The Application of Low-Intensity Pulsed Ultrasound (LIPUS) in Dentofacial Deformity

2021 Grants

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FollowUp Form

Award Information

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Title of Project*

The Application of Low-Intensity Pulsed Ultrasound (LIPUS) in Dentofacial Deformity

Award Type

Biomedical Research Award (BRA)

Period of AAOF Support

July 1, 2021 through June 30, 2023

Institution

University of North Carolina Chapel HIll

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)

Collin McKinney, BS

Amount of Funding

$30,000.00

Abstract

(add specific directions for each type here)

Respond to the following questions:

Detailed results and inferences:*

If the work has been published, please attach a pdf of manuscript below by clicking "Upload a file".
OR
Use the text box below to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should also be attached by clicking "Upload a file".

Final Report for AAOF BRA - Rishma Shah.pdf
Please see attached file for complete report - thank you.

Were the original, specific aims of the proposal realized?*

The original Aim 1 was realized. Aim 2 was modified following analysis of the results - we have secured additional grant funding to continue with RNAseq investigations to generate preliminary data for a NIDCR R01 application.

Were the results published?*

Yes

Have the results of this proposal been presented?*

Yes

To what extent have you used, or how do you intend to use, AAOF funding to further your career?*

Please see the attached report which outlines how my career progression has been supported by the AAOF, and for this, I am eternally grateful.

Accounting: Were there any leftover funds?

$0.00

Published

Citations*

You indicated results have been published. Please list the cited reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers

• Portillo G, Sanchez D, Sun K, Shah R*. Enhanced Skeletal Muscle Regeneration Using Low-intensity Pulsed Ultrasound. Tissue Eng 2023;29(Iss 9-10):257-305.
• Young M, Sanchez D, Pramojaney M, KcKinney C, Shah R*. Low-Intensity Pulsed Ultrasound (LIPUS) Promotes Murine Skeletal Muscle Regeneration. J Dent Res 2021;100(Spec Iss B):2267 (IADR/AADR/CADR).

Was AAOF support acknowledged?

If so, please describe:

The AAOF was acknowledged as sponsors of the work.

Presented

Please list titles, author or co-authors of these presentation/s, year and locations:*

• Portillo G, Sanchez D, Sun K, Shah R*. Enhanced Skeletal Muscle Regeneration Using Low-intensity Pulsed Ultrasound. Presented at the TERMIS-AM annual meeting.
• Young M, Sanchez D, Pramojaney M, McKinney C, Shah R*. Low-Intensity Pulsed Ultrasound (LIPUS) Promotes Murine Skeletal Muscle Regeneration. Presented at the AADR/IADR annual session. Awarded NC-AADR Bloc Travel Award.
• Young M, Sanchez D, Pramojaney M, McKinney C, Shah R*. Low-Intensity Pulsed Ultrasound (LIPUS) Effect on Murine Skeletal Muscle Regeneration. Presented at the UNC-CH Adams School of Dentistry Research Day. Awarded Turner Award, NC-AADR

Was AAOF support acknowledged?

If so, please describe:

The AAOF was acknowledged as sponsors of the work.

Internal Review

Reviewer Comments

Reviewer Status*

File Attachment Summary

Applicant File Uploads

- Final Report for AAOF BRA - Rishma Shah.pdf

The Application of Low-Intensity Pulsed Ultrasound (LIPUS) in Dentofacial Deformity
FINAL REPORT FOR AAOF BRA

1. Hypothesis and Specific Aims

Our **long-term goal** is to improve outcomes through the development of innovative therapies for the management of dentofacial and craniofacial deformity. In this study, we proposed to study the role of low-intensity pulsed ultrasound (LIPUS) in skeletal muscle regeneration and adaptation *in vitro*, and understand its potential to help engineer craniofacial muscle tissue for restoration of defects. In the US, LIPUS was approved for the accelerated healing of specific fresh bone fractures in 1994, and treatment of non-union of bone fractures in 2000. Although LIPUS is used for muscle injuries, little research exists on the muscle response. Therefore, our study **hypothesis** was that faster skeletal muscle regeneration and adaptation is supported by application of LIPUS, and the following **specific aims** were used to answer our hypothesis:

- **Aim 1: Modification of bioreactors and optimization of LIPUS parameters**
  The goals of Aim 1 were to: **(1)** Undertake modification of our bioreactors for the delivery of ultrasound and mechanical loading, and measurement of tissue impedance and contraction; **(2)** Identify optimal LIPUS parameters for muscle tissue regeneration *in vitro*.

