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The effect of electroporation on cellular dedifferentiation in *Holothuria glaberrima*

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List of abbreviations

AM	Adjacent to the rudiment
BrdU	5-bromo-2-deoxyuridine
DAPI	6-diamidino-2-phenylindole
Dpe	Days post evisceration
dsRNAs	Double-stranded ribonucleic acids
ECM	Extracellular matrix
FOV	Field of view
H. glaberrima	*Holothuria glaberrima*
MM	Medial mesentery
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
PBS	Phosphate-buffered saline
PM	Proximal to the body wall
RISC	Ribonucleic acid-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
S.E.M.	Standard error of the mean
siRNAs	Small- interfering ribonucleic acids
SLSs	Spindle-like-structures
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nicked labeling

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Abstract

The initial step in many regenerative events is the dedifferentiation of cells close to the injury site; this process is characterized by the formation of spindle-like-structures (SLSs). If the dedifferentiation process can be duplicated in-vitro by using electroporation it would allow for a thorough study of the mechanisms involved in regeneration. We hypothesized that electroporation would cause the mesenterial muscle cells to dedifferentiate, replicating the initial cellular response of the regenerative process. For this, sea cucumbers were collected, eviscerated and placed in seawater aquaria for 4 days. Sea cucumbers were dissected, regenerating intestine tissues were collected, electroporated and placed in media culture for 4 hours, 24 hours and 4 days. By immunohistochemistry we were able to study the processes of muscle dedifferentiation, cell proliferation, and apoptosis. No significant differences in muscle dedifferentiation were detected after 4 hours in supplemented media. However, at 24 hours and at 4 days, a significant increase in dedifferentiation was observed. No significant differences in either proliferation or apoptosis were found. Results suggest that electroporation indeed induces muscle cells to dedifferentiate, which would allow us to study regeneration while surpassing other processes associated with studying regeneration in-vivo.

Chapter 1: Introduction

Regeneration has been a topic of interest since the 1700s when studies regarding the regenerative capacities of the hydra started (Lenhoff & Lenhoff, 1986). There are a great number of questions encountered when studying regeneration, such as the mechanisms that allow this process to take place, the relationship between regeneration and disease, and others. Specifically, there is a big interest in the identification of genes involved in the process of regeneration. Some of the genes involved in the process of regeneration of lost tissues or organs take part in different sections of the organisms and answer to different cellular and molecular responses. Therefore, it is essential to understand the role of a specific gene during this process to comprehend how it is carried out. A way in which we can study this effect is by manipulating the expression of said gene and observing its response. This can be done using a gene knockdown, in which one can diminish the expression of a gene of interest and study the effects it has throughout the process of regeneration. An example of a way in which one can see if a specific gene is essential and if it takes part in a specific stage of this process is by using techniques such as RNA interference (RNAi) since this tool allows for the modification of the expression of a gene in vivo or in vitro.

The method of RNAi has both advantages and drawbacks. An advantage being that it is specific and relatively easy, while the drawbacks are that to be able to generate a knock-down (not a knock-out) using RNAi, a transfection method is needed to allow for the RNAi to cross the cellular membrane and enter the cell, which it otherwise would not be able to pass since the membrane is impermeable to

molecules such as drugs, toxins and RNAi (Mocellin & Provenzano 2004). Various transfection methods are currently being used; among them are the use of the agent lipofectamine, and the technique we have used in this project: electroporation. Electroporation is a genetic tool that temporarily opens the pores of a cellular membrane and allows the entrance of substances such as toxins, drugs, and DNA into the cell.

Various animal models are used to study regeneration; among them, it is known that echinoderms have an impressive regenerative capacity. Both the phylum Echinodermata—where echinoderms are located—and the phylum Chordata—where vertebrates are located—belong to the deuterostome family, therefore a high number of genes are preserved among species. This makes *H. glaberrima* a good model of study. *H. glaberrima* has a remarkable regeneration capacity, it can eviscerate most of its internal organs, including the digestive tube, the respiratory trees, and the hemal system and regenerate the lost organs in approximately a month following evisceration. Therefore, the extraordinary regenerative capacities and its evolutionary relationship makes the study of this organism's relevant to understand the cellular and molecular processes of organ regeneration. Understanding the role of the different genes that are involved in this process of regeneration and the different cellular responses such as dedifferentiation, proliferation, and apoptosis, would serve as a gateway for regenerative medicine.

In the present study, we focus on the effects of dedifferentiation, proliferation and apoptosis, all different cellular responses that an organism

undergoes during the initial stages of regeneration of an organ or a tissue after being submitted to electroporation, to see if this experimental methodology affects these processes. To achieve our goal, intestines were dissected, electroporated, cultured in supplemented media for different time points: 4 hours, 24 hours, and 4 days, and cryosectioned; immunohistochemistry and other microscopic analyses was done to study the different cellular responses to see the effect that electroporation is causing.

Chapter 2: Literature revision

To study the molecular basis of regeneration, different genes and proteins must be studied to understand their roles in the cell processes that take place, such as dedifferentiation, proliferation, and apoptosis. There are multiple ways in which the function of genes and/or proteins can be modified to be able to study them. One way is through pharmacological drugs, where one can inhibit enzymatic activity modifying the possible genes or protein functions. However, these methods do not provide specific results because one is unable to control if other pathways or proteins are affected. RNAi is a post-transcriptional process that serves as a more specific way to modify gene expression. Messenger RNA (mRNA) is degraded by introducing double-stranded (ds) RNA into cells or tissue, which results in the corresponding protein being depleted or 'knocked down'. This occurs by binding small regulatory RNAs to mRNA for destruction in a sequence-dependent manner. The outcome is that the gene of interest is silenced, thereby allowing us to study the defects associated with the loss of protein function (Maine, Eleanor 2008). This

method used for targeted gene silencing could also be used to identify the genes that interact in the process of organogenesis.

The mechanisms of RNAi have been well studied. In brief, when the RNAi is introduced into the cell the dsRNA is cleaved by Dicer, an endonuclease, into small-interfering (si) RNAs of approximately 21 to 23 base pair fragments that hybridize with the target mRNA and initiate its destruction. These associate with the RNA-induced silencing complex (RISC), which are then led to its target mRNAs by simultaneously scanning the complementary mRNA sequence for homology to the small, unwound, RNA fragment. When guided to the mRNA it is then cleaved by the RISC enzymatic activity promoting then the destruction of the mRNA through the enzymatic activity integral to the complex (Gewirtz, 2007). In other words, this process turns down the expression of specific genes by the interference of RNA with a specific messenger RNA, which either blocks translation or targets messenger RNA for degradation. This process results in a lack of protein production. Electroporation may have other potential transfection uses such as transiently transfecting and down-regulating some of the proteins in the stem cells (Arabsolghar and Rasti, 2012).

Modifying gene expression could help us understand the different pathways for diseases such as cancer, genetic disorders, autoimmune diseases, viral infections, etc. by developing RNAi as a tool that can help us improve the treatment of different diseases. For instance, it could be used as cancer therapy: a siRNA would be designed to inactivate multiple gene mutations in oncogenes and tumor suppressor genes. To recapitulate, RNAi is an invaluable tool that results in the form of post-

transcriptional gene silencing. It could be used to study gene function, thereby increasing what is currently known in functional genomic studies and revolutionizing different areas of research.

A group of scientists at John Hopkins used electroporation to transfect adult mouse dorsal root ganglion neurons in-vivo to study peripheral axon regeneration. They were able to study the function of adult sensory neurons by experiments where they were able to generate gain- and loss-of-function experiments by delivering a gene into embryonic neurons (Zhou, Feng-quan 2014). Another group at Harvard Medical School introduced genes into postnatal rat retina and embryonic mouse brain by using the temporal and spatial regulation of RNAi to generate a conditional regulation of gene expression from electroporated plasmids. Using electroporation and site-specific recombination systems they were able to lead experiments that included cell-type-specific gene misexpression, conditional RNAi, and fate mapping of progenitor and precursor cells (Matsuda & Cepko 2007). In addition to allowing the introduction of exogenous molecules into the cell membrane, the activation of dedifferentiation without the introduction of DNA has also been reported (Blackiston, McLaughlin & Levin 2009).

There seems to be a great interest in comprehending the genes that are active during regeneration, and RNAi seems to be the way to modulate the genes to study the gene activation network that is necessary to reform the lost tissue/organ. Several genes are known to be essential in this process such as the candidate gene Wnt, which has an important role in embryonic development, but there are still questions left unanswered (Stoick-Cooper 2006). In summary, by using RNAi in

combination with the transfection methods of electroporation, the study of the role of specific genes could be possible in processes such as organogenesis.

Regeneration is a process by which plants or animals can re-form or restore tissue or an organ that has been lost after the damage has occurred. It is an example of postembryonic morphogenesis that involves the recognition of tissue loss, where an organism can restore the relevant structure (Brockes, J. P., & Kumar, A. 2008) One of the reasons why *H. glaberrima* makes such a good model of study is because it allows us to study the regeneration of the whole intestine organ. The anatomy of the intestine of this sea cucumber at the macroscopic level can be divided into three parts: descending intestine, ascending intestine and second descending intestine. The mesentery is a continuous structure along the digestive tract that connects the intestinal system to the body wall. At the histological level, the digestive tract has three main tissues: the mesothelium, a connective tissue layer, and the luminal epithelium. The mesothelium includes the coelomic epithelium or serosa and muscle layer, the connective tissue layer is also called the submucosa and the luminal epithelium is also called the mucosa. The mesothelium outer cells are the peritoneocytes. These are monociliated coelomic cells that make up most of the external layer; the internal layer is composed of monocytes of muscle cells (García-Arrarás, Lázaro-Peña & Díaz-Balzac 2018).

One of the topics heavily studied in the regeneration field is how the cells behave to allow for regeneration to occur. The cells involved in the formation of a new organ after an animal has encountered injury or trauma may originate from either one of two different processes: (1) proliferation of multipotent stem cells

followed by differentiation or (2) dedifferentiation of mature cells followed by proliferation and then differentiation (García-Arrarás et al. 2011). The process in which regeneration occurs at the cellular level depends on the organism and the capacity to regenerate the specific tissue.

