THE EFFECTS OF NEUREGULIN-1 ON MICROGLIAL CELL STATE

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By

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APPROVED

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Abstract

The damage a stroke causes to a patient increases over time due to inflammatory responses around the region of initial cell death. If this inflammatory response can be controlled, the total amount of damage from a stroke may be reduced. Microglial cells act as a main form of active immune defense in the brain. Neuregulin-1 is a drug shown in previous studies to have anti-inflammatory and neuroprotective roles, especially in mice with induced strokes. Application of neuregulin-1 to the microglia cell culture may induce anti-inflammatory phenotypes. During a stroke, microglia cells are activated. This state is replicated by using LPS, a component of gram-negative bacterial cell walls that strongly activate immune cells such as microglia. The levels of the protein act as a measurement to determine the degree of effectiveness of neuregulin-1 influencing the microglia anti-inflammatory cell state when compared to a control without neuregulin-1. Using LPS and neuregulin-1, four conditions are setup for the microglial cells with and without LPS and neuregulin-1. For the conditions involving neuregulin-1, although their apparent density is increased, there is no detectable protein concentration. For the LPS condition, some cells appear to remain in the ramified/inactivated form, indicating the LPS concentration may be too low for activation. The lack of a measurable protein concentration may be due to the prevalence of dead cells.

Acknowledgments

I would like to thank Dr. Byron Ford for allowing me into his lab for a unique, clinically-relevant lab experience which I hope to carry on the knowledge into medical school. Moreover, I am thankful for the opportunity to learn about and experience lab techniques in a professional setting, allowing me to apply what I have learned through my courses. Moreover, I would like to thank Catherine Augello for guiding me through the process of this project, suggesting protocols which help me attain relevant information, and ensuring my technique is perfected.

Table of Contents

Introduction

In order to fully appreciate stroke research, it is important to acknowledge the severity of stroke. In 2017, a study by the American Heart Association approximated about 795,000 strokes each year in the US alone occur and is the leading cause of disability (Benjamin et al.). However, not all strokes are the same, and one form is much more prevalent over the other. Of the two major types of strokes, hemorrhagic strokes occur in about 13% of the population that suffers from stroke. (Benjamin et al., 2017). This form of stroke occurs when a blood vessel bursts and the surrounding region is denied a consistent flow of blood. The more prominent form, ischemic strokes, are responsible for roughly 87% of all strokes, and are the focus of this research. Unlike hemorrhagic strokes, ischemic strokes involve the clotting of a blood vessel in the brain, denying access to blood in the local region.

One reason why stroke causes a large amount of brain damage is not because of a direct cause from the stroke, but from genes introduced related to oxidative stress, apoptosis, and inflammation. (Pulliam et al., 2013). The damaged area directly caused by the stroke is known as the core but can indirectly damage an area as large as the penumbra if left untreated. (Figures 1 and 2). Fortunately, Neuregulin-1 treatment has shown a large decrease in total subcortical infarct volume by about 40% in rats by blocking microglial infiltration and astrocytic activation. (Xu, Jiang, Ford, Ford, 2004).

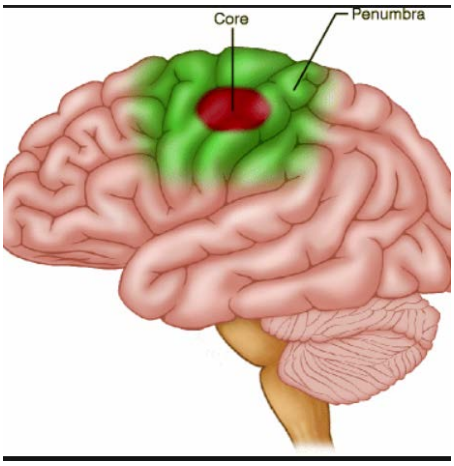

Figure 1. The core is the region of the brain immediately damaged by a stroke. Over time, the damage may spread to the entire penumbra. (Image Source: https://radiologykey.com/stroke-and-its-imaging-evaluation/)

