CHAPTER-3

TO STUDY THE EFFECTS OF CURCUMIN ON LEVOPHED/ NE INDUCED CARDIOTOXICITY

3.1. Overview of the chapter

Levophed is an anti-hypotensive drug, also known as Norepinephrine (NE), and reported to have cardiotoxic side-effects in the form of cardiac hypertrophy, ECM remodeling, oxidative stress etc. In the present chapter, cardiotoxicity of NE was studied by various qualitative and quantitative assays. H9C2 cardiomyoblasts were used to study NE induced cardiotoxicity by analyzing dose mediated morphological alterations, caspase expression, mitochondrial membrane disruption and free radical generation. Transition dose of NE from compensatory hypertrophy towards cardiac cell death was optimized and validated by various cell viability assays including MTT, Trypan blue dye-exclusion and FACS-calibur assays. Further, the effects of Curcumin on NE induced cardiac stress were studied in detail by various microscopic, biochemical and molecular assays. This chapter is structured as:

3.2 Introduction

3.3 Experimental Design

3.3.1 To study NE induced cardiotoxicity

3.3.2 To study the cellular transition of cardiomyoblasts towards death

3.3.3 To study the effects of Curcumin on NE induced cardiac stress

3.4 Results

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3.2. Introduction

Levophed (or NE bitartrate) is a well-known anti-hypotensive drug that is generally used for treating acute hypotensive conditions that may arise as a result of septic shock or during surgical procedures on critical care patients [211-212]. It is also available for non-critical patients with persistent low blood pressure complaints. NE is considered to be the safest and more effective vasopressor agent as compared to the other available options including dopamine, phenylephrine etc. [213-214]. However, it has been observed that the effects of NE on cardiac functioning and output are uncertain in septic patients [215]. NE-induced cardiac complications were first reported in 1970 where hypertrophic heart was witnessed upon NE-induction in dog [216]. Later, studies validated the role of NE in developing various complications including cardiac hypertrophy, bradycardia etc., where extended exposure may also lead to cardiac cell death and heart failure. However, till date, the distinguished mechanism of NE-induced cardiac complications is not clearly defined [217-223]. As NE is a neurotransmitter and a stress hormone, its physiological concentration also increases under stress conditions, and administration of this drug during sepsis may further results in developing concentration mediated cardiac complications in patients. As the therapeutic applications of Levophed outweights the possible associated cardiac risks, its use cannot be avoided in clinical emergencies, but, interventions for over-coming these toxic effects on heart should be developed. For this, deriving the exact mechanism of action for NE induced cardiac stress is a critical step and should be elucidated in detail. Also, heart failure is known to be associated with increased NE levels in plasma, and hence studying the concentration-mediated effects may serve as a platform to develop advanced therapeutic approaches for preventing the adverse effects [224].

Use of NE as well as elevated levels in plasma have also been reported to induce metastasis and cancer in breast and colon cells by stimulating β-adrenergic receptor [225-226]. This makes it even more significant to check and regulate the NE physiological levels and other concentration mediated events in the body.

In the present chapter, concentration mediated effects of NE were initially studied on H9C2 cardiomyoblasts followed by establishing the concentration dependent molecular switch from compensatory hypertrophy to apoptotic cellular death upon NE induced stress. Effects of Curcumin
on the NE induced cardiac stress were also studied in detail using different molecular and biochemical assays.

### 3.3 Experimental Design

In the present chapter, experiments were conducted in three different phases to study the different mode of deleterious effects of NE on cardiac cells and effects of Curcumin on NE induced cardiac stress responses.

#### 3.3.1 To study NE induced cardiotoxicity

Different doses of NE ranging from 2.5 µM to 150 µM concentrations were used in this study. Untreated (UT) cardiomyoblasts were used as control cells.

