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Renal tissue toxicity and miRNA 21, miRNA122, and miRNA221 tissue level alterations in rats administered 4-Nitroquinoline

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Abstract

A widespread variety of toxicants modify miRNAs profiles in target tissues. Five rat treatment groups were selected for this study, each consisting of five animals and an additional one left untreated used as a control. Administration of the carcinogen 4-Nitroquinoline (4-NQO) lasted for 5 months with four weeks intervals separating the successive groups. When the carcinogen intake period ended, the animals were euthanized and renal tissue was collected for both histopathological and molecular investigations. The results showed no significant difference (p=0.65) between the animal groups that showed kidney tissue toxicity and those that did not. Conversely, a statistically significant difference emerged if the mean ΔCt of the gene of interest (miRNA-21) in the treated group (6.95±1.23) was compared to that of the control group (4.42±0.34) (p=0.000). A significant difference was also observed when the comparison involved miRNA-122 and miRNA-221 whose ΔCt values for the treated and control groups were 2.28±0.2, 1.19±0.51 (p=0.04);0.39±0.08,1.52±0.06 (p=0.02), respectively. In conclusion: The findings of the present work revealed variable degrees of renal tissue injury despite non-significant; but it emphasizes the significant alterations of the miRNA tissue levels, the latter can indicate the adoption of specific miRNAs as vital markers denoting the prediction and/or the diagnosis of tissue injury after exposure to certain chemicals.

Keywords: miRNA 21, miRNA122, miRNA221, 4 nitroquinoline, carcinogenesis

سمية النسيج الكلوي وتغيير مستويات الاحماض النووية الرايبوزية الدقيقة (miRNA-21, miRNA-122, miRNA-221) في النسيج لفئران جرعت بمادة 4-Nitroquinoline

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الخلاصة

تؤدي أنواع واسعة الانتشار من المواد السامة الى تغيير في تعبير الاحماض النووية الرايبوزية الدقيقة في انسجتها المستهدفة. أجريت هذه الدراسة على خمسة مجاميع علاجية من الفئران وكل مجموعة تألفت من خمسة فئران بالإضافة الى فأر سادس ترك بدون معالجة لاستخدامه كمجموعة سيطرة. تم تجريع مادة 4-

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Nitroquinoline المسرطنة لمدة خمسة اشهر بواقع تجريع كل أربعة أسابيع. وعند انتهاء فترة إعطاء المادة المسرطنة تم اماتة الحيوانات بطريقة رحيمة وتم جمع النسيج الكلوي لأجراء الفحوصات النسيجية المرضية والفحوصات الجزيئية عليه. أظهرت النتائج عدم وجود فروق معنوية ($p=0.65$) بين مجاميع الحيوانات التي أظهرت تسمم النسيج الكلوي وبين المجاميع التي لم تظهر ذلك. وعلى العكس فقد أظهرت النتائج فروقا" معنوية بين المجاميع عند حساب ΔCt لجين miRNA−21 في المجاميع المعالجة ومقارنتها مع مجاميع السيطرة. كذلك لوحظ وجود فرق معنوي عند حساب ΔCt ومقارنتها لمستويات miRNA−122 و miRNA− 221 . وكأستنتاج من نتائج الدراسة الحالية فقد وجدت درجات مختلفة من التلف النسيجي وبرغم كونه غير معنوي لكنه يؤكد التغييرات المعنوية في مستويات الاحماض النووية الرايبوزية الدقيقة miRNAs في الانسجة والذي قد يكشف تبني لبعض miRNAs كمعلمات مهمة للغاية للدلالة على التنبؤ وتشخيص تلف الانسجة بعد التعرض لبعض المواد الكيميائية.

Introduction

MicroRNAs (miRNAs) are tiny non-coding RNAs which play a key role in regulating biological activities in plants and animals. They exert their action by adjusting the expression of genes at the posttranscriptional level by attaching to messenger RNAs and either preventing protein synthesis or altering the messenger RNA stability. Due to the outstanding biological contribution of miRNAs, it is of prodigious awareness to investigate their cellular expression level. Moreover, miRNAs have been linked with a group of illnesses and cancers [1] and the expression of miRNA can aid in the diagnosis and prognosis of mankind's illnesses [2,3].

Toxicant-caused alterations in miRNA expression reveal indicators of the appraisal of toxic properties on various organs and body systems. Hence, miRNAs are well-thought-out to be prognostic indicators or biomarkers of tissue damage due to exposure to toxicants [4]. As miRNAs manipulate the expression of mRNA, their changed transcriptional levels are supportive of clarifying and outlining contrary consequent paths of definite toxic materials [5].

