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Delayed Xenograft Transplantation of Mouse Olfactory Ensheathing Cells in Adult Rats

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Abstract

Regeneration of the central nervous system after injury using appropriate cells for transplantation is a controversial issue. Accessibility of allograft olfactory ensheathing cells to transplant in the spinal cord of patients is not applicable. Therefore, in this study, an attempt has been made to xenotransplant cells from mouse into a corticospinal tract lesion in a rat in order to achieve a plausible preclinical approach for future application to a clinical study. Adult rats were trained to use their forepaws for retrieving. The dorsal corticospinal tract was lesioned by a stereotactic radio-frequency lesion maker at the level of the first/second cervical segments. Rats that had shown no forepaw retrieval by 8 weeks were xenotransplanted with a suspension of cultured olfactory ensheathing cells derived from the mouse olfactory bulb. Starting between 1 and 3 weeks, 10 rats with transplants bridging the lesion site resumed ipsilateral forepaw reaching. After transplanting cells into the lesion side, the cross and horizontal sections of GFAP and NF staining of 10 animals that have the Directed Forepaw Reaching (DFR) function returned showed the regenerated CST fibers in the lesion area after 8 weeks postoperative. Xenotrasplant of olfactory ensheathing cells from the mouse olfactory bulb into a rat corticospinal tract lesion was promising and positive. Animals that had difficulty in Directed Forepaw Reaching had returned the function 8 weeks postoperatively.

Keywords: Xenograft; Olfactory Ensheating Cells; Transplantation; Corticospinal Tract; Repair; Rat.

1. Introduction

Olfactory Ensheathing Cells (OEC) are located in the olfactory epithelium (OE) and the outer layer nerve (OLN) in the primary olfactory system (POS) [1]. The similarity of OEC to non-myelinating Schwann cells (SCs) in the PNS, in which they ensheat axons without forming myelin, has caused scientists to introduce them as olfactory Schwann cells [2]. OECs give stem cell-like properties to OE, which leads to regrowth of axons in the PNS (OE) and in the CNS olfactory bulb (OB) every four weeks [3]. It has been reported previously that directed forepaw retrieval by the ipsilateral forepaw is intervened by delayed allograft transplant of OEC in a rat with a unilateral lesion of the dorsal corticospinal tract (CST) [4]. The possible importance of such a method to human spinal cord injuries is the repair of the delayed cervical spinal cord by 3 months [5]. However, as recent studies show, the use of auto- and allo-transplants of OECs is not practicable for therapeutic use due to the inaccessibility of the olfactory bulb and the unavailability of donors [6]. Also, although some scientists use periphery-derived OECs, Olfactory Mucosa Cells (OMCs), obtained from a small biopsy of olfactory mucosa lamina propria from external nares (nostrils), it is not an efficient alternative source, is not feasible, and does not have enough cell numbers as the auto- and allo-transplantation of OM in a solid piece as well as cultured OM cells [4]. A recent study indicated that OEC significantly improved motor function after SCI, but had no effect on allodynia and might lead to a comparative exacerbation of hyperalgesia [7]. In this study, we look at the

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xenotransplant of olfactory ensheathing cells derived mice olfactory bulb as the OEC cell source in the rat unilateral corticospinal tract lesion model to retrieve Directed Forepaw Reaching (DFR) with the hope of future therapeutic use of the xenotransplant source of OEC cells as an alternative stem cell source to solve some problems such as the inaccessibility and unavailability of cell numbers in auto-transplantation of OEC and OM and the difficulty in finding a donor for allograft transplantation.

2. Materials and Methods

In our study, animals were used in accordance with the UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act 1989, with the ethical approval of the University College London, Institute of Neurology.

