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(RESEARCH ARTICLE)

Effect of azacytidine administration with/without folic acid on the histology and genomic DNA methylation of mice liver

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Abstract

Azacitidine is a cytosine analog and antineoplastic agent used in the therapy of myelodysplastic syndromes with mild to moderate liver injuries. Accordingly, this research implicated to evaluate the epigenetic effect of azacytidine on mice liver as a model organism and to determine the role of folic acid administration as a protective methyl donor micronutrient. Thus, forty-eight mice were divided into four groups, reliant on the kind of treatment they received, where animals received calculated doses of azacytidine and folic acid twice a week for three months. Then, animals were killed, and their livers were autopsied and examined for histopathological abnormalities, followed by a genetic assessment for the DNA banding pattern profile of the excised livers. It was found that the anticancer drug azacytidine processes a hepatotoxic effect, and it profoundly modifies the DNA banding pattern. While the combination of folic acid with the administration of azacytidine might induce a protective effect against azacytidine toxic effects, these findings might unravel essential insights and highlight new potential cancer therapeutics.

Keywords: Azacytidine; Folic acid; Histology; Genomic DNA; Mice liver; RAPD-PCR

1. Introduction

DNA methylation is a molecular epigenetic heritable enzymatic modification, which results from the addition of a methyl group into the carbon-5 of cytosine [1, 2, 3]. This modification plays an essential role in gene expression, where cytosine analogs such as azacytidine could adapt to stress the expression of the genome through changing the methylation pattern and chromatin structure [4, 5, 6]. Azacytidine acts as an analog, incorporating itself into the genome during DNA replication and leading to non-targeted partial demethylation across the genome in all sequence contexts [7,8,9]. Besides, azacytidine acts to inhibit the action of DNA methyltransferases in the cell, contributing to wide genomic demethylation in tissues [3, 5, 10]. This s-triazine analog was first isolated from the culture filtrate of *Streptoverticillium ladakanus* and received approval by the U.S.A. Food and Drug Administration (FDA) on May 19, 2004, for the treatment of lymphatic leukemia [11,12]. It possesses a broad spectrum of biological effects wherein mammalian tissues it is phosphorylated to 5-azacytidine 5'-phosphates and incorporated into different species of RNAs and DNA [13, 14]. Following the administration of azacytidine, a rapid breakdown of liver polyribosomes has been observed; furthermore, it interferes with different induced liver enzymes [9, 10]. However, it was found that the cytostatic effect of this analog is reduced upon the administration of methyl-donor micronutrients [11, 12, 13].

Therefore, this research was suggested to consider the following objectives:

- The histopathological effect of azacytidine on the liver tissue of mice.
- Application of folic acid for the reduction of azacytidine cytotoxic effect
- The epigenetic impact of administrating azacytidine and folic acid on the methylation pattern of the liver DNA.

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2. Material and methods

2.1. Animals of the study

A total of forty-eight mice of Mus musculus strain were obtained from Al-Nahrain Center of Biotechnology/ Baghdad/ Iraq during 2019. They aged (4-6) weeks and weighed about (23-25 gm). Experimental animals were housed and fed according to the procedure mentioned by Tawfeeq, 2014 [15].

2.2. The solution of azacytidine

Treatment solution of 5 - azacytidine (Sigma-Aldrich/Germany) was prepared according to Argemí et al., 2009 [16] by dissolving (0.2 mg) of 5-azacytidine powder in (100ml) normal saline, sterilized by filtrations and used immediately for intraperitoneal injection of the experimental animals.

2.3. The solution of folic acid

This solution was prepared according to Hernandez-Diaz et al.,2000 [17] by dissolving (1 g) of folic acid (Sigma-Aldrich/Germany) in (100 ml) of distilled water, sterilized by filtration and used immediately for the treatment of the experimental animals.

2.4. Mice treatment with azacytidine and folic acid

All animal studies conducted were approved by the ethical committee of the Medical Laboratory Techniques Dep., and the procedures followed the Guide of Experimental Animals [18] as follows:

2.4.1. Treatment of mice with 5- azacytidine

- Twelve adult mice animals of (1:1) male to female ratio were selected.
- Animals received intraperitoneal injections of (0.1 ml) of (8 mg / m2 BSA) of 5-azacytidine, twice a week for three months according to the procedure of Bigatti et al., 2010 [19].
- The LD50 of 5- azacytidine defined as the dose, which caused the death of 50% of animals of the treatment group.

2.4.2. Treatment of mice with folic acid

- Twelve adult mice animals of (1:1) male to female ratio were selected.
- Oral doses of (0.1 ml) of folic acid (8 mg/ m2 BSA) was given to the treatment group two times a week for three months.
- The control group was intraperitoneally injected with (0.1 ml) of distilled water for the same treatment period mentioned above.

