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Original Research

Effects of Aqua-acupuncture on Wound Healing in Rainbow Trout

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ABSTRACT
This study investigated the effect of a single aqua-acupuncture (aqua-AP) treatment on wound healing after surgical ventral incision in rainbow trout, *Oncorhynchus mykiss*. Three experiments were conducted using 20 fish per experiment (10 aqua-AP, 10 non-acupuncture): 1) ventral incision closed with two sutures and insertion of an acoustic transmitter, 2) ventral incision closed with two sutures (no transmitter), and 3) unclosed ventral incision. Aqua-acupuncture was performed by injecting a 0.25 mL bleb of vitamin B12 - saline mixture intramuscularly at 4 points around the surgical site. Statistical differences between aqua-AP and non-acupuncture for survival, transmitter retention, wound closure and redness (inflammation) were conducted ($p<0.05$). Experiment 1 had significantly lower ($p=0.007$) redness scores with aqua-AP treatment when compared to non-acupuncture. In the second and third experiments, there were no significant differences in wound closure times or redness, and acoustic transmitter retention was not significantly different between treatments. Experiment 3 had granuloma formation (50% both groups) in place of skin edge closure at 6 weeks. By study termination (12 weeks), only 1 aqua-AP fish still had a granuloma compared to 4 fish in non-acupuncture ($p<0.04$). There were no significant differences in wound closure times (aqua-AP versus non-acupuncture). While the overall results of this study were inconclusive for wound healing time, the decreased redness (Experiment 1) and improved granuloma resolution (Experiment 3) suggest that there may be potential benefit from aqua-AP use in rainbow trout wound healing. Aqua-AP appears safe to use on fish with future research directed at technique refinement.

Keywords: acupuncture, aqua-acupuncture, *Oncorhynchus mykiss*, rainbow trout, surgery

ABBREVIATIONS: AP: acupuncture; aqua-AP: aqua-acupuncture

Wound healing is a complicated process that has been studied extensively in both mammalian and fish species.[1-3] The inflammatory response in fish is similar to mammals, following the same expression of pro-inflammatory cytokines, vasodilation, and chemotactic influx of leucocytes from the blood to the wound site.[4] Unlike mammals, fish skin lacks a keratin protective layer. Instead the epithelial cells of fish are in direct contact with the environment.[5] Fish, therefore in comparison to non-aquatic animals, must have faster regeneration of the dermal and epidermal layers for protection from waterborne pathogens and to maintain osmotic balance.[6]

Acupuncture (AP) involves the insertion of thin needles at strategic anatomic points to treat various health conditions, including wound healing.[7] In Asia, acupuncture has been an integral part of traditional Chinese medicine for thousands of years.[8] It increases blood circulation to affected areas, with subsequent increase of neuropeptides, cytokines, and other vasoactive substances.[9] Acupuncture also enhances wound healing accelerators such as fibroblast growth factors and platelet-derived growth factors in cats and mice.[8,10] It promotes more rapid wound closure, reduces edema and reduces wound associated hematomas in mammals.[10,11]

While there are a variety of acupuncture techniques, aqua-acupuncture (aqua-AP) may be most useful for fish. It is a stimulation technique where a liquid agent (often vitamin B12) is injected into an acupoint.[12] In aqua-AP, the needles do not need to be maintained for stimulation. Rather, stimulation is created through the spatial configuration changes at the acupoint caused by the injected liquid substrate.[13]

Despite its potential benefits, aqua-AP has not been examined in fishery and aquaria science. The objective of this study was to investigate the potential of aqua-AP

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performed at the time of surgery to promote incision closure/wound healing in rainbow trout, *Oncorhynchus mykiss.* The study hypothesis was that the incision wound healing outcome data would be improved in the aqua-AP groups when compared to the non-AP groups.

