# Vitamin $D_3$ 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:**

**Introduction:** Increasing evidence demonstrated that there are altered levels of both pro- and anti-inflammatory cytokines in children with autism spectrum disorder (ASD) and pointed out that immune dysfunction may also relate to social deficits. This study investigated 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.

**Material & 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. We evaluated the serum levels of IL-6 and IL-10 and 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 ($p < 0.001$).

**Conclusion**: It is recommended that aqua-based exercise programs combined with vitamin D supplementation maximize the improvement of social and communication dysfunction in children with ASD.

**Keywords**: Swimming, vitamin D supplementation, Interleukin-6, Interleukin-10, Children with autism.

## 1. Introduction

It is estimated that approximately 10 per 10,000 of the child population in Iran are affected by autism spectrum disorders (ASDs). These are a multifactorial determined group of neurodevelopmental disorders characterized by deficits in communication and social interaction, and by restricted, repetitive patterns of behaviors (1). The etiology of ASDs is largely 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 (3-5). Cytokines, a superfamily of proteins, are involved in average growth and development, neuronal migration, and synaptic plasticity (6). In addition, cytokines are essential in various other processes, such as regulating hematopoiesis inflammation and in the proliferation and differentiation of immune cells (7). Disruption of normal cytokine-mediated signals can be deleterious to the developing processes in the infant (8).

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) (9). IL-6 also has anti-inflammatory effects and stimulates the production of classic anti-inflammatory cytokines such as interleukin-10 (IL-10) (10). 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 (11). 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 (12).

Increasing evidence points to altered levels of both pro- and anti-inflammatory cytokines in ASD. Studies indicate significantly elevated levels of IL-6 (3, 5, 13) and altered levels of IL-10 (2, 12, 14) in individuals with ASD compared with typically developing controls.

It has also been reported that elevated serum levels of both IL-6 and peroxiredoxins Prx2 and Prx5, involved in modulating oxidative stress, were associated with elevated ASD severity (15). Social communication is one of the main diagnostic features of ASD. It is defined as specific behaviors that lead to proper social interactions, including smiling, adequate eye contact, responding to questions, initiating and maintaining social interactions, and interpreting verbal and nonverbal social cues, emotions, and facial expressions (16). Previous studies pointed out that immune dysfunction may also 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 (17, 18).

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 (16). Numerous interventions have been suggested to improve social dysfunction, such as pharmacological treatment (19), behavioral strategies (20), multivitamin supplementation (21), and participation in physical exercise interventions (16, 22, 23).

Some studies indicated that decreased levels of vitamin D are highly common among children with ASD (24). Vitamin D deficiency may be another potential risk factor for impaired social and communicative functioning (25), weakness of the immune system, and increased inflammation (26). Optimal levels of vitamin D ($>30$ and $<100$ ng/ml) are vital to preserve neurological development, protect brain function via neurotrophic actions, calcium signaling, neuronal differentiation, maturation, and growth (24), modulate immune function, and reduce the production of some cytokines, including IL-6 (26).

Albeit the beneficial effect of vitamin D on ASD-related traits (26, 27), there are conflicting results regarding the effect of vitamin D supplementation on inflammatory markers. Zheng et al. found that following vitamin D treatment, no significant change in the serum level of IL-6 and IL-10 was observed (28). 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 Patients (29).

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 of children with ASD. Improvements have been documented in trials of physical (30, 31), physiological (32), and behavioral (22, 23, 31, 33-36) 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 8 weeks of aquatic training resulted in a significant decrease in serum levels of IL-6 and IL-10 in patients with MS (37). 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 the colon shortening, splenic enlargement, 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 (38).

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

## 2. Materials and methods

### *Participants:*

Participants in this study were 52 children with ASD from Autism Love Rain Institute between July 2018 and January 2019 who received similar treatment procedures. Inclusion criteria were: (1) ASD diagnosed by a psychiatrist (based on Diagnostic and Statistical Manual of Mental Disorders-Fifth Edition (DSM-V)), (2) male gender, (3) aged 6 to14 years, (4) lack of comorbid disability (including 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.