#### 3.3.2 To study the cellular transition of cardiomyoblasts towards death

Following three different doses of NE were derived and selected for the experiments:

- i) Hypertrophic dose (2.5 µM)
- ii) Transition dose (50 µM)
- iii) Apoptotic dose (100 µM)
- iv) Control UT cells

#### 3.3.3 To study the effects of Curcumin on NE induced cardiac stress

NE hypertrophic and transition doses were further selected to study the effects of Curcumin. Cardio-protective doses of Curcumin for these different NE doses were individually derived and effects of Curcumin were validated in following experimental sets:

- i) Control untreated;
- ii) 2.5 µM NE;
- iii) 2.5 µM NE with 10 µM Curcumin;
- iv) 50 µM NE;
- v) 50 µM NE with 15 µM Curcumin;
- vi) 15 µM Curcumin alone.
3.4. Results

We observed concentration mediated deleterious side effects of NE induction in cardiomyocytes as confirmed by concentration and time dependent MTT cell viability assay including lower to higher concentrations at three different time points of 24, 48 and 72 hours. More than 90% cell viability was witnessed upto the concentration of 10 µM at all the time points. At 50 µM concentration, this viability decreased significantly to 65% and 60% at 48 and 72 hours respectively. Less than 50% viability at the 90 µM norepinephrine was observed that further reduced to 20% at the maximum concentration of 150 µM (Fig. 3.1). For further experiments, induction time of 48 hours was selected because prolonged incubation may induced other toxicity like nutrition depletion and altered pH in the culture plates. NE induced morphological alterations were further studied by microscopic analysis for the increasing concentrations. We observed significantly enlarged cells at the concentration of 2.5 µM. With further increase in concentration, significant increase in death and more number of rounding-off of cells was observed. Significant cytoplasmic granulation was observed at the dose of 30 µM and excessive cell death as well as decrease in cell density and proliferation was observed above 70 µM concentration. (Fig. 3.2). To quantitate the cellular proliferation, altered morphology, and reduced average cell size, NIH Image J Software and cell counter were used. Approximately 2-fold increase in cell size was observed in hypertrophic cells. This decreased significantly at transition and apoptotic doses. (Fig. 3.3). The inverse relationship was observed between cellular size and drug concentrations were observed above 30 µM concentration where size decreased with increasing in concentration. These norepinephrine effects on the proliferation of cardiomyoblasts were also studied by measuring cellular density using cell counter. Observed decrease in cellular density further validated our above results (Fig. 3.4). Significant decrease in percent dead cells and increased TUNEL positive cells with increasing concentrations were witnessed thereby validating our previous results as well as establish the manifestation of concentration mediated norepinephrine induced cardiac alterations (Fig. 3.5). Quantitative analysis of apoptotic death at higher NE concentrations was observed by FACS-calibur using Annexin-V/PI stains and significant shift in the cells towards altered morphology and death was observed (Fig. 3.6 and 3.7).

