The Study of Regeneration in Posterior Part of Aporrectodea Caliginosa

Parnian Jamshidi and Zahra Pishkahi
Farzanegan 3 high school, Karaj, Iran
Email: {parnian.jamshidi7, pishkahi.zahra}@gmail.com

Latifeh Karimzadeh, Masoumehzaman Alizadehnohi, and Mohammad Nabiuni
Department of Cell and Molecular Biology, Faculty of Biological Sciences, Kharazmi University Tehran, Iran
Email: latifehkarimzadeh@gmail.com, masoumehzamanalizadeh@yahoo.com, nabiuni@khu.ac.ir

***Abstract*—Aim: Many organisms have the ability to regenerate tissue to some extent from the healing of wounds to replace the entire organs. Aporrectodea caliginosa is an Oligochaeta species are prime organisms for tissue regeneration and stem cells studies due to their extensive capacity to regenerate. Taking notice of its high potential as a new material for regenerative and stem cells study, detailed studies were undertaken on the regeneration of Aporrectodea caliginosa. Methods: 80 Aporrectodea caliginosa were acquired from Karaj in Alborz province of Iran and kept in six situations different in temperature, humidity, pH, structure of soil and section plane. Posterior part of the worms seated in the medium and kept up to four weeks. At the end of each week the samples were collected for further studies such as histological experiments and morphological studies. Result and Conclusion: The data have shown that anterior part from a new tail regenerate like other species but regeneration in posterior part is limited to wound healing in section plane and could regenerate a new head in posterior part. Even more the regeneration in Aporrectodea caliginosa regulated by both internal and external environmental factors.**

***Index terms*—Aporrectodea caliginosa, regeneration, stem cells, posterior part regeneration**

I. Introduction

The wild range of developmental biology is the study of process by which a single egg can grow and develop to become a perfect organism, which is called embryogenesis, the other subdivision of this study is the process that is involved in post embryogenesis which is named regeneration. In this field of study, determination and establishment of body axes, regional localization, Organization and expression of the genes which have essential role in nourogenesis and segmentation will perform again. Actually the common aim of embryogenesis (sexual reproduction), and post embryogenesis (asexual reproduction and regeneration) is to create a perfect organism [1]-[4].

In the family of Lumbricidae, regeneration required the present of huge amount of regenerative stem cells to rebuild anterior mesoderm, even more, dedifferentiation of epidermis and intestine is needed for the process of rebuilding ectodermis and endodermis, nevertheless, regeneration of the posterior organs exclusively is performed by stem cells [4].

Earthworm plays the important role as key organism in terrestrial ecosystems to improve the agricultural soils by feeding and dunging it, which is caused the increase in the amount of potassium, phosphorous and nitrate and reduce in the amount of dirt in the soils [5]. In addition, the sticky mucous which is excreted by the earthworm adhesive the soil particles and produce the soil aggregate. Even more, from the 1984 and according to the OECD committee, earthworm has known as an international index to realize the amount of pollutant, because of their ability to accumulate the heavy metal in its body [6]. However among the whole phylum of Annelida, Oligochaeta has the most capability to regenerate, even more, the earthworm (Aporrectodea caliginosa) is an epigenic species in Karaj, then we used A. caliginosa in this investigation.

Nowadays, the use of stem cells for the therapeutic purpose and investigation process has been increased and introduction of Lumbricidae as a useful source of stem cell, and the notable role of earthworm in agriculture is no longer unknown. Then in this study we tried to investigate the process of posterior region regeneration according to the species characteristic .On the contrary to the other spices, *A. caliginosa* has capability to reproduce the anterior region from the cutting segment.

II. Materials and Methods

A total of 80 adult worms were collected in different location in Karaj (Alborz province), which the morphological characters were as followed :There was no specific head and the body consist of 80-120 segments .The genital belt was located over the 31-37 segments which it carries the eggs until they hatch .The ventral surface was darker than the dorsal surface ,and the month ,which is hanging by a piece of meat to the mouth, is located in the first segment . The whole body cover by transparent and clear cuticle and each segment except the first and the last, has 4 pairs of hair-like in their ventral

