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Review Article

Flow Cytometry: A versatile and Powerful Tool for Drug Discovery and Development

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Abstract

Flow cytometry, a pivotal tool in clinical and research labs since the discovery of cell markers in the mid-1970s, plays a crucial role across all phases of drug discovery. Modern flow cytometers can detect rare cell types relevant to disease pathogenesis, measure numerous parameters simultaneously, thus, offer versatility in drug screening. In drug discovery studies, flow cytometry contributes to the assessment of drug pharmacokinetics, pharmacodynamics and safety in animal models and clinical trials. It can also be used to monitor drug efficacy and identify biomarkers for diagnosis and prognosis.

In essence, flow cytometry is a versatile, instrumental technique that supports drug discovery from target identification through to clinical development, limited only by the creativity of the researcher and the availability of fluorescent labels or specific size/scatter related findings. This review article focuses on the use of flow cytometry in drug discovery and drug development studies, summarizing not only conventional assays such as immunophenotyping, measurement of programmed cell death pathways and cell division to provide insights into drug effects and patient responses, but also novel approaches including mass cytometry, spectral cytometry, and droplet cytometry.

Keywords: Flow cytometry, drug discovery, drug development, mass cytometry, spectral cytometry

1. Introduction

Flow cytometry has been an indispensable tool for clinical and research laboratories for many decades now. After the discovery of cell markers in mid-1970's, use of flow cytometry has become an important part for all phases of drug discovery. It is known that only approximately 0.1% of new drug candidates can survive from preclinical research to marketing approval stage (1). The need for better assays and tools for evaluation and characterization of these drug candidates is still present and flow cytometry is one of the essential instruments in drug screening studies. Flow cytometry can be applied to various stages and aspects of drug discovery and development such as target identification, screening, mechanisms of action, biomarker discovery, toxicity testing and clinical trials (2–5).

It has some advantages over other methods by being multiparameter, high throughput availability and single cell analysis.

Modern flow cytometers can measure from 10 to >40 colors, providing the ability to measure many parameters simultaneously on each cell. Using bead technologies, cytometers can also measure soluble proteins such as cytokines, chemokines, enzymes, and all types of proteins in soluble format (6). Recent advances in imaging flow systems, mass spectrometry cytometers, spectral cytometers and the development of high throughput screening cytometry systems have increased the capabilities of single cell profiling for systems level approaches to drug discovery.

Target identification is the first step in the drug discovery process, where a disease is elucidated and potential targets for intervention are selected. Flow cytometry allows the analysis of cell populations and the detection of rare cell types that may be involved in disease pathogenesis. Examples of the use of flow cytometry include the identification and isolation of specific cells such as stem cells, cancer cells, different subtypes of immune cells that express specific markers or receptors that can be targeted by drugs. Cytometry systems can be used to screen libraries of small molecules or antibodies for their binding affinity and specificity to the target cells, using fluorescent tags or labels to identify novel ligands or modulators of the target molecules.

Target validation is the next step in the drug discovery process where the selected targets are confirmed to be relevant and essential for the disease. Flow cytometry is a useful tool for the functional characterization of the target molecules and their role in the disease mechanism. It can be used to measure the activation, signaling, proliferation, differentiation, various cell death modalities such as apoptosis, or migration of target cells in response to modulation of the target molecules by drugs or other agents (7). Cytometry can also be used to assess the effects of gene knockdown or knockout of target molecules on target cells using techniques such as RNA interference or CRISPR-Cas9. Once a target is identified and validated, the drug candidates must be optimized to achieve the desired outcome with high specificity and low toxicity. At this stage, cytometry can be used for optimization of the drug properties and the selection of best candidates for further development. With various assays, potency, efficacy, selectivity, and kinetics of the drug candidates can be determined by cytometry.

Flow cytometry can be used to assess the pharmacokinetics, pharmacodynamics, and safety of drug candidates in animal models for biodistribution, pharmacological imaging and toxicity testing. It can also be used in the clinical development phase of drugs being tested for efficacy and safety in human trials. Monitoring the effects of drugs and identifying biomarkers for diagnosis and prognosis are important areas of application for

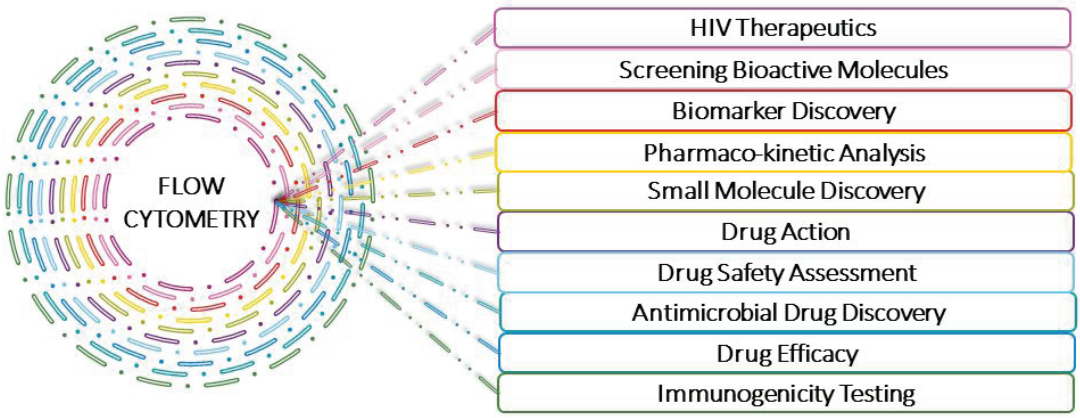

Figure 1. Use of flow cytometry at different stages of drug discovery and development

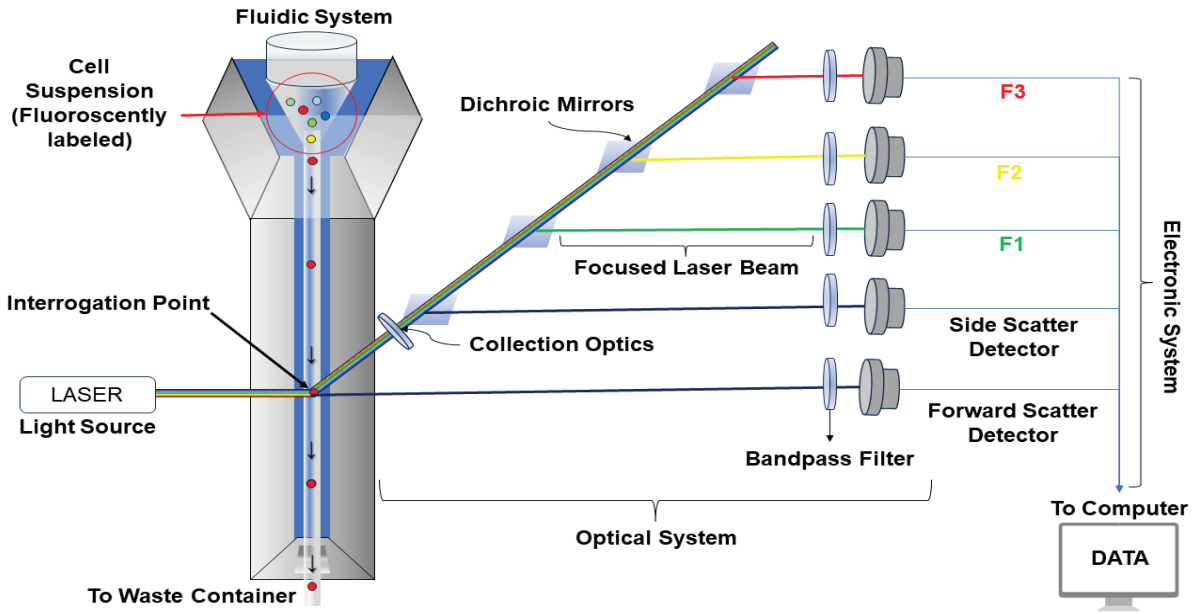

Figure 2. Schematic drawing of conventional flow cytometry (10,11). Illustration is adapted by Furkan AYDIN.

flow cytometry. It can be used to measure changes in target cells or other cells in blood or body fluids by measuring parameters such as blood cell count, immunophenotype, function, DNA cell content and response to drugs.

Hence, flow cytometry is a versatile and powerful tool that supports the drug discovery process from target identification through to clinical development. The parameters measured are only limited by the imagination of the researcher or clinician if there is a fluorescent label or size/scatter related specific finding about the event being evaluated (**Fig 1**). It is an essential technique in the discovery and development of new drugs for various diseases.

It should be noted that flow cytometry is used in different analytical areas, including research, preclinical applications, and routine methods provided as a medical laboratory service, all of which should follow standardization and validation procedures (8). However, method validation in flow cytometry is challenging due to the complexity of the technology and is expected to become even more complex in the future as high-parameter research methods such as clustering become routine applications (9).

2. Flow cytometry methods in drug discovery

2.1. Immunophenotyping

Immunophenotyping is an important technique in immunology that characterizes and identifies cell populations based on the expression of surface markers or antigens. This method uses monoclonal antibodies specific for certain cell surface proteins, allowing the discrimination and classification of different cell types within a sample. Immunophenotyping plays a fundamental role in understanding the complexity of the immune system, aiding in the diagnosis of disease, monitoring treatment response and elucidating immune-related disorders (12). This technique has been extensively used in flow cytometry, allowing the simultaneous analysis of multiple markers on individual cells, providing detailed insights into the composition and functionality of immune cells (13). In addition, recent advances in high-dimensional technologies, such as mass cytometry, have further improved the precision and depth of immunophenotyping analyses, facilitating comprehensive immune profiling and advancing our understanding of immune system dynamics (14–16).

In drug discovery studies, immunophenotyping is often used to assess the impact of potential therapeutic agents on the immune system and to identify drug candidates that modulate immune

cell responses (14,17). In addition, the combination of immunophenotyping with advanced high-dimensional approaches such as RNA sequencing has the potential to revolutionize the drug discovery landscape by providing unparalleled insights into the intricate workings of the immune system and its modulation by pharmacological agents (18,19). Single-cell resolution analyses have revealed heterogeneity within immune cell populations, shedding light on previously unexplored cellular subsets and their differential responses to therapeutic interventions (20).

2.2. Evaluation of proliferation

The measurement of cell proliferation by flow cytometry encompasses a range of methods designed to comprehensively analyze and quantify cell division dynamics within heterogeneous cell populations (21–23). Furthermore, the incorporation of multiple fluorochromes into flow cytometry assays allows the simultaneous assessment of proliferation markers alongside other cellular characteristics, providing a multi-faceted understanding of cell behavior (24–26).

A widely used technique involves the incorporation of nucleotide analogues such as bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) during the DNA synthesis phase of the cell cycle (27,28). In this method, cells are incubated with these analogs, which are incorporated into newly synthesized DNA. After incubation, the cells are fixed, permeabilized and exposed to specific antibodies conjugated to fluorescent markers, allowing the identification and quantification of cells actively synthesizing DNA. This BrdU/EdU incorporation assay is critical in determining the proportion of cells in S phase, providing a snapshot of the actively dividing cell population within a sample.

Another widely used technique for assessing cell proliferation by flow cytometry is DNA content analysis, which relies on staining cellular DNA with fluorescent dyes such as propidium iodide (PI) or Hoechst (21). After fixation, permeabilization and nuclear staining, different phases of the cell cycle are distinguished according to their DNA content, allowing researchers to study the distribution of the cell cycle between the G0/G1, S and G2/M phases, providing crucial information about cell cycle distribution and proliferation rates within a population.

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye widely used in biomedical applications to track and quantify cell division (29). Upon cellular uptake, CFSE is cleaved into its fluorescent form by intracellular esterases, resulting in its covalent binding to intracellular molecules (30). The fluorescence of CFSE halves with each cell division, allowing the precise determination of the number of divisions a population of cells has undergone. The dye's ability to stain different cell types without affecting cell viability has made it a valuable tool in the study of lymphocyte proliferation, immune responses, and cell kinetics. Today, CFSE-based assays in flow cytometry are widely used to characterize the dynamics of cell proliferation and differentiation in both in vitro and in vivo experimental settings. There are also other dyes on the market such as CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific), Tag-it Violet™ Proliferation and Cell Tracking Dye (BioLegend Inc.) and Violet Proliferation Dye 450 (VPD450) (Beckton Dickinson Biosciences), all of which work on a similar principle to CFSE staining. Besides, cell proliferation can also be assessed using flow cytometry by intracellular staining to evaluate proliferation markers such as Ki-67 or proliferating cell nuclear antigen (PCNA), which will be discussed in more detail (31,32).

