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Dose dependent effect of 5-aza-cytidine on mRNA expression of DNA methyl transferases (DNMT1, DNMT3a and DNMT3b) and histone deacetylase (HDAC) in buffalo skin fibroblast cells

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ABSTRACT

Epigenetic processes modulate gene expression patterns without modifying primary DNA sequence and have profound effects on the cellular repertoire of expressed genes. DNA methylation, histone modification and genomic imprinting are interrelated forms of epigenetic inheritance. Modulation of chromatin structure through histone methylation, acetylation/deacetylation is known to be one of the major mechanisms involved in the regulation of gene expression. Most cloned animals have defects in their epigenetic marks, mainly in their particular patterns of DNA methylation and histone modification. The present investigation was undertaken in the cultured buffalo skin fibroblast cells treated with 5-aza-cytidine to study the dose dependent effect of 5-aza-cytidine on mRNA expression of DNA methyl transferases (DNMT1, DNMT3a and DNMT3b) and Histone deacetylase (HDAC). Reduction in the expression of DNMT1, HDAC1 was observed in skin fibroblast cell when treated with higher doses of 5-aza-cytidine. However, expression of DNMT3a and DNMT3b mRNA increased with higher doses of the drug.

Key words: 5-Aza-cytidine, Buffalo skin fibroblast cells, DNA methyltransferases, Histone deacetylase1

Epigenetic mechanism plays an important role in cell cycle control, apoptosis, developmental and differentiation pathways, DNA damage repair and cell adhesion and migration (Baylin and Ohm 2006). DNA methylation and histone modification mediate epigenetic changes. DNA methylation, a major epigenetic modifications of the mammalian genome, is implicated in genomic imprinting, X chromosome activation, embryonic development, transcriptional repression of certain genes and in carcinogenesis (Razin and Cedar 1991). Methylation is carried out by a family of DNA methyl transferases DNMTs (DNMT1, DNMT3A and DNMT3B) (Goll and Bestor 2005), and main enzyme responsible for replicating DNA methylation patterns is DNMT1 and in contrast, DNMT3A and DNMT3B are responsible for *de novo* methylation, in which a methyl group is transferred to the carbon position of cytosine from methyl donor S-adenosyl-L-methionine (Okano *et al*. 1999). Post-translational modification of histones was also identified as important epigenetic mechanism (He and Lehming 2003) and is a primary regulator of gene expression (Marks *et al*. 2001). Alteration in gene expression encoding HAT and HDAC was clearly linked to carcinogenesis (Zhu *et al*. 2004, Mai *et al*. 2005, Ouaissi *et al*. 2008, Ropero *et al*. 2008) and moreover, aberrant expression of HDAC enzymes was linked to prognosis in a variety of cancers (Ouaissi *et al*. 2008, Weichert *et al*. 2008a, Weichert *et al*. 2008b).

Cytosine methylation and histone acetylation are reversible and may be altered by biochemical and biological manipulation, which makes it an attractive target for therapeutic intervention. Demethylation and consequent reactivation of tumor suppressor genes are rational approaches being used in cancer treatment (Issa 2005, Issa *et al*. 2005, Momparler 2005). Methyl transferase inhibitors such as 5-aza-cytidine and 5-aza-2-deoxycytidine are analogues of cytosine having similar inhibition mechanism (Christman 2002, Stresemann *et al*. 2006) and have undergone the most preclinical and clinical testing (Santini *et al*. 2001). 5-aza-cytidine was evaluated in clinical trials as a cancer therapeutic agent for treatment of acute myeloid leukemia and myelodysplastic syndrome (Santini *et al*. 2001, Kornblith *et al*. 2002, Silverman *et al*. 2002). Demethylation

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of genomic DNA as a secondary consequence by incorporation of 5-aza-cytidine into DNA forming covalent adducts with cellular DNMT1, thereby depleting the cells from enzyme activity and causing demethylation of genomic DNA as a secondary consequence (Christman 2002). In various *in vitro* experiments, 5-aza-cytidine treatment leads to re-expression of former silenced genes (Christman 2002). The resulting DNA hypomethylation has been linked to the induction of cellular differentiation *in vitro* (Petti *et al*. 1993) and altered expression of genes involved in tumor suppression (Christman 2002).