Manuscript received July 5, 2013; revised September 4, 2013

doi: 10.12720/jomb.3.1.45-49

and lateral lobes which are made by chitin. With notice to the previous studies by Liebmann (1942), which is the different season have no effect on Annelida regeneration, so the earthworm has been collected at all forth seasons [7]. In addition, the identical length has been considered in order to achieve the optimum condition of the regeneration. The samples were divided into seven experimental groups for our investigations, group one, two and three kept in different temperature, humidity and pH conditions respectively (15, 20 & 25 ° C) , (30, 50, 70 ml/200gr of soil), [8]-[10]. In the forth group, the amount of clay was different (one or two unit of soil added to the ordinary soil). In the fifth and sixth groups the amount of sand and humus were different respectively. In the last group, cutting the worm was performed from the different part of the body segment (20, 21 of the head and 20 segment from the tail) to define the optimum condition of regeneration, then try to determine the optimum condition for regenerating the tail sectors and the whole groups divided into forth different group in forth weeks, at the end of each week, samples were observed with loop microscope (Jenus), then, samples were placed in Bouin`s fixative for histological preparation; fixed samples were kept in alcohol solution of 20 to 100% for a period of 45 minutes to dehydrated, and then in alcohol / Toluene (50:50) and Toluene (three times) for clearing, and finally blocked in paraffin. Samples were sectioned about 7 micron thickness and then placed on slides, which is previously coated with gelatin, before hand for staining with Hematoxylin- Eosin for histological observation, the data were analyzed by one- way ANOVA and INSTAT soft ware and the results were taken from at least three independent experiments, performed in triplicates. Values of $p \leq 0.05$ were considered statistically significant.

III. Results

On the one hand the temperature for the optimum condition according to the previous studies [11] were defined 20 ° C which has shown the significant increase to comparison with control group ($p<0.01$), although the change in temperature to15 ° C was not significant (Fig. 1).

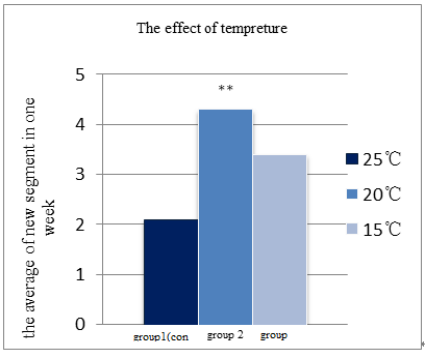

Figure 1. The chart has demonstrated the regeneration of lost segments in different temperatures in A. caliginosa, the rate of regeneration in 20 ° C has a meaningful increase to the control group **(**P<0.01).**

Moisture plays an essential role in the earthworm life not only as an important factor in its embryogenesis and regeneration but also as an inevitable factor for breathing and excreting [12], therefore in this investigation the effect of moisture on regeneration of the lost segment were studied and the 70% of humidity in the soil were defined as a optimum condition for reproducing the lost segment. On the contrary, the significant reduction has shown in the group of 30% humidity of the soil which, the rate of regeneration was not demonstrated the significant value ($p<0.001$) (Fig. 2).

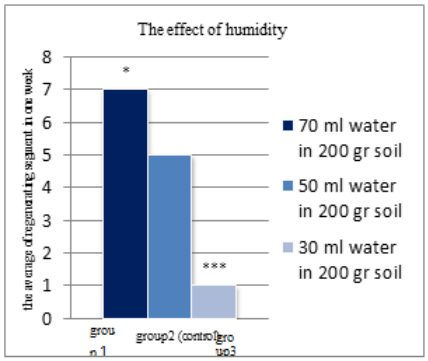

Figure 2. The chart has demonstrated the effect of humidity on A. caliginosa regeneration **(* P<0.05, ***P<0.001)**

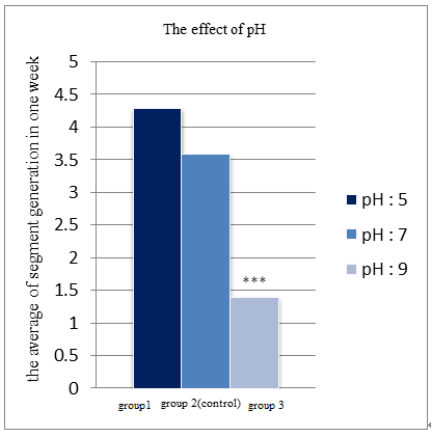

Figure 3. The chart has shown the rate of regeneration segments in different pH.

Due to pervious study of Tian based on increasing in the number of earth worm (*Hyperiodrilus africanus, Eudrilus eugeniae)* in pH: 5 [13]. The change in pH was considered as an effective factor in the process of earthworm regeneration, the observation has been shown the significant reduction of regeneration in alkaline soil ($P<0.001$). However the decrease in soil pH did not have a meaningful effect on earthworm regeneration. (Fig. 3).Even more, counting the renewed segments in samples has shown that, the soil with less amount of clay can provide the optimum condition for regeneration in earthworm. If the amount of clay become double can cause the significant reduction in the process of regeneration (P, 0.001). (Fig. 4).

