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AIMS

EURAS Journal of Health (EJOH) is a peer-reviewed international scientific open access periodical published in accordance with independent, unbiased, and double-blind peer-review principles. It publishes two issues per year. The publication language of the journal is English. The journal is the official publication of the Eurasian Universities Union (EURAS).

EJOH aims to contribute to the literature by publishing manuscripts of highest scientific level in all fields of health including medicine with clinical and basic fields, nursing, physiotherapy, audiology, nutrition and dietetics, dentistry, public health, epidemiology, and all relevant disciplines.

SCOPE

The journal targets all healthcare professionals in all health disciplines and publishes original experimental and clinical research articles, case reports, reviews of experts in a specific field, letters to the editors and brief reports on new methods or techniques or preliminary results of original studies. The journal conforms to the Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice).

From The Editor

Welcome to the 2nd issue of EURAS Journal of Health (EJOH)!

As we all know about the current crisis all over the world due to the ongoing COVID-19 pandemic, we included in this issue a splendid review on COVID-19, in addition to valuable research articles in pharmacology, oncology, dentistry, nutrition and gynecology. Thanks to all our authors, reviewers and editors.

We continue to invite and welcome works from our international colleagues and look forward to the future submissions.

Thanks for joining us.

With best regards,
Prof. Zeynep Çiğdem KAYACAN, M.D.

Review Article

Nanotechnology and COVID-19: Potential Application for Treatment

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Abstract

The novel coronavirus (2019-nCoV) emerged in China at the end of 2019 and then spread worldwide, particularly to Italy, Spain, the USA, and Iran. Currently, Coronavirus Disease 2019 (COVID-19) is a main public health issue. As of April 21, 2020, more than two million confirmed cases of COVID-19 with more than 170,000 deaths have been reported in 210 countries by WHO. The 2019-nCoV can be spread by direct contact or droplets between humans and shows great potential for a pandemic. At present, there is no particular antiviral therapy for 2019-nCoV-infected persons. However, a wide range of therapeutic agents are being examined. The capabilities inherent to nanotechnology hold a large guarantee in presenting innovative approaches in the field of COVID-19 prevention, diagnosis, and cure. We in this article discuss how nanotechnology can improve the treatment of persons infected with the COVID-19 virus.

Keywords: *2019-nCoV, COVID-19, Coronavirus, Nanotechnology.*

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Introduction

The novel coronavirus disease (2019-nCoV), called by the World Health Organization (WHO) as Coronavirus Disease 2019 (COVID-19), was first reported on December 31, 2019 (Wuhan, China) (1). Compared to the other known coronaviruses like as the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV), the mortality rate of COVID-19 is considerably lower; however, it is more transmissible (2), so far, it has spread to many countries around the world, infected more than two million people, and it has led to the death of tens of thousands of people. Coronaviruses are a big family of viruses that attack the upper and lower respiratory tracts in humans, leading to a range of disease from the common cold to very severe, even fatal forms. But, it is the third time in the 21st century that a coronavirus explosion turns into a global health emergency. More than hundreds of coronaviruses have until been known, most of that is transferable between animals like pigs, camels, and bats, however, in several cases, a genetic mutation is all these viruses require to spread to humans (3). So far, seven coronaviruses have been diagnosed with human diseases, four of which are NL63, OC43, 229E, and HKU1, which cause moderate to severe disease, and the other three are even fatal (4). The first was SARS-CoV, which appeared in late 2002 and disappeared in 2004; the second is MERS-CoV, which appeared in 2012 and yet spread between camels (2, 5); and the third is SARS-CoV-2 that causes COVID-19 – first reported in China and according to numerous researchers, is leading the world toward the grips of a pandemic (6). The SARS-CoV-2 virus is in the form of spheres with a diameter of 125 nm, with lipid-based viral envelopes and positive-sense single-stranded RNA genomes. The SARS-CoV-2 virus has four kinds of structural proteins: envelope (E), spike (S), nucleocapsid (N), and membrane (M) proteins (6, 7), which the S protein has a vital role in connecting the virus to its host's cells and facilitating it to penetrate the cells (5). The most frequent symptoms and signs of COVID-19 are fever, cough, and dyspnoea. After a week or more, it could cause shortness of breath, about 20% of the patients requiring hospital cure. In many patients, particularly the elderly and those with chronic health conditions, the early signs could develop to pneumonia, with chest tightness, and shortness of breath (4). On the other hand, the quickly increasing fatality tolls of COVID-19 have been an alarm for global health. Many researchers have lately turned their focus to this growing threat and a global attempt is underway to stop its spread. At present, there is no particular antiviral treatment accessible for COVID-19, however, a broad range of pharmaceutical agents are being examined. Nanotechnology holds a large guarantee in presenting innovative approaches to a broad range of problems regarding the prevention, diagnosis, and cure of COVID-19, in which nanotechnologists certainly play a vital role and bear their social responsibility. In the meantime, among a variety

of fields of technology and science, nanotechnology has huge probability to be of massive help in prevention, diagnosis, and cure of COVID-19. At prevention step, nano-fiber based facial respirators, with the help of nanotechnology-enabled extremely effectual antimicrobial and antiviral disinfectants have been the first personal protective ways that could prevent the extension of the virus; also, widespread research is underway to expand a vaccine for COVID-19 based on different nano-materials. In diagnostics, nanotechnology has shown significant promise in designing sensors for developing rapid-response COVID-19 tests. Last but not least, at the curing phase, nano-medicines have been at the core of many researchers' attention, several of that are at present being studied in clinical trials. Given the wide abilities of nanotechnology, it is probable that innovations in this field might have an important effect on problems associated with COVID-19. This review briefly addresses the potential application of nanotechnology for the treatment of COVID-19 disease.

Entry mechanism and replication of Coronavirus

Coronavirus spike glycoprotein has been shown as an important determinative of virus entry into host cells (2). The SARS-CoV and SARS-CoV-2 spike glycoproteins bind to the angiotensin-converting enzyme II (ACE2) cellular receptor (8, 9), also, SARS-CoV and MERS-CoV binds CD209L and dipeptidyl peptidase 4 (DPP4) cellular receptors respectively (10, 11). It is recognized that SARS-CoV entry into cells is mainly achieved by direct membrane fusion between the virus and the membrane (12). An important proteolytic cleavage event at the SARS-CoV S protein at position (S20) has been reported to mediate membrane fusion and viral infection (13). The MERS-CoV as well as the development of abnormal two-phase furin activation for membrane fusion (14). In addition to membrane fusion, clathrin-dependent and -independent endocytosis also mediates SARS-CoV entry (15). Once the virus enters the cells, the genome is released into the cytoplasm space host and translates into two structural proteins and polyproteins, which then begin to replicate the viral RNA genome (16). Delayed envelope glycoproteins are inserted into the membrane of the endoplasmic reticulum or Golgi and are nucleocapsidis created by a mixture of genetic and nucleocapsid protein. After that, the viral cells germinate into the endoplasmic reticulum-Golgi intermediate compartment. Eventually, vesicles, including virus cells, coalesce with the membrane to release the coronavirus (2). The 2019-nCoV employed the same cell entry receptor ACE2 as the SARS-CoV. Peng Zhou et al. showed that 2019-nCoV is capable to employ all ACE2 proteins, but no ACE2 mouse, as an entry receptor to enter into cells with ACE2 expression, but not cells which did not express ACE2, demonstrating that ACE2 is almost the cell receptor through which 2019-nCoV enters the cell. The COVID-19 binds to ACE2 via S-protein on its surface. During infection, the S-protein is divided into S1 and S2. S1 subunit includes the receptor-binding domain that lets coronaviruses to

straightly bind to the peptidase domain of ACE2. S2 then probably has a function in membrane fusion. Peng Zhou et al. also showed that 2019-nCoV does not employ other coronavirus receptors, like DPP4 and aminopeptidase N (7).

COVID-19 Challenge

Several issues make 2019-nCoV mainly worrying. Being a novel virus, there is no acquired immunity; 40 candidate vaccines are at present in the study phase, but specialists agree that no extensively utilizable vaccine will be accessible for at least 12 to 18 months. The case-fatality rate, that by meaning is computed only based on identified patients and is so presently hard to estimate correctly, appears to be about 4%. Here are some reasons why the 2019-nCoV appears to be a threat. First, it can kill older people with existing health problems as well as healthy ones. The information up to now shows that the virus has a fatality risk of about 4%; this rate is many times more severe than usual seasonal influenza. Second, COVID-19 is relatively efficiently transmitted (17). The average infected person spreads the disease to three or four others. That's an exponential growth rate. There is as well strong proof that it could be spread by persons who are just mildly disease or not even showing signs. This represents a challenge because it would be hard to identify patients that need to be tested for the disease because they have no symptoms or signs, but their capacity to transmit the disease would permit for amplification in an uninfected population [18]. This means 2019-nCoV will be more difficult to contain than SARS, which was only transmitted by those showing signs and were much fewer proficiently transmitted (17). So, rapid treatments are vital interventions for COVID-19.

Current treatments of COVID-19 and their limitations

As mentioned above, there is no special antiviral treatment for COVID-19. Scholars are attempting to find therapeutics to treat patients with this infection. Researchers so far have examined several drug candidates, from different classes, including protease inhibitors, nucleoside analogs, neuraminidase inhibitors, polymerase inhibitors, and DNA synthesis inhibitors, that may have potential efficacy for treatment of COVID-19 (19, 20). Currently, several drugs including ritonavir/lopinavir, darunavir, emtricitabine/tenofovir, ruxolitinib, remdesivir, favipiravir, and chloroquine are undergoing clinical trials to evaluate their efficacy to treat COVID-19 and have been achieved several promising results so far (Table1). Lim et al. showed that using lopinavir/ritonavir in patients with COVID-19 causes a reduction in viral loads, therefore improving clinical symptoms during the treatment (21). Recently, Wang et al. showed in vitro which chloroquine and remdesivir were highly efficient in control of COVID-19 (22). The Clinical Medical Research Center of the National Infectious Diseases and the Third People's Hospital of Shenzhen initiated a clinical trial on favipiravir to treat COVID-19 on February 14, 2020. Their results showed favipiravir had

more strong antiviral activity than that of ritonavir/lopinavir on patient with COVID-19.

Also, Chen et al. reported that favipiravir, compared to arbidol, does not significantly improve rate of clinical recovery at day 7. In this clinical trial, favipiravir showed remarkably improvement in time-to-relief for cough and fever (23).

In recent days, an international team reported that human recombinant soluble ACE2 (hrsACE2) can remarkably inhibit primary stages of SARS-CoV-2 infection. Angiotensin-converting enzyme 2 (ACE2) has been reported as an entry receptor for SARS-CoV-2 infection and it has been proposed which blocking this interaction might be used in treatment of persons with COVID-19 infection (24).

Table 1. Current therapeutics used in treatment of COVID-19.

Candidate Therapeutics	Class	Mechanism of Action	Currently being trialled COVID-19?
Corticosteroids	Steroid hormones	Inhibition of the effector function of Th2 cells, eosinophils, and epithelial cells (25)	Yes NCT04244591
Chloroquine	Heme polymerase inhibitor, Antimalarial agent	Inhibition of glycosylation of newly synthesized proteins in many viruses (26), Increase endosomal pH needed for virus/cell fusion (27)	Yes (27) NCT04261517 ChiCTR2000029542, ChiCTR2000029559, ChiCTR2000029609, ChiCTR2000029740, ChiCTR2000029760, ChiCTR2000029761, ChiCTR2000029762, ChiCTR2000029803, ChiCTR2000029826, ChiCTR2000029837, ChiCTR2000029868, ChiCTR2000029898, ChiCTR2000029899, ChiCTR2000029935, ChiCTR2000029939

Ritonavir+ Lopinavir (Kaletra)	Protease inhibitors, Antiretroviral	Inhibits virus-specific processing of viral Gag-Pol and Gag polyproteins in cells infected with virus through inhibition of viral proteases (28)	Yes NCT04255017 ChiCTR2000048824, ChiCTR2000048919, ChiCTR2000048809, ChiCTR2000048991, ChiCTR2000048992, ChiCTR2000049015, ChiCTR2000049065
IFNα2b (PegIntron®, Sylatron®, IntronA®)	Type I interferon made by leukocytes during viral infection	Inhibition of viral replication, Stimulation of innate antiviral responses in patients infected with virus (29)	Yes ChiCTR2000048684
Emtricitabine+ tenofovir (Truvada)	Non-nucleoside reverse transcriptase inhibitor + DNA synthesis inhibitor	Incorporated into the viral DNA strand by virus reverse transcriptase and terminates DNA chain elongation (28)	Yes ChiCTR2000048919
Ruxolitinib (Jakafi or Jakavi)	Janus kinase (JAK) 1 and 2 inhibitor	Inhibition of virus replication in lymphocytes and macrophages (30)	Yes ChiCTR2000049088

Darunavir (with cobicistat) (Prezista®/ Prezcobix® and Generic)	Protease inhibitors, Antiretroviral	Inhibits virus-specific processing of viral Gag-Pol and Gag polyproteins in cells infected with virus through inhibition of viral proteases (28)	Yes ChiCTR2000048992, NCT04252274
Baloxavir marboxil (Xofluza)	Endonuclease inhibitor, Antiviral	Inhibition of cap-dependent endonuclease of influenza virus (31)	Yes ChiCTR2000049013
Favipiravir (or T-705 or Avigan)	Experimental antiviral drug. Pyrazinecarboxamide derivative viral RNA polymerase inhibitor.	Inhibition of the RNA dependent RNA polymerase of RNA viruses (32)	Yes ChiCTR2000049015, ChiCTR2000049013, ChiCTR2000049042
Arbidol (Umifenovir)	Russian-made small indole-derivative molecule. Antiviral	Inhibition of the different steps of the viral life cycle (33)	Yes ChiCTR2000049069, ChiCTR2000049065, NCT04252885
Novaferon, Nova	Recombinant protein produced by DNA-shuffling of IFN-α	Inhibition of viral replication (34)	Yes ChiCTR2000049065, ChiCTR2000048809

GS-5734/ Remdesivir	Nucleoside inhibitor	Incorporation into nascent viral RNA chain and pre-mature termination of RNA chain elongation (22)	Yes NCT04252664, NCT04257656

Despite the promising effects of the drugs used to treat COVID-19, several challenges remain with current treatments. One of the challenges is the administration of the drug by the oral route that can create both topical and systemic side effects. The drug delivery to the lung to treat pulmonary diseases by oral therapy requires high doses of drug, also rapid clearance of drugs leads to a need for daily treatment with several drug candidates for the long periods (35). The other significant challenge in viral infectious diseases is emergence of drug resistance (28), which may also occur in patients with COVID-19 disease.

Potential applications of Nanotechnology in treatment of COVID-19

Nanotechnology offers promising solutions to overcome the challenges of oral administration of drug and to treat drug resistance. Nanotechnology-based drug delivery systems (nano-DDS) have several unique physical properties, such as high water-solubility, sensitivity to temperature, simple surface modification, controlled release capability of encapsulated drugs, and high surface-area-to-volume ratios (36). The medical scientists can use these unique properties to overcome the challenges related to enhanced drug resistance of infectious agents.

Nano-DDS can be adapted (i) to increase the solubility of drugs, (ii) to protect drugs from degradation, (iii) to reduce drug-associated toxicity, (iv) to modulate drugs release, (v) to enhance cellular uptake of drugs and their blood circulation time, and (vi) to target drugs to affected cells, tissues, and organs (37-40).

Unlike oral administration, systemic delivery of therapeutics via lungs allows that therapeutic can bypass harsh conditions of digestive tract and avoid first-pass metabolism (41). Also, compared to the digestive system, the lungs show a low level of enzymatic activity (42).