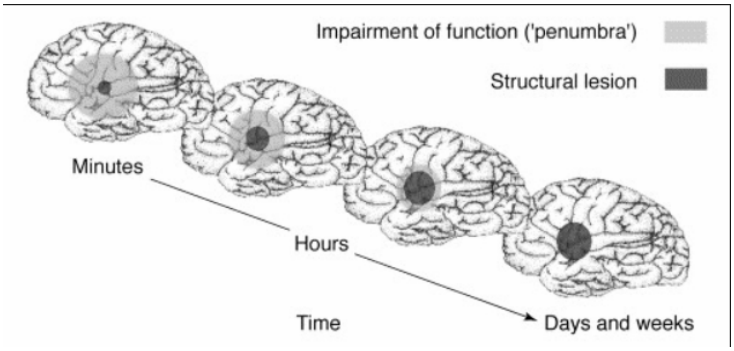

Figure 2. An illustration of the amount of time typically required to expand in the penumbra. (Image Source: http://www.cell.com/trends/neurosciences/fulltext/S0166-2236(99)01401-0)

If Neuregulin-1 (NRG-1) promotes anti-inflammatory and neuroprotective roles in rats, then would there be a way to study this effect in vitro? A study by Xu and colleges (2005) demonstrated that the inflammatory response in macrophages was inhibited by NRG-1 application. This suggests an in vitro study is possible. Xu and colleagues (2004) demonstrated the activation of astrocytes and microglial infiltration were partly responsible for the death

associated in the penumbra region of a stroke. Because of this, microglia are identified as a target for experimental cell-line studies.

In this study, the protein content and cell status of microglia are analyzed with and without NRG-1. The cell line SIM-A9 is chosen as it is an immortalized mouse microglial cell line. Microglial cells act as the main form of active immune defense in the brain; surveying and adapting to whichever form that is best suited to protect the brain (Nimmerjahn, Kirchhoff, & Helmchen, 2005). As demonstrated by Xu and colleagues (2004), microglial cells become activated during a stroke. Figure 3 illustrates the form of microglial cells when activated, where the bottom is activated, and the top is inactivated.

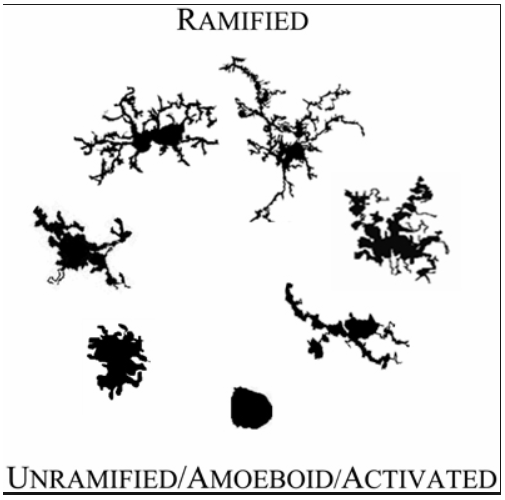

Figure 3. The shape of microglial cells is dependent on its activation state. Fully ramified cells are inactivated, and amoeboid cells are activated. (Image Source: http://jonlieffmd.com/blog/are-microglia-the-most-intelligent-brain-cells)

Unlike a stroke, in vitro cell-line studies do not have a blood vessel that can be blocked to induce stroke. Fortunately, activation of microglia is achieved by using lipopolysaccharide (LPS), a highly activating component of the gram-negative bacterial cell wall. (Werts et al., 2001). LPS is also known as “endotoxin,” and helps illustrate why microglia, the main cell type

for active immune defense in the brain, may be activated. Figure 4 shows how LPS affects microglial cells to become more activated/amoeboid.

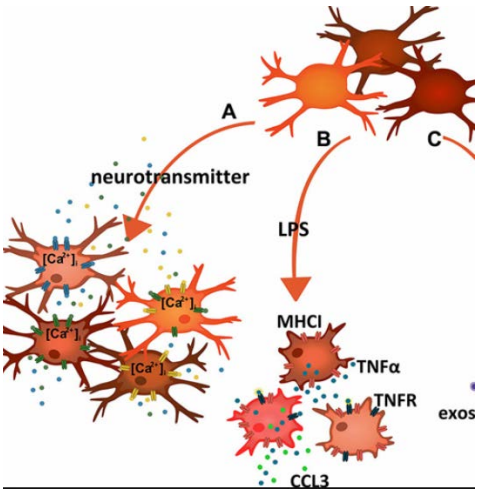

Figure 4. Inactivated microglial cells may be activated with LPS, simulating stroke. (Image Source: https://www.frontiersin.org/articles/10.3389/fncel.2014.00101/full)

Because of these studies, an experiment using the SIM-A9 mouse microglial cell line will be used to study the effects of NRG-1. LPS can stimulate a stroke and create conditions where NRG-1 is applied to both activated and inactivated microglial cells. Based on previous studies, it is expected that the cells treated with NRG-1 and LPS will proliferate when compared to those treated with only LPS. Any cells treated with LPS are expected to take a more activated form than the cells untreated with LPS. The protein content will be analyzed and expected to be different for each of the four conditions with NRG-1 and LPS.