2.3. Toxicity measurements

Over the past decade, the mechanisms underlying cell death, which include a wide range of signaling cascades that regulate the initiation, execution and post-mortem disposal of cells have become a focus of interest for many researchers in drug discovery studies (33). Here, we aimed to summarize the flow cytometric applications aiming to measure different modes of programmed cell death pathways. It should also be noted that in addition to programmed cell death pathways, cell death and viability can also be measured with flow cytometry by labelling dead cells with fluorescent dyes that are excluded from viable cells due to membrane integrity such as 7-Aminoactinomycin (7-AAD), ethidium homodimer-III or 4′,6-diamidino-2-phenylindole (DAPI); these intercalating dyes can also be combined with cell-permeable viability indicators

to differentiate between healthy and dead cell populations, although these stains do not provide information on the mode of cell death.

2.3.1. Apoptosis

Relying on the simultaneous measurement of membrane permeability and morphological changes observed during the course of apoptosis, Annexin V/Propidium Iodide (PI) staining is a widely used method in flow cytometry for evaluating this pathway (34,35). Briefly, this method is based on the principle that during apoptosis, phosphatidylserines located at the inner leaflet of the plasma membrane are translocated to the outer leaflet, where they can be detected by Annexin V. At the same time, PI is used to stain necrotic cells with increased membrane permeability, allowing to distinguish between apoptotic and necrotic cells. Since PI is excluded from live or early apoptotic cells due to the presence of an intact plasma membrane, but can enter late apoptotic and necrotic cells due to the loss of membrane integrity, Annexin V/PI staining can detect early apoptotic, late apoptotic, necrotic along with viable cells (36). Currently, there are several protocols available for Annexin V/PI staining in flow cytometry to detect apoptosis. The most common procedure involves staining the cells with Annexin V and PI, followed by flow cytometry analysis to differentiate between healthy, apoptotic, and necrotic cells (37,38). A modified version of the protocol involves RNase treatment steps to limit the number of false-positive staining events (36). When running Annexin V/PI analysis, it should be kept in mind that the presence of calcium in the cell suspension is critical for Annexin V binding, thus, avoiding buffers containing calcium chelators and maintaining the calcium concentration is important to eliminate the risk of false results; in addition, ideal calcium concentration may be determined for different cell types for optimum results (39)

Activation of endonucleases that specifically cleave chromosomal DNA at internucleosomal regions is a hallmark of apoptosis. Based on fluorochrome-labeling of 3′-OH termini of DNA strand breaks *in situ* with exogenous terminal deoxynucleotidyl transferase (TdT) is named as Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay, and considered as the gold standard for detecting apoptosis (40,41). Moreover, by combining a DNA stains such as PI or DAPI followed by multi-parametric analysis of cells by flow cytometry enables evaluating the correlation between apoptosis and cell division (40).

Additionally, apoptosis can also be assessed by measuring intracellular proteins known to be involved in the apoptotic process: this can be achieved either by measuring active forms of the proteins (42,43), evaluating caspase activity with cell-permeable fluorogenic caspase substrates (44), or fluorochrome-linked caspase inhibitors (45). These approaches can be combined with Annexin V/PI staining or TUNEL assay to provide a better insight into the apoptotic process.

2.3.2. Autophagy

Autophagy is an evolutionarily conserved pathway for clearing cytosolic aggregated proteins, damaged organelles, invading microorganisms as well as maintaining metabolic balance (46). Modulation of autophagy has been shown to hold great potential in the treatment of various diseases, thus is receiving interest in drug discovery studies (46,47).

Being located in the autophagosomes of cells undergoing the autophagic process, microtubule-associated protein 1A/1B-light chain 3 (LC3-II) is the most prominent biological marker for autophagy detection. Currently there are various studies reporting flow cytometry based LC3-II measurements to evaluate autophagy, which is either performed by labelling the protein with an antibody, or using transduced cells that express green fluorescence protein-tagged LC3 (48–50). Along with LC3-II, other proteins that play a role in autophagy such as Beclin-1 can also be investigated simultaneously (51,52). Alternatively, autophagy can also be measured by investigating the levels of selective autophagy substrates, which are usually recognized by autophagic proteins, and their degradation can be measured using lysosomal inhibitors (53). A well-known example for such substrates is SQSTM1/p62, though it should be noted that its expression is regulated at the transcriptional level, thus, its mRNA levels should also be evaluated to eliminate

the possibility of the involvement of transcriptional induction.

While autophagic markers can provide a general insight to autophagy, assessment of autophagic cargo flux is needed for specific and accurate detection of autophagic activity. For investigating monitoring bulk and selective autophagy, employing the fluorescent coral protein Keima is another approach (54). This protein is excited at 440 nm and 586 nm under neutral and acidic conditions, respectively, allowing to monitor its delivery from the cytosol to lysosomes (53). However, this probe is not suitable to be used in fixed samples and can only be combined with LC3-II staining if transduced cells are used.

In addition to the methods mentioned above, there are commercial kits such as CYTO-ID® Autophagy detection kit (Enzo Life Sciences), FlowCellect™ Autophagy LC3 Antibody-based Assay Kit (Merck Millipore) and Autophagy Assay Kit (Abcam) where the cells can be subsequently evaluated with flow cytometry.

2.3.3. Ferroptosis

First proposed in 2012, ferroptosis is a form of regulated cell death characterized by iron-dependent lipid peroxidation, leading to the accumulation of reactive oxygen species (ROS) and subsequent cell demise. This process involves the dysregulation of cellular redox balance and antioxidant defense mechanisms, ultimately resulting in oxidative damage to cell membranes (55,56). Ferroptotic pathway is distinguished by the loss of lipid peroxide repair capacity by the phospholipid hydroperoxidase GPX4, the presence of redox-active iron, and the oxidation of polyunsaturated fatty acid (PUFA)-containing phospholipids (57). Ferroptosis has been shown to overcome drug resistance in cancer (55) in addition to holding importance in neurodegeneration, stroke, traumatic brain injury, ischemia-reperfusion injury, cardiomyopathy, and kidney degeneration (58). However, a significant challenge in assessing the therapeutic possibilities of ferroptosis lies in accurately identifying under pathological conditions.

Since ferroptosis was identified relatively recently, assays regarding its evaluation are not as well established as apoptosis. In flow cytometry, BODIPY™ 581/591 C11 probe is widely used to detect ferroptosis by determining the amount of lipid peroxides in cellular membranes (59,60). Another probe is Liperfluo which directly reacts with lipid hydroperoxides to yield fluorescent Liperfluo-OX which is excited and emitted at 524 and 535 nm wavelengths, respectively (61). Certain proteins including long-chain-fatty-acid—CoA ligase 4 (ACSL4), Transferrin receptor protein 1 (TFRC, CD71), Cyclooxygenase 2 (PTGS2), or glutathione specific gamma-glutamylcyclotransferase 1 (CHAC1) protein expressions have been shown to be enhanced; autophagy also promotes ferroptosis, and evaluating LC3-II may reflect the degree of ferroptotic damage, which can all be measured via flow cytometry (62).

2.3.4. Pyroptosis

Pyroptosis is a type of inflammatory programmed cell death which is induced by caspase cleavage and gasdermins and results in the secretion of inflammatory mediators interleukin 1β and interleukin 18 (63–65). This cell death pathway has been reported to be involved in carcinogenesis as well as autoimmune, neurodegenerative or allergic diseases (66–69). Therefore, pyroptosis is considered as an attractive target for treating a wide array of diseases (70,71).

In flow cytometry, activation of pyroptotic pathway can be detected via evaluating gasdermin D and active caspase-1 levels, or secretion of IL-1β and IL-18. For caspase-1, intracellular staining can be performed by fixing and permeabilizing the cell membrane, followed by labelling an appropriate antibody of the target, or with a commercial kit that includes a fluorescent caspase-1 substrate such as FAM-FLICA™ Caspase-1 Assay Kit (72,73). For detecting IL-1β and IL-18, inflammatory mediators released as a result of pyroptosis, commercial bead-based flow cytometric arrays which are suitable for blood and plasma samples in addition to cell culture media (74).

2.4. Flow-fluorescence *in situ* hybridization (Flow-FISH)

High-throughput analyses evaluating gene expression are widely used in early drug discovery to identify disease-associated genes. To further characterize the expression of selected genes, in situ hybridization (ISH) using RNA probes (riboprobes) is a powerful tool to localize mRNA expression at the cellular level, which is especially beneficial for

investigating novel drug targets (75). Flow-FISH (Fluorescence In Situ Hybridization) is a technique that combines the principles of fluorescence in situ hybridization with flow cytometry, allowing for the simultaneous detection and quantification of specific nucleic acid sequences within individual cells within a heterogeneous population (76). In this method, cells are first treated with fluorescently labeled nucleic acid probes that target complementary sequences of interest (77). These probes hybridize to their specific targets within the cells. Subsequently, flow cytometry is employed to analyze the labeled cells, measuring the fluorescence signals emitted by the hybridized probes. Moreover, by combining flow-FISH with fluorescence-activated cell sorting (FACS), the target cell populations can be obtained and investigated at the single stage (78).

Flow-FISH enables the examination of genetic or RNA content at a single-cell resolution within a mixed population, providing insights into cellular heterogeneity, gene expression patterns, and telomere length (76,79). Taking typically 1-2 days, flow-FISH is faster compared to conventional methods which involve culturing cells (80). In addition, in terms of evaluating telomere length, flow-FISH was reported to be more accurate, reproducible, sensitive, and specific in comparison with quantitative PCR (qPCR) (81). Likewise, even though sensitivity of monochrome multiplex–quantitative polymerase chain reaction (MM–qPCR) and flow-FISH was found to be similar, specificity of MM–qPCR was reported to be significantly lower compared with flow-FISH (82).

2.5. Receptor-ligand binding assays

Ligand binding assays in flow cytometry encompass methodologies to investigate molecular interactions between ligands and their receptors expressed on the cell surface or within the cellular milieu (83). These assays utilize fluorescently labeled ligands or antibodies to detect and quantify specific binding events. The integration of flow cytometry with ligand binding assays offers a powerful means to examine receptor-ligand interactions at a single-cell level with high sensitivity and multiparametric analysis capabilities. Moreover, they can be combined with other cellular markers to determine specific events in a heterogenous cell suspension (84).

Flow cytometry-based binding assays serve as a cornerstone in characterizing product activity by probing its interaction with a specific cell surface receptor: in the case of monoclonal antibodies (mAbs) aimed at obstructing ligand-receptor binding, employing *in vitro* binding assays emerges as a potent surrogate for assessing the therapeutic mAbs' potency to illuminate its efficacy by gauging its ability to impede the crucial interaction between the ligand and its cellular receptor (85). Summarizing, these assays play a pivotal role in the realm of drug development, offering a crucial avenue for assessing candidate therapeutic agents that specifically target receptors or ligands; in addition to evaluating their specificity, this process can significantly contribute to refining and perfecting the design of pharmaceutical interventions, charting a course towards more precise and effective treatments.

2.6. Receptor occupancy assays

Implementing the precision medicine framework in drug development requires the integration of a range of information to facilitate more informed decision-making regarding target selection and a better understanding of the pharmacological aspects of the drug, including bioavailability, pharmacodynamics, and pharmacokinetics(86). For developing biologically-based therapeutics, measuring the binding of the biotherapeutic to its cellular target, Receptor Occupancy (RO), can aid in determining the Minimal Biological Effect Level (MABEL) as well as revealing optimal dosing and administration schedules(86,87). In addition, data generated in RO assays can be used in safety assessment as long-term maximum RO can be a hallmark of overdosing or long-term binding, which can lead to serious side effects and even toxicity. RO assays measure unbound or free receptors, total available receptors and/or the fraction of bound receptors (**Fig 3**). These assays are largely applied it evaluating therapies directed at immune conditions as well as targeted oncology therapy, making flow cytometry an ideal instrument for performing RO assays. Moreover, with flow cytometry, target engagement on specific cell subsets within heterogeneous mixtures can be achieved (4).

