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NON -THERMAL PLASMA APPLICATIONS: EFFECT OF ALKALINE WATER ON CALCIUM OXALATE STONES INDUCED IN MALE ALBINO RATS

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ABSTRACT

Background: Kidney stones are one of the most painful urologic disorders and have beset humans for centuries. About 75% of urinary stones are predominantly composed of calcium oxalate. The initial therapy for prevention of any type of kidney stones formation is increasing fluid intake. As suitable liquid intake affects both the urine concentration and frequency with which, solid micro particles are expelled from the urinary systems there is an ongoing search for alternatives to conventional liquids. Plasma-activated water (PAW), which, is produced by different types of non-thermal plasma devices, have recently attracted particular interest as a promising method for biomedical applications. This study investigates the effect of alkaline (PAW) as well as commercial alkaline water on the formation of calcium oxalate stones induced in albino rats. **Methods:** forty adult male albino rats, used in the present study, were divided into 4 equal groups; Normal (G1) drinking tap water only, the remaining 3groups received calcium oxalate inducing treatment for 4 weeks, comprised of (0.75ml v\v), ethylene glycol mixed with designated drinking water; i.e. group (G2) drink ethylene glycol mixed with tap water only and served as diseases control. Meanwhile, group (G3) Plasma-activated water and commercial alkaline water (G4) served as treatment groups, which received ethylene glycol mixed with alkaline (PAW) and commercial alkaline water respectively. After 4 weeks of the experiment, urine samples of all rats were collected. Subsequently, rats were sacrificed and blood samples were assembled for analysis of kidney and liver functions, as well as CBC and serum K. Furthermore, histological study for kidney tissue were carried out. **Results:** plasma-activated water prevent calcium oxalate stones formation, also, decrease adverse effects of ethylene glycol on liver functions compared with others groups. In conclusion, alkaline plasma-activated water has higher ability, rather than commercial alkaline water, to reduce the formation of calcium oxalate stones as well as protect liver from harmful effects of ethylene glycol, due to its alkalinity and unique properties.

KEYWORDS: Atmsphoric Plasma Jet, Plasma Activated Water (PAW), Alkaline Water, Calcium Oxalate Stones, Ethylene Glycol, Urine Analysis, Kidney Functions.

1. INTRODUCTION

Studies have reported the presence of kidney calculi in about a 7,000-year old Egyptian mummy.[1] Calcium oxalate is the most prevailing type of urinary stones. It take place in about 70–80% of the urinary stones populations. In the absence of medical therapy, kidney stones is a recurrent disease, with a propagation of 50% over ten years.[2] In most cases, the calculi are very small and can pass out of the body without any troubles. However, if a calculi blocks the flowing of urine, intensive pain may result, and instant medical treatment will be needed.[3] Unrestrained passage of calculi may be aided by rising pH, anti-calcifying activity and volume of urine. Which make equilibrium between the promoter and inhibitor of the crystallization in urine, that affects the nucleation, aggregation and growth of crystals, which known as (Crystallization inhibition activity).[4]

Several treatments have been used for ages to treat urinary stones. In almost all cases management of urinary stones involves surgical as well as medical approaches, i.e., antibiotics, percutaneous nephrolithotomy and extracorporeal shock wave lithotripsy.[5]

Yet, these therapeutic approaches are comparatively painful, expensive and require proficient hands with availability of suitable tools. Hence, it was important to search for other treatments showing anti-urolithiatic activity and without harmful effects.[6] Nutrition

treatments may have ability in decreasing formation and the repetition rate of renal stones without harmful effects. The best nutrition is intrinsically related to water. The guideline distinct between qualitative and quantitative components of water intake. The human water requirements as well as water quality for nutritional health pointing out mainly to the protection from the double burden of nutrition-linked diseases.[7] As a few water intake is an intensive risk factor for urinary stones formation, leading to a low urine volume and, consequently, higher urinary concentricity of lithogenic substances.[8]

All kind of drinks have this beneficial effect, except some sport drinks and few juices, because of their high content of carbohydrates as well as oxalate.[9] Water contains numerous inorganic nutrients, amongst these, calcium (Ca), magnesium (Mg) and sodium (Na), are the most substantial from a nutrition perspective, as water (rather than a food), is its main dietary source.[10] In view of above mentioned of inorganic nutrients in water, it can be anticipated that magnesium as well as pH can do a fine kinetic control on the crystallization and aggregation of calcium oxalate monohydrates crystals. As, magnesium could displace calcium ions that, affecting the dissolution of the calcium oxalate mono hydrates with a potential role played by pH.[11]