Local delivery of therapeutics to lungs has many advantages as it improves the efficiency of the treatment by enhanced accumulation of therapeutics in the lungs, also, reduces systemic side-effects of drugs on other tissues and organs through reduction of required dosage of delivered drugs and prevention of their penetration into the systemic circulation (43).

The different types of nanocarriers such as polymeric and solid lipid nanoparticles, liposomes, and micelles have been used for delivering therapeutics to the lung due to their flexible and controllable biological properties. These nanocarriers can be administered through distinct routes, including oral, inhalation and intravenous (44). The administration route is as significant as drug formulation for reaching therapeutic goals. Table 2 lists several nano-DDS that are used for treatment of pulmonary infections.

In recent years, nanoparticle DDS has been designed for improvement of therapeutic stability, efficacy, safety, and patient compliance (45). The therapeutic benefits of nanoparticles (NPs) as drug carrier are well controlled release properties, high bioavailability, low toxicity, high stability, feasibility of incorporation of both hydrophobic and hydrophilic therapeutics, and high encapsulation efficacy for therapeutics (46, 47). The development of nanoparticle DDS is one of the best ways to gain higher drug levels in the lungs, drug-loaded NPs can be used for direct pulmonary delivery via the aerosol route (48).

The inhalational delivery of a drug in form of suspension aerosol or inhaled dry powder has two potential limitations including, limited bioavailability of drug because of its microparticulate nature and poor solubility that leads to restricted dissolution and diffusion of a drug at the site of action, and reduced residence time of drug due to its fast alveolar macrophage uptake and ciliary clearance that results in elimination of long-term effects of drug. In contrast, inhalational delivery of a drug in form of nanosuspensions and nanoparticles has several potential advantages including, fast start of action of the drug because of its rapid dissolution and diffusion, prolonged drug residence time at the site of action due to enhanced adherence of nanoparticulate drug to mucosal surfaces, and lower phagocytic clearance of NPs compared to microparticles (49).

The effective pulmonary delivery of an inhalable therapeutic depends on many factors, such as shape, size, and surface features (42). These factors affect all aspects of pulmonary delivery of drug, such as deposition in respiratory tract, dissolution in lung lining fluid and clearance process (50).

Polymeric NPs are achieving fast importance to delivery of drug to the lungs. Various polymers have been studied for pulmonary applications. Polymers possess multiple benefits, including high capacity for encapsulation of drugs and their protection from degradation, and extended delivery of drug for a long time (51). The polymers include poly(lactic-*co*-glycolic acid) PLGA, poly(ε caprolactone) PCL, poly(lactic acid) PLA, chitosan, alginate, and gelatin are commonly used for therapeutic purposes (52, 53). By modifying chemical and surface properties of polymers, they can be biodegradable (54, 55). Several studies have been carried out using polymeric NPs to delivery of anti-infectious drugs to the lungs (Table 2).

Solid lipid nanoparticles (SLNs) are other group of NPs that have widely been investigated for potential pulmonary drug delivery. SLNs are nanocrystalline suspensions in water, prepared from phospholipids, physiological lipids, and primarily triglycerides (56). SLNs are popular for pulmonary delivery of drug since the formulations are prepared with the use of physiological components (57). The SLNs have advantages like good tolerability, scaling-up possibility, low toxicity, the ability to encapsulate hydrophilic/hydrophobic drugs, and increased stability of encapsulated drugs (58). To treatment of lung infections, different anti-infectious drugs have been incorporated in SLNs (Table 2).

Liposomes are a popular DDS for pulmonary drug delivery, as they are formed mainly from phospholipids, that are inherent in lungs (59). Liposomes possess properties like capacity to incorporate of drugs in core or within their membrane bilayer, ability to decrease toxicity of drugs and change their pharmacokinetics, and potential for sustained release of drugs to increase their effect over an extended time period, which make them attractive drug nanocarriers for the treatment of pulmonary infections (60, 61). Liposomal DDS using both lipophilic and hydrophilic drugs was used to treatment of lung infections through the inhalation route (43).

So far, liposome has been one of the most promising nanocarriers to pulmonary antibiotics delivery, with several antibiotic formulations in clinical trials (44). Conventional liposomes are easily uptake through macrophages cells of the reticuloendothelial system. Thus they form a worth delivery vehicle to target high antibiotics doses to sites where the bacteria reside (62). Also, the half-life antibiotics may extend in the body due to sustained release of these drugs from liposomes (62).

Nebulized liposomes to target drugs to the lung have been well described (63, 64). It was shown which nebulization of liposomal dispersions permitted penetration into the lung peripheral region (65). Efficient chemotherapy of pulmonary infections can be performed by drug targeting to alveolar macrophages by using aerosolized liposomes (65). However, physical and chemical long-term stability of liposome suspensions during storage, such as fusion, leakage, and aggregation, is one of key limitations in the development of these nanocarriers (66, 67).

Also, liposomes can be used as a carrier to target cell- and organ-specific using ligands. Although, therapeutic applications of ligand-anchored liposomes that are injected intravenously, can become restricted because of some factors, including leakage of contents of liposomes before they reach to the target site, and uptake of liposomes through macrophages of the spleen and liver [64]. The anti-infectious drugs which have been encapsulated as liposomal formulations are listed in Table 2.

Polymeric micelles because of their nanoscopic core-shell structure are considered as suitable carriers for delivery of poor water-soluble drugs like antibiotics and antifungal into the lung (68). The delivery of anti-infection drugs to the lung by polymeric micelles mentioned in Table 2.

Table 2: Examples of nano DDSs developed for delivery of anti-infectious drugs to the lungs.

Nano DDS	Therapeutic agent	Route of administration	Organism	Purpose/Effect of nanoformulation
PLGA nanoparticles	Isoniazid Rifampicin Pyrazinamide	Inhalation	Mycobacterium tuberculosis	To improve the bioavailability of antitubercular drugs, To prolong half-life of drugs (69)
PLGA nanoparticles	Voriconazole	Inhalation	Not tested	To improve pulmonary delivery (70)
PLGA nanoparticles	Rifampicin	Inhalation	Mycobacterium tuberculosis	To increase uptake of drug (71)
Lection –PLGA nanoparticles	Isoniazid Rifampicin Pyrazinamide	Inhalation	Mycobacterium tuberculosis	To decrease the drug dosage frequency, To improve patient compliance in chemotherapy of TB (72)
Alginate/chitosan nanoparticles	Isoniazid Rifampicin Pyrazinamide	Inhalation	Mycobacterium tuberculosis	The controlled release of antitubercular drugs (48)
Mannitol:Lectin nanoparticles	Iitraconazole	Inhalation	Aspergillus fumigatus	To improve the bioavailability of drug (50)
Polysorbate 80: poloxamer 407 nanoparticles	Iitraconazole	Inhalation	Aspergillus fumigatus	To increase water solubility of drug (73)

Polysorbate 80: poloxamer 407 nanoparticles	Itraconazole	Inhalation	Aspergillus fumigatus	Improved survival and limited invasive disease (74)
Cyclodextrin complexes	Rifampicin	Inhalation	Acinetobacter baumannii	To increase water solubility, To maximize the active drug amount, To optimize lung pharmacokinetic profile of drug (75)
Soli lipid nanoparticles	Isoniazid Rifampicin Pyrazinamide	Inhalation	Mycobacterium tuberculosis	Improved drug bioavailability and mean residence time, Reducing the dosing frequency (58)
Soli lipid nanoparticles	Amikacin	Inhalation	Not tested	To increase concentration of drug in the lungs (76)
Soli lipid nanoparticles	Budesonide	Inhalation	Not tested	To increase water solubility and adsorption of drug (77)
Soli lipid nanoparticles	Rifampicin	Inhalation	Mycobacterium tuberculosis	To reduce of toxicity of drug (56)
Liposome	Amphotericin B	Inhalation	Aspergillus fumigatus	To improve the drug chemotherapy efficacy (78)
Liposome	Amphotericin B	Intravenous	Candidosis Aspergillosis	To improve the drug chemotherapy efficacy, To reduce acute and chronic toxicities of drug (79)

Liposome	Ciprofloxacin	Inhalation	Not tested	To improve the drug chemotherapy efficacy against intracellular parasites (80)
Liposome	Ciprofloxacin	Inhalation	Francisella tularensis	Higher serum levels of drug, Prolonged drug retention in lung (81)
Liposome	Ciprofloxacin	Inhalation	Not tested	To delivery high drug concentration to target site, Reducing the local irritation (82)
Liposome	Rifampicin	Inhalation	Mycobacterium avium	To reduce of dosage of drug (65)
Liposome	Rifampicin	Inhalation	Mycobacterium tuberculosis	To improve the chemotherapy efficacy of drug (64)
Liposome	Tobramycin	Intratracheal	Pseudomonas aeruginosa	Improved efficacy after multiple treatments, Prolonged drug retention in lung (83)
Liposome	Tobramycin	Intratracheal	Burkholdera cepacia	Prolonged efficacy of drug (84)
Liposome	Polymyxin B	Intratracheal	Pseudomonas aeruginosa	To increase pulmonary level of drug, To decrease of toxic effect of drug (85)
Liposome	Cyclosporine A	Inhalation	Mycobacterium tuberculosis	To deposit of drug to the peripheral lung (86)

Liposome	Isoniazid	Inhalation	Mycobacterium tuberculosis	To increase pulmonary level of drug (87)
Chitosan-based micelles	Amphotericin B	Inhalation	Candida spp Aspergillus spp	To improve water solubility, To reduce adverse effects, To reduce the aggregation state of drug (68)

Conclusion and future perspectives

As shown in this review, remarkable works have been done in nano-formulation therapeutics for treatment of the lung infections. Nanotechnology presents an excellent opportunity for the basic improvement of current treatments and development of novel therapeutic options for lung infections formerly thought impossible or difficult to treat. Nonetheless, we are yet in the primary stages of nanomedicine in respiratory infections care, which requires physicochemical and nanotoxicological analysis for possible human applications. At present, we are entering a modern world where nanotherapeutics will change the way we practice respiratory medicine. Nanotherapeutics offer improved clinical efficacy for patients, especially to those patients who are currently treatment-resistant to conventionally administered therapeutics.

According to the above content and the potential applications of nanotechnology to delivery of anti-infection drugs to the lungs, current and potential therapeutics of COVID-19 can be encapsulated into nanocarriers and delivered to the lungs through the respiratory tract.

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Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Neuroprotective Effect of the Recombinant Human Erythropoietin in the Entorhinal Cortex and Thalamus of Rats Exposed to Focal Cerebral Ischemia

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Abstract

Objective: Neuroprotective potential of recombinant human erythropoietin (rhEpo) was reported in various experimental models of brain damage but the exact mechanism of its effect is still unclear. In the present study, the effect of rhEpo administration on the level of neuronal loss and neurodegenerative changes in the dorsolateral band of the entorhinal cortex and ventral posteriomedial nucleus of the thalamus in rats following focal cerebral ischemia was examined.

Methods: Focal cerebral ischemia was induced in male Hanover Wistar rats (250-350 g) by right middle cerebral artery occlusion (MCAO) model for 1 h. After 23 h of reperfusion, ischemic animals were sacrificed and the neuronal damage was detected using the Fluoro Jade B fluorescent staining to detect neurodegeneration, together with NeuN immunostaining used to detect neuronal loss. Ischemic animals received either vehicle or rhEpo (5000 IU/kg, intraperitoneally) 3 hrs after MCAO, and were sacrificed 21 h later. Sham operated; vehicle treated animals served as the control group.

Results: Administration of rhEpo significantly increased the NeuN immunoreactivity in the entorhinal cortex compared to the neuronal loss detected in ischemic, non-treated animals and decreased the number of Fluoro Jade B positive neurons in comparison to neuronal damage detected in ischemic, non-treated animals. The effect of rhEpo treatment in thalamus was not significant.

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Conclusion: Our results indicated that focal cerebral ischemia caused neuronal damage in the entorhinal cortex and that rhEpo treatment was effective in preventing above mentioned alterations.

Keywords: *recombinant human erythropoietin (rhEpo), focal cerebral ischemia, neuroprotection, neuronal loss, neurodegeneration*

Introduction

Acute ischemic stroke is one of the major causes of death and disabilities worldwide. The majority of ischemic human strokes are caused by middle cerebral artery occlusion (MCAO) (1). The MCAO model, experimentally induced in rodents by the intraluminal suture method, is well-standardized animal model of focal cerebral ischemia. This model effectively mimic clinical changes that occur during and after human ischemic stroke (2). In addition, it is considered as a reliable model to test a complex pathophysiology of cerebral ischemia such as a potential neuroprotective effect of various drugs.

It is well known that during focal cerebral ischemia neurons and glial cells are severely damaged by compelx cascade of pathophysiological events starting with periinfarct depolarization and overproduction of reactive oxygen species (ROS). Consequent oxidative stress followed by more delayed postischemic inflammation cause mitochondrial damage, activation of apoptotic proteins, cytochrome c release, and caspase activation. These events finally result in apoptotic cell death within several days/weeks after cerebral ischemia and are involved in the progression and expansion of brain injury (3,4).

Thus, the administration of drugs that have potential to control one or more elements of the above mentioned pathophysiological cascade during focal cerebral ischemia is important for achieving neuroprotection against ischemia/reperfusion injury.

Erythropoietin (Epo) is hematopoietic growth factor mostly secreted by the kidneys in response to tissue hypoxia (5). Its crucial role in the regulation of process of erythropoiesis stimulation is well known, but a significant interest in its effect came after it was found that Epo and erythropoietin receptors (EpoR) are expressed locally in response to the brain ischemia. It is assumed that they act as potential endogenous neuroprotectants excreted in response to oxidative stress, especially in the penumbra. This pointed out possibility that exogenous administration of rhEpo could contribute to endogenous neuroprotective response of ischemic brain tissue (6,7). Effective clinical application for the treatment of anemia in renal failure and cancer was well described (8,9) but it was shown

also that rhEpo acts as multifunctional drug that could potentiate endogenous neuroprotective effects as a consequence of activation of various biochemical pathways. This provide anti-apoptotic, anti-oxidant and anti-inflammatory effect as well as the stimulation of angiogenic and neurogenic mechanisms (10). The capability of rhEpo to cross the blood-brain barrier after systemic administration, its acceptable therapeutic window and good clinical tolerability are additional advantages for its potential application in human stroke therapy (11,12). However, the exact mechanisms of its neuroprotection are still not completely understood and outcomes from conducted clinical trials are controversial (13,14).

In this study, in order to evaluate the potential neuroprotective effect of rhEpo against ischemia/reperfusion injury, we examined the effects of a) focal cerebral ischemia/reperfusion on the potential neuronal loss and neurodegenerative changes in the entorhinal cortex and thalamus of rats exposed to transient middle cerebral artery occlusion and b) systemic administration of 5000 IU/kg of rhEpo on above mentioned parameters.

Materials and Methods

Experimental animals

Male Hannover-Wistar rats, weighing 250-350 g were used in this study. Rats were maintained on a 12 h light-dark cycle and allowed free access to food and water. All experiments were performed between 9 a.m. and 3 p.m. in a silent room, at a temperature of 22–24°C. All experimental procedures involving animals were approved by the Faculty's Ethical Committee and were carried out in accordance with the Croatian laws and rules (NN135/06; NN 37/13; 55/13) and with the guidelines set by the European Community Council Directive (2010/63/EU). All efforts were made to minimize animals' suffering and to reduce the number of animals used in experiments. This research was supported by University of Rijeka under project uniri-biomed-18-115 1253.