To understand the molecular switch from compensatory responses towards death, further cell viability assays were done and a transition dose. The concentration dependent response of NE for
the lower doses ranging from 2.5 µM to 20 µM was studied initially. The viability of the cells
determined by MTT assay was reduced considerably at 20 µM NE. However, it was still above
85% (Fig. 3.8 A). Further we conducted the assay for cells treated with higher concentrations of
NE (25 to 150 µM) which showed a concentration dependent decrease in cell viability with a
modest decline (~60%) at 50 µM and a sharp reduction (~40%) at 100 µM NE (Fig. 3.8 B). Trypan
blue staining further validated reduction in cell viability up to 60% at 50 µM and approximately
40% at 100 µM NE, thereby confirming dose dependent transition due to cell stress (Fig. 3.9). High
concentrations of NE also showed a significant increase in the number of TUNEL positive cells.
The apoptotic effect was found to be insignificant (~10%) in 2.5 µM NE treated cells. However, a
significant increase in apoptosis of up to ~45% and ~80% in 50 µM and 100 µM NE was observed
respectively (Fig. 3.10). Based on the above analysis, 50 µM NE was selected as a concentration
for the transition of compensatory to deleterious phase and 100 µM as an apoptotic concentration.
Microscopic studies showed that NE exerts an increase in cell size at 2.5 µM, condensed cells with
altered morphology along with nuclear deformities at 50 µM concentration and cell shrinkage with
a reduction in cell volume as well as number in 100 µM NE treated cells. Quantitative estimation
of the experimental sets further validated the similar observations (Fig. 3.11-3.12). Further, nuclear
morphology of the cells analyzed by DAPI and PI staining showed a hypertrophic increase in
nuclear size at 2.5 µM NE whereas cells treated with higher concentrations modulated the nuclear
structure and integrity. However the nuclear stress was higher on the cells treated with 100 µM NE
as compared to 50 µM NE (Fig. 3.13-3.14)
Semi-quantitative PCR displayed down-regulation of hypertrophic marker and up-regulation of
inflammatory responses at this transition dose, where 2.5 fold up-regulated expression of β-MyHC
at 2.5 µM NE treatment as compared to control, that subsequently decreased at transition (50 µM)
and apoptotic concentrations (100 µM), in comparison to the hypertrophic dose was witnessed. On
the other hand, TNF-α mRNA expression was significantly increased by two fold in 100 µM NE
induced set with reference to control, thus confirming extensive cell death (Fig. 3.15). Involvement
of mitochondria was studied by Rhodamine 123 and DCFH-DA staining, where altered
mitochondrial potential was witnessed at the transition dose and further exaggerated at the
apoptotic dose (Fig. 3.16). These alterations were validated by mitochondria mediated ROS
generation during cells transiting towards death. Intracellular ROS was significantly increased with
increase in stress where ROS levels at 50 µM NE were significantly higher there by confirming NE
induced cardiac stress (Fig. 3.17). These mitochondrial mediated events were validated by increase caspase activation at the transition dose. The activity of apoptotic effectors caspase 8 and caspase 9 was found to be increased in 50 μM NE treated cells indicating the activation of mitochondria mediated apoptotic pathways at the transition concentration of NE which was further exaggerated (three fold and two fold for caspase 8 and 9 respectively) at 100 μM NE concentration in comparison to UT cells (Fig. 3.18). ECM alterations while cells undergoing transition towards cell death were studied and Verhoeff-Van Gieson staining revealed elevated intracellular collagen fibrils at hypertrophic dose whereas higher concentrations led to a substantial decrease in the collagen as compared to hypertrophic dose. A significant two folds increase in collagen content was observed at 2.5 μM NE in contrast to control. The effect was normalized at 50 μM NE and further decreased in 100 μM NE treated cells suggesting the loss of compensatory feature of cells at higher concentrations and at 50 μM NE as a compensatory phase shift (Fig. 3.19). PicroSirius Red Collagen Staining further validated the same observations (Fig. 3.20). To validate the adrenergic receptor mediated effects of NE, Propranolol was used as a positive control and in its presence, NE induced effects were seen to be reduced significantly (Fig. 3.21).

Effect of Curcumin on NE induced cardiotoxicity were studied next and two different concentrations for NE were selected for further studies- hypertrophic and transition dose. Cardioprotective doses of Curcumin for these two different NE concentrations were derived first by MTT cell viability assay. Concentrations of 10 µM and 15 µM Curcumin for NE hypertrophic and transition doses were selected respectively (Fig. 3.22). These concentrations were then validated by trypan blue assay and used for further experiments (Fig. 3.23). Morphological alterations upon NE and Curcumin treatment were studied at cellular and nuclear level. NE induced morphological alterations were observed to be reduced or prevented significantly in presence of derived Curcumin doses (Fig. 3.24). Curcumin treatment also displayed significant reduction in NE induced collagen imbalance in hypertrophic cells (Fig. 3.25). These Curcumin mediated effects were validated by biomarker expression studies where significant down-regulation of hypertrophic and inflammatory biomarkers was witnessed at both hypertrophic and transition doses (Fig. 3.26).

Hence, overall preventive effect of Curcumin was observed in NE induced hypertrophy and cellular transition towards death.
Fig. 3.1. **NE induced concentration and time dependent cell death**: MTT cell cytotoxicity assay for increasing NE concentrations ranging from 2.5-150 µM for 24, 48 and 72 hours. Concentration dependent significant increase in cell death was observed. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). (*Jain and Rani, 2018, *In vitro Cell Dev Biol-Animal, 54(2):92-98 [227]).
**Fig. 3.2. NE concentration induced morphological alterations in cardiomyoblasts:** Cells were treated for 48 hours with increasing doses of NE and morphological alterations and effect of NE on overall cell proliferation and death was captured at 100 X magnification. Scale bar corresponds to 6 µm (Jain and Rani, 2018, In vitro Cell Dev Biol-Animal, 54(2):92-98 [227]).

**Fig. 3.3. Quantitative growth inhibition analysis upon NE treatment:** Effect of increasing NE concentration on cell proliferation and density was analyzed by Countess® II Automated Cell Counter. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*P<0.05). (Jain and Rani, 2018, In vitro Cell Dev Biol-Animal, 54(2):92-98 [227]).
**Fig. 3.4. Alterations in cellular size analysis upon NE treatment:** Average cell size upon different NE treatments was quantified by NIH Image J Software and plotted as a bar graph (µm²/cell). One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*P<0.05). (*Jain and Rani, 2018, *In vitro Cell Dev Biol-Animal, 54(2):92-98 [227]).

