Glutathione peroxidase-1 and klotho gene expression in acute renal failure rats and its association with creatinine and urea levels.

Fatma, E. El-gendy [a], Hemeda, SH. A. [b], Sosa, G.A.[c] and Naglaa, F. Al Hussenii [d]

[a] *Animal Wealth Development Dept., Fac. of Vet. Med., Benha Univ.* [b] *Animal Wealth Development Dept., Fac. of Vet. Med., Alexandria Univ.* [c] *Theriogenology Dept., Fac. of Vet. Med., Benha Univ.* [d] *Biochemistry & Mol. Biology. Fac. of Medicine- Benha Univ.*

A B S T R A C T

The main objectives of this work was to study the effect of glycerol induced acute renal injury on Glutathione peroxidase (GPx-1) and klotho gene expression which used as early indicator for acute renal failure. Twelve male albino rats divided into two groups were included in this study. Blood samples were taken for assessment of creatinine and blood urea nitrogen. Kidney tissue specimens were processed for mRNA gene expression study of Glutathione peroxidase (GPx-1) and klotho genes by real time polymerase chain reaction (RT-PCR). The creatinine and blood urea nitrogen significantly increased (4.97 ± 0.75 and 242.03 ± 41.93) compared with control group (0.67 ± 0.06 and 24.43 ± 2.18). Moreover, the renal Glutathione peroxidase-1 and klotho gene expression was found to be decreased in the glycerol treated group (affected group), to 0.899 and 0.91fold. Also a correlation between gene expression of Glutathione peroxidase-1and klotho gene with the level of creatinine and blood urea nitrogen was detected.

Keywords: Glutathione peroxidase-1. klotho. Correlations. RT-PCR

1. INTRODUCTION

Glycerol-induced renal failure causing severe muscle injury (rhabdomyolysis), accompanied by the release of myoglobin that becomes deposited in the kidney and causing renal failure after 24 hrs from injection. The magnitude of elevation of serum creatinine (SCr) and Blood urea nitrogen (BUN) sufficient to diagnose acute renal failure ARF. Kim et al., 2010, Kaul, and Ruhela (2012). Glutathione peroxidase-1 play a critical role as antioxidant defense system by catalyzing detoxification of hydrogen peroxide (H_2O_2) and organic hydroperoxides (Srikanta et al., 2012). Klotho gene is an anti-aging gene highly expressed in the kidney. The soluble form of klotho functions as an endocrine substance that exerts multiple actions including the modulation of renal solute transport and the protection of the kidney

(Hu et al., 2012). The objective of this study was to evaluate the effect of glycerol induced acute renal failure on expression level of Glutathione peroxidase, klotho gene and on kidney function (creatinine and blood urea nitrogen) and determine correlation between gene expression of Glutathione peroxidase-1, klotho, and level of blood urea and creatinine.

2. MATERIAL AND METHODS

2.1. Experimental animals

Twelve male albino rats were used in this study. They weighted (180-200 g) at the beginning of the experiment. Rats were obtained from Lab of animal care centre, Faculty of Veterinary Medicine (Benha University). Animals were kept for one week before use to acclimatize to the laboratory conditions. The management

was kept constant throughout the experimental period. Water and normal balanced ration was offered ad-libitum, and was renewed every day. Cages were cleaned regularly in which rat were moved to completely clean cages two times a week. Animals were divided into 2 groups, each 6 animals as follows: Group I (Control group): Without any treatment. Group II (Affected group): Glycerol (50%, 8 ml/kg) was injected intra muscular distributed equally in both hind limbs in one dose. (Savic et al., (2002.) After 48 hrs from the glycerol injection, the rats of two groups were sacrificed, Kidney specimens were taken for assessment of gene expression of Glutathione peroxidase (GPx-1) and klotho genes by real time polymerase chain reaction (RT-PCR).

2.2. Kidney functions.

Blood samples were obtained from the venous plexus to determine the serum levels of blood urea nitrogen and creatinine by a colorimetric kinetic method (Henry et al., 1974).