Experimental design

The rats were randomly divided into three experimental groups. The first group was sham control, vehicle-treated (n = 4), the second group was MCAO, vehicle-treated (n = 3), and the third group was rhEPO (Eprex 2000 IU/0,5 ml, Janssen Biologics B.V., Leiden, Netherlands) treated (n = 4). The vehicle or rhEPO were administered at 3 h following ischemia induction, rats were sacrificed, and their brains were isolated 24 h post-MCAO induction.

Surgical procedure for the transient middle cerebral artery occlusion

Rats were anesthetized with 350 mg/kg of chloral hydrate administered intraperitoneally (i.p.). The cerebral ischemia-reperfusion injury was performed

by the right MCAO for 1 h. MCAO was performed by intraluminal nylon suture occlusion method as described by Longa et al. (15). and with modifications suggested by Belayev et al. (16). Briefly, under an operating microscope, the right common carotid artery was exposed and carefully dissected from the surrounding nerves and fascia. The internal carotid artery was isolated and separated from the adjacent vagal nerve, and the pterygopalatine artery was ligated close to its origin with a 5-0 nylon suture. Next, a 4-0 silk suture was tied loosely around the mobilized external carotid artery stump, and a 3-0 monofilament nylon suture of approximately 4 cm length, prepared by blunting the tip of the suture by heating it near a flame, was inserted through the proximal external carotid artery into the internal carotid artery and subsequently into the circle of Willis, effectively occluding the middle cerebral artery (MCA). The suture was inserted about 18–20 mm from the bifurcation of the common carotid artery after which the neck incision was closed. The body temperature was maintained at 37±0.5°C with a heating pad and rectal probe. After 1 h of MCAO, the intraluminal suture was carefully removed, and the internal carotid artery was reperfused. Sham-operated, vehicle-treated rats served as the control groups. In these animals, the internal carotid artery was only isolated, but the MCA was not occluded. Once awakened from the anesthesia, the animals were returned to their home cages. Animals of all experimental groups were sacrificed 24 h after the induction of MCAO or sham experimental procedure.

Histological and immunohistochemical analyses

Histological and immunohistochemical analyses were performed in the dorsolateral band of the entorhinal cortex and ventral posteriomedial nucleus of the thalamus in rats of control, vehicle treated as well as ischemic animals that were injected with either vehicle or rhEpo. For these analyses, animals were perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline. Brains were removed and stored in the fixative solution for 20 h at 4°C and subsequently embedded in paraffin.

For the neuronal loss determination, immunohistochemical detection of NeuN stained cells was performed on 3 μm paraffin sections. Following deparaffinization and rehydration of the sections, antigen retrieval was achieved by microwaving slides in citric acid buffer (10 mM, pH 6.0). Following the blocking step with 5% normal rabbit serum and 1% bovine serum albumin in TBS-Triton X-100 (0.025%), sections were incubated with mouse monoclonal anti-NeuN primary antibody (Millipore, Billerica, MA, USA), overnight at 4°C. The next day biotinylated secondary rabbit anti-mouse antibody (Dako Cytomation, Glostrup, Denmark) was applied for 2 h, followed by 30 min incubation with red fluorescence emitting DyLight 594™-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA).

Microphotographs of the designated brain regions were obtained using the Olympus BX 51microscope equipped with an Olympus DP 70 digital camera (Olympus, Japan).

Fluoro-Jade B fluorescent staining was used to study neurodegenerative changes in the investigated brain regions. Brain slices were first deparaffinized in xylene and then rehydrated in decreasing concentrations of ethanol to deionized water (dH2O). Slides were then subjected to 0.06% potassium permanganate solution for 10 minutes, rinsed twice with dH2O for 1 minute, and incubated in 0.001% Fluoro-Jade B (Millipore, Billerica, MA) solution diluted in 0.1% acetic acid, for 20 minutes in the dark. Slides were washed 3 times and air-dried at room temperature (RT) overnight. The next day, after clearing in xylene, the slides were mounted in Entellan (Merck Millipore) and coverslipped.

Photo acquisition and image analysis

In the histological analyses, all slides were examined by epifluorescence microscopy using the appropriate light filter cubes (Olympus BX51 microscope and Olympus DP 71 CCD digital camera; Tokyo, Japan). Microphotographs of the coronal sections of the rat brain were taken at approximately -3.12 to -3.60 mm relative to bregma, according to Paxinos and Watson (2005) (17). All the image processing and analyses were performed manually by a blind investigator by using the ImageJ software (NIH, Bethesda, MD, USA). For the thalamus, photographs were taken within the ventral posteromedial thalamic nucleus, and for the entorhinal cortex, the dorsolateral band was visualized.

For the quantification of the number of NeuN-immuno stained nuclei and the intensity of the Fluoro-Jade B staining, brain sections were photographed at ×400 magnification. NeuN positive cells were counted within the visual field that was 0.143 mm2 in size. Quantification of the Fluoro-Jade B staining intensity was performed by calculating the densitometric mean of fluorescence intensity. Within each image, background fluorescent intensity was subtracted and mean gray intensity determined which resulted in the measurement of degenerating neurons within that field.

Statistical analyses

All the statistical analyses were performed using Statistica software version 13.0 (StatSoft Inc., Tulsa, OK, USA). Statistical significance for the number of NeuN positive cells, and the Fluoro Jade B intensity measurement were calculated according to the one-way analysis of variance, followed by Duncan's multiple range post-hoc test. All the results are expressed as means ± standard error of means (SEM). In all the comparisons $p<0.05$ was considered to indicate statistical significance.

Results

We examined the effects of focal cerebral ischemia/reperfusion as well as the influence of i.p. administration of 5000 IU/kg rhEpo injected 3 h after ischemic procedure on the neuronal loss and neurodegenerative changes in entorhinal cortex and thalamus of rats exposed to transient middle cerebral artery occlusion.

Neuronal loss

Figure 1 shows the NeuN immunoreactivity and the results of the semiquantitative analysis of number of NeuN positive cells in the dorsolateral band of the entorhinal cortex and ventral posteriomedial nucleus of the thalamus in rats of the control group and ischemic rats treated with either vehicle or rhEpo at 3 h after the induction of ischemia. Representative microphotographs show decreased NeuN immunoreactivity in the examined region of entorhinal cortex of the ischemic, vehicle treated animal compared to the rat of the control group. It is also apparent that the rhEpo treatment caused an increase in the NeuN immunostaining in the enthorinal cortex of the MCAO exposed animals (Figure 1a).. An overall ANOVA reveal a statistically significant difference among tested groups in enthorinal cortex [$F(2,8) = 26{,}447$; $p<0{,}05$] (Figure 1b).. Namely, the number of NeuN labeled cells in the enthorinal cortex was decreased in MCAO/Veh animals in comparison to the control group, (443.68±57.75 cells/mm 2 in the MCAO/Veh animals versus 599.86±36.42 cells/mm 2 in the control animals). In the ischemic animals treated with rhEpo, the number of NeuN positive cells was 513.51±24.97 cells/mm 2 and it showed significant difference in comparison to the control or MCAO/Veh animals (Figure 1b). In our experimental conditions, an overall ANOVA did not reveal a statistically significant effect of the treatments on the number of NeuN immunopositive cells in thalamus [$F(2,8) = 2{,}173$; $p>0{,}05$] (Figure 1c and 1d)..

Neurodegeneration

The presence and extent of neurodegenerative changes were analyzed throughout the dorsolateral band of the entorhinal cortex and the ventral posteromedial nucleus of the thalamus using the Fluoro-Jade B histofluorescence. Within the both examined brain regions of the ischemic, vehicle-treated animals, Fluoro-Jade B positive staining was detected in neuronal cell bodies and neuronal fibers (Figure 2a), contrary to the sections from the animal of the control group in which no degenerative changes were detected. From the microphotographs of the brain sections of the rhEpo injected ischemic rats, it is apparent that the investigated drug caused a slight decrease in the intensity of the Fluoro Jade B staining in both examined regions, but particularly in the dorsolateral entorhinal cortex. The quantification of the Fluoro-Jade B staining intensity in the dorsolateral entorhinal cortex revealed significant neurodegeneration in the animals exposed to MCAO

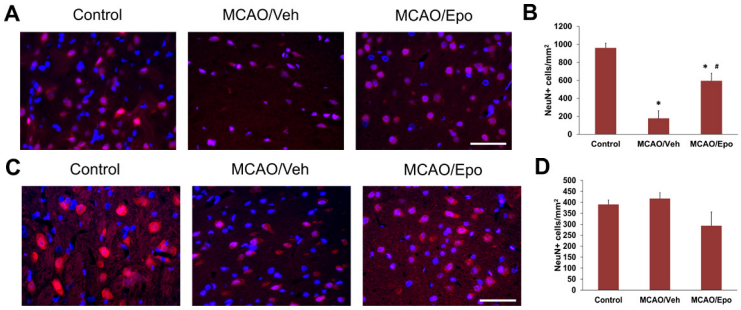

Figure 1. *The effect of recombinant human erythropoietin treatment on the neuronal loss measurement in the dorsolateral band of the entorhinal cortex (A, B) and ventral posteriomedial nucleus of the thalamus (C, D) of rats exposed to transient cerebral ischemia. Representative microphotographs of the investigated regions of the entorhinal cortex (A) and the thalamus (C), immunofluorescently labeled with NeuN neuronal marker (red) from the rats sacrificed at 24 h after the induction of 1 h middle cerebral artery occlusion (MCAO) or sham operation. DAPI fluorescent dye (blue) was used as the nuclear counterstain. Sham-operated, vehicle-treated animals (Control) and the ischemic rats, treated with either vehicle (MCAO/Veh) or rhEpo (MCAO/Epo), were intraperitoneally injected at 3 h after the onset of the ischemic procedure or the end of the sham operation. Scale bar: 200 µm. Quantification of neuronal loss in the dorsolateral band of the entorhinal cortex (B) and ventral posteromedial nucleus of the thalamus (D) was done by counting the NeuN positive cells. Each value represents the mean ± S.E.M. (N = 3-4). *P < 0.05, significantly different from the Control group; #P < 0.05, significantly different from the MCAO/Veh group.*

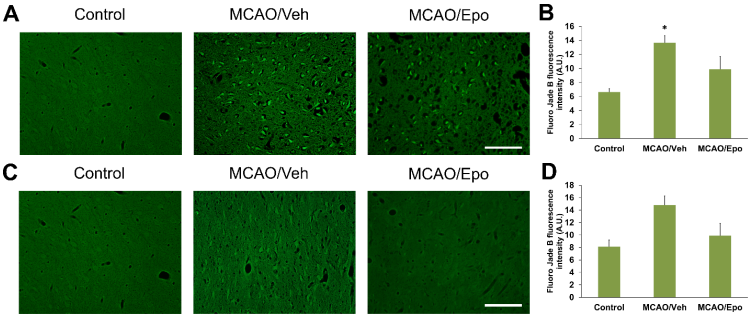

Figure 2. *The effect of recombinant human erythropoietin treatment on the levels of neurodegenerative changes in the dorsolateral band of the entorhinal cortex (A, B) and ventral posteriomedial nucleus of the thalamus (C, D) of rats exposed to transient cerebral ischemia. Representative microphotographs of the investigated regions of the entorhinal cortex (A) and the thalamus (C), from the brain sections stained with Fluoro Jade B fluorescent dye. Rats were sacrificed at 24 h after the induction of 1 h middle cerebral artery occlusion (MCAO) or sham operation. Sham-operated, vehicle-treated animals (Control) and the ischemic rats, treated with either vehicle (MCAO/Veh) or rhEpo (MCAO/Epo), were intraperitoneally injected at 3 h after the onset of the ischemic procedure or the end of the sham operation. Scale bar: 200 µm. Fluoro Jade B staining in the dorsolateral band of the entorhinal cortex (B) and ventral posteromedial nucleus of the thalamus (D) was quantified as a measure of their fluorescence intensity and presented as arbitrary units (A.U.). Each value represents the mean ± S.E.M. (N = 3-4). *P < 0.05, significantly different from the Control group.*

($p<0.05$) (Figure 2b) in comparison to the control animals. Also, rhEpo treatment caused a decrease in the Fluoro- Jade B staining intensity in this region but it was not significantly different from values determined in either control or MCAO/Veh animals [$F(2,8)=6,6075;p<0.05$]. In the examined thalamic region, results of the quantitative analyses were evaluated statistically but the overall ANOVA did not reveal significant differences in the levels of Fluoro Jade B staining intensities between the investigated experimental groups [$F(2,8)=4,1727;p<0,05$] (Figure 2c and 2d).

Discussion

In our study, we have examined extent of neuronal damage i.e., neuronal loss and/or neurodegeneration in the dorsolateral band of the entorhinal cortex and ventral posteriomedial nucleus of the thalamus 24 h after induction of 1 h MCAO. In addition, the effect of rhEpo administration 3 h after induction of ischemia on the above-mentioned parameters was measured.

The results of presented experiments showed that focal cerebral ischemia induced by MCAO caused a statistically significant neuronal loss in dorsolateral cortex of experimental animals. Namely, registered decrease in NeuN staining in dorsolateral cortex of MCAO/vehicle treated animals was statistically significant in comparison to control animals. Our findings are in accordance with different published results. Thus, it was shown that MCAO followed by 24 h of reperfusion causes significant neuronal damage in various brain regions. The predominant affected areas are mostly striatum and cortex due to the lack of collateral blood supply (2,18,19). It was described also that immunoreactivity for NeuN decrease significantly following MCAO (20), causing massive cortico-striatal lesion with the peak of the damage 12-24 h following reperfusion (20,21). Neuronal loss in our experimental model is in good correlation with statistically significant increase in neurodegeneration that we have found in experimental animals exposed to MCAO. Fluoro-Jade B positive neuronal cells in dorsolateral cortex revealed significant degeneration in ischemic animals exposed to MCAO in comparison to control animals according to Liu et al. (20). Duckworth et al. (22) have shown that a time course of escalating cortical neuronal degeneration was evident from 10 min to 7 days with a peak after 4 days following MCAO. On the contrary, Onken et al. (23) published results that have shown no Fluoro Jade staining 24 h after ischemic injury. They detected degenerative neurons 48-72 h post ischemia in different brain regions like hippocampus, striatum, and cortex. Similar neuronal damage in entorhinal cortex was described even up to 6 months following MCAO (24). Differences in time related neuronal damage following focal cerebral ischemia described above are mostly caused by different brain region sensitivity and suture placement technique. In conclusion,

in our experimental model significant neuronal damage i.e., neuronal loss and neurodegeneration of the dorsolateral cortical neurons are described within first 24 h after 1 h MCAO.

In contrast to above mentioned findings, in our experimental model we did not find a significant neuronal damage, manifested neither by neuronal loss nor extent of neurodegeneration in the ventral posteriomedial nucleus of the thalamus of rats exposed to MCAO in comparison to control animals. The thalamus is a deep brain structure important for maintenance of normal neurological functions and transmission of various signals (25). In most animal models including MCAO, the thalamus is spared from the acute ischemic damage. Namely, the thalamus is primarily supplied by branches of the posterior cerebral arteries with minor contribution from the internal carotid artery. So, after MCAO, the brain damage is predominantly registered in regions supplied by the middle cerebral artery like cortex, striatum or globus pallidus. But it was shown also that brain damage following MCAO could occur even in deep regions such as thalamus because of the occlusion of deep and small cerebral arteries arising directly from the internal carotid artery (25,26), proximally to the origin of middle cerebral artery. Consequently, this region could still be affected due to synaptic connections with the primary injury site. It was described that the widespread corticostriatal damage could result in retrograde and anterograde degeneration, from the perilesional cortex via the descending axons to the thalamus (27). This is probable explanation for results published by various authors demonstrating delayed neuronal damage even in the thalamus following MCAO (25,28). It was described that MCAO induced neuronal damage in regions within middle cerebral artery zone of irrigation, causes widespread edema that leads to secondary, delayed neuronal damage in the thalamus beginning 24 hours after induction of ischemia (27,29,30). This delayed response of thalamic neuronal tissue is probable explanation for lack of significant neuronal damage in thalamus region in our experimental model.

