Published in final edited form as:

Otolaryngol Head Neck Surg. 2011 December ; 145(6): 1007–1015. doi:10.1177/0194599811416778.

Role of Stromal Cell-Derived Factor-1 Expression in the Injured Mouse Auditory Nerve

Lauren A. Kilpatrick, MD¹, Juhong Zhu², Fu-Shing Lee, PhD¹, and Hainan Lang, MD, PhD²

¹Department of Otolaryngology—Head and Neck Surgery, Medical University of South Carolina, Charleston, South Carolina, USA

²Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina, USA

Abstract

Objective—The degeneration of hair cells and spiral ganglion neurons (SGNs) is an important pathologic process in the development of sensorineural hearing loss. In a murine model, predictable and reproducible damage to SGNs occurs through the application of ouabain to the round window. Recent evidence has shown that the chemokine stromal cell–derived factor-1 (SDF-1) is a potent chemoattractant of hematopoietic stem cells (HSCs) and provides trophic support to injured tissues during development and maturation. The hypothesis for the current study is that expression of SDF-1 plays an important role in protecting SGNs and preventing further degeneration in the setting of cochlear injury.

Study Design—Prospective, controlled.

Setting—Academic research laboratory.

Subject and Methods—Auditory brainstem response (ABR) and the expression of SDF-1 mRNA and protein were examined 1, 3, 7, 14, and 30 days after application of ouabain in 35 adult mice.

Results—Following ouabain application, real-time reverse-transcription polymerase chain reaction for SDF demonstrates increased mRNA expression following ouabain injury in

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Corresponding Author: Lauren A. Kilpatrick, MD, Department of Otolaryngology—Head and Neck Surgery, Medical University of South Carolina, 135 Rutledge Ave MSC 550, Charleston, SC 29425, USA, kilpatri@musc.edu.

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Author Contributions

Lauren A. Kilpatrick, primary author, conception, data acquisition and analysis, revision, final approval; Juhong Zhu, data acquisition, revision, final approval; Fu-Shing Lee, data acquisition and analysis, revision, final approval; Hainan Lang, conception, data acquisition and analysis, revision, final approval.

This article was presented at the 2010 AAO-HNSF Annual Meeting & OTO EXPO; September 26–29, 2010; Boston, Massachusetts.

Disclosures

Competing interests: None.

Sponsorships: None.

Funding source: This study was supported by National Institutes of Health grant numbers DC00422 (H.L.) and DC07506 (H.L.) and American Academy of Otolaryngology—Head and Neck Surgery grant number CORE 130165 (L.A.K.).

nontransplanted mice. A significant increase in SDF protein expression was also observed using immunolabeling techniques and Western blot analysis.

Conclusions—SDF-1 expression is increased in the auditory nerve following cochlear injury. Further knowledge about the cochlear microenvironment, including SDF-1, is critical to maximizing HSC engraftment in the injured cochlea and providing a therapeutic option for sensorineural hearing loss.

Keywords

ouabain; SDF-1; CXCL-12; stem cell; neural degeneration

Spiral ganglion neuron (SGN) degeneration due to age or direct injury to the cochlea secondary to noise, oto-toxic drugs, or genetic mutations has been well described.^{1–4} This degeneration may occur as a primary event or be due to sensory hair cell loss. Of the afferent auditory neurons, 90% to 95% are type I neurons, which synapse directly onto the inner hair cells, whereas the remaining 5% to 10% are type II neurons, which innervate the outer hair cells.^{5–7} In 1997, Dodson⁸ demonstrated that the loss of type I neurons in the spiral ganglion after introduction of intracochlear gentamicin occurs via apoptosis. However, most studies suggest that auditory nerve degeneration takes place over months to years, thus making it difficult to study in vivo. The application of ouabain to the round window (RW) membrane of the cochlea in gerbils and mice has been shown to result in a rapid loss of type I SGNs with little effect on the morphology and function of the sensory hair cells.^{9–12} Thus, the ouabain-induced auditory neuropathy model is ideal for investigating potential therapies for reversing sensorineural hearing loss.