4-NQO is a synthetic molecule derivative of quinolone, whose carcinogenic properties were initially defined by a team of researchers [6]. 4-NQO is well-thought-out a tobacco-like carcinogenic substance that stimulates intracellular oxidative stress, leading to the formation of reactive oxygen species (ROS), products of metabolism which attach to DNA and result in extensive damage [7]. Consequently, 4-NQO is a carcinogenic chemical defined as a DNA-reactive genotoxin [8]. Its carcinogenic and mutagenic attributes elucidate why it is usually utilized in numerous genotoxicity analyses [9].

Even though most investigational information using 4-NQO both topically and in drinking water has all together specified no significant general variation in the treated subjects [10], others revealed a drop in body weight improvement [11]. The outcomes of the study of general toxicity including 4-Nitroquinolin could benefit from notifying and regulating the associated features that might arise throughout the experimentation and can likewise add to the consideration of many of the modes of action of this chemical. Accordingly, the purpose of the current work was to explore the histopathological and molecular changes in renal tissues indicating systemic toxicity in rats orally administrated 4-NQO.

Materials and methods

The experiment was planned as prospective animal research. Animal exposure to the carcinogen (4-Nitroquinolone, 4-NQO) was performed at the animal house which belongs to the College of Sciences /University of Duhok. Histological preparation and examination of kidney tissue was done by a specialist. Thirty Albino Wistar rats aged 2 months were selected for the experiment. Animals were divided into five equal groups, each including 5 animals and an additional one as a control. Their weights ranged from 190 to 250 gm at the beginning of the work. The carcinogen powder was dissolved in Dimethyl Sulfoxide (DMSO) to obtain

a final concentration of 50 µg/mL corresponded to 50 ppm given to the animals ad-libitum by drinking water and according to the specified time period. The rats were assigned into five treatment groups, each group consisted of 5 animals for a treatment plus one left untreated as control. The total number of animals was 30 divided into five treatment categories. Animals were divided into five equal groups, each including 5 animals and an additional one as a control. They were given the carcinogen in a final concentration of 50 ppm. The first group has drenched with the carcinogen for 4 weeks and then euthanized, and the second group was given the carcinogen for 8 weeks and then sacrificed, the drenching regimen continues separated by the same 4 weeks lapse period between the groups to the end of the experiment culminating in a total of 20 weeks drenching time. The uptake of the carcinogen continued for five months and animals were sacrificed followed by kidney tissue collection. From each euthanized animal, two kidney tissue samples were obtained. One sample was soaked in formalin and stored for histopathological investigation; the other sample was transferred to an Eppendorf tube containing 0.5 ml TRIzol™ and stored at -20 ^{o}C for later extraction of RNA. The steps of tissue processing and staining (Hematoxylin and eosin**)** strictly followed former literature. Tissues of approximately 2 mm in thickness were processed over one night, using the automatic processor machine (Leica), then stained with Hematoxylin and eosin, and assessed by the microscope [12].

Molecular investigation

Total RNA was extracted from renal tissue samples (20-30 mg). Extraction proceeded by using the TRIzol® LS Reagent according to the procedure provided by the manufacturing company.

Micro-RNA Quantitation by Qubit 4.0.

The method is extremely discriminatory for miRNA over other RNA types and precise for the primary range of concentrations from 10 pg/µL to 100 ng/µL.

Synthesis of the complementary DNA (cDNA)

The primers were obtained from Macrogen® (S. Korea). Table 1 indicates the single-stranded primers used for the synthesis of the cDNAs corresponding to the particular miRNA, namely miRNA-21, miRNA-122, miRNA-221, and U6 which was used as a housekeeping gene.

Table 1-Primers utilized for the synthesis of cDNAs

Name of Primer	Sequence	Reference
miR-21	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA ^{RT}	(Yu et al., 2017) [13]
miR-122	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCAAACAC ^{RT}	(Wang et al., 2018) [14]
miR-221	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGAAACCC ^{RT}	(Gong and Gong, 2018) [15]
U6	CGCTTCACGAATTTGCGTGTCAT ^{RT}	(Wang et al., 2018) [14]

Complementary DNA was synthesized by utilizing total RNA as a template and the use of ProtoScript® First Strand cDNA Synthesis Kit according to the instructions of the manufacturing company. The following program was applied for the generation of cDNAs (Table 2).