In the last decade, plasma-activated water (PAW), or most generally plasma-activated liquid (PAL) has received a lot of interest from both the plasma biomedical applications and agriculture field. PAW refers to the liquid that, contains reactive species, mostly reactive oxygen and nitrogen species (RONS) which, generated by the interaction of afterglow non-thermal plasma with the water. In physical field, "plasma" is the fourth state of matter; meanwhile in biology and medicine plasma refers to the non-cellular liquid component of blood. Interestingly, the term "plasma" has been reported by Irving Langmuir to emphasize that, the properties of ionic liquids, that ubiquitous in medicine as well as biology are analogues to plasma in the physical field.[12]

PAW was generated by using the atmospheric non-thermal plasma jet device under this study, which emitted different kinds of active species including (N, O, O_3) due to the carrier gas and air environments.[13] According to (Shaimaa et al., 2020), these active species play the main role to give PAW unique properties.

PAW has been found to have antimicrobial effects,[14,15] which is reported to occur due to the synergetic effects between RONS and/or pH of the liquid,[16] Plasma-activated buffered solution and media of cell culture have also been studied for therapeutically purposes,[17] and its potency in cancer treatment has been shown,[18] Also, in the field of agriculture with PAW, the plants growth as well as amelioration of seeds germination have been targeted.[19]

PAW, in addition, has been supplemented orally for albino rats with long periods (60 days) without harmful effects.[20] Based on recent findings of our studies, PAW has unique properties and does not have adverse effects on rats, even for long periods of time. Therefore, it is tempting to employ these advantages of PAW in different biomedical applications. One of these properties is the alkalinity of PAW.

PAW was generated in nuclear research center, Egyptian atomic energy authority, using atmospheric plasma jet operated with compressed air.[20] A number of studies reported *in vitro* applications,[21] while, evaluation of PAW impact on calcium oxalate stones formation *in vivo* are very rare. So, this study is designed to illustrate the effects of alkaline PAW as well as commercial alkaline water on calcium oxalate stones formations, kidney, liver as well as potassium and blood pH levels in albino rats.

2. MATERIAL AND METHODS

2.1 Experimental animals

Forty adult male albino rats weighing between 160-180g were used in this study. Animals were allowed for acclimatization 2 weeks under standard laboratory conditions, i.e.; room temperature of 28 ± 2°C,and a 12:12h light / dark cycle. Experiments were performed in accordance with the Committee for the Purpose of Control and Supervision of experimental animals. The study protocol was approved by the Institutional Animal Ethics Committee.

2.2 *Chemicals:* All chemicals were of high analytical grade and were obtained from different commercial sources include (Spinreact, Biomed, Spectrum and Diamond, from their branches in Cairo, Egypt).

2.3 The Atmospheric Non-Thermal Plasma Jet (ANPJ) Device that used in this study

The ANPJ device under consideration has been constructed and operated in the Plasma and Nuclear Fusion Dept., Nuclear Research Center, Egyptian Atomic Energy Authority to initiate first step for research in the field of biomedical applications fig.(1).

Fig. (1): A photograph of ANPJ device setup, PAW generation and rats drinking different types of water.

2.4 Setup the optimum condition for ANPJ water treatment

Non-thermal atmospheric plasma jet was generated using an experimental setup similar to the one previously described by.[22] The feed Gas is air of flow rate 12 L/min, and discharge was generated by applying alternating voltage of 6 kV (peak to peak) from a 20 Hz power supply. The distance between the cathode surface and the water sample surface was fixed at 2 cm using a special holder. Corresponding exposure plasma dose is estimated to be (0.425 J/cm^2).[23]

2.5 Water sources

A- Plasma activated water (PAW) was obtained by exposing tap water to the 0.7 mm-length non-thermal plasma jet for 10 minutes according to the findings reported elsewhere (Shaimaa et al., 2020).

B- Commercial alkaline water is supplied from the commercially available bottled alkaline water.