Methods

Original samples of SIM-A9 mouse microglial cells were ordered and stored in liquid Nitrogen until they were ready to be revived. These cells were frozen in serum with DMEM/F12 and 10% DMSO. The DMEM/F12 was used to support the growth of mammalian cells, and DMSO prevents the formation of ice crystals that would otherwise rupture the cells. Once a new cell line was required, the cells were heated in a 37℃ water bath until liquid, then plated into a

75mm culture flask. To grow and maintain the culture, the cells were supplemented with about 8mL of media containing DMEM/F12, 5% heat-inactivated donor horse serum, 10% heat-inactivated fetal bovine serum, and 1.5 micrograms/mL penicillin/streptomycin/neomycin. The serums provided growth factors and nutrients to the cells that allow growth. Because growing cells requires sterile conditions, antibiotics were added and we worked under a sterile fume hood. The cells were attached to the surface of the flask in an incubator at 37°C overnight. Once the cells attached, they began to show flattened and ramified characteristics when viewed with 100x magnification.

Now, these cells can be used for either maintenance or for well-plating. For an earlier cell line, maintenance protocols were used for additional cell growth because the well-plating protocol was not ready. For maintenance, every couple days the old serum-containing media was replaced with new serum-containing media. This was done by aspirating the old media and adding 8mL of new media. Before removing the old media, the cell confluency was checked by looking at the cells under 100x magnification and estimating the percentage of area covered by the cells. When this approximated percentage is about 80%, then the cells are ready to be split.

Cell splitting is a protocol that detaches and dilutes the cells from the flask to be regrown in a new flask. This is an important step as at a high confluency where cell crowding becomes an issue, and may cause cells to enter the stationary phase of cell growth; the log growth phase allows for the best data when applying the excitation conditions. To split the cells, the old media was aspirated, and the cells were washed with cold PBS to expose the binding sites on the cells to the Trypsin. The cells were gently shaken for 30 seconds, then aspirated the PBS and added a Trypsin/EDTA solution of 0.25% to allow Trypsin to remove the cells from the surface of the flask. EDTA was used to weaken interactions by removing Calcium and Magnesium. The flask

was gently shaken until most of the cells have been detached and floated in the solution. Detached cells should have a more spherical and shadowy appearance when viewed with 100x magnification. Once the flask showed about 5% confluency, the cells were transferred to a 15mL centrifuge flask, and run at 200g for 3 minutes. The supernatant was removed, and the cells were resuspended in 10mL media. Using a new 75mm culture flask, 0.5mL of the resuspended cells were added to about 8 mL media for a 5% dilution. The remaining 9.5mL of cells were discarded by using proper sterilization techniques (10% bleach solution was added). The cells attached in this new flask overnight in an incubator at 37℃.

When the cells were ready to be plated in the wells, the same protocol for cell splitting was used, except added serum-free media when resuspending the cells. Serum-free media help ensure the proper seeding density when plating. Once the cells were resuspended in serum-free media, 4 microliters of the cells were added onto a slide and counted in a hemocytometer. For a 12-well plate, the cells need to be $1.0x10^5$ cells/mL, but the hemocytometer showed a count between $0.4x10^5$, and $1.2x10^5$ depending on the area counted, 1mL of this dilution was added to each well. The cells sat overnight in an incubator at 37℃. Figure5 shows these cells attached to a well.

