Cell viability in three *ex vivo* rat models of spinal cord injury

RUNNING TITLE: Cell viability in *ex vivo* SCI models

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ABSTRACT

Spinal cord injury (SCI) is a devastating disorder that has poor prognosis of recovery. Animal models of SCI are useful to understand the pathophysiology of SCI and the potential use of therapeutic strategies for human SCI. *Ex vivo* models of central nervous system (CNS) trauma, particularly mechanical trauma, have become important tools to compliment *in vivo* models of injury in order to reproduce the sequelae of human CNS injury. *Ex vivo* organotypic slice cultures (OSCs) provide a reliable model platform for the study of cell dynamics and therapeutic intervention following SCI. In addition, these *ex vivo* models support the 3R's concept of replacement, reduction and refinement of animal use in SCI research. *Ex vivo* models cannot be used to monitor functional recovery, nor do they have the intact blood supply of the *in vivo* model systems. However, the *ex vivo* models appear to reproduce many of the post traumatic events including acute and secondary injury mechanisms. Several well-established OSC models have been developed over the past few years for experimental spinal injuries *ex vivo* in order to understand the biological response to injury. In this study we investigated cell viability in three *ex vivo* OSC models of SCI: stab injury, transection injury and contusion injury. Injury was inflicted in postnatal day 4 rat spinal cord slices. Stab injury was performed using a needle on transverse slices of spinal cord. Transection injury was performed on longitudinal slices of spinal cord using a double blade technique. Contusion injury was performed on longitudinal slices of spinal cord using an Infinite Horizon impactor device. At days 3 and 10 post-injury, viability was measured using dual staining for Propidium Iodide (PI) and Fluorescein Diacetate (FDA). In all *ex vivo* SCI models, the slices showed more live cells than dead cells over 10 days in culture, with higher cell viability in control slices compared to injured slices. Although no change in cell

viability was observed between time points in stab and contusion injured OSCs; a reduction in cell viability was observed over time in transection injured OSCs. Taken together, *ex vivo* SCI models are a useful and reliable research tool that reduces the cost and time involved in carrying out animal studies. The use of OSC models provide a simple way to study the cellular consequences following SCI and they can also be used to investigate potential therapeutics regimes for the treatment of SCI.

KEYWORDS: *Ex vivo* slice culture, spinal cord injury, stab injury, transection injury, contusion injury, cell viability

INTRODUCTION

Cell death is one of the consequences of traumatic SCI, with both neurons and supporting glial cells affected (Beattie *et al*., 2002). Numerous studies have reported spinal cord tissue samples isolated from embryonic or newborn rats (Sypecka *et al*., 2015, Pohland *et al*., 2015, Weightman *et al*., 2014, Hashemian *et al*., 2014, Gerardo-Nava *et al*., 2014, Pinkernelle *et al*., 2013, Gerardo-Nava *et al*., 2013, Ravikumar *et al*., 2012, Kim *et al*., 2010, Cho *et al*., 2009, Krassioukov *et al*., 2002). The organotypic slice culture (OSC) model appears to reproduce much of the post traumatic events following injury including acute and secondary injury mechanisms. It has been reported that there is a significant difference in function, circuitry connections and regenerative capacities in the immature spinal cord compared to adult (Krassioukov *et al*., 2002). The heterogeneous populations of cells found *in vivo* are maintained within OSCs with three dimensional connections between neurons and supporting cells (Pohland *et al*., 2015, Pellowska *et al*., 2015, Weightman *et al*., 2014, Hashemian *et al*., 2014, Gerardo-Nava *et al*., 2014, Pinkernelle *et al*., 2013, Gerardo-Nava *et al*., 2013, Ravikumar *et al*., 2012, Kim *et al*., 2010, Cho *et al*., 2009) to avoid hypoxia and necrosis of the central tissue (Krassioukov *et al*., 2002).

A SCI caused by a stab injury is a rare type of injury in patients, resulting in partial or, in some rare cases, the complete transection of the spinal cord (O'Neill *et al*., 2004). The reproducible way to model a spinal cord stab injury is to cut the corticospinal tract (CST) at the dorsal column of the spinal cord using a sharp object (Suzuki *et al*., 2010). The stab injury of the dorsal CST results in loss of locomotion of hind limbs and depending on the level of the injury, forelimbs can also be affected (McCaughey *et al*., 2016, O'Neill *et al*., 2004). Transection injury of the spinal cord is rarely encountered in clinical practice, however it is a widely used SCI model because it is a reproducible and reliable model for investigation of regeneration, degeneration, neuroplasticity

