JJBS

Volume 16, Number 4,December 2023
ISSN 1995-6673
Pages 641 – 647
https://doi.org/10.54319/jjbs/160409

Jordan Journal of Biological Sciences

Renal Calculi Composition in Alkaptonuria: Insights on Etiology

Nesrin Riad Mwafi [1,2*], Amjad Asri Al-Tarawneh[3], Ibrahim Naji Tarawneh [4], Mohannad Fayiz Alqedrh [5], Ahmad Ibrahim Alsbou' [6],Lekaa Ja'far Al Mughrabi F [6], Ali Mohammad Khlaifat[7], Muhamad Odeh Al-Limoun [8],Khaled Mohammad khleifat[8]

[1] Department of Biochemistry and Molecular Biology, Faculty of Medicine, Mutah University, Al-karak 61710, Jordan; [2] Faculty of Allied Medical Sciences, Mutah University, Al-karak 61710, Jordan; [3] Prince Faisal Center for Dead Sea, Environmental and Energy Research, Mutah University, Al-karak 61710, Jordan; [4]Department of Chemistry, Faculty of Science, Al-Balqa Applied University, Al-Salt 19117, Jordan; [5]Department of general surgery, Albasheer hospital, Amman 11151, Jordan; [6] Faculty of medicine, Mutah University, Al-karak 61710, Jordan; [7] Department of Nursing, Faculty of Prince Aysha for Applied Health and Nursing, Al-Hussein Bin Talal University, Ma'an 71111, Jordan; [8]Department of Biological Sciences, Faculty of Science, Mutah University, Al-karak 61710, Jordan.

Received: October 5, 2022; Revised: April 6, 2023; Accepted: April 12, 2023

Abstract

Alkaptonuria is a rare genetic disease caused by defect in the phenylalanine and tyrosine metabolism due to a deficiency in homogentisate 1,2-dioxygenase enzyme. The formation of ochronotic brownish-black pigment is the hallmark of the disease and at later stage AKU patients complain of recurrent renal calculi formation. In this report, we describe the case of 48 years old man who is previously diagnosed with alkaptonuria and is suffering from recurrent renal stones formation. We analysed the chemical composition of his AKU-stones by Fourier transform infrared spectroscopy (FTIR) and Inductively coupled plasma-mass spectrometry (ICP-MS) respectively and compared them with non-AKU stones obtained from 48 years old non-AKU man. Our results showed that sulphur content was 33 folds higher in AKU stones compared to non-AKU sample. This provides an evidence that sulphur-rich protein is a critical component for stone formation and growth from calcium oxalate monohydrate (COM) crystals. Accordingly, a model was proposed for the formation and growth of ochronotic renal stones in AKU patients with protein stimulated COM aggregation based on the generation of adducts between benzoquinone acetate and sulphur-rich proteins. Overall, the outcomes of this study shed a new light on the disease pathogenesis at the molecular level and opened new perspective which might orient the physicians to find a convenient therapeutic intervention or at least prophylactic measures to avoid recurrent formation of calculi in AKU patients.

Keywords: Alkaptonuria, Homogentisate 1,2-dioxygenase enzyme, Ochronosis, Renal stones.

1. Introduction

Alkaptonuria (AKU), also called the black bone disease, was originally defined as an inborn error of metabolism by Garrod in 1902(Garrod, 1902; Phornphutkul *et al*., 2002). It is an autosomal metabolic disease inherited in a recessive mode and characterized by generation and accumulation of homogentisic acid (HGA)in the blood and urine due to abnormal metabolism of phenylalanine and tyrosine(Alsbou and Mwafi, 2013; Mwafi *et al*., 2021b). This happens due to mutations inhomogentisate 1,2-dioxygenase(*HGD*) gene located on the longarm of chromosome 3 and subsequent deficiency in functional HGD enzyme(Khalil *et al*., 2021; Mwafi *et al*., 2021a). Although AKU is globally classified as a rare disease (incidence of 1:250,000), its prevalence is considered high in certain ethnicity such as Slovakian, Dominican, Indian and Jordanian population(Alsbou and Mwafi, 2013; Al-Shagahin *et al*., 2019).

