Effects of 5 Aza 2′ Deoxycytidine on Proliferation and Differentiation of Embryonic Chick Caudal Region Chondrocytes in Culture

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Abstract: DNA methylation is one of the epigenetic mechanisms which have been implicated in cellular differentiation, ageing and disease development. The effect of hypomethylating drug 5-aza-2′deoxycytidine (5-aza dC) on the biosynthetic profile of caudal region chondrocytes from chick sternum was studied in detail. The chondrocytes in culture were treated with varying doses of 5-aza dC for 48h and maintained subsequently without the treatment and harvested at selected time points for analysis of growth and differentiation status. 15μg/ml of 5-aza dC showed optimum Concentration at which there was a significant increase in DNA synthesis and RNA synthesis as per cell basis. There was also a significant increase in total protein synthesis and collagen synthesis as per cell basis at this concentration. This optimal concentration also showed to up regulate the gene expression of Type X collagen and alkaline phosphatase, which are the marker of hypertrophic chondrocyte expression. These results further support the notion that methylation is the major epigenetic factor controlling the differentiation and maturation of chondrocytes.

Keywords: ALP (alkaline phosphatase); OA (osteoarthritis); 5-aza dC (5-aza-2′deoxycytidine); epigenetics

1. Introduction

Skeletogenesis in vertebrate is characterized by a precise temporal and spatial coordination of growth and differentiation with concomitant reorganization of preexisting structural elements consist of   primarily the extracellular matrix (ECM).This is particularly manifested in the growth of long bone called endochondral ossification (EO), where chondrocytes pass through discrete stages of proliferation, maturation, and hypertrophy and eventually calcification.   The exact molecular mechanism governing the articular cartilage remains oblivious in skeletal biology. This process can be observed in cephalic region of embryonic chick sternum, where the morphogenetic changes in the matrix may be followed by both morphologically and biochemically [1-3]. For example the chick sterna prior to day 16 /17 are mainly composed of closely packed small rounded chondrocytes. These cells usually synthesize predominantly Collagen type II, type IX (and type XI collagens [4, 5]. Starting from day 17 of chick development distinct morphological changes occurs in the chondrocyte morphology in the cephalic region of chick sternum. The chondrocytes become hydrated and markedly increase in size due to cytoplasmic and nuclear swelling. In this hypertrophic stage of the chondrocyte, the cells remain biochemically active and secrete Type X collagen which is considered to be the biochemical marker of hypertrophic chondrocytes   [6]. As hypertrophy progresses, type X collagen synthesis increases to about 45% of total collagen produced   [3]. The marked increase in

type X collagen is accompanied by a relative decrease in other collagens [7] suggesting the change in the extracellular matrix. While in the caudal region of chick sterna the chondrocytes remain rounded and maintains their resting cell phenotype. This is also true in superficial articular cartilage where the chondrocytes remain in their rounded resting state morphology secreting basically the cartilage specific collagen (Type II) as the main biosynthetic product as well as cartilage specific proteoglycans (PGs) which helps in the maintenance of normal articular cartilage functions in the healthy individuals. However this balance is disrupted in osteoarthritis (OA) where there is extensive breakdown of extracellular matrix by aggregenase and collagenases.

During OA increasing number of chondrocytes from articular cartilage undergoes a remarkable phenotypic modulation turning them into hypertrophic chondrocytes as described above. These hypertrophic cells secrete type X collagen and alkaline phosphatase enzyme [8, 9] as well as different cartilage degrading proteases which results in the progression of OA. The maintaining of the normal chondrocytes as that of adult somatic cells is stabilized by epigenetic factors. This raises the possibility that the destabilization of chondrocyte morphology could be the result of change in its epigenetic status.   The role of epigenetic modification controlling the chondrocyte hypertrophy is being suggested [10, 11] but still poorly understood.

In order to study the molecular mechanisms that control the differentiation of chondrocytes, the hypomethylating drug 5-aza-2´deoxycytidine was used in the chick sternal chondrocytes in cultures. This study aimed to find out the optimum dosage of the drug and the effective duration for the treatment of chondrocytes. The effects on the pretreatment of   chondrocytes with this de-methylation drug were further investigated by keeping these cells in long term cultures and studying their gene expression.

