COMPARISON OF APOPTOSIS IN CONTRALATERAL RENAL TUBULAR CELLS IN *ORYCTALAGUS CUNICULUS* DUE TO ARTIFICIAL UNILATERAL URETERAL OBSTRUCTION, WITH AND WITHOUT VERAPAMIL

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ABSTRACT

Objective: *To study the effects of verapamil on the kidney affected by contralateral obstruction of the ureter.* ***Material & method:*** *The right ureter of oryctalagus cuniculus rabbits, were obstructed surgically with a silk suture, and kept alive for 14 days. The control group was obstructed without other treatment, the study group was given verapamil from day 7 until day 14 of the obstruction. A third group was obstructed and given verapamil from day 0 through day 14. The fourth was given a sham operation as control. The contralateral kidney of all groups were removed after 14 days and processed with ApopTag. The number of apoptotic tubular cells was then reevaluated and compared in each group.* ***Results:*** *The highest increase in apoptotic cells was in the obstructed group without verapamil, and significantly increased compared to control (p<0,001). The groups which received verapamil had a lower increase of apoptotic tubular cells. In the group with verapamil for 14 days it was lower than the group which received verapamil only for 7 days. Both the 7 to 14 and the 0 to 14 groups were significantly lower than the group without verapamil (p=0,035 and p<0,001 respectively).* ***Conclusion:*** *verapamil has a protective effect on the contralateral kidney by inhibiting apoptosis caused by unilateral ureter obstruction. While the definitive treatment for urinary obstruction is to relieve it, verapamil can protect the kidney in the mean time.*

Keywords: *Obstructive uropathy, verapamil, apoptosis.*

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INTRODUCTION

Urine obstruction within urinary tract may occur either in upper or lower tract, acute or chronic, total or partial, unilateral or bilateral.[1] Each cause may present with particular symptoms, although all causes will lead to the same outcome in the function and abnormalities of the kidney. It decreases renal function, either reversibly or irreversibly.[2] Long term total obstruction may result in progressive lost of the nephron and causes atrophy of the medulla and cortex.[3]

Infection and ischemia accelerate obstruction-resulted renal damage and inhibit renal relieve after the removal of obstruction. Studies in rats subjected to unilateral ureteral obstruction revealed increased cell proliferation and apoptosis in the kidney.[4] Therefore, ureteral obstruction induced pathological and functional changes of the kidney, necessitating early detection and management to maintain the function of the kidney before the occurrence of irreversible renal damage.[5]

The damage of renal functional unit or nephron as well as the reduction of renal mass in ureteral obstruction occurs due to the death of tubular cells. Rather than necrosis, most of these events take place through apoptotic mechanism.[6]

The apoptosis of tubular cells develops rapidly after ureteral obstruction with ligation. The peak occurs between day 7 and 24 post-obstruction, and it becomes established thereafter. Initially, apoptosis only involves dilated ducts, but it subsequently involves other tubules as well. Common apoptotic mechanism pathway has been widely studied and can be elaborated satisfactorily. However, particular molecular control of apoptosis in obstructive uropathy has not been disclosed.

Apoptosis is a programmed cell death, which is actually a normal process in growth and development as well as in homeostasis. Cells die due to various stimuli, and the process of death runs orderly and controlled. Apoptosis involves regulation within the cell itself, so that apoptosis is also designated as cellular suicide. Apoptosis may occur resulting from various stimuli to the cells, such as DNA damage from radiative ionization, virus, stress cell, or extrinsic markers through the binding of ligands to the "death receptors". Apoptosis is mediated by caspase protein groups, which are breaking the components of cell structure, such as DNA repair, or activating DNAse that breaks down DNA structure.[8]

Verapamil is a calcium channel blocker of diphenylalkylamine that has protective nature against renal tubular damage. Verapamil inhibits the entrance of calcium ion into the cells through voltage-gate channel, which results in preglomerular vasodilatation and increased renal blood flow. These effects slow down the progressiveness of damage in renal cells, as observed in cisplatin-caused nephrotoxicity, which becomes lower after the administration of verapamil.[9] Burke et al (1984) found the protective effect of calcium channel blocker to alleviate renal damage due to norepinephrine-induced ischemia.[10]

