IRIS
Journal for Young Scientists
ISSN 2278 – 618X (Print)
ISSN 2278 – 6384 (Online)

http://www.patnawomenscollege.in/journal

Comparison of Regeneration in Adult and Juvenile *Eisenia fetida* and Effect of Riboflavin on Regeneration

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Received : November 2016
Accepted : March 2017
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Abstract : *Regeneration in adult and juvenile earthworm, Eisenia fetida was studied by amputating at different regions. The rate of regeneration was faster in juveniles as compared to adults. Further, the role of coelomic fluid and riboflavin on the rate of regeneration was verified. Regeneration was delayed in the earthworms from which coelomic fluid was extracted before amputation, suggesting coelomic fluid was important for regeneration activity. Infrared (IR) spectra of riboflavin, coelomic fluid, skin of earthworm and metabolite of bacteria found in the gut of earthworm were found to be similar, suggesting the presence of riboflavin in the body of earthworm. The study concluded that riboflavin could be used in regenerative medicine.*

Keywords: *Regeneration, Amputation, Coelomic fluid, Riboflavin, FTIR.*

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Introduction :

Regeneration is a process in which damaged tissue grows again. Earthworms are unique and valuable model to study regeneration (Park et al., 2013). Among earthworms, the lumbricid earthworm *Eisenia fetida*, has been commonly used for research into regeneration, because it is easy to culture and handle them in the laboratory.

The promotive role of vitamins in growth and development has been found by earlier workers in mice (Maden et al., 1998). Riboflavin (vitamin B2) is now recognized as a potentiator of immunocompetence and tissue regeneration capacity in earthworms and other organisms (Plytycz and Morgan, 2011; Johnson et al., 2012).

There may be differences both in the survival and rate of regeneration between juvenile and clitellar adult earthworms (Xiao et al., 2011). The role of coelomic fluid and riboflavin on the regeneration in *Eisenia fetida* has not been studied so far. Therefore, the present study was undertaken to assess the regeneration capacity of *Eisenia fetida,* and to find out the possible difference in the rates of regeneration and survival in the adult and juvenile worms. The roles of coelomic fluid and riboflavin on the rate of regeneration were also studied.

Materials and Methods :

Hundred healthy adult and juvenile earthworms (*Eisenia fetida)* were taken and maintained in pot and cup culture in the college laboratory.

Out of these, worms were taken and grouped into four categories. Each group had forty worms. Group A constituted the worms which were amputated at 10^{th} segment from their head end, Group B worms were computed at 10^{th} segments from the tail end, Group C and Group D constituted the median amputated worms (cut at 26^{th} segment and 32^{nd} from head-end respectively). For amputation, the worm was placed on a chilled slide and the chosen segment was amputated using a surgical blade. Only one part of amputated worm was kept in one cup. The cup was accordingly labeled and kept in a tray.

For testing the role of commercial riboflavin on regeneration, earthworms were taken and grouped into four categories. Group-I constituted control, amputated at 10^{th} segment from the head end, Group-II constituted worms with coelomic fluid extracted before amputation, Group-III constituted worms with coelomic fluid extracted before amputation and 1 mg riboflavin applied daily on cut part. Group-IV constituted worms similar to control but riboflavin applied daily (extra dose).

Fourier Transform Infrared Spectroscopy (FTIR) analysis was performed on the riboflavin sample to study (i) The presence of riboflavin in the coelomic fluid, (ii) Skin and metabolite of bacteria from the earthworm's gut. Analysis was done to show the primary source of the riboflavin (i.e. the gut bacteria), accumulation in the coelomic fluid and skin of *Eisenia fetida*.

Results and Discussion :

Four distinct stages were distinguished during the course of regeneration in earthworm. After about three days, 'bud' appeared (Fig. 1) followed by 'blastema' formation (Fig. 2) after about five days. The third stage 'pigmentation and segmentation' (Fig. 3), and the fourth and final stage of regeneration i.e. 'complete regeneration' (Fig. 4) took 27 to 40 days depending upon the site of amputation.