2.3. Assessment of Glutathione peroxidase (GPx-1) and klotho genes expressions compared with β- actin as endogenous control.

2.3.1. Tissue handling:

Kidney biopsies were taken and immediately placed at -80 °C for further processing. Total RNA extraction was done by using total RNA Purification Kit from Jena Bioscience GmbH, according to the manufacturer instructions with about 30 mg tissue sample collected in a micro centrifuge tube, 300 µl of lysis buffer was added with β mercapto ethanol (β ME). The sample was homogenized using motor driven grinder.

2.3.2. U.V. Spectro photometric Quantification and purity of RNA:

The concentration of RNA in each sample was measured by Nano Drop Spectrophotometer at A_{260} and A_{280}. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. Concentration of RNA sample at A_{260} was measured 44 µg/ml (Wiffinger et al., 1997). Pure RNA has an A_{260}/A_{280} ratio of 1.9.

2.3.3. Two steps RT-PCR:

First step: template RNA (5ul) and distilled water (15 ul) were added to Maxime RT pre mix tube. cDNA synthesis (Reverse transcription) reaction using G-storm thermalcycler (Ingland) was performed at a temperature of 45°C for 60 min followed by RTase inactivation step at 95°C for 5 min . This reactant was diluted by adding 30 ml nuclease free water. *Second step:* RT-PCR was done using ABI 7900HT fast real time PCR (applied Biosystem USA), the prepared reaction components were done in 96 well PCR plate (micro Amp ® 90 well optical reaction plate with Barcode, code 128). Multiplex reaction was done using qPCR SYPER Green Master from (Jena Bioscience GmbH), using real time cycler conditions using real time cycler conditions as showed in table 1. The primers sequences for all genes was shown in table 2.

2.4. Data analysis

According to the relative quantification (RQ) manager program software (ABI 7900HT), the data are produced as sigmoid shaped amplification plots in which the number of cycle is plotted against fluorescence (when using linear scale). Because the samples of control group used as calibrators, the expression levels are set to 1. However, because the gene expression levels were plotted as log10 values (log10 of 1 is 0), the expression level of the calibrator samples appear as 0 in the graph. Because the relative quantities of the Glutathione peroxidase-1 and klotho genes are normalized against the relative quantities of the endogenous control β-actin gene, and fold expression changes are calculated using the equation $2^{-\Delta\Delta CT}$

2.5. Statistical analysis:

The collected data were statistically analyzed using the statistics package for social sciences (SPSS) (Anova) and

Microsoft office Excel. The data presented as means ± Standard error (SE). Differences are considered as statistically significant for P values less than 0.05.

3. RESULTS

3.1.Kidney functions (creatinine and Blood urea nitrogen(BUN).

Table 3 showed that the serum creatinine was significantly increased in glycerol treated group (4.97± 0.75) than control groups (0.67 ± 0.06). In addition, BUN was significantly increased in glycerol induced renal failure 242.03 ± 41.93, than control group (24.43 ± 2.18).

3.2.Gene expression of Glutathione peroxidase-1 and klotho genes:

Gene expression data is represented in figure 1, 2 and table 4. The expression level of GPx-1 and klotho mRNA for glycerol treated group was indicated by pink and green colour, this color also indicated Relative quantification (RQ). Control (a) samples used as a calibrator, the expression levels are set to one, and found that the expression levels of GPx-1 and klotho genes were decreased to 0.899 fold and 0.91 fold in kidney samples of glycerol treated group. The obtained data in table 5 showed that the means of Log 10 relative units of GPx-1 mRNA expressions levels in control and glycerol treated groups are 5.17 ±0.08 and 5.11 ± 0.08 respectively. In addition, the means of Log 10 relative units of klotho mRNA expressions levels in control and glycerol groups are 5.12 ± 0.08 and 5.08± 0.08 respectively.

