Electrophysiological assessment of retinal functions by ERG in Ischemia/Reperfusion (I/R) *Allium cepa* pre-treated mice

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KEY WORDS

Allium cepa
pretreatment
ERG
PPA
Retina

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ABSTRACT

Retinal disorders are the one of the most challenging and complex degenerative diseases that need to be addressed because of rapid increase in the number of affected individuals. Most of the available treatments strategies are inadequate to exert permanent solution to the patients. Therefore, as an alternative approach we wanted to test the efficacy of *Allium cepa* (*A. cepa*) in an Ischemia/Reperfusion (I/R) mouse model. We orally administered the aqueous extract of *A. cepa* at different dosages 100 mg/kg, 200 mg/kg, 300 mg/kg 24 hrs prior to the surgery. Electroretinogram (ERG) analysis was carried out at 7 day, 21 day, and 28 day after the surgery. ERG recording depicted that *A. cepa* administration is able to increase the implicit time but not at the statistically significant level for which larger sample size and deeper analysis is required.

doi: 10.38205/imcr.010118

Introduction

Repeated failure in treating different neurodegenerative diseases have left no option for the researchers to think about the alternative approaches. Fruits and vegetables are the excellent alternative source to treat such disorders (1). Different studies suggest the potential role of fruits and vegetables in reducing the risk of degenerative diseases (2, 3). Flavonoids, a polyphenolic secondary metabolite are known to have antioxidant, anti-inflammatory, and anti-microbial properties. These flavonoids have been used in different *in vitro* and *in vivo* models and several studies suggests its protective role in degenerative diseases (4, 5).

Eye related disorders are most common degenerative disorders and have very limited scope of permanent cure. However, to overcome this, various traditional approaches like herbal remedy and homeopathy have been employed by ophthalmologist (6). Scientific studies have reported preventive role of onion on different eye related disorders like; Cataract (7, 8), Age-related Macular Degeneration (AMD) (9).

Onion bulbs (*Allium cepa L.*) are the rich source of flavonoid, especially in quercetin. Different groups have elucidated the potential biological activity of *A. cepa* as neuroprotective, antioxidant, antiallergic, and anti-inflammatory (5, 10, 11). Published literature explains that onion extract (OE) lowers the total cholesterol content in rabbits (12) and in rats (13) fed with high cholesterol diet. Protective role of ethanolic extract of *A. cepa* was found in case of cognition impairments in streptozotocin induced rat diabetes model (14). The neuroprotective effect of flavonoid is known to exert by two processes: First, by interacting with lipid kinase thereby leading to the inhibition of the apoptosis. Second, by benefiting the vascular system (15).

Retinal ischemia which is a leading cause of blindness, results due to insufficient supply of blood to the retina and is known to be associated with several eye related disorders like glaucoma, diabetic retinopathy, and optic retinopathy (16). There have not been many studies on neuroprotective role of onion in rescuing the retinal ganglion cell death resulting due to retinal ischemia. Therefore, in the present study we have investigated the efficacy of aqueous extract of *Allium cepa* in rescuing the retinal functions depicted by electroretinogram (ERG) analysis. Ischemia/Reperfusion (I/R) induced retina injured mouse model was used to alter the retinal functions thereby affecting the vision.

ERG is a non-invasive technique used to depict the retinal functions. The ability of ERG to detect and isolate various signals from a different set of retinal neurons makes it a important tool for electrophysiological measures and this can be achieved by controlling/changing the stimulus, light intensity or adaptation, and parameters for data processing (17). Data acquisition in the form of wave pattern is a means to represent and distinguish different retinal cells activity. The wave pattern generally starts in the following pattern: “a-wave” is the first negative deflection and represents the primary retinal neurons (photoreceptors: rods and cones); “b-wave” is the

positive peak which represents the bipolar cells (17); c-wave originates from retinal pigment epithelium (RPE) cells; oscillatory potentials (OP) originates from inner retina/amacrine cells (18). We used scotopic ERG (dark-adapted mice) for this purpose.

Methods

Animals

A C-57BL/6J male 8- to 10-week old mouse was used for the experimentation purpose. The weight of the mice ranges from 25 g–30 g. Ethical approval was obtained from animal ethical committee (IAEC) of Post Graduate Institute of Medical Education & Research (PGIMER), Chandigarh, India. The animals were kept in the sterile cages and temperature and humidity controlled facility of PGIMER animal house with no restriction to food and water. A 12 hr light/dark cycle was followed. The animals were divided into 4 groups **(Figure 1)**.

Experimental groups

The complete experiment was carried according to the GLP guidelines at Neuroscience Research Laboratory (19, 20). The *A. cepa* extract preparation is detailed in our previous publication (21). Different dosages of *A. cepa* (100 mg/kg, 200 mg/kg, 300 mg/kg) was administered 24 hours prior to the surgery. Animals were divided into 4 different groups: group 1 (Injury alone), group 2 (Injury + 100 mg/kg *A. cepa* pretreatment), group 3 (Injury + 200 mg/kg *A. cepa* pretreatment), and group 4 (Injury + 300 mg/kg *A. cepa* pretreatment).