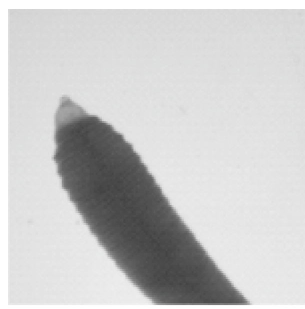

Fig. 1. Bud formation

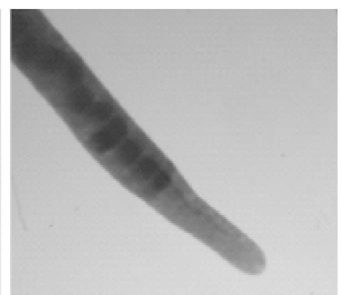

Fig. 2. Blastema formation

Fig. 3. Pigmentation and segmentation

Fig. 4. Complete regeneration

Time taken for complete regeneration was significantly more in adults as compared to juveniles when the earthworms were cut at 10^{th} segment from head as well from tail end (Tables 1 and 2). So, the rate of regeneration was faster in juvenile as compared to that in adult. Equimeric regeneration was observed in these cases. Similar reporting was done by Xiao et al.,(2011) and Rashmi et al., (2015). However, there was no significant difference in the regeneration time between adult and juvenile when cut was made before clitellum (i.e. at 26th segment) or after the clitellum (i.e. at 32nd segment) (Tables 3 -6).

Time taken for regeneration of posterior ten segments was significantly less in adults as compared to anterior ten segments (Table 7). There was no significant difference in juveniles (Table 8). When the earthworm was cut at 26th segment (i.e. before clitellum region) and at 32nd segment (i.e. after clitellum region), the time taken for regeneration by posterior segments was significantly less as compared to anterior segments (Tables 9-12). Complete but hypomeric regeneration was seen in the posterior parts of these worms. Similarly, Savigny (1826) also found hypomeric and equimeric regeneration in earthworm. The anterior part of earthworm cut at 26th segment did not develop clitellum. Similarly, the posterior part of earthworm cut at 32nd segment also did not develop clitellum.

When coelomic fluid was extracted, time taken for regeneration was found to be significantly more as compared to control. When riboflavin was applied to such worms, the regeneration time matched with the control. When extra dose of riboflavin was added to control worms, regeneration was significantly faster (Table 13).This shows that riboflavin promotes the rate of regeneration in the earthworm *Eisenia fetida* (Samuel et al., 2012)

Table 1. Time taken (in days) for regeneration when anterior 10 segments from head end were cut. Values are Mean ± S.E.

	Adult (N=3)	Juvenile (N=3)
Bud	3.16 ± 0.27	2.02 ± 0.02
Blastema	6.10 ± 0.25	5.32 ± 0.24
Pigmentation and Segmentation	16.53 ± 0.29	15.53 ± 0.29
Complete Regeneration	27.23 ± 0.28	24.89 ± 0.14*

* P<0.05

Table 2. Time taken (in days) for regeneration when 10 segments from tail-end were cut. Values are Mean ± S.E.

	Adult (N=3)	Juvenile (N=3)
Bud	3.36 ± 0.31	2.63 ± 0.27
Blastema	5.66 ± 0.28	5.36 ± 0.27
Pigmentation and Segmentation	16.27 ± 0.31	15.60 ± 0.26
Complete Regeneration	25.96 ± 0.08	24.33 ± 0.33*

* P<0.05

Table 3. Time taken (in days) for regeneration in the anterior part (cut at 26th segment i.e. before clitellum). Values are Mean ± S.E.

	Adult (N=3)	Juvenile (N=3)
Bud	3.16 ± 0.04	3.08 ± 0.04
Blastema	5.63 ± 0.21	5.56 ± 0.28
Pigmentation and Segmentation	33.96 ± 0.08	33.90 ± 0.05
Complete Regeneration	40.93 ± 0.06	40.96 ± 0.08

Table 4. Time taken (in days) for regeneration in the posterior part (cut at 26th segment i.e. before clitellum). Values are Mean ± S.E.

	Adult (N=3)	Juvenile (N=3)
Bud	3.10 ± 0.04	3.01 ± 0.01
Blastema	5.66 ± 0.13	5.70 ± 0.15
Pigmentation and Segmentation	20.67 ± 0.33	20.33 ± 0.33
Complete Regeneration	30.02 ± 0.07	29.97 ± 0.03

Table 5. Time taken (in days) for regeneration in the anterior part (cut at 32nd segment i.e. after clitellum). Values are Mean ± S.E.

	Adult (N=3)	Juvenile (N=3)
Bud	2.99 ± 0.05	3.05 ± 0.05
Blastema	5.00 ± 0.05	4.96 ± 0.03
Pigmentation and Segmentation	34.39 ± 0.31	34.43 ± 0.20
Complete Regeneration	40.88 ± 0.07	40.80 ± 0.11

Table 6. Time taken (in days) for regeneration) in the posterior part (cut at 32nd segment i.e. after clitellum). Values are Mean ± S.E.

