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In Vitro Models of Spinal Cord Injury

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Abstract

Living organisms are extremely complex functional systems. At present, there are many *in vivo* models of spinal cord injury (SCI) that allow the modeling of any type of central nervous system (CNS) injury, however, with some disadvantages. The production of injury models can be a highly invasive and time-consuming process and requires high technical requirements, and costly financial issues should also be taken into account. Of course, a large number of animals have been used to obtain the relevant data of statistical significance. All of these aspects can be reduced by carrying out experiments in *in vitro* conditions. The primary advantage of *in vitro* method is that it simplifies the system under study. There are two major groups of *in vitro* model in use: cell culture and organotypic slice (OTS) culture. OTS is an intermediate system of the screening of *in vitro* cell culture and animal models and represents the *in vitro* system preserving the basic tissue architecture that able to closely mimic the cellular and physiological characteristics *in vivo*. *In vitro* models are the preferred methods for the study of acute or subacute pathophysiology after a trauma stimulus, enabling precise control on the extracellular environment, easy and repeatable access to the cells.

Keywords: *in vitro* model, spinal cord injury (SCI), cell culture, co-culture, organotypic slice (OTS) culture

1. Introduction

Spinal cord injury (SCI) is a critical incurable difficulty affecting the quality of life of patients and society equally. The spinal cord injury interferes predominantly with motor function defects, which substantially restrict normal daily activities, thus leading to serious psycho-

logical trauma. Today, the majority of patients are left paralyzed with unsuccessful treatment currently available. Many strategies, including surgical, transplantations, pharmacological, neurophysiological, and others approaches, have been used and examined in attempts to develop new and successful therapies that will allow patients to get the valuable life they lived before the injury [1].

First, before devising a new treatment for spinal cord injury, it is also important to know the processes of nerve tissue regeneration, neurogenesis, and gliogenesis, which can be influenced in many ways. The answer to these needs, a growing number of animal models have been introduced and utilized. The creation of *in vivo* injury models requires many invasive interventions and is time consuming. The financial costs of such models as well as technical equipment are often high. However, the biggest problem of the *in vivo* models is the utilization of large amounts of experimental animals. All of these facts can be reduced by carrying out experiments in *in vitro* conditions. *In vitro* experimental models are needed to understand the cellular mechanisms and pathophysiology of the injured spinal cord (SC) and to provide evidence for further treatment. This is also the right place to mention the principles of the 3Rs (Replacement, Reduction, and Refinement). The principles of the 3Rs were developed as a framework for humane animal research and became embedded in national and international legislation regulating the use of animals in scientific procedures in most world countries. There are two major groups of *in vitro* model in use: cell culture and organotypic slice (OTS) culture.

2. Cell cultures

After central nervous system (CNS) injury, the acute primary and the chronic secondary damage takes place. After initial mechanical insult, a rapid deformation of CNS tissue happens, causing an immediate cell death in the epicenter of injury site followed by a cascade of processes leading to the secondary damage. Traumatic injury disrupts spinal white matter tracts resulting in the loss of sensory and motor function. This loss of function is generally permanent due to the limited regenerative capacity of the CNS [2]. Cells used for *in vitro* modeling should have morphological, functional qualities, and relevant requirements depending on disease, injury, and ultimate goal of the proposed experiments. The commonly used *in vitro* cell culture models of SCI include:

- i. Primary isolated cells, neurons, oligodendrocytes, astrocytes, or microglia cells [3, 4]. Primary cells are obtained from the dissociated neural tissues, spinal cord, or brain and are usually cultivated immediately after the isolation as “primo-cultures.” The origin of these cells could be embryonal, fetal, neonatal, or adult [5, 6]. It is well known that younger cells are more potent according to their strong proliferative activity, in contrary to terminally differentiated poor proliferating capacity owning adult cells [6]. By using the primary cultures, we can investigate the behavior and intercommunication of the individual or mixed cell populations. These populations preserve the main characteristics similar to the *in vivo* examined types of cells [7]. Very often used are also the immortalized cell lines, which are in fact the primary cells with genetical modification.