MATERIALS AND METHODS

Three experiments were conducted at McNenny State Fish Hatchery (Spearfish, South Dakota, USA). The hatchery is supplied with well water with quality parameters at the time of the experiments that included: 11˚C; total hardness as $CaCO_3$, 360 mg/L; alkalinity as $CaCO_3$, 210 mg/L; pH 7.6; and total dissolved solids, 390 mg/L. All experiments used adult Arlee strain rainbow trout, received as eggs from Ennis National Fish Hatchery[a] and reared in a common tank. At experiment start, subject fish had a mean (±SE) initial length of 253±3 mm and weight of 211±9 g. The 3 experiments (n=20 fish/experiment) were: 1) ventral incision closed with two sutures and insertion of an acoustic transmitter, 2) ventral incision closed with two sutures and no insertion of acoustic transmitter, and 3) unclosed ventral incision and no insertion of acoustic transmitter (Table 1). Each experiment consisted of two treatment groups: subject fish not treated with aqua-AP (n=10) and subject fish treated with aqua-AP to promote incision closure (n=10). The three experiments were based on three commonly encountered scenarios in fisheries and aquaria sciences. Acoustic transmitters are commonly used by fisheries professionals to track movement and migration patterns of fish.

Pre-aquapuncture Methods Common to All Experiments

Before surgery, each fish was anesthetized to stage IV anesthesia with 60 mg/L MS-222 Syncaine[b].[14] All surgeries were performed by a trained surgeon using standard published technique.[15] Once anesthetized, a Floy Tag[c] was inserted directly below the dorsal fin on the left side. Different colors were used for each treatment so group identification of a fish could be maintained throughout the study. Further identification of separate individuals within a treatment group was not performed. Immediately after Floy Tag insertion, each fish was placed in a V-shaped trough, with their gills constantly flushed with water containing anesthetic. A 10 mm incision was then made cranial to the pelvic groove, slightly off the mid-ventral line, and just deep enough to open into the peritoneal cavity.

Methods Unique to Each Experiment

Experiment 1: A dummy acoustic transmitter[d] (9 × 24 mm, 3.6 g weight in air) was inserted through the incision into the peritoneal cavity. Each transmitter was soaked in iodine for disinfection prior to insertion. Two absorbable sutures[e] were placed in a simple interrupted pattern to close the ventral surgical incision. Mean (±SE) tag-to-body ratio was 1.8±0.06 %, well below the suggested 2 % tag to body-weight guideline.[16]

Experiment 2: The methods were identical to the first experiment except that a dummy transmitter was not inserted.

Experiment 3: The methods were identical to the first two experiments except neither a dummy transmitter nor sutures were used.

Aqua-acupuncture: The acupuncture method, Circling the Dragon, was performed during the surgical process at the incision site by a certified veterinary acupuncturist.[17] The technique was performed using a 25-gauge needle to inject a 0.25 mL bleb of a 1:1 vitamin B12[f] - saline mixture intramuscularly at four points (two on each side of the incision) around the surgical site. This was performed quickly and added no more than 10 seconds to the surgical procedure. One treatment group (n=10) from each of the 3 experiments received aqua-AP during the surgical process.

Table 1: Experimental design for each of 3 different experiments evaluating the use of aqua-acupuncture (aqua-AP) during surgery on rainbow trout and study findings for length of surgery site healing (weeks) and individuals with granuloma formation.

Experiment	Acoustic Tag	Sutures	Aqua-AP	50% Closure (weeks) mean±SE	100% Closure (weeks) mean±SE	Granulomas	
						6 wks	12 wks
1 (n=20)	Yes (n=20)	Yes (n=20)	Yes (n=10)	1.0±0.0	3.2±0.7	0/10	0/10
			No (n=10)	1.0±0.0	3.8±0.7	0/10	0/10
2 (n=20)	No (n=20)	Yes (n=20)	Yes (n=10)	1.6±0.4	2.2±0.2	1/10	0/10
			No (n=10)	1.0±0.0	1.6±0.6	0/10	0/10
3 (n=20)	No (n=20)	No (n=20)	Yes (n=10)	12.0±0.0 no closure	12.0±0.0 no closure	5/10	1/10
			No (n=10)	12.0±0.0 no closure	12.0±0.0 no closure	5/10	4/10

