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The usage of Resveratrol for eye pain after refractive surgery

by

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An undergraduate honors thesis submitted in partial fulfillment of the

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Abstract

LASEK and PRK are common laser corrective eye procedures that aim to correct the refractive errors of the eye, although these procedures do carry the potential of post-operation complications of dry eye and symptoms of pain and photophobia as a result of corneal innervation damage.

Resveratrol was tested on the surface of the cornea to see if the drug would be effective at attenuating post-corrective eye pain. Male and female rats underwent a unilateral corneal abrasion surgery that aimed to mimic the post-op symptoms of PRK. The rats either received the resveratrol drug treatment or a vehicle formulation. The Rat Grimace Scale was used to measure spontaneous pain by grading the severity of orbital tightening, where a higher orbital tightening score represents a higher value of pain. Results showed that there was no significant difference between resveratrol and the vehicle formulation on orbital tightening. There was however a differential response to resveratrol between males and females. Male orbital tightening scores were significantly higher than females, most apparent at 72 hours and 1-week post-abrasion. Histological analysis of the β -Tubulin and CGRP in the cornea was also performed to examine the volume of corneal nerves and their peptide content. The histological assessment did not show a significant difference in β -Tubulin and CGRP levels between the vehicle and resveratrol groups. The findings of this study does not provide support for the use of resveratrol as a post-corrective eye surgery therapeutic.

Introduction

As an alternative to eyeglasses or contact lenses, many patients that suffer from refractive errors of the eye elect to undergo laser corrective eye surgery. Becoming increasingly popular for patients who are not viable candidates for LASIK are procedures such as photorefractive keratectomy (PRK) and laser-assisted sub-epithelial keratectomy (LASEK). Both of these procedures involve the removal of the corneal epithelium with alcohol to access the underlying corneal stromal tissue for ablation with excimer lasers. A consequence of these operations is the potential development of dry eye and associated symptoms of pain, irritation, and photophobia (Herrmann et al, 2015). Ocular pain is mediated by corneal epithelial nerve endings that have their cell bodies in the trigeminal ganglion. Persistent pain may emerge as a result of spontaneous neural activity from the interaction of the abnormally regenerated epithelial nerves with adjacent nerve endings (Ebrahimiadib et al., 2020). In some cases, these acute conditions could become permanent conditions such as with chronic dry eye. Patients that develop chronic dry eye disease exhibit reduced tear secretion which can lead to inflammation and peripheral nerve damage. Inflammation has been shown to increase sensitization of pain sensory neurons (hyperalgesia), and if inflammation were to persist it can lead to changes of gene expression at the trigeminal ganglion effecting their excitability and firing (Belmonte et al. 2017).

Current treatment for post-operative pain consists of usage of non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, although the efficacy of these drugs have been called to question. Treatment with NSAIDs carries side effects of gastric distress, while the use of opioids carries high risk for addiction. Currently there are opportunities in the pharmaceutical field for the development of new

therapeutic treatments that are both beneficial in alleviating symptoms, while also limiting potential harmful side effects.

Prior research has shown the benefits of the drug, resveratrol on the healing process. In mice hind paw incision models, the drug has shown to be able to reduce acute pain without impairing wound healing (Dasgupta and Milbrandt, 2007) and additionally, it was able to prevent the transition into the chronic pain state (Tillu et al., 2012; Burton et al, 2017). Resveratrol, a polyphenol that originates from plant sources is an activator of the enzyme AMP-activated protein kinase (AMPK) which modulates excitability of peripheral nociceptors. Activation of AMPK leads to suppression of proteins such as rapamycin (mTOR) and kinases like mitogen activated protein kinase pathways (MAPK) which are important for sensitization and pain development (Tillu et al., 2012). As a potentially viable treatment in reducing acute spontaneous pain, the effectiveness of resveratrol was tested in a rodent model of PRK.

