Annexure – X

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ASSESSMENT CERTIFICATE
(to be submitted with the proposal)

It is certified that the proposal entitled "Chick embryonic pluripotent stem cells as a model to study muscular dystrophy in vitro" by Dr. U. K. Gowri Kumari, Department of Zoology has been assessed by the committee consisting the following members for submission to the University Grants Commission, New Delhi for financial support under the scheme of Major Research Projects:

Details of Expert Committee: 1. (Dr. R.B. SUBRAMANIAN) [Professor, S.P. University, Anand]

2. (Dr. K.C. Patel) [Professor, S.P. University, Anand]

The proposal is as per the guidelines.

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FINAL REPORT

Chick embryonic pluripotent stem cells as a model to study muscular dystrophy *in-vitro*

Muscular dystrophy (MD) is an X-linked recessive disorder related to muscle weakness. A gene complex associated with sarcolemma in myofibres, includes dystrophin, dystroglycan, sarcoglycan, etc. The dystroglycan complex interacts with laminin and dystrophin so it has the role in mediating a connection between actin cytoskeleton and extracellular matrix (ECM) (Henry and Campbell, 1996). The naturally occurring murine model of MD has a mutation in large gene coding for protein dystrophin. The condition is also leading to hypoglycosylation of α-dystroglycan (α-DG) in muscle and brain (Jimenez-Mallebrera *et al*., 2005). The condition ultimately leads to muscle loss and may lead to death. At present several therapeutic options under investigation include adult-derived stem cells' transplantation to counter MD. In spite of the enormous progress in the molecular understanding of MD, there is still no cure. Therefore, the present study aims at understanding the MD by inducing the condition in the muscle cell line differentiated from the avian pluripotent stem cells *in vitro*.

Embryonic Stem Cells (ESCs) as model

The stem cells are capable of undergoing differentiation into several cell types. The true ECSs can give rise to somatic as well as germ cells *in vitro*. Blastoderm derived ESCs of chick also have capacity to form different cell types via Embryoid Bodies' (EB) formation (Petitte *et al*., 2004). These cells because of the relative closeness to mammals in phylogeny share lot if similar features *in vitro* to the murine and human ESCs. There has been a significant progress in developing *in vitro* models from murine and human samples derived from patients. However, the progress in deriving models from chick embryos has not been explored. Methods for isolating ESCs from early embryos of chick are been studied thoroughly (Chapman et al., 2001; Hiriuchi *et al*., Horiuchi *et al*., 2011).

Myogenic differentiation steps *in vitro*

ESCs need to undergo several stages of differentiation to myogenic cells. The protocols are studied for human and murine ESC for the process (Salani *et al.*, 2012). There are two methods available. One is obtaining Multipotent Mesenchymal Precursors (MMPs) and conversion of MMPs to myogenic cells. The other method is of obtaining Embryoid Bodies (EBs) and conversion of those into myogenic precursor cells. Mouse ESCs (mESCs) and human ESCs (hESCs) are converted to EBs by hanging drop cell culture method. The EBs are precursors to various multipotent cell lines. EB formation by this method takes 4-5 days. Amino acids like Insuline, Transferrin and Selenium, growth factors like bFGF (basic Fibroblast Growth Factor), EGF (Epithelial Grwoth Factor), etc are added for further differentiation into myocytes (Rohwedel *et al*., 1998). Regarding human EBs, Zheng achieved generation by varying the concentration of serum (Zheng *et al*., 2006). Serum decrease permits a progressive proliferative decline and stimulates myogenic differentiation through cellular fusion. This cellular mechanism is also enhanced by addition of some specific factors such as dexamethasone, ITS, glutamine, epidermal growth factor (EGF), and 5-AZA [61]. The specific contribution of these substances to the differentiation process is not completely understood, although some mechanisms have been identified. For example, dexamethasone, a potent synthetic member of the glucocorticoid class, enhances myogenic differentiation through the synthesis of dysferlin and sarcolemmal and structural proteins in addition to the up-regulation of specific myogenic transcription factors, as shown in experiments with the C2C12 cell line (Belanto *et al*., 2010)

Advantages of cESC as models

Chicks are close to mammals in phyogenetic history. Therefore, they are frequently used as models to study several aspects of vertebrate embryology and development. Chicks are easily available and less expensive models. Isolation of cells from these do not involve many ethical issues. The mammalian models specially the ones derived from patients require high cost and ethical permissions. The attempt is to develop a less expensive still relevant model for study of MD *in vitro* for preclinical studies as well as for study of muscle pathological conditions.

Methodology

Procuring eggs

Fertile Rhode Island Red (RIR) eggs were procured from government intensive poultry farm just after they were laid.

Harvest and culture of cESC

The eggs were wiped with Povidone-Iodine and all the procedures were carried under hood in Laminar Air Flow.

The cells were isolated from stage X of chick embryo according to EGK stages, which are unincubated eggs. The embryo was isolated with the help of filter paper ring. The rings were made of Whatman paper number 3 (cat no. 1003-125) by punching the paper and cut the circle or square around the hole (fig.1).

The general method for harvesting cESC is described by Horiuchi and group. Egg is broken and content is poured in 90 mm glass dish. The embryo must be placed on the upper side. The filter paper ring is put on the embryo such that the hole is just above it. The ring is cut from outer edge along with the vitelline membrane onto which the embryo is stuck. The embryo along with the paper ring is placed in 60 mm dish which is having PBS (calcium and magnesium free, laboratory made). The embryo is washed thoroughly to get rid of yolk as much as possible as yolk attracts contaminants into culture plate.

There are two regions in the embryo at this stage- area pellucida and area opaca (fig. 2). Both these areas are comprised of the cells which are pluripotent. The isolated and washed embryo is harvested with the help of 1000 micro litre's micropipette. It is added in the micro centrifuge tube with DMEM low glucose with antibiotics. Cells from four such embryos will be added to 500 micro litre of media mix which contain 10% FBS (Gibco, catalogue number 26140111) and 1x antibiotics (Gibco, 15240062). The cells were counted using haemocytometer. Each embryo is comprised of $2x10^4$ cells/ml. The cells can remain in pluripotent state for about 7-10 days according to seeding density. The higher seeding density yields early embryoid body formation.

Embryoid body formation

The cells which are not plated onto the feeder layer get converted into embryoid bodies in 10-11 days post plating. Thus, the pluripotent cells were maintained in undifferentiated state onto the feeder layer developed as described in the next point. However, for the differentiation process some of the stock pluripotent cells are taken and used for hanging drop cell culture to make even sized embryoid bodies. In case of normal plating, the cells form embryoid bodies with different sizes which show high variability of differentiation process further.

Hanging drop cell culture

The type of culture is performed when the pluripotent cells' density reaches $2x10^6$ cells/ml. 20 µl of 1 ml of such cell suspension (in media mix) is taken and placed on the lid of a 60 mm culture dish (fig. 3). Four such drops can be placed in one lid. The dish is poured with PBS to keep the humidity high and reduce evaporation of small amount of media. The lid is placed on top of the dish and incubated till embryoid bodies are formed (Teng Y, 2015).

Differentiation

As soon as the embryoid bodies start to appear, they are collected and placed in a differentiation media in 35 mm tissue culture grade dishes. The differentiation media compositions were tried with various ratios of bFGF, ITS, horse serum and chicken serum in the medium DMEM/low glucose. Effects of addition of Horse and chicken serum were used from 2% to 20%, ITS from 2% to 15% and bFGF from 2 to 20% were checked based on literature survey for differentiation (Salani et al. 2011; Lawson and Purslow, 2015). Media mix was added every 48 hours to avoid nutritional deficiency. However, the embryoid bodies were not pelleted and disturbed, the media was only added and never replaced. RNA was isolated and gene expression for myogenesis related genes was checked after 10 days of addition of differentiation media. DMEM/low glucose with 2% horse serum, 10% bFGF and 5% ITS resulted in optimum cell differentiation.

Antibody mediated dystrophy - induction

The anti-dystroglycan antibody, IIH6 (DSHB, University of Iowa) was used to target the α-dystroglycan and severe the linkage between the dystrophin and laminin α-2 in

the cultured cESMC. Cells were divided into different groups: untreated control (UC), supernatant treated control (STC), isotype control (IC) and treatment group (T). The UC cells received no experimental treatment. The STC culture was treated with 10% supernatant alone of IIH6 Ab solution prepared by centrifugation of antibody solution mixed with 50% ammonium sulphate at 5000 rpm for 20 minutes at 4^0C, so as to rule out any non-specific effects of antibody solution or the preservatives present in it (Grodzki and Berenstein 2010). The IC culture was treated with IgM isotype (Sigma-Aldrich, USA); the dose chosen was similar to that of the IIH6 Ab used in the T group. In the T group of culture, for initial standardisation of antibody-blockade, different culture plates were added with the antibody on third day of fusion and were tested with different concentrations of the antibody: 2.1, 1.05, 0.525 and 0.350µg/ml media mix.

Trypan blue cellular exclusion test

10 µl media containing the detached cells from each group of cultured cells was taken for the test at the intervals of 8 hours for 24 hours post-treatment. From these cell suspensions, percentage non-viable cells were counted with the help of trypan blue exclusion test. 0.04% trypan blue (in PBS) was added in 1:1 ratio to collected cell suspension and 10µl of this mixture was immediately taken on haemocytometer for calculating the ratio of dead (blue) vs. live (colourless) cells (Strober 2015). Values of percentage nonviable cells were further tested by performing two-way ANOVA (GraphPad Software Inc., USA).

Amongst all the antibody treatment doses, 0.525 µg/ml concentration of the IIH6 antibody was selected for further observations, as the said dose seemed to induce morphological changes similar to dystrophic phenotype and the cell deaths were relatively less. Media mix was not changed during differentiation phase. However, at every 48hours' interval, media mix was added to compensate the loss of evaporated media.