One potential strategy for replacement and regeneration of degenerated neural tissue in the inner ear is the application of stem/progenitor cell transplantation. Attempts to introduce neural tissue or stem cells directly into the inner ear of several animal models with auditory nerve degeneration have been variably successful; however, it appears that acute injury before transplant increases the survival rate of transplanted cells.^{13,14} Despite the advances in stem/progenitor cell transplantation and its role in the regeneration of damaged SGNs, little is known about the injury-induced microenvironment and how changes contribute to the engraftment and differentiation capacity of stem cells transplanted into the inner ear. Investigation into the expression of growth factors, cytokines, and so on is warranted to further characterize the postinjury microenvironment and its role in the survival of transplanted cells.

Hematopoietic stem cells (HSCs) are defined by their ability to repopulate previously irradiated bone marrow (BM) and blood lineages. It is well established that BM-derived stem cells can migrate and yield cells of both a hematopoietic lineage and mesenchymal lineage.^{15–18} Using the Y-chromosome or green fluorescent protein (GFP) as a marker of donor cells in transplantation studies, investigators have demonstrated that pericryptal myofibroblasts in the intestine and colon, fibrocytes in wounded skin, myofibroblasts in liver fibrosis, and fibroblasts in pulmonary fibrosis are derived from BM.^{19–22} Our recent study demonstrates that HSCs can engraft into the auditory ganglion of the adult mouse inner ear and differentiate into nonsensory cochlear cells.²³

There is a large body of literature supporting the concept that tissue injury enhances the homing, survival, engraftment, and differentiation of stem cells.^{24–26} Moreover, Harris et al²⁷ and Park et al²⁸ have documented stem cell recruitment and differentiation among cells of neural lineage, specifically following injury to the retinal pigment epithelium (RPE) or after acute brain injury, respectively.

Recent studies have revealed that homing, survival, and engraftment of stem cells into injured tissues and organs are mainly controlled by interactions between CXC chemokine receptor 4 (CXCR4) and stromal cell–derived factor (SDF)–1 (or CXC chemokine ligand–12, CXCL-12).^{29–32} The upregulation of SDF-1 was found in damaged organs, including brain, heart, liver, and kidney.^{29,33–35} The upregulation of SDF-1 directly corresponded to increased migration and homing of circulating CXCR4⁺ stem/progenitor cells to ischemic tissues.³⁶

The current study aims to examine the expression of SDF-1 and determine if SGN injury significantly upregulates SDF-1 expression in the auditory nerve.

Methods

Animals

A colony of adult CBA/CaJ mice was established with original breeding pairs purchased from the Jackson Laboratory (Bar Harbor, Maine) and bred in-house in a low-noise environment at the Animal Research Facility of the Medical University of South Carolina. Mice of both genders, aged 2 to 12 months and weighing 16 to 35 g, were used in the study.

All aspects of the animal research were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Before data acquisition, mice were examined for signs of external ear canal and middle ear obstruction. Mice with any symptoms of ear infection were excluded from the study.

Using auditory brainstem response (ABR) thresholds, data typically cover a range of ± 10 dB. Using a simple 2-tailed *t* test, a 95% confidence interval for the mean in a population of *n* animals yields the following: Data range = $10 = t_{n,0.05} (SD/\sqrt{n})$, in which *SD* is the standard deviation and $t_{n,0.05}$ is the *t* value with the degrees of freedom equal to *n* – 2. In these experiments, $t_{n,0.05}$ is approximately 2.2; with relatively large *SD*s in these studies, *n* = 7 per experiment results in a reasonable confidence interval (CI) while keeping animal usage to a minimum.

Physiological Procedures

Mice were anesthetized by an intraperitoneal injection of xylazine (20 mg/kg) and ketamine (100 mg/kg) and placed in a head holder in a sound isolation room. Young adult CBA/CaJ mice underwent physiological measurements before and after ouabain treatment. Auditory brainstem responses were recorded via customized needle electrodes inserted at the vertex (+) and test side mastoid (–), with a ground in the control side leg. The acoustic stimuli were generated using Tucker Davis Technologies equipment III (Tucker-Davis Technologies,

Gainesville, Florida) and a SigGen software package (Tucker-Davis Technologies). Signals were delivered into the ear canal through a 10-mm-long (3- to 5-mm diameter) plastic tube. ABR thresholds, defined as the lowest sound levels at which the response peaks are clearly present as read by the eye from stacked wave forms, were obtained. ABRs were evoked at half-octave frequencies from 4 to 45 kHz with 5-ms duration tone pips with $\cos^2$ rise per fall times of 0.5 ms delivered at 31 per second. Sound levels were reduced in 5-dB increments from 90 to 10 dB sound pressure level (SPL) below thresholds. Physiological results were analyzed for individual frequencies and then averaged for each of these frequencies from 4.0 to 40.0 kHz.