C-pH Value: pH electrode is used to measure the pH value within a liquid.[24]

2.6. Induction of calcium oxalate stones

Water that was mixed with ethylene glycol (0.75% v/v), was drank to all studied groups (see next subsection) except normal group (NG) for induction of calcium oxalate stones till the 4 weeks.[25]

2.7. Experimental Groups

This study was designed to evaluate the effect of alkaline PAW and commercial alkaline water on calcium oxalate stones formation in male albino rats. Therefore, the rats were divided in to equal four groups.

Group 1: Negative control group, healthy and untreated rats served as a normal control and maintained on regular rat food and drinking tap water only.

Group 2: Ethylene glycol group, rats were drunk tap water mixed with ethylene glycol (0.75 ml v\ v).

Group 3: Plasma activated water group (PAW), rats were drunk PAW water mixed with ethylene glycol (0.75 ml v\ v).

Group 4: Alkaline water group, rats were drunk commercial alkaline water mixed with ethylene glycol (0.75 ml v\ v).

Rats drank water for 4 weeks, then urine samples were collected. After that, rats were sacrificed and blood samples as well as, kidney tissue were taken to biochemical and histological analysis respectively.

2.8 Samples collection

2. 8.1 Urine collection and analysis

All animals were kept in individual metabolic cages and 24 h urine samples were collected on 29th day. Rats had free access to drink water during the urine collection period. Urine samples were used for microscopic analysis.

2.8.2 Blood sampling

Two ml of blood was collected into fresh vials containing anticoagulant and the rest of blood was left to coagulate and serum was separated by centrifugation at2000 rpm for 3 min.

2.9 Biochemical Analysis

Kidney function includes urea & creatinine were analyzed by using diagnostic kit methods. Meanwhile, K level, and pH of blood, were measured by using arterial blood gases **(ABG Roch analyzer),** to evaluate impact of PAW and commercial alkaline water on formation of calcium oxalate stones in all groups. Moreover, liver function includes (ALT, AST, Alkaline phosphatase (Alkaline ph.) albumin and Total protein) were detected by using diagnostic kit methods, to evaluate the effect of both PAW and commercial alkaline water against ethylene glycol adverse effects.

2.10 Histopathological study

To emphasize the presence of urolithiasis, rats were sacrificed and their kidneys were isolated and used for histopathological studies. The kidneys were washed and after that fixed with 10 % formalin (pH 7.4), then soaked in paraffin, cut at 5µm intervals and the slides were stained with hematoxylin and eosin stains. Slides of tissue were photographed using optical microscopy and observed the pathological changes, then images were taken at (H&E X200).[26]

2.11. Statistical analysis

Data of the present study were statistically evaluated by use of one-way ANOVA, followed by post hoc Duncan's test using version 16 of SPSS software. The values were considered significant when P< 0.05.[27]

3. RESULTS

The data of this study showed significant increase in alkalinity of tap water after exposure to plasma jet to generate of PAW, as PAW pH (8) compared with tap water only pH (7.65). Meanwhile the pH of commercial water was (7.9) as shown in fig. (2). Also, Ethylene glycol administration in a dose of 0.75%v/v in experimental animals induced significant changes in urine, biochemical parameter as well as histopathology analysis which will be covered in detail.

3.1. Effect of ethylene glycol in calcium oxalate deposition on urine

The microscopic examination of urine samples for all groups under this study showed that, significant presence of calcium oxalate mono hydrates crystals in ethylene glycol group "G2" compared with normal "G1" and PAW "G3" groups, which showed completely absent of crystals. Meanwhile in commercial alkaline water group "G4" there were few crystals as shown in fig. (3).

3.2. Effect of ethylene glycol in kidney functions, potassium and blood pH.

Data of this study showed, a significant increase in creatinine and urea levels in ethylene glycol group (G2) by percent of change 124% and 138% respectively, compared with negative control group (G1). While, showed a significant decrease in urea and creatinine in (G3) by percent of change -45% and -48% respectively, compared with (G2). Whereas, in (G4), a slight decrease in urea and creatinine is shown compared with (G2). This study also, showed that, there are significant increase in K level in (G2) by percent of change7.8% compared with (G1). While, there is a significant decrease in K level in (G3) by 5.2% compared with (G2). Moreover, there is a significant decrease in blood pH in G2 compared with G1, while, a significant increase in pH in G3 is shown, compared with G2 as shown in table (1).