Myotube morphometry and contractibility assays

For morphometry, twelve-well plates were seeded with cESMC for each of the UC, STC, IC and T group (0.525 µg/ml). Further, three fields of view (under 10x objective) were selected randomly from each well for counting numbers of myotubes. For

widths, five randomly selected myotubes were considered per well. Myotube diameters were calculated by ISCapture software (Tucsen Photonics, China). One way ANOVA was performed for understanding the significant deviation in average numbers and widths of myotubes of treated cultures relative to the control cultures.
Since there were no significant differences in above two phenomena among the three control groups, all further experiments were carried with only two groups IC and T (0.525 µg/ml). For testing the contractibility, 12-well plates were seeded with cells, treated (one with isotype control and one with IIH6) and grown till myotube stage, 80mM KCl was added to each well at a time and time taken for starting the contractions in each was noted (Paulino et al. 2003). Independent sample t-test was performed to look for any significant variation in the means of contraction times among the groups. After formation of myofibres, number of myofibres with normal and dystrophic phenotypes was calculated and plotted in a graph after performing student's t-test (GraphPad Software Inc., USA).

LADD and DAPI staining

To understand whether the antibody blockade can hinder myoblasts fusion, on the very day of seeding the cells were set as IC and T groups. LADD multiple staining was done three days post-seeding according to the protocol described by Rhys McColl and co-workers (McColl et al. 2016). The cells were washed with PBS thrice to get rid of media and were fixed in 70% ethanol. Later, the stain was added at final volume of 500 µl which was prepared from 0.50 g toluidine blue (Sigma-Aldrich, USA) and 0.15 g fuchsin (Sigma- Aldrich, USA) in a final volume of 100 ml of 30% ethanol. The cells were kept immersed in the stain for 5 minutes. Excess stain was removed with the help of PBS. All the culture plates were observed under phase contrast inverted microscope (Lawrence & Mayo, India) LM-52-3501. Fusion index was calculated by counting cells undergoing fusion in ten fields (two from each well) under 20x objective view. The myoblast fusion was analysed by calculating fusion index [(percentage cells fusing/total cells in field)*100]. Data was analysed using independent sample t- test to identify significant mean differences. For DAPI staining, the cells were treated with 30nM DAPI solution (prepared in PBS). Thirty minutes after adding this solution, the images were taken by Leica EZ camera. The staining procedure was carried out in dark.

Detection of cell death

IC and T group cultures were seeded over a coverslip and were washed with PBS twice to get rid of media completely. Coverslips were immersed in 1 ml PBS added with 80 µl EtBr/Acridine orange mix prepared from 100 µg/ml concentrations of each dye (Ribble et al. 2005). After five minutes of incubation, apoptotic cells (orange coloured) were scored under fluorescent microscope (Leica DM2500). Images were taken by Leica EZ camera. Twenty fields were scored under 40x objective view and calculated percentage of apoptotic cells in each. Further, student's t- test was performed to check the significant variation among the two groups.

Cleaved Caspase-3, β-dystroglycan and MyoD localization

The cells were seeded on APES coated slides for Immunocytochemistry. The whole protocol was carried out by keeping coverslips upright in the petridishes of 35mm diameter. Coverslips were rinsed with PBST and fixed with chilled methanol for five minutes. Then they were washed thrice with PBS. Antigen retrieval was carried out by adding preheated retrieval buffer (100 mM Tris buffer in 5% Urea solution) at 95°C on the coverslips for 10 minutes. Again three subsequent washes with PBS were given for five minutes each. The coverslips were then incubated with permeabilization buffer for 10 minutes at room temperature. Three PBS washes of five minutes each were given again. Blocking was performed by incubating in blocking buffer for 1hr at room temperature. The cells were incubated in primary monoclonal cleaved Caspase-3 antibody (Sigma Aldrich, USA), diluted 1:500 in PBST, for one hour at room temperature. For MyoD localisation primary monoclonal antibody (DSHB, University of Iowa) was used. Primary anti-β-dystroglycan (DSHB, University of Iowa) was diluted 50 times in PBST for its localization. Excess antibodies were removed by giving three PBS washes for 5 minutes each. Samples were then incubated in FITC-conjugated secondary goat anti- rabbit IgG antibody (GeNei, India) diluted 200 times with PBST, for one hour at room temperature in dark for cleaved Caspase-3 localization. For MyoD and β-dystroglycan localisation, *anti-*mouse secondary antibody conjugated with FITC (*Genei*, India) was used. Excess antibodies were washed off by washing thrice (for 5 minutes each time) with PBS. The fluorescence was observed and captured with the help of fluorescent microscope and camera. Images were merged using ImageJ software.

Western blot of active Caspase-3

Protein expression status of cleaved Caspase-3 was checked by Western blot method. Protein was isolated from the approx. 10^8 cells in the lysis buffer (50mM tris pH 7.5, 200mM NaCl, 10mM $CaCl_2$ and 1% triton-X 100)**.** The lysed mixture was centrifuged further at 12000 rpm for 10 minutes and the supernatant was then used for protein estimation done by Bradford method (Bradford, 1976). 30 microgram of protein was then allowed to electrophorese through SDS-PAGE, on 12 % gel to separate the constituent proteins, which were then transferred on PVDF membrane by blotting method. The membrane was stained next, with Ponceau to observe the quality of transfer and then subjected to cleaved Caspase-3 specific primary antibody (Sigma Aldrich, USA), overnight. Further following the complete protocol of blotting and due exposure to the secondary antibody (goat anti- rabbit IgG antibody, GeNei, India) exhibited clear bands of protein, which were tested for their band intensities, using Doc-ItLs software (DBA Analytik Jena US) and the obtained values were normalised with the corresponding β- actin band intensities.

Gene expression patterns of myogenesis related genes

The UC, IC and T groups were separately harvested in TRIzol reagent (Invitrogen) at secondary myotube stage for RNA isolation. The process was followed by decanting media followed by PBS (Calcium-Magnesium free) washing and then addition of TRIzol. Cells added with TRIzol were detached with the help of sterile cell scraper (Tarsons, India). Three dishes confluent with cells were utilized for RNA isolation in 1 ml TRIzol reagent. RNA was reverse transcribed to cDNA by High Capacity cDNA Reverse Transcriptase Kit (applied biosystems, USA) according to manufacturer's instruction. These were screened for gene expression of MYOD1, TGFB1, MYF5, LAMA2 and MYOG with the help of polymerase chain reaction with an initial denaturation at 94ºC for 3 minutes, followed by 32 cycles of 10s at 94ºC (denaturation), 20s at 60ºC (annealing), and 20s at 72ºC (extension). 18s rRNA was used as a house keeping gene. Following were the primer sequences:

MYOD1: fwd: CGGAATCACCAAATGACCCAA; rev: ATCTGGGCTCCACTGTCACT
TGFB1: fwd: TCGACTTCCGCAAGGATCTG; rev: CCCGGGTTGTGTTGGTTGTA
MYF5: fwd: CCAGGAGCTCTTGAGGGAAC; rev: AGTCCGCCATCACATCGGAG
LAMA2: fwd: TCCCCTCTTGATTCGTGTGC; rev: AAGCCAGAGTCAGCCATTGT

MYOG: fwd: CATCCAGTACATCGAGCGCC; rev: GCTCAGGAGGTGATCTGCG
18s rRNA: fwd: GGCCGTTCTTAGTTGGTGGA; rev: CAATCTCGGGTGGCTGAAC

Real Time RT-PCR

Real time Reverse Transcriptase PCR was performed on a Lightcycler96 (Roche Diagnostics, Switzerland) for MYOD1, TGFB1, MYF5, LAMA2 and MYOG to identify relative quantities of these in isotype-control cells and IIH6 treated cells. The program was set as follows: 3 minutes at 95^{o}C, 35 cycles (each cycle of 10 seconds at 95^{o}C, 10 seconds at 60^{o}C and 10 seconds at 72^{o}C). Melting curves for each well was used to confirm the specificity of the products. 18s rRNA was used as an internal loading control. Mean Cq values of control gene expressions were normalised with internal control gene expression for each group (control and treated). Fold change in expression of both the genes compared to control group was calculated using $2^{-\Delta\Delta Cq}$ values as described by Livak and Schmittgen (Livak and Schmittgen 2001). Data was analysed by student's t test for significance of mean differences.

Statistical operations

All the above mentioned statistical analysis was carried out using GraphPad Prism v5.03 (GraphPad Software Inc., USA).

Results

Chicken ESC maintenance *in vitro*

The cells were maintained in the standardised culture condition of DMEM low glucose with 10% FBS for 10 days. Further they detach and form EB (fig. 6). EBs were further converted to myogenic cells with the help of ITS and bFGF as described in methodology section. The process was prevented by co-culture with feeder layer developed from fibroblast cells isolated from chick for keeping a stock of cESCs on hand (fig. 5). The pluripotency was confirmed by amplification of specific products for Nanog and Oct4 (fig. 4).

IIH6 antibody mediated blockade of laminin α2 and α-dystroglycan

Addition of different concentrations of antibody led to modulated severity of phenotypic abnormalities. For the T group 0.525µg/ml was finalized based on the following results.

Trypan blue exclusion test

All the selected concentrations of IIH6 treatment caused significant cell death in cultured cESMCs. 2.1 µg/ml of IIH6 caused more than 50% mortality in the duration of 24 hours of treatment. This fast progression of cell death is not ideal for the model system of dystrophy as increasing cell death evokes immediate inflammation and early breakdown of an *in vitro* system, making it of no use for further experimentation. About 50% cells became nonviable in second treatment group with 1.05 µg/ml IIH6, making it unsuitable again. The next two doses decreased viability in a gradual manner and nearly 10% or less cells died in a day's time. The lowest concentration caused too less mortality. Therefore, the T group was set to receive 0.525 µg/ml concentration. The cell deaths recorded in all the different doses of the T group were significantly higher than that in the IC group given 2.1 µg/ml of IgM isotype antibody (fig. 7).

Myotube morphometry and contractibility

Both myotube numbers and widths showed that the selected dose of IIH6 caused significant changes in morphological features of the cultured cESMCs. Average number of myotubes was found to be around 11 in UC, STC and IC cultures while in

the T group, the number was significantly reduced (***p≤0.001) (fig. 8). Mean myotube widths of T group cultures showed statistically no significant variation relative to the controls (fig. 9), however, there was an aberrance observed amongst the myotubes of T group where the upper and lower limits of myotube diameters showed noticeable variations.

