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Project on assessment of effects of the pharmacological agents in the modulation of regenerative abilities in Eisenia Fetida

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ABSTRACT

Regeneration is repair and regrowth of the lost tissues or organs in response to injury. To unravel the process of regeneration, it is really important to understand the repair mechanism and proteins involved in the process of tissue repair as well as regrowth, factors affecting them and the regulation of those factors. The present study was conducted to firstly observe the modulation of those proteins using different pharmacological agents to check on the regenerative ability of Eisenia fetida by targeting oct ¾, Nanog and lin28. These genes and their regulatory product proteins respectively were found to be modulated using pharmacological agents like isotroin and Metformin. The other proteins which were involved in the regeneration process were expected to get illuminated in protein profiling on using these agents. By using Metformin and Isotroin which were involved in the regulation of proteins like oct-4 and lin-28 which were themselves involved in regeneration, we could detect the upregulation rather the complete production of other proteins like lumbrokinase, gag, CRT which were concluded to be responsible for the regeneration in Eisenia fetida.

Keywords— *Regeneration, Eisenia Fetida, lesion, Nanog, Lin 28, Isotroin, Metformin, Regulation of proteins, Lumbrokinase, Gag, CRT*

1. INTRODUCTION

Regeneration of complex structures after injury requires dramatic changes in cellular behavior. Regenerating tissues initiate a program that includes diverse processes such as wound healing, cell death, dedifferentiation, and stem (or progenitor) cell proliferation. Furthermore, newly regenerated tissues must integrate polarity and positional identity cues with preexisting body structures Dedifferentiation is an important biological phenomenon whereby cells regress from a specialized function to a simpler state reminiscent of stem cells. Stem cells are self-renewing cells capable of giving rise to differentiated cells when supplied with the appropriate factors. Stem cells that are derived by dedifferentiation of one's own cells. (1)

When earthworm is lesioned, it is capable of regenerating structurally and functionally in a span of 48 to 72 hours. According to a paper published in Elsevier, stem cell pluripotency factors can induce somatic cells to form induced pluripotent stem cells, which are involved in cell reprogramming and dedifferentiation. There is limited information available about the factors responsible for this regeneration in the earthworm. As per the information already available on the factors involved in the regeneration of the stem cells included oct4, lin28, nanog, sox2, c-myc. Oct-3/4 was identified as a novel Oct family protein specifically expressed in EC cells, early embryos, and germ cells(2–4). Lin28a, a highly conserved RNA-binding protein expressed during embryogenesis, plays roles in development, pluripotency, and metabolism. Lin28a bound to and enhanced the translation of mRNAs for several metabolic enzymes, thereby increasing glycolysis and oxidative phosphorylation (OxPhos). Lin28a-mediated enhancement of tissue repair was negated by OxPhos inhibition, whereas a pharmacologically induced increase in OxPhos enhanced repair. Thus, Lin28a enhances tissue repair in some adult tissues by reprogramming cellular bioenergetics(5).

According to the papers already published on how these proteins were modulated using some pharmacological agent, data about usage of Metformin and Isotroin drug to modulate the factors like lin-28 and oct-4 was found to be involved in the regeneration. This study was conducted to target another such factor involved in the regeneration of earthworm in a similar way by using Metformin and Isotroin(13-cis- retinoic acid)(6–11).

2. MATERIALS AND METHODS

2.1 Acclimatization of the earthworm

Temperature: 25-29 degree Celsius while performing the tests, earthworms are supposed to be kept separately to avoid the errors might occur due to presence so some components in the soil which might affect the test results. So, they are transferred to the cups

made. A plastic cup is taken and small holes are made at the bottom of it. A tissue paper is taken and folded in a way which makes the bedding for the earthworm inside the cup. This tissue is made moist which will serve as a food source for the survival of the earthworm. Another cup with no holes at its bottom is also filled with some water and wet tissue and the first cup is placed inside the one with no holes at the bottom in order to maintain the moisture and prevent the escape of earthworms from the bottom of the first cup. Both the cups are covered on top with thin cotton material with the help of a rubber band. One earthworm per cup is placed to get acclimatized and such many cups are prepared as per the need of a number of earthworms in the experiment. Acclimatization usually takes 2-3 days, after which those acclimatized earthworms are used for the test purposes.

2.2 Protocol for Anesthetising the Earthworm

Pour 50% chloroform a bigger beaker. Stuff tissues in the smaller beaker and put the sample organism on it. Add this smaller beaker to the bigger beaker and cover this with a lid. Keep this lid for 30secs and remove the smaller beaker out and with the help of forceps and remove the sample organism out.

2.3 Protocol for testing the toxicity

Toxicity was tested for both the drug with keeping the concentration 2000mg/kg. The batches of 24, 48 and 72 hours are observed for the mortality and the survival rate. If the negligible or no toxicity is seen with the currently used concentration, 1/10 of it is used for performing the actual test else 1/10 of it is used for the next iteration to check the toxicity.

2.4 Use of Metformin to Alter the Regeneration in Earthworm

One Metformin tablet equivalent to 500mg metformin was used which actually weighed 550mg. According to the toxicity test carried out, the concentration of metformin used was 200mg/kg. For the groups made based on controls (control with only cut, control with the only drug) and the test - for 24, 48 and 72 hours, 30 earthworms were required to be exposed to the drug and hence the calculations were made according to the weight of those 30 earthworms which came up to 5.20 g. 200mg of the drug was required for 1000 g of the earthworms.