Table 2- Complementary DNA synthesis program

Step	Time (minutes)	Temperature (°C)
Hold	5	25
Hold	60	42
Hold	20	65
Hold	∞	4

Real time-quantitative PCR (RT-qPCR)

The resultant cDNA were used as templates for the amplification by quantitative-real time PCR (qRT-PCR). Four primes' sets (Forward and reverse) were used for the amplification of the cDNA of interest (Table 3).

Table 3-Primers used for the amplification of DNA in qRT-PCR

Primers	Sequence	Reference
miR-21	F 3´ CGGCTAGCTTATCAGACTGA 5´ R 3´ GTGCAGGGTCCGAGGT 5´	(Yu et al., 2017) [13]
miR-122	F 3´ TGCGGTTGGAGTGTGACAATGG 5´ R 3´CAGTGCAGGGTCCGAGGT 5´	(Wang et al., 2018) [14]
miR-221	F 3´ GGAGCTACATTGTCTGCTGG 5´ R 3´ CAGTGCGTGTCGTGGAGT 5´	(Gong and Gong, 2018) [15]
U6	F 3´ CTCGCTTCGGCAGCACA 5´ R 3´AACGCTTCACGAATTTGCGT 5´	(Wang et al., 2018) [14]

The reaction components with their relevant volumes are demonstrated in Table 4.

Table 4-The reaction components of qRT-PCR

Component	20 ul Reaction
Forward primer (10 µM)	1ul
Reverse primer (10 µM)	1ul
Template DNA	5ul
Luna Universal qPCR Master Mix	10 ul
Nuclease-free Water	3ul

The qRT-PCR reaction conditions were programmed as shown in Table 5. The analysis of the resulting amplification figures was performed as per Livak formula [16].

Table 5-Running conditions for the qRT-PCR

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95°C	60 seconds	1
Denaturation Extension	95°C 60°C	15 seconds 30 seconds (+plate read)	40-45
Melt Curve	60°-95°C	40 minutes	1

SPSS (IBM Corporation, New York, NY, USA) statistical package (Version 22.0) was used to analyse the results' Data.

Results

Histopathological sectioning and staining of kidney tissues with H and E revealed the presence of the so-called acute tubular necrosis as an indication of tissue toxicity, all the five

treated groups showed varying degrees of this sign (Figure 2). Figure 1 shows the normal histology of the rat kidney.

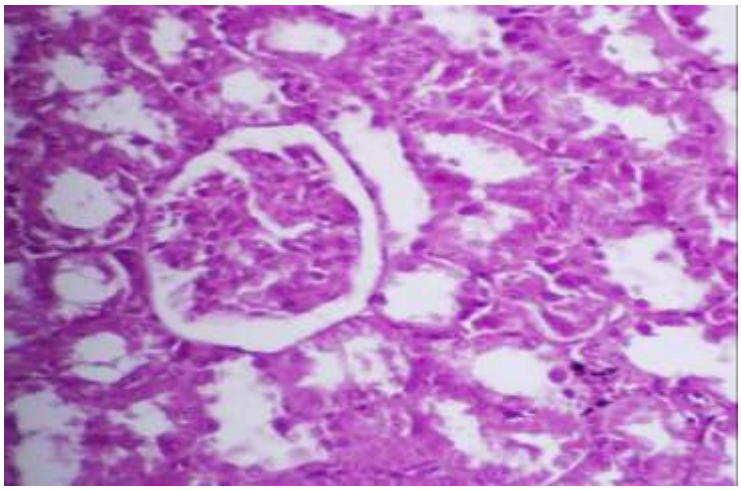

Figure 1-Normal Kidney histology in the control group (H & E at 40x).