**Fig. 3.5. Cellular death characterization by TUNEL assay:** Mean percentage of TUNEL positive cells were counted at all individual NE concentrations and with plotted as % dead cells. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*P<0.05). (*Jain and Rani, 2018, *In vitro Cell Dev Biol-Animal, 54(2):92-98 [227]).
Fig. 3.6. Quantitative analysis of apoptotic damage by flow cytometer analysis using Annexin/PI stains: Cardiac cells were stained with Annexin-V and PI for increasing NE concentrations ranging from 2.5-150 μM. Percentage cellular death was observed by flow channel 1 and 2 of FACS-Calibur. (Jain and Rani, 2018, In vitro Cell Dev Biol-Animal, 54(2):92-98 [227]).
Fig. 3.7. Graphical representation of Annexin-V/PI staining by flow cytometry data: Percent apoptotic cells as obtained in the box plots of Fig. 3.6. were plotted against NE concentrations. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*P<0.05). (Jain and Rani, 2018, In vitro Cell Dev Biol-Animal, 54(2):92-98 [227]).
Fig. 3.8. MTT assay for lower and higher concentration ranges of NE: (A) NE concentrations ranging from 2.5-20 μM were used. (B) NE concentrations ranging from 25 to 150 μM were used. Cell death was although significant at all concentrations above 15 μM NE, but shows sharp changes at 50 μM and 100 μM NE. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).

Fig. 3.9. Trypan blue staining with derived hypertrophic, transition and apoptotic doses of NE: To validate the cell death upon different NE concentration as derived from MTT cell viability assay, trypan blue dye exclusion assay was done and quantified using spectrophotometer. Significantly higher cellular death was higher at 50 μM that further increased at 100 μM NE as compared to the untreated (UT) control cells. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.10. TUNEL assay for detection of apoptotic cell death in cardiomyoblasts upon NE treatment: Fluorescent micrographs were recorded and mean percentage of TUNEL positive cells was evaluated in the four pre-defined experimental groups. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). Images were captured at 100 X magnification and scale bar corresponds to 10 µm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.11. NE induced morphological alterations in cardiomyoblasts at different concentrations: H9C2 cells were treated with NE for 48 hours at different concentrations and captured at 100X magnification. Scale bar corresponds to 10 µm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).

Fig. 3.12. Haematoxylin-Eosin staining for altered cellular morphology upon NE treatment:

Increase in cellular size was seen at hypertrophic concentration and the cell fragments/debris were observed at higher concentrations. Graph displays the quantification of eluted stain. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05) from the pre-defined experimental sets. Images were captured at 40 X and scale bar corresponds to 25 µm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.13. Nuclear staining using DAPI stain in NE treated cells: Nuclear alterations were observed in cells treated with different NE concentrations. The fluorescence micrographs were captured at 40X magnification under DAPI filter. Fluorescence intensity was measured by spectrofluorometer. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). Scale bar corresponds to 25 μm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.14. **Nuclear staining using PI stain in NE treated cells:** Nuclear alterations were recorded in the form of fluorescence micrographs at 40X magnification and fluorescence intensity was measured by spectrofluorometer. The data was analyzed from the individual experiments. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p* < 0.05). Scale bar corresponds to 25 μm (*Jain et al*, 2015, *Chemico Biol Int*, 225:54–62 [228]).
Fig. 3.15. Expression of marker genes for hypertrophy and cell death upon NE treatment:
RT-PCR analysis for β-MHC and TNF-α expression was done for the pre-defined experimental
groups. β-actin was used as a loading control. Fold Change with respect to control was calculated
after normalization using Image J software and plotted. Two-way ANOVA and student’s t-test
were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain et al,
2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.16. Rhodamine 123 staining of cardiac cells for alterations in mitochondrial membrane potential upon NE treatment: Images were captured at 40X magnification and mitochondrial stress was witnessed at all the three concentrations of NE as indicated by arrows. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained. Scale bar corresponds to 25 µm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.17. **Intracellular ROS staining by DCFH-DA staining of H9C2 Cells:** ROS production in the adherent cells upon different NE treatments was done and the total fluorescence of the eluted stain from the detached and adhered cells was measured by spectrofluorometer. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). Images were captured at 40X magnification and scale bar corresponds to 25 µm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.18. Caspase 8 and 9 staining of NE treated H9C2 cells: Activation of cell death pathways mediated by mitochondria was validated by Caspase 8 and 9 staining after NE treatment. FACS acquisition was done using flow channel-1 for 1000 events. (Jain et al, 2015, *Chemico Biol Int*, 225:54–62 [228]).
Fig. 3.19. Analysis of ECM remodeling by Verhoeff van Gieson stain: Comparison of collagen content was done in all the experimental cells at 100 X. The NaOH eluted stains were estimated by colorimetric method and expressed as μg/ml of collagen concentration as shown by histogram. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p≤0.05). Scale bar corresponds to 10 μm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.20. Analysis of ECM remodeling by Picrosirius stain: Collagen content was also estimated in different experimental sets at 20X magnification using picrosirius stain. Collagen concentration was quantified and expressed as μg/ml in the histogram. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). Scale bar corresponds to 50 μm (Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.21. Effect of Propranolol on NE induced cells to validate beta adrenergic receptor dependent activation of cell death: (A) Morphological analysis of cardiac cells displaying no significant cell death upon Propranolol treatment. (B) MTT assay indicates reduced cell death on using different concentrations of Propranolol with and without NE. (C) RT-PCR analysis showing the reversal of NE induced cell death and bringing it back to the hypertrophic phase indicating dependence on adrenergic receptor signaling. (D) DCFH-DA staining of H9C2 with NE and Propranolol showing opposing effects of NE and Propranolol. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained *(P<0.05). Images were captured at 40X magnification and scale bar corresponds to 25 µm. *(Jain et al, 2015, Chemico Biol Int, 225:54–62 [228]).
Fig. 3.22. Dose optimization of Curcumin for NE induced cardiotoxicity at different doses: Cardio-protective doses of Curcumin for selected NE hypertrophic dose was derived as 10 µM Curcumin and for transition dose as 15 µM Curcumin. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*P<0.05).
Fig. 3.23. Confirmation of cell viability of Curcumin optimized doses with different NE treated groups in finalized experimental sets: (1) Untreated Control Cells (2) 2.5 µM NE (Hypertrophy) (3) 2.5 µM NE + 10 µM Curcumin (4) 50 µM NE (Transition) (5) 50 µM NE + 15 µM Curcumin (6) 15 µM Curcumin alone.
Fig. 3.24. Cellular and nuclear alterations upon Curcumin treatment in NE induced cells:
(A) Bright field images (B) Giemsa staining (C) DAPI nuclear staining. Curcumin was shown to suppress NE induced hypertrophy as well as restrict the transition of the cells towards death in H9C2 cells as evident from the different morphological analysis at cellular and nuclear levels. Images were captured at 40X and 100X magnification and scale bar corresponds to 15 µm and 6 µm respectively.
Fig. 3.25. **Effect of Curcumin on collagen content in NE treated cells:** Verhoeff van Gieson stain was used to study the effect of Curcumin on NE induced collagen content and ECM remodeling. Images were captured at 100X magnification and scale bar corresponds to 10 µm.
**Fig. 3.26. Expression of Hypertrophic and death markers upon Curcumin treatment:** β-MHC and TNF-α expression were studied by RT-PCR in different experimental sets as follows:

A: Untreated Control Cells; B: 2.5 µM NE (Hypertrophy); C: 2.5 µM NE 10 µM Curc; D: 50 µM NE (Transition); E: 50 µM NE 15 µM Curcumin; F: 15 µM Curcumin. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*P<0.05).
3.5. Discussion