3.3.Corelations between blood urea nitrogen, creatinine levels and, Glutathione peroxidase-1 and klotho gene expression:

Table 6 revealed that, there is a significant positive correlation (0.96) between BUN and creatinine (fig. 3). The correlation between GPx-1 gene expression and blood level of creatinine and BUN was non-significant negative correlation (-0.303 and - 0.36) fig. 4, 5. In addition, there were non-significant negative correlation between klotho gene expression with creatinine and BUN (-0.284 and - 0.344) as shown in fig. 6, 7. A significant positive correlation was found between klotho gene expression and GPx-1 gene expression (1.00) fig. (8).

Table (1): Real time thermal cycle condition.

Step	Time	Temperature.	Cycles
Initial denaturation	5 min	95°C	1 cycle
Denaturation	30 sec	95°C	
Annealing	1 min	55°C	35cycle
Extension	30 sec	75°C	

Table (2): Primers sequences of β –actin as internal control, Glutathione peroxidase (GPx-1) and Klotho.

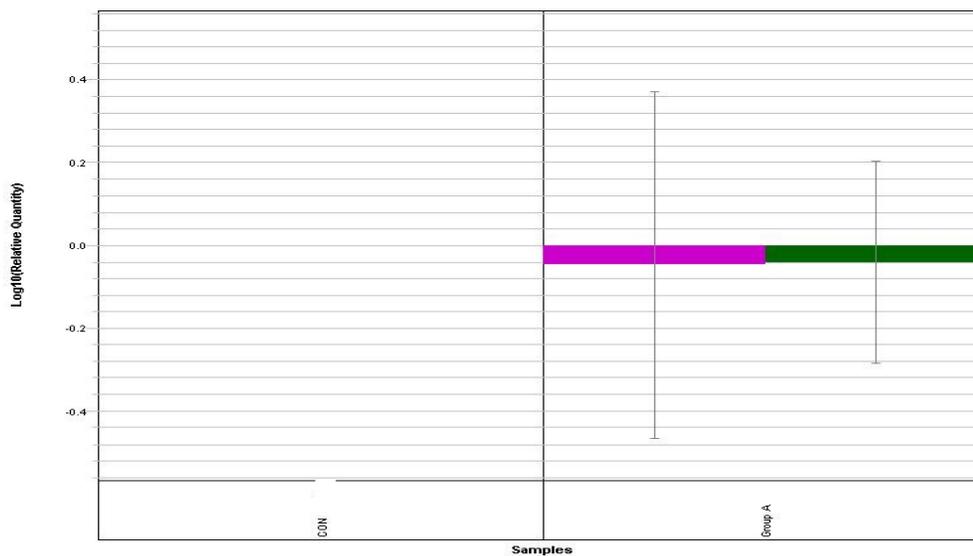| Genes	5\ to 3\ sequence forward	5\ to 3\ sequence Reverse
β-actin	CCC ATT GAA CAC GGC ATT G	GTA CGA CCA GAG GCA TAC A
GPx-1	ATG TCT GCT GCT CGG CTC TC	GTT GCT AGG CTG CTT GGA CAG
Klotho gene	CGT GAA TGA GGC TCT GAA AGC	GAG CGG TCA CTA AGC GAA TAC G

Table (3): Mean ± Standard error of serum creatinine and blood urea nitrogen of control and glycerol treated groups.

Symbol	Groups	Duration	No. of animals	Mean ± SE of creatinine	Mean ± SE of BUN
a	Control	-	6	0.67±0 .06[b]	24.43±2.18[b]
A	50% glycerol	48 hrs	6	4.97± 0.75[a]	242.03±41.93[a]

Means ± SE having different letters within the same column are significantly different at the level of $P < 0.05$

Table (4): Glutathione peroxidase -1 and Klotho m-RNA expression in kidney samples of control and glycerol (ARF) group.