Although there is considerable literature available on the possible antitumor mode of action of aza drugs, their exact role in the methyl transferase as well as histone deacetylase in buffalo skin fibroblast cells is unknown. Our investigation was undertaken to study the dose dependent effect of 5-aza-cytidine on mRNA expression of methyltransferases DNMT1, DNMT3A, DNMT3B and HDAC1 in buffalo skin fibroblast cell culture using quantitative real-time polymerase chain reaction (qPCR).

MATERIALS AND METHODS

Freshly biopsied tissue was obtained from adult ear of female Murrah buffalo from Karnal district of Haryana, India and transported in culture media (DMEM + Ham's F-12 nutrient mixture + L-glutamine + 10% FBS + penstrep) at 37ºC within 2–3 h. The tissue piece was transferred in to a fresh culture medium and was scraped from both sides to remove epidermal layer and was cut into small pieces. This was placed about 1 mm apart in 25 cm² tissue culture flask with 500 µl of culture medium containing 15% FBS. They were incubated for 24 h at 37°C in humidified air with 5% CO_2. After 24 h, 3.5 ml of complete culture medium containing 15% FBS was added gently in contamination free culture flask without disturbing the attached tissue pieces in the flask. The culture medium was replaced after every 3 – 4 days. Once the cells reaches 20 – 25% confluency around the tissue pieces, the tissues were removed from the flask and were reseeded in to fresh flasks to generate more number of primary cells. After removing tissues, the cells were allowed to grow up to 75%–80% confluency and the cells were harvested as per Kubota *et al*. (2000). After harvesting, the cells were seeded in a fresh 25 cm² flask in duplicates at a seeding density of 3,200 cells/cm² in 4 ml culture media and were labeled as passage number 1. Primary cultures of skin fibroblast were subjected to multiple passaging till 15th passage. Skin fibroblast cells growing in the tissue culture flasks were harvested at late log phase when 75–80% confluency was observed. The spent media was removed and the cell monolayer was washed with 2 ml of DPBS-A. To each tissue culture flask, 1 ml of cold (4°C) trypsin-EDTA was added to make wet layer over the monolayer. Flasks were kept 10 min at 37°C for rounding and detachment of cells. Once the cells were rounded after detachment, 2 ml of complete medium was added to stop the enzyme activity of trypsin-EDTA. After rinsing with medium, cells were collected in a 15 ml tube and centrifuged for 10 min at 1,000 rpm. After centrifugation, supernatant was removed and the cell pellet was resuspended in 1ml of culture media, which was used for cell counting and further treatment with different doses of 5-aza-cytidine. The cell viability, cell proliferation rate (CPR), mean population doubling time (MPDT), and cumulative population doublings (CPD) were calculated during different passages. The viability of cells was recorded at each passage using dye-exclusion method as already described (Freshney 2000). Equal volumes of cell suspension and trypan blue dye (0.4%) was mixed and 10 µl of this suspension was loaded in haemocytometer and cells were counted in 5 squares (4 WBC squares and 1 RBC square). After staining, cells with blue stains were counted as non-viable and viability percentage was calculated.

The cell concentration per ml and viability was calculated as follows:

Cell concentration per ml = Total no. of cells in 10 squares × dilution factor × 10^3

% Dead cells = Dead cell count /Dead cells + Viable cells ×100

% Viability = 100 (Total cells) –% Dead cells

Eighty thousand primary cells (3,200 cells/cm^2) were seeded in 4 ml culture medium in tissue culture flask in duplicate and were given passage no. 1. These cells were harvested at 75 – 80% confluency and at each harvest, passage number was increased by 1 and cells were subcultured and harvested till 15 passages. From the data on cell count at the time of seeding, harvesting and duration of culture for each passage, the cell proliferation rate (r) per day was calculated (Phillips and Cristofalo 1989).