Then, for 5.20 g the drug required was calculated to be 1.04 mg. Since 0.55g of the tablet had 0.5g of metformin, we took 1.144mg of the tablet powder after crushing the tablet. The concentration of metformin which was subjected to earthworm was now 0.0011g/10ml.The earth worms of the group "control with the only drug (no cut)" were exposed to drug and were put back in their cups for the observation. A cut was made on the dorsal side of the earthworm at the 22nd segment from the clitellum. The earthworm of the group "control with only cut and no drug" were put in their respective cup for further observation. The earthworms of the group "test" were exposed to the drug after making a lesion on them and were put back to the cups under observation. After 24 Hours, the 3 groups (two controls and one test) were observed for their mortality, weight and manually visible Regeneration Rate in the test samples with compared to the controls. Half the number of the earthworms of each group –for 24 hours batch were stored in the formal saline (10% formalin) for performing Histology on them and the others were homogenized in 10% normal saline solution (w.r.t weight) at -20 degree C to perform differential protein profiling. The earthworms from batches for 48 and 72 hours were also observed and the "test" group as well as "control with only drug" group were exposed to the same concentration of metformin. The above steps were carried out for 48 and 72 hours batch till day three (that is 72 hours after the lesion was made).

2.5 Use of Isotroin to Alter the Regeneration in Earthworm

One isotroin capsule equivalent to 10mg isotroin was used. According to the toxicity test carried out, the concentration of isotroin used was 200mg/kg. For the groups made based on controls (control with only cut, control with the only drug) and the test - for 24,48 and 72 hours, 30 earthworms were required to be exposed to the drug and hence the calculations were made according to the weight of those 30 earthworms which came up to 3.50gms. 200mg of the drug was required for 1000 gms of the earthworms. Then, for 3.20 gms the drug required was calculated to be 0.7 mg. 0.7 gms of isotroin was weighed by piercing the capsule and Concentration which was made to 0.0007g/10 ml by diluting it with distilled water and the same protocol was followed as the above for metformin.

3. PROTOCOL FOR HISTOLOGY

3.1 The Paraffin Method

All histological procedures can be divided into a similar series of steps. For the paraffin method these steps are as follows:
The earthworm was cleanly washed with tap water to remove any dirty on its body. Sacrifice the earthworm in 10% Formaldehyde Solution in 20º C for 24 hours for fixation. After fixation, the tissue was washed with distilled water and put in 60% Iso-propyl alcohol for 1 hour for dehydration. Similarly, the steps were performed in 70%, 80%, 90% and 100 % (two times) each at the incubation of 1 hour at 60º C temperature. After dehydration, the tissues were allowed for clearing the ethanol by put in xylene for 45 min. After clearing, the tissues were put in a wax 1 at 60º C for 2 hours then transfer the tissues to wax 2 for overnight and in wax 3 each for 2 hours. Using two L-shaped equipment a paraffin block was prepared and in that the processes tissue was embedded for sectioning. Section paraffin blocks at desires thickness (usually 5-7µm) on a microtome and the ribbon was carefully transferred to an Egg albumin: Glycerol (50:50) coated slide overlaid with distilled water. Place the slide in 60º C for half an hour to melt the paraffin then deparaffinize slides in two changes of xylene for 5 minutes each. Transfer the slide to isopropyl alcohol for 2 minutes before washing the deparaffinize slides in water. The deparaffinized slide washed in water was stained with haematoxylin (which stains nucleus) for 6-8 minutes and the excess stains were removed by water wash followed by acid alcohol treatment (99 ml of 70% isopropyl alcohol and 1ml of concentrated HCL) for 30 seconds. The excess stains were further removed by keeping the slide for blooming in running tap water for overnight. The nucleus stained slide was counter stained with eosin (which stains cytoplasm) for 30 seconds and the excess stains were removed by dipping in water and isopropyl alcohol. Then the slide was dipped in xylene and the slides were mounted by overlaid with DPX followed by careful plotting of the cover slip. The setup is undisturbed for 1 day to dry and after that, it is ready for microscopic examination.

Chloroform-methanol extraction of proteins for orbitrap differential protein profiling detergents, like SDS, and salts, like NaCl, can disrupt liquid chromatography/tandem mass spectrometry (LC-MS/MS) studies by interfering with chemistry and clogging columns and spray needles. Precipitation with chloroform and methanol results in a dry protein material, free of salt and detergent, which perform sweetly during these critical steps.

To 400 µL protein sample (~400ug protein) in an Eppendorf tube:
Add 1600 µL methanol and vortex thoroughly. Add 400 µL chloroform and vortex. Add 1200 µL H_2O—mixture will become cloudy with precipitate—and vortex. Centrifuge 1 minute @ 14,000g. The result is three layers: a large aqueous layer on top, a circular flake of protein in the interphase, and a smaller chloroform layer at the bottom. Remove the top aqueous layer carefully, trying not to disturb the protein flake. Add 400 µL methanol and vortex. Centrifuge 5 minutes @ 20,000g, which will slam dandruffy precipitate against the tube wall. Remove as much methanol as possible. Be careful, because the pellet is delicate. You should be able to remove all but a few µL of methanol with care, which will speed drying. Dry under vacuum.

3.2 Protocol for orbitrap differential protein profiling

After protein extraction, 50ug of protein sample was taken for in solution digestion. Reduction, blocking of free cydteins groups and trypsin Digestion. Protein samples (50 µg) were reduced with 50 mM DTT at 60°C for 1 h and the cysteine- groups were blocked using a 50 mM IAA solution at room temperature for 30 min. The protein samples were then subjected to trypsin (Pierce) digestion by adding trypsin in 1:30 ratio (Trypsin: Protein) at 37°C in a dry bath for 16 hours. After trypsinization, samples were dried in speed vac and reconstituted in 20ul of Milliq water with 0.1% formic acid and desalting was performed.

3.3 Ziptipping Protocol

- C18 Tips were first conditioned with 100% Acetonitrile solution thrice.
- Washing was given with 100ul of 0.1% formic acid water solution twice.
- The sample was passed 7-8 times through the activated tips.
- Bound peptides were then eluted with 100ul of 40% ACN, 50% ACN and 80%
- ACN+0.1% FA respectively.
- The desalted samples were then dried in speed vac and finally reconstituted in 20ul of 0.1%FA in water and then subjected to LCMS/MS.