Groups	Description	GPx-1 gene expression	Klotho gene expression
a	control	1	1
A	Glycerol (ARF) group	0.899	0.91

Group A= 50% glycerol treated group.   GPX-1 gene expression.   Klotho gene expression

Figure (1):- Gene expression levels of Gpx-1 and klotho m- RNA in kidney for control samples as a calibrator group and glycerol group (A).
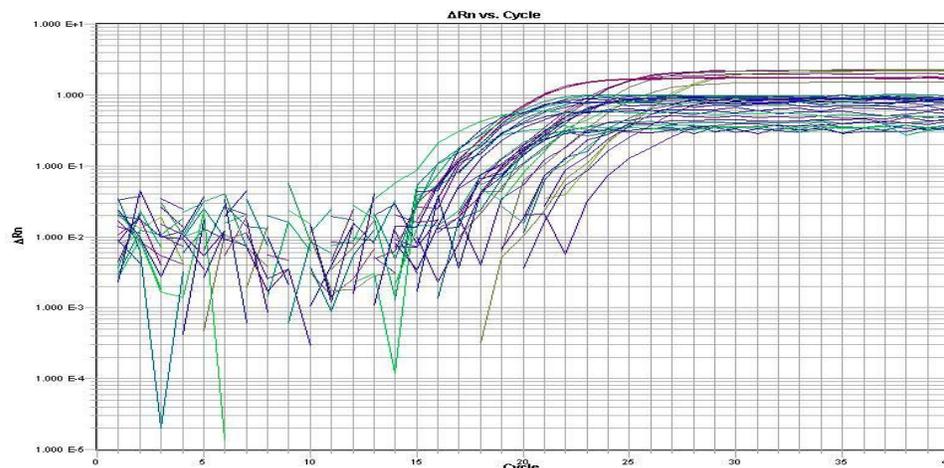
Figure (2): Amplification plot curves for all detectors (β-Actin, GPx-1 and klotho genes) by Δ Rn against cycles of the kidney samples of control and treated groups. (Curves by ABI 7900 Real Time).

Table (5): Mean ± Standard error of Log10 relative units of   GPx-1 mRNA expressions in kidney of control and treated groups.

Symbol	Groups	Dose	Duration	No. of animals	GPx-1 Gene expression	Klotho Gene expression
a	Control	-	-	6	5.17 ± 0.08^a	5.12 ± 0.08^a
A	50% glycerol	8 ml/kg, B.wt	48 hrs	6	5.11 ± 0.08^a	5.08 ± 0.08^a

Means ± SE having different letters within the same column are significantly different at the level of $P < 0.05$.

Table (6): Correlations between blood urea nitrogen, creatinine, Glutathione peroxidase-1 and klotho gene expression. [**] Correlation was significant at level $P < 0.01$

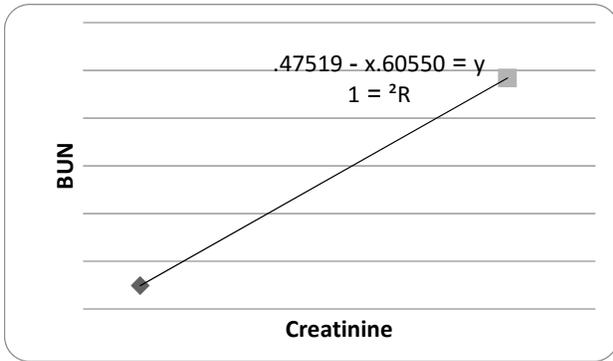| Correlations	Creatinine	Blood urea nitrogen	Glutathione peroxidase-1gene expression	Klotho gene expression
Creatinine	1			
Blood urea nitrogen	0.96^{**}	1		
Glutathione peroxidase-1gene expression	- 0.303	- 0.36	1	
Klotho gene expression	- 0.284	- 0.344	1.000^{**}	1

