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Research Article

STUDY OF HEART REGENERATION IN TOAD TADPOLES UNDER THE INFLUENCE OF *TERMINALIA ARJUNA*

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ABSTRACT
Terminalia arjuna (Arjuna) accelerated regeneration of heart in tadpoles of the toad, *Bufo melanostictus.* Experiment was designed in two phases: In first phase tip of heart was amputated and animals were reared in *T. arjuna* solution. In second phase excised ventricular tips were pooled and meshed, considered as explants tissue. This meshed cardiac tissue extract was implanted into a pit made on mid lateral side of tadpoles. The implanted tissue showed good regeneration and rhythmic beating in treated tadpoles. The percentage of cardiac regeneration in both mode of experiments was found higher in *T. arjuna* treated tadpoles in comparison to that of untreated control tadpoles.

Keywords: *Terminalia Arjuna, Proliferation, Heart Regeneration*

INTRODUCTION
A number of medicinal plants have been described in ancient system of Indian medicine which has been claimed to have beneficial cardiac effect; *Terminalia arjuna* is one of them (Sharma, 1996). *Terminalia arjuna* belongs to the combretaceae family and is a deciduous tree of 20-30 meter height found throughout India (Dwivedi and Jauhari, 1997). It is full of medicinal properties and clinical evaluation indicates its beneficial effects in the treatment of coronary artery disease, heart failure and possibly hyper cholesterolemia. A high degree of antioxidant activity of bark of *Terminalia* has been documented (Malik *et al.,* 2009; Gauthaman *et al.,* 2001), which might have important contribution to its cardio protective effect. Experimental and clinical studies have shown that the dried bark powder of *Terminalia arjuna* has significant protective effects in ischemic heart diseases (Tripathi, 1993; Dwivedi, 1994; Kumar *et al.,* 2009). The alcoholic extract of the bark of plant contains a large amount of flavones and tannins, which possess significant antioxidant activity against induced ischemic heart diseases (Karthikeyan *et al.,* 2003; Ramesh *et al.,* 2004; Mana *et al.,* 2006; Sinha *et al.,* 2008; Singh *et al.,* 2008; Kumar *et al.,* 2009). Several workers reported positive effect of *T. arjuna* on inflammation and wound healing. Its alcoholic extract significantly increases the tensile strength of the incisions wounds and the percentage of epithelialization of wounds (Rane and Mengi 2003; Choudhari and Mengi 2006). Patnaik *et al.,* (2007) reported that triterpenoid glycoside from the bark of *T. arjuna* species could be a contributing factors leading to healing of growth of muscle tissue and healing wound in operated frog. The present study was under taken to evaluate the effect of *T. arjuna* on cardiac tissue regeneration in situ as well as in transplantation set up in tadpoles of the toad *Bufo melanostictus.*

MATERIALS AND METHODS
Young (3 toe stage) & mature (5 toe stage) tadpoles of toad *Bufo melanostictus* were used for experiments. Tadpoles were maintained in aquaria at room temperature (35*C-37*C) and fed on half boiled spinach leaves.

Animals were anaesthetized in 1:2000 MS222 solutions for 3-5 minutes before operation and fixation. The experiment was designed into two series I and II. Series I was concerned with the study of heart regeneration in situ (in vivo study).

While series II was concerned with the study of fate of meshed cardiac tissue implants in the pit made on mid lateral position of the tail of host tadpoles. In both cases out of these operated tadpoles, half were treated with Arjuna solution and remaining half were kept untreated and served as control.

Operation

Anesthetized tadpoles were put on moist cotton in petriplate with ventral side up. 20% of ventricle apex part of heart was amputated with irridectomy scissors (Figure 1). Sterilized forceps were used to penetrate the skin, muscles and pericardial sac. Operated animals were reared in respective solution.

Preparation of Explants

Ventricle part of 5 donor young (3 toe stage) tadpoles were taken out and pooled and meshed in 2ml of saline solution that was treated as explant tissue.

Preparation of Recipients

Young (3 toe stage) and mature (5 toe stage) tadpoles were used as recipients.

The animals were anesthetized (1:2000 MS222 solution Ethyl-m-amino benzoate methane sulfonate) before operation.

A pit of about 1mm depth was made by a sharp sterile needle at mid lateral position of the tail of recipient tadpoles.

Implantation

A pin head size meshed cardiac explant tissue implanted into the pit on recipient tadpole tail (Figure 8).