Previous studies suggest that the regeneration process of the sea cucumber *H. glaberrima* takes place through dedifferentiation (Candelaria et al. 2006). Dedifferentiation is "a process in which a cell loses its specialized morphology, function, and biochemistry to initiate cell division and reverts to a less differentiated state prior to redifferentiate again" (Hall 2005). This is a process that many animal species have to undergo before beginning to regenerate an injured or lost organ, they later proliferate eventually becoming the cells that will be involved in the regeneration process. The dedifferentiation capacity of a cell is still being studied but is though to maintain a memory of the tissue or function to which it was specialized (Kragl et al. 2009). This means that the dedifferentiated cells often differentiate into the same cell type as the original cell.

Chapter 3: Theory

Animal Model

The sea cucumber *H. glaberrima* is known to regenerate most of its internal organs after evisceration. When they eviscerate they eject their viscera through their cloaca, which includes their intestinal system, respiratory tree, hemal system, and gonads. The intestine system takes around 21 days to regenerate and look like a functioning intestine organ. This event takes place in different stages: wound

healing, blastema formation, lumen formation, layer proportioning stage, and enlargement. These different stages involve the organization of specific cells and tissues into what becomes this new organ (García- Arrarás, Lázaro Peña & Díaz-Balzac 2018).

The regeneration of *H. glaberrima* takes place by a thickening of the free mesentery edge, which will end up giving rise to the regenerated intestine by allowing the invasion of this thickening by mucosal epithelium. Previous experiments show that changes in the extracellular matrix (ECM) are also associated with intestine regeneration and that they involve the proteolytic activities of MMPs (Quiñones at al, 2002). Studies were later done to understand these changes in depth where disorganization of the muscle layer with the myocyte dedifferentiation was observed (Candelaria et al. 2006). Muscle cells are fragmenting and the contractile apparatus is disintegrating, expelling the spindle-like-structures (SLSs) that characterized the dedifferentiation response that occurs during regeneration. A gradient of muscle dedifferentiation is found throughout the mesentery where near the blastema-like-structure more SLSs are observed, and near the wall-like structure more muscle fibers are observed. There have been other studies done in echinoderms that also suggest that muscle is dedifferentiating by producing SLSs. Therefore, SLSs are suggested to be a definite signal of muscle dedifferentiation (Dolmatov 1992).

Dedifferentiation

Dedifferentiation is an important biological phenomenon whereby cells regress from a specialized function to a simpler state reminiscent of stem cells (Cai, Fu & Sheng 2007). Even though dedifferentiation is the first cellular response that occurs in the mesentery adjacent to the injury during the process of intestine regeneration in *H. glaberrima*, multiple cellular responses can give rise to new tissue (Candelaria et al. 2006)

Dedifferentiation seems to be characterized by the fragmentation of the muscle cells and by the degradation of the contractile apparatus, these structures that are degraded are ejected from the cell in membrane-bound structures, SLSs (García-Arrarás, Lázaro-Peña & Díaz-Balzac 2018).

A gradient is observed along the regenerating intestine explant where more SLSs are observed near the blastema, while muscle fibers are mostly focused near the wall-like structure. Studies done in the laboratory suggest that evisceration induces the first cellular response, dedifferentiation of the muscle cells of the mesentery, close to where the rupture occurred near the mesentery (García-Arrarás, Lázaro-Peña & Díaz-Balzac 2018).

Electroporation

The electroporation technique is usually used as a tool to briefly open up the pores of a cellular membrane to introduce small molecules, such as DNA or drugs, which typically could not enter the cell membrane. However, electroporation is

thought to induce dedifferentiation, similar to the first cellular response that occurs at the beginning of regeneration.

Newts are another example of organisms that depend on the process of dedifferentiation to be able to regenerate, and it has been studied that after differentiation the nuclei of the myofibers are removed from the cell cycle, while before dedifferentiation the myonuclei once again enter the cell cycle. Electroporation can be used as a transfection tool; it has been shown to cause cellular dedifferentiation in newts limbs. Atkinson and colleagues showed that electroporating the limb of a newt presents a dedifferentiation response indistinguishable from that of injury–associated regeneration (2006). They not only suggested that their response is indistinguishable but also proposed that the time course for both processes followed similar patterns, starting at about 5 days, up until 3 weeks. They also showed that the changes in gene expression were similar during amputation and electroporation. Taking this into account, they wanted to observe if through electroporation the myonuclei of skeletal muscle were capable of reentering the cell cycle. Results suggest that electroporation promoted the reentering of the cell cycle in mammalian skeletal muscle (Miyoshi et al. 2012).

Chapter 4: Hypothesis

Studies show that electroporation, an electric field sufficient to open the pores of a membrane, initiates a dedifferentiation response in an organ capable of regeneration characterized by cell cycle reentry of appendage cells if the voltage emitted is not sufficient to cause necrosis or apoptosis (Atkinson et at. 2006). We

hypothesize that electroporation causes the intestinal rudiment cells to dedifferentiate in *H. glaberrima*. In this way, electroporation duplicates the initial cellular response of the regenerative process. Dedifferentiation is characterized by the formation of spindle-like-structures, therefore we think that during regeneration muscle fibers are dedifferentiating into spindle-like-structures (Candelaria et al. 2006).

Chapter 5: Justification

Many animals have the power to regenerate, and scientists can use them as tools that can help the field of regenerative medicine. While adult human regeneration is limited, we can use different animal models to study the mechanisms that occur in order to be able to undergo the regenerative response to form new organs. Not only that, but we can develop new techniques that can be translated into medicine. While humans, or mammals for that manner, are not able to regenerate full organs such as axolotls or planarians, they have been known to regenerate the tips of their fingers. Beginning to understand how this process takes place can help us understand how the human body works. How this process occurs is still not fully understood, and there is still a whole field of regenerative medicine left with a lot of promise. It is important to note that only the small tips at the end of the fingertips can regenerate, therefore the regeneration capacity is very limited in humans (Illingworth 1974). This phenomenon is also observed in mice, where adult mice can regenerate their paws to their original form, but there has to be a small section of this tissue to allow the regeneration process to occur (Takeo 2013).

Understanding the cellular and molecular processes is a vital part of developing new treatments.

While studying regeneration in our model of study, *Holothuria glaberrima*, we can study different cellular responses that take part during regeneration such as dedifferentiation, proliferation, apoptosis, and migration. If electroporation causes the intestinal rudiment cells to dedifferentiate, it would bring in a new approach that could allow us to study different cellular and molecular processes such as gene expression and the processes by which the cells differentiate to acquire their new phenotypes. The study of the mechanisms that the organism undergoes in-vivo is marred by complex tissue interactions, therefore if the electroporation technique produces a dedifferentiation response indistinguishable from that of the cellular dedifferentiation response that occurs as the first response of the regeneration process it would allow us to study this process in- vitro. This tool could serve as a basis for many in vivo applications that still need to be better understood, such as electrochemotherapy and gene therapy (Chen et al. 2006). Electroporation could be used as a transfection tool and as a gateway for new studies that could enhance the regeneration potential in the research that is currently being conducted, including identifying the genes that are associated with regeneration and its mechanisms.

Chapter 6: Materials and methods

A. Sea cucumber *Holothuria glaberrima*

The sea cucumber *Holothuria glaberrima* used as the model of study were collected from the rocky shores of Piñones, Puerto Rico. Sea cucumbers were gathered and left in seawater aquaria with constant oxygenation.

B. Evisceration and tissue sections

The sea cucumbers were eviscerated before dissection by injecting 0.35 M KCl solution (3-5 ml) into the coelomic cavity. They were left in seawater aquaria for four more days. Animal maintenance and dissection procedures have been discussed previously (García-Arrarás et al. 1998). The animals were then placed in ice for an hour and cut by the longitudinal section of the body wall. The mesentery and the regenerating intestine were dissected, collected and placed in an antibiotic solution containing penicillin/streptomycin (10mg/mL), neomycin (10mg/mL) and amphotericin (2.5 mg/mL) for two hours. The antibiotic solution was changed twice during this lapse of time, once every hour.

C. Electroporation

After the two hours in the antibiotic solution, tissue explants were electroporated using the ECM Electroporation System in electroporation cuvettes. The intestinal tissues that were electroporated were taken individually from the antibiotic solution to the Disposable Electroporation Cuvettes PlusTM with 5 µL of Nuclease-Free Duplex Buffer, making sure the tissue touch both parts of the cuvette

using parameters chosen in previous studies: 35V, 25mV pulse and 975 interval pulse for ten pulses, specific to intestine explants.

The intestine tissues that were used as control went through the same procedure, but in this case, no electroporation was applied. The control tissues were transferred directly from the antibiotic solution to the same Nuclease-Free Duplex Buffer that was used with the electroporated tissues, ten seconds passed before placing them in the explant culture.

D. Tissue culture

After electroporating the tissue, the explants were placed in 4-well-plates with a supplemented media that included 500uL penicillin, 250uL gentamicin, 50uL amphotericin B, 500uL nonessential amino acid, 500uL sodium pyruvate, and 50uL alpha-tocopherol-acetate. This supplemented media also contained a solution of 50M solution of BrdU. The tissues were placed at room temp (25°C) in an incubator chamber for three different time points: four hours, twenty-four hours and four days.

E. Fixation and histology

When the tissues were taken out of the media they were fixed in 4% paraformaldehyde at 4°C for 24 hours, they were washed 3 times for 15 minutes with 0.1 M phosphate-buffered saline (PBS), and placed in a 30% sucrose solution at 4°C. The tissues were embedded using Tissue Tek and cut in a cryostat. Transversal

cryostat tissue sections of 20 μm in width were made and mounted on poly-lysine-treated slides.

F. Immunohistochemistry protocol

1. Dedifferentiation

The explant tissue sections, both electroporated and control, were treated with Phalloidin TRITC (1:2500) for one hour. They were then washed 3 times with 0.1 M PBS for 15 minutes and finally, stained using a mounting media that contained DAPI (1/50)-PBS/Glycerol mounted medium and sealed.

