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The Role of Testosterone Hormone on Crystal Formation Through the Renal Expression of Osteopontin in Male Wistar Rat

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ABSTRACT

This study aims to investigate the role of testosterone hormone on crystal formation through osteopontin expression. This was an experimental study with post-test only control design. A total of 12 rats aged 10 weeks were divided into two groups which are male rats group fed with water containing EG (Ethylene Glycol) 0.75% and male rats group fed with water containing EG (Ethylene Glycol) 0.75% and were injected with testosterone hormone. Furthermore, the rats were observed and fed with standard diet for 30 days. Then, both groups were terminated. The kidneys were extracted and examined for Osteopontin expression. Histopathology examination was also performed in both groups The result of the study showed that the mean kidney osteopontin expression in male rats fed with water containing EG 0.75% and received testosterone hormone injection were significantly higher (29.16 ± 9.072) compared to male rat groups fed with water containing EG 0.75% alone (11.42 ± 3.692) with p value 0.001. Histopathology examination with HE stain revealed that both groups demonstrated the presence of stone in kidney tubules, kidney swelling and pelvic ectasis. This feature suggested the presentation of hydronephrosis as a result of stone obstruction. As a conclusion, this study shed light on the role of hormonal sex of testosterone involving in crystal formation through osteopontin expression.

Keywords: urolithiasis, testosterone, sex hormone, osteopontin

INTRODUCTION

An epidemiological study conducted in 2005 by the Japanese Society on Urolithiasis Research found that the incidence of upper urinary tract stones had a 2.4-fold greater risk in men than women, in which most stones consisted of calcium oxalate stones. The male-to-female ratio of patients with urolithiasis in some countries has been reported around 2:1 to 3:1 in recent years (1–3). Although the pathogenesis of urolithiasis appears to be multifactorial and complex, sex hormone-derived are thought to influence the incidence. In addition, several previous reports have concluded that testosterone induces urolithiasis whereas estrogen inhibits (4–8).

Several studies have suggested that the explanation on this phenomenon was due to variations in citrate excretion, as 24-hour urinary citrate excretion was found to be higher in women than men (9–12). Excreted urinary citrate, which inhibits crystal growth and aggregation, is present at higher concentrations in female urine and estrogen replacement increases urinary citrate excretion in postmenopausal women (13,14). Differences in citrate metabolism are widely recognized as the cause of gender-based differences in the incidence of urolithiasis.

In another study also reported by Richardson observed that the liver of male rats had higher activity of glycolic acid oxidase enzyme, which caused normal male

rats to secrete more oxalic acid than castrated male rats and normal female rats (15). These observations led Finlayson to postulate that lower serum testosterone levels might contribute to some protection against oxalate stone disease in women (16). Sex differences in the formation of urolithiasis have also been reported in experimental observations in which male rats fed ethylene glycol were more prone to form urinary stones than female rats, but no explanation for this interesting phenomenon was offered. Osteopontin, a highly acidic phosphorylated glycoprotein, was originally isolated from mineralized bone matrix and has recently been found in the kidney and in organic matrix kidney stones (17). This macromolecule is known to inhibit calcium oxalate crystallization in vitro and block the adhesion of calcium oxalate monohydrate crystals to renal tubular cells (18,19). Osteopontin expression has been reported to be increased in experimental models of urolithiasis suggesting that it influences stone formation in vivo (5,20). We hypothesized that sex hormones play a role in stone formation via lithogenic factors, including osteopontin. In this study, we assessed the effect of the sex hormone testosterone on urinary stone formation through the expression of renal osteopontin in ethylene glycol-treated rat models.

