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Concussion-related visual memory and reaction time impairment in college athletes improved after osteopathic manipulative medicine: a randomized clinical trial

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Abstract

Context: Concussion is an acute, transient disruption in brain function due to head injury. Previous studies suggest osteopathic manipulative medicine (OMM) improved recovery from concussion.

Objectives: The hypothesis was that new-onset impairments (NOI) of neurological functions identified by Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT) will improve more so after OMM than after concussion-education.

Methods: College athletes presenting to the outpatient academic healthcare center (AHCC) with concussion due to head injury within the preceding 2 weeks were recruited for this IRB-approved, randomized, single-blinded trial. Consented men and women were randomized into groups receiving two OMM treatments or two concussion-education sessions to control for social effects. Preseason, Baseline, ImPACT was compared to Post-Injury scores to determine NOI. Baseline, Post-Injury, and Post-Interventions ImPACTs were compared by analysis of variance (ANOVA, $\alpha \le 0.05$). Post-Injury correlations and mean changes in King-Devick (KD) scores were analyzed.

Results: Post-Injury NOI were found in 77.8% (14/18) men and 85.7% (6/7) women, including ImPACT subscore indices for verbal and visual memory, processing speed (PS), and reaction time (RT). Of those with NOI, mean visual memory recovered by 50.0% following one and by 104.9% (p=0.032) following two OMM treatments in men and by 82.8% (p=0.046) following one treatment in women. Following two interventions, the mean RT in men receiving OMM improved by 0.10 more than education (p=0.0496). The effect sizes of OMM were large (Cohen's d=1.33) on visual memory and small (Cohen's d=0.31) on RT.

Conclusions: The NOI in visual memory and RT following concussion significantly improved in the OMM group compared to the education group. Integrating OMM utilizing physical exam and this treatment was a safe individualized approach in athletes with acute uncomplicated concussions. Further research is warranted to improve the utilization of OMM for individuals with concussion.

Keywords: concussion; manual therapy; osteopathic medicine; placebo effect; traumatic brain injury; visual memory.

The Centers for Disease Control and Prevention (CDC) estimated that 300,000 sports-related traumatic brain injuries (TBI) occur annually within the United States [1–3]. A concussion is a mild TBI caused by acute injury impacting the head directly, or a blow to the body with an impulsive force transmitted to the head, followed by central neurologic symptoms lasting days, or up to 6 months [2]. The mechanical forces (acceleration, deceleration, rotational, and shear forces) cause an immediate primary brain injury without macroscopic damage [4–8]. Frequently, neuronal cell membrane disruption and axonal stretch occur [6–8]. The secondary injury phase, which can occur for hours, involves the progression of cellular and molecular damage [6–10]. Changes in cell membrane potential lead to ionic shifts, neurotransmitter redistribution, and increased oxidative

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stress [6–10]. The release of free radicals causes inflammation in the meninges and brain parenchyma, potentiating any neurological disruption over time [6–10]. These may contribute to the clinical manifestation of neurological signs, including cognitive impairment [1–10]. The perturbations of cellular physiology, or functional injury, as well as the microstructural injury (that which is not readily evident on computed tomography [CT]) to the brain involve neurometabolic changes [8]. The ongoing neurometabolic changes found in animal models and human studies involve bioenergetic impediments, cytoskeletal and axonal alterations, impairments in neurotransmission, vulnerability to delayed cell death, and potentially chronic dysfunction (as in persistent postconcussion symptoms or postconcussion syndrome) [8]. Evidence supporting osteopathic manipulative medicine (OMM) in concussion management has grown, but studies have not established how OMM may aid in the recovery of cognitive impairments.