Surgery

Combination of Xylazine (50 mg/ml) / Ketamine (1:4 ratio) was used to anesthetize the mice. Intraperitoneal injection (IP) was administrated. Approximately 1.5 cm incision was made around the neck region. Initially, the CCA was exposed by retracting the muscles. The bifurcation was exposed and ECA was

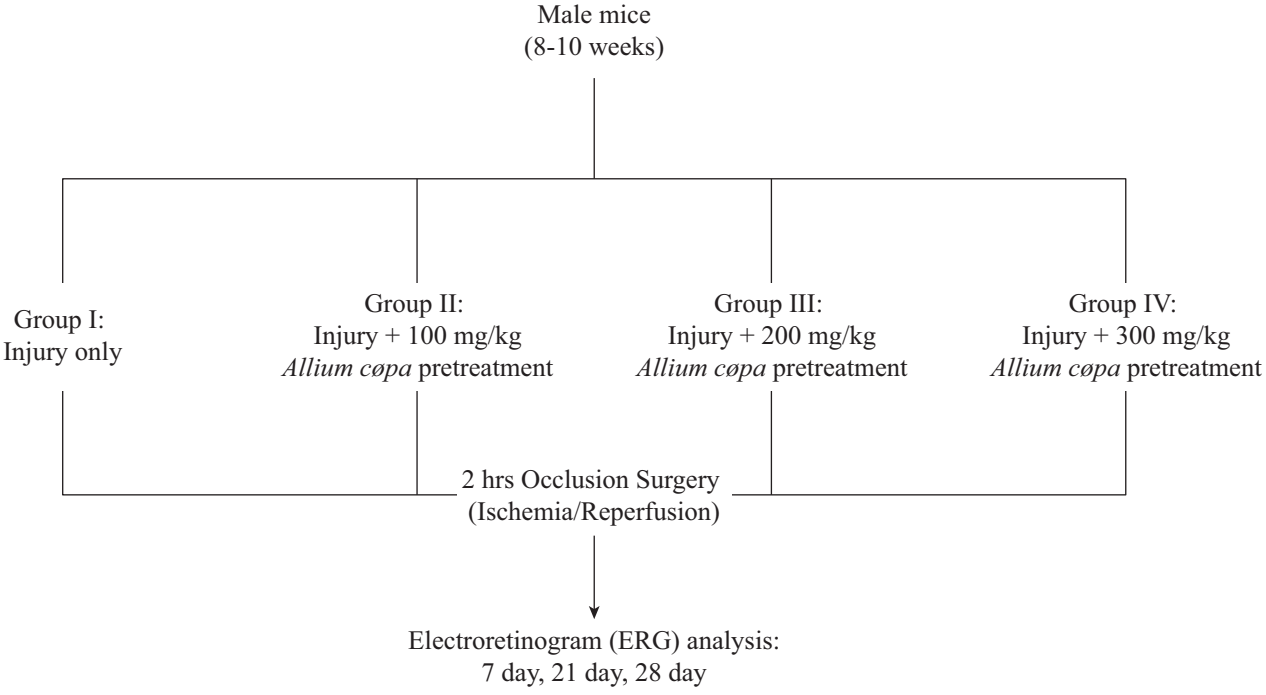

Fig. 1: Representation of the study design.

ligated with a fine suture. Further, the bifurcation of ICA was traced down and PPA was ligated with a 7.0 mm ethicon suture. The ligation was maintained for 2 hrs after that the ligated sutures were removed and mice was allowed to reperfuse (7 day, 21 day, and 28 day) under the sterile condition. The surgery was carried out under the Leica Stereozoom microscope.

Electroretinography recordings

Scotopic electroretinography (ERG) was performed to evaluate the function of retina caused due to 2 hrs of ischemia. The experiment was carried out using iWork ERG instrument (Dover, USA). The ERG data was recorded and evaluated using Labscribe software. ERG was done for all the 4 groups. This experiment was performed once the 24 hrs dark adaptation completed. Anesthesia was administrated intraperitoneally and the animals were kept on heating pad. Tropicamide and methylcellulose eye drops were used to dilate the pupils. Ground electrode was placed on tail, negative electrode in between the ears, and positive electrode was connected to the cornea (19). The readings were taken with flashes of light.

Right eye represents the 'test eye' whereas the left eye was taken as the 'control eye'. Later on, the amplitudes of different wave were measured.

Results

Table 1 represents the implicit time and amplitude of both a- and b- waves depicted by ERG. Scotopic ERG was performed on 24 hrs dark adapted mice. We recorded four important parameters, i.e. implicit time to a-wave, implicit time to b-wave, amplitude of a-wave, and amplitude of b-wave. At 7 day, the implicit time to a-wave was found to be 45 ± 5.78 for Injury alone, 35 ± 8.01 for 100 mg/kg pretreated, 44.85 ± 2.86 for 200 mg/kg pretreated, and 45.45 ± 1.72 for 300 mg/kg pretreated group. We found that at 21 day the mean implicit time to a wave was highest 43.55 ± 1.72 for 200 mg/kg, whereas for injury alone it was 42.25 ± 4.51, for 100 mg/kg group it was 42.27 ± 1.62 and for 300 mg/kg it was 36.95 ± 6.75. With the increase in time points the we observed that at 28 day the implicit time to a wave also increased i.e. 51.13 ± 3.12, 43.33 ± 0.85, 44.33 ± 1.84, 46.2 ± 0.76 for injury alone, 100 mg/kg, 200 mg/kg, and 300 mg/kg, respectively. Positive wave i.e. the b-wave implicit time was found to be 88.27 ± 9.26 for injury alone at 7 day, whereas for 21 day it was 77.25 ± 7.67, and for 28 day it recorded 102.27 ± 5.72. For *A. cepa* pretreatment group's implicit time to b wave at 7 day was 88.8 ± 7.17 for 100 mg/kg, 90.8 ± 2.19 for 200 mg/kg and 92.3 ± 1.45