Cell proliferation rate (r) = $3.32 \times (\text{Log } N_H - \text{Log } N_I)/ T_2\text{-}T_1$

where, NH, no. of cells harvested; N_I, No. of cells seeded; T_1, time at seeding (h); and T_2, time till harvesting (h).

The mean population doubling time (MPDT) is expressed as the time taken to double the existing cell population and was calculated (Phillips and Cristofalo 1989).

$$MPDT = 24/r$$

Where, r is the cell proliferation rate.

The cumulative population doubling for each cell was calculated ((Phillips and Cristofalo 1989, Hayflick 1973):

$$CPD = T_2 - T_1/ r$$

where, T2-T1, duration in passage; and r, cell proliferation rate.

The data generated on cell proliferation rate, mean population doubling time, cumulative population doublings and cell viability during 15 passages were grouped into passage intervals of 1–3, 4–6, 7–9, 10–12 and 13–15.

To study the effect of passaging on growth pattern of skin fibroblast, growth curve studies were conducted at 5th, 15th and 25th passage. The cells from 4th, 14th and 24th passage of the cell line were harvested by trypsin-EDTA method as described earlier. For each experiment, 40,000 cells were

Table 1. Oligonucleotide primer sequences, expected amplicon size and annealing temperatures used for real-time PCR assays

Genes	Primer sequence	Size (bp)	Annealing temperature
DNMT1(AY244709)	For: CACCGTTCCTTGTATGC Rev: ATGGGCTGCTCGTCACTG	126	55°C
DNMT3a(AY271298)	For: GACAAGAATGCCACCAAAGC Rev: ATCCACCAAGACACAATGCG	134	55°C
DNMT3b(AY244710)	For: TACGACGACGACGGCTAC Rev: CAGACACTCCACGCAGAAG	108	55°C
HDAC1(BT030718)	For: AAGGAAGAGAAGCAAGAAGCC Rev: GGTTGTGGGATAAAGGTAGGG	108	56°C
GAPDH(NM_001034034)	For: TCTGTTGTGGATCTGACCTG Rev: CCAGCATCGAAGGTAGAAGAG	172	55°C

DNMT1, DNA methyl transferase 1; DNMT3a, DNA methyl transferase 3a; DNMT3b, DNA methyl transferase 3b; HDAC1, histone deacetylase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

seeded in each of 8 tissue culture flasks (25 cm^2) in 5 ml culture medium. Of these, 1 flask was harvested at a regular interval of 24 h for 8 consecutive days. The cell count was made using haemocytometer. The cell count data was plotted against time (days) using MS-Excel 2000. The growth curve for a given cell line was compared at different stages of multiplication at early (5^{th}), mid (10^{th}) and late (15^{th}) passages.

Skin fibroblasts were treated with different doses of 5-aza-cytidine (0.25, 0.5, 1.0 and 1.5 µmol). Total RNA was isolated by Cell to cDNA II kit from the cell line when they have reached nearly 80% confluency of sizable population of dividing cells. Total RNA was quantitated using a picodrop spectrophotometer. Following DNase treatment, RNA was reverse transcribed in to first-strand cDNA using oligo dT primer and Cell to cDNA II kit.

Primer sequences for the genes DNMT1, DNMT3a, DNMT3b, HDAC1 and GAPDH were designed online using Beacon Designer 7.0 (Table 1). Gene transcripts were quantified by real time PCR using the Mx3000p Stratagene System. For each assay, a master mix was prepared that contained SYBR Green, 10 pMol /µl forward and 10 pMol / µl reverse primers and nuclease free water. Thermal cycling conditions applied to each assay consisted of an initial denaturation step at 95°C for 10 min followed by 45 cycles of denaturation, annealing and extension. The quantification of gene transcripts such as DNMT1, DNMT3a, DNMT3b and HDAC1 was carried out in 3 replicates. GAPDH was amplified for every sample as a housekeeping gene for normalization.