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Post-Surgery Common Methods

All fish were placed in the same concrete raceway (5.2 m long × 2.4 m wide and 0.5 m deep) for the duration of the study. Fish were fed a floating feed[g] at a rate slightly above satiation. Dissolved oxygen in the raceway was maintained at or above 6.0 mg/L. Once a week, the fish were anesthetized, and their surgical incision/wound formation were photographed. At the conclusion of the study, the acoustically tagged fish were euthanized with a lethal dose of MS-222[b] and necropsied to determine acoustic tag retention. Surgical site photographs were viewed with knowledge of the treatment group and graded by five experienced fisheries professionals. Incisions were graded based on closure and redness using an adaptation of the Paukert et al. method as described by Kientz (Table 2; Figures 1-3).[18,19]

Data Analysis

All data were analyzed using the SPSS[h] statistical analysis computer program. Chi-square analysis was conducted to identify differences between aqua-AP and non-AP treatments for survival and tag retention. Wound closure data was analyzed using one-way analysis of variance (ANOVA). A repeated measures ANOVA with Greenhouse-Geisser correction was used to analyze wound redness data between treatments over time. If a significant difference was observed, a one-way ANOVA test was performed for each week. Significance was pre-determined at $p<0.05$.

Table 2: Scoring criteria used to determine post-surgical incision healing and site closure; based on Paukert method[18]

Score	Incision/Wound Redness	Incision/Wound Closure
0	No redness present	Complete closure
1	Redness localized to incision/suture site	Closure of <50%
2	Redness extended beyond incision/suture site	No closure

Figure 1: Photographs of Experiment 1 fish incision sites; Left (non-aquapuncture, Week 3): redness around sutures and graded as Redness = 1, Closure = 1; Right (aquapuncture, Week 11): sutures retained in a healed site and graded as Redness = 0, Closure = 0

Figure 2: Photographs of Experiment 3 incision site (same fish, Aqua-AP Group); Left picture - Week 6 and Right picture - Week 12; Note a granuloma has formed (left) and graded as Redness = 1, Closure = 1; on right, granuloma resolution, site graded as Redness =0, Closure = 0

Figure 3: Photographs of Experiment 3 incision site (same fish, Non-AP Group); Left picture - Week 6 and Right picture - Week 12; Note a granuloma has formed (left) and graded as Redness=1, Closure = 2 (no closure); on right, granuloma still present, site graded Redness=0, Closure=2 (no closure)

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RESULTS

Mortality and Subject Data Removal

One mortality occurred in Experiment 1 in the non-AP group by second sampling, with cause of death undetermined. This was the only mortality in the study and survival was not significantly different among the treatments in any of the experiments (p=0.42, χ^2=7.089).

In Experiment 2, the data from 1 aqua-AP fish was removed prior to analysis. The fish developed a granuloma, which made determination of wound closure unattainable with the grading method used in this study. In Experiment 3, granuloma formation was equally distributed among both treatment groups, so statistics were still used. Both Experiment 1 (non-AP group) and Experiment 2 (aqua-AP group) had reduced group numbers (n=9) used for outcome data statistics. All other study groups had all fish complete the study (n=10) and their outcome data was used for statistical evaluation.

Experiment 1

The mean (±SE) time to achieve 50% closure was 1.0±0.0 week for both aqua-AP and non-AP treated groups (Table 1). Complete wound closure (100%) was slightly shorter in the aqua-AP group at 3.2±0.07 weeks when compared to the non-AP group at 3.8±0.07, although not statistically different in this small study (p=0.6). Fish in the aqua-AP treatment had significantly lower mean redness scores at the end of the 12-week experiment compared to those not receiving aqua-AP (F=5.074; p=0.007) (Figure 4). In addition, the aqua-AP treatment had significantly lower wound redness scores on weeks 2, 3, 4, 5, 8, 9, and 10 compared to the non-aqua-AP treatment. No granulomas were formed in Experiment 1. Additionally, acoustic transmitter retention was not significantly different at 100% in the non-AP treatment compared to 90% in the aqua-AP treatment (p=0.3, χ^2=1.053).