2.5. Experimental design

Mice of the study were divided into four groups, according to (Table 1) after the procedure mentioned by Tawfeeq, 2005 [20]. Treatment doses were calculated to bodyweight according to the FDA measurements [21].

Table 1 Treatment procedure of the study

Group No. n =12 mice	Treatment Material	Concentration mg /m2 BSA*	Dose Duration Week/month	Route of administration
G1	Distilled water	0	Twice / 3 month	IP injection
G2	Folic acid	8	Twice / 3 month	Oral
G3	Azacytidine	8	Twice /3 month	IP injection
G4	Azacytidine +folic acid	8	Twice /3 month	IP injection + Oral

*azacytidine and folic acid doses were calculated to bodyweight Index according to the FDA measurement [21].

Mice were observed for phenotypic changes during the treatment period, and four mice (two males and two females) from each treatment group were killed at the end of each month, and their liver organs were assessed histologically.

2.6. Histological evaluation of mice liver

Excised liver organs from the treatment and control groups were fixed in 10% formalin and embedded in paraffin. Then, tissue sections were cut at 5µm by Microtome (BIOBASE/ Meihua /China), mounted on slides after staining with Hematoxylin Eosin Y solutions (H & E Stain Kit/TissuePro/USA) according to Tawfeeq, 2014 [15]. Sections were examined by a light microscope (Optika/Italy) and photographed by mounted Camera (Optica- Italy 4083-B5). Significant histopathological abnormalities were evaluated and recorded accordingly.

2.7. Genetic assessment of liver DNA methylation pattern

2.7.1. Extraction of DNA

DNA extraction was performed using the DNA isolation kit (Genomic Prep /Amersham Biosciences/UK) following the instructions of the manufacturer. Then extracted DNA was resuspended in Tris-EDTA, and the quantity of the extracted DNA checked by spectrophotometer (Schimadzo/Japan) as described by Le et al., 2011[22]. For this analysis,

2.7.2. Analysis of DNA methylation pattern

The five methyl Cytosin levels were relatively quantified after the enzymatic hydrolysis of the genomic DNA with HpaII and MspI restriction enzymes (1U/1µg DNA/Thermo Scientific/France). RAPD-PCR (Eppendorf /Germany) primer set (Ready-To-Go RAPD- PCR analysis kit/ Amersham Biosciences/ UK) used as instructed by the manufacturer company. Three samples of liver DNA (azacytidine, folic acid, and control) were subjected to PCR with a single primer concentration of 25 pmol in a reaction volume of 25 µl and a DNA marker of (pUC19 DNA MspI/HpaII Marker/ Thermo Scientific/ France). Each change observed in RAPD profiles was scored as described by Enan, 2006 [23].

3. Results and discussion

The DNA methyltransferase (DNMT) inhibitory drug called 5-azacytidine that induces DNA hypomethylation has been used since the 1970s for the treatment of acute leukemia and is still being used as a part of the treatment regimen for Myelodysplastic syndromes (MDS), liver and pancreas cancers [24, 25, 26]. This chemical analog of cytidine is incorporated in DNA and RNA, resulting in cytotoxic effects on hematopoietic cells in the bone marrow and hepatocytes at high doses [10, 11, 12]. Since the liver plays a pivotal role in regulating various physiological processes in the body and any hepato-damage inflicts grave consequences [25]. Thus, the combination of any agent that could act in synergy with azacytidine to reduce its hepatotoxic effect would be essential. Therefore, this study implicated in evaluating the impact of azacytidine on the liver DNA methylation with/without folic acid as a methyl donor.

Accordingly, forty-eight mice were divided into four groups, reliant on the kind of treatment they received. Then, after three months of treatment, animals were killed, and their livers were autopsied and examined with naked eyes; results were shown in (Table 2).

Table 2 Macroscopic changes observed in the livers of the study groups

Group No.	Treatment Material	Concentration mg /m2 BSA*	Dose Duration	Route of administration
G1	Distilled water	0	Twice / 3 month	Normal
G2	Folic acid	8	Twice / 3 month	Normal
G3	Azacytidine	8	Twice /3 month	Congested & Enlarged
G4	Azacytidine +folic acid	8	Twice /3 month	Normal

Results showed congestion and enlargement in the livers of (G3) animals, which received intraperitoneal injections of (8 mg /m^2) BSA of azacytidine twice a week for three months.While the macroscopic appearance of the other groups of (G1, G2, and G4) appeared normal.

On the other hand, the histological analysis of the autopsied liver organs of the treatment groups showed normal liver histology (Figure 1a&1b) with radial hepatic cord and clear hepatic sinusoid for both the control group (G1), and the group which received oral doses of (8 mg / m^2 BSA) for three months.

