

Vitamin D₃ Supplementation and Aquatic Exercise Combination as a Safe- Efficient Therapeutic Strategy to Ameliorate Interleukin-6 and 10, and Social Interaction in Children with Autism

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ABSTRACT

Objectives

Increasing evidence demonstrated that there are altered levels of both pro-and anti-inflammatory cytokines in autism spectrum disorder (ASD) and pointed out that immune dysfunction may also relate to social deficits. This study aimed to investigate the effect of aquatic exercise combined with vitamin D supplementation on social interaction and two related cytokines (Interleukin-6 and Interleukin-10) in children with ASD.

Materials & Methods

Forty boys with ASD (mean age: 10.90; age range: 6–14 years) were randomly assigned to the three interventions (groups 1, 2, and 3) and one control group (each 10 participants). Participants in the group 1 and 3 received a 10-week aquatic exercise program. Subjects in groups 2 and 3 took orally 50,000 IU of vitamin D₃/week. This study evaluated the serum levels of IL-6 and IL-10, as well as the participants' social interaction at baseline and post-intervention.

Results

Compared to the control group, all three interventions improved social skills scores ($p < 0.001$). Surprisingly, the combination strategy could significantly reduce IL-6 and increase IL-10 serum levels in children with ASD.

Conclusion

Aqua-based exercise programs combined with vitamin D supplementation are recommended to benefit children with ASD and improve social and communication dysfunction.

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Introduction

The prevalence of autism spectrum disorders (ASDs) in Iran is estimated to be approximately ten cases per 10,000 children (1). These disorders are complex and influenced by various factors. They manifest as communication and social interaction deficits, along with repetitive and restricted behavioral patterns. The etiology of ASDs is mainly unknown, possibly attributable to a combination of genetic vulnerabilities and environmental factors. Recently, immune dysregulation has been related to derailed neurodevelopment, suggesting a role for the immune system in the pathology of ASD (2).

Several research groups have shown immunological disturbances and cytokine abnormalities in the peripheral blood of individuals with ASD (3, 4). Cytokines, a superfamily of proteins, are involved in normal growth and development, neuronal migration, and synaptic plasticity. In addition, cytokines play an essential role in various other processes, such as regulating hematopoiesis and inflammation and in the proliferation and differentiation of immune cells. Disruption of normal cytokine-mediated signals can be deleterious to the developing processes in the infant (5).

Interleukin-6 (IL-6), a 26 kDa glycoprotein, is an inflammatory cytokine and a key mediator of neurogenesis, glycogenesis, cell growth, cell survival, myelination, and demyelination in the central nervous system (CNS). Furthermore, IL-6 has anti-inflammatory effects and stimulates the production of classic anti-inflammatory cytokines such as interleukin-10 (IL-10) (6). IL-10, a multifunctional cytokine, is the most substantial central anti-inflammatory cytokine in the human immune system and suppresses pro-inflammatory cytokines derived from monocytes/

macrophages such as IL-6. These cytokines go together in a complex interplay with immune cells and messengers of the neuroendocrine system, including neurotransmitters and hormones. Consequently, they have modulatory effects on the activity of serotonin transporters and the diurnal secretion of hormones of the hypothalamic-pituitary-adrenal (HPA) axis (4).

Increasing evidence points to altered levels of both pro- and anti-inflammatory cytokines in ASD. Studies indicate significantly elevated levels of IL-6 (3, 7) and altered levels of IL-10 (2, 4) in individuals with ASD compared with typically developing controls.

Social communication is one of the main diagnostic features of ASD. It is defined as specific behaviors leading to proper social interactions, including smiling, adequate eye contact, responding to questions, initiating and maintaining social interactions, and interpreting both verbal and nonverbal social cues, emotions, and facial expressions (8). Furthermore, previous studies pointed out that immune dysfunction may relate to social deficits. For example, Ashwood et al. found that there are trends for associations between more impaired social interactions measured by Autism Diagnostic Interview-Revised (ADI-R) and plasma IL-10 levels, suggesting an underlying defect in immune function (3). Moreover, the results of another study revealed that the ratio between the inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 (IL-6:IL-10) was correlated with social scores in individuals with ASD (9).

