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MAESTRÍA EN BIOCIENCIAS

Efecto del $CdCl_2$ sobre la expresión de DNA metiltransferasas y la metilación de los genes RASSF1A, MGMT y SOX17 en células HepG2

T E S I S

QUE PARA OBTENER EL GRADO DE MAESTRÍA EN BIOCIENCIAS

P R E S E N T A:

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APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 11 días del mes de enero de dos mil diecinueve, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Biociencias, para examinar la tesis titulada "Efecto del $CdCl_2$ sobre la expresión de DNA metiltransferasas y la metilación de los genes RASSF1A, MGMT y SOX17 en células HepG2", presentada por el alumna **Sonia Ivette Alcocer Lorenzo**, para obtener el Grado de Maestría en Biociencias. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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La presente investigación se realizó en el Laboratorio de Investigación de Citopatología e Histoquímica, en el Laboratorio de Epigenética del Cáncer de la Facultad de Ciencias Químico Biológicas, de la Universidad Autónoma de Guerrero (FCQB-UAGro) y en el Laboratorio de Morfología Celular de la Escuela Superior de Medicina en el Instituto Politécnico Nacional.

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Durante el proyecto que se realizó la C. Sonia Ivette Alcocer Lorenzo fue beneficiaria de la beca otorgada por el Consejo Nacional de Ciencia Tecnología (CONACYT), con número de CVU: 828171, perteneciente al programa de Maestría en Biociencias, UAGro.

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Efecto del $CdCl_2$ sobre la expresión de DNA metiltransferasas y la metilación de los genes RASSF1A, MGMT y SOX17 en células HepG2

CONTENTS

Effect of exposure to low-doses of cadmium on the methylation of RASSF1A, MGMT and SOX17 genes in HepG2 cells

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Highlights

- Cd induces hypermethylation of Sox17 gene in HepG2 cells.
- The DNMTs expression is modulated by exposure to low-dose of Cd.
- Sox17 hypermethylation can be a good epigenetic biomarker induced by exposure to Cd in low-doses.

ABSTRACT

Cadmium (Cd) is a highly toxic heavy metal, established as carcinogen. There is evidence of Cd mediated epigenetic modifications, such as changes in methylation, resulting in alteration in target gene expression. This could be attributed to changes in the expression of DNA methyltransferases (DNMTs), enzymes responsible for methylation. In the present study, we tested to effect of low-doses Cd exposure on methylation of MGMT, RASSF1A and SOX17 genes and the expression of DNMTs (DNMT1, DNMTA/3B) in HepG2 cells. Methylation-specific PCR (MSP) assay revealed that there was no significant difference in the methylation of the MGMT and RASSF1A, however the hypermethylation was observed in the SOX17 gene. We suggest that is caused by the effect of Cd on the expression of DNMTs. These findings indicate that exposure to low doses of Cd can lead to the epigenetic silencing of tumor-related genes, such as SOX17, which can serve as a biomarker for exposure to Cd at low-dose.

Key words: Cadmium, Methylation, DNA methyltransferase, HepG2 cells

1. INTRODUCTION

Cadmium (Cd) is a heavy metal, that has spread widely in the environment (Martelli *et al*. 2006), it has been categorized as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2012), and the main sources of exposure are through diet and tobacco consumption (ATSDR 2012; ESFSA 2009). Cd can easily reach micromolar concentrations, that can affect target organs like the liver (Cartularo *et al.* 2015).

The mechanisms by which Cd induces cancer are not fully understood, however, it has been reported that this metal can promote carcinogenesis through epigenetic mechanisms such as DNA methylation (Arita and Costa 2009). DNA methylation, is the covalent addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring (Rosales-Reynoso et al. 2016), it is catalyzed by DNA methyltransferases (DNMTs), which encompasses DNMT1 (maintenance), DNMTA and DNMT3B (*de novo*) (Zhang and Xu 2017). Although it is not clear how the expression levels of DNMTs are altered by the treatment with Cd, it has been documented that it can modulate the expression of DNMTs and generate aberrant DNA methylation (Cavagnari 2012; Gopalakrishnan *et al.* 2008). Several studies have linked the aberrant hypermethylation of promoter CpG islands to the over-expression of DNMTs (Doi *et al*. 2011; Zhang *et al.* 2009). The effects of DNA methylation depend on the sites that are methylated, the silencing by hypermethylation of some tumor suppressor genes, has been associated with carcinogenesis (Zhang *et al.* 2009), this has been demonstrated in DNA damage repair genes, cell cycle regulation genes, and cell signal transduction genes, among others (Fu *et al.* 2015). The genes studied in the present work have potential relevance to carcinogenesis, for instance, the gene of the Association Domain 1A (RASSF1A) is an important tumor suppressor gene (Choy *et al*. 2005), O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair gene (Johannessen *et al.* 2018) and the transcription factor SOX17 is involved in the regulation of embryonic development and in the determination of cell fate (Li *et al.* 2018). The hypermethylation of these genes has been correlated with tumor progression and poor prognosis in the pathogenesis of human cancer (Cartularo *et al*. 2015). There has been interest in evaluating if its inactivation can occur by exposure to some pollutants such as Cd. The objective of this study was to analyze the effect of low-doses exposure to Cd on the methylation of the promoters of MGMT, RASSF1A and SOX17 and their relationship to changes in the

expression of DNMTs in the HepG2 cell line. The concentrations of Cd exposition used in the present study are rarely examined in the effects on methylation of target genes. Even small changes in methylation induced by environmental factors (such as Cd) might influence genetic instability (Wodarz 2014). Therefore, the results of our study may help to better understand the mechanisms of cadmium toxicity.