NOTE: For LC-MS/MS analysis, the protein sample should have a minimum amount of salt.

Instrument Name: QExactive Plus, Thermo Scientific Data Acquisition software: Thermo XCalibur Data Analysis software: Thermo Proteome Discoverer Version: 2.2 Acquired raw files were searched against database Eisenia fetida using 1% FDR and precursor and fragment mass tolerance of 10ppm and 0.05 Da respectively.

4. OBSERVATIONS

Toxicity tests were performed on the acclimatized earthworms but there was no mortality or toxicity seen on them and hence the further experiments were conducted with a $1/10^{th}$ concentration of the toxicity test concentration used.
To confirm regeneration and find out factors affecting its two tests were employed.
(a) Histology
(b) Differential Protein profiling

Firstly, let us consider visual observations made on the earthworms under test. Each control group had and the test group had 4 & 6 earthworms respectively.

5. HISTOLOGY RESULTS

5.1 Controls without the effect of drugs

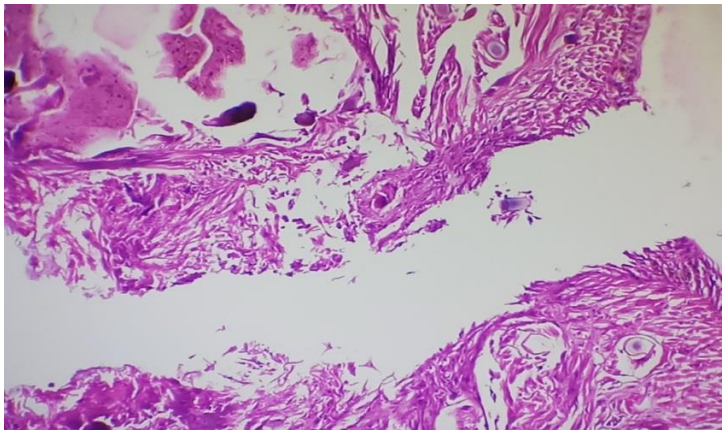

Fig. 1: Control without drug after 24 hours

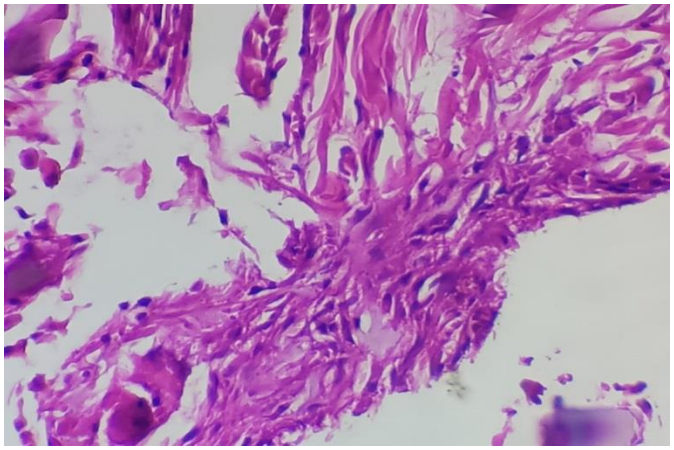

