Investigating the Effects of Human Serum Albumin and Mouse Serum on Proliferation of Neural Precursor Cells

Abstract

Organismal aging is correlated with reduced cell performance. Our research investigated the response of cells to the age of the environment, as this could help develop techniques to combat aging's negative consequences. Researchers have planned a surgery in which half a mouse's blood is replaced with "neutral" blood. To understand whether this could yield positive results, we investigated two important neutral blood components: human serum albumin (HSA) and blood serum (young (YS) and old (OS)).

We concentrated on the brain, so our in-vitro investigation used neural precursor cells (NPCs). We exposed NPCs to HSA and serum, ranging from 0% to 4%. We then immunostained the cells with BrdU (a marker of cell proliferation). This allowed us to visually quantify the percentage of multiplying cells under each set of conditions. We did 4 repetitions to minimize variability and firmly establish trends.

We found that albumin consistently encourages cell proliferation, while serum impaired cell growth, OS more so than YS. Small quantities of serum may be marginally beneficial, but their potential for harm is much greater. Overall, the systematic, thorough approach of this research allows researchers to build on their surgical and human plasmapheresis models, leading to greater efficiency in future anti-aging investigations.

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1 Introduction

It is well established that increased age of an organism is directly correlated with reduced performance of a variety of cell types, including T-cells, hepatic cells, neurons, and stem cells.[3,4,5] These impacts extend beyond the cellular level and eventually affect tissues and entire organs. The resulting effects on the human body include age-related diseases, such as immunodeficiency and inflammation, which arise from changes in cell behavior.[5] One branch of research investigating aging and its consequences, from the cellular to the organismal level, has focused on the environment of cells and tissues. Investigating the relationship between cells' deterioration and the age of their environment could provide insight into the factors governing the growth and development of maturing cells, allowing researchers to combat the negative consequences of aging at the source.

Some anti-aging investigations have involved the process of surgical parabiosis, the technique in which two organisms are connected permanently via blood vessels such that their mixed blood intermingles. This process reveals how one animal's cells, tissues, and organs behave in the presence of substances dissolved in a different animal's blood. In a previous experiment using heterochronic parabiosis, one mouse 3 months of age (young) and another mouse 23 months of age (old) were linked and they functioned as one entity.[1] The study found that the cells and tissues of the old mouse saw increased performance while opposite effects took place in those of the young mouse.[1] Specifically for the brain, the organ around which this report is centered, neurogenesis, neuronal plasticity, and cognition all deteriorated in the young mouse's brain and improved in the old mouse's brain after parabiosis.[6,7] These researchers quantified cell performance using Ki67, a common cell marker protein for proliferation.[1,18] Despite these developments, the complexity of parabiosis made it difficult to isolate and study a single variable in depth.[1] Specifically, the shared organs and circulatory system of the animals after the permanent parabiosis procedure allowed them to eventually adapt to each other's presence, and this change during the experiment introduced uncertainty regarding the cause behind each observed effect.[11] To refine the investigation, researchers at our mentor's institution developed

a unique blood exchange system, which allowed animals to be temporarily connected, reducing the above-mentioned uncertainty.[1]

Our mentor's research discovered that when differently-aged mice shared blood but not organs, the young mice saw muscle and liver cell impairment while the old mice saw some rejuvenation.[1] Stem cells in young animals are better able to divide than the ones in older animals, so the frequency of cell division was the defining indicator of age in this research. However, in the brain, the young mice experienced rapid, significant negative impacts on hippocampal neurogenesis due to the blood from the older mouse but the old mice didn't see an impact either way.[1] These effects were quantified using the rate of cell proliferation (percentage of cells in a given sample that have divided after a certain period of time). Considering the results of this blood-exchange, it is unclear why the young mouse brain tissue was negatively affected. There are 3 possible explanations:

1. Inhibitory factors were introduced to the young mouse from the old mouse's blood.
2. Regenerative factors present in the young blood were lost.
3. A combination of the previous two.

Additionally, why didn't the old mouse's neurogenesis or cognition benefit? Building upon the results observed in these parabiosis and blood-exchange experiments will refine our understanding of the aging process and the connection between a cell's environment and its growth. Gaining a thorough understanding could point to techniques to counteract the effects of aging more effectively, or at least mitigate the symptoms of age-related diseases.

One possible experiment to help answer the questions introduced by the heterochronic blood-exchange research would be to replace one of the animals with "neutrally aged" blood solution.[11] Instead of a mouse-mouse blood-exchange, we would exchange mouse blood with neutral aged blood. The replacement neutral blood used in the procedure would contain intact red blood cells, white blood cells, half the normal platelet and plasma contents, and the remainder would consist of purified mouse serum albumin. We can create the neutral blood by first reverse-engineering a sample of mouse blood and then reconstituting the components after replacing half the plasma with albumin and saline. This proposed in-vivo procedure isolates a single variable (age of the mouse in the experiment) and we can observe the results in its brain. For example, if we were to perform the procedure on an old mouse, half of its blood volume would be replaced with

neutral blood solution. If we observed beneficial impacts in the old mouse's brain, then we could attribute the outcome to the presence of inhibitory factors in old mouse blood (half of which left the mouse during the procedure). If we were to see little to no rejuvenation, then since the neutral blood has no "young" factors (only albumin), the previous heterochronic blood-exchange results might have resulted from pro-proliferative factors in the young mouse's blood. Beneficial impacts are quantified using the rate of cell proliferation at a given location.

Our research focused on an in-vitro model to establish the viability and preliminary expectations from the neutral blood exchange procedure in which the mouse would have half of its plasma content replaced with albumin and saline solution. In our cell cultures, we used human serum albumin (HSA, or albumin for short) instead of mouse serum albumin for logistical reasons. We prefer to use mouse serum albumin in-vivo to avoid immunorejection by the live animal. However, the two types of albumin are expected to affect cells in the same way due to their similar structure, so we opted to use HSA in our in-vitro study (HSA and albumin are synonymous from this point forward).