2. Proliferation

Explant tissue sections, both electroporated and control, were first washed with 1% Triton 100x (made with PBS) for 15 minutes, washed 2 times with 0.1 M PBS for 15 minutes, washed with 0.05 M HCl for 1 hour and lastly, with 0.1 M PBS for 15 minutes. Goat serum (1:50) was added to the slides for 1 hour, excess goat serum was removed. Sections were incubated with the primary antibody, anti-5-bromo-2-deoxyuridine (BrdU), in a humid chamber, overnight at room temperature.

The next day the slides were washed three times with 0.1 M of PBS for 15 minutes, incubated with secondary antibody Goat anti mouse (GAM) CY3 (1:1000) for one hour in a humid chamber, washed three times with 0.1 M PBS for fifteen minutes, and lastly, the slides were stained using a glycerol-buffered mounting media that contained DAPI (1/50)-PBS/Glycerol mounted medium and sealed.

3. Apoptosis

A TUNEL Assay Kit, which works by labeling DNA breaks using a TdT enzyme, was used to determine if electroporation had an effect on cell apoptosis. Slides were washed with TBS once for 5 minutes. The tissue was covered with 30uL of ProteinaseK in TRIS (1:100) for 5 minutes. The slides were washed again with TBS two times for 10 minutes.

For a positive control, the slides were covered with DNAase and incubated for 15 minutes. They were then washed with TBS once for 5 minutes.

Tissues were covered with 30uL of 1x TdT Equilibration Buffer (1:5) with distilled water and incubated at room temperature for 10-30 minutes while the labeling mixture was prepared. After removing the TdT Equilibration Buffer, a 30uL/sample of TdT labeling Rxn Mixture was applied to the tissues. Per 30uL/sample the TdT labeling Rxn Mixture was vortexed and transferred to a clean tube placed in ice. A total of 28.5uL of Fluorescein FraGel Tdt Labeling was mixed with 1.5uL TdT enzyme.

The negative control was prepared using 28.5mL of Fluorescein FraGel TdT Labelling Mix and 1.5uL of distilled water.

The slides were placed in a humid chamber at 37^{o}C for 1-1.5 hours. They were incubated in TBS 1X for 1 minute twice at room temperature. The remaining liquid was removed and the cover slides were placed using Fluorescein FraGel Mounting Media following the protocol of slide preparation in the laboratory.

G. Cell quantification and statistical analysis

Cells were quantified using the 100x objective on the fluorescent microscope to take images of specific regions of the mesentery or of the overall tissue. Images were analyzed qualitatively and quantitatively. The pictures were taken to obtain an overall response for dedifferentiation, proliferation, and apoptosis, while additional pictures were taken taking into consideration using spatial bias to quantify the dedifferentiation response in specific regions.

For evaluation of statistical differences between controls and experimental groups in relation to the effect of electroporation two statistical tests were used: t-test analysis and ANOVA follows of Turkey's test. All values are reported as mean ± S.E.M., and a statistical difference was defined as a p-value < 0.05. These analyses were performed using the software Prism 8.

Chapter 7: Results

A. Dedifferentiation

I. Regenerated intestine tissue 4 days after evisceration, 4 hours in supplemented media culture

Analysis

Through a qualitative analysis of the images taken using a fluorescent microscope, no remarkable differences were observed regarding the presence of SLSs and muscle fibers when comparing control tissue (**1A**) with tissue 4 hours after electroporation (**1B**). To measure the effect of electroporation on the regenerated

intestine tissue after 4 hours in supplemented media, the cellular results were quantified (**1C-1H**).

SLSs, muscle fibers and cell nuclei per field of view

These cellular responses were quantified per field of view (FOV) to see if electroporating and incubating the tissues for 4 hours in supplemented media had an effect on the total number of SLSs, muscle fibers, and cell nuclei.

Cells were quantified throughout the tissue by using DAPI staining as a marker to determine the number of cell nuclei per FOV (**1C**). The control tissue averaged a total of 66.00 cell nuclei per FOV, while the electroporated tissue averaged a total of 45.34. No significant differences were observed.

SLSs were quantified throughout the tissue using Phalloidin staining as a marker (**1D**). The control tissue averaged a total of 9.83 SLSs per FOV, while the electroporated tissue averaged a total of 21.17. No significant differences were observed.

Muscle fibers were quantified throughout the tissue using Phalloidin staining (**1E**). The control tissue averaged a total of 8.00 muscle fibers per FOV, while the electroporated tissue averaged a total of 4.00. No significant differences were observed.

SLSs/cell nuclei and muscle fibers/cell nuclei

To see if there was a significant difference regarding the number of SLSs and muscle fibers in proportion to the cells, the cell nuclei were quantified to obtain a

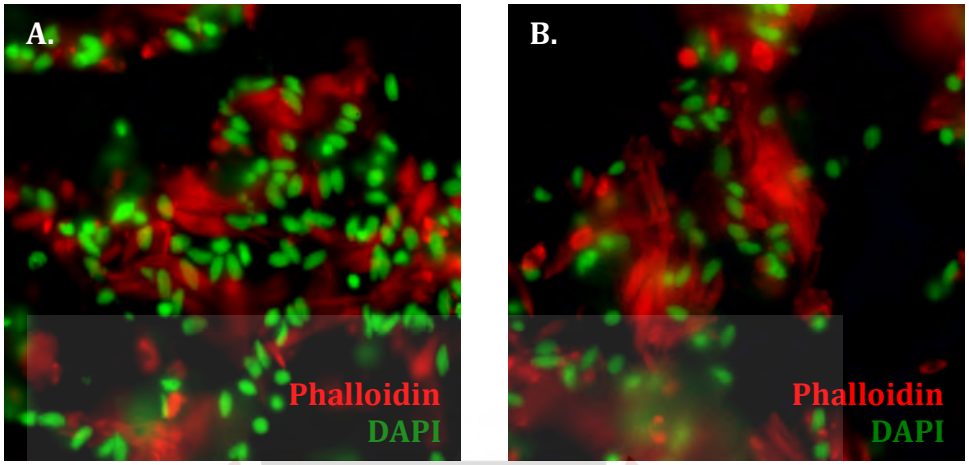
percentage normalized by using cell nuclei DAPI+ staining. The total amount of Phalloidin+ SLSs was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage (**1F**). The control tissue accounted for an average of 17.50% SLSs, while the percentage of electroporated tissue averaged a total of 31.48%. No significant differences were observed.

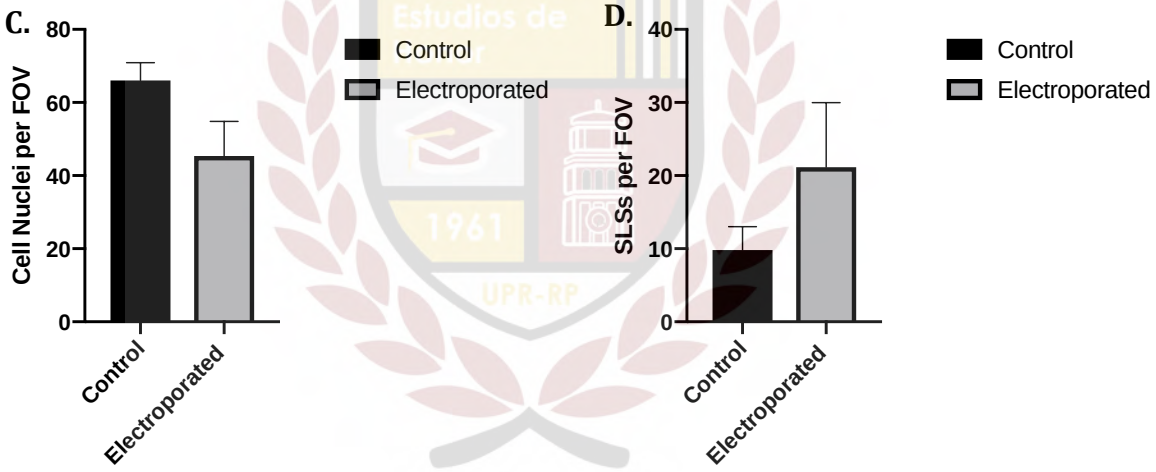
Muscle fibers were quantified to obtain a percentage normalized by using cell nuclei. The total amount of Phalloidin+ muscle fibers was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage (**1G**). The percentage of muscle fibers over cell nuclei in the control tissue accounted for 17.78%, while in the electroporated tissue the total percentage accounted to 24.03%. No significant differences were observed.

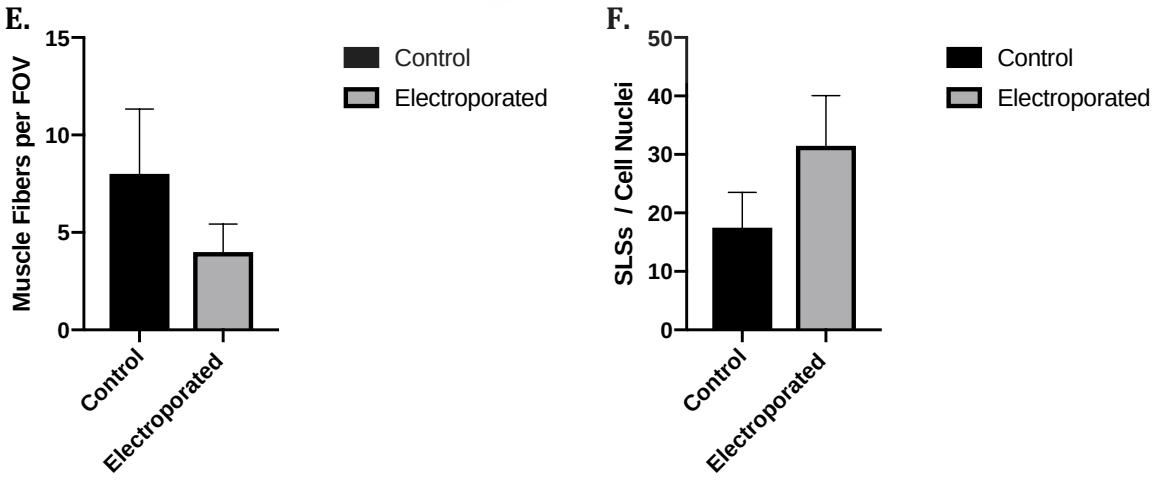
Dedifferentiation ratio quantification

By quantifying the total amount of SLSs and muscle fibers in the overall regenerated intestine tissue, a ratio was obtained in order to measure the dedifferentiation response (**1H**). The control tissue had a ratio of 1.66, while the electroporated tissue accounted for a 5.47 dedifferentiation ratio. No significant differences were observed.