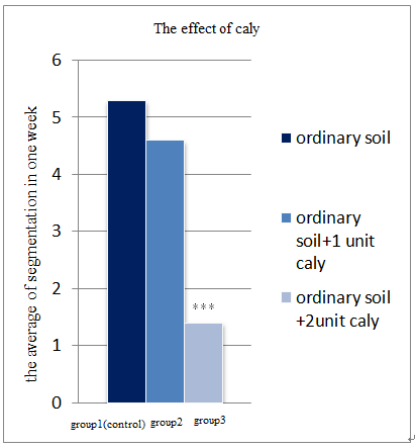

Figure 4. The chart has demonestrated the rate of regeneration segmnets in the soil with different amount of clay.

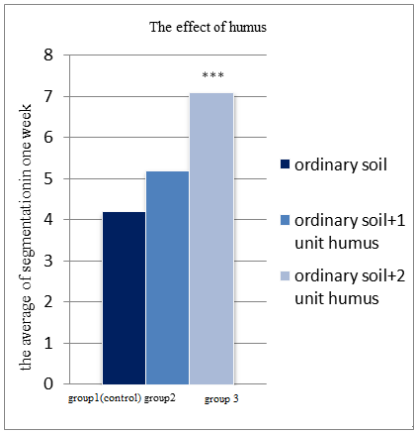

Figure 5. The chart has demonstrated the rate of regeneration segments in the soil with different amount of humus.

To amend the agricultural soil by earthworm there are two approaches, the first one by adding the earthworm directly to the soil, and the second way by adding Vermicompost. Nowadays the agricultural soil is full of soil waste, cow dung, domestic sewage, humus, and other organic materials is caused the increase in vermicompst, and indicate the biological activity in these worms. As a result it seems adding humus to their environment, not only improve the plant growth but also cause the increase in the number of the worm and have positive effect on their regeneration process. The survey results showed that the soil with humus unit as expected shows a significant increase in the recovery rate of worms (Fig. 5).

As a result of variant type of soil in different agricultural farm in Alborz province (Karaj), the different rate of regeneration was observed in the worm which was tested in the Lab situation; ordinary soil was collected and about two or three unit of sand was added which, one unit sand made a significant increase in the rate of earthworm regeneration. Another remarkable result is to increase the amount of sand in the soil at a rate of two units, which made a significant decreases in regeneration of earthworm .Thus one of the critical parameters in the regeneration of the earthworm, is the soil texture in the worm's environment (Fig. 6).

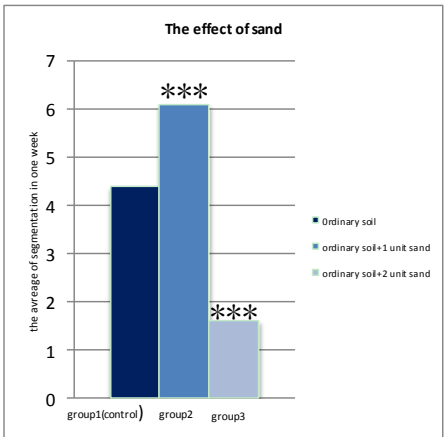

Figure 6. The chart has demonstrated the rate of regeneration in the soil with different amount of sand.

In the final trial, the histological tissue imaging and comparison between the new built-in tissues, which possess the less amount of melanin ,indicate that the part of blastoma repaired itself in the way of Epimorphic regeneration, because the posterior sections of the worm, blades with a thin wall of muscles were observed which are gradually thicken through the first to forth week, that demonstrated the reorganization of the tissues and organs which is not the alternative tissue as a result of elongation(Fig. 7, Fig. 8).In addition, the worms, from the tail section, regenerated the segments which had ability to search , navigate and react to the environmental stimulant and, even more they survive for one month (Fig. 9).

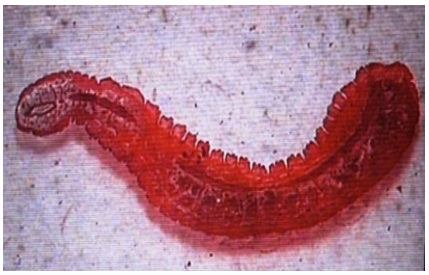

Figure 7. Photomicrograph of the A.caliginosa at the first week after cutting. The left side shows the new head and the right side shows the healing of the cutting section. Magnification 40x.