2.1. Cell Culture

The instruments were autoclaved and left in the flow hood. The growing medium was made by (50:50) DMEM/F12 (Invitrogen, UK) media with penicillin p/s and 10% foetal calf serum (FCS) and left with a few dishes of hank on the cold plate in the flow hood. Animals were placed under terminal anaesthesia and decapitated in a CO_2 chamber. The olfactory bulb was divided and placed in a 60 mm dish having hanks (HBSS) (Life Technologies, UK) [8]. Under the dissecting microscope, as much of the meninges and cartilage were removed as possible using no 5 tweezers. The glomeruli were dissected out of the olfactory bulbs by slicing the bulbs in half down the center. The white matter was detached, and the tissue was washed mildly in hanks and transferred into a 15 ml tube with 2 ml of hanks with 200 µl of 1% of 25% Trypsin-EDTA (Worthington, UK) and gone in oven at (37˚C, 5% CO_2) for 15 mins by spending small blue curved tweezers and a scalpel [9]. After trypsinization, 10 ml of growing medium were added to stop the trypsinization process. After the tissue was settled, the media was removed, and only 1.5 ml of media in the bottom, and 2 µl of DNAse (Invitrogen, UK) solution was added and triturated by the pipette tip until no big lumps remained. More media was added, and if there were any lumps at the bottom, the cell suspension was transferred to a fresh tube spined at 1200 rpm for 5 min, the supernatant was removed, and 2 ml of medium was re-suspended and plated on PdL coated dishes. Cultures are fed and the medium is changed after 4-5 days and thereafter every 2-3 days for 16–18 days.

2.2. Surgical Procedure

The first stage of the procedure was to build up a constant and reproducible unilateral corticospinal tract lesion animal model using a radio-frequency lesion generator (Lesion Generator Mode, REG-3C RF, Burlington, Massachusetts, USA). To produce complete loss of directed forepaw retrieval (DFR) on one side, the lesion has to destroy all CST and some part of grey matter [10]. 93 adult female rats (180-210 g body weight) of a locally inbred Albino Swiss Strain (AS) were used, and all the procedures were performed under UK Home Office regulation and the Animal (Scientific Procedures) Act 1986 [11]. The pretrained rats were shaved, the skin was cleaned by Tamodine wound cleanser (Vetark professional, UK), at the neck area, and anaesthetised under a low concentration of isoflurane (1.5 or 2%, Primal company), and as anaesthetised rats were straddled in a stereotaxic frame with the neck flexed and the head steadied with ear plugs and an incisor bar [11]. Anaesthesia was adequately maintained with isoflurane-in oxygen on the anaesthesia system. Throughout the whole process, the head of the animal was situated firmly in a stereotaxic apparatus in the correct position (incisor bar 5mm above the interaural line) [12], and the dorsal exterior of the spinal cord at the level of the first and second cervical segments was exposed over the middle line incision of the skin, splitting the supraspinal muscles in the midlin [13].

After locating the occipital bone, the atlanto-occipital membrane was incised. The cervical atlas and axis were opened, and a small needle was used to separate two segments. The dura matter was torn up with micro-dissecting tweezers, and cerebral spinal fluid (CFS) came out. The presence of the median sagittal venous pattern can be the indicator middle line; however, it is not absolutely reliable and may cause some error at lesion position. The KCTE-TC-S electrode (Cosman, Germany), using a straight RF tip of 0.25 mm diameter and 1.8 mm tip length, was implanted to a depth of 1.5–2 mm at a point 0.5 mm lateral to the midline. As the electrode has a built-in thermocouple (TC) temperature sensor, we set the tip temperature at 60 ˚C then insert the electrode after the tip temperature reaches to 60 ˚C, lesion is made for 30 seconds. The electrode was withdrawn gently. The animal supraspinal muscles and outer most layer of skin was sutured with violet braided absorbable sutures (Ethicon, Belgium). After the procedure, the animals were kept distinctly and preserved on a heating mat during the recovery period [8]. Analgesics (0.025 ml of Carprofen, 50 mg/ml, s.c.) were administered every day for the 3 days after surgery, and the lesion size was dependent on two constraints: tip temperature and time [8]. We have done the experimental procedure on 50 animals, changing the tip temperature ranging from 30-80 ˚C and time of 10-60 second. Operated rats were under the care and prescription of the 0.0192 mls analgesia drug Carprieve 5% w/v (Norbrook, UK) subcutaneously for three days. The histological analysis and behaviour test proved the consistent lesion size with the knock-out function at 60 ˚C tip temperature and 30 sec lesion time.