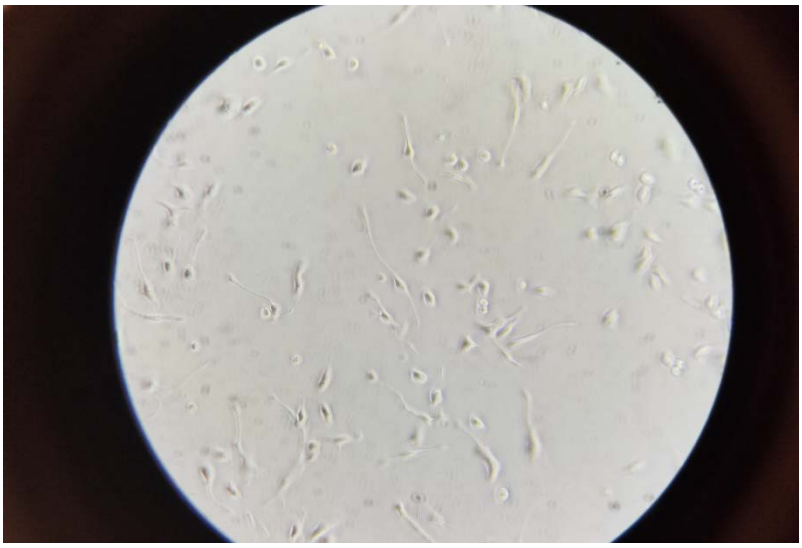

Figure 5. Overnight growth of SIM-A9 cells plated onto a 12-well plate. Their ramified form indicated inactivation.

Now, all 12 wells were aspirated to remove the serum-free media. In 3 wells, 1mL of serum-free media containing 100ng/mL NRG-1, 5ng/mL LPS, and 1% penicillin/streptomycin by volume was added. In another 3 wells, 1mL of solution containing the same concentration of NRG-1 and antibiotics was added. In another 3 wells, 1mL of a solution with the same concentration of LPS and antibiotics were added. In the last 3 wells, 1mL of serum-free media with antibiotics was added as a control. These cells were exposed to the conditions for 2 days in the incubator at 37℃.

When the cells were exposed to the conditions for 2 days, the media was aspirated from each well, and each well was washed with PBS once for 30 seconds, then aspirated. 200 microliters of M-PER containing 2 microliters protease inhibitor was added to each well. M-PER is used to extract protein from mammalian cells by using a detergent to allow for protein analysis. The wells were then shaken for 5 minutes. Each well was transferred into a microcentrifuge tube and centrifuged at 14000x g for 9 minutes. The supernatant was then used to run a Bradford Protein Assay

A 96-well plate was used for the Bradford assay, applying 5 microliters of the 8 standards of BSA for protein concentrations, then 5 microliters of the 12 supernatants collected. 250 microliters of the Bradford 1X dye Reagent was added using an 8-channel 300 microliter pipette. The plate stayed 5 minutes in room temperature, then was recorded on the plate recorder. Bradford dye is used to visualize a protein concentration measurable by the plate recorder.