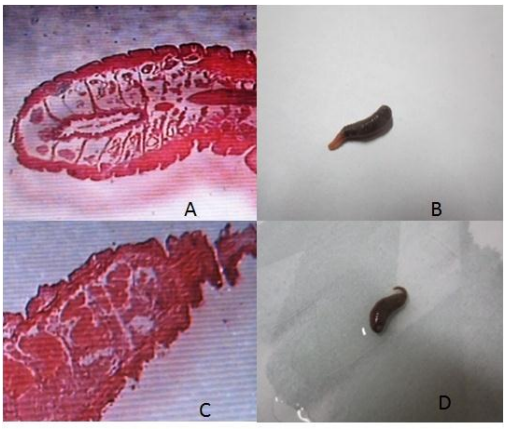

Figure 8. The picture has demonstrated the worms (A. caliginosa) after cutting of 20 segments and shown the new head which is regenerated at the opposite point of cut. A and B show the worm after one week, C and D after four week.

Figure 9. The section of the tail of A.caliginosa the thick section showed the part which cutting was occurred. This part of worm just closed and healed and had less amount of melanin. The opposite section has a smaller diameter and elongated which had more melanin and the ability of search.

IV. Discussion

Fragmentation in Annelida follows several different approaches; one of the well known ways is spontaneous fragmentation or Autotomy which is happened in the ordinary situation for the earthworm in the soil [13]. In another way which is named induced fragmentation that is performed by decapitation and electric shock, Autotomy will occur after 24 hours of cutting [13], [14]. In Autotomy (induced or spontaneous) in the Fission Zone, the circular body wall muscles contract and as a result the worm will divide into half [15]. To avoid Autotomy in the samples, the cut were performed between 20-21 segments from the head and 20 segments from the tail. In this fragmentation the importance of seven segments from the head which is considered as a head part of the body with the reproductive organs for regeneration were taken in to consideration, even more the existence of growth zone and pygidium in the posterior region was considered in order to evaluate the external factors in renewing a lost part. Regeneration in Annelida has been reported in different approaches, in Enchytraeus japonensis even a three pieces of worm can recreate a new one in seven days which the worm shows two different approaches simultaneously: Epimorphic Regeneration or Epimorphosis and Morphallactic Regeneration or Morphallaxis [15]. In Lumbriculus variegates even a three segments have ability to regenerate a new worm [4]. As a result of the effect of Fission Zone in regeneration and the existence of several fission zones in one worm, the number of segments and the distance of section from fission zone in the samples were considered. Therefore it can be stated that Fission zone inhibits the survival of head segments and the existence of the head inhibits the formation of the head at the posterior region. Based on the previous studies we assumed that the last 20 segments of the worm because of having fission zone have potential ability to create the head, and the first 20 segments as a result of having head and non attendance of fission zone can regenerate the tail part and the result not like the Kawamoto 2005 investigation, does not have two head.In addition Glutamine synthetase gene detection can also display the stem cell proliferative activity. This gene, in regenerating the Zenopus tail, repair of motor nerve injury in rat and in intestinal crypt region is widely expressed. Niva2008 investigated the gene expression in Enchytraeus japonesis, examined and found its expression in epidermal cells and regenerating blastoma shows substantially increased [1]. These cells were very active in our first week of the quarter volume ratio which the cell with higher ratio of nucleus and cytoplasm with high ability of division was observed. It is assumed that this Blastoma stem cells leaded into nerve cell differentiation in the fourth week and the latest searching environmental characteristic, is provided by the neural tissue.

V. Conclosion

Regeneration in earth worm (A. caliginosa) is an Epimorphic Regeneration, which is very sensitive to the environmental situations, and at the optimum condition, regeneration of the posterior segments occur at the opposite point of cutting, which is involved the formation of new head. Therefore, the anterior –posterior axes which lead to the formation of fetal organs along this axis, has potential to be renewed due to the existence of stem cells, which help the worm to regenerate the lost organs, however at the tail segments of the worm the nervous, digestive and respiratory structures does not exist. This kind of regeneration could be happened due to the low level of differentiation in these organisms to the comparison with higher level organisms in the evolutionary tree. Thus, it should be stated that the rebuilding of the cutting part organs in Annelida regeneration is one of their exclusive species characteristics.