In order to model the brain, the organ of interest for our report, we used hippocampal neural precursor cells (NPCs) to mimic the brain's behavior in-vitro. Mouse blood serum is identical to plasma but contains more growth factors and fewer clotting factors, so mouse serum was used in the in-vitro studies as a substitute for plasma. Albumin refers to the protein that transports a variety of solutes through the blood and contributes to osmoregulation. HSA was used because previous research suggests that it may encourage cell growth; it plays a role in the transport of ligands, amino acids, ions, hormones and such solutes.[15,16] The previous research combined with the plan for future experiments such as the neutral blood exchange led us to investigate a basic question: If the idea behind the in-vivo experiment is to replace half of a mouse's plasma content with albumin and saline, then what would happen if we did the same in-vitro, swapping serum for plasma? What effects will we observe in the NPCs, and how do these results connect to what was seen in the parabiosis and heterochronic blood exchange research?

Our first idea was to carry out a halfway replacement in-vitro, so the cells' medium contains just as much serum as albumin, as compared to all serum. We soon realized that our proposed procedure would confound our understanding of the results. The half-serum, half-albumin

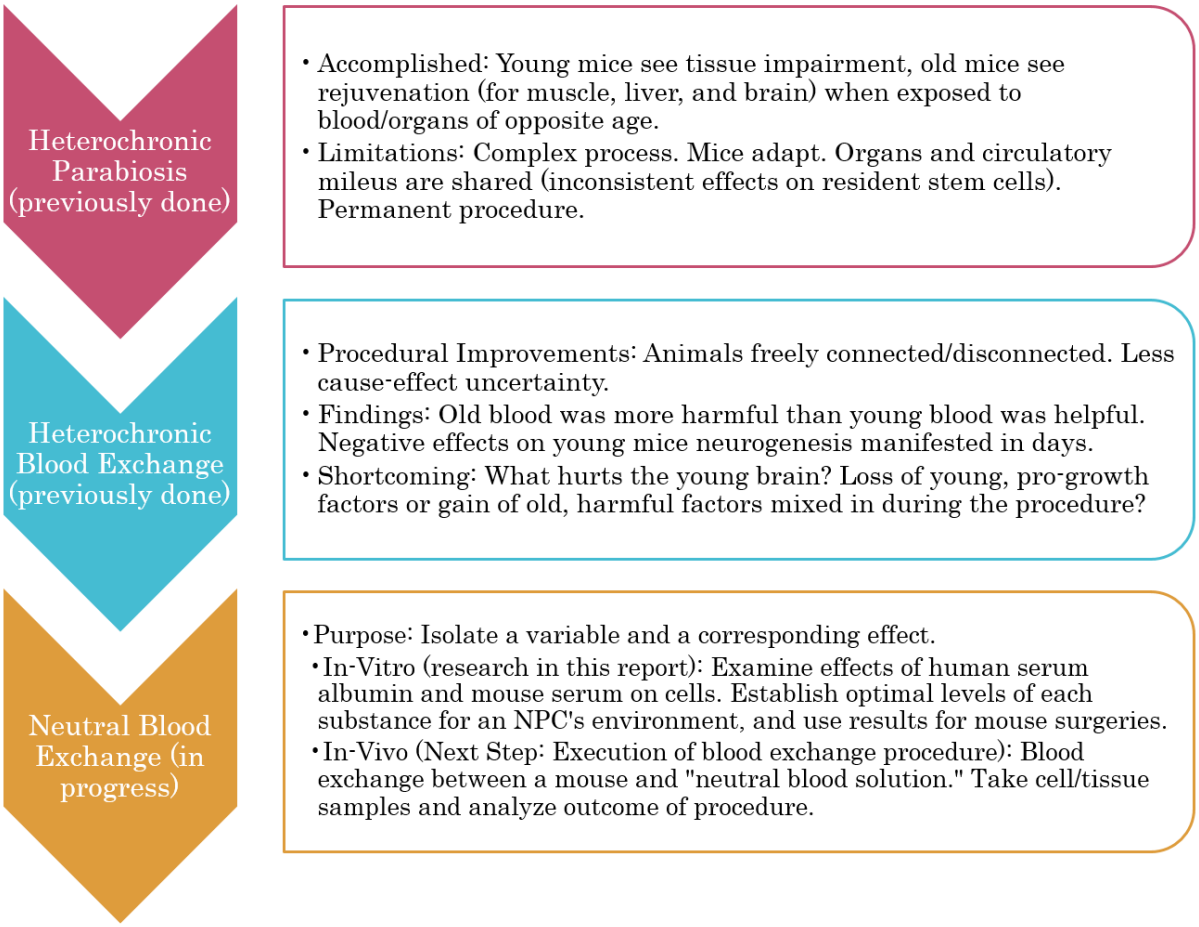


Figure 1: Visual representation of the logic behind the progression from previous research to that presented in this report. Each one builds on the findings of the previous while adjusting for limitations and answering new questions.

switch is a two-variable experiment. If the replacement increased cell proliferation, it would be difficult, if at all possible, to discern whether the benefits arose from reducing the serum, adding albumin, or both. Although albumin may benefit cell proliferation, we must confirm these findings for three reasons. First, the neural precursor cells may behave differently from more traditional culture cells such as myoblasts, fibroblasts, or endothelial cells which were used in the published research. Second, the albumin may behave or interact with cells differently in the presence of serum. Finally, the effects of albumin on the growth of cells in culture is not well explored. We therefore began by carrying out albumin against serum dilutions to clearly establish the impact of these substances on the degree of a neural precursor cell's proliferation. We began with this set of experiments because we hoped it would give us a clear idea regarding what to expect from the in-vivo experiments, and any discrepancies between the in-vitro and in-vivo models could be further investigated. A summary of the research described plan is in

Figure 1, which clarifies what has been done, the motivation behind what is in progress, and the developments made from one step to the next.

These experiments were designed to investigate the potential for mitigating or reversing the negative consequences of aging, including cell function deterioration which manifests in tissue degradation, immunodeficiency, and autoimmune diseases. The neural precursor cells are designed to mimic the function of brain cells (and we can infer from the results how the brain itself would behave under these conditions). Any findings could yield insight into potential methods of controlling age-related diseases and could also point to promising avenues of further research.