2. MATERIALS AND METHODS.

2.1 Reagents.

Cells culture reagents; Dulbecco´s Modified Eagle´s Medium-high glucose; Trypsin-EDTA solution; Penicillin-Streptomycin; $CdCl_2$ analytical grade; Bromuro de 3-[4,5-dimetiltiazol-2-il]-2,5-difenil-tetrazol (MTT); Cloroform: Isoamyl alcohol and Sodium bicarbonate were obtained from Sigma-Aldrich Co. (San Lui MO). Fetal bovine serum was obtained from Byproducts. TRIzol RNA extraction was obtained from Life Technologies, USA. Vero SYBR Green 1-Step qRT-PCR ROX Mik Kit was obtained from Thermo Scientific. Wizard Genomic DNA Purification Kit was obtained from Promega. 5-mC DNA ELISA Kit was obtained from Zymo Research Corp (Irvine, CA, USA). Amplitaq Gold 360 Master Mix was obtained from Applied Biosystems. DNMT Activity Quantification Kit (colorimetric) was obtained from ABCAM.

2.2 Cell culture and exposition to Cd.

Human HepG2 cells were obtained from the ATCC (American Type Culture Collection). Cells were thawed and plated in plastic culture dishes (Corning, NY) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (all obtained from Sigma-Aldrich Co.). They were incubated in air at 37 °C with 95% humidity, 5% CO2 and used on the seventh day after culture. Before any treatment, cells were seeded onto 24-well plates and incubated for 24 h at a density of 1 × 105 cells/well and the enriched medium was replaced by low-serum DMEM media (0.5% FBS) and then treated as indicated in the figure legends. An aqueous sterile stock solution of $CdCl_2$ (1mM

Sigma-Aldrich Co.) was prepared and diluted with DMEM/0.5% FBS to obtain the desired final concentrations (0.5, 0.8, 1 y 3 μM).

2.3 MTT reduction assay

Cell viability was measured after treatment with by the MTT reduction assay [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma-Aldrich Co.) performed as described by Mosmann (Mosmann 1983). Cells were seeded on 24-well plates in 300 μl of culture media and incubated for 24, 48, 72 y 96 h with 0.5, 0.8, 1 y 3 μM of $CdCl_2$ at 37C in a 5% CO2 atmosphere. After, cells were treated with MTT (5 mg/ml) for approximately 4 h. An isopropanol was added to lyse the cells and solubilize the crystals. The optical density was determined at 545-630 nm using an ELISA plate reader Stat Fax 2100 (Awareness Technology, Palm City, FL). The results are presented as the percentage of live cells (% control) with MTT response.

2.4 Cell morphology analysis

For morphological examination HepG2 cells were plated in 12-well plates, and images were obtained using an EVOS® FL Auto Imaging System inversted microscope. Three areas with approximately equal cell densities were identified in each well, and images of each of these areas were captured

2.5 Extraction and purification of nucleic acids

Total extraction of RNA from the cell line after treatment with Cd from was performed with TRIzol reagent (Life Technologies, USA). The extraction of DNA it was done with the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. The integrity of both nucleic acids was determined by electrophoresis in a 1 % agarose gel. The concentration of RNA and DNA was evaluated by spectrophotometry using NanoDrop 2000c (Thermo Scientific, Wilmington, USA).

2.6 Methylation-specific PCR (MSP)

Methylation of the MGMT, RASSF1A and SOX17 genes was analyzed using a bisulfite conversion reaction, where an unmethylated cytosine is converted into uracil, followed by PCR amplification. DNA HepG2 cells was treated with bisulfite using an EZ DNA Methylation-Gold™ kit (Zymo Research Corp., Irvine, CA, USA), according to the manufacturer's protocol. MSP primer sequences are shown in Table 1. MSP was performed in a total of 10 µL, containing 2 µL of bisulfite-treated DNA (100 ng), 250 nM (1 µL) of each primers and 5 µL AmpliTaq Gold360 Master Mix (Applied Biosystems) and under the following amplification conditions: denaturation 95°C for 10 min, 40 cycles of amplification: 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, and a final extension of 72°C for 10 min. The reactions were done in Eppendorf Mastercycler EP Gradient 96 Thermal cycler (Applied Biosystems).

2.7 RT-qPCR

The expression levels of DNMT1, DNMT3A and DNMT3B were analyzed by real-time PCR. 100 ng of total RNA were used in each RT-qPCR assay. Reverse transcription and quantitative PCR were performed with Vero SYBER Green 1-Step Kit qRT-PCR ROX Mix (Thermo scientific), according to the manufacturer's protocol. In all cases, the conditions of reverse transcription and amplifications were:15 min at 50°C and 95°C for 5 min; 40 cycles of amplification: 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. A melt curve stage was added. The reactions were done in CFX^{TM} 96 Real-Time PCR Detection system Biorad. Data were normalized using GAPDH as an internal control and relative expression differences were calculated using the 2-ΔΔCt method. Primers sequences are shown in Table 1.