The effect of electroporation on cellular dedifferentiation in regenerated intestine tissue- 4 hours after electroporation







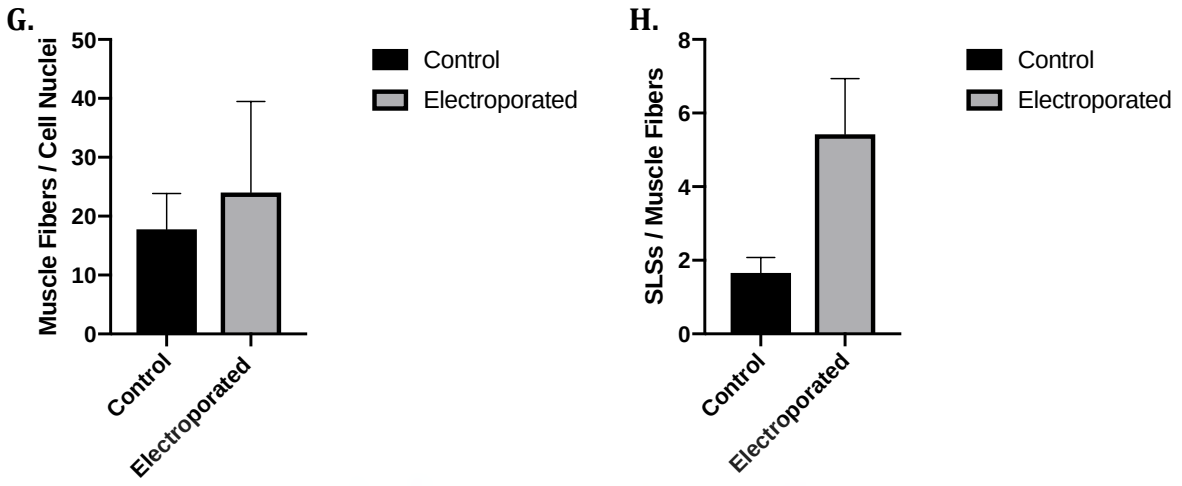


Figure 1. Labeling of cell nuclei and spindle-like-structures (SLSs) to determine cellular dedifferentiation 4 hours after electroporation. (A-B) Immunohistochemistry of the cellular dedifferentiation response did not demonstrate a difference regarding the amount of both SLSs and muscle fibers in the control regenerated (non-electroporated intact) intestine tissue **(A)** and electroporated regenerated intestine tissue **(B)**. SLSs and muscle fibers were labeled with rhodamine-labeled phalloidin (red) and cell nuclei were labeled with DAPI (green). **(C-E)** The cellular responses were quantified per FOV to see if electroporating and incubating the tissues for 4 hours in supplemented media had an effect on the total number of SLSs **(C)**, muscle fibers **(D)**, and cell nuclei **(E)**. **(F-G)** SLSs and muscle fibers were normalized using DAPI and expressed as a percentage of SLSs **(F)** and muscle fibers **(G)**. **(H)** The expression of SLS and muscle fibers were divided to obtain a dedifferentiation response ratio that would allow the quantification of the effect of electroporation. Data expressed as mean ± S.E.M. Analyzed using t-test, two tailed; n=4 per group, *$p < 0.05$.

II. Regenerated intestine tissue 4 days after evisceration, 24 hours in supplemented media culture

A. Overall regenerated intestine tissue analysis

Analysis

Through a qualitative analysis of the images taken using a fluorescent microscope, a difference was observed regarding the presence of SLSs when comparing control tissue (**2A**) with tissue 24 hours after electroporated (**2B**). More SLSs seemed to be present in the electroporated regenerated intestine tissue left 24 hours in supplemented media, but in order to measure the effect the cellular results were quantified (**2C-2H**).

SLSs, muscle fibers and cell nuclei per field of view

These cellular responses were quantified per FOV to see if electroporating and incubating the tissues for 24 hours in supplemented media had an effect on the total number of SLSs, muscle fibers, and cell nuclei.

Cells were quantified throughout the tissue by using DAPI staining to determine the number of cell nuclei per FOV (**2C**). The control tissue averaged a total of 28.89 cell nuclei per FOV, while the electroporated tissue averaged a total of 44.15. A significant difference was observed.

SLSs were quantified throughout the tissue using Phalloidin staining (**2D**). The control tissue averaged a total of 5.52 SLSs per FOV, while the electroporated tissue averaged a total of 14.26. A significant difference was observed.

Muscle fibers were quantified throughout the tissue using Phalloidin staining (**2E**). The control tissue averaged a total of 8.52 muscle fibers per FOV, while the electroporated tissue averaged a total of 12.00. No significant differences were observed.

SLSs/cell nuclei and muscle fibers/cell nuclei

To see if there was a significant difference regarding the number of SLSs and muscle fibers in proportion to the cells, the cells were quantified to obtain a percentage normalized by using cell nuclei DAPI+ staining. The total amount of Phalloidin+ SLSs was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage (**2F**). The control tissue accounted for an average of 21.48% SLSs, while the percentage of electroporated tissue averaged a total of 34.06%. A significant difference was observed.

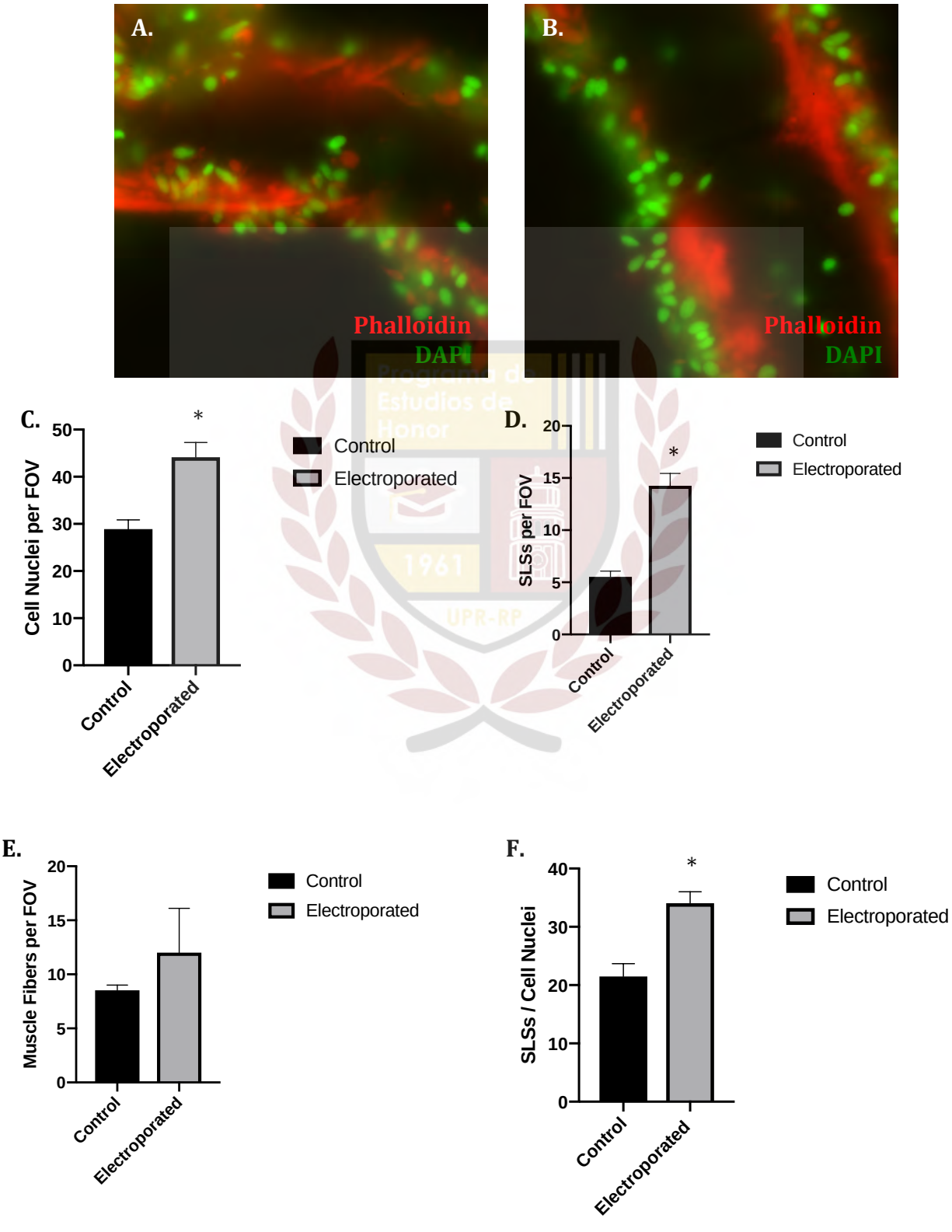
Muscle fibers were quantified to obtain a percentage normalized by using DAPI staining. The total amount of Phalloidin+ muscle fibers was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage (**2G**). The percentage of muscle fibers over cell nuclei in the control tissue accounted for 36.97%, while in the electroporated tissue the total percentage accounted to 41.03%. No significant differences were observed.

Dedifferentiation ratio quantification

By quantifying the total amount of SLSs and muscle fibers in the overall regenerated intestine tissue a ratio was obtained in order to measure the

dedifferentiation response (**1H**). The control tissue had a ratio of 3.60, while he electroporated tissue accounted for a 8.12 dedifferentiation ratio. A significant difference was observed.

