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Study of segmental regeneration and healing of ventral nerve cord in *Eisenia fetida*

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Abstract : *The regeneration in a lumbricid earthworm, Eisenia fetida was studied by amputating the earthworms at different regions. Also, the healing of Ventral Nerve Cord (VNC) was studied in them by using Rapid Escape Response (RER) Assay. Further the role of an extracellular pathway, namely the Wnt/β- catenin in the regeneration of earthworms was verified using the chemicals Lithium Chloride (LiCl) and Sodium Chloride (NaCl).*

Keywords : *Regeneration , Amputation , Ventral Nerve Cord, Rapid Escape Response , Wnt/β- cateninin.*

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Introduction :

Earthworms are extremely important in the formation and maintenance of soil structure and turnover of dead organic matter. Therefore, the factors that affect their survival are important in their ecology. Regeneration is a process in which damaged tissues grow again. Annelids exhibit extensive variation in their regenerative ability (Bely and Sikes, 2010). Earthworms are unique and valuable model to study regeneration (Park et.al., 2013). Among earthworms, the lumbricid earthworm *Eisenia fetida* has been used commonly for research, because it is easy to culture and handle them in the laboratory.

The earthworm, *Eisenia fetida* shows sensitivity towards various chemicals in the environment (Edwards and Bohlen, 1996). Ventral Nerve Cord (VNC) is a vital part of the nervous system of the earthworm that is involved in motor coordination. It consists of the medial giant axon (MGA) and two electrically coupled lateral giant axon (LGA). Rapid Escape Response (RER) in *Eisenia fetida* can be used to study the regeneration in the Ventral Nerve Cord (VNC) (Drewes et al., 1988).

The VNC is responsible for effecting sudden violent contractions of body in response to alarms.

A major question that remains incompletely answered involves the identification of the extracellular signals that regulate the formation or activation of stem cells during regeneration. Several studies have documented expression of Wnt ligands and components of the β-catenin signalling pathway in regenerating amphibian and fish appendages (Caubit et al., 1997a,b; Poss et al., 2000), and other studies have suggested that Wnt/β-catenin signalling is functionally involved in the proliferation of cells during regeneration of mammalian muscle, liver and bone (Polesskaya et al., 2003; Sodhi et al., 2005; Zhong et al., 2006). Wnt is resultant from a fusion of the name of the *Drosophila* segment polarity gene *wingless* and the name of the vertebrate homolog, *integrated* or *int-1* (Wodarz and Nusse, 1998). However, whether Wnt/β-catenin signalling plays an important role in the epimorphic/true regeneration has not been tested.

Wnt/β-catenin signalling regulates progenitor cell-fate and proliferation during embryonic development and in adult tissue homeostasis (Logan and Nusse, 2004; Reya and Clevers, 2005), raising the possibility that it is also involved in progenitor cell function during regeneration.

The present study was undertaken to assess the regeneration capacity of *Eisenia fetida* and to find out the possible role of Wnt signalling pathway on the regeneration of earthworms by using the chemicals Lithium Chloride (LiCl) and Sodium Chloride (NaCl).

Materials and Methods :

Eighty adult healthy earthworms (*Eisenia fetida)* were taken and maintained in cup and bottle culture in the college laboratory.

Out of these forty-five worms were taken and grouped into four categories. Group A constituted the worms which were amputated at 6th segment from the head end, Group B at 5th segment from the tail end, Group C constituted the anterior parts of median amputated worms (cut at intersegmental groove of 30-31 segment from the head end) possessing both mouth and clitellum and Group D constituted the posterior parts of median amputated worms (cut at intersegmental groove of 30-31 segment from the head end) possessing neither the mouth nor the clitellum.

For amputation, the worm was placed on a chilled slide and the segment to be amputated was located using a hand lens. Then using a surgical blade a sharp cut was made.