- ii. Co-culture of neuronal cells with different cell types, which are present in the glial scar [8, 9]. *In vitro* cultures offer simplified, high throughput systems to study disease, drug toxicity, and biological processes by controlling environmental factors and directly measuring cell responses [10].

2.1. *In vitro* model of glial scar

The characteristic manifestation of the secondary reactive phase is scar formation at the initial impact site, which represents the major obstruction to CNS regeneration. The glial scar is rich in different cells (microglia and reactive astrocytes), myelin-associated inhibitors, and it physically surrounds the damaged tissue. To study the components of the glial scar, many models of co-cultivation of neurons with others glial scar situated cells have been developed [8, 9, 11]. By combining two pivotal aspects of CNS damage, mechanical injury and co-cultivation with meningeal cells in *in vitro* scar formation model, it has been shown that astrocytes increase the expression of GFAP (glial fibrillary acidic protein), as well as the scar-associated markers, e.g., phosphacan, neurocan, and tenascin. Subsequent short-time co-cultivation with different developmental neuronal populations led to significantly reduced neurite growth in this scar-like model [9]. After spinal cord injury, demyelination of spared white matter significantly hinders spontaneous function recovery [12]. To understand the processes related to this phenomenon, a wide range of developed cultures in which the many stages of myelination can be followed over time are used [13–15]. These myelinating cultures involve plating dissociated rat spinal cord cells onto a confluent monolayer of neurosphere-derived astrocytes. Sorensen et al. [16] compared the different types of cells in the process of myelination promotion of CNS axons. The mixed cell population of dissociated embryonal spinal cord was cultivated on olfactory ensheathing cells (OECs), Schwann cells (SchCs), or neurosphere-derived astrocytes to study the mutual interactions in context to affect the myelination. Myelin internodes and nodes of Ranvier were often found only in the cells cultivated onto neurosphere-derived astrocytes. This model is very useful to investigate the CNS axonal myelination because it recapitulates the processes occurring in CNS *in vivo*. On the basis of these cultures, it has been shown that astrocytes have a direct role in promoting myelination either by releasing a soluble factor or by cell–cell contact [16]. Also, the phenotype of astrocytes has a crucial role in determining their effects on myelination. Myelination is poor when the myelinating cultures are plated on quiescent astrocytes but enhanced when they are plated on a more reactive/activated phenotype. Interestingly, quiescent astrocytes secreted CXCL10, which did not appear to directly affect the purified oligodendrocyte progenitor cell differentiation but had the effect on the ability of oligodendrocytes to ensheath axons [13]. Astrocytes exhibit dynamic cell process movement and changes in their membrane topography as they interact with axons and oligodendrocytes during the process of myelination [15]. These observations may have important implications with respect to the development of therapeutic strategies to promote CNS remyelination in demyelinating diseases. Also other models have been developed to investigate and to understand the molecular mechanisms of CNS axon regeneration. The process of axon regeneration can be monitored by neurite outgrowth assays, which often represent the phenotypic expression of regeneration progress. In neurite outgrowth assays, the neurons are cultivated on an adherent substrate, and the

neurite growth is evaluated in specific medium conditions for extended periods of time, and then the overall length of the radial outgrowth is measured. Primary neurons (neurons derived from dorsal roots ganglion, cerebellum, cortex, or hippocampus) are considered more biologically relevant to neurons *in vivo* instead of induced pluripotent stem cells or any special cell lines [17].