The effect of electroporation on cellular dedifferentiation in regenerated intestine tissue- 24 after electroporation



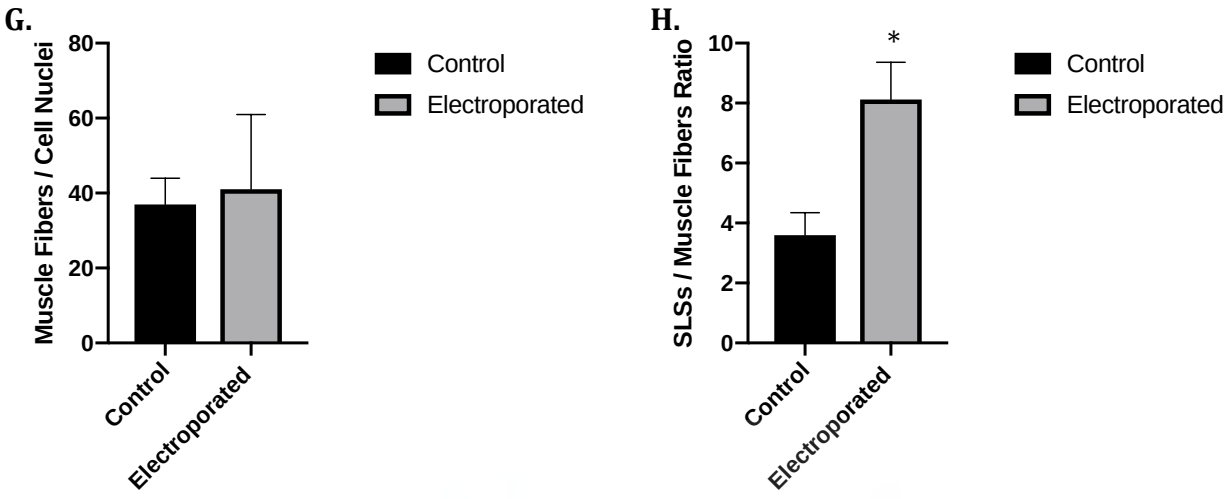


Figure 2. Labeling of cell nuclei and spindle-like-structures (SLSs) to determine cellular dedifferentiation 24 hours after electroporation. (A-B) Immunohistochemistry of the cellular dedifferentiation response presented a difference regarding the amount of SLSs when comparing the control regenerated (non-electroporated intact) intestine tissue **(A)** and electroporated regenerated intestine tissue **(B)**. SLSs and muscle fibers were labeled with rhodamine-labeled phalloidin (red) and cell nuclei were labeled with DAPI (green). **(C-E)** The cellular responses were quantified per FOV to see if electroporating and incubating the tissues for 24 hours in supplemented media had an effect on the total number of SLSs **(C)**, muscle fibers **(D)**, and cell nuclei **(E)**. **(F-G)** SLSs and muscle fibers were normalized using DAPI and expressed as a percentage of SLSs **(F)** and muscle fibers **(G)**. **(H)** The expression of SLS and muscle fibers were divided to obtain a dedifferentiation response ratio that would allow the quantification of the effect of electroporation. Data expressed as mean ± S.E.M. Analyzed using t-test, two tailed; n=3 per group, *p < 0.05.

B. Spatial localization of the effect of electroporation on regenerated intestine tissue

Analysis

Through a qualitative analysis of the images taken using a fluorescent microscope, a difference was observed regarding the presence of SLSs when comparing control tissue (**3A-3C**) with tissue 24 hours after electroporation (**3D-F**). Regenerated intestine tissues were stained in order to analyze if tissue electroporation is affecting the dedifferentiation response, but this time taking into consideration spatial localization: proximal to the body wall (PM), medial mesentery (MM) and adjacent to the rudiment (AM).

The control and electroporated tissue at the AM was mostly made up of SLSs, nearly no muscle fibers could be observed (**3A, 3D**). The control and electroporated tissue MM was mostly made up of a combination of SLSs and muscle fibers (**3B, 3E**). The control and electroporated tissue at the PM showed mostly muscle fibers, a very limited amount of SLSs were present (**3C, 3F**). By analyzing the images taking into consideration spatial localization and the dedifferentiation gradient (**3G-3O**) that has been studied we would be able to analyze if the effect was all throughout the tissue or corresponding to specific parts of the regenerated intestine explant.

SLSs/cell nuclei and muscle fibers/cell nuclei

To see if there was a significant difference regarding the number of SLSs and muscle fibers in proportion to the cells, the cell nuclei were quantified to obtain a percentage normalized by using cell nuclei DAPI+ staining. The total amount of

Phalloidin+ SLSs was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage taking into consideration spatial localization (**3G-I**). In the AM section the control tissue had 49.00% of SLSs, while the electroporated tissue had 45.33% (**3G**). In the MM section the control tissue had 8.33% of SLSs, while the electroporated tissue had 8.33% (**3H**). In the PM section the control tissue had 7% of SLSs, while the electroporated tissue had 27.33% (**3I**). No significant differences were observed in either three of the sections.

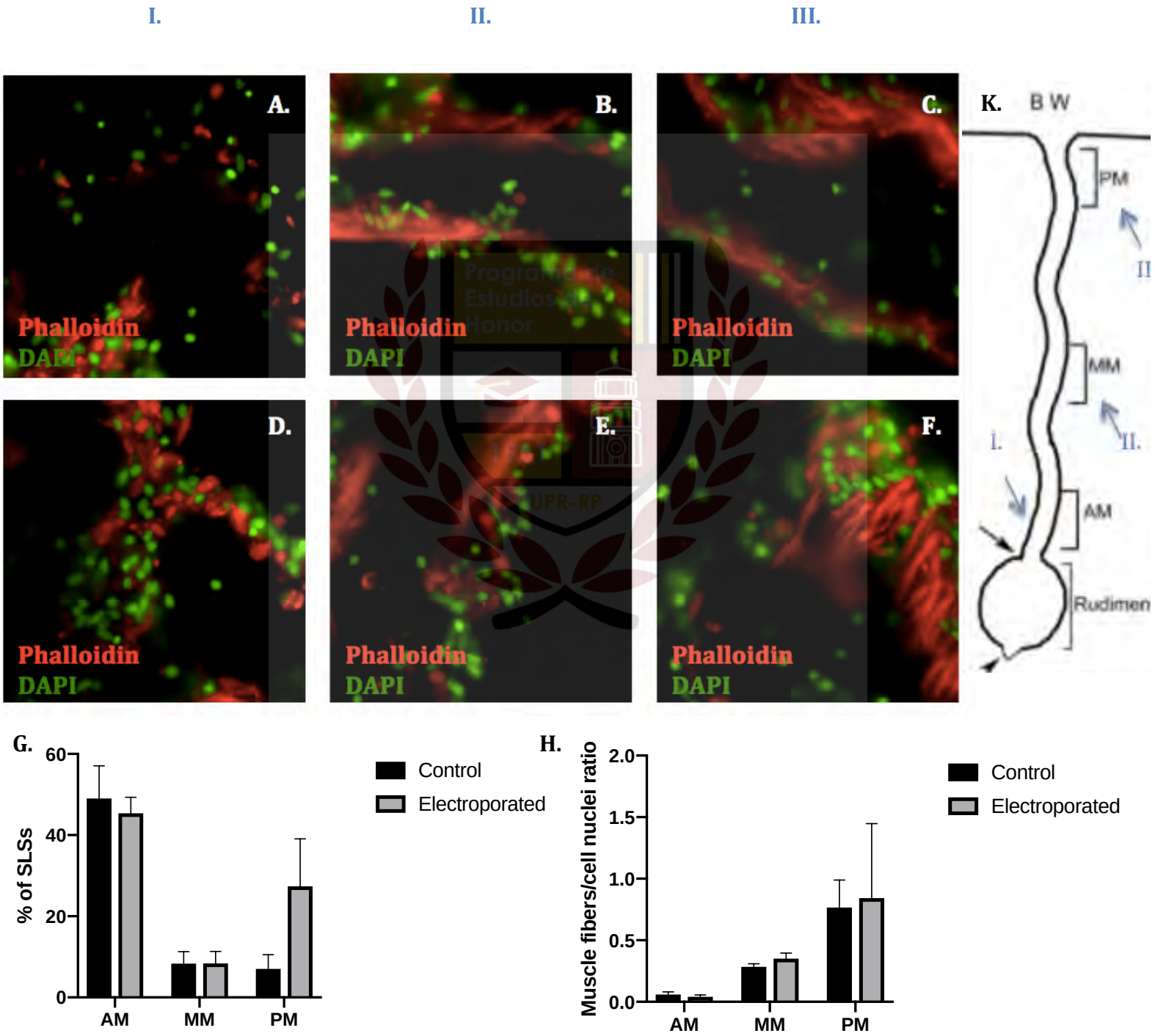
The same was done in order to quantify the amount of muscle fibers in relation to the cell nuclei. Muscle fibers were quantified to obtain a percentage normalized by using cell nuclei. The total amount of Phalloidin+ muscle fibers was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage (1J-L). In the AM section the control tissue had 6.00% of SLSs, while the electroporated tissue had 4.00% (3J). In the MM section the control tissue had 28.33% of SLSs, while the electroporated tissue had 35.00% (3K). In the PM section the control tissue had 76.67% of SLSs, while the electroporated tissue had 84.33% (3L). No significant differences were observed in either three of the sections.

Dedifferentiation ratio quantification

By quantifying the total amount of SLSs and muscle fibers in the overall regenerated intestine tissue a ratio was obtained in order to measure the dedifferentiation response (1H). In the AM section the control tissue had a dedifferentiation ratio of 9.56, while the electroporated tissue had a ratio of 19.63 (3M).. In the MM section the control tissue had a dedifferentiation ratio of 0.96,

while the electroporated tissue had a ratio of 2.14 (**3N**). In the PM section the control tissue had a dedifferentiation ratio of 0.27, while the electroporated tissue had a ratio of 2.60 (**3O**). No significant differences were observed in either three of the sections.

