Iran Red Crescent Med J. 2014 October; 16(10): e15605. DOI: 10.5812/ircmj.15605

Published online 2014 October 5. **Research Article**

Investigating Antithyroid Effects of Propylthiouracil on the Ischemia and Reperfusion Injury in Rat' Kidney and Determining the Role of Nitric Oxide in Mediating this Effect

Shahnaz Tofangchiha [1]; Seyed Mir Mansoor Moazen Jamshidi [2]; Hamed Emami [3]; Banafshe Dormanesh [4,*]

[1]Department of Internal Medicine, AJA University of Medical Sciences, Tehran, IR Iran
[2]Department of Orthopedic Surgery, Mousavi Hospital, Zanjan University of Medical Sciences, Zanjan, IR Iran
[3]Tehran University of Medical Sciences, Tehran, IR Iran
[4]Department of Pediatric Nephrology, AJA University of Medical Sciences, Tehran, IR Iran

*Corresponding Author: Banafshe Dormanesh, Department of Pediatric Nephrology, AJA University of Medical Sciences, Tehran, IR Iran. Tel: +98-2143825616, E-mail: Dormanesh68@yahoo.com

Received: October 23, 2013; **Revised:** January 28, 2014; **Accepted:** February 22, 2014

Background: Renal ischemia/reperfusion injury (IRI) is a major problem in renal transplantation, which occurs during the process of organ retrieval and storage, and is closely associated with acute rejection episodes and late allograft failure. Recent studies have revealed a new phenomenon called "chemical preconditioning" that can induce tolerance against the ischemic stress via a variety of proposed pathways especially nitric oxide (NO) system. Propylthiouracil (PTU) is suggested to modulate the intracellular NO signaling.

Objectives: In this study, we investigated the preconditioning properties of chronic pretreatment with PTU in preventing renal IRI. In addition, we evaluated the involvement of NO pathway.

Materials and Methods: Sixty adult male Wistar rats were allocated into six groups. All groups underwent right nephrectomy 15 days before intervention. In groups 1 (Chronic PTU + L-NG-nitro arginine methyl ester [L-NAME]) and 2 (Chronic PTU) oral PTU (500 mg/L in water) treatment was started 15 days before right nephrectomy to achieve the therapeutic plasma level of PTU. Fourteen days after nephrectomy, animals received either L-NAME (10 mg/kg) or its vehicle and renal IRI was induced 45 minutes later. Groups 3 and 4 (Control) received respectively L-NAME (10 mg/kg) and its vehicle 45 minutes before IRI. The last two groups were normal sham operated rats and PTU + sham. Rats were killed 24 hours after IRI. The blood samples were collected and assessed for serum blood urea nitrogen (BUN) and creatinine (Cr) level, and tissue samples were fixed in formalin for histopathologic scoring of tubular damage (H-score).

Results: The mean BUN, Cr, and H-score of control group were 176.66 ± 12.24 mmol/L, 4.45 ± 0.44 μmol/L, and 83.5% ± 3.5%, respectively. Chronic pretreatment with PTU significantly improved BUN (40.4 ± 6.1 mmol/L), Cr (0.96 ± 0.068 μmol/L), and H-score (7.83% ± 4.02%) in IRI animals in comparison to those that were not treated with chronic PTU ($P < 0.001$) and L-NAME; however, it did not completely reversed the chronic PTU-induced protection (BUN, 93.33 ± 12.22 mmol/L; Cr, 2.7 ± 1.15 μmol/L, and H-score, 24.83% ± 3.5%). There was no significant difference between rats that were treated with L-NAME alone (group 5) and the control group.

Conclusions: Our study demonstrates that preconditioning of kidney with chronic PTU administration protects renal tissue against IRI and this phenomenon was mediated through NO system. The results suggest a potential indication for using PTU to protect the kidney before transplantations and to reduce the risk of tissue rejection afterwards.