2 Methods

2.1 Dilutions of Albumin against Serum and Young Serum against Old Serum

2.1.1 96-well Plate Preparation

Neural precursor cells (NPCs) were harvested from young mice and cultured in the laboratory for use in cell culture. These NPCs were plated in growth medium on a 10cm diameter cell culture plate at 20% confluence, which is a measure of the percentage of a plate's area covered by cells. The NPC growth medium formula consisted of 50% each of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F10, a nutrient medium that supports cell growth in serum-free environments. 1% penicillin and streptomycin antibiotics and 0.5% N2 supplement were added to the basal medium. N2 contains some components found to replace serum, including insulin, transferrin, and antioxidants.[9,11] There were no fibroblast-growth-factors (FGF) in the medium because we did not want the NPCs to grow at their optimal rate. We were not certain what the effects of serum and albumin would be on proliferation, so the lack of FGF leaves room for these compounds to either increase or decrease proliferation relative to the baseline. The NPCs grew in the petri dish until they reached 80% confluence.

The cells were harvested off of the petri dish by washing with phosphate-buffered-saline (PBS) and adding 0.05% trypsin. The trypsinization helps detach the cells. However, because concentrated trypsin can digest cell contents, the cells were quickly triturated and added to 2.5 mL of a different growth medium. We used this medium because it included bovine growth

serum, which has trypsin inhibitors. The cells and medium were transferred to a 15 mL tube and the cells were pelleted in a centrifuge. The cells were re-suspended in NPC growth medium. A 96-well plate was coated with extracellular matrix (ECM) in preparation for the addition of cells. ECM enhances cell adhesion onto the bottom surface.

The NPCs were transferred to the 96-well plate at 20% confluence. This plate was then separated into different regions, each receiving different proportions of young serum (YS), old serum (OS), and HSA, ranging from 0% to 4%. 4% was tested to be the optimal maximum quantity that allows us to examine a range of values with narrow enough increments to establish a trend.[11] Each well had a capacity of 150 µL, so a 4% albumin, 2% YS well contained 6 µL of albumin, 3 µL YS, and 141 µL NPC medium. The left half of the plate determined the combined effects of HSA and serum on overall cell proliferation. The right half varied both YS and OS to examine how they affect proliferation when present together in an environment. Tables 1 and 2 depict the layouts of the left and right halves of the 96-well plate.

		1	2	3	4	5	6	7
		YS(%)			OS(%)			
	HSA(%)	4%	2%	1%	4%	2%	1%	No Serum
A	4%							
B	2%							
C	1%							
D	No HSA							
E	4%							
F	2%							
G	1%							
H	No HSA							

Table 1: The left half of the 96-well plate used in the in-vitro study. The quantities of HSA and serum were simultaneously varied to observe the effects of those variables on overall cell proliferation. The YS and OS were manipulated independently so there was no cross-interaction between them. The "No Serum" column and the "No HSA" rows were used as negative controls. The top and bottom halves of the plate acted as duplicates to increase the size of the data set and help discern experimental errors that may be present for a given row.

2.1.2 Determination of Cell Proliferation by Uptake of BrdU

Once the cells were added to the plate, they were left in the incubator overnight to allow sufficient time to adhere to the ECM and react to the substances in their environment. 100 µL of 3M HCL was added to each well to denature and split the DNA. The acid helped antibodies and staining indicators access the DNA which was previously coiled around histones; the HCL

		8	9	10	11	12
		OS (%)				Growth Medium
	YS(%)	4%	2%	1%	No OS	
A	4%					BrdU+
B	2%					BrdU+
C	1%					BrdU+
D	No YS					BrdU+
E	4%					BrdU-
F	2%					BrdU-
G	1%					BrdU-
H	No YS					BrdU-

Table 2: The right half of the 96-well plate used in the in-vitro study. This side was used to vary the relative quantities of YS and OS. There was no HSA added to these wells. The bottom half served a duplicate of the top half. The Growth Medium column acted as a positive control for cell proliferation and BrdU, explained in the following subsections.

was also added before the immunostaining process described in the next subsection. Bromodeoxyuridine (BrdU) is an indicator commonly used to detect cell proliferation in cells. It is typically used to highlight regions of activity in live tissues, but the function is the same when used in cell cultures.[8] BrdU mimics the structure of deoxythymidine. When a cell undergoes mitosis, it replicates its DNA. The similarity in structure between deoxythymidine and BrdU causes DNA polymerase, a key component in DNA replication, to occasionally match a BrdU molecule to a deoxyadenine. BrdU was thus incorporated into the DNA of the daughter cells after mitosis. When the cells were examined using fluorescence microscopy, the BrdU highlighted the cells that had multiplied. The BrdU was added to the cells at a concentration of 10 $\mu g/mL$ about 6 hours before the 96-well plate was fixed. The NPCs were fixed in formaldehyde for 10 minutes and then in ethanol for 20 minutes. To ensure that the BrdU worked, the top four growth medium wells received it while the bottom four did not. This column therefore acted as the positive control for BrdU function, since all the column 12 wells contained proliferating cells but only the wells that contained BrdU would have fluorescent cells.

2.1.3 Immunostaining

Once the cells were fixed to the plate, we immunostained them using primary and secondary antibodies. Immunostaining entails the use of antibodies to identify certain compounds in cells. The primary antibody (PA, Rat anti-BrdU) was added to the wells at 0.5 $\mu g/\mu L$. This compound was given sufficient time to bind to the BrdU in the cells' DNA. In cells that did not incorporate