and tissue engineering applications (Cheriyan *et al*., 2014). Two types of transection injury are commonly used in animal models of SCI: full transection and partial transection (hemisection injury). Since the injury model is easy to perform and is reproducible, this injury model has been reported in a variety of species including rat, mice, cats, dogs and primates (Cheriyan *et al*., 2014). The contusion injury model is the most clinically relevant model of SCI and is commonly used in animal models of SCI (Adamchik *et al*., 2000, Krassioukov *et al*., 2002, Sieg *et al*., 1999). This injury model is similar to the compressive trauma method *in vitro*, however this method utilises dropping a weight from the prescribed height onto organotypic cultures of spinal cord. The severity of the impact is in proportion to the mass of the weight and the height of the drop. A recent study reported by Krassioukov *et al* (2002) used a pin drop that weighed 0.2g and was dropped from 1.7 cm height onto the centre of OSC slices. Other studies have used similar weight drop apparatus in OSC of brain tissue (Adamchik *et al*., 2000, Sieg *et al*., 1999). These *in vitro* weight drop models bear some resemblance to the models used in *in vivo* SCI in rats and mice, i.e. the New York University (NYU) Impactor, Infinite horizon (IH) and Ohio State University (OSU) Impactor (Cheriyan *et al*., 2014).

The viability of cells in organotypic cultures is a very important parameter to consider when culturing ex vivo slice cultures as this indicates the health of the tissue slices. The investigation of live and dead cells described in this manuscript can be tested using a simple cell viability assay. Dual staining using a live and dead assay containing Fluorescein Diacetate (FDA) and Propidium Iodide (PI) was used in this study to examine cell viability within spinal cord slices over 10 days in culture. The time window was chosen as day 3 and 10 post-injury in this study due to most secondary mechanism events of cell death following ex vivo occurred at this time window (Cho et al., 2009). A slightly longer period of time was used by Gerardo-Nava (2014)

who kept the slices in culture between 7-14 days to investigate the interaction between axons in postnatal spinal cord. Slice thickness of the spinal cord tissue is recommended at 350–400µm as this has been reported to avoid hypoxia and necrosis of the central tissue (Krassioukov et al., 2002). In this study, two different spinal cord slice orientations were chosen, transverse and longitudinal. The slice orientation cut for stab injury was transverse due to the clear visibility of dorsal white matter in this orientation versus longitudinal slices. In the transverse slices we aimed to create a stab injury at the CST in the dorsal white matter and it is much easier to observe the CST in transverse slices (Suzuki et al., 2010). For transection and contusion induced injury, a longitudinal slice orientation was chosen as this is a suitable orientation for disruption of the long axons that extend along the long axis of the cord and also to observe the mixed population of cells in grey and white matter of spinal cords.

The aim of this study was to develop reproducible *ex vivo* models of SCI and compare the cell viability of the spinal cord slices. In this paper, we described the development of three *ex vivo* OSC models of SCI: stab injury, transection injury and contusion injury. We attempt to mimic injury models *in vivo* by refining these *ex vivo* organotypic spinal culture and carried out the investigation on the viability of the OSCs using dual staining of PI and FDA.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (Charles River UK Ltd, Margate, UK) were used in this study. All housing and surgical procedures carried out in this study were approval by the Animal Care Research Ethics Committee (ACREC) at the National University of Ireland Galway. Postnatal day (P) 4 rat pups

were sacrificed by anaesthesia with 5% Isoflurane followed by decapitation using a guillotine (Stoelting Co, Germany). The bodies were kept in sterile dishes on ice prior to spinal cord harvesting. Spinal cord isolation was performed in a class II biological safety cabinet under aseptic conditions. The skin was incised using a sterile blade #10 (Swann-Morton, England) along the midline of the dorsum. A small transverse incision was made on the sacral vertebrae. The spinal cords were flushed from the vertebral column using a 1ml syringe fitted with an 18G needle and filled with 1X ice-cold Phosphate Buffered Saline (PBS; pH 7.4) (Kennedy *et al*., 2013). The spinal cords were suspended in ice-cold artificial cerebrospinal fluid (aCSF; pH 7.4) composed of 126mM NaCl, 2.5mM KCl, 1.25mM $NaH_2PO_4.H_2O$, 2mM $CaCl_2.2H_2O$, 2mM $MgSO_4.7H_2O$ and 10mM glucose (all from Sigma Aldrich, Ireland). The meninges was gently dissected away using sterile fine forceps in ice-cold aCSF using a stereomicroscope (Wild MZ32, Switzerland). The intact spinal cords were cut into smaller segments (approximately 1 cm). Three different spinal cord injury models were carried out on the spinal cords after they were chopped on a tissue chopper: stab injury, transection injury and contusion injury. For each injury model, spinal cords were isolated from three litters of P4 rat pups (average litter size 12 pups per litter). For each injury model, the information below details the experiment performed when using one litter.