Keywords: Ischemia; Reperfusion; Rat, Renal; Transplantation

1. Background

Ischemia/reperfusion injury (IRI) is one of the clinical challenges that can lead to various organs failure such as heart, intestine, brain, and kidney (1-3). The IRI can result in catastrophic structural and performance failures in various cells and tissues during surgical operations such as vascular surgeries as well as organ and tissue transplantation. During kidney transplantation, IRI inevitably occur in all stages of harvesting, storage, and engraftment. This phenomenon might lead to serious complications such as reduction of the lifetime of the transplanted organ (kidney, in our example) and increased risk of early and late rejection (4-6). Appropriate organs for donation are scarce, patients' need for organ transplantation is increasing, and this type of treatment is costly. Therefore, efforts to minimize the injuries to the transplanted tissue and prolong the transplanted organ and patients' survival are worth of attention. In that regard, several efforts have been made to increase the ability of the grafted tissue against the tissue injury caused by ischemia/reperfusion (IR). Previous studies have shown that an organ's exposure with a transient ischemia and reperfusion period increase its resistance against further and later IRI, a phenomenon that is

dubbed ischemic preconditioning (7, 8). In addition, various pharmacologic interventions have been proposed to counter and reduce IRI some of which include use of cyclosporine, vitamin E, melatonin, ozone, and many other interventions called pharmacologic preconditioning (9-12). Nonetheless, none of the medicines has found clinical application yet due to low efficacy and safety as well as their unclear pharmacologic profile. Hence, it seems that the drug's efficacy in protecting body tissues against IRI along with their safety profile is considered an important criterion to select these medicines. Propylthiouracil (PTU) has been widely used since 1947 and is approved by FDA for treating patients with hypothyroid. The only serious complication of this medicine has been liver injury in some cases; however, it has been considered an appropriate medicine for patients with hypothyroidism, especially for those with Grave's disease (13,14). PTU exerts its antithyroid effects through inhibiting iodine oxidation and ionization of monoiodotyrosine, preventing the coupling stage in the process of thyroxin production, and inhibition of peripheral conversion of T4 into T3 (15). Other paths on which PTU can have influence are not thoroughly known; however, there is evidence of PTU's effects on opening nonspecific calcium channels of renal cells mitochondria (16) and reducing the oxidative stress (17). Many studies indicated the PTU protective effects on the vascular beds through nitric oxide (NO) (18, 19). Some studies showed that PTU could have protective effects against IRI in brain and heart (17, 20) Considering the significance of the IRI, particularly in the field of organ and tissue transplantation, and the great number of transplantation in general and particularly in kidney transplantation, research on reducing and alleviating the complications is indispensable. In addition, based on different investigations, PTU might have positive effects on IRI in kidney.

2. Objectives

In this study, we intend to assess the PTU effects on the kidney tissue through planning, designing, and creating an animal model with IRI. Moreover, we plan to explain the role of NO as a mediator for this effect.

3. Materials and Methods

3.1. Population, Samples, Sampling Method, and Sample Size

It should be noted that our study had an experimental nature and was done on an animal IRI model. Based on the previous investigations, rats were used as the model of the study. The animal was killed by CO_2 chamber 24 hours after the reperfusion. The thorax and abdomen of the animal was opened and blood samples were taken from its heart and the biopsy samples were taken from the kidney. The obtained blood samples were immediately centrifuged and the separated plasma was delivered to the laboratory to assess the level of blood urea nitrogen (BUN) and creatinine (Cr). The kidney sample was put in 10% formalin solution and delivered to the pathologist for the histopathologic analyses. A part of kidney sample was snap-frozen in liquid nitrogen and then were kept in freezer in the -80℃ to assess the level of released NO and nitric oxide synthase (NOS) via Western blotting.

3.2. Procedure

In this study, we used male Wistar rats, weighing 250 to 300 g. Creation of the kidney IRI model was done in the following fashion. At first, the rats underwent right nephrectomy. This was done to omit the compensatory and interfering effects of the healthy kidney on the kidney performance in I/R assessment stage. Two weeks after right nephrectomy, the left kidney was exposed to IRI through a surgery. The rat was anesthetized by administration of a mixture of ketamine and chlorpromazine. The right flank region was shaved for nephrectomy and left flank region for creating the IRI. Then under sterile conditions, a 2-cm incision was created on the abdomen skin in parallel with paravertebral muscles and the kidney was exposed. In the nephrectomy stage, the right kidney base was ligated and the nephrectomy was done. In the IRI stage, the left kidney pedicle was clamped using a nontraumatic vascular clamp for a specific period. Thereafter, the clamp was opened and kidney reperfusion started. Later on, the abdominal muscles and skin were sewed separately. After 24 hours of reperfusion, another rat was killed and blood and biopsy samples were taken. Firstly, the rat was killed in a CO_2 chamber. The thorax was immediately opened and blood was taken from the heart of the rat. The blood samples were centrifuged for 15 minutes and at 2000 rpm. The separated serum was kept in the -20℃ until the biochemical analyses for BUN and Cr levels. Next, the kidney of the rat was excised and kept in 10% formalin solution. Afterwards, the samples were sliced and embedded in paraffin and 5-μm incisions were made for nuclear staining with hematoxylin and eosin. A pathologist, who was unaware of the conducted and planned interventions, analyzed the prepared slides. In the histopathologic analyses, based on three criteria of tabular dilatation, sloughing, and existence of naked basement membrane, the percentage of tissue injury was determined. In this study, in phase 1, the ischemia effect on the kidney tissue injury was measured. The ischemia was induced for 45, 60, and 75 minutes in three separate groups. After 24 hours, the effect of ischemia timing was compared and contrasted with Sham group.