Methods:

Subjects and Experimental Design

Rats underwent a corneal abrasion surgery (Hegarty et al, 2018) where heptanol was used to remove the corneal epithelium from the left eye. The procedure aimed to mimic the effects of corrective eye surgery by producing acute pain, as well as chronic dry eye symptoms. Three different concentrations of resveratrol were tested in different groups of rats (1%, 2%, 4%) and final testing used a 4% concentration of the drug. Resveratrol emulsion was applied to the ocular surface 24 hours prior to surgery, 10 minutes after surgery, and 24 hours after surgery - a timeframe that is

feasible for human ocular surgeries. This study utilized both male and female rats, tested and graded under the same conditions to ensure that the effects of resveratrol are consistent in both sexes. In addition to the treatment groups, this study employed a control group that received no corneal abrasion and treatment on the eye, and a vehicle group where the rats underwent corneal abrasion surgery, but received a vehicle emulsion on the eye rather than resveratrol.

Behavioral Assessments

To evaluate spontaneous pain after corneal abrasion, the Rat Grimace Scale (Sotocinal et al. 2011) was used which rates the severity of behavioral pain through the displayed degree of orbital tightening (OT). The grading scale ranges from 0-2 where 0 represents no tightening, 1 is moderate, and 2 is obvious and clear orbital tightening. Each of the rats were graded at baseline prior to surgery and treatment, as well as at additional time points post-operation to evaluate for changes in pain.

Histological Assessments

Immunohistochemical analysis of corneal tissue was performed to examine changes in corneal innervation, as well as peptide content within the nerve fibers. Antibodies against β -Tubulin and Calcitonin gene-related peptide (CGRP) were used for these studies. β -Tubulin is a microtubule that help make up the structure and morphology of neurons. Within the β -Tubulin family are three classes: Class I (TUBB1), Class II (TUBB2), and Class III (TUBB3) that are strongly expressed in neurons (Niwa et al, 2013). Since β -Tubulin plays a vital role in the compositional structure of neurons, it is expected that β -Tubulin levels will decrease after neuronal injury. CGRP is a

neuropeptide whose expression has been seen to be upregulated after the occurrence of peripheral nerve injury. The continuous expression of CGRP has been associated with nerve regeneration (Chen et al. 2010).

Control and experimental rats were perfused with 4% paraformaldehyde and corneas were dissected and placed in 0.1 M phosphate buffer (PB), pH 7.4 until immunoprocessing. Corneas were rinsed in 0.1 M PB and 0.1 M Tris-buffered saline (TS), pH 7.6, blocked in 0.1% bovine serum albumin, 0.25% Triton-X in TS and then incubated for 3 nights at 4°C in a cocktail of primary antibodies raised against β -tubulin and CGRP. Corneas were then rinsed and incubated in a cocktail of secondary antibodies conjugated with fluorophores for 2 hours at room temperature. The fluorescent-labeled markers of interest were visualized using a Zeiss LSM 900 confocal microscope. The resultant confocal scans were then analyzed using Bitplane Imaris software where the corneal epithelium was traced and volume of β -tubulin-labeled nerves and CGRP-labeled nerves were measured.

Results

Differences in orbital tightening scores between males vs. females when treated with resveratrol

We found that there was an increase in OT at 24 hours' after corneal abrasion compared to baseline in both the vehicle and treatment groups (Figure 1A & 1B) which is consistent with the expectation that corneal injury evokes acute pain, but also shows that the drug did not reduce acute nociceptive responses. The subsequent grading periods showed a contrast between the resveratrol-treated male and female groups in regards to how OT scores changed over time. In the female group, it was

observed that when rats were treated with resveratrol there is a greater decrease in OT when compared to the vehicle. For female rats treated with resveratrol, at 72 hours, OT values were significantly lower than at 24 hours ($p < 0.05$), and were comparable to OT scores at the one-week mark in the vehicle group. At the one-week time point for resveratrol treated female rats, OT scores were similar to baseline (pre-injury) values.

Within the male groups, rats that received the vehicle formulation displayed a similar pattern of OT scores over time to their female vehicle counterparts. However, in resveratrol treated male rats, the OT scores stayed consistently higher than the vehicle treated rats. From 24 – 72 hours, OT scores in the male resveratrol treated group stayed approximately at the same value, followed by a minor decrease at the one-week mark.