While flow cytometry stands as a valuable tool in evaluating receptor density and occupancy during both preclinical and clinical pharmacodynamic studies, it's crucial to perform optimization, standardization and validation studies as it is the

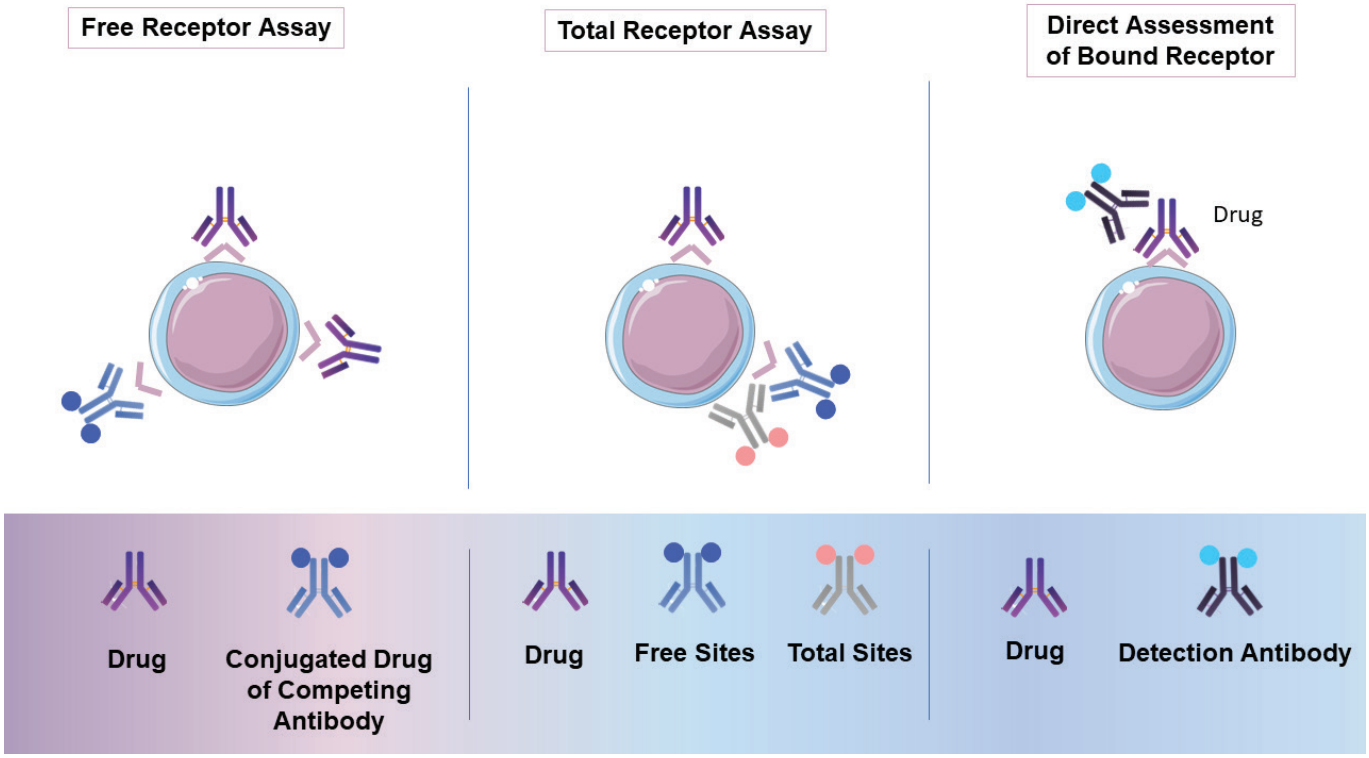

Figure 3. Three different formats of Receptor Occupancy (RO) assays (88). Free receptor assays quantify the unbound fraction of receptors in the presence of a drug, which is achieved after incubating with a fluorochrome-conjugated antibody, especially designed to compete with the drug for occupancy at the identical epitope. These assays are commonly employed to ascertain dosage levels. Total receptor assays, on the other hand, evaluate the combined presence of both unbound and bound receptors on cells These assays employ a dual-antibody approach: one antibody competes with the drug for binding, while another targets a distinct epitope on the receptor. This method is employed when anticipating the drug's potential impact on the up- or down-regulation of the receptor when administered at specific concentrations, and successful execution of this method relies upon the availability of a non-competing antibody. Finally, direct assessment of bound receptor assays are preferred in scenarios where receptor expression remains low or the target cells are scarce. Within this modality, the antibody directly engages with the drug, amplifying the detection of drug molecules bound to the receptor and thereby significantly enhancing assay sensitivity.

initial step to ensure consistency and reliability in the assessment process (4,9,87). Methods regarding flow cytometric RO assays remain challenging to develop due to various obstacles including low antigen expression, rare frequency of the target population, presence of soluble targets, and stability of reagents in addition to samples.

2.7. Enzyme activity assays

Enzyme activity assays serve as a cornerstone in understanding biological processes, providing insights into enzymatic reactions' kinetics and mechanisms. Traditionally, these assays rely on bulk measurements, providing averaged values for a population of cells while to directly explore how certain enzymes relate to different cell types and their functions, it's essential to use single-cell enzyme activity assays which enable investigating enzymes within individual cells, providing insights into their roles across various cell types and functions (89,90). Within this context, flow cytometry allows the assessment of enzymatic activity present in individual cells, uncovering heterogeneity and variations that might be obscured in bulk measurements. This single-cell resolution offers valuable insights into cellular heterogeneity and the diverse roles enzymes play within different cellular subpopulations (91). Furthermore, cell sorting enables isolation of cells based on their enzymatic activity levels, and by coupling flow cytometry with cell sorting capabilities, researchers can isolate subpopulations exhibiting specific enzymatic activities for further downstream analyses (92).

When measuring enzymatic activity by flow cytometry, fluorochrome-labelled antibodies can be used to measure enzyme concentration as an indirect indicator of enzyme activity, or the generation of a fluorescent product instead of solely relying on antibody-based assessments can be employed.

There are also studies where fluorogenic substrates originally developed for imaging and fluorimetry have been adapted to flow cytometry format (93).

2.8. Intracellular protein staining

Intracellular protein staining using flow cytometry represents a pivotal technique in modern cellular biology, enabling the examination and quantification of specific proteins within individual cells (94–96). However, according to the nature of the protein-of-interest, the protocol that should be applied should be chosen carefully. Measurement of secreted proteins such as cytokines and chemokines consist of four steps: (1) *in vitro* activation, (2) fixation, (3) permeabilization, and (4) immunostaining. For analyzing a specific cell subset in a heterogeneous cell population, cells surface markers are also stained simultaneously with intracellular cytokines. For stimulating cytokine secretion, *in vitro* cellular activation is generally achieved by phorbol-12-myristate-13-acetate (PMA) and ionomycin, or via antigens while inhibitors of cytokine secretion, Brefeldin A or monensin are used to increase intracellular cytokine concentration in order to increase Signal-to-Noise ratio (97,98). Nevertheless, both inhibitors exert cytotoxic effects and thus, should not be incubated more than 12 hours though cytokine accumulation generally reaches to its maximum within 2 to 4 hours after treatment with these inhibitors, but ideal incubation durations should be determined according to the cytokines that will be evaluated. For measuring cytokines with dim and low-frequency such as IL-4, IL-5, and IL-13, fluorochromes with high signal-to-noise ratios such as phycoerythrin (PE) and allophycocyanin (APC) are recommended.

For fixation and permeabilization, aldehyde-based fixatives such as paraformaldehyde (PFA) and saponin are commonly preferred. However, when evaluating nuclear antigens along with cytokines, saponin will not allow nuclear staining, and permeabilization buffers may not enable cytokine staining as the PFA/saponin system (98). In addition, number of cells that will be acquired to obtain statistically significant numbers of cytokine-positive events should be determined (99). When using PMA and ionomycin for cell activation, around 15% of CD4+ cells exhibit IFN-γ positivity, and about 4% show IL-13 positivity in healthy donors, thus, lower number of PBMC (e.g., 20,000 total events) can provide an ample count of cytokine-positive events. Nevertheless, with antigen-specific activation, these frequencies usually range much lower, from 0.001% to 0.10%. Hence, researchers must collect a sufficient number of cells during flow cytometry to achieve statistically significant counts of cytokine-positive events(98).

Besides secreted proteins, flow cytometry can also be used for evaluating cytoplasmic and nuclear proteins. This method is especially feasible for investigating heterogeneous cell populations such as peripheral blood mononuclear cells (PBMC), murine splenocytes, bone marrow cells as these samples have mainly remained outside the scope of biochemical analysis. Similar to evaluating cytokines, intracellular proteins require a fixation step which is followed by permeabilization to provide access to the cytoplasm. To date, extensive studies have been carried out to determine the ideal fixation and permeabilization reagents (100–102), and many epitopes have been reported to be successfully stained including enzymes (103), native and phosphorylated proteins (104–106), viral particles (107), and immunoglobulins (108). In this context, 'phosphoflow', the staining of phosphorylated proteins, has been studied for more than two decades and has outlined essential requirements for the effective detection of intracellular phosphoproteins. Firstly, rapid fixation of cells is essential to preserve the authentic phosphorylation state of the proteins of interest. Second, efficient cell permeabilization is necessary to allow specific antibodies to access the targeted epitopes within the cells. Thirdly, the antibodies used for staining both cell surface proteins to distinguish subsets and phosphorylated components within intracellular proteins should accurately identify and securely bind to epitopes altered by the fixation process (109). Aiming to develop a general protocol for phosphoflow, the first effective and reproducible approach for intracellular phosphoprotein detection using flow cytometry was reported in 2003, where the authors have investigated the ideal stimulation, fixation and permeabilization conditions(109). Over the past decade, collaborative efforts between academic and industrial researchers have led to the development of refined fixation and permeabilization methods specifically tailored to the detection of various phosphoproteins,

which some of them now commercially available. Moreover, small molecule fluorescent dyes such as fluorescein isothiocyanate (FITC), Alexa Fluor 488® and Alexa Fluor 647®, have proven to be superior for phosphoflow applications thanks to their small size that facilitates antibody entry into cells in addition to their minimal interference with the ability of antibodies to bind to their target proteins (110). In summary, it can be concluded that advances in both reagents and assay techniques will significantly expand the application of phosphoflow beyond preclinical research as this approach holds great promise for monitoring immune responses and assessing immune status following vaccination and immunomodulatory interventions. Additional investigations, including adherence to Good Laboratory Practice (GLP) standards, are essential for the incorporation of phosphoflow cytometry into clinical applications (111). Another critical area for further exploration is to assess the potential of phosphoflow as a sensitive tool for tracking antigen-specific T-cell responses, particularly in the development of vaccines and immunotherapies for diseases such as HIV and cancer. There's also a need to understand how immunomodulators and immune response suppressors affect intracellular signaling pathways downstream of T cell and cytokine receptors in people with cancer, chronic viral infections and autoimmune diseases (112).

2.9. Analysis of extracellular vesicles (EVs)

Extracellular vesicles (EVs) are small lipid bilayer compartments released by cells that contain nucleic acids, proteins and lipids. Long thought to be as insignificant as platelet dust, recent studies have shown that EVs play a role in cell-to-cell signaling and can serve as drug delivery vectors and disease biomarkers (113,114). Moreover, their ability to target specific cells also positions them as potential drug delivery vehicles (115). EVs of different origins have the ability to influence both normal physiological and disease-related processes. Despite their significant potential, the current methods used in EV research are somewhat limited. Currently, EVs are predominantly studied using nanoparticle tracking analysis and bulk molecular techniques such as Western blot. However, these methods fall short of fully dissecting the observed heterogeneity of EVs as visualized by electron microscopy (EM). While EM is instrumental in revealing the diversity within EV samples, its limitations hinder the performance of more complex and quantitative EV analyses.

Studying EVs using flow cytometry presents challenges due to their sizes being too small to detect (116). A common strategy for analyzing EVs on conventional flow cytometers is to attach these structures to beads of a size that ensures accurate detection on the flow cytometer. In one of the first studies to use this method, the researchers have isolated and characterized exosomes between 30 and 100 nm in cell culture supernatants by binding them to latex beads (117). However, advances in flow cytometry have made it possible to isolate EVs without beads: in a recent study, researchers removed plasma proteins to isolate EVs by size exclusion chromatography followed by flow cytometric analysis, and 95% of the samples were positive for the exosome marker CD63 and contained neural cell adhesion molecule (NCAM) (118).

EV analysis is a challenging area of cytometry, but there are ongoing efforts to develop new stains and diagnostic tools for the identification of EVs and their drug cargo. Guidelines for the identification of these small vesicles have been developed by interested research groups (119).

2.10. Microbial applications

Flow cytometry is a good tool for evaluation of the effect of anti-microbial drugs. Effective antifungal drugs can inhibit the fungal growth or may change the fungal cytological features. In addition to phenotypic profiling studies, utilization of imaging flow cytometers (IFC) have the potential of improving and enhancing cytological profiling. IFC combines the abilities of flow cytometry and fluorescent microscopy with advanced data processing algorithms. With this method, measurement of different components of the cell, size, shape, texture, nuclear DNA morphology, integrity of cell wall, membrane permeability, surface and intra cellular markers are also possible (120–123). There are studies for development of highly sensitive nano level detection systems for high throughput systems but due to low amount of sample and limitations in cell numbers they have not yet reached to daily use stage (124).