The bluish-black discoloration of the connective tissues, a phenomenon called ochronosis, is the hallmark of the disease which occurs due to the deposition of the oxidized polymeric products of homogentisic acid in tendons, ligaments,heart valves, sclera, auricular and articular cartilages, bone of large joints and intervertebral discs(Albatayneh *et al*., 2019). Consequently, multiple degenerative changes, inflammation and calcificationwill occur resulting in severe spondyloarthropathy and osteoarthritis(Wu, 2018). Rarely patients are recognised in early childhood due to diaper black staining and the disease diagnosis remains delayed until the patient is refereed to orthopaedic clinic complaining of severe low backache, pain in the knee, hip and/or shoulder joints(da Silva Martins Ferreira *et al*., 2014; Mirzashahi, 2016). These symptoms usually start in the third or fourth decades of life depending on the degree of the disease severity score(Koh*et al*., 1994). As a general rule, the disease is suspected in the presence of the triad of: brownish-black turning of urine if left standing or upon alkalinization, ochronosis and extensive degenerative changes and arthritis in the large joints and spine of young adults(Raina *et al*., 2008; Doganavsargil*et al*., 2015; Gil *et al*., 2016). In later stages of the disease, AKU patients complain of recurrent kidney stones formation with the function of kidneys being conserved (Wolff *et al*., 2015 ; Al-Tarawneh

* Corresponding author. e-mail: drnesrin@mutah.edu.jo.

et al., 2023).Few studies reported the chemical composition of AKUrenal calculi and the analysis of stones with efficient physical methods such as ICP-MS are poorly documented in the literature. The aim of this study is to investigate the composition of renal stones collected from AKU patient and compare them with renal stones obtained from non-AKU patient. The calculi were analysed using FTIR and ICP-MS techniques. Our results afford insight into stone etiology and pathogenesis in AKU patients and suggest a critical role of sulphur-containing proteins in the growth and formation of kidney stones in AKU compared to non-AKU patient.

2. Materials and Methods

2.1. Sample collection

Renal stones were obtained from AKU (48 years male) and non-AKU (48 years male) patientsafter subjection to the process of lithotripsy in the King Hussein Medical City. Both patients were matched in gender and age. Informed consents were obtained from patients and the study was approved by the Research Ethics Committee in faculty of medicine/ Mutah University. The study protocol was in accordance with the Declaration of Helsinki and its contemporary amendments. AKU patient was diagnosed based on family history and clinical examination. The black urine was confirmed by overnight standing of urine sample and by black ring formation upon addition of few drops of 10% $FeCl_3$ solution to fresh urine sample collected from the AKU patient (Figure 1, C-H). Moreover, the existence of homogentisic acid was confirmed by measuring urinary homogentisic acid level (1.2g/24h) using gas chromatography-mass spectrometry (GC-MS) analysis.

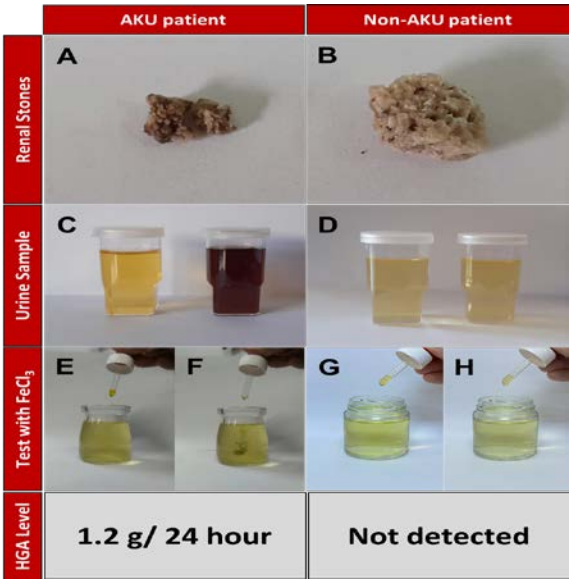

Figure 1. The black colour of renal stones and urine sample collected from AKU patient compared to non-AKU patient. (A) COM category of AKU calculi with budding appearance compared to (B) COD crystals with beige colour. (C) In AKU patient, the urine turns black in colour on 24-48 hours of standing (D) but the colour is not changed in non-AKU urine. (E) upon addition of few drops of 10% $FeCl_3$ solution to fresh AKU urine sample, (F) a black ring will appear due to the oxidation of HGA, (G,H) a phenomenon which is not detected in non-AKU sample. HGA level measured by GC-MS was detected only in AKU urine sample.