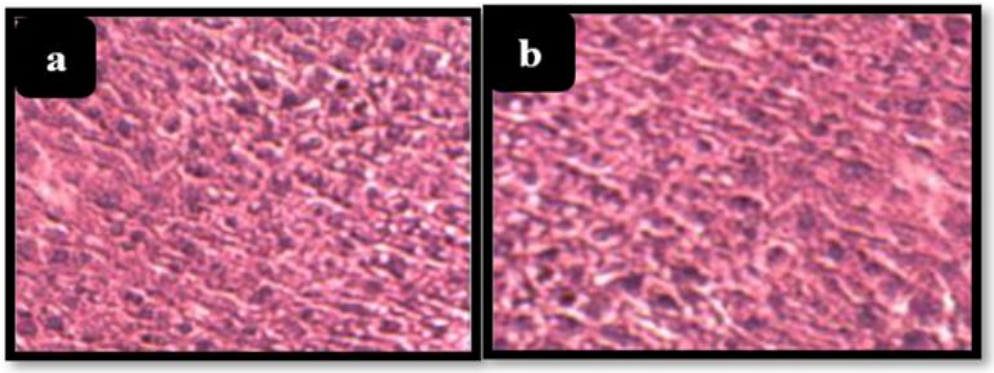

Figure 1 Histological sections of mouse liver showing; a- Typical liver histology section of (G1) control group at (40X), b- Normal liver section of (G2) folic acid-treated group at (40X).

So far, the oral administration of folic acid for three months did not induce a cytotoxic effect on the hepatocytes as Ebaid *et al*., 2013 [27] had declared a protective effect of folic acid against different hepatotoxic substances. Yet, the histological examination of livers tissues excised from mice treated with (8 mg / m^2 BSA) of azacytidine for three months showed a significant increase ($P \leq 0.05$) in the histological changes ranged from mild to severe injuries as shown in (Figure 2).

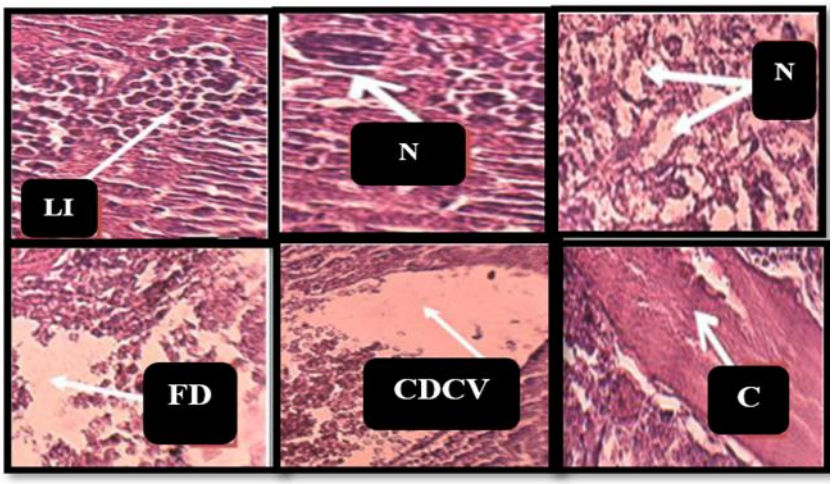

Figure 2 Liver section of mice livers (G3) treated with (8 mg / m^2 BSA) of azacytidine for three months showing; lymphocyte infiltration (LI), cellular necrosis (N), Fatty Degeneration(FD), congestion and dilatation of hepatic vein (CDCV), and congestion (C), H. &E. Staining (40X).

Results of (Figure 2) had shown that the continued treatment of animals with azacytidine for three months had resulted in the congestion and enlargement of the livers of the treated animals beside to the necrosis, lymphocyte infiltration, enlargement of Kupffer cells and the congestion and dalitation of the hepatic vein with the complete loss of hepatic architecture. The same result was asserted by Kiziltepe *et al*., 2007 and Poirier *et al*., 2014 [28, 29], where they found that azacytidine induces synergistic cytotoxicity on hepatocytes and interferes with cholesterol and lipid metabolism. On the other hand, it was found that the liver sections of mice animals treated with both azacytidine and folic acid did not show any histopathological abnormalities which ascertained the result obtained by Ebaid *et al*., 2013 [27], as shown in (Figure 3).

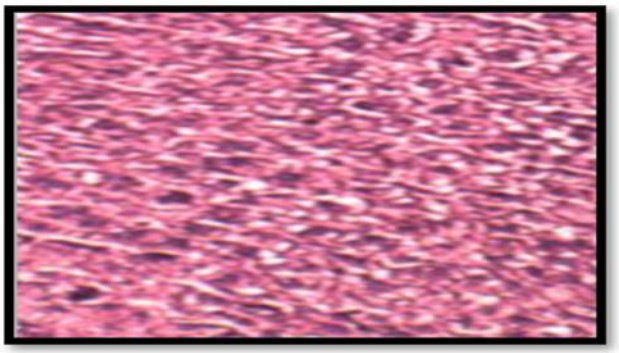

Figure 3 Histological sections of mouse liver of (G4) treatment group showing typical liver histology.