Fig. 2: Control without drug after 24 hours 400X

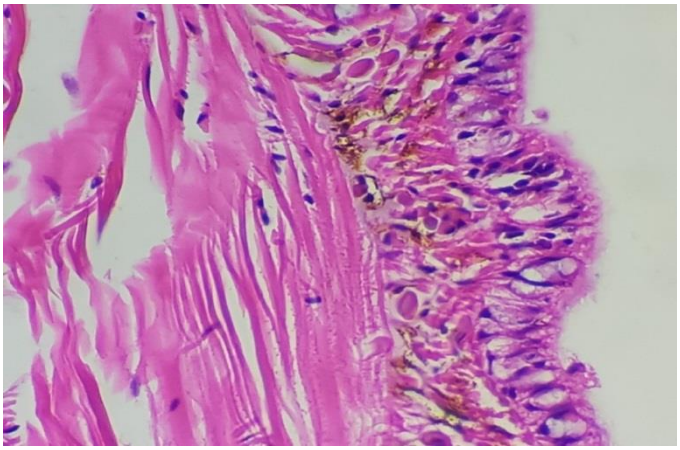

Fig. 3: Control without drug after 72 hours 400X

5.2 Effect of metformin on the lesion of earthworm

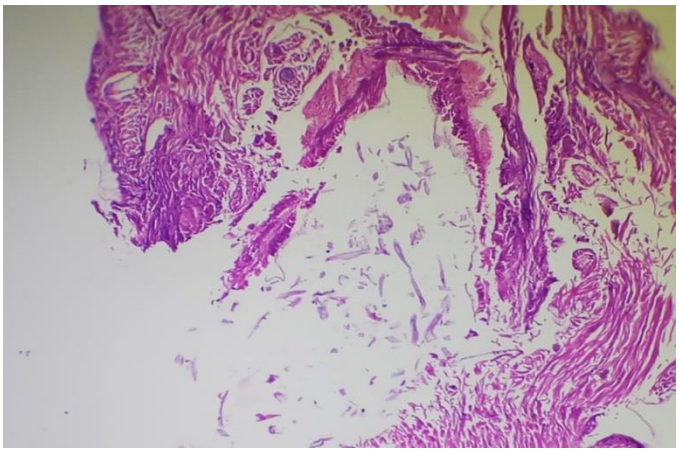

Fig. 4: Test group after 24 hours

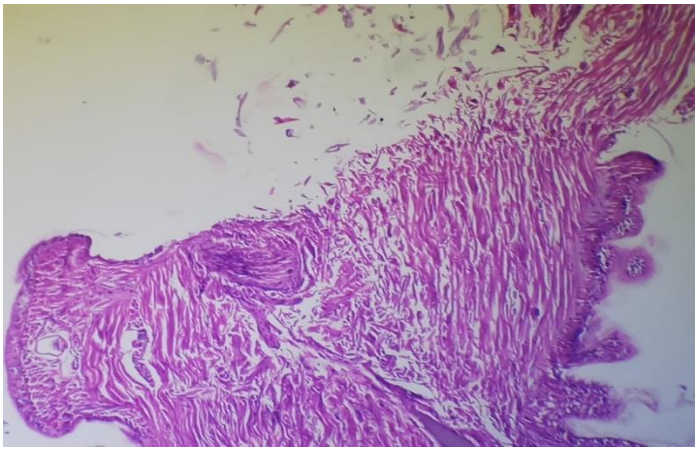

Fig. 5: Test group after 24 hours 400X

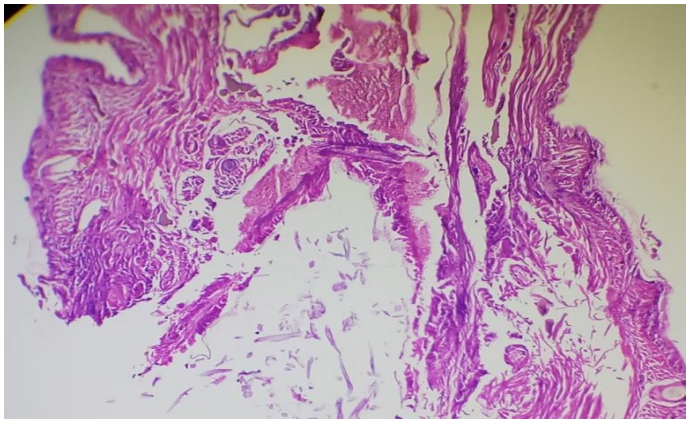

Fig. 6: Test group after 24 hours 400X

Inference: From the histology report after 24 hours, there is an expansion of spaces in between the longitudinal muscular cells seen for the recruitment of granular cells resembling myoblastoma cells which further gets differentiated.

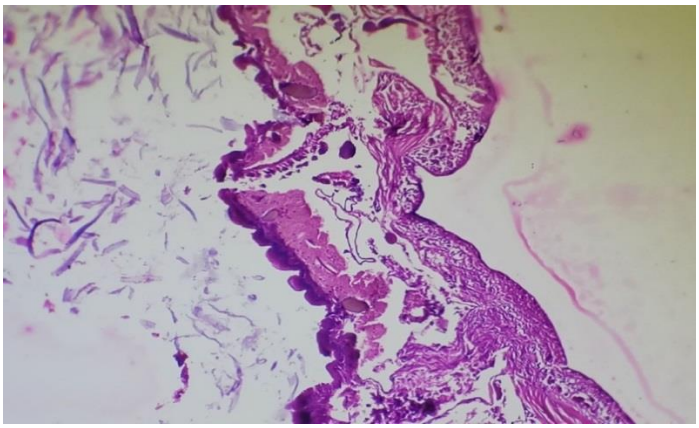

Fig. 7: Test group after 72 hours

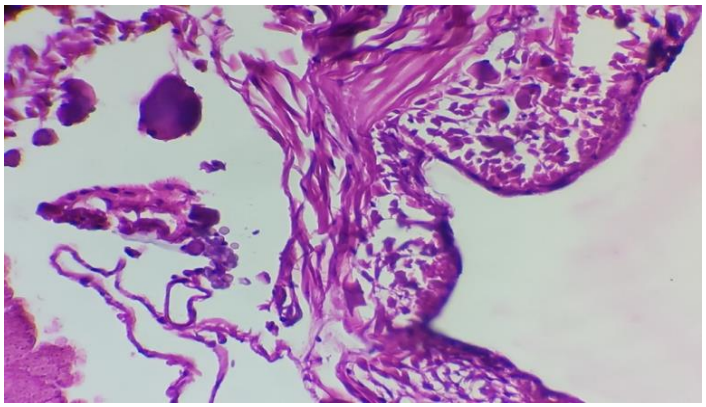

Fig. 8: Test group after 72 hours 400X

Inference: Partial damage with cloudy swelling is seen at the lesion area and which shows the differentiation of granular cells into longitudinal muscle cells making the side of lesion thicker than the side opposite to it which has no cut on it and only the scar is remaining in the wound region.