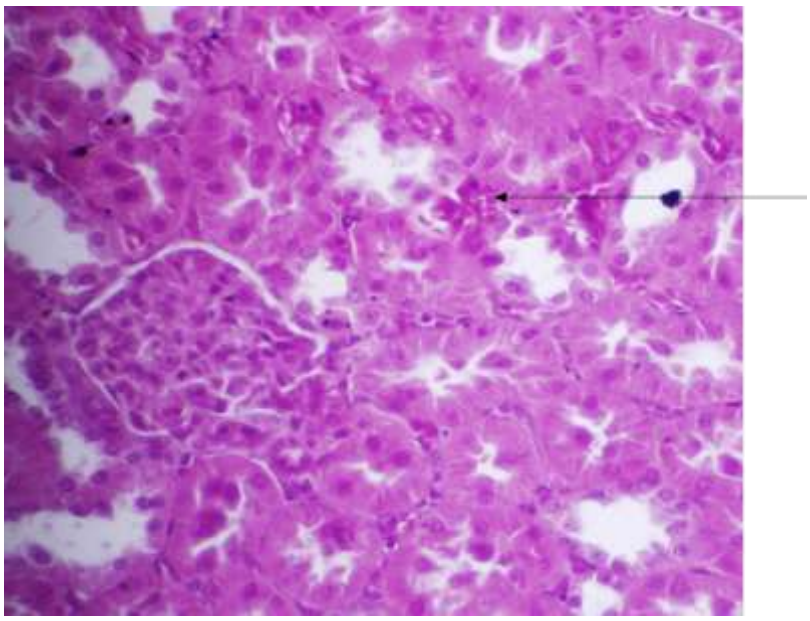

Figure 2- Histopathologic specimen of a rat kidney presenting ~~an~~ acute tubular necrosis. The arrow indicates the sloughing of the epithelial lining of the renal tubules away from the basement membrane. (H & E at 40x).

The result of the current investigation showed no significant difference when the groups showing tissue toxicity were compared with those who did not (Table 6).

Table 6-Tissue toxicity observed in the treatment groups

Tissue toxicity	Group 1	Group 2	Group 3	Group 4	Group 5	P value
Yes	3	3	2	4	4	0.65
No	2	2	3	1	1	

*Significant at $p \leq 0.05$

The results of the current study showed that the fold change of miRNA-21 was the highest in animal number eight (upregulated) which has been drenched for eight weeks in comparison

to the control. While it was the lowest (downregulated) in animal number two which has been drenched for four weeks only. Despite downregulation, the response was inconsistent in animals numbered 19, 20 and 25, 26; a Consistent downregulated response was observed in animals numbered 13 and 14 (Figure 3).

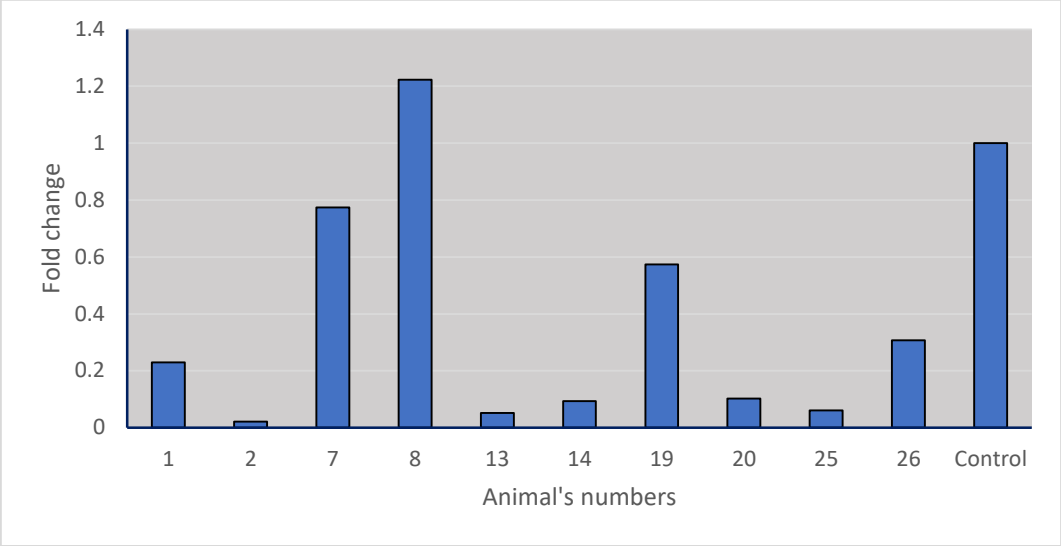

Figure 3-The fold changes are shown by the rat animals indicating miRNA-21 expressions

The highest fold change was reported for animal numbered eight treated with the carcinogen for eight weeks while the lowest fold change was observed in animal numbered two given the carcinogen for four weeks only. Inconsistent responses magnitude was noticed in all animal groups except for animals numbered 13 and 14who showed nearly the same level of downregulation. The aforementioned results concerned miRNA-122 (Figure 4)

