IN VITRO STUDY OF CARDIAC TISSUE REGENERATION IN TADPOLES OF THE TOAD, BUFO MELANOSTICTUS

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ABSTRACT
Present findings provide valuable basic information on cardiac tissue regeneration in culture medium. Meshed ventricular tissue of toad tadpoles was employed as explant and inoculated in culture medium supplemented with vitamin A, showed good regeneration ability with rhythmic beating. Vitamin A was found good model to accelerate the percentage of cardiac tissue regeneration. The culture system appears to be a suitable for investigating the changes occurred during regeneration and differentiation of cardiac muscles.

Keywords: In vitro, Cardiac Tissue Regeneration, Vitamin A.

INTRODUCTION
Regeneration is a complex process by which animals restore the shape, structure and function of body part lost. The body is equipped with several strategies to regenerate, including the rearrangement of pre-existing tissue, the activation of resident stem cells and the regression of specialized cells or tissues to simpler form by the process known as dedifferentiation. These strategies are directed toward the rebuilding of the appropriate tissue and organ structure. But this regeneration capacity varies in different organisms. The rate of cardiac regeneration from fish to amphibian to mammals demonstrates a decreasing trend high in fish, moderate in amphibians and limited in mammals. It is known that zebrafish possesses the significant ability to restore injured heart (Poss et al., 2007, 2010; Kikuchi et al., 2011a &b, Jopling et al., 2010). The regenerative ability is also observed in response to a variety of injuries, including cryoprobe induced injury (Chablais et al., 2011; Gonzalez-Rosa et al., 2000; Schnabel et al., 2011) and genetic ablation of cardiomyocytes (CMs) by transgenic induction of toxin expression (Wang et al., 2011).

The high regenerative ability of amphibians provides a valuable model system to gain basic information on regeneration that may be transferable to human trauma (Jangir et al., 2012, 2013, 2014; Poss 2010). Recently Jangir et al., (2012, 2013) reported heart regeneration in toad and frog tadpoles under the influence of vitamin A in situ as well as in transplantation set up at ectopic site. The cellular and molecular mechanisms that control the regenerative capacity of the toad tadpoles heart are not still clear. The repair process is associated with the proliferation of cardiomyocytes, which are characterized by a partial disassembly of sarcomeric structures (Bader and Oberpriller, 1978; Tate and Oberpriller 1989).

However, it was not clear whether different subsets of cardiomyocyte or cardiomyocyte progenitor cells contribute to heart repair in vivo and whether all cardiomyocytes own an inherent plasticity that might enable them to contribute to regenerative processes. It is also not known whether changes in cardiomyocytes morphology during cardiac repair reflect an obligatory de-programming and/or dedifferentiation step of cardiomyocytes that is necessary for regeneration or simply represent a feature of dividing cardiomyocytes that reduce the mechanical constraints of a dense myofibril network.

Laube et al., (2006) reported that dedifferentiation and reprogramming are not necessarily a pre requisite for cell proliferation because cultured newt cardiomyocytes reentered the cell cycle in vitro without the loss of sarcomeric proteins. They also reported that cardiac regeneration in newts is based on dedifferentiation of mature cardiomyocytes which enables them to respond efficiently to the induction of DNA synthesis and to proliferate efficiently.
Kikuchi et al., (2011) suggested that heart regeneration in zebrafish occurred through the activation of cardiomyocytes proliferation in areas of trauma. They revealed that within three hours of ventricular injury, the entire endocardium undergoes morphological changes and induces expression of the retinoic acid (RA) synthesizing enzyme raldh2. This enzyme localized at injury site and accelerate cardiogenesis i.e. regenerative cardiomyocyte proliferation begins. Recently Jangir et al., (2013) also reported accelerating influence of vitamin A on heart regeneration in toad/frog tadpoles in situ as well as in transplantation setup. The present study has been undertaken to report toad tadpoles cardiac tissue regeneration in culture medium. Vitamin A was found a good model to accelerate cardiac tissue regeneration in culture medium under the influence of vitamin A. A better understanding of cardiac tissue regeneration in culture medium might open the way for heart regeneration in higher animals.

MATERIALS AND METHODS

Animals Employed
In all experiments (three toe stage) young tadpoles of the toad, *Bufo melanostictus* were used. All tadpoles were obtained from a single pair. For experiment purpose, tadpoles were anaesthetized with MS222, 1:2000 before operation. The operation was done under stereoscopic binocular microscope.