*Aquatic exercise program:*

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 basic swimming skills, 15-minute free swim, and a 5-minute cooldown (30). To ensure safety and follow the schedule of training techniques, five certified and qualified swimming trainers who had previous experience teaching swimming to children with autism were instructed. The child-to-trainer ratio was 2:1. At each session, one parent was present to help the interventional program when needed.

*Vitamin $D_3$ supplementation:*

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

*The combined intervention:*

The aquatic + supplementation intervention was administered for participants of the combination group. They received both aquatic exercise and vitamin D supplementation similar to the previous two groups.

*Control Group:*

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 treatment from the institute. All children completed the interventional approaches in full.

***Measures***

*Gilliam Autism Rating Scale:*

We utilized the social interaction subscale of the Gilliam Autism Rating Scale-Second Edition (GARS-2) (39), 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 6 hours, parents/ caregivers are asked to rate the individual (16). We 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. According to the manufacturer's instructions, Serum IL-6 and IL-10 were measured by a human ELISA kit (Diaclone; France). The sensitivity or the minimum detectable dose of IL-6 and IL-10 was 2 and 4.9 pg/mL, respectively. Each measurement was duplicated, and all samples were run on the same assay. The analytical sensitivity was 0.1 pg/mL.

*Procedures:*

The Committee approved all procedures for Ethical Considerations in Human Experimentation of Medicine College, Azad University of Rasht Branch (IR.IAU.RASHT.REC.1396.99), with the Iranian registry of clinical trials (IRCT20180503039517N1). Parents signed written informed consent before the study. All children orally expressed their assent.

This study was semi-experimental research with pre and post-tests. Parents signed informed consent and completed their child's social interaction status at first and at the end of the study. Participants' anthropometric features such as weight, standing height, and BMI were measured in a standardized method with light clothes and no shoes. BMI was calculated (weight (kg) /height (m2)) (31). An experienced physician conducted screening of all participants' eligibility for performing the aquatic exercise protocol. All participants completed the protocols in full.

*Statistical analysis:*

The demographic data were reported as mean and standard deviation (as shown in Table 1). Data normality and homogeneity of variances were evaluated by the Shapiro-Wilk and Leven's tests, respectively. Analysis of covariance was to adjust for confounding variables. When the F ratio was significant, the Bonferroni post-hoc test was used for pairwise comparisons. Effect sizes were reported as partial eta squared $\eta 2p$ 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 $p < 0.05$.

## 3. 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. There was no statistically significant difference between the research groups at the beginning of the study (P>0.05).

Table 1 Participants characteristics (age, height, weight, BMI, vitamin D and autism severity) at baseline (n=10 in each groups)

| | 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

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 that there is no statistically significant difference in research variables in the groups, at baseline.

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 | $\eta 2_p$ | |
| | 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 |

*Social interaction scores:*

According to the analysis of Covariance, there was a significant difference in mean interaction scores [F (3, 35) =32.49, p=0.001, η2p=0.736)] between the groups whilst adjusting for the pretest. Post hoc tests showed there was 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).

*IL-6 serum levels:*

The results demonstrated that there was a significant difference in mean IL-6 [$F_{(3, 35)}$ =106.21, p=0.001, $\eta^2p$ =0.901)] between the groups whilst adjusting for pretest of IL-6. Post hoc tests showed there was 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).

*IL-10 serum levels:*

According to analysis of Covariance, there was a significant difference in mean IL-10 [$F_{(3, 35)}$ =27.50, p=0.001, $\eta^2p$ =0.702)] between the groups, whilst adjusting for 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).

