Nephrocalcinosis in rabbits – a case study

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Summary

Varying degrees of kidney calcification have been found in New Zealand White rabbits used for *in vitro* physiological studies in our animal unit over the past 4 years. Histologically, the lesion is characterised by the deposition of calcified deposits inside tubular structures in the cortical and (cortico)medullary region of the rabbit kidney. The kidney calcifications could be so severe, that the isolation of renal resistance vessels, dissected for intended *in vitro* studies, was no longer possible. Because 40% of the rabbit kidneys had to be discarded at the start of the year 2000, since then, a routine histological check of all rabbit kidneys used until 1 January 2004, was performed. The yearly incidence of numbers of animals having kidneys with calcified deposits in cortex and/or medulla as compared to the total number of rabbits used, ranged from 16 % to 63%. Kidney calcifications were seen in both sexes. A large interindividual variation in the degree of nephrocalcinosis was found. Of the dietary factors involved in the ethiopathogenesis in rats, phosphorus (P) concentration is an important determinant for kidney calcification. Also in rabbits it has been proven that dietary phosphate supplements and increased dietary P-levels in semipurified diets will lead to kidney calcifications in higher frequencies and degrees of severity. The recommended dietary P-level for growing rabbits is 0.22%, according to the National Research Council guidelines of 1977. Commercial, natural-ingredient rabbit diets always have dietary P levels that exceed 0.22%. It is therefore considered recommendable to lower dietary P concentration in marketed rabbit diets, in order to reduce or prevent nephrocalcinosis in rabbits.

Introduction

Since the year 2000, kidney calcifications have been detected in New Zealand White rabbits (from HsdIf:NZW, Harlan, Holland) used at our laboratory (pictures 1 and 2). Intratubular calcified deposits could be found in the cortex (picture 1), the medulla (picture 2) and the corticomedullary region. The disorder occurred in both sexes in animals of about 1.5-2.5 kg of body weight (3-4 months of age) in variable frequencies and degrees of severity among individuals. The kidney deposits were found bilaterally. In case of severe calcifications, microdissection of the renal resistance vessels (12-20 µm in diameter) intended to be used for further in vitro studies, could not be performed. At the start of the year 2000, in 14 out of 35 rabbits used, i.e. 40%, the kidneys were so heavily calcified that the renal resistance vessels could not be isolated for the intended aim. From that time on, all rabbit kidneys were sampled until 1 January 2004, in order to analyse them histologically for the presence and severity of kidney calcification. By defining and identifying the problem and attempting to understand the ethiopathology, possible solutions could

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be found which would aid in preventing an unnecessary increased use in numbers of rabbits (Reduction). As a variable degree of kidney calcification can also interfere with experimental results, causing an increasing standard deviation, preventive measures will also help to reduce the numbers of animals needed to prove statistical significance.

Materials and Methods

Male and female New Zealand White rabbits (from HdsIf:NZW, Harlan, Horst, The Netherlands and Gannat, France) had a body weight of 1.5-2.5 kg (3-4 months of age) on arrival. The rabbits had a defined health status as given on http://www.harlan.com. On arrival, the rabbits were housed under standardised housing conditions, as described before, except for the dietary regimen *(Ritskes-Hoitinga & Schlederman, 1999)*, and were fed a restricted amount of rabbit diet once a day at 2 pm (about 115 g of Altromin 3123: catalogue values of Ca 0.8%, and P 0.6%; Altromin diet produced by Chr. Petersen A/S, Ringsted, Denmark). At the breeder the animals had been fed with Harlan Teklad 2030 (catalogue values of Ca 1.0%, and P 0.6%; Harlan UK, Bicester, UK). Batches of both these types of diets were analysed for Ca and P, as described earlier *(Hoek et al., 1988)*. Both diets had a similar total P-level of about 0.60% (wt/wt). Ca-level in the Harlan Teklad batch was 1.1%, and in the batch of Altromin diet 0.9%. After variously a week to a month in our laboratory, the rabbits were euthanised (by stunning followed by exsanguination) and a transverse slice of one of the kidneys about 0.5 cm thick was cut in the middle and fixed in formalin. 5 µm sections were stained with haematoxylin-eosin and scored for the presence of calcified deposits.