Spatial localization of the effect of electroporation on cellular dedifferentiation in regenerated intestine tissue- 24 hours after electroporation



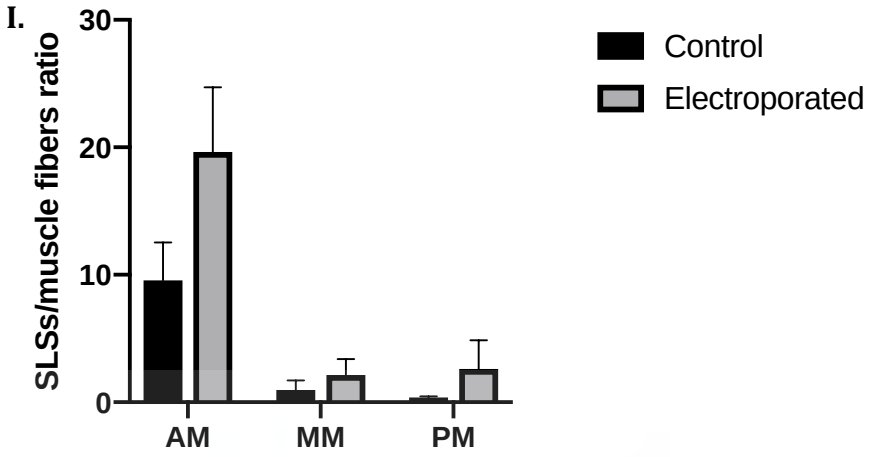


Figure 3. Labeling of cell nuclei and spindle-like-structures (SLSs) to determine cellular dedifferentiation 24 hours after electroporation depending on spatial localization. (A-F) Immunohistochemistry of the cellular dedifferentiation response did not present a remarkable difference regarding the amount of SLSs when comparing the control regenerated (non-electroporated intact) intestine tissue **(A-C)** and electroporated regenerated intestine tissue **(D-F)**. SLSs and muscle fibers were labeled with rhodamine-labeled phalloidin (red) and cell nuclei were labeled with DAPI staining (green). **(G)** There was no significant difference on the % of SLSs when comparing control tissue with electroporating in regards of the three different tissue areas analyzed. **(H)** There was no significant difference on the muscle fiber/cell nuclei ratio when comparing control tissue with electroporating in regards of the three different tissue areas analyzed. **(I)** There was no significant difference on the SLSs/muscle fiber dedifferentiation ratio when comparing control tissue with electroporating in regards of the three different tissue areas analyzed. **(K)** A transversal cut of the regenerated intestine specifying the areas by sections (BM= body wall, PM= proximal to the body wall, MM= medial to the mesentery, and AM= adjacent to the rudiment). Data expressed as mean ± S.E.M. Analyzed using t-test, two tailed; n=3 per group, *$p < 0.05$.

III. Regenerated intestine tissue 4 days after evisceration, 4 days in supplemented media culture

Analysis

Through a qualitative analysis of the images taken using a fluorescent microscope, a difference was observed regarding the presence of SLSs when comparing control tissue (**4A**) with tissue 4 days after electroporation (**4B**). More SLSs seemed to be present in the electroporated regenerated intestine tissue left 4 days in supplemented media, but in order to measure the effect the cellular results were quantified (**4C-4H**).

SLSs, muscle fibers and cell nuclei per field of view

These cellular responses were quantified per FOV to see if electroporating and incubating the tissues for 4 days of supplemented media had an effect on the total number of SLSs, muscle fibers, and cell nuclei.

Cells were quantified throughout the tissue by using DAPI staining to determine the number of cell nuclei per FOV (**4C**). The control tissue averaged a total of 42.89 cell nuclei per FOV, while the electroporated tissue averaged a total of 33.78. No significant differences were observed.

SLSs were quantified throughout the tissue using Phalloidin staining (**4D**). The control tissue averaged a total of 4.89 SLSs per FOV, while the electroporated tissue averaged a total of 17.89. A significant difference was obtained.

Muscle fibers were quantified throughout the tissue using Phalloidin staining (**4E**). The control tissue averaged a total of 12.11 muscle fibers per FOV, while the

electroporated tissue averaged a total of 3.44. No significant differences were observed.

SLSs/cell nuclei and muscle fibers/cell nuclei

To see if there was a significant difference regarding the number of SLSs and muscle fibers in proportion to the cells, the cells were quantified to obtain a percentage normalized by using cell nuclei DAPI+ staining. The total amount of Phalloidin+ SLSs was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage (**4F**). The control tissue accounted for an average of 14.23% SLSs, while the percentage of electroporated tissue averaged a total of 67.85%. A significant difference was not observed.

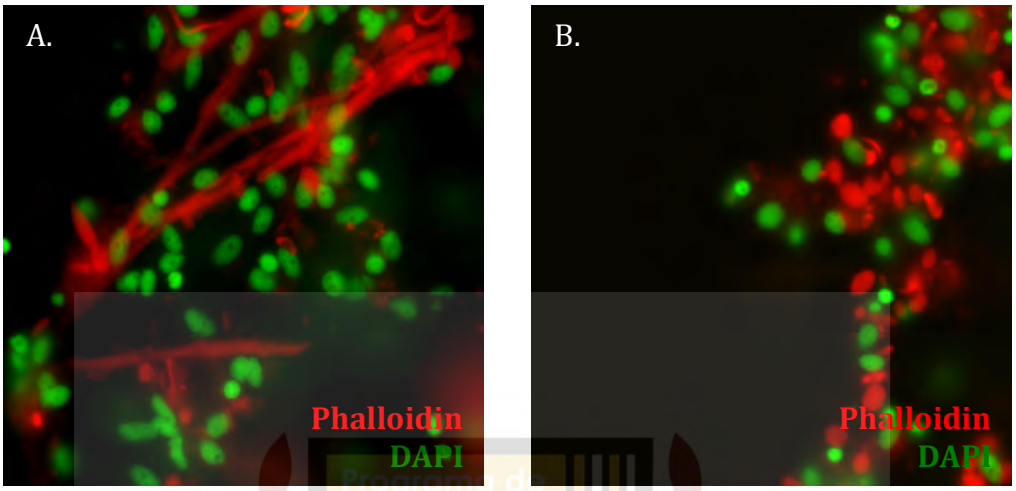
Muscle fibers were quantified to obtain a percentage normalized by using DAPI staining. The total amount of Phalloidin+ muscle fibers was divided over the total amount of cell nuclei and multiplied by 100 to obtain a percentage (**4G**). The percentage of muscle fibers over cell nuclei in the control tissue accounted for 35.40%, while in the electroporated tissue the total percentage accounted to 17.49%. A significant difference was obtained.

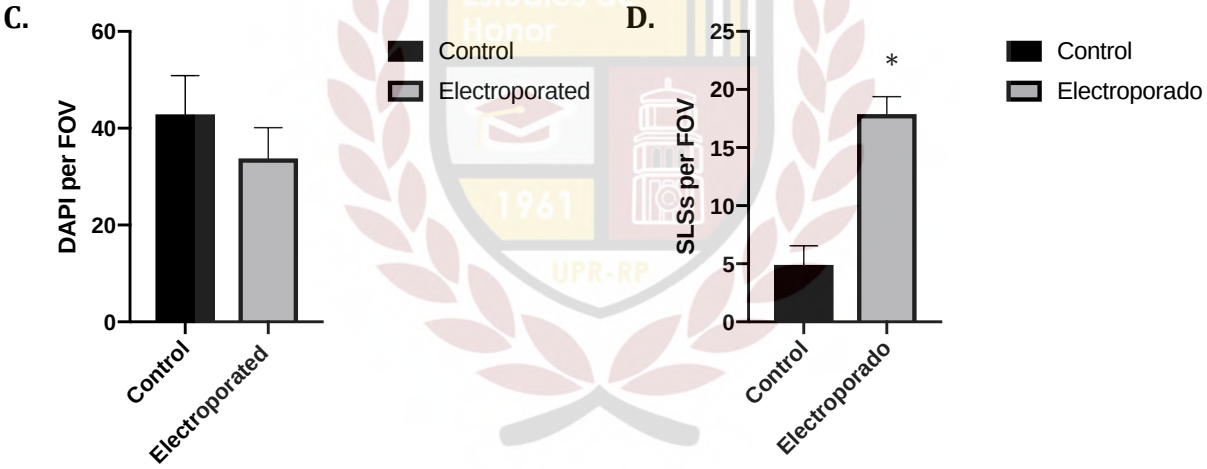
Dedifferentiation ratio quantification

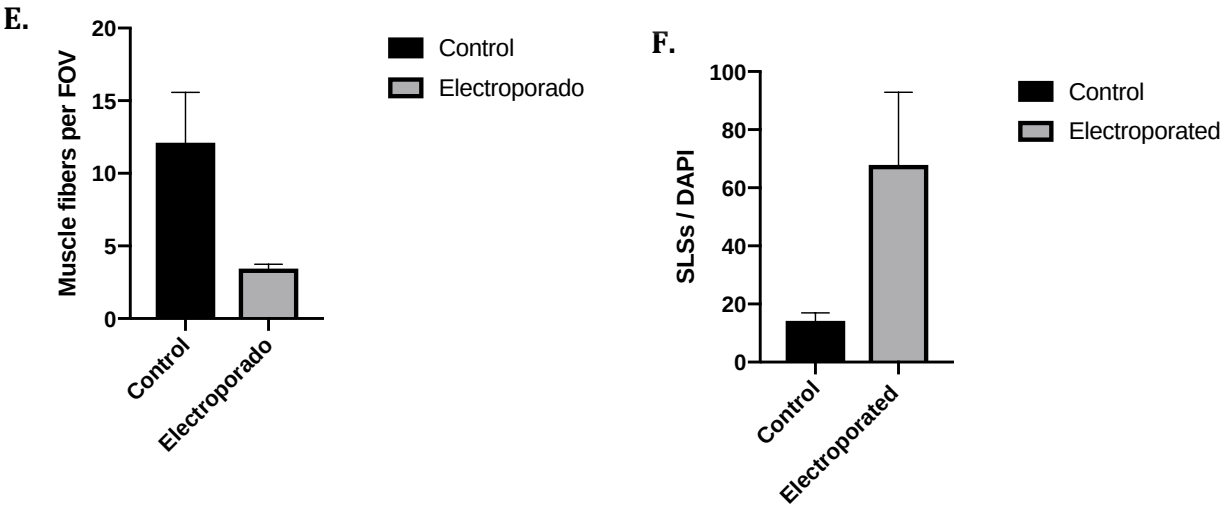
By quantifying the total amount of SLSs and muscle fibers in the overall regenerated intestine tissue, taking into consideration the tissue as a whole, a ratio was obtained in order to measure the dedifferentiation response (**4H**). The control tissue had a ratio amounting to a 0.41 dedifferentiation ratio, while the

electroporated tissue accounted for a 8.9 dedifferentiation ratio. A significant difference was obtained.