	Adult (N=3)	Juvenile (N=3)
Bud	3.00 ± 0.04	3.04 ± 0.04
Blastema	5.01 ± 0.10	4.97 ± 0.05
Pigmentation and Segmentation	23.03 ± 0.07	23.00 ± 0.04
Complete Regeneration	31.91 ± 0.04	31.86 ± 0.07

Table 7. Number of days required for regeneration in anterior vs posterior cut regions of adult earthworm. Values are Mean ± S.E.

	Anterior 10 segments cut (N=3)	Posterior 10 segments cut (N=3)
Bud	2.85 ± 0.08	3.36 ± 0.31
Blastema	6.10 ± 0.25	5.67 ± 0.28
Pigmentation and Segmentation	16.53 ± 0.29	16.27 ± 0.31
Complete Regeneration	27.00 ± 0.05	25.95 ± 0.10*

* P<0.05

Table 8. Number of days required for regeneration in anterior vs posterior cut regions of juvenile earthworm. Values are Mean ± S.E.

	Anterior 10 segments cut (N=3)	Posterior 10 segments cut (N=3)
Bud	2.33 ± 0.28	2.63 ± 0.27
Blastema	5.30 ± 0.25	5.33 ± 0.28
Pigmentation and Segmentation	15.53 ± 0.29	15.62 ± 0.26
Complete Regeneration	24.90 ± 0.15	24.29 ± 0.31

Table 9. Number of days required for regeneration in anterior vs posterior parts of adult earthworm cut at 26th segment (i.e. before clitellum). Values are Mean ± S.E.

	Anterior Part (N=3)	Posterior Part (N=3)
Bud	3.16 ± 0.04	3.10 ± 0.04
Blastema	5.63 ± 0.21	5.66 ± 0.13
Pigmentation and Segmentation	33.96 ± 0.08	20.67 ± 0.33*
Complete Regeneration	40.93 ± 0.06	30.02 ± 0.07*

* P<0.05

Table 10. Number of days required for regeneration in anterior vs posterior parts of juvenile earthworm cut at 26th segment (i.e. before clitellum). Values are Mean ± S.E.

	Anterior Part (N=3)	Posterior Part (N=3)
Bud	3.08 ± 0.04	3.01 ± 0.01
Blastema	5.56 ± 0.28	5.70 ± 0.15
Pigmentation and Segmentation Complete	33.90 ± 0.05	20.33 ± 0.33*
Regeneration	40.96 ± 0.08	29.97 ± 0.03*

* P<0.05

Table 11. Number of days required for regeneration in anterior vs posterior parts of adult earthworm cut at 32nd segment (i.e. after clitellum). Values are Mean ± S.E.

	Anterior Part (N=3)	Posterior Part (N=3)
Bud	2.99 ± 0.05	3.00 ± 0.04
Blastema	5.00 ± 0.05	5.01 ± 0.10
Pigmentation and Segmentation Complete	34.39 ± 0.31	23.03 ± 0.07*
Regeneration	40.88 ± 0.07	31.91 ± 0.04*

* P<0.05

Table 12. Number of days required for regeneration in anterior vs posterior parts of juvenile earthworm cut at 32nd segment (i.e. after clitellum). Values are Mean ± S.E.

	Anterior Part (N=3)	Posterior Part (N=3)
Bud	3.05 ± 0.05	3.04 ± 0.04
Blastema	4.96 ± 0.03	4.97 ± 0.05
Pigmentation and Segmentation Complete	34.43 ± 0.20	23.00 ± 0.04*
Regeneration	40.80 ± 0.11	31.86 ± 0.07*

* P<0.05

Infrared (IR) spectra of riboflavin, coelomic fluid, skin of earthworm and metabolite of bacteria found in the gut of earthworm are shown in Figs. 5-8. Vibrational frequencies were similar in all the four spectra and were found between 1000 and 1700 cm^{-1}. Riboflavin producing gram positive, rod shaped bacteria of *Bacillus* sp. was obtained from the gut of *Eisenia fetida.* They were distinctly yellow- coloured.

Table 13. Time taken for regeneration (in days) when anterior 10 segments were cut from head end of adults. Values are Mean ± S.E.

	Control (N=3)	CF Extracted (N=3)	Riboflavin– CF (N=3)	Riboflavin + CF (N=3)
Bud	3.19±0.25	4.08±0.04*	3.16±0.16	2.14±0.07*
Blastema	6.12±0.25	8.01±0.06*	6.86±0.07	5.08±0.04*
Pigmentation and Segmentation	16.53±0.29	17.99±0.05*	16.11±0.07	13.99±0.05*
Complete Regeneration	26.99±0.05	28.98±0.04*	27.08±0.04	21.75±0.29*