Table 1. Primer sequences for the different genes evaluated in this study

GENE	SEQUENCE	TM °C
RT-qPCR		
DNMT1	F5´- GGTTCTTCCTCCTGGAGAATGT	60
	R5´- GTCTGGGCCACGCCGTACTG	
DNMT3B	F5´- ACCACCTGCTGAATTACTCACG	60
	R5´- GATGGCATCAATCATCATGG	
DNMT3A	F5´- GGTGCTGTCTCTCTTTGATG	60
	R5´- ATGCTTCTGTGTGACGCTG	
GAPDH	F5´- GACCCCTTCATTGACCTCAAC	60
	R5´- TGGCAGTGATGGCATGGAC	
MSP		
MGMT	F5´-TTTCGACGTTCGTAGGTTTTCG	56
	R5´- GCACTCTTCCGAAAACGAAACG	
RASSF1A	F5´-GTGTTAACGCGTTGCGTATC	60
	R5´- AACCCCGCGAACTAAAAACGA	
SOX17	F5´-GGAGATTCGCGTAGTTTTCG	62
	R5´- AACCCGACCATCACCGCG	

MSP: Methylation-specific PCR. **TM °C:** Annealing temp.

2.8 Statistical analysis

Statistical analysis of all data was performed with SIGMAPLOT V 10.0 computer software and all data were assessed ANOVA. All data were expressed as the mean± S.D. $p < 0.05$ was considered significant difference.

3. RESULTS

To investigate the effects of exposure to low-doses of Cd on target gene methylation, we used HepG2 cells which were continuously cultured for 24, 48, 72 and 96 h in the presence of Cd (0.5, 0.8 1, 3 μM). Because these exposure concentrations have been poorly studied, it was necessary to determine the effect of Cd at low-doses on the viability of HepG2 cells. The HepG2 cells viability exposed to Cd is presented in Figure 1A, we can observe that the concentration of 0.8 μM was cytotoxic in more than 20% of the cells at 72 and 96 h of exposure, this decrease was dependent on the concentration (48 hr-LD_{50} of 2.0 μM, Table 2. $HepG_2$ cells exposed to 1 and 3 μM of Cd showed a decrease in viability of 40% and 60% respectively ($p < 0.05$) compared to control cells. Moreover, the Cd treated cells exhibited changes in their morphology such as structural changes, loss of the cytoplasm nucleus relation, and these changes were more evident at 48 h of exposure when compared control cells (Fig. 1B). These results indicate that Cd at low-doses had significant cytotoxic effect significant causing cell mortalities in HepG2 cells compared to the control.