2.2 *In vitro*

The majority of SCI occurring in human is due to the compression of spinal cord; therefore most *in vitro* models have been designed to mimic this type of injury. To simulate the spinal cord injury *in vitro*, the confluent layer of astrocytes and dissociated embryonic spinal cord cells plated onto them are cut with scalpel blade, the so-called scratch model. Such treatment creates an empty area without any cells—lesion. With an increasing time of cultivation, the situation around the injury becomes comparable to the SCI *in vivo*. The microglial cells and astrocytes start to migrate to the injury site. Conversely, the decrease in neurite density and the process of demyelination start as the consequences of disrupting confluent cell layer. The main purpose of this model is to find out, to recognize any factors, molecules, biomaterials, even cells that can contribute, may be, to accelerate the potential regeneration of remaining cells destroyed by injury. The closure of this lesion could be modified using different Rho/ROCK inhibitors [11] or by cAMP modulators, which promote neurite outgrowth and myelination [5]. *In vitro* models are the preferred methods for the study of acute or sub-acute pathophysiology after a trauma stimulus enabling the precise control of the extracellular environment, easy and repeatable access to the cells.

To imitate the SCI in our experimental model, the P1 rat spinal cord cells were mechanically scratched across the cell monolayer with a pipette tip. The result of this handling was the linear cell-free area/lesion which varied between 400 - 600 μm in width. After washing out the scraped cells with PBS (phosphate-buffered saline), cells were maintained for up to 10 days in culture. During the cultivation time, the cultures remained viable. Cell migration or wound closure of the scratched surface was observed and photographed at daily intervals. To investigate the potential stimulative effect of mesenchymal stem cells (MSCs) on the neural cells, we treated such injured cultures with conditioned medium obtained from rat bone marrow MSCs. In fact, MSCs cannot differentiate into fully functional neurons, or into others neural cells; the supporting effect of neuroregeneration and neuroprotection is mediated by specific neurotrophic factors and cytokines produced by MSCs [18]. All experiments conformed to the Slovak Law for Animal Protection No. 377/2012 and 436/2012 transposed from the European Communities Council Directive (2010/63/EU) and were approved by the Institutional Ethical Committee for animal research. Our preliminary data showed that the cell-free area in injured group treated with MSC conditioned medium became filled with reactive astrocytes, microglia cells, and oligodendrocyte in a shorter time when compared to untreated controls (**Figure 1**).

Figure 1. Fluorescent microscopy images of spinal cord culture injured by mechanical insult, control group (A) and group treated (B) with conditioned medium obtained from MSCs. The images were taken on day 2 after injury. Cells were stained with GFAP/astrocytes/green antibody, Iba/microglia cells/red antibody and DAPI/nuclear/blue marker, and (C) the detail of microglia cells infiltrating the lesion on day 2. Scale bar is 100 μm.

The cell culture approaches lack the complexity and physiological relevance of *in vivo* system. However, animal studies offer complexity, which cannot be modeled *in vitro* and which is also difficult to control and manipulate and therefore results in data that must be extrapolated to human systems [10].

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Organotypic cultures are whole slices of tissue cultured without dissociation of the individual cells. Organotypic slices (OTSs) preserve the basic structural and connective organization of their original tissue (organotypic) and represent an interim system sharing the properties of the cell culture *in vitro* and an animal *in vivo* model. The OTS culture allows long-term maintenance of tissue architecture in “dish” [19].

OTS preparation is carried out according to the following steps. Briefly, after tissue removal under sterile conditions, the tissue is cut into 150–400 μm thick sections using a tissue chopper or vibratome. After washing, the slices are attached to a substrate and cultivated under appropriate conditions for several weeks. Today, we know many ways of OTS preparation depending on the final thickness of the slices and the survival time in culture. In *roller-tube*

neurons, and that H_2O_2 -mediated cell death was significantly alleviated by antioxidants and independent of calcium influx in both glial and neuronal populations [36].