While testing the contractibility, addition of KCl to the culture media led to spontaneous contraction of cells in less than 20 seconds in all the UC, STC and IC culture wells. The T group deferred showing a highly significant decrease in the contraction time (***p ≤0.001) (fig. 10).

7-8 days post-fusion, the morphology of myofibres in the T group showed very evident alterations which included: wavy margins, splitting in between and at the ends of myofibres, breakage, damage, bifurcations and heavy cell death; while such changes were not observed in the control phenotypes (fig. 11A-G). Typically the myofibres also lost the normally seen directionality while growing (fig. 11H and I). Percentage of cells bearing normal morphology reduced in the treated group significantly (fig. 11J).

LADD and DAPI staining

The LADD staining enabled a clear visualization of the cell status, where the IC group showed a clear fusion of myoblasts into myotubes with multinucleate condition (fig. 12A and C). The T group showed fewer numbers of myoblasts fusing into myotubes and most of the cells being in uninucleate condition (fig. 12B and D). The Fusion index of the T group cells showed a sharp decline, relative to the IC group (***p ≤0.001) (fig 12E). Multinucleation of fused myocytes was distinct in IC group whereas the T group showed separate cells. This was evident when the cells were stained with a nuclear stain DAPI (fig 12F).

Detection of cell death by EtBr/AO staining

Analysis by fluorescence based technique showed that the T group cells showed characteristic dystrophic cell death. At primary myotube stage, the T group showed both the apoptosis and necrosis, visible by red and orange fluorescence respectively (fig 13A-13E). When apoptotic and total number of cells were counted, it was found

that about 30% cells were seen to be apoptotic on an average in T group while the apoptotic index was below 10% in IC group (fig 13E).

Cleaved Caspase-3, β-dystroglycan and MyoD1 localization by immunostaining

Localization of cleaved Caspase-3 through immunostaining in the treated cells further supported the earlier result indicating that, blocking dystroglycan can switch on apoptotic pathway during early myogenesis. Large amount of cells showed the presence of cleaved Caspase-3 which adduced the atrophic state in T group compared to the control (fig 14). There were fewer signals from cells for protein MyoD1 in treatment when compared to control groups at the stage when fusion starts (fig 15). This is the stage when MyoD1 gets highest expressed in these cells (Abmayr and Pavlath 2012). β-dystroglycan localization revealed that unlike control cells, the treated myocytes showed decreased immunofluorescence. β-dystroglycan is known to be held at cell membrane by α-dystroglycan which further connects to laminin. Blockage of laminin and α-dystroglycan bond seems to cause delocalization of β-dystroglycan in the treated myocytes (fig 16).

Western blot analysis of cleaved Caspase-3

The Western blot analysis for cleaved Caspase-3 shows significant increase in expression under the effect of the treatment. This is in accordance with the localisation results, further enforcing the results obtained.

Gene expression patterns of MYOD1, TGFB1, MYF5, LAMA2 and MYOG

While looking for the expression pattern of the characteristic dystrophic genes in the primary myotubes of control and treated cultures, we found an upregulation of TGFB1 levels in T group relative to the IC. Also, a sharp downregulation of genes related to muscle sculpture- MYOD1, MYF5, LAMA2 and MYOG was noticed in the T group (Fig. 17). The control muscle cells contained high level of LAMA2 which was diminished by 0.05 folds in treated group. There were 0.009, 0.5 and 0.06 fold reduction in the levels of MYOD1, MYF5 and MYOG respectively in the T group compared to the IC group (Fig. 17).

Discussion

The development of the complex muscle tissue at the molecular level has been studied with the help of various animal models like Drosophila, Zebra fish, chick and mouse (Nunes et al. 2005). Use of chick as model to study myogenesis has a long history in developmental biology research (Dennis et al. 1984; Masaki 1974). The chick embryonic skeletal muscle cells were used as an *in vitro* model to study effect of vitamin-E in prevention of cell death (Nunes et al. 2005). An earlier report described the method for isolation and culture of muscle cells from breast regions of developing chick (Godinho 2006). Here, we attempted to culture *in vitro*, the muscle cells which could feature the condition of muscle dystrophy. The source was chick embryonic cells which gave the advantages of ease of availability, collection, and manipulation of cells. The blockade of the connection between the extracellular matrix and cytoskeletal proteins of muscles by antibody IIH6 lead to impairment of the connection between dystrophin and laminin, which resulted in the muscle cells, phenotypically similar to dystrophic muscle. A similar method of alteration of muscle cell morphology was earlier shown in the mouse skeletal muscle cells (Brown et al. 1999).

The phenotype of the targeted and cultured cells in the current experiment showed morphological variations similar to the MD condition *in vivo* or in few other animal models for MD: i.e. similar muscle cell phenotype was reported in patients suffering from muscle dystrophy (Isaacs et al. 1973; Schmalbruch 1976). Also, similar decrease in number and diameter as well as splitting of the myofibres was also observed in the dystrophic mice derived myofibres (Hernández-Ochoa et al. 2015). The variations observed in the diameter and the huge upper and lower limit-differences are characteristic features of dystrophic muscles. Thinner and few myotubes is the characteristic feature of dystrophy *in vivo* as well. These thinner and fewer myotubes might as well be responsible for impaired signal transmission and contraction. Our model also showed cell death and clumps of the dead cells in between the growing myofibres, disrupted basement membrane, multi-directional myofibres- the typical characters of myopathies as described in earlier reports (Brown et al. 1999; Williamson et al. 1997).

Further in an earlier *in vitro* study the muscle cells from the hind limb of mice when treated with IIH6 antibody to target α-dystroglycan, it was reported that the procedure did not affect the fusion or myotube formation process and showed no indications of apoptosis till 7 days post-fusion (Brown et al. 1999). In contrary, we observed that the formation of myotubes was delayed and high level of apoptosis was induced by the blockade of α-dystroglycan within 3 to 7 days of fusion process. There was almost 50% decrease in proportion of the cells undergoing fusion in treated cells when compared to control. This observation accentuates the role of α-dystroglycan in initial myogenesis in the chick embryo and further the above varied observation might even indicate variations in activation of specific molecular pathways in different species as well.

Laminin α-2 is an extracellular matrix protein and is known to hold the muscle tissue integrity by reconciling the attachment and organization of the cells. A deficiency of laminin α-2 was found to be typical to most of the cases of muscle dystrophy (Jimenez-Mallebrera et al. 2005). In concurrence to this occurrence, in our experiment, the dystrophied-like cells *in vitro* showed a decreased expression of LAMA2 gene. Additionally, our study showed an increase in the expression level of TGF-β, which is a known regulator of fibrosis and inflammation. Muscles from DMD patients are known to express more TGF-β, which stimulates the myoblasts to form myofibroblasts (Li et al. 2004). The heightened levels of TGF-β, extra cellular matrix components get deposited, resulting in fibrotic changes and damage to membrane integrity of the muscle cell. Our model showed rise in the level of TGFB1 gene as well. Two other genes, MYOD1 and MYOG which got modulated by IIH6 treatment in our model are widely known to regulate cell numbers and differentiation in muscle cells. In case of reprogramming during myogenic regeneration, MYOD1 gene remains upregulated in these tissues (Bentzinger et al. 2012).

Disorganisation of costameres in case of dystrophin gene defects are known to cause the inappropriate ROS generation and membrane leaks (Goldstein and McNally 2010). Our results showed that several of the dystrophy induced myofibres ruptured at various points along the lengths. Similar descriptions were reported earlier, where even low level mechanical pressure could bring about the membrane rupture in case of dystrophy (Friedrich et al. 2008; Biondi et al. 2013).

Currently available models including mdx mice are known to exhibit different disease progression than that in human at both phenotypic and molecular level. Moreover, there are alternative pathways like the utrophin upregulation when dystrophin is knocked off in mice (McGreevy et al. 2015). Even the closely related model GRMD were found to escape from disease progression via Jagged1-Notch pathway (Vieira et al. 2015). Above cases show how different species can evade the dystrophic condition even in absence of dystrophin gene mutations, unlike human.

Collectively, all the above discussed models to muscle dystrophy have been quite efficiently able to analyse the disease pathology at various level, nevertheless, there remains a limitation. Among all these, the cells from the chick embryo as discussed here can be manipulated to resemble the muscle dystrophy condition *in vitro* with much ease and involves minimal animal sacrifice and ethical concerns. The morphological alterations, contractibility, atrophy and gene expression of the altered *in vitro* muscle cells we report here, shares high similarities to the MD condition. It could be an efficient model with low cost of development and maintenance, as it does not need many expensive growth factors and regulators. Owing to these advantages we believe that these MD like cell culture system would be helpful in bringing further advancement in understanding the early molecular changes of this disease and also during preliminary drug evaluation.

