Local Anesthetics Can Affect the Efficacy of Telomerase-Positive Stem Cells

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Abstract

Early clinical studies with telomerase-positive stem cells demonstrated no response when these stem cells were mixed with lidocaine prior to clinical treatments in multiple individuals. Their stem cells demonstrated a positive response when mixed with normal sterile saline utilizing alternative routes of implantation. We hypothesized that lidocaine killed the stem cells before injection and that dead stem cells would give no response. We tested five local anesthetics, e.g., bupivacaine, lidocaine, marcaine, novocaine and procaine, with sterile saline in a series of blinded experiments to determine their ability to affect the viability of telomerase-positive stem cells. A mixture of TSCs, PSCs and MesoSCs were utilized from five individuals, three males and two females. Trypan blue was used to distinguish live versus dead PSCs and MesoSCs. The number of dead cells divided by total number of cells and multiplied by 100 was used for each test solution to determine their respective % kill ratio. Sample size was n=180 for each test solution. Lidocaine demonstrated a 100% kill ratio; novocaine and procaine demonstrated a 50% kill ratio and marcaine, bupivacaine and sterile saline demonstrated a 0% kill ratio. The
results showed that lidocaine should not be used with telomerase-positive stem cells for clinical treatments.

**Keywords**

Adult; Stem Cell; Telomerase; Local Anesthetics; Regenerative Medicine

**Introduction**

Direct injections of stem cells for regenerative medicine necessitate the use of a local anesthetic. The local anesthetic is used to deaden the area before insertion of a large bore (18G) needle to quickly inject a bolus of stem cells into a damaged tissue. Because of its numbing properties, lidocaine has been the preferred choice, as the local anesthetic, to be used for regenerative medicine.

Early clinical studies demonstrated an absence of a functional response when telomerase-positive stem cells, e.g., Totipotent Stem Cells (TSCs), Pluripotent Stem Cells (PSCs) and Mesodermal Stem Cells (MesoSCs), were mixed with lidocaine prior to direct injection into damaged tissues in multiple individuals. These individual’s same telomerase-positive stem cells would demonstrate a positive functional response when mixed with sterile saline and using alternative routes of implantation [1-3]. It was hypothesized that lidocaine killed the stem cells and that dead stem cells would not show a positive functional response. Five local anesthetics were examined, e.g., bupivacaine, lidocaine, marcaine, novocaine and procaine and sterile saline (as the blinded negative staining control) in a series of blinded experiments to ascertain their ability to affect the viability of telomerase-positive stem cells.

**Materials and Methods**

Five individuals, three males and two females, contributed their telomerase-positive stem cells for these experiments. The stem cell contributions were blinded with respect to gender. A mixed population of TSCs, PSCs and MesoSCs was utilized. Isolated and segregated stem cell samples were stored in sterile saline at 4°C until examination on day 1, day 3 and day 5 after harvest from the individual. The telomerase-positive stem cells from each individual were diluted to 1000 PSCs and MesoSCs per 100 microliter aliquots.

Five local anesthetics, e.g., bupivacaine, lidocaine, marcaine, novocaine and procaine and sterile saline (as the negative staining control) were examined as the blinded test solutions. The test solutions were examined at full strength and at step-down dilutions by a factor of 10, from $10^{-1}$ to $10^{-12}$ dilution. One hundred microliters of each blinded dilution of the blinded test solutions was repeated in triplicate (from gender-blinded stem cells on days 1, 3 and 5 post-harvest). One hundred microliters of gender-blinded stem cells were added to 100 microliters
of blinded test solution at a particular blinded dilution, triturated 5 times, 100 microliters of trypan blue added, triturated 5 times and 50 microliters of the cell/test solution/stain removed to a hemocytometer for counting. Trypan blue positive PSCs and MesoSCs and total number of PSCs and MesoSCs were counted for each sample examined, i.e., blinded dilution of blinded test solution. The percent kill ratio ([Trypan blue positive PSCs and MesoSCs per total number of PSCs and MesoSCs] x 100) was determined for each sample examined (n=180). The normally trypan blue positive totipotent stem cells served as the positive staining control in each sample examined. An aliquot of non-blinded sterile saline served as the negative staining control on each day that the post-harvest stem cells were examined.

Results

TSCs were trypan blue positive in each sample (positive staining control), while stem cells mixed with non-blinded normal sterile saline were trypan blue negative (negative staining control). Lidocaine demonstrated a 100% kill ratio; novocaine and procaine demonstrated a 50% kill ratio; and sterile saline, marcaine and bupivacaine demonstrated a 0% kill ratio.