3. Recent technological developments

What we have discussed so far has focused on classical cytometry systems. Recent developments in cytometry systems including high-throughput flow cytometry, Mass-TOF, droplet flow cytometry, and spectral cytometry, will enhance the use of cytometric methods.

3.1. High-throughput screening of compound libraries

While flow cytometry is a widely validated technique used in preclinical development from target selection and validation to mechanism of action identification, its applicability to drug screening has been very limited due to the lower number of assays that can be performed per patient sample and the lack of automation (125). High throughput screening systems can assist in drug screening by reducing reagent costs, compound usage and cell consumption through the ability to sample small volumes with negligible waste. With these systems, many compounds can be tested in a short time in 96, 384 or even larger capacity plates, and various high-throughput flow cytometry (HTFC) systems with fast autosampler devices tailored to traditional flow cytometers have been reported by different research groups. HTFC is an advanced method that uses automated sample processing and offers a promising way to screen large compound libraries. Recently, a novel system called HyperCyt has emerged that streamlines automated HTFC analysis by rapidly aspirating cell samples from microplate wells and transporting them to the flow cytometer (126,127). Since its commercial launch in 2006, the HyperCyt sampling technology has been attracting attention, particularly from companies favoring flow cytometry for a range of research objectives, including SiRNA-based functional genomics screens, hybridoma screens for therapeutic antibodies and immune cell analysis from primary tissues.

High-throughput flow cytometry platforms are likely to benefit from two recent trends in drug discovery. Firstly, there's a growing focus on moving away from target-centric screening towards target-agnostic or mechanism-informed phenotypic screening approaches. Second, large pharmaceutical companies are increasingly investing in the development of biologic drugs, particularly novel antibodies. These trends have likely contributed to the increased utility and interest in high-throughput flow cytometry systems. The appeal of HyperCyt lay not only in its faster sample processing, but also in its ability to handle smaller sample volumes while mitigating cell loss, a common problem with conventional flow cytometry due to dead volume during sample backflushing (127).

3.2. Mass cytometry

Mass cytometry is a modern variation of flow cytometry diverging from the traditional approach by replacing fluorescent labels with heavy metal ion tags on antibodies. This technique employs heavy metal isotopes not found in biological systems, solving the issue of overlapping fluorescence spectra and allowing for a greater number of parameters to be analyzed at once in comparison to conventional cytometry methods. In mass cytometry, cells are treated with a mixture of probes and antibodies, each labeled with a distinct non-radioactive heavy metal isotope.

For single-cell analysis, cell suspensions are aerosolized so that each droplet contains only one cell. These individual cells are then sent through argon (Ar) plasma, which atomizes and ionizes the sample, transforming each cell into an ion cloud of the elements present it contains. A quadrupole, acting as a high-pass filter eliminates lower-mass ions of biological origin, primarily of biological origin, leaving a cloud of ions tied to the isotope-conjugated probes.

Within the Time of Flight (TOF) chamber, ions are sorted by their mass-to-charge ratio. When these ions hit the detector, their counts are amplified and converted into electrical signals. Practical limitations, such as the availability of isotopes with sufficient purity and antibody conjugation chemistries, restrict applications to approximately 60 parameters per mass cytometry panel, as opposed to the theoretical claim of 120 parameters.

An alternative single-cell technology with even greater dimensionality is single-cell RNA sequencing (scRNAseq), providing a quantitative measure of gene expression levels per cell. While scRNAseq is a potent genomic tool for dissecting cell populations, it is constrained by higher costs and can only be run on a limited number of single

cells. In contrast, mass cytometry experiments can acquire data on a much larger scale, ranging from 10^6 to 10^7 cells. Despite offering more reliable results, the cost of disposables remains a hindrance to the widespread adoption of these systems (15). Mass cytometry cannot provide information about the cell size, internal complexity and autofluorescence profile which can be counted as a drawback depending on what the researchers are looking for (128).

Mass cytometry data can be analyzed by using SPADE Cluster Analysis, Principal Component Analysis and Boolean Gating (129,130).

3.3. Spectral cytometry

Spectral cytometry, a technique developed based on conventional flow cytometry, employs a spectrograph and a multichannel detector system, such as CCD. In contrast to conventional flow cytometry optics, which observe photons based on their wavelength, spectral flow cytometers disperse photons according to their wavelength. Utilizing a prism to disperse emitted light, this technique offers the advantage of using a more extensive array of colors for a sample, yielding accurate results with reduced reliance on color compensation (131). Just like conventional cytometers, careful panel selection is crucial for optimal performance in spectral cytometers. Another beneficial feature is the automated subtraction of cellular autofluorescence. In studies comparing mass cytometry and spectral cytometry results, researchers underscore the significance of selecting an efficient panel (128,132).

3.4. Droplet cytometry

Cytometry experts have extensively investigated this method, using traditional cytometers for its application. The technique involves droplet microfluidics to encase individual cells in tiny, picoliter-scale microdroplets. Among the droplet types suitable for cytometry in aqueous environments are double emulsion droplets (water-in-oil-in-water) and hydrogel-based droplets. Integrating flow cytometry with these droplets enhances cell characterization beyond traditional markers, facilitating simultaneous analysis of genomes, epigenomes, or transcriptomes of single cells encapsulated in droplets. This approach has led to significant breakthroughs, including enzyme and protein evolution, analysis of cell variance under drug exposure, detection of rare cells in microbial populations, pinpointing antibiotic resistance genes, and discovering new biomarkers (133).

4. Conclusion

In summary, we wanted to highlight the importance of the described techniques throughout different phases of drug development. To ensure dependable outcomes, it is imperative to have proficient and experienced users who adhere to the fundamental principles of flow cytometry and possess the expertise to select appropriate reagents. The possibilities that can be explored through cytometry are only constrained by one's imagination.

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Conflict of Interest

Authors have no conflict of interest to declare.

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Original Article

The Antioxidative, Antimicrobial Activity and HPLC Analysis of *Ornithogalum pyrenaicum*

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Abstract

Introduction: The aim of this study was to specify the antioxidant, antimicrobial activity and phenolic contents of *Ornithogalum pyrenaicum.*

Methods: The antioxidant capacity of *O. pyrenaicum* was determined by 2,2-diphenylpicrylhydrazyl (DPPH) method and ferric reduction antioxidant power by FRAP method. The total phenolic content (TPC) was of the samples was determined using spectrophotometric method. The phenolic contents the samples were analyzed by reverse phase-high performance liquid chromatography (RP-HPLC). Antimicrobial activity was researched on 9 microorganism by agar diffusion method.

Results: As a result of the study, high phenolic contents and strong antioxidant capacitiy were observed. Phenolic compounds were detected as *p-coumaric* acid and benzoic acid. Additionally, it was determined that *O. pyrenacium* had considerable antimicrobial activity on *Yersinia pseudotuberculosis, Staphylococcus aureus* and moderate activity on *Pseudomonas aeruginosa* and *Mycobacterium smegmatis.*

Conclusion: In conclusion, *O. pyrenaicum* extract could be evaluated in the pharmaceutical and cosmetic fields due to their antioxidant and antimicrobial potential and phenolic compounds.

Keywords: Antimicrobial activity, antioxidant activity, *Ornithogalum pyrenaicum*, RP-HPLC, total phenolic content

1. Introduction

The genus *Ornithogalum* L. comprises approximately 200 species. *Ornithogalum* L. which belong to the Liliaceae family are deployed along soft climates in Africa, Asia, and Europe (1). The genus is defined among this genus 36 species are found in Turkey (2). *Ornithogalum* species are used for various medical purposes. These species are well-known to have antimicrobial, anticarsonogenic, antioxidant and cytotoxic properties due to their various phytochemical components (3-5).

Natural antioxidants plants are of great interest in order to protect the human body against the attack of free radicals today (6). Phenolic compounds are the essential sources of antioxidant impacts in plant products. These products can prevent many radical diseases such as cancer, diabetes, Alzheimer's and heart disease by preventing free radical reactions (7).

Plants have unlimited components which are aromatic substances, most phenol or oxygen-substituted derivatives (8), most of these components have antimicrobial activity. For example caffeic acid is effective against viruses (9), bacteria (9, 10, 11) and fungi (9, 12). Catechol and pyrogallol are both compounds that have been shown to be toxic against microorganisms. The number of hydroxyl groups in the phenol group is thought to be associated with their toxicity to microorganisms. There is also proof that increased hydroxylation increases toxicity (13).

Nevertheless, there is insufficient information examining the phenolic contents and biological activity of *O. pyrenaicum* in the literature. That is why antioxidant, and antimicrobial activitiy of the plant were examined in present study. Additionally, phenolic profiles of the plant extracts was determined using HPLC.

2. Methods

2.1. Chemicals and instrumentation

The chemicals used in biological activity and HPLC studies are as follows: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), and sodium carbonate (Na_2CO_3) were bought from Sigma. Butylated hydroxytoluene (BHT), and Folin-Ciocalteau reagent were provided from Fluka and Supelco, respectively. Methanol and acetic acid were purchased from Merck. Ampicillin and Fluconazole used in antimicrobial activity were purchased from Mustafa Nevzat and Phizer, respectively. BMG LABTECH SPECTROstar® Nano spectrophotometer was used for antioxidant activity studies. Evaporation processes were realised by Heidolph (Schwabach, Germany) rotary evaporator system. HPLC studies were performed HPLC system (Shimadzu Corporation, Kyoto, Japan).

2.2. Plant material and preparation of samples

Ornithogalum pyrenaicum were collected from Dikmen, Akçaalan Region / Sinop in Turkey in May 2014 and identified by Prof. Zeki AYTAC and Murat EKICI (herbarium number: 26706). The aerial parts of *O. pyrenaicum* were dried in the ambient conditions and grinded. Then, 50 g of grinded plant mixed with 500 mL methanol and extracted shaking incubator overnight at room temperature (25 °C). This process was repeated three times and solvent was removed by a rotary evaporator. The methanolic extract was stored at +4 °C till all analyses. This extract obtained was used for biological studies. For HPLC study, this extract was prepared HPLC purity methanol to 10 mg/mL and filtered by membranes filter and stored at +4°C.

2.3. Antioxidant activity

The methanolic extract was prepared at a concentration of 10 mg/mL for TPC and FRAP studies. TPC in extract was specified using the Folin method (14). Gallic acid was prepared as standard in a range of concentrations (1000, 500, 250, 125, 62.5, 31.25 ve 15.63 µg/mL). In this study, the extract, Folin reagent, and Na_2CO_3 were mixed in test tubes and incubated at 20 °C and in dark conditions for about 2 hours. The absorbances were read at 760 nm using spectrophotometer. Whole processes was repeated in triplicate.

In order to determine the ferric reduction antioxidant power, FRAP method was performed as stated in the literature (15). Trolox was prepared as standard in a range of concentrations (62.5, 125, 250, 500, 1000 µM). The methanolic extract and FRAP reagent was added in test tubes. All samples were incubated in dark conditions at 37 °C during 20 minutes. And then, the absorbance was read 595 nm againist a blank. Whole processes was repeated in triplicate.

DPPH method was applied to investigate the radical scavenging effect of the plant (16). BHT was prepared as standard and methonolic extract

were prepared in a range of concentrations (0.005, 0.025, 0.0125, 0.00625 mg/mL). DPPH and extract were added in test tubes at the same proportions and mixed. After the test tubes were incubated in the darkness during 50 minutes, the absorbances of the samples were read at 517 nm. Whole processes was repeated in triplicate.

2.4. Antimicrobial actvity

2.4.1. Test microorganisms

The studied microorganisms were provided from Refik Saydam Hifzissihha Institute (Ankara, Turkey). *Bacillus cereus* 709 ROMA, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Mycobacterium smegmatis* ATCC607, *Pseudomonas auroginosa* ATCC 43288, *Staphylococcus aureus* ATCC 25923, and *Yersinia pseudotuberculosis* ATCC 911 were chosen as test bacteria. *Candida albicans* ATCC 60193 and *Saccharomyces cerevisiae* RSKK 251 were chosen as yeast.

2.4.2. Antimicrobial assay

In order to detect the antimicrobial activity, some modifications were made in agar disk diffusion method (17). All bacteria were suspended in MH broth except M. smegmatis, which was augment in BHA. For yeast-like fungi, Sabouraud Dextrose Agar (SDA) was used. Each microorganism was diluted. A "flood inoculum" was applied to the surface of Mueller Hinton Agar (MHA) and SDA and then dried. Wells with a diameter of 5 millimeters were opened from the agar using a sterile cork borer and 50 µL of extract was added to the wells. The plates were then incubated at 35°C for 18 hours. Antimicrobial activity was studied by comparing the zone of inhibition with the test organism. In this study, ampicillin, fluconazole and streptomycin as the standard drug and dimethylsulfoxide as the control were preferred.