Empirical evidence shows that social interaction deficits in children with ASD contribute significantly to the withdrawal of the family and community, academic and occupational failure, aggression, depression and anxiety problems later

in life, insufficiency of learning opportunities and independence, more stereotypic behaviors, and exposed to social isolation (8). Numerous studies have been suggested to participate in physical exercise interventions in order to improve social dysfunction (8, 10, 11).

Some studies indicated that decreased levels of vitamin D is highly common among children with ASD (12, 13). Vitamin D deficiency may be another potential risk factor for impaired social and communicative functioning, weakness of the immune system and increase of inflammation (14). Optimal levels of vitamin D (>30 and <100 ng/ml) are vital to preserve the neurological development, to protect brain function via neurotrophic actions, calcium signaling, neuronal differentiation, maturation and growth (Petruzzelli et al., 2020), to modulate immune function, and to reduce the production of some cytokines, including IL-6 (14).

Although vitamin D has a beneficial effect on ASD-related traits (14, 15), conflicting results exist regarding its effect on inflammatory markers. Zheng et al. found that following vitamin D treatment, no significant change in the serum levels of IL-6 and IL-10 was observed (16). However, Karonova et al. discovered a decrease in IL-6 levels and an increase in IL-10 levels after 24 weeks of vitamin D supplementation in diabetic neuropathy (DN) patients (17).

In addition, swimming-based exercise interventions are considered to be beneficial, healthy, and safe for children with special needs, including those with ASD. Numerous studies have shown that aquatic training programs can reduce a range of problems for children with ASD. Improvements have been documented in trials of physical (18), physiological (19), and behavioral (10, 11, 19-21) interventions. To

date, no study has examined the effects of aqua-based exercise on serum levels of IL-6 and IL-10 in the ASD population. Few studies have examined the effectiveness of aquatic exercises on inflammatory cytokine levels in other diseases. Bezheh, Soltani, and Khaleghzade investigated the effects of aquatic aerobic exercise on IL-6 and IL-10 in men with multiple sclerosis (MS). They found that eight weeks of aquatic training resulted in a significant decrease in serum levels of IL-6 and IL-10 in patients with MS (22). In a rat study, Qin et al. examined the efficacy of swimming on inflammation in rats who had been given dextran sulfate sodium (DSS)-induced chronic colitis. They demonstrated that swimming improved colon shortening, splenic enlargement, and serum LDH release and resulted in reduced levels of pro-inflammatory cytokines like IL-6 and increased levels of anti-inflammatory cytokines such as interleukin-10 (23).

To our knowledge, this is the first study to compare the influence of ten weeks of aquatic exercises versus vitamin D supplementation on cytokines related to the social interaction of children with ASD. Therefore, the present study sought to produce further intuition in this field by comparing aquatic exercise training, vitamin D supplementation, and the combination of both to understand which strategies are the most effective in ameliorating social interaction and the serum levels of IL-6 and IL-10 in children with ASD.

Materials & Methods

Participants in this study were fifty-two children with ASD from Autism Love Rain Institute between July 2018 and January 2019 who received similar treatment procedures.

The inclusion criteria were:

1. ASD is diagnosed by a psychiatrist (based on

the Diagnostic and Statistical Manual of Mental Disorders-Fifth Edition (DSM-V)).

2. Male gender.

3. Aged 6 to 14 years.

4. Lack of comorbid disability (Spina Bifida, cerebral palsy, phenylketonuria, and other neurocognitive and neurologic disorders).

5. Ability to perform the aquatic exercise program.

6. Parental informed consent.

Exclusion criteria were (1) being absent more than two times in training sessions, (2) any change in medication, dietary, supplementation, or behavior treatment during the study, and (3) being ill during the study.