In the present chapter, cardiotoxic effects of a well-known anti-hypotensive drug- Levophed or NE were studied in detail. NE is a neurotransmitter and its transmission gets activated in response to stress conditions leading to elevated physiological concentration. In addition to this, when extrinsic NE is administered to hypotensive septic patients, its cumulative concentration increases exponentially within the system leading to various deleterious effects and limits the therapeutic efficacy of NE as an anti-hypotensive drug. NE concentration mediated effects are reported to have severe cardiotoxic side effects that may ultimately lead to heart failure [224]. Alternative approaches are need to be developed to reduce these NE effects and supplementing natural cardio-protective compounds holds a great potential in this regard. This approach should not alter or compromise the actual anti-hypotensive effects but significantly reduce the associated cardiac toxicity [229-230]. In this regard, we studied the effects of Curcumin, polyphenol present in Curcuma longa (turmeric) NE induced cardiac stress. We firstly studied NE mediated concentration and time dependent cellular responses in H9C2 cardiomyoblasts in detail to identify the critical doses of NE to be regulated in cardiac pathologies and establish a link between NE concentration and the mediated toxicity. Physiological NE levels above the hypertrophic concentration have been reported to exert deleterious and significant stress and range between 10 to 100 μM concentrations. These concentrations represent cell death and imitate end-stage heart failure in vitro [231]. Therefore, this range of concentrations were screened initially in detail in the present chapter for all the stress-related studies.

Concentration and time dependent cell viability assays using MTT dye were conducted initially for broad range of NE concentrations ranging from 2.5 to 150 μM NE for three time points of 24, 48, and 72 hours. Cell viability upto 90% was observed till the concentration of 10 μM for the three time-points. This represented the compensatory responses in cells as no significant cell death is observed till now. However, significant reduction in cell viability was observed when the concentration was increased upto 50 μM NE. This concentration can be considered as a transition check-point for the shift in cells from hypertrophy towards cell death. This viability further decreased exponentially at 90 and 150 μM NE concentrations (Fig. 3.1). From this, we derived the optimum induction time of 48 hours for all the further experiments, as nutrition depletion and pH alterations beyond this time may induce additional stress on cardiomyoblasts and alter the actual
drug stress to be studied. As morphological alterations are the first sign of stress within the cells, these alterations were further studied by microscopic analysis for the increasing concentrations. Significant increase in cellular size was observed at the hypertrophic concentration that further decreased with higher number of rounded-off of cells with increasing in concentration (Fig. 3.2). Change in cellular size was quantitated by using the captured morphology images with NIH Image J Software and average cell size was then calculated (Fig. 3.3). This inverse relationship between cardiomyocyte size and NE concentrations were also supported by growth inhibition analysis where proliferation of cells and density were measured and significant decrease was witnessed with increasing NE concentrations (Fig. 3.4). NE concentration-dependent cellular death was also validated by TUNEL assay where significant increase in TUNEL positive cells was witnessed with increasing NE concentrations. Increase in percent dead cells as observed by TUNEL assay further validated the occurrence of concentration-mediated norepinephrine-induced cardiac alterations (Fig. 3.5). To further validated the above results, and quantitate cellular death in cardiomyoblasts, Annexin-V was used and apoptotic cells were analyzed by FACS-calibur with increasing NE concentrations. Significant characteristic shift in the cardiomyocytes towards altered morphology and death was witnessed (Fig. 3.6 and 3.7). This validated the above observations suggesting an important role of concentration mediated effects induced in cardiomyocytes. Also, as Annexin-V is specific for apoptotic cell death, this explains the concentration dependent differences observed in cell death in both the assays.

The observed strident decrease in the cell viability in a concentration dependent manner indicates its significant role in inducing pathophysiological alterations in cardiac cells. As cardiomyoblasts are terminally differentiated cells and in presence of any kind of temporary stress, compensatory responses are witnessed in form of hypertrophy. Once a stress is given to terminally differentiated cardiac myocytes in a concentration dependent manner, cell tries to sustain the load initially (hypertrophic phase) but further increase of stress leads to a transit (transition phase) of cardiac myocytes from hypertrophy to toxicity induced progressive cell death (death phase). We further studied concentration dependent switch from compensatory NE induced hypertrophy to apoptotic cell death to derive the transition concentration of NE.