Results

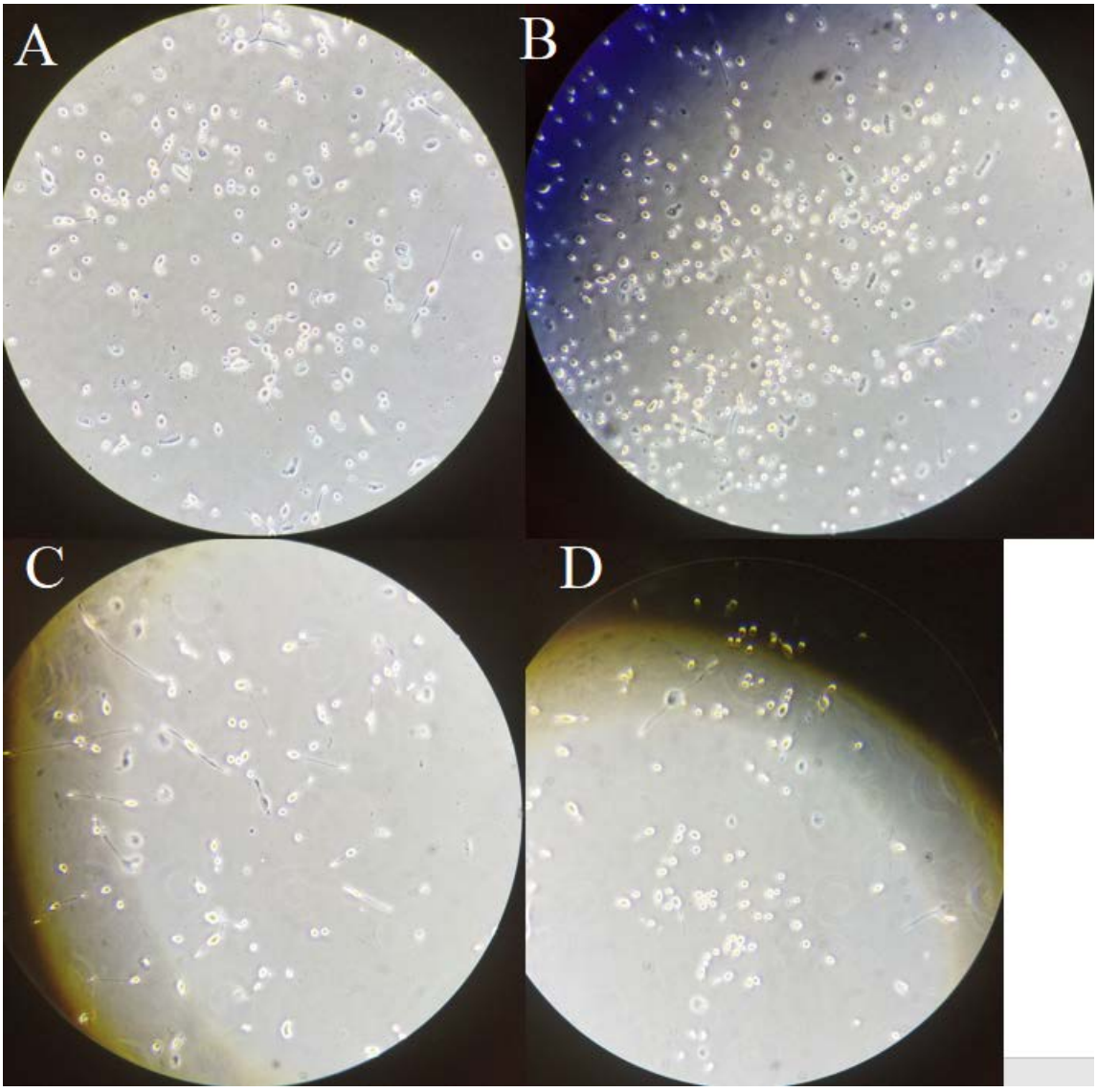

Figure 6. Growth after 2 days of exposure time to (A) LPS and NRG-1, (B) NRG-1, (C) LPS, and (D) control.

After applying the three excitatory conditions and control to the 12 wells, a 2-day exposure time revealed growth patterns and microglial states as shown by Figure 6. Conditions C and D show about 100 cells each in equal areas (counted manually and rounded to account for human error). These two wells displayed cells in both activated and inactivated forms. Condition A shows about 200 cells that are mostly spherical/activated, and condition B shows about 400 cells that are also mostly spherical/activated. All conditions showed cells with signs of death,

shown by the brown dot on spherical, dislodged cells. When shaken, those cells move in the liquid, indicating they are dislodged.

The Bradford Protein Assay (Figure 7) could not detect any protein from the 5 microliter samples used in the 96-well plate, with exception to the third control, which yielded a protein concentration of 0.019 (unknown units).

	A	B	C	D	E	F
41	Sample Report:					
42	Sample ID	Well	Replicates	Mean	Conc	SD (Conc)
43	1	D5	0.471	0.47	(-)	0.004
44		D6	0.468			
45	2	E5	0.448	0.451	(-)	0.008
46		E6	0.454			
47	3	F5	0.453	0.454	(-)	0.004
48		F6	0.456			
49	-1	D3	0.436	0.438	(-)	0.004
50		D4	0.439			
51	-2	E3	0.451	0.448	(-)	0.008
52		E4	0.445			
53	-3	F3	0.504	0.481	(-)	0.059
54		F4	0.457			
55	-1	A5	0.481	0.482	(-)	0.001
56		A6	0.482			
57	-2	B5	0.487	0.488	(-)	0.003
58		B6	0.489			
59	-3	C5	0.482	0.49	(-)	0.02
60		C6	0.498			
61	1	A3	0.469	0.476	(-)	0.018
62		A4	0.483			
63	2	B3	0.503	0.493	(-)	0.025
64		B4	0.483			
65	3	C3	0.529	0.526	0.019	0.009
66		C4	0.522			

Figure 7. The Bradford protein assay highlights the concentration of protein in each well. Column E displays the concentration for only one well in the control condition. All other concentrations were too low to be detected.

Discussion

Conclusion

From the analysis of the 4 conditions in Figure 6, the apparent cell density of the conditions treated with NRG-1 (A and B) were much higher than those not treated with NRG-1 (C and D). However, because the protein concentrations from the Bradford Assay were too low

to be detected, it is inconclusive if NRG-1 influences cell proliferation. Moreover, by comparing conditions C and D, condition C still shows microglial activation despite the addition of LPS, so the concentration of LPS may have been too low to be effective.