Apoptosis resulting from ureteral obstruction involves mediators and enzymes, particularly the ones belonging to Renin-Angiotensin system. Angiotensin II has a character to promote apoptosis. Higher activity of angiotensin converting enzyme (ACE) is found in obstructed kidney. It is also found that ACE level in renal tissue is equal to serum ACE level.[11]

Because the enzyme that promotes renal cell apoptosis is circulating systemically, the contralateral kidney is also exposed. Adi et al (2007) found an increase of the number of apoptotic tubular cells in contralateral kidney in unilateral artificial ureteral obstruction after the obstruction lasting for 14 days.[12]

OBJECTIVE

To compare the increase of the number of apoptotic cells in contralateral renal tubule of *Oryctalagus cuniculus* due to artificial unilateral total ureteral obstruction with verapamil administration and that of artificial unilateral total ureteral obstruction without verapamil.

MATERIAL & METHOD

This was a quasi-experimental study using male rabbits (*Oryctalagus cuniculus*) as experimental animals that were treated with total unilateral ureteral ligation for 2 weeks and administered with verapamil. After nephrectomy was performed, apoptosis examination was conducted to the contralateral kidney. The length of the treatment for 2 weeks was based on the study by Adi et al (2007) who found significant number of apoptotic cells in contralateral kidney, as compared to that of control, after unilateral ureteral obstruction for 14 days.[12]

As many as 56 male rabbits were divided randomly into 4 groups, each comprising 14 rabbits. Prior to the treatment, the four groups were subjected to weight measurement. In group A, the rabbits were operated by ligating total unilateral ureter. Two weeks thereafter contralateral nephrectomy was performed and immunohistochemical examination with ApopTag was conducted to observe the number of apoptosis in contralateral kidney. In group B, the rabbits were operated by ligating total unilateral ureter and verapamil was given from day 7 to 14, and then they were subjected to nephrectomy and immunohistochemical examination with ApopTag to observe apoptosis in contralateral kidney. In group C, the rabbits were operated by ligating total unilateral ureter and verapamil was given from day 0 to 14, and then they were subjected to nephrectomy and

immunohistochemical examination with ApopTag to observe apoptosis in contralateral kidney. In group D, the rabbits were subjected to sham operation, and two weeks later they were subjected to nephrectomy and immunohistochemical examination with ApopTag to observe apoptosis in contralateral kidney (2 weeks control group).

Prior to the operation, the rabbits were fasted (except drinking) for 5-6 hours. The rabbits were given with 100 mg/kg BW ampicillin before operation. One and a half before being anesthetized, 1-3 mg/kg BW atropine was given intramuscularly. Ketamine in a dose of 40 mg/kg BW was injected intramuscularly and combined with paraldehyde 0,5 mg/kg BW to keep the anesthetic effect longer. After being anesthetized, the rabbits were positioned supine in such a way that the exposure of lower abdomen is sufficient for midline incision.

Asceptic procedure was performed in operation field and the surrounding area using 10% povidone iodine, operation field was draped with sterile cloth. Midline skin incision was on lower abdomen was made 5 cm, and deepened layer by layer. Thereafter, distal ureter was identified, and all groups were subjected to total ureteral ligation by ligating the ureter using silk thread size 4-0. Bleeding was treated and operation wound was closed layer by layer. After the rabbits recovered, 200 mg ampicilin was given intravenously through external ear.

The rabbits were reared as usual and verapamil was given for 7 days starting from day 7 in group B, and for 14 days in group C. On treatment day 14, the four groups were subjected to nephrectomy and immunohistochemical examination. Sliced renal tissue was stained with manual kit that was able to detect cell apoptosis. The trade mark of the kit was ApopTag with selling code of S 7101. The preparations were examined with light microscope in 400 times magnification, in 10 visual fields (high power) in each slide. The apoptotic cells would look dark with pycnotic nuclei.

Obtained results were tabulated statistically. Data were presented descriptively and analyzed using comparative test to compare the difference of unilateral total ureteral obstruction without verapamil and that with verapamil in its effect on the increase of the number of apoptotic contralateral renal tubular cells. The significance level was alpha = 0,05.