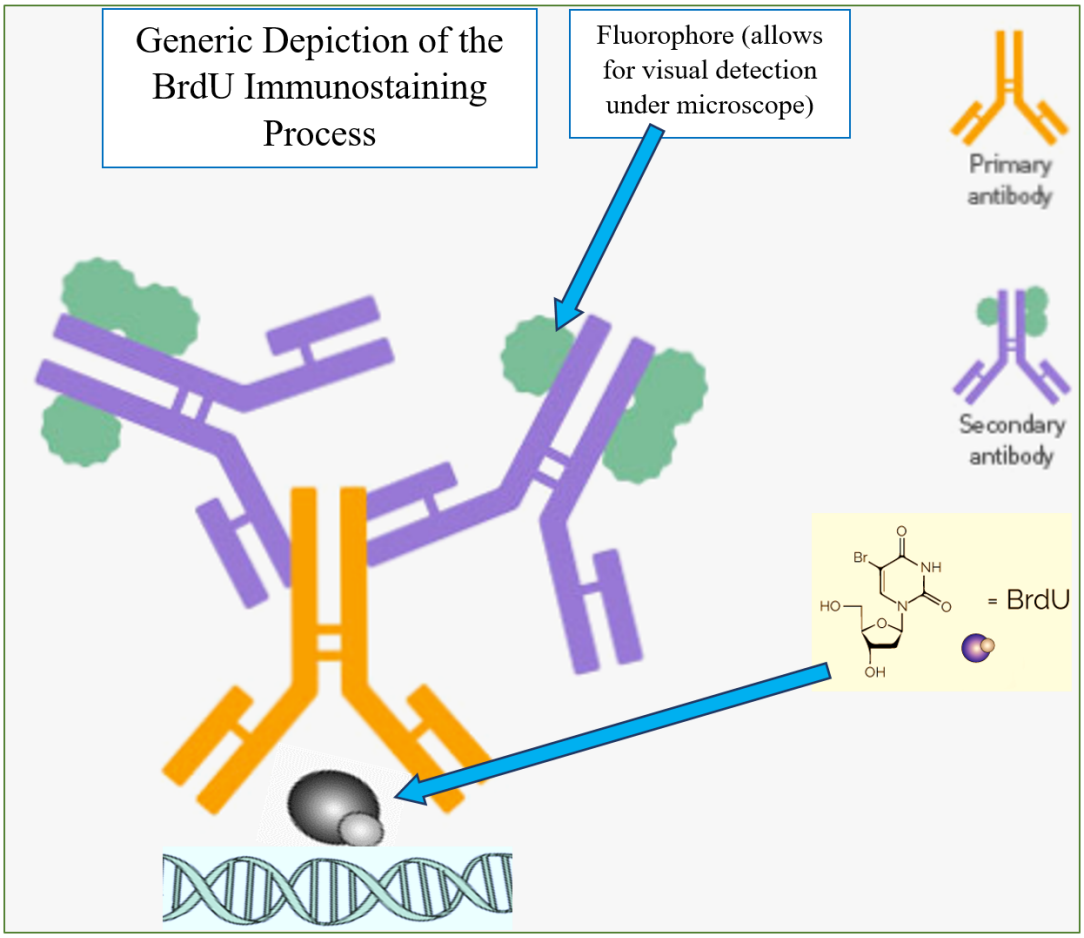


Figure 2: Visual depiction of the BrdU immunostaining process. The PA binds to BrdU, then the SA binds to PA, and the fluorophore is activated under the fluorescent light of the microscope. This allows for visual classification of cells.[17]

BrdU into their DNA, it was washed out with PBS; therefore, primary antibody would only attach to cells that proliferated since only those cells would be BrdU positive (BrdU+). The wells were then washed in the secondary antibody (SA), donkey anti-rat, which bound to the primary antibody. The SA is linked to a fluorophore that fluoresces red under the microscope, making it possible to image the wells. It is important to note that Hoechst DNA staining dye, which binds to nuclear DNA and fluoresces blue under UV light, was added at 0.5 $\mu g/mL$ along with the secondary antibody. This compound made it possible to identify the nuclei and determine if there was corresponding fluorescence from any given cell. Figure 2 depicts a simplified version of the immunostaining process.

Once the 96-well plate was immunostained, it was imaged under the microscope. A visual estimation of the percentage of fluorescence for any given well was first carried out through a method of random sampling. Over 100 cells were chosen from random fields of view from

any given well and the percentage of those cells that was fluorescent was determined. Two researchers performed independent counts to produce two sets of visual data. In essence, the identical top and bottom halves and the two separate trials yield 4 sets of corresponding data.

3 Results and Discussion

3.1 Dilution of Albumin against Serum

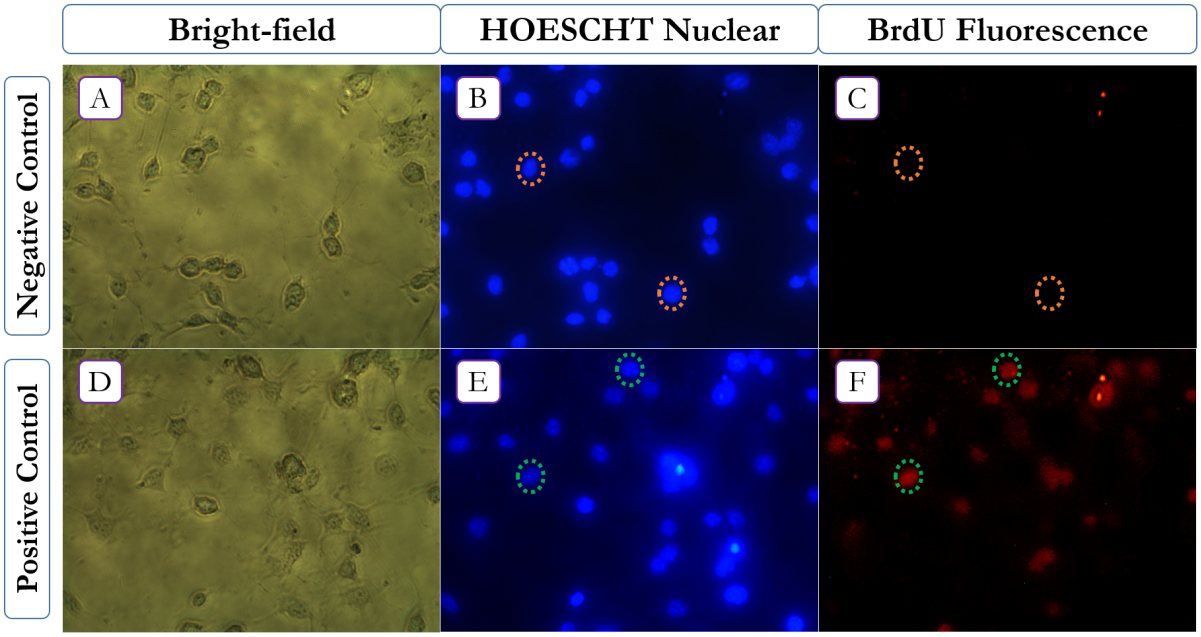


Figure 3: Sample Images for BrdU+ and BrdU- neural precursor cells (NPCs). These images were taken from the growth medium control wells, with the positive control images taken from well C12 (See Table 1) and the negative control images taken from well H12 (See Table 2). The nuclei of cells from well H12 are not fluorescent, because the BrdU, which marks proliferating cells, was never added to those wells. The images in Figures 3C and 3F have been identically contrast-adjusted in ImageJ for enhanced visualization. The dashed outlines indicate example cells which were considered BrdU+ and BrdU- (green for positive, orange for negative).