Preparation of Explants
Meshed ventricle tissue of young tadpole was employed as explant. For this purpose three toe stage young tadpoles were immersed in 1% Euclorine solution for 30 sec., rinsed thrice in sterile Holtfreter’s solution and anaesthetized with MS 222 (Sandoz) 1:2000. Operations were carried out in sterile Holtfreter’s solution containing 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Fungizone (GIBCO). During operation, a small cut was made on anterior ventral surface of young tadpole to expose the heart and then the tip of ventricle was incised. The ventricular tips from ten young tadpoles were pooled and meshed in Leibovitz (L-15) culture medium. This meshed cardiac tissue (ventricle tissue) was treated as explant. The removed meshed tissue was rinsed four times in Leibovitz (L-15) diluted with sterile water (2:1) and containing 100 U/ml penicillin and 100 µg/ml streptomycin.

Culture Medium
Leibovitz (L-15) diluted with sterile water (2:1) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% inactivated foetal calf serum was used.

Culture Method
Meshed cardiac tissues as explant were placed in a plastic organ culture dish (35x10 mm Falcon plastics) with culture medium. Vitamin A (0.1 ml of vitamin A (15 IU/ml) in 100 ml culture medium) was supplemented to the culture medium for treated group. The culture medium was renewed on every 2nd day. Cultures were terminated after 5, 10, 15 and 40 days of inoculation.

Histological Method
Culture were fixe in 95% chilled ethanol (at 4°C), embedded in paraffin, cut into 7 µm, serial sections and stained with Haematoxyline and counter stain with eosin.

RESULTS AND DISCUSSION

Results
The results obtained from in vitro study are presented in the Table 1. The results clearly demonstrate the ability of cardiac tissue regeneration in anuran tadpoles in culture medium. Vitamin A was found to enhance the percentage of cardiac tissue regeneration. It was 55% in Vitamin A treated cases in comparison to 35% that of untreated control group explants. The present description is mainly concerned with the changes occurred in cultured explant tissues. The inoculated explants both vitamin A treated and untreated controls were preserved at different time intervals viz. day 5, 10, 15 and day 40 for histological evaluation. About one half of the meshed tissue cells were found to attached with bottom of plastic dishes/cavity blocks. Some of them were suspending in culture medium. By day 5 undifferentiated cells were reported among meshed explants (Figure 2).
### Table 1: Influence of Vitamin A on meshed cardiac tissue regeneration in culture medium

<table>
<thead>
<tr>
<th>Mode of Experiment</th>
<th>Group</th>
<th>Days of Culture</th>
<th>No. of Culture vessels examined</th>
<th>Status of Cardiac tissue regeneration(No.)</th>
<th>Percentage of cardiac tissue regeneration</th>
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<td><strong>Meshed cardiac tissue (tip of ventricle) inoculated in culture medium</strong></td>
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Figure 1: Schematic diagram showing the process of meshed cardiac tissue regeneration in culture medium

a) Figure showing showing level of amputation through ventricle.
(b) Incised ventricle tip inoculated in culture medium.
(c) Preparation of cellular meshed extract of cardiac tissue (ventricle part) as explants.
(d) Formation of undifferentiated cells from meshed cardiac tissue into the culture medium.
(e) Differentiation of newly formed cells into cardiomyocytes.
(f) Differentiation of cardiomyofibrils.
(g) Differentiation of cardiomyocytes into functional cardiac muscle

Figure 2: Microphotograph of a meshed cardiac tissue explants in culture medium supplemented with Vitamin A showing accumulation of undifferentiated cells on 5 of inoculation. Note- cultivated explants underwent dedifferentiation and forming new cells (40x)

Figure 3: Microphotograph of a section passing through 7 day old explant tissue after inoculation in culture medium supplemented with Vitamin A showing aggregation of newly formed cells from meshed cardiac tissue (100x)
Figures 4 and 5: Microphotograph of sections passing through the 15 days old explants after inoculation in culture medium supplemented with Vitamin A showing differentiation of cardiomyocytes (100x)

Figure 6: Microphotograph of a section passing through the 40 days old explant after inoculation in culture medium supplemented with Vitamin A showing complete regeneration of cardiac tissue (ventricular part) (100x)

Figure 7: Photograph of a regenerated explant ventricular tissue on day 40 after inoculation in culture medium supplemented with Vitamin A. (40x) Note: regenerated cardiac tissue showing rhythmic beating

Legends:
RExVT - Regenerated explant ventricular tissue
UNC - Undifferentiated newly formed cells
MCT - Meshed cardiac tissue
ANC - Aggregation of newly formed cells
CM - Cardiomyocytes
CMF - Cardiomyofibril
CdM - Cardiac Muscles