References

1. Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell [Internet]. 1987 Dec [cited 2017 Feb 16];51(6):987–1000. Available from: http://linkinghub.elsevier.com/retrieve/pii/009286748790585X
2. Wright WE, Sassoon DA, Lin VK. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell [Internet]. 1989 Feb [cited 2017 Feb 16];56(4):607–17. Available from: http://linkinghub.elsevier.com/retrieve/pii/0092867489905837
3. Braun T, Bober E, Buschhausen-Denker G, Kohtz S, Grzeschik KH, Arnold HH, et al. Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. EMBO J [Internet]. 1989;8(12):3617–25. 4. Taylor DJ, Frank J. Structure and Function of the. 2007;(Chapter 21):59–85.
5. Alan E. H. Emery, Francesco Muntoni RCMQ. Duchenne Muscular Dystrophy. 2015. p. 320.
6. Theadom A, Balalla S. Prevalence of Muscular Dystrophies : A Systematic Literature Review. 2014;259–68.
7. McGreevy JW, Hakim CH, McIntosh MA, Duan D. Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. Dis Model Mech [Internet]. 2015;8(3):195–213. Available from: http://dmm.biologists.org/content/8/3/195.full.pdf
8. Manning J, O'Malley D. What has the mdx mouse model of duchenne muscular dystrophy contributed to our understanding of this disease? Vol. 36, Journal of Muscle Research and Cell Motility. 2015. p. 155–67.
9. Brown SC, Fassati A, Popplewell L, Page AM, Henry MD, Campbell KP, et al. Dystrophic phenotype induced in vitro by antibody blockade of muscle α -dystroglycan-laminin interaction. J Cell Sci. 1999;112:209–16.
10. Salani S, Donadoni C, Rizzo F, Bresolin N, Comi GP, Corti S. Generation of skeletal muscle cells from embryonic and induced pluripotent stem cells as an in vitro model and for therapy of muscular dystrophies. J Cell Mol Med. 2012;16(7):1353–64.
11. Godinho RO. In vitro development of skeletal muscle fiber. Braz J morphol Sci

[Internet]. 2006;23(2):173–86. Available from: http://jms.org.br/PDF/v23n2a02.pdf

12. Rhee D, Sanger JM, Sanger JW. The Premyofibril : Evidence for Its Role in M yofi bri l logenesis. 1994;24.
13. Williamson RA, Henry MD, Daniels KJ, Hrstka RF, Lee JC, Sunada Y, et al. Dystroglycan is essential for early embryonic development: disruption of Reichert' s membrane in Dag1-null mice. Hum Mol Genet. 1997;6(6):831–41.
14. Yoshida M, Suzuki A, Yamamoto H, Noguchi S, Mizuno Y, Ozawa E. Dissociation of the complex of dystrophin and its associated proteins into several unique groups by rt -octyl P-D-glucoside. 1994;1061:1055–61.
15. V. Hamburger HLH. A series of normal stages in the development of the chick embryo. Dev Dyn. 1951;88(1):49–92.
16. Hillier LW, Miller W, Birney E, Warren W, Hardison RC, Ponting CP, et al. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature. 2004;432(7018):695–716.
17. Ciglar L, Furlong EEM. Conservation and divergence in developmental networks : a view from Drosophila myogenesis. 2009;754–60.
18. Genetics C. The molecular regulation of myogenesis Developmental Biology : Frontiers for Clinical Genetics The molecular regulation of myogenesis. 2014;(August).
19. Abmayr SM, Pavlath GK. Myoblast fusion: lessons from flies and mice. Development. 2012;139(4):641–56.
20. Masaki T. Immunochemical Cardiac , Fast Comparison Slow of Myosins and from Chicken Muscle * Smooth slow The properties of anti-myosin have been investigated in particular detail , but the re sults of these experiments are rather compli cated . Usually several prec. 1974;76(2):441–9.
21. Dennis JE, Shimizu T, Reinach FC, Fischman DA. Localization of C-protein isoforms in chicken skeletal muscle: Ultrastructural detection using monoclonal antibodies. J Cell Biol. 1984;98(4):1514–22.
22. Biochemistry C, Part P, Federal ICU, Oliveira R, Universidade G. Vitamin E prevents cell death induced by mild oxidative stress in chicken skeletal muscle cells skeletal muscle cells. 2015;(July).
23. Hernandez-Ochoa EO, Pratt SJP, Garcia-Pelagio KP, Schneider MF, Lovering

RM. Disruption of action potential and calcium signaling properties in malformed myofibers from dystrophin-deficient mice. Physiol Rep [Internet]. 2015;3(4):e12366–e12366. Available from: http://physreports.physiology.org/cgi/doi/10.14814/phy2.12366

24. Isaacs ER, Bradley WG, Henderson G. Longitudinal fibre splitting in muscular dystrophy: a serial cinematographic study. J Neurol Neurosurg Psychiatry [Internet]. 1973;36(5):813–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4753877
25. Schmalbruch H. Muscle fibre splitting and regeneration in diseased human muscle. Neuropathol Appl Neurobiol [Internet]. 1976 Jan [cited 2017 Feb 16];2(1):3–19. Available from: http://doi.wiley.com/10.1111/j.1365-2990.1976.tb00558.x
26. Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, et al. Transforming Growth Factor-β1 Induces the Differentiation of Myogenic Cells into Fibrotic Cells in Injured Skeletal Muscle: A Key Event in Muscle Fibrogenesis. Am J Pathol. 2004;164(3):1007–19.
27. Goldstein JA, Mcnally EM. Mechanisms of muscle weakness in muscular dystrophy. 2010;0–5.
28. Friedrich O, Wegner F Von, Chamberlain JS, Fink RHA, Rohrbach P. L-Type Ca 2 + Channel Function Is Linked to Dystrophin Expression in Mammalian Muscle. 2008;3(3).
29. Smolina N, Kostareva A, Bruton J, Karpushev A, Sjoberg G, Sejersen T. Primary Murine Myotubes as a Model for Investigating Muscular Dystrophy. Biomed Res Int [Internet]. 2015;2015:594751. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4561302&tool=pmcentrez&rendertype=abstract
30. Kamdar F, Doyle MJ, Chapman C, Lohr J, Koyano Nakagawa N, Garry DJ. In Vitro Modeling of Duchenne Muscular Dystrophy (DMD) Cardiomyopathy Using Human Induced Pluripotent Stem Cells (hiPSC). J Hear Lung Transplant. 2013;32(4).

FIGURES

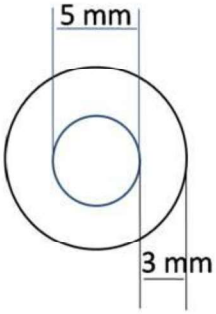

Fig. 1: filter ring dimensions

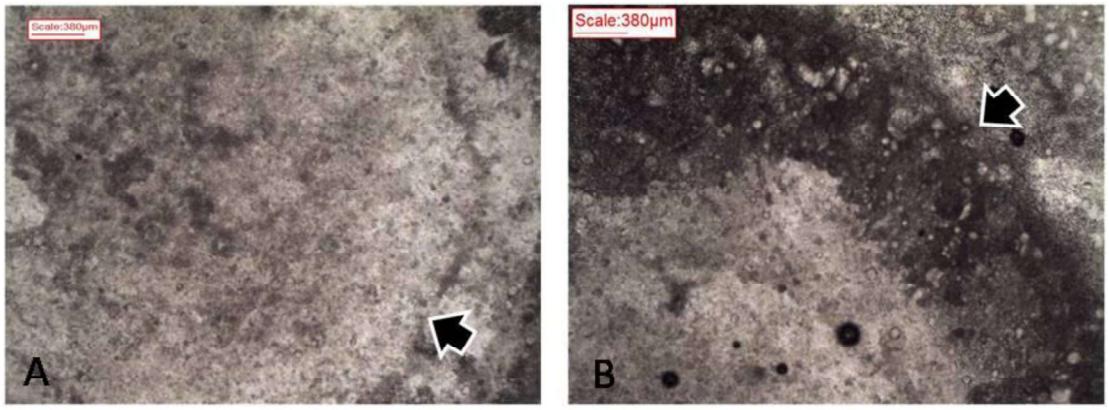

Fig. 2: Areas of blastoderm at EGKX: A: Area pellucida; B: Area opaca

Fig. 3: drop of cell suspension onto the lid roof. Lid is then put on the PBS filled dish

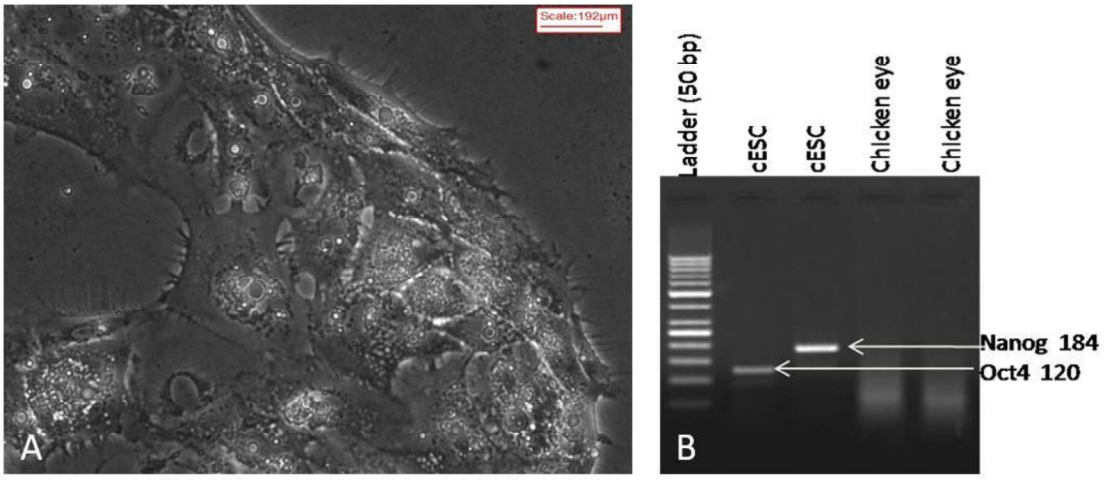

Fig. 4: A: embryonic pluripotent cells typically with ceellular processes. B: Gel image showing Nanog and Oct4 expression in samples of stem cells and absence of the same in the differentiated cells

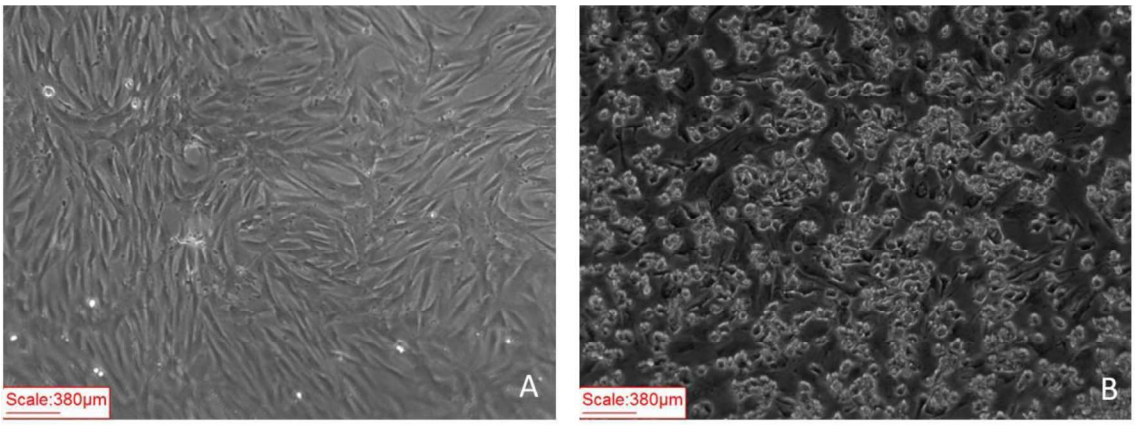

Fig. 5: A: embryonic fibroblastic cells in confluent state; B: embryonic stem cells cultured over the fibroblastic bed in the vessels