Diagnosis and assessment of neurologic function in concussion include a physical exam for clinical signs of trauma and neurological changes as well as standardized indices for neurocognition and patient-rating of symptoms [2–4]. The computerized Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT) measures visual memory (Visual), verbal memory (Verbal), visual-motor processing speed (PS), reaction time (RT), impulse control (IC), and patient-rating of symptom severity (SS) [11, 12]. It is a well-established, validated tool widely utilized for measuring cognitive function following concussion injury. The ImPACT had 81.9% sensitivity and 89.4% specificity in detecting concussion-related new-onset impairments (NOI) when administered during the preparticipation sports physical to establish Baseline, and, again, after sustaining a concussion (Post-Injury), compared to normalized, validated age-gender–matched demographic data [11, 12]. The demographic data section of ImPACT includes relevant sport, medical, and concussion history information. A previous study demonstrated that impairments as measured by ImPACT and their resolution among young adults were associated with deficits and resolution on the King-Devick test (KD) [13]. The KD utilizes timed, visual-spatial test-cards validated for screening adolescents and adults at the sport sidelines following head injury. The KD was found to have high interrater-reliability (0.97 intraclass correlation coefficient), 86% sensitivity, and 90% specificity for concussion when utilized by healthcare or nonhealthcare providers following head injury [13–15].

Due to the variance in the effects of concussions, treatment approaches vary [2, 4, 7]. Current practice guidelines (CPG) recommend rest until the injured person is asymptomatic followed by a gradual return-to-play [16, 17]. Indications for impairment-specific rehabilitation are not always initially apparent [17]. Impaired patients may start rehabilitation before or after the expected recovery time (adults 10–14 days; children 4 weeks) [1, 17]. Rehabilitation programs may integrate OMM [18–21]. In a prospective, randomized study of acute concussion in 30 participants ages 18–26 years, there was a significant decrease in symptom number (p=0.002) and SS (p=0.001) after one session in the OMM group on the validated, self-rating scale Sport Concussion Assessment Tool, fifth edition (SCAT5) [18]. In a retrospective chart review of 26 patient charts that had a diagnosis of concussion sustained during athletics and a completed SCAT2, paired sample *t* tests revealed significant improvements in 10 (45.4%) of the 22 symptoms ($p<0.05$) as well as the total score after osteopathic manipulative treatment (OMT) [19]. Symptoms were also found to be safely improved in previous case reports [20, 21].

The primary injury from nonpenetrating impacts may be biomechanically evaluated by osteopathic structural examination (OSE) of the cranium and the rest of the body for clinical somatic dysfunctions (SD) [18–24]. Cranial SD have previously been reported to improve after the manual techniques of OMM [22, 23]. Mild and moderate TBI studies suggest that compressions and deformations of the skull, affecting bone and connective tissue microarchitecture, are found in concussion patients impacted at velocities relevant to college sports [25–27]. The cerebral ventricles, vasculature, and meninges delineate fluid compartments and maintain homeostasis. They are required for healthy central nervous system (CNS) function, but they may also be distorted during concussions, thereby altering the fluid-structural-interface [23–28]. Further research with large sample sizes of humans is necessary to definitively characterize the effects of concussion or OMM on the head. In general, the forces utilized to perform OMM are directed to reduce SD with a goal of restoring optimal function of the natural skeletal, fascial, and circulatory physiology. In this manner, OMM has been applied to affect the drainage of the lymphatic vessels (glymphatics) and dural venous sinuses [7, 18].

The objective of this study was to assess the presence of and recovery from NOI in concussions among college athletes receiving OMM. To account for potential placebo effects from patient-physician interaction, concussion patient-education (CEd) was utilized [29–31]. The hypothesis was that there will be participants with ≥1 NOI, identifiable by changes in ImPACT indices following concussion, who will demonstrate significantly greater recovery after receiving OMM than after CEd.

Methods

The randomized, single-blinded trial of OMM vs CEd (New York Institute of Technology Institutional Review Board-approved: BHS1139; clinicaltrials.gov: NCT02750566) was conducted at the outpatient academic healthcare center (AHCC) in college athletes. Power analysis was performed prior to data collection utilizing preliminary data measuring symptom number (Cohen's d=1) [18], indicating that 34 participants would be required to detect the effect size with statistical power of 80% ($\alpha \le 0.05$). Inclusion criteria were males or females 18–50 years of age who were clinically diagnosed by a licensed family practice (FP) physician or neurologist as having a concussion from a head injury within the prior 2 weeks. Exclusion criteria included the diagnosis of any emergency condition by a physician, current or previous neurodegeneration or spinal cord injury, inability to complete assessments, absolute contraindications to OMM (e.g., skull fracture, intracranial hemorrhage, cervical fracture, dissection, stroke), concussion-related loss of consciousness for ≥2 min, seizures, intractable vomiting, pregnancy, or paralysis (self-reported or witnessed). Participants were further excluded if their pre-to-post-Injury ImPACT indices did not show a concussion-related NOI.