RESULTS

Bodyweight in rabbits with 14-day ureteral ligation without verapamil (comprising 11 rabbits) had mean bodyweight of 1590,91 $\pm$ 86,076. In rabbits with 14-day ureteral ligation with verapamil 7 - 14 (comprising 8 rabbits), the mean bodyweight was 1568,75 $\pm$ 96,130. In the third group, rabbits with ureteral ligation for 14 days with verapamil 0 - 14 (consisted of 14 rabbits) had mean bodyweight of 1610,71 $\pm$ 78,883, and in control group/SHAM (there were 12 rabbits), the mean bodyweight was 1529,17 $\pm$ 72,169.

The result of this estimation indicated that data between groups had homogeneous variance and the result of F test revealed significance level higher than 0,05, leading to a conclusion that there was no difference in bodyweight between the treatment groups. On the other words, the bodyweight in this study was not an influential confounding variable. Apoptotic cells in 14-day ligated rabbits without verapamil had the highest mean of cell number, 7,62 $\pm$ 4,16 (Table 1). Table 2 shows that in four data groups, two data groups had normal distribution ($p>0,05$), and two other groups had no normal distribution.

Although outlier data had been eliminated, abnormally distributed data remained present in the data of rabbits with 14-day ureteral ligation with verapamil and control/SHAM rabbits. Therefore, the subsequent statistical test was the non-parametric test (Table 3).

Table 1. Description of apoptotic cells in contralateral renal tubule.

Groups	N	Mean	Std. Deviation
Rabbits with 14-day ureteral ligation without verapamil	11	7,62	4,16
Rabbits with 14-day ureteral ligation with verapamil7-14	8	4,92	3,92
Rabbits with 14-day ureteral ligation without verapamil 0-14	14	2,61	3,43
Control/SHAM rabbits	12	0,0033	0,20
Total	45	3,5511	4,25

Table 2. Data normality test stage 1, apoptotic cells in contralateral renal tubule in all treatment groups.

Groups	Kolmogrov Smirnov	Sig.	Notes
Rabbits with 14-day ureteral ligation without verapamil	0,858	0,054	Normai
Rabbits with 14-day ureteral ligation with verapamil7-14	0,838	0,072	Normal
Rabbits with 14-day ureteral ligation without verapamil 0-14	0,596	0,001	Abnormal
Control/SHAM rabbits	0,592	0,001	Abnormal

Table 3. Data normality test stage 1, apoptotic cells in contralateral renal tubule in all treatment groups.

Groups	Kolmogrov Smirnov	Sig.	Notes
Rabbits with 14-day ureteral ligation without verapamil	0,858	0,054	Normai
Rabbits with 14-day ureteral ligation with verapamil7-14	0,929	0,545	Normal
Rabbits with 14-day ureteral ligation without verapamil 0-14	0,848	0,027	Abnormal
Control/SHAM rabbits	0,793	0,008	Abnormal

Table 4. Homogeneity and comparative test of apoptotic cells in contralateral renal tubule in all treatment groups.

Statistical test	Result	Notes
Chi square test	33,201	Significantly
Significance	0,001	different

Assessment using Kruskal Wallis revealed significant value of 0,001 ($p<0,05$), demonstrating difference between treatment groups in the mean of apoptotic cells in kidney contralateral to the obstructed kidney. From these results, the number apoptotic cells in rabbits with ureteral ligation for 14 days without verapamil was higher than that in rabbits with ureteral ligation for 14 days with verapamil 7-14, which was higher than that in rabbits with ureteral ligation for 14 days with verapamil 0-14, and the latter was higher than that in control/SHAM group. After being determined that there was differences altogether, there must be minimally 1 pair of different groups.

Table 5 shows that the mean of apoptotic cells in rabbits with ligated ureter for 14 days without verapamil was different from those in other groups, except from that in rabbits with ligated ureter for 14 days with verapamil 7-14. Whereas, the mean of apoptotic cells in rabbits with ligated ureter for 14 days with verapamil 7-14 days was different from that in rabbits with ligated ureter for 14 days with verapamil 0-14 days and that of control group. The mean of apoptotic cells in rabbits with ligated ureter for 14 days with verapamil 0-14 was different from that in control group.

Renal tissues from all groups were processed with hematoxylin-eosin staining. No necrotic cells in all experimental rabbits group were observed.

Table 5. Advanced or post-hoc test with Mann Whitney apoptotic cells in contralateral renal tubule in all treatment groups.

