International Journal of Applied Research 2015; 1(11): 1138-1141

International Journal of Applied Research

ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2015; 1(11): 1138-1141
www.allresearchjournal.com
Received: 20-09-2015
Accepted: 16-10-2015

Dr. Archana Khandelwal
Department of Zoology, SMM Govt. Girls College, Bhilwara, Rajasthan, India

Correspondence
Dr. Archana Khandelwal
Department of Zoology, SMM Govt. Girls College, Bhilwara, Rajasthan, India

Investigation on limb and finger regenerative ability in mice after amputation through various proximo distal level at prenatal stage using embryo-culture technique

Dr. Archana Khandelwal

Abstract
Regeneration process of the limb and finger regeneration following may be related to the age of host. When both stages of (prenatal and postnatal) were compared after surgical amputation regeneration ability in prenatal stage was found greater than the postnatal stage. Present study carried out using embryo culture technique shows that when the embryo's limb and finger was amputated at various proximo-distal levels and kept in a culture medium for 48 hours, there was repair and growth of skeletal muscle elements, as indicated by the accumulation of newly formed chondrocyte cells in the distal end of the amputated limb. Additionally, the Disto-proximal cut and finger stump showed a thin layer of wound epidermis covering the amputated surface of the stump. These findings suggest that there is some capacity for regeneration of amputated hindlimbs and fingers, although the extent of regeneration is limited. However, it is important to note that this study was conducted on prenatal mice and further research is needed to determine whether these findings apply to other mammalian species, including humans. Overall, this study provides valuable insight into the regenerative ability of neonatal mice limbs and highlights the need for further research in this area.

Keywords: Regeneration, amputation, embryo culture

Introduction
It is well known that anuran tadpoles can regenerate their lost limb elements and this power is gradually decline in the limb segment the Disto-proximally and ultimately disappear during metamorphosis. Since the discovery of phenomenon of regeneration of several reasons have been suggested for the loss of power of limb regeneration in adult frogs, reptiles, birds and mammals - Schmidt, A.J. (1968) [3], Goss R.J. (1969) [4], Polezhaev, L.V. (1972) [5], Wallace, H.(1981) [11]. In mammals, studies concerning restoration of lost limb and digit have been performed on opossum, rat and mice. This has been comprehensively reviewed by Polezhaev, L.V. (1972) [5]. The studies carried out by Rajan, K.T., H.J. Merker and M. Wilkins (1980) [11] on culture method, the human fetal were kept In-Vitro conditions for 12 days, and results show that the digits grow rapidly and the head elongated by 60% after 6 days of culture. In the inception, it was believed that mammals, human beings do not regenerate their extremities in the manner as Anuran Tadpoles and Urodeles do. Studies by Illingworth C.M. (1974) [8] show that the fingertip of young children has remarkable capacity to regenerate. In his experiment, Deuchar', E.M. (1976) [9], the hindlimb of 11 1/2 days old mammalian embryos were kept for 44 hours In-Vitro conditions. Considering above, the present studies on limbs and fingers regeneration in mice, was undertaken to investigate the regenerative ability in mice at prenatal stage after amputation of hindlimb and finger, through various proximo distal level using In-Vitro culture technique. Using this embryo culture technique by New, D.A.T. (1966) [2], young embryos grew well in static medium i.e., simple watch glass method. By this method, it was possible to maintain mice embryos' following amputation for a period of 2 to 3 days *In vitro* conditions. Due to technical difficulties, the experiments on regenerative ability in amniotic embryos have been limited.

Material and Method

The present studies were carried out on the closely inbred Swiss albino mice (order-Rodentia, Family Muridae), obtained from the animal House of the department of zoology, University of Rajasthan, Jaipur. To get regular supply of mice of different pre-natal and postnatal stages, 3 - 4 healthy (weight 25 ± 2) females were placed with a healthy mail for mating in breading cage. The breeding cage were kept in the animal room under normal light and dark conditions. All the animals were fed on synthetic diet, the mice feed supplemented with germinating grams, wheat, seasonal vegetables and water *ad libitum*. Investigation on the regenerative ability at embryonic stage were carried out on 16th day gestation embryo. For this purpose, operation procedure was used

Operation procedure for the pregnant females:
For the Embryo culture technique, pregnant females on 16th day gestation were selected from the breeding cages. Anesthesia was given to the animals by placing cotton soaked with ether near the nostrils of the animals. After anesthesia, pregnant female was placed on the operation table in the sterilized chamber. The hair from the ventral side of the abdomen were removed by shaving them. The abdomen was cut open with a longitudinal incision of about 2 cm through the ventral body wall. The visceral organs were exposed, and then uterine horn bearing the embryos were located in the abdominal cavity (Figure 1).

