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Optimization of Alkaline Protease Enzyme Using Statistical Methods

Marzieh Keyhanian[1*]

1. Master of Microbial Biotechnology

* Corresponding author: Marzieh Keyhanian
Email: kavir00133@yahoo.com

***Abstract*— the extensive usage of proteases in industries such as pharmacy, nutritious, weaving, paper production, curriery, detergent and silver recycling in crystallography (X-ray) has caused a serious attention to the production of protease enzymes. Bacillus species are been used as suitable microscopic organisms for the production of enzymes [1-2]. The design of experiments is a strong tool for improving the quality of products and eliminating the causes of quality losses, especially in the pre-production stages or in the provision of services. Today, there are new methods for performing the design of experiments. These methods have been enhanced by foresighting suitable models in comparing data collection, known as modern test design techniques [3]. The purpose of this review essay is to optimize the production protease enzyme by using the Taguchi statistical method and the effects of different sources of Carbon, Nitrogen, mineral elements, and creating different conditions such a pH, temperature, the duration of incubation and aeration in the production of protease [4].**

Keywords: Protease, Different techniques of experiment designing, Taguchi method, Optimization of Alkaline Protease Production Factors.

Introduction

Proteases are proteolytic or peptidases enzymes that have proteolytic activity. These enzymes perform protein catabolism by peptide banding hydrolysis; secretion of proteases by the bacterium may be as exotoxin which is the cause of the disease and the destruction of the cell's external coverage [1-2].

The division of proteases is based on the existing amino acid in the active station and is divided into six groups including serine protease, threonine protease, cysteine protease, aspartate protease and metallo proteases. Serine proteases have a wide range of substrates and a remarkable stereotypic activity for the steric substrate. Cysteine proteases were also identified for the first time in a tobacco plant [1].

The usage of proteases

Proteases have an extensive usage in the industry; they are included 60% of commercial enzymes and have 1/3 of the world market commercial detergents. These enzymes generate about $1.6 billion annually in the United States which it's 29% is for human's food, 15% for livestock feed , 56% of it has usage for other industries like pharmacy ,nutritious, weaving, paper production, curriery, detergent and silver recycling in crystallography(X-ray). The optimized pH of protease is between 9 and 12.The tolerance of various pH in protease depends on the available serine in active station. Proteases are produced in plants, animals and microorganisms in high amount. In protease plants such as papain from papaya tree, keratiniases are produced in some vegetal plants and bromelain from pineapple tree, and in animals proteases such as trypsin (boar pancreas), chymotrypsin (cow pancreas) and pepsin (calf stomach) are produced [1-2].

The ATCC (American Type Culture Collection) presents a catalog of bacteria and protease producing phages. Proteases are produced by Bacillus, such as : Bacillus licheniformis , Bacillus horikoshi, Bacillus sphaericus, Bacillus formis, Bacillus alkalifilus and Bacillus subtilis ,other bacteria such as Alcaligenes faecalis, pseudomonas fluorescens, Aeromonas hyphilia, and molds such as Aspergillus, Penicillium, Rhizopus and Fusarium oxysporum.[1-2-3-4].

Designing medium for protease enhancement

Designing experiments in a traditional way is a statistical approach. This method was broached by Fisher in England in 1920.For years in a row; he was responsible for analyzing the statistical data of an agricultural station in London. Fisher developed an analysis of variance (ANOVA) in an effort to improve agricultural products. The method of designing the experiments is a powerful tool for improving the quality of products and eliminating the causes of quality losses, especially in the pre-production stages or the provision of services. Nowadays, new methods have been developed to carry out the design of experiments. These methods have been upgraded by generating appropriate forecast models in comparison with data collection and are known as modern experiment designing techniques [5].

Different techniques of experiment designing

In general, experiment designing can be done in three ways: One-factor-at- a- time, Full factorial and fractional factorial designs.

One-factor-at-a-time (OFAT) method

The simplest test mode is based on one factor on the product. This method is also called one-factor method. In this way other factors are kept constant, a factor changes and at the end the best amount with an optimal response is selected. In this experiment every single factor is repeated. Before OFTA designing, there is a need for a general diagnosis to be analyzed. The limitations of this method can be noted as: if there is an interaction between the variables, the probability of reaching optimal conditions or even near them is very low. On the other hand the total time required is long, because the experiments are carried out consecutively [6].

Full factorial method

In the Full factorial method, the effects of the variables are simultaneously checked. In this method, all possible states between the variables and their levels are tested. In this way, the exact answer is obtained but because of expenditure lots of time, energy and cost, using of this method in experiment design is not recommended. The number of experiments is obtained from: N=Lm .For example, to examine the effect of four factors on two levels, the number of required test is 24 equals with 16 tests. This method is suitable for cases with the low number of experiments [6].

Fractional of full factorial method

Experts have developed the statistics of performance plans that are known as fractional business plans. In this method, a number of possible combinations of variables are selected and the best possible conditions are obtained after the tests are performed by evaluating the obtained answers. There may be no optimal conditions between the tested modes. If the initial study to determine the variables and their interrelations between them is done carefully, the obtained answer is optimal. Of course in this method, some interactions are eliminated in order to simplify, so the best answer may not be obtained but if the assumptions applied are close to the truth, the answer is close to the optimal answer. In this method, in addition to the lower number of experiments, due to the possibility of performing parallel experiments, the overall time also greatly decreases [6].

Taguchi method

The basis of this method was presented by Dr. Genechi Taguchi which was later used as Taguchi method to optimize researches in engineering processes. Recently, this method has been used in other areas of biotechnology, including some genetic engineering techniques. In this method a collection of tables is called orthogonal arrays for designing experiments.

The characteristics of these orthogonal arrays are that each test series has a different composition than others, and there is a balance between levels or in the other words, the variables are distributed equally in one level. The experiments are based on these arrays and are performed randomly and repeatedly to reduce errors caused by uncontrollable factors. Of course, if these factors are well known, they can be examined with a separate orthogonal array (external array). Analyzing the results in this method, evaluating the main effects and interaction can evaluate the contribution of each factor in the response. Analysis of variance is statistical [5].

In order to determine the level of each factor, degree of freedom, sum of squares, variance or mean square, the ratio of variance and total squared net are used. When the number of experiments is low, analysis of variance of manual methods is possible using existing formulas. The Qualitek-4 statistical software designed specifically for statistical analysis of the responses by the Taguchi method is proposed for a large number of experiments [5].

Advantages and limitations in the Taguchi method

To highlight the advantages and limitations of the Taguchi method, it is better to compare this method, which is a fraction of the full factorial method, with other methods, namely, the full factorial method and the one-factor method at one time. The main advantage of statistical methods in comparison to the one-factor method at one time is that in the statistical methods, the overall design of the experiments is carried

out in one step and at the beginning, and then the tests are carried out simultaneously. If, in one factor at a time method, at each stage, at first thinking about what should be tested must be done, then several tests are carried out and this process continues into several stages. It may be possible to make statistical analysis, also briefly, in the calculation of error and variance in experiments of one factor at a time, but accurate analysis of the results is not feasible.