After insertion of the cardiac tissue explant, skin flap was covered over it. Half of the operated tadpoles were reared in *T. arjuna* solution for first 3 days and then transferred to tap water.

Remaining operated animals were not treated with *T. arjuna*.

For histological purpose operated tadpoles of both control and *T. arjuna* treated groups were preserved in Bouin's solution for 24 hours on day 5, 10 and 20.

Preparation of T. arjuna Extract

Powdered form of *T. arjuna*'s bark (50g) was mixed with 2 liter of tap water and kept for 2 hours. The solution was boiled in pressure cooker.
The vapours of such solution were condensed to get its watery form and were collected in sterilized beaker.
This extract was treated as standards solution. To make the working solution, 5 ml of standard solution was mixed with 500 ml of tap water. Half of the operated animals were reared in the *T. arjuna* solution which was considered as a treated group.

RESULTS AND DISCUSSION

Result

The results obtained are presented in Table 1. *Termenalia arjuna* induced cardiac tissue regeneration in both mode of experiments in vivo (FigUREs 2,3,4,5,6 &7) and transplantation set up (Figures 8,9,10 &11).

The results show that heart regeneration ability is found in tadpoles of the toad, *Bufo meleanostictus*. However, it declined with the age of animals.

It was 40% in 3 to stage young tadpoles of untreated group S_IC1, while 30% in 5 to stage mature tadpoles S_IC2 of control group where as in *T. arjuna* treated tadpoles it was 70% in 3 to stage tadpoles (S_IT1) and 40 % in 5 to stage tadpoles (S_IT2) {Table 1}.

Similar declined trend was observed in transplantation setup.

It was 30 % in 3 to stage tadpoles ($S_{II}C3$), where as in 5 to stage it was 20 % only ($S_{II}C4$). In case of *T. arjuna* treated tadpoles, it was 60 % in 3 to stage ($S_{II}T3$) and 40% in 5 to stage tadpoles ($S_{II}T4$) respectively (Table 1).

Table 1: The influence of *T. arjuna* on heart regeneration in tadpoles of the toad, *Bufo melanostictus*

Series	Developmental Stage on the day of operation	Group	No. of animals employed	Day of preservation	No. of animals preserved	Percentage of Regeneration	
						Regenerates (lost part)	Non regenerates
SI: Heart Regenera-tion *in vivo* (Tadpoles with amputated heart)	Young (3 toe stage) tadpoles	C1 : (Control operated animals reared in water D0 - D20 =Water)	40	5 10 20	10 10 20	40%	60%
		T1 : *T. arjuna* treated (D0 - D3 = Arjuna D4 - D20 =Water)	40	5 10 20	10 10 20	70%	30%
	Mature (5 toe stage) tadpoles	C2 : (Control operated animals reared in water D0 - D20 =Water)	40	5 10 20	10 10 20	30%	70%
		T2 : *T. arjuna* treated (D0 - D3 = *T. arjuna* D4 - D20 =Water)	40	5 10 20	10 10 20	40%	60%
SII: Ectopic heart regenera-tion (Tadpoles with meshed cardiac tissue explants in a pit made on their tail)	Young (3 toe stage) tadpoles	C3 : Control (operated animals reared in water D0 - D20 =Water)	40	5 10 20	10 10 20	30%	70%
		T3 : *T. arjuna* treated (D0 - D3 = *T. arjuna* D4 - D20 =Water)	40	5 10 20	10 10 20	60%	40%
	Mature (5 toe stage) tadpoles	C4 : Control (operated animals reared in water D0 - D20 =Water)	40	5 10 20	10 10 20	20%	80%
		T4 : Arjuna treated D0 - D3 = Arjuna D4 - D20 =Water	40	5 10 20	10 10 20	40%	60%

Histological study revealed that after operation of ventricle tip, some of cells accumulated at amputation site by day 5. By day 7 cardiomyocytes become deteched from one another creating large intercellular space (Figure 5). By day 10-15 after operation the number of dedifferentiated cells increased and blastema like structure formed. The exact source of origin of blastema cells is not clear. These blasemal cells may originated either from dedifferentiation of injured cardiac tissue or from the resident progenitor cells. Later on blastemal cells differentiated to form myocardial architecture (Figure 6). By day 20 amputated heart showed little or no scar in the wounded area in *T. arjuna* treated tadpoles (Figures 4 & 7). Almost myocardium was looking normal by day 20. Consequently complete re growth of amputated region was reported in most of the case of *T. arjuna* treated animals.
Promising results obtained from the tadpoles of series II. It was observed that engrafted cardiac tissue extract lost its identity in most of the control recipient operated tadpoles.