Table 1: Electroretinogram (ERG) analysis for Injury alone, and *A. cepa* pretreated groups (100 mg/kg, 200 mg/kg, 300 mg/kg) at different time points (7 day, 21 day, 28 day) (A). a-wave implicit time; b-wave implicit time (B). a-wave amplitude; b-wave amplitude. The data is represented as mean ± SE. For Injury alone group sample size was (7 day (n = 3); 21 day (n = 4); 28 day (n = 3)); for 100 mg/kg pretreated group (7 day (n = 4); 21 day (n = 3); 28 day (n = 3)); for 200 mg/kg pretreated group (7 day (n = 4); 21 day (n = 4); 28 day (n = 3)); for 300 mg/kg pretreated group (7 day (n = 4); 21 day (n = 4); 28 day (n = 4)).

A.	Implicit Time a (Time in ms ± SE)				Implicit Time b (Time in ms ± SE)			
Time/Groups	**Injury**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**Injury**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**
7 day	45 ± 5.78	35 ± 8.01	44.85 ± 2.86	45.45 ± 1.72	88.27 ± 9.26	88.8 ± 7.17	90.8 ± 2.19	92.3 ± 1.46
21 day	42.25 ± 4.51	42.27 ± 1.62	43.55 ± 1.72	36.95 ± 6.75	77.25 ± 7.67	87.2 ± 8.25	86.4 ± 3.89	88.3 ± 8.09
28 day	51.13 ± 3.12	43.33 ± 0.85	44.33 ± 1.84	46.2 ± 0.76	102.27 ± 5.72	79 ± 4.06	82 ± 4.56	89.6 ± 5.09

B.	Amplitude a (Time in ms ± SE)				Amplitude b (Time in ms ± SE)			
Time/Groups	**Injury**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**Injury**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**
7 day	−0.198 ± 0.031	−0.192 ± 0.045	−0.339 ± 0.071	−0.595 ± 0.013	0.0163 ± 0.016	0.021 ± 0.03	0.007 ± 0.02	0.086 ± 0.007
21 day	−0.1025 ± 0.048	−0.363 ± 0.05	−0.205 ± 0.031	−0.407 ± 0.024	0.0295 ± 0.024	0.0673 ± 0.026	0.0617 ± 0.012	0.078 ± 0.020
28 day	−0.124 ± 0.017	−0.188 ± 0.026	−0.302 ± 0.029	−0.356 ± 0.062	0.004 ± 0.038	0.0163 ± 0.006	0.1076 ± 0.035	0.074 ± 0.044

for 300 mg/kg. At 21 day we found slight decrease in the implicit time to b-wave i.e. 87.2 ± 8.25 (100 mg/kg), 86.4 ± 3.89 (200 mg/kg), 88.3 ± 8.09 (300 mg/kg). Similar trends where observed for 28 day where mean implicit time to b-wave was recorded as follows: 79 ± 4.06, 82 ± 4.56, and 89.6 ± 5.09 for 100 mg/kg, 200 mg/kg, 300 mg/kg respectively.

Further, the amplitude for both the waves was recorded. The amplitude of a-wave for injury alone was −0.198 ± 0.031 (7 day), −0.1025 ± 0.048 (21 day), −0.124 ± 0.017 (28 day); for 100 mg/kg pretreatment group it was -0.192 ± 0.045 (7 day), −0.363 ± 0.05 (21 day), −0.188 ± 0.026 (28 day); for 200 mg/kg pretreatment group it was −0.339 ± 0.071 (7 day), −0.205 ± 0.031 (21 day), −0.302 ± 0.029 (28 day); and for 300 mg/kg pretreatment group it −0.595 ± 0.013 (7 day), −0.407 ± 0.024 (21 day), −0.356 ± 0.062 (28 day). Similarly, the mean amplitude of b-wave was as follows: 0.0163 ± 0.016, 0.0295 ± 0.024, 0.004 ± 0.038 for injury alone group; 0.021 ± 0.03, 0.0673 ± 0.026, 0.0163 ± 0.006 for 100 mg/kg group; 0.007 ± 0.02, 0.0617 ± 0.012, 0.1076 ± 0.035 for 200 mg/kg group; 0.086 ± 0.007, 0.078 ± 0.020, 0.074 ± 0.044 for 300 mg/kg *A. cepa* pretreated group at 7, 21, and 28 day time points respectively. The details of significance level have been incorporated in the supplementary file (Supplementary Table 1).