As it is known, in the Taguchi method, the number of experiments required to study the main effects of factors independently is much less than the full factorial method and is almost lesser than the one-Factor at a time method. Of course, as mentioned, if there is a need of reviewing interaction effects between factors in the Taguchi method, the number of required experiments will be increased. It should be noted that despite the fact that all the mutual effects in the full factorial method can be investigated, since the degree of influence of the factors and interactions are not equal to each other and only some of them have a significant effect and should be investigated, performing experiments in abundant number, is only a waste of time and money. Sometimes, large number of tests also reduces their accuracy. The thing that distinguishes Taguchi method from the others, even other fractional methods of factorial, is reducing the change in quality due to the presence of intrusive (uncontrollable) factors by taking them into experiments without deleting them. Usually in industrial processes, eliminating these factors involves a large amount of money. Using the Taguchi method, it is not necessary to spend on eliminating these factors, and their effects are made by adjusting the levels and controlling the change of other factors. For this purpose, the optimal point for the factor is usually determined in such a way that, due to its minor changes, there will be no significant changes in the final quality. To eliminate the effects of disturbing factors, it is possible to design experiments for effective factors in different conditions for disturbing and repetitive factors. Another advantage of the Taguchi method, as already mentioned, is the possibility of examining the effects of disturbing agents simultaneously and in foreign array. In this way, the optimal conditions are determined with minimum variability against the change of controllable and uncontrollable factors (disturbance). Subsequently, the cost of guaranteeing the quality of goods decreases in industrial production processes. In the Taguchi method and possibly other complete fractional factor methods, given that statistical control of the process is a method for ensuring or developing quality and applied online, if the effective factors have already been identified, statistical control of the process for these factors can be done mainly. In the discussion of "experiment designing", the main advantage of the Taguchi method is its application in cases where different variables do not have the same levels, and each one is examined at different levels. The most important limitation of the Taguchi method is that it can only be used in design. In other words, once the industrial process is launched, the Taguchi method cannot be used during the work to increase product quality or to stabilize against external factors in that process, unless the development of the industrial process is contemplated. Another limitation is Taguchi's method of using it for discrete variables. In other words, this method is not suitable for optimizing continuous variables [7-8-29].

Optimization of Alkaline Protease Production Factors

Compounds of the growth medium of a bacterium that differs from one organism to another, plays an important role in the production of extracellular alkaline protease, and therefore the compositions required for the culture medium with their concentrations should be optimally optimized. The researchers' efforts mainly focus on the effects of different nutrients of carbon and nitrogen as substrates that affect the enzyme's efficiency, the need for bivalent metallic ions in the culture medium and the optimization of fermentative and environmental parameters such as pH, temperature and aeration rate. No growth medium has been created for the best protease production from various microbial sources, and each organism has special conditions for maximum enzyme efficiency. The production of the enzyme shows a definite

relationship due to the growth stage of the organism. Protease synthesis in Bacillus species is controlled by complex mechanisms that are in the phase of growth and stagnation[9]. the secretion of proteases is highly dependent on the growth medium and the factors affecting the optimal production for other species are different. Therefore, it is important to determine the most suitable culture and environment parameters in order to maximize growth [10]. Production of extracellular protease in microorganisms is heavily influenced by the components of the culture medium, such as variation in the C / N ratio, the presence of some metabolizable sugars such as glucose and metal ions. In addition, a number of other physical factors such as aeration, temperature, PH, incubation period, and inoculum concentration also affect the amount of protease production. [9]. Temperature: Temperature is a vital parameter that varies from an organism to another for maximize cell growth and enzyme production. The temperature control mechanism for producing the enzyme is not easily understandable. Although studies by Frankena et al. showed that there is a link between enzyme synthesis and energy metabolism in Bacillus, which is controlled by temperature and oxygen uptake [11]. The temperature affects the production of the enzyme by altering the physical properties of the membrane, which has been shown by Usharani and Muthuraj on Bacillus laterosporous [12].

The optimum temperature for the alkaline protease produced by Bacillus has been reported. The optimal temperature for the production of protease by Bacillus cereus and Bacillus coagulants is 30 ° C and the lowest optimum temperature for Bacillus seroclaning and Cinerea Bacillus is 28 ° C. The temperature of 37 ° C has been reported as the optimum temperature for the production of protease by a number of Bacillus species such as Amovivorus Bacillus[11]. The data from the study on PE-11 Bacillus subtilis showed that the maximum production of protease at 47 ° C and the best temperature for producing protease was 60 ° C. The results show that the maximum protease production for PCSIR EA-3 Bacillus is at 35 ° C, and this bacillus cannot produce protease below 25 ° C. On the other hand, a significant reduction in the production of the enzyme was observed at 40 ° C, and this bacillus did not produce any proteases at 50 ° C [13]. In another study on Bacillus SP MIG showed that the optimum temperature for producing the enzyme was 30 ° C, and the increase in temperature would reduce the production of the enzyme. High temperatures at 40 and 45 degrees cause a loss of 37 to 77 percent of the enzyme[14]. In another study on Bacillus SP MIG showed that the optimum temperature for producing the enzyme was 30 ° C, and the increase in temperature would reduce the production of the enzyme. High temperatures at 40 and 45 degrees cause a loss of 37 to 77 percent of the enzyme[14]. The effect of temperature on N2 Bacillus subtilis also showed that the highest production of protease is at 40 ° C and the minimum production is at 80 ° C[15] Ray et al. (1992) reported that Temperature can regulate the synthesis and secretion of extracellular protease by microorganisms [14].

pH: pH is the most important physical factor determining the rate of biological process [15]. The production of alkaline protease by microbial strains strongly depends on the extracellular pH, since this parameter significantly affects the enzymatic processes and the transmission of various components throughout the cell membrane [16]. Bacillus subtilis Bacillus subtilis needs an alkaline pH to grow and produce enzymes and can grow Above the 12 to 5 / 6 range. Maximum enzyme production was observed at pH 10.5. Similar effects on protease production were achieved by researchers: Tari (2006), Banerjee (1999) and Shikha et al. (2007). Protease higher than pH: 12 will not be produced. The highest production of protease by brevis B., B.pantotheneticus and Bacillus spl18 was at pH 10.5, 10, 10, respectively. The maximum production of protease in Ve1 Bacillus and Bacillus subtilis species has been reported at an optimum pH of 9. Bacillus subtilis has a wide range of pH and, according to researches, it seems that the protease enzyme is stable against pH changes. Similar results have been reported by Genckal for l21 Bacillus sp [15]. Sarkar et al. reported that the maximum amount of enzyme production was obtained when the pH of the culture medium was about 6, whereas for Bacillus subtilis 38, the maximum production of protease was reported at pH 6.5. According to the results of Qadar et al. (2009), the maximum production of protease is at pH 7 and the

enzyme activity gradually drops in pH above 5.7 and below 5.6. It is likely that pH above 8-7/5 leads to accumulation of metabolites, which, as a result, deactivates the enzyme [12]. According to the findings of the Sepahi and colleagues, protease activity is different with the initial pH of the culture medium. The highest level of protease activity was detected in cultures that grow at pH 8 [17]. In the same study, Das and Prasad stateded the best pH for production of protease [16]. The effect of pH on Bacillus subtilis N2 showed that favorable pH for the production of protease is 9 and production reduces at higher pH. According to researches by mukesh et al. In 2012, Tsujibo et al. (1999) at higher pHs, the growth rate decreases and the enzyme will be deactivated [14]. Also, according to Rao et al., Cell growth of microorganisms and protease production depend on the initial pH of the culture medium [18]. Maximum enzyme production is reported by Bacillus sp CAMA4 at optimized pH 8. Similar results were obtained by Padmapriya et al. (2012) in the production and purification of protease from marine bacillus species, Geethanjah and Subash in optimizing the production of Bacillus subtilis protease.In agreement with this result they have a same report that pH8 is optimal for producing the maximum enzyme [19].