Forty participants met the inclusion criteria and agreed to participate in the study. They were randomly assigned to one of the four groups: the aquatic exercise (N=10), supplementation (N=10), combination of aquatic exercise+ supplementation (N=10), and control (N=10) groups. The allocation procedure for the research groups was performed by an uninvolved third-party researcher who had no stake in the outcome and analysis of this study. Participants were designated to either group based on simple randomization and a computerized random distribution stratified by gender and autism severity, pre-arranged and performed with closed envelopes. In this way, both participants and researchers were blinded to the intervention. The aquatic exercise program participants received a 10-week group aquatic exercise program (20 sessions, 2 sessions/week) in the Arsen swimming pool in Rasht, Iran. Each training session lasted 60 min, including a 5-minute warm-up, 15-minute orientation training, 20-minute of basic swimming skills, a 15-minute free swim, and a 5-minute cooldown (18). Five certified and qualified swimming trainers with previous experience teaching swimming to children with

autism were instructed to ensure safety and follow the schedule of training techniques. The child-to-trainer ratio was 2:1. At each session, one parent was present to help the interventional program when needed.

Since all children in this study had vitamin D deficiency, participants in the vitamin D group received a therapeutic oral dosage of 50,000 IU vitamin D₃ (provided by Dana Pharmaceutical Company) after dinner once a week, prescribed by a specialist physician.

The aquatic + supplementation intervention was administered to participants of the combination group. Similar to the previous two groups, they received both aquatic exercise and vitamin D supplementation.

Participants in the control group did not receive any intervention and were asked not to participate in new exercise programs during the study. However, they were not passive and received occupational, game, and educational treatments from the institute.

All children completed the interventional approaches in full.

The present study utilized the social interaction subscale of the Gilliam Autism Rating Scale-Second Edition (GARS-2), a 14-item informant, to describe specific, measurable, and observable social behaviors. It includes observations, parent or teacher interviews, and questions completed by the examiner according to their interpretation. Caregivers (or parents/teachers) are asked to score on a 4-point Likert scale ranging from never observed to frequently observed for each item that best expresses the child's specific social behavior. The subscale items are about avoiding eye contact, staring/looking unhappy when praised, resisting physical contact, not imitating, Withdrawing/remaining aloof, being unreasonably fearful, no

affection, no recognition, laughing, giggling, crying inappropriately, misusing toys/objects, doing things repetitively/ritualistically, being upset when routines changed, having tantrums when given commands, and lining up objects and becoming upset when disturbed. The higher the score, the higher the level of social deficiency. Based on the frequency of occurrence of each social behavior under ordinary circumstances in a 6-hour period, parents/caregivers are asked to rate the individual (8). This study used the total raw score of the social interaction subscale.

Blood samples were collected twice before the beginning and the end of the study at the laboratory following 12 hours of overnight fasting between 7 and 9 a.m. The serum was separated and stored at 70°C until analysis. The serum concentration of 25 (OH) D₃ was assessed by a Diasorin kit (DiaSorin Corporation, Stillwater, MN, USA) according to the manufacturer's instructions. Serums IL-6 and IL-10 were measured using a human ELISA kit (Diacclone; France), according to the manufacturer's instructions. The sensitivity or the minimum detectable dose of IL-6 and IL-10 was 2 and 4.9 pg/mL, respectively. Each

sample measurement was performed in duplicate, and all samples were run on the same assay. The analytical sensitivity was 0.1 pg/mL.

This study was semi-experimental research with pre and post-tests. Parents signed informed consent forms and completed their child's social interaction status at the beginning and end of the study. Participants' anthropometric features such as weight, standing height, and BMI were measured using a standardized method, with light clothes and no shoes. BMI was calculated (weight (kg) /height (m²)). An experienced physician conducted a screening of all participants' eligibility for performing the aquatic exercise protocol. All participants completed the protocols in full.