Discussion

Retinal ischemia is a serious complication associated with glaucoma, diabetic retinopathy, and optic retinopathy (16). The associated condition may lead to blindness if appropriate and adequate treatments are not given on time. We have established a retinal ischemia mouse model by ligating two important arteries i.e. ophthalmic artery (PPA) and ECA (20). It's a 2 hr occlusion model followed by reperfusion for 3 different time points: 7, 21, and 28-day. Because of the repeated failure (24) and side effects (25) of the available commercial drugs, we wanted to test the alternative approach of testing

A. cepa (onion) as the pretreatment strategy to explore if it is able to improve the vision or not. Apart from its many biological benefits in the form of antioxidant, anti-inflammatory, and neuroprotective activity (as shown by previous studies) its common availability makes it an important biological product to be tested for its efficacy (5, 10, 11). For this, 3 different concentrations of aqueous extract of commonly used red onion was tested i.e. 100 mg/kg, 200 mg/kg and 300 mg/kg. The functional efficacy of *A. cepa* in the form of retinal wave analysis is very important in retinal degeneration cases and to the best of our knowledge it has not been previously reported in such models. According to standardized protocols the ERG recordings are depicted by three important factors- the instrument setup, intensity of the light stimulus, and animal state (26).

ERG recording depicted that implicit time to a-wave decreased in all the *A. cepa* administered groups except for slight increase in 300 mg/kg in comparison to the Injury alone group for 7-day, however, this decrease was not statistically significant. Similarly, the implicit time to b-wave for both 21-day and 28-day increased throughout the *A. cepa* administered groups in comparison to the injury alone group. However, for 28-day, the implicit time to b-wave was highest for injury alone group followed by 300 mg/kg. From this, it can be said that A.cepa administration is able to increase the implicit time but not at the statistically significant level for which larger sample size and deeper analysis is required. Delay in implicit time a-wave have previously been reported in diseased cases (27). Similarly, reduction in a-wave amplitude has also been reported in certain cases. Mixed results were obtained in case of both amplitude a- and b- wave. Amplitude a-wave was recorded to be decreased in most of the *A. cepa* administered groups with respect to the injury alone group. Further, b-wave amplitude was highest at 7 day for 100 mg/kg; at 21 day for 300 mg/kg, and at 28 day for 200 mg/kg. So, from this data it can be concluded that *A. cepa* may able to improve the retinal functions depicted by ERG analysis.

Limitations of the study

Though we have tried to minimise the limitation, factors such as intensity of light stimulus, temperature/humidity, anesthesia, ocular environment, adaptive state, and other technical difficulties affecting the ERG recordings cannot be ruled out. Large sample size and more group like: Injury+ PBS/solvent comparison is needed.

Acknowledgments

Authors would like to acknowledge the Ministry of AYUSH, Govt. of India for providing the funding (Z.28015/106/2014-HP-C(EMR)-AYUSH-A).

Authors Contribution

SK: Experimentation, data analysis, original writing
AA: Conceptualization, editing of the manuscript, securing funding.
RS: Co-conceptualization
SK: Co-conceptualization
VS: Experimentation

Ethical statement

All experiments were performed after getting the approval from Institutional Animal Ethical Committee (IAEC) via approval no: 67/IAEC/390R.

Conflicts of interests

The authors declare that they have no conflict of interest.

Source of funding

Thanks to ministry of AYUSH file No. (Z.28015/106/2014-HPC (EMR)-AYUSH-A) Government of India

Received Date: 10-02-20; Revised Date: 12-02-20

Accepted Date: 26-02-20

References

1. Essa MM, Akbar M, Guillemin G. The Benefits of Natural Products for Neurodegenerative Diseases: Springer; 2016.
2. Sofi F, Dinu MR. Nutrition and prevention of chronic-degenerative diseases. Agriculture and agricultural science procedia. 2016;8: 713–7.
3. Jiang X, Huang J, Song D, Deng R, Wei J, Zhang Z. Increased Consumption of Fruit and Vegetables Is Related to a Reduced Risk of Cognitive Impairment and Dementia: Meta-Analysis. Frontiers in aging neuroscience. 2017;9:18.
4. Sarchielli E, Morelli A, Guarnieri G, Iorizzi M, Sgambati E. Neuroprotective effects of quercetin 4'-O-β-d-diglucoside on human striatal precursor cells in nutrient deprivation condition. Acta histochemica. 2018;120(2):122–8.
5. Shri R, Bora KS. Neuroprotective effect of methanolic extracts of *Allium cepa* on ischemia and reperfusion-induced cerebral injury. Fitoterapia. 2008;79(2):86–96.
6. Nejabat M, Salehi A, Noorani Azad P, Ashraf MJ. Effects of onion juice on the normal flora of eyelids and conjunctiva in an animal model. Jundishapur journal of microbiology. 2014;7(5):e9678–e.
7. Javadzadeh A, Ghorbanihaghjo A, Bonyadi S, Rashidi MR, Mesgari M, Rashtchizadeh N, et al. Preventive effect of onion juice on selenite-induced experimental cataract. Indian journal of ophthalmology. 2009;57(3):185.
8. Stefek M, Karasu C. Eye lens in aging and diabetes: effect of quercetin. Rejuvenation research. 2011;14(5):525–34.
9. Agte V, Tarwadi K. The importance of nutrition in the prevention of ocular disease with special reference to cataract. Ophthalmic research. 2010;44(3):166–72.
10. Sato A, Zhang T, Yonekura L, Tamura H. Antiallergic activities of eleven onions (*Allium cepa*) were attributed to quercetin 4'-glucoside using QuEChERS method and Pearson's correlation coefficient. Journal of Functional Foods. 2015;14:581–9.
11. Singh T, Goel RK. Neuroprotective effect of *Allium cepa* L. in aluminium chloride induced neurotoxicity. Neurotoxicology. 2015;49:1–7.
12. Movahedian A, Sadeghi H, Ghannadi A, Gharavi M, Azarpajooh S. Hypolipidemic activity of Allium porrum L. in cholesterol-fed rabbits. Journal of medicinal food. 2006;9(1):98–101.
13. Kumari K, Augusti K. Lipid lowering effect of S-methyl cysteine sulfoxide from *Allium cepa* Linn in high cholesterol diet fed rats. Journal of ethnopharmacology. 2007;109(3):367–71.