2. Materials and Methods

2.1: Chondrocytes culture: About ten sterna of day 17th chick embryos were dissected and removed from the surrounding tissues. The cephalic and Caudal (one third part of the tip region) of sterna were dissected as described by [12]. Tissues were finely diced and pre- incubated for 30 min at 37ºC with Bacterial collagenase type 1A (10mg/ml) purchased from Sigma chemical Co., Dorset U.K. and Trypsin (0.4mg/ml) GIBCO (BRL)   in minimal essential medium. The resulting supernatant containing the perichondria fibroblasts were removed and tissues were further incubated for 60min under the same conditions as above. Released chondrocytes were washed three times with DMEM +10% FCS GIBCO (BRL). Chondrocytes were cultured in 75cm^2 plastic tissue culture dishes or in multiwall tissue culture plates with the seeding density of 1 X 10^5 cells/cm^2. All the cultures were incubated at 37^{0}C in an atmosphere of 95% air and 5% CO$_2$ in DMEM which was buffered with 10mM HEPES and 0.37% sodium bicarbonate and supplemented with 100μg/ml Ascorbate, 100 units/ml penicillin, 0.1mg/ml streptomycin100μg/ml glutamine and 10% fetal calf serum (FCS).

2.2: Treatment of chondrocyte culture:

One hour after plating cultures were treated in triplicates with   0,2.5, 5,10,15 and 20 µg/ml of   5-aza dC (purchased from Sigma chemical Co., Dorset U.K) in the culture medium which contains DMEM + 10% FCS as described above for 48h. The medium was then removed and the cultures were maintained in DMEM +10%FCS + 100µg/ml ascorbate, 100 units/ml penicillin, and 0.1mg/ml streptomycin and 100µg/ml glutamine. The medium was replaced after every other day throughout the culture period. Control cultures were set up as described above without the pretreatment with 5-aza dC.

2.3: Estimation of DNA Content:

The DNA content of the cell pellet was determined by [13]. Cell pellet was suspended in 200µl of fresh extraction buffer (0.01Tris-HCl pH 7.4) and an equal volume of percholoric acid added prior to incubation at 4°C for 30min.The cell pellet was centrifuged at 10,000g for 10min, supernatant discarded and insoluble pellet hydrolyzed with 250µl of 1M percholoric acid at 70°C for 30 min. Hydro-lysate was than cooled and 0.5ml of freshly prepared chromogenic reagent was added and incubated at 37° C overnight. The absorbance was read at 600nm against a blank reagent. Calf thymus DNA standard ranging from 0.5 to 100µg were assayed with each series of sample.

2.4: Assay of DNA synthesis

The cells were incubated with varying concentration of 5-aza-2′deoxycytidine as described in section **2.2** for 48h above and then incubated for 24h in DMEM containing 0.01% transferrin and 4mM amethopterin in dimethyl formamide to inhibit *de-nova* thymidine synthesis. A pulse of [^{3}H] thymidine (5µCi/ml) was introduce for 90min. The wells were then washed with 1.5ml of ice cold phosphate buffer solution (PBS) followed by overnight precipitation of the nucleic acid with 5% ice cold TCA in the presence of cold salmon sperm DNA. The precipitate were washed twice with 1.5ml ice cold 95% ethanol and then solubilized in 2ml of 0.1M sodium hydroxide (NaOH). A 100µl aliquot of the solubilized precipitate was mixed with 3ml of scintillant and counted using a Packard Scintillation counter. Cell number was determined on a coulter counter after briefly trypsinising cells with trypsin /EDTA(0.25M) salt solution.

