  mg/24h in the HP(-) and  $77.21 \pm 78.69$  mg/24h in the HP(+) group ( $p > 0.05$ ). During the removal period, oxalate excretion decreased dramatically in the HP(-) group to  $19.1 \pm 10.48$  mg/24h, while in group which continued to be fed with 4% HP (HP(+) group) oxalate excretion increased even more to  $116.48 \pm 55.34$  mg/24h at the end of the experiment ( $p > 0.05$ ). This was significantly higher ( $p > 0.05$ ). The results for oxalate excretion with urine are shown in Figure 2.



**Figure 2.** Effect of HP administration on oxalate excretion. In all statistical analysis,  $p < 0.05$  was taken as the level of significance

### Determination of blood oxalates levels

The presence of plasma oxalates in samples was measured using Trinity Biotech Oxalate reagents (Kit #.591 D Trinity Biotech, Ireland). The amount of oxalates were expressed in mmol/L. The level of plasma oxalates changed with HP challenge. In animals treated with HP for the whole study, the plasma oxalate level changed from  $0.026 \pm 0.016$  mmol/L to  $0.059 \pm 0.038$  mmol/L ( $p < 0.05$ ). In the group without 4% HP in diet during last 2 weeks, the plasma oxalate level changed from  $0.027 \pm 0.018$  mmol/L to  $0.033 \pm 0.015$  mmol/L and was almost 50% lower than in the group treated with 4% HP for 2 weeks ( $p < 0.05$ ).

### Oxalate/creatinine ratio

There were differences in this ratio during the experiment between groups. The higher value for Ox/Cr ratio in group treated with 4% HP for 4 weeks is the result of higher oxalate level in urine in that group ( $p < 0.05$ ). Urinary oxalate/creatinine ratio presented on Figure 3.

### Creatinine clearance

There were no differences in creatinine clearance between groups during the course of experiment ( $p < 0.05$ ). Creatinine clearance is presented in Figure 4.

### Urinary and plasma creatinine

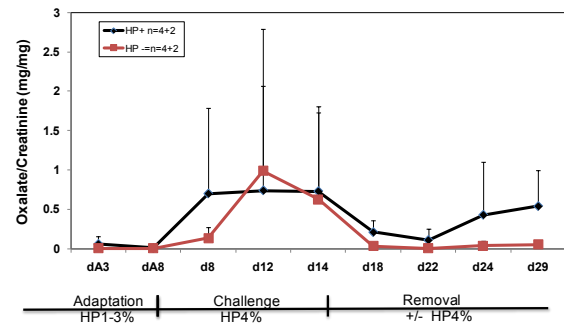
Results for urinary and plasma creatinine is presented in Figure 5 and 6 respectively ( $p < 0.05$ ).

### Body weight

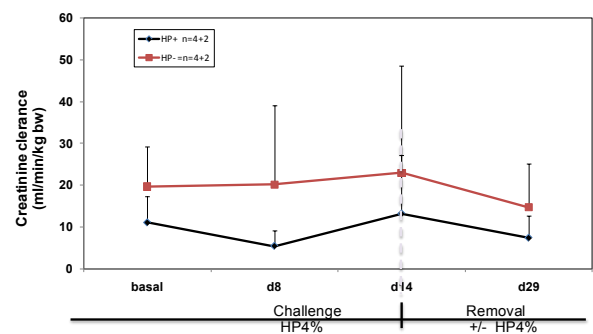
Pigs had normal growth and food intake suggesting that the given dose of 4% HP was well tolerated, but was high enough to induced significant and reversible hyperoxaluria without hyperoxalemia ( $p < 0.05$ ). The body weight of pigs during the experiment are shown in Figure 7.