For testing the possible role of Wnt signalling pathway on regeneration, again, forty-five earthworms were taken and amputated at similar segments as done for the study of normal regeneration. They were grouped similarly into four groups, A,B,C and D and five each were treated with different concentrations (0.5 Molar, 0.4 Molar and 0.3 Molar) of Lithium Chloride (LiCl) and Sodium Chloride (NaCl) once daily with the help of a brush.

For giving a lesion on Ventral Nerve Cord (VNC), an earthworm was placed ventrally on a chilled slide and an incision was made transversally to the longitudinal axis of the worm. For testing the Rapid Escape Response (RER) of these worms, two milli litre (ml) of salt solutions (10 milli Molar, 100 milli Molar, and 200 milli Molar) were taken in three different test tubes. Thereafter, each worm (both lesioned and control) was immersed entirely in the salt solution of above mentioned concentrations one by one and the time taken by it to climb out of the test tube was noted down with the help of a stopwatch. When the time taken by the earthworm to climb out of the test tube was less than two minutes then, it was taken as a positive result. On the other hand, when the worm

failed to climb out of the salt solution, the result was taken as negative. Same procedure was followed for both the control (without VNC cut) and VNC lesioned worms. Five readings were taken for each earthworm.

All the observations were noted down daily in a log book.

Results and Discussion :

The regeneration process in *Eisenia fetida* followed a definite pattern and accordingly a time schedule. It began with a small rounded or conical bud formation. This bud lacked pigmentation and appeared white. This bud was called bulb (Fig 1). This bulb appeared after an average of four days in the worms of Group A, after less than three days in the worms of Group C and D, and after three days in the worms of Group B (Tables 1 and 2). The bulb then elongated gradually to form a blastema (Fig 2) after six days in Group A, five days in Group C and D and less than five days in Group B worms. Meanwhile the colour of the outgrowth became reddish and segmentation appeared. Finally regeneration got completed in 28 days in Group A and 26 days in Group B worms. The time taken for the appearance of bulb and complete regeneration was significantly greater in the worms in which anterior six segments were cut from the mouth end (Group A) as compared to the worms in which posterior five segments were cut from the tail end (Group B) (Table 1). Here we can say that regeneration in Group B worms was faster than in Group A. This result was consistent with the work done by Xiao et al., (2011) where they claimed that the posterior segments had a greater regeneration capacity than the anterior segments. Similarly, number of days required for the growth of blastema and complete regeneration was significantly greater in the anterior cut part (Group C) versus posterior cut part (Group D) when an earthworm was cut into two pieces in the middle region (Table 2). However, while the Group D showed restoration of all its lost segments except the clitellum in about 32 days (Fig 3), Group C worms developed a blastema of about 55-60 mm, orangish-red in colour, faintly segmented (Fig 4) but this blastema never fully developed to form segments like the original ones.

There was no overshooting in the number of segments because the number of regenerated segments was equal to the number of segments amputated in all the worms of Group A to D. The findings of this study differ from that of Gates (1950), who obtained hypomeric and hypermeric conditions along with equimeric condition in his regeneration experiments in *Eisenia fetida*.

The time taken for regeneration was significantly higher in Lithium Chloride (LiCl) treated worms as compared to control and Sodium Chloride (NaCl) treated ones, indicating that Lithium Chloride (LiCl) hampered the regeneration process (Table 3-6). It is known from earlier works that Lithium Chloride (LiCl) inhibits the Wnt signal transduction pathways, most probably by inhibiting the GSK-3 enzyme (Klein and Melton, 1996). This, in effect, shows that Wnt/β-catenin pathway has a role in proliferation and hence regeneration.