5.3 Effect of Isotroin on the lesion of earthworm

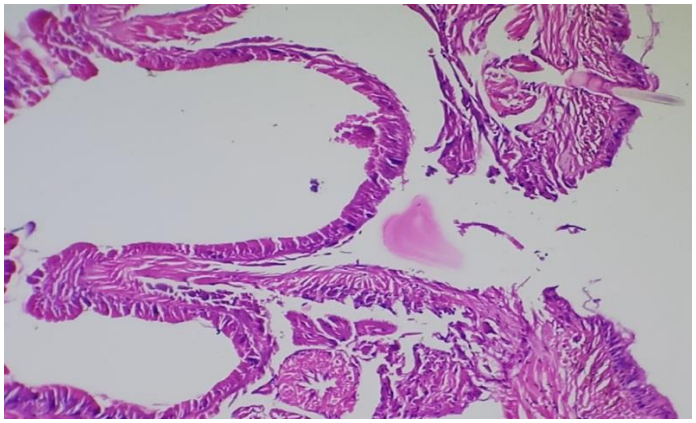

Fig. 9: Test group after 24 hours

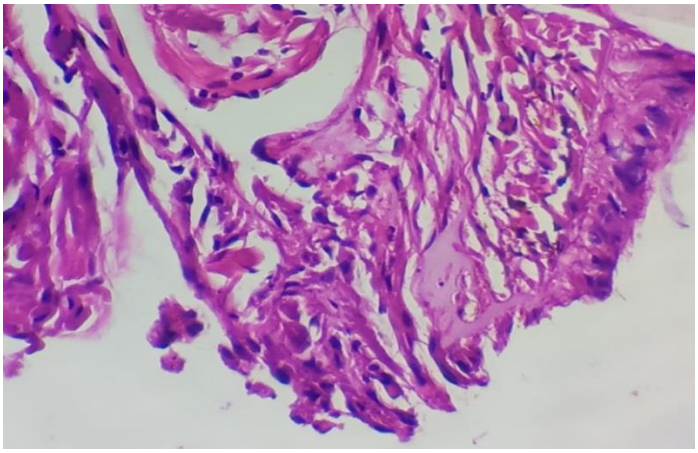

Fig. 10: Test group after 24 hours 400X

Inference: No granular cells were seen near the lesion, only the space made for the granular cells to travel near the cut area is remaining and the epithelial, circular muscle cells are well defined along with a normal sized layer of longitudinal muscle cells.

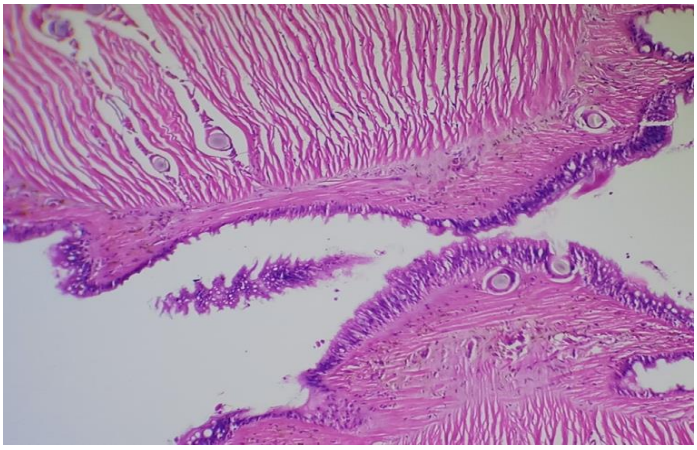

Fig. 11: Test group after 72 hours

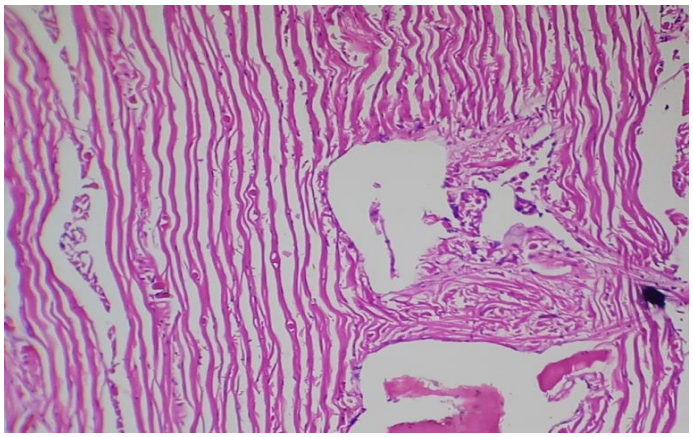

Fig. 12: Test group after 72 hours 400X

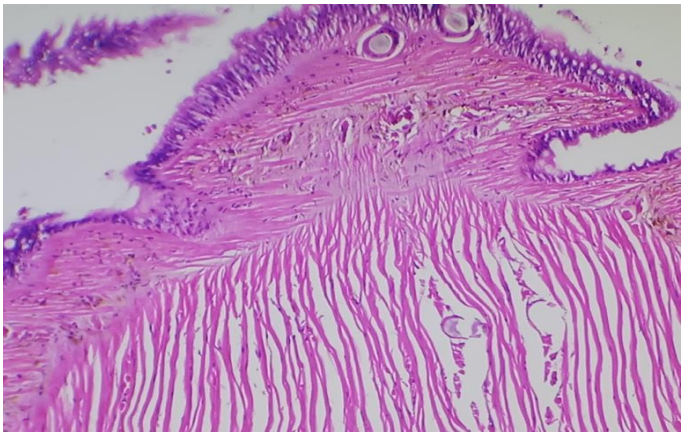

Fig. 13: Test group after 72 hours 400X

Inference: The space left vacant for the recruitment of granular cells at the site of lesion was taken up by the longitudinal cells by a rapid proliferation.

Table 1: Protein profiling of metformin 24 hours test

1	Protein FDR Conf	Master	Accession	Description	Exp. q-value: Cor	Sum PEP Score	Coverage [%]
2	High	Master Protein	A0A2I7YV73	Actin OS=Eisenia fetida OX=	0	8.497	22
29	High	Master Protein	G3M459	Catalase OS=Eisenia fetida C	0	7.184	15
54	High	Master Protein	Q6T375	Lumbrokinase-4 OS=Eisenia	0	4.656	14
64	High	Master Protein	O15991	Lombricine kinase OS=Eisen	0	4.583	13
82	High	Master Protein	Q2I6A1	Ubiquitin (Fragment) OS=Eis	0	1.406	29
91	High	Master Protein	Q5CD25	Valosin containing protein-1	0	0.764	2
96	High	Master Protein	Q8IT81	Myosin essential light chain	0	0.758	3
101	High	Master Protein	Q0PGR9	Fibrinolytic protease 0 (Frag	0	0.717	3
106	High	Master Protein	B6E9G3	Histone H3 (Fragment) OS=E	0	0.645	6
111	High	Master Protein	E9NPR6	Heat shock protein 70 OS=E	0	0.521	2
116	Low	Master Protein	Q5CD24	Valosin containing protein-2	0.133	0.359	3