### Macroscopic examination of the kidney

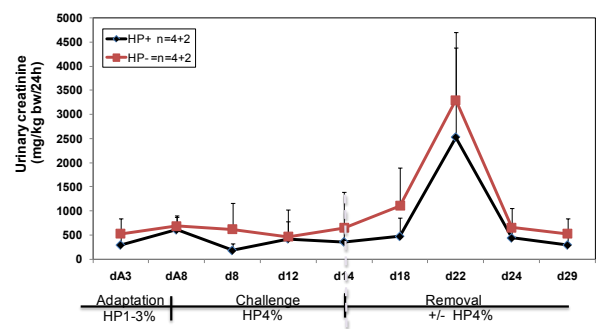
In pigs which obtained a diet containing 4% HP for 4 weeks, kidneys were found to be fibrotic with enlarged



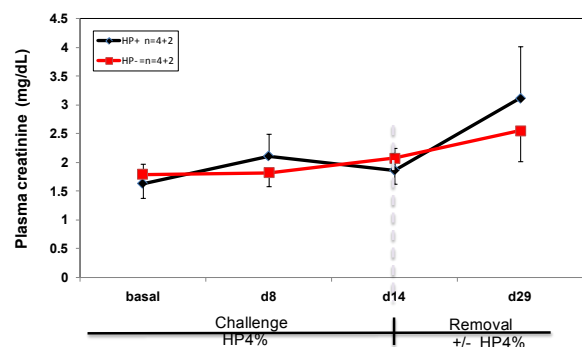
**Figure 3.** Oxalate /creatinine ratio in urine. In all statistical analysis,  $p < 0.05$  was taken as the level of significance



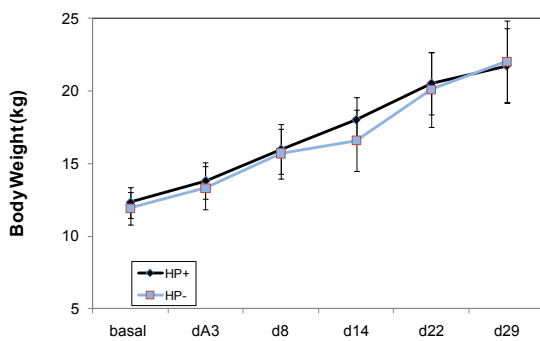
**Figure 4.** Creatinine clearance. In all statistical analysis,  $p < 0.05$  was taken as the level of significance



**Figure 5.** Urinary Creatinine. In all statistical analysis,  $p < 0.05$  was taken as the level of significance



**Figure 6.** Plasma Creatinine. In all statistical analysis,  $p < 0.05$  was taken as the level of significance



**Figure 7.** Body Weight changes. In all statistical analysis,  $p < 0.05$  was taken as the level of significance

cavities and hemorrhages. Urinary stones were visible with the naked eye. Stone size was varied and ranged from 3 to 4 mm. In all these kidneys, crystalline forms were present. In stone-free kidneys, no crystalline forms were found. Kidneys kept their natural morphological features. Stone-free kidneys were isolated from animals which did not get HP in the last 2 weeks of the experiment.

## Discussion

Swine models have proven useful in biomedical research because of the similarities in organ structure and function to humans. It is important to address that at a functional level, human and pigs share many similarities with regard to genitourinary structures. In addition to being multipyramidal structures, human and swine have comparable maximal urinary concentration, glomerular filtration rate and total renal blood flow characteristics. Pig models of hyperoxaluria and calcium oxalate stone disease after feeding with hydroxyproline was describe by others.<sup>8-10</sup> The purpose of this study was to develop a model of hyperoxaluria disease by feeding young growing pigs with 4% HP mixed with regular feed and to determine if hyperoxaluria is reversible if HP is removed from the diet 7–14 days after the start of the study. Dietary hydroxyproline is a precursor of oxalate in the pig model. An increase in urinary oxalate excretion can be achieved with adequate dose of HP in diet.