Whereas in *T. arjuna* treated host tadpoles, engraft tissue showed good vascularization and even normal cardiac beating in most of cases (Figures 9 &10).

It seems that Arjuna enhanced the differentiation process of implanted meshed cardiac tissue even at ectopic site (tail), consequently transplantated cardiac tissue differentiated like miniature ectopic heart on the tail (Figures 10 &11). Matter of curiosity was synchronization between beating of implanted cardiac and blood supply at ectopic site. Some explants in *T. arjuna* solution treated host tadpoles showed normal histological features of ventricle tissue on day 20 post transplantation (Figure 11).

Discussion

The results of present study showed that *Termenalia arjuna* induces cardiac tissue regeneration in both mode of experiments (in vivo and transplantation set up).

The cardiac tissue regeneration capacity declined with the age of animals in both untreated and arjuna treated groups. Similar results have also been reported by Jangir *et al.,* (2013a&b, 2014) in cardiac tissue regeneration in toad and frog tadpoles.

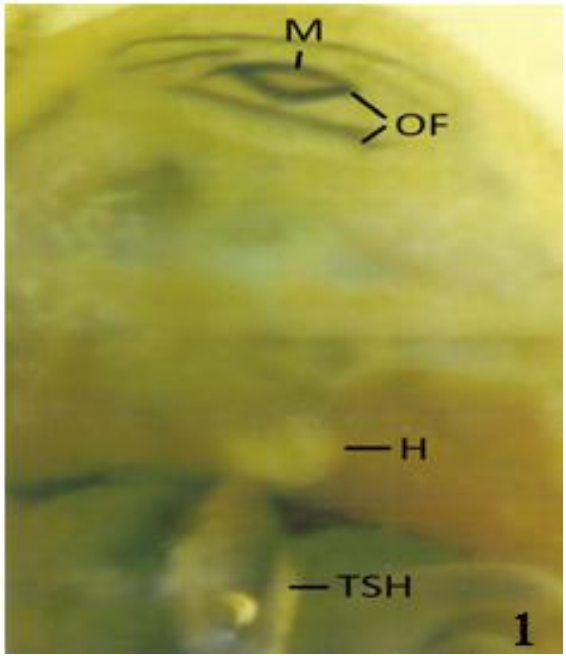

Figure 1: Photograph showing level of amputation (Surgical removal of the tip of the ventricle region of the toad tadpole, (20 X)

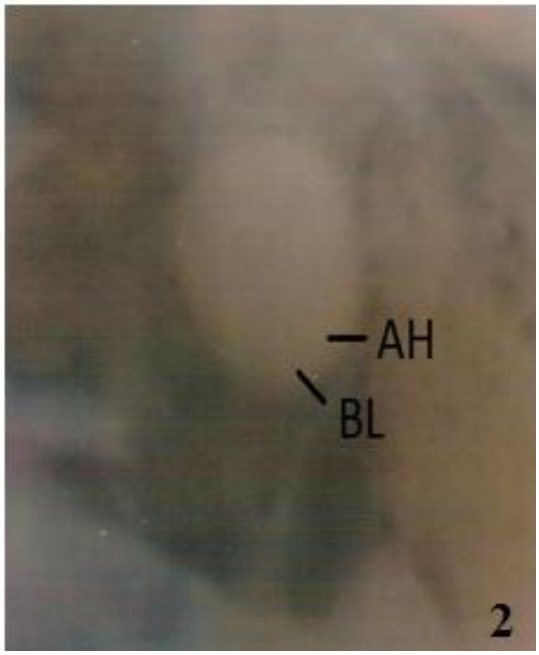

Figure 2: Photograph of amputated heart of *T.arjuna* treated group young tadpole (5 days old) showing blastema formation at wounded site, (20 X)

Figure 3: Photograph of amputated heart of *T.arjuna* treated young tadpole (10 days old) showing regeneration of lost ventricle part, (20X)