The demographic data were reported as mean and standard deviation (as shown in Table 1). Data normality and homogeneity of variances were evaluated using Shapiro-Wilk and Leven's tests, respectively. The Analysis of Covariance (ANCOVA) was used to adjust for confounding variables. The F ratio was significant, the Bonferroni post-hoc test was used for pairwise comparisons. Effect sizes were reported as partial

Table 1. Participants characteristics (age, height, weight, BMI, vitamin D and autism severity) at baseline

	AG (n = 10)	SG (n = 10)	COG (n = 10)	CG (n = 10)
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Age (years)	11.00 (2.00)	11.30 (2.54)	10.70 (2.45)	10.60 (2.36)
Height (m)	1.52 (0.14)	1.44 (0.11)	1.52 (0.14)	1.53 (0.15)
Weight (kg)	61.90 (17.51)	60.80 (10.40)	61.90 (17.22)	60.30 (15.13)
BMI (kg/m ²)	26.10 (5.30)	29.85 (8.93)	26.10 (5.30)	25.41 (4.14)
Autism severity (total score)	46.00 (13.03)	46.60 (11.11)	46.01 (13.03)	49.30 (15.24)
Vitamin D (ng/ml)	10.70 (3.16)	11.50 (3.20)	11.30 (3.33)	11.80 (2.97)

AG: Aquatic group; SG: supplementation group; COG: Combination group; CG: Control group

eta squared η^2_p for ANCOVA evaluations. All statistical analyses were performed using SPSS® 26.0 (IBM Corporation, Armonk NY, USA) for Windows® with a significance level set at $p < 0.05$.

Results

In total, 40 children with ASD (mean age 10.90 ± 2.27 years) were recruited for this study. Table 1 shows the demographic, vitamin D levels, and autism severity (using GARS-2) information for the four groups at baseline assessment. No statistically significant difference was found between the research groups at the beginning of the study ($P > 0.05$).

Table 2 shows the research variables (social interaction raw scores, IL-6, and IL-10 serum levels) for the research groups at baseline and post-intervention. The results indicated no statistically significant difference in the groups' research variables at baseline.

According to ANCOVA, a significant difference in mean interaction scores [$F(3, 35) = 32.49$, $p = 0.001$, $\eta^2_p = 0.736$] was observed between the groups when adjusting for the pretest. Post hoc tests showed a significant difference between combined and control groups ($p = 0.001$),

combined and supplementation groups ($p = 0.025$), aquatic and control groups ($p = 0.011$), and supplementation and control groups ($p = 0.008$).

The results demonstrated a significant difference in mean IL-6 [$F(3, 35) = 106.21$, $p = 0.001$, $\eta^2_p = 0.901$] between the groups, adjusting for the pretest of IL-6. Post hoc tests showed a significant difference between combined and control groups ($p = 0.001$), combined and supplementation groups ($p = 0.001$), and combined and aquatic groups ($p = 0.001$).

According to the ANCOVA, a significant difference in mean IL-10 [$F(3, 35) = 27.50$, $p = 0.001$, $\eta^2_p = 0.702$] was found between the groups while adjusting for the pretest of IL-10. Post hoc tests showed a significant difference between combined and control groups ($p = 0.001$), combined and supplementation groups ($p = 0.001$), and combined and aquatic groups ($p = 0.012$).

Discussion

Children with ASD have been identified to have altered levels of both pro-and anti-inflammatory cytokines that probably impact the main features of ASD. Although many studies have been focused on people with ASD, to the best of our

Table 2. IL-6, IL-10 and social interaction values in four research groups

Variables	AG		SG		COG		CG		Statistics ANCOVA			Group differences
	Mean (SD)		Mean (SD)		Mean (SD)		Mean (SD)		$F_{(3,35)}$	p	η^2_p	
	Pre test	Post test	Pre test	Post test	Pre test	Post test	Pre test	Post test				
IL-6 (pg/ml)	13.56 (1.61)	12.40 (1.50)	12.50 (1.95)	12.40 (1.83)	13.56 (1.61)	7.50 (1.26)	11.80 (3.11)	11.90 (2.76)	106.21*	0.001	0.901	COG>AG>SG>CG
IL-10 (pg/ml)	9.60 (2.11)	10.50 (2.06)	10.70 (1.63)	11.60 (1.34)	8.90 (2.60)	14.90 (1.79)	9.50 (2.99)	9.70 (2.66)	27.50*	0.001	0.702	COG>AG>SG>CG
Social interaction	13.00 (2.58)	8.50 (1.58)	12.20 (3.32)	9.00 (2.16)	13.00 (2.58)	7.70 (1.56)	13.90 (3.14)	13.60 (2.78)	32.49*	0.001	0.736	COG > AG > SG > CG