Carbon source: A carbon source is needed for all biosynthetic activities that results in production, such as formation of the product and cell survival. Carbohydrates are the oldest sources of carbon and energy for microbial fermentation. Between carbohydrates, the most common are sugars, which are used. Most microorganisms are able to take glucose. Polysaccharides such as starch and cellulose are used by a limited number of microorganisms. Hydrolysis of pectin is done by some bacterias and molds. A limited number of microprocessors use fats to supply their energy sources if they do not have the proper carbon source, such as sugar. In general, aerobic microorganisms hydrolyze fats more than anaerobic. In the absence of more suitable energy source, microorganisms use proteins hydrolysis, peptides, and amine acids to supply their energy sources [20]. The demand and the need for different sources of carbon vary from organism to the other organism and Even within the same species isolated from different sources is different. Glucose is the best carbon source for the production of protease from the Bacillus CAMA14 species.These findings are matching with reports that are offered for the production of proteas [19]. Carbon sources significantly affect the production of enzymes, and the most commonly used substrate has been reported as casein [8]. According to the studies conducted on Bacillus Sp MIG, it has been found that adding wheat bran, starch molasses, or maltose increases enzyme production, since wheat bran is one of the inexpensive and available resources and has a great importance in the medium. It is estimated that the wheat bran price for a liter of culture medium is $ 0.002. Studies done by Chauhan and Gupta (2004) and Fang et al. In 2001 on alkaline protease showed that adding starch to the medium induces enzyme synthesis [13]. In a study by Ciahi and Jebel Ali, it was shown that maltose and galactose use has a better growth outcome than starch consumption; although starch was the best substrate for the production of enzymes, it was suggested that favorable conditions for the production of protease are not necessarily the optimal conditions for growth. These observations are in contradiction with previous studies by Camlia Rocha et al. which showed that starch is the best source of carbon for the growth and production of protease [12]. A study on carbon sources such as maltose, glucose, lactose, starch and fructose on Ns Bacillus subtilis was investigated to produce alkaline protease and revealed that the maximum production of enzymes is due to glucose consumption. Samarntarn et al. Reported in 1999 that the production of Protease is high by microbes in the presence of carbohydrate sources, especially lactose [14].

Nitrogen source: Nitrogen forms 4 to 14 percent of the dry weight of bacteria and fungi. A wide range of organic and inorganic compounds can be used to estimate the need for microorganisms to nitrogen. The revived nitrogen element is in an amino group. Most prokaryotes can absorb and regenerate the oxidized forms of these elements, named nitrate. The most suitable source of nitrogen for microorganisms is ammonium minerals. Some microprocessors also have the ability to use N2. Most industrial microorganisms can consume organic and non-organic sources. Non-organic nitrogen is used in the form

of ammonium salts, which are often ammonium sulfate and phosphate hydrogen, diammonium or ammonia. Ammonia is also used to regulate fermentation pH. Nitrogen sources include amine acids, proteins and urea. Nitrogen is often used in crude forms that are commonly used by other factories such as corn grinding, extracts, yeasts, peptons and soybeans [20]. Pure amine acids are used only in certain conditions, which are commonly used as precursors for specific products. Bacillus lichen formis can use amino and immunogenic nitrogen, arginine, asparagine and glutamine forms by the enzymes of arginine deaminase, arginase, asperginaz and glutaminase. The use of yeast extract as a source of nitrogen has a beneficial effect on the production of protease [20]. The use of other organic compounds as a source of nitrogen, such as polypheton, also increases the production of protease.In Bacillus lichen formis and Bacillus coagulans, 57 production decreases during sporulation after 24 hours. Production starts in liquid medium and reaches its maximum after 44 hours of production [21]. Meat extracts are also used in Bacillus species as sources of nitrogen. Including inorganic nitrogen sources wich are used to produce protease enzyme , ammonium carbonate can also be mentioned [21]. In microorganisms, organic and inorganic nitrogen metabolizes the primary production of amino acids, nucleic acids, proteins, and cell wall components. The production of alkaline protease strongly depends on the availability of carbon and nitrogen sources in the culture medium. Both sources regularly affect the synthesis of the enzyme, in the study of protease production by Bacillus subtilis, various sources of nitrogen including yeast extract, meat extract, soybean peptone, ammonium sulfate and casein were used. Among the various sources of nitrogen, casein was the best source for inducing alkaline protease production. Between sources of mineral nitrogen, nitrate sodium also is the best source of nitrogen for the production of alkaline proteases,While other ammonium compounds inhibit the production of enzymes. These results are corresponded with recent findings on Bacillus species for casein, ammonium compounds and sodium nitrate [15]. Patel et al. and Prakasham reported that production of protease in Bacillus sp and Bacillus subtilis increases with organic nitrogen sources such as peptone. While the study on Bacillus subtilis revealed that peptone surprisingly inhibited the production of alkaline protease [15]. In research by patel et al. (2005) and Krishnaveri et al. (2013), it was found that yeast extract inhibited the production of alkaline protease by Bacillus subtilis.While yeast extract showed the maximum effect on increasing the production of enzymes in Bacillus sp and Bacillus licheniformis [2-22]. In the research done about bacillus sp MIG, it was found that yeast extract is the best source of organic nitrogen and sodium nitrate and potassium as the best sources of inorganic nitrogen [13]. Soybeans, casein hydrolyzate, and casein support the enzyme's high activity. Peptone and urea decrease the activity of the enzyme by 20 to 47 percent. Using 1% fill in the culture medium with 1% wheat shell protects the enzyme production, but the incubation period lasts 4 days [13]. Naile and johnvesly (2010) reported that protease production by Bacillus sp.JB-99 was supported by nitrogen nitrogen, sodium nitrate and potassium, while ammonium nitrogen completely inhibits the production of the enzyme.

The effect of the source of peptone nitrogen on the produced protease by Bacillus SP.PCSIR EA-3 was interesting, and it was observed that the best source of nitrogen is peptone between other sources, followed by it there are: the extract of meat and yeast extract [12]. The results showed that corn steep liquer causes maximum production of protease and growth in Bacillus sp.CR-179. The use of corn in the culture medium decreases the cost of enzyme production [17].

In a research conducted on Ns Bacillus subtilis, it was found that organic nitrogen sources increase protease production relative to mineral nitrogen sources. From the sources of organic nitrogen, the extract of meat had the highest production [14].

Incubation time: The production of protease increases at the end of the logarithmic phase and the early phases of stagnation, but it's highest amount is in the early phase of stagnation, and production decreases with the death of the cells. Synchronization of sporulation and production of protease has been observed in

other studies [23]. Understanding cell growth period in relation to the production of crude enzymes may be an important factor for identifying the suitable incubation period for maximum productivity of enzymes. In a study on Bacillus sp CAMA 14, the highest enzyme production was obtained in 48 hours of cell growth. And after this time, due to the decrease in microbial growth that is available due to reduced resources, and the increase in the cell death, the enzyme production decreases [19]. The incubation period plays a significant role in the maximum production of enzymes. According to the reports, Bacillus subtilis, PE-11 showed maximum production of protease in 48 hours after inoculation, while 3411 Bacillus subtilis showed the maximum production in 72 hours and Bacillus sp. K-30 showed up to 96 hours. Studies on Bacillus sp PCSIR EA-3 showed that the production of protease depends on cell growth. Cells began to multiply within 6 hours of incubation and peaked at 48 hours. The cells then entered the inertia phase and then decreased. Maximum enzyme production was obtained at the end of the logarithmic phase and the beginning of the resting phase, and then the number of available organisms decreased [5]. The role of incubation time with different species of Bacillus has been studied. According to Krishnaveni et al. (2012), the highest protease production for firmus TAP5 Bacillus was observed between 60-64 hours. The researcher also found 48 hours to produce the maximum protease in RMK Bacillus subtilis [16]. Tari and Genckal reported a maximum 96-hour production of protease in Bacillus species 21 and 18 [24].