101

Figure (3): Correlation between blood urea nitrogen and creatinine (Significant positive correlation).
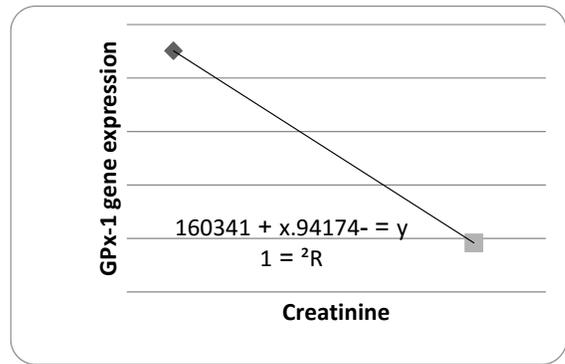

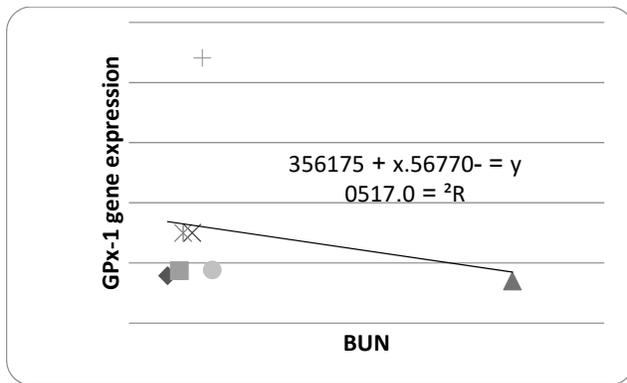Figure (4): Correlation between GPx-1 and creatinine (non-significant negative correlation).

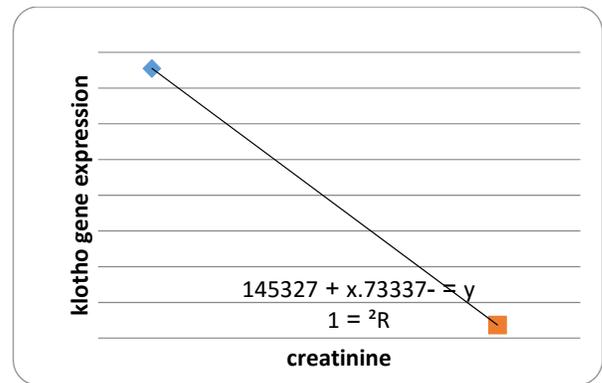Figure (5): Correlation between GPx-1 gene expression and BUN (non-significant negative correlation).

Figure (6): Correlation between klotho gene expression and creatinine gene expression (non-significant negative correlation).
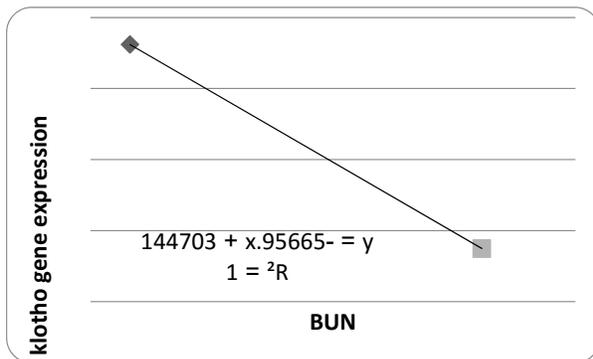

Figure (7): Correlation between klotho gene expression and BUN (non-significant negative correlation).
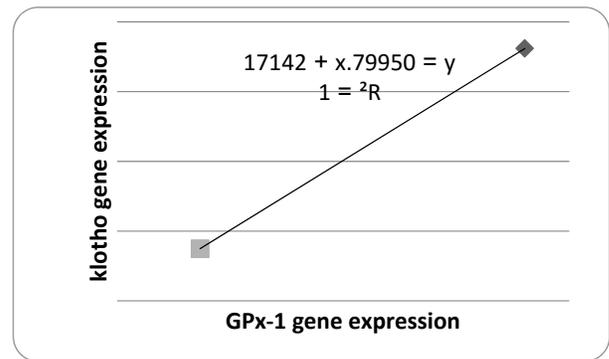

Figure (8): Correlation between klotho and GPx-1 gene expression (significant positive correlation).