Table 2: Protein profiling of metformin 72 hours test

1	Checked	Protein FDR Conf	Master	Accession	Description	Exp. q-value: Cor	Sum PEP Score	Coverage [%]
2	FALSE	High	Master Protein	O15991	Lombricine kinas	0	29.27	40
44	FALSE	High	Master Protein	O18425	Lysenin-related p	0	11.25	27
75	FALSE	High	Master Protein	A8ILP4	Lumbrokinase (F	0	11.243	23
89	FALSE	High	Master Protein	G3M459	Catalase OS=Eise	0	10.166	21
119	FALSE	High	Master Protein	A0A2I7YV73	Actin OS=Eisenia	0	9.958	29
148	FALSE	High	Master Protein	O18423	Lysenin OS=Eiser	0	5.172	15
163	FALSE	High	Master Protein	Q0PGR9	Fibrinolytic prote	0	3.942	10
172	FALSE	High	Master Protein	B6E9G3	Histone H3 (Frag	0	2.828	17
181	FALSE	High	Master Protein	Q2I6A1	Ubiquitin (Fragm	0	2.794	13
186	FALSE	High	Master Protein	Q8IT81	Myosin essential	0	1.575	18
197	FALSE	High	Master Protein	Q5MBA0	Lumbrokinase (F	0	1.238	5
202	FALSE	High	Master Protein	Q2I6A7	Calmodulin (Frag	0	0.741	19
207	FALSE	High	Master Protein	M9VY46	Lysozyme OS=Eis	0	0.73	5
212	FALSE	High	Master Protein	A0A2I7YV74	Heat Shock Prote	0	0.395	2
217	FALSE	High	Master Protein	A0A2I7YUY6	Piwi2 OS=Eisenia	0	0.263	2
222	FALSE	Low	Master Protein	A0A0C5B368	CYP450 family 27	0.08	0.252	14
227	FALSE	Low	Master Protein	Q6T374	Lumbrokinase-5	0.154	0.232	11
232	FALSE	Low	Master Protein	A0A0K2RV70	1,3-beta-glucans	0.148	0.232	4
237	FALSE	Low	Master Protein	B5AXP6	Peptidylprolyl isc	0.241	0.215	15

Table 3: Protein profiling of Isotroin 24 hours test

1	Checked	Protein FDR Conf	Master	Accession	Description	Exp. q-value: Cor	Sum PEP Score	Coverage [%]
2	FALSE	High	Master Protein	O15991	Lombricine kinas	0	61.18	50
74	FALSE	High	Master Protein	G3M459	Catalase OS=Eise	0	27.47	29
116	FALSE	High	Master Protein	A0A2I7YV73	Actin OS=Eisenia	0	26.441	39
161	FALSE	High	Master Protein	Q0PGR9	Fibrinolytic prote	0	16.794	38
182	FALSE	High	Master Protein	E9NPR6	Heat shock prote	0	3.149	9
196	FALSE	High	Master Protein	A0A2I7YV10	Glyceraldehyde-3	0	5.111	16
210	FALSE	High	Master Protein	A1YB06	Calreticulin OS=E	0	11.261	22
236	FALSE	High	Master Protein	H6VSD9	Cytochrome c ox	0	5.568	17
248	FALSE	High	Master Protein	A0A2I7YUY6	Piwi2 OS=Eisenia	0	0.967	7
259	FALSE	High	Master Protein	Q2I6A1	Ubiquitin (Fragm	0	6.021	36
270	FALSE	High	Master Protein	A0A0P0YK20	Endoglucanase C	0	2.16	4
278	FALSE	High	Master Protein	A0A2I7YV68	CuZn superoxide	0	4.556	15
286	FALSE	High	Master Protein	A0A2I7YV74	Heat Shock Prote	0	0.999	4
298	FALSE	High	Master Protein	Q5MBA0	Lumbrokinase (F	0	5.285	14
306	FALSE	High	Master Protein	M9VY46	Lysozyme OS=Eis	0	1.102	5
311	FALSE	Low	Master Protein	D3WK90	PlcR OS=Bacillus	0.094	0.298	11
316	FALSE	Low	Master Protein	Q5CD25	Valosin containir	0.25	0.224	1
321	FALSE	High	Master Protein	A8ILP4	Lumbrokinase (F	0	10.318	23
333	FALSE	Low	Master Protein	A0A0R4Y8R8	Cytochrome c ox	0.058	0.356	4
338	FALSE	High	Master Protein	B6E9G3	Histone H3 (Frag	0	1.794	10
343	FALSE	High	Master Protein	Q2I6A7	Calmodulin (Frag	0	1.113	19

Table 4: Protein profiling of Isotroin 72 hours test

1	Checked	Protein FDR Conf	Master	Accession	Description	Exp. q-value: Cor	Sum PEP Score
2	FALSE	High	Master Protein	A0A2I7YV73	Actin OS=Eisenia	0	27.14
55	FALSE	High	Master Protein	O15991	Lombricine kinas	0	23.888
100	FALSE	High	Master Protein	G3M459	Catalase OS=Eise	0	10.275
133	FALSE	High	Master Protein	Q8IT81	Myosin essential	0	3.597
148	FALSE	High	Master Protein	O18425	Lysenin-related p	0	7.369
168	FALSE	High	Master Protein	Q2I6A1	Ubiquitin (Fragm	0	3.129
179	FALSE	High	Master Protein	E9NPR6	Heat shock prote	0	2.345
190	FALSE	High	Master Protein	A0A2I7YV10	Glyceraldehyde-3	0	4.094
191	FALSE	High	Master Protein	A0A142IHP8	Gag protein (Frag	0	0.846
203	FALSE	High	Master Protein	A0A0A7DI07	Cytochrome c ox	0	0.796
211	FALSE	High	Master Protein	Q6T376	Lumbrokinase-3	0	1.665
219	FALSE	High	Master Protein	B7SHS5	Coactosin-like pr	0	1.236
227	FALSE	High	Master Protein	O18423	Lysenin OS=Eiser	0	3.29
241	FALSE	Low	Master Protein	A0A088BZG9	Phosphodiestera	0.093	0.276
246	FALSE	High	Master Protein	M9VY46	Lysozyme OS=Eis	0	1.38
251	FALSE	High	Master Protein	Q1ZZB7	Fibrinolytic prote	0	2.201
256	FALSE	Low	Master Protein	A0A2I7YV08	Poly[ADP-ribose]	0.159	0.254
261	FALSE	High	Master Protein	Q8I6N3	ARSP1 OS=Eiseni	0	2.742
269	FALSE	High	Master Protein	Q2I696	Beta-adrenergic	0	0.559
274	FALSE	High	Master Protein	B6E9G3	Histone H3 (Frag	0	0.414
279	FALSE	High	Master Protein	A8ILP4	Lumbrokinase (F	0	3.379