2.3. Immunohistochemistry of Complete Corticospinal Tract Lesion

The lesion area was studied by immunohistochemistry technique looking at axons, astrocyte scar tissue, and myelination. Neurofilament staining (Abcam, Cambridge, UK) was performed to check if all the axons were destroyed on one side, anti-Glial Fibrillary Acidic Protein Clone GA5 (Chemicon, UK) to look at astrocyte behaviour after a lesion in the corticospinal tract, and Myelin Protein Zero Antibody-Neuronal Marker P0 (Novus Biological, UK) to stain peripheral type myelination in the lesion area. Animals were perfused and tissue was cut, as explained in Section 1.2. In each case, 16 µ cryostat sections were fixed in 4% PFA for 30 minutes. Sections were blocked in 2% milk-PBS in 1% Triton for 30 minutes and then incubated in 1/500 primary antibodies of monoclonal anti-neurofilament, anti-GFAP, and anti-P0 at 4 ˚C [14]. All primary antibodies were diluted at 2% milk-PBS with 1% Triton for 2 hours at room temperature, and the next day the slides were washed away in PBS for 30 minutes and exposed to 1/500 diluted biotinylated anti-mouse secondary antibody (Alexa Fluor 546 goat anti-mouse) for all three antibodies [15]. The sections were mounted with Fluoromount mounting media (Sigma, UK). Immunohistochemistry analysis confirmed that the axons were destroyed at the completed lesion, astrocytes proliferated around the lesion core, and there were no astrocytes in the lesion epicentre. Also, peripheral type myelinated can be seen around the lesion centre and peripheral root.

2.4. Pre-Operative Ipsilateral Paw Reaching Training

Here we use a preclinical animal model to assess forelimb activity, impairment, and recovery by training rats for reach-to-eat ability [16]. AS Female adult rats with a body weight of 180–200 g were located in the testing cage to ensure that the animals had a slot in which to reach food pellets [17]. The cage was made of clear Plexiglas and Perspex and 25 x 35 x 30 cm in size. Each slit is about 15 centimetres above the cage base, 2 cm in weight, and 3 cm in length. Animals were trained by the daily presentation of fragments of noodles for two days before starting training. After they were fed by forceps to reach food pellets (dried spaghetti) four times a week during the two weeks prior to surgery, they learned how to reach by using both forehands (forepaw). The results were recorded for 50 times of pa retrieval (locating the food, advancing the lime, grasping the noodle, bringing the food to the mouth, and returning to the start point). Those rats with the record of both paws reaching no less than 15 times each pa was chosen for the CST lesion operation. In general, after skilled forelimb reaching, a total of 93 AS rats were able to use both pa to reach the noodle and were therefore ready to go under CST lesion operation.

2.5. CST Lesion Post- Operative Skilled Forepaw Reaching Assessment

Three days after surgery, those operated rats recovered from surgery and had shown no abnormalities. They were assessed for pa reaching 50 times their task three times a week for a period of 4 weeks. The movement of each pa was scored as a retrieval if the limb was advanced directly through the slot towards the pellet, even though no grasping was done. The complete lesion with paw knocked-out function was determined if no retrieval was recorded by the limb of the operated CST lesion. By the end of 4 weeks, if the knocked-out limb had not shown any signs of retrieval, the animals were going under the second operation of olfactory ensheathing cell transplantation. In total, 20 rats have shown complete lesions and functional deficits on the operated CST lesion side.