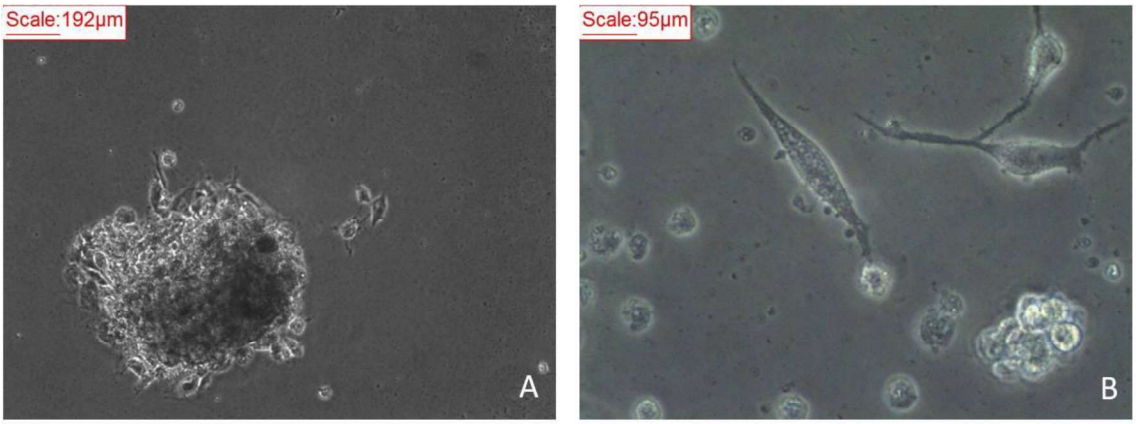

Fig. 6: A: embryonicd body formed out of cESCs in hangind drop cell culture; B: Myogenic cell derived from cESCs

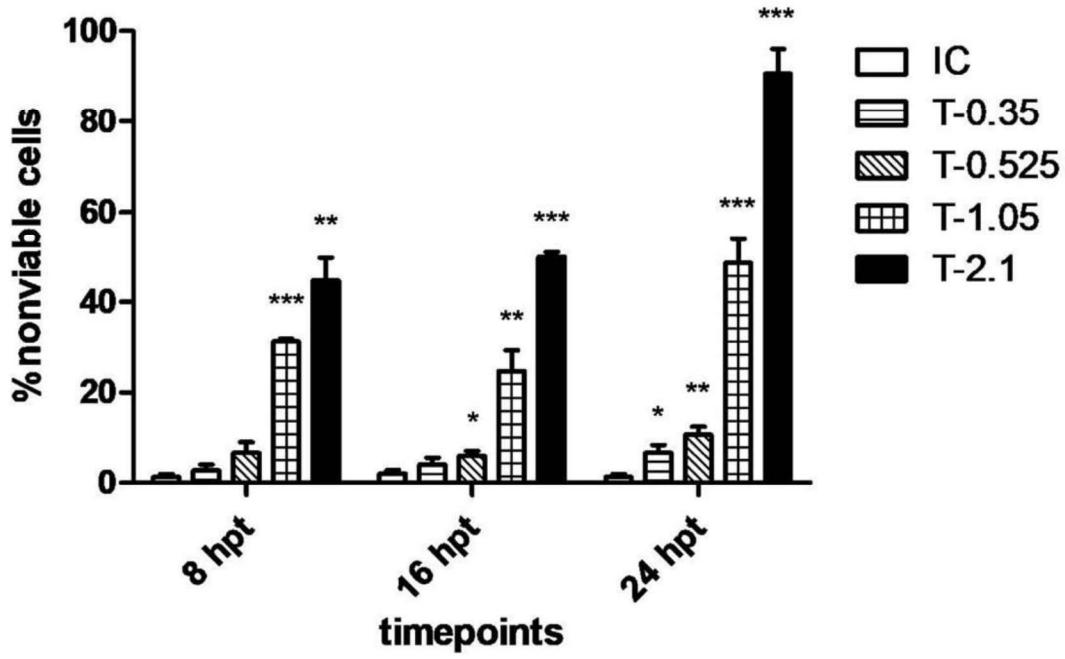

Fig. 7: Percentage nonviable cells in various treatment groups viz. isotype control, 0.35, 0.525, 1.05 and 2.1µg/ml doses of IIH6. *p value≤0.05, ** p value≤0.01, *** p value≤0.001. hpt- hours post-treatment

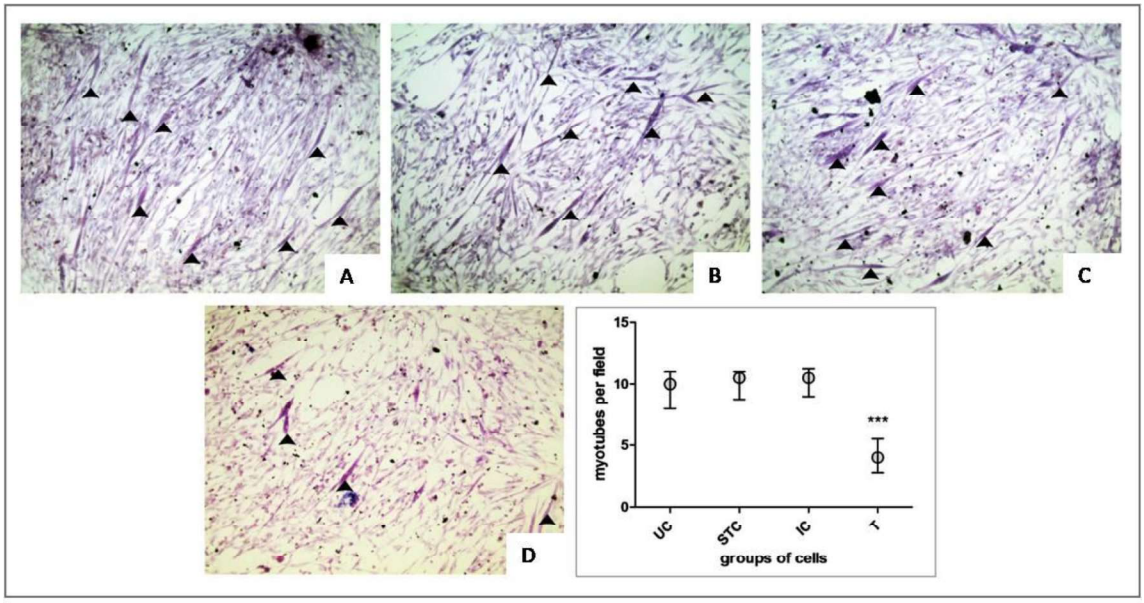

Fig. 8: Variation in myotube numbers. Black arrow-heads point at the myotube in each of the images. A: 100x field view of UC group with numerous myotubes stained with LADD for ease of evaluation. B: representative view from one of the culture vessels treated with supernatant of IIH6 (STC group) showing number of myotubes per view. C: cells of IC group showing abundant myotubes. D: Remarkable reduction in numbers of myotubes in treated cells. Graph: The values when analysed by performing one way ANOVA, showed that only treated cells had significantly less numbers of myotubes per field. There was no significant difference among all the control groups. *** p value≤0.001.

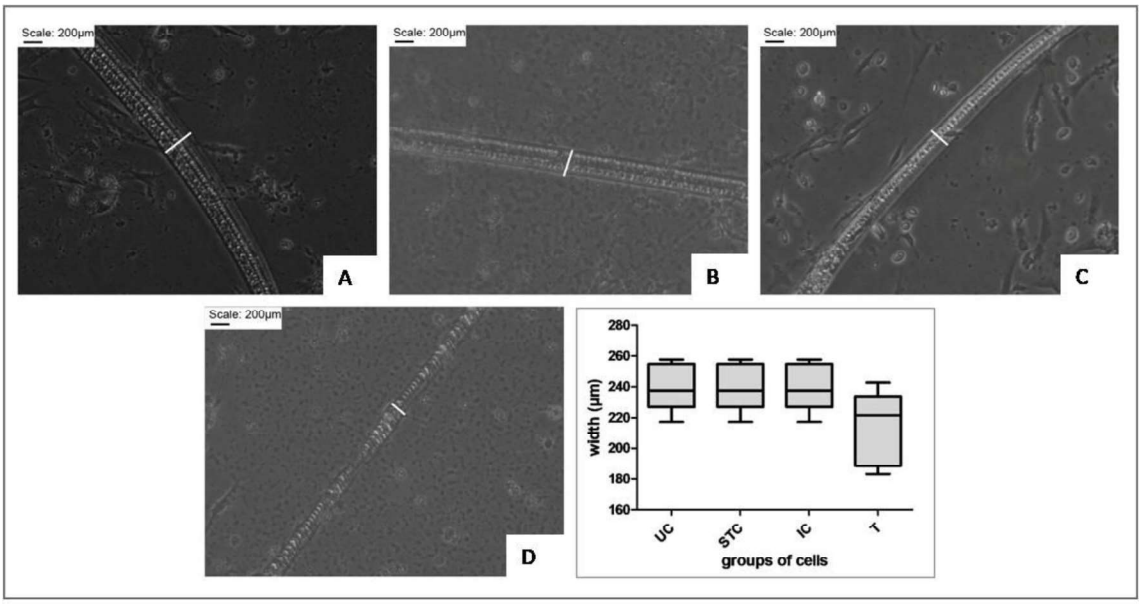

Fig. 9: Myotube widths. Each of the A, B, C and D images are representing the normal phenotypic diameter of myofibres grown in the cESMC cultures belonging to UC, STC, IC and T respectively. Graph: The diameter values are plotted along with mean in the box plot. High internal variation in widths of treated myotubes led to insignificant difference in mean of treated vs. control cultures. However, such variation is characteristic of MD condition.