2.2. FTIR spectroscopy

Fourier transform infrared spectroscopy (FTIR) was performed using Bruker FTIR spectrometer (Bruker, Bremen-Germany) equipped with OPUS 7.5 program. The spectra were acquired in the middle IR range (i.e. 400 to 4000 cm^{-1}). The concentration of potassium bromide (KBr) was in the range of 0.2-1% of the sample through the preparation process of the pellets. The dried FTIR grade potassium bromide was transferred from the oven into a mortar in which 1-2 % of the powdered AKU, and non-AKU samples were mixed separately and again grounded into a fine powder. Finally, the pellet of the homogenous sample and potassium bromide were obtained. The pellets were placed in the transmission holder in front of the IR beam. Finally, the AKU and non-AKU renal stone components were evaluated according to the absorbed wavelength.

2.3. Elemental analysis using ICP-MS

One ml of external standard (Bismuth 200 ppm) was added to 0.1 g of each sample into teflon vials. The samples were digested by concentrated HNO_3, HCl, and HF with mixing ratio of 2:6:2 (v/v) respectively for 5 min at 75 ^oC, 10 min at 100 ^oC, 10 min at 120 ^oC, 10 min at 150 ^oC, and 20 min at 200 ^oC using hotplate. The samples were filtered with syringe filters of 0.45µm pore size (Sartorius, Göttingen/Germany). The filtrate was diluted to 50 ml with 2% nitric acid and then analyzed for elements by means of inductively coupled plasma-massspectrometry abbreviated as ICP-MS (ELAN 9000, Perkin-Elmer SCIEX).

2.4. Kidney stones analysis

The stones were analyzed by kidney stones kit "URISTONE3" (Ben Biochemical Enterprise, Italy)for semiquantitative colorimetric determination of the chemical class of the collected calculi. Initially, the stones were crushed in a mortar. The well mixed powder was transferred to a sample tube. Five drops of Reagent 1 (H_2SO_4) were added to dissolve the powder. Then, 10 ml distilled water was added. After gentle mixing, the sample was transferred to 50 ml tube and distilled water is added up to the mark of 50 ml. The sample was distributed in 7 test tubes, and the colorimetric reaction was performed according to the instructions of URISTONE3 kit.

3. Results

3.1. Renal stones morphology and colour

In the current study, renal calculi were collected from AKU and non-AKU patients. It was observed that there are differences both in colour and morphology between AKU and non-AKU kidney stones (Figure 1, A &B). AKU stones are dark brown to black in colour due to the presence of the ochronotic pigment supposed to be composed of the oxidation product of HGA. However, non-AKU renal stones featured brighter colour ranging from white to beige. Surprisingly, chemical analysis of stones by URISTONE3 kit revealed that both are majorly composed of calcium oxalate salt. The non-AKUrenal stone is classified as IIabased on the classification previously reported in literature (Daudon *et al*., 2008a ; Daudon *et al*., 2016a). However, the dissimilarity in the morphology could be related to the other components in

stone composition as well as the mechanism involved in the stone formation. Therefore, the stones were analysed by means of physical methods namely FTIR spectroscopy and ICP-MS for elemental analysis to identify the nature of chemical constituents and to semi-quantify their respective proportion within the calculi.