The hypoxic environment in SCI has been shown to inhibit recovery. One of the promising treatments for hypoxic ischemia sustained during SCI is gene therapy. Vascular endothelial growth factor (VEGF) increasingly has gained attention as a potential factor in gene therapy to replace or improve damaged neurons in SCI because of its role in neurogenesis and neuroprotection, astrocyte proliferation, and neurite outgrowth. The controlled release of VEGF in hypoxia-inducible VEGF gene delivery system in the OTS-SC may prove to be useful for providing neuroprotection and in stimulating the neural growth in the hypoxic environment of the injured spinal cord [37]. The principal feature of acute SCI pathophysiology is excitotoxicity that is considered the main contributor to the clinical outcome. An established *in vitro* SCI model using excitotoxic damage evoked by transient kainate application to organotypic slice cultures (with predominant neuro- rather than glio-toxicity) closely mimics the *in vivo* pathophysiology of SCI. The results from such model indicate that, after an excitotoxic stimulus, extracellular S100b, a useful biomarker in serum or cerebrospinal fluid for brain or spinal cord injury, was significantly elevated in association with ongoing neuronal damage and unscathed glia [38]. The application of glutamate is believed to mimic excitotoxicity that occurs as a consequence of ischemic stroke. Also, glutamate-induced excitotoxicity is a major contributor to motor neuron (MN) degeneration in disorders such as amyotrophic lateral sclerosis, stroke, and spinal cord injury. On the basis of using OTS from different parts of SC (i.e., cervical, thoracic, and lumbar), it is found out that the MN susceptibility to glutamate-induced excitotoxicity differs within diverse populations of MNs. This sensitivity is in correlation to the segmental variable expression of glutamate receptor sub-types in MN populations. In the cervical and lumbar parts, the AMPA receptor is in dominance, whereas in thoracic segments, the NMDA receptors predominate. These results should be taken into account by devising a new drug screening experiment with cell culture system [39].

4.2. Mechanical insults

Adamchik et al. [24] described two original models of moderate mechanical trauma containing the induced primary and secondary traumatic damage in organotypic slice cultures. The primary trauma injury was achieved by quickly rolling a stainless steel cylinder (weight, 0.9 g; diameter, 5 mm; length, 7 mm) twice on the organotypic slice. To model the secondary trauma injury, a weight of 0.137 g was dropped from a height of 2 mm on a localized area of the organotypic slice. The primary damage delivered by rotating the steel cylinder on sections induces injury following the head accidents, while the damage caused in this model for secondary injury, limited initially to a small area of the section, enables to monitor the expansion of dying cells.

Almost all SCI occurring in human are due to the compression of spinal cord; therefore, most *in vitro* models have been designed to mimic this type of injury. For that reason, an *in vitro* model of neurotrauma was developed and assessed by using organotypic slice culture of adult mice spinal cord to facilitate the investigation of primary and secondary mechanisms of cell death that occur after mechanical SCI [25]. The mechanical trauma was achieved by using a

weight drop model of injury, where an impactor (pin) with a weight of 0.2g was dropped from 1.7 cm onto the center of the culture slice. They modified previously described methods for generating murine spinal trauma in OTS [40]. In contrary to other models, where the OTS-SC from embryonic or neonatal mice was used, the modified model used the OTS from adult animals according to the different regenerative capabilities.

Organotypic slice cultures of spinal cord have been used in neuroscience research for a long time, but classically, these cultures were cut in the transverse plane. The transverse slices could be, however, obtained from only a single part of the spinal cord. In order to reflect the spinal cord microenvironment, the longitudinal cultures are required to maintain the basic cytoarchitectonic organization of the intact spinal cord. In contrary to the previous model, Bonnici and Kapfhammer [41] used the spinal cord slices for their study that were cut not in the transverse but in the sagittal longitudinal plane such that several spinal cord segments were included in the slice culture. This OTS-SC model is well suited for the study of axonal growth because the typical ventro-dorsal polarity of the SC is maintained after a culture period of 2 weeks, and intrinsic spinal cord axons formed a strong fiber tract extending along the longitudinal axis of the slice. The axons became myelinated during the culture period, and synaptic contacts were present in these cultures. After mechanical lesions originated by completely transecting the SC tissue with a scalpel blade, the remaining spinal cord neurons had a considerable capability to regenerate the axons. The amount of renewed axons infiltrating the wound site decreased with increasing cultivation time and maturation of the culture. This indicates that the cellular differentiation and cytoarchitectonic organization of the spinal cord neurons are similar to that of slice cultures of the transverse plane and reflects the *in vivo* situation well [41]. Some scientists use the OTS for the study of radiation effect on cell damage. By determining the time-dependent course of choline uptake in mature organotypic slice cultures of rabbit and assessing the effects of continuous and single high-dose irradiation on choline uptake, the results demonstrate that the delivery of continuous but relatively low-dose rate gamma irradiation is more efficacious than single high-dose external irradiation on high-affinity choline uptake in nervous tissues [28].