Participants were new or established patients to the AHCC Sports Medicine Program, which serves three universities. Physicians directly recruited participants from November 24, 2015, to January 30, 2018, screened for eligibility, and obtained informed consent (paper format). Both groups followed concussion CPG. On the first visit, eligibility-screened, consented participants were evaluated with the primary outcome measures (Injury ImPACT and KD), and they provided their medical history and Baseline ImPACT. They were randomly assigned by blocks of two to OMM or CEd. Then, participants had their first-intervention on this first visit. They were instructed to follow the recommendation of rest and then return to the AHCC within 72 h. All participants repeated ImPACT and KD immediately before the second intervention (Post-1 Intervention). To measure the effects of the second intervention, ImPACT and KD were repeated on the third visit (6–7 days after their first visit). Investigators assessing outcomes were blinded to grouping. Outcome measurements and interventions were conducted with one participant per patient room. All participants were instructed to continue the CPG of rest until cleared by their sports medicine physician for return-to-play. Safety and side-effects of OMM were assessed.

The OMM group received an OSE and 30-min treatment performed by an neuromusculoskeletal medicine (NMM)/OMM or family practice (FP)/OMT board-certified physician as previously described (Supplemental Table 1) [18]. In Part 1, a whole-body OSE (cervical, thoracic, lumbar, and sacral spine; head, thorax, external pelvis, diaphragms, and extremities) was performed with participants lying supine in a dimly lit room. The vault hold was utilized to determine the sphenobasilar synchondrosis SD [24]. Examination of the cranium and face also included sutures, occipitoatlantal joints, cranial strain patterns, and cranial rhythmic impulse [18, 22–24]. The SD and OMM performed were documented. In Part 2, the initial treatment guidelines were based on individual SD pertinent to the mechanism of injury causing the concussion in order to alleviate biophysical restrictions that were clinically felt to limit physiologic functions [18, 22–24]. Physicians' options to appropriately address SD included cranial-OMM, face decompression, balanced ligamentous tension, muscle energy techniques, facilitated positional release, articulatory, high-velocity low-amplitude, and counterstrain. In Part 3, with the primary goal of improving circulation, specific techniques for improving glymphatic, lymphatic, and venous drainage of the head and neck were performed in the same manner on all OMM group participants [7, 18].

The CEd group received 30 min of education including CDC materials about recognizing cognitive, behavioral, and physical signs; diagnosis, risk factors, and predictors; recommendations for recovery-management; and expectations for return-to-play/return-to-work [1]. Questions that were addressed, comments, and observations were documented on standardized forms.

The ImPACTs were compared to previously validated, normative (age-gender–matched) performance ranges of *very-superior*, *superior*, *above-average*, *average*, *below-average*, *borderline*, and *below-borderline* for each indexed category. For clinical relevance, pre-sport (Baseline) to concussion (Injury) ImPACT and performance rank level differences were compared to determine if individuals had NOI compared to Baseline [11, 32]. The percentage of participants with NOI was calculated, and only these participants were included in further analyses. Qualitative measures of past medical history and history of present illness were evaluated for potential differences in subtypes and confounding variables. The Shapiro-Wilk test determined that ImPACT indices had a normal distribution. Changes in means (Reliable Change 90% Confidence Intervals [RCI]) from Baseline to Injury, Post-1 Intervention, and Post-2 Intervention ImPACT-indices were calculated (Reliable Change methodology reduces measurement error in test interpretation and identifies potentially clinically significant changes) [32]. Changes in mean ImPACT indices were compared by two-tailed, repeated-measures analysis of variance (ANOVA). Differences between the groups at Baseline, Injury, Post-1 Intervention, and Post-2 Intervention ImPACT data were also tested utilizing generalized estimating equations (GEE) for repeated measures to accommodate attrition for the final-outcome measurements (Post-2 Interventions). The nonparametric, related-samples Wilcoxon signed-rank test was utilized to test the null hypothesis that the median of the differences between the Injury and Post-2 Intervention within-group outcome equals 0.