3.2. FTIR spectroscopy

Fourier transform infrared spectroscopy represents a conventional analytical technique from which information about the composition of renal stones of AKU patient could be achieved rapidly. Infrared spectra give information about kind of functional groups involved in the structural formula rather than defining the exact chemical composition. Samples of AKU and non-AKU renal stones were analysed using FTIR spectrum, in which the band assignment was done according to peer-reviewed papers (George, 2004). Figure 2(A & B) shows the recorded mid-infrared spectra of AKU and non-AKU samples, respectively. The two main observed differences between AKU and non-AKU FTIR transmittance spectra are the downstream peak shift from 1644 cm^{-1} to1621cm^{-1} and the shape and strength of peak detected around 3477 cm^{-1}.

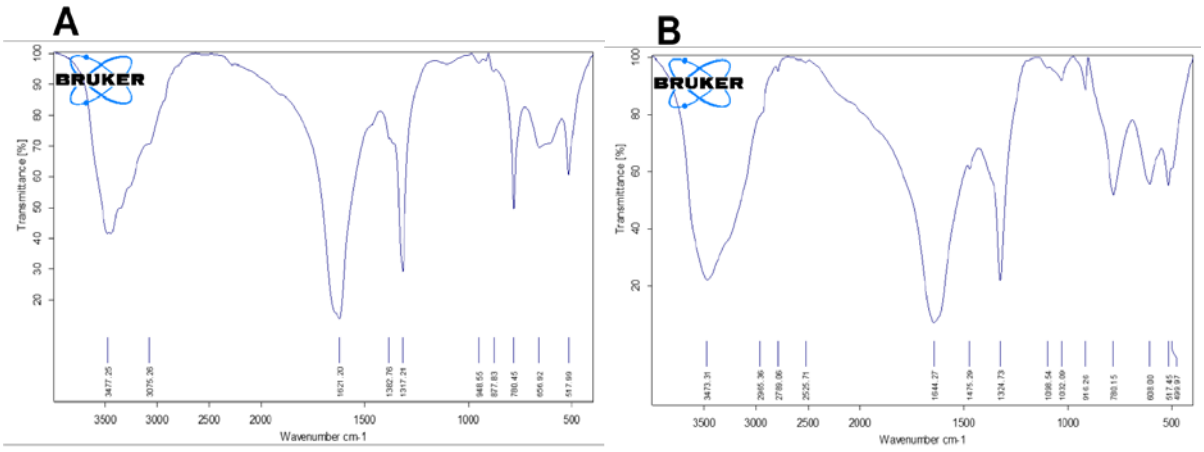

Figure 2. FTIR transmittance spectrum for AKU (A) and non-AKU (B) renal stones. (A) The peak appeared at 3477 cm^{-1} with transmittance of 40% and two spikes is indicative of N-H bond and the second distinctive peak at 1621cm^{-1} indicates Amide I rich with β-Sheet aggregates. (B) The peak appeared at 3473 cm^{-1} is strong (with transmittance of 20%) broad, smooth and it is indicative of alcoholic O-H bond. Moreover, there is an upstream shift in the second distinctive peak from 1621cm^{-1} to 1644 cm^{-1} indicates less β-Sheet aggregates.

3.3. Elemental analysis by ICP-MS

To measure the trace level of elements (range: ppm = parts per million) found in biological samples such as kidney stones, inductively coupled plasma-mass-spectrometry (ICP-MS) was used. ICP-MS is rapid and sensitive technique with minimal elemental interference. It is a suitable method for elemental analysis of very small sample size such as renal stones. The sample was prepared according to the standard protocol of solid ICP-MS sample preparation. After digestion, the sample was converted to aerosol and transported directly to the plasma with inert gas. The results are illustrated in Table 1 and Figure 3. The concentrations of twelve elements in AKU and non-AKU renal stone samples were expressed in ppm (horizontal line) and the relative amount in percentage (vertical line) as depicted in Figure 3. Additionally, the ratio of each element to the total amount was calculated in both types of renal stone samples (Table 1). The obtained results showed a marked difference of approximately 33 times in sulphur content between AKU and non-AKU samples.

Table 1. Ratio of elements in AKU and non-AKU renal stones calculated by dividing each element concentration on total elements quantity.