14. Tamtaji OR, Hosseinzadeh H, Talaei SA, Behnam M, Firoozeh SMT, Taghizadeh M, et al. Protective Effects of Red Onion (*Allium cepa*) Ethanolic Extract on Learning and Memory Impairments in Animal Models of Diabetes. Galen Medical Journal. 2017;6(3): 249–57.
15. Vauzour D, Vafeiadou K, Rodriguez-Mateos A, Rendeiro C, Spencer JPE. The neuroprotective potential of flavonoids: a multiplicity of effects. Genes & nutrition. 2008;3(3-4):115–26.
16. Minhas G, Morishita R, Anand A. Preclinical models to investigate retinal ischemia: advances and drawbacks. Frontiers in neurology. 2012;3:75.
17. Tomiyama Y, Fujita K, Nishiguchi KM, Tokashiki N, Daigaku R, Tabata K, et al. Measurement of electroretinograms and visually evoked potentials in awake moving mice. PloS one. 2016;11(6).
18. Hema L. Ramkumar KDE, Koushik Tripathy, Peter A. Karth, Usha Rajapuram Kumar, and Vinay A. Shah. Electroretinogram: EyeWiki; [updated 15 November 2019. Available from: https://eyewiki.aao.org/Electroretinogram.
19. Neuroscience Research Lab DoN, Post Graduate Institute of Medical Education and Research, Chandigarh. Redefining Quality Standards in Basic Research Investigations by Broadening the Purview of GLP: Quality Council of India.
20. Bammidi S, Sharma K, Tyagi R, Sharma NK, Anand A. Eye Genetics: The Road Ahead to Quality Standards. Advances in Vision Research, Volume I: Springer; 2017. p. 473–80.
21. Kumar S, Modgil S, Bammidi S, Minhas G, Shri R, Kaushik S, et al. *Allium cepa* exerts neuroprotective effect on retinal ganglion cells of pterygopalatine artery (PPA) ligated mice. J Ayurveda Integr Med. 2020.
22. Modgil S, Cameotra SS, Sharma VL, Anand A. Early Life Pb Exposure and its Effect on Later Life Retinal Degeneration. Journal of cellular biochemistry. 2017.
23. Minhas G, Prabhakar S, Morishita R, Shimamura M, Bansal R, Anand A. Transplantation of lineage-negative stem cells in pterygopalatine artery ligation induced retinal ischemia–reperfusion injury in mice. Molecular and cellular biochemistry. 2017;429(1–2):123–36.
24. Kumar S, Modgil S, Bammidi S, Minhas G, Shri R, Kaushik S, et al. *Allium cepa* exerts neuroprotective effect on retinal ganglion cells of pterygopalatine artery (PPA) ligated mice. Journal of Ayurveda and Integrative Medicine. 2020.
25. Farkouh A, Frigo P, Czejka M. Systemic side effects of eye drops: a pharmacokinetic perspective. Clinical Ophthalmology (Auckland, NZ). 2016;10:2433.
26. Brandli A, Stone J. Using the electroretinogram to assess function in the rodent retina and the protective effects of remote limb ischemic preconditioning. JoVE (Journal of Visualized Experiments). 2015(100):e52658.
27. Hancock HA, Kraft TW. Oscillatory potential analysis and ERGs of normal and diabetic rats. Investigative ophthalmology & visual science. 2004;45(3):1002-8.

Supplementary Table 1

POST HOC TESTS

1. IMPLICIT TIME A-WAVE

Multiple Comparisons

1.1. Dependent Variable: Implicit Time A-Wave: 7 Day

7 Day	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	10.0000	7.6475	.646	−15.089	35.089
		Two Hundred	.1500	7.6475	1.000	−24.939	25.239
		Three Hundred	−.4500	7.6475	1.000	−25.539	24.639
	Hundred	Injury	−10.0000	7.6475	.646	−35.089	15.089
		Two Hundred	−9.8500	7.0803	.602	−33.077	13.377
		Three Hundred	−10.4500	7.0803	.557	−33.677	12.777
	Two Hundred	Injury	−.1500	7.6475	1.000	−25.239	24.939
		Hundred	9.8500	7.0803	.602	−13.377	33.077
		Three Hundred	−.6000	7.0803	1.000	−23.827	22.627
	Three Hundred	Injury	.4500	7.6475	1.000	−24.639	25.539
		Hundred	10.4500	7.0803	.557	−12.777	33.677
		Two Hundred	.6000	7.0803	1.000	−22.627	23.827
Dunnett t (2-sided)[a]	Injury	Three Hundred	−.4500	7.6475	1.000	−21.281	20.381
	Hundred	Three Hundred	−10.4500	7.0803	.364	−29.735	8.835
	Two Hundred	Three Hundred	−.6000	7.0803	1.000	−19.885	18.685