The KD composite score was the sum of three test-card time-scores. Baseline KDs were not performed. Differences in the KD means from Post-Injury to Post-1 Intervention and Injury to Post-2 Interventions were compared between OMM and CEd utilizing a paired *t*-test. Spearman's rho (r_s) two-tailed correlations of Injury ImPACT sleep symptoms rating scale with other indices or KD were tested for linear relationships (r_s Strength: +1–1 Perfect; +0.9–0.7 Strong; +0.6–0.4 Moderate; +0.3–0.1 Weak; 0 None) [14, 33]. The variables were independent of one another and not influenced by any other observations. Statistical tests were performed utilizing the Statistical Package for Social Sciences-25 (SPSS-25, $\alpha \le 0.05$). Effect sizes were measured by Cohen's d.

Results

There were 55 patients who presented complaining of head injury and symptoms including headache, amnesia, visual disturbance, nausea, dizziness, balance disturbance, sleep disturbance, photophobia, phonophobia, fogginess, lethargy, and/or tinnitus. Among those, 22 were ineligible (Figure 1). Baseline (Pre-season) ImPACT were obtained with consent for 18 men and 7 women with a mean age of 20 (±1.8) years, range 18–26 years. The mean time between

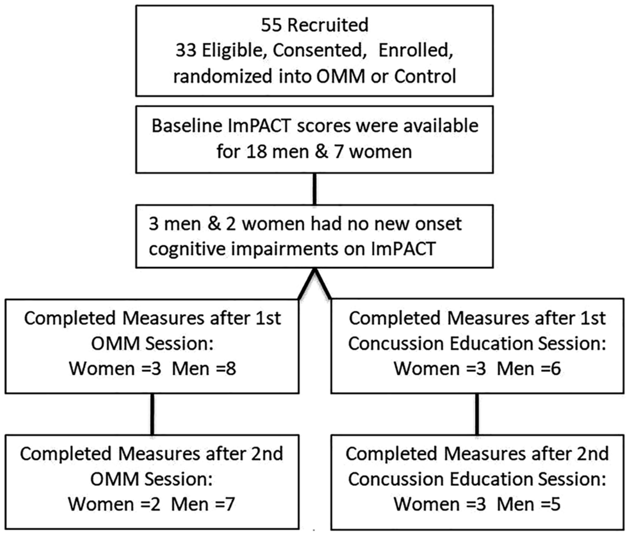

Figure 1: Research participant flow chart. ImPACT, immediate post-concussion assessment and cognitive testing; OMM, osteopathic manipulative medicine.

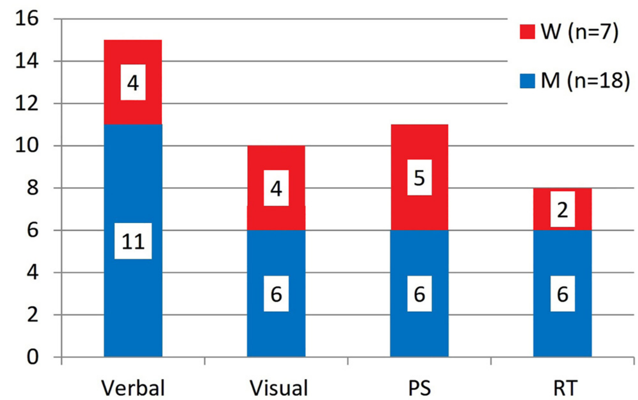

Figure 2: Effects of concussion injury on baseline ImPACT scores. The number of NOI on the ImPACT indices, verbal memory, visual memory, visual-motor PS, and RT following head injury in men (M) and women (W) are shown. ImPACT, immediate post-concussion assessment and cognitive testing; NOI, new-onset impairments; PS, processing speed; RT; reaction time.