- **Aim 2: Investigate *in vitro* effects of LIPUS on skeletal muscle regeneration and adaptation**
  The goals of Aim 2 were to: **(1)** Investigate human craniofacial muscle-derived cell response to LIPUS; **(2)** Investigate response of mechanically-loaded skeletal muscle constructs exposed to LIPUS.

2. Studies and Results

- **Aim 1: Modification of bioreactors and optimization of LIPUS parameters**

Clinical application of LIPUS involves a 20-minute period once a day at 1-3MHz, pulsed at 1kHz and intensity of 30mW/cm^2. We successfully modified our bioreactors and incorporated transducers to deliver ultrasound at a frequency of 1.7MHz based on availability of transducers (**Fig 1**). Mathematical mapping validated consistent frequency throughout the base of the petri dishes used to contain muscle cells (**Fig 2**). There was a slight drop-off at the edges, however, this was deemed negligible.

To determine the effect of 1.7mHz LIPUS, we applied 20 minutes of LIPUS daily over a 5-day period to the C2C12 mouse muscle cell line in 2D culture. Control cells were not exposed to LIPUS. The 5-day period was sufficient to determine cell proliferation and differentiation within a starting cell population of 10^4 cells/petri dish. We found greater cell proliferation over time in both LIPUS-exposed cells and control cells. However, there were greater numbers of cells in the LIPUS-exposed cells by day 5 albeit statistically non-significant (**Fig 3**).

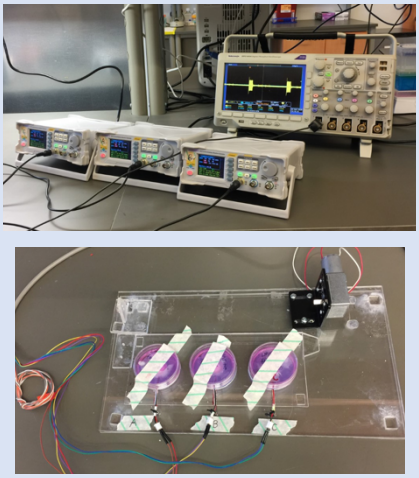

Fig 1. Bioreactor with transducers and signal generators

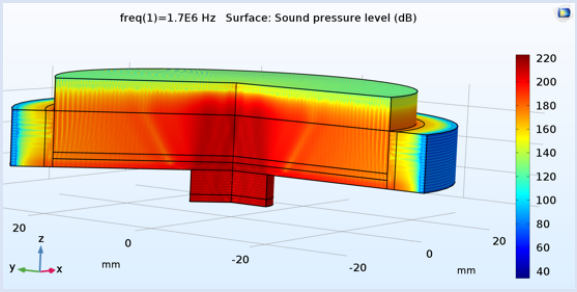

Fig 2. Mathematical mapping of ultrasound frequency throughout the petri dish used for cell culture. The ultrasound signal reached throughout the base albeit a little concentrated at the core and reduced at the extreme edge.

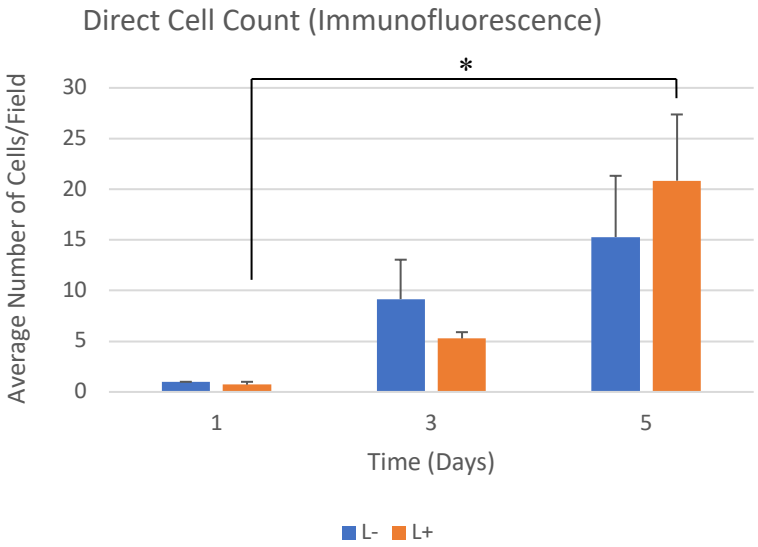

Fig 3. Muscle cell proliferation over a 5-day period. The general trend was increase in cell number with time, with C2C12 mouse muscle cells exposed to LIPUS demonstrating greater numbers at day 5. (*P<0.05)