1.2. Dependent Variable: Implicit Time A-Wave: 21 Day

21 Day	(I) Group2	(J) Group 2	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	−.01667	6.68074	1.000	−21.9335	21.9001
		Two Hundred	−1.30000	6.18516	.997	−21.5910	18.9910
		Three Hundred	5.30000	6.18516	.863	−14.9910	25.5910
	Hundred	Injury	.01667	6.68074	1.000	−21.9001	21.9335
		Two Hundred	−1.28333	6.68074	.998	−23.2001	20.6335
		Three Hundred	5.31667	6.68074	.887	−16.6001	27.2335
	Two Hundred	Injury	1.30000	6.18516	.997	−18.9910	21.5910
		Hundred	1.28333	6.68074	.998	−20.6335	23.2001
		Three Hundred	6.60000	6.18516	.770	−13.6910	26.8910
	Three Hundred	Injury	−5.30000	6.18516	.863	−25.5910	14.9910
		Hundred	−5.31667	6.68074	.887	−27.2335	16.6001
		Two Hundred	−6.60000	6.18516	.770	−26.8910	13.6910
Dunnett t (2-sided)[a]	Injury	Three Hundred	5.30000	6.18516	.736	−11.5473	22.1473
	Hundred	Three Hundred	5.31667	6.68074	.774	−12.8805	23.5138
	Two Hundred	Three Hundred	6.60000	6.18516	.601	−10.2473	23.4473

1.3. Dependent Variable: Implicit Time A-Wave: 28 Day

28 Day	(I) Group3	(J) Group 3	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	7.80000	2.58314	.085	–.9932	16.5932
		Two Hundred	6.80000	2.58314	.145	–1.9932	15.5932
		Three Hundred	4.93333	2.41630	.308	–3.2919	13.1586
	Hundred	Injury	–7.80000	2.58314	.085	–16.5932	.9932
		Two Hundred	–1.00000	2.58314	.984	–9.7932	7.7932
		Three Hundred	–2.86667	2.41630	.711	–11.0919	5.3586
	Two Hundred	Injury	–6.80000	2.58314	.145	–15.5932	1.9932
		Hundred	1.00000	2.58314	.984	–7.7932	9.7932
		Three Hundred	–1.86667	2.41630	.895	–10.0919	6.3586
	Three Hundred	Injury	–4.93333	2.41630	.308	–13.1586	3.2919
		Hundred	2.86667	2.41630	.711	–5.3586	11.0919
		Two Hundred	1.86667	2.41630	.895	–6.3586	10.0919
Dunnett t (2–sided)[a]	Injury	Three Hundred	4.93333	2.41630	.171	–1.9099	11.7766
	Hundred	Three Hundred	–2.86667	2.41630	.540	–9.7099	3.9766
	Two Hundred	Three Hundred	–1.86667	2.41630	.796	–8.7099	4.9766

2. IMPLICIT TIME B-WAVE

2.1. Dependent Variable: Implicit Time B-Wave: 7 Day

7 Day	(I) Group1	(J) Group 1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	–.53333	8.02095	1.000	–26.8468	25.7802
		Two Hundred	–2.53333	8.02095	.991	–28.8468	23.7802
		Three Hundred	–4.03333	8.02095	.967	–30.3468	22.2802
	Hundred	Injury	.53333	8.02095	1.000	–25.7802	26.8468
		Two Hundred	–2.00000	7.42596	.995	–26.3616	22.3616
		Three Hundred	–3.50000	7.42596	.973	–27.8616	20.8616
	Two Hundred	Injury	2.53333	8.02095	.991	–23.7802	28.8468
		Hundred	2.00000	7.42596	.995	–22.3616	26.3616
		Three Hundred	–1.50000	7.42596	.998	–25.8616	22.8616
	Three Hundred	Injury	4.03333	8.02095	.967	–22.2802	30.3468
		Hundred	3.50000	7.42596	.973	–20.8616	27.8616
		Two Hundred	1.50000	7.42596	.998	–22.8616	25.8616
Dunnett t 2-sided)[a]	Injury	Three Hundred	–4.03333	8.02095	.924	–25.8810	17.8143
	Hundred	Three Hundred	–3.50000	7.42596	.936	–23.7270	16.7270
	Two Hundred	Three Hundred	–1.50000	7.42596	.994	–21.7270	18.7270

2.2. Dependent Variable: Implicit Time B-Wave: 21 Day

21 Day	(I) Group2	(J) Group 2	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Upper Bound	Lower Bound
Scheffe	Injury	Hundred	−9.95000	10.50654	.826	−44.4177	24.5177
		Two Hundred	−9.15000	9.72716	.828	−41.0609	22.7609
		Three Hundred	−11.05000	9.72716	.736	−42.9609	20.8609
	Hundred	Injury	9.95000	10.50654	.826	−24.5177	44.4177
		Two Hundred	.80000	10.50654	1.000	−33.6677	35.2677
		Three Hundred	−1.10000	10.50654	1.000	−35.5677	33.3677
	Two Hundred	Injury	9.15000	9.72716	.828	−22.7609	41.0609
		Hundred	−.80000	10.50654	1.000	−35.2677	33.6677
		Three Hundred	−1.90000	9.72716	.998	−33.8109	30.0109
	Three Hundred	Injury	11.05000	9.72716	.736	−20.8609	42.9609
		Hundred	1.10000	10.50654	1.000	−33.3677	35.5677
		Two Hundred	1.90000	9.72716	.998	−30.0109	33.8109
Dunnett t (2-sided)[a]	Injury	Three Hundred	−11.05000	9.72716	.557	−37.5451	15.4451
	Hundred	Three Hundred	−1.10000	10.50654	.999	−29.7180	27.5180
	Two Hundred	Three Hundred	−1.90000	9.72716	.995	−28.3951	24.5951