The effect of electroporation on cellular dedifferentiation in regenerated intestine tissue- 4 days after electroporation







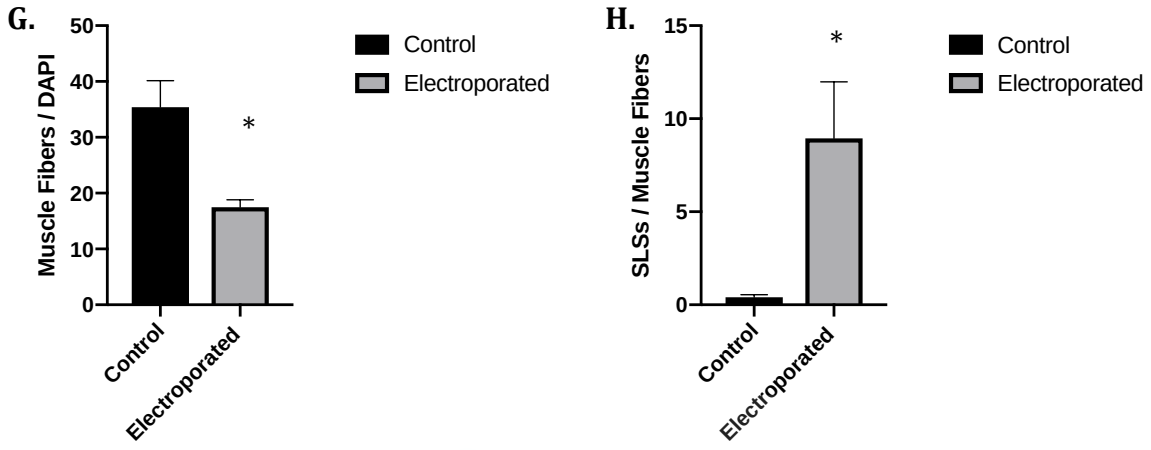


Figure 4. Labeling of cell nuclei and spindle-like-structures (SLSs) to determine cellular dedifferentiation 4 days after electroporation. (A-B) Immunohistochemistry of the cellular dedifferentiation response presented a difference regarding the amount of SLSs when comparing the control regenerated (non-electroporated intact) intestine tissue **(A)** and electroporated regenerated intestine tissue **(B)**. SLSs and muscle fibers were labeled with rhodamine-labeled phalloidin (red) and cell nuclei were labeled with DAPI (green). **(C-E)** The cellular responses were quantified per FOV to see if electroporating and incubating the tissues for 4 days in supplemented media had an effect on the total number of SLSs **(C)**, muscle fibers **(D)**, and cell nuclei **(E)**. **(F-G)** SLSs and muscle fibers were normalized using DAPI and expressed as a percentage of SLSs **(F)** and muscle fibers **(G)**. **(H)** The expression of SLS and muscle fibers were divided to obtain a dedifferentiation response ratio that would allow the quantification of the effect of electroporation. Data expressed as mean ± S.E.M. Analyzed using t-test, two tailed; n=3 per group, *$p < 0.05$.

B. Proliferation

Regenerated intestine tissue 4 days after evisceration, 24 hours in supplemented media culture

Analysis

Through a qualitative analysis of the images taken using a fluorescent microscope, no remarkable differences were observed regarding the proliferation of the control tissue (**5A**) with tissue 24 hours after electroporation (**5B**). To measure the effect of electroporation on proliferation in the regenerated intestine tissue after 24 hours in supplemented media, the cellular results were quantified (**5C**).

Percentage of proliferating cells

To see if electroporating the regenerated intestine tissue tissues had an effect on cellular proliferation BrdU + cells were quantified and normalized in respect to cell nuclei by using DAPI and multiplied by a 100 to obtain a percentage. The percentage of proliferating cells in the control tissue was 2.79%, while it was 7.89% in the electroporated tissue. No significant differences were observed.

Electroporation has no effect on cellular proliferation in regenerated intestine explant culture 24 hours in supplemented media

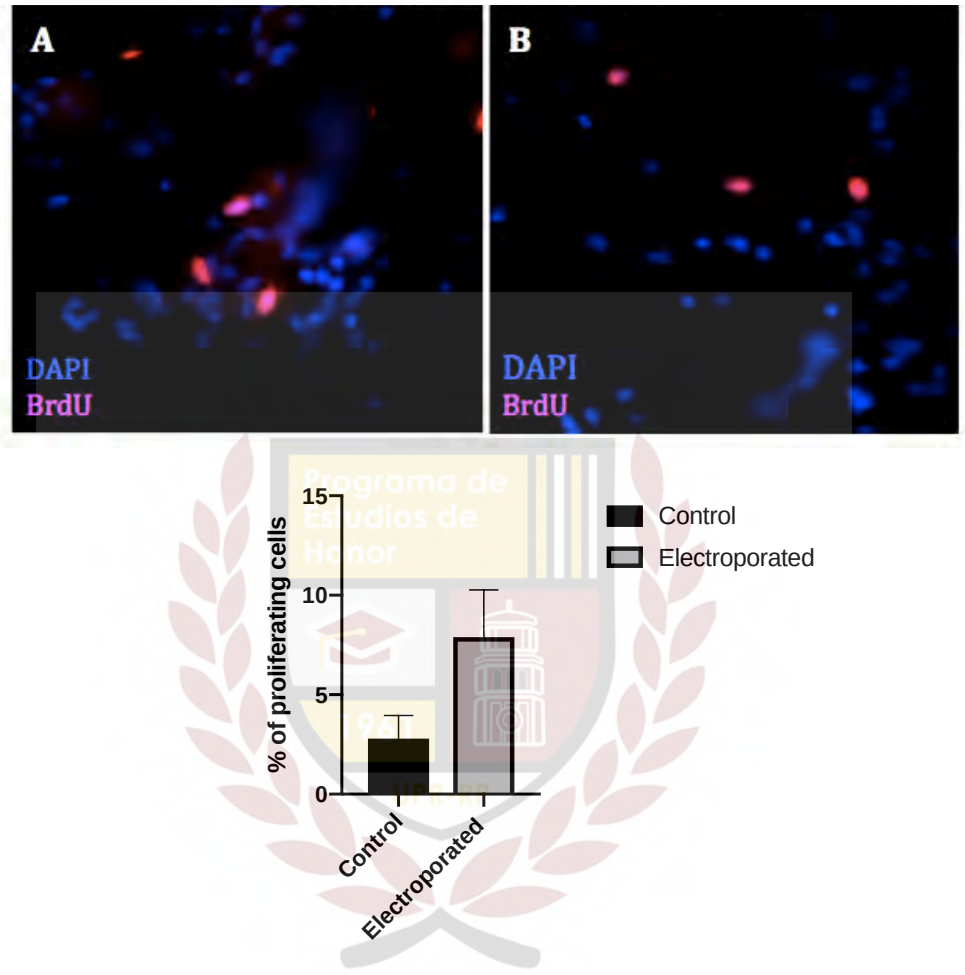


Figure 5. Labeling of cell nuclei (blue) and proliferating cells (magenta) to determine the effect of electroporation on cellular proliferation. (A-B) Immunohistochemistry of the proliferation response showed that electroporation had no effect on this process when comparing control regenerated (non-electroporated intact) intestine tissue **(A)** and electroporated regenerated intestine tissue **(B)**. **(C)** Proliferating cells were normalized using cell nuclei and a percentage of proliferating cells were obtained. Data expressed as mean ± S.E.M. Analyzed using t-test, two tailed; n=3 per group, *p < 0.05.

C. Apoptosis

Regenerated intestine tissue 4 days after evisceration, 24 hours in supplemented media culture

Analysis

Through a qualitative analysis of the images taken using a fluorescent microscope, no remarkable differences were observed regarding apoptosis of the control tissue (**6A**) when compared with tissue 24 hours after electroporation (**6B**). To measure the effect of electroporation on apoptosis in the regenerated intestine tissue after 24 hours in supplemented media, the cellular results were quantified (**6C**).

Percentage of cells undergoing cellular death

In order to see if electroporating the regenerated intestine tissue explants had an effect on cellular apoptosis. TUNEL+ cells were quantified and normalized in respect to cell nuclei by using DAPI, and a percentage was obtained (**6C**). The control tissue showed a total of 3.43, while the electroporated tissue a total of 3.29. No significant differences were observed.

Electroporation has no effect on cellular apoptosis in regenerated intestine explant culture- 24 hours in supplemented media

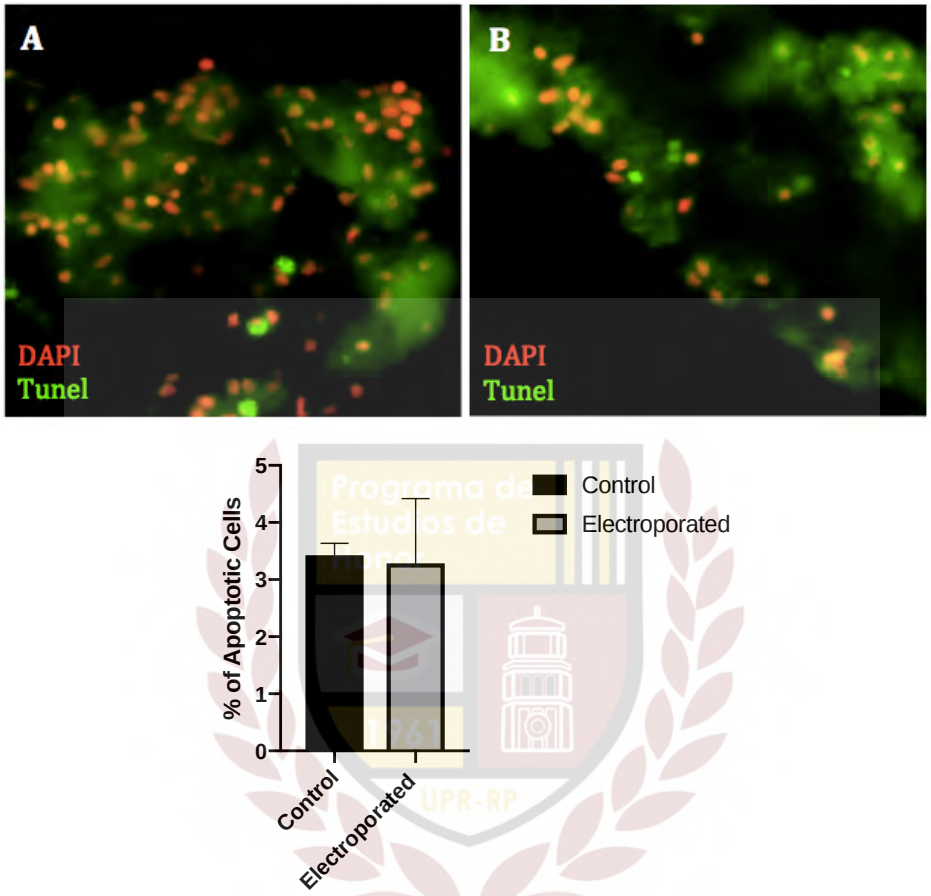


Figure 6. Labeling of cell nuclei (red) and apoptotic cells (green) to determine the effect of electroporation on cellular apoptosis. (A-B) Immunohistochemistry of the programmed cell death response showed that electroporation had no effect on apoptosis when comparing control regenerated (non-electroporated intact) intestine tissue **(A)** and electroporated regenerated intestine tissue **(B)**. **(C)** Apoptotic cells were normalized using cell nuclei and a percentage of apoptotic cells were obtained. Data expressed as mean ± S.E.M. Analyzed using t-test, two tailed; n=3 per group, *p < 0.05.