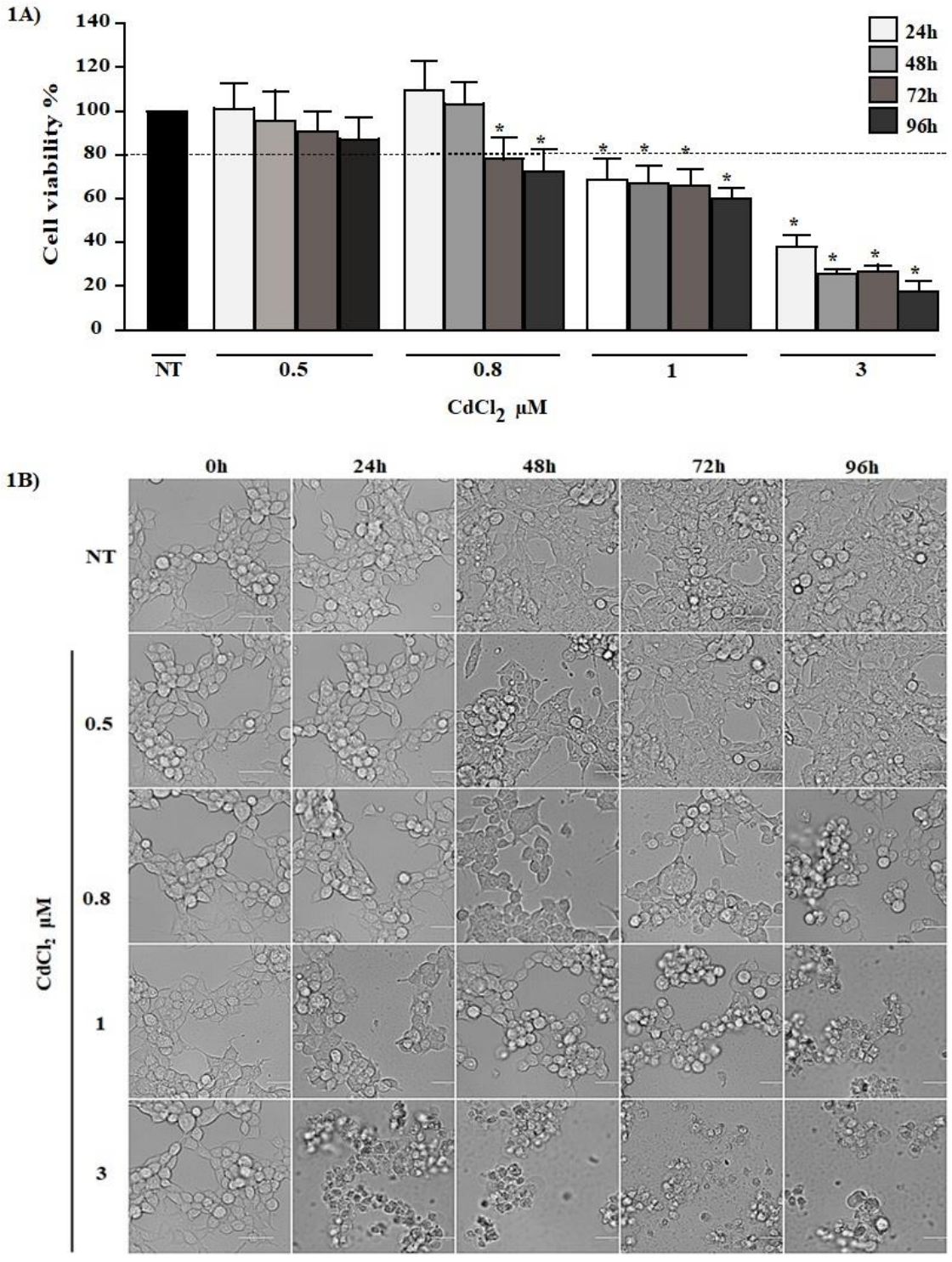

Figure 1. Cytotoxic effect and changes in the morphology induced by Cd exposure in HepG2 cells. The cells were exposed to different concentrations of Cd (0.5, 0.8, 1 and 3 µM) by 24, 48, 72 and 96 h. (A) Cytotoxic effect of Cd in HepG2 cells; The cytotoxicity was determined by the methyl tetrazolium method (MTT). (B) Changes in the morphology in HepG2 cells induced by Cd exposure. The experiments were carried out in triplicate and the data are expressed in percentage with respect to NT and each bar represents the average of three independent experiments in triplicate (mean ± E.S.); * $p < 0.05$ (t-Student test). NT, Not treated

Table 2. Time-response relationship of the effect of $CdCl_2$ on HepG2 cells.

	$CdCl_2$	
Exposure time	**$IC_{50\ \mu M}$**	**Confidence Interval 95%**
24 h	2.3 μM	90.27296 -117.8754
48 h	2.0 μM	91.73579 -116.9254
72 h	1.9 μM	89.2057 -108.3697
96 h	1.72 μM	88.18517 - 107.8917

The cells were exposed to different concentrations of Cd (0.5, 0.8, 1 and 3 μM) by 24, 48, 72 and 96 h. IC-50 concentrations were calculated using regression analysis (R2). **IC:** inhibitory concentration.

To investigate effects of Cd on target gene methylation, HepG2 cells were exposed at concentrations of 1 and 3 μM for 48 h, and then level of methylation of the MGMT, RASSF1A and SOX17 genes was measured. Our results showed, no significant changes in the methylation of the MGMT and RASSF1A genes in HepG2 cells exposed to 1 and 3 μM of Cd (Fig. 2), while for the SOX17 gene showed important changes. The methylation status of the transcription factor SOX17, increased more than 100% in HepG2 cells exposed to 3 μM of Cd compared to the cells of control. This result confirms that exposure to low-doses of Cd can modify the methylation status of target gene promoters such as SOX17.