Results

From each animal, one histological kidney section was scored for the presence of calcified deposits in cortex and medulla (Figures 1 and 2). Table 1 shows the incidences and the mean scores for the cortex and medulla in each registration year. The severity scores were defined as follows: Score 0 = no calcified deposits found; score 1 = a few calcified deposits present throughout the entire kidney section; score 2 = multiple deposits; score 3 = a band of calcification present throughout the entire kidney section. The results over all animals shows that the incidence could vary from 5 out of 31 rabbits (16%) showing deposits in the cortical area in the remainder of the year 2000 as the lowest value, to 22 out of 35 rabbits (63%) having deposits in the

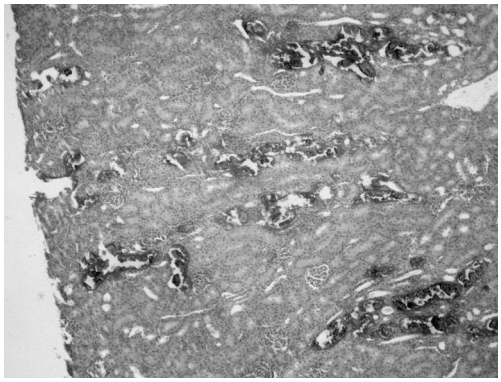

Figure 1. Cortical area of a HE-stained slide of the rabbit kidney demonstrating several calcified deposits.

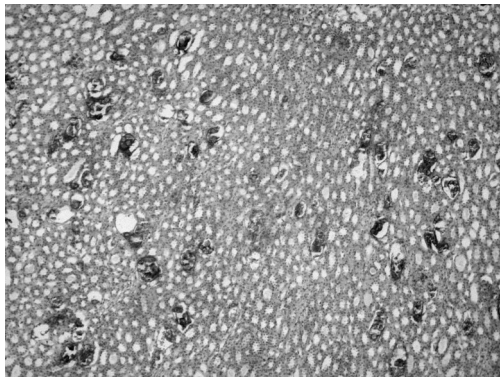

Figure 2. HE-stained slide of the medullary area in rabbit kidney with multiple calcified deposits.

Table 1. Incidences and severity scores in rabbit kidneys during the years 2000-2003.

	Nephrocalcinosis in cortex (C) and medullary (M) region	total over all animals	males	females	gender not registered
2000	C Mean score	0.4	0.4	0.6	0.0
	C Incidence	5 / 31	2 / 7	4 / 15	0 / 9
	M mean score	0.5	0.3	0.6	0.0
	M Incidence	12 / 31	2 / 7	7 / 15	0 / 9
2001	C mean score	0.4	0.4	-	0.6
	C incidence	23 / 91	16 / 69	-	7 / 22
	M mean score	0.6	0.5	-	0.7
	M incidence	39 / 91	29 / 69	-	10 / 22
2002	C mean score	0.6	0.5	-	1.1
	C incidence	24 / 59	19 / 51	-	5 / 8
	M mean score	0.9	0.9	-	1.3
	M incidence	32 / 59	27 / 51	-	5 / 8
2003	C mean score	0.5	0.2	0.5	1.3
	C incidence	11 / 35	3 / 21	2 / 6	6 / 8
	M mean score	0.9	0.6	0.9	1.8
	M incidence	22 / 35	11 / 21	3 / 6	8 / 8

Score 0 = no calcified deposits; Score 1 = a few calcified deposits; Score 2 = multiple deposits; Score 3 = a band of calcification throughout the entire kidney section.

medullary region in the year 2003. Mean severity scores varied from 0.4 to 0.9. Individual scores could range from 0 to 3, both in the cortex and medulla (results not shown). The gender had not always been registered, but upon comparing those animals where the sex was known, no clear indication of a sex difference in the sensitivity towards the development of kidney calcifications was found.

Histopathological evaluation revealed the presence of distorted, dilated and basophilic tubules and inflammatory responses in the cortex in conjunction with calcified deposits.