MATERIAL AND METHODS

Animal Study Groups and Treatments

All experimental and animal care protocols were in accordance to the guidelines provided by our university. Male wistar rat were fed with standard commercial diet. They were divided into 2 groups (n = 6 each group) as follows: male wistar rat fed with water containing EG 0.75% and male wistar rat fed with water containing EG 0.75% with testosterone injection The appropriate dosages of sex hormones were determined based on previous report. After treatment, the rats were euthanized with an excessive dose of anesthesia, and the kidneys were immediately excised. The kidneys were cut longitudinally, fixed in 4% paraformaldehyde, and embedded in paraffin. All specimens were stored for later examination.

Confirmation of Kidney Crystal Deposits

The excised kidneys embedded in formalin were cut into 5 mm sections, stained with hematoxylin and eosin, and mounted on slides. Crystal deposits were visually examined under a microscope.

Examination of Kidney Osteopontin (OPN) Expression

Kidney OPN expression were measured using an ELISA method according to manufacturer's instruction. The ELISA is designed to detect rat OPN in kidney tissue. The plate has been pre-coated with rat OPN antibody. OPN present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Rat OPN Antibody is added and binds to OPN in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated OPN antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Rat OPN. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Statistical analysis

SPSS version 20 was used in the statistical analysis of this study. Due to the outcome data was in normal distribution, independent t-test was used in comparing between nominal and numeric data. P value < 0.05 was considered to be statistically significant.

RESULTS

Animal Study Groups and Treatments

This is an experimental study with a *post-test only control group design*, in which the dependent variable was examined after treatment. A total of 12 white rats (Rattus Norvegicus) male wistar strain were used in this experimental study with 120 days of age as samples. Samples were allocated into two groups consisting of a male group fed

with 0.75% Ethylene Glycol drinking water and a male group fed with 0.75% EG drinking water + injected testosterone hormone. The expression of Osteopontin was then examined using ELISA method from the rat kidney tissue.

Baseline characteristics of the sample

This study used 2 groups of male rats consisting of a group of rats that received EG intervention with testosterone injection and a group of rats that received EG intervention without testosterone injection. The two groups had no difference in baseline and final body weight.

Table 1. Baseline characteristics of the sample

Variabel	Male Experimental Groups n=6	Male Control Groups n=6
[a] Weight (g) baseline	220,3 ± 4,71	217,8 ± 5,89
[a] Weight (g) final	203,8 ± 5.12	209,6 ± 7,19

Descriptive Analysis on Kidney Osteopontin Expression

The results of the analysis of osteopontin levels in kidney tissue showed that the average level of renal osteopontin in the male rat group fed with 0.75% EG drinking water with testosterone injection was significantly higher (29.16 ± 9.072) compared to the male rat group fed with only 0.75 EG % drinking water without testosterone injection (11.42 ± 3.692) with p value 0.001.

Table 2. Descriptive analysis on Kidney Osteopontin Expression

Rats	N	Mean Osteopontin Expression (mg/dl) ± SD	*p
Male EG 0,75%	6	11.42 ± 3.692	**0.001**
Male EG 0,75% + Testosterone hormone	6	29.163 ± 9.072	

Kidney Stone Formation

Histopathological examination of the kidneys with HE staining in both groups of rats showed the appearance of stones in the kidney tubules. Kidneys in male Wistar rats induced with Ethylene Glycol experienced swelling and dilation of the renal pelvis. This presents a feature of hydronephrosis that shows evidence of the obstructive effect of stone formation.