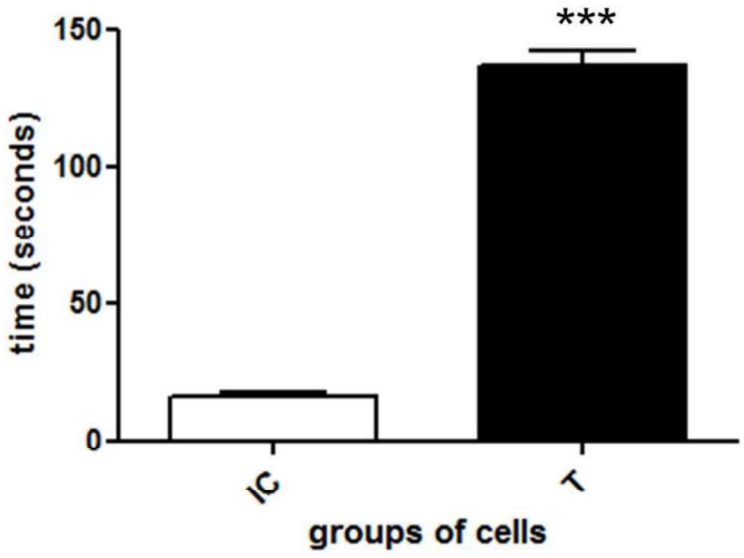

Fig. 10: Time taken by cultures for exhibiting contraction. *** p value≤0.001

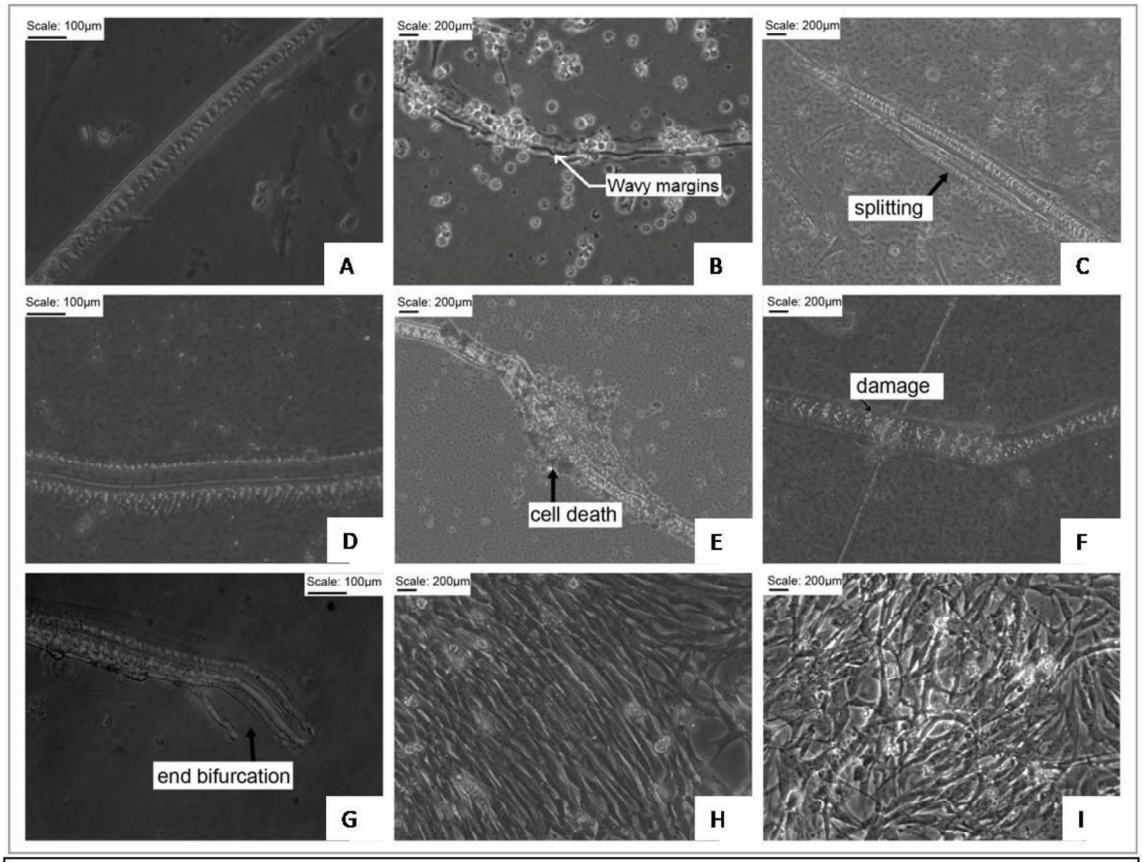

Fig. 11:Morphological features of IC and T cultures. A: control morphology at 400X magnification, scale: 100 µm; B: wavy margins of myotubes formed in treated culture, scale: 200 µm; C: central splitting in developed myofibre of treated culture, scale: 200 µm; 6D: Disruption along sarcolemma of myofibre in treated culture, scale: 200 µm; E: Cell death in treated myotube, scale: 200 µm; F: Myofibre damaged along its length in treatment group, scale: 200 µm; G: Treated myofibre end-splitting, scale: 200 µm; H: IC group cells showing normal morphology of myotubes growing in a unidirectional manner, scale: 200 µm; I: treated myotubes growing in various directions unlike normal growth, scale: 200 µm.

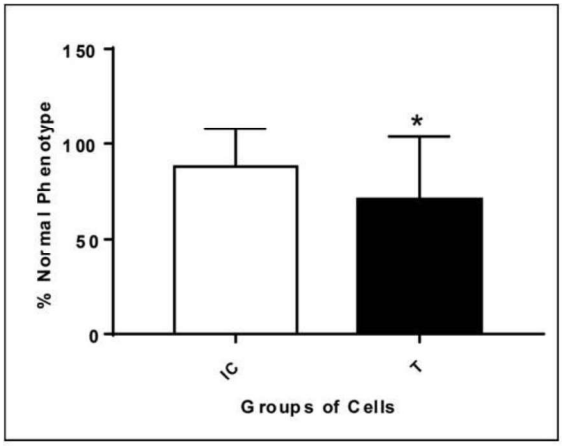

Fig. 11J:graph showing the reduction in normal phenotype of myotubes in treated group when compared to isotype control. *p value≤0.05

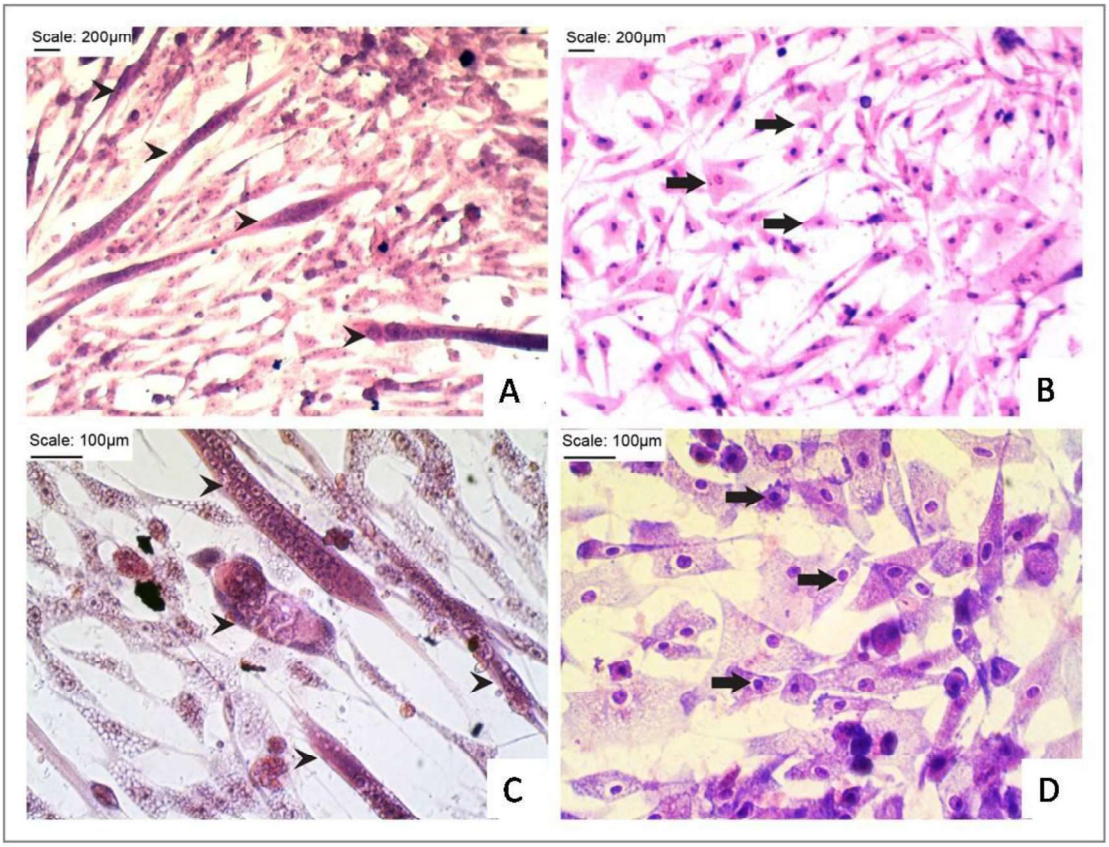

Fig. 12:LADD stained control and treated cells. A and C images represent control group with majority of cells undergoing fusion. The arrowheads show the formed myotubes with typical lighter multinuclei of fusing cells bearing darkly stained cytoplasm; B and D images represent the treated cultures wherein arrows show dark nuclei of cells in lightly stained cytoplasm. Scale: 200 µm for A & B; 100 µm for other images in the panel.