A two-way repeated measures ANOVA with Holm-Sidak post-hoc comparisons did not show a significant difference for vehicle vs. resveratrol in either male or female groups. Whereas a two-way repeated measures ANOVA conducted between the resveratrol groups of male vs. female revealed a significant level of difference at 72 hours and 1-week post-operation where at these time periods, male groups showed significantly higher OT scores in comparison to females ($p < .001$) (Figure 1C).

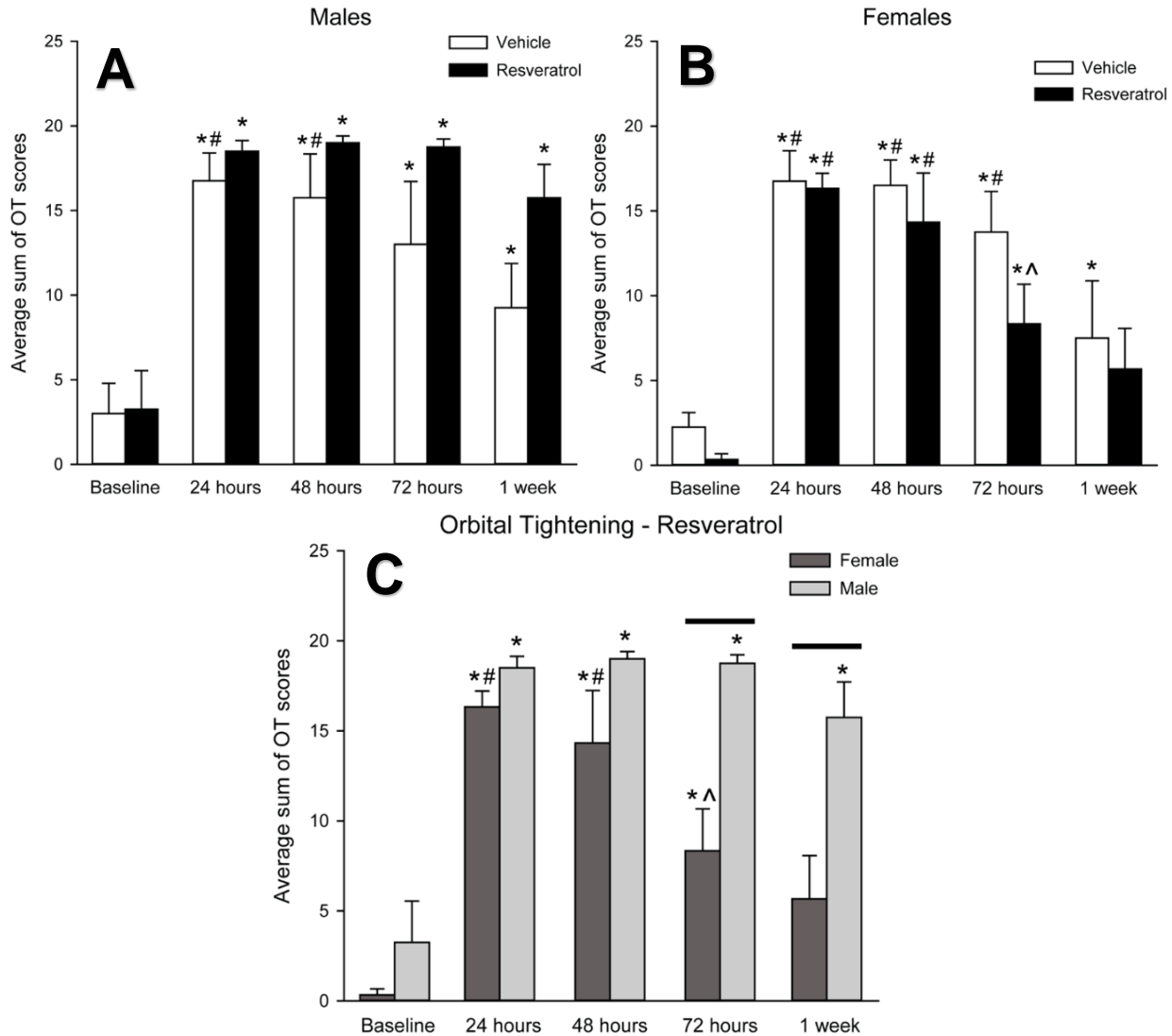


Figure 1: Effect of resveratrol on OT scores over time (A) OT scores for treatment vs. vehicle in male rats (n = 4 Vehicle; 4 Resveratrol) (* p < 0.05 vs. Baseline; # p < 0.05 vs 1 week). **(B)** OT scores for treatment vs. vehicle in female rats (n = 4 Vehicle; 3 Resveratrol.) (* p < 0.05 vs. Baseline; # p < 0.05 vs 1 week; ^ p < 0.05 vs 24 hours) **(C)** Comparison of male vs. female treatment groups results from figure 1A and 1B. A two-way repeated measures ANOVA with Holm-Sidak and pairwise multiple comparison post hoc test was used to determine differences between treatment groups. There was no effect of treatment group (Vehicle vs Resveratrol) in either the male or female groups. There were significant differences between males vs. females at 72 hours and 1 week (— p < 0.001.)

Immunohistochemistry