Experiment 2

The mean (±SE) time to achieve 50% closure for trout receiving aqua-AP was 1.6±0.4 weeks, which was not significantly different than the 1.0±0.0 weeks for the non-aqua-AP trout (Table 1). Similarly, the 2.2±0.2 weeks to complete wound closure in the aqua-AP group was not significantly longer than the 1.6±0.6 weeks for the non-aqua-AP group. There was no significant difference for wound redness scores over the 12-week period (F=2.111; p=0.119) (Figure 5). One fish in the aqua-AP group developed a granuloma by week 3 (persisted until week 9) and all data for this fish was removed from statistical analysis.

Experiment 3

Neither 50% nor complete wound closure was significantly different between treatments (Table 1). These results were skewed by the presence of numerous granulomas. In both treatments, 50% of the fish developed a granuloma by week 6. By week 12, only 1 fish receiving aqua-AP had a granuloma remaining, while 4 fish not receiving aqua-AP still had granulomas. Depending on the reviewer's perception, granulomas may have been graded as an unclosed wound. There was no significant difference over time between the treatments in wound redness scores over the 12-week trial (F=0.27; p=0.66) (Figure 6).

Experiments 1-3

Combining all experiments, 2.5% (1 of 40) sutured fish developed a granuloma. In contrast, 50% (10 of 20) of un-sutured fish developed granulomas. Resolution of granulomas by study termination (week 12) was 83% for fish receiving aqua-AP and 20% for fish not receiving aqua-AP (p=0.04). There were no adverse events documented during the study associated with the use of aqua-AP technique on study fish.

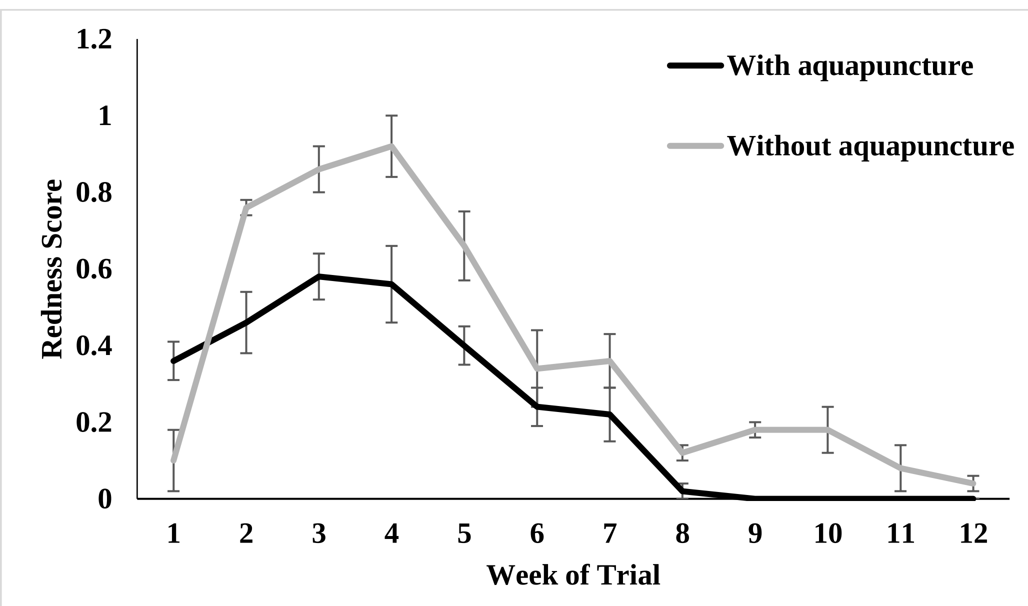

Figure 4: Experiment 1 mean (±SE) wound redness scores for rainbow trout subjected to acoustic transmitter insertion surgery with sutures and with or without aqua-AP. The aqua-AP treatment had significantly lower mean redness scores over the 12-week period compared to the treatment without aqua-AP (F=5.074; p=0.007). Significant differences occurred on weeks 1, 2, 3, 4, 5, 8, 9, and 10.