Data were analysed by one-way ANOVA using SPSS 7.5. After testing for normality and equal variance, one-way ANOVA followed by post-hoc multiple pair wise comparisons using LSD and Duncan's multiple range tests were employed to determine the difference in the gene expression pattern between 5-aza-cytidine treated and non-treated skin fibroblast cells. Differences were considered to be significant at $P \leq 0.05$. If the n-fold difference relative to the calibrator for each treated skin fibroblast cells equivalent to non-treated skin fibroblast cells did not fall within the confidence interval for the competent donor cells, they were considered abnormal, either up regulated or down regulated.

RESULTS AND DISCUSSION

The pure fibroblast cultures were obtained by fifth passage and no traces of epithelial cell contamination were found. The fibroblast cell line completed an average of 46.06 CPDs during 10 passages. The average duration of one passage was lowest (108.2h) during 3–4 passage and highest (140.00 h) during 1–2 passage (Table 2). The MPDT was lowest

Table 2. Effect of passaging on cell proliferation rate (CPR), population doubling time (PDT) and viability of skin fibroblast cultured cells

Passage No.	Average duration of passage (h)	CPD	PDT (h)	CPR	Viability (%)
P-1–2 (6)	144	27.36	24.74	0.97	99.80 ± 0.24
P-3–4 (3)	108	30.43	26.08	0.92	99.73 ± 0.37
P-4–6 (4)	120	34.89	27.90	0.86	99.92 ± 0.28
P-7–8 (4)	129.42	42.30	30.76	0.78	98.84 ± 0.65
P-9-10 (4)	111.84	46.06	31.57	0.76	97.69 ± 0.26

CPD, Cumulative population doublings; PDT, population doubling time; CPR, cell proliferation rate. Figures in parenthesis indicate the number of observations. Data in the form of mean ± SE for viability and is in mean for others.

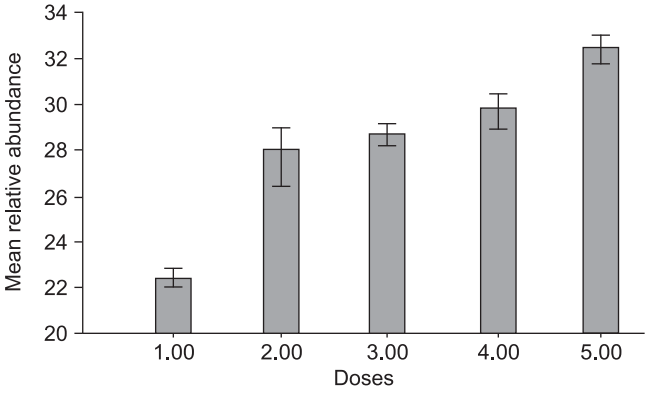

Fig. 1. Relative abundance of DNMT1 mRNA of skin fibroblast at 5-AzaC dilutions of 0.25 µmol, 0.5 µmol, 1.0 µmol, 5 µmol belong to group 1, 2, 3 and 4 respectively and 5th is control.

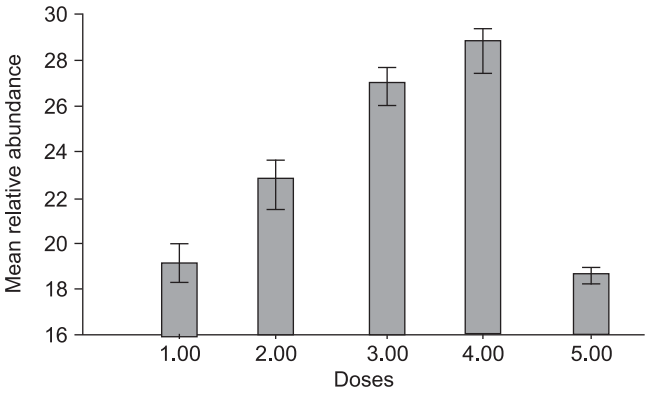

Fig. 2. Relative abundance of DNMT3a mRNA of skin fibroblast at 5-AzaC dilutions of 0.25 µmol, 0.5 µmol, 1.0 µmol, 5 µmol belong to group 1, 2, 3 and 4 respectively and 5th is control.

