Genotoxic Effect of Arsenic Trioxide on Rats Treated with Alpha Lipoic Acid.

Khalil H. Aljeboori[1], Khalil G. chelab[2]
[1] college of dentistry, Al-iraqia university/Iraq
[2] college of veterinary medicine, Al-qadissiyah university, Iraq.

Abstract
This study was designed to identify and investigate the genotoxic effect of arsenic trioxide on the oncogenes cyclin D1 and p27 kip1 in treated rats with arsenic trioxide for this reason, three groups of rats, 2 weeks old, first treated with Arsenic trioxide (ATO) (1.5mg/ kg Bw), the second group treated with ATO and Alpha lipoic acid (ALA) (100 mg/ kg Bw) and 3rd group (control) treated with phosphate buffer saline (pbs) orally) and the experiment and treatment were continued for 3 monthes. The results showed that the 1st group received ATO demonstrated increase ($p \leq 0.05$) in cyclin D1 mRNA gene expression level (8.932926 ± 1.6916) as compared with control group (1±0) and a decrease in the expression level of p27 kip1 mRNA gene (0.167883 ±0.01620) compared with control. The 2nd group that received both ATO and ALA showed the opposite results a significant decreased in cyclin D1 m RNA gene expression level (4.135597 ± 0.6603) compared to the control group and an increase level ($p \leq 0.05$) of p27kip1 m RNA gene expression level (3.592233± 0.3266) compared with control group. Conclusion ATO have a genotoxic effect on rat tissue through increase expression level of cyclin D1 and decrease expression level of p27kip1 mRNA and the genotoxic effect of ATO was ameliorated by ALA with opposite result level of cyclin D1 and p27kip1.
Keywords: Arsenic trioxide, alpha lipoic acid genotoxicity.

1. Introduction

Arsenic is a carcinogen to human and both animals [(1)] and [(2)].
There are many different mechanisms of action by which arsenic cause cancer including genotoxicity cell proliferation altered DNA repair and DNA methylated oxidative stress [(3),] co- carcinogenesis and tumor promotion [(4).] the oxidative damage is considered to play an important role in arsenic carcinogenesis [(5)]. The genotoxic effect of arsenic trioxide can be considered as important mechanism by which arsenic induce tumor growth via the direct effect on the oncogenes that controlled the cell cycle proliferation [(6)] through increase cellular proliferation via decreasing $p27^{kip1}$ level and an increase level of cyclin D1 over expression in mouse mammary tumor [(7)] and chemical carcinogen induced mouse skin tumor [(8) (9)]. The important effect of Arsenic trioxide in living body, this study aimed at to investigate the genotoxic effect of arsenic trioxide on the oncogenes cyclin D1 and $p27^{kip1}$ mRNA in rats using quantitative real time- PCR (QTR- PCR).

2. Materials and methods

Three group of rats 8 week old, each group (15 rat) were divided into:
First group: Received 1.5mg/kg BWt) Arsenic trioxide orally and daily.
2nd group: received alpha lipoic Acid (100mg/kgBWt) and Arsenic trioxide (1.5 mg/kg BWt)orally.
3rd group: received phosphate buffer solinine (Pbs). The expression and treatment continued for 3months at the end of experiment all the animals were sacrificed and small pieces of liver tissues (0.25-0.5cm^3) dipped in liquid nitrogen (-196c) and then put in epindroff tubes contained DEPEC water and sent polymerase chain reaction unit for determination gene expression of cyclin D1 and $p27^{kip1}$ mRNA by quantitative Real time –PCR (QTR-PCR).
Quantitative Reverse Transcription Real – Time PCR:
Quantitative reverse transcription Real- Time PCR (technique) was performed for estimation of relative quantification (gene expression analysis) for cyclin D1 and $p27^{kip1}$ in the treatment and control groups.
Total RNA extraction
Total RNA were extracted from rat liver tissue by using (TRIzol® reagent kit) and done according to company instructions as following steps.