Discussion

Kidney calcifications were found in New Zealand White rabbits in variable incidences and severities over the past 4 years. In some cases the degree of calcification was so severe, that the renal resistance vessels could not be isolated for *in vitro* studies. As this led to an increased use of animals, which is in conflict with the concept of Reduction, it was important to define ethiological factors, in order to prevent/reduce this disorder. Even though a smaller degree of nephrocalcinosis (NC) may not prevent the succesful isolation of the renal resistance vessels, there may be a risk of interference with the results. In rats it is well-known that NC can interfere with kidney function *(Ritskes-Hoitinga et al., 1989)* and can prolong single-nephron passage time *(Al-Modhefer et al., 1986)*. Also in rabbits increased serum creatinine levels have been measured *(Cramer et al., 1998a)* in association with NC induced by oral phosphate supplements, which is an indication of a disturbed kidney function. Kidney histology shows also several characteristics that indicate that the function can become influenced as a result of calcified deposits: tubulus dilatation, basophilia, degeneration and inflammatory responses in the cortex occurred in association with calcifications. This can be regarded as an adaptive or reactive process in response to the presence of calcified deposits.

In rabbits, phosphate-induced calcified deposits occurred in the tubules in the medulla, corticomedullary area and the cortex (pictures 1 and 2; Cramer et al., 1998b; Ritskes-Hoitinga et al., 2004), whereas in rats it is only seen in the corticomedullary junction *(Ritskes-Hoitinga et al., 1989)*. In rabbits both sexes can become affected, which is in contrast to the rat, where virtually only females are affected. In rats oestrogens are involved in the etiology *(Geary and Cousins, 1969; Ritskes-Hoitinga & Beynen, 1992)*. Whether this is also true for rabbits, is not clear.

By giving rabbits oral phosphate supplements from the age of 5-6 weeks, 16 out of 20 rabbits had developed NC by 3 weeks, and in the remainder of the rabbits NC had developed after 6 weeks *(Cramer et al., 1998b)*. When the rabbits arrived at our unit, they were 3-4 months of age. One rabbit was killed immediately after arrival (age in between 12-16 weeks) and showed clear presence of nephrocalcinosis. The others were killed one week to one month after arrival, i.e. at 13-20 weeks of age. The age at which NC was found at our laboratory appears similar to what Cramer et al. (1998b) have found.

In rats, nephrocalcinosis is related to genetic, hormonal and dietary factors *(Ritskes-Hoitinga & Beynen, 1992)*. In rabbits, dietary factors are also known to be involved in the etiology: thus Cheeke (1994) noted that prolonged intake of high calcium diets (4%) may cause calcification of soft tissues such as aorta and kidney. The localisation of these calcifications is not further specified. In rabbits, calcium is absorbed very efficiently and the excess excreted in the urine, rather than via the bile, as is typical in other species. Cheeke (1994) also noted that reducing the dietary calcium level to 0.4-0.5% for rabbits kept under maintenance conditions helps to reduce kidney and urinary tract calcium deposits but did not discuss the role of dietary phosphorus. In a study with semipurified diets it was found that a dietary Ca level of about 0.45% in combination with increasing P-levels of 0.1, 0.2, 0.4 and 0.8%, led to increased incidences and severities of NC, both in the cortex and medulla *(Ritskes-Hoitinga et al., 2004)*. Table 2 shows the results of this study, including the chemical analysis of the % Ca in the kidney. Also Cramer et al. (1998a) demonstrated that oral phosphate supplements caused increased levels of NC. This implies that increased dietary P

Table 2. Nephrocalcinosis (NC) in New Zealand White rabbits after an 8-week feeding period with purified diets, differing only in dietary P-level. Dietary Ca level was about 0.45 % (Ritskes-Hoitinga et al. 2004).

dietary P-level (%)	0.1	0.2	0.4	0.8
Parameter				
NC-score cortex	0.0	0.3	0.5	1.8
Incidence	0/8	2/8	3/8	8/8
NC-score medulla	0.4	0.5	1.8	1.9
Incidence	3/8	3/8	8/8	8/8
% Ca in kidney	0.05 ± 0.01	0.07 ± 0.04	0.34 ± 0.65	1.40 ± 1.51

