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Evaluation of four animal models of intrarenal calcium deposition and assessment of the influence of dietary supplementation with essential fatty acids on calcification

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Abstract Firstly, to determine a satisfactory animal model for induction of intrarenal calcification, a study of four previously described animal models of intrarenal calcification was carried out which showed that intraperitoneal injection of 10% calcium gluconate into female Sprague-Dawley rats was most effective. We then investigated the hypothesis that dietary supplementation with essential fatty acids could reduce the intrarenal calcification developing as a result of intraperitoneal calcium injection. Using a combination of fish oil and evening primrose oil, we demonstrated a significant difference in renal parenchymal calcification, which was 940 ± 240 μg Ca/g dry weight renal parenchyma in unsupplemented animals and 320–370 ± 55–65 μg Ca/g dry weight renal parenchyma in supplemented animals (means ± SEM, $P < 0.005$). It was also demonstrated that there was synergism between eicosapentaenoic acid (EPA) and γ-linolenic acid (GLA): dietary supplementation with a combined oil preparation containing 27 mg/ml EPA and 67 mg/ml GLA mixed as 2% with food was as effective as oils containing either 400 mg/ml EPA or 80 mg/ml GLA mixed as 4% of food.

Key words Intrarenal calcium deposition · Dietary supplementation · Essential fatty acids · Calcification

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Several theories have been proposed to explain the aetiology of recurrent renal stone formation (nephrolithiasis) but none fully explains all the facets of this multifactorial disease. The two most important theories are that urinary physicochemical factors favour the precipitation of stone-forming salts within the urine and that a structural abnormality within the kidney occurring at either or both the macro- or microscopic levels provides a site for stone formation. Structural causes include defective drainage of the kidney (e.g. caliectasis and hydronephrosis) and calculus-forming material deposition within the renal substance (e.g. nephrocalcinosis) [1]. An example of a physicochemical factor is hypercalciuria, which is found in approximately 70% of recurrent calcium stone formers [6].

Prostaglandins have been shown to be intimately involved in the renal handling of calcium [5], and the prevention of intrarenal calcification with prostaglandin synthetase inhibitors [e.g. the non-steroidal anti-inflammatory drugs (NSAIDs), indomethacin and flurbiprofen] in experimental studies in rats [5, 7, 8] indicate that they must also play a role in intranephric calcium salt deposition. Unfortunately the side effects of NSAIDs make them unsuitable for long-term renal stone prophylaxis. Essential fatty acids (EFAs) are the building blocks for prostaglandin synthesis and changes in the EFA content of the diet have been shown to result in the production of an alternative series of prostaglandins with a different range of actions [10, 17, 20, 25].

The Greenland Eskimos and coastal Japanese are reported to have a very low incidence of diseases common to Western man, including renal stone disease [2, 16, 22–24, 26, 30, 31, 33]. This has been attributed to their diet, which is rich in the EFA eicosapentaenoic acid (EPA) [9]. Studies of the Eskimo also indicate that, due to an inborn variation in plasma phospholipid

Table 1 Retissue calcium content (evaluation of animal models of intrarenal calcification)

Intrarenal calcification induction method	Renal tissue (mean ± SD) mg/g
Group 1: controls	0.19 ± 0.014
Group 2: calcium gluconate	2.39 ± 0.44*
Group 3: sodium phosphate	0.23 ± 0.03 NS
Group 4: oxamide	0.18 ± 0.03 NS
Group 5: sodium oxalate	Abandoned

* $P < 0.001$ tested by t-test and Mann-Whitney U test, comparing test group with Controls

metabolism, they have high levels of dihomo-γ-linolenic acid (DGLA) in addition to high levels of EPA [11, 18, 19]. The EFA profile of the Eskimo can be mimicked in Caucasian subjects by dietary supplementation with EPA and γ-linolenic acid (GLA) (which is rapidly converted in the body to DGLA) [18, 19]. Marine fish oil is a rich source of EPA and evening primrose oil a source of GLA.

This study relates entirely to the 'anatomical' theory of renal stone aetiology that calcium is deposited in the renal parenchyma and describes firstly how we determined which of four previously described animal models reliably resulted in intrarenal calcium deposition and then, using this model, examined the effect of dietary supplementation with fish oil (EPA) and evening primrose oil (GLA) on renal parenchymal calcification. We defined a satisfactory model as one which did not induce any signs of suffering in the animals; and which resulted in a significant increase in renal calcium content when compared to controls, demonstrable by both biochemical and histological techniques.