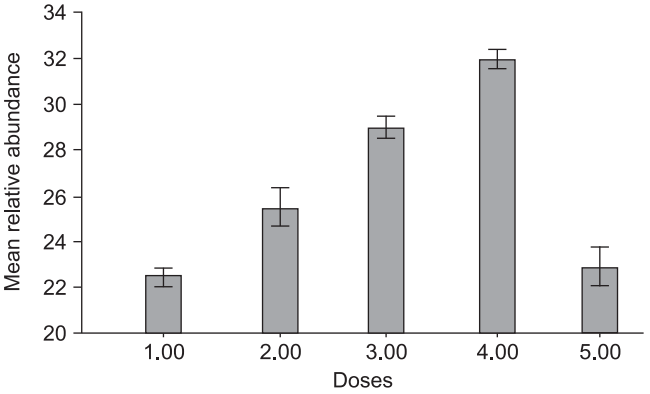

Fig. 3. Relative abundance of DNMT3b mRNA of skin fibroblast at 5-AzaC dilutions of 0.25 µmol, 0.5 µmol, 1.0 µmol, 5 µmol belong to group 1, 2, 3 and 4 respectively and 5th is control.

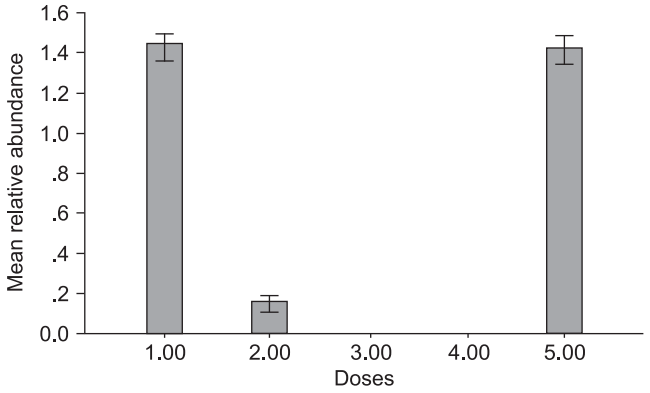

Fig. 4. Relative abundance of HDAC1 mRNA of skin fibroblast at 5-AzaC dilutions of 0.25 µmol, 0.5 µmol, 1.0 µmol, 5 µmol belong to group 1, 2, 3 and 4 respectively and 5th is control.

during 1–2 passage (24.74h) but increased at later passages. The CPR was 0.97/day in early passage (1–2) but in subsequent passages ranged between 0.76–0.92 (P 9–10)/day. The viability of cultured cells varied from 97.69 to 99.92% in different passages. The data was arranged into 5 class intervals of 2 passages (Table 2).

Effects of 5-aza-cytidine on mRNA expression in skin fibroblast cells: The relative abundance of DNMT1 mRNA decreased significantly with the treatment of 5-aza-cytidine (Fig. 1) when compared to control. Both the higher doses (such as 1 and 5 µmol) and lower doses (such as 0.25 and 0.50 µmol) showed down regulation of expression as compared to control and the very low dose 0.25 µmol affected the most expression of DNMT1 and hence, this was dose dependent. Treatment with 5-aza-cytidine (0.5, 1.0, 5.0 µ mol) dose dependently increased the mRNA expression of DNMT3a (Fig. 2) and DNMT3b (Fig. 3) except the lower dose, which showed similar as compared to control. The relative abundance of HDAC1 mRNA decreased with higher doses (0.5, 1.0 and 5.0 µmol) as compared to control, whereas the lower dose 0.25 µmol did not produce any significant effect (Fig. 4). The doses 1.0 and 5.0 µmol induced the silencing of expression of HDAC1 and restoration of transcriptional machinery.