4. DISCUSSION

Down regulation in Glutathione peroxidase -1 gene expression in glycerol induced renal failure group agree with Baliga et al., (1997) who found that, the oxidative stress increasing in ARF is one of the key underlying mechanisms in nephropathy pathogenesis. Impairment in the antioxidant defense mechanism, including increased lipid peroxidation and inactivation of antioxidant enzymes including catalase, superoxide dismutase and GPx. Also Ghatak et al., (1999) found that a significant reduction in plasma scavenging enzyme activities as GPx in patients with oxidative stress. Decreases in klotho gene expression in glycerol induced renal failure group (A) agree with Ohyama et al., (1998) suggesting that the expression of klotho is modulated by acute inflammatory stress in vivo. Similarly Saito et al., (2003) and Mitobe et al., (2005) were proved that the oxidative stress can decrease klotho mRNA and protein in a cultured cell line, and increased Tumor necrosis factor (TNF) and interferon-γ (IFN-γ) in acute kidney injury lead to klotho gene down-regulation. In addition Sugiura et al., (2005) reported that the renal klotho mRNA and protein expressions were significantly reduced in the rats with renal failure assessed by real-time PCR or western blotting. The elevation in urea and creatinine in glucerol induced renal failure may be attributed to the increase in protein catabolism superimposed to glomerular or tubular destructive changes (Ravel, 1984; Abdel Moneim et al., 1999). Furthermore, Dubrow and Flamenbaum, (1983); Zager, (1996) found that the intramuscular administration of hypertonic glycerol induces renal injury represents the integrated effects of three major pathophysiologic mechanisms: renal vasoconstriction, direct cytotoxicity and cast formation lead to both direct toxic and hemodynamic abnormalities resulting in acute renal failure and elevation of creatinine and BUN. Moreover Kim et al., (2010) reported that the administration of

glycerol led to a rise in BUN from 16 mg/dl (baseline) to 145 mg/dl and a rise in serum creatinine from 0.5 mg/dl (baseline) to 4.0 mg/dl at 24 hrs after glycerol injection compared with controls. Correlations between the different parameters (blood urea nitrogen, creatinine, Glutathione peroxidase-1 and klotho gene expression) agree with Koh et al.,(2001) and Imura et al., (2004) who reported severely reduced production of klotho in urine and kidneys (messenger RNA and protein) by Western blot of patients with acute renal failure, and klotho levels have been shown to correlate negatively with plasma creatinine levels. Also the previous results come in harmony with the results obtained by El-Far et al., (2005) found that presence of significant negative correlation between glutathione peroxidase -1 activity and serum creatinine level. Furthermore, a highly significant negative correlation was found between glutathione peroxidase -1 and blood urea nitrogen. Moreover, Shih and Yen (2007) were evaluated the positive correlation between anti-aging gene (klotho) and antioxidant status and many important antioxidant enzymes such as Glutathione peroxidase.

5. REFERENCES.

Abdel-moneim, A., El-zyat. E., Ahmed, O. 1999. Studies on the effects of glimepiride and some antidiabetic plants on protein metabolism of streptozotocin diabetic rats. J. Egypt. Ger. Soc. Zool., 28:79-98.

Baliga, R., Ueda, N., Walker, P.D., Shah, S.V. 1997. Oxidant mechanisms in toxic acute renal failure. Am. J. Kidney Dis. 29(3):465-77.

Dubrow, A., Flamenbaum, W. 1983. Acute Renal Failure Associated with Myoglobinuria and Hemoglobinuria. In: Acute Renal Failure, Soles, K. and A. Whelton (Eds.). Marcel Dekker, New York 279-293.

El-Far, M. A., Bakr, M. A. , Farahat, S. E. , Abd El-Fattah, E.A. 2005. Glutathione peroxidase activity in patients with renal

disorders. Clinical Exp. Nephrol. 9(2):127-158.