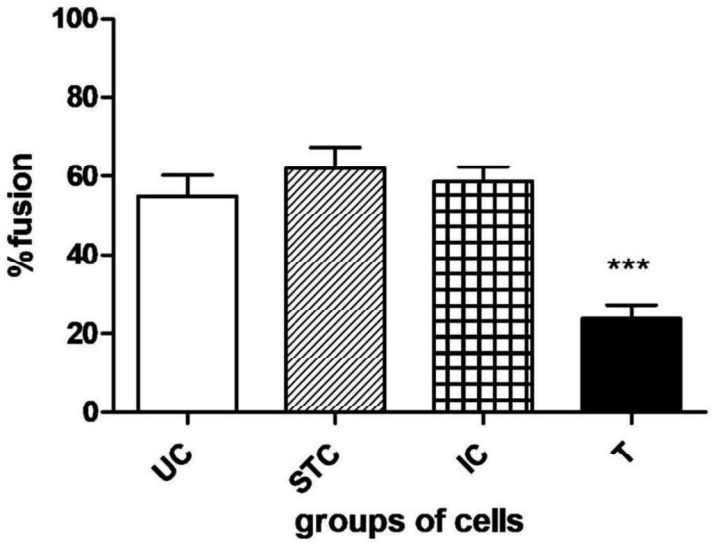

Fig. 12E: Fusion index calculated on the basis of LADD staining shows the heavy reduction in fusing cell numbers in treated group when compared to all other groups which show no significance differences among themselves. *** p values≤0.001.

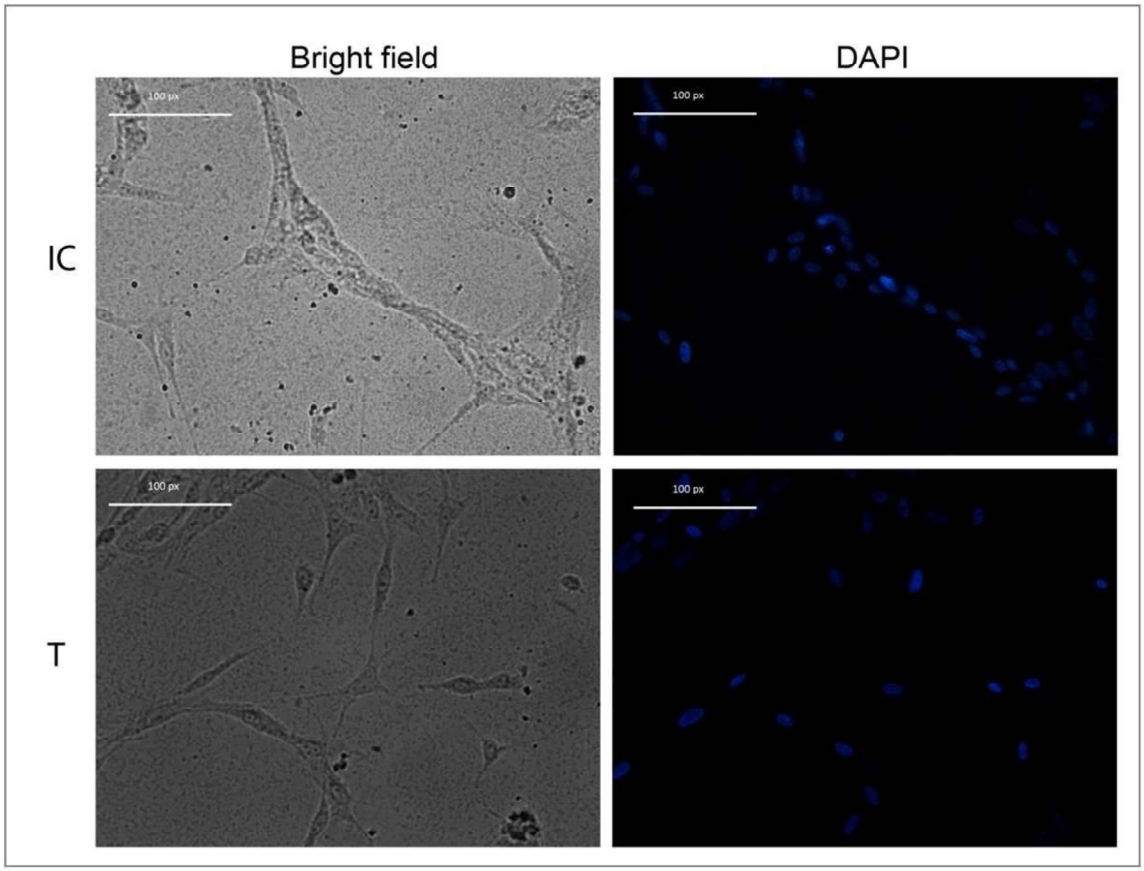

Fig. 12F: DAPI staining for fusing cells of IC group and separate cells of treated culture, scale: 100 px.

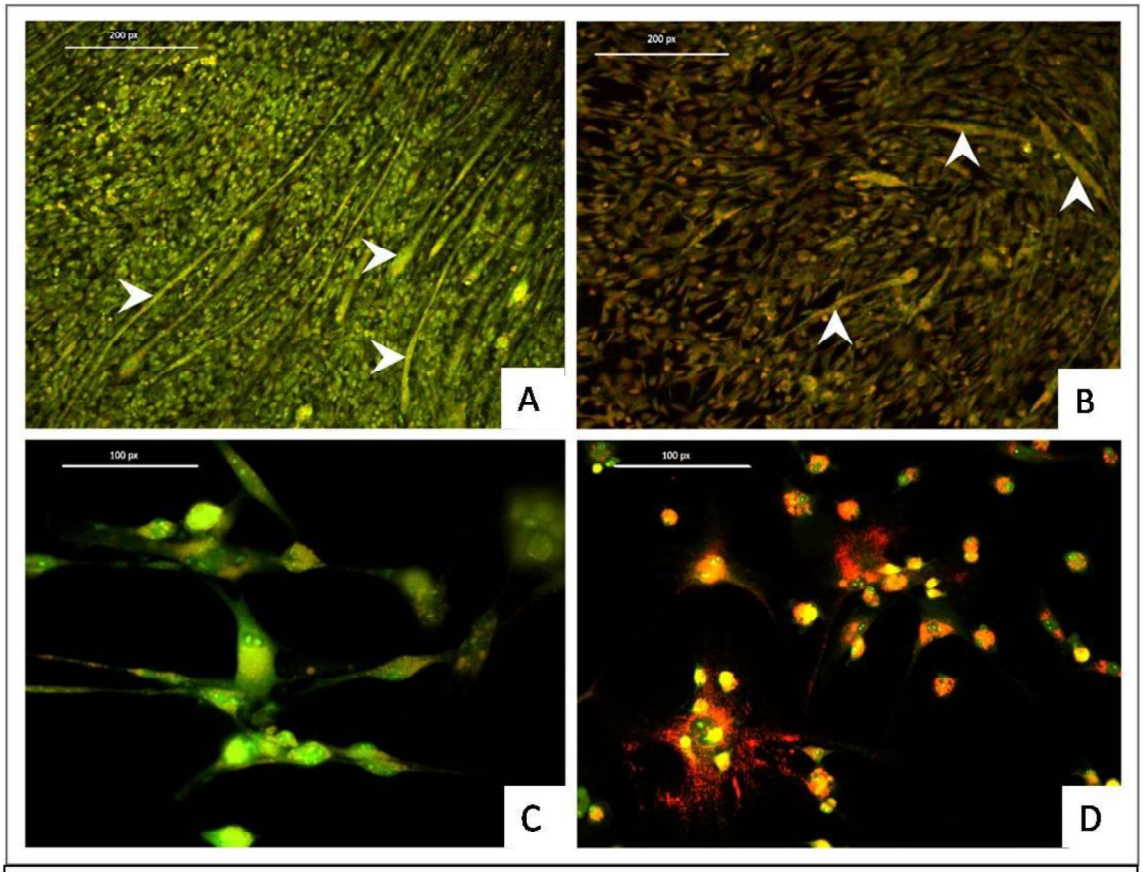

Fig. 13: EtBr/AO staining in IC vs. T cells. A and C show control cells with minimum amount of apoptotic orange fluorescence and maximum amount of viable green phenotype. 8B and 8D show high amount of cells exhibiting apoptotic and necrotic phenotype. First and second row of images are 200x and 400x field views respectively. White arrow heads specifically mark the myotubes in normal (A) and apoptotic (B) states.

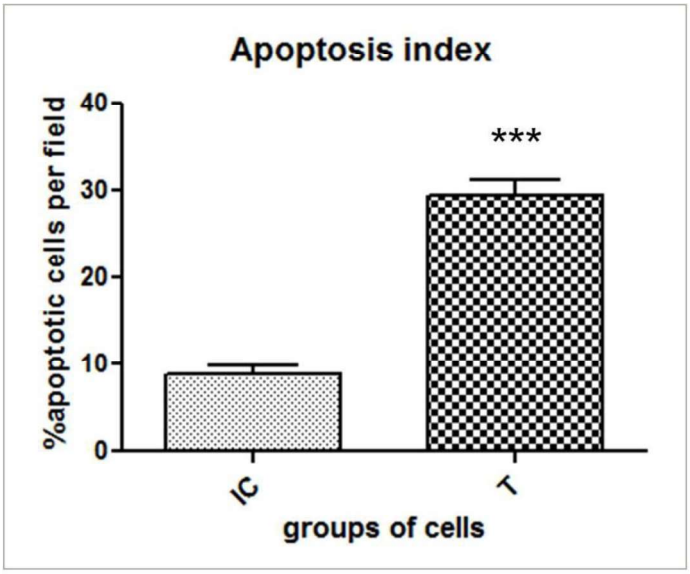

Fig. 13E: Apoptotic index in IC and T groups exhibiting typical rise in apoptotic cells in developed model system. *** p values0.001.