2.5. Determination of phenolic content by HPLC

HPLC analysis to detect the phenolic compounds were implemented as regardes the method employed by Aliyazıcıoglu et al (18). The standards used in this HPLC study are given in Table 1. In this study, a reverse phase column (250 × 4.6 mm i.d, 5 µm) and a gradient program with two solvents system were preferred. The solvent system was prepared as 2% acetic acid in water and 5% acetic acid in acetonitrile:water (1:1). The flow rate was adjusted to 1.2 ml per minute and all signals were determined by diode array detector (DAD) (18).

Table 1. Phenolic standards used in HPLC analysis

Benzoic acid
Caffeic acid
Chlorogenic acid
Gallic acid
Ferulic acid
Protocatechuic acid
Protocatechuic aldehyde
Sinapic acid
Syring aldehyde
Vanillic acid
Vanillin
p-coumaric acid
p-OH benzoic acid

3. Results

3.1. Antioxidant activities of *O. pyrenaicum* methanolic extract

Antioxidant activity of *O. pyrenaicum* methanolic extract was identified by using three different methods i.e. total phenolic contents (TPC), 2,2-diphenylpicrylhydrazyl (DPPH), and reducing antioxidant power (FRAP). TPC and FRAP results of the methanolic extract were found as 5.7 ± 0.2645 mg of GAE/g sample and 129 ± 3.7859 µM Trolox/g sample, respectively. According to DPPH assay result, SC_{50} value of the plant was 2.2186 ± 0.0418 mg/mL. The radical scavenging capacity of extract was lower than that of BHT (0.0099 ± 0.0002 mg/mL). The methanolic extract of *O. pyrenaicum* was exhibited potent antioxidant activity. All the results of antioxidant activities of

the plant are presented in Table 2.

3.2. Antimicrobial activities of *O. pyrenaicum* methanolic extract

The extract of the *O. pyrenaicum* (10 mg/mL) showed antimicrobial effects against *M. smegmatis*, *P. aeruginosa*, *S. aureus*, and *Y. pseudotuberculosis* (Table 3).

3.3. HPLC chromatogram of *Ornithogalum pyrenaicum* methanolic extract

Chromatograms of the all standards and the plant extract of the *O. pyrenaicum* have been given in Figure 1 and 2. In the extract of the *O. pyrenacium*, *p*-coumaric acid (0.6039 mg/g) and benzoic acid (8.1926 mg/g) were detected as phenolic compounds.

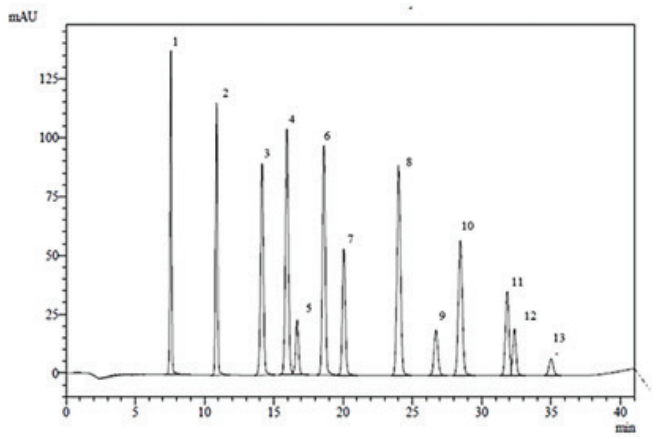

Figure 1. RP-HPLC chromatogram of phenolic standards investigated in O. pyrenacium. Peak identification: (1) gallic acid, (2) protocatechuic acid, (3) protocatechuic aldehyde, (4) p-OH benzoic acid, (5) chlorogenic acid, (6) vanillic acid, (7) caffeic acid, (8) vanillin, (9) syringaldehyde, (10) p-coumaric acid, (11) ferulic acid, (12) sinapic acid, (13) benzoic acid [18].

Table 2. The antioxidant activities of *Ornithogalum pyrenaicum* extract

Test Compounds	TPC[1]	FRAP[2]	DPPH[3]
Methanolic extract	5,7 ± 0,2645	129 ± 3,7859	2,2186 ± 0,0418
BHT			0,0099 ± 0,0002

[1]TPC stated in mg of gallic acid equivalent (GAE) per gram of dry plant weight.

[2] FRAP value stated as µM trolox equivalents (TE) per gram of dry plant weight.

[3]Concentration of test specimen (mg/mL) required to produce 50% scavenging (SC_{50}) of the DPPH radical.

Table 3. Inhibition zone values of extract of *Ornithogalum pyrenaicum*

Tested Compounds	Microorganisms and Inhibition Zone (mm)								
	Gram negative			Gram positive			No gram	Yeast Like Fungi	
	Ec	Pa	Yp	Ef	Bc	Sa	Ms	Ca	Sc
O. pyrenaicum	-	6	10	-	-	10	6	-	-
Ampicillin	10	18	10	35	15	10	-	-	-
Fluconazole							-	25	25
Streptomycin	-	-	-	-	-	-	35	-	-

Ec: *E. coli* ATCC 25922, Pa: *P. aeruginosa* ATCC 43288, Yp: *Y. pseudotuberculosis* ATCC 911, Ef: *E. faecalis* ATCC 29212, Bc: *B. cereus* 709 Roma, Sa: *S. aureus* ATCC 25923, Ms: *M. smegmatis* ATCC607, Ca: *C. albicans* ATCC 60193, Sc: *S. cerevisiae* RSKK 251, (-): no activity

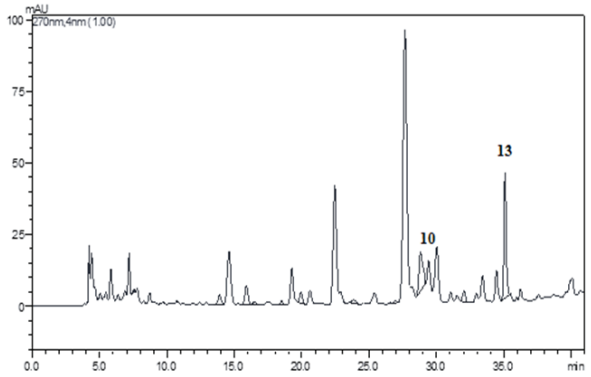

Figure 2. RP-HPLC chromatogram of *O. pyrenacium*.

4. Discussion

Antioxidant substances can reduce damages caused by free radicals which are known to have harmful effects on health. The antioxidant effects of herbal products are mainly due to their phenolic content. The protective effects of phenolic compounds against many diseases (i.e. cardiovascular diseases, cancer, diabetes mellitus, neurodegenerative diseases and osteoporosis) have shown in different studies (19-21).

The antioxidant activity of *O. pyrenacium* was examined and also HPLC studies were performed to determine phenolic content. Herewith, it was determined that due to its rich phenolic content, the plant showed strong antioxidant activity. In the study, benzoic acid and *p*-coumaric acid were found as major phenolic constituents of *O. pyrenacium*. Benzoic acid derivatives have antioxidant, antipyretic, antirheumatic, bacteriocidal and fungicidal and analgesic properties due to their phenolic groups. Also benzoic acid derivatives are a group of chemicals widely used as preservatives in the cosmetic and pharmaceutical industries (22).

Coumaric acid is a compound with antioxidant properties that suppress nitrosamine formation and thus reduce the risk of developing stomach cancer (23).

In a study by Chen et al. was found that *O. caudatum* Ait has possessed considerable antioxidant activity (24). In a biological activity study performed with *O. narbonse*, antioxidant and tyrosinase inhibition activity and phenolic profiles were researched by using different anatomical parts of the plant. In this study, the highest activity was observed in the bulb samples of the plant. *O. narbonse* was also found to be rich in phenolic content (2).

In a study using *O. sintenisii* L. was found that the bulb and aerial parts of this plant were demonstrated good antioxidant properties (25). When compared with these studies in the literature, it can be said that the plant shows potent antioxidant characteristics. However, in this study has found that *O. pyrenacium* has not effective on tyrosinase enzyme.

Interest in extracts of the plants are gradually increasing in the protection of foods and in the production of natural origin medicines. Because the side and toxic effects of sentetic medicines have giving damages to human healthy (26). These days, long duration treatment with certain antibiotics can lead to detrimental side effects. These side effects can be mitigated with phenolic compounds that are antioxidant sources. In addition, these compounds can be used as antimicrobial agents. In this study, antimicrobial properties of the plant were examined and the extract of the *O. pyrenacium* showed antimicrobial effects against *M. smegmatis*, *P. aeruginosa*, *S. aureus*, and *Y. pseudotuberculosis*. *Y. pseudotuberculosis* is a pathogen that causes symptoms such as swelling of lymph nodes, septicemia, typhoidal clinical effects in humans (27). *P. aeruginosa* is a gram-negative bacterium. These bacteria cause urinary tract, eye and ear infections. It also causes diseases such as burn and wound infections, meningitis and bronchitis (28). Spread infections lead to by *M. smegmatis* are frequently associated with immunosuppression (29). When the antimicrobial effect of the plant is taken into consideration, it is thought that it can be used from the plant in the production of drugs in diseases caused by these bacteria.

5. Conclusion

Consequently, it was shown that *O. pyrenaicum* posses significiant antimicrobial and antioxidant properties in the present study. The phenolic components which have antioxidant properties were analysed from the plant. So that *O. pyrenaicum* may represent an artificial source of antimicrobial

and antioxidant agents. There are not enough studies about *O. pyrenaicum*. In our opinion, the study is important in terms of pioneering further studies.

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Conflicts of interest: The authors have no conflicts of interest to declare.

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Original Article

The Effect of Thyroid Dysfunction on Tissue Factor Level and Activity in Rats

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Abstract

Introduction: Tissue factor (TF) is a cellular element that initiates the coagulation cascade. Hypothyroidism and hyperthyroidism are the most common thyroid dysfunctions and several coagulation and hemostatic abnormalities occur in thyroid disorders. The study aims to investigate the effects of thyroid dysfunction on TF activity in the tissues, such as brain, liver, and kidney tissues, and on TF levels in sera of rats.

Methods: Thirty rats were divided equally into 3 groups: 1. Controls, 2. Hypothyroid group, 3. Hyperthyroid group. Methimazole (75 mg/100 g diet) was added to the diet of the hypothyroid group, and L-thyroxine (0.4 mg/100 g diet) was added to the diet of the hyperthyroid group to obtain experimental groups. Controls were fed with standard chow. TF activities were determined in brain, liver and kidney tissues, while TF levels were investigated in sera.

Results: In brain, liver, and kidney tissues, significant decreases were observed in TF activities of both hypothyroid and hyperthyroid rats compared to the controls. Sera TF levels increased significantly in both hypo- and hyperthyroid rats than those of controls.

Conclusion: Coagulation abnormalities can be developed and coronary artery diseases might be triggered by hypo- and hyperthyroidism.

Keywords: Tissue factor, hypothyroid, hyperthyroid, coagulation, thrombosis

1. Introduction

Tissue factor (TF), also known as Factor III (FIII), is a cellular element that initiates the coagulation cascade in physiological and also pathological conditions. It is a membrane-embedded protein in contact with the extracellular and intracellular environment (1). Several tissues, blood, and body fluids have TF activity and following vascular damage, TF performs its physiological function in the circulation. Under physiological conditions, TF, found in the vascular system and in case of damage,

contacts the epithelial surface and is activated with calcium to initiate the coagulation mechanism (2). TF-initiated coagulation has a crucial role in the pathophysiology of diseases and it has been revealed that there are changes in TF activity in many diseases, such as diabetes, hyperlipidemia, and atherosclerosis (3-5). Thrombosis, a primary cause of morbidity, can be developed by the presence of blood or plasma-derived TF in the circulation, and the increase in TF expression is considered as a factor causing atherosclerosis (6).

Thyroid hormones play an important role in the repair after injury in several tissues and organs. There may be a common mechanism of repair in the organism which can be regulated by thyroid hormone because thyroid hormone is effective in the regulation of DNA repair following the damage (7). Hypothyroidism and hyperthyroidism are the most common thyroid dysfunctions worldwide and these disorders affect the regulation of blood cells. Besides, several coagulation and hemostatic abnormalities occur in thyroid disorders; hypothyroidism patients are at risk for developing hemorrhage, atherosclerosis, and cardiovascular diseases. In addition to that, hyperthyroidism patients display a tendency to develop thrombotic complications (8). Especially altered free thyroxine levels lead to thrombotic tendency due to impaired dysfunction of coagulation factors in blood circulation (9).