Therapeutic strategies against cerebral ischemia are still very limited. The only clinically approved drug against acute ischemic stroke is tissue plasminogen activator (tPA) (31). However, tPA's narrow therapeutic time window (up to 4.5 hours after the induction of stroke) significantly reduces its' therapeutic potential in the treatment against focal cerebral ischemia (5). Various potential neuroprotective approaches have failed to reduce neuronal damage, neurological deficits, and mortality after cerebral ischemia, which leads to conclusion that discovery of novel therapies against focal cerebral ischemia are of the highest importance. It was shown that one of the promising therapeutic approaches in preventing neuronal damage might be administration of rhEpo (3,6,7). According to Brines & Cerami, several authors have confirmed that a different doses

(500-5000 IU/kg) of rhEPO, administered systemically in rodents or primates, were reaching neuroprotective concentrations within various brain regions approximately 1 h after drug application (32). However, most of the experimental research in various animal models that examined the neuroprotective effects of different doses of rhEpo on brain damage, proposed 5000 IU/kg of rhEpo as optimal dosage (32). It was also shown that rhEpo applied at various time-periods ranging from pre-treatment to up to 24 h after ischemia, exerts the best results when applied within the first 3 h after induction of ischemia (18).

Administration of 5000 IU/kg of rhEpo in our experimental conditions, 3 h after induction of 1h MCAO, significantly preserved the neuronal loss in dorsolateral cortex of ischemic animals in comparison to ischemic, vehicle-treated rats. Namely, the number of NeuN staining neurons in ischemic animals treated with rhEpo is significantly higher in comparison to ischemic, vehicle treated animals. Administration of the drug was also effective in decreasing the number of cortical neurons undergoing neurodegenerative changes of ischemic animals although this result did not reach statistical significance in comparison to ischemic, vehicle treated animals. Administration or rhEpo did not reveal any significant changes in neuronal damage manifested as neuronal loss and/or neurodegeneration in thalamus of ischemic rats.

Neuroprotective effect of rhEpo application was revealed also by other authors although in different experimental conditions and in various brain regions. Various authors have shown that application of 5000 IU/kg of rhEPO either before or even up to 12 h after induction of 1 h MCAO, significantly reduced the size of the brain infarction and extent of inflammation and apoptosis (33) in the ischemic penumbra in rats. In addition, it was demonstrated that rhEpo administration significantly attenuates the neuronal loss in various brain regions after focal transient cerebral ischemia (34). Moreover, administration of the drug exerts decrease in various stages of complex pathophysiological cascades following cerebral ischemia (12,18). Juneman et al. (3) and Im et al. (35) have shown also that reduction of cerebral edema by administration of rhEpo exerts its indirect or secondary neuroprotective effect. Namely, it was shown that administration of rhEpo in rodents caused decrease in swelling of ischemic tissue such as impairment of microcirculation in the critically hypoperfused penumbral area. All these results have shown that rhEpo is drug with neuroprotective potential against cerebral ischemia and that further experiments are needed to confirm these results.

Conclusion

Our experimental results clearly showed that focal cerebral ischemia induced by MCAO caused significant neuronal damage in the dorsolateral band of the

entorhinal cortex. Contrary to neuronal loss and neurodegenerative changes in dorsolateral cortex, absence of such results in ventral posteriomedial nucleus of the thalamus is probably due to delayed neuronal response to ischemic changes caused by MCAO in thalamic brain region. Postischemic administration of rhEpo significantly reduced neuronal loss and caused moderate decrease in neurodegeneration. These results clearly indicated neuroprotective potential of rhEpo administered after induction of MCAO.

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Metformin-Loaded Nanostructured Plga: A New Strategy for Enhancing Efficacy of Metformin in Breast Cancer Treatment

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Abstract

Objective: Nano-formulation approaches would be more efficient in decreasing drug toxicity, preventing tumor development in relation to free form of drug.

Methods: Therefore, the purpose of this study is to construct poly lactic-co-glycolic acid (PLGA) nanoparticles (NPs) loaded with Metformin (MET) to investigate their cytotoxicity as well as their impact on expression levels of apoptosis related genes in MDA-MB-231 breast cancer cells. Field Emission Scanning Electron Microscope (FE-SEM) and Fourier-transform infrared spectroscopy (FTIR) characterized the synthesized NPs. Then, MTT assay was used to evaluate and compare the cytotoxic effect of various concentrations of the chemotherapeutic molecules in pure and nano-formulated forms after 48 h exposure time. Moreover, the mRNA levels of apoptosis related genes expression were studied by quantitative PCR.

Results: By encapsulating MET into PLGA, the cytotoxic efficiency of the compounds considerably augmented for all concentrations. Furthermore, the results demonstrated that MET-loaded NPs could induce apoptosis in MDA-MB-231 breast cancer cell by upregulation of caspase-3, caspase-7 and BAX, along with Bcl-XL, hTERT and Cyclin D1 down regulation.

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Conclusion: Ultimately, this study revealed that the combination of MET–PLGANPs with current cancer therapies holds promise for the potential of breast cancer treatment.

Keywords: *Metformin, PLGA, Nanoparticles, MDA-MB-231, Apoptosis*

Introduction

Nowadays, cancer is a major global health issue around the world (1)**.** Breast cancer is considered the most prevalent malignancy, with an estimated 2.3 million new cases (11.7%) in 2020 and a leading health challenge among females. According to some estimates in recent years, it is responsible for 6.9% of mortality cases in female breast cancers (2). Despite the significant progression in therapeutic strategies for breast cancer, many patients suffer from relapse and metastasis. The etiology of insufficient therapies can be related to ambiguous insight into molecular mechanisms involved in breast cancer initiation, progression, and metastasis. Multiple genomic and epigenetic events have long been considered hallmarks for the initiation of cancer formation and progression of the disease (3). Current approaches of cancer therapy including radiotherapy and chemotherapy are restricted because of owning various frustrating and unfavorable adverse effects such as unwanted toxicity on the normal cells. Therefore, it is essential that innovative strategies and approaches be developed to effective treatment of human breast cancer (4).

Currently, numerous non-hazardous natural compound have been derived, and their ability in moderating cancer relapse and improvement of treatment have been assessed (5). Some naturals compound like MET is promising products for therapeutic efficiency.

Metformin is a semi-synthetic oral hypoglycemic drug which mostly moderates blood glucose by activating of the vital AMPK/mTOR/p70S6K pathway, inhibiting hepatic glucose output and increasing glucose uptake (6). The anti-cancer ability of MET in various cancer cells including prostate, breast, ovary, cervix cancer, and leukemia incidents both *in vivo* and *in vitro* have been studied and recorded (7-9). In spite of the recognized efficacy of MET in treating cancer, the use of this drug in medication is related to some major challenges such as poor biological availability, short half-life, low solubility, low duration of stability in the bloodstream and degradation (10, 11). To overcome these limitations, use of nano-formulation system that allows controlled and sustained release of nano-formulated drugs can be a hopeful way to rise the activity and transfer of natural compounds to target cells.

Polymeric particles are a distinctive type of nanomaterials that have found many new applications in delivery systems for in situ controlled release

of therapeutic applications (12). Nanoparticle drug delivery supplies numerous advantages, due to high encapsulation efficiency, protected drug from degradation, improved biodistribution, delivered, and controlled drug release (5, 13)

In the current study, we aimed to encapsulate MET into PLGA NPs and investigate the improvement of its anti-cancer effects on MDA-MB-231 breast cancer cells through modulating the expression of apoptosis related gens, as the important molecules involved in tumorigenesis.

Materials and Methods

Synthesis of NPs

The improved w/o/w produced MET-encapsulated PLGA NPs with slight modifications (9) . Briefly, a premixed emulsion of PLGA (100 mg) and MET (6 mg) in DCM-Methanol (4:1) was added to the 2% PVA. The mixture was emulsified by sonication at 70,000 rpm for 1 min to produce w/o/w emulsion. Then, a rotary evaporator under a low vacuum was used to evaporate the DCM. The NPs formed by this method were gathered via three cycles of centrifugating at 12,500 g for 15 min.

Nanoparticle characterization

To characterize the surface charge (zeta potential), hydrodynamic particle size (nm) and polydispersity index (PDI) of designed NPs, dynamic light scattering (DLS) system (Zetasizer Nano ZS; Malvern Instrument) possessing a helium-neon laser beam at 633 nm wavelength. Furthermore, FE-SEM (Hitachi Ltd., Japan) was exploited to investigate the surface morphological features and Shape of prepared NPs. Also, to further evaluate MET's successful loading on prepared NPs, free MET and NPs, FTIR spectroscopy was utilized to investigate the chemical configuration of the samples in the range of 400–4000 cm^{-1}.

In vitro release study

The MET release pattern from PLGA NPs was obtained through immersing 80 mg of NPs into 10 mL of PBS (pH 7.4, 37 °C) under shaking at 200 rpm. At certain time intervals, 1 mL of incubation solution was transferred for UV-Vis spectroscopy absorbance measurement at 234 nm and replaced with 1 mL of fresh PBS. The cumulative drug release data were plotted as a function of time:

$$\text{Cumulative amount of release (\%)} = Ct / (C\infty \times 100)$$

where Ct is the quantity of MET discharged at time t and C∞ refers to total quantity of MET loaded in 100 mg of NPs

Cell line maintenance

MDA-MB- 231 breast cancer cell line was gifted from researcher at our university. MDA-MB- 231 cell were grown in RMPI-640 supplemented with 5% FBS (v/v) and 1% pen/strep (v/v). The medium was replaced every other to remove floating debris and the flasks were fed with fresh medium

In vitro cytotoxicity

To evaluate the effect of synthesized NPs in increasing the cytotoxicity of MET on MDA-MB- 231, MTT assay was done. First, MDA-MB- 231, at a density of 8,000 cells per well, were seeded into 96-well plates and incubated for 24 h to become confluent. Then, the cells were treated with various concentrations of MET and MET-NPs and incubated for 24, 48, and 72 h. Then, the medium was aspirated and 50 µL MTT solution (2 mg/ml, Sigma Aldrich) was added to each well. Next, MTT solution was discarded and replaced with DMSO to dissolve the formazan crystals. The absorbance of solubilized formazan in 96-well plates was measured at a wavelength of 490 nm using an ELISA plate reader (Dynex MRX).

Real-time PCR assay

According to the manufacturer instructions, after exposure of A549 cells with different concentrations of the NPs for 48 h, the total RNA was isolated using TRIzol reagent (Invitrogen, USA). Then, a Nanodrop was applied to determine the purity and quantity of total RNA. RevertAid™ First Strand cDNA synthesis kit (Thermo Fisher Scientific, MA, USA) was used for synthesizing cDNA from 1 µg RNA. Next, the quantitative PCR (qPCR) was carried out in a Mic qPCR Cycler (BioMolecular Systems, Australia) by applying SYBR Green Premix PCR Master Mix (Roche, Mannheim, Germany), specific primers, and cDNA. The housekeeping GAPDH gene was applied as the internal control, and the quantification of the samples was analyzed using the $2^{-\Delta\Delta ct}$ method**.**

Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed using chi-square to determine the statistical significance between the two means evaluated at $p<0.05$. All experiments were replicated at least three times. Analyses were conducted in GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA).

Results

Characterization of drug-loaded NPs

The O/W single-emulsion solvent-evaporation process was successfully used for the formulation of the MET-PLGA NPs. The particle size distribution of NPs was assessed by a dynamic light scattering (DLS) method. According to the Table 1,

the typical particles size, polydispersity, and zeta potential of NPs was 150±1.17 nm, 0.175, and zeta potential of -7.3±0.27 mV, respectively. Drug encapsulated NPs presented larger diameter and a smaller polydispersity than blank NPs. This may include drugs that have amphiphilic copolymers that are completely bound.

Table 1. Mean (±SD) particle diameter, Polydispersity (±SD) and zeta potential of drug loaded PLGA-PEG NPs.

Formulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)
PLGA NPs	150±1.17	0.175	-7.33±0.27
MET-loaded PLGA/ PEG NPs	190±5.56	0.166	-6.2±3.1

The morphological characterization of MET NPs was studied by SEM. The images of the drug-loaded NPs revealed their regular spherical shape (Figure 1). The surface morphology of NPs was smooth with a regular spherical shape. The size distribution of NPs was narrow with a mean particle diameter ~ 80 nm. The ranges of drug encapsulation efficiencies (EE) were about 76.8 % with a loading capacity of 12.3 ± 2.2, respectively.

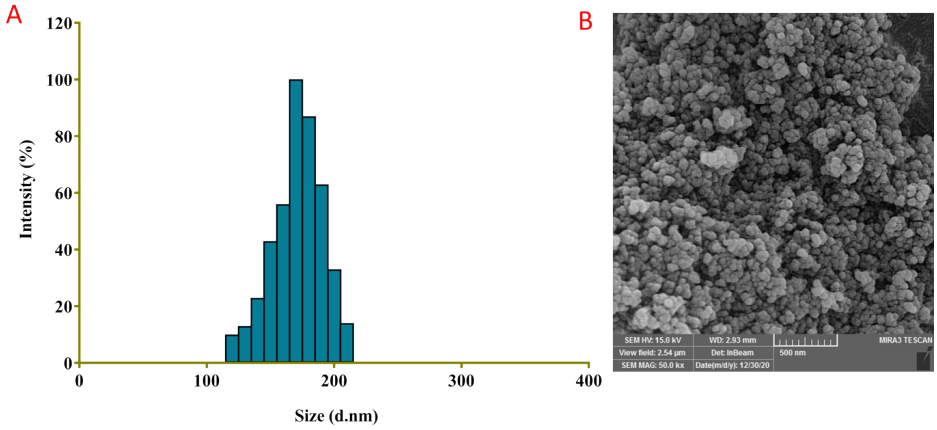

Figure 1. *A DLS histogram showing the size distribution of MET NPs. The average size ranged from 130–200 nm. B Field emission scanning electron microscopy (FE-SEM) image of surface morphology of MET-Cur NPs*

FTIR was applied to describe the functional groups present in pour and drugs loaded NPs (Fig. 2). The FTIR spectrum of pure MET showed two typical peaks at 3369 cm^{-1} and 3294 cm^{-1} relatives to the N–H primary stretching

vibrational mode and a peak at 3155 cm^{-1} because of the N–H secondary stretching, and characteristic peaks at 1626 cm-1 and 1567 cm^{-1}, attributed to C-N stretching. The physical mixture spectrum can be considered as the sum of pure MET and PLGA spectra. The spectrum of MET-loaded NPs shows the typical bands at 3300, 3200, 1625, 1583 cm^{-1} which are assigned to N-H asymmetric stretching, N-H symmetric stretching, C=N stretching and N-H bending groups. The characteristic spectral peaks corresponding to MET functional groups were present in nano-formulations. Also, all the characteristic absorption bands of MET functional groups in the drug loaded polymer, indicating the MET encapsulation into NPs

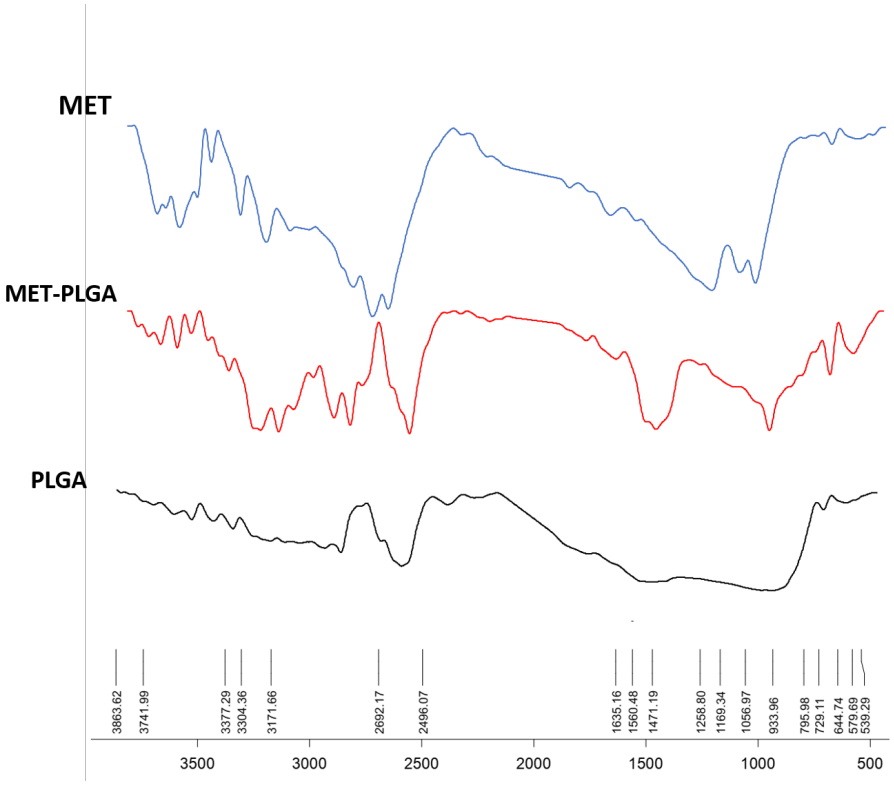

Figure 2. *Infrared spectra of MET, PLGA, MET NPs.*

Drug release

Cumulative MET release from MET-NPs was assessed for 140 h at 37 °C. As shown in Fig. 3, MET-NPs exhibited an initial rapid drug discharge pursued by slower constant release rates, which was consistent with the previously reported release kinetics of MET from PLGA NPs. In the first 48 and 72 h, roughly 55.3% and 72.4% of MET were released from the PLGA NPs, respectively.