5. Age of donor animal

The age of the donor animal influences the viability of OTS, the degree of tissue organization, and the successfulness of experiment at all. Early postnatal periods (P0–P9) are ideally suited for culturing [26, 39, 42]. Since most diseases of the spinal cord concern the adults, some organotypic spinal cord slice culture models from grown rats were established [25, 26]. But the isolation of the cord from adult individuals is, however, much more problematic. The fully myelinated spinal cord is very sensitive to any damage, and cutting it with tissue chopper causes injury to about 60% of slices. The slice culture survives about 7 days maintaining proper parallel fiber tract architecture. Also, the constant high vitality and tissue organization are preserved up to 4–5 weeks in neonatal slices, while the vitality of adult slice cultures decreases significantly upon the first 5 days of cultivation [26]. On the contrary, spinal cord organotypic culture from adult mouse can be used to produce neurospheres that can be further differen-

tiated into astrocytes and neuronal cells of different phenotypes. Although, it is not clear what exactly triggers the formation of neurospheres, one explanation could be, that neuronal loss and/or long-term culturing of spinal cord slices resets the internal cell program and stimulates proliferation and further specification of neuronal stem cells (NSCs) [43]. Most researchers did not use cultures from animals older than P6, because neuronal survival in such cultures was more variable and a longitudinal fiber tract did not develop consistently [41]. The same goes for the transverse spinal cord slice cultures, in which neuronal survival also appears to be declining in cultures derived from older postnatal animals [44].

6. Utilization of organotypic slices

Because the OTS system is accessible to experimental manipulations, OTSs represent a suitable model which may be used for different research projects not only for investigation of various parameters accompanying the CNS injury but also for the development of therapeutic application for such injury. Spinal cord injury leads to the death of various cell types, including neurons, oligodendrocytes, astrocytes, and precursor cells. A high percentage of astrocytes and oligodendrocytes in the white matter at the site of injury die within a short time after injury leading to axonal demyelination. Grafts of cells used as a treatment or as the source of supporting factors can be directly transplanted in the slices and in this way, the graft-host interaction, behavior, and fate of grafted cells can be analyzed. The injured spinal cord microenvironment is very inhospitable for survival and differentiation of transplanted or endogenous NSCs. To mimic NSC grafting into the spinal cord, NSCs were seeded on top of cultured spinal cord slices with the Hamilton syringe between 7 and 10 days after the initial culture. In such culture system, possible effects of environmental manipulation on biological behavior of either exogenous grafted NSCs or endogenous neural progenitor cells in a complex tissue environment similar to that of *in vivo* system were studied. The majority of focally applied exogenous NSCs survived up to 4 weeks, and dispersing cells did not show any preference to either the white or the gray matter. Cultured spinal cord slices retained the non-neurogenic characteristics of *in vivo* spinal cord tissue since they did not support neuronal differentiation of either exogenous NSCs or endogenous neural progenitors [42]. Also, it is very much preferred to use the OTS derived from animals with some genetic alteration, which is manifested in special disease features. For example, to study the process of the myelination and myelin support, the OTSs obtained from the cerebellum of shiverer mice were used. These mice are genetically modified in myelin basic protein (MBP) gene resulting in wide CNS dysmyelination. After the transplantation of oligodendrocyte precursors into such modified sections, the direct observation of myelin produced by grafted cells is possible. This system is more advantageous in comparison with neuron-oligodendrocyte cell co-cultures for its propinquity with *in vivo* system [45].