Ghatak, A., Brar, M. J. S. , Agarwal, A., Goel, N., Rastogi, A. K., Vaish, A. K., Sircar, A. R. , Chandra, M. 1999. Oxygen free radical system in heart failure and therapeutic role of oral vitamin E. Int. J. Cardiol. 57:119 –127.

Henry, J.B., Todd, Sanford, Davidsohn, 1974. Clinical diagnosis and measurement by laboratory method W.B. Saunders and Co Philadelphia, 260.

Hu, M.C., Kuro, O.M., Moe, O.W. 2012. The emerging role of Klotho in clinical nephrology. Adv. Exp. Med .Biol. 27:2650–2657.

Imura, A., Iwano, A., Tohyama, O. 2004. Secreted Klotho protein in sera and CSF implication for post-translational cleavage in release of Klotho protein from cell membrane. FEBS Lett. 565:143-147.

Kaul, A., Ruhela, V. 2012. Approach to a patient with acute kidney injury. Clin. Quer. Nephrol. (0101)6–12.

Kim, J.H., Lee, S.S., Jung, M.H., Yeo, H.D. Kim, H., Yang, J., Roh, G.S., Chang, S.H. , Park, D. 2010. N-acetylcysteine attenuates glycerol-induced acute kidney injury by regulating MAPKs and Bcl-2 family proteins. Nephrol Dial Transplant 25:1435–1443.

Koh, N., Fujimori, T., Nishiguchi, S. 2001. Severely reduced production of klotho in human chronic renal failure kidney. Biochem Biophys Res. Commun 280:1015-1020.

Mitobe, M., Yoshida, T., Sugiura, H., Shirota, S., Tsuchiya, K., Nihei, H. 2005. Oxidative stress decreases klotho expression in a mouse kidney cell line. Nephron Exp. Nephrol. 101:67–74.

Ohyama, Y., Kurabayashi, M., Masuda, H., Nakamura, T., Aihara Y., Kaname ,T., Suga, T., Arai, M., Aizawa, H., Matsumura, Y., Kuro-o, M., Nabeshima, Y., Nagail, R. 1998. Molecular cloning of rat klotho cDNA markedly decreased expression of klotho by acute inflammatory stress. Biochem. Biophys. Res. Commun. 251:920–925.

Ravel, R. 1984. Renal Function Tests. In: Clinical Laboratory Medicine: Clinical Application of Laboratory Data, Ravel, R. (Ed.). 4th Edn., Year Book Medical Publishers Inc., 129-140.

Saito, K., Ishizaka, N., Mitani, H., Ohno, M., Nagai, R. 2003. Iron chelation and a free radical scavenger suppress angiotensin II-induced down regulation of klotho, an anti-aging gene, in rat. FEBS Lett. 551:58–62.

Savic, V., Vlahovic, P., Djordjevic, V., Mitic-Zlatkovic, M., Avramovic, V., Stefanovic, V. 2002. Nephro-protective effects of pentoxifyl line in experimental myoglobinuric acute renal failure. Pathologie Biol. 50:599–607.

Shih, P., Yen, G.2007. Differential expressions of antioxidant status in aging rats: the role of transcriptional factor Nrf2 and MAPK signaling pathway. Biogerontol. 8:71–80.

Srikanta, J., Gagan, C., Jagneshwar, D. 2012. Expression of antioxidant genes in renal cortex of PTU-induced hypothyroid rats: effect of vitamin E and curcumin, Academic J. Molecul. Biol. Rep. 39:1193.

Sugiura, H., Yoshida, T., Tsuchiya, K., Mitobe, M., Nishimura, S., Shirota, S., Akiba, T., Nihei, H. 2005. Klotho reduces apoptosis in experimental ischaemic acute renal failure. Nephrol Dial Transplant 20:2636–2645.

Wiffinger, W.W., Mackey, K., Chomczynski, P. 1997. Effects of PH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotech. 22:474-481.

Zager, R. 1996. Rabdomyolysis and myoglobinuric acute renal failure. Kidney Int., 49:314-326.