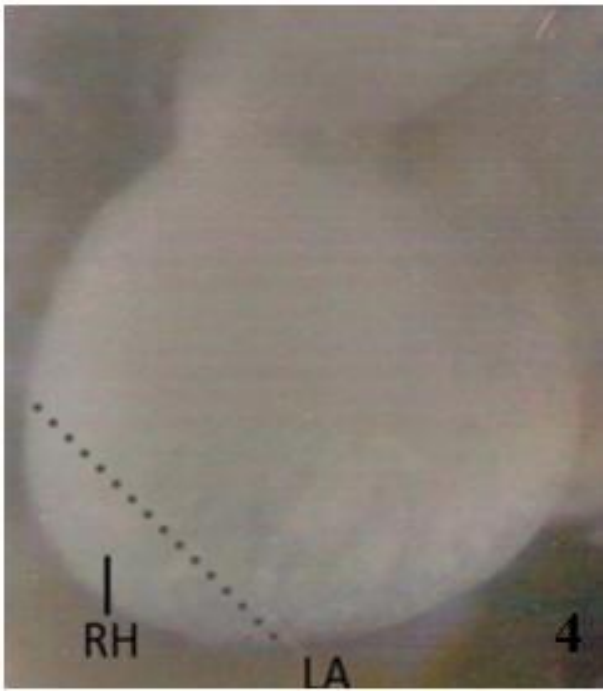

Figure 4: Photograph showing com- plete regeneration of heart in *T.arjuna* treated tadpole 20th day post amputation (20X)

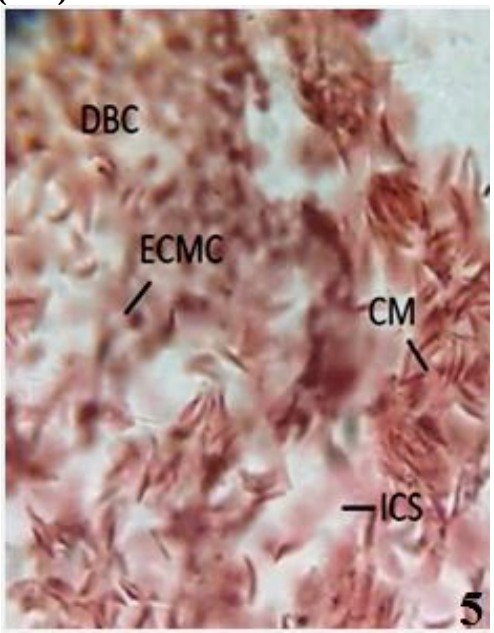

Figure 5: Microphotograph of a section passing through the am- putated heart of *T.arjuna* treated young tadpoles (5 days old) showing dedifferentiation of cardiac tissue in to cardiomyocytes,(100X)

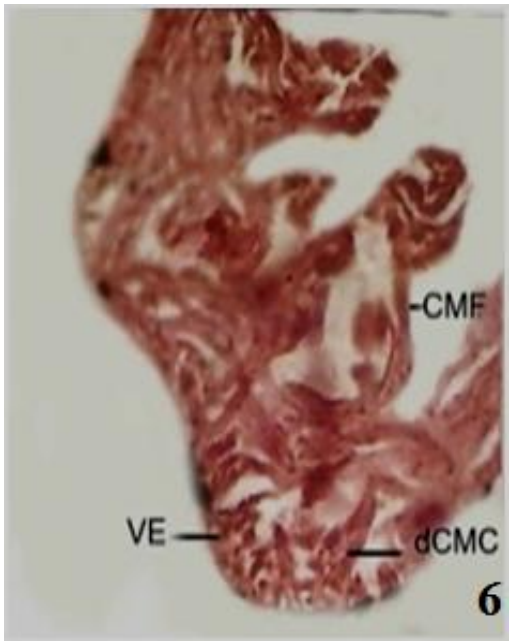

Figure 6: Microphotograph of a section passing through the am- putated heart of *T.arjuna* treated young tadpoles (10 days old) showing differentiation of cardiomyocytes into cardiomyo-fibril (100X)