Groups	Rabbits with 14 day ureteral ligation without verapamil	Rabbits with 14 day ureteral ligation with verapamil 7-14	Rabbits with 14 day ureteral ligation with verapamil 0-14	Control/ SHAM rabbits
Rabbits with 14-day ureteral ligation without verapamil	-	0,035*	0,001*	0,001*
Rabbits with 14-day ureteral ligation with verapamil 7-14	-	-	0,046*	0,001*
Rabbits with 14-day ureteral ligation with verapamil 0-14	-	-	-	0,001*
Control/SHAM rabbits	-	-	-	-

Notes: ± = Ligated ureter was the ureter of contralateral kidney
* = Significantly different

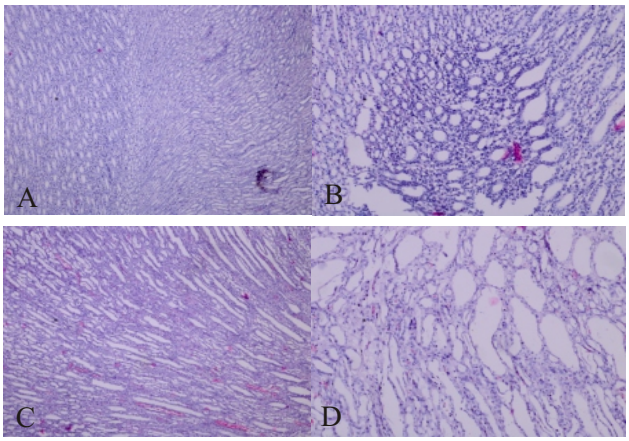

Figure 1. Slide photos in each group with hematoxylin-eosin staining, magnification 100x, no necrotic cells observed.

DISCUSSION

Obtained data showed that there was significant difference between rabbits with contralateral unilateral ureteral obstruction and those without obstruction (control). This result showed that obstructed kidney also affects the contralateral kidney, which, in this study, particularly in regard with the number of apoptotic cells in renal tubule. A study by Adi (2007) found similar result that the tubular apoptotic cells due to obstructed contralateral kidney increased significantly in the length of obstruction for 7 days.[12]

Rabbits were subjected to contralateral ureteral obstruction, and on day 7 verapamil was started to be administered up to the day 14. This was performed to imitate conditions that may occur in human. In most of the patients with ureteral obstruction, the exact time of the onset of obstruction is not known, and those patients begin to search for medication after the emergence of the symptoms. In group receiving verapamil after obstruction lasted for 7 days, the increase of the number of apoptotic cells was less than that in group with obstruction but without verapamil. The difference between these groups was significant. This indicated that verapamil administration, although the obstruction has lasted for 7 days, is still beneficial for slowing down the apoptotic rate in order not to reach the level in obstruction group without verapamil. Extraploted to human beings, it still can be expected that verapamil can be used for patients with urinary tract obstruction, where in clinical practice it is not easy to find when and how long the obstruction has occurred.

In group receiving verapamil after artificial obstruction up to 14 days, the increase of the number of apoptotic cells was lower than that in obstruction group without verapamil. The difference between these groups was significant. This finding demonstrates verapamil capacity in preventing apoptosis due to ureteral obstruction in contralateral kidney. This confirmed the previous study by Topcu (2008), who gave verapamil since the first day of obstruction up to day 7, but conducted his research only to ipsilateral kidney.[9] Contralateral kidney received exposure to systemic angiotensin due to obstruction, and triggers the cascade of free radicals formation through vasoconstriction, which can be prevented with verapamil. The author has not found

similar studies discussing especially verapamil for contralateral kidney. In human, the prevention of renal apoptosis with verapamil from the first day of obstruction can only be performed in cases where the onset of obstruction can be precisely recognized, such as that in iatrogenic ureteral ligation.