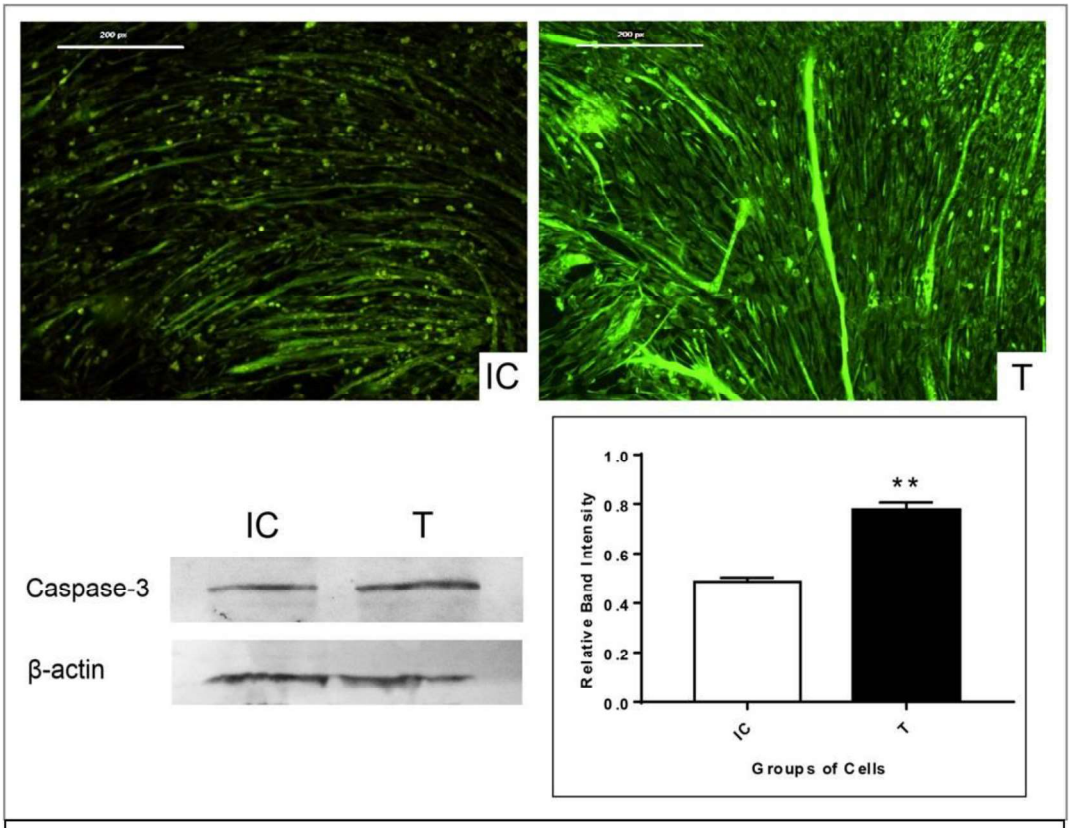

Fig. 14: Immunolocalization of cleaved Caspase-3 in control and treated cells. The treated myotubes intensively stained with cleaved Caspase-3 antibodies when compared to control myoutubes. 100x field view, scale: 200 px. Western blots of cleaved Caspase-3 relative to the internal control β-actin showed significant increase of cleaved Caspase-3 in treated cells when compared to control. ** p value≤0.01

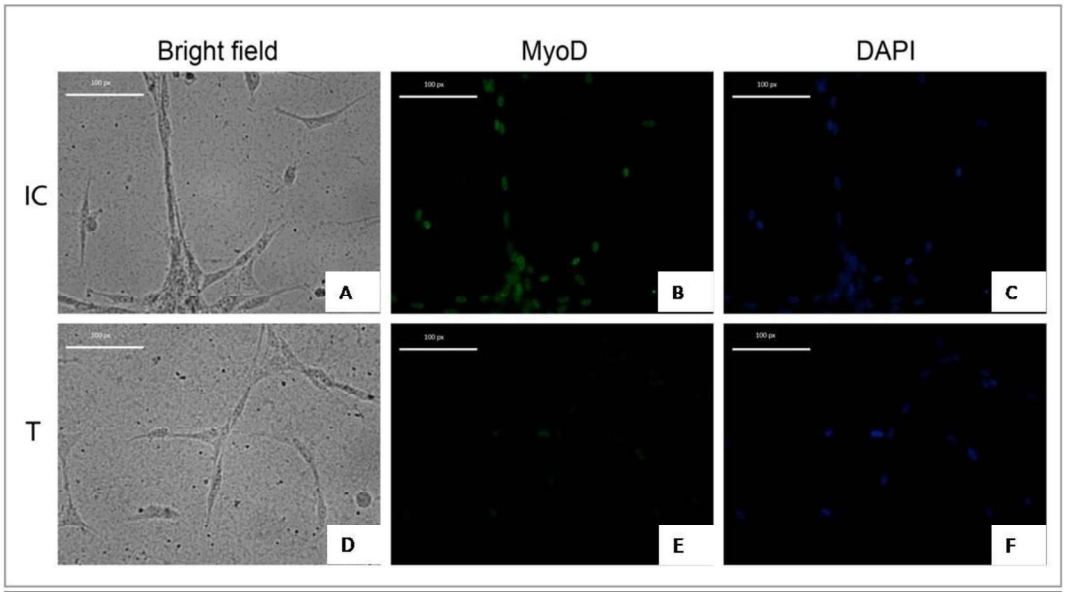

Fig. 15: Immunolocalization of MyoD1 protein in IC vs T group. The upper panel (A, B and C) shows the control cells stained brightly with MyoD antibodies. The lower panel comprising of D, E and F show treatment group cells wherein, very few cells have got immunostained with MyoD antibody. 400x field view, scale: 100 px for each image.

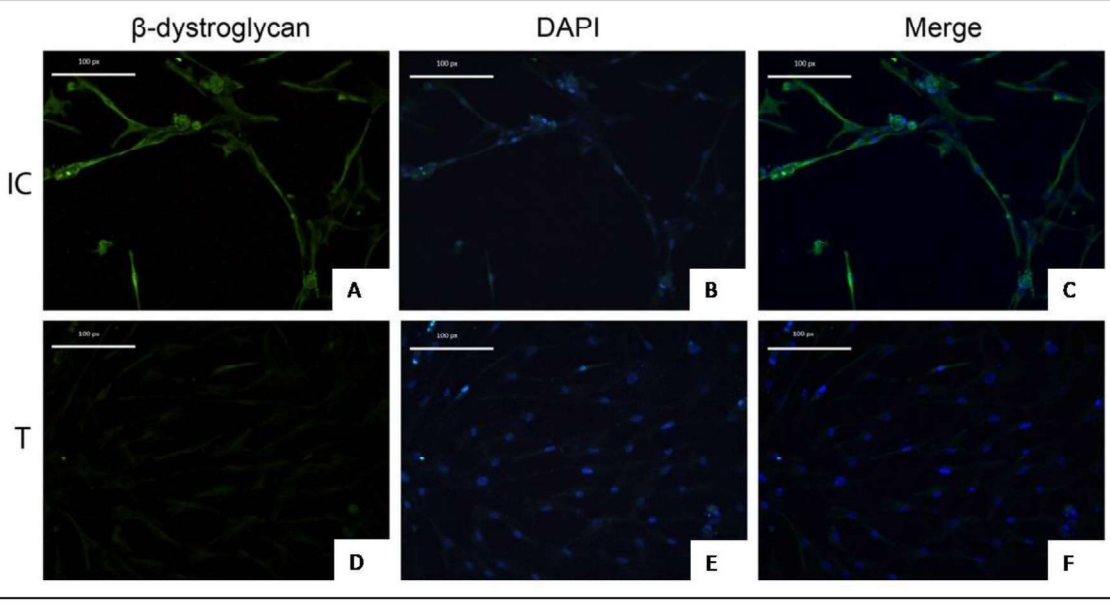

Fig. 16: Immunolocalization of β-dystroglycan. The upper panel (A, B and C) includes control cells which have caught the staining intensively more than the treated cells placed in lower panel (D, E and F). B and E are respective counterstained images for A and D cells. Scale: 100 px.

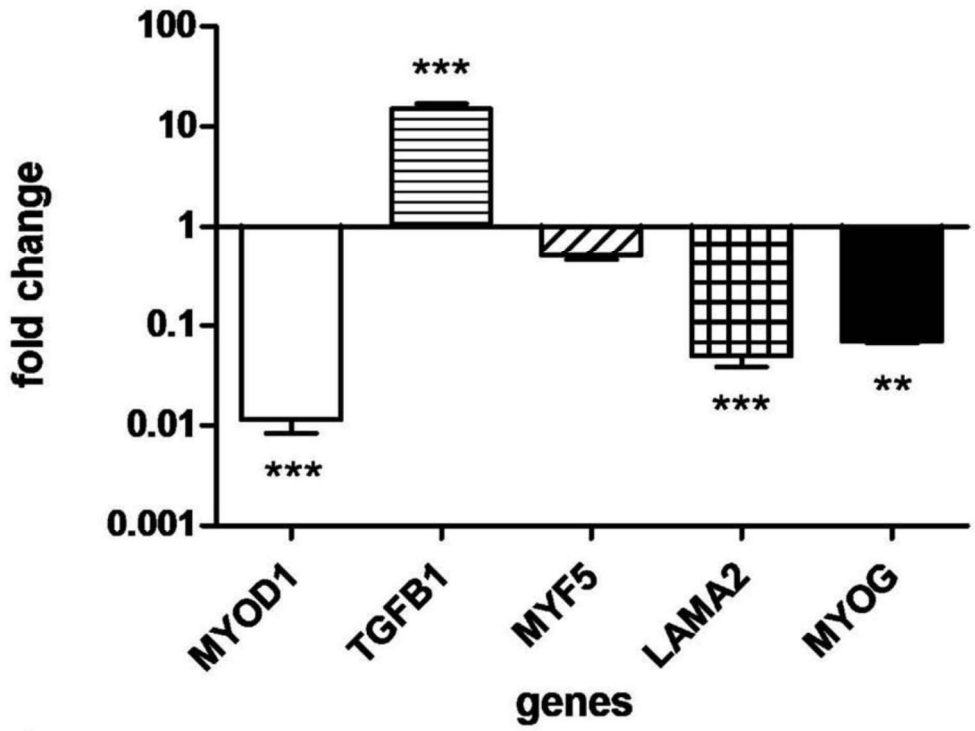

Fig. 17:Quantitative difference in gene expression pattern between control and treated groups. **p value≤0.01, ***p value≤0.001.