Discussion

Telomerase-positive stem cells have been identified as a conserved population of cells in amphibians, reptiles, avians, mice, rats, rabbits, dogs, cats, sheep, goats, pigs, cows, bears, horses and humans and represent less than 10% of the total number of stem cells in the body [4]. Telomerase-positive stem cells reside in the connective tissues of an individual in their native state as quiescent, undifferentiated stem cells [5]. There are five populations of endogenous adult-derived telomerase-positive stem cells in the body, e.g., Totipotent Stem Cells (TSCs), that will form every cell type of the body across of all three embryonic germ layers (ectoderm, mesoderm and endoderm), as well as forming gametes (sperm and ova) and the nucleus pulposus of the intervertebral disc (the only derivative of the notochord in a postnatal individual); Pluripotent Stem Cells (PSCs), that will form all cell types of the body, EXCEPT gametes (sperm and ova) and the nucleus pulposus of the intervertebral disc; Ectodermal Stem Cells (EctoSCs), that will form all tissues derived from the ectodermal embryonic germ layer, e.g., all cells belonging the central nervous system, peripheral nervous system, cranial nerves, anterior and posterior pituitary, derivatives of neural crest, ganglia, adrenal medulla and all cells and structures associated with the epidermis (glands that secrete their contents to the outside of the body, hair, enamel of teeth, eye structures, lower 1/3 of urethra, etc.); Mesodermal Stem Cells (MesoSCs, e.g., pluripotent mesenchymal stem cells), that will form all tissues derived from the mesodermal embryonic germ layer, e.g., three types of muscle, two types of fat, five types of cartilage, two types of bone, tendons, ligaments, organ capsules, organ trabeculae, all blood cells, multiple types of blood vessels, lymphatic vessels,
all immune cells, lymphoid nodes, free lymphatic nodules, Peyer’s Patches, GALT (Gut Associated Lymphatic Tissue), appendix, spleen, kidney, ureters, urinary bladder, upper 2/3’s of urethra, testicle (without sperm) and ovary (without ova) [4]; and Endodermal Stem Cells (EndoSCs), that will form all tissues of the endodermal embryonic germ layer lineage, e.g., lining of the respiratory system, lining of the gastrointestinal system, glands that secrete their contents to the inside, gall bladder, pancreas, liver, thyroid, parathyroid and adrenal cortex [4,6].

Based on their respective ability to regenerate multiple cell types and other unique characteristics, the telomerase-positive TSCs, PSCs and MesoSCs are utilized for our clinical studies [4]. TSCs are 0.1-2 microns in size and comprise 0.1% of all stem cells in the body. TSCs respond to tissue damage chemokines to migrate anywhere in the body and they can migrate over long distances in a relatively short period of time (nose to cauda equina in 45 minutes). TSCs can propagate both inside the body and outside the body in suspension or on a type-I collagen substratum. TSCs do not understand the concept of ‘contact inhibition’ and form multiple layers of cells in culture. Being telomerase-positive, TSCs essentially have an unlimited proliferation potential as long as they remain undifferentiated. They respond to local environmental cues, i.e., exosomes released by the intact and damaged tissues, to differentiate into what is missing and/or damaged. Once TSCs begin to differentiate and lose the telomerase enzyme, they assume a biological clock of 70 population doublings before pre-programmed senescence and cell death occurs. TSCs are naturally trypan blue positive, because they lack the machinery found in larger more differentiated cells that pump the trypan blue dye out of the cells.

PSCs are >2 to <10 microns in size and they comprise 0.9% of all stem cells in the body. PSCs respond to tissue damage chemokines to migrate anywhere in the body. PSCs can propagate both inside the body and outside the body on a type-I collagen substratum. Similar to TSCs, they do not understand the concept of ‘contact inhibition’ and form multiple layers of cells in culture. Being telomerase-positive, PSCs essentially have an unlimited proliferation potential as long as they remain undifferentiated. They respond to local environmental cues, i.e., exosomes released by the intact and damaged tissues, to differentiate into what is missing or damaged. Once PSCs begin to differentiate and lose the telomerase enzyme, they assume a biological clock of 70 population doublings before pre-programmed senescence and cell death occurs. PSCs are naturally trypan blue negative, because they have the machinery found in larger more differentiated cells that pump the trypan blue dye out of the cells.