Yet, the results of (Table 3) expressed the percentages of histopathological abnormalities encountered during the examination of the excised livers of the treatment groups. These results agree with the results obtained by Tawfeeq, 2005 [20], where the researcher had long-established the antagonist effect of the folic acid on the hepatotoxic effect of the administration of 5-azacytidine on white mice liver.

Table 3 Percentage of the scored histological changes in the livers of the treatment groups

Group no.	Treatment	Percentage of histological changes				
		LI%	N%	FD%	CDCV%	C%
G1	Distilled water	0	0	0	0	0
G2	Folic acid	0	0	0	0	0
G3	Azacytidine	73	85	45	22	18
G4	Azacytidine + folic acid	3	2	1	0	0

In accordance, genomic DNA was extracted and purified from mouse livers of the treatment groups. Then, equal concentrations of DNA from the three liver samples were digested with (5 µl) of each enzyme of HpaII or MspI. The digested products were analyzed on a gel of (1.6%) of agarose concentration, and the results were shown in (Figure 4).

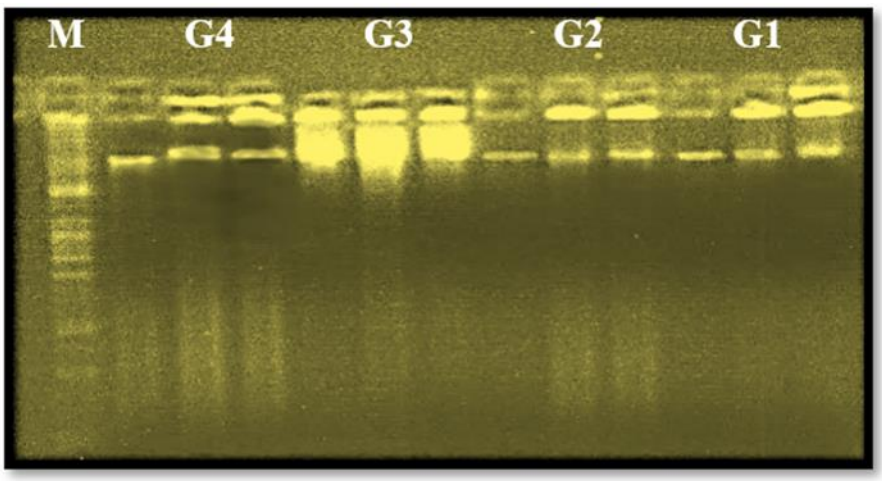

Figure 4 Gel pattern of mouse liver DNA (10 µg) treated with HpaII or MspI restriction enzymes and incubated overnight at 37 ºC. Where, M; marker DNA; G1; control DNA; G2; folic acid-treated group; G3; azacytidine treated-group; G4; azacytidine and folic acid-treated group.

The careful examination of (Figure 4) showed differences in the molecular size of the bands and the intensity of the amplified bands of (G1, G2, and G4) lanes, and (G3) lanes where lanes (G1, G2, and G4) showed the amplification of (11.497 bp) molecular size bands whereas lanes of (G3) did not produce distinguished bands upon digestion with the restriction enzymes. This result agrees with the result obtained by Kiziltepe *et al*., 2007 [27], where they found that azacytidine induces mediated DNA double-strand break responses which might affect the banding pattern profile of the isolated DNA.

In this study, the RAPD-PCR technique with two methylation-sensitive restriction enzymes was used to analyze the genomic instability. Banding profile showed no change in the bands of the (G4) treatment group of azacytidine and folic acid, while the (G_3) group showed a significant difference in the banding pattern. These results, however, might indicate a probable protective role played by folic acid for the maintenance of gene expression stability, as proclaimed by Barnett *et al*., 2015 [30].

4. Conclusion

The anticancer drug azacytidine acts through an incompletely understood mechanism where it processes a hepatotoxic effect, and it profoundly modifies the DNA banding pattern. While the combination of folic acid with the administration of azacytidine might induce a protective effect against azacytidine toxic effects, these findings might unravel essential insights and highlight new potential cancer therapeutics.

Compliance with ethical standards

Acknowledgments

The present work was carried out at Al-Nahrain Center of Biotechnology / Baghdad / Iraq.

Disclosure of conflict of interest

The author declares no conflict of interest.

Statement of ethical approval

The present study work was approved by the ethical committee of the Medical Laboratory Techniques Department.

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