Surgical Procedures

The mouse injury model was modified from previous work in our laboratory on gerbils.^{9,10} Mice were anesthetized by intraperitoneal injection of xylazine (20 mg/kg) and ketamine (100 mg/kg). Body temperature was maintained between 36°C and 38°C using a heating pad. The right ear served as the operative ear in all cases. Using sterile procedures, the bulla was approached via a postauricular incision, and a small perforation in the bulla was created to expose the round window niche. With a 26-gauge needle and tuberculin syringe under direct microscopic guidance, approximately 5 to 10 μ L of 1 mM ouabain solution in normal saline (O-3125; Sigma, St Louis, Missouri) was applied to the round window niche to fill the niche completely. In 15-minute increments, the ouabain solution was removed by wicking with a small piece of sterile filter paper and then reapplied. After a total of 60 minutes of exposure, the ouabain was removed and incisions were closed with sutures. The animals were allowed to recover for 1, 3, 7, 14, and 30 days after ouabain exposure.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Following treatment with ouabain, mice were allowed to recover for 1, 3, 7, 14, and 30 days (3–6 experiments per group). Both control (left ear) and treatment (right ear) cochleas were harvested using micro-instruments cleaned with RNase Zap (Qiagen, Germantown, Maryland). Microdissection was performed in 3 mL of RNeasy lysis solution (Qiagen). Total RNA of spiral ganglia was subsequently isolated using the protocol from the RNeasy Micro Handbook (Qiagen).

The total RNA from the treatment and control specimens was subjected to real-time reverse-transcription polymerase chain reaction (RT-PCR) according to protocol from the QuantiTect Reverse Transcription Handbook (Qiagen), with a reaction volume of 20 μ L. Forward and reverse primers (Qiagen) for 18S (QT01036875; Qiagen) and CXCL12 (QT00161112; Qiagen) were used. To assess the precision of the quantitative PCR results, 3 reactions were performed for each cDNA sample and primer set. Positive and negative controls were also run in triplicate. Each sample showed a particular threshold cycle (Ct) value in amplification, which is representative of the relative abundance of the gene in the sample RNA. The Ct value of each sample in the examined gene is normalized with the value of the endogenous control gene, 18S. The relative fold change in gene expression was obtained by comparing Ct data of ouabain-treated ears to that of controls.

Morphological and Immunohistochemical Analysis

For immunohistochemistry, anesthetized animals (1, 3, 7, 14, or 30 days after ouabain treatment) were perfused via cardiac catheter with 10 mL of normal saline containing 0.1% sodium nitrite and then with 4% paraformaldehyde as fixative, decalcified with EDTA, cryoprotected in 30% sucrose in phosphate-buffered saline (PBS), and embedded in Tissue-Tek OCT compound (Electron Microscopy Science, Ft Washington, Pennsylvania). Cochlear tissues were incubated overnight at 4°C with a primary antibody; primary antibodies used included rabbit anti-SDF-1 (SC-28876; Santa Cruz Biotechnology, Santa Cruz, California), rabbit anti-class III β -tubulin (MRB-435p; Covance, Emeryville, California), and mouse anti-myelin basic protein (ab24567; Abcam, Cambridge, Massachusetts). Control staining for primary antibodies was performed. Secondary antibodies were biotinylated, and binding was detected with fluorescent (fluorescein isothiocyanate [FITC])–conjugated avidin D (Vector, Burlingame, California).

The sections were examined with a Zeiss Axio Observer or a Zeiss LSM5 Pascal confocal microscope (Carl Zeiss, Oberkochen, Germany). The captured images were processed using AxioVision 4.8 (Carl Zeiss) and Zeiss LSM Image Browser Version 2.0.70 (Carl Zeiss). Adobe Photoshop CS2 (Adobe, San Jose, California) was used to adjust brightness, contrast, and sharpness of images with identical settings for all panels. Alterations were not performed on images used for quantitative purposes.