Element Name	non-AKU renal stones		AKU renal stones	
	Element Conc. [Mean+SD]	Element ratio [%]	Element Conc. [Mean+SD]	Element ratio [%]
Ca	304485 ± 44333	88.35	26836 ± 3907	79.12
Fe	785 ± 85	0.23	56 ± 6	0.17
K	294 ± 27	0.085	281 ± 26	0.83
Mg	1210 ± 190	0.35	293 ± 46	0.87
Na	1974 ± 177	0.57	783 ± 70	2.31
P	34421 ± 4027	9.99	2877 ± 337	8.49
S	836 ± 94	0.24	2678 ± 300	7.90
Zn	589 ± 59	0.17	45 ± 4	0.13
Ba	3 ± 0.3	0.0009	1 ± 0.1	0.003
Cr	3 ± 0.5	0.0009	2 ± 0.3	0.006
Pb	9 ± 0.6	0.003	4 ± 0.3	0.012
Ti	23 ± 2	0.007	28 ± 3	0.083
Total	344632	100%	33884	100%

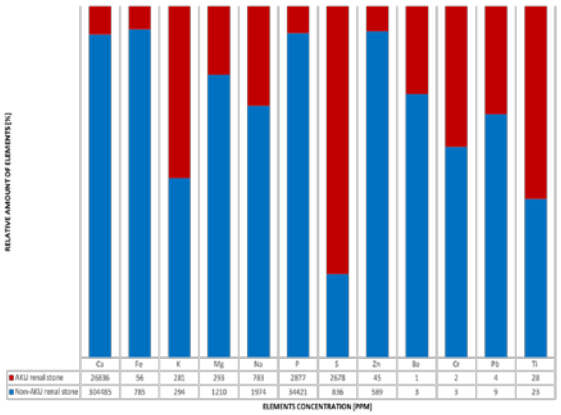

Figure 3.Comparison between the elements content in renal stones of AKU and non-AKU patients using ICP-MS method of analysis

4. Discussion

The comparison between AKU and non-AKU renal stones reveals differences both in color and morphology. In fact, 80% of renal stones are primarily composed of calcium oxalate crystals of two main types: calcium oxalate monohydrate (COM) known as whewellite and calcium oxalate dihydrate (COD) known as weddellite (Zhang *et al*., 2021). Studies reported that COM stones are formed in hyperoxaluria condition and when calcium to oxalate ratio is low(Giordani *et al*., 2003; Trinchieri *et al*., 2005). On the other hand, hypercalciuria and higher calcium to oxalate ratio are predominately associated with COD class of calcium oxalate stones(Bazin *et al*., 2016; Daudon *et al*., 2016b). Our results demonstrated that AKU and non-AKU stones fall under the categories of COM and COD crystals, respectively. AKU stones are distinguished with the budding appearance and dark-brown rough surface. The dark color can be explained due to the incorporation of HGA-oxidized pigments within the stones during their growth and formation process. Several studies stated that the color of the stones generally reflects the lithogenic activity of the disease in which light colour stones are generated when crystallization process is very active(Daudon *et al*., 2008a;Daudon *et al*., 2008b). Consequently, the dark colour of AKU stones indicates that the lithogenic mechanism is slow and intermittent in AKU patients. This is in agreement with the mechanism implicated in the development and appearance of AKU symptoms as it is accumulative process highly dependent on the build-up of HGA level in various tissues of human body (Zatkova *et al*., 2020). In the study of evaluation of factors that affect COM and COD fragmented calculi regrowth, Costa-Bauzá and his co-authors found that COM and COD are formed under pH conditions of 5.5 and 6.5 respectively (Costa-Bauzá *et al*., 2006). In AKU patients, homogentisic acid is normally excreted in the urine by renal glomerular filtration and tubular secretion leading to slightly acidic urine in AKU patients with average pH = 5.6 (Tokuhara *et al*., 2018; Ranganath *et al*.,2020).In addition to the stone colour and morphology, AKU acidic urine condition provides another evidence that AKU renal stones fall under the category of COM crystals compared to COD stones formed in non-AKU calculi.