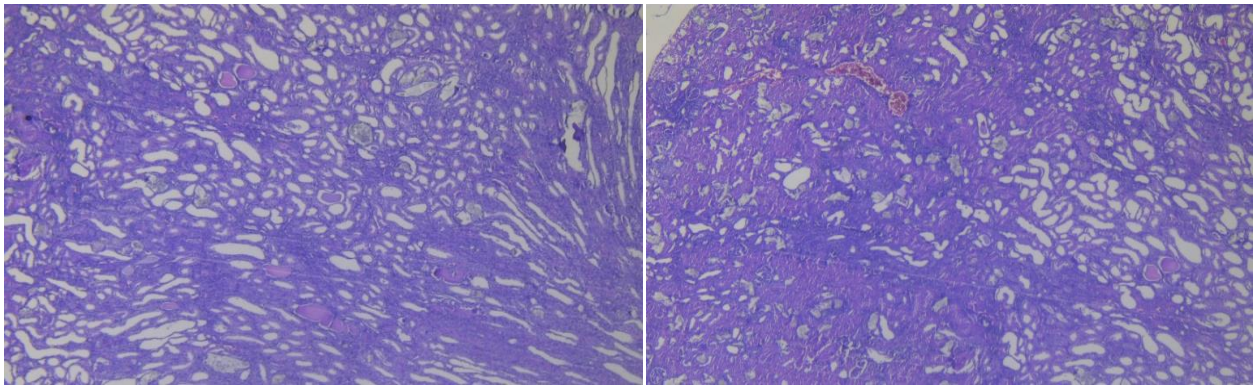

Figure 1. Histopathological examination revealed stone formation in male wistar rat fed with water containing Ethylene Glycol 0.75%

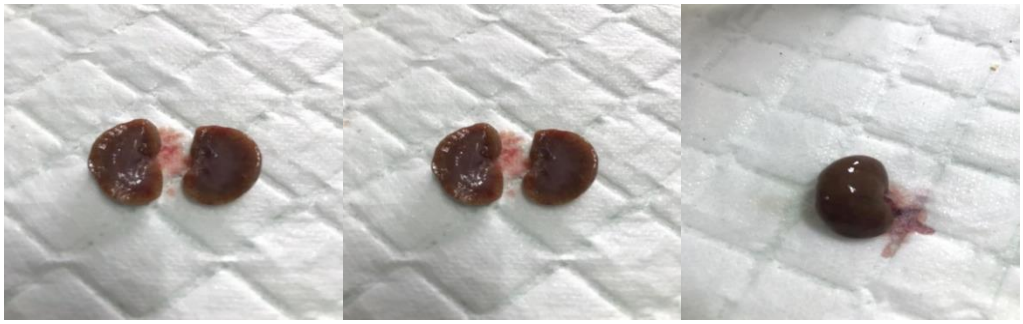

Figure 2. Rat kidney revealed the presence of swelling (hidonephrosis)

DISCUSSION

In this experimental study, hyperoxaluria model was induced by the administration of 0.75 % Ethylene Glycol (EG) drinking water for 1 month. This method of induction for hyperoxaluria has been previously demonstrated by Yagisawa et al. in which hypercalciuria in experimental mice was induced by administering 0.75% EG in drinking water (21). Since osteopontin expression has been reported to influence and is associated with crystal deposition in the kidney, hyperoxaluria without crystal deposition is considered an ideal condition for evaluating the role of sex hormones in lithogenesis of urine through osteopontin expression (22). Thus, the relationship between osteopontin and kidney stone crystal deposition was analyzed in this study. In this study, it was reported that the expression of renal osteopontin was significantly higher in the group of male rats receiving 0.75% EG with the injection of testosterone hormone compared to the group of male rats receiving 0.75% EG without testosterone injection.

Calcium oxalate stones are the most common types of stones in humans and their incidence has increased rapidly in recent years. Although the formation of calcium oxalate stones in the upper urinary tract is related to lifestyle and diet, the mechanism underlying stone formation remains unclear (23). It is known that urinary tract stones are a disease that is more dominant in men. Several experimental studies have been conducted to explain the differences in the prevalence of urolithiasis in males and females (24). A previous study reported that male castration decreased urinary oxalate excretion and crystal deposition in a mouse model of urinary stones (21). Moreover, Yagisawa et al reported that exogenous testosterone implanted in mice was able to increase urinary oxalate excretion. This report shows that testosterone plays an important role in oxalate metabolism and urinary oxalate excretion. Until now, the mechanism underlying the increased urinary oxalate excretion by testosterone is still not clearly understood (25).