Metal ions: Microorganisms require specific mineral elements for growth and metabolism. Usually enough amounts of cobalt, copper, iron, manganese, molybdenum, and zinc in water are present as impurities in other components of the medium. For example, corn kernels contain large amounts of mineral elements that usually eliminate the need for low-energy elements. In some materials, the amount of calcium, magnesium, phosphorus, potassium, sulfur and chlorine is low for the needs of bacteria. So these materials are added to the environment in the form of pure salts. Metal ions, which bind to the active site of the enzyme, cause protein stability. For example, in neutral proteases like thermolysis, which binds to the active site by bacterial heat broth bacillusthemoplatonic 58, activates the protease enzyme stability. The presence of copper and zinc ions in this bacterium also leads to the persistence of alkaline phosphatase. In subtilisin BPN, the presence of calcium ions in the N terminal section results in the stability of protease enzyme. Other ions, such as Ba2 +, Co2 +, Hg2 + and Mn2 +, also contribute to the protease enzyme stability. Excessive ion uptake reduces the activity of alkaline proteases. The use of Mn ion in the culture medium of Bacillus stearotermophilus 59 F1 increases the activity of this bacterium. Increasing the concentration of calcium in the cytoplasmic membrane to more than 2 μmol also results in the production of spores, given that detergents use triphosphate as a softening factor. The use of proteases that require calcium is not appropriate [23] in a study on Bacillus sp MIG, the effect of various metal ions on enzyme activity was investigated. Mercury and silver ions inhibit enzyme activity by about 70-50%. Metal ions such as Mn2 +, Mg2 +, CO2 +, Ca2 + increase or sustain the activity of the enzyme, and these cations play an important role in the stability of the protease structure and enzyme protection against thermal degradation [13]. Between metal ions, Magnesium chloride increases the production of protease in NS Bacillus subtilis [14].

Inoculum amount: The amount of inoculum has a critical role in the production of protease enzyme. In the study on Bacillus subtilis, the amount of inoculum was reported as 5%. In parallel to these results, the inoculation rate for Bacillus subtilis L18 was reported as 5%. The amount of this inoculation was reported 2% in Bacillus species, according to Chauhan and Gupta (2004) reports [15]. The results on Bacillus sp.CAMA14 showed that the best inoculation rate was 2.5%. Further increasement in inoculation rate for microorganisms is not desirable due to changes in the availability of oxygen and competition for access to limited resources that ultimately reduce protease production. These findings are similar to those of Elibol et al. (2005) and Kalaiarasi and Sunitha (2009) who reported that 2.5% of the inoculum for the production of protease is desirable [14].

Aeration and stirring: The change in stirring rate affects the amount of mixing in the flask or bioreactor, as well as the availability of nutrients. The desired yield of alkaline protease for B.subtilis ATCC 14416 and B. licheniformis was reported to be 200 rpm [25-26].

The rate of aeration indirectly affects the level of insoluble oxygen in the medium. The maximum aeration rate in Bacillus tequilensis and B. proteolyticus CFR3001 and Bacillus and Bacillus circulans MTCC 7942 was reported as 100 rpm. These results indicate that oxygen supply is an important limiting factor for the growth and synthesis of protease [27].

Conclusion

Taguchi statistical method is a successful statistical method for determining the important link between the culture medium and culture factors for the production of protease enzyme. Selection of suitable sources for increasing the production of protease enzyme is one of the reasons for increasing the production of this enzyme. Due to the widespread application of proteases in the industry, such as the food industry, the industries include pharmacy, knitting, paper production, curriery (used in soaking, disinfecting, fat removing and tanning) , detergent and silver recycling in crystallography (X-ray) The need for this enzyme is rising in the world daily.

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The Effect of Impulsivity Level on Performance Indicators of Beginner Volleyball Players

Marjan Hamoun Navard [1*]
1. Master of Motor Behavior, Kharazmi University of Tehran, Iran

Abbas Bahram [2]
2. Professor of Motor Behavior, Kharazmi University of Tehran, Iran

Rasoul Yaali [3]
3. Assistant Professor of Motor Behavior, Kharazmi University of Tehran, Iran

* Corresponding author: Marjan Hamoun Navard
Email: marjan_hnd@yahoo.com

***Abstract*— Impulsivity affects cognitive and behavioral aspects. Because in volleyball, the player must decide at the moment and implement its skill, and most of the outcome (outcome) of his performance is functional of his correct decision, his impulsive level can be important. The main purpose of this research was to compare the performance indicators of beginner volleyball players with different impulsivity levels based on Bart (2004). The statistical population of this study was volleyball player girls at Karaj clubs. Among these people were selected by about 300 people. The data gathering tool was Bart's impulsivity questionnaire (2004), Duda and Nicholls Sport Questionnaire (1992) and coach checklist, which measures the indicators of error number and risk of riskaceability. In order to test the hypotheses one-way multivariate analysis of variance (MANOVA) and at a significant level of 0.05 were used with SPSS software. According to the results of this study, there is a significant difference between the high, low and moderate levels in the risk level of players, the number of errors and the sports success of volleyball players. But there was no significant difference between high, low and moderate levels for technical performance of players (1 to 10 questions of instructor checklist including fitness, receiving, pass, defense, reaction time, game intelligence, etc.). In the sports variable, players with high impulsivity players had a significant difference with players with low and moderate impulsivity levels, but there was no significant difference between moderate and low levels in sport success. In variables of risk and number of errors, low impulsivity players had a significant difference with players with high and moderate impulsivity levels, but there was no significant difference between high and moderate levels in riskaceability and error number.**

Keywords*— *impulsivity, risk level, sports success, error number, volleyball

Introduction

Sports performance is influenced by various factors, including physical, psychological and technical icons. The relationship between different characteristics with the function of athletes has long been considered by researchers long, but still finding indicators that can improve directly or indirectly the athlete's performance, it has attractive and importance for coaches. Identification of the role of physical

variables has been studied enough in various fields of sports sciences, but the study of psychological indicators affecting exercise performance is less investigated. Different sports fields depend on the nature of the field, the environment that is performed and the individual characteristics of the athlete, in a variety of ways, are influenced by the psychological characteristics of the athlete (1).

High-level performance requires that the athlete has a favorable control over its functional resources. In volleyball, due to the need for accuracy and speed, more techniques that should be implemented during the competition and the importance of decision making in the rate of success, the impact of indicators Psychologically special importance (2). One of the indicators that play an important role in decisions during the game is impulsivity. The impulsivity is typically an inability to wait, the preference for high-risk results, expresses tendency to action without measurement, lack of sensitivity to the consequences and / or inability to prevent inappropriate behaviors (3). Impulsivity is one of the determinant features that affect motor function and learning. Impulsivity is a pattern of behavior that manifests itself in various forms. Bart, for example, suggests that three classes in impulsivity include; There is movement (thoughtless action), attention (lack of focus on the task at hand) and unplanned (focus on presenting future results without account) (4, 5). All three impulsive categories introduced by Barthes can have specific functions in open sports skills such as volleyball. Besharat et al., (2014) showed that there is a positive correlation between the dimensions of impulsivity and sports success in collision sports athletes and the sports success of players can be predicted in terms of motor and cognitive impulsivity (6). Eysenck et al. (1993) characterized impulsivity with unplanned risky behaviors and rapid and hasty arrangement of the mind, and considered impulsivity in relation to risk-taking, lack of planning, and quick decision-making (7). Giustiniani et al. (2019) showed that impulsivity is an important element in the decision-making process and impulsivity is associated with risk-taking and lack of reflection between environmental stimuli and behavioral response when making decisions (8). According to the findings of Tzagarakis et al. (2013), participants with high levels of impulsivity had greater errors in their performance (9).