In accordance with this information, the study aims to investigate the effects of thyroid dysfunction on TF activity in the brain, liver, and kidney tissues and TF levels in sera of rats.

2. Methods

2.1. Study group

Thirty Wistar Albino rats were divided equally into 3 groups, as follows: 1. Controls, 2. Hypothyroid group, 3. Hyperthyroid group, Methimazole (75 mg/100 g diet) was added to the diet of the hypothyroid group, and L-thyroxine (0.4 mg/100 g diet) was added to the diet of the hyperthyroid group to obtain experimental groups for 3 months. Controls were fed with standard chow during the study. All animals were sacrificed, and blood and tissues, such as the brain, liver, and kidney were taken from animals at the end of the study. The blood was centrifugated and sera were taken, also the tissues were homogenized with saline (0.9% NaCl), and 10% (w/v) homogenates were done for biochemical analyses.

2.2. Biochemical analysis

Homogenates made from brain, liver and kidney were used for the analysis of TF activity, and also TF levels were determined in the sera of the rats. TF activity was done according to Quick's one-stage method (10). Pooled plasma collected from healthy subjects used for the determination of TF activity and it was performed by mixing 0.1 mL tissue homogenate with 0.1 mL of 0.02 M $CaCl_2$, with the clotting reaction being started on addition of 0.1 ml of plasma. All reagents were brought to the reaction temperature (37 ◦C) before mixture. TF levels were determined by using a commercially available enzyme-linked immunosorbent assay (catalog no: E90524Ra).

2.3. Statistical analysis

Statistical analysis of the results was carried out using GraphPad Prism 5.0 (GraphPad Software, USA). One way ANOVA, Kruskal Wallis and Post-hoc Dunn tests were used for the comparison of the groups and a $p < 0.05$ value was defined as significant.

3. Results

Brain, liver, and kidney TF activity and sera TF levels of the rats were shown in Table 1. The clotting time is inversely proportional to the TF activity. In brain, liver, and kidney tissues, significant decreases were observed in TF activities of both hypothyroid ($p<0.05$, $p<0.001$, $p>0.001$, respectively) and hyperthyroid rats ($p<0.05$, $p<0.01$, $p<0.001$, respectively) compared to the controls. Also, sera TF levels increased significantly in both hypothyroid and hyperthyroid rats than those of controls ($p<0.05$, $p<0.001$).

Table 1. Brain, liver and kidney TF activity and sera TF level of the rats

		Controls	Hypothyroid	Hyperthyroid
TF activity (sec.)	Brain	34,03±3,38	39,41±3,04*	39,03±4,20*
	Liver	96,86±2,20	103,50±1,75***	101,6±2,03**
	Kidney	39,02±1,06	48,46±1,76***	44,33±1,41***
TF level (pg/mL)	Sera	47,97±0,51	113,00±1,15***	86,36±0,50*

Values are given as mean±standard deviation. TF: Tissue factor, *p<0.05, **p<0.01, ***p<0.001 significant compared to control group.

4. Discussion

Thyroid dysfunction and thyroid autoimmunity may lead to several hemostatic disorders, such as thromboembolism, hemorrhage, and some laboratory abnormalities, affecting physiologic hemostasis (11). Excessive or insufficient release of thyroid hormones affects thrombocyte function, and regulation of coagulation factors. Also, changed blood viscosity may be effective in the emergence of coagulopathies caused by thyroid disease (12). High levels of procoagulant factors create a prethrombotic environment in the blood, and this situation increases the hemostatic risk aspect of atherosclerosis, and thrombin formation accelerates. Although the relationship between thyroid insufficiency and the hemostatic system has been studied for many years, it is still not well understood.

There are conflicting results regarding hypothyroidism; a tendency to bleed has been reported in some hypothyroid cases, on the contrary, a tendency to the development of atherosclerosis, its associated complications or thrombus are also mentioned (13-17). However, to generalize, hypothyroid and coagulation-related studies are in the direction of hypercoagulability. Studies have reported that fibrinogen and D-dimer are increased in hypothyroidism and this situation tends to cause hemostatic hypercoagulation in hypothyroidism, and such cases are at risk of cardiovascular disease (18,19). Although similar findings to fibrinogen deficiency were observed in the decrease in TF, none of these effects were determined in factor XI deficiency (20). In addition, it was reported that thrombus formation accelerated when TF is encountered, especially in the damage of vessels with high flow rates such as the carotid artery (21).

Thrombocytes are important components of coagulation. Hyperthyroidism may cause a disruption in thrombocytes, thus it is a risk factor for thrombosis. Besides, increased fT4 level is associated with elevated concentrations of Von Willebrand factor (VWF), which promotes platelet aggregation and FVIII (22). Also, a hyperdynamic circulatory in a hyperthyroidism state can stimulate the endothelium to disruptions in endothelial function, and due to increased synthesis of the endothelial proteins via a thyroid hormone-responsive element, a tendency to endothelial dysfunction can be developed (8,23). Considering the previous results, it can be suggested that thyroid hormones may affect the regulation of TF activity and/or inhibition from the endothelium.

5. Conclusion

In the present study, TF activities in brain, liver, and kidney tissues of the hypothyroid and hyperthyroid groups decreased significantly than those of controls and showed different values from each other, which may be a result of TF having tissue-specific functions. Since TF activity is inversely proportional to the clotting time, the lengthening of the clotting time is a manifestation of decreased TF activity. Under normal conditions, endothelial cells do not express TF, but in the case of endothelial dysfunction, TF appears on the cell surface. TF is also a potent cofactor and receptor for circulating FVII. The extrinsic and intrinsic systems are stimulated after TF comes into contact with blood. Thrombin, formed by TF activation, activates the platelets by stimulating them and accelerates the

adhesion, secretion, and aggregation of platelets. In this case, atherothrombosis and hypercoagulation are both triggers and consequences of each other. We found the decreased activity of TF and increased levels of TF in both hypothyroid and hyperthyroid groups. These results can be a compensation mechanism of the organism for the irregularities in the coagulation. Also, it may be a hemostatic mechanism to protect these organs. This situation may indicate a tendency to hypercoagulation in thyroid dysfunction. In this case, we can conclude that coagulation abnormalities can be developed and coronary artery diseases might be triggered by hypo- and hyperthyroidism.

Conflict of Interest: Authors state that there are no conflicts of interest in the manuscript, including financial, consultant, institutional, and other relationships that might lead to bias or a conflict of interest.

Ethics approval: The study was approved by the Ethics Committee of the Marmara University (71. 2009. mar).

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Original Article

The Relationship Between Temporomandibular Joint Disorder Level and Clinical Parameters in Patients with Fibromyalgia Syndrome

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Abstract

Introduction: The aim of this study was to investigate the relationship between fibromyalgia disease activity and temporomandibular joint disorder (TMD) level in patients with fibromyalgia, as well as TMD-related symptoms of cervical muscle endurance, cervical pain, anxiety and kinesiophobia.

Method: The study comprised 50 participants, consisting of 3 males and 47 females, all diagnosed with TMD and fibromyalgia. The severity of TMD was gauged through the Fonseca Amnestic Questionnaire (FAQ), while the functional status of the subjects was evaluated using the Fibromyalgia Impact Questionnaire (FIQ). Cervical muscle endurance was measured via the Cervical Flexor Muscle Endurance Test (CFMET). Additionally, cervical pain, kinesiophobia, and anxiety levels were assessed utilizing the Visual Analog Scale (VAS), Tampa Scale for Kinesiophobia (TSK), and Beck Anxiety Scale (BAS), respectively.

Results: A statistically significant positive correlation was observed between the level of TMD and the functional status of the patients ($p < 0.05$). Conversely, no statistically significant correlation was discerned between TMD and the variables of VAS, CFMET, TSK, and BAQ ($p > 0.05$). Notably, a significant correlation was detected between TMD and the disease activity among fibromyalgia patients exhibiting TMD. However, no statistically significant correlation was identified between head and neck complications, kinesiophobia, and anxiety within the fibromyalgia patient cohort.

Conclusion: Although it is recognized that these clinical parameters in the context of fibromyalgia do not depend solely on the level of TMD, it is imperative that they be evaluated collectively when evaluating the patient.

Keywords: Anxiety, fibromyalgia, kinesiophobia, pain, temporomandibular disorder.

1. Introduction

Fibromyalgia syndrome (FMS) is a syndrome characterised by chronic widespread pain and multiple symptoms, including fatigue, sleep disturbances, cognitive dysfunction and depressive episodes. It occurs in all populations worldwide,

with a prevalence of 2% to 4% in the general population (1). The prevalence is higher in women and increases with age (2). Common disorders associated with FMS include chronic fatigue syndrome, irritable bowel syndrome (IBS), irritable bladder syndrome or interstitial cystitis, and temporomandibular disorder (TMD) (3).

TMD encompasses a spectrum of pathological conditions characterized by pain, limited jaw mobility, and tenderness in the temporomandibular joint and/or associated masticatory muscles, often extending to the preauricular region (4). This disorder represents a musculoskeletal dysfunction of the masticatory system, impacting over a quarter of the general population (5). Moreover, TMD frequently co-occurs with various symptoms affecting the craniofacial and cervical regions, including headaches, otologic symptoms, and cervical spine manifestations (6). Investigations have explored the interrelation between masticatory motor system pain and cervical spine disorders, suggesting a bidirectional causative relationship (7,8). Notably, elevated levels of kinesiophobia have been observed in both painful and painless TMD cases (9). Cluster analyses employing the Tampa Scale for Kinesiophobia (TSK) have delineated subgroups within TMD cohorts characterized by heightened kinesiophobia, often concomitant with elevated anxiety levels (10). Chronic TMD patients frequently report symptoms indicative of psychological distress, including depression, anxiety, poor sleep quality, and low energy (11).

FMS and TMD have some common clinical characteristics, encompassing prolonged duration, incompletely elucidated pathophysiology, significant physical and psychological ramifications, and common predisposing factors (12). It is postulated that there may exist shared underlying mechanisms contributing to pain in both TMD and FMS (13). FMS is linked with various comorbidities, including chronic and widespread musculoskeletal pain, depressive symptoms, sleep disturbances, heightened stress responses, and central sensitization, which serve as predisposing and precipitating factors for TMD and elucidate the elevated incidence of TMD signs and symptoms among individuals with FMS (14,15).

Given the elevated occurrence of TMD among FMS patients, the recognition of TMD indicators and manifestations should be integrated into the diagnostic framework for FMS to improve pain management in these patients. Nevertheless, research investigating the correlation between TMD severity and clinical parameters characteristic of TMD among individuals with FMS remains limited. Hence, the primary objective of this study was to explore the association between TMD severity and fibromyalgia disease activity, alongside assessments of cervical muscle endurance, cervical pain, anxiety, and kinesiophobia, within the FMS patient population.

2. Methods

This study was conducted with 50 patients between aged between 18 and 65 years, all of whom had received a clinical diagnosis of fibromyalgia. Prior to the study, ethical approval was obtained from Uskudar University Non-Interventional Studies Ethics Committee with decision number 61351342/September 2021-39. Patients were informed before the study and signed an informed consent form.

Inclusion criteria encompassed individuals aged 18 to 65 years, previously diagnosed with FMS, and exhibiting symptoms indicative of TMD, as evidenced by a Fonseca Anamnestic Index (FAI) score exceeding 15 points (16).

Exclusion criteria included individuals with a history of cardiovascular, pulmonary, neurological or psychiatric disorders, malignancy, pregnancy, or difficulties in cooperation.

2.1. Data Collection Tools

The socio-demographic data of the individuals participating in the study was recorded by taking detailed anamnesis face-to-face. The presence and levels of TMD were determined with the Fonseca Amnestic Questionnaire (FAQ). FAQ is a questionnaire developed by Fonseca et al. in 1994 to classify TMD (17). The Turkish validity and reliability study of the questionnaire was

conducted by Kaynak et al. in 2018 (18). The questionnaire consists of 10 questions answered as yes, no or sometimes. The total score is calculated as 10 points for yes, 5 points for sometimes, and 0 points for no. A score range of 0-15 points indicates no TMD, 20-40 points indicates mild TMD, 45-65 points indicates moderate TMD, and 70-100 points indicates severe TMD (17).