3. Result

The cross and horizontal sections of GFAP and NF staining of 10 animals that have the DFR function return show the regenerated CST fibers in the lesion area after 8 weeks postoperatively. This finding is confirmed by the previous research done on xenotransplantation of transgenic pig olfactory ensheathing cells into transacted dorsal column lesions of the spinal cord of an immunosuppressed rat, which showed axonal regeneration [18]. Figure 1 shows the elongated, NF-stained axons on the right-hand side of the lesion; however, the CST fibers have not been traced to look at the terminal points in gray matter. Figure 1 shows the cross section of the GFAP-labeled astrocytes and NF-stained axons in the lesion area, which is almost 1% of total axons. Also, the histology images show that the animals, after two weeks, start immunorjecting the xenotransplanted mice OECs in the grafted tissue. Among the five groups of animals, the one that had a survival time of 4 days and 1 week did not show any immunorejection, the GFP-labeled OEC cells can be visualized, and there is no inflammation sign, which supports the finding that transplantation of OEC in experimental animals improves the retinal immune environment [19]. Also, it proves the study by Zhang et al. showing the inflammation in the spinal cord was decreased after intervenous transplantation of OEC [20]. However, groups 3, 4, and 5 animals with a survival time of 10–14 days (3, 4, and 5 weeks) show an immune reaction to xenogenic transplant of OEC-degenerated cells and the central necrosis and lymphocyte infiltration in the lesion area. Moreover, the images of postoperative xenotransplanted OECs under daily subcutaneous injection of 100 ml at a dosage of 6 mg/kg/day avoid immunorejection (Figure 2), which shows the misplacement of GFP-labeled transplanted OECs with a survival time of 4 weeks and no sign of inflammation.