When deciding to perform motor skills, human information processing takes place in both overt and covert ways. In the obvious method, it means that the processing is conscious, controlled and conscious, and it is more of a chain method. In the hidden method, unconscious processing is automatic and direct and provides the possibility of parallel processing (10). These two different information processing systems are directly affected by impulsive behaviors. Given that impulsivity in conditions of arousal and activation can affect self-control, so impulsivity is one of the characteristics that can be effective for the quality of decisions and ultimately the performance of athletes. Because impulsivity affects the cognitive and behavioral aspects, and given that in volleyball, the player must decide in the moment and apply his skill, and much of the final result (consequence) of his performance depends on his correct decision, the level his impulsiveness can be significant. Impulsivity, especially in sports with a changing environment (open skill) such as volleyball, plays a key role in optimizing performance by influencing attention needs and decision making. In this regard, the difference between information processing systems and other psychological factors can play a special role. Individual differences between athletes with different skill levels can also affect the athlete's technical performance along with other factors. Research findings have shown that there is a significant relationship between impulsivity and athletic proficiency (11) and participation in competitive sports that require momentary decisions during the game probably requires an optimal level of impulsivity. Impulsivity plays a role in open and closed skills (10) and in collision and non-collision sports (12) and is one of the effective factors in determining the success of athletes (12). Play post in team sports also requires different levels of impulsivity (10). Even impulsivity at a lower level than competitive sports and in the field of physical activity has played a significant role (13).

Despite many studies on impulsivity and related bio-psychosocial variables in recent years, many questions remain unanswered. Various studies in different contexts have examined the role of impulsivity in learning and performing sports skills. For example, Lage et al. (2011) observed a significant

relationship in handball (14). The present study intends to compare the performance of people with different levels of impulsivity in another way and with a causal-comparative method to clarify the possible differences between people with different levels of impulsivity. Since no such comparison has been made in volleyball and volleyball, unlike handball, is a non-collision sport, the present study was conducted to compare the performance indices of beginner volleyball players with different levels of high, medium and low impulsivity.

Methodology

The present study has a descriptive strategy and a causal-comparative design whose data were collected by questionnaire and non-clinical method. The current statistical population is beginner female volleyball players who have attended volleyball training in Karaj clubs and have at least three years of experience in specialized volleyball training and education. About 300 people were selected from these available people and participated in the study voluntarily. After fulfilling the conditions for entering the research, the subjects were placed in one of the high, medium and low impulsivity groups based on the scores obtained from the impulsivity questionnaire, and then the questionnaires related to measuring the research variables were completed online by the players. In this study, Barratt (2004) questionnaire was used to measure the impulsivity variable (4). This questionnaire includes 30 items and three subscales including unplanned, motor impulsivity and cognitive impulsivity. Previously, Besharat et al. (2007) and Javid et al. (2012) have confirmed the validity and reliability of this tool (15). Also, to evaluate the technical performance of the players in this study, based on previous studies, forms and tests were used to evaluate the performance of the players. Accordingly, for the field of volleyball, according to previous studies (2), the desired variables were extracted and checklists were compiled and used, and based on the opinion of volleyball experts, its formal validity was confirmed. Also, the convergence validity coefficient was calculated for this questionnaire and then its reliability was calculated using Cronbach's alpha coefficient and SPSS software, which was 0.829, so considering that it is more than 0.7, the questionnaire has the necessary reliability. In addition, Duda and Nicholls self-report questionnaire, 1992, was used to measure the variable of sports success, which has 13 questions and two dimensions of task orientation (7 questions) and self-control (6 questions). The mentioned questionnaire has already been used by Shamshiri (1373) on student-athletes in Tehran and Keshtmand (1383) on student-athletes in Kermanshah and Bahrami, Yousefi (1383) on wrestling athletes in Lorestan province and has been validated and reliable (16, 17). It has also been validated using Cronbach's alpha and has been reported at 0.8%. In this study, multivariate analysis of variance (MANOVA) was used and a significance level of 0.05 was determined.

Results

According to the results of descriptive statistics and demographic characteristics of the participants in this study, most participants (about 61%) were in the age range of 15 to 20 years. More than half of the participants had more than 3 years of experience in this sport. Also, 28% of the players participating in the passer activity and most of them (37%) were 161 to 170 cm tall. 31% of them weighed between 61 and 70 kg.

For cognitive impulsivity, most participants chose the option most often (52%). For motor impulsivity, the highest frequency was sometimes related to the option (78%). For non-planning, the option was often

the most frequent (80%). Impulsivity level of most participants (92%) was moderate, 5% had low impulsivity level and only 3% of participants had high impulsivity level.

Table 1 shows the descriptive characteristics of the research variables.

Table1. Descriptive properties of variables

Variables	Impulsivity level	Mean	Standard deviation	Number
Sports success	Down	3.93	.258	15
	medium	3.91	.557	277
	Top	4.50	.535	8
	Total	3.92	.552	300
Number of technical errors	Down	3.13	1.125	15
	medium	2.76	1.117	277
	Top	2.25	1.282	8
	Total	2.77	1.124	300
Risk level	Down	3.00	.378	15
	medium	2.56	.687	277
	Top	2.50	.756	8
	Total	2.58	.682	300
Technical performance of players	Down	3.20	.775	15
	medium	3.26	.792	277
	Top	3.38	.744	8
	Total	3.26	.788	300

To investigate the hypothesis of homogeneity of the covariance matrix, the Mbox test was used and the results of this test are presented in Table 2. The null hypothesis in this test is the homogeneity of the covariance matrix of the dependent variables. According to the obtained result (p-value> 0.05), the hypothesis of zero confirmation and homogeneity of the covariance matrix of dependent variables is confirmed.

Table2. Mbox test results

Box's M	28.513
F	1.188
df1	20
df2	1504.028
Sig.	0.256

Table 3 shows the results of the Leven test to examine the equality of variances in different groups:

Table3. levene test results for equality of variances

Variables	levene	df1	df2	p-value
Sports success	2.906	2	297	.056
Number of technical errors	.295	2	297	.745
Risk level	3.707	2	288.526	.226

Technical performance of players	.040	2	297	.961

Given that the p-value is greater than 0.05, so the assumption of equality of variances is confirmed.

Table 4 presents the results of multivariate analysis of variance (MANOVA) statistical test:

Table4. Results of multivariate analysis of variance on the mean scores of the studied variables

Variable	the amount	F	Hypothesis df	Error df	p-value	Eta Squared
Impulsivity	.945	2.094[b]	8.000	588.000	.035	.028

As can be seen in Table 4, in the second row, the result of the Wilkes lambda test is shown. In this table, the p-value is equal to 0.035, which is less than the alpha value (0.05) and is therefore significant. In other words, the effect of impulsivity level group on the linear composition of dependent variables is significant (p-value <0.05). This test allows the use of multivariate analysis of variance and shows that there is a significant difference between at least one of the variables in players with high, medium and low level of impulsivity. The square of Eta shows that the difference between the three groups with respect to the dependent variables is significant in total and the amount of this difference is approximately 28%.