1- The tissue was homogenized by using micropestle in 1 ml of TRIzol® reagent.
2- 200µl chloroform was added to each tube and shaken vigorously for 15 seconds.
3- The mixture was incubated on ice for 5 minutes. Then centrifuged at 12000 rpm, 4C°, for 15 minutes.
4- Supernatant was transferred into a new epindorf tube, and 500ul isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 12,000 rpm, 4C° for 10 minutes.
5- Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4C° for 5 minutes.
6- The supernatant was discarded and the RNA pellet was left to air to dry.
7- 50µl DEPC water was added to each sample to dissolve the RNA pellet, Then, the extracted RNA sample was kept at -20.

Assessing RNA yield and quality

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The extracted total RNA was assessed and measurement by Nano drop spectrophotometer (THERMO. USA), There are two quality controls performed on extracted RNA. First one is to determine the quantity of RNA (ng/µl), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nano drop machine as follows:

1- After opening up the Nano drop software, chosen the appropriate application (Nucleic acid, RNA).
2- A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet lµl of ddH20 onto the surface of the lower measurement pedestal.
3- The sampling arm was lowered and clicking OK to initialized the Nano drop, then cleaning off the pedestals and 1µl of the appropriate blanking solution was added as blank solution which is same elution buffer of RNA samples.
4- After that, the pedestals are cleaned and pipet lul of RNA sample for measurement.
5- The purity of RNA, also determined by reading the absorbance in Nano drop spectrophotometer at 260 nm and 280 nm, so the RNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

DNase Treatment:

The extracted RNA were treated with DNase I enzyme to remove the amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by promega company, USA instructions as follow:

mix	Volume
Total RNA 100ng/ µl	10µl
DNase I enzyme	1µl
10X buffer	4 µl
DEPC water	5 µl
Total	20 µl

After that, The mixture was incubated at 37C° for 30 minutes. Then, lul (25mm) EDTA was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

cDNA synthesis

DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as following:

RT master mix	Volume
Total RNA 100ng/ µl	10µl
Random Hexamer primer	1µl
DEPC water	9 µl
Total	20 µl

This RT PreMix was placed in AccuPower RocketScript RT PreMix tubes that contains lyophilized Reverse transcription enzyme at form. Then dissolved completely by vortex and briefly spinning down.

The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Table (1): Shows the thermocycler steps to convert RNA to cDNA.		
Step	Temperature	Time
CDNA synthesis (RT step)	50Co	1 hour
Heat inactivation	95 Co	5 minutes

Quantitative Real-Time PCR (qPCR) master mix preparation:

qPCR master mix was prepared by using AccuPower™ Green Star Real-Time PCR kit that dependent syber green dye detection of gene amplification in Real-Time PCR system and include the follow:

qPCR master mix	Volume
cDNA template (100ng)	3µl
Forward primer(10pmol)	1µl
Reverse primer (10pmol)	1µl
DEPC water	15 µl
total	20 µl

After that, these qPCR master mix component that mentioned above Accopwer Green star qPCR premix standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in Exicycler Real-Time PCR system.

After that, the qPCR plate was loaded and the following thermocycler protocol in the following table (2):

Table (2): Shows the thermocycler protocol in Real-Time PCR system.			
qPCR	Temperature	Time	Repeat cycle
Initial Denaturation	95Co	5min	1
Denaturation	95 Co	20sec	45
Annealing\Extention Detection(scan)	55 Co	30sec	
melting	60-95 Co	0.5sec	1

Data analysis of qRT-PCR

The data results of a RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method described by [10]. The relative

quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a Reference Gene was used as following equations:

Gene	Test (treatment group)	Cal. Control group
Target gene	CT (target, test)	CT (target, cal)
Reference gene	CT (ref, test)	CT (ref, cal)

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

- ΔCT (calibrator) = CT (ref, calibrator) - CT (target, calibrator) Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:
- ΔCT (Test) = CT (ref, test) – CT (target, test)
- ΔΔCT =ΔCT (test) – ΔCT (calibrator)
- fold change = 2-ΔΔCT
- ratio (reference/target) = 2CT (reference) - CT (target), the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample