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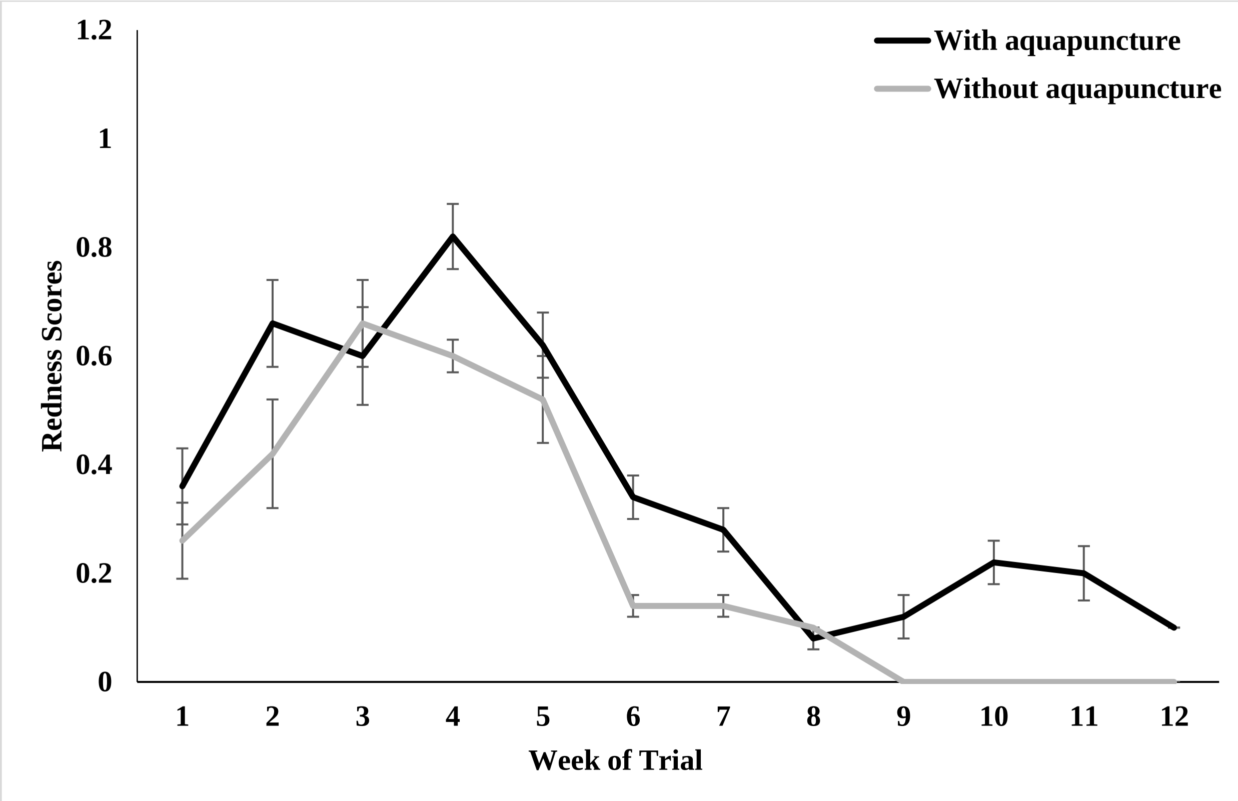

Figure 5: Experiment 2 mean (±SE) wound redness scores for rainbow trout subjected to a sutured incision with or without aqua-AP. There was no significant difference in wound redness scores over the 12-week period (F=2.111; p=0.119).