Fig. 1

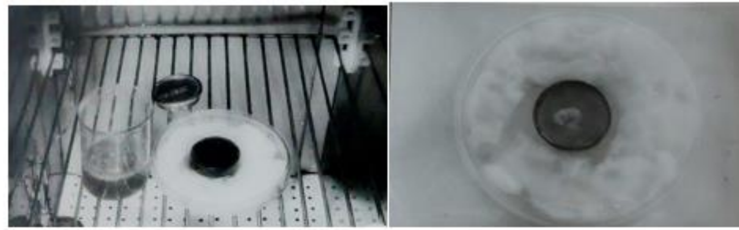

Fig. 2(a) and Fig 2(b)

Fig 2(a) showing Embryo culture - on 16th day the gestation was cultured in the petri dish culture chamber in a CO2 incubator after finger amputation. Fig 2(b) showing Petri dish culture chamber - showing explant in a watch glass with culture medium surrounded by the wet cotton bed
The above steps were followed for the embryo culture method to allow a post amputation development in the embryos.

Embryo-Culture Method

The general procedure of operation of the pregnant females of mice (16th day gestation) to obtain the embryos was followed as has been described above. The embryos were separated from the uterine horn with as much of placental tissues as possible and then the amnion was removed. These naked (without amnion) embryos were placed into the embryo culture medium in a petri dish. The limb was amputated through the proximal fingers and middle fingers. Precaution was taken to minimize the injury to the placenta and to the embryo itself.
After amputation, embryos were cultured by the modified method of New (1966) as follows:

Preparation of Culture Medium

Powdered MEM (minimum essential medium) with Hanks salts (Himedia, Bombay) was dissolved in the required amount of triple distilled water in a sterilized conical flask. This medium was supplemented with hepes buffer (10 mm), penicillin (100 IU/ ml), streptomycin sulphate (100 mg/ml), sodium pyruvate (5.6 mg/100 ml), glucose (100 mg/100 ml) nahco3, (300 mg/100 ml), Insulin (0.04 units/ml). The ph of culture media was adjusted to 7.4. The media was then filtered with the help of membrane filter (5μ, pore size) fixed in holder, attached to a glass syringe.

Culture Method

In a sterilized petri dish (2" size) 1% solution of agar (Riedel) was poured and it was allowed to cool down to get a thin layer of agar jelly at the inner side of the petri dish. In this petri dish coated with a layer of agar, 4 ml culture media or 2 ml culture media and 2 ml of sterilized mouse serum was poured. In this medium one embryo was placed as shown in figure 2. This small petri dish was then placed in a bigger petri dish (6" sizes) with a lid. The embryo explant floats in culture medium. In the bigger petri dish cotton wetted with 0.9% saline was kept to maintain humidity in the petri dish in the culture chamber. The cultures were incubated for 48 Hours in 60 to 70% oxygen and 3 - 4% CO_2 in a CO_2 incubator (IEC. Bombay) as shown in Fig. 2(a). Ten embryos were cultured in separate petri dishes after amputation of their fingers. After 48 hours of post amputation developments the embryos were fixed or 6% formalin for further investigations. The hind limbs were secured from the base and observe morphological changes some representative cases were processed for histologic observation although the culture method facilities surgical manipulations of the embryos more conveniently but some limitations in this technique. Firstly, most suitable chemical define media or sera does not provide natural conditions as *in vivo* conditions. Secondly, extra embryonic membranes are greatly damaged while preparing the fetus for the culture. Thirdly, to maintain a good culture, certain factors such as Co2/O2 supply atmosphere temperature and pressure etc. Have to be regulate very carefully and a slight disturbance may lead to fetal death or abnormal development.