Fibromyalgia Impact Questionnaire (FIQ) was used to assess the functional status of the participants. The FIQ is a 21-question questionnaire that assesses how patients' pain status has affected their activities of daily living, physical functioning, social life, sleep quality, pain status, and mood in the last 1 week. The maximum score is 100, and a score of 70 and higher indicates severe fibromyalgia (19). The questionnaire was developed in 1991 and a Turkish validity and reliability study was conducted (20).

The cervical muscle endurance was evaluated with the Cervical Flexor Muscle Endurance Test (CFMET). Patients were asked to lie supine, with hands on the abdomen, in a resting position, with their heads in a chin-tuck position, and perform an isometric contraction. The assessor placed his/her hand on the occiput of the patient. The patient was asked to hold the head 2.5 cm above the resting position while maintaining the chin-tuck position, and the duration of the head elevation was recorded. The test was terminated when the chin-tuck position was broken. The test was repeated twice, and a 5-minute rest time was given between each test. The mean of the two tests was recorded in seconds (21).

The Visual Analogue Scale (VAS) was used to assess pain in the cervical region. A 10-cm-long straight line is drawn, and "I have intense pain" and "I have no pain" are written at the beginning and end of the study, respectively. The side without pain is considered "0," and the side with pain is considered "10.". The patient was asked to mark the most appropriate place for the pain condition. The distance from the marked place to the starting point was measured with a ruler and recorded (22).

TSK, which was developed in 1991 and has Turkish validity and reliability, was used to measure the fear of movement or injury in individuals. The scale consists of 17 questions, and 4-point Likert scoring is used (1=strongly disagree, 4=completely agree). The total score is calculated by scoring the questions in the 4th, 8th, 12th, and 16th items in reverse order. A high score on the scale indicates a high severity of kinesiophobia (23, 24).

Beck Anxiety Scale (BAS) was used for anxiety assessment. The degree of anxiety is determined according to the total score. Each question in the BAS is scored between 0 and 3 (0=Not at all, 3=I had a lot of difficulty) It is a 4-point Likert-type scale. It consists of 21 questions. According to the score obtained, a 0–17 point range indicates low severity, an 18–24 point range indicates moderate severity, and 25 and higher scores indicate a high degree of anxiety (25). Turkish validity and reliability were performed by Ulusoy et al. (26).

2.2. Statistical Analysis

IBM SPSS 26 package programme was used for data analysis. "Kolmogorov-Smirnov Test" was performed to test whether the data of the research variables show normal distribution. Frequency analysis was performed to determine the demographic characteristics of the participants in the study, the characteristics of the answers given to the scales used in the study and the distribution of the participants according to the TMD categories. Spearman Correlation Analysis was conducted with the purpose of elucidating potential correlations between the level of TMD and variables such as VAS scores and cervical muscle endurance. Pearson Correlation Analysis was performed to determine whether there was a relationship between TMD level and kinesiophobia, fibromyalgia and anxiety levels. Significance level $p<0.05$ was accepted.

3. Results

Demographic characteristics and TMD levels of the individuals who participated in the study are shown in Table 1.

The standard deviation, median, minimum and maximum values of VAS, TSK, FIQ, FAQ BAS and CFMET were shown in Table 2.

As seen in Table 3, a positive and moderately statistically significant relationship was found between TMD level and fibromyalgia level (r=.330; p<0.05) There was no statistically significant correlation between the level of TMD and cervical muscle endurance, cervival pain, kinesiophobia, anxiety levels.

Table 1. Demographic characteristics of study participants (N=50)

Variables	Mean ± sd (min max) N (%)	
Age		41,38 ±1,63 (24-62)
Gender	Male Female	3 (6) 47 (94)
Mild TMD (20-40 Points)		7
Moderately(45-65 Points)		23
Severe (70-100 points)		20

Table 2. General information on research variables

Assessments	Mean ± SD	Median	Min.	Max.
VAS	6,72 ±,23	7	3	10
TSK	44,52 ±78	44,5	29	55
FIQ	63,32±2,32	59	17	96
FAQ	63,60±2,59	65	25	95
BAS	22,70±0,51	23	16	29
CFMET (sec)	20,0±,45	19	14	28

SD: Standard Deviation, sec: second

Table 3. Comparison of the relationship between TMD level and research variables

	r	P
FIQ	0,330*	0,019
VAS	0,271**	0,057
TSK	0,122*	0,322
BAS	0,119*	0,41
CFMET	-0,127**	0,378

FIQ: Fibromyalgia Impact Questionnaire; VAS: Visual Analogue Scale; TSK-SV: Tampa Scale for Kinesiophobia questionnaire; BAS: Beck Anxiety Scale; CFMET: Cervical Flexor Muscle Endurance Test; p<0.05 statistical significance level **r: pearson coefficient*; ***rho: spearman coefficient*

4. Discussion

The findings of this study, undertaken among individuals diagnosed with TMD concomitant with FMS, revealed a notable association between the severity of TMD and the activity level of fibromyalgia disease. However, no statistically significant correlations were observed between the degree of TMD severity and other parameters examined, including cervical muscle endurance, pain intensity, kinesiophobia, and anxiety levels.

FMS is more common in middle-aged women than in men, and most commonly between the ages of 20 and 50 (27). Similar to the study in the literature, 94% of the 50 individuals diagnosed with fibromyalgia in the present study were women with a mean age of 41.38±1.63 years.

Considering that the most common symptoms in patients with FMS with a high frequency of TMD are muscle pain, temporomandibular joint pain, and muscle tenderness on palpation, it is thought that fibromyalgia syndrome may be an etiological or aggravating factor for TMD or may represent a general vulnerability to pain disorders (28). In the present study, the cervical muscle pain of the participants was questioned with VAS, and the mean score was 6.72±0.23. A low-level correlation close to statistical significance was found between TMD level and pain.

Kinesiophobia is defined as the fear of avoiding movement due to pain caused by trauma and the possibility of experiencing pain again (29).When the literature is reviewed, there are many studies showing that fibromyalgia syndrome causes kinesiophobia in patients (30–32). In another study in which it was investigated that TMD also causes kinesiophobia, it was also reported that TMD patients developed fear of movement due to problems such as limitation of movement in the head and neck region and locking in the jaw (33). No study investigating kinesiophobia in patients with fibromyalgia syndrome with TMD was found in the literature. In the present study, the mean total score of the participants on the kinesiophobia scale was 44.52±.078. No significant relationship was found between TMD and the kinesiophobia levels of the participants.

In the literature, there is no study on the relationship between TMD and cervical deep flexor muscle endurance in patients with fibromyalgia syndrome. In one study, cervical endurance was evaluated with the test used in the present study, and a decrease in endurance was found in participants with neck pain (34). In another study, cervical endurance was evaluated in individuals with TMD, and it was found to be affected (35). In the present study, the duration of keeping the head 2.5 cm above the resting position while maintaining the chin-tuck position was measured, and the mean was found to be 20±0.45 seconds. No significant correlation was found between TMD levels and the duration of neck elevation.

FMS is characterised by widespread and chronic musculoskeletal pain throughout the body, which may include pain in the masticatory muscles and temporomandibular joints in some patients, and these patients are defined as having temporomandibular disorders (36, 37). In the literature, there is a high prevalence of TMD in patients with FMS. Albayrak et al. found the prevalence of TMD in individuals with fibromyalgia to be 83.8%, and Erbaşar Hasanoğlu et al. found the prevalence of fibromyalgia in patients with TMD to be 60% (38, 39). This study included fibromyalgia patients with TMD, and 14% of patients with fibromyalgia had mild, 46% had moderate, and 40% had severe TMD. It was also found that those with severe TMD had higher fibromyalgia disease activity, and the level of fibromyalgia increased as the level of TMD increased.

5. Conclusion

The results suggest that there is a relationship between TMD and FMS, that the prevalence of TMD is high in patients with FMS, and that signs and symptoms of TMD should be considered in the diagnosis of FMS to improve pain management in these patients.

Limitations

This study is subject to some limitations; The most important of these is the inclusion of a limited number of studies on fibromyalgia patients diagnosed with TMD. Large-scale studies are recommended to ensure broader applicability of the findings. Moreover, while the FAQ serves as a preliminary diagnostic tool in determining the presence of TMD, a definitive diagnosis by a qualified dentist will increase the robustness of the study. Additionally, the lack of inquiry regarding the duration of TMD symptoms among the patients participating in the study constitutes another limitation that could potentially affect the study results.

Conflict of Interest: Authors state that there are no conflicts of interest in the manuscript, including financial, consultant, institutional, and other relationships that might lead to bias or a conflict of interest.

Ethics approval: The approval of this study, which was conducted in accordance with the ethical rules communicated in The World Medical Association (WMA) Declaration of Helsinki, was approved by the Uskudar University Non-Interventional Research Ethics Committee with the decision dated September 2021-39 and numbered 61351342

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Original Article

The Effects of High-Dose Whey Protein Concentrate Intake on Hepatorenal and Intestinal Tissues

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Abstract

Introduction: This study aims to compare the antioxidant effects of high doses of whey protein (WP) concentrate intake on liver, kidney, and intestinal tissues.

Methods: 18 rats were divided into three groups control (n=6), control+ 8 g/kg WP (n=6), and control + 2 g/kg WP (n=6). 8 g/kg WP group was fed with whey protein added rat chow. 2 g/kg WP group, addition to their standard ad libitum feed, received the whey protein concentrate by oral gavage. On day 10, liver, kidney and intestinal tissues were removed. Lipid peroxidation, glutathione (GSH) levels, superoxide dismutase (SOD) and glutathione-S- transferase (GST) activities were determined in liver, kidney and intestine tissues.

Results: GSH, SOD, and GST activities increased in whey protein-administered groups. Liver glutathione level was higher in the 2 g/kg WP compared to the 8 g/kg WP group. There were no significant differences in intestinal glutathione levels between the groups. Kidney GST activity decreased in the kidney and intestine tissues of the 8 g/kg WP group compared to the 2 g/kg WP group. SOD activity was higher in all tissues in the 2 g/kg WP group compared to the other groups.

Conclusion: As a result, both whey protein treatments showed different antioxidant effects in the tissues examined. High-dose whey protein application showed lower antioxidant capacity compared to the optimal whey protein dose.

Keywords: High dose whey protein, antioxidant activity, liver, kidney, intestine

1. Introduction

Whey proteins are a group of highly beneficial proteins derived from the liquid component of milk that separates during cheese production. These proteins hold considerable importance due to their amino acid composition, which gives them

the highest biological value among all protein sources (1). Whey protein-rich diets offer protection from some human diseases; however, there is no study on whether their effect changes when taken with food. Whey proteins are not only an excellent source of high-quality nutrients but are also quickly absorbed and utilized by the human body. They provide numerous health benefits, such as promoting muscle growth, supporting weight management, reducing inflammation, and improving digestion (2).

Whey proteins have a high level of radical scavenging action, which can be attributed to the numerous peptides included in them. Previous research has shown that whey proteins can inhibit oxidative damage in the iron-catalyzed liposome oxidation system (3). One of the advantages of using whey protein as a source of antioxidants is its high bioavailability. Whey protein is easily absorbed by the body and can be quickly transported to cells, where it can exert its antioxidant effects (4). They have rich cysteine amino acid content, which has a thiol group that interacts with glycine and glutamate to make GSH, the principal antioxidant of the cells. GSH detoxifies toxic substances such as toxic metals, lipid peroxides, and prostaglandins. Cysteine controls GSH concentrations; hence, supplementing the diet with whey protein high in cysteine can increase GSH production (5). Whey proteins are digested more slowly than casein and remain in the intestines for a longer period compared to casein. As a result, whey protein is more beneficial to the body and has the potential to promote human health, and it may be employed as a treatment method for oxidative stress-related disorders (6).

A high-protein diet can help to lose weight and improve insulin sensitivity, but it's unclear if the kind of protein to be taken affects these benefits. The high dose whey proteins usage may have negative effects on health. The negative consequences include a rise in acne, microbiota malfunction, and changes to the kidneys' normal metabolic process (7). Therefore, it is crucial to reasearch how different doses of whey proteins affect particular tissues. In this study, the antioxidant effects of high-dose whey protein application on liver, kidney and intestinal tissues were investigated.

2. Methods

2.1. Preparation of Whey Protein Added Rat Chow

Rat chows were pulverized using a laboratory grinder. 100 g of powdered chow was mixed with 200 mL of whey protein concentrate (Tazelen, Kaanlar Food Industry and Trade, Turkey). The mixture, which was brought to the consistency of dough, was given its first shape. The prepared chow was dried using a lyophilizer at -50^{o}C. 158 g of chow containing 20 g of protein was obtained. This dried chow was given to the rats. The feed consumption of the rats was monitored.