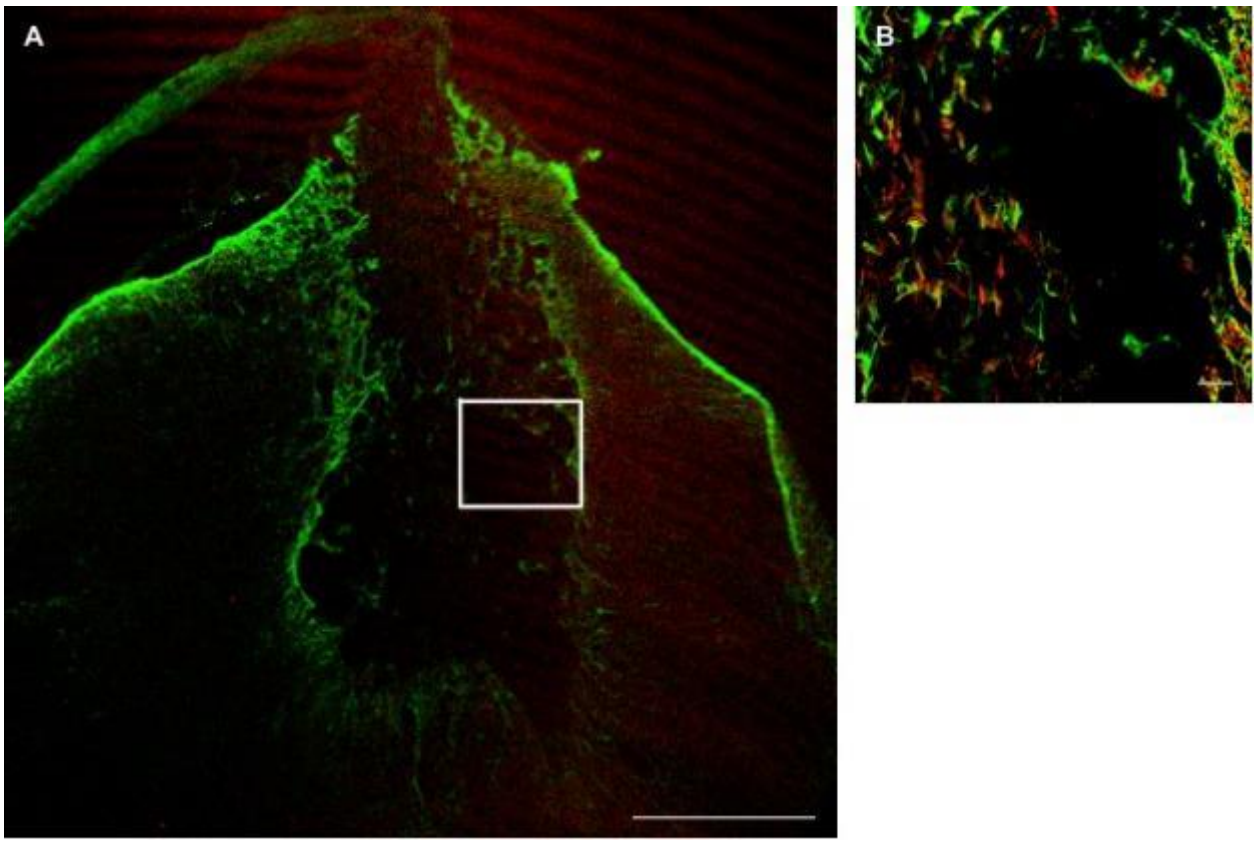

Figure 1. A) The regenerated Axon after transplant of olfactory ensheathing cells. B) The image of astrocytes immunostained with an antibody against glial fibrillary acidic protein (GFAP) and axons labeled with red labelled neurofilament antibody, 20µm-thickness. Survival time: 4 months, Scale bar: A; 150µm B; 60µm.

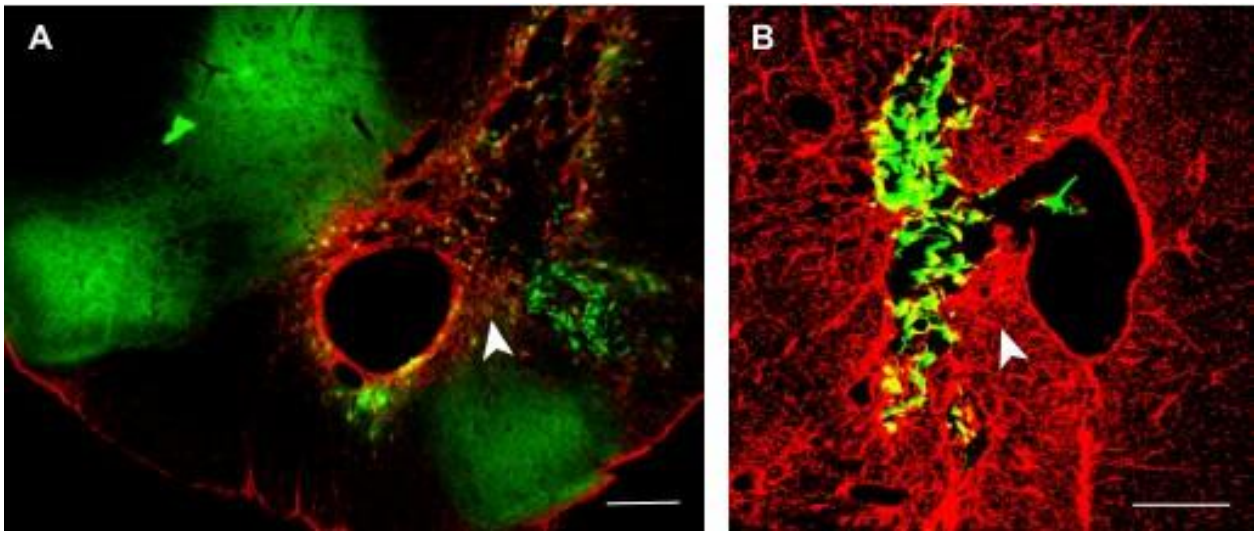

Figure 2. The 20 µm thickness coronal section of transplanted CST lesion failure. A). The image shows the GFP labelled grafted OECs at the dorsolateral side of lesion. B). The enlarged view of transplanted side shows the distance between OEC transplanted and the lesion. Surviving time: 4 weeks; scale bar: A, 100 µm; B, 50 µm.

The numerical show the rising curve of DFR over the 8 weeks starts reaching from first week in most animals and second and third weeks in 3 rats reaching to almost 50- 85% of the total counting of 30 by the end of 8 weeks (Figure 3). The rate of recovery (DFR) is related to the percentage of spared CST [8]. Our result shows the total number of DFR by the ipsilateral forepaw is well correlated with ($R^2 = 0.79$) spared axons, which confirms the result of a previous study on delayed transplantation of OECs that promoted sparing of supraspinal axons in the contused adult rat spinal cord, which showed the 7-day OECs-medium group animal showed twice as many labelled neurons in the brain compared with the 7-day medium-only control group [21].