The impact of individual cells on the regeneration of damaged CNS tissue can be studied not only by direct transplantation of cells to OTS but also by the so-called co-culture system, where the exogenous populations are cultivated underneath the slice co-cultures without direct contact using a Transwell system. The cultured OTSs are then influenced by factors released

from exogenous, co-cultured cells. Indirect co-cultivation of exogenous cells along with OTS-SC permits the analysis of the factors secreted into the medium by both cells and the spinal cord tissue and the determination of their mutual influence. For example, MSCs, the promising candidates for neuroregenerative cell therapies, promote neuronal regeneration by significantly enhanced fiber outgrowth, an effect that may be mediated by a higher expression of brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor [46]. So, the role of the MSCs is in establishing the microenvironment to be more favorable for tissue repair.

Cho et al. [47] demonstrated that the treatment of lumbar slices of spinal cord with lysolecithin caused an important degree of cell death and demyelination of nerve fibers. However, after the co-cultivation of lumbar sections with human MSCs (hMSCs), the earlier observed effects were elevated to a significant range. Since the spinal cord slice culture is totally isolated and does not have any circulating immune cells originated from the host, it excludes the possibility of any systemic immune response to transplanted human cells. The results demonstrated that direct hMSC transplantation into the demyelinated organotypic spinal cord slice culture alter the tissue microenvironment and clearly increased the survival of endogenous cells and promoted axonal outgrowth of nerves in this organotypic spinal cord slice culture. So, the OTS-SC cultures provide the opportunity to investigate the underlying mechanisms of nerve regeneration after injury in the adult nervous system and to identify the factors which enhance axonal outgrowth as well as cell survival. The ease by which organotypic slice cultures can be manipulated and inspected also make them interesting tools for studies of cell proliferation, chronic CNS astrogliosis [48] and migration [49].

The OTS could be applied also to study the neurons and glial damage during bacterial infection. In the course of pneumococcal meningitis, both spontaneous and antibiotic-induced lysis of bacteria occurs. During this process, not only cell wall constituents but also nucleic acids of the bacteria are liberated. Therefore, organotypic hippocampal slices were utilized for the investigation of possible toxic effects of complete bacteria causing meningitis or their components on neuronal and glial tissue in the absence of leukocytes. The neuronal tissue of an infected host is damaged as a result of the intense inflammatory response against the attacking bacteria. Though OTS closely mimics the *in vivo* system, during the actual cultivation, it represents only the part of more complex unit derived from, which is closed without any circulating immune cells. According to this, by the measurement of neuron-specific enolase in cultivation medium, as an additional indicator of neuronal damage, it was found out that not only heat-inactivated whole bacteria but also diverse bacterial elements were capable of inducing nervous tissue damage, such as bacterial walls and nucleic acids [50]. Also, the organotypic brain slice cultures of bovine origin are a suitable model to study aspects of host-pathogen interaction in listeric encephalitis and potentially in other neuroinfectious diseases [51]. To evaluate the effect of HIV-1 virus on neural cells, a method for culturing human fetal organotypic brain slices in the presence of live virus was developed. The brain-derived OTSs were maintained on membranes, and co-cultured with HIV-1-infected T-cells on the well bottom. Thus, OTSs were exposed to live virus during whole cultivation time, HIV-1 proteins, and other molecules produced by the infected T-cells. The results obtained from this model indicate the gliosis observed *in vivo*. This valuable method can be used to model the dynamics

and the microenvironment of brain tissue exposed to HIV-1 and can eventually be used to test therapies directed at preventing HIV-1-induced neural damage in CNS [52].

Within the wide range of available OTSs derived from different CNS sections, the OTS-SC system represents an attractive model with some benefits, when compared to generally used *in vitro* and *in vivo* models. OTS-SC can be kept under cultivation conditions for a long time. During the whole cultivation time, this system enables quick and direct evaluation and identification of huge diapason of different cellular, molecular relations involved in regeneration processes. Moreover, OTS-SCs can be utilized to improve the *ex vivo* neurological disorders and thus represent an intermediate step between *in vitro* experiments and animal models [53].