Chapter 8: Discussion

This work suggests that electroporation is causing an effect on the regenerated intestine tissue regarding at least one of the cellular processes that occurs during regeneration, cellular dedifferentiation. We also showed that the effect of electroporation on cellular dedifferentiation is time-dependent since at 4 hours after electroporation there is not a significant increase on the SLSs/muscle fibers ratio used to define the process of dedifferentiation, but at 24 hours and 4 days a significant increase on the dedifferentiation ratio is observed. The SLSs/muscle fibers ratio was used to quantify this cellular process since previous studies define dedifferentiation as a disorganization of the muscle tissue at the microscopic level and the trademark of this process as the formation of SLSs (García-Arrarás & Dolmatov 2010). As the time after electroporation increases a greater number of SLSs can be observed, while at the same time a decrease in the number of muscle fibers, thus the increase of the SLS/muscle ratio.

The differentiation process occurs in a temporal manner and a spatial gradient can be observed starting in the mesentery at 1 dpe and continuing for two weeks following evisceration. SLSs increase in the mesentery, initially at the tip that used to be attached to the intestine and as time passes towards the body wall as muscle cells disappear from the areas adjacent to the regenerating structure (Pasten et al. 2014). Regenerated intestine tissues were studied as a whole and at different areas where dedifferentiation is known to follow a pattern. Our original hypothesis was that electroporation would cause an increase in the dedifferentiation response, replicating the initial response that is seen during regeneration of the intestine

tissue. The experimental results suggest that the hypothesis is correct, but this depend on the time it takes in order to see an increase in the numbers of SLSs and a decrease in the number of muscle fibers. Thus, as tissues are left in supplemented media, the increase in dedifferentiation as quantified by the number of SLSs per muscle cell was made more pronounced as they were left more time in culture.

Two of the other cellular responses that occur during regeneration are proliferation and apoptosis. These processes are necessary for regeneration to take place and if electroporation affected these responses it would be hard to use the electroporated regenerated intestine tissue as an in-vitro model to study other cellular processes that are associated with regeneration. In our experiments, the 24-hour time point was used to study these two responses since we had sufficient data to support these responses. No effect was seen regarding the process of proliferation, nor apoptosis, which seems to suggest that electroporation is not affecting either processes. The results of electroporation in the three responses are summarized in **Table 1** and on figure **(6A-C)**. Using electroporation to replicate these three initial processes that are crucial during regeneration would allow us to study other processes associated such as genetic expression and other processes by which cells acquire a new phenotype leading to regeneration.

Results are similar to studies that demonstrate that electroporation, an electric field sufficient to open the pores of a membrane, initiates a dedifferentiation response in an organ that is capable of regeneration if the voltage is enough to cause the temporary opening of the membrane but is not sufficient to cause necrosis or apoptosis such as what was seen in newts where electroporation also induced a

dedifferentiation response (Atkinson et at. 2006). Electroporation has also been seen to induce the degenerative/regenerative response in skeletal muscle, where it had been previously been accepted that myofibers were not able to dedifferentiation after an injury had occurred in the muscle (Miyoshi 2012).

These results provide important information regarding the cellular processes associated with regeneration and provide information regarding the potential of electroporation to induce a response that is crucial for this process to occur. This work presents a new perspective concerning the field of regenerative medicine.

Review of the effect of electroporation on cellular dedifferentiation in a time dependent manner

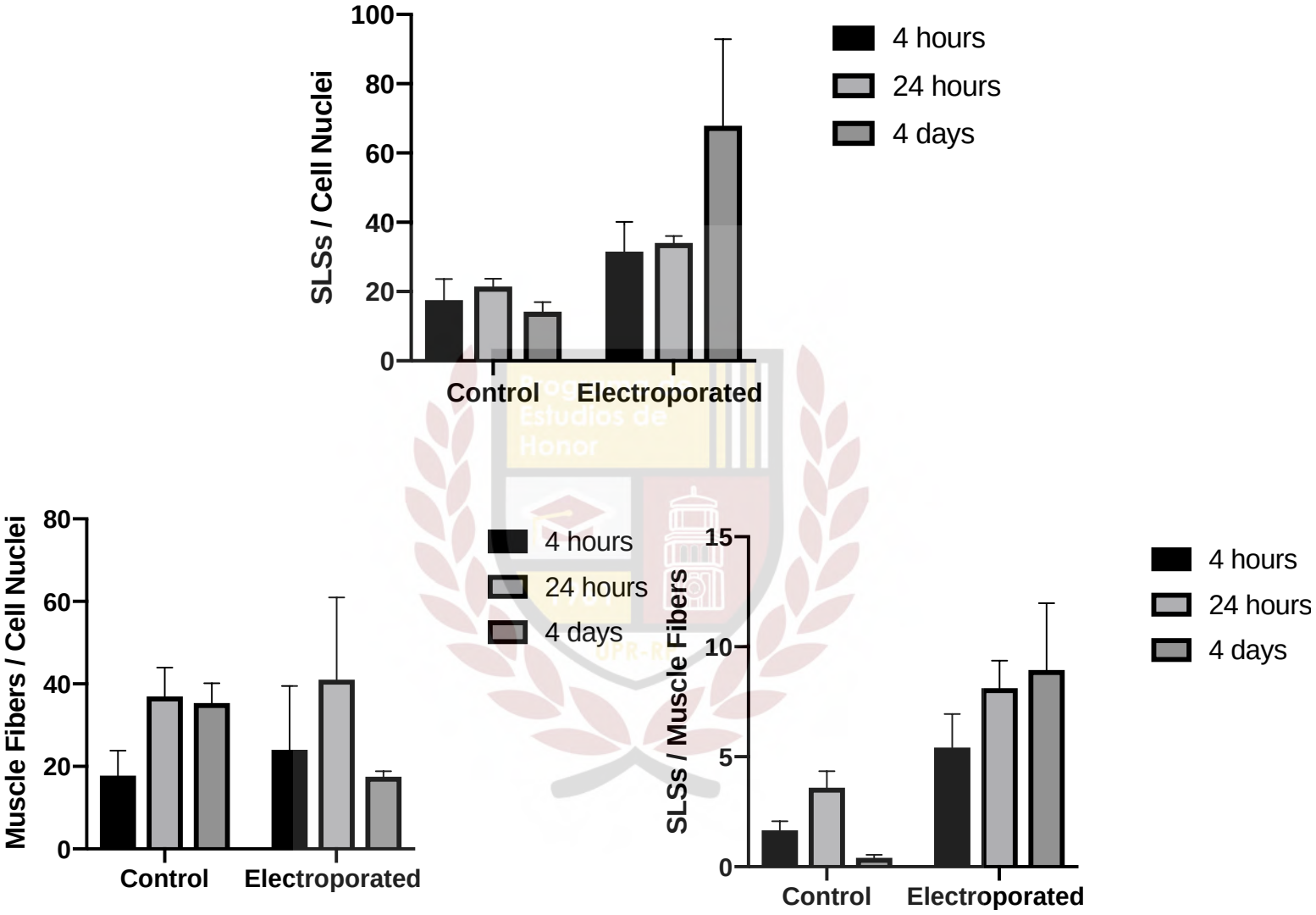


Figure 7. Summary of the effect of electroporation on cellular dedifferentiation regarding the different time-points after electroporation: 4 hours, 24 hours and 4 days (A) An increase is seen in the percentage of SLSs normalized with cell nuclei in the tissue that has been electroporated when compared with control (non-electroporated intact) intestine tissue. **(B)** Percentage of muscle fibers normalized with cell nuclei. **(C)** An increase is seen in the SLSs/muscle fiber dedifferentiation ratio on the electroporated tissue when compared with the (non-electroporated intact) intestine tissue. Data expressed as mean ± S.E.M. Analyzed using t-test, two tailed; n=3 per group, *p < 0.05.

Time in supplemented media:	Dedifferentiation	Proliferation	Apoptosis
4 hours	No effect	X	X
24 hours	Increases	No effect	No effect
4 days	Increases	X	X

Table 1: The effect of electroporation 4 hours, 24 hours and 4 days after electroporation Different cellular processes that are known to be important during the first steps of regeneration were studied and are summarized in order to understand the effect that electroporating the regenerated intestine tissue caused.

Chapter 9: Future work

Even though we were able to answer our questions, and prove our hypothesis to be true some questions are still unanswered. Why is electroporation causing the cells to dedifferentiate? What occurs during these processes that is changing the course of the cellular events? Electroporation alters the cell membrane and opens the pores of the cell allowing an exchange of molecules that otherwise would not have that increase of access through the membrane. Other processes could also be studied such as size of the intestinal rudiment and migration, and see if this technique is inducing a change. A different approach could be taken to understand the processes that are taking place in order to increase dedifferentiation. Also, by using electroporation as a transfection tool different

specific genes could be studied using RNAi in order to understand their role during regeneration.

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