We examined corneal tissues at 1-week post-abrasion in vehicle and resveratrol-treated rats, as well as control animals that received no surgery and no eye treatment. The corneal tissue analysis showed that in female rats, the % β -Tubulin volume at 1-week post-abrasion showed a decrease in both the vehicle and resveratrol group when compared to the control ($p < 0.05$) (Figure 2A). The male group also showed lower values of % β -Tubulin volume in the vehicle and resveratrol groups, although the reduction in nerve density was less pronounced than the female group. For both male and female groups, a t-test showed no significant differences between vehicle and resveratrol.

The volume of CGRP within the β -Tubulin volume for each animal was examined (Figure 2C, D). %CGRP volume of the female group showed a significant decrease in the vehicle and resveratrol groups vs. the control ($p < 0.05$) (Figure 2C). In comparison, the male group showed no significant change in CGRP volume when treated with the vehicle or resveratrol when compared to the control. (Figure 2D). A t-test showed that there were no significant differences between vehicle vs. resveratrol in either male and female groups.

A two-way ANOVA was used to examine if there were any differences in β -Tubulin or CGRP when comparing the male drug vs. female drug groups. For the % β -Tubulin, no interaction was found between sex vs treatment. As for %CGRP volume, a two-way ANOVA was attempted but normality and equal variance tests both failed, likely due to the high variance in the male drug group. These findings show that corneal abrasion caused a loss of nerve fibers that was still present one

week later in females, and resveratrol treatment did not cause any changes in the loss of corneal nerves.