3.2.1. Operated Control-Sham

In this group, all the stages of surgery were conducted except kidney pedicle clamping (IRI).

3.2.2. Ischemia/Reperfusion Injury-45

In this group, the kidney was exposed to ischemia for 45 minutes.

3.2.3. Ischemia/Reperfusion Injury-60

In this group, the kidney was exposed to ischemia for 60 minutes.

3.2.4. Ischemia/Reperfusion Injury-75

In this group, the kidney was exposed to ischemia for 75 minutes. The best time for the further stages was selected. In the further stages of the study, to assess and evaluate the effects of chronic treatment with PTU on the injured kidney, phase 2 of the study was planned, which included the following four groups:

3.2.4.1. Propylthiouracil Plus Sham

In this group, 500 mg/L of PTU was added to the drinking water of the rats for 30 days and after 15 days of starting chronic treatment with, the animals underwent nephrectomy and after 30 days (15 days after the nephrectomy), the rats underwent Sham surgery.

3.2.4.2. Propylthiouracil Plus Ischemia/Reperfusion-20

In this group, 500 mg/L of PTU was added to the drinking water of the rats for 30 days and after 15 days of starting treatment, the animals underwent nephrectomy and after 30 days (15 days after the nephrectomy), the rats underwent I/R surgery.

3.2.4.3. L-NG-nitro arginine methyl Plus Propylthiouracil

In this group, 45 minutes before IRI, 10 mg/kg of L-NG-nitro arginine methyl (L-NAME), as a nonspecific NOS inhibitor, was injected intraperitoneally. In this stage, the effects of L-NAME on the effect of PTU use on the IRI were assessed.

3.2.4.4. L-NG-nitro arginine methyl Plus Ischemia/Reperfusion Injury

In this group, 45 minutes before the IRI, 10 mg/kg of L-NAME drug was injected intraperitoneally. Therefore, the study groups include the followings:

Sham control, IRI-45, IRI-60 Phase I: IRI-75, Groups, PTU, Phase II: PTU + IRI, PTU + L-NAME, IRI + L-NAME

3.3. Data Analyses

The observed differences between the BUN and Cr in different groups was analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) via Tukey's Post hoc and One-way ANOVA ($\alpha < 0.05$). The level of tissue injury was presented in percent. The difference between groups was assessed by Tukey's Post hoc and one-way ANOVA.

3.4. Research Ethical Considerations

The animals in the study were treated based on the University Animal Care Committee Protocol, as well as NIH US (Publication 86-23, revised 1985).The rats were kept anesthetized during the surgery to prevent their suffering. They were kept at proper temperature and regarding their food and water, they received appropriate treatment. The hygienic status of where they were kept was highly suitable, particularly in the surgery place. To collect data, rats were killed using CO_2 chamber.

4. Results

4.1. Phase 1 Results

The values of BUN, Cr, and the percentage of injured tissue in the control group were 0.75 ± 0.09 mg/dL, 30.0 ± 3.74 mg/dL, and 0%, respectively. In IRI-45 group, the values of BUN, Cr, and the percentage of injured tissue were respectively 0.75 ± 0.12 mg/dL, 30.16 ± 9.94 mg/dL, and 1.66% ± 2.58%, which showed no significant difference with the control group. In IRI-60 group, the values of BUN, Cr, and the percentage of injured tissue were 4.45 ± 0.44 mg/dL, 176.66 ± 12.24 mg/dL and 83.50% ± 3.56%, respectively, showing significant difference with control group ($P < 0.001$). In IRI-75 group, the values of BUN, Cr, and the percentage of injured tissue were 4.88 ± 0.24 mg/dL, 209.0 ± 15.11 mg/dL, and 85.16% ± 3.65%, respectively, showing significant difference with control group ($P < 0.001$). In this final group, the BUN value was significantly higher than IRI-60 group ($P < 0.05$) (Figures 1, 2 and 3). Considering these results, 60-minute time was considered for the later stages (Table 1).