Broad and narrow range of NE were studied by MTT assay was done where 50 µM NE concentration has proved as checkpoint for the transit phase. As a transition checkpoint, 50 µM
concentration of NE was chosen in our study since it caused a steep decline in viable cell number but still maintained the cell viability above 60% suggesting the initiation of stress mediated events which further leads to extensive cell death at additional higher concentrations. The higher concentration, 100 µM NE, was chosen as an apoptotic dose based on our results as well as previous studies done on rat cardiomyocytes and on H9C2 cell lines (Fig. 3.8 A and B). A time dependent study (24, 48, 72 hours) for cell viability showed an insignificant effect of NE at 24 hours whereas at 72 hours, organelle damage and cell death were observed even in the control cells. This led to conduct the study with treatment duration of 48 hours. These observed cell viability was also confirmed by Trypan blue dye exclusion assay and three different doses were then derived for further studies, namely hypertrophic dose at 2.5 µM, transition dose at 50 µM and apoptotic dose at 100 µM NE concentrations (Fig. 3.9). This transition or switch of cardiomyoblasts towards cellular death was then validated by various qualitative and quantitative assays. TUNEL assay displayed less increase in number of TUNEL positive cells at the transition dose, that is, upto 45% as compared to significantly higher increase at apoptotic dose upto 80% (Fig. 3.10). Presence of TUNEL positive cells at concentrations of 50 µM and 100 µM NE clearly depict the apoptosis in rat cardiac myocytes. The transition to the death phase is apparent at the concentration of 50 µM NE and has laid a platform for studying other associated downstream and upstream pathways associated with the transition of cells towards death phase.

NE induced concentration dependent cellular and nuclear morphological alterations were studied by microscopic studies. Increased number of dead cells were observed at the transition dose and cellular size was also reduced as compared to the hypertrophic dose. However these effects were further exaggerated at apoptotic dose (Fig. 3.11). Quantitative estimation of the stain from cells treated with haematoxylin-eosin stain also validated these observations (Fig. 3.12). DAPI and PI stains were used to study the nuclear alterations and increased nuclear size was observed at hypertrophic dose that reduced at transition dose but characteristic altered nuclear structure and integrity was witnessed at higher NE concentrations (Fig. 3.13 and 3.14). Changes in cellular & nuclear morphology further suggests that 50 µM NE concentration is sufficient to initiate cell toxicity but lesser cell death, while 100 µM NE concentration adequately leads to loss of cell viability, changes in nuclear morphology, irregular outer edges around the nucleus and fragmented
nuclear bodies. These observations distinctly indicated the transition of cardiomyocytes from hypertrophy towards cell death in a concentration dependent manner.

Expression of hypertrophic and cell death markers β-MyHC and TNF-α was studied by semi-quantitative PCR. The results showed an enhanced expression of β-MyHC, a well-documented marker of pathological hypertrophy only at hypertrophic dose and decreases subsequently. It was also observed that the expression of TNF-α, a known pleiotropic cytokine for inducing cell death in stressed myocardium. The study demonstrated that at lower concentrations, cells express the hypertrophic genes bringing a compensatory/healthy response, whereas at higher concentrations the hypertrophic genes are down-regulated and cells expresses the apoptotic genes in a concentration dependent manner leading to irreparable cell death suggesting that the emphasis should be given for the transition phase and should be targeted in vitro to prevent cell death during the cardiac pathologies. (Figure 3.15). These observations further validated the cells transiting towards death from compensatory responses in vitro.

To further understand the involvement of mitochondria in response to stress, mitochondrial integrity was analyzed which correlates with changes in membrane potential of the cells in response to the activation of cell death pathways upon NE exposure. Our data showed that NE mediated stress lead to changes in membrane potential suggesting a role of mitochondrial mediated pathways, ROS generation and caspases activation with progressive NE stress, which is involved in various pathological conditions involving apoptosis and further leading to cardiac cell death and heart failure (Fig. 3.16). Following this, intracellular ROS levels of adhered cells were estimated to further understand the stress mediated transitions. A significant increase in intensity of the DCFH-DA stain was observed in 2.5 µM NE treated cells as compared to adhered control cells, that was further reduced in response to higher NE concentrations. However, the net fluorescence intensity showed a direct relation between ROS production and NE concentrations where maximum net fluorescence was observed at 100 µM NE concentration indicating mitochondrial mediated ROS production in NE mediated stress responses (Fig. 3.17). The increase in ROS levels have been known to play a central role in cells transiting towards death and in cardiac system, it may leads to cardiomyocyte death or heart failure [232]. Hence, NE concentration dependent changes were ROS mediated, whereas at higher concentrations the intracellular ROS production increased significantly which might have led to the activation of cell death pathways.
The caspase dependent signaling has been reported in the progression of cardiomyocyte hypertrophy. Role of mitochondria in cellular transition was also confirmed by the activation of programmed cell death markers - caspases. We observed the activation of intrinsic caspases at 50 μM NE leading to transition of compensatory to apoptotic phase. Caspase 8 and 9 are initiator caspases involved in starting the process of apoptosis and their expression was significantly increased at the transition dose. (Fig. 3.18). This expression of caspases was further exaggerated at the apoptotic dose of NE and it is well supported by the reported literature [220].