injury and first-visit was 3.4 (±2.0) days, range 0–7 days. At Baseline, the mean (CI) in men and women, respectively, were: Verbal 83.4 (6.9); 94.1 (3.6), Visual 76.3 (5.8); 83.9 (5.0), PS 37.1 (4.6); 43.2 (2.4), RT 0.63 (0.1); 0.62 (0.1), IC 5.8 (1.5); 2.6 (0.9), and SS 5.8 (4.7); 2.6 (2.2). There was no significant within-group variability or outlier for ImPACT or KD. There were *Low-Average* to *Impairment*-level scores on Verbal in 6 men, 1 woman; Visual 3 men; PS 3 men; and RT 7 men, 4 women at Baseline. The IC ranges were men 2–13; women 1–4.

Concussion-injury scores

Injury ImPACT indicated that there were NOI of: Verbal in 61.1% (11) men; 57.1% (4) women, Visual 33.3% (6) men; 57.1% (4) women, PS 33.3% (6) men; 71.4% (5) women, and RT 33.3% (6) men; 28.6% (2) women, totaling 77.8% (14/18) men and 85.7% (6/7) women (Figure 2). The difference in means (RCI) from Baseline to Injury (Figure 3) showed decreased Verbal, Visual, and PS as well as increased RT (<0.1), IC, and SS in men and women. Injury IC ranged 0–19 men; 0–10 women. The OMM group of men had the most worsened IC after Injury. Among men, Injury IC significantly correlated with the ImPACT SS item "Getting more sleep than usual" (r_s=0.597; p=0.02; n=14). Among women, Injury "Getting more sleep than usual" significantly correlated with KD (r_s=0.595; p=0.04; n=12). There were no other significant correlations with sleep symptoms.

The NOI identified in Injury ImPACT significantly improved in 83.3% (5/6) women and 64.3% (9/14) men. There were no significant declines after OMM. There were no adverse reactions reported by participants when asked at each visit in both groups. Following one intervention (Post-1 Intervention), there were significant between-group differences suggesting an improvement of Visual 82.33 (RCI 83.20; 81.47) after OMM (n=3) compared by ANOVA to CEd 59.67 (RCI 62.80; 56.54) (n=3; p=0.046) in women as well as of IC after OMM -2.4 (n=8) compared to CEd 1.0 (n=6; p=0.021) in men relative to Baseline (Figure 3). There was a significant between-group difference in Women's Verbal 89.67 (RCI 95.07; 84.27) with recovery after OMM of 1.2 compared to CEd −3.2 (85.00 RCI 91.98; 78.02; p=0.030) (Figure 3), and recovery was better in the CEd group (Cohen's d=0.64 men and women) after the first and second interventions.

Following two interventions, there was a significant improvement to −14.3 relative to Baseline Visual in men receiving OMM 75.29 (RCI 85.81; 64.77) compared by ANOVA to CEd −2.8 (p=0.032). Men receiving OMM had a significant recovery of 68.0% in RT, 0.63 (RCI 0.67; 0.59), compared by ANOVA to CEd (p=0.050). The attrition included OMM men (n=6) and OMM women (n=2) group sizes for Post-2 Interventions measures at the third visit. Utilizing the GEE test to account for the attrition, there were significant between-group differences in Visual (p=0.030), PS (p=0.001), and RT (p=0.045) in men as well as in PS (p<0.001) in women.