4.2. Phase 2 Results

These results are shown in Table 2. The values of BUN, Cr, and the percentage of the injured tissue in the group of chronic treatment with PTU, which had undergone the sham surgery, had no significant difference with the sham group. The chronic treatment of rats with PTU had significantly decreased the values of BUN, Cr, and the percentage of tissue injury (40.4 ± 6.1 mg/dL, 0.96 ± 0.068 mg/dL, and 7.83% ± 4.02%, respectively) in comparison with IRI ($P < 0.001$). In chronic treatment with PTU, the injection of L-NAME could reduce the protective effects of PTU. The amounts of BUN, Cr, and percentage of injured tissue were 93.33 ± 12.22 mg/dL, 2.7 ± 1.15 mg/dL, and 24.83% ± 3.54%, respectively. These amounts were significantly lower than IRI group ($P < 0.05$), and showed that the protective effects of chronic treatment with PTU was not completely inhibited by blocking the NO pathway. In addition, the L-NAME, per se, had no significant effect on IRI (Figures 1 , 2 and 3).

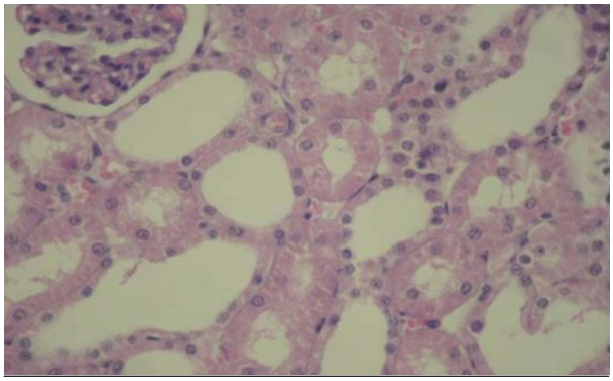

Figure 1. Pathologic figure of Normal Rat

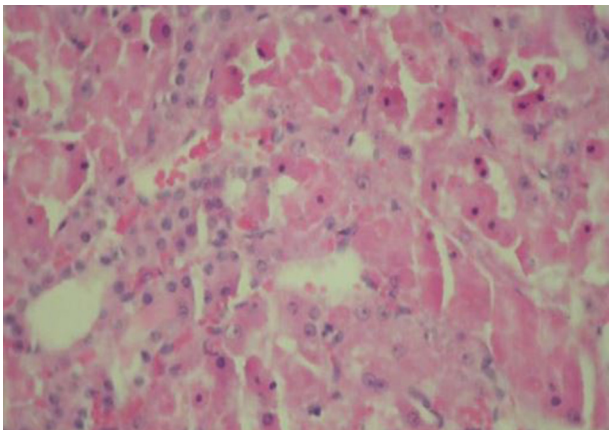

Figure 2. Pathologic figure in Sample With Sixty Minutes Ischemia and Twenty-Four Hours Reperfusion

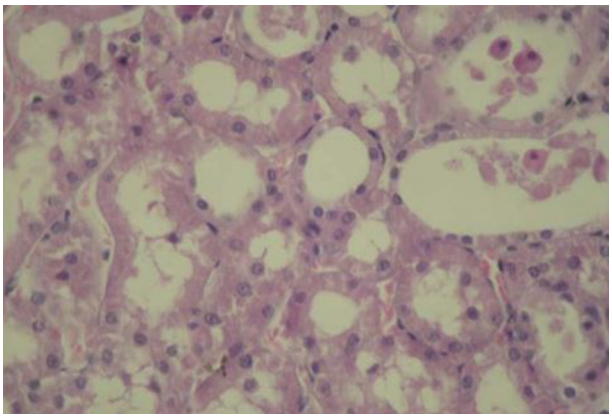

Figure 3. Pathologic figure in Sample With Chronic Use of Propylthiouracil Sixty Minutes Ischemia and Twenty-Four Hours Reperfusion

Table 1. Determining the Effects of Ischemia on the Kidney Tissue Injury Level [a,b]

	Injured Tissue, %	BUN, mmol/L	Cr, µmol/L
Sham	0.00 ± 0.00	30.00 ± 3.74	0.75 ± 0.09
IRI-45	1.66 ± 2.58	30.16 ± 9.94	0.75 ± 0.12
IRI-60	83.50 ± 3.56	176.66 ± 12.24	4.45 ± 0.44
IRI-75	85.16 ± 3.65	209.00 ± 15.11	4.88 ± 0.24

[a] Abbreviation: BUN, blood urea nitrogen; Cr, creatinine; and IRI, ischemia/reperfusion injury.
[b] Data are presented as mean ± SD.