Table5. Results of multivariate analysis of variance on the scores of dependent variables in players with high, low and medium impulsivity

Variables	df	Mean Square	F	p-value	Eta Squared
Sports success	2	1.372	4.604	.011	.030
Number of technical errors	2	2.079	1.654	.033	.011
Risk level	2	1.427	3.108	.046	.020
Technical performance of players	2	.080	.128	.880	.001

According to Table 5, a value of 0.88 for p-value and F = 0.128 shows that the level of impulsivity has no significant effect on the dependent variable of technical performance (p-value> 0.05). Therefore, the results of the first hypothesis do not confirm and there is no significant difference between the technical performance of beginner volleyball players with high, medium and low level of impulsivity. There is a significant difference between the risk level of players with high, medium and low level of impulsivity, in other words, the level of impulsivity has a significant effect on the dependent variable of risk level (p-value <0.05) where F = 3.108, p-value = 0.046 and the effect size is 0.02, so the null hypothesis is rejected and the second hypothesis of the research is confirmed. There is a significant difference between the number of technical errors of players with high, medium and low level of impulsivity, in other words, the level of impulsivity has a significant effect on the dependent variable number of technical errors of players (p-value <0.05) where F = 1.654, P-value = 0.033 and the effect size is 0.02, so the null hypothesis is rejected and the third hypothesis of the research is confirmed. There is a significant difference between the sports success of players with high, medium and low level of impulsivity, in other words, the level of impulsivity has a significant effect on the dependent variable of sports success of players (p-value <0.05) where F = 4.604 and p -value = 0.011 and the effect size is 0.03, so the null hypothesis is rejected and the fourth hypothesis of the research is confirmed.

Discussion and conclusion

The aim of this study was to compare the performance indices of beginner volleyball players with different levels of impulsivity based on Bart's theory. In this study, impulsivity as a behavioral model based on Barratt (2004) model in three levels of movement (thoughtless action), attention (lack of focus on the current task), and lack of planning or unplanned (focus on presentation without Account of future results) was examined (4). In general, the results of this study showed that there is a significant difference between the participating players with different levels of impulsivity (high, low and medium) in the variables of sport success, number of errors and risk. The difference was that in the sports success variable, players with high levels of impulsivity had a significant difference with players with low and medium levels of impulsivity, but there was no significant difference between average and low levels of sports success and they acted the same. In the variables of risk level and number of errors, players with low level of impulsivity had a significant difference with players with high and medium levels of impulsivity, but between high and medium levels in the amount of risk and number of errors were not observed and acted the same. The results of this study indicate that impulsivity affects the cognitive and behavioral aspects, therefore, given that in volleyball, the player must decide at the moment and apply his skill, and most of the final result (consequence) of his performance. It depends on his correct decision, his level of impulsivity can be important.

The following are the results of rejecting or confirming each of the research hypotheses:

Hypothesis 1: There is no significant difference between the technical performance of beginner volleyball players with high, medium and low level of impulsivity:

The results of rejecting the main hypothesis of the research are not in line with the findings of Ghahramani et al. (2015), because they showed that football players in different positions, goalkeeper, defender, midfielder and striker have different levels of impulsivity (10). This can be the result of years of practice based on post-game requirements and the need for cognitive processing and decision-making in various positions. According to Table 4, there is a significant difference between the risk level of players with high, medium and low level of impulsivity.

Hypothesis 2: There is no significant difference between the risk level of beginner volleyball players with high, medium and low level of impulsivity during the game:

The results of confirming the second hypothesis are consistent with the research of Giustiniani et al., (2019) they also showed that there is a strong and negative relationship between impulsivity and risk-taking (8). Also, according to Eysenck et al. (1993), impulsivity is characterized by unplanned risky behaviors and rapid and hasty arrangement of the mind and impulsivity is considered in relation to risk-taking, lack of planning and quick decision-making (7).

Hypothesis 3: There is no significant difference between the number of technical errors of beginner volleyball players with high, medium and low level of impulsivity during the game:

The results showed that there is a significant difference between the number of technical errors of players with high, medium and low level of impulsivity. The results of confirming this hypothesis are in line with the findings of the research of Lage et al. (2011). They showed that there is a significant relationship between impulsivity test scores and mistakes made, mistakes made and offensive mistakes in handball (14). The difference in the variables of risk level and number of errors in different levels of impulsivity can be explained by the fact that when accuracy and planning in performing movements and paying attention to sensitive situations, can reduce risk and error rate in the game. Players with high impulsivity will lack performance techniques in competitive back-and-forth positions, which in most cases require high precision with limb coordination and agility and a strong focus on movement patterns. , Because they need to think and react quickly and accurately, so reducing the level of impulsivity increases the

amount of error and risk. Chamberlain and Sahakian (2007) also showed that impulsivity is characterized by a failure to control an impulse that is potentially dangerous to the individual or those around her.

Hypothesis 4: There is no significant difference between the sports success of beginner volleyball players with high, medium and low level of impulsivity:

According to Table 4, there is a significant difference between the sports success of players with high, medium and low level of impulsivity, in other words, the level of impulsivity has a significant effect on the dependent variable of sports success of players. The results of this finding are in line with the findings of Reeve (2007) and Besharat et al. (2014). Reeve showed that impulsivity had a significant relationship with participants' gender, SAT, tested items, success items, and not with race, age, and accuracy (18). According to the findings of Besharat et al., In athletes of collision disciplines, impulsivity and in athletes of non-collision disciplines, self-regulation is one of the determinants of sports success. Peltola (1992) also showed that by identifying the characteristics of athletes of different levels, the ground is prepared for guiding and growing athletes towards peak performance. The difference in the variable of sporting success at different levels can be explained by the fact that the time limit for organizing the performance and skills of players in the successful implementation of different positions in volleyball requires timely decisions and quick reactions. Increasing this skill will increase the success of the player's sport. Also, considering the effect of the level of impulsivity on the success of players, it can be said that in addition to physical and tactical abilities and specialized skills, abilities and personality traits are undoubtedly one of the factors affecting the success and development of sports. It is suggested that in addition to the talent identification stage, the level of impulsivity, as one of the indicators of players' performance, be periodically evaluated to determine the current status of athletes. In fact, by identifying players at higher levels of impulsivity, the ground for their growth and guidance is provided and the loss of capital and frustration of athletes is prevented.

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The Effect of Water Exercise on Pain and Disability in Patients with Non-Specific Chronic Back Pain and Its Relationship with Changes in Inflammatory Factors

Mohammad Ali Kohanpour [1]
1. Department of Physical Education and Sports Sciences of Zand Shiraz University, Iran

Saeideh Najd [2*]
2. Senior expert in corrective movements, Ferdowsi University of Mashhad, Iran

Monireh Khademi [3]
3. Senior expert in sports pathology and corrective movements, Binaloud Institute of Higher Education, Mashhad,Iran

Sirous Farsi [4*]
4. Department of Physical Education and Sports Sciences, Islamic Azad University, Marvdasht Branch, Iran

* Corresponding author: Sirous Farsi
Email: sirous.farsi@gmail.com

***Abstract*— the aim of this study was to investigate the effect of exercise in water on pain and the disability of patients with non-specific chronic back pain and its relationship with changes in inflammatory factors. The statistical sample of the research included 20 women suffering from non-specific chronic back pain purposefully selected and randomly assigned to three groups: exercise in water and control (10 people in each group). The exercise group participated in a course of 8-week program, while the control group received no intervention and had their normal routine activities. 48 hours before and 48 hours after the intervention, pain and disability and serum levels of CRP and TNF-α were measured. In order to examine and compare the changes of the variables, mixed analysis of variance was used. The results showed that pain and disability as well as serum levels of CRP and TNF-α in the training group were significantly reduced compared to the control group (P<0.05). Also, the results indicated that there was a positive significant relationship between all the variables (P<0.05). It seems that eight weeks of training in water leads to improvement of pain and disability in patients with non-specific chronic back pain. In the meantime, the reduction of inflammation caused by these exercises probably plays a role. However, we need more studies in this field.**

Keywords*— *Non-specific chronic back pain, inflammation, pain, exercise in water, CRP

Introduction

Work-related low back pain is the most common debilitating musculoskeletal injury in the world and can negatively affect working quality. Most of the medical costs are related to patients with chronic pain. This situation ultimately leads to reduced productivity and increased sick leave. Therefore, its economic dimension has drawn the attention of managers. So these people should quickly recover and return to work to prevent further economic loss (1-4). Low back pain is more common in women than men (5) and is one of the most common reasons for absences from work and using health insurance and health services.