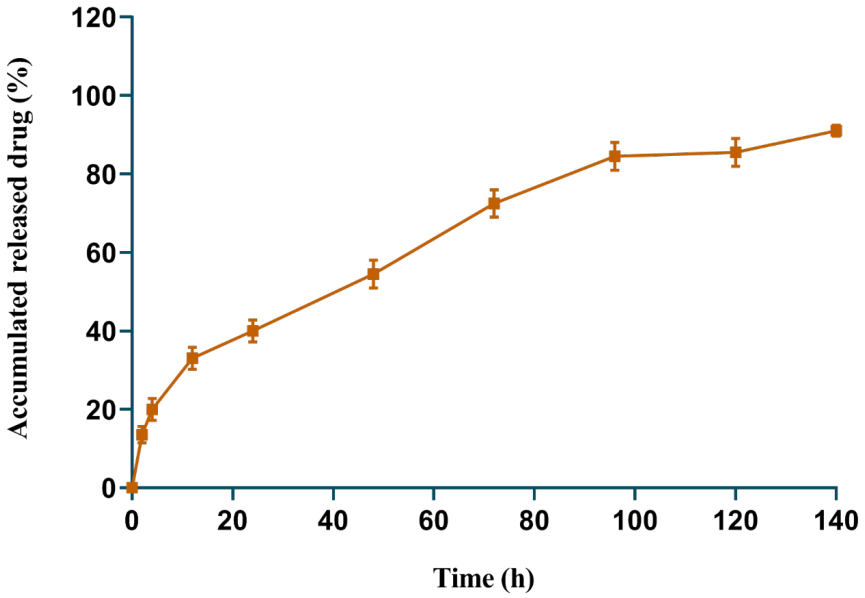

Figure 3. *Cumulative release (%) behavior of MET from NPs in phosphate buffered saline (pH 7.4).*

Cell cytotoxicity and synergistic analysis

To evaluate the growth inhibition effect of MET and MET-PLGA NPs, MTT assay was applied using different doses (0-30 mM) of free MET and MET-PLGANPs on MDA-MB-231 breast cancer cells during 24, 48, and 72 h (Fig. 4 and Fig. 5). Cells without treatment were used as control groups.

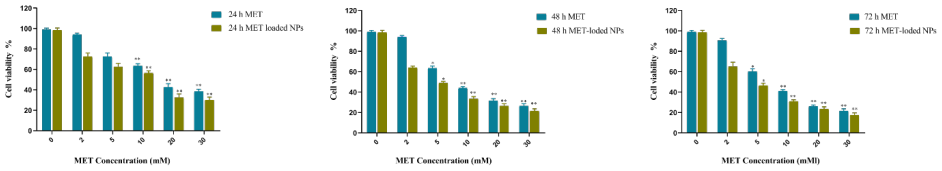

Figure 4. *The in vitro cytotoxicity by MTT assay. The viability of MDA-MB-231 cells receiving various treatments of free MET and MET–NPs, Cur–NPs. Error bars indicate standard deviations. (* p value < 0.05, ** p value < 0.001).*

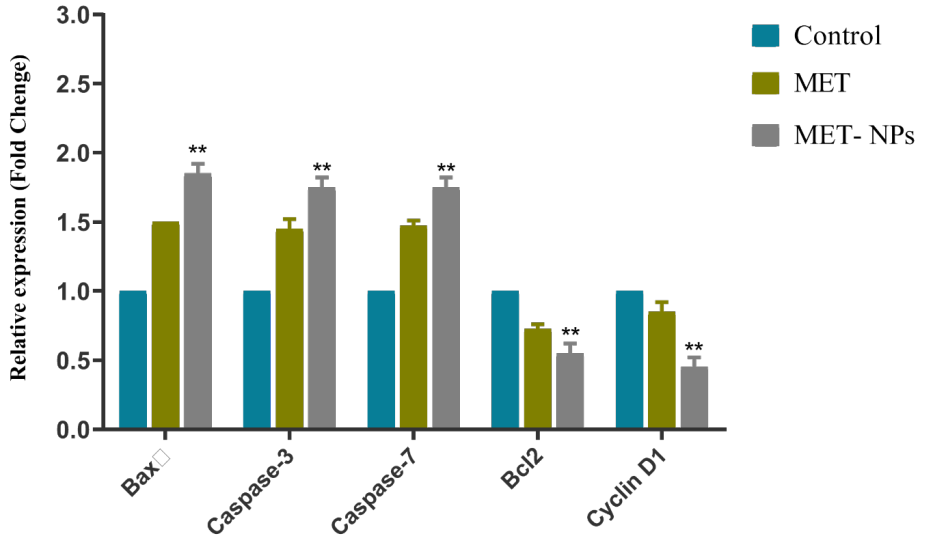

Figure 5. *Relative mRNA expression levels of CyclinD1, P53 and apoptosis related genes in MDA-MB-231 cancer cell line treated with by free and nano-formulated form of MET. *p<0.05, **p<0.01 and ***p<0.001 vs. control was considered significant.*

Gene expression findings

The expression of apoptosis related genes including caspase-3 caspase-7, bcl-2, Bax, Cyclin D1 in MDA-MB-231 cancer cell lines after exposure to MET and MET-PLGA NPs for 48 of incubation was determined by real-time PCR. GAPDH region normalized and calculated by the $2^{-\Delta\Delta ct}$ method to assess the alterations in expression levels of apoptosis related genes between the control and treated MDA-MB-231cancer. Our results showed that MET in free and nano-capsulated forms altered the expression levels of mentioned genes more than free MET and untreated cells. Moreover, our results displayed, caspase-7, caspase-3, and Bax (apoptosis markers) mRNA levels were meaningfully increased in the cells treated with nano-formulated forms of MET compared to control.

Discussion

As previously described, release of drug from nanoparticles indicates the combined effects of swelling, matrix erosion, degradation processes and in situ cross-linking (14). The sustained and sequential release pattern of chemotherapeutic agents could be valuable for inducing apoptosis and attaining an effective approach in cancer treatment (15).

As revealed in Fig. 4 in both free and nano forms of MET showed a concentration-dependent growth inhibitory effect on MDA-MB-231 cells. Remarkably, MET-loaded NPs exploited so much higher cytotoxicity relative to the free form of MET against the MDA-MB-231 cells. Improved growth inhibitory activity of drugs in nano-capsulated form might be distributed to the controlled release of drugs from the PLGA NPs and several procedures that included in the high cellular uptake of encapsulated drugs and as a result, leads to the effective discharge of drugs from the PLGA NPs into the cancer cells cytosol.

In one study, the effect of MET- NPs showed a greater inhibitory effect than pure MET by reducing the expression of the hTERT gene in T47D and MDA-MB-231 cell lines in *in vitro* [16]. Liou and co-colleges showed that MET and SIL in combination form prevent the survival of human cervical cancer cells (17).

Bcl-2 is a member of a substantial family of apoptosis regulatory proteins, which regulate apoptosis by either inducing proapoptotic proteins or inhibiting anti-apoptotic proteins(18). Caspases as a unique family of cysteine proteases are the executive proteins. Caspase-3 and caspase-9 are a family of protease enzymes that have a key role in both intrinsic and extrinsic apoptotic pathways (19). The p53 is a tumor suppressor encoded by the gene TP53, and has properties of a transcriptional activator, and it seems to act a role in the regulatory control of normal cellular proliferation. It is also noteworthy that, p53 contributes directly in the intrinsic path of apoptosis by interacting with the Bcl-2 family that prompt mitochondrial outer membrane permeabilization (20). These observations led to the suggestion that P53/BCL2/BAX apoptosis signaling pathway plays an essential role as a coordinator of apoptosis.

Various studies in consistent with our study have shown that free MET and MET-NPs as well as in combined with other chemotherapeutic molecules can alter the expression levels of apoptosis genes (16, 21). In the preliminary study conducted in our group, it was found that MET in combination with SIL exhibits synergistic antiproliferative effects via down-regulating Cyclin D1 and hTERT (16). Moreover, in another study conducted by our group, it was shown that MET loaded into polymeric PLGA-PEG NPs may be a convenient drug delivery system to enhance its anticancer effects for ovarian cancer therapy(9).

In the present study, we proved that delivery of MET in nanoparticles, which are identified as appropriate delivery system for in situ drug delivery and formulation of anticancer drug with low solubility in water leads to strong anti-proliferative effect on lung carcinoma cells.

Conclusions

In this study, we had successfully fabricated a PLGA-PEG NPs as vehicle to nanocarrier structure to deliver of MET into breast cancer cells for natural based compound chemotherapy. According to the achieved results, MET loaded PLGA-PEG NPs where be able of killing MDA-MB-231 breast cancer cells much more than free drug, which is attributing to the sustained and controlled release. Furthermore, MET strongly prevent proliferation of cell and induces apoptosis via reducing of cyclin D1 and bcl-2 mRNA expression levels. In addition, MET increases the RNA transcription level of caspase-3, caspase-7 and Bax as well as p53, resulting in growth inhibition and apoptosis induction. This preliminary study showed that the loading of the chemotherapeutic molecules into PLGA might lead to developing novel and safe drug nano-delivery systems to treat breast cancer effectively.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Use of Behavior Management Techniques by Dental Practitioners During the Treatment of Pediatric Patients from Different Age Groups

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Abstract

Objective: Behavior management is widely agreed to be a key factor in the care of children in pediatric dentistry. It is important dentists have a wide range of behavior guidance techniques to meet the needs of the individual child. To investigate the dental practitioners` use of the non-pharmacological behavior management techniques in attending pediatric dental patients from different age groups.

Methods: An anonymous, self-completed mailed survey was sent to 200 dentists. The recorded information included items on the frequency of using different non-pharmacological behavior management techniques and factors influencing their use, socio-demographic questions, working experience, specialty status. Descriptive statistics were generated to estimate demographic data and the frequency of using behavior management techniques

Results: Distraction was practiced by all participants mainly for patients less than 3 years (43.22%). Positive reinforcement and Stop signals have been chosen for middle-aged children. One-third of practitioners selected Voice control techniques in each patient`s age group. The least employment of non-pharmacological techniques by respondents was for the restraint, used mainly for the very young patients (7.63%), and Latent inhibition, predominantly in children 6-12 years old. Almost all practitioners reported being influenced by children`s emotional state, their past dental experience and the age in the selection of a behavior management technique.

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Conclusion: The results of the present study highlighted the use of a variety of non-pharmacological behavior management techniques among dental specialists, although few acknowledged having adequate skills to apply the techniques. The choice of the technique was mainly influenced by the children`s factors.

Keywords: *behavior management techniques, age, pediatric dentistry*

Introduction

Behavior management is widely agreed to be a key factor in the care of children in pediatric dentistry. Therefore, it is one of the cornerstones of the specialty [1]. The child`s behavior on each dental visit depends on the variables such as age, parental behavior, parental anxiety, medical history, type of dental procedure, behavior guidance and the procedural techniques followed by the dentist [2]. As the etiology of dental anxiety is multi-factorial, it is important that dentists have a wide range of behavior management techniques (BMTs) to meet the needs of the individual child and are tolerant and flexible in the implementation of these techniques [3]. The American Academy of Pediatric Dentistry (AAPD) has issued a set of guidelines on behavior guidance for pediatric dental patients [4]. BMTs have been classified as pharmacological as opposed to non-pharmacological, communicative (communication) versus advanced BMTs and universally accepted ones, as well as informal and common-sense techniques versus formal relaxation techniques [1,5]. The AAPD has classified BMTs into basic and advanced techniques. BMTs are a set of procedures employed by dental practitioners used to alleviate anxiety, establish communication, instill a positive attitude and enable performing quality oral health care safely and efficiently for children [6]. Children exhibit different attitudes and temperaments, which are influenced by their differences in physical, emotional, and social development. Thus, to communicate successfully with a child, it is necessary to understand his or her intellectual level and how cognitive processes work associated with the relevant age period. As per Jean Piaget's theory of cognitive development, a child's way of thinking about and viewing the world is quite different at the different age stages in its development [7]. Research on the association between the application of the BMTs and the proper age period of the child`s development for their use can enable the pediatric dentists to better understand them and deliver improved quality of care. Few publications reporting the application of BMTs were retrieved despite its importance in creating a positive attitude towards dentistry. Therefore, the objective of the study was to investigate the dental practitioners` use of the non-pharmacological BMTs in attending pediatric dental patients from different age groups.

Methods

The cross-sectional study consisted of an anonymous, self-completed mailed survey. Potential subjects were sent an email describing the study and inviting their participation. The participants were randomly selected from the official register of the Bulgarian Dental Organization in Plovdiv, Bulgaria. Two hundred dentists were invited to participate in the study, extrapolated using a randomized program from the complete email list of the scientific society`s members. The mail included a brief cover letter explaining the purpose of the survey. It stressed the anonymity of the survey and that the responses would be aggregated. The surveys were mailed within three weeks. The study was conducted in September 2020 and consisted of two sections, including multiple-choice and close-ended questions. Section I included demographic questions, including gender, age, work setting, experience, specialty status-general practitioner versus specialist. From section II an information concerning the frequency of using different non-pharmacological BMTs during child age periods and factors influencing the choice for their use was collected. To limit the survey to dental practitioners who provide dental care to children, the first question was `Do you provide dental care to children at your dental office?`. In case of a negative answer, the respondent was excluded from the study.