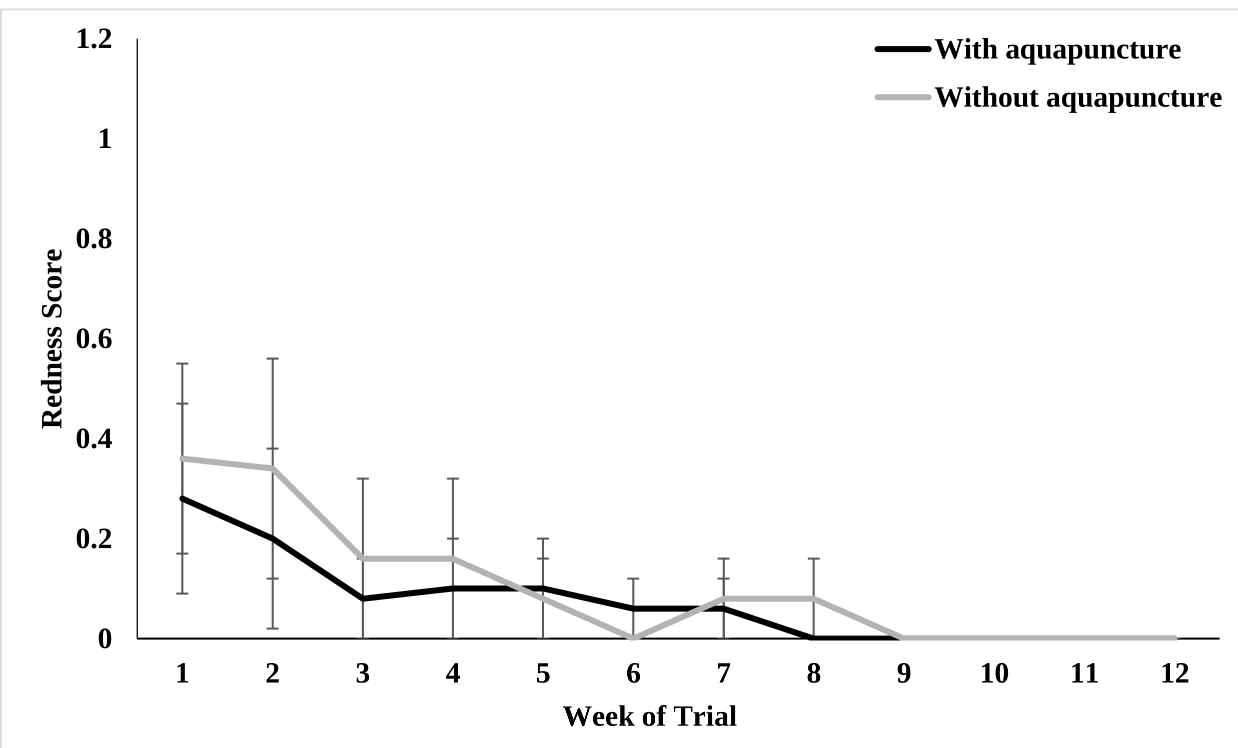

Figure 6: Experiment 3 mean (±SE) wound redness scores for rainbow trout subjected to a non-sutured incision with or without aqua-AP. There was no significant difference in wound redness scores over the 12-week period (F=0.27; p=0.66).

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DISCUSSION

Aqua-acupuncture as a form of traditional Chinese medicine, has been effectively used to treat wounds in humans, rats, and mice.[12,17,20] While fish integument is different from mammals, the inflammatory response is similar; making it plausible to investigate its benefit for fish.[4,5] Three experimental scenarios which included the use of aqua-AP following a ventral surgical incision were explored in rainbow trout. Outcome data included wound closure time, post-surgical inflammation (i.e. tissue reddening), and resolution of surgical site granulomas. Two statistically significant findings were demonstrated in the study. In Experiment 1, lower mean redness scores (inflammation) were present in the aqua-AP group compared to the non-acupuncture group (p<0.007); and in Experiment 3, resolution of surgical wound granulomas was greater for the aqua-AP group (83%) versus non-acupuncture group (20%, p<0.04). These findings partially satisfied the study hypothesis.

Although not statistically significant, complete closure of incision wounds in the first experiment were slightly shorter in the aqua-AP group. This, however, was offset by non-statistical slightly longer wound closure times in Experiment 2. The difference between the groups, transmitter insertion (shorter aqua-AP group healing time) and no transmitter (longer aqua-AP group healing time) would not seem to explain the difference in results. One plausible explanation is that study fish were evaluated as a group rather than followed as individuals. Outliers with pre-existing conditions may have existed in either group but would not be identified. With small group numbers (n=9), this may lead to confounding results. Support that the shorter healing time in Experiment 1 may be valid is the associated statistically significant decreased redness scores (inflammation) in the aqua-AP group and the improved resolution of granulomas associated with aqua-AP in Experiment 3.