Table 5: Protein profiling of 24 hours control group

1	Checked	Protein FDR Conf	Master	Accession	Description	Exp. q-value: Cor	Sum PEP Score	Coverage [%]
2	FALSE	High	Master Protein	A0A2I7YV73	Actin OS=Eisenia	0	21.555	30
40	FALSE	High	Master Protein	Q2I6A1	Ubiquitin (Fragm	0	5.873	28
49	FALSE	High	Master Protein	O15991	Lombricine kinas	0	5.629	10
60	FALSE	High	Master Protein	A0A2I7YV10	Glyceraldehyde-3	0	2.073	3
65	FALSE	High	Master Protein	Q8IT81	Myosin essential	0	1.158	5
70	FALSE	High	Master Protein	A0A088BZ09	Uncharacterized	0	0.509	15
75	FALSE	High	Master Protein	Q0PGR9	Fibrinolytic prote	0	0.509	3
80	FALSE	High	Master Protein	A0A2I7YUY6	Piwi2 OS=Eisenia	0	0.271	1
85	FALSE	High	Master Protein	G3M459	Catalase OS=Eise	0	0.271	3
90	FALSE	High	Master Protein	A0A088BZM2	Neurocalcin-like	0	0.271	15

Table 6: Protein profiling of 72 hours control group

1	Checked	Protein FDR Conf	Master	Accession	Description	Exp. q-value: Cor	Sum PEP Score	Coverage [%]
2	FALSE	High	Master Protein	A0A2I7YV73	Actin OS=Eisenia	0	12.191	13
19	FALSE	High	Master Protein	O15991	Lombricine kinas	0	6.76	6
27	FALSE	High	Master Protein	Q6T376	Lumbrokinase-3	0	5.173	6
32	FALSE	High	Master Protein	B6E9G3	Histone H3 (Frag	0	4.177	16
43	FALSE	High	Master Protein	Q8IT81	Myosin essential	0	3.53	11
51	FALSE	High	Master Protein	Q2I6A1	Ubiquitin (Fragm	0	3.277	30
59	FALSE	High	Master Protein	Q2I6A7	Calmodulin (Frag	0	3.152	19
64	FALSE	High	Master Protein	A0A088BZ80	Uncharacterized	0	0.279	4
69	FALSE	High	Master Protein	A0A088BZ09	Uncharacterized	0	0.142	24
74	FALSE	High	Master Protein	A0A2I7YUY6	Piwi2 OS=Eisenia	0	0.142	2

Table 7: Protein profiling of normal earthworm

1	Checked	Protein FDR Conf	Master	Accession	Description	Exp. q-value: Cor	Sum PEP Score	Coverage [%]
2	FALSE	High	Master Protein	O15991	Lombricine kinas	0	18.376	35
46	FALSE	High	Master Protein	A0A2I7YV73	Actin OS=Eisenia	0	15.721	27
76	FALSE	High	Master Protein	A8ILP4	Lumbrokinase (F	0	12.241	29
91	FALSE	High	Master Protein	Q8I6N3	ARSP1 OS=Eiseni	0	9.631	20
103	FALSE	High	Master Protein	G3M459	Catalase OS=Eise	0	7.227	11
120	FALSE	High	Master Protein	O18425	Lysenin-related p	0	6.446	17
141	FALSE	High	Master Protein	Q8IT81	Myosin essential	0	5.948	21
158	FALSE	High	Master Protein	Q2I6A1	Ubiquitin (Fragm	0	4.452	28
166	FALSE	High	Master Protein	A0A2I7YV10	Glyceraldehyde-3	0	3.502	9
177	FALSE	High	Master Protein	O18423	Lysenin OS=Eiser	0	2.936	10
189	FALSE	High	Master Protein	Q1ZZB7	Fibrinolytic prote	0	2.514	5
194	FALSE	High	Master Protein	Q6T376	Lumbrokinase-3	0	2.514	6
199	FALSE	High	Master Protein	Q5CD25	Valosin containir	0	2.113	3
207	FALSE	High	Master Protein	E9NPR6	Heat shock prote	0	1.585	3
215	FALSE	High	Master Protein	M9VY46	Lysozyme OS=Eis	0	1.358	5
220	FALSE	High	Master Protein	Q0PGR9	Fibrinolytic prote	0	0.792	3
225	FALSE	High	Master Protein	D3WK94	Prohead proteas	0	0.528	3
230	FALSE	High	Master Protein	A0A088BZ09	Uncharacterized	0	0.467	15
235	FALSE	High	Master Protein	A0A088BZG9	Phosphodiestera	0	0.299	4
240	FALSE	High	Master Protein	A0A2I7YV82	Adiponectin OS=	0	0.299	2
245	FALSE	Low	Master Protein	B7SHS5	Coactosin-like pr	0.132	0.163	6
250	FALSE	Low	Master Protein	A0A0K0WWI5	Cytochrome c ox	0.135	0.163	3

6. DISCUSSION

Metformin, an FDA-approved diabetes drug, promotes proliferation, self-renewal, and differentiation of newly proliferated cells by activating the AMPK-aPKC-CBP pathway(12). Half of the treated earthworms with metformin, after storing in the formalin were sent for Histology from each of the test groups as well as the control groups.