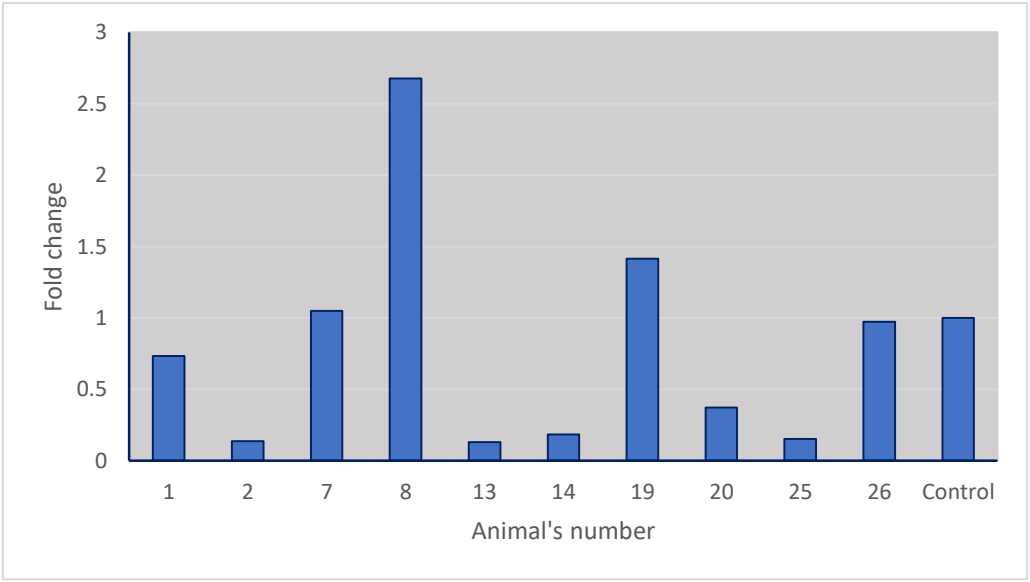

Figure 4-The magnitude of fold changes in the expression of miRNA-122.

The outcomes of the current investigation showed that the animal numbered eight, who has been drenched the 4-nitroquinoline for eight weeks, had the highest fold change followed by the animal numbered 19 consuming the carcinogen for 16 consecutive weeks as far as

miRNA-221 was taken into consideration. Except for the above-mentioned two animals, the rest of the rats showed nearly a consistent downregulated response (Figure 5).

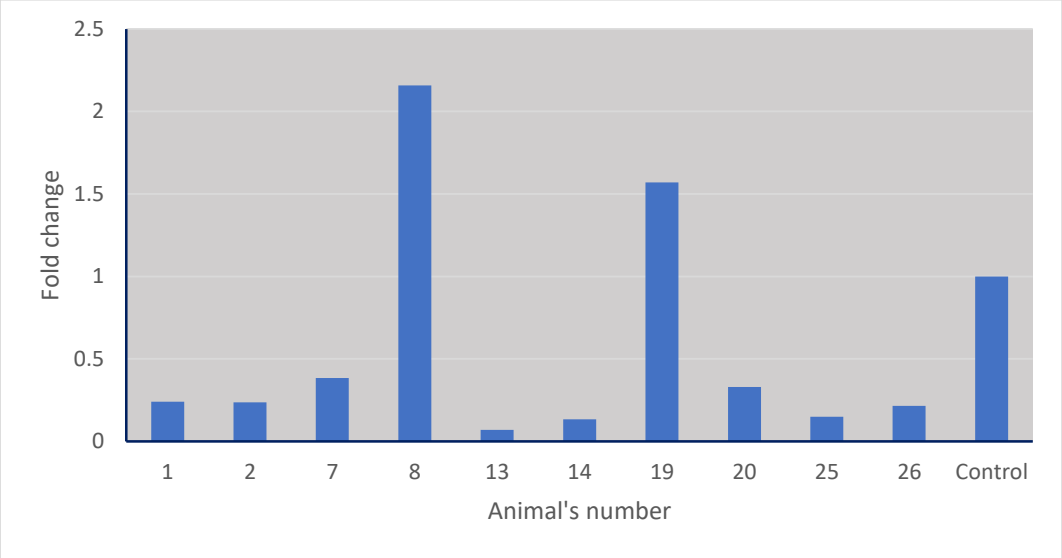

Figure 5-fold changes in the miRNA-221 expression by experimental animals

The mean ΔCt of the gene of interest (miRNA-21) in the treated group was 6.95±1.23 and differed significantly from that of the control group (4.42±0.34) (p=0.000). Significant difference was also attained when the comparison involved miRNA-122 and miRNA-221 whose ΔCt values for the treated and control groups were 2.28±0.2, 1.19±0.51; 0.39±0.08,1.52±0.06, respectively. The p-value was 0.04 for the comparison of miRNA-122 while it was 0.02 when miRNA-221 test results were considered (Table 7).