Akionowldments

The basic idea of this project has been raised at the Farzaneghan 3 High school, and the primary stages was performed at this school, and then the rest stages were performed at the Research Center for Cell and Developmental Biology and Animal's Lab Unit at Kharazmi University (TMU). The authors would like to thank the manager and all the members of this center.In addition the outhors would like to thank Dr.Farhad Mashayekhi and MS Mahnaz Taghavi for their help and support.

Rferences

[1] C. C. Niva, J. M. Lee, and M. Myohara, "Glutamine synthetase gene expression during the regeneration of the annelid Enchytraeus japonensis," *Dev Genes Evol.*, vol. 218, pp. 39-46, 2008.

[2] M. Grdisa, "Mechanism of wound healing in annelids," *Idaho State Journal*, vol. 7, pp. 192-197, 2010.

[3] M. Myohara, "Differential tissue development during embryogenesis and regeneration in an annelid," *Developmental Dynamics*., vol. 231, pp. 349–358, 2004.

[4] C. Yoshida and T. N. Shin, "Stem cell system in asexual and sexual reproduction of Enchytraeus japonensis (Oligochaeta, Annelida)," *Develop Growth Differ*., vol. 52, pp. 43–55, 2010.

[5] C. Edwards's, "Historical overview of vermicomposting," *Biocycle*, vol. 36, pp. 56-58, 1995.

[6] OECD, "Guidelines for testing of chemicals. Test 207: Earthworm acute toxicity tests," *Organization for Economic Co-Operation and Development (OECD)*, pp. 13-16, Paris, 1984.
[7] E. Liebmann, "The correlation between sexual reproduction and regeneration in a series of Oligochaeta," *Journal of Experimental Zoology*, vol. 3, no. 91, pp. 373–38, 1942.
[8] W. H. Elmer and F. J. Ferrandino, "Suppression of verticillium wilts of eggplant with earthworms," *Plant Disease*, vol. 93, pp. 485-489, 2009.
[9] C. Hong, S. Takahashi, M. Imamura, E. Okutani, *et al.*, "Earthworm fibrinolytic enzyme: Anti-tumor activity on human hematoma cells in vitro and in vivo," *Chinese Medical Journal*, vol. 10, no. 120, pp. 898-904, 2007.
[10] Y. M. Chang, Y. T. Shih, Y. S. Chen, C. L. Liu, *et al.*, "Schwann cell migration induced by earthworm extract via activation of PAS and MMP2/9 mediated through ERK1/2 and P38," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, P.12, 2011.
[11] W. H. Elmer and F. J. Ferrandino, "Suppression of verticillium wilts of eggplant with earthworms," *Plant Disease*, vol. 93, pp. 485-489, 2009.
[12] R. Sherman, *Raising Earthworms Successfully*, North Carolina State University, Raleigh, North Carolina Cooperative Extension Service. Publication Number, EBAE 103-83, 2003.
[13] G. Tians, J. A. Olimah, G. OAdeoye, and B. T. Kang, "Regeneration of earthworm populations in a degraded soil by natural and planted fallows under humid tropical conditions," *Soil Sci. Soc. Am. J.*, vol. 64, pp. 222–228, 2000.
[14] K. Inomata, F. Kobari, C. Yoshida-Noro, M. Myohara, and S. Tochinai, "Possible neural control of asexually reproductive fragmentation in Enchytraeus japonensis (Oligochaeta, Enchytraeidae)", *Invert Report. Develop.*, vol. 37, pp. 35-42, 2000.
[15] C. Yoshida-Noro, M. Myohara, F. Kobari, and S. Tochinai, "Nervous system dynamics during fragmentation and regeneration in Enchytraeus japonensis (Oligochaeta, Annelid)," *Dev Genes Evol.*, vol. 210, pp. 311–319, 2000.
[16] S. Kawamoto, C. Yoshida-Noro, and S. Tochinai, "Bipolar head regeneration induced by artificial amputation in Enchytraeus japonensis (Annelida, Oligochaeta)," *J. Exp. Zool. A Comp. Exp. Biol*, vol. 303, pp. 615–627, 2005.
[17] Y. Yan-qiu, C. Hong, H. Jin, and Z. Zhi-guo, "Effect of earthworm fibrinolysin on growth inhibition and induction of apoptosis in human gastric carcinoma cell line MGC803," *Journal of Southeast University* (Medical Science Edition), vol.1, pp. 898-904, 2007.
[18] M. Grdisa, M. Popovic, and T. Hrzenjak, "Glycolipoprotein extract G-90 from earthworm Eisenia foetida exerts some antioxidative activity," *Comparative Biochemistry and Physiology*, vol. 128, pp. 821-825, 2001.
[19] S. Wei, X. Yin, Y. Kou, and B. Jiang, "Lumbricus extract promotes the regeneration of injured peripheral nerve in rats," *Journal of Ethnopharmacology*, vol. 123, pp. 51–54, 2009.
[20] M. Ueda, K. Noda, M. Nakazawa, K. Miyatake, *et al.*, "A novel anti-plant viral protein from coelomic fluid of the earthworm Eisenia foetida: Purification, characterization and its identification as a serine protease," *Comparative Biochemistry and Physiology*, vol. 151, pp. 381–385, 2008.
[21] L. P. Canellas, F. L. Olivares, L. A. Okorokova-Facanha, and A. Rocha Facanha, "Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+ -ATPase activity in maize roots," *Plant-Physiology*, vol. 130, pp. 1951–1957, 2002.
[22] X. Y. Li, Y. R. Luo, M. X. Yun, J. Wang, and J. J. Wang, "Effects of 1methyl-3-octylimidazolium bromide on the anti-oxidant system of earthworm," *Chemosphere*, vol. 78, pp. 853–858, 2010.
[23] M. Myohara, C. Yoshida-Noro, F. Kobari, and S. Tochinai, "Fragmenting oligochaete Enchytraeus japonensis: A new material for regeneration study," *Develop. Growth Differ*, vol. 41, pp. 549–555, 1999.
[24] E. Liebmann, "New light on regeneration of Elsenia foetida," *Jour Morph*., vol. 73, pp. 583-610, 1943.
[25] T. H. Struck, N. Schult, T. Kusen, E. Hickman, *et al.*, "Annelid phylogeny and the status of Sipuncula and Echiura," *BMC Evol Biol*, vol. 7, no. 57, pp. pp.1-11, 2007.
[26] A. E. Bely and G. A. Wray, "Evolution of regeneration and fission in annelids: Insights from engrailed- and orthodenticle-class gene expression," *Development*, vol. 28, no. 14, pp. 2781–279, 2001.
[27] A. E. Bely, "Evolutionary loss of animal regeneration: Pattern and process," *Integrative and Comparative Biology*, vol. 4, no. 50, pp. 515-527, 2010.
[28] M. Agosto, K. A. H. Mikkola, A. J. Hartenstein, V. Hartenstein, and U. Banerjee, "The hematopoietic stem cell and its niche: A comparative view," *Genes Dev.*, vol. 21, pp. 3044–3060, 2007.
[29] A. E. Bely and J. M. Sikes, "Latent regeneration abilities persist following recent evolutionary loss in asexual annelids," *Proc. Natl. Acad. Sci. USA*, vol. 107, pp. 1464-1469, 2010.