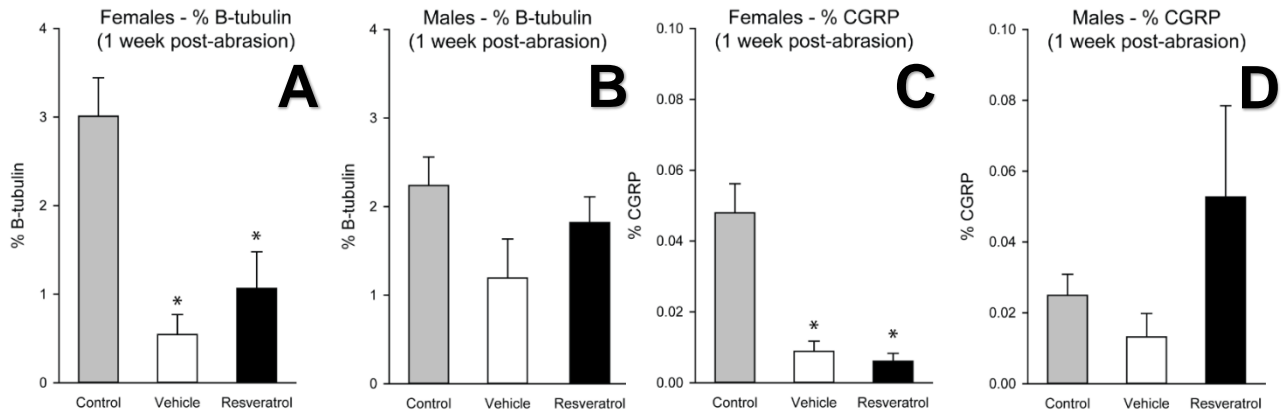


Figure 2: Levels of β -Tubulin and CGRP volume % measured at 1-week post-abrasion. (A) β -Tubulin nerve density for female rats (* $p < 0.05$ vs. Control). **(B)** β -Tubulin nerve density for male rats **(C)** CGRP for female rats (* $p < 0.05$ vs. Control) **(D)** CGRP for male rats. A t-test or Mann-Whitney Rank Sum test was used to compare resveratrol vs. vehicle for each group. There were no significant differences between vehicle and resveratrol for any metric. A sex vs. treatment two-way ANOVA was attempted for β -Tubulin and CGRP, respectively. β -Tubulin volume showed no interaction between sex vs treatment, while normality and equal variance tests both failed in the CGRP test.

Discussion:

Refractive eye surgeries such as PRK and LASEK have become increasingly popular for patients. These surgeries involve the removal of the corneal epithelium which can lead to the development of post-op conditions of pain, discomfort, and photophobia. Prior studies have shown that resveratrol is effective in mice hind paw incision models in limiting acute pain and development in the chronic pain state (Dasgupta and Milbrandt, 2007; Tillu et al., 2012). Seeing resveratrol's usefulness, we wished to see similar results when resveratrol is used on the eye after an abrasion surgery.

The behavioral results were ultimately disappointing. The usage of resveratrol on the eye did not show an improvement of acute pain, and as for the attenuation of pain over time, there did not seem to be a difference when compared to the vehicle groups. Furthermore, it would be advisory for males to limit the use of resveratrol not only because there does not appear to be a noticeable benefit, but the drug could be harmful as it can amplify the duration of pain. Deviations from the results of prior studies could be from differential activation of various pain pathways in the eye in comparison to other areas of the body. Whether the AMPK pathway and associated downstream proteins and kinases (Tillu et al., 2012) are still involved, or if there are other processes at play could be a topic of further study. The effect of resveratrol on pain pathways could also explain the differences in sex response as proteins associated with the transmission of pain are upregulated rather than suppressed in males, explaining the increased OT scores. Another plausible explanation is that the amount of uptake of the resveratrol emulsion into the eye could also have affected the results. It is possible that the mechanism of drug uptake in male and female rats could have resulted in greater or less absorption of the drug.

This study utilized the Rat Grimace Scale (Sotocinal et al. 2011) as a means of measuring behavioral displays of spontaneous pain. The application of this grading scale does have some merits. The first being its main grading criteria, orbital tightening, is applicable towards actual human behavior. Post-op, many human patients display similar eye squinting activity as a result of photophobia. Secondly, this grading model is able to limit the need for excess handling and harm for the rats in comparison to other methods that focus on evoked pain.

The histological analysis displayed a reduction of β -Tubulin nerve density and CGRP peptide in non-control groups, although there wasn't a substantial change in the regeneration of nerve density or difference in CGRP count when resveratrol was used. Data analysis was used to see if the histological data could provide a link to the sex difference seen in the behavioral data. The tests though did not appear to show an effect of sex on treatment.

Overall, taking into account behavioral and histological assessments, the data does not provide a compelling argument for the usage of resveratrol on the cornea. While the usage of resveratrol has been shown to provide beneficial healing effects in prior studies, the extension of its usage to the cornea cannot be recommended. Research on other compounds that could be helpful as a therapeutic for post-corrective eye surgery pain is still worthwhile. Compounds such as vitamin D may play an important role for in modulating corneal wound healing (Reins et al, 2016), and could be a worthwhile compound to further examine.

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