When compared to published studies the 1.0 week to 50% wound closure and 3.2 weeks to 100% wound closure times in the Experiment 1 aqua-AP treated fish are shorter than those times reported in other similar studies. Kelican observed 50% wound closure in 2.6 weeks and 100% wound closure in 4.5 weeks in identical surgery methodology without aqua-AP.[21] Kientz achieved 100% wound closure in four weeks with similarly sized rainbow trout.[19]

The presence of granulomas in this study was unexpected and particularly complicated data analysis in Experiment 3 where they occurred in 50% of the fish. Depending on reviewer perception, granulomas may be graded as wound inflammation. Wound site inflammation can be affected by temperature, suture retention, suture material, and fish species.[22,23] Fish with sutures in this study (Experiments 1 and 2) recorded no wound inflammation by week 9, and only 1 fish out of 40 had granuloma formation. When compared to other published studies with surgery protocols similar to the present study, resolution of inflammation for sutured fish in the present study was 5 weeks earlier than results by Kientz but 4 weeks later than Huysman and Kelican.[19,21,24]

Granuloma occurrence is difficult to predict but may be associated with multiple factors such as: inflammatory response to a foreign body (i.e. suture material), variable surgeon technique, and/or immune response to un-apposed edges of an incision (healing by secondary intention).[22,25,26] The most likely of these scenarios was lack of incision suturing in Experiment 3, as the same surgeon performed all surgeries and sutured fish had fewer granulomas, demonstrating good tolerance of the suture material. Sutures are typically required to prevent infiltration of water or pathogens into the body cavity post-surgery.[27] Their use to create primary intention healing is suggested by many.[28-30] This is supported by study results demonstrating lack of granulomas when primary union (i.e. sutures) were used in Experiment 1 and 2, compared to secondary healing used in Experiment 3. Of interest, aqua-AP demonstrated resolution of granulomas by study termination (week 12) in 83% of Experiment 3 fish versus 20% for fish not receiving aqua-AP (p=0.04).

This study was limited by a few factors. One of the primary challenges was small group size which affects the quality of statistical information when making group-based estimates. Additionally there was lack of blinding of some experts grading lesions (i.e. knowledge of experimental groups) which can introduce bias either for or against experimental results. As previously stated, surgeon-to-surgeon variability can alter results of surgical studies in fish.[26] This was minimized by using only one trained surgeon during the surgical process. Other limitations included suture technique and material. Closing wounds in fish with sutures can increase wound site inflammation, especially when sutures are retained for extended periods of time.[22] Such inflammation can be reduced by using the correct suture material, which in rainbow trout, is an absorbable monofilament.[23,31,32] Finally, only a single aqua-AP treatment was used in this study. This is the only practical option in a fisheries science setting, however, acupuncture is usually done consecutively for an improved cumulative effect.[33,34] Further studies examining multiple aqua-AP treatments should be performed and may be of particular interest in locations where such multiple treatments would be practical and feasible, such as zoos or aquariums.

In conclusion, while the overall results of this initial study examining wound closure time with aqua-AP in rainbow trout was inconclusive, aqua-AP did reduce wound site inflammation in Experiment 1 and had quicker resolution of granulomas in Experiment 3. The results of this study provide a foundation for future studies on aqua-AP and wound healing in teleost fish. Additional experimentation should occur with different species of fish, different wound locations, multiple aqua-AP injections, more aqua-AP locations, and using different amounts of vitamin B12.

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Declaration of Interest and Funding

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this paper and the authors did not receive any specific grant or funding from any organization in the public, commercial or non-profit sectors.

FOOTNOTES

[a] Ennis National Fish Hatchery, 180 Fish Hatchery Rd, Ennis, MT, USA
[b] Tricaine Methanesulfonate, MS-222; Snydel, Ferndale, WA, USA
[c] Floy Tag & Mfg, Inc. Seattle, WA, USA
[d] VEMCO, Belford, Novia Scotia, Canada
[e] Oasis Nylon Monofilament sutures, 4-0, Glendora, CA, USA
[f] Vitamin B12 Injection 1000 mcg, shopmedvet.com, Mettawa, IL, USA
[g] 4.5-mm floating Oncor 80, Skretting, Tooele, UT, USA
[h] 24.0; IBM, Armonk, NY, USA

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