The clinical study was conducted in accordance with the conditions and principles of the Declaration of Helsinki, the existing EU Clinical Trial Directive (EC) No. 2001/20/EC, the recommendations of the Ethical Committee at the Medical University of Plovdiv, Bulgaria and the International ethical and scientific quality standard for designing, recording and reporting trials that involve the participation of human subjects - Good Clinical Practices (GCP). Ethical approval was obtained from the University Research Ethics Committee before circulating the questionnaire (Document No.P-1371/30.04.2018).

The sample size was calculated using an online calculator (www.raosoft.com/samplesize.html). Based on the calculation with 5% margin of error, 95% confidence level, and 80% response distribution, at least 173 dental practitioners were needed out of a total of 581 dentists involved [8]. By the end of the data collection phase, 118 completed questionnaires were collected from the participants.

The obtained data were tabulated, processed and analyzed using SPSS (Statistical Package for Social Science software) version 21.0 (IBM, USA). Descriptive statistics were generated to estimate demographic data and the frequency of using BMTs.

Results

Out of the 200 surveys that were mailed, 118 subjects (59% response rate) were included in the statistical analysis for this study. The sample size was n=118 dentists. Demographic information about responders and their practices is reported in Table 1.

Table 1. Demographic and practice information of survey practitioners, n=118

	n	Percentage of responders
Gender		
Male	47	39.8%
Female	71	60.2%
Total years in practice		
< 5 years	23	19.5%
5 - 10 years	54	45.8%
10 - 20 years	26	22.0%
> 20 years	15	12.7%
Specialty status		
General practitioner	69	58.5%
Another specialty, not including pediatric dentistry	40	33.9%
Pediatric dentistry	9	7.6%
Location of facility		
Urban	113	95.8%
Rural	5	4.2%
Received formal training on BMT		
Yes	22	18.6%
No	96	81.4%

Overall, the mean age of 118 subjects responding to this item was 36.75±9.16 years old. Subjects were asked to indicate one of four categories of total years in practice (0-5 years, 5-10 years, 10-20 years and over 20 years). The largest group had 5-10 years of clinical experience, while the other groups were reasonably well distributed. Female respondents outnumbered males 1.5:1. 113 dentists (95.8%) were working in urban located facilities. A large portion, 81.4%, reported to have not been received formal training on BMT.

Table 2 summarizes the usage of different BMTs for different age periods. Responses regarding the general use of BMTs were as follows: Nonverbal communication, Tell-show-do (TSD), Voice control, Positive and Negative reinforcement, Distraction and Stop signals, Modelling, Desensitization, Cognitive restructuring, Parental presence/absence, Latent inhibition, Restraint.

Table 2. Frequency and percentage of BMT for each patient`s age group, n=118

BMTs	0-3 years	3-6 years	6-12 years	>12 years
Nonverbal communication	16.95% (20)	9.32% (11)	8.47% (10)	8.47% (10)
Tell-show-do (TSD)	33.89% (40)	51.69% (61)	24.58% (29)	22.03% (26)
Voice control	22.03% (26)	27.12% (32)	30.51% (36)	24.58% (29)
Positive reinforcement	38.14% (45)	62.71% (74)	52.54% (62)	49.15% (58)
Negative reinforcement	0.85% (1)	5.08% (6)	12.71% (15)	13.60% (16)
Distraction	43.22% (51)	23.73% (28)	15.25% (18)	11.86% (14)
Stop signals	15.25% (18)	46.61% (55)	58.47% (69)	52.54% (62)
Modelling	11.02% (13)	11.02% (13)	26.27% (31)	21.19% (25)
Desensitization	15.25% (18)	15.25% (18)	1.69% (2)	0.85% (1)
Cognitive restructuring	1.69% (2)	2.54% (3)	4.24% (5)	8.47% (10)
Parental presence/absence	20.34% (24)	18.64% (22)	13.60% (16)	11.86% (14)
Latent inhibition	-	0.85% (1)	3.39% (4)	1.69% (2)
Restraint	7.63% (9)	-	0.85% (1)	-

The majority of practitioners responded that with all aged groups, except for 0-3 years, they use: Positive reinforcement, Stop signals and Voice control. TSD was selected by 51.69% of participants to be used for 3-6 years patients. Positive reinforcement and Stop signals have been chosen for middle-aged children, about 60% indicated their use of Positive reinforcement for patients 6-12 and about 50% employed both techniques for patients older than 12 years. Distraction techniques were mainly selected for the very young patients, with 43.22% responded using

the technique for patients 0-3 years, one quarter employed it for 3-6 years, and less than 15 % - in patients older than 6 years. One-third of practitioners selected Voice control techniques in each patient`s age group. The least employment of non-pharmacological BMTs by respondents was for the restraint, used mainly for the very young patients (7.63%), and Latent inhibition, predominantly in children 6-12 years old. Techniques percentages for different ages are described in table 2.

Almost all practitioners who participated in the current study reported being influenced by children`s emotional state, their past dental experience and the age in the selection of a BMT during handling of a particular child. Parents` preferences were reported by only 2.54% of the practitioners to influence their choice of a BMT (Table 3).

Table 3. Factors influencing the choice of particular BMTs while handling a child dental patient

Influencing factors	percent	n
Past dental experience	77.97%	92
Oral health	22.03%	26
Emotional state	82.20%	97
Social status	16.95%	20
Medical history	15.25%	18
Child`s age	73.73%	87
Parents` dental anxiety	33.89%	40
Parents` preferences for a BMT	2.54%	3

Discussion

The response rate to this survey (59%) is an indication of the interest that dental practitioners have in the topic of behavior management of child dental patients. The great majority of respondents in our investigation employed communicative BMTs, particularly with children under 12 years of age. Levy and Domoto found that Positive reinforcement, Distraction and TSD were used by a high percentage of dental practitioners in the state of Washington. In contrast to the present study, they also found that Nonverbal communication in the form of touching and stroking a child`s hand or arm was employed by 83% of pediatric dental practitioners [9].

AAPD indicates TSD and Positive reinforcement are two of the most successful yet simple basic BMTs which can be used with all pediatric patients regardless of their cooperation level [4]. In the present study, these two BMTs were found to be the most popular techniques in the age of 3-6 years. A recent survey of members of AAPD reported similar popularity (99%) with both techniques

(4,10). TSD is one of the most used techniques for patients 3-8 years, as it is safe, non-invasive and being acceptable among practitioners and parents (1,4,6,11). In line with the present study, Rajasekhar et al. reported that 43.1% of participants in their study opted for TSD for building rapport with children of age 4-7 years (2). As the child reaches 3-6 years, they represent objects symbolically and attain imaginary means of thinking. They are intensely curious about the dentist`s office and eager to learn about the novel things around (12). Hence, explaining the treatment and allowing them to handle and test the instruments for work would be a motivating factor in managing these children (13).

Positive reinforcement was reported as highly effective in children of 6-12 years by respondents in the present study, as the child derives from a sense of the industry and accomplishment during this stage of development. Peretz et al. also consider that receiving Positive reinforcement will facilitate positive dental attitudes in pediatric dental patients and promote future dental attendance (14). However, a theme related to the suitability and the personal value attached to receiving Positive reinforcement emerged.

Stop signals were reported as the most accepted BMT by 9-11-year-old children in an exploratory study investigating children's perceptions of dental BMTs in 2013. In line with these results, the vast majority of the respondents in our research determined Signaling as the most commonly used and most effective BMT in the treatment of children during the period of middle childhood (6-12 years). There is a dearth of literature reporting its use among dental practitioners [15]. Australian dentists were the only ones to report allowing the child to exercise some form of control over the treatment. As benefits of its use, the authors considered the provision of control aiding patient`s active role during treatment, relief of worry, distress and physical discomfort treatment (12).

Distraction is also a simple and effective BMT that could be used with any child, regardless of their cooperation level (4). The results of the current study are in line with other reported surveys where Distraction has been reported to be used by the majority of respondents during the treatment of children under 3 years (13,16). Concerning the attitude related to Distraction, more than half of the practitioners in India have responded positively to its use during the treatment of 2-7-year-old children (2). In contrast, Nazal et al. demonstrated that the routine use of such technique is less than TSD and Positive reinforcement (17).

Although some authors in the USA and Arabian region reported high-frequency use of Voice control (92%), in the present study only one-third of the respondents selected it for anxiety reduction during treatment of pediatric dental patients (13,17). Our study corresponds with a clear trend indicating a decline in the use of Voice control among dentists (18). This is consistent with a continual

decline in the acceptance of Voice control as an appropriate BMT among parents [19,20] Kuhn and Allen opined that the utilization pattern of BMT has changed over the past three decades. Parents` acceptability, legal/ethical concerns, accessibility and feasibility of certain BMTs are the factors most often cited for these changes (21).

The children of age group 0-3 years are emotionally attached to their parents and depict separation anxiety. Therefore, parental presence in the dental operatory is a crucial factor among these children during treatment. Practitioners should get accustomed to this added involvement of parents and welcome the queries and concerns (22). Nonetheless, 20.34% of the respondents in the current study use the Parental presence/absence technique while treating children under 3 years. The present results show a trend indicating a decrease in the use of Parental presence/absence technique with the increasing age of patients. In Egypt, almost all practitioners (93.10%) prefer this technique for the management of uncooperative 0-2 years patients, as the major problem in providing treatment for them are their infantile behavior and immature communication ability (6, 23). While in India, less than half of the respondents (40.2%) rely on parental presence while treating children of 0-2 years (2). A cross-sectional study in 2018 indicated that dentists utilized parental absence with approximately 34% of patients from all age groups (18). Probably similar usage across all age patients` groups responds with the parental demand for presence in the operatory. Several studies have reported that contemporary parents have a clear preference to be present in the operatory for all types of dental procedures – a trend reported in various countries and cultures (14,22). However, Parental absence can be an effective BMT and have high parental acceptance if discussed with parents before the treatment (24,25).

In past studies, the use of physical restraint was reported by more than 80% of respondents (13). Restraint techniques are recommended in specific situations (4). A study in the Arabian region showed a wide use of protective stabilization whose results are within the range reported by members of the AAPD – 68-73% (15,19). The least employment of non-pharmacological BMTs by the respondents in our study was for the restraint, used mainly for the very young patients (7.63%) – 0-3 years. In line with the current study, the use of restraint has been reported as the least used BMT amongst UK dentists (26). The present results showed that Latent inhibition has also been used by very few practitioners mainly for patients during the period of middle childhood. The specific indications, preparation and time consumption required for such techniques are likely reasons for the low frequency of use reported in the current and a previous study (17). In 2016 Williams found that dental practitioners are least familiar with this technique, as it is a psychological technique that is not a traditional part of dental curricula (16).

The analysis of the present results demonstrates that personal factors associated with the physical, emotional and psychological health of the child mainly influence dental practitioners` choice of BMT to be used in a particular pediatric dental patient. This indicates that the child`s emotion and presenting behavior in the dental setting are important. Of the individual-level factors of the children, the previous dental experience was reported by the majority of authors to influence their choice that underlines the importance of proper child management in pediatric dentistry (27). In line with the present findings, Oredugba et al. and Kawia et al. reported that a major factor influencing the choice of BMT was also the child`s age (27,28). Unlike the present results, Carr et al. reported the reason for the use of most BMT to be parental influence (29). Generally, the personal factors of the child were reported by more dentists than socioeconomic status and medical history to influence their choice for a BMT to be applied.

The information of the participants collected during the study will be kept strictly confidential and will not be disclosed to third parties. Confidentiality will be guaranteed by a coded ID number, access will be granted exclusively to the study investigators.

Conclusion

The results of the present study highlighted the use of a variety of non-pharmacological BMTs among dental specialists, although few acknowledged having adequate skills to apply the techniques. Distraction is the best BMT for patients under 3 years, while Positive reinforcement was the most effective in 3-6 years old children. Almost all respondents have rated Stop signals as the most commonly used non-pharmacological BMTs for patients older than 6 years. The choice of the technique was mainly influenced by the personal factors associated with the physical and phycological health of the child.

A limitation of the present study which is worth mentioning is the small sample size. Since the responsibility rate was lower than the ideal one, it had not been possible to survey the estimated larger sample size. Therefore, the chance of assuming as true a false premise has increased.

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A Comparison of Anxiety Level and Nutritional Habits of University Students During Exam and Non-Exam Periods

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Abstract

Objective: Stress and anxiety play an active role in determining the nutritional habits of an individual. This study was aimed to determine the relation between anxiety levels and nutritional habits of university students during exam and non-exam periods.

Methods: The study was conducted on a total of 259 students (131 females and 128 males) enrolled in the first grade of the Faculty of Health Sciences in a private university in Istanbul, during the 2019-2020 academic year. Participants' energy and macro-nutrient consumption were calculated using BeBis software program (8.1 version) and 3-day food consumption records. Turkish version of State-Trait Anxiety Inventory was used to analyse the anxiety levels. Obtained data was evaluated by SPSS (22.0 version) statistical package program. Anova and t-test were applied to determine the difference between energy and food consumption between the two genders during exam and non-exam periods ($p<0.05$).

Results: While there was no significant difference between number of meals consumed in a day or skipping of meals between males and females ($p> 0.05$), breakfast was the most skipped meal (28.96%), the most common reason being lack of time (20.46%). Percent of energy coming from carbohydrates, fats and proteins did not show a gender difference during examination times ($p>0.05$). However, in normal times, males were found to consume more fats and proteins whereas females consumed more carbohydrates ($p<0.05$). Frequency of consumption of tea-coffee, sugary and fried foods increased prior to examination period ($p<0.001$). State Anxiety Scores (SAI) decreased by 11 points during

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examination period in general (mean=34.08±8.58). State and trait anxiety scores were significantly higher in females ($p<0.05$).

Conclusion: Students consumed energy dense foods during/prior to examination periods related to anxiety levels. Providing students with adequate knowledge regarding balanced nutrition and psychological support during stressful periods are important for physical, spiritual and social development.

Keywords: *Anxiety Scale, Nutrition, Nutritional Habits, Exam Period, Non-Exam Period,*

Introduction

Adequate and balanced nutrition is very important in young adults due to rapid physical growth as well as pubertal and cognitive development. Food habits in young adults such as university students are different from general population due to time constraints, peer influence, palatability and therefore prefer foods which are easy to access, economic, tasty and easy to prepare (1). Unhealthy food habits as skipping of meals, high intake of sugary beverages, fried and salty food and low intake of fresh vegetables, wholegrains and legumes have led to increased incidence of obesity and other health problems in young adults in recent years (2). Females were more likely to choose healthier foods as fresh fruits and vegetables in accordance with their higher nutrition literacy status as compared to males (3,4). However, in other cases females tended to consume more carbohydrates, fatty foods or snack items under stress as compared to males (5,6). Males on the other hand were likely to purchase alcoholic or energy beverages (7,8). Economic factors and education levels were also reported to affect health behaviors as eating habits, physical activity, smoking and consumption of alcohol and drugs in young individuals (9). Therefore, it may be suggested that nutritional habits in young adults may be negatively influenced by various genetic, sociodemographic, and environmental factors.

Anxiety is defined as the emotion experienced when an individual encounters a new situation and does not know how to react to it (10). Anxiety was reported to affect an individual both physiologically and psychologically. Examination or test anxiety was encountered widely among young individuals especially if the success was related to their career choices and opportunities. While intermediate exam anxiety positively affected academic success, high-level anxiety was found to have negative effects on it (11). Although test anxiety is managed differently among individuals, it is found to affect food habits, physical activity, smoking, drinking and caffeine consumption in young adults. In addition, increased consumption of foods rich in carbohydrates and fat content whereas decreased

consumption of fruits and vegetables was noted among students leading to weight gain during these periods (11, 12).