We used RT-PCR and western blots to determine the expression of genes and proteins respectively that are associated with muscle cell differentiation and muscle fiber type. The myogenic regulatory factors (MRFs), *MYF5*, *MYOD1*, *MYOG*, *MRF4*, demonstrate temporal expression and are determinants of muscle fiber formation[1]. Muscle fiber types determine contractile properties and overall muscle function. Regenerating muscle fibers express the perinatal and embryonic fiber types followed by fast muscle fiber types. Further maturation of the fibers results in a mix of slow and fast muscle fibers. Unique to the craniofacial adult muscle, there is persistent expression of the perinatal, embryonic, and α-cardiac fiber types that allow for rapid adaptation to a continually changing environment[2]. We found *MYOG* gene expression at day 3 was expressed at greater levels in cells exposed to LIPUS indicating greater muscle fiber formation (**Fig 4**). Embryonic fiber type gene expression was greater at day 3 in control cells,

however this gene expression increased in control and LIPUS-exposed cells by day 5 with no statistically significant difference.

Myogenin protein expression was expressed at day 1 in both groups indicating muscle fiber formation. This decreased over the 5-day period as per the expected temporal expression in regenerating muscle (**Fig 5**). The later increase in *MYOG* gene expression may suggest further cell commitment to differentiation. Embryonic muscle fiber (MyHC3) protein expression was greater in cells exposed to LIPUS by day 3.

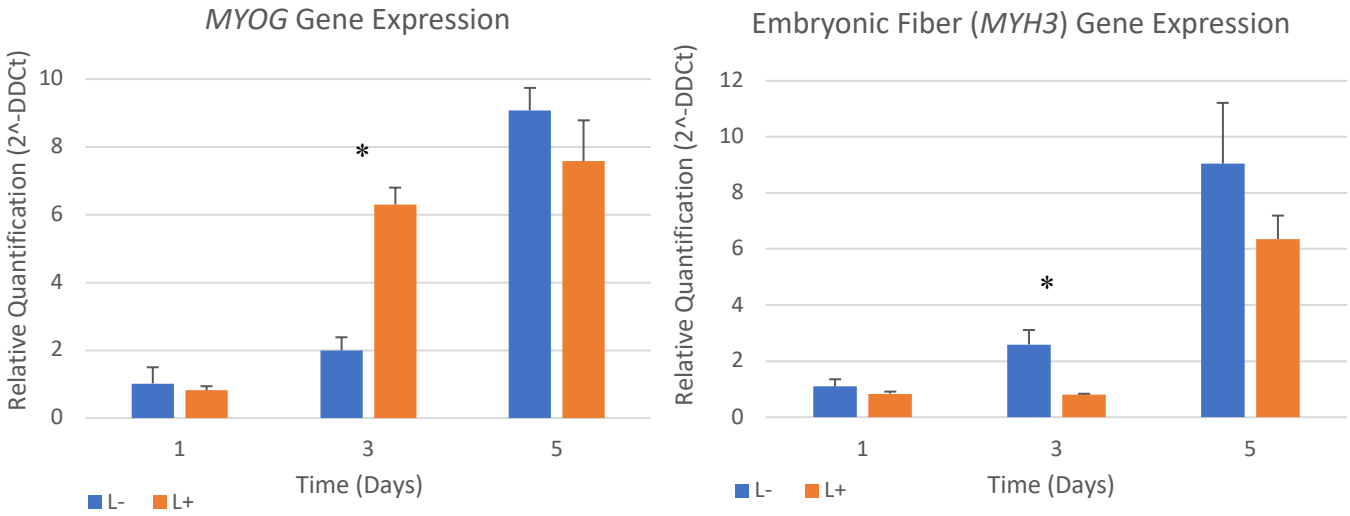

Fig 4. Gene expression in cells exposed to LIPUS and control cells that were not exposed to LIPUS. There was earlier and greater muscle fiber formation by day 3 in cells exposed to LIPUS as indicated by *MYOG* gene expression. Cells in both groups expressed increased *MYH3* gene expression by day 5. (*P<0.05)