In this study, hyperoxaluria modeling was carried out through the induction of 0.75% EG which was administered as drinking water for experimental rats. This study modeled hyperoxaluria rats using ethylene glycol at a concentration of 0.75%. After 30 days, all experimental rats given ethylene glycol solution showed the signs of urinary tract stones in the kidneys. Because several types of kidney stones were derived from calcium oxalate, an effective model of chronic hyperoxaluria was established in mice through several agents. One of them is ethylene glycol (EG), which may cause crystalluria and deposition of calcium oxalate crystals in the renal parenchyma. Previous studies have also demonstrated that chronic modeling of nephrocalcinosis could be produced in rats by deposition of calcium oxalate crystals by administration of ethylene glycol (EG) at concentrations above 0.75%. Previous studies reported that oxalate excretion in rats treated with ethylene glycol was significantly different in terms of urine levels. In male rats, urinary oxalate excretion was significantly greater in normal male rats than in castrated male rats (21). These results were consistently reported by previous studies which reported that the effect of oxalate metabolism and urinary oxalate excretion by ethylene glycol was influenced by the testosterone hormone in which testosterone hormone may aggravate urinary oxalate excretion (26).

Ethylene glycol is a liquid that is colorless, odorless, has a sweet taste and is non-volatile (27). The mechanism of EG which may cause an increase in oxalate levels in the urine is through metabolism in the liver by the enzyme nicotinamide adenine dinucleotide (NAD). The first step is the oxidation of ethylene glycol to glycolaldehyde by alcohol dehydrogenase. Furthermore, glycolaldehyde is oxidized to glycolic acid and finally to oxalic acid. About 80% of absorbed EG is metabolized in the liver, with the remainder excreted via the renal system. Renal calcium oxalate

deposition induced by ethylene glycol is associated with proximal tubular cell necrosis leading to the sequential production of several metabolites (glycolaldehyde, glycolate, glyoxylate and oxalate) and accumulation of oxalate crystals in the tubular lumen (28).

In this study, it was found that the group of rats fed with EG 0.75% drinking water receiving testosterone injection had significantly higher renal osteopontin levels (mean 29,163 ± 9,072) than the group of rats given EG 0.75% without testosterone injection (mean 11.42 ± 3,692). with a p-value of 0.001. Osteopontin is a macromolecule that plays a role in the pathogenesis of urinary tract stone formation, although the role of osteopontin is still not clearly understood. Until now, the role of osteopontin is still unclear with some studies reporting its role as a promotor of stone formation and other studies reporting its role as an inhibitor of stone formation.

The increase in OPN in rats with kidney stones induced by hyperoxaluria can be explained by 2 mechanisms, the first that the interaction between crystals and the membrane stimulates the production of OPN mRNA and protein which in turn triggers the adhesion of crystals to the renal epithelium and facilitates crystal aggregation causing blockade from the tubular lumen. This theory supports the role of OPN as a promoter of stone formation (29). Reports from previous studies found that hyperoxaluria and calcium oxalate crystal deposition in mice generally upregulate OPN expression (30). Several in vitro studies have demonstrated the role of osteopontin in crystallization formation in the Madin-Darby model of canine kidney cells (31). The expression of osteopontin could be examined in normal kidneys and increased in kidneys with kidney stones (32). The study conducted by Kleinman et al. in 2004, modeling hyperoxaluria for 4 weeks was associated with increased OPN expression by immunohistochemical examination in a rat animal model (33). Khan reported that both oxalate and oxalate stone crystals are substances that may damage the renal epithelial tissue thereby increasing the expression of osteopontin (34). The study also found that the increase in osteopontin expression increased in proportion to urinary oxalate excretion. The previous study reported by Mohamaden et al. reported that there was a significant increase in osteopontin expression in hyperoxaluria rats (22). On the other hand, Umekawa et al. failed to prove upregulation of OPN in rats treated with glyoxylic acid which is a precursor of other types of oxalate (20). Osteopontin expression was evaluated in distal tubular cells from healthy kidneys and was markedly increased in stone-forming kidneys induced with glyoxylic acid. OPN expression was not detected in the glomerulus, proximal tubule or collecting duct in healthy kidneys but instead, OPN was synthesized in renal tubular cells and secreted into the urine by epithelial cells (35). This finding is consistent with previous studies (36). The mechanism of action of OPN resulting in stone formation is that OPN is reported to be a promoter of the stone formation process based on OPN levels in *stone forming* and is a promoter of the adhesion of calcium oxalate crystals in *Madin-Darby Canine Kidney* (MDCK) cells (22).