To identify the chemical composition of renal stones, FTIR was performed, and the obtained peaks were compared to infrared spectroscopy correlation tables as functional groups give different peaks in different substances(George, 2004). Regarding the fingerprint region of IR spectrum located between 500 and 1500 cm^{-1}, both AKU and non-AKU stones exhibit partial similarity and matching in some peaks. It is known that fingerprint region of certain compound is unique and can be used to distinguish it from other compounds. Additionally, AKU-renal stone spectrum displays a lower intensity at 517 and 780 cm^{-1} (O-C=O bending) compared to the healthy renal stone. This was in agreement with the study of Bazin*et al*., 2016 who found a difference between IR spectrum of COM and COD crystals of renal stones(Bazin *et al*., 2016). The authors reported differences in peaks strength and shape at the region of 517-780 cm^{-1} with COD spectrum at this region being similar to our non-AKU spectrum and COM spectrum being identical to our AKU spectrum (Bazin *et al*., 2016). These results additionally confirm the previous morphology findings in which AKU and non-AKU stones were classified as COM and COD crystals, respectively.

The discrete formation of these bands is important for distinguishing AKU from non-AKU renal stones.

However, few information can be extracted from fingerprint region to differentiate between the types of functional groups in the two kinds of renal stones included in this study. Therefore, the diagnostic region located between 1500-4000 cm^{-1} is more informative. In this study, the most important spectral characteristic has been chosen to give a good distinguishing bands between the two different categories of stones. In fact, the five bands between 3477 – 3047 cm^{-1} and the band at 1380 cm^{-1},which are due to symmetric and asymmetric OH, stretch and out of plane OH bending respectively, are not found in a healthy renal stone spectrum (Figure 2). This finding could be explained by the presence of higher amounts of HGA and its oxidized products in AKU sample. In general, by comparing the peaks in Figure 2, two preliminary information are extracted. Firstly, in non-AKU sample, the peak at 3470 cm^{-1} is smooth, broad and strong (20% transmittance). This peak is indicative of alcoholic OH. On the other hand, similar peak appears in AKU sample, but it is broad with two spikes and weaker than that appeared in non-AKU sample (40% transmittance). This peak is typical for amide N-H group rather than alcoholic O-H group. Secondly, there was a downstream shift in the peak appearing at wave number of 1644 cm^{-1} in non-AKU to wave number of 1621 cm^{-1} in AKU sample. This downstream shift is indicative of Amide I and representative for protein secondary structure(Barth, 2007). As a matter of fact, the downshift from 1644 (Figure 2 B) to 1621 cm^{-1} (Figure 2 A) indicates the presence of more β-Sheet aggregates in AKU-renal stone (Giorgini *et al.*, 2010 ; Miller *et al.*, 2013; Boulet-Audet *et al*, 2014). β-Sheets consist of extended polypeptide strands (β-strands) connected by a network of hydrogen bonds and occur widely in proteins. Although the importance of β-sheets in the folded structures of proteins has long been recognized, there is a growing recognition of the importance of intermolecular interactions among β-sheets. Intermolecular interactions between the hydrogen-bonding edges of β-sheets constitute a fundamental form of biomolecular recognition and are involved in protein quaternary structure, protein-protein interactions, and peptide and protein aggregation. The importance of β-sheet interactions in biological processes makes them potential targets for intervention in diseases such as AIDS, cancer, Alzheimer's (Nowick, 2008; Miller *et al*., 2013) and could be for AKU disease.

However, AKU renal stones are expected to be rich with protein which is precipitated and participated in the growth and formation of AKU renal stones. To get more evidence of this suspicion, elemental analysis was performed for both samples using ICP-MS technique.