3.3 Impact of ethylene glycol in liver functions

Data of present study postulated that, ethylene glycol has harmful effect on liver enzymes as there were significant increasing in level of (Alt, AST and Alkaline. Ph.), by percent of change 55, 53 and18% respectively, compared with normal group G1. Moreover, there is significant decrease in total protein in ethylene glycol group G2 by -11% compared with normal control group. Meanwhile, there are significant decrease in liver enzymes (ALT, AST and Alkaline Ph.) in G3 group by -16.6, -16 and-18% respectively and G4 by -7.14, -9.3 and -12.5% respectively compared with ethylene glycol G2 group.

In addition, there was significant increase in total protein in both G3 and G4 by 6.3% and 7.9 % compared with G2, as shown in table (2).

3.4. Effect of different treatments on histological study of kidney tissue

The histological examinations of kidney tissues with Hematoxylin and Eosin stains in the different studied groups confirmed the biochemical study in all studied groups, as shown in fig.(3). Normal group, "A" Kidneys showing normal renal histology; note the normal renal glomeruli and the normal renal tubules, While in "B", ethylene glycol group kidneys showing massive degenerative changes in the renal glomeruli. Whereas, In both "C" PAW as well as, "D" commercial alkaline water groups, kidneys showing healthy renal glomeruli (thick arrows)as well as healthy renal tubules (thin arrows) Whereas, In both "C" PAW as well as, "D" commercial alkaline water groups, kidneys showing healthy renal glomeruli as well as healthy renal tubules than control positive group "G2" (H&E X200).

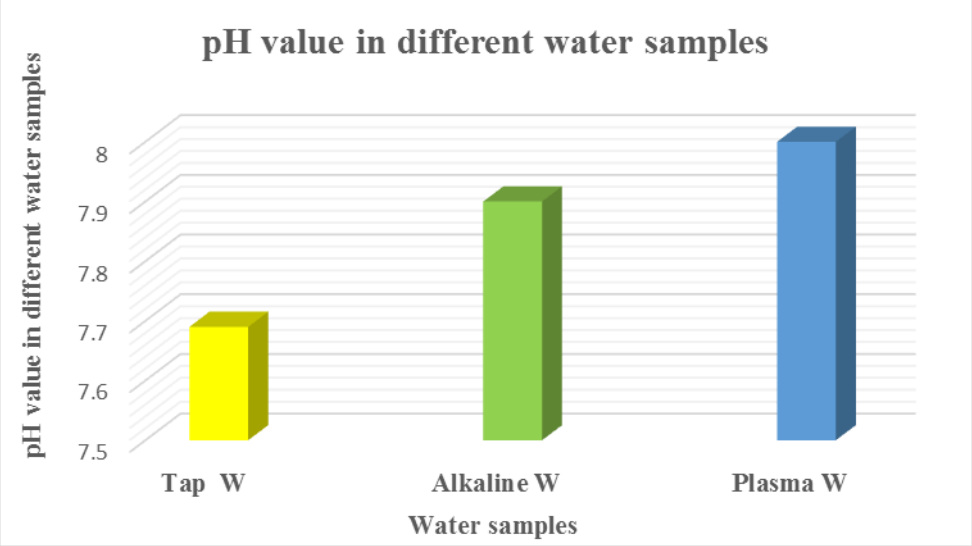

Fig. (2): showing pH value of different water samples.