These cells might be developed or originated from meshed explant through dedifferentiation. Later on undifferentiated cells found to aggregate on certain foci and showing further differentiation. Number of cells increased and most of them become elliptical myocytes (Figure 3). By day 15-20, these cardiomyocytes differentiated into a network of cardiomyofibrils (Figure 4). By day 40 of inoculation,
well developed cardiac muscles were reported in vitamin-A treated explants. The newly developed and/or regenerated tissues were having normal cardiac muscle architecture (Figures 5, 6). Similar histological events were reported in both treated as well as untreated explants like enlargement of nuclei, nucleoli, cell elongation, formation of myofibrils and then cardiac muscles. In some cases nacrotic cells have also been observed. Some tissues so formed in culture medium could not be identified. Particularly in vitamin A treated group a nodulated structure (cardiac patches) developed showing normal rhythmic beating (Figure 7). Histological observations give clear evidence of cardiac tissue regenerative ability under the influence of vitamin A in culture medium. The process leading to the formation of cardiac patches was similar regardless of the chemical employed although vitamin A always showed higher growth rate and percentage of regeneration (Table 1).

**Discussion**

The results obtained under the present experimental conditions show that in culture medium, meshed ventricular tissue can regenerate into normal beating cardiac patches. Vitamin A was found to accelerate the percentage of cardiac tissue regeneration in culture medium. In our previous findings, we reported accelerating effect of vitamin A on heart regeneration in vivo as well as in transplantation set up in tadpoles of frog and toad (Jangir et al., 2012, 2013). How vitamin A affects cardiac regeneration is still not clear. However, Chytill and Ong (1984) and Maden (1988) suggested that retinoids enter the cells via some surface receptor or by lipophilic intercalation through the membranes and then bind to cytoplasmic binding proteins (RABP). Perkovich et al., (1987) also reported two such binding proteins one for retinoic acid, cellular retinoic acid binding protein (CRABP) and one for retinal, cellular retinal binding protein (CRBP). The complex then transported to the nuclei where it ultimately alters the pattern of gene activity. Maden (2000) also suggested that in nucleus RA acts as a ligand to activate families of transcription factor. Thus it can be suggested that retinoid play an important role in transmission of their legends from the cell membrane to the nucleus where the pattern of gene activity may be altered. Okabayashi et al., (2009) established a culture system for the differentiation of mouse ES cells in to cardiac muscles cells by applying retinoic acid (derivatives that activate retinoic acid receptors RAR and RXR). They reported enhanced differentiation of cardiac muscle cells by adding RXR specific agonist (PA024). On the other hand, differentiation of cardiac muscle cells was inhibited by the addition of RXR specific antagonist (PA452). These results suggest that RXR-mediated signaling have crucial role in the effects of retinoic acid signaling on the differentiation cardiac muscle cells.

Kikuchi et al., (2011a and b) reported accumulation of retinoic acid (RA) synthesizing enzyme (raldh2) which activates cardiomyocytes proliferation in the area of trauma during zebrafish heart regeneration. At the injury site if supplemented with raldh2-expressing epicardial cells then cardiogenesis begins. On the other hand, induced transgenic inhibition of RA receptors or expression of an RA-degrading enzyme blocked regenerative cardiomyocyte proliferation. They further suggested that injury- responsive source of RA in zebrafish, indicate key roles for cardiac cells in targeting RA synthesis to damaged heart tissue and promoting cardiomyocyte proliferation. Although the molecular mechanism by which the endocardium is activated and induces raldh2 is not yet clear, it needs further clarification. One possibility may be growth factors or cytokines which are released after injury and impact the permeability of endocardial cells, with raldh2 induction being a response to this change.

Lien et al., (2012) revealed that during zebrafish heart regeneration cardiomyocytes initiate DNA synthesis and cell proliferation. Several workers have studied that vitamin A promoted and enhanced dedifferentiation and proliferation of cells during limb lens and heart regeneration in toad tadpoles (Jangir and Niazi 1979; Jangir et al., 2005, 2012, 2013, 2014; Sharma et al., 2010).

Ieda et al., (2010) revealed that some transcriptional factors (TFs) like GATA4, Mef2c and Tbx5 are essential for cardiac myocyte differentiation for direct reprogramming of heart fibroblasts to cardiomyocyte formation in vitro. Vitamin A might have induced these transcription factors and make the meshed explants capable to differentiate into rhythmic beating cardiac patches.

Sucov et al., (2009) suggested that during development, the epicardium has been shown to stimulate myocardial cell proliferation through the secretion of trophic factors. It was hoped that during regeneration,
epicardial derived cells (EPDCs) might served as progenitor cell source to repopulate the damaged heart (Porrello 2011; Jopling et al., 2010). From the present in vitro study it can be presumed that cardiomyocytes may constitute the ideal donor cells for repairing injured heart, as they possess the necessary structural and physiological attributes to integrate functional cells. It can also be suggested that cell based cardiac repair offers the promise of rebuilding the injured heart from its component parts.

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