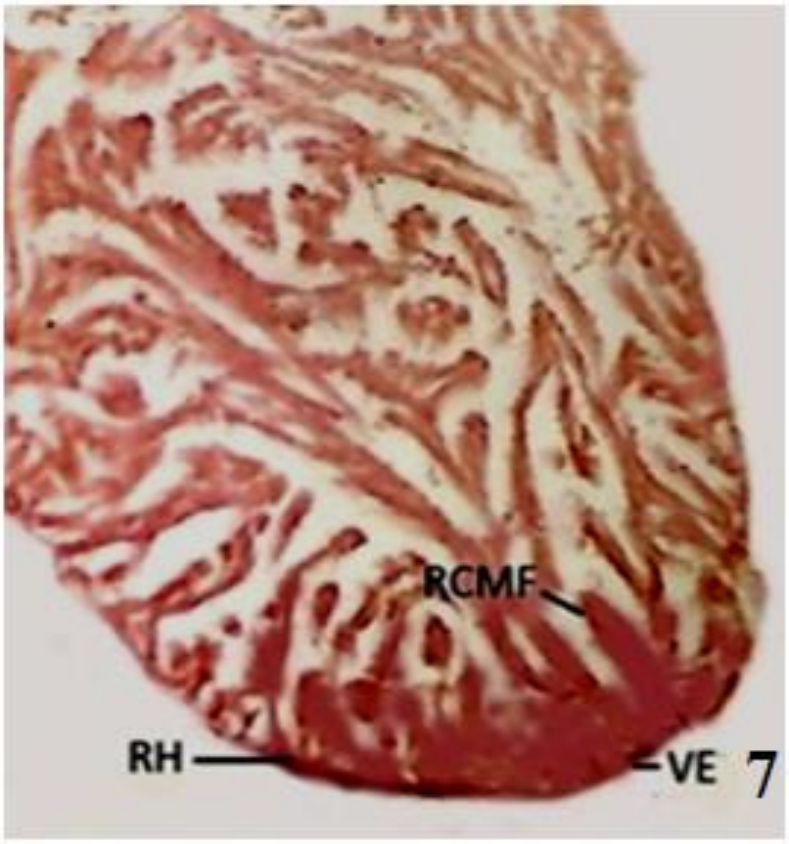

Figure 7: Microphotograph of a section passing through the amputated heart of *T.arjuna* treated young tadpoles (20 days old) showing complete regeneration of lost part of the ventricle, (100 X)

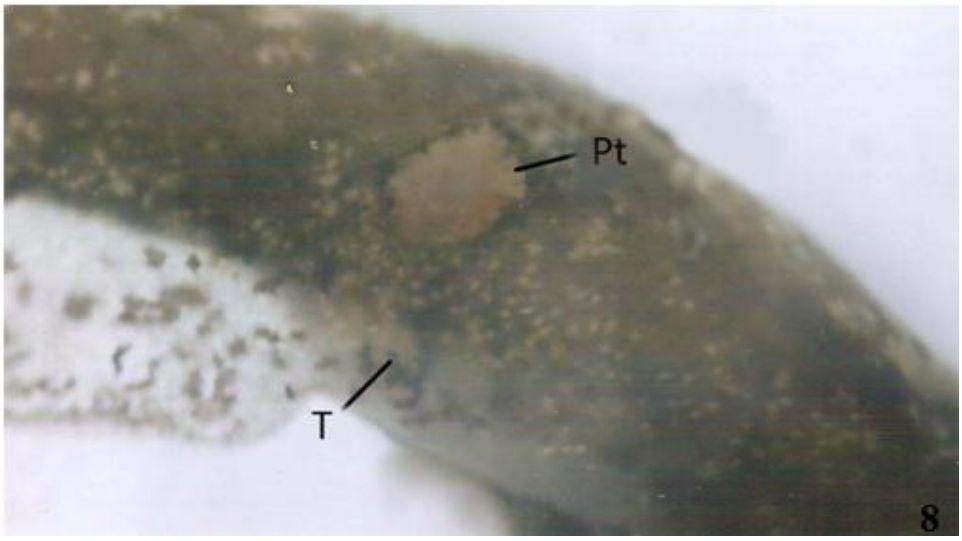

Figure 8: Photograph showing the site of implantation of messed cardiac tissue into a pit made on mid-lateral position of tail of the host tadpole (20X)