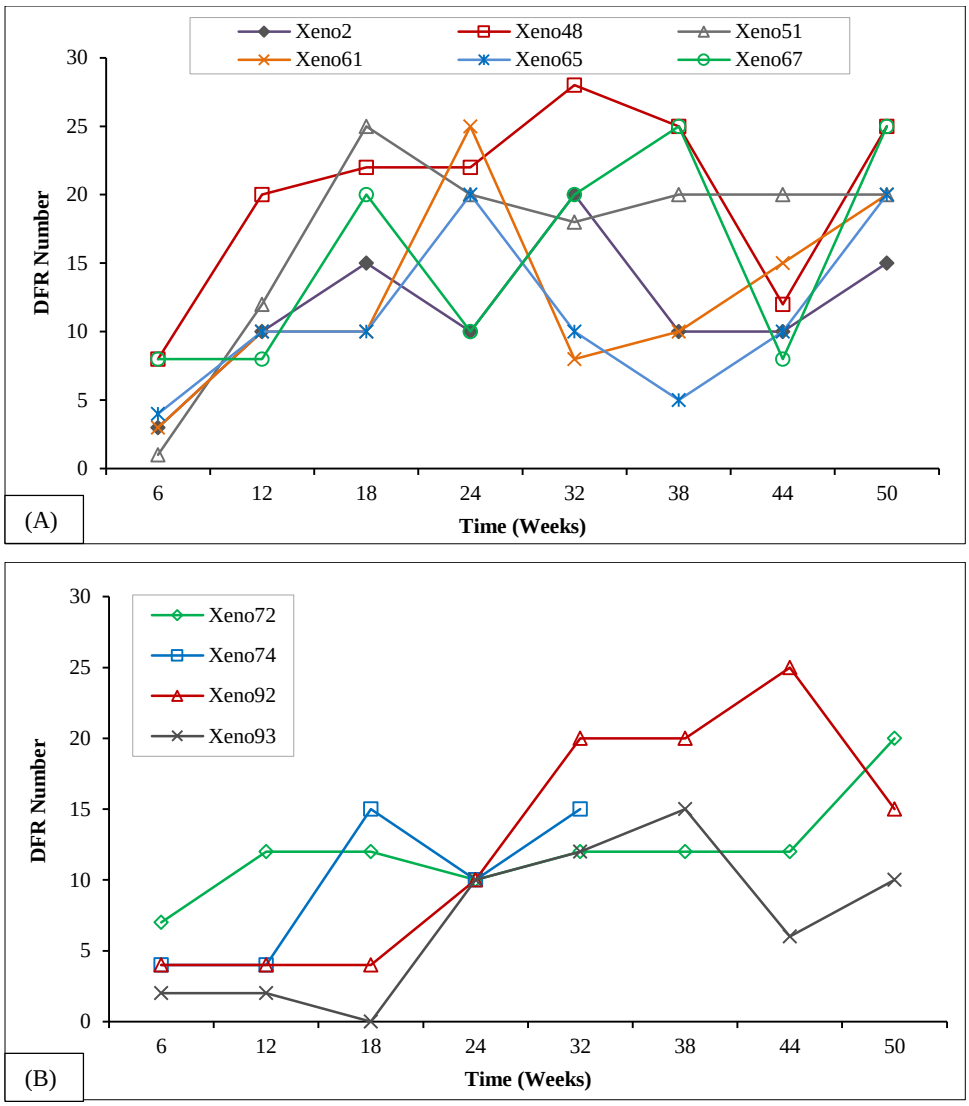

Figure 3. (A) and (B) Changes with time in weeks (x-axis) in the number of retriel (y-axis) performed by the forepaw ipsilateral to the lesion in each testing period of a total of 50 retrievals by both forepaw