Spinal cord stab injury model

Transverse sections of spinal cord were chopped at 350 µm thickness on the McIIwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., USA) and tissue slices were pooled together in a petri dish containing aCSF. The tissue slices were then transferred to 30mm diameter cell culture inserts (Merck Millipore, Germany) and cultured in 6 well trays (5 slices per insert). Slices were

maintained in 1ml of spinal cord slice culture medium consisting of: 48% MEM, 25 mM Hepes, 25% heat-inactivated horse serum, 2 mM glutamine, 1% Penicillin Streptomycin, 1% N-Acetyl L-Cystein and 25% Hanks Balanced Salt Solution (all from Sigma Aldrich, Ireland) at 37^{o}C in a 5% humidified CO_2 atmosphere. After 4 days in culture, the tissue slices were separated into a control (uninjured; 6 spinal cord tissue transverse slices) and an injury group (6 spinal cord tissue transverse slices). A stab injury was created in the slices within the injury group using a sterile 27G needle targeting the corticospinal tract of the slices located within the dorsal white matter. The anatomical region of the rat corticospinal tract/dorsal white matter region was defined based on the proportion of white matter and gray matter in the transverse slices observed using the stereomicroscope (Steward and Willenberg, 2017). At 3 days and 10 days post injury, medium was aspirated from culture wells and the slices were fixed with 4% paraformaldehyde (PFA) for 24 hours at 4^{o}C (3 spinal cord tissue slices per time point in the control group and 3 spinal cord tissue slices per time point in the injured group).

Spinal cord transection injury model

Longitudinal sections of spinal cord were chopped at 350 µm thickness using the McIIwain tissue chopper. All tissue slices were pooled together in a petri dish. The spinal cord tissue slices were then transferred to 30 mm diameter cell culture inserts (Merck Millipore, Germany). Two slices per insert were cultured at 37^{o}C in a 5% humidified CO_2 atmosphere in 6 well trays containing 1ml spinal cord slice culture medium (see preceeding section for list of culture medium ingredients). After 4 days in culture, the tissue slices were separated into a control group (uninjured, 6 slices) and injured group (6 slices). A transection injury was performed on the spinal

cord slices within injured groups midway along the length of the tissue slices using two sterile scalpel blades #10 (Swann-Morton, England) attached to a scalpel handle. These blades were 460 µm distance apart. Spinal cord slices were fixed with 4% PFA at day 3 and day 10 post injury (3 spinal cord tissue slices per time point in the control group and 3 spinal cord tissue slices per time point in the injured group).

Spinal cord contusion injury model

Longitudinal tissue slices were prepared as detailed in the methods section above and spinal cord tissue slices were pooled together in a petri dish. The tissue slices were then transferred to a 30 mm diameter cell culture inserts (1 slice per insert) and cultured at 37^{o}C in a 5% humidified CO_2 atmosphere in 6 well trays containing 1ml spinal cord slice culture medium. After 4 days in culture, the tissue slices were separated into a control group (uninjured, 6 slices) and injured group (6 slices). In the injury group, a contusion injury was performed on the slices midway along the length of the spinal cord slices using an Infinite Horizon (IH) Impactor device (Precision Systems and Instrumentation, USA). A sterile mouse impactor rod with a 1.25mm diameter tip was dropped onto the spinal cord slices at a force of 50 kilodynes (kdyn). The spinal cord slices on the inserts were positioned in the centre of the platform and aligned parallel to the position of the impactor rod so as to ensure the rod hit the spinal cord slices. The impactor rod was lowered to 5 mm above the slices prior to injury. Spinal cord slices were fixed with 4% PFA at day 3 and day 10 post injury (3 spinal cord tissue slices per time point in the control group and 3 spinal cord tissue slices per time point in the injured group).

Cell viability assay

Control and injured slices from each SCI model were subjected to dual staining for dead cells using PI (Invitrogen, UK) and live cells using FDA (Sigma Aldrich, Ireland). For each injury model, three control and three injured spinal cord slices were examined at 3 days post injury and 10 days post injury. A scalpel blade was used to cut around the edge of the insert mesh in order to remove the insert mesh (containing the spinal cord tissue slices) from the insert ring. Each insert mesh containing the spinal cord tissue slices (2 mm x 2 mm) could fit inside the 24 well tray wells. The tissue slices on insert meshes were washed with 1X PBS three times (5 mins per wash). The slices were incubated with 50 µg/ml PI and 50 µg/ml FDA for 30 seconds at room temperature. The slices were then inverted and placed into 35 mm glass bottom dishes (WillCo Well BV, Netherlands) in 500µl 1X PBS during confocal imaging.

Imaging

Images were captured at 10X and 20X magnifications using an Andor spinning disc confocal microscope (Andor Technology Ltd, UK). To examine the stained cells, confocal z-stack images were captured 1 µm apart from the top to the bottom of the spinal cord slices. The red and green channels were used to visualize stained cells at 488 nm and 561 nm emission wavelength respectively. All the imaging was carried out using the same exposure time and emission gain for all the spinal cord slices. One image per slice was obtained from control and one image per slice from injured slices for each animal model.