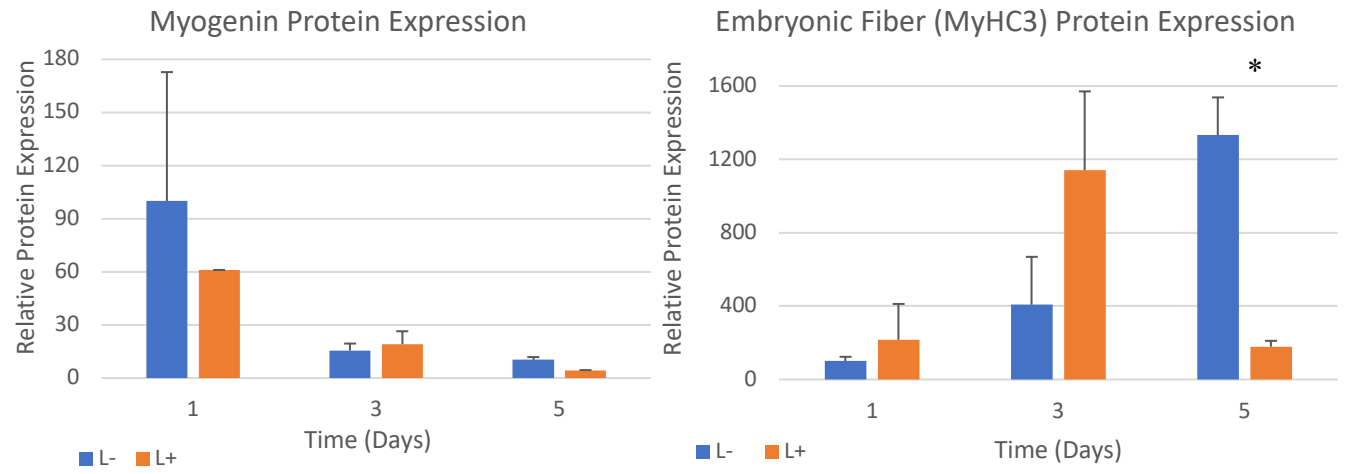

Fig 5. Protein expression in cells exposed to LIPUS and control cells that were not exposed to LIPUS. There was muscle fiber formation by day 1 in both groups as indicated by myogenin protein expression. MyHC3 expression was greater by day 3 in cells exposed to LIPUS. (*P<0.05)

- **Aim 2: Investigate *in vitro* effects of LIPUS on skeletal muscle regeneration and adaptation**

With limited availability of ultrasound transducers as a result of the COVID pandemic and the positive results obtained from Aim 1, we decided to not investigate other ultrasound frequencies and continue with investigation of primary masseter muscle-derived cells.

Cells were extracted from mouse masseter muscle and characterized for muscle fiber formation. Thereafter, we investigated cell proliferation and differentiation in 2D culture. We found cell proliferation increased in both groups over a 5-day period (**Fig 6**). Compared to the C2C12 cell line, we found *MYOG* gene expression had greatest increase by day 3 in both groups followed by a decrease – again, this was consistent with the temporal expression seen in regenerating muscle (**Fig 7**). Expression of the fast fiber types (*MYH1* and *MYH2*) increased from days 1 to 3 in both groups. The *MYH1* gene expression decreased by day 5 in both groups, however, this was less in the cells exposed to LIPUS. *MYH2* gene expression decreased in the control cells by day 5, but increased in cells exposed to LIPUS.

It seems that LIPUS has a positive effect on muscle cell proliferation and differentiation. Our experiments have been carried out in 2D. Discussion of our findings with our consultants and colleagues has informed our future direction. It seems prudent to investigate the cell response in 3D scaffolds without any other influences (i.e. mechanical loading), and the use of bulk RNAseq to identify markers of muscle and tendon formation has been suggested. We have secured additional funding supported by these study findings to undertake bulk RNAseq experiments. Thereafter, the overall findings will support a future R01 application to the NIH NIDCR.