Within-OMM group means significantly increased by 11.0 (p=0.047) in women and 9.9 (p=0.030) in men for Visual, decreased by 0.07 (p=0.015) in men for RT, and decreased by 16.0 (p=0.045) in women and 13.4 (p=0.021) in men for

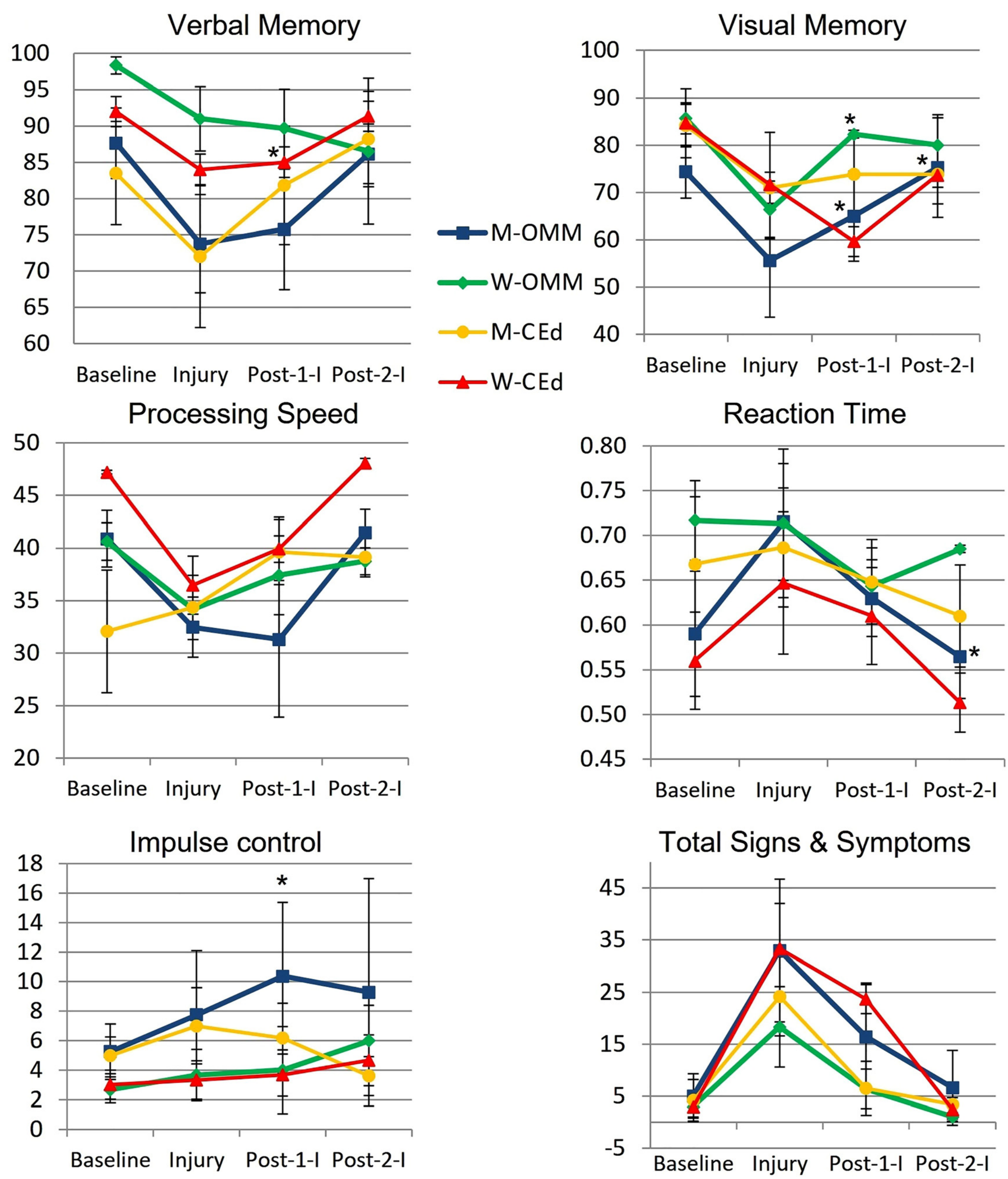

Figure 3: Reliable changes in the mean ImPACT scores in men (M) and in women (W) for verbal memory, visual memory, visual motor PS, RT, impulse control, and total symptoms indices. Index scores are plotted for baseline (pre-season), after concussion (injury), after one OMM or concussion education (CEd) intervention (Post-1 intervention), and after two OMM or CEd interventions (Post-2 intervention). Error bars indicate the SEM. *p ≤ 0.05 for between-group differences in the mean changes from baseline. CEd, concussion education; IC, impulse control; ImPACT, immediate post-concussion assessment and cognitive testing; OMM, osteopathic manipulative medicine; PS, processing speed; RT, reaction time; SEM, standard error of the mean.