Image analysis

Image analysis was performed to measure the FDA and PI staining. In the uninjured/control tissue slices the central region of the spinal cord tissue slices was examined. In the injured slices a scar zone, defined as a zone that fell in 100 µm radius from the edge of the lesion, was examined. For each image captured, 6 regions on the image were magnified and examined. All the images were prepared and examined at 120% magnification from the original image (100%). A square point grid was placed randomly on the top of the images using computer-generated grids and this grid was used to count the number of points hitting labelled cells. The entire field of view was counted and recorded in Microsoft Excel 2016 (Microsoft Office, USA). Cells were counted positive if the cells lay on intersection points on the grid. All the images were counted with the help of the ImageJ Cell Counter tool. The cells were counted if they stained red (dead cells) and green (live cells). ImageJ software was used to split the two channels and these were measured separately using the de-interleave ImageJ editing tool. The images were inverted to black and white and contrast/brightness levels were adjusted. The average number of cells stained with FDA and PI was calculated in the projected confocal image stacks.

Statistics

All data collected was saved in Microsoft Excel 2016 (Microsoft Office, USA). The mean number of live and dead cells was calculated in each image and the results were expressed as mean ± standard error of the mean (SEM). All the results were illustrated using Graphpad Prism software (Prism 7, USA). Statistical analysis was carried out using Minitab software (Minitab Incorporation, USA). To test for differences between the parameters examined Two-way Analysis

of Variance (ANOVA) were performed followed by Multiple comparison Tukey's test. Statistical significance was set at probability (p) $\leq$ 0.05.

RESULTS

Examination of all *ex vivo* SCI models showed more live cells than dead cells in both control and injured spinal cord slices at each time point. Cell viability was examined within control and injured spinal cord slices at day 3 and day 10 following stab injury (Figure 1A-D). The number of FDA and PI stained cells was counted in the projected confocal image stacks (Figure 1E). When the control and injured groups were compared to each other, higher levels of dead cells were observed in injured slices compared to control slices at both time points post-injury and an increase (non-significant) was also observed in live cells in injured slices compared to control slices (Figure 1E). There was no difference in the numbers of cells when cells were examined between day 3 and 10.

In contusion injured spinal cord slices the FDA and PI stained cells were observed (Figure 2A-D). Some of the live cells at the lesion site appeared to resemble neurons (see Figure 2D). Immunohistochemical staining for markers of neurons confirmed the presence of many axons at the edge of the lesion site (Supplementary figure 1). Similar cell viability findings were observed in the contused spinal cord slices as those observed in stab injured slices, with a higher levels of dead cells observed in injured slices at both time points compared to control and no change observed over time (Figure 2E).

In the transection injury model cell viability was observed at each time point (Figure 3A-D). The proportion of dead cells was higher in transection injured slices than in the control slices at day 3 post-injury with no change observed at day 10 (Figure 3E).

DISCUSSION

Cell viability within three *ex vivo* models of rat SCI was investigated here at day 3 and day 10 post injury. These *ex vivo* models support the 3R's concept of replacement, reduction and refinement of animal use in SCI research. Observation of cellular changes in *ex vivo* slices allows heterogeneous cell populations to be studied. Neurons and supporting cells are maintained with 3-dimensional connections within OSCs (Doussau *et al*., 2017). In OSCs, one can manipulate treatment strategies to observe the effect of treatments in 3-dimensional microenvironments. Viability of cells in OSCs is a very important parameter to consider when culturing *ex vivo* slice cultures, as cell viability indicates whether the slices are healthy and reliable enough for experimentation. In this study, dual staining using FDA and PI was carried out to examine cell viability within spinal cord slices over 10 days in culture. The 10 day time period allowed investigation of how the environment changes following SCI in *ex vivo* OSCs. This time window was similar to that of other researchers who examined the primary and secondary mechanisms of cell death following *ex vivo* injury (Cho *et al*., 2009; Gerardo-Nava *et al*., 2014). We have also investigated secondary injury mechanisms such as scarring in these model systems (data submitted for publication).

The selection of the slices used in this study, either transverse slices for use in the stab injury model or longitudinal for use in the transection or contusion injury model, was carefully carried out. An attempt was made to have an equal proportion of both white and grey matter within the slices. In our hands, we managed to obtain eight good slices from one spinal cord. Weightman *et al*. (2014) recommended that the best strategy for selection of slices was to discard the extreme

lateral margins of the spinal cord due to presence of fragmented tissue and that the slices should be examined on a dissecting microscope to confirm their intactness (Weightman *et al*., 2014).