As described by Flores-Otero et al,³⁷ quantitative analysis of protein expression was determined as antibody luminance. Photographic exposures (200 ms exposure length) and adjustments were maintained throughout all images to allow accurate luminance measurements. Within the spiral ganglion, calculations were obtained from equally representative areas (radius 38.5 μ m) after conversion of the image to grayscale.

Protein Isolation and Western Blot Analysis

Inner ear tissues were dissected from the temporal bones of control and 3-, 7-, 14-, and 30-day posttreatment mouse ears (3–7 experiments per group, 2 isolated auditory nerve fractions per experiment). After removing the cochlear lateral wall and organ of Corti, the auditory nerve was separated from the vestibular branch of the VIII nerve at the inferior aspect of the internal acoustic meatus. The isolated specimens were extracted in cold lysis buffer (Cell Signaling, Danvers, Massachusetts). Protein lysates were boiled for 5 minutes, subjected to 6% to 18% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 30 μ g protein/lane in protein loading buffer), and electro-transferred onto an Immune-Blot PVDF membrane (Bio-Rad, Hercules, California). Membranes were blocked with bovine serum albumin (BSA)/Tris-buffered saline with Tween (TBST; 5% BSA/Tris-buffered [50 mM HCl-Tris, pH 7.5], 150 mM NaCl, 0.02% Tween-20) for 2 hours and then incubated with primary antibody for 3 hours. The blots were washed 3 times (10 minutes each) with TBST. To detect the antigen complexes, membranes were incubated with alkaline phosphatase-conjugated secondary antibody (1:6000; Promega, Madison, Wisconsin) for 2 hours, rinsed with TBST, and then incubated with 5-bromo-4-chloro-3-indolyl phosphate disodium salt/nitrotetrazolium blue chloride (Sigma).

Data Analysis

Unless otherwise specified, all data in the figures are presented as mean $\pm$ SD. Data for the ABR thresholds and the fold changes of SDF mRNA and protein expression were analyzed by 1-sample *t* test (SPSS, Inc, an IBM Company, Chicago, Illinois). A value of $P < .05$ was considered statistically significant. Control samples were the untreated ear from the same animal unless otherwise indicated.

Results

Hearing Loss and Type I SGN Degeneration after Ouabain Exposure

As demonstrated previously, the application of ouabain to the round window of young adult CBA/CaJ mice quickly induced a severe hearing loss and rapid degeneration of SGNs (Figure 1). ABR wave I thresholds were dramatically increased across all frequencies as early as 1 day after ouabain exposure. Seven days following exposure, ABR responses were largely absent; these responses did not recover as late as 30 days posttreatment (data not shown). Figure 2 demonstrates the specific loss of type I SGNs in these mice following exposure to ouabain.

SDF-1 Expression in Normal and Injured Auditory Nerve

SDF-1 mRNA expression in normal and ouabain-treated ears was examined and quantified by RT-PCR. As shown in Figure 3, mRNA expression of SDF-1 at 1, 3, 7, 14, and 30 days after ouabain exposure was significantly greater than that of controls by 1-sample *t* testing ($P = .0237$, $P = .0103$, $P = .004$, $P = .0192$, and $P = 0.0329$ for 1, 3, 7, 14, and 30 days, respectively).

Protein expression of SDF-1 was evaluated and measured in terms of luminance factor analysis. Figures 4 and 5 depict the spiral ganglion expression pattern of SDF-1 in the cytoplasm of cells in the auditory nerve; Figure 4 shows a control (A, C) and ouabain-treated (B, D) ear 3 days after exposure with clearly increased cytoplasmic expression of SDF-1 under immunofluorescent staining. When converted to grayscale, there was increased protein expression of SDF-1 comparing a control (A) and damaged (B) ear, as shown in Figure 5, 1 day after exposure. Figure 6 shows overall SDF staining intensity or “luminance” across all turns. As with mRNA expression, controls had lower levels of luminance than ouabain-treated ears; however, there appeared to be no difference among turns with respect to SDF-1 protein expression. Protein expression of SDF-1 is further plotted in Figure 7; the “luminance factor” of protein expression described the SDF-1 expression of ouabain-treated ears compared to control such that any factor change >1 revealed increased protein expression. Luminance factor appeared to increase for all time points following ouabain exposure. *P* values for luminance factor measurements are listed in Table I.