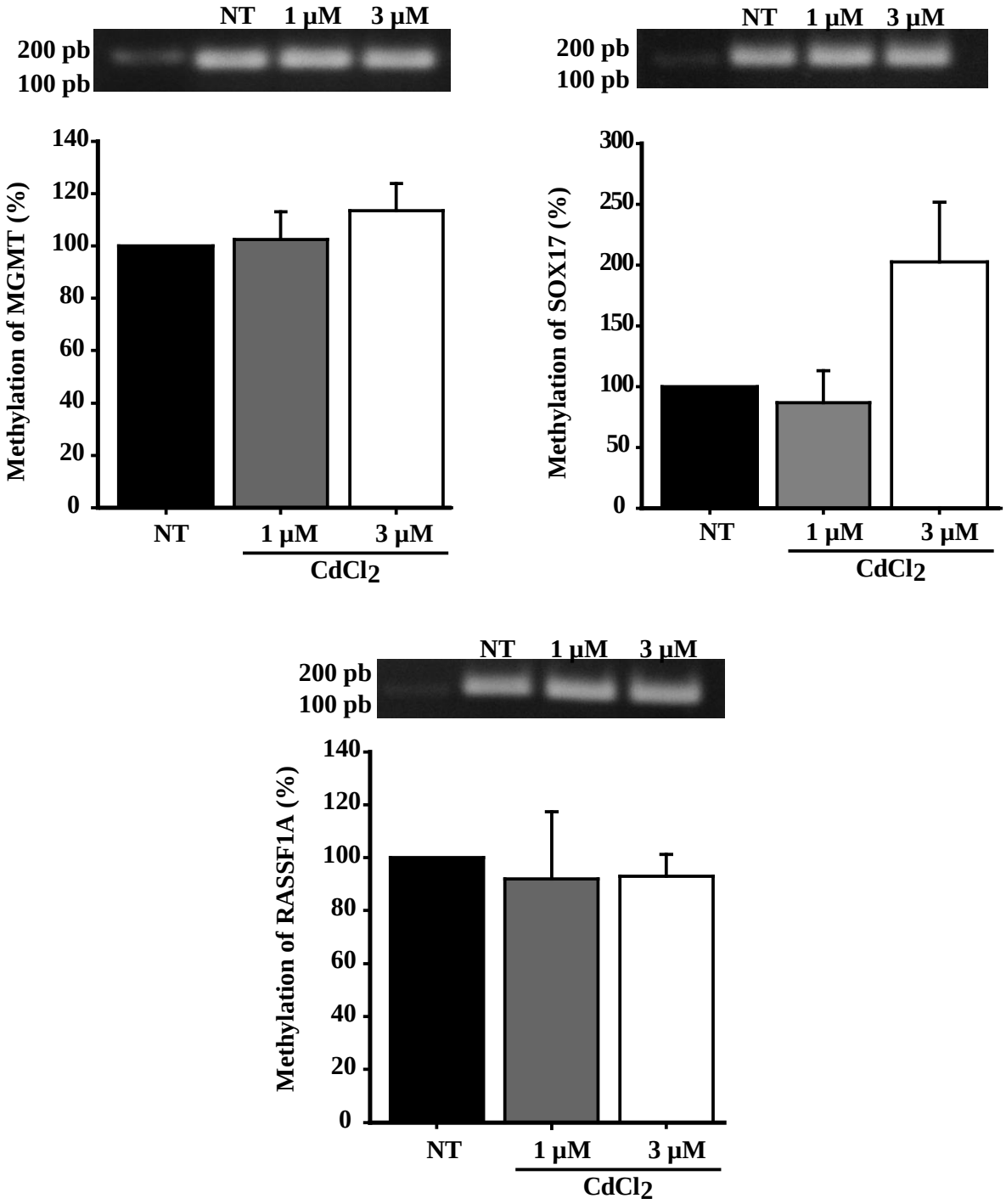

Figure 2. Effect of Cd exposure in the methylation of the promoters of the genes MGMT, RASSF1A and SOX17 in the HepG2 cell line. The cells were exposed to 1 and 3 µM of Cd for 48 h and the methylation was evaluated by PCR MSP. The experiments were carried out in triplicate and the data are expressed in percentage with respect to NT and each bar represents the average of three independent experiments in triplicate (mean ± E.S.); * p <0.05 (t-Student test). NT, Not treated.

To investigate whether the hypermethylation of SOX17 it's related with changes in expression of DNMT´s we measured the levels of DNMT1, DNMT3A and DNMT3B mRNA in HepG2 cells exposed to Cd (0.5, 0.8 1, 3 μM for 48 h). DNMT1 gen was found to be upregulated and the genes DNMT3A y DNMT3B downregulated in the cells HepG2 exposed to Cd (Fig. 3). DNMT1 levels decreased by approximately 40% when the cells were exposed to 0.5 μM of Cd ($p < 0.05$), however, whith 0.8 μM of Cd the mRNA levels were increased in a concentration-dependent manner. No obvious changes in the expression of DNMT3A were observed in HepG2 cells exposed to Cd at low concentrations, however at 3 μM a significant decrease in mRNA levels was observed with respect to untreated cells. Likewise, a significant decrease of approximately 50% in the DNMT3B expression by exposure to 0.8 μM to 3 μM of Cd during 48 h was observed ($p < 0.05$). These results indicate that DNMT1, DNMT3A and DNMT3B expression could be modulated by exposure to low-doses of Cd.