Score 0 = no calcified deposits; Score 1 = a few calcified deposits; Score 2 = multiple deposits;
Score 3 = a band of calcification throughout the entire kidney section.
The score is an average for 8 animals for each dietary group. Kidney sections were stained by Von Kossa.
% Ca in kidney is the chemically analysed amount of calcium in the entire kidney (wt/wt).

levels will lead to a higher incidence and degree of kidney calcifications and that P is involved in the ethiology.

The high interindividual variation in incidence and severity found in the rabbits used at our laboratory is probably the result from the interaction between dietary and genetic factors, in conjunction with other unknown etiological factors. As natural ingredient chows were used both by the breeder and user, many unknown dietary factors can influence and modify the process. Analyses of catalogue values of Ca and P levels in commercial rabbit diets (Altromin 2010, 2020, 2120, 2230, 2420; Harlan Teklad 2030, 2031; SDS rabbit maintenance (RABMA) and standard rabbit diet; LabDiet 5304, 5321, 5322, 5325, 5326, 5P25, 5P26; Zeigler lab research diets formula nrs. 42180000, 42190000, 42210000, 41900000, 41320000) for (breeding and) growth and maintenance revealed that Ca-levels can vary from 0.78 to 1.26% and P-levels from 0.40 to 0.80%. Analysis of batches of two natural-ingredient diets used at the breeder and our unit revealed Ca-levels of 1.1% and 0.9%, respectively, and P levels of 0.6% in both diets.

Not much is known about the availability of dietary P to the rabbit, but due to the high microbial activity in the large intestine, it is believed that all P is available to the rabbit *(National Research Council, 1977)*. This is in contrast to the situation in e.g. rats and humans, where phytate P is not available. The recommended dietary P-level for the growing rabbit is currently 0.22%, whereas dietary Ca level should be 0.34-0.40% *(National Research Council, 1977)*. On the basis of a study using semipurified diets, probably even a dietary P level as low as 0.1% P is still sufficient for the growing rabbit, as there was no evidence of a deleterious effect on growth and bone development, while kidney calcifications were virtually prevented *(Ritskes-Hoitinga et al., 2004)*. The current reommended dietary P level of 0.22% for rabbits should probably be regarded as a maximum advisable concentration, and a lower P level may be more optimal. From this it seems plausible to conclude that the dietary P-levels of 0.6%, as found in natural-ingredient rabbit diets on the market, ought to be reduced.

An interaction with the genetic background, may be one of the reasons for the large individual variation in the degree of NC found in the rabbits at our laboratory. As New Zealand White rabbits are from an outbred stock, each individual rabbit will have a different genetic background. From rat studies it is well-known that there is a clear genetic variation in the susceptibility of inbred strains *(Ritskes-Hoitinga et al., 1992)*, which is demonstrated only when dietary P concentrations are sufficiently high. NC was prevented in 10 different inbred strains, when the dietary P level was sufficiently low (0.2%) *(Ritskes-Hoitinga et al., 1992)*. Also in outbred female Wistar rats, NC is virtually avoided on a diet containing 0.2% P *(Ritskes-Hoitinga et al. 1991, 1993)*. When dietary P levels are sufficiently low, the genetic background is irrelevant, as the disorder cannot occur.

In conclusion, it is expected that the numbers of rabbits used for *in vitro* physiological studies of kidneys could be reduced by lowering the dietary P levels in natural-ingredient diets. How much lower the dietary P level in natural ingredient diets should become, remains to be established, but the current information indicates that a dietary P level of 0.22 % as recommended by the National Research Council (1977) should be regarded as a maximum advisable concentration. By preventing/ reducing NC, a reduction in the numbers of rabbits used becomes achievable, as (1) more kidneys are suitable for the intended *in vitro* purpose, and (2) a reduced variation between animals can create statistically significant results with a smaller number of animals used.

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