MesoSCs are 10-12 microns in size and they comprise 3% of all stem cells in the body. MesoSCs respond to tissue damage chemokines to migrate anywhere in the body. MesoSCs can propagate both inside the body and outside the body on a type-I collagen substratum. They somewhat understand the concept of ‘contact inhibition’. They will stop proliferating once they reach a single layer of confluent cells. However, they do not die, but enter a quiescent state and can be revived at any time by feeding with fresh complete medium. Being telomerase-
positive, MesoSCs essentially have an unlimited proliferation potential as long as they remain undifferentiated. They respond to local environmental cues, i.e., exosomes released by the intact and damaged tissues, to differentiate into what is missing or damaged with respect to the mesodermal germ layer lineage only. They do not transdifferentiate into ectodermal or endodermal lineage cell types. Once MesoSCs begin to differentiate and lose the telomerase enzyme, they assume a biological clock of 70 population doublings before pre-programmed senescence and cell death occurs. MesoSCs are naturally trypan blue negative, because they have the machinery found in larger more differentiated cells that pump the trypan blue dye out of the cells.

The telomerase-positive TSCs, PSCs and MesoSCs have been tested in pre-clinical animal models of induced disease, e.g., Parkinson disease, cardiovascular disease, pulmonary disease, type-I diabetes and orthopedic injuries [3,7-10] as well as in human clinical models of Parkinson disease, myocardial infarction, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, celiac disease, systemic lupus erythematosus and orthopedic injuries [11-17]. It is this last group, orthopedic injuries that brought us to the current study.

In initial studies when TSCs, PSCs and MesoSCs were combined with lidocaine before direct injection into damaged tissues there was no discernible response, i.e., a decrease in symptoms and/or an increase in function. It was also noted that when TSCs, PSCs and MesoSCs from these same individuals were added to sterile normal saline that there was a reduction of symptoms and an increase in function, when alternative routes of delivery were utilized. This led to the conclusion that lidocaine somehow neutralized the activity of the telomerase-positive stem cells, either before, during, or after the injection. A hypothesis was generated, “lidocaine kills telomerase positive stem cells before direct injection preventing them from reducing symptoms and increasing function in the affected organ”.

This hypothesis was examined using multiple blinded permutations. The TSCs, PSCs and MesoSCs were derived from five donors, e.g., three males and two females. The separate cell processing procedures performed for each gender-blinded donor were identical to the current procedures used to isolate telomerase-positive stem cells for clinical transplant. The donors were blinded to determine if any gender-specific issues existed. It was found that there were no differences between genders with respect to the telomerase-positive stem cells isolated.

Donors were instructed to ingest Combinatorial Nutraceuticals (CN) daily for 30 days. CN stimulates telomerase-positive stem cells to proliferate inside the individual’s connective tissues throughout their body. Eighteen hours before harvest the donors ingested Glacial Caps (GC). GC causes the connective tissue-resident telomerase-positive stem cells to mobilize into the blood stream. At harvest, 300-400cc’s of blood is removed by venipuncture. The harvested blood was given to the isolators (blinded as to who did the stem cell processing) to separate the telomerase-positive stem cells from the red blood cells, white blood cells and platelets using FDA-mandated minimal manipulative procedures, e.g., zeta potential, gravity and differential centrifugation using serum gradients, sterile normal saline gradients and sterile distilled water

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gradients and operating under standardized General Manufacturing Protocols (GMP) [11-17]. The isolators were blinded with respect to their identity to determine if there were any differences when different individuals were using the same stem cell processing protocol. We detected no differences between the isolators.

The telomerase-positive stem cells from each individual were further segregated into individual populations of TSCs, PSCs and MesoSCs using variations of the above procedures and activated. The TSCs, PSCs and MesoSCs were then recombined into a single population and stored at 4°C until examined on days 1, 3 and 5 after harvest. We wanted to determine if any differences occurred with increased storage time at 4°C. Earlier viability by propagation animal studies noted that TSCs could survive longer than 30-days at 4°C in sterile saline; PSCs could last up to 14-days at 4°C in sterile saline; and MesoSCs could survive up to 7-days at 4°C in sterile saline [4]. Five days was within the seven to 14-day window for MesoSCs and PSCs for the animal telomerase-positive stem cells. No differences in viability were noted to have occurred between one, three, or five days in storage at 4°C for the human telomerase-positive stem cells.

The stem cell population from each individual was diluted to 1000 PSCs and MesoSCs per 100 microliter aliquots and blinded a third time. Trypan blue was used distinguish live (Trypan blue-negative) PSCs and MesoSCs from dead (Trypan blue-positive) PSCs and MesoSCs. Trypan blue positive TSCs were used as the positive staining control within each sample, while an aliquot of non-blinded sterile saline served as the negative staining control on each day the samples were examined. There was foreknowledge that one of the blinded test solutions was normal sterile saline and therefore should have a 0% kill ratio. However, due to the blinded portion of the experiment, the identity of which dilution samples were saline was unknown until the codes were revealed. As it was, three of the blinded test solutions demonstrated a 0% kill ratio.