Table 2. Determining the Effects Propylthiouracil on the Level of Ischemia/Reperfusion injury and Role of Nitric Oxide [a,b]

	Injured Tissue, %	BUN, mmol/L	Cr, µmol/L
Sham	0.00 ± 0.00	30.00 ± 3.74	0.75 ± 0.09
IRI-60	83.50 ± 3.56	176.66 ± 2.24	4.45 ± 0.44
PTU + Sham	0.00 ± 0.00	30.16 ± 4.49	0.77 ± 0.08
PTU + IRI	7.83 ± 4.02	40.40 ± 6.10	0.96 ± 0.06
PTU + L-NAME + IRI	24.83 ± 3.54	93.33 ± 12.22	2.70 ± 1.15
L-NAME + IRI	79.50 ± 5.20	178.16 ± 6.16	4.46 ± 0.38

[a] Abbreviation: BUN, blood urea nitrogen; Cr, creatinine; IRI, ischemia/reperfusion injury; PTU, propylthiouracil; and L-NAME, L-NG-nitro arginine methyl ester.
[b] Data are presented as mean ± SD.

5. Discussion

The results of this study showed that chronic treatment with PTU could protect the kidney against IRI, improve the performance of kidneys, and reduce the tissue injury. In addition, the present study shows that the NO-related mechanisms had a key role in the protective effect of PTU. The IRI is one of the inevitable complications of kidney transplantation. In addition to the directly induced injury on the kidney by IR, this phenomenon increases transplantation rejection by increasing the immunogenicity of tissue through increasing the surface antigens including major histocompatibility complex II (MHC-II) (4-10). In order to increase the survival of transplantation, organ, and patient, increasing the resistance of the tissue against ischemic injuries is logical. Hence, several interventions including various pharmacologic interventions have been examined to reduce the kidney IRI (9-11). The results of this study indicate that PTU could be used as a simple and practical preconditioning drug. In this study, the used dosages created a safe serum level with regard to side effects. Regarding the long history of PTU use in treating patients with hyperthyroidism as well as its approved safety, PTU might be clinically used. Specifically, considering the protective effect of chronic treatment with PTU on kidney IRI, this method might be used to reduce the tissue injury and minimize the risk of tissue rejection in the kidney transplantation process. According to our findings, the protective effects of PTU against IRI could be inhibited by L-NAME administration and blocking the NO synthesis pathway, which showed that the NO-related mechanisms had a major role in the protective effects of PTU against kidney tissue injury. It should be mentioned that there is great controversy regarding the role of NO in the kidney injury. In a study it was indicated that the Sodium nitroprusside (NO giver) and L-arginine (the NO precursor) led to increased and intensified IRI in the proximal tubules of rats. In addition, inhibition of NOS led to intensified injury to kidney, which prevents the full return of blood circulation into the kidney. They concluded that the reduction of NO level inside the tissue might be among the major reasons of tissue injury (21). Nevertheless, the majority of research and available data confirm and emphasize on the protective effects of NO in kidney injuries (22, 23). The analysis of our results indicates that the protective effect of chronic treatment with PTU was relatively inhibited by use of L-NAME. Therefore, other pathways and protective mechanisms other than NO pathway might be involved in the chronic treatment with PTU. These possibilities, however, were not considered in the present study, and settling the issue requires further and in-depth studies to determine the precise mechanisms of protective effects of PTU against kidney injury. Finally, the results of the present study showed that chronic treatment with PTU has acceptable protective effects against kidney injury in the rat animal models. In addition, the NO-related mechanisms might have a major role in the PTU's protective effects on kidney. In

the next phase of the study, PCR and Western blotting of kidney will be used to determine the contribution of various subtypes of NOS.

5.1. Suggestions

The results of this research suggested that the use of PTU, as a chronic treatment, was a simple, yet practical intervention, which can lead to promising results in protecting the kidney tissue, especially in case of kidney transplantation, to reduce the tissue injury and minimize the possibility of tissue rejection and graft failure.

Acknowledgements

We thank all the personnel who helped us accomplish this project and AJA University for support.

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