In all three *ex vivo* SCI models, the slices showed more live cells than dead cells over the 10 days in culture, with higher cell viability in control slices compared to injured slices. These viable cells may be resident cells that have survived the injury, newly generated cells from proliferating cells within the tissue slice and/or cells that have migrated into the slice from adjacent regions. The FDA and PI stains were not specific to any particular cell type, however, we observed some FDA-tagged live cells that appeared like neurons in Figure 3D. The survival of neurons is a major challenge in OSCs. Since the OSCs are an axotomised system, the neurons lose their target innervation and this later causes neuronal death (Humpel, 2015). However, some of the neurons in the OSCs maintain short axonal connections to other neurons, therefore, the axotomy allows one to study neuronal sprouting and reactive synaptogenesis as shown by Gähwiler *et al*. (1997) in hippocampal slices. The contusion injury model showed an increase (albeit non-significant) in live cells between day 3 and day 10 (Figure 2E), which may be due to the difference in the inflicted injury itself as no difference in cell viability was observed between time points in the stab/transection injury models. In our study, we used spinal cord slices derived from P4 rat pups for both transverse and longitudinal oriented slices. It has been reported that neurons are more stable in OSCs when isolated from P6 or younger aged pups, as neurons tend to be more variable with increased age and decline in culture (Bonnici and Kapfhammer, 2008).

The contusion model is the most clinically relevant model to human SCI (Cheriyan *et al*., 2014). This model is relevant to investigate the pathophysiology following acute injury. We isolated longitudinal spinal cord slices and induced a contusion injury using the IH impactor device. Our results concur with other studies using the same setting of 50-kdyn force impact and

mouse impactor tips indicating a moderate injury of contusion injury (Bastien *et al*., 2015). After 3 days and 10 days in culture, the high numbers of live cells in the slices indicated the cells survived after the impact, although dead cells were also observed (Figure 2E).

The idea of using slice cultures is to eliminate confounding factors found *in vivo*, thus helping to elucidate the mechanisms underlying a mechanically induced trauma. The OSC model appears to reproduce much of the post traumatic events including acute and secondary injury mechanisms (Krassioukov et al., 2002, Pinkernelle et al., 2013, Pohland et al., 2015, Weightman et al., 2014). It serves to control the extracellular environment and has the promise of lower economical cost and faster discovery (Morrison et al., 1998). However, the use of *ex vivo* culture system is subjected to some limitations. First, this culture system does not have any blood flow within capillaries. Therefore, the role of monocytes derived from blood circulation following injury cannot be studied in this *ex vivo* system. The accumulation of microglia/macrophages occurs at the edges of slices due to damage caused by the dissection and tissue chopping procedures. However, we optimised our culture protocol by delaying any treatment until day 4 post injury to avoid the involvement of immune cells activated during the tissue harvest procedure. Another limitation to the use of OSCs is that the cells within the slices lose their target innervation as the slices are an axotomized system (Humpel, 2015).

In summary, all the *ex vivo* models of SCI presented here have fulfilled the 3R's concept of replacement, reduction and refinement of animal use in SCI research models. Each model is a consistent, easily reproducible and graded injury that represents SCI pathology. Cell viability appeared robust within these OSCs, albeit with a reduction in live cells observed over time in culture within the stab and transection models. These *ex vivo* model systems are very useful for testing potential treatments before moving to larger studies involving animals.

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AUTHOR CONTRIBUTIONS

Azim Patar: contributed to concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript, revision of the manuscript and approval of the article

Peter Dockery contributed to data analysis/interpretation and approval of the article

Linda Howard contributed to concept/design, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article.

Siobhan S. McMahon contributed to concept/design, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article.

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FIGURE LEGENDS

Figure 1: Live dead assay assessment in stab injured spinal cord *ex vivo* slices. Photomicrographs show dual staining for live cells with FDA (green) and dead cells with PI (red) at day 3 (A, B) and day 10 (C, D) in control and stab injured slices respectively. Graph shows the number of live and dead cells in the control and injured spinal cord slices (E). Mean ± SEM. The mean differences were analysed using Two-way ANOVA. n=3 litters (12 pups per litter) which relates to 6 slices per control group per time-point and 6 slices per injured group per time-point for each litter. + = $p \leq 0.05$. Scale bar = 200 µm