SS after one treatment (Post-1 OMM). The Verbal (p=0.018), Visual (p=0.018), PS (p=0.018), RT (p=0.028), and S (p=0.018) improved significantly within the OMM men from Injury to Post-2 OMM on Wilcoxon signed-rank testing. The effect sizes for improvements found after one OMM intervention among men and women combined were 1.33 Visual, 0.42 PS, and 0.31 RT. There were also significant within-CEd group (n=6 women; 7 men) improvements for Verbal 3.2 (p=0.047) men, RT 0.06 (p=0.036) men, and SS 12.6 (p=0.030) women; 13.8 (p=0.020) men.

Women's OMM-group KD improved significantly more so, 8.6 (95% CI 4.67; 12.90) p<0.001, at the post-1 OMM visit

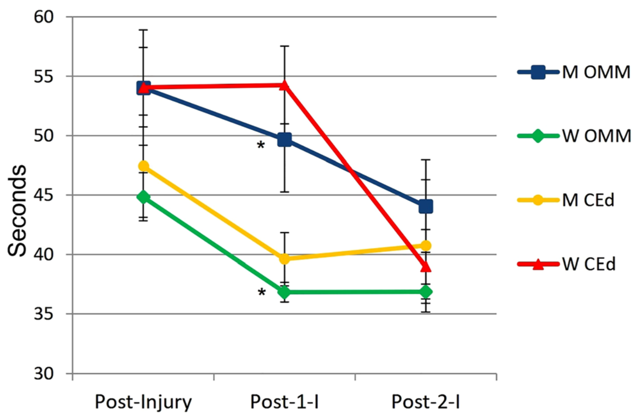

Figure 4: Mean KD times in subjects having had NOI. The post-injury, Post-1 intervention (post-1-i), and Post-2 interventions (post-2-i) mean KD scores (SEM) depicted for the OMM and CEd groups of men (M) and women (W). *p≤0.05 between-group differences. CEd, concussion education; KD, king-Devick; NOI, new-onset impairments; OMM, osteopathic manipulative medicine; SEM, standard error of the mean.

compared to CEd (Figure 4). Changes in men's KD from Injury to post-1 OMM were −4.3 (95% CI -2.4; 22.5) compared to CEd −7.8 (95% CI -0.8; 20.9) p=0.045. Combining men and women, the KD improved by 13.4% OMM and 8.0% CEd after one intervention and by 17.9% OMM and 21.0% CEd after two interventions.

Discussion

Adult patients with concussions with a higher symptom burden after injury appear to suffer prolonged symptoms [34]. Although previous studies utilizing OMM demonstrated safety and benefits for concussion symptoms, benefits in ImPACT after OMM have not been established. The hypothesis was that there will be participants with ≥1 NOI identifiable by reliable changes in ImPACT following concussion who will demonstrate significantly greater recovery after receiving OMM than after CEd. The results support the safety of this OMM approach in acute concussions with NOI in ImPACT. They contribute to the determination of an optimal treatment protocol, dose timing and frequency, and potential therapeutic endpoints. Participants had significantly greater recovery of Visual and RT after OMM (Supplemental Table 2).

Improvements in other ImPACT indices were less clear, especially in the women's groups, which had attrition at the last visit. There was a medium effect size for one CEd intervention on Verbal. The PS returned to Baseline in the OMM group but exceeded Baseline in the CEd group. Other reports of randomized trials of OMM in concussion utilized participants with and without ImPACT NOI [18–21], demonstrating safety [18–21] and investigating changes in symptoms after one treatment [18, 20, 21]. While comparing OMM to the CPG could have demonstrated that OMM was inferior, equivalent, or superior to the comparator [29], the current CPG emphasizes rest. Previous reports suggested that adjunctive OMM was superior to rest [19–21], and the prescription of rest lacked potential placebo effects. The placebo effect appears to consistently be ≤35% of any treatment effect [29]. These results are consistent with other studies, which suggested that OMM is safe and beneficial [18–21]. The underlying mechanisms of OMM may include relieving ongoing osseus strains affecting both bone and connective tissue microarchitecture as well as improving the drainage of neurometabolic waste products through the glymphatic system and/or venous sinuses, as described in Hitscherich et al. in 2016 [7].