2.5: Assay of RNA synthesis.
The cells were incubated with varying concentrations of 5-aza-2′deoxycytidine as described in Section **2.2** above and then incubated with [^{3}H] uridine (5µCi/ml) for 24h in DMEM, 10% FCS and 100µg/ml ascorbate/glutamine and 0.01% transferrin. The wells were then washed with 1.5ml of ice cold PBS followed by overnight precipitation of the nucleic acid with 5% ice cold trichloric acid (TCA) in the presence of cold salmon sperm DNA. The precipitate were washed twice with 1.5ml ice cold 95% ethanol and then solubilized in 2ml of 0.1M NaOH. A 100µl aliquot of the solubilized precipitate was mixed with 3ml of scintillant and counted

using a Packard Scintillation counter. Cell number was determined on a coulter counter after briefly trypsinising cells with 0.25% trypsin / 0.25mMEDTA.

2.6: Hydroxyproline assay:

Hydroxyproline assay was done by two methods as was described by [14]. The percentage of proline and hydroxyproline was also measured by amino acid analysis. Triplicate samples were used to assay hydroxyproline.   The other Triplicate samples were used to measure the cell count at the end of the experiment.   The Cells were maintained for 7 days prior to labeling with 1μCi/ml of   L-[5-^{3}H] proline in the presence of DMEM which contains 10% FCS 50μg/ml of βAPN and 100μg/ml of ascorbate /glutamine for 24h. Proteinase inhibitors were added as described before at the end of incubation and the medium samples were then dialyzed against running water for at least 48h. Cell surface were washed once with PBS and scraped off in 500μl PBS and then dialyzed against running tap water. The medium and cell layer/matrix proteins were hydrolyzed in 6M HCl at 120°C overnight. The hydrolyzed were evaporated to dryness under vacuum, the residue dissolved in 1ml of 1M HCl and the radioactivity present in 100μl aliquot measured on the scintillation counter. Hydrolyzed containing about 15,000dpm was loaded onto a column analysis on amino acid analyzer. The samples were eluted with a citrate buffer pH3.0. About 1ml fractions were collected and counted on scintillation counter. [^{3}H] proline and [^{3}H] hydroxyproline peaks obtained were used to calculate percentage hydroxylation.

2.7: Western blotting:

Medium proteins from control and treated chondrocyte cultures were recovered by 30% ammonium sulphate precipitation. The precipitate was dialyzed extensively against PBS buffer and   mixed with 4X sample buffer, boiled (5 min), then run on 8% SDS/PAGE and transferred to poly(vinylidene difluoride) membrane. Membranes were blocked (30 min) in 5% dried milk, then treated with primary Ab (1 h), washed 3 times (PBS/0.05% Tween 20), and incubated (1 h) with secondary horseradish peroxidase-conjugated Ab. Signal was developed with enhanced chemiluminescence and visualized by autoradiography.

2.8: Alkaline phosphatase assay:

Alkaline phosphatase was determined by a modification of the method of Lowry (1955). Chondrocytes were washed with PBS three times. 500μl of ice cold extraction buffer which contains 0.01M Tris-HCl was added to the petri dishes and the cells were lysed by freezing in a liquid ethanol-dry-ice bath and thawed at 37^{0}C for three times. The cell lysate was centrifuged at 10,000g for 20min at 4°C and cell pellet used to estimate the DNA content while supernatant were removed for alkaline phosphatase activity. Alkaline phosphatase activity was assayed by measuring the release of p-nitrophenol from p-nitrophenylphosphate at 37°C. The assay mixture (100μl) contains 0.2M diethanolamine-HCl (pH 9.8), 1mM MgCl2 and 1mg/ml p-nitrophenylphosphate.

2.9: Statistical Analysis

All data are expressed as mean ±SD. The Student's two-tailed unpaired *t* test was used to determine statistical significance of the difference between the pretreated samples vs untreated or control samples.

3. **Results**

The chondrocytes were pre-treated with varying doses of 5-aza-2′deoxycytidine for 48h and then maintained in the normal culture conditions for up to three weeks.   The control and pretreated chondrocytes cultures were grown on multiwall culture plates in triplicates. They were released from the ECM by trypsinisation and collagenase digestion and counted on automated coulter counter as well as viable cell count by trypan blue exclusion test. A dose dependent cytotoxic effect of 5-aza dC was observed at Day 7th in culture (Fig 1).