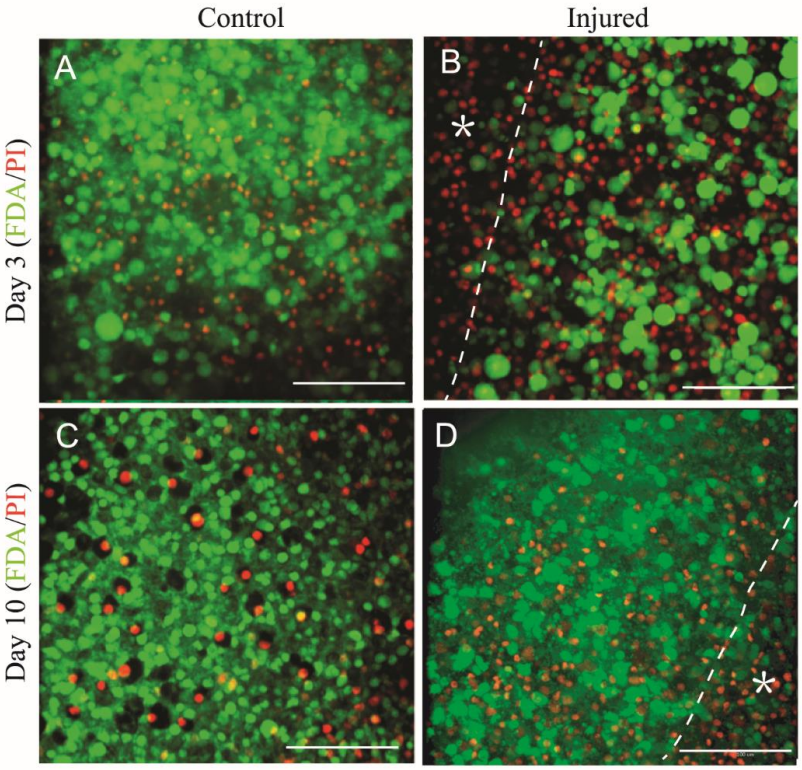
Figure 2: Live dead assay assessment in contusion injured spinal cord *ex vivo* slices. Photomicrographs show dual staining for live cells with FDA (green) and dead cells with PI (red) at day 3 (A, B) and day 10 (C, D) in control and contusion injured slices respectively. Graph shows the number of live and dead cells in the control and injured spinal cord slices (E). Mean ± SEM. The mean differences were analysed using Two-way ANOVA. n=3 litters (12 pups per litter) which relates to 6 slices per control group per time-point and 6 slices per injured group per time-point for each litter. + = $p \leq 0.05$. * =injury site; Scale bar = 200 µm

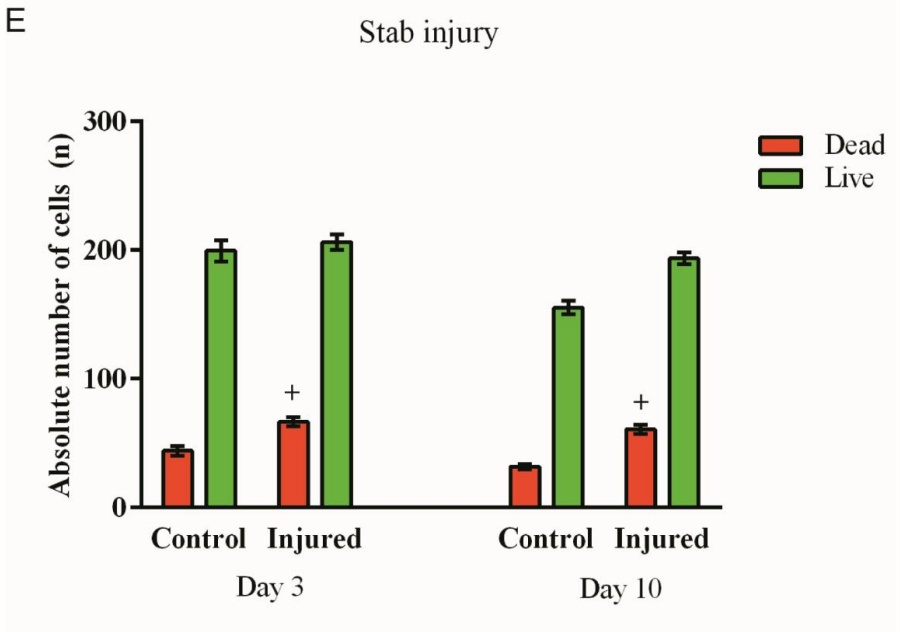
Figure 3: Live dead assay assessment in transection injured spinal cord *ex vivo* slices. Photomicrographs show dual staining for live cells with FDA (green) and dead cells with PI (red) at day 3 (A, B) and day 10 (C, D) in control and transection injured slices respectively. Graph shows the number of live and dead cells in the control and injured spinal cord slices (E). Mean ± SEM. The mean differences were analysed using Two-way ANOVA. n=3 litters (12 pups per litter)

which relates to 6 slices per control group per time-point and 6 slices per injured group per time-point for each litter. + = $p \leq 0.05$. * =injury site; Scale bar = 200 µm.

Supplementary figure 1: Distribution of white matter and gray matter in contused tissue slices. Photomicrographs show dual immunostaining of ßIII Tubulin (neurofilaments) and NeuN (nuclei of neurons) on day 3. Dashed line separates the IZ and tissue region near IZ; White asterisk, IZ= injury zone; * =injury site; scale bar = 200 µm.