Western blot analysis demonstrating the expression changes of SDF-1 proteins isolated from ears 3 and 7 days after ouabain treatment is shown in Figure 8. The bands in the treatment groups demonstrate increased expression of SDF-1 after ouabain exposure.

Discussion

Type I SGN Degeneration and Hearing Loss

The CBA/CaJ mouse is considered the “gold-standard” strain for maintenance of excellent hearing throughout life.^{38–40} The application of ouabain to this mouse strain again demonstrated a reproducible model for sensorineural hearing loss. Ouabain is a cardiac glycoside similar to digoxin and inhibits plasma membrane Na⁺/K⁺ ATPase. Our previous studies in the adult gerbil and more recently in the adult mouse have demonstrated that ouabain is able to selectively and permanently induce the degeneration of type I SGNs.^{9–10,41} As the primary transmitters of auditory information, the loss of type I SGNs resulted in significant loss of auditory function across all time points tested. As determined in prior studies, ouabain’s effect on type I SGNs can be attributed to the induction of apoptosis.^{9,42} This apoptotic cascade starts nearly immediately following exposure, as seen in the threshold shifts at day 1, and is nearly complete as early as day 3 after ouabain exposure.⁴¹ Although toxic to type I SGNs, ouabain has relatively little effect on type II SGNs, efferent fibers, and sensory hair^{9,10,41}; this same pattern has been described by other groups with respect to noise trauma, ototoxic lesions, or cochlear nerve injury.^{3,5}

SDF-1 Upregulation in the Injured Auditory Nerve

Numerous studies have previously described the unique capabilities and roles of SDF-1 and its receptor CXCR4. SDF-1 expression in embryonic stem cells is critical to their survival and migration such that deletions of the chemokine or its receptor result in deficits of B-lymphopoiesis and myelopoiesis, cardiogenesis, angiogenesis, neurogenesis, and germ cell migration and development.^{43–50} Hematopoiesis is highly dependent on SDF-1/CXCR4 signaling as well. A SDF-1-mediated chemoattractant relationship retains HSCs in BM in adult mice^{51–54}; this relationship is clinically used today such that CXCR4 antagonism is a therapeutic method to mobilize HSCs from BM during HSC harvest.⁵⁵

With respect to the auditory nerve, our data indicate that increased SDF-1 expression occurs rapidly following ouabain exposure at both mRNA and protein levels. Protein expression of SDF-1 by luminance factor measures appeared to increase across all time points after cochlear injury, but several time point indices did not achieve statistical significance. Because chemokines are diffusible secreted proteins, some SDF-1 protein may be lost during the preparation of immunostaining procedures. This theory is supported by the Western blot analysis of isolated auditory nerves showing increases in SDF-1 expression 3 and 7 days after ouabain exposure. Overall, our data showed an upregulation of SDF-1 expression in the injured auditory nerve shortly after ouabain exposure due to SGN degeneration. Although prior research has described high SDF-1 expression in neuronal cells and endothelial cells, ouabain selectively damages SGNs, and thus increased SDF-1 expression after ouabain exposure is attributed to neuronal damage.^{56,57} These results suggest that SDF-1 may play an important role in preventing further SGN degeneration or promoting repair after injury. Furthermore, SDF-1 may serve to supplement this protection by enhancing HSC engraftment in the injured site as seen in other tissues and organs.^{29,33–36} Further experiments are needed to determine if the upregulation of SDF-1 expression correlates with increased HSC engraftment in the injured auditory nerve.

Tan et al⁵⁸ have described both HSC engraftment and SDF-1 expression in the ear. After acoustic trauma, previously transplanted mice were observed for engraftment and SDF-1 patterns of expression. SDF-1 expression localized to the type II fibrocyte region of the spiral ligament with minimal expression in other regions of the cochlea, including the organ of Corti. Our expression patterns are similar with a low level of expression in the organ of Corti. Their findings differ from this study in that SDF-1 expression was determined to be most prominent within 1 week after trauma, although our results indicate that SDF-1 mRNA and protein expression is increased for at least 14 days after ouabain exposure. This difference may be attributed to different injury patterns and involved cell types.