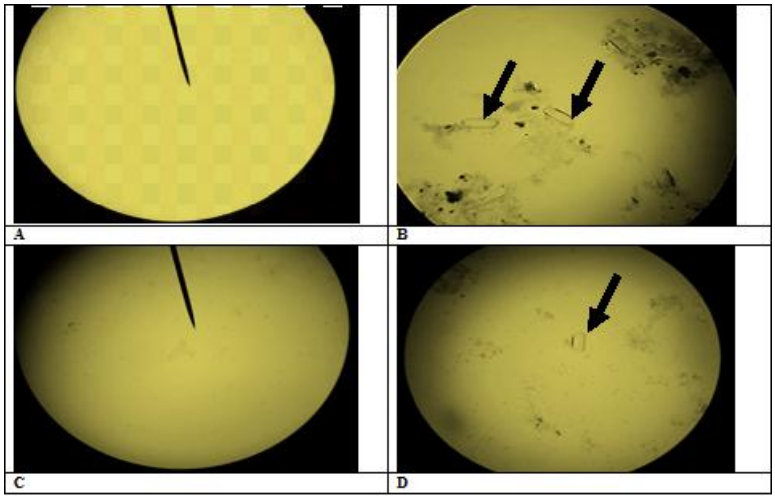

Fig. 3: (A, B, C and D) showing urine analysis in all groups, in group (1) normal group, fig (3A) showing clear urine sample. Meanwhile, in ethylene glycol (G2), fig. (3B), showing calcium oxalate mono hydrates crystals deposition and aggregation of oxalate. While in (G3) fig. (3C), there is clear urine sample. However, in (G4), fig. (3D), there was few calcium oxalate mono hydrates compared with (G2) as shown in fig. (3 B).

Table (1): Effect of different treatments on kidney functions, potassium and blood pH in all groups.

Groups		Urea(mg/dl)	Creat.(mg/dl)	K mmol/L	Blood pH
Control "G1"group	Mean ±SD	24.5 ± 2	0.63±0.08	7.05 ± **0** .04	7.34 ±0.05
	%change	-----	-----	-----	-----
Ethylene glycol group "G2"	Mean ±SD	55±3$^{\#\#}$	1.5 ±0.08$^{\#\#}$	7.6 ±0.07$^{\#}$	7.21 ±0.6$^{\#}$
	%change	124.5 %	138%	7.8 %	-1.7 %
Plasma activated water+ Ethylene glycol "G3"	Mean ±SD	30 ±5 **	0.78± 0.07**	7.2 ±0.05*	7.53 ±0.14 *
	%change	-45%	- 48%	-5.2 %	4.4%
Alkaline water + Ethylene glycol Group "G4"	Mean ±SD	42 ± 3**	1. 2 ± 0.12*	7.4 ±**0**.02*	7.39 ±0.04
	%change	-23.6 %	-20 %	-2.6%	2.4%

Numerical data were expressed as mean ± SD. Positive control group (G2) is compared with normal control group (G1) (#), alkaline plasma activated water (G3) and commercial alkaline water (G4) groups compared with positive control group (G2) (*). P value < 0.05 **considered significant. Percent of change is the harmful effects of ethylene glycol on (G2) compared with (G1), as well as the protective effect of PAW (G3) and commercial alkaline water (G4) against ethylene glycol harmful effects compared with (G2).**

Table (2): Effects of different treatments on Liver functions in all groups.

Groups		Total P. "g/dl"	Albumin "g/dl"	Alk. Ph (U/l)	AST (U/l)	ALT (U/l)
Control "G1"	Mean ±SD	7.1±**0.5**	4.3±**0.2**	74±**3**	28±**3**	27±**1.6**
	%change	-----	-----	-----		
Ethylene glycol "G2"	Mean ±SD	6.3±**0.1**$^{\#\#}$	3.95±**0.1**	88±**2**$^{\#}$	43±**1.5**$^{\#\#}$	42±**3**$^{\#\#}$
	%change	-11.2%	-8.3%	18.9%	53.5%	55%
Plasma activated water+ Ethylene glycol "G3"	Mean ±SD	6.7±**0.3***	4±**0.4**	72±**2****	36±**2***	35±**1.4***
	%change	6.3%	1.2%	-18%	-16.2 %	-16.6 %
Alkaline water + Ethylene glycol "G4"	Mean ±SD	6.8±**0.2***	3.9±**0.3**	77±**1.7** *	39±**1.4***	39±**1.42***
	%change	7.9%	-1.2%	-12.5%	-9.3%	-7.14%