Our results detected a marked difference in sulphur content which is 33 folds higher in AKU stones compared to non-AKU sample. Similarly, Millucci *et al*., 2014 reported the presence of sulphur in ochronotic cardiac valve tissue obtained from AKU patient compared to non-AKU sample (Millucci *et al*., 2014). This finding strongly supports our result and suggests sulphur as one of several components of AKU ochronotic pigment. Although ochronosis or the black discoloration of tissues is the hallmark of AKU, the nature of this pigment and the exact mechanism implicated in the dark colour appearance of biological samples (e.g. ligament, tendon, bone, cartilage, renal stones, etc) obtained from AKU patients are still ambiguous(Braconi *et al*., 2015).Preliminary studies suggested the spontaneous oxidation of homogentisic acid (HGA) into 1,4-benzoquinone-2-acetic acid (BQA) followed by polymerization and subsequent formation of melanin-like pigment (Zannoni *et al*., 1969;Hegedus and Nayak 1994). It was found that BQA is highly reactive metabolite and can form adducts with compounds containing free sulfhydryl or amino groups(Lustberg *et al*., 1971) such as cysteine thiols and lysine amines; the nucleophilic sites on proteins (Fisher *et al*., 2014; Li *et al*., 2005). Indeed, BQA can bind single amino acids as well as whole protein (Morrison *et al*., 1969). Moreover, BQA-protein binding induces structural and functional modification of the bound proteins enhancing their aggregation and subsequent precipitation, a mechanism proposed to contribute to ochronotic pigment formation in AKU patients (Nicolis *et al*., 2013; Millucci *et al*., 2014). Our finding is in agreement with Wolff *et al*., 2015 who detected high protein content among the chemical composition of AKU renal stones analysed by X-ray diffraction(Wolff *et al*., 2015)and Ranganath *et al*., 2019 who proposed the presence of HGA-bound protein in the ochronotic pigment deposited in tissues (Ranganath *et al*., 2019).

Our data recognised a quite similar ratio of calcium and phosphate both in AKU and non-AKU samples. This can be justified because ochronotic pigment is known to induce damage of connective tissues (e.g. cartilages, joints, bone) and enhance bone resorption process (Wolff *et al*., 2015) Consequently, higher amounts of calcium and phosphate will be excreted in the renal filtrate of AKU patients. In addition, our results reported higher percentages of Na^+ and K^+ elements in AKU compared to non-AKU samples. Accordingly, our study suggests a stone formation model in AKU patients based on the precipitation of HGA as Na^+ and K^+ salts in the kidney followed by auto-oxidation to the corresponding BQA molecules which subsequently co-precipitate with free cysteine amino acids, cysteine-containing proteins or any free sulfhydryl containing compounds found in the renal system and resulting in the formation and development of renal stones (see Figure 4). This model is strongly supported by finding that AKU stones are highly enriched in sulphur (33 folds).

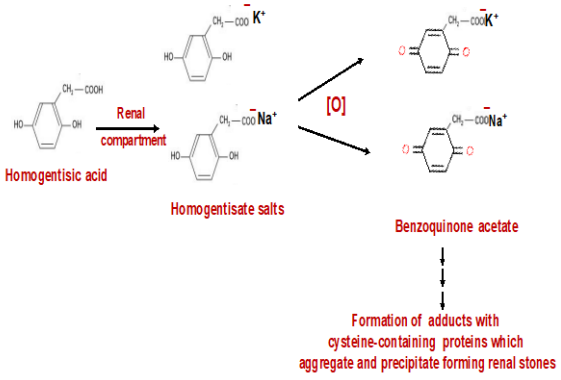

Figure 4. The proposed mechanism for the formation of ochronotic renal stones in AKU patients. The homogentisate salts in renal compartment undergo spontaneous oxidation to the corresponding benzoquinone acetate which can bind sulphur containing proteins resulting in precipitation of the modified adducts and subsequent formation of stones.

5. Conclusion

The identification of ochronotic pigment constituents is challenging, and further work is required to understand how does circulating HGA in AKU patients can interact with biological molecules to uncover the exact molecular mechanism underlying renal stones formation. Our findings show that sulphur-rich proteins are major components of AKU renal stones. However, knowing the composition of the calculus is fundamental because the nature of AKU stones in fact helps the physicians to find a convenient therapeutic interventions or at least guide the development of more effective stone preventive measures to avoid recurrent formation of calculi. Further studies are needed to understand the influence of sulphur-containing proteins on calcium oxalate (COM) stone formation and growth in AKU patients.

Acknowledgments

This study was Funded by the Deanship of Scientific Research, Mutah University,Al-Karak 61710, Jordan. Grant Number (422/2021).

Competing interests

The authors declare no competing interests.

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