This study was aimed to determine the relation between anxiety levels and nutritional habits of university students during exam and non-exam periods.

Methods

Study Participants

The study was conducted on a total of 259 students (131 females and 128 males) enrolled in the first grade of the Faculty of Health Sciences in a private university in Istanbul, Turkey during the 2019-2020 academic year. The study was conducted between January 2020-March 2020. Care was taken to enable a uniform distribution with respect to the students' gender and fields of study (Nutrition and Dietetics, Nursing, Physiotherapy, Health Management, Social Services, Child Development, Odiology). The total population of students enrolled in the Faculty of Health Sciences in the academic year 2019-2020 were 760 approximately. The experimental sample was calculated to be 255 according to stratified sampling method (confidence level 95%, margin of error 5%). The participants were selected on a random basis after taking their voluntary approval.

Ethical issues

The study was conducted in accordance with ethical standards of the responsible committee on human experimentation (institutional and national) and with Helsinki Declaration. Ethical approval for this study was acquired by the university's ethical committee dated on January 14, 2020 (Document No: B.30. AYD.0.00.00-500.06.04/220-2019/152).

Data Collection

Data was collected by a means of a questionnaires conducted by face-to-face interview technique.

Demographic Information Form

The individuals were asked to fill in general information about themselves as sex, age, weight (kg), height (m) and the department of the faculty that they were enrolled in. Body Mass Index (BMI was calculated from body weight and height measurements using the formula body weight / height2). BMI was assessed as per below: (15)

BMI <18 kg / m^2: underweight

18 <BMI <24.99 kg / m^2: normal weight

24.99 <BMI <29.99 kg / m^2: overweight-slightly obese

Dietary Habits

In the dietary habits section, questions related to eating habits of an individual were examined, such as the number of meals consumed per day, skipping meal habits, preferences of food types at meals, food preferences during examination periods. In addition, during the examination period and non-examination period. Food consumption frequency and a 3-day food consumption record was taken for all individuals during examination and normal periods, to calculate and compare the macro and micro-nutrients during these periods. The book "Food and Food Photo Catalog: Measures and Amounts" was used to determine the portions and amounts of foods consumed.

The participants were also questioned regarding their appetite (hunger and desire to eat) during meals in order to evaluate the reason for skipping meals. In addition, the students were also asked about consumption of caffeinated beverages and cigarettes during normal and examination periods.

Using the food consumption records, macro and micro-nutrients intake amounts were calculated using the BeBiS (Nutrition Information Systems) program.

Measurement of Anxiety: State Trait Anxiety Inventory (STAI)

Turkish version (Oner and Le Compte, 1983) of State-Trait Anxiety Inventory (STAI) (Spielberger et al, 1970) was used to analyse the anxiety levels of participants. The tool comprised of two sub-scales each consisting of 20 items evaluating state (SAI) and trait (TAI) anxiety levels using 4-point likert scale (not at all, somewhat, moderately so, very much so). The first part of the scale (SAI) indicated the anxiety level of the individual at that moment ("I am calm right now") and the responses were scored as 1=Not at all, 2=Somewhat, 3=Moderately so, 4=Very much so. The second part of the scale (TAI) indicated the continuous anxiety levels ("Usually I am in the mood") of individuals with the same kind of likert scale, however, the responses were scored as 0=not at all, 1=Somewhat, 2=Moderately so, 3=Very much so, respectively. Total scores vary between 20-80 points and higher scores indicated greater degree of anxiety.

Statistical Analysis

Obtained data were analyzed by the SPSS 22.0 software program. The means, standard deviations, frequencies and percentage values of demographic variables,

STAI scores and intake of nutrients by participants were calculated. As the data was found to be normally distributed, t-test and one-way analysis of variance (ANOVA) were applied to measure the effect of demographic data on the scales. Independent t-test was applied to compare the data between males and females for nutritient intakes and STAI scores during exam and non-exam periods. Level of significance was defined as $p<0.05$.

Results

The study was conducted on 259 students (131 females, 128 males) whose average age was calculated as 21.31±1.49 years. Mean height and weight of female participants were found to be 165.98±5.96 cm and 59.21±10.20 kg, whereas for males the values were 179.37±6.74 cm and 78.00±11.77 kg respectively. On categorizing the participants according to BMI ranges, 17.1% of participants were in the 24.99 - 29.99 range considered to be overweight or slightly obese. On the other hand, 67.6% of the participants were within BMI range of 19.0 - 24.99 and were considered normal.

Food habits of participants were enquired and recorded as per statements during non-exam periods. On enquiring about the number of meals that participants consumed in a day, 31.7% stated that they consumed three meals, 29.3 % four meals and 10.2 % consumed two meals in a day. Gender difference was not found to be significant ($p>0.05$). 56.8% of participants spent more than 500 TL and 30.9% spent 250-500 TL for personal food expenditures in a month. Regarding appetite during meals, %50.4 stated that they had adequate appetite whereas 45.6% mentioned that they lacked appetite during certain meals. Most of the participants (57.1%) drank 6-10 cups of water whereas 18.9 % consumed more than 10 cups and 23.9 consumed less than 5 cups of water in a day.

Of the participants, 76.4% consumed 1-5 cups of other beverages whereas 18.1% did not consume any other beverage apart from water. 96.1% of the student ate outside, 63.3% ate outside on 1-5 days, 20.5% 6-10 days, 12.7% on more than 10 days in a month. Regarding meals eaten outside, 59.07% ate lunch and 42.86% ate dinner outside. 63.0% of the students preferred fast food whereas 19.8% preferred home-made meals. Regarding eating late nights or on waking up in the middle of night, 39% responded negatively whereas 31.3% mentioned that they did so sometimes and 29.7% mentioned that they did it on a regular basis. Regarding vitamin and mineral supplements, 64.9% replied in negative, 14.7% sometimes took supplements 20.5% took supplements on a regular basis.

Regarding skipping of meals, 67.2% of females and 69.5% of male participants stated to skipped at least one meal in a day. Breakfast was the

most skipped meal (38.22%) followed by lunch (35.14%). The main reason for skipping meals stated by both genders was lack of time (39.0%) followed by lack of appetite (14.29%).

All the above questioned were repeated to the participants during exam periods. Independent t-test and ANOVA were performed to compare and evaluate the responses provided by males and females at two different times (exam and non-exam) to understand the effect of exam related anxiety on the food habits of participants (Table 2).

On analysing the snacking habits of participants, carbonated sugary drinks were the most consumed beverage (40.15%) followed by buttermilk (31.66%). Chocolates were found to be the most consumed item (54.83%) followed by nuts (44.79%), fruits (42.47%) and cookies (40.54%). Table 1 shows energy, carbohydrate (CHO), protein and fat consumption of participants in normal times.

Table 1. Energy, CHO, Protein and Fat consumption during non-exam times

	Gender	N	Mean	S.D.	t	p
Enerji (kcal)	Female	131	1390.57	388.67755	2.022	0.44
	Male	128	1283.51	459.64735		
CHO (g)	Female	131	159.587	56.92048	3.643	**0.000***
	Male	128	134.976	51.71989		
CHO (%)	Female	131	46.6641	7.11727	6.094	**0.000***
	Male	128	39.2422	11.84795		
Protein (g)	Female	131	57.3198	19.87399	0.855	0,393
	Male	128	55.0914	22.04717		
Protein (%)	Female	131	16.4427	3.02594	-3.230	**0.001***
	Male	128	17.6313	2.89506		
Fat (g)	Female	131	56.2061	18.08225	-0.639	0,523
	Male	128	58.3313	33.37331		
Fat (%)	Female	131	36.5344	5.85767	-3.031	**0.003***
	Male	128	38.9344	6.83438		

*p<0.05 (significant)

Regarding consumption of alcoholic and caffeinated beverages, 90.7% of participants consumed coffee, 90.6 % tea and 50.6 % alcohol on an occasional basis. 80.7 % of the students stated that they consumed 1-5 cups of coffee in a day, 69.9% stated consuming 1-5 cups of tea on a daily basis. Regarding alcohol, 80% of the students consumed alcohol 1-5 days in a month and12.3% consumed 6-10 days in a month.

On conducting the State Anxiety Inventory (SAI) during non-exam time, mean SAI score of participants was calculated as 34.08 ± 8.58 (Minimum 16 - Maximum 71). The scores were found to be 11 points lower than those calculated during exam times. Independent t-test was conducted to evaluate the difference between scores obtained by male and female participants. SAI score was found to be significantly higher in females during both exam and non-exam times ($p<0.05$).

Table 2 compares food habits, macro-nutrient intakes and SAI scores of male and female participants during exam and non-exam periods.

Table 2. Food habits, macro-nutrient intakes and SAI scores of participants

Items		N	Mean	S.D.	t	p
Amount (TL) spent on food per month	Exam Period	259	2.44	0.70	0.885	0.377
	Non-Exam Period	259	2.39	0.69		
Number of meals consumed in a day	Exam Period	259	3.76	1.14	-1.678	0.094
	Non-Exam Period	259	3.93	1.06		
Skipping Meals	Exam Period	259	1.32	0.47	-0.279	0.780
	Non-Exam Period	256	1.33	0.47		
Appetite	Exam Period	258	1.53	0.57	0.571	0.568
	Non-Exam Period	259	1.51	0.59		
Cups of water consumed in a day	Exam Period	259	1.95	0.65	-0.477	0.633
	Non-Exam Period	259	1.98	0.63		
Other beverages (Cups) consumed in a day	Exam Period	259	0.87	0.47	-0.184	0.854
	Non-Exam Period	259	0.88	0.49		
Eating of meals outside	Exam Period	256	1.04	0.19	0.027	0.979
	Non-Exam Period	259	1.04	0.19		
Frequency of meals taken	Exam Period	251	1.47	0.72	2.176	**0.030***
	Non-Exam Period	259	1.34	0.67		

Meal consumed outside	Exam Period	248	1.40	0.77	-0.360	0.719
	Non-Exam Period	259	1.42	0.82		
Consumption of coffee (yes/no)	Exam Period	259	1.09	0.29	-1.393	0.164
	Non-Exam Period	259	1.13	0.34		
Cups of coffee consumed in a day	Exam Period	259	1.04	0.51	1.237	0.217
	Non-Exam Period	259	0.98	0.55		
Consumption of tea (yes/no)	Exam Period	258	1.15	0.36	-0.227	0.821
	Non-Exam Period	259	1.15	0.36		
Cups of coffee consumed in a day	Exam Period	259	1.02	0.61	-0.075	0.940
	Non-Exam Period	259	1.03	0.56		
Consumption of food before sleep or middle of night.	Exam Period	259	2.02	0.78	-1.766	0.078
	Non-Exam Period	259	2.13	0.71		
Intake of vitamin/ mineral supplements	Exam Period	259	1.94	0.59	-1.882	0.060
	Non-Exam Period	259	2.04	0.58		
Energy (kcal)	Exam Period	257	1374.62	485.12	0.918	0.359
	Non-Exam Period	259	1337.67	427.78		
CHO (g)	Exam Period	257	213.37	917.71	1.154	0.249
	Non-Exam Period	259	147.42	55.69		
CHO (%)	Exam Period	257	46.74	8.34	4.505	**0.000***
	Non-Exam Period	259	43.00	10.41		
PRO (g)	Exam Period	257	54.15	19.99	-1.149	0.251
	Non-Exam Period	259	56.22	20.97		
PRO (%)	Exam Period	257	16.35	3.62	-2.331	**0.020***
	Non-Exam Period	259	17.03	3.02		

LIPID (g)	Exam Period	257	57.03	22.37	-0.104	0.917
	Non-Exam Period	259	57.26	26.72		
SAI (State Anxiety Inventory)	Exam Period	259	45.22	12.61	11.745	**0.000***
	Non-Exam Period	259	34.08	8.58		

* p<0.05 (significant)

As per results, AI scores of all participants, frequency of meals and CHO % were consumed significantly higher during exam times (p<0.05). On the other hand, percentage of protein consumed was significantly lower during exam time (p<0.05). (Table 2)

On comparing the SAI scores obtained by males (47.03±12.76) and females (44.42±12.45), no significant difference was found among the means of the two (p>0.05).

Table 3 below shows the Trait Anxiety Inventory (TAI) scores for male and female participants. This score indicates the continuous stress levels of the participants. Mean score obtained by the students was found to be 39.23±9.13. The lowest score was 4 and the highest score was 67. Mean TAI score of females was 41.01±9.97 whereas for males the score was 37.42±7.80. As per the results, TAI scores were found to be significantly higher in females. (p<0.05) (Table 3)

Table 3. TAI scores of Male and Female Participants

	Gender	N	Mean	S.D.	t	p
TAI Scores	Female	131	41.01	9.97	3.226	0.001
	Male	128	37.42	7.80		

Food consumption frequency of certain foods as milk products, meat, fruits and vegetables, grains, tea, coffee, cereals, legumes, sugary items, fatty and fried foods and energy beverages during exam periods and non-exam periods were enquired to the participants. Consumption of tea, coffee (63%), fatty fried foods (48.6%) and sugary items (64.1%) increased substantially during exam periods. On the other hand, consumption of fruits, vegetables, meat and milk products, legumes and grains were not affected during exam and non-exam periods.

Discussion

The prevalence of obesity in Turkish adolescents have increased from 0.6% to 7.3% with an 11.6-fold increase between the periods 1990-1995 to 2011-2015.

As per the latest Turkish national health survey results, prevalence of obesity has increased in both genders; However, boys were more likely to be obese than girls (16,17). In another study conducted on university students, 33.5% of males and 24.8% of females were found to be in overweight range (18). In this study, 17.1% of the students had a BMI in the 24.99 - 29.99 range of overweight or slightly obese. However, gender difference was not significant ($p>0.05$).

In this study, 31.7 % of participants consumed three and 29.3% four meals in a day, slightly higher than Ayhan et al's study in Bursa Uludag University in which 20.4% were reported to have consumed three and 5.4% four meals a day. In this study, there was no gender difference and effect of exam periods on meal consumption was not found to be significant ($p>0.05$) (19).

Normally, 67.2% of females and 69.5% of male participants stated to skipped at least one meal in a day. Breakfast was the most skipped meal (38.22%) followed by lunch (35.14%) in this study. Similarly, in a study conducted at Atatürk University students in Erzurum, morning meals were reported to be skipped the most and evening meals the least (20). In a study conducted in Ankara by Zemzemoglu et al, the major reason stated by the students (47.4%) for skipping meal was lack of time. This finding was similar to results of this study as 39% state the same reason for skipping meals. Choice of beverages by students in Zemzemoglu et al's study was similar to this study in the sense that coke, tea, coffee, buttermilk were the most popular drinks between meals (21).

In this study, eating outside (96.1%) and fast-food consumption were high among students (63.3%) during exam and non-exam times. In another study conducted on university students in Burdur, 62.5% of participants preferred fast food and 74.1% ate outside on a regular basis (22).

In this study, while 90% of the students consumed tea and coffee in general, students stated that they increased consumption of tea and coffee during exam period, in a study, conducted by Budak et al, 80.5% of the students were reported to consume coffee and 97.4% black tea (23).

In this study, the energy intake in exam and non-exam periods were 1374.62±485.12 and 1337.67±427.78 kcal respectively. These amounts were comparatively less as compared to a study conducted in Bingol University students where average daily energy intake was reported to be 3175.5±776.4 kcal in men and 2583.8±703.6 kcal in women. In addition, 44.05% of the foods consumed by the students contained carbohydrates, 43.35% fat and 11.5% protein (24). On comparing with his study however, the consumed CHO, protein and fat percentages of participants were similar. In this study, only carbohydrate (%) intake increased significantly during exam period ($p<0.05$) and protein

(%) decreased significantly during exam period ($p<0.05$). Furthermore, it was observed that women intake less energy than men during the non-examination period, but the amount of carbohydrate that women took in this period was higher than men, and that men consumed more protein than women (Table1). Energy consumption varies to a great extent among young population groups. In a study conducted on 450 students in Ankara preparing for university entrance exam, the mean energy consumption was found to be 1738.2 kcal varying between 696.2 - 4294.1 kcal/d (25).