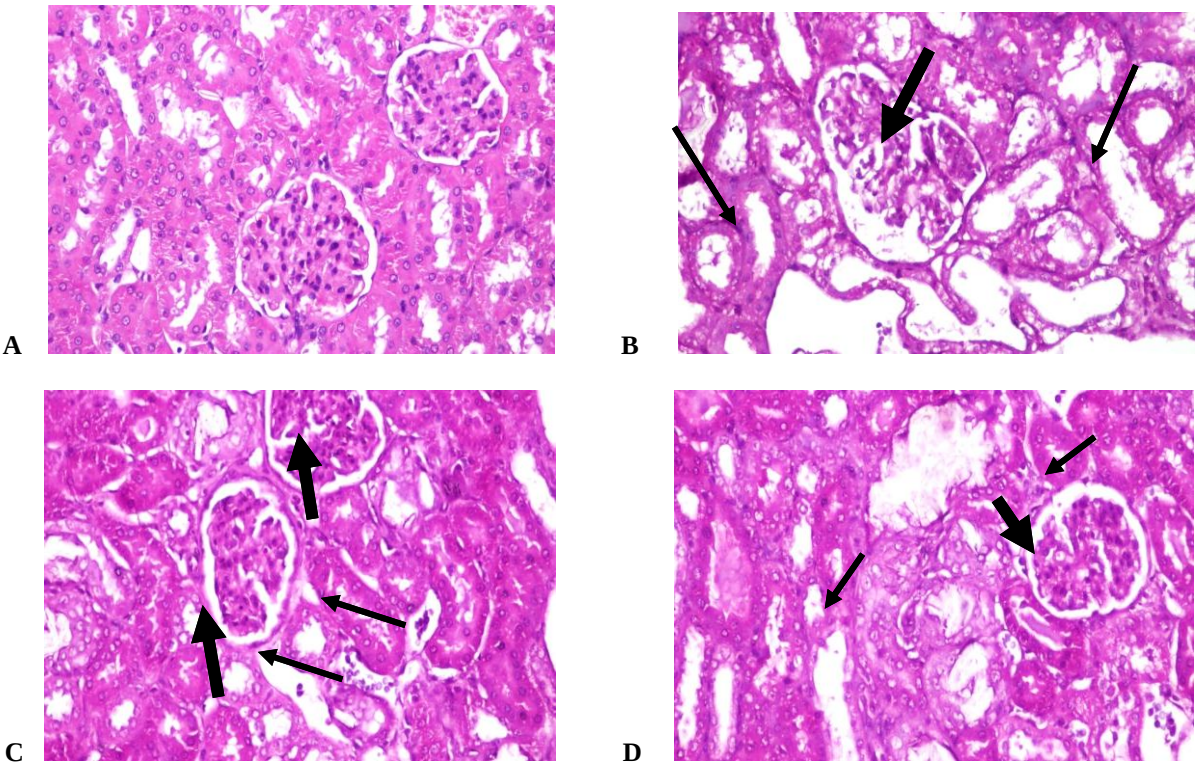

Fig. 4: (A, B, C and D) showing kidney histopatholigical study, as "A" normal group, Kidneys showing normal renal histology; note the normal renal glomeruli) and the normal renal tubules. Commercial alkaline water+ ethylene glycol group "G4" Kidneys showing massive degenerative changes in the renal glomeruli. In both "C" PAW and "D" commercial alkaline groups, kidneys showing healthy renal glomeruli (thick arrows) and healthy renal tubules (thin arrows), than control positive group "B", (H&E X200).

4. DISCUSSION

Previous studies showed that, human can survive without food for a month, but they can survive without water for only seven days.[28] Because water have vital importance to public health as water is not only a food in itself, but also a defined chemical molecule, which comprise a core nutrient essential for health as well as survival.[29] Also, water is a basic need and is the major player in carrying out all biological functions in the living body.[30] All types of water have special advantages over others with variety range of pH, electrolytes as well as minerals.[31] In addition, because pH can affect the level of metal corrosion and disinfection efficiency, hence, it may have indirect effects on biological functions. Although pH usually has no direct effect on water consumers, but it is one of the main important operational water-quality parameters. Moreover, accurate attention to pH control is important at all stages of water treatment to assure satisfactory water disinfection as well as clarification. The optimum pH will be variable in different supplies, as it is related to the water composition, but is often in the range 6.5–9.5.[32] On the other hand, alkaline water is drinkable and recommended in the marketing literature as being useful in different treatments such as, gastro-intestinal problems, hypertension, diabetes as well as cancer.[33] As a response, the present study deals with effect of alkaline plasma-activated water and commercial alkaline water on inhibition or decrease of calcium oxalate stones, as well as the extent of its ability against the harmful effects of ethylene glycol on liver functions.