According to the research, 70 to 85 percent of people have experienced back pain during their lives, and 80% of them have reported recurrence (6, 7). Because of the complexity of the mechanism of this type of pain, there is no proven method of treatment (8). Recent studies have shown that flexibility, strength, and endurance exercises, referred to as conventional methods of exercise therapy, are used to reduce pain and improve function in patients with chronic low back pain (9-11). The main causes of back pain are not clear yet but it seems that in most cases, back pain is caused by muscle weakness and awkward posture of the body (12). Research has shown changes in the degree of lumbar lordosis, abdominal muscle weakness, posterior lumbar muscle weakness, and loss of muscle endurance of thigh are the main factor contributing to low back pain (13, 14). The decrease in trunk muscle endurance is one of the most common findings in patients with back pain. Trunk flexor-extensor muscles are among postural muscles of body which act against gravity to keep the body in an upright position and control it when bending (15, 16).

According to many researchers, decreased endurance of these muscles leads to early fatigue, increased pressure, force on passive tissues of lumbar spine, damage to these tissues, and finally the incidence of low back pain (15-17). Similarly, the results of EMG tests prove increased muscle fatigue in people with low back pain compared to healthy individuals (18, 19). Farahpour e. al (2005) examined muscle endurance in patients with chronic low back pain and the changes in different modes of therapy and showed that patients with low back pain have significantly less strong trunk flexor-extensor muscles in the pre-test compared to normal individuals (17). Thinness and atrophy of trunk muscles in patients with low back pain compared to healthy individuals could be among the causes of lower muscular endurance in these people. Hides et al. (1994) showed in their study that in patients with low back pain, multifidus muscle cross-sectional area is 31% lower than in healthy individuals (20). Verbunt et al. (2003) also state that back muscles as postural and body maintenance muscles contract more than other muscles and are more prone to atrophy and weakness (21). So, it can be a cause of reduced endurance and fatigue of the muscles and the subsequent incidence of back pain and disability in such patients. Therefore, it can be helpful to enhance muscle endurance and delay the onset of fatigue. So far, several methods for the treatment of patients with chronic low back pain have been considered including using pain medication, muscle relaxants, therapeutic yoga, stretching and flexibility exercises, stability exercises, massage therapy, and therapeutic exercise (13, 22, 25). Exercise therapy is a common practice in the treatment of patients with chronic low back pain (24, 26, 27). Hayden et al have found exercise therapy effective in the treatment of back pain (10). The importance of exercise in patients with low back pain is so considerable that different sport programs have been offered by researchers (10). Overall, the results show exercise therapy can reduce pain, increase muscle endurance, and improve the performance of patients with low back pain (28). By reviewing the literature of the studies on low back pain, it seems that in most studies, common exercises that can affect back pain are separately studied. Few studies have focused on a combination of several methods. Physiotherapy exercises have always been used for treatment.

Various studies show that there is a possibility of increasing inflammatory indices in people with back pain. C-reactive protein (CRP) is among the indicators that have been mentioned to be increased in people with back pain in some studies. Various findings indicate that IL-6, which is considered to be one of the factors that increase CRP, increases significantly in people with back pain (29). On the other hand, due to the effectiveness of IL-6 on CRP, this reactive protein also increases significantly and leads to inflammation in the lower back. It seems that CRP, which increases the activity of prostanoids and leukotrienes, leads to inflammation, followed by pain, and part of the pain in the lower back in people with lower back pain is caused by inflammation (29, 30). Sturmer et al. (2005) also found CRP effective in inflammation and sciatica pains (31). Considering the role of physical activity in reducing inflammatory factors, it seems that the use of sports activities is effective in this field (10). Various studies have reported a significant decrease in CRP following physical activities (32). However, Nickolas et al. (2004) investigated the effect of 18 months of combined resistance and aerobic training in elderly obese men and women with symptoms of

knee osteoarthritis and reported that the level of inflammatory markers including CRP did not change significantly (33). On the other hand, exercise in water is one of the therapeutic methods that is used in all therapeutic fields recently. Water pressure on the body, joints, muscles and internal organs during exercise relaxes the whole body like a good massage and prevents bruises. Also, due to the reduction of pressure inside the disc, the size of the intervertebral space may increase, which often allows patients with back problems to feel less discomfort during exercise (34). In the use of water therapy, no pressure is applied to the damaged tissue and the person continues to exercise while he is really far from the injury. One of the major benefits of water treatment is reducing the forces related to weight bearing (34).

Considering the conflicting results and since no study has investigated the effect of exercise in water on pain and disability related to inflammation in patients with non-specific chronic low back pain, the aim of this research was to determine the effect of exercise in water on pain and the disability of patients with non-specific chronic back pain and its relationship with changes in inflammatory factors.

Methodology

The current research was a clinical trial type.

The statistical sample of the research included 20 women suffering from non-specific chronic back pain purposefully selected and randomly assigned to two groups: exercise in water and control (10 people in each group).

After the aim of the research and all the steps were described in details for the participants, all of them volunteered and completed a written consent form. Criteria for inclusion of patients in the study included being female, suffering from chronic non-specific low back pain, having low back pain of at least 3 months, a reduction in trunk flexor-extensor muscle endurance, and being aged between 20 and 45 years. Exclusion criteria included having acute and subacute low back pain, being pregnant, having a history of diseases, injuries and disorders affecting the underlying back pain such as a herniated disc, arthritis, sciatica, narrowing of vertebral canal, osteoporosis, the existence of bone Spurs in lumbar spine, hip and vertebral fractures, previous surgery, tumor, infection, ponytail syndrome, lumbar, scoliosis, flat back, kyphosis and history of any medication or health measures to remove low back pain. In addition, all the subjects were examined by a specialist, their MRI images were studied, and based on the criteria of inclusion they were homogenized.

The exercises were done for 8 weeks and 3 sessions per week in the swimming pool with a temperature of 29±1 and an ambient temperature of 32 degrees Celsius. An orientation session was held to familiarize patients with the pool environment, exercises, hydrotherapy tools, training and emphasis on proper posture and the use of deep muscles during exercises. In this session, the patient was introduced to the RPE 6 to 20 assessment method to evaluate the intensity of aerobic exercise more accurately. Each training session was 55-60 minutes and was done under the direct supervision of the hydrotherapist. Each training session consisted of 10 minutes of warm-up, 15-20 minutes of strength training, 20-25 minutes of endurance training, 5 minutes of balance training, and 10 minutes of cooling down (stretching exercises) (35).