3. Results and Discussion

The results of this experiment revealed that the first group: that received (1.5 mg/kgBw) Arsenic trioxide (ATO), showed an increase level of expression of cyclin D1 m RNA ($P \leq 0.05$) 8.932926 ± 1.6919 compared to expression level in control (1±0), Table (3) whereas decrease level of expression of $p27^{kip1}$ m RNA in the same period of exposure (3months) to ATO (0.167883±0.01620) compared with control (1±0). (Table 3)

Table (3): showed the effect of arsenic trioxide and alpha lipoic acid treatment the gene expression of cyclin D1 and P27kip-1 in rat Livers:		
Stastistical reading	Mean ± se	Groups
a a	cyclidin D1 = 8.932926 ± 1.6919 P27kip1 = 0.167883 ± 0.01620	First group ATO alone
b b	cyclin D1 = 4.135597 ± 0.6603 P27kip-1 = 3.592233 ± 0.3226	2nd group ATO and ALA
c c	cyclin D1 = 1 ± 0 P27kip1 = 1 ± 0	3rd group Control group

The similar letters refers to non significant differences while the different letters refers to the significant differences at ($p \leq 0.05$) for ATO and ALA receiving groups.
The 2nd group that received ATO and alphalipoic acid (ALA) (100mg/kgBw) for 3 months showed that decrease level of cyclin D1 ($P \leq 0.05$) 4.135597 ± 0.6603 compared to the control group (1±0), table (3) But the 2nd oncogene $p27^{kip1}$ m RNA level showed increase ($p \leq 0.05$) 3.592233 ± 0.3266 compared to the control (1 ± 0.5) at the same time of exposure.
From the results of this experiment we demonstrated the important role of arsenic trioxide to induce the tumor and these results also provided with the histopathological lesions seen in liver sections through the initiation of dysplastic lesions in liver cells exposed to ATO which is transformed into tumor cells (hepatocellnlacarcinoma). This evidence of dysplastic changes in liver tissue agreed with other studies [(11) (12)] who showed the same effects due to amplification and over expression of the cyclin D1 gene which demonstrated with a variety of human cancers including esophageal tumors [(7)] in human and in mouse mammary tumors, and chemical carcinogen induce mouse skin tumor [(8) (9)]. Other studies showed that the arsenite exposed cells can exhibit an increased expression of the positive regulators for proliferation of (c- myc and E_2F-1) and a decreased expression of negative regulators of proliferation MAP kinase phosphates -1 and $P27^{kip1}$ [(4) (6)] provided that the arsenic trioxide can be cause cellular proliferation via decrease $P27^{kip1}$ level. Recent studies showed that inorganic arsenic exposure in drinking water of pregnant mouse result high incidence of hepatocellular carcinoma in the offspring when they reach the adulthood [(13)]. this experiment revealed that the liver is a potential target organ of arsenic carcinogenesis in both human and animal species, similar finding reported by [(14)].
This study also hypothesized that arsenic toxicity and carcinogenic which occur in liver section via histopathological examination may involve perturbation of the oncogenes which demonstrated in this study (cyclin D1 and $P27^{kip-1}$).
The gene expression of cyclin D1 m RNA decreased ($p \leq 0.05$), and increased gene expression $P27^{kip1}$ m RNA in the 2nd group received both ATO and alpha lipoic acid (ALA) togather compared to first group level of expression of both genes (cyclin D1 and $P27^{kip-1}$).
The reason of this variation in both first and 2nd groups indicate that protective effect of alpha lipoic acid against genotoxic effect of Arsenic trioxide to induce proliferative and carcinogenic effect opposite the anti-proliferation and ant carcinogenic effect of ALA through decreased expression level cyclin D1 and increase $P27^{kip-1}$ m RNA.
Similar results agreed with [(15)] who reported that the anti- neoplastic effect of ALA in number of systemic tumors [(15)] through increase expression level of $P27^{kip-1}$ and accentuated apoptosis effect in breast malignancy[(16)].

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