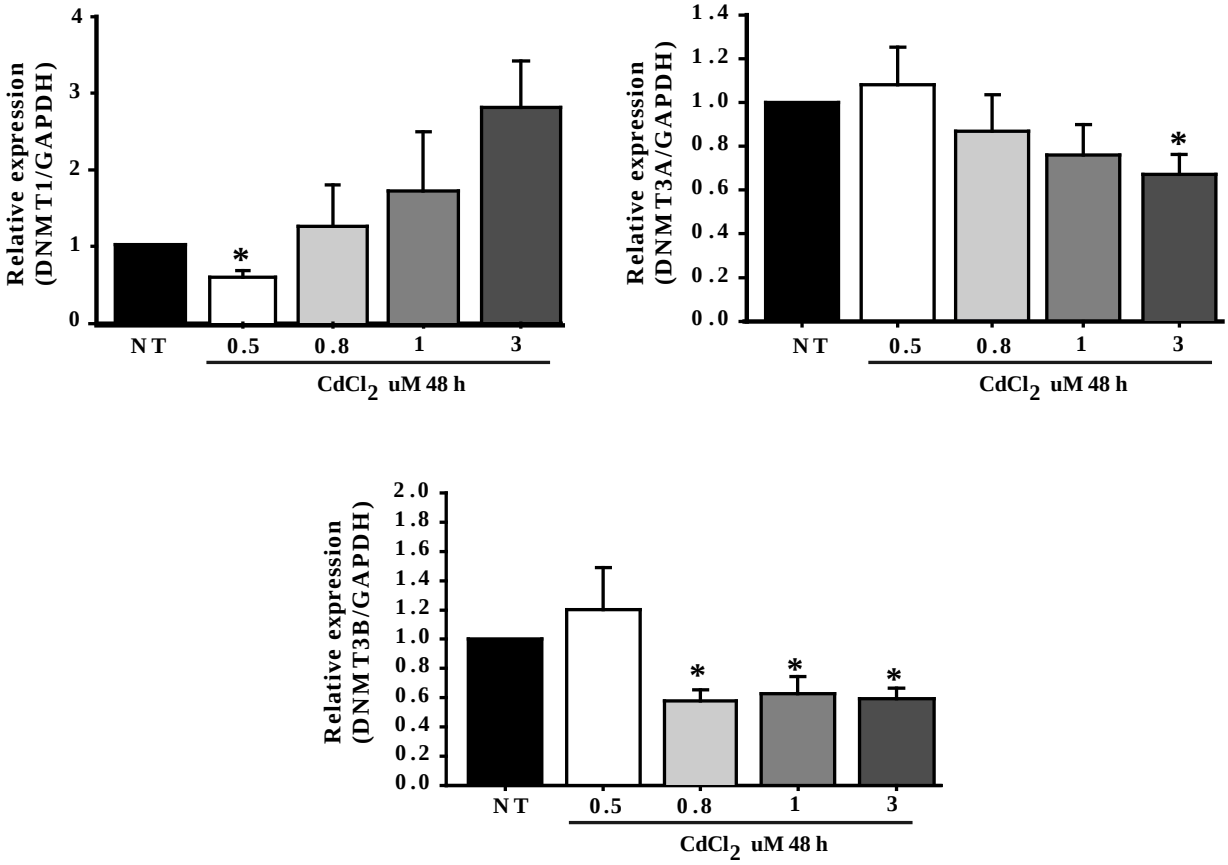

Figure 3. Effect of Cd exposure on the expression of DNMT1, DNMT3A and DNMT3B in the HepG2 cell line. The cells were exposed to 0.5, 0.8 1 and 3 μM of Cd for 48 h and the mRNA expression was measured with RT-PCR. The experiments were carried out in triplicate and the data are expressed in percentage with respect to NT and each bar represents the average of three independent experiments in triplicate (mean ± E.S.); * $p < 0.05$ (t-Student test). NT, Not treated

4. DISCUSSION

The present study demonstrates that Cd exposure in low doses causes hypermethylation of transcription factor SOX17 and differential changes in the expression of DNMTs genes in HepG2 cells. Cd is a heavy metal, has spread widely in the environment (Martelli *et al.* 2006). The liver is one of the main organs involved in the elimination of this metal and it is especially sensitive to its toxic effects (Souza-Arroyo *et al.* 2013). In this study the cytotoxic effect of exposure to low-doses of Cd was evaluated, because was of our interest to observe the effects at biologically relevant concentrations, due to the characteristic of bioaccumulation that presents the Cd (Sarkar *et al*. 2013). The main source of exposure in the general population is orally through diet, mainly due to the consumption of foods contaminated with Cd (Pérez-García and Azcona-Cruz 2012). As mentioned by Sakar *et al.,* 2013, the average Cd intake of food generally varies between 8 and 25 µg per day, of which approximately 0.5 to 1.0 µg is retained and distributed in the body. Specifically, in liver tissue has been found to bioaccumulate concentrations ranging from 8.81µg/g (Hayashi *et al*. 2012), 12 µg/g up to 60 µg/g (Baba *et al.* 2013), comparable to 0.07, 0.1 and 0.5 µM respectively and this can change depending on the degree of exposure. Low-doses of exposure were used in this work (0.5, 0.8, 1 and 3 µM), comparable with those that have been reported to bioaccumulate in the liver (Baba *et al*. 2013; Cartularo *et al*. 2015; Hayashi *et a*l. 2012). There was a gradual decrease in the viability of cells, with increasing concentrations of Cd and time to exposure in HepG2 cells (Fig. 1A). We showed that concentrations of 1 and 3 µM caused cell mortalities 40 and 60% respectively at all exposure times compared to the control ($p<0.05$). Consistent with our report, many studies also indicated that Cd induces cytotoxic effects and hepatocellular damage by an imbalance in the cellular redox status which leads to oxidative stress (EO)(Souza-Arroyo *et al.* 2013). Low-doses of Cd lead to proliferation or delayed apoptosis, intermediate concentrations of 10 µM Cd cause various types of apoptotic cell death, and very high concentrations (>50 µM Cd) lead to necrosis (Nair *et al*. 2013).

The human exposure to Cd is associated with cancer (IARC, 2012). Currently, the focus of mechanists, is the investigation of epigenetic modifications mediated by Cd, such as changes in DNA methylation (Hirao-Suzuki *et al*. 2018). Methylation of DNA is an epigenetic modification that can play an important role in the control of gene expression in mammalian cells, and aberrant DNA methylation is important factor in cancer progression (Kondilis-

Mangum and Wade 2013). Epigenetic silencing due to hypermethylation of tumor-related genes is known to play critical roles in the initiation and progression of cancer; this has been demonstrated in DNA damage repair genes, cell cycle regulation genes, and cell signal transduction genes, among others (Fu *et al*. 2015; Skipper *et al.* 2016).

Methylation status of cancer-related genes is considered to be a promising biomarker for the early diagnosis and prognosis of tumors (Fu *et al.* 2015). The genes studied in this work have potential relevance to carcinogenesis. For instance, in this study investigated the methylation status of RASSF1A, an important tumor suppressor gene, involved in cell cycle regulation, microtubule stabilization, cellular adhesion and motility, and cell apoptosis (Zhuang *et al.* 2019), studies have indicated that methylation of the promoter of the tumor suppressor gene RASSF1A reduces its expression, which may play an important role in carcinogenesis (Kawai *et al.* 2010; Ye *et al*. 2017). A study conducted showed that exposure to Cd can progressively reduce the expression of the RASSF1A tumor suppressor gene (Benbrahim-Tallaa *et al.,* 2007). However, it is necessary to determine if the regulation to the down is given by epigenetic mechanisms such as promoter methylation of said gene. In this study the methylation of the RASSF1A promoter was evaluated, however, as shown in the figure 3, no significant changes were observed in the methylation patterns in HepG2 cells exposed to 1 and 3 μM of Cd by 48 h. Another gene studied was MGMT, it is having a decisive role in the protection of mammalian cells against the genotoxic effects of alkylating carcinogens (Lavon *et al*. 2007). The silencing of the MGMT tumor suppressor gene by promoter methylation commonly occurs in human cancers (Cabrini *et al*. 2015); however, when evaluating the effect of Cd on MGMT methylation patterns, as with the RASSF1A gene, no differences were found, this could indicate that the down-regulation of MGMT, could be by other epigenetic pathways, and by means of phosphorylation of proteins (Liu and Gerson 2006), nevertheless more studies are necessary.