The escape reflex circuitry of earthworms is derived from more than 100 sets of segmentally arranged, homologous neurons located within the ventral Nerve Cord (VNC) (Drewes et. al., 1988). In the experiment of Drewes et. al., (1988), Rapid Escape Response (RER) was induced by using multi-channel electrode arrays etched onto a printed circuit board (O'Gara *et. al.,* 1982). In the present experiment the stimulus for RER was Sodium Chloride (NaCl) solution which served as gustatory repellant for the worm. One of the notable features of the escape response was that it was associated with muscular contractions and these contractions were mediated from the anterior end

by the medial giant axon (MGF) and from the posterior end by the lateral giant axon (LGF) (Bullock, 1945; Rushton, 1946). Locomotion in earthworm is brought about by coordinated reciprocal contractions of longitudinal and circular muscle bands which lie in the body wall. Hence, the control worm in which VNC was intact, was able to climb out of the test tube within two minutes. But when VNC was injured, as in the lesioned worm, it could not climb out of the test tube. This showed that the motor coordination in such worms had become dysfunctional.

The Rapid Escape Response (RER) assay on lesioned worms showed that it took about few hours (4-5 hours) to two days (48 hours) for the Ventral Nerve Cord (VNC) to be restored depending upon the extent of injury. Similar results were reported by Drewes et al., (1988). The medial and lateral giant nerve fibers in the earthworm, *Eisenia fetida,* regenerate cell-specific connections and recover through-conduction capabilities in as little as 1-2 days after ventral nerve cord (VNC) transaction (Drewes et. al., 1988). Once the VNC was expected to get healed, the worms started behaving normally i.e., the worms climbed out of the test tube within two minutes.(Table 7).

Table 1. Number of days (Mean±S.E.) required for regeneration in anterior (six segments cut) v/s posterior (five segments cut).

	Group A	Group B
	Anterior (6 Segments Cut) (n=15)	Posterior (5 Segments Cut) (n=15)
Appearance of bulb	3.93 ± 0.38	3.06 ± 0.26*
Appearance of blastema	5.93 ± 0.38	5.06 ± 0.26
Pigmentation & segmentation	16.33 ± 0.12	16.27 ± 0.18
Complete regeneration	27.4 ± 0.25	25.93 ± 0.33*

* Significant difference at P<0.05

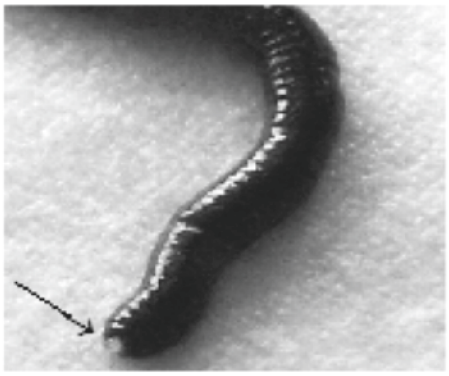

Fig 1. Appearance of bud

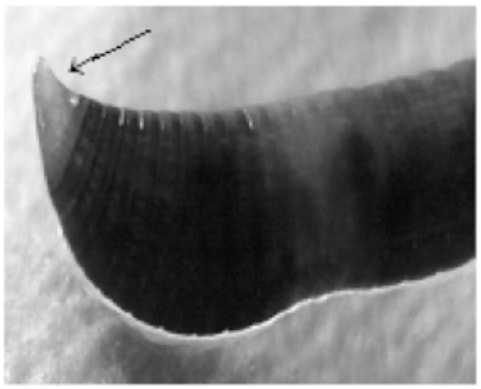

Fig 2. Appearance of blastem

Fig 3. Complete regeneration in Group D worms

Fig 4. Regeneration in Group C worms after 40 days of amputation

Table 2. Number of days (Mean ±S.E.) required for regeneration in anterior cut part (with mouth and clitellum) v/s posterior cut part (without mouth and clitellum)

	Group C	Group D
	Anterior part (n=14)	Posterior part (n=14)
Appearance of bulb	2.71±0.12	2.71±0.12
Appearance of blastema	4.71±0.12	4.71±0.12
Pigmentation &segmentation	34.71±0.12	23.07±0.22*
Complete regeneration	40.29±0.46	32.07±0.22*

* Significant difference at P<0.05

Table 3: Number of days (Mean ±S.E.) required for regeneration in worms with anterior six segments cut from head end (Group A) and treated with NaCl (n=5)