Mohammad Nabiuni was born at 1967 in province Qom the city near the capital city Tehran. He got his BSc in General Biology, and then he continued his MSc education in Developmental Biology at Tarbiat Moallem University. He got his PhD in Cell and Developmental Biology from the Manchester University (England) at 2006.He has published more than one hundred and fifty articles in neuroscience, cancer and Developmental biology, nowadays he is an associate professor at Kharazmi University (Tarbiat Moallem University).

Parnian Jamshidi was born in 1995 in the province Kermanshah and her friend Zahra pishkahi also was born in 1995 in the province Karaj. They both study at the National Organization for Development of Exceptional Talent. They have started their study on earthworm since five years ago, They selected as the best young researcher at 2010 and 2012 by NODET, and finally at 2013 they successfully register their new way of earthworm regeneration at Khwarizmi International Award.

Masoumehzaman Alizadehnohi was born at 1978 on Tehran, after interance exam she was accepted in General Biology at Ferdwsi University in Mashhad, and then she has continued her education in Developmental Biology at Kharazmi University. She has published two articles in Cancer Biology under supervision of Dr.Nabiuni, and she became participated in two recent researches in(the effect of dissolved nitrate on pregnant mic and their off spring) and the present paper.

Latifeh Karimzadeh was born at 1985 in province Shahrekord .She achieved her BSc in Animal Biology from Shahid Chamran University in Ahvaz at 2006 and then she continued her education in Developmental Biology in Kharazmi University and got her MSc at 2010, her studies in Toxicology and reproduction ,leaded to five articles so far. Nowadays she is one of the instructors at the center of Cell and Developmental Biology in animal lab.