The methylation status of SOX17 gene promoter was evaluated and they were observed important changes, the methylation status of SOX17, increased more than 100% in HepG2 cells exposed to 3 μM of Cd compared to the cells of control. SRY-box 17 (SOX17) is a transcription factor which is involved in a variety of developmental processes and can act as

an antagonist of canonical Wnt/beta-catenin signaling pathway (Fu *et al.* 2010). Additionally, SOX17 induced the cell cycle arrest at the transition from the G0/G1 phase to the S phase (Li *et al*. 2018). SOX17 repression is associated with promoter region hypermethylation in HCC cells (Jia *et al.* 2010). There is no direct evidence as to whether Cd can evoke hypermethylation of SOX17 promoter region, however our result suggests that the exposure to 3 µM of Cd can increase almost double (Fig. 2) methylation of the SOX17 promoter. SOX17 promoter methylation can provide important prognostic information in cancer (Engert *et al.* 2013). Hypermethylation of the SOX17 promoter is correlated with poor prognosis in several cancers (Fu *et al.* 2010). Many studies have demonstrated that the Sox17 gene can perform tumor suppression functions. Thus, SOX17 gene silencing due to promoter methylation may deactivate its tumor suppressor role (Fu *et al*. 2015). Thus, DNA methylation is considered as a promising tumor biomarker for early detection and prognosis and extremely interesting for therapy approaches (Costa *et al.* 2007). There is no clear mechanism explaining the mechanism by which Cd generates hypermethylation in the SOX17 promoter; however, many studies have shown that hypermethylation in the initial exon or intron plays an important role in the epigenetic regulation of a variety of genes (Yuan *et al.* 2013). Nishida and Kudo, (2013) suggest that the oxidative stress (EO) generated by Cd can increase or decrease the binding and function of DNMTs to the promoters of the genes, and change the methylation, however, more studies are needed that contribute evidence of Cd exposure and methylation in gene promoters (Nishida and Kudo 2013).

DNA hypermethylation together with up-regulation of DNMTs may provide a unique set of biomarkers to specifically identify cadmium-induced cancers (Szyf 2011). It is suggested that DNMT1, DNMT3A and DNMT3B, could be regulated by exposure to low doses of Cd (Jiang *et al.* 2008). In the present study, exposure of HepG2 cells to Cd increases mRNA level of DNMT1, but not DNMT3A and DNMT3B (Figure 2). With respect to the maintenance enzyme of methylation, DNMT1, the levels of expression were increased in a dose-dependent manner. DNMT1 expression is critical to the maintenance of Cd-induced aberrant DNA hypermethylation. It is believed that increased expression of DNMT1 it is due to the constant presence during S-phase correlates to its maintenance of DNA methylation patterns during replication (Kinney and Pradhan 2011). Therefore, the up-regulation of

DNMT1 expression results in a relative increase methylation (Zhang *et al.* 2009). We showed a significant decrease in mRNA levels of DNMT3A in the cells exposed to 3 µM of Cd, an there is a significant decrease of approximately 50% in the expression of DNMT3B by exposure to Cd from 0.8 µM to 3 µM for 48 hours ($p < 0.05$), these results are similar to those reported by Huumonen *et al*., (2014), they observed Cd affect the DNMTs status directly after exposure as indicated by decreased mRNA levels of the de novo DNMT3A and DNMT3B (Huumonen *et al.* 2014). In this regard Reveron-Gómez., (2011) refers that DNMT3A and DNMT3B are expressed mostly in embryonic stem cells, however, they are found low level in some tissues (Reverón-Gómez 2011).

There are several mechanisms by which mammalian cells regulate DNMT levels (Denis *et al*. 2011; Kinney and Pradhan 2011) . These regulatory processes can be disrupted in disease or by environmental factors or pollutants like the Cd, resulting in altered DNMT expression and aberrant DNA methylation patterns (Brocato and Costa 2013). The transcription of DNMT1, DNMT3a and DNMT3b was reported to be stimulated by the transcription factors Sp1 and Sp3 (Sato *et al*. 2006). We do not know whether $CdCl_2$ regulates DNMTs expression by this route. DNMT1 mRNA expression is also regulated by transcriptional repression, p53 represses DNMT1 transcription, and this repression is abrogated by the specificity protein 1 (Sp1) transcription factor (Torrisani *et al.* 2007). Thus, the p53/Sp1 ratio can determine whether DNMT1 is activated or inactivated (Calzone *et al.* 2008). The p53 protein causes G1/S arrest of the cell cycle in response to many types of cellular stress, including DNA damage, which can be generated indirectly by the EO generated by exposure to Cd (McCabe *et al.* 2005). Nishida and Kudo, (2013) suggest that the EO generated by Cd can increase or decrease the union and function of DNMTs (Nishida and Kudo 2013).

Other additional mechanism for regulation of DNMT3B mRNA levels are the knockout of Vezf1, a zinc finger DNA binding protein, results in decreased DNMT3B mRNA levels (Gowher *et al*. 2008), this could explain the results obtained in this study, the cells exposed to concentrations of 0.8, 1 and 3 µM of Cd, showed a decrease in the expression of DNMT3B, and this occurs due to the bioavailable Cd imitates metals that are essential as Zn^{2+} (Nair *et al.* 2013), leading to the decrease of proteins as Vezf1 thus generating a decrease of

DNMT3B (Lopez de Silanes *et al*. 2009). However, more studies are needed that provide evidence of the mechanism by which contaminants such as Cd can modify the expression of DNMTs.