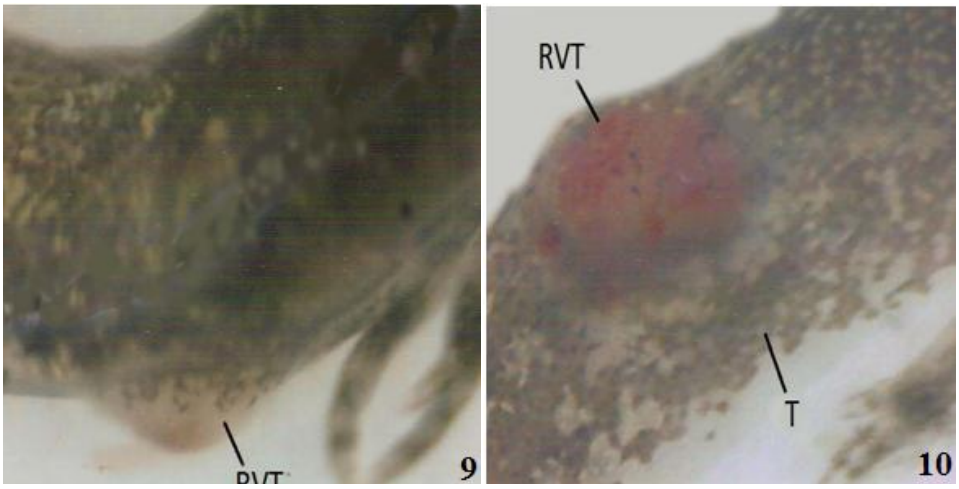

Figure 9 and 10: Photographs showing development & differentiation of cardiac implants at ectopic site (mid-lateral position of tail) (20X)

Sumitra *et al.,* (2001) also observed the cardiao protective effects of arjunolic acid by showing its effect on induced myocardial necrosis in rat. This cardio protective effect of arjunolic acid has been possibly attributed to the protective effect against the damaged caused by myocardial necrosis. The cardio protective effect of arjunolic acid can be attributed to its powerful antioxidant, free radical scavenging and metal chelating properties. The mechanism of *T. arjuna* effect on cardiac tissue regeneration is still exactly not clear. However, Rane and Mengi (2003), reported that both ethanolic and hydroalcohalic extract of the bark of *T. arjuna* significantly increase the tensile strength of the incision wounds and increase in the percentage of reduction in wound size in comparison to control. The estimated increase in hydroxiproline content of the granulation tissue of the excision wound indicated rapid collagen turnover thus, leading to rapid healing of wounds. It is also reported that the aqueous extract of Arjuna's bark significantly prevented the oxidative stress, decline in endogenous antioxidant level and also prevent myocardial changes induced by oxidative insult (Kumar *et al.,* 2009). The alcoholic extract of *T. arjuna* enhance the cardiac intracellular antioxidant activity and protect heart from induced oxidative stress (Sinha *et al.,* 2008; Singh *et al.,* 2008; Manna *et al.,* 2006; Nammi *et al.,* 2003; Karthikeyan *et al.,* 2003). In vivo ischemic- reperfusion injury induced oxidative stress, tissue injury of heart were prevented in the *T. arjuna* treated rabbit hearts which provide scientific basis for the putative therapeutic effect of *T. arjuna* in ischemic heart disease (Ramesh *et al.,* 2004).

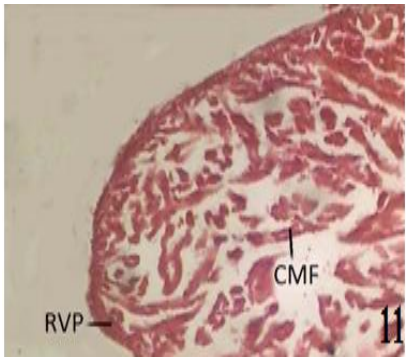

Figure 11: Microphotograph of a section passing through differentiated implanted cardiac tissue on recipient *T.arjuna* treated tadpole's tail showing regeneration and differentiation of cardiac tissue (100X)

Legends

M	:	Mouth	RVT: Regenerated
OF	:	Oral fringe	ventricle tissue
H	:	Heart	RVP: Regenerated
TSH	:	Tip of syringe supporting heart	ventricle part
AH	:	Amputated heart	
BL	:	Blastema	
RH	:	Regenerated heart	
LA	:	Level of amputation	
DBC	:	Dedifferentiated blastema cells	
ECMC	:	Early cardiomyocytes	
CM	:	Cardiomyocytes	
ICS	:	Intercellular space	
CMF	:	Cardiomyofibril	
dCMC	:	differentiating cardiomyocytes	
VE	:	Ventricle epithelium	
RCMF	:	Regenerated cardiomyofibril	
Pt	:	Pit for transplantation	
T	:	Tail	

In present study, Arjuna might have enhanced dedifferentiation of myocardial tissue cells of tadpoles of *Bufo melanostictus* resulting in regeneration of lost ventricle part of infracted heart. This suggests that *T. arjuna* may act as a good model employed for investigation of the molecular mechanisms responsible for cardiac tissue regeneration. Heart regeneration appears to be a suitable system for such investigation. This discovery opens the possibility that researchers may one day enhance the endogenous regenerative capacity of mammals including man by inducing cellular dedifferentiation invivo.

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