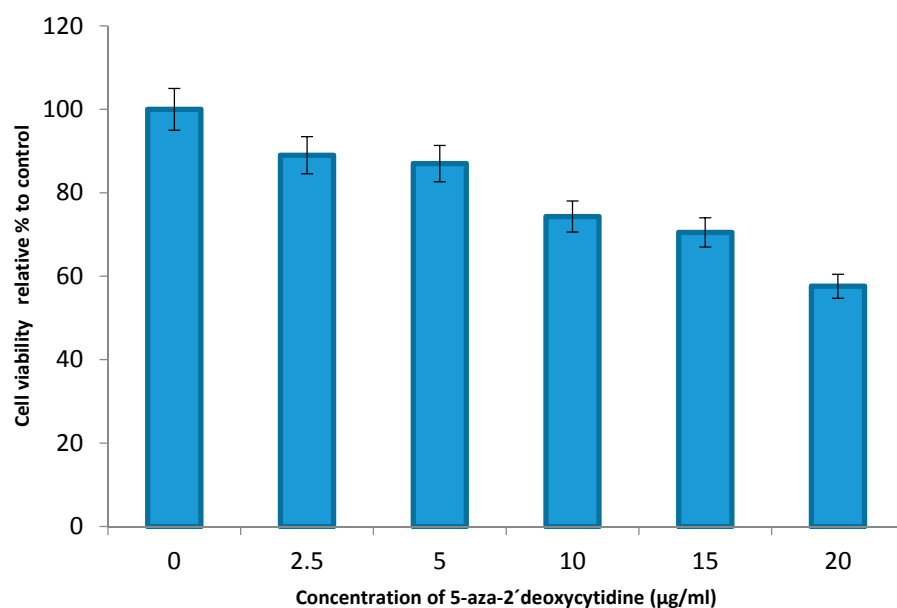



Fig 1: The Chick sternal chondrocytes grown in the absence and presence of varing doses of   5-azadC for 48h and than cultured without any treatments for 7 days. On Day 7th the cells were trysinized briefly and counted on outomated counter as well as viable cell count by trypan blue exclusion dye method   and expressed as % viability relative to control. For each condition three random fields of cells from triplicate wells were counted. Values are normalized by the value in control group.

The total DNA synthesis was assessed at various doses of 5-aza dC after 72h in culture as described

in materials and method section, a dose dependent increase in DNA synthesis on per cell basis was

observed (Fig 2A). The maximum incorporation was observed at 15μg/ml which shows a fivefold

increase in DNA synthesis. In other experiments (results not shown) pre-treated cells with 15 μg/ml

of 5-aza dC   resulted in a two fold increase in DNA content after 7th day in culture as compared to

the control cultures.

(A)   (B)

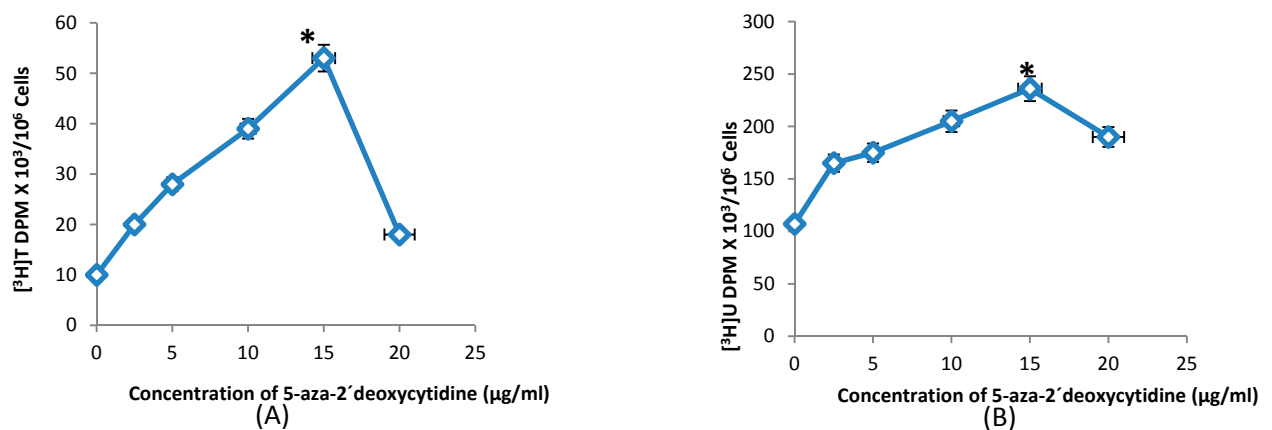



Fig 2.Dose dependent increase in [³H] thymidine and [³H] uridine Incorporation:

A: Embryonic Chick sternal Caudal region chondrocytes were grown in the presence of varying doses of 5azadC for 48h in multiwall plates in triplicate followed by a pulse with [³H] thymidine for 24h in the absence of the treatments. Total [³H] thymidine incorporation in the triplicate control and pretreated cultures (±SD) were expressed as DPM/10⁶ cells. *P< 0.0001 as compared to the control samples.**B**: Control and pretreated chondrocytes were pulsed with [³H]uridine as described in Materials and methods. Total [³H]Uridine incorporation into the control and pretreated cultures in triplicates (±SD) were expressed as DPM/10⁶ cells. *P<0.0001 as compared to the control samples.

When the total RNA synthesis was measured after 72h in culture at various concentrations of

5-aza-2´deoxycytidine, again maximum incorporation of [³H] Uridine was observed at 15μg/ml.

which exhibited 1.5 fold increases in total RNA synthesis per cell basis when compared to cells

without any pre- treatment (Fig 2B).

(A)   (B)

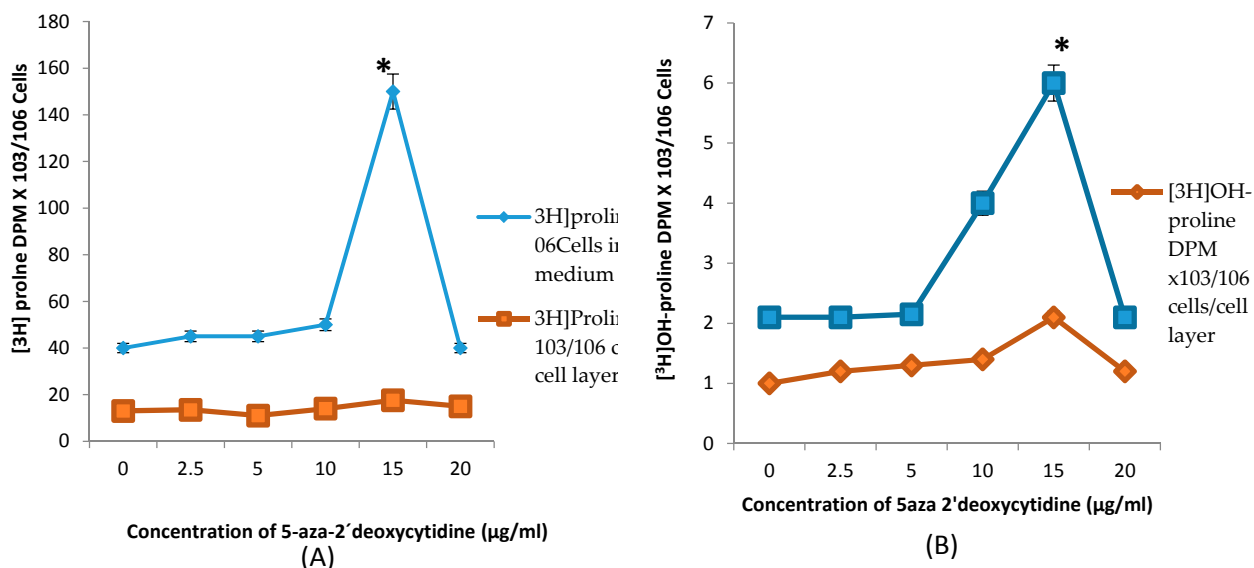



Fig 3: Dose dependent Increase in [³H] proline and [³H] hydroxyl-proline incorporation:The control and pretreated cultures were maintained in the normal culture medium as described in the materials and methods section to Day 7th , after that they were labeled with [³H] proline for 24h. Medium and Cell/matrix proteins were hydrolyzed with 6M HCl after dialysis against running tap water. Hydrolyzed was rotary evaporated and dissolved in 1M HCl and counted on scintillation counter. [³H] proline incorporation was expressed as DPM/10⁶ Cells. For hydroxyproline assay about 1500cpm was applied onto an amino acid analyzer.**A**: Incorporation of [³H] proline into collagenous and non-collagenous medium and cell lysate proteins in triplicate samples (±SD) at day 7th in culture with varying concentration of 5-aza 2'deoxycytidine. *P< 0.0001 when compared to the control samples.**B:** Incorporation of [³H]OH proline into the collagenous medium and Cell lysate proteins in triplicate samples (±SD) at day 7th in culture. *P< 0.0001 when compared to the control samples

The influence of 5-aza-2′deoxycytidine on total protein synthesis was also assessed at day 7th by analyzing the incorporation of [³H] proline per cell into non diffusible macromolecule. The results showed a dose dependent increase in protein synthesis (Fig 3A). In order to determine [³H] proline incorporation into collagenous protein, medium hydro-lysate were subjected to amino acid analyzer in which hydroxyl[³H] proline was measured and plotted as a function of various concentration of 5-aza dC (Fig 3B). Again maximum incorporation was shown at 15µg/ml with the rate of synthesis of collagenous protein increasing about four fold at this concentration. Total [³H] proline incorporation into the cell layer/matrix also indicated a dose dependent increase in both collagenous and non-collagenous protein synthesis. (Fig 3A, B)

As 15µg/ml of 5-aza-2′deoxycytidine was the optimum concentration for chondrocytes proliferation and collagen synthesis, so this concentration was further used to study the gene expression of various collagen types.   The biosynthetic profile showed   that in the pretreated cultures type X collagen represented 25% of the total collagen secreted in the medium as compared to the control cultures where Type X constituted only about 3% of the total collagen being secreted into the medium.   The relative proportion of Type II and Type I collagens were also very different in the control and treated cultures (Fig 4 A, B).