48 hours before and 48 hours after the intervention, the level of pain (Quebec Standard Pain Questionnaire) and disability (Oswestry Disability Questionnaire) and were measured. Also, a blood sample was collected to measure CRP and TNF levels in a state of 12 hours of fasting. TNF-α levels were measured with a kit from Diaclone, France, with a sensitivity of 8pg/ml. C-reactive protein (CRP) levels were obtained by ELISA method using a commercial ELISA kit from Ontario Canada Company with a sensitivity of 10ng/ml. Quebec pain questionnaire consists of 25 five-item questions (minimum 0 and maximum 4) which scores

the pain perceived while doing daily routines between 0 and 100. Scoring 0 implies complete health, 1-25 mild pain, 26-50 moderate pain, 51-75 strong pain, and 75-100 severe pain causing trouble for the patient (36). Oswestry questionnaire measures functional ability of patients by ten 6-option sections in the field of tolerance and coping with pain, personal care, lifting objects, walking, sitting, standing, sleeping, social life, travel, and change in the degree of pain. At worst disability conditions, score 5 is given to each section and the total score of the ten sections is 50. Total disability is obtained by multiplying the score in each section by 2. In fact, the range of scores is between 0 and 100. Thus, a score of zero indicates perfect health and a pain-free functionality, 1-25 mild disability, 26-50 moderate disability, 51-75 high disability, and 75-100 severe disability causing trouble for the patient (37). It should be noted that in this study, samples were selected among those who scored over 25 for pain and disability. Previous studies have approved and confirmed the validity and reliability of Quebec and Oswestry questionnaires to assess pain and disability in daily activities and the reliability of them has been reported 84% (38).

To analyze the data, descriptive and inferential statistics were used. In descriptive statistics, mean and standard deviation were used. In inferential statistics, In order to investigate and compare the changes of the variables, the statistical method of mixed variance analysis was used. The significant level was considered $P \leq 0.05$ and the SPSS software (version 16) was used to perform statistical operations.

Results

The results of mixed analysis of variance are reported in Table 1. The results of the Pearson correlation coefficient test are also presented in Table 2. The results showed that pain and disability as well as serum levels of CRP and TNF-α in the training group were significantly reduced compared to the control group ($P<0.05$). Also, the results indicated that there was a positive significant relationship between all the variables ($P<0.05$).

Table1. Results of mixed variance analysis

Variables	Groups	Before	After	F	P	Effect size
Pain	Training	39.40 ± 11.68	28.80 ± 11.26	8.91	0.008 *	0.33
	Control	46.90 ± 12.52	44.40 ± 11.57			
Disability	Training	54.30 ± 9.25	42.60 ± 10.78	10.19	0.005 *	0.36
	Control	52.70 ± 7.37	49.90 ± 8.37			
CRP (ng/ml)	Training	1879.60 ± 376.90	1390.30 ± 298.93	9.34	0.007 *	0.34
	Control	2139.20 ± 450.16	2094.70 ± 501.30			
TNF-α (pg/ml)	Training	11.21 ± 1.65	8.98 ± 1.45	9.21	0.007 *	0.33
	Control	9.99 ± 1.19	10.15 ± 1.07			

*significant at the level of $P \leq 0.05$

Table2. Pearson correlation coefficient test results

Correlation matrix	Pain	Disability	CRP	TNF-α
Pain	-	r= 0.88 p= 0.001 *	r= 0.55 p= 0.011 *	r= 0.56 p= 0.010 *

Disability	r= 0.88 p= 0.001 *	-	r= 0.59 p= 0.006 *	r= 0.63 p= 0.003 *
CRP	r= 0.55 p= 0.011 *	r= 0.59 p= 0.006 *	-	r= 0.44 p= 0.047 *
TNF-α	r= 0.56 p= 0.010 *	r= 0.63 p= 0.003 *	r= 0.44 p= 0.047 *	-

*significant at the level of P≤0.05

Discussion

Based on the findings of the present study, eight weeks of training in water led to a significant reduction in pain and disability, as well as the serum levels of CRP and TNF. Also, there was a significant positive relationship between changes in pain and disability with changes in inflammatory factors, so that with the reduction of these inflammatory factors, the pain and disability of patients with non-specific chronic back pain also decreased significantly.

Considering the impact of exercise on reducing pain and disability, the results of the present study are in line with the results of Barr (39). Previous research has showed that patients with chronic low back pain have weakness and atrophy of deep and central muscles, especially abdominal and multi-headed lumbar muscles (40). Also, it is aid that in patients with chronic low back pain, muscle dysfunction may be due to deformation and change of one of neuromuscular control mechanisms affecting trunk stability and efficiency in motion. Exercise may reduce pain and increase performance by increasing strength, endurance, flexibility, coordination, static and dynamic stability, neuromuscular control, motor control, correcting movement patterns and relaxation of muscles (39, 41).

Motealeh (2005) stated that endurance and coordination exercises and a combination of them improve pain and disability in patients with chronic low back pain and believes using a combination of endurance and coordination exercises is more effective in reducing pain and disability (42). Farahpour et al (2005) also pointed out that a 12-week physical therapy decreased the pain and disability of patients with chronic low back pain with the weakness of the flexor-extensor muscles of the trunk (17). However, the research done by Grifka (2006) reported contrasting results (43). Rainville et al (2004) pointed out that there is no evidence that exercise therapy increases back pain or disability of patients and suggested that therapeutic exercise and endurance activities reduce the risk of injuries and lower back pain. They believe these exercises can be used for treating patients as they increase the flexibility of muscles, improve their performance, and reduce pain (24). Differences in different findings are probably due to differences in training protocols.

Also, the reduction of inflammatory factors due to exercise is consistent with the results of previous studies (33, 44). CRP is a general measure of body inflammation and can be caused by various reasons. The possible important role of CRP such as binding to the phospholipids of damaged cells to activate some compounds and increasing the uptake of these cells by macrophages, activating endothelial cells to grow molecules, reducing the expression and biological availability of endothelial oxidase synthase And the reduction of endothelial expression of nitric oxide compounds is considered for it (45). Various findings show that IL-6 is one of the factors that increase CRP and its levels increase significantly in people with back pain (29). On the other hand, due to the effectiveness of IL-6 on CRP, this reactive protein also increases significantly and leads to inflammation in the lower back area (30). It seems that CRP leads to inflammation, followed by pain, by increasing the activity of prostanoid and leukotrienes, and includes part of the pains in the lower back in people with lower back pain (29, 30).

From different studies, it can be seen that sports activity leads to the reduction of CRP values through

different mechanisms. Fat tissue values, body mass index and ratio of waist circumference to hip circumference are among the effective indicators in the secretion of inflammatory indicators. Many researchers believe that an increase in the amount of body fat reserves causes an increase in serum CRP, so there is a possibility that sports activity is effective in reducing CRP levels through the reduction of fat reserves (46). However, some researchers have also reported that sports activity without significant reduction of body fat had a significant effect on CRP values and its reduction (47). On the other hand, the effect of inflammatory cytokines such as TNF-α on CRP also seems to be important. TNF-α is among the cytokines that are released from fat tissue and other tissues and leads to the increase of IL-6. However, it has been found that exercise has an effective role in reducing TNF-α levels and increasing IL-6. Therefore, changing the amount of inflammatory cytokines due to sports activity is also effective in reducing CRP (48).

Conclusion

It seems that eight weeks of training in water leads to improvement of pain and disability in patients with non-specific chronic back pain. In the meantime, the reduction of inflammation caused by these exercises probably plays a role. However, we need more studies in this field.

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