Collagen imbalance is one of the most important cellular mechanisms responsible for transformation of compensatory myocardial hypertrophy to deleterious cellular death. Total collagen content in cells transiting towards death was also analyzed by Verhoeff van Gieson and picrosirius staining. Significant reduction up to two folds was witnessed at the transition dose as compared to the hypertrophic dose (Fig. 3.19-3.20). The increase in intracellular collagen fibril content at the concentration of 2.5 μM suggests that the cardiac cells respond initially to compensate the low NE stress but higher stress suppresses the production of collagen in the cell. This leads to an imbalance in collagen production and breakdown by matrix metallo proteinases causing changes of the myocardial ECM with the transition of hypertrophy to cardiac cell death.

To further understand if NE induced cell death was adrenergic receptor (AR) mediated mechanism or a general cytotoxic effects of high NE and its metabolites, studies with a beta AR blocker, Propranolol was conducted which confirmed that NE exerted β-AR mediated cell death in H9C2 cardiac cell lines. In its presence, reversal of NE mediated effects were observed as confirmed by morphological, cell viability, expression analysis ROS production studies (Fig. 3.21). These observations confirmed that NE induced cell death is adrenergic receptor mediated.

Hence we concluded that NE induced transition events in cardiomyoblasts from compensatory hypertrophic responses to cell death are mitochondrial mediated where increases ROS generation and activation of apoptosis results in cardiomyocyte death. Cell death is a later and the ultimate event succeeding the compensatory hypertrophy. The transition in between is critical which could shift the balance on the either end. The study of a transition phase is important to develop novel therapeutics which suppress the transition and prevent the cells from further damage and death.
In this regard, we further extended our findings to study the effect of Curcumin with hypertrophic and transition doses of NE. Apoptotic dose was not taken as at this dose, cardiomyoblasts have lost the capacity to be revived back as they are terminally differentiated cells. Hence, the transition of the cells should be targeted therapeutically as well. The two NE doses were taken and effect of Curcumin was studied individually by MTT cell viability assay. Cardio-protective dose of Curcumin for hypertrophy and transition were derived as 10 and 15 µM respectively (Fig. 3.22). This dose was further validated by trypan blue dye exclusion assay and the experimental sets for further studies were derived (Fig. 3.23). Morphological alterations in NE induced cells upon Curcumin treatment were studied at cellular and nuclear level by microscopic assays using Giemsa and DAPI nuclear specific stain respectively. Curcumin treatment displayed significant reduction in NE induced hypertrophy as well as cell death at 2.5 µM NE. The Curcumin treatment also displayed significant reversal NE induced cell death and transition at 50 µM concentration (Fig. 3.24 A and B). Disintegrated nuclear morphology was also found to be resisted upon respective Curcumin treatments, thereby suggesting protective effects of Curcumin in NE induced cardiac stress (Fig. 3.24 C).

Curcumin treatment also displayed significant reduction in NE induced Collagen content at hypertrophic dose thereby suggesting ECM remodeling mediated protective effects (Fig. 3.25). To validate these observations, semi-quantitative expression studies were conducted for hypertrophic and inflammatory markers- β-MHC and TNF-α. Curcumin treatment significantly reduced the hypertrophy and inflammatory responses at 2.5 µM NE. Exaggerated inflammation was also significantly reduced when cells were treated with Curcumin and 50 µM NE (Fig. 3.26). These observations confirmed the protective effects of Curcumin in cardiomyoblasts, where it reduced the initial compensatory stress responses as well as the transition of cells towards death.
3.6. Conclusion

Following conclusions can be derived from the present chapter:

- Levophed induces concentration-mediated deleterious effects in cardiomyoblasts.
- At 50 µM NE concentration, there occurs a transition from cellular hypertrophy towards death. This dose should be studied as a transition check-point to prevent cells from undergoing irreparable cellular death.
- NE mediated cardiac stress is adrenergic receptor mediated.
- Curcumin suppresses NE induced transition of cells from compensatory hypertrophy to cell death in terminally differentiated cardiac cells.