2.3. Dependent Variable: Implicit Time B-Wave: 28 Day

28 Day	(I) Group3	(J) Group 3	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Upper Bound	Lower Bound
Scheffe	Injury	Hundred	23.26667	7.36009	.070	−1.7875	48.3209
		Two Hundred	20.26667	7.36009	.123	−4.7875	45.3209
		Three Hundred	12.66667	6.88473	.388	−10.7694	36.1027
	Hundred	Injury	−23.26667	7.36009	.070	−48.3209	1.7875
		Two Hundred	−3.00000	7.36009	.982	−28.0542	22.0542
		Three Hundred	−10.60000	6.88473	.529	−34.0361	12.8361
	Two Hundred	Injury	−20.26667	7.36009	.123	−45.3209	4.7875
		Hundred	3.00000	7.36009	.982	−22.0542	28.0542
		Three Hundred	−7.60000	6.88473	.752	−31.0361	15.8361
	Three Hundred	Injury	−12.66667	6.88473	.388	−36.1027	10.7694
		Hundred	10.60000	6.88473	.529	−12.8361	34.0361
		Two Hundred	7.60000	6.88473	.752	−15.8361	31.0361
Dunnett t (2-sided)[a]	Injury	Three Hundred	12.66667	6.88473	.230	−6.8318	32.1651
	Hundred	Three Hundred	−10.60000	6.88473	.349	−30.0984	8.8984
	Two Hundred	Three Hundred	−7.60000	6.88473	.591	−27.0984	11.8984

3. AMPLITUDE a-WAVE

3.1. Dependent Variable: Amplitude a-Wave: 7 Day

7 Day	(I) Group 1	(J) Group 1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	–.0062500	.0701467	1.000	–.236373	.223873
		Two Hundred	.1410000	.0701467	.310	–.089123	.371123
		Three Hundred	.3965000*	.0701467	.001	.166377	.626623
	Hundred	Injury	.0062500	.0701467	1.000	–.223873	.236373
		Two Hundred	.1472500	.0649432	.222	–.065802	.360302
		Three Hundred	.4027500*	.0649432	.001	.189698	.615802
	Two Hundred	Injury	–.1410000	.0701467	.310	–.371123	.089123
		Hundred	–.1472500	.0649432	.222	–.360302	.065802
		Three Hundred	.2555000*	.0649432	.018	.042448	.468552
	Three Hundred	Injury	–.3965000*	.0701467	.001	–.626623	–.166377
		Hundred	–.4027500*	.0649432	.001	–.615802	–.189698
		Two Hundred	–.2555000*	.0649432	.018	–.468552	–.042448
Dunnett t (2-sided)[b]	Injury	Three Hundred	.3965000*	.0701467	.000	.205433	.587567
	Hundred	Three Hundred	.4027500*	.0649432	.000	.225856	.579644
	Two Hundred	Three Hundred	.2555000*	.0649432	.006	.078606	.432394

*. The mean difference is significant at the 0.05 level.

3.2. Dependent Variable: Amplitude a-Wave: 21 Day

21 Day	(I) Group 2	(J) Group 2	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	.2615000*	.0570859	.007	.074224	.448776
		Two Hundred	.1032500	.0528513	.332	–.070134	.276634
		Three Hundred	.3055000*	.0528513	.001	.132116	.478884
	Hundred	Injury	-.2615000*	.0570859	.007	–.448776	–.074224
		Two Hundred	-.1582500	.0570859	.108	–.345526	.029026
		Three Hundred	.0440000	.0570859	.896	–.143276	.231276
	Two Hundred	Injury	-.1032500	.0528513	.332	–.276634	.070134
		Hundred	.1582500	.0570859	.108	–.029026	.345526
		Three Hundred	.2022500*	.0528513	.021	.028866	.375634
	Three Hundred	Injury	-.3055000*	.0528513	.001	–.478884	–.132116
		Hundred	-.0440000	.0570859	.896	–.231276	.143276
		Two Hundred	-.2022500*	.0528513	.021	–.375634	–.028866
Dunnett t (2-sided)[b]	Injury	Three Hundred	.3055000*	.0528513	.000	.161542	.449458
	Hundred	Three Hundred	.0440000	.0570859	.789	–.111492	.199492
	Two Hundred	Three Hundred	.2022500*	.0528513	.007	.058292	.346208

*. The mean difference is significant at the 0.05 level.