Figure 1

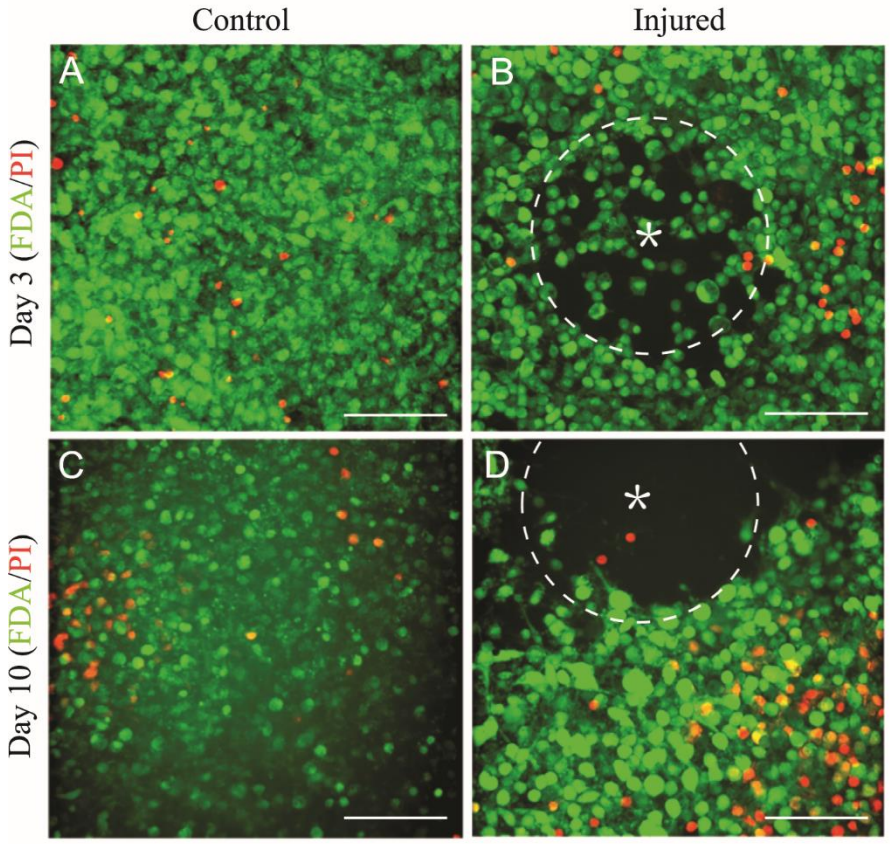




435

436

Figure 2


Control
Injured
A
B
C
D
Day 3 (FDA/PI)
Day 10 (FDA/PI)