In another study, OPN was reported as an inhibitor with its effect as an inhibitor on the formation/aggregation/growth of calcium oxalate crystals in vitro. While high expression of OPN has been found in various calcified regions in humans, OPN has been reported to inhibit the maturation of hydroxyapatite crystals. It has been reported that OPN exists in free form in liquids, OPN inhibits crystal aggregation/growth, but when OPN adheres to certain substances, calcium oxalate crystals may aggregate and grow on the OPN. Renal tubular cells are considered a potential attachment site for OPN in urinary stone formation. Thus, OPN has a double property: the release and accumulation of calcium depends on the ambient conditions. The study conducted by Konya et al. 2003

explained that OPN adhering to the surface of collagen granules, adhesion/aggregation of seed crystals and adhesion of newly formed crystals can cause crystal deposition. This explains that the solid form of OPN enhances the formation and aggregation of calcium oxalate crystals (37). Grohe et al. In his research, also explained that OPN is an endogenous agent that inhibits stone formation by converting crystallization of calcium oxalate into a type of COD (calcium oxalate dihydrate) which possess the property of lower adhesion to renal epithelial cells than the COM phase (calcium oxalate monohydrate). It is explained that COM is more commonly found in kidney stones and COD is more excreted in urine. It has been previously reported that the structural properties of COM crystals tend to be retained in the kidney where they act as nuclei for stone formation, while COD decreases their likelihood of adhering to renal cells. Increased COD formation compared to COM acts as a protector against stone formation (38).

Infiltration of inflammatory cells is a common characteristic in damaged tissues (39). Renal damage due to the deposition of COM crystals in the kidney is capable in inducing an inflammatory response. Injured tubular cells and macrophage cells may produce various cytokines including OPN in kidney tissue in hyperoxaluric mice to attract and retain inflammatory cells in the inflamed area which in turn coats COM crystals and mediates their adhesion to macrophages and multinucleated interstitial cells in facilitation of crystal release and tissue repair. OPN also plays a role in helping macrophages to engulf crystals and protecting surrounding healthy tissue from the cytotoxic effects of cytokines such as NO produced by macrophages (40).

Testosterone has been known to increase hepatic levels of glycolic acid oxidase (GAO) which is an important enzyme in the urinary oxalate synthesis metabolic pathway that may cause hyperoxaluria (41). Urinary oxalate excretion increased 12.8 times after 4 weeks with EG therapy and it was concluded that dihydrotestosterone (DHT) partially contributed to the increase in excessive hyperoxaluria (42). In this study, it was found that rats fed with 0.75% EG drinking water receiving testosterone injection possessed significantly higher levels of osteopontin compared to rats fed with 0.75% EG drinking water without testosterone injection. However, this result is not in accordance with previous research conducted by Yagisawa et al. who reported that testosterone increases kidney stone formation by inhibiting the expression of osteopontin in the kidney and increasing urinary oxalate excretion (21).

CONCLUSION

As a conclusion, testosterone hormone may significantly promote stone formation via an increase in kidney osteopontin expression in hyperoxaluria induced Ethylene glycol rat model. This study provided evidence on the role of hormonal sex of testosterone in the promotion of kidney stone through the expression of kidney osteopontin.

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