In summary, this study shows that cochlear injury correlates with increased SDF-1 expression. Together with previous HSC studies in other tissues and organs, our results suggest that SDF-1 may serve to promote homing and engraftment of HSCs to the cochlea following injury. Although further research into the microenvironment and HSC engraftment pattern of the cochlea after injury is warranted, this study suggests that SDF-1 expression may serve a protective role in the cochlea to promote regeneration or repair after injury; moreover, optimizing SDF-1 expression following cochlear injury may improve HSC engraftment and/or differentiation to further develop a treatment model for hearing loss.

Acknowledgments

The authors thank Dr Vinu Jyothi for assistance in ABR measurements, Dr Manna Li and Nancy M. Smythe for help with histological observations, and Drs Richard A. Schmiedt and Bradley A. Schulte for critical comments.

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Figure 1.

Auditory brainstem response (ABR) in ouabain mouse models. Pretreatment ABR for CBA/CaJ mice Ln2.2 and Ln3.8 demonstrated normal ABR thresholds (approximately 20–30 dB). A progressive increase in click ABR threshold is seen 1 and 3 days after ouabain treatment.

Figure 2.

Spiral ganglion neuron (SGN) loss in ouabain mouse model. (A–C) Dual labeling with anti-TuJ1 III β -tubulin (TuJ1, red) and anti-myelin basic protein (MBP, green) in a control mouse. TuJ1 antibody preferentially labels the cytoplasm of large type I neurons. MBP labeling of the myelin sheaths of Schwann cells in a honeycomb-like pattern in the spiral ganglion. (D–F) Dual labeling with anti-TuJ1 (red) and anti-MBP (green) in a mouse 3 days after ouabain application. The arrows in Figure 2D and F represent surviving type II SGNs following ouabain exposure. Scale depicts 15 μ m.

Figure 3.

Stromal cell-derived factor-1 (SDF-1) mRNA expression in ouabain mouse model.

Following ouabain application, there was a significant increase in SDF-1 mRNA expression in the injured cochlea at all time points.

Figure 4. Stromal cell-derived factor-1 (SDF-1) protein expression (green fluorescent protein). Double labeling for SDF-1 (green) and nuclear stain PI (red) showed increased cytoplasmic expression of SDF-1 following ouabain application. Scale depicts 10 μ m.

Figure 5. Stromal cell-derived factor-1 (SDF-1) protein expression (grayscale). Increased SDF-1 expression following ouabain application is seen 1 day after exposure after conversion of images to grayscale. Scale depicts 10 μ m.

Figure 6.

Stromal cell-derived factor-1 (SDF-1) protein expression depicted as grayscale luminance in the apical, middle, and basal turns of the cochlea after ouabain exposure. Asterisks indicate a significant increase in SDF-1 protein expression ($P < .05$).

Figure 7.

Stromal cell-derived factor-1 (SDF-1) protein expression depicted as mean factor change of grayscale luminance after ouabain exposure in the apical, middle, and basal turns of the cochlea. Asterisks indicate a significant increase in SDF-1 protein expression ($P < .05$).

Figure 8. Stromal cell-derived factor-1 (SDF-1) protein expression depicted using Western blot analysis. Increased SDF-1 expression is observed at 3 and 7 days after ouabain exposure.

Table 1

Significance (*P* Values) for Stromal Cell–Derived Factor-1 Protein Expression as Luminance Intensity following Cochlear Injury

Day after Ouabain Exposure	Apical Turn	Middle Turn	Basal Turn
1	.01662 (n = 6)	.005216 (n = 6)	.005087 (n = 6)
3	.05878 (n = 6)	.070215 (n = 7)	.112268 (n = 7)
7	.018789 (n = 3)	.024565 (n = 3)	.110447 (n = 3)
14	.027775 (n = 4)	.062304 (n = 4)	.102597 (n = 4)
30	.140867 (n = 3)	.336725 (n = 3)	.116126 (n = 3)

The bold values in Table 1 indicate statistically significant values.