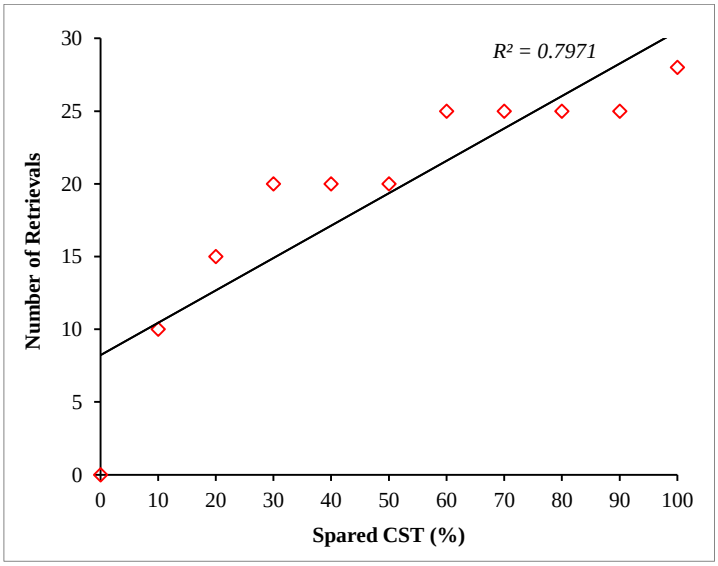

Figure 4. A and B Changes with time in weeks (*x*-axis) in the number of retrievals (y-axis) performed by the forepaw ipsilateral to the lesion in each testing period (of a total of 50 retrievals by both forepaws). The rate of recovery of DFR over the 8 postoperative weeks is proportional to the amount of the CST spared by the lesion. Significant correlation ($R^2 = 0.79$) between the total number of retrievals by the ipsilateral forepaw (y-axis) and the proportion of the CST spared (x-axis).

4. Discussion

The total of 93 animals after being trained for the DFR task underwent unilateral CST lesion operation using a 0.25-diameter built-in thermocouple (TC) temperature sensor electrode. In total, 20 rats lost the ability to retrieve their paw unilaterally. As the behaviour test results show, these animals lost the ability to reach their paw through the cage slot to grasp the noodle when the number of times they reached was counted 30 times, three times a week over the 8 weeks postoperatively. Therefore, animals show 0 times of retrieval. This result correlates with the histology analysis of lesioned tissue. The lesion we produced at the CST tract unilaterally destroyed dorsal CST fibers, and slight damage to ascending (sensory) dorsal columns totally damaged ipsilateral DFR function, with no return of function for the maximum period of 12 months. The loss of DFR is associated with impairment of the rostral motor neuron innervating proximal muscle, which leads to failure in the ability of proximal muscular to extend the forelimb through the slit and would not cause any failure in grooming, handling food, grasping, and walking of the affected forelimb on the proteoglycan effect on repair of laminin–mediated axon growth [22]. According to the observations we made in the present study, the lesion we produced at the CST tract unilaterally destroyed dorsal CST fibers and slight damage to ascending (sensory) dorsal columns, totally damaging ipsilateral DFR function, with no function returning for a maximum period of 12 months [22].

In future clinical trials of spinal cord injury, xenograft transplant of OECs will be more efficient than allogenic transplant of human OECs from the patient's olfactory bulb, which is not feasible, and the auto-transplant or allotransplant of olfactory mucosa cells is not accessible due to the difficulty of finding donors and the problem with the cell number of cultured tissue. In the present study, we designed a rat delayed xenograft transplant model to investigate regeneration of CST fibers and DFR function return after transplanting cells from a mouse bulb and the effect of stopping the immunosuppressive agent (cyclosporine) on DFR function. Recovery of function accrued if the transplanted OECs were injected unilaterally in the CST lesion area and the immune attack was suppressed by injecting immusuppressant reagent regularly. After OEC xenotransplant of a total of 39 AS rats, 29 rats did not show any DFR function recovery of the lesioned paw due to either misplacement of the cell transplant in the CST dorsal column (Figure 1), the cells being injected 1-2 mm away from the dorsolateral side of the lesion, or the immunorejection of CST cells, which can be due to the error in the injection of an immunosuppressant. Of those xenotransplanted rats, only 10 rats have recovered from DFR deficit and being able to reach their lesioned paw through the aperture to grasp a noodle, and their DFR ability continued rising up to 8 weeks after transplantation. As the immunohistochemistry analysis proved the xenotransplanted OEC cells migrate caudally into the distal CST, and by 10 days post-surgery, they form ~100-150 μm elongated bridge alongside the nerve axis, which allows the CST fibres to cross the lesion area and terminate in the dorsal horn (Figures 2A and 2B).