Results of the present study showed that, tap water has significant increase in pH value after exposure to plasma jet for ten minutes to generate PAW compared with tap water without exposure to plasma as (8 and 7.65) respectively, as shown in fig. (2). According to shaimaa et al., 2020, it this may be attributed to the conversion of H_2O_2 which generated from exposure of tap water to plasma jet into OH in water according to the equation (1): $H_2O_2 + h\nu \rightarrow OH\bullet + OH$.[34] Also, the present study showed that ethylene glycol lead to deposition of calcium oxalate crystals in urine of G2. It is true that calculi formation in Ethylene Glycol intake animals is due to hyperoxaluria that, causes increased renal retention as well as excretion of oxalate as explained elsewhere.[35] As the mode of EG action is that: oxalic acid is one of the major metabolites of ethylene glycol, and can bind-with calcium in renal tubular epithelium to form a precipitate of Calcium oxalate (Caox). Moreover, impaired in kidney functions is noticed via a significant increase in levels of creatinine and urea as well as potassium that detected in G2. It may be attributed to administration of ethylene glycol, as acute overdose lead to a renal failure which, is linked with the presence and accumulations of calcium oxalate stones in the kidney tissue as suggested by.[36] The biochemical mechanisms for these processes are related to calcium oxalate stones

formation in G2, and renal tubule degeneration is believed to happen as the calcium oxalate crystals can either cause biochemical damage to cells and/or physical obstructions to renal tubules with concomitant interstitial inflammation and stimulation of oxidative stress, which exacerbates the toxicity by impairing renal urodynamic and hemodynamics.[37] Moreover, Potassium (K), levels were elevated in G2 compared to other groups, it may be as a result of, a membrane Na+ K+ ATPase, that plays a major role in active transport of Na+ and K+ ions via the plasma membrane.[38] The enzyme Na+K+ ATPase utilizes the energy derived from ATP hydrolysis to pump out Na+ from inside the cell to transfer K+ from outside to cytosol. In this study, Na+ K+ ATPase activity may be markedly decreased in kidney and liver of ethylene glycol induced calcium oxalate in rats as a result to increased oxalate formation, as oxalate is known to interfere with a broad spectrum of solute transport processes in the renal tubule and the reduction of Na+ K+ ATPase activity.[39]

Meanwhile, in case of PAW there is a protective effect against calcium oxalate stones formation. This was clear by the absence of deposition of calcium oxalate crystal in urine of rats. Also, a significant decrease in Urea and Creatinine levels as well as potassium are detected in PAW and commercial alkaline water compared with G2. It may be related to the alkalinity of PAW and Commercial alkaline water, as the alkaline water may play important role against calcium oxalate formation according to,[40] who showed that, magnesium could replace calcium ions affecting the dissolving of the later with a possible role played by alkaline pH. In addition, this may be related to presence of dissolved oxygen in PAW (Shaimaa et al., 2020), which has anti-inflammatory effects, according to,[41] who showed that oxygen nano-bubble water (ONB) fundamentally affected kidney stones formation (Calcium oxalate) in the kidney of rat by decreasing renal tubular cell injury. Hence ONB water is an essential prophylactic agent for calcium oxalate stones. Moreover, in G2 there were significant adverse effects on rats liver, it is scientifically recognized that, any drug administered will exert its action at the hepatic level,[42] In case of ethylene glycol, Oxalate lead to peroxidation of membrane lipids as well as oxidation of proteins, which, initiates the loss of membrane integrity and activities of liver membrane bound enzymes.[43] But both PAW and commercially alkaline water groups showed some protection of liver functions compared with G2. It may be related to, the Alkalinity of drinking water that, plays a major role in ridding the body of toxins,[44] Also, the Alkalinity of water decrease liver cirrhosis according to,[33] who showed that improvement of liver cirrhosis and hepatitis in patient drinking electrolyzed reduced alkaline water due to the water's alkalinity.

Conclusions and future work

Favorable health effects of rats could be obtained by consumption of both PAW and commercial alkaline water. As, the findings of this study indicate that ingesting alkaline water especially (PAW), proved to be effective as anti-urolithiasis (calcium oxalate mono hydrates) stones formation as well as to reduce adverse effects of ethylene glycol in liver, and may have many other beneficial effects. Hence, it is, somehow, necessary to speak about conducting numerous researches to find out the ambiguities in PAW properties including (affectivity, safety, viability and economic). Also, it is of importance to find out how to benefit from these ambiguities in medical fields.

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Compliance with ethical standards

Conflict of interest, all authors declare that they have no conflict of Interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Also, all procedures performed in studies were in accordance with the ethical standards of the institutional and national research committee.

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