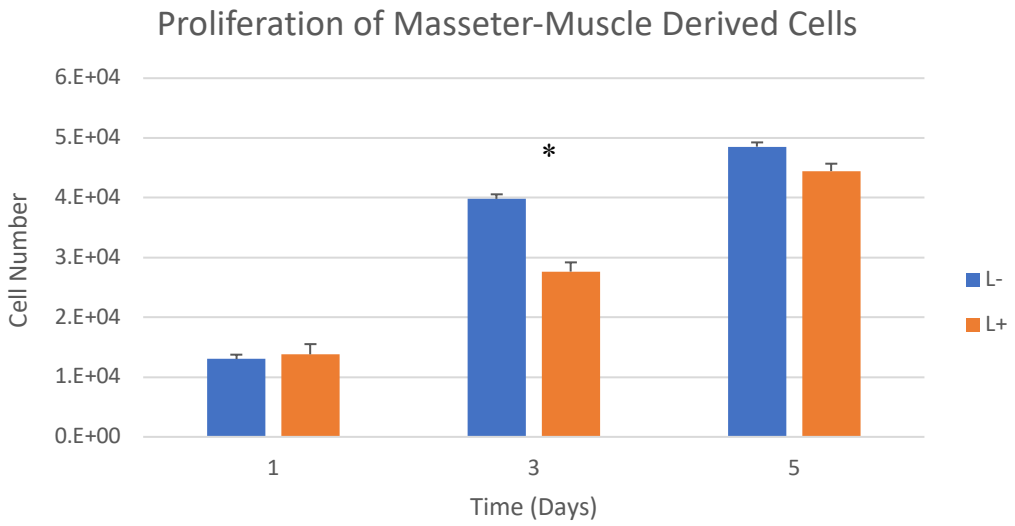

Fig 6. Mouse masseter muscle-derived cell proliferation over a 5-day period. The general trend was increase in cell number with time, with control cells demonstrating greater numbers on day 3 compared to cells exposed to LIPUS. There was no difference between the groups by day 5. (*P<0.05)