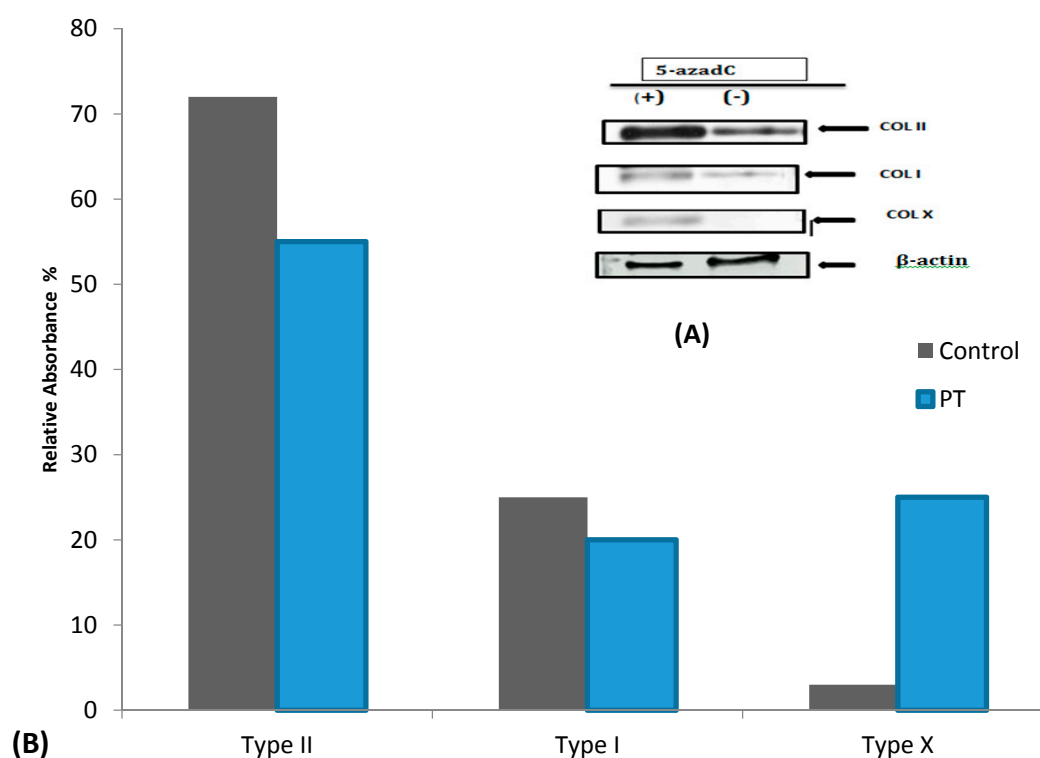
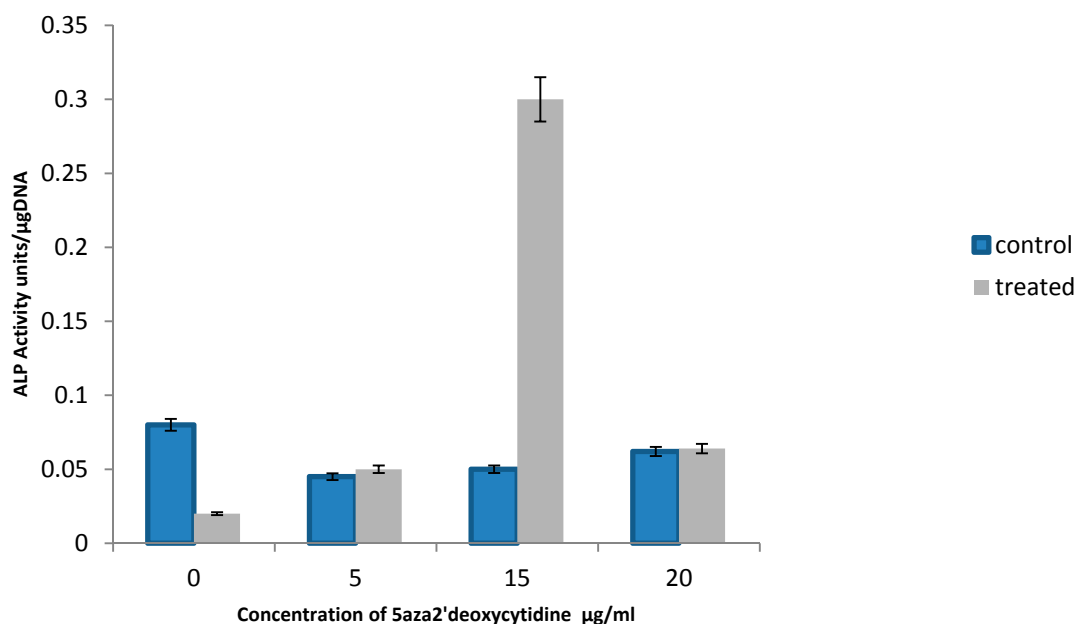



Fig 4: A:Western Blot analysis for Collagen types I, II and X for C (control) and PT (pretreated cells):   Medium proteins from 7-day control and pretreated cultures were extracted by 30% ammonium sulphate precipitation and run on 8% SDS-PAGE. The proteins were blotted onto nitrocellulose membrane and immunoblotted with antibodies against collagen Type I, Type II, Type X and β actin. **B: Histogram of the laser densitometry scans of the western blot in fig 3A.** Laser densitometry quantification for collagen type II, type I and Type X and beta actin as housekeeping gene, using LKB 2202 ultrascan laser densitometer. The results are expressed as relative absorbance in percentage and the amount of Type II, Type I collagen and type X collagen were quantitated   with respect to these types of collagen secreted into the medium.

Alkaline phosphatase enzyme activity which is a biomarker of the mineralization process was also investigated.   The caudal region chondrocytes were treated with various concentration of 5-aza dC

for 48h and then maintained in normal culture conditions as described before for up to 14th days. The amount of the enzyme released into the medium was assessed, which showed a dose dependent increase in alkaline phosphatase activity (Fig 5 A).