knowledge, no study has tested the effects of an aqua-based training program and the combination of aquatic exercise + supplementation on serum levels of IL-6 and IL-10 in the ASD population. This study aimed to compare the effect of three interventional strategies, including an aquatic exercise program, vitamin D₃ supplementation, and the combination of aquatic exercise plus vitamin D₃ supplementation on serum levels of IL-6 and IL-10, cytokines related to social behaviors, among children with ASD aged 6-14 years.

The main findings of the present study were that all interventions lasting for ten weeks improved the social interaction scores of children diagnosed with ASD, compared to the control condition. Interestingly, the results showed that the combined intervention (aquatic exercise+vitamin D supplementation) had the highest effect on reducing social interaction scores. This is the first study reporting the beneficial effects of aquatic training and vitamin D combination intervention on ASD symptoms, as confirmed by a remarkable decrease in the social interaction subscale of GARS-2. These findings align with previous research results that revealed aqua-based intervention could significantly improve social and communication skills in children with ASD (10, 20, 24).

As previously mentioned, social interaction and skill deficits in children with ASD significantly affect academic and occupational underachievement, lack of learning opportunities and independence, the occurrence of stereotypy, and their parents' psychological well-being and quality of life (8, 10). Aquatic exercise is based on buoyancy, viscosity, resistance, and hydrostatic principles, providing multisensory stimuli through water temperature, weight relief,

and atrial input. It may cause active movement, strengthen muscle relaxation, improve blood circulation, and consequently facilitate motor skills based on individual abilities (24). On the other hand, research has also shown that the water environment, due to its pleasant nature, has advantageous effects on the autonomic nervous system, reduces stereotyped behaviors (25), stimulates the sensory and emotional system (11), increases social motivation, eye contact, and social interaction (21), and as a result communication skills increase in children with ASD.

Regarding the vitamin D₃ supplementation, the obtained results are consistent with the results of Feng et al. and Javadfar et al. They found significant changes in clinical traits of ASD following vitamin D supplementation and showed that there are statistically significant, negative associations between vitamin D₃ levels and ASD symptoms (14, 15). Many mechanisms could be responsible for the association between vitamin D levels and ASD. In humans, vitamin D is obtained from two primary sources of sunlight and food and has several important functions. Vitamin D plays not only an essential role in regulating calcium and phosphate metabolism but also in neural development, immune regulation (including the brain's immune system), anti-oxidation, anti-apoptosis, neural differentiation, and gene regulation (14, 26). Cannell proposed that vitamin D can reduce the severity of autism symptoms through anti-inflammatory actions, increased T-regulatory cells, and anti-immune and glutathione-regulating effects. Therefore, it helps to reduce the risk of ASD, and possibly increase the quality of life in individuals with ASD (26). Of course, the mechanism of vitamin D deficiency in the ASD population needs to be further investigated in future studies.