Figure 3 shows a representative sample of cells which were considered positive and negative for proliferation during the manual counting process. Figure 4 shows the cumulative results from the visual estimation of the left half of the plate. In addition to the data from the left half, appropriate control data were included from the right half as well. For example, the results for the "4% YS and 0% Albumin" included data from well A11 as seen in Table 2 which similarly contained 4% YS, no albumin, and no old serum.

Standard deviation calculations were carried out and these error margins are indicated in Figure 4. These deviations are small relative to the overall percentages of proliferation. Despite occasional overlap between error bars, the large sample size of over 400 cells increases our

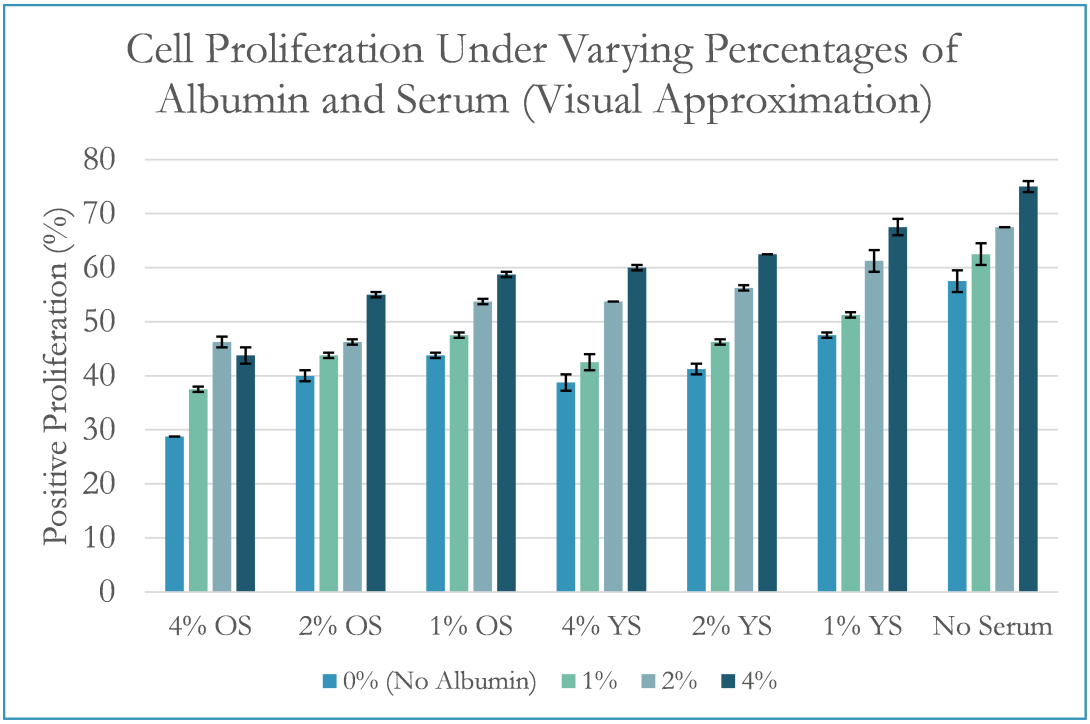


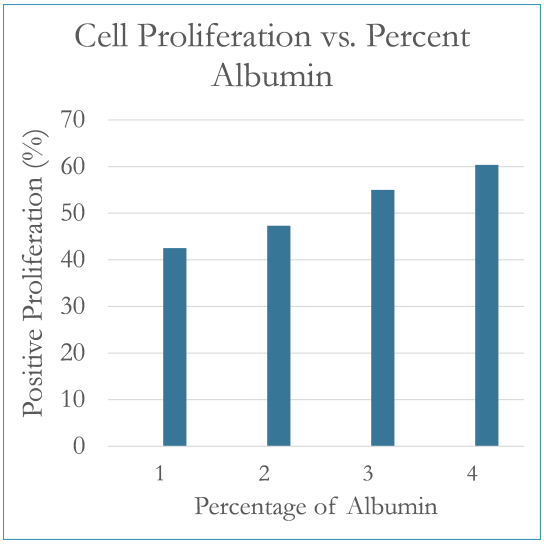
Figure 4: A compilation of the visual estimates of the cell proliferation in the left half of the 96-well plate. The percentage of albumin was varied as different percentages of young or old mouse serum were added to the well. Error bars represent 1 standard deviation and the minimal overlap between error bars suggests that our findings are statistically significant.

confidence in the results.

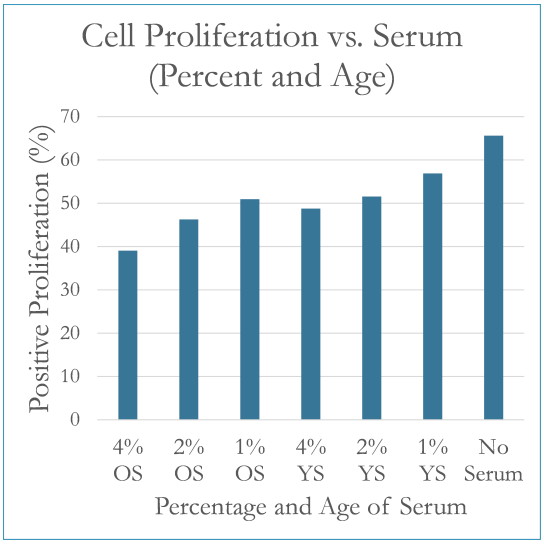
Figure 4 shows a few key trends. Firstly, the greater the percent of albumin in the well for any given percentage of either young or old mouse serum, the greater the percent proliferation. This trend holds across all but the 4% OS cluster, in which 2% albumin encouraged slightly more proliferation than 4%. However, this single deviation is not concerning since there is a stochastic element to the proliferation in any given well. The average proliferation for each albumin percentage irrespective of serum age or quantity is summarized in Figure 5a. The robust data set lends even more confidence in these results.