7. Experimental study

The aim of our study was: (i) to imitate the spinal cord injury (SCI) in organotypic spinal cord slice (OTS-SC) and (ii) to treat these injured OTS-SCs (OTS-SCIs) in the presence of rat mesenchymal stem cells (rMSCs). We imitate the SCI in the OTS-SC not by direct mechanical impact, but the injury was induced by OTS-SC treated with conditioned medium obtained from injured spinal cord (CM-SCI). CM-SCI represents a cocktail of cytokines and chemokines released by central lesion segments of injured spinal cord. We examined the impact of CM-SCI, as well as rMSCs on behavior of microglia cells, astrocytes, and neurons in the organotypic spinal cord slice cultures.

7.1. Methods

The SCI was induced by modified balloon-compression technique in adult rats [54], and after 3 days of animal survival, the central lesion segments were dissected and cultured for 24 h *in vitro* to obtain CM-SCI used for OTS-SC treatment. Similarly, we obtained the conditioned medium from control-intact thoracic spinal cord (CM-SC), which in our experiment served as a source of control-conditioned medium. The OTS-SCs were prepared from lumbar SCs of naive adult male rats. The isolated SCs were transversely cut with a McIlwain tissue chopper into 400 μ m OTS-SC sections and cultivated for an adaptation for 5 days in 1ml/well of culture media containing DMEM, 10% fetal bovine serum, and 1% penicillin–streptomycin and incubated at 37°C in humidified atmosphere with 5% of CO₂ using standard interface method. The study was performed with the approval and guidelines of the Institutional Animal Care and Use Committee of the Slovak Academy of Sciences and with the European Communities Council Directive (2010/63/EU) regarding the use of animals in Research, Slovak Law for Animal Protection No. 377/2012 and 436/2012.

7.2. *In vitro* model of spinal cord injury and treatment

After adaptation, OTS-SCs were divided into four groups according to the treatment and cultivated for the next 3 days. (i) **OTS-SC** (OTS treated with CM-SC), (ii) **OTS-SCI** (OTS treated

with CM-SCI) (iii) **OTS-SC + MSCs** (OTS co-cultured with rMSCs in CM-SC), and (iv) **OTS-SCI + MSCs** (OTS co-cultured with rMSCs in CM-SCI) (**Figure 3**).

Figure 3. The design of the experiment. After adaptation, OTS-SCs were divided into four groups according to the treatment and cultivated for the next three days. Control group is represented by OTS-SCs treated with conditioned medium obtained from intact spinal cord. OTS-SCs treated with conditioned medium from injured spinal cord represent *in vitro* model of spinal cord injury. Both groups were co-cultured with mesenchymal stem cells to investigate the potential influence of these cells on behavior of different cell population in OTSs.

Rat MSCs were isolated from bone marrow of the long bones (femur, tibia) of adult male Wistar rats (290–320 g). To confirm the phenotypic characteristics of rMSC of the third passage, the surface markers (CD29, CD90, and CD45) were analyzed by flow cytometry [55]. The co-culture of rMSCs with OTS-SCs started at the same time with CM-SCI or CM-SC influence. On the basis of immunohistochemistry analyses for cell markers (Iba-microglial cells, GFAP-astrocytes, NeuN-neurons), after 3 days of treatment, OTS-SCs were analyzed using a Leica SP5X confocal microscope and an inverted Nikon ECLIPSE Ti fluorescence microscope. We examined the percentage of NeuN and Iba positive cells calculated from the total number of DAPI (4',6-diamidino-2-phenylindole) - positive cells in 10 random visual fields (600 × 600 μm) in OTSs. The quantification of immunofluorescence intensity of GFAP positive cells was also analyzed by Image J software according to the previous protocol [56]. Data are presented as mean ± SEM. Statistical differences between groups were evaluated with ANOVA, and values of * $p < 0.05$ and ** $p < 0.01$ were considered to be statistically significant.