Part 1: evaluation of animal models of intrarenal calcification

Materials and methods

The animals used for this experiment were female Sprague-Dawley rats (A Tuck & Son, Essex, UK) aged 8 weeks and of average weight 200 g. They were housed in the Animal Unit at the University Hospital of Wales and maintained in a temperature-controlled room (71° C, relative humidity 55%) and kept in a 12:12 light-dark cycle (lights on at 7.00 a.m.). Water was available ad libitum and, except for group 4, the animals were fed on a standard maintenance diet. Since this was only a pilot study, the absolute minimum number of animals required to obtain a meaningful result were used. There were four animals in groups 1, 2, 3 and 4, and five animals in group 5. The animal groups were treated as follows:

Group 1. Controls.

Group 2. Intraperitoneal injection of 1.5 ml 10% calcium gluconate (containing 0.337 mmol of calcium) for 10 days [3, 7, 12, 14].

Group 3. Intraperitoneal injection of 0.5 M neutral sodium phosphate (15.5 mg phosphate/100 g body weight/24 h) for 10 days [15, 34].

Group 4. Oxamide (amino-oxalic acid: $H_2NCOCONH_2$) 1.2 g/100 g diet for 3 days [32].

Group 5. Single intraperitoneal injection of 2.5% disodium oxalate ($Na_2C_2O_4$) in 0.9% saline (1.5 ml) [21].

At the end of the experimental period the animals were put to death and their kidneys were analysed. Histologically, a qualitative analysis of calcium deposition was carried out by optical microscopic evaluation of sections stained by the Von Kossa method. Quantitative assessment of renal parenchyma calcium content was carried out by acid digestion of renal tissue, followed by atomic absorption spectrophotometry using a standard method [4].

Results

The animals in groups 2 and 3, given calcium gluconate and sodium phosphate respectively, tolerated the injections well. The animals in group 4 did not like the taste of oxamide and consequently only ate small amounts of oxamide-supplemented feed. All of the animals in this group lost weight until placed back on their normal diet. In group 5, following the intra-peritoneal injection of sodium oxalate, the animals rapidly became unwell and to prevent unnecessary suffering were put to death immediately. This was thought to be due to the acid pH of the oxalate solution and a further test on one animal was made using sodium oxalate solution buffered to pH 7.4, which resulted in a decrease in the post-injection symptoms. However, the animal still did not tolerate the injection well so was killed immediately and the sodium oxalate method was abandoned.

Histologically, renal parenchymal calcification was demonstrated by Von Kossa staining. Mean tissue levels of calcium determined by atomic absorption spectroscopy are shown in Table 1. Tissue calcium was significantly higher in the calcium gluconate injected animals (group 2) when compared to controls ($t = 14.1$, $P = < 0.001$) and were also significantly higher than those in groups 3 ($t = 13.9$, $P = < 0.001$) and 4 ($t = 14.2$, $P = < 0.001$). In groups 3 and 4, no calcification could be seen on histological section of the kidneys and tissue calcium was not significantly higher than in control animals.

The experiment demonstrated that intraperitoneal injection of calcium gluconate for 10 days is the most suitable model of intrarenal calcification. Therefore this model was used to determine whether the dietary supplementation of essential fatty acids could protect against the development of intrarenal calcification.

Part 2: assessment of the influence of dietary supplementation with essential fatty acids on renal calcium deposition

Materials and methods

Five groups of six female Sprague-Dawley rats of average weight 200 g were studied. All groups were fed on a standard maintenance

Table 2 Renal tissue calcium content (assessment of the influence of dietary supplementation with essential fatty acids on renal calcium deposition)

Group	Renal tissue (mean ± SD) mg/g
Group 1: untreated controls	0.074 ± 0.005
Group 2: calcification controls	0.940 ± 0.580
Group 3: 4% EPA	0.370 ± 0.150*
Group 4: 1% GLA	0.350 ± 0.130**
Group 5: 2% EPA/GLA	0.320 ± 0.160**

* $P < 0.01$, ** $P < 0.001$ tested by t-test and Mann-Whitney U test, comparing test group (3, 4, 5) with calcification controls

diet which was powdered and mixed with tap water (50:50 feed/water by weight). EFA supplementation was provided by mixing the various oils with the diet on a weight/weight basis. The concentrations of oils were determined from experiments performed previously on an animal model of hypercalciuria (paper in preparation). EFAs were fed to groups 3–5 from day 1 and intraperitoneal calcium gluconate injections were given to groups 2–5 for 10 days from day 14. All animals were put to death after 24 days and their kidneys were harvested and analysed biochemically and histologically for calcium as above. The animal groups were treated as follows:

Group 1. Maintenance diet only (untreated controls).

Group 2. Maintenance diet plus i.p. calcium gluconate (calcification controls).

Group 3. Maintenance diet + 4% EPA (Marine 40) plus i.p. calcium gluconate.

Group 4. Maintenance diet + 1% GLA (Efamol) plus i.p. calcium gluconate.