Secondly, relative to the control cluster (far right) which received no serum, adding serum (along with albumin) seems to lower the degree of cell proliferation, with old serum having more degenerative impacts than young serum. The average proliferation for wells that contained no serum was greater than all other conditions. Increasing the percentage of YS from 1% to 4% decreased proliferation across the board when albumin was also added. Similar conclusions can be made for the OS clusters. Figure 5b depicts this trend.

Combining these results, we conclude that the addition of OS is more inhibitory to prolifer-



(a) Cell proliferation as a function of percentage albumin, regardless of amount or age of serum. There is a consistent increase in proliferation as albumin percentages are increased, supporting the conclusion that albumin acts as a pro-proliferative component of a cell's environment. The far right bar represents the control value for proliferation when no albumin is present.



(b) Cell proliferation as a function of serum, regardless of percent albumin. Old serum impairs cell growth more than young serum. For example, comparing the 1% OS and the 1% YS bars, the cells in YS proliferated more than those in OS. Overall, serum decreases proliferation relative to the control (far right) with greater quantities of serum having more pronounced effects.

Figure 5: Summary of information seen in Figure 4. When we isolate the effects of albumin only (left) and serum only (right) on cell proliferation, the individual effects are more clear, and these trends are consistent with those seen previously. Error bars were not included since the raw data is identical to that for Figure 4.

ation than the addition of YS. This makes sense because if OS contains inhibitory factors that accumulate with age, then adding them to a cell's environment will make it less active and less likely to proliferate. Additionally, increasing albumin up to 4% has consistently positive impacts on the degree of proliferation. We are investigating the viability of using neutral blood plasma in place of mouse blood, and the neutral solution is a combination of red blood cells, white blood cells, half of the platelets found in unaltered serum, and albumin. These results suggest that if the old serum is removed and replaced with this albumin solution, then the combined pro-proliferative effects will lead to increased cellular regeneration.

3.2 Dilution of Young Serum against Old Serum

Figure 6 depicts the results of the YS against OS dilutions. BrdU+ and BrdU- cells were classified in accordance with the samples shown in Figure 3. In these right-half wells, there was no albumin; the purpose was to observe how the two ages of serum interacted with each other, and to account for any nuances or complexities that may arise when the two are mixed.

For certain wells, appropriate data was included from the left half of the plate, as explained previously. For example, well D10 (no YS, 1% OS, 0% albumin) included data from well D6 which contained the same components.

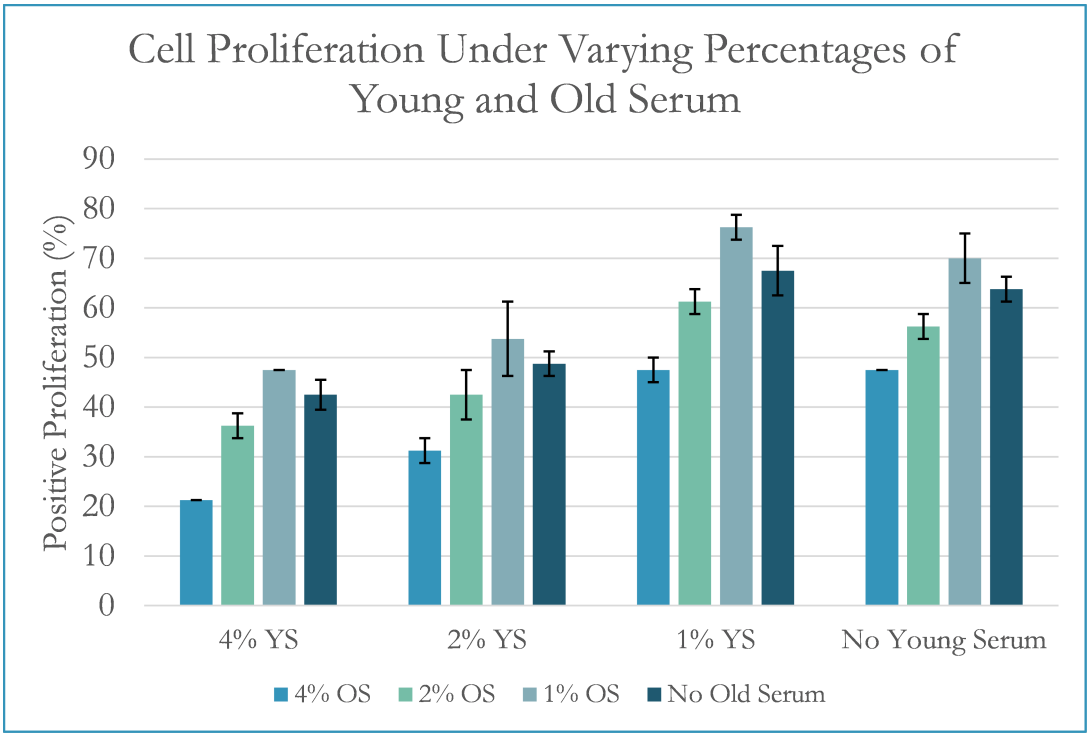


Figure 6: Dilutions of young serum against old serum. As a general trend, when more serum is added to the environment of NPCs, the worse they tend to perform, which agrees with Figure 4 and the albumin experiment results. However, when small quantities of OS are added to wells that have YS (of any amount), proliferation increased slightly relative to wells which contained no OS. Error bars represent 1 standard deviation and we conclude that these findings are statistically significant from the minimal overlap between error bars.