4.   Discussion

The involvement of DNA methylation in controlling the differentiation of stem cells has been reported recently in a number of studies, the most relevant is the pretreatment   of bone marrow derived mesenchymal   stem cells with 5 aza cytidine resulted in the osteogenic gene expression [15].   The treatment of cultured cells with 5-Azacytidine results in replacement of cytosine bases in genomic DNA with this analogue, which cannot be methylated by methyl transferase and thus perturbs the methylation pattern of cytidine (CpG islands) present in various target gene promoters. Under normal conditions repression of hyper methylated genes primarily occurs via transcription

factor competition with methyl –CpG binding proteins for binding to regulatory sites in the DNA, thus removing the methylated CpG target with azacytidine treatment facilitates transcription factor access to previously occluded sites in the DNA relieving transcription repression [16, 17].

Since preservation of normal pattern of methylation is crucial for appropriate mammalian development. Azacytidine treatment represents a potent modulator of the developmental process and it has been exploited in a number of different primary culture systems including mouse embryonic fibroblasts [18] and eryhroleukemia cells [19].

In this study the effect of hypomethylating drug 5-aza-2′deoxycytidine (Decitabine) recently approved by FDA to treat myelodysplastic syndrome was investigated on the cultured chondrocytes.   Most of the earlier studies had used 15μg/ml of this drug as the treatment dose [4, 24]. This study investigated the detailed effect of the varying doses of this drug and further emphasized that the optimal concentration of this drug was indeed 15μg/ml.

It is interesting to note that unlike 5-azacytidine, which can intercalate in both DNA and RNA could be effective in inducing differentiation and morphological changes to the cells at any stage of cell cycle and development. 5-aza 2′deoxycytidine has the ability to only intercalate in DNA, so it is most effective at the replicative stage of cells. This replaces normal cytosine and forms irreversible adducts with DMTs, thus inhibiting genome wide methylation [21].   Once the methylation pattern of the growing cells was altered by the treatment of the demethylation drug, growing in its absence would make no difference to the cells as the change in pattern of methylation was conserved and successfully maintained in their daughter cells [22, 23].

This has been demonstrated in this study which showed that the optimum time of treatment was just after plating the primary culture on the culture dishes as the first few cell divisions in the presence of this drug were crucial for the epigenetic imprinting of the cells. After that they were kept in normal culture medium similar to that of control cells.

We observed that there was a significant increase in DNA synthesis as per cell basis in the pretreated cultures as compared to the control. There was also a genome wide enhancement of

transcription as well as translation as that reflected in overall increase in RNA synthesis and protein synthesis in the pretreated cultures when compared to the control cultures. We found that pretreated of chondrocytes induced the expressions of collagen type X with the subsequent induction of ALP expression. The up-regulation of marker genes of hypertrophy (type X collagen) and mineralization (ALP) of chondrocytes demonstrated that the chondrocytes underwent differentiation in the manner similar to endochondral bone formation which occurs in the growth plate *in-vivo*. This study further emphasized the need for looking at epigenetic mechanism at molecular levels in detail and explores its significance in various pathological conditions of the bone.

5. Conclusions

Cells of a multicellular organism are genetically homogeneous but structurally and functionally heterogeneous owing to the differential expression of genes. Many of these differences in gene expression arise during development and are subsequently retained through mitosis. Over the past few years research was focused on the molecular mechanisms that mediate these epigenetics phenomena. In this study we have investigated the effect of demethylation drug 5aza-dC (methyltransferase inhibitor) in promoting proliferation and differentiation of chondrocytes in culture. Our results showed that pretreatment of chondrocytes in culture could results in upregulation of hypertrophic marker genes, thus initiating the differentiating pathways. The cell culture system described here provides an excellent tool to determine the role of methylation in differentiation and maturation of chondrocytes, as well as it can be exploited to study the role of methylation in gene expression of various types of cells in culture.

Acknowledgments: The author extents their appreciation to the research Centre, King Saud University.

Conflicts of Interest: There is no conflict of interest to declare.

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