Group 5. Maintenance diet + 2% EPA/GLA (Efamol Marine) plus i.p. calcium gluconate.

Efamol (evening primrose oil) contains 80 mg/Ml GLA and 700 mg/Ml linolenic acid (LA). Marine 40 (fish oil) contains 400 mg/Ml EPA and 73 mg/Ml docosahexaenoic acid (DHA). Efamol Marine contains EPA (27 mg/Ml) and GLA (67 mg/Ml).

Results

Histological examination using Von Kossa staining demonstrated no calcification in group 1 (untreated controls). In group 2 (calcification controls), calcification was present in the basement membrane of the cortical tubules of all animals. In the oil supplementation groups (3, 4 and 5), no histological evidence of renal parenchymal calcification could be seen. Renal tissue calcium contents for the five groups are presented in Table 2.

Discussion

In each of the treatment groups (3, 4 and 5), histological examination revealed no evidence of calcification and, therefore, no difference in the degree of protection from intrarenal calcification between each group. The renal tissue calcium content results confirm that intra-peritoneal injection of calcium gluconate produced a significant increase in renal tissue calcium (comparing untreated control group with unsupplemented calcium-injected control group, $P = 0.001$). Each of the EFA-supplemented groups had significantly lower levels of renal tissue calcium than the unsupplemented calcium-injected control group (group 3, $P = 0.01$; group 4, $P = 0.001$; group 5, $P = < 0.005$) and, although the EPA/GLA mixture (group 5) appeared to give better protection, there were no statistically significant differences between the three treatment groups.

The reduction in renal parenchymal calcification seen in this study is similar to that reported by others who used dietary supplementation with EPA alone. Buck et al. [9] demonstrated significantly reduced levels of renal tissue calcium [0.12 ± 0.017 mmol/g dry weight (mean ± SEM) compared to 0.195 ± 0.015 mmol/g dry weight, $P < 0.02$] in female PVG (piebald Virol Glaxo) rats, gavage fed 1 ml Maxepa (180 mg/ml EPA and 120 mg/ml DHA), when compared to control rats which received no dietary supplement. Rothwell [29] performed similar experiments using male Sprague-Dawley rats, again administering calcium gluconate by daily i.p. injection. In the treatment group the animals were gavage fed 1 ml Maxepa and in the placebo group olive oil. Tissue calcium levels were not measured, but the degree of calcification at light microscopy was reported to be reduced in the treatment group when compared to placebo. The placebo used by Rothwell more closely resembles fish oil in consistency but was not used in this study as it contains linoleic acid, albeit in relatively small amounts, and linoleic acid has been shown to increase prostaglandin synthesis [25, 27, 13, 28].

The proposed mechanism of action for EFAs in the prevention of experimentally induced intrarenal calcification is firstly through their inhibitory effect upon the production of the dienoic series of prostaglandins synthesised from arachidonic acid, in particular PGE_2. This series of prostaglandins have been shown to be actively involved in the renal handling of calcium so that inhibition of their synthesis should exert the same effect as the administration of NSAIDs and so reduce intranephric calcium deposition [6, 7]. Secondly, fish oils inhibit the synthesis of leukotrienes, which are mediators of the inflammatory process and, thus, inhibition of their synthesis may reduce the inflammation associated with dystrophic intrarenal calcification. Renal tissue damaged by inflammation is more susceptible to further calcium deposition, and a vicious cycle may be initiated. This cycle can perhaps be prevented or interrupted by inhibiting the synthesis of the prostaglandins and leukotrienes that are involved in propagating this process. This study suggests that this hypothesis may be valid for the rat but obviously cannot be directly extrapolated to the human because

there may be interspecies differences in EFA metabolism. Further, this study cannot indicate whether patients with recurrent renal stones have an anomaly of EFA metabolism or whether they have a different anomaly that would be overcome by the changes induced by altered EFA concentrations in their diet.

The synergism between EPA and GLA (group 5) is particularly important: although only containing a fraction of the EPA administered to group 3 (2% of an oil containing 27 mg/ml EPA and 67 mg/ml GLA compared to 4% of an oil containing 400 mg/ml EPA) or the GLA administered to group 4 (i.e. 2% of an oil containing 27 mg/ml EPA and 67 mg/ml GLA compared to 4% of an oil containing 80 mg/ml GLA), the combination oil was as effective at reducing intrarenal calcification as seen from the renal tissue calcium concentrations. This supports the theory that supplementing GLA as well as EPA is important and that perhaps the most effective dietary supplement is that containing a combination of EPA and GLA.

We therefore conclude that the prevention of intrarenal calcification in rats given dietary supplementation with essential fatty acids is an interesting and potentially useful effect which warrants further investigation to determine firstly whether a similar effect can be found in humans and secondly whether this may have any therapeutic applications.

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