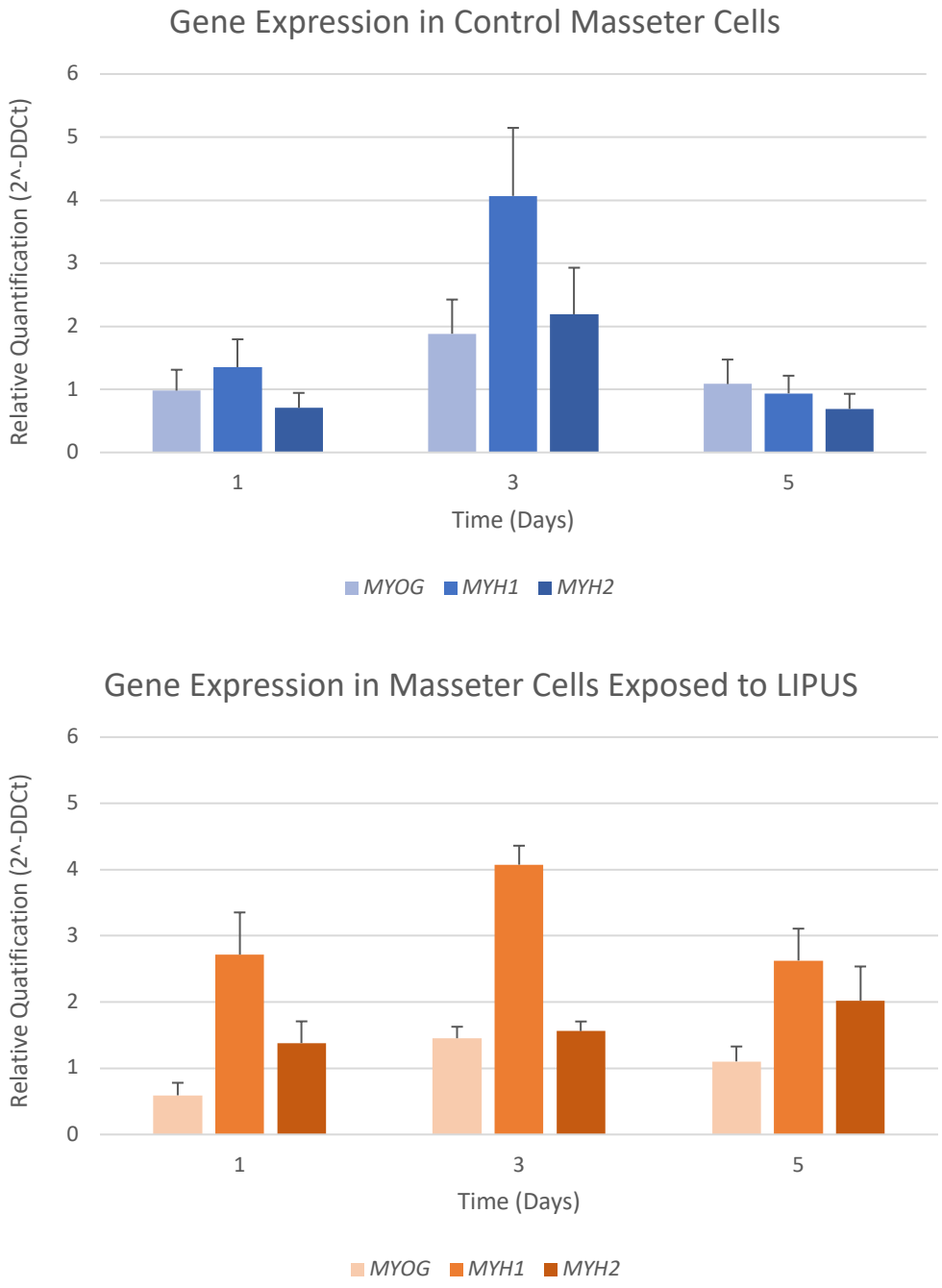

Fig 7. Gene expression in mouse masseter muscle-derived cells over a 5-day period. There was the expected temporal expression of the *MYOG* gene in cells exposed to LIPUS and control cells. Expression of the fast fiber type genes was noted in both groups consistent with regenerating muscle. It was noted the *MYH2* gene expression increased by day 5 in cells exposed to LIPUS whereas levels decreased in the control cells.

3. Impact

a. Grants

In Preparation

Feb 2024 NIDCR R01 Award
"Application of Low-Intensity Pulsed Ultrasound for Rapid Prototyping of Craniofacial Skeletal Muscle"
PI: **Shah R**
20% effort. Total Award: ~$2,600,000.

Awarded

2022-2023 NCTraCS Institute, Pilot Award #550KR282119
"Application of Ultrasound for Craniofacial Skeletal Muscle Regeneration"
PI: **Shah R**
1% effort. Total Award: $50,000.

b. Publications and Presentations

- Portillo G, Sanchez D, Sun K, **Shah R***. Enhanced Skeletal Muscle Regeneration Using Low-intensity Pulsed Ultrasound. Tissue Eng 2023;29(Iss 9-10):257-305.
  Presented at the TERMIS-AM annual meeting
- Young M, Sanchez D, Pramojaney M, McKinney C, **Shah R***. Low-Intensity Pulsed Ultrasound (LIPUS) Promotes Murine Skeletal Muscle Regeneration. J Dent Res 2021;100(Spec Iss B):2267 (IADR/AADR/CADR).
  Awarded NC-AADR Bloc Travel Award
  Presented at the AADR/IADR annual session
- Young M, Sanchez D, Pramojaney M, McKinney C, **Shah R***. Low-Intensity Pulsed Ultrasound (LIPUS) Effect on Murine Skeletal Muscle Regeneration. Dental Research Day, UNC Adams School of Dentistry, UNC-CH, Chapel Hill, NC.
  Awarded Turner Award, NC-AADR
  Presented at the UNC-CH Adams School of Dentistry Research Day

4. A Note of Gratitude

Funding from the AAOF has been invaluable to support the purchase of research supplies and protection of my time to undertake the above outlined work. I am very grateful for the award and the multiple achievements that it has facilitated. Thank you!

References

[1] Asfour, H. A., Allouh, M. Z., & Said, R. S. (2018). Myogenic regulatory factors: The orchestrators of myogenesis after 30 years of discovery. *Experimental biology and medicine (Maywood, N.J.)*, *243*(2), 118–128. https://doi.org/10.1177/1535370217749494

[2] Sciote, J. J., Horton, M. J., Rowlerson, A. M., & Link, J. (2003). Specialized cranial muscles: how different are they from limb and abdominal muscles?. *Cells, tissues, organs*, *174*(1-2), 73–86. https://doi.org/10.1159/000070576