## 4. Discussion

Children with ASD have been identified to have altered levels of both pro- and anti-inflammatory cytokines that probably impact on main features of ASD. Although many researches have been focused on people with ASD, to the best of our knowledge, there are no study testing the effects of an aqua-based training program and the combination of aquatic exercise + Vit D 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 $D_3$ supplementation, and the combination of aquatic exercise plus vitamin $D_3$ 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) significantly reduced social interaction scores. This is the first study reporting the beneficial effects of aquatic training and vitamin D3 combination intervention on ASD symptoms, as confirmed by a remarkable decrease in the social interaction subscale of GARS-2. This finding aligns with previous research results that revealed aqua-based intervention could significantly improve social and communication skills in children with ASD (22, 33, 34).

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 (16, 22). Aquatic exercise is based on the buoyancy, viscosity, resistance, and hydrostatic principles, which provide multisensory stimuli through water temperature, weight relief, and atrial input (31) and may cause active movement, strengthen muscle relaxation, improve blood circulation, and consequently, facilitate motor skills based on individual abilities (34). 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 (40), stimulates the sensory and emotional system (22), increases social motivation, eye contact, and social interaction (35), and as a result communication skills increase in children with ASD.

Regarding the vitamin $D_3$ supplementation, our result is 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 $D_3$ levels and ASD symptoms (26, 27). Many mechanisms could be responsible for the association between vitamin D levels and ASD. In humans, vitamin D is obtained from two main sources of sunlight and food and has several important functions. Vitamin D acts not only important role in regulating calcium and phosphate metabolism, but also in neural development, immune regulation (including brain immune system), anti-oxidation, anti-apoptosis, neural differentiation and gene regulation (26, 41). Cannell proposed that vitamin D can reduce the severity of autism symptoms through anti-inflammatory actions, increased T-regulatory cells, 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 (41). Of course, the mechanism of vitamin D deficiency in the ASD population needs to be further investigated in future studies.

Moreover, we figured out that both vitamin $D_3$ supplementation and aquatic exercise interventions did not induce a significant change in serum IL-6 and IL-10 levels in children with ASD. Surprisingly, our findings indicated that 10 weeks of aquatic exercise + vitamin $D_3$ supplementation strategy could reduce the serum level of IL-6 and increase IL-10 serum concentration in children with ASD. To date, there is little or no study to examine the influence of this combination therapy on IL-6 and IL-10 in the ASD population; therefore, it is hard to compare our findings to earlier studies.

A few studies have exclusively assessed the effect of swimming or vitamin D interventions on inflammatory markers in different patient subjects. The result of our study is 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 (38). But, our findings are partly in agreement with Bezheh's study. Bezheh et al. found that 8 weeks of aquatic training significantly decreased serum levels of IL-6 and IL-10 in MS patients (37). 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. This trial demonstrated that vitamin D supplementation did not result in a statistically significant decrease in serum IL-6 levels in individuals with ASD (26). 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 (42). Several studies have shown that regular and long-term physical activity reduces the concentration of IL-6 (37, 43). In other words, it has been reported that there is a negative correlation between regular physical activity and baseline IL-6 levels (44).

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, especially glycogen, long-term aerobic exercise can deplete these stores and reduce interleukin-6 (43). In the present study, a significant decrease in IL-6 levels was observed as a result of aquatic exercise, which is in line with the findings of previous studies. Besides, regular physical activity stimulates immune cells to release anti-inflammatory cytokines, including IL-10 (45). In turn, IL-10 has an inhibitory effect on the activity of T-regulatory cells and reduces interleukin-6 levels (46).

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 (26). It seems that 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 set up the applicability of the current results to young and adult individuals of both genders with ASD through replication studies.

## 5. Conclusion

The present investigation demonstrated that 10 weeks of aquatic exercises, vitamin D supplementation, and a combination of these interventions contribute to improved scores of social interaction, IL-6, and IL-10 serum levels in 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 to maximize improving social and communication dysfunction in children with ASD.

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