The greatest increase in IC after concussion was in the men's OMM group, and they continued to have worse IC than the CEd group. The significant positive correlations of ImPACT "getting more sleep than usual" scores with KD and IC were moderate. Response inhibition/delay is represented by the IC index [11]. The Baseline and Injury IC values in this study were below the level expected among those having Left-Right confusion (IC>20). The KD measures afferent visual function, fixation, and saccadic eye movements, which require multiple brain regions and cranial nerves. Dysfunction of these regions/nerves in concussion causes slower KD performance [14, 15]. A worsening of KD by ≥5 s in competitive fighters was previously reported only found after head trauma [14, 15]. Mean KD in the present study improved by >5 s in each group. However, preinjury KD was not measured, thereby limiting interpretation. While there was a significant difference between groups after one intervention, confidence may be poor due to attrition.

There are limitations to the generalizability of this study, which did not investigate long-term outcomes or severe TBI. There was a small sample size based on the preliminary assessment of symptoms, and the inclusion criteria were further narrowed by sex and NOI on ImPACT in a population of otherwise healthy young student athletes. Pregnancy was an exclusion criterion because progesterone levels have previously been found to be significantly related to concussion recovery [35]. The CEd group did not receive an OSE. The outcomes after OMM in our study may be due to relief from tissue restrictions or tissue distortions of the concussion injury. The relationship between the area of the head hit and the type of cranial SD has been investigated [22]. Although the OMM physician followed guidelines previously outlined by the author [7, 18], the approach varied in the applied techniques and regions treated specific to individual presentation. This variability is similar to how OMM is applied for personalized patient care

[20, 21, 36]. By alleviating restrictions to the clearance of substances, OMM had the potential to also reduce secondary injury when utilized acutely in concussion (when not contraindicated). However, this study did not distinguish between potential improvements in primary and secondary injuries.

Future studies would benefit from sham control, menstrual history, endocrine levels, and biomarkers for concussion and social-placebo effects. Additional measures are necessary to fully understand the mechanisms of OMM's effect, to improve providers' targeting to the individual's impairment, and to evaluate the long-term effects of OMM in concussion. ImPACT "getting more sleep than usual" scores positively and significantly correlated with KD and IC in some groups. OMM may have aided in the recovery of brain regions and/or nerves involved in afferent visual function, fixation and/or saccadic eye movements, but these relationships need further investigation. These effect sizes could be utilized to determine the sample sizes needed for future studies of an OMM sequence targeted to address specific NOI.

Conclusions

There were NOI of Verbal, Visual, and PS indices as well as worsened IC and SS indices in >50% of men and women after concussion, and RTs increased by <0.1 s. The results of this randomized, single-blinded trial testing OMM as an adjunct to the CPG of rest further supported its safety 0–12 days after concussions having NOI in ImPACT indices.

Recovery of Visual improved significantly more in OMM than CEd groups in men and women. The effect size on Visual was large in the OMM group. Although RT improved to better than Baseline in both groups, it significantly improved in men after OMM by 0.10 more than CEd. Effect sizes of one OMM treatment in men and women for PS and RT were small. The primary results supported the application of OMM to improve the recovery of concussion-related cognitive impairments in Visual and RT ImPACT indices. Further research is needed to optimize the utilization of OMM for individuals after concussion.

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Competing interests: None reported.

Ethical approval: This study was approved by the New York Institute of Technology Institutional Review Board (BHS1139) and was registered with ClinicalTrials.gov (NCT02750566).

Informed consent: All participants in this study provided written informed consent prior to participation.

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