3.3. Dependent Variable: Amplitude a-Wave: 28 Day

28 Day	(I) Group3	(J) Group 3	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	.0640000	.0637237	.800	–.152920	.280920
		Two Hundred	.1776667	.0637237	.117	–.039253	.394586
		Three Hundred	.2315000*	.0596081	.026	.028590	.434410
	Hundred	Injury	–.0640000	.0637237	.800	–.280920	.152920
		Two Hundred	.1136667	.0637237	.413	–.103253	.330586
		Three Hundred	.1675000	.0596081	.114	–.035410	.370410
	Two Hundred	Injury	–.1776667	.0637237	.117	–.394586	.039253
		Hundred	–.1136667	.0637237	.413	–.330586	.103253
		Three Hundred	.0538333	.0596081	.844	–.149076	.256743
	Three Hundred	Injury	–.2315000*	.0596081	.026	–.434410	–.028590
		Hundred	–.1675000	.0596081	.114	–.370410	.035410
		Two Hundred	–.0538333	.0596081	.844	–.256743	.149076
Dunnett t (2-sided)[b]	Injury	Three Hundred	.2315000*	.0596081	.010	.062682	.400318
	Hundred	Three Hundred	.1675000	.0596081	.052	–.001318	.336318
	Two Hundred	Three Hundred	.0538333	.0596081	.717	–.114985	.222651

*. The mean difference is significant at the 0.05 level.

4. AMPLITUDE b-WAVE

4.1. Dependent Variable: Amplitude b-Wave: 7 Day

7 Day	(I) Group1	(J) Group 1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	–.0046667	.0343660	.999	–.117407	.108074
		Two Hundred	.0093333	.0343660	.994	–.103407	.122074
		Three Hundred	–.0696667	.0343660	.303	–.182407	.043074
	Hundred	Injury	.0046667	.0343660	.999	–.108074	.117407
		Two Hundred	.0140000	.0318167	.978	–.090378	.118378
		Three Hundred	–.0650000	.0318167	.297	–.169378	.039378
	Two Hundred	Injury	–.0093333	.0343660	.994	–.122074	.103407
		Hundred	–.0140000	.0318167	.978	–.118378	.090378
		Three Hundred	–.0790000	.0318167	.165	–.183378	.025378
	Three Hundred	Injury	.0696667	.0343660	.303	–.043074	.182407
		Hundred	.0650000	.0318167	.297	–.039378	.169378
		Two Hundred	.0790000	.0318167	.165	–.025378	.183378
Dunnett t (2-sided)[a]	Injury	Three Hundred	–.0696667	.0343660	.160	–.163274	.023940
	Hundred	Three Hundred	–.0650000	.0318167	.156	–.151663	.021663
	Two Hundred	Three Hundred	–.0790000	.0318167	.075	–.165663	.007663

4.2. Dependent Variable: Amplitude b-Wave: 21 Day

21 Day	(I) Group 2	(J) Group 2	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	–.0378333	.0306341	.685	–.138331	.062665
		Two Hundred	–.0322500	.0283616	.735	–.125293	.060793
		Three Hundred	–.0485000	.0283616	.439	–.141543	.044543
	Hundred	Injury	.0378333	.0306341	.685	–.062665	.138331
		Two Hundred	.0055833	.0306341	.998	–.094915	.106081
		Three Hundred	–.0106667	.0306341	.989	–.111165	.089831
	Two Hundred	Injury	.0322500	.0283616	.735	–.060793	.125293
		Hundred	–.0055833	.0306341	.998	–.106081	.094915
		Three Hundred	–.0162500	.0283616	.953	–.109293	.076793
	Three Hundred	Injury	.0485000	.0283616	.439	–.044543	.141543
		Hundred	.0106667	.0306341	.989	–.089831	.111165
		Two Hundred	.0162500	.0283616	.953	–.076793	.109293
Dunnett t (2-sided)[a]	Injury	Three Hundred	–.0485000	.0283616	.261	–.125752	.028752
	Hundred	Three Hundred	–.0106667	.0306341	.972	–.094109	.072775
	Two Hundred	Three Hundred	-.0162500	.0283616	.894	–.093502	.061002

4.3. Dependent Variable: Amplitude b-Wave: 28 Day

28 Day	(I) Group 3	(J) Group 3	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	Injury	Hundred	–.0123333	.0541912	.997	–.196804	.172137
		Two Hundred	–.1036667	.0541912	.358	–.288137	.080804
		Three Hundred	–.0700000	.0506913	.611	–.242556	.102556
	Hundred	Injury	.0123333	.0541912	.997	–.172137	.196804
		Two Hundred	–.0913333	.0541912	.458	–.275804	.093137
		Three Hundred	–.0576667	.0506913	.736	–.230223	.114890
	Two Hundred	Injury	.1036667	.0541912	.358	–.080804	.288137
		Hundred	.0913333	.0541912	.458	–.093137	.275804
		Three Hundred	.0336667	.0506913	.929	–.138890	.206223
	Three Hundred	Injury	.0700000	.0506913	.611	–.102556	.242556
		Hundred	.0576667	.0506913	.736	–.114890	.230223
		Two Hundred	–.0336667	.0506913	.929	–.206223	.138890
Dunnett t (2-sided)[a]	Injury	Three Hundred	–.0700000	.0506913	.429	–.213564	.073564
	Hundred	Three Hundred	–.0576667	.0506913	.570	–.201231	.085898
	Two Hundred	Three Hundred	.0336667	.0506913	.855	–.109898	.177231

[a] Dunnett t-tests treat one group as a control, and compare all other groups against it.