The increase of the number of apoptotic cells in contralateral renal tubule was lower than that in group receiving verapamil since day 0, compared to that receiving verapamil after 7 day obstruction. This difference was statistically significant. Both groups were also significantly lower than obstruction group without verapamil. The result indicated that verapamil administration was beneficial for reducing the increasing rate of apoptosis in tubular cells, and the earlier the administration, the lower the increase of apoptotic cells. Other studies on the prevention of obstruction-resulted apoptosis showed that calcium channel blocker or angiotensin II blocker were given since the onset of obstruction, even before the obstruction.[9,10]

Results obtained in this study revealed that treatment group receiving verapamil still had significant difference compared to that of control group. This result showed that verapamil administration, even since the first day of obstruction, does not prevent apoptosis significantly, resembling the kidney unaffected with contralateral renal obstruction. The administration of calcium channel blocker can be expected to prevent the apoptosis of tubular cells due to obstructed contralateral kidney by preventing the vasoconstriction that results in the increase of free radical concentration, which is triggering apoptosis. The vasoconstriction of renal blood vessel occurs due to the increase of angiotensin II level, while angiotensin II itself is directly included in cellular apoptosis-triggering agents. If apoptosis still occurs in the contralateral renal tubular cells in obstruction group receiving verapamil, this may result from significant direct effect of angiotensin II, even though vasoconstriction-resulted apoptosis has been prevented.

Further studies on the prevention of renal tubular cell apoptosis due to ureteral obstruction can be performed to find the effect of angiotensin II-formation inhibitor (Angiotensin Converting Enzyme Inhibitor), or angiotensin II blocker and compared it with the effect of verapamil and its combination.

Comparative study on the number of apoptotic cells in contralateral kidney as compared to that of ipsilateral kidney was performed along with the studies comparing the number of apoptotic cells due to unilateral artificial ureteral obstruction with and without verapamil in obstructed ipsilateral kidney in *Oryctalagus cuniculus* rabbits. As seen in table 1, it was indicated that in ipsilateral kidney, the number of apoptotic cells in contralateral kidney in rabbits with unilateral ureteral obstruction without verapamil was 7,62 ± 4,16 as compared to 12,46 ± 12,6. From this result, it can be seen that the apoptotic rate of contralateral kidney is lower, in accordance with the concept that the effect of obstruction on contralateral renal apoptosis occurs indirectly through a systemic mediator.

Interesting result can be seen in rabbits with ureteral obstruction that received verapamil, both from day 0 and day 0 up to day 14. In obstruction group with verapamil 7-14 days, the number of apoptotic cells in contralateral kidney was higher than that in ipsilateral kidney, which was 4,92 ± 3,9 compared to 2,89 ± 1,8. Similar finding was also found in obstruction group with verapamil day 0 to 14, which was 2,61 ± 3,43 in contralateral group compared to 2,73 ± 2,10 in ipsilateral group.

The effect of obstruction that results in apoptosis in contralateral renal tubular cells in non-verapamil group was not as remarkable as that in ipsilateral kidney. However, by the administration of verapamil, the number of apoptotic cells in contralateral kidney remained higher than that in ipsilateral kidney. This finding lead to an assumption that the mechanism of apoptosis in obstructed ipsilateral and contralateral kidney is affected by apoptosis activator through different mechanism. If in ipsilateral kidney verapamil is able to significantly reduce the number of apoptotic cells,[13] the apoptotic mechanism through vasoconstrictive pathway up to the occurrence of DNA damage can be regarded as more predominant compared to other mechanism. Whereas, in contralateral kidney verapamil is able to reduce the

apoptotic rate of renal tubular cells. However, the cell number remains higher than that in ipsilateral kidney. This shows that apoptotic mechanism pathway inhibited by verapamil is possibly not the predominant one.

It can also be assumed that, since in contralateral kidney the number of apoptotic cells is lower than that in ipsilateral kidney, the apoptotic rate of tubular cells reaches its peak faster. These assumptions require further investigation comparing the number of apoptotic cells in kidney tubule with more detailed time frame, and comparing various apoptotic mechanisms. The number of apoptotic cells in control group, either from right or left kidney, showed almost similar result, 0,38 ± 0,30 for right kidney and 0,38 ± 0,20 for left kidney. It can be concluded that the normal apoptotic rate in the kidney of *Oryctalagus cuniculus* rabbits is about 0.38.

CONCLUSION

Verapamil has a protective effect on the contralateral kidney by inhibiting apoptosis caused by unilateral ureter obstruction. While the definitive treatment for urinary obstruction is to relieve it, verapamil can protect the kidney in the mean time.

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