The earthworm treated with metformin after 24 hours showed expansion of spaces in between the longitudinal muscular cells for the recruitment of granular cells resembling myoblastoma cells which further gets differentiated. Where in the control of 24 hours group such recruitment of granular cells was not seen. After 72hours in the test (the earthworm samples which were treated by metformin), Partial damage with cloudy swelling was seen at the lesion area and which showed the differentiation of granular cells into longitudinal muscle cells making the side of lesion thicker than the side opposite to it which had no cut on it and only the scar was remaining in the wound region ,clearly showing regeneration rapid than the one in control.

Also, in the manual observation in a similar way, the same recovery and regeneration were seen faster in the tests than in control. The factors affecting the regeneration got cleared after we checked and compared the data obtain from protein profiling of the other half samples, homogenised and given for orbi-trap LSMS. The protein "lumbrokinase-4" of the family Lumbrokinase (LK) was seen in only the samples treated with Metformin (24 hours and 72 hours). Therapeutic effects of lumbrokinase, a group of enzymes extracted from the earthworm, on peripheral-nerve regeneration using well-defined sciatic nerve lesion paradigms in diabetic rats is already observed and proved in previously done researches (according to the literature preferred) and It is found that lumbrokinase therapy could improve the rats' circulatory blood flow and promote the regeneration of axons in a silicone rubber conduit after nerve transection(13).

In tests, some other proteins were also detected which were not present in the respective control. Those proteins were Histon H3, Heat shock protein, valocine containing protein. Where HSP70 has been proved to accelerate wound closure through the stimulation of macrophage-mediated phagocytosis of wound debris(14). This indicates We could successfully target the other factors (lumbrokinase) involved in the regeneration with the pharmacological agent used (metformin). The dosage given to the earthworm was optimum enough to generate detectable rate of regeneration and the factors involved in it. Metformin-induced the production of lumbrokinase (LK) and HSP70. Metformin is responsible for repair and regeneration in *Eisenia fetida* production of Lumbrokinase protein.

Next, the pharmacological agent used was Isotroin. In the histology of samples treated with isotroin showed No granular cells near the lesion, only the space made for the granular cells to travel near the cut area was remaining and the epithelial, circular muscle cells are well defined along with a normal sized layer of longitudinal muscle cells just after the 24 hours after the wound was made on the earthworm. After 72 hours of lesion made, The space left vacant for the recruitment of granular cells at the site of lesion was taken up by the longitudinal cells by a rapid proliferation. The rate of proliferation was justified by the results obtained in protein profiling.

In protein profiling of the homogenized sample from where the protein was extracted and given for orbitrap LSMS, A protein named "Calreticulin(CRT)" and lumbrokinase were found which was completely absent in the protein profiling data obtained for the control of 24 as well as 72 hours control. CRT is the first potential topical biotherapeutic for chronic wounds that promotes the healing process through broad and diverse biological effects including attracting cells to resurface and repair the wound defect, and in fighting infection. Furthermore, CRT has unique tissue regenerative qualities including hair follicle neogenesis. Calreticulin (CRT) is expressed in many cancer cells and plays a role to promote macrophages to engulf hazardous cancerous cells. [47]

We can conclude that CRT production was an induced effect of the drug isotroin since it is absent in the control. On comparing the histology results and protein profiling results for the drug isotroin, we can draw a conclusion that the regeneration caused due to production of lumbrokinase was at a faster rate than normal which made a need of translocation of "coactosin like protein " and CRT which is responsible for the controlled cell proliferation and causing differentiation to reduce the risk of cancer respectively (15). Due to the high chances of mutations in the rapid cell proliferation and division process. So, CRT can be expected to be the protein-making a "Stop Button" for the proliferation cells at a higher rate to not enter the cancer cycle and differentiate into normal cells.

Which clearly describes that within 24hours after the lesion and exposure to the drug isotroin the proliferation rate was high enough for the cells to produce coactosin like protein and CRT to control it. Lysozyme proteins were also produced only tests samples and were absent in contols indicated it was induced to produce due to the effect of isotroin to facilitate tissue repair by first clearing out the area of cell debris formed due to the lesion. In the results obtained from protein profiling for the tests after 72 hours contained Gag protein along with the CRT and lumbrokinase proteins.

GAG-binding proteins are well known to play roles in more than one of the cited systems. For example, fibroblast growth factor (FGF)-2 (also known as basic FGF, bFGF) plays a function in vascular biology (angiogenesis), cancer biology (tumor growth), cytology (cell growth and differentiation), and histology (tissue development and regeneration(16). Thus, after 72 hours three proteins (lumbrokinase, gag, CRT) contributing to the fastest rate of regeneration were found to be active in the test samples and were completely absent in the controls.

This indicates: We could successfully target the other factors (lumbrokinase, gag, CRT) involved in the regeneration with the pharmacological agent used (Isotroin). The dosage given to the earthworm was optimum enough to generate detectable rate of regeneration and the factors involved in it. Isotroin induced the production of lumbrokinase(LK), gag, CRT, lysozyme, coactosin like protein. Isotroin is responsible for repair and regeneration in *Eisenia fetida* production of lumbrokinase, gag, CRT.

Even when the two drugs were compared, looking at the number of proteins getting produced for enhancing tissue repair and regeneration were seen more in isotroin (lumbrokinase, gag, CRT) where only one was found in metformin(lumbrokinase). Also, while comparing the histology results of both the drugs, we could see that the longitudinal muscles cells had taken over the vacant spaces which were made to facilitate regeneration first in the earthworms treated with isotroin than metformin.

Thus, By using Metformin and Isotroin which were involved in the regulation of proteins like oct-4 and lin-28 which were itself involved in regeneration, we could detect the upregulation rather the complete production (since did not find in any controls) of other proteins like lumbrokinase and lumbrokinase, gag, CRT respectively which were responsible for the regeneration in *Eisenia fetida.*

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