5. CONCLUSION

Exposure to low doses of Cd can lead to the epigenetic silencing of tumor suppressor genes such as SOX17. HepG2 cells showed hypermethylation of SOX17 that may be due to up-regulated mRNA expression levels of DNMT1. The results observed in the present work, can provide a new perspective on the mechanisms of carcinogenesis, toxicity, and identification of genes with aberrant methylation induced by Cd. However, the precise events that explain the changes in methylation and the expression of the DNMT remain uncertain.

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8. ANEXOS

ANEXO 1

To evaluate the expression of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) in the HepG2 cell line exposed to $CdCl_2$, as a first instance the cells were exposed to different concentrations of $CdCl_2$ (0, 0.5, 0.8, 1 and 3 μM) by 48 h. The RNA was extracted by the TRIzol method, the integrity of the RNA was analyzed by visualization on a 1% agarose gel (Figure 4), with GelRed® and visualized in a ChemiDoc MP system (BioRad).

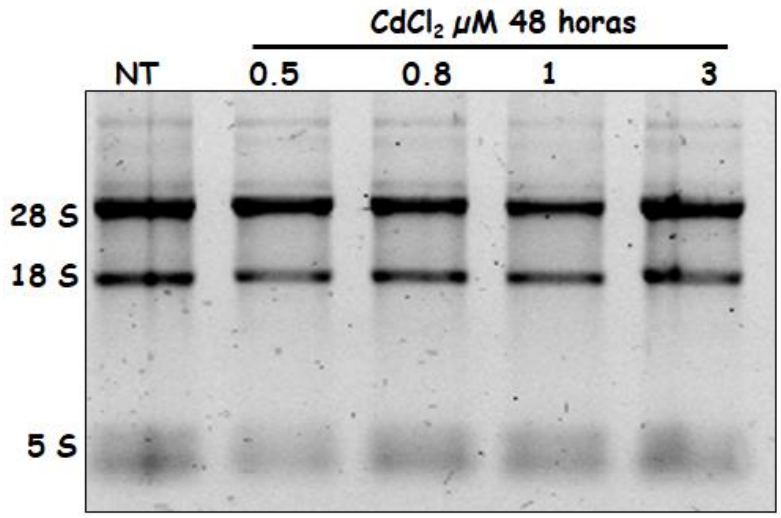

Figure 4. Integrity of mRNA from HepG2 cells exposed to $CdCl_2$. Electrophoresis in 1% agarose gel, where RNAm of HepG2 cells treated with 0.5, 0.8, 1 and 3 μM of $CdCl_2$ for 48 h was observed. The two bands 28S and 18S correspond to the major and minor subunit of the ribosomal RNA, which indicate an optimal RNA.

ANEXO 2

In order to evaluate the alignment and amplification of the DNA methyltransferase genes, endpoint PCR was performed confirming the expected PCR product (Figure 5). It is important to mention that in addition to evaluating the efficiency of amplification, this test was important to discern the expression of each of the DNMTs in their basal state in the HepG2 cell line, because there are no reports that indicate it. In each reaction, cDNA from untreated HepG2 cells was used and as an internal control cDNA from cell line K562. Each PCR run included a denaturation phase at 94 ° C for 10 min, an alignment phase (60 ° C) for 30 seconds for DNMTA and 45 seconds for GAPDH and an elongation phase at 72 ° C for 30 and 60 seconds respectively.

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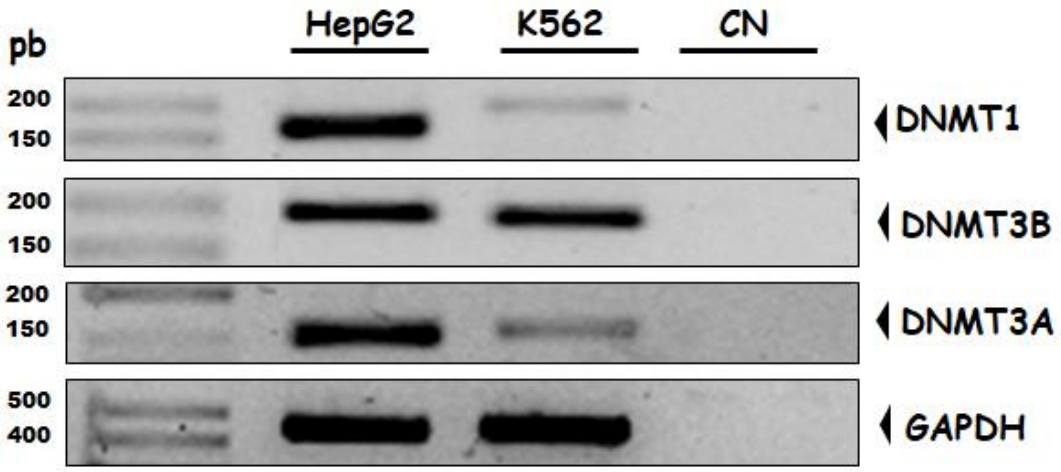

Figure 5. Verification of primer sequences. Electrophoresis in 2.5% agarose gel with TBE 1X Buffer; PCR product of the gene of DNMT1 (145pb), DNMT3A (178bp) and DNMT3B (146bp) to evaluate the expression of DNMTs in HepG2 cells, the K562 cell line that was used as an internal control. CN: Negative control in which cDNA was omitted