Figure 6 agrees with Figures 4 and 5b in terms of the effect on NPCs when serum is added to the environment. As a general trend, when more serum is added, the cell proliferation decreases relative to the baseline. As an extreme example, consider the 4% OS bar in the 4% YS cluster. The proliferation is around 20%, whereas that of the control (far-right bar in the far-right cluster) is around 65%.

While these results generally agree with the previous findings from the dilutions of albumin against serum regarding the negative effects of serum, there may be a subtle interplay between the two categories of serum that the other experiment wouldn't reveal. An analysis of Figure 4 yielded that adding serum of any kind or quantity (when albumin was also present) reliably decreased proliferation, with OS having more degenerative effects than YS. However, in this case, adding small quantities of serum (of any kind) slightly increased proliferation. For example,

take the 4% YS cluster: the 1% OS increased proliferation by 5% relative to the baseline, but adding 2% and 4% had adverse effects. This observation holds across all the clusters.

These results, when taken alone, suggest that adding 1% each of YS and OS to a cell's environment will benefit its growth most. However, these results don't point to the addition of serum as a significant factor benefiting cell growth. In fact, the potential for serum to harm cells is much greater. Although there appears to be slight improvement when small quantities of serum are added to the environment, the negative effects that result from greater quantities are much more pronounced. Increasing albumin seems to be the key to increasing cell proliferation relative to the control.

4 Conclusion

The work done in this report serves as a significant step towards understanding the effects of various substances on the proliferation of cells. In this case, hippocampal neural precursor cells were cultured in the presence of human serum albumin and/or mouse serum of two ages (YS and OS). The findings are summarized below.

1. Human Serum Albumin is a consistently effective pro-regenerative supplement for NPCs even in the presence of serum.
2. While small quantities of serum may slightly benefit NPC growth, adding large quantities to a cell's environment (greater than 3-4%) markedly decreases their rate of proliferation. OS is more harmful than YS.
3. There is no significant cross-reaction between YS and OS; their combined effects mimic the sum of their individual effects as seen in the albumin experiments.

Knowing the effect of albumin on cell proliferation is so important because the in-vivo mouse surgeries described earlier in the report are contingent on knowing the potential of albumin to affect cells at high concentrations. Albumin is a significant component of the neutral blood solution, so it was necessary to establish how albumin would impact the cells and tissues of an animal. Furthermore, the specific design of this experiment allows us to account for any potential differences in the effects of albumin on brain cells in differently-aged mice. Without the research in this report, we don't have a complete, research-supported understanding of this phenomenon, so further investigative experiments or surgical procedures on mice, and eventually humans,

could not be conducted effectively.

By establishing the effects of mouse serum and albumin on cell behavior, we understand the ideal circumstances under which cells thrive. This information allows us to formulate the ideal neutral blood solution. Prior to conducting this research, it would not have been possible to predict the specific response of cells under varying percentages of young and old mouse serum, but these findings provide a baseline from which further refinement specific to the in-vivo procedure can be made.

Of course, the research in this report was conducted in controlled settings, and it is possible that confounding variables introduced when working with actual animals could cause the findings to deviate from the predictions. But more than the specific quantitative outcomes, the fundamental trends that we observed for both the albumin and the serum dilutions increase our confidence that the mice and cells will react in similar ways. The trends we observed were consistent, and the replicability of the results is another testament to the significance of the findings and research methods of this report. Standard deviation calculations across all the individual trials showed little overlap between the error margins, suggesting that the trends we see were not due to chance variability and our findings have statistical significance. According to these results, the brains of the old mice may actually see some improvement from the neutral blood exchange. In light of the heterochronic blood exchange, in which there was detriment to the young mice brains and no corresponding improvement in the old brains, these findings are especially encouraging. The brain is an especially tricky organ to work with, since the rate of neurogenesis is remarkably slow relative to that of myogenesis or hepatogenesis.[13] This aspect of NPC behavior makes this report's findings ever-more pertinent.

For the reasons described above, this research informs future experiments, allowing researchers to formulate a detailed plan of action for the neutral blood exchange to effectively tackle the problem of aging and its consequences.

5 Future Work

Although this research clarifies several aspects of the in-vivo procedure and its feasibility, the fundamental question remains: Which of the three explanations best explains the observations in the parabiosis and heterochronic blood exchange? Was it the loss of "young factors" or

introduction of "old" factors that causes degeneration in the young mouse brain? We also need to investigate why the old mouse didn't experience increased neurogenesis to complement the impairment in the young mouse.

There are several possible avenues of research that should be pursued in order to more fully understand the interaction of albumin and serum with a cell. First, a bio-molecular approach to investigating the interaction between these compounds and cells may point to intra-cellular biochemical signaling cascades that govern the change in cell behavior. The albumin could interact with specific signaling molecules that ultimately causes the increase in proliferation. The albumin may also effectively increase a cell's exposure to ions and other beneficial substances already present in the environment by providing a transport mechanism. The NPCs themselves actually secrete growth factors (albeit fairly slowly) to enhance proliferation of surrounding cells, so albumin may increase the cell receptor's exposure to these factors.

Alternatively, an analysis of the changes in cell's genomic or proteomic qualities could show a change in cell behavior encoded within the DNA specifically for the presence of albumin. Finally, beyond these secondary approaches to understanding these phenomena, we could be even more confident in our results by repeating the in-vitro experiments with plasma instead of serum. Although plasma is harder to obtain than serum and slightly worse for cells, it will mimic the proposed in-vivo experiments more closely. These are all factors that should be considered as part of the overarching anti-aging research efforts.

Having conducted this in-vitro study, the next step would be to carry out the actual neutral blood exchange procedure described in the Introduction section. Although complications may arise with the use of a live animal, we expect that the in-vitro study accurately models in-vivo outcomes. With this hypothesis, we can compare the findings from our in-vitro research to the in-vivo surgery and account for any differences we may observe. Furthermore, we can design future experiments and gain a more complete understanding of a cell's behavior and also establish the relationship between the components of a cell's environment and the growth of that cell.

Having observed the effects of purified albumin and mouse blood serum on neural precursor cell proliferation, we can investigate behavior when human blood serum is added to the wells.