Moreover, this study figured out that both vitamin D₃ supplementation and aquatic exercise interventions did not induce a significant change in serum IL-6 and IL-10 levels in children with ASD. Surprisingly, these findings indicated that ten weeks of aquatic exercise + vitamin D₃ supplementation strategy could reduce the serum level of IL-6 and increase IL-10 serum concentration in children with ASD. To date, little or no study exists to examine the influence of this combination therapy on IL-6 and IL-10 in the ASD population. Therefore, comparing these findings to those of earlier studies takes much work. A small number of studies have exclusively assessed the effect of swimming or vitamin D interventions on inflammatory markers in different patients' subjects. The results of the present study are consistent with the findings of Qin et al. They recommended that swimming inhibited pro-inflammatory cytokines like IL-6 and increased interleukin-10 levels in rats with chronic colitis (23). Nevertheless, the obtained findings are partly in agreement with Bezheh's study. Bezheh et al. found that eight weeks of aquatic training significantly decreased serum levels of IL-6 and IL-10 in MS patients (22). The possible reason for this discrepancy can be due to different ages and types of disease from the present study.

In ASD children, there is just one randomized, placebo-controlled trial by Javadfar et al., who evaluated the influence of 15-week vitamin D supplementation on serum levels of IL-6 in children with ASD. The results of this trial demonstrated that vitamin D supplementation did not result in a statistically significant decrease in serum IL-6 levels in individuals with ASD (14). This inconsistency might be clarified due to various age ranges (3 to 13 years old) and

receiving different dosages of vitamin D (300 IU/kg up to a maximum of 6000 IU daily).

Cytokines are proteins that affect the proliferation, differentiation, and function of immune cells and other systems in the body. They can be secreted by different cells, including neutrophils, activated macrophages, fibroblasts, endothelial cells, and as a result of motor unit contractions (27). Several studies have shown that regular and long-term physical activity reduces the concentration of IL-6 (22, 28). In other words, it has been reported that a negative correlation was observed between regular physical activity and baseline IL-6 levels (29).

The precise mechanism underlying exercise-induced immunomodulation is not yet completely understood. Since long-term exercise affects body composition, and carbohydrate and fat metabolism, one of the possible mechanisms for decreased serum levels of IL-6 could be fat reduction because adipose tissue is one of the main sources of IL-6 production. Moreover, because IL-6 is associated with muscle fuel stores, specifically glycogen, long-term aerobic exercise can deplete these stores and reduce interleukin-6 (28). In the present study, a significant decrease in IL-6 levels was observed due to aquatic exercise, which aligns with previous studies' findings. Besides, regular physical activity stimulates immune cells to release anti-inflammatory cytokines, including IL-10. In turn, IL-10 has an inhibitory effect on the activity of T-regulatory cells and reduces interleukin-6 levels (30).

On the other hand, the anti-inflammatory effects of vitamin D could decrease the level of reactive oxygen species (ROS), increase cellular glutathione, and significantly decrease IL-6 gene expression [29]. Seemingly, vitamin D supplementation in combination with aquatic

exercise training could be a more effective intervention to ameliorate inflammatory markers in children with ASD.

The limitations of this study should be considered. The small number of samples, age range (6-14 years), and single gender of participants (all male) limit generalization. Future research with longer periods and a large number of both genders is warranted to establish the applicability of the current results to young and adult individuals of both genders with ASD through replication studies.

In Conclusion

The present investigation demonstrated that ten weeks of aquatic exercises, vitamin D supplementation, and a combination of these two interventions contribute to improvements in the scores of social interaction, IL-6, and IL-10 serum levels of children with ASD. Since these interventional approaches are safe, non-expensive, and effective therapies to improve ASD-related traits and, consequently, improve the quality of life in individuals with ASD and their families, it is recommended that parents, educators, and physicians benefit aqua-based exercise programs combined to vitamin D supplementation in order to maximize improving social and communication dysfunction in children with ASD.

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number was IR.IAU.RASHT.REC.1396.99; Also, Iranian Registry of clinical trial number was IRCT20180503039517N1

Author's Contribution

Soleyman Ansari, Fahimeh Adibsaber: has contributed for study concept, conducting research project, development of original idea, writing the manuscript, collecting data and statistical analysis.

Alireza Elmieh: Study concept, help in writing of manuscript, development of original idea, edition of manuscript.

Babak Barkadehi : Statistical analysis and edition of manuscript.

All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest

The authors declare that no conflict of or competing interests existed or occurred in the conduction of this manuscript.

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