Fixation Schedule

The hindlimb were severed from the mid ankle or finger, and fixed in 6% formalin and then observed for morphological changes. Some representative cases were followed for histological examination. The staining is followed by decalcification of tissues for histological examination. For staining modified Azan Staining Technique by Domagk, L. (1948) [1] were used.

Observation and results of embryo culture technique

After using embryo culture method, the embryos grown in culture medium for 48 hours following amputation of middle fingers II, III and IV through the proximal phalanges show wound healing around the cut end of phalanges. The skeletal elements of the amputated fingers show repair and growth as a result of accumulation of newly formed cartilage (Fig. 3). The tissue present around the amputated fingers have also regenerated (Figs. 4 and 5). In the embryos in which fingers (II, III and IV) were amputated through the proximal, proximal and middle phalanx respectively. The wound is covered by a thin wound epidermis. Toe indentation is absent in these cases. In these, dermis is absent in the anterior region. The epidermal cells are loosely arranged (Figs. 6). The basement membrane is also absent in the anterior region. The shaft of amputated Phalanges shows repair and growth as a result of accumulation of chondrocytes at the cut end of the stump. In these cases, also the tissues, present around the shaft of phalanges show regenerative developments.

In the embryo, in which amputation of fingers were made through the base of proximal phalanges a cartilaginous mass has accumulated at the amputated site

Surrounded by the wound epidermis (Figs. 7 and 8). In these the cartilaginous differentiation does not take place but the toe indentation is clear as observed after 48 hours of development.

Post amputation developmental changes in the hindlimb of 16th day gestation embryos (pre-natal stage) of mice amputated through the proximo phalanges and allowed to develop embryo culture. Observed after 48 hours of amputation.

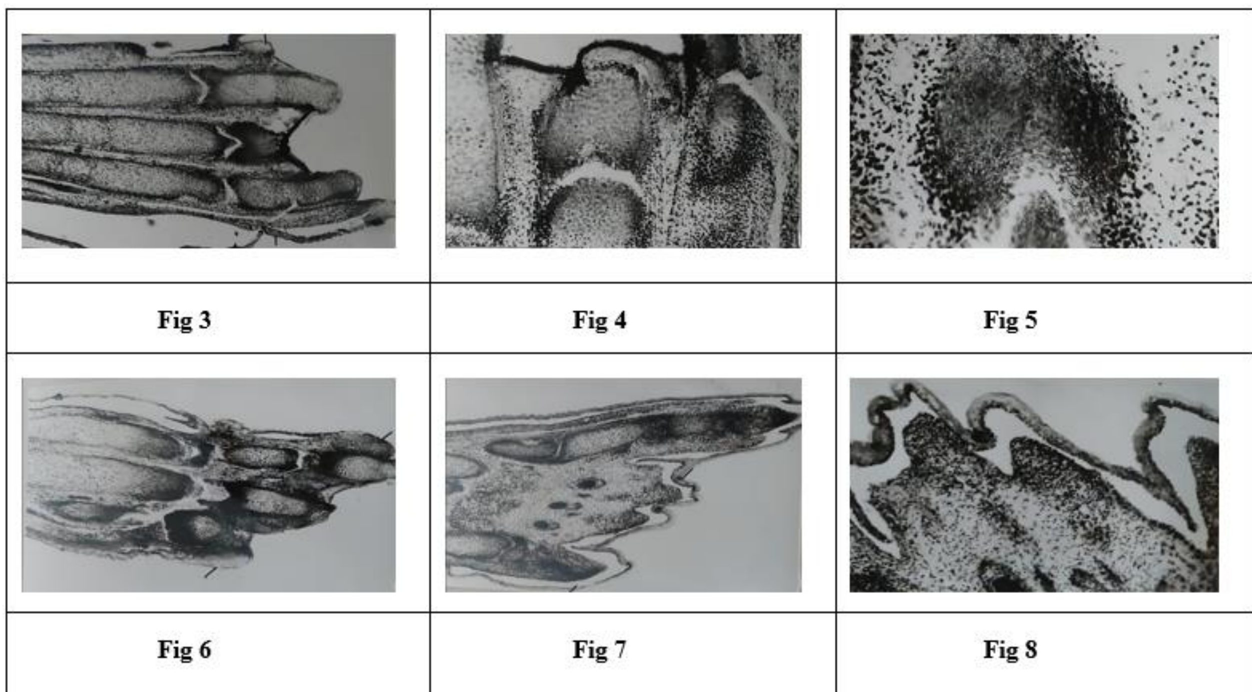

Fig. 3 L.S. showing wound healing. Fig. 4 L.S. showing repair and growth Fig. 5 Showing accumulation of chondrocytes Fig. 6 L.S. showing loosely arranged epidermal cells. Figure 7&8 L.S. showing repair and growth of the phalanges by accumulation of cartilaginous cells.