* P<0.05 w.r.t control

CF= coelomic fluid

Riboflavin – CF=coelomic fluid extracted and 1 mg riboflavin added

Riboflavin + CF= coelomic fluid intact and 1 mg riboflavin added

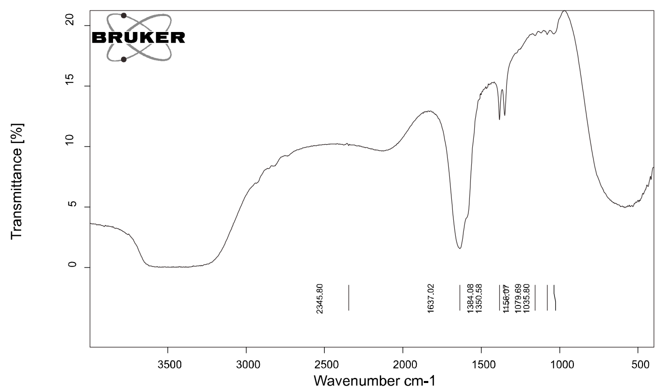

Fig. 5. FTIR spectrum of Riboflavin

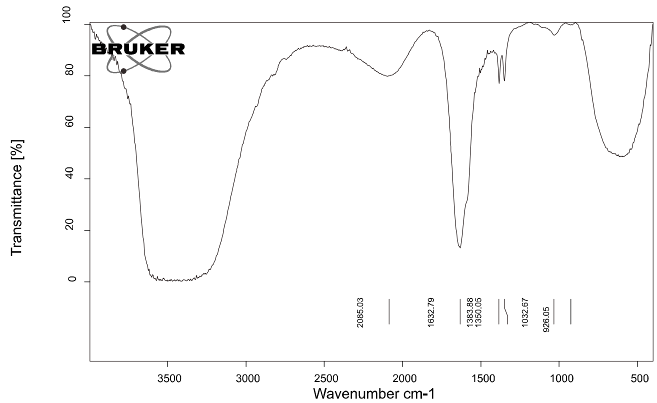

Fig 6. FTIR spectrum of coelomic fluid of earthworm

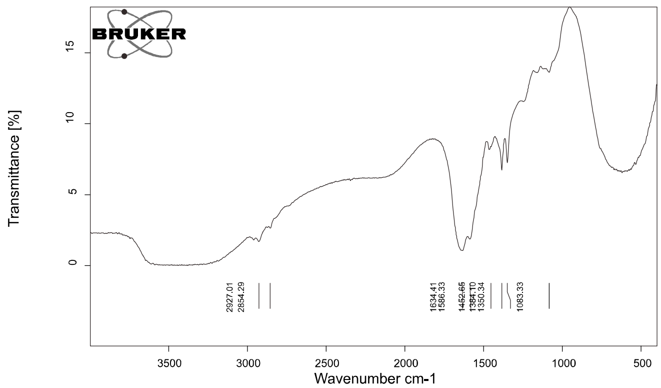

Fig 7. FTIR spectrum of skin of earthworm

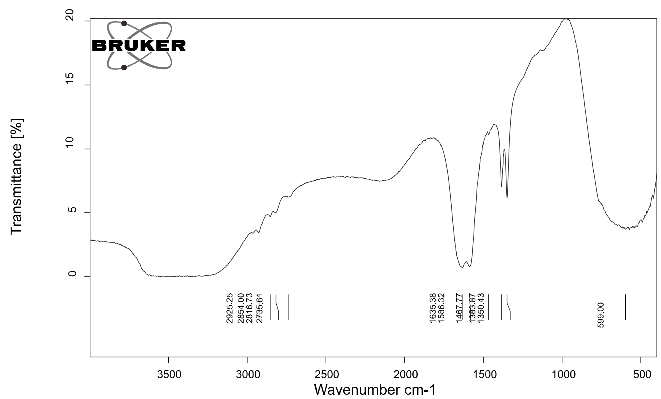

Fig 8. FTIR spectrum of gut bacteria (*Bacillus* sp.) of earthworm

Conclusion :

The time taken for complete regeneration in juvenile *Eisenia fetida* was faster as compared to that of adult for anterior as well as posterior region regeneration. Time taken for regeneration of posterior ten segments was significantly less in adults as compared to its anterior ten segments. But the rate of regeneration of median region was almost similar in both adult and juvenile. Riboflavin promoted the rate of regeneration in earthworm *Eisenia fetida.* The role of coelomic fluid was important in the process of regeneration as the rate of regeneration was delayed when coelomic fluid was extracted out of the body of *Eisenia fetida.*

Acknowledgements :

We express our sincere thanks to our respected Principal, Dr. Sister Marie Jessie A.C.

We are also grateful to Dr. M. C Arunan of HBCSE, Mumbai and CUBE mailing group for their valuable suggestions.

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