The data obtained points out that hyperoxaluria caused by uptake of dietary HP can be spontaneously ceased by as yet undescribed mechanisms. Elevated oxalate levels in urine or in blood or presence of HP in the gut switch on natural mechanisms of elimination of oxalate from the body. This probably explains the origin of individual variation of oxalate in urine. The mechanism can be related to specific gut bacteria overgrowth, which utilizes oxalate, or to a natural mechanism of mobilization of Ca from bones to produce salt in the gut. Participation of gut bacteria (e.g. *Oxalobacter*) in these mechanisms is not excluded. Low levels of oxalate (Ox) in the blood reflects low levels of Ox in the gut and con-

sequently low numbers of bacteria utilizing oxalate as a substrate. Enhanced levels of oxalate in blood affect gut levels. Elevated levels of Ox in gut provokes the corresponding oxalate dependent bacteria to grow and utilize oxalate; this can eliminate Ox from blood and, in consequence, from urine.<sup>11,12</sup>

Enhanced absorption of oxalate can result in urolithiasis, nephrocalcinosis, metabolic acidosis, hematuria, pyelonephritis, hydronephrosis, and renal failure. In primary hyperoxaluria, there is a systemic deposition of calcium oxalate in almost all body tissues including kidneys, heart, bone, cartilage, teeth, vasculature, and brain.<sup>8,7,13</sup>

In the most popular model, ethylene glycol, a precursor of oxalate, is given to animals specially rodents in their drinking water. Ethylene glycol consumption causes development of hyperoxaluria, leads to crystalluria and CaOx crystal deposition in the kidneys. Mandel et al., observed that feeding pigs with 10% HP induced hyperoxaluria, which reached a maximum at day 6 and leveled off until the end of the study despite further increases in HP in the diet to 20%. Addition of such high HP doses results in hyperoxaluria, CaOx crystalluria, nephrolithiasis, metabolic acidosis, and may lead to multiorgan injuries. Additionally, crystalline calcium phosphate deposition in kidneys and ureters leads to hypercalciuria. Modification of the diet by high dosing of HP causes appetite loss resulting in lowering the weight gain, and may also interfere the water intake and urine excretion.<sup>14,15</sup> Such disorders may have an impact on the results of the studies. Mild reversible hyperoxaluria has important implications since it presents a model that can be used for testing future therapies. Also mild hyperoxaluria will not switch mechanisms of oxalate elimination. Taking into account potential complications of more toxic responses seen with agents such as ethylene glycol and high doses of HP, we induced mild hyperoxaluria by administrating a diet enriched with 4% HP. One group of 6 pigs administrated with a 4% HP diet for 2 weeks and the other cohort of 6 pigs continued on a diet supplemented with 4% HP for next 2 weeks. Our intention was to develop a model of hyperoxaluria disease by feeding young growing pigs with 4% HP mixed with regular feed and to determine if hyperoxaluria is reversible when HP is removed from the diet 14 days after treating with 4% HP.

We reported that administration of a diet enriched with 4% hydroxyproline to growing pigs induces reversible hyperoxaluria and promotes formation of CaOx crystal deposits and morphological changes in the pigs kidneys. Our study shows that supplementation with 4% HP did not affect the weight gain, water and feed intake, urine excretion or creatinine clearance. It has significant meaning, when taking under consideration overall condition of animals.

## Conclusion

Proposed porcine models of reversible hyperoxaluria show that in animals after 2 weeks of HP administration, urine oxalate level return normal when pigs are not treated with HP for next 2 weeks. Longer exposure of animals on HP treatment lasting 4 weeks caused elevated hyperoxaluria and manifestation of morphological lesions on kidneys with crystals, sand and stone formation. Normal growth of pigs suggests that the given dose of 4% HP was well tolerated, but was high enough to induced significant and reversible hyperoxaluria. This presented model mimics oxalate urolithiasis in man and can be used for testing of different medical substances preventing stone formation in kidneys. This model is attractive because of its reversibility.

## Compliance with ethical standards

*Conflict of interest:* The authors declare that they have no conflicts of interest.

*Funding:* None

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