(Short lines indicate approximate level of amputation)

Result

Most of the studies related to culture of mammalian embryos have been designed to study differentiation, growth and morphogenesis of an embryos under *in vitro* condition, the result of present study Clearly indicates that fingers of 16th day gestation embryos possess capacity of restitution of lost limb elements although limited. 48 hours after amputation have demonstrated that chondrocytes cells have accumulated at the end of amputated end of the finger. Observation provides further interest for the investigation related to studies on mammalian limb regeneration.

Discussion

The present studies about In-Vitro culture method were different from earlier work by New, D.A.T. (1966) [2], Cockroft, D.L. (1973) [7] in two respects. Firstly, in present study Hepes buffer. (Concentration 10mm) [4-(2 hydroxy ethyl -1 piperazine thane – sulphonic acid] was used in place of bicarbonate buffer. Secondly thin layer of agar gel was used in petri dish to prevent sticking of embryos to the glass surface.

New and Steele(1972) [6] reported that this watch glass culture techniques holds good for the younger embryos up to somite stage however in present study even older embryo (16th day gestation) were cultured successfully for about 48 hours without the floating medium condition. Normal development of embryo under culture condition depends upon the culture media. In comparison to heterogeneous sera, rabbit sera, fowl sera, and the synthetic media sera, the synthetic media sera supplement by glucose sodium, pyruvate and insulin was found better for culture

The culture method facilitates surgical manipulation of the embryos, some limitations in this technology. Firstly, more suitable chemical media or Shera does not provide natural conditions as *in vitro* conditions. Secondly, extraembryonic membrane is greatly damaged while preparing the fetus for the culture. Thirdly, to maintain a good culture certain factors such as CO2/O2 supply, atmospheric temperature and

pressure etc. have to be regulated. A slight difference may lead to fetal death or abnormal development.

Conclusion

Although due to technical difficulties and lack of facilities related to the maintenance of embryos for longer period following limbs and finger amputation. The growth and morphogenesis of the regenerated part could not be further developed. Efforts are being made in this direction for technical advancement of the embryo culture. So that amputated embryos can be maintained for longer period to study. The result of present study is encouraging for further study about the phenomenon of limb regeneration in mammals.

References

1. Domagk L. Kernechtrot-Anilineblau-Orange G. In: Mikroskopische Technique. B. Romeis, Oldenburg, Munchen; c1948. p. 346.
2. New DAT. Development of rat embryo cultured in blood sera. J. Reprod. Fert. 1966;12:509-524.
3. Schmidt AJ. Cellular biology of vertebrate regeneration and repair. University Chicago Press, Chicago; c1968.
4. Goss RJ. Principles of regeneration. Academic press, New York; c1969.
5. Polezhaev LV. Organ regeneration in Animals: Recovery of organ regeneration ability in animals (Charles C. Thomas. Springfeld and Illinois); c1972.
6. New and Steele, Improved development of rat "egg cylinders" *in vitro* as a result of fusion of the heart primordia. Nature, Lond. New Biology. 1972;237:150.
7. Cockroft DL. Development in culture of rat foetuses explanted at 11.5 and 13.5 days of gestation. J. Embryol. Exp. Morph. 1973;29:473.
8. Illingworth CM. Trapped fingers and amputated fingertips in children. J Pediat. Surg. 1974;9:853-858.
9. Deuchar' EM. Regeneration of amputated limb-buds in early rat embryos. J Embryol. Exp. Morphol. 1976;35(2):45-354.
10. Rajan KT, Merker HJ, Wilkins M. Observations on human digits *in vitro* and its possible role in evaluating teratogens. Teratology of the limbs- Walter de Guyter and Co., Berlin New York; c1980.
11. Wallace H. vertebrate limb regeneration publ. John. Willey and sons; c1981.