Table 7-The comparison of mean ΔCt values for the miRNAs of interest

miRNA	Mean ΔCt treated±SD	Mean ΔCt control±SD	p-value
miRNA-21	**6.95±1.23**	**4.42±0.34**	0.000*
miRNA-122	**2.28±0.2**	**1.19±0.51**	0.04*
miRNA-221	**0.39±0.08**	**1.52±0.06**	0.02*

*Significant at p≤0.05

Discussion

In the present work, the objective was to assess the toxic renal consequence of 4-NQO administration, in order to inspect probable renal involvement due to this chemical along with the induction of hypothetically malignant manifestations of the oral cavity.

The results of the current work were consistent with others who presented the histology of the kidneys of the rats. The histological investigation of rats' renal tissue in the control group exhibited regular renal characteristics when examined by a microscope. While, histological examination of renal tissue from 4-NQO administered rats revealed disintegration of the glomerulus, reduction of capsular distances, and mild distention of distal and proximal tubules [17]. Moreover, the present work results were also in line with what was previously stated, histo-morphological modifications were likewise noticed in the kidneys, principally in the distal tubular part [18].

New tissue damage biomarkers have lately been acknowledged that outperform or improve the value of the ordinary safety biomarkers. These novel biomarkers had improved specificity and/or sensitivity in investigating drug-associated tissue injury in diverse tissues, encompassing kidney, liver, and skeletal muscle. Amongst these novel biomarkers, microRNAs (miRNAs) are one sort principally that have gained plentiful consideration in recent years [19].

In the early 2000s, a serious remark was that some miRNAs occurred in a tissue-specific pattern. Notably, miR-122 was one of the initial to be recognized that was individually found within liver hepatocytes [20]. Investigation using targeted qPCR and sequencing methods in humans acknowledged numerous further candidates that existed only within a specified tissue, growing the list of tissue-associated miRNAs [21].

MiRNAs satisfy vital characteristics of diagnostic approaches for example i) non or slightly invasiveness for sample access, ii) standardized, economical quantitative, and robust investigation, iii) quick reversal of the procedure outcome and iv) generally as miRNAs offer a wide-ranging picture of the continuing physiologic functions in tissues and cells [22]. Additionally, miRNAs were noticed to pose tissue-specific expression patterns (Guo et al., 2014), and they are seldom vanished over an evolutionary period once attained and might thus show well-preserved roles among model organisms and human beings [23].

Synthesis of miRNAs is reactive to severe biological signals, regularly unveiling variations former to additional obvious pathophysiological fluctuations, and they can appear in dissimilar biological environments [24]. The existence of these molecules in body fluids changes and look, in certain situations, to follow thoroughly with devastation and healing of the disturbed tissue or cell, at the same time anther groups of miRNAs continue in spite of recovery signs [25]. In the present investigation, we preferred to demonstrate the occurrence of miRNA in the tissue of interest as far as its accessible because it will give better insight into what is happening in situ before attaining a biofluid in a detectable concentration.

The result of the present study supported the previous view that certain miRNA biomarkers can be exclusive to kidney tubular cells in experimental animals. For instance, gentamicin introduction works in rats recognized miRNAs miR-203, miR-320, and let-7d as possible biomarkers for the epithelial injury of renal tubular [26]. In the same context but with different miRNAs, the current study demonstrated that the alteration of miRNA-21, miRNA-122, and miRNA-221 was associated with the necrosis of tubules recognized by histopathology. Our findings also gain support from earlier research where miRNAs have been utilized to examine drug-induced injury [27].

The miRNA approaches can diverge from investigator to investigator, involving the extraction of total RNA from the biological specimens, the qPCR platform, and the normalization of the endpoint miRNA indicator. These systematic changes render it challenging to straightly compare data across independent research works and highlight the necessity for a reliable and standardized manner of miRNA assessments [19]. Our point of view is quite similar to the later comment which may explain different research outputs and/or the emphasis on inconsistent miRNAs within the same organ to refer to tissue toxicity.

Conclusion: miRNAs can be considered reliable tissue toxicity markers and their profile may be linked with conventional diagnostic methods to have a clear view of the ongoing processes inside the tissue.

Ethical clearance

This research was ethically approved by the Research Ethical Committees of the Ministry of Environmental and Health and the Ministry of Higher Education and Scientific Research, Iraq.

Conflict of interest:

The authors declare that they have no conflict of interest.

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