While a greater density of cells is expected to produce more protein, this did not seem to be the case for the conditions with NRG-1. Upon looking more closely at the condition of the cells excited with NRG-1 and LPS compared to a maintenance culture not exposed to NRG-1 and LPS (Figure 8), the former condition shows cells which display characteristics of poor cell health: round, detached shape with a brown dot. Compared to the maintenance culture, the cells are spherical because they were recently resuspended with Trypsin/EDTA, but they also lack the brown dot. Since the wells exposed to NRG contained mostly dead cells, the lack of detectable protein concentration was justified.

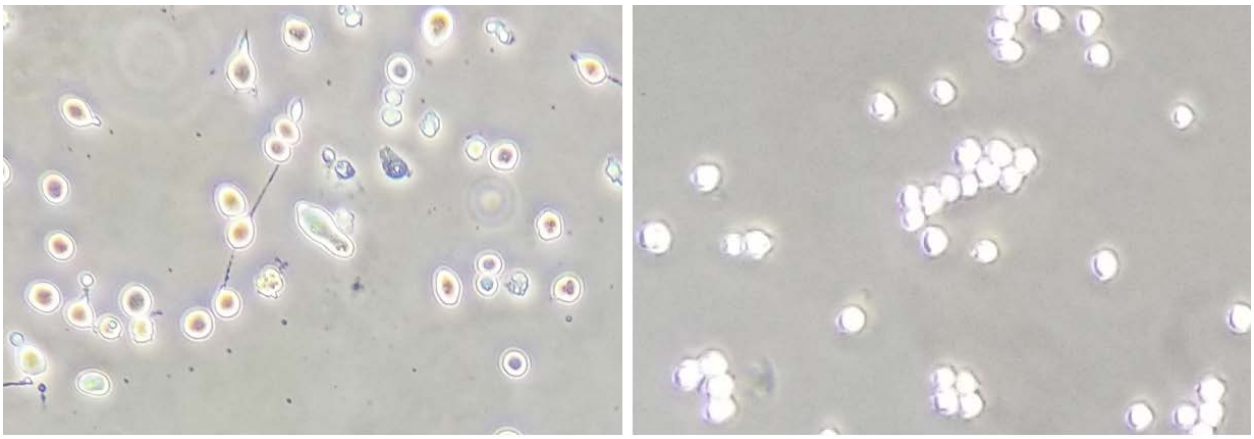

Figure 8. A comparison between the SIM-A9 cells exposed to both NRG-1 and LPS (left) compared to a different line of SIM-A9 cells not exposed to NRG-1 or LPS (right). The shape and color of the cells indicate health.

Error Analysis

In this experiment, SIM-A9 cells were plated exclusively with itself, and with no other cells were present. In the brain, a variety of cells are present, and does not reflect the environment presented in the experiment. To obtain better data, it may be reasonable to include a

variety of cells growing alongside the microglial cells. While we can study microglial cells by isolating them, their functions may be different without the surrounding neurons.

Moreover, the low cell density may have been problematic for the growth and proliferation of the microglial cells. When plating the cells, it was suggested to use the seeding density of 1.0×10^5, but the reading for cell density measured anywhere between 0.4×10^5 and 1.2×10^5 depending where on the slide the density was taken. The low cell density may have caused the microglial cells to lose contact with other microglial cells. Because microglial cells are naturally in contact with other cells, removing contact may cause a different expression of proteins which may lead to death.

Comparing conditions C and D in Figure 6, condition C appeared to display cells similar in activation to those of D. This seems to suggest the current concentration of LPS was not high enough to cause activation of the microglial cells, and a higher concentration is needed.

Lastly, when splitting the SIM-A9 cells into the 12-well plate, confluency was below 70%, indicating the cells may have been in early log phase or late lag phase, which may have reduced the effectiveness of the exciting conditions.

Future Directions

While this experiment can improve, it opens opportunities for future research. With the knowledge from this experiment, future research can include a variety of cells to better mimic the condition of the brain. This may allow for more accurate data as microglial cells will be interacting with a variety of cells as they normally do. Furthermore, this study examined a single level of each variable. With future research, a varying level of LPS and NRG-1 may be added to determine what works best.

Once a higher concentration of protein can be attained, future research can employ the use of Western Blotting protocols, analyzing specific proteins of interest across the various conditions. Once a protein of interest is identified, cross-referencing with animal models of similar research would allow for correlational analysis that help strengthen the hypothesis.

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