2.2. Animals and Experimental Design

Sprague Dawley rats (male, 200–300 g, n=18) were kept in standard animal laboratory conditions with light and dark (12h/12h) cycles. Control group rats (n=6) were fed with standard rat chow and tapped water ad libitum. Control+whey with chow (8 g/kg WP) group rats (n=6) were fed with prepared whey protein added chow and tap water ad libitum. 2 g/kg WP group rats (n=6) were fed with rat chow tap water ad libitum and also was given whey protein concentrate (2 g/kg).

2.3. Determination of the Lipid Peroxidation and Glutathione (GSH) Levels

Hepatorenal and intestinal malondialdehyde (MDA) levels were determined by the method of Ledwozyw for the detection of lipid peroxidation (8). In summary, the compound that is produced by boiling tissue homogenate with thiobarbituric

acid is extracted using n-butanol. The variance in optical density at 532 nm is calculated using the MDA level which serves as an indicator of lipid peroxidation. The results were presented as nmol MDA/g tissue. The GSH level in these tissues was measured by the method of Beutler (9). The process involves reducing Ellman's reagent with SH groups to generate 5,5′-dithiobis (2-nitrobenzoic acid), which has a yellow color and is measured spectrophotometrically at 412 nm.

2.4. Determination of the Superoxide dismutase (SOD) and Glutathione-S-Transferase (GST) Activities

Superoxide dismutase (SOD) activity was determined by the capacity of riboflavin-sensitized o- dianisidine to increase the rate of photooxidation (10). 2.8 mL 50 mM potassium phosphate (pH = 7.8) with 0.1 mM EDTA, 0.2 mM riboflavin in 10 mM potassium phosphate (pH 7.5), 0.1 mL 6 mM *o*-dianisidin, and tissue extract were mixed. Cuvettes with all their components were illuminated with 20-W Slylvania Grow Lux fluorescent tubes maintaining the temperature of 37°C. Absorbance was measured at 460 nm and the result expressed in U SOD per g tisssue. Glutathione-S-transferase (GST) activity was determined by measuring the absorbance at 340 nm of the product produced by the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) (11). Glutathione and 1-chloro2,4-dinitro-benzene conjugation product was measured at 340 nm. The GST activity was calculated using the extinction coefficient (9.6 mM-1 cm-1) obtained for the compound produced by the conjugation of glutathione and 1-chloro-2,4-dinitrobenzene. The results were expressed in U/g tissue.

2.5. Statistical Analysis

Analysis of variance (ANOVA) and Tukey, multiple comparison tests, were carried out using GraphPad Prism 9.0 (California, USA). $p < 0.05$ was considered significant.

3. Results

MDA levels in all tissues did not change significantly with whey protein applications in all groups (Fig. 1). Glutathione levels increased in liver and kidney tissues but did not change in the intestine tissue both in the 8 g/kg WP and 2 g/kg WP groups compared to the control group. Liver GSH level was also significantly higher than the 8 g/kg WP group in the 2 g/kg WP group (Fig. 2). Liver and intestine SOD activities increased both in the 8 g/kg WP and 2 g/kg WP groups compared to the control group. In the 2 g/kg WP group, intestinal SOD activity was also significantly higher than the 8 g/kg WP group (Fig. 3). GST activity did not significantly change in liver tissue in all groups. Kidney and intestine GST activities decreased in the 8 g/kg WP groups compared to the control group. In the 2 g/kg WP group, intestinal GST activity was also significantly higher than in the 8 g/kg WP group (Fig. 4).

4. Discussion

This study aims to investigate the antioxidant effects of high-dose whey protein concentrate on the liver, kidney, and intestinal tissues of healthy rats. Whey proteins have antioxidant characteristics due to their high concentrations of bioactive peptides and amino acids, including cysteine, methionine, and glutamine. These components are thought to defend against lipid peroxidation via different pathways.

Adding 1% whey protein hydrolyzate to the beverage has been shown to increase the antioxidant activity of the beverage (12). Mann et al. revealed that flavoured milk drinks fortified with 1% or 2% whey protein hydrolysate showed antioxidant activity (13). Contrary to this Garcia-Casas et al. reported that adding whey protein to a beverage rich in polyphenols with antioxidant

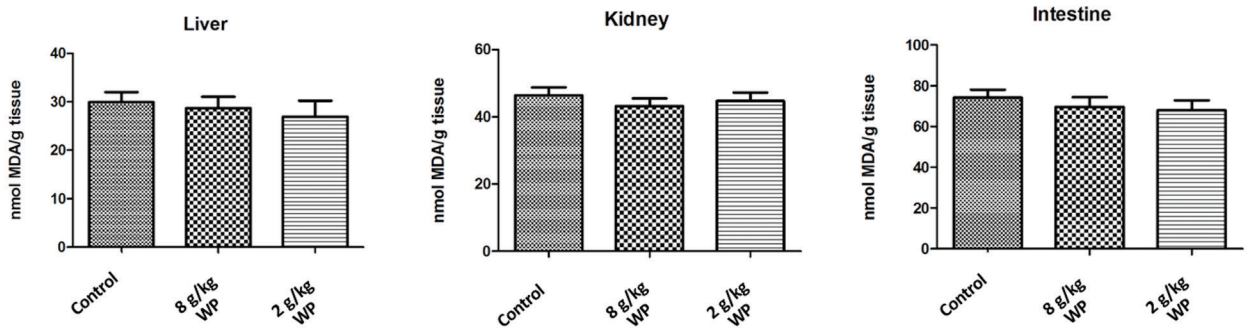

Figure 1: Malondialdehyde levels of liver, kidney and intestine tissues

MDA: Malondialdehyde, **WP:** Whey protein

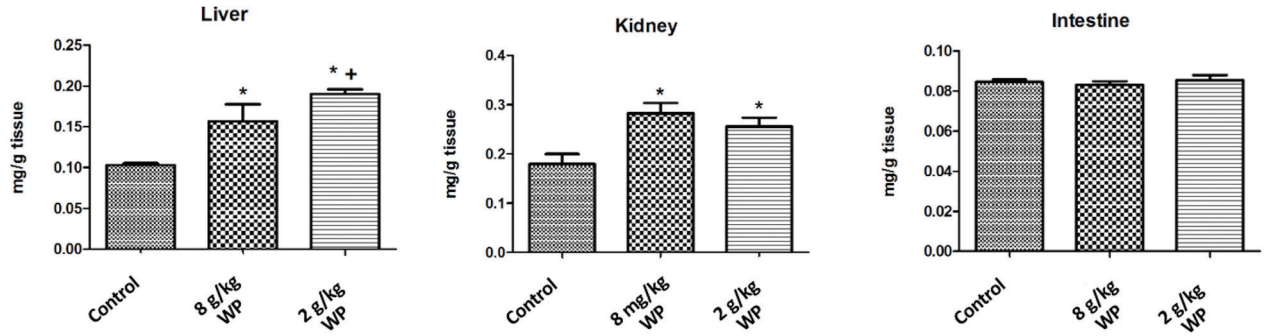

Figure 2: Glutathione levels of liver, kidney and intestine tissues

GSH: Glutathione, **WP:** Whey protein

*: p<0.05 compared to control group

+: p<0.05 compared to 8 mg/kg whey protein group

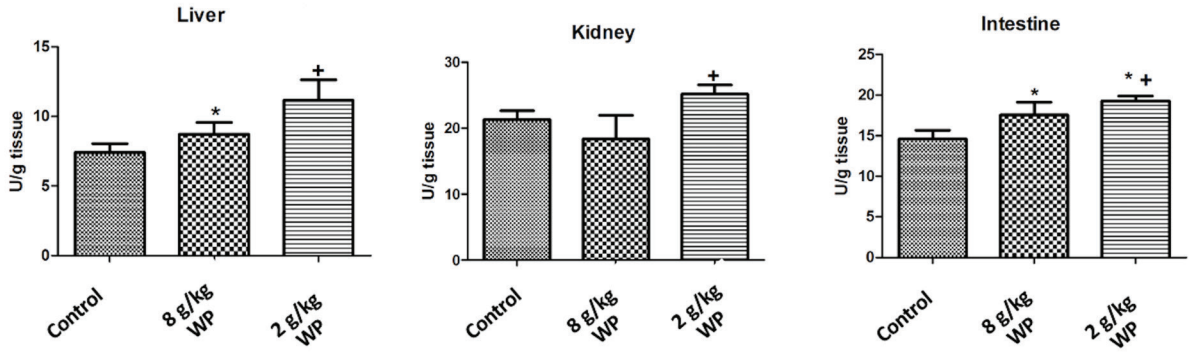

Figure 3: Superoxide dismutase activity of liver, kidney and intestine tissues

SOD: Superoxide dismutase, **WP:** Whey protein

*: p<0.05 compared to control group

+: p<0.05 compared to 8 mg/kg whey protein group

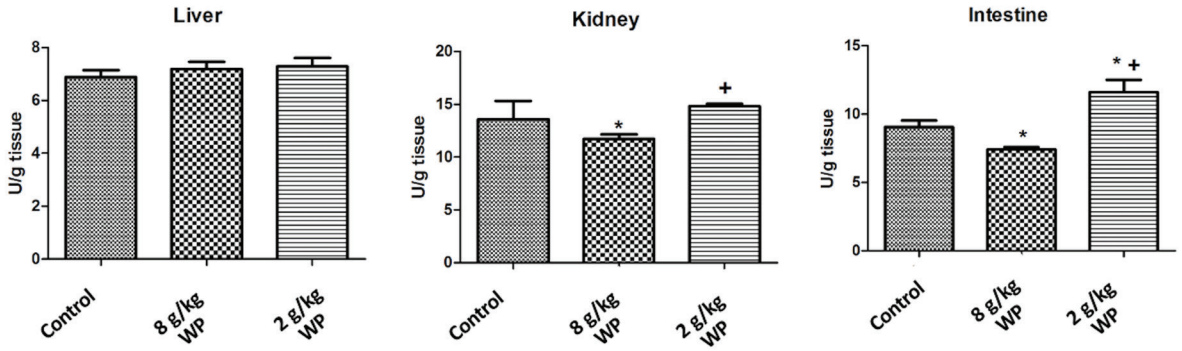

Figure 4: Glutathione-S-transferase activity of liver, kidney and intestine tissues

GST: Glutathion-S-transferase, **WP:** Whey protein

*: $p<0.05$ compared to control group

+: $p<0.05$ compared to 8 mg/kg whey protein group

properties does not create additional antioxidant activity (14). The optimal dosage of whey proteins that should be taken depends on the objectives of individuals, the amount of physical activity, and the composition of the body. However, some studies suggest that a dose of 20 to 25 g of whey protein per day is sufficient to achieve the proposed benefits, whereas doses above 40 g/day were associated with adverse effects on the organism (7). The optimal dose of whey proteins needs to be detected with experimental and scientific data. Experimental animal models and biochemical and histological examination of tissues may also provide valuable insights into the potential effects of whey protein administration.

Whey protein or its hydrolysates have been found to reduce lipid peroxidation caused by exercise, alcohol use, or diabetes (15). Furthermore, peptides derived from whey proteins have antioxidant activity as they can bind pro-oxidant metals or directly can scavenge free radicals (16). Whey protein's efficacy in preventing lipid peroxidation appears to be regulated by parameters such as concentration, degree of hydrolysis, and oxidative state. Since peptides with lower molecular weight can easily bind to lipids, it is hypothesized that they may have higher antioxidant potential (17).

In the present study, high dose whey protein administration did not increase lipid peroxidation in liver, kidney and intestinal tissues compared to the control group. While liver and kidney GSH levels increased with whey protein application, no change was detected in intestinal GSH levels. This result suggests that high doses of whey proteins are less effective on intestinal tissue compared to their effects on the liver and kidney. Liver and intestine SOD activities increased with both doses of whey proteins, but renal SDO activity did not change compared to the control group. Low-dose whey protein increased SOD activity in all tissues compared to high-dose whey protein. Liver GST activity did not change in all groups. Kidney and intestine GST activities decreased with the administration of high dose of whey protein compared to the control group. In low dose whey protein group, intestinal GST activity was also significantly higher than in the high-dose whey protein group.

5. Conclusion

In conclusion, a high dose of whey protein did not show the expected high antioxidant activity. It has

been determined that whey protein consumed at the optimum dose increases the antioxidant activity in the liver, kidney and intestinal tissue better than the high dose.

Conflict of Interest

The authors have no conflict of interest to disclose.

Ethics Approval

The experimental protocol of this study was approved by the Marmara University Animal Care and Use Committee (Approval date: May 26, 2021, Approval code: 502021.mar).

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