A separate pilot clinical trial (not at our mentor's institution) is currently conducting plasmapheresis experimental trials on individuals that are middle aged and older. The new study is modeled off of the similar, previously conducted Grifols clinical trial.[12] The humans in the new study consented to a study in which half of the plasma volume of their blood would be replaced with 5% HSA and saline solution by circulating the blood through an apheresis machine (this procedure is called plasmapheresis).

The plasmapheresis procedure will remove half of the plasma from the patient's blood and replace this portion with 5% albumin and saline solution. Only the albumin and plasma concentrations will change; the quantity of other blood components will remain the same. After the procedure is completed, the patient will be given a dose of IVIG antibiotics to replace the antibodies that were suspended in the plasma (which was removed by the apheresis machine).

Samples will be collected from the human at various points. The List 1 corresponds with column numbers in Table 3. Samples 1-6 are from Subject 1. Table 4 uses the Pre-P.P samples for each subject.

Plate Labels 1.

1. Pre-P.P: Taken immediately before the procedure.
2. Post-P.P: Taken after the plasma replacement but before the dose of IVIG.
3. Post-IVIG: Taken after the IVIG is administered.
4. IVIG: In pure form.
5. Post-P.P + IVIG: Individual samples of 2 and 4 are mixed.
6. Pre-P.P 1 month before: Serum drawn from Subject 1, one month before their procedure.
7. Pre-P.P 1 month before: For Subject 7.
8. Pre-P.P 1 month before: For Subject 8.
9. Pre-P.P 1 month before: For Subject 9.
10. FGF Dilutions: Test the effects of FGF on NPC growth.
11. HSA dilutions: Observe effects of albumin; compare to the findings of this report.
12. Growth Medium: Positive proliferation controls. Only top half received BrdU.

Using serum from actual humans would give us a very close approximation of the behavior of cells and tissues in a person's body. Here, we will be investigating similar questions as before, using serum from differently-aged humans instead of mice. The plan is as follows: Two 96-well plates will be prepared and immunostained as described previously. The proposed layout of each is depicted in Tables 3 and 4. Instead of visually estimating the percentage of cells positive for BrdU at the microscope, we can instead use an automated process. After completing our data analysis, we determined that ImageJ could handle large sets of images efficiently, so we decided

to use this software for future experiments. By using ImageJ software for more accurate, reliable results, we can reduce time taken for quantification as a whole.

		Subject 1						Sub. 7	Sub. 8	Sub. 9	Investigative Controls		
		1	2	3	4	5	6	7	8	9	10	11	12
A	4%												+BrdU
B	2%												+BrdU
C	1%												+BrdU
D	0%												+BrdU
E	4%												-BrdU
F	2%												-BrdU
G	1%												-BrdU
H	0%												-BrdU

Table 3: Proposed layout of Plate 1 for the second set of experiments. See List 1, which provides a description of the samples in each column. These plates will contain human serum samples, collected from individuals that consented to the plasmapheresis procedure. Columns 1 through 9 will contain serum samples. Samples 1-6 will be taken from the same individual at different points in the plasmapheresis procedure. Samples 7-9 will be taken from 3 separate individuals ranging in age from middle-age to old. Column 10 will contain dilutions of FGF to establish the cell proliferation under different quantities ranging from 0 $\mathrm{ng/mL}$ to 20 $\mathrm{ng/mL}$. Column 11 will help ensure that the albumin induces the same effect in these cells as it did in our research. Column 12 will act as positive and negative controls for growth and function of BrdU.

		1	2	3	4	5	6	7	8	9	10	11	12
		Subject 1			Subject 7			Subject 8			Subject 9		
	↓ HSA% → Serum%	4%	2%	1%	4%	2%	1%	4%	2%	1%	4%	2%	1%
A	4%												
B	2%												
C	1%												
D	0%												
E	4%												
F	2%												
G	1%												
H	0% (Control Row	GM; -FGF; +BrdU			GM; +FGF; +BrdU			GM; +FGF; -BrdU					

Table 4: Proposed layout of Plate 2 for the second set of experiments. This plate is designed to investigate the effects of different amounts of serum on cell proliferation. It is similar in several ways to the plate discussed in this report, except we will deal with 4 different sera, the specific ages of which are currently unknown. The bottom row has two different controls; the first set of three wells will establish the effect of FGF. The second and third sets will show that the BrdU works as expected.

Table 3 is designed to observe the effect of the plasmapheresis procedure on a human patient from whom the serum samples will be collected and analyzed. The five Subject 1 wells allow for a direct comparison of the cell proliferations at different times during the procedure. Table 4 will investigate the effects of increasing quantities of human serum on NPCs. We may or may not see a correlation between the age of the subject and the extent of impairment to cell

proliferation relative to the control.

This research with human serum samples will enable us to compare the quality of a patient's serum over time as a series of plasmapheresis treatments are carried out. We could use proteomic analyses of the different serum samples to determine what actually changes in the contents of the serum over time. Such time-based investigations could allow us to create quantitative trends, giving us even more insight into the aging process. These are all potential avenues of further research that can and should be pursued to advance our understanding of the aging process.

If we were to repeat our own in-vitro albumin and serum research again, we would invest more time into finding an automated method to count cells. Our preliminary search has found that ImageJ can rapidly process images, with the added benefit that the image analysis will not remove information from the image (resolution-wise or RGB value-wise). Additionally, the presence of albumin seems to alter the effect of serum on cell proliferation, although not by much. To confirm that the two compounds are somehow related or unrelated, we would conduct dilutions with finer differences in the quantities of serum (perhaps ranging from 0% to 6% in 0.5% increments). In a real-world application, every marginal increase in beneficial impacts of albumin could hold significance, so it would be worthwhile to complete the picture of how each substance affects cell proliferation.

Overall, the research in this report acted as a critical step in a long-term process. The findings have important repercussions in the future of anti-aging experimentation and help us understand the connection between a cell's behavior and its environment. It also enables immediate further research to be conducted more efficiently, and we have greater faith in the outcome of the in-vivo surgeries once these findings are incorporated into the methodology.

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