In the present investigation, skin fibroblast cells were harvested at 70–80% confluence by loosening network of cells by washing monolayer with Ca^{++} and Mg^{++} free Dulbecco's phosphate buffered saline (DPBS-A), followed by treatment with dissociation solution trypsin-EDTA. Use of trypsin-EDTA solution for detachment and rounding of skin fibroblasts was also reported earlier (Bayreuther 1988, Katska 2002). Full or over confluent cultures, often resulted into slow growth in subsequent passages probably due to long lag phase. This observation was in agreement with results of Kubota *et al*. (2000) for ear skin fibroblasts. In this study, viability of cells varied from 95 to 100% in different passages across different cell lines of different cell types. This showed that culture conditions were healthy for growing cells. Similar cell viability in skin fibroblast cells were reported in different passages (Hill *et al*. 2001). The overall average cumulative population doublings (CPDs) completed in 10 passages by cell lines derived from skin

fibroblast cells were 46.06. These CPDs during the entire culture period reflect mitotic potential of cell line (Giraldo *et al*. 2006) and final population doubling number achieved by diploid cultured cells can be correlated to lifespan of animal species from which the cells were derived (Hayflick 1965). The cell proliferation rates of skin fibroblast cell were lower in early passages and showed an increasing trend as the culture progressed (7–10 passages). The higher cell proliferation rates during early passages could be due to some degree of mixture of epithelial type cells in skin fibroblast culture. The reduction of cell proliferation rates in later passages could also be due to the replicative aging of cells.

Several studies demonstrated that Dnmt1 and Dnmt3 families of methyltransferases directly interact with one or more HDACs. DNMT1 bound HDAC1 and HDAC2 via its N-terminal regulatory region, and DNMT3a, DNMT3b, and DNMT3L bound HDAC1 through their ATRX-homology domain (Aapola 2002).

In this study, we analyzed the gene expression patterns of HDAC1, DNMT1, DNMT3a and DNMT3b mRNA transcripts in buffalo skin fibroblast. Reduction in the expression of HDAC1, DNMT1 was observed in skin fibroblast cell when treated with higher doses of 5-azacytidine. However, expression of DNMT3a and DNMT3b mRNA increased with 0.5 µmol, 1.0 µmol, and 5.0 µmol respectively and it is noticed that the relative abundance (RA) of DNMT3b is higher than DNMT3a (Bird 1996). Patterns imposed on the genome by the *de novo* DNMTs at defined developmental time points are maintained by DNMT1 and lead to predetermined programs of gene expression during embryo development (Jirtle 2011). The expressions of HDAC1 decreased with increase in doses from 0.25 µmol to 0.50 µmol, while at higher doses of 1 µmol and 5 µmol the chemical 5-azacytidine induced the silenced state and thus helped in gene silencing, as 5-azacytidine is a methyltransferase inhibitor, it works by inhibiting the expression of methyltransferases when compared to control. As DNMT1 and HDAC1 works together in transcriptional silencing, here the silencing of HDAC1 by 5-AzaC at dose level of 1 µmol and 5 µmol halts the methylation machinery. In future, further studies to evaluate the effect of drug, one need to measure either the enzyme activity for DNMT or HDAC1 or assess its final outcome, like level of DNA/histone methylation or histone acetylation following treatment with epigenetic drugs or evaluate the expression of pluripotent genes following treatment, like Oct 4, Nanog, Sox2 etc. Also, additional experiments involving the analysis of apoptotic cell death, cell cycle status; methylation levels etc., in treated and untreated cells may be undertaken in future.

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