As the results show, although we stopped daily cyclosporine injections by the end of 3 weeks, the DFR function remained constant as it was prior to stopping cyclosporine. However, no GFP labelled OEC was visualized under florescent microscope due to immunorejection (Figures 2A and 2B). This immunohistochemistry results correlate with numerical data of behaviour test as you can see in (Figures 3A and 3B) the number of DFR retrieval after transplanting OEC starts increasing and reached to maximum number of 20 -30 during the first 3 weeks post-operative under cyclosporine injection and remains spontaneous up to 4 weeks after stopping cyclosporine injection and although the histology image proved that by stopping cyclosporine xenotransplanted OECs being rejected at week 8 of posts surgery , the immunorejection of grafted tissue did not effect on DFR Recovery and numerical data shows that the animals did not shows deficit only in their paw reaching task and the number of DFR remains spontaneous between 10-30 . This result is also consistent with the study on the facial nerve xenograft model [23]. Histology analysis shows that ~ 1% of the total 50,000 myelinated CST fibres on OEC cells transplanted animals (Figure 3B) shows that regenerated fibers are ~ 1% of the total 50,000 myelinated CST fibers on OEC cells transplanted animal and this result is correlated with the number of spared CST fibers with $R^2 = 0.79$ (Figure 4). These results are also consistent with previous studies [24]. Our observation of cross-species transplantation immunorejection indicates that transplanted rats start immurejecting OEC cells by 10 days postoperatively [13].

5. Conclusion

The xenotransplan of OEC does recover paw reaching task in unilateral corticospinal tract lesion in a rat animal model on a large scale. However, it is still difficult to choose the right source and method of cell production. As our study has proven, primary culture of OECs is not the ideal transplant material in SCI because they normally survive for a few weeks, they get contaminated, they are limited in number, and it is difficult to generate a pure cell population. The other problem is the use of an immunosuppressive drug such as cyclosporine in the case of a non-synergic cell transplant, which may cause various side effects such as nephrotoxicity, hypertension, neurotoxicity, hirsutism, and gum hypertrophy [25, 26]. Therefore, it would be better if we could replace different methods with an immunosuppressive drug to avoid an immune attack. The future plan is to produce a transgenic homogenous OEC clonal cell line (nOEC) and investigate whether nOEC have the same characteristic and property in repair of SCI as primary OEC cell culture or not, and whether we can avoid immunorejection by transplanting cells derived from transgenic mice expressing

human complement inhibitory protein, hCD59 (membrane cofactor protein), which mediates the removal of microorganisms and the clearance of modified self-cells that appear to have an immunosuppressive effect in transgenic expressing human complement inhibitory protein [27]. Then hopefully, they will be able to apply it in future clinical therapy.

6. Declarations

6.1. Data Availability Statement

The data presented in this study are available on request from the corresponding author.

6.2. Funding

The study was supported by the UK Stem cell Foundation and Nicholls Spinal Injury Foundation (MR/J015369/1).

6.3. Acknowledgements

I am grateful to all of those with whom I have had the pleasure to work during this and other related projects.

6.4. Ethical Approval and Consent of Participate

In our study animal were used in accordance with the UK Home Office regulations for the care and use of laboratory animal, the UK Animals (Scientific Procedures), Act 1989, with the ethical approval of the University College London, Institute of Neurology.

6.5. Informed Consent Statement

Not applicable.

6.6. Declaration of Competing Interest

The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the author.

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