The test solutions consisted of sterile saline (blinded) and each of five local anesthetics, e.g., bupivacaine, lidocaine, marcaine, novocaine and procaine (also blinded). They were tested at full strength (pharmacological dose of 10⁻¹) and at step-down dilutions by a factor of 10, from 10⁻¹ to 10⁻¹² dilution (presumed physiological dose at 10⁻¹² factor of 10 dilution). Each dilution for each blinded test solution from 10⁻¹ to 10⁻¹² was also blinded. Each blinded dilution was repeated in triplicate (on day’s one, three and five post-harvest). We wanted to know what affect, if any, a physiological dose would have on the viability of the stem cells. We had assumed that the starting point for the assumed linear decrease in percentage kill ration for each anesthetic would be somewhere between 10⁻¹ and 10⁻¹² factor of ten dilutions, once the codes were revealed. That was not the case. There were only three results, either 100% kill ratio (lidocaine), 50% kill ratio (novocaine and procaine), or 0% kill ratio (bupivacaine, marcaine and sterile saline) for all blinded dilutions tested. Either the physiological dose is lower than a 10⁻¹² factor of ten dilution for each anesthetic, or the telomerase-positive stem cells are super sensitive to the anesthetic with respect to their viability. Either case does not matter, just that
lidocaine is not the preferred choice for direct injections of telomerase-positive stem cells into damaged tissues.

One hundred microliters of stem cells were added to 100 microliters of test solution, triturated 5 times, 100 microliters of Trypan blue added, triturated 5 times and 50 microliters of the solution removed to a hemocytometer for counting Trypan blue positive PSCs and MesoSCs versus total number of PSCs and MesoSCs, to determine percent kill ratio (Trypan blue positive cells/total number of cells x 100). Sample size for each blinded test solution was n=180. The sample size was maximized to ensure that statistics could performed to determine significant differences between the anesthetics and their respective dilutions. That was not necessary as the results demonstrated once the codes were revealed.

It is unknown at this time whether other telomerase-positive stem cells, e.g., Embryonic Stem Cells (ESCs), Induced Pluripotent Stem Cells (iPSCs), Ectodermal Stem Cells (EctoSCs) or Endodermal Stem Cells (EndoSCs) have their efficacy reduced or eliminated when using with local anesthetics for regenerative medicine. It is also unknown at this time whether telomerase-negative stem cells, e.g., Mesenchymal Stem Cells (MSCs) derived from bone marrow, adipose tissue, umbilical cord, placenta, amnion, or other tissue sources, Mature Adult Progenitor Cells (MAPCs), Bone Marrow Stromal Cells (BMSCs), Very Small Embryonic Like Stem Cells (VSEL-SCs), Medicinal Signaling Cells (renamed MSCs), Multilineage Differentiating Stress Enduring Cells (MUSE), or other stem cell types have their efficacy reduced or eliminated when using with local anesthetics for regenerative medicine. If there is a disconnect in efficacy between the respective stem cells suspended in normal sterile saline versus being suspended in either a local anesthetic or injected immediately after injection of a local anesthetic, then this issue should be addressed, “does the local anesthetic being utilized reduce or eliminate the efficacy of the stem cell when using local anesthetics for regenerative medicine”. If a disconnect is seen between saline and a local anesthetic, it may explain to some degree the reason for no response of the tissues to the stem cell or adverse events that occurred when using local anesthetic with the stem cells.

In the current study, lidocaine demonstrated a 100% kill ratio for telomerase-positive PSCs and MesoSCs; novocaine (generic name) / procaine (trade name) demonstrated a 50% kill ratio; bupivacaine (generic name) / marcaine (trade name) and sterile normal saline, demonstrated a 0% kill ratio at all dilutions tested. The results proved the hypothesis that lidocaine kills telomerase-positive stem cells before injection and therefore should not be used with TSCs, PSCs and MesoSCs for direct injection into damaged tissues for regenerative medicine. Currently, either bupivacaine or marcaine are being used, depending on the preference of the treating physician, for the direct injections of telomerase-positive TSCs, PSCs and MesoSCs into damaged tissues for regenerative medicine [17].
**Conclusion**

Based on the results from previous studies, we hypothesized that lidocaine killed telomerase-positive stem cells before injection and that dead stem cells would give no response. We tested five local anesthetics, e.g., bupivacaine, lidocaine, marcaine, novocaine and procaine, with sterile saline in a series of blinded experiments to determine their ability to affect the viability of telomerase-positive stem cells. Sample size was n=180 for each test solution. Lidocaine demonstrated a 100% kill ratio; novocaine and procaine demonstrated a 50% kill ratio; and marcaine, bupivacaine and sterile saline demonstrated a 0% kill ratio. The results showed that lidocaine should not be used with adult-derived telomerase-positive stem cells for clinical treatments.

**References**