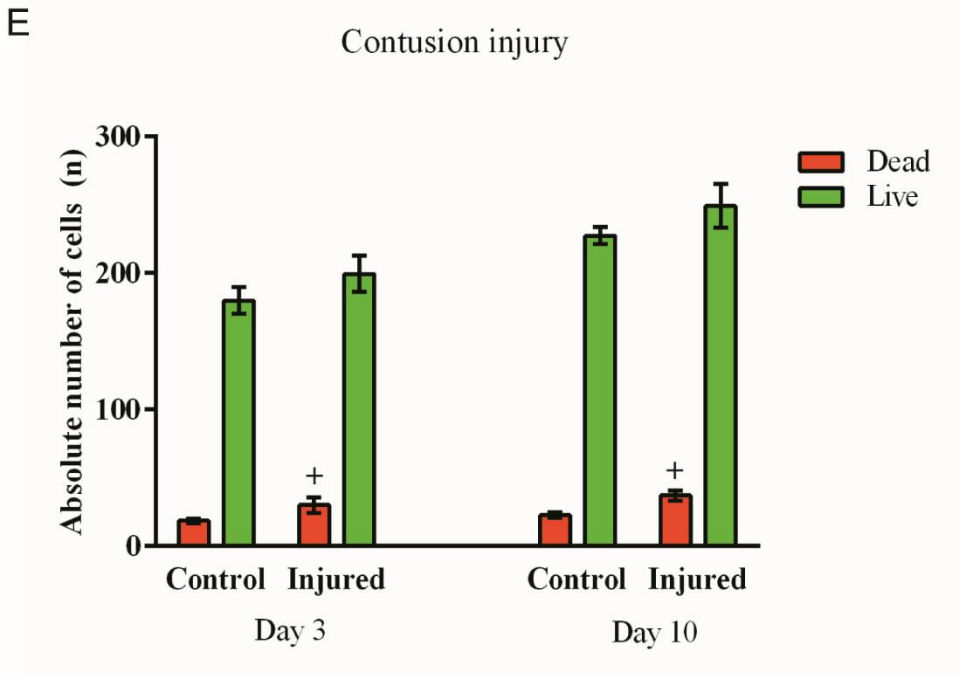
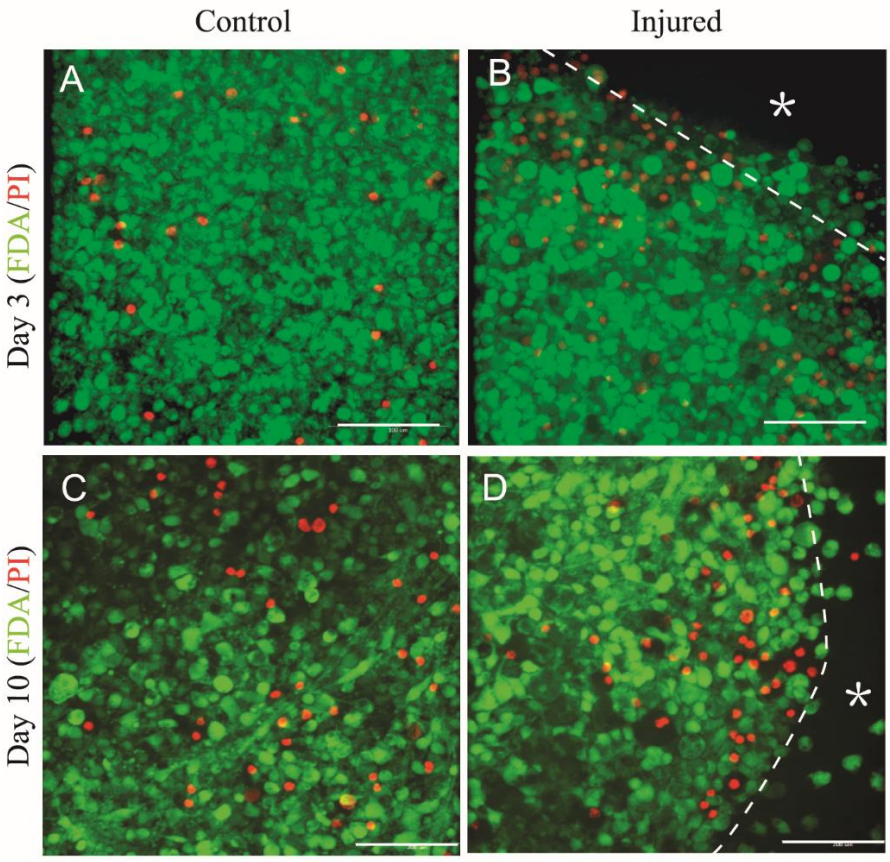
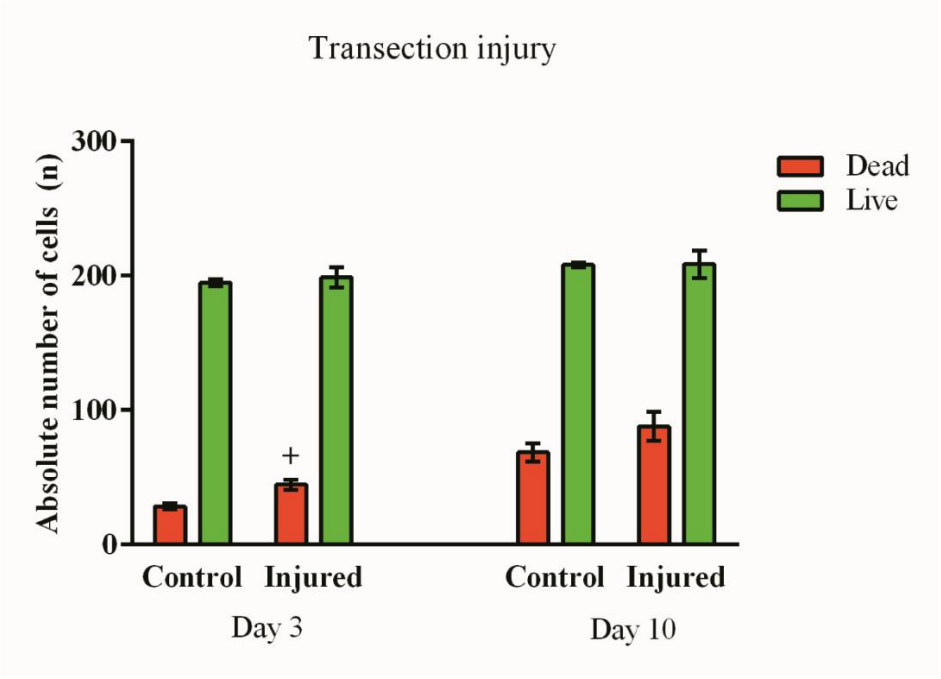

E
Contusion injury
Absolute number of cells (n)
300
200
100
0
Dead
Live
Control
Injured
Control
Injured
Day 3
Day 10

Figure 3


Control
Injured
A
B
C
D
*
*
Day 3 (FDA/PI)
Day 10 (FDA/PI)

E


Transection injury
Absolute number of cells (n)
300
200
100
0
Dead
Live
+
Control
Injured
Control
Injured
Day 3
Day 10

Supplementary figure 1