Concentration of NaCl	Appearance of bulb	Appearance of blastema	Pigmentation & segmentation	Complete regeneration
0.5M	3.6 ± 0.4	6.6 ± 0.74	14.4 ± 0.8	27.6 ± 0.24
0.4M	3.4 ± 0.24	6.6 ± 0.67	15.0 ± 0.7	27.4 ± 0.24
0.3M	4.0 ± 0.31	6.2 ± 0.37	15.0 ± 0.4	25.4 ± 0.4

Table 4. Number of days (Mean ±S.E.) required for regeneration in worms with anterior six segments cut from head end (Group A) and treated with LiCl (n=5)

Concentration of LiCl	Appearance of bulb	Appearance of blastema	Pigmentation & segmentation	Complete regeneration
0.5M	6.2 ± 0.2*	8.6 ± 0.24*	13.2 ± 0.3	32.0 ± 0.44*
0.4M	5.8 ± 0.37*	7.6 ± 0.24	13.0 ± 0.3*	32.6 ± 0.24*
0.3M	5.0 ± 0.31	7.4 ± 0.24*	14.2 ± 0.37	31.0 ± 0.44*

* Significant at P<0.05 with respect to NaCl treated worms

Table 5: Number of days (Mean ±S.E.) required for regeneration in worms with five segments cut from tail end (Group B) and treated with NaCl (n=5)

Concentration of NaCl	Appearance of bulb	Appearance of blastema	Pigmentation & segmentation	Complete regeneration
0.5M	3.8 ± 0.48	5.4 ± 0.2	13.8 ± 0.8	25.8 ± 0.2
0.4M	3.8 ± 0.37	4.8 ± 0.37	13.2 ± 0.58	24.6 ± 0.4
0.3M	3.6 ± 0.24	5.2 ± 0.58	13.2± 0.73	23.0 ± 0.44

Table 6. Number of days (Mean ±S.E.) required for regeneration in worms with five segments cut from tail end (Group B) and treated with LiCl (n=5)

Concentration of LiCl	Appearance of bulb	Appearance of blastema	Pigmentation & segmentation	Complete regeneration
0.5M	6.8 ± 0.37*	8.6 ± 0.24*	17.4 ± 0.2*	30.6 ± 0.4*
0.4M	6.0 ± 0.31*	8.4 ± 0.24*	16.8 ± 0.5*	32.4 ± 0.4*
0.3M	6.2 ± 0.37*	8.4 ± 0.24*	16.4 ± 0.5*	32.2 ± 0.37*

* Significant at P<0.05 with respect to NaCl treated worms

Table 7. RER Assay on control and lesioned (VNC cut) worms.

	Time taken to escape (in seconds)		
Concentration of salt solution	Control worms (n=10)	Lesioned worms (n=6)	
		Just after incision of VNC	After healing (after 48 hours of incision of VNC)
10mM	0±0.00 (did not escape in 2 minutes)	0±0.00 (did not escape in 2 minutes)	0±0.00 (did not escape in 2 minutes)
100mM	58.4 ± 7.33	0±0.00 (did not escape in 2 minutes)	93.16 ± 4.94*
200 mM	42 ± 9.01	0±0.00 (did not escape in 2 minutes)	50.5 ± 4.07

Values are Mean ± S.E.

* Significant at P<0.05 with respect to control

Conclusion :

It was found that the regeneration capacity in earthworm depended upon the segment or the region where the earthworm was amputated. There was no overshooting in the number of segments after regeneration. The Ventral Nerve Cord (VNC) was deemed to get healed within few hours (4-5 hours) to two days (48 hours). The rate of regeneration in Lithium Chloride (LiCl) treated

worms was slower as compared to the control worms, which signified that Wnt signalling pathway may have a role behind regeneration in earthworms, as Lithium Chloride (LiCl) is a known inhibitor of Wnt signalling pathway.

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