In this study, the Trait Anxiety Scale (TAI) was used to evaluate the continuous anxiety score of the students. The mean score of the students was found to be 39.23±9.13. The lowest score was 4 and the highest score was 67. The mean TAI score of female students (41.01±9.97) was significantly higher as compared to males (37.42±7.80) ($p<0.05$) (Table 3). In a similar study conducted on another group of university students, the mean SAI score of students was found to be 57±5.25. In the normal period, it was observed that the anxiety levels of men were moderate and the anxiety levels of women were high (26). In this study, SAI scores during non-exam time, mean SAI score of participants was calculated as 34.08 ± 8,58. The scores were found to be 11 points lower than those calculated during exam times. SAI score was found to be significantly higher in females during both exam and non-exam times ($p<0.05$). In another study conducted by Bayindir et al on-university students preparing for exams, (27) TAI scores of female students were found to be significantly higher (47.2±6.35) as compared to males (45.4±6.85). In a study carried out on 4850 university students in Bursa Turkey, 29.6% and 36.7% of the students reported state (SAI) and trait (TAI) anxiety scores of more than 45 points, respectively (28).

Conclusion

Nutritional habits of young adults as university students are affected by several factors as gender, age, socio-demographic characteristics, social circle and so on. Stress and anxiety factors have been found to affect eating habits of students. The study demonstrated that state and trait anxiety levels were higher in females as compared to males in exam and non-exam times. Individuals tended to consume food items as high sugar and fatty fried foods, tea, coffee and other caffeinated beverages during exam times. At the same time students tended to skip meal eat outside and prefer fast food during exam and non-exam periods. Consumption of dairy products, meat, fresh fruits and vegetables, grains and legumes remained unchanged.

Considering the rising obesity among adolescents and youth being a crucial stage for adopting dietary habits that may continue throughout lifetime,

increase in awareness among this age group regarding healthy eating and dietary quality is of special importance from the perspective of health and prevention of obesity related chronic diseases. The evidence stresses on increasing public awareness, recommends health practitioners and policymakers to consider public health strategies and regulations that focus on increasing nutrition education, knowledge and health awareness in the society.

Conflict of Interest and Acknowledgement

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Routine Cervical Dilatation at Cesarean Section and Its Influence on Pain Scores and Post-Operative Morbidity: A Randomized Controlled Study

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Abstract

Objective: We hypothesized that, collection of blood in the intrauterine cavity and distended uterus in women after elective caesarean section could lead to increasing pain. The aim of this study was to identify the effect of routine cervical dilation during elective caesarean section on infectious morbidity, postpartum hemorrhage, and pain after labor by using visual analogue scale (VAS).

Methods: A case-control study was conducted on 245 pregnant women who had elective cesarean section at Bakırköy Dr. Sadi Konuk Teaching and Research Hospital, Istanbul. The cases had intraoperative digital cervical dilatation at elective cesarean section while the controls did not.

Results: There was a significant reduction in hemoglobin levels postoperatively in each group (p=0.001 for both groups), but the level of reduction was not statistically different between the groups (p = 0.37). The duration of operation was not statistically different between the two groups. In addition, there were no statistically significant difference in febrile morbidity (p =0.478), endometritis (p = 0.311), wound infection (p = 0.297) and UTI (p =0.479). Mean postoperative endometrial cavity thickness of the dilated group was significantly less than that of the non-dilated group (7.8 ± 4.1, 6.8 ± 3.8, respectively, p=0.044). We found VAS scores of dilated group to be significantly less than those of the non-dilated group (5.4 ± 3.0, 4.6 ± 2.8, respectively, p=0.023)

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Conclusion: In conclusion, cervical dilatation during cesarean section does not increase the incidence of postoperative endometritis or wound site infection, but it may decrease postoperative pain. However, larger studies on homogenous patient groups are required for generalization of these results.

Keywords: *Cervical dilation, elective cesarean section, endometrial thickness, infectious morbidity, visual analogue score*

Introduction

Cesarean section (CS), the most common obstetric surgery, can be either an elective or an emergency procedure. The surgical technique varies amongst centers and individual surgeons. Infectious morbidity is the most frequent complication of CS(1). Strategies to minimize postoperative morbidities includes modifications in surgical technique, methods of placental delivery, changing gloves and altering the uterine position during repair of the uterine incision (2-6).

A woman's cervix is firm and undilated at the beginning of pregnancy and some obstetricians consider undilated cervix at pre-labor to result in obstruction of blood and lochia drainage (7). Collection of lochia or debris may lead to postpartum hemorrhage and endometritis. To circumvent this, obstetricians routinely dilate the cervix from above. Finger, sponge forceps, or other instruments can be used for dilatation. In contrast, mechanical cervical dilatation during CS may result in cervical trauma and increase the risk of infection. Endometritis appears to result from contamination of uterus, abdominal cavity and the abdominal incision with ascending vaginal flora bacteria (8).

We hypothesized that, collection of blood in the intrauterine cavity and distended uterus in women after elective caesarean section could lead to increasing pain. Previously published studies have evaluated infectious morbidity and postpartum hemorrhage after the dilatation of the cervix during elective CS. However, none of these have evaluated effect of dilatation on pain. The aim of this study was to identify the effect of routine cervical dilation during elective caesarean section on infectious morbidity, postpartum hemorrhage, and pain after labor by using visual analogue scale (VAS) (9).

Materials and Methods

This prospective randomized study was carried out at Bakırköy Dr. Sadi Konuk Teaching and Research Hospital from January 2013 to March 2015. A total of 285 cases of elective caesarean delivery were enrolled in this study after obtaining informed consent. The Non-invasive Human Research Ethics

Committee and Local Health Ministry Authority approved the study (Approval number: 2012-14-06, ClinicalTrials.gov Identifier: NCT01954719). Patients were randomized using computer-generated random numbers into either intraoperative cervical dilatation (Group A, n=143) or no intraoperative cervical dilatation (Group B, n=142) groups. The investigator was not blinded to the procedure allocation. The trial profile is shown in Figure 1.

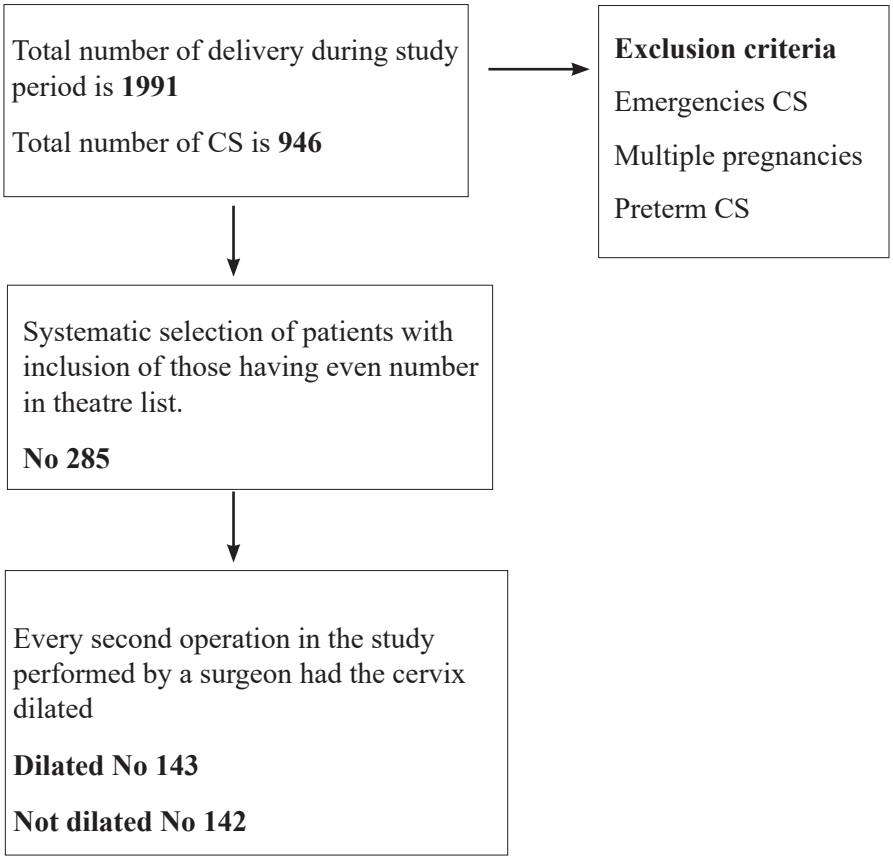

Figure 1. *Consort diagram for randomization*

All participants in the included studies were undergoing elective caesarean section at gestational ages more than 37 weeks. The exclusion criteria were: women undergoing CS due to multiple pregnancies, preterm births, rupture of membranes, fever on admission, chorioamnionitis, need for blood transfusion before or during caesarean section, use of antibiotics during the last 24 h, women who felt labor pain before their previous cesarean operations.

Cesarean operations were performed by the same operators. Before CS, a Foley catheter was inserted and the abdomen was cleaned with povidone iodine solution. Intravenous 1 g cefazolin was administered to each participant when the umbilical cord was clamped. Except for digital cervical dilatation, all surgical techniques and suture materials were same in both groups. This included pfannenstiel incision, blunt entry into the peritoneal cavity, lower segment transverse uterine incision digitally expanded to deliver the fetus a placenta. In the cervical dilatation group, the surgeon performed the cervical dilatation by inserting the double-gloved index finger into the cervical canal of the patients after the extraction of placenta and membranes. The outer glove was removed after this procedure. Suturing of the uterine incision was performed without exteriorization of the uterus. The abdominal wall was closed in two layers. Skin incisions were closed.

Postoperative patient care was the same for both groups. The temperature was measured every4 h using sublingual digital devices. Clinical signs of urinary tract infection (UTI) were checked and urinalysis was performed. At 8 h postoperatively, urinary Foley catheters were removed and oral fluid intake was started. All patients had their complete blood count tested prior to surgery and 24h postoperatively. All patients were monitored for clinical signs of post-operative infectious morbidity including febrile morbidity, wound infection, endometritis and UTI. Febrile morbidity was defined as an axillary temperature of 38°C or more measured twice or more after 24 h post-delivery. Wound infection refers to clinical evidence of purulent or serous discharge with induration, warmth and tenderness or bacteriological evidence on a swab of suspicious area of the wound. Endometritis was defined as axillary temperature greater than 38°C associated with purulent, foul smelling vaginal discharge or uterine tenderness on bimanual examination. UTI was suspected in the presence of symptoms and confirmatory diagnosis made when culture of midstream urine specimen revealed significant bacteriuria (>100,000 organisms/mL). All the patients were followed up with specific attention to the clinical aspects of endometritis, such as foul-smelling vaginal discharge, uterine tenderness. Wound infection was detected clinically by presence of smelly discharge, erythema, induration, and tenderness. Blood loss during CS was estimated using a drop in packed cell volume 24 hours postoperatively.

At 24 h post-operation, endometrial cavity thickness was measured using transabdominal ultrasound. The measurement involved both the decidual thickness and intracavitary fluid collection. Patients without infection or other complications were discharged on the third postoperative day

Patients were informed verbally and in a written form about the timing of postpartum control examinations and the need to contact the physician in case of adverse events, such as findings of infection including fever, foul smelling vaginal discharge or abnormal bleeding after discharge from the hospital. They were invited to Bakırköy Dr. Sadi Konuk Teaching and Research Hospital Obstetrics and Gynecology clinic on the 7th day and at 6 weeks after delivery for control examination and for detection of complications including infections in the puerperium period. On the 7th day after operation, patients' pain levels were evaluated by using VAS (9). It is self-completed by the patients. The patients were asked to place a line to the perpendicular VAS line at the point that describing their pain intensity.

The primary outcome was rate of post-partum endometritis, postpartum hemorrhage and pain. Secondary outcomes that were analyzed included wound infection, febrile morbidity and infectious morbidity. Blood loss at surgery and operative time were compared between the two groups.

Sample size estimation

Our primary outcome was comparing of VAS scores between the two groups. Using the program 'MedCalc', we calculated that a sample size of 141 subjects would be required for each group. Influence was 0.33 with a sample size of 141 in each group that would provide 80% power to detect a 95% difference in VAS scores. Assuming a 10% dropout rate, 128 women were required in each study group.

Statistical analysis

Data were analyzed using descriptive statistical methods (mean, standard deviation, median, frequency, rate, Minimum, Maximum). The distribution of variables was assessed by Kolmogorov-Smirnov test. Mann-Whitney U test was used for comparison of independent two groups. Wilcoxon test was used for comparison of two dependent groups. Statistical analyses were performed using Statistics Package for Social Sciences version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

A total of 245 women were included in this study. Of these 245 women, 143 women had intraoperative digital cervical dilatation and 142 women with no intraoperative cervical dilatation. The mean age of patients in the cervical dilatation group and the non-dilated group was 29.8 ± 5.4 and 30.4 ± 5.9, respectively. There were no statistically significant differences between the two

groups with regard to maternal age, gravidity, parity, gestational age on the day of cesarean, birth weight, preoperative temperature, duration of hospitalization and 1st and 5th minute Apgar scores of the babies. Furthermore, there was no statistically significant difference in the mean baseline pre-operative hemoglobin blood level between the two groups (11.0 ± 1.6 vs. 11.1± 1.4, p = 0.516). Please refer to Table 1.

Table 1. Demographic and clinical properties of patients

Variable	**Cervical dilatation (n = 143) (mean±SD)**	**No Cervical dilatation (n = 142) (mean±SD)**	**p**
Age	29.8 ± 5.4	30.4±5.9	0.364
Gravidy	2.5±1.2	2.7±1.3	0.083
Parity	2.0±1.1	2.0±1.2	0.924
Gestational age at delivery	37.4±3.0	37.4±2.5	0.212
Birth weight (kg)	3001±778	3078±705	0.771
Preoperative hemoglobin blood level (g/L)	11.0±1.6	11.1±1.4	0.516
Preoperative temperature (°C)	36.4±0.3	36.5±0.6	0.327
First minute Apgar score	7.6±1.9	7.7±1.5	0.524
Fifth minute Apgar score	9.1±1.5	9.0±1.1	0.121
SD: Standard deviation			

As shown in Table 2, there was a significant reduction in hemoglobin levels postoperatively in each group (p= 0.001 for both groups), but the level of reduction was not statistically different between the groups (p = 0.37). In other words, preoperative and postoperative hemoglobin levels of the groups did not reveal a significant difference. The duration of operation was not statistically different between the two groups. In addition, there were no statistically significant difference in febrile morbidity (p =0.478), endometritis (p = 0.311), wound infection (p = 0.297) and UTI (p =0.479). Mean postoperative endometrial cavity thickness of the dilated group was significantly less than that of the non-dilated group (7.8 ± 4.1, 6.8 ± 3.8, respectively, p=0.044). We found the VAS scores of dilated group to be significantly less than those of the non-dilated group (5.4 ± 3.0, 4.6 ± 2.8, respectively, p=0.023) as seen in Table2.

Tables 2. Intra-operative and post-operative outcomes

Variable	Cervical dilatation (n = 143) (mean±SD)		No Cervical dilatation (n = 142) (mean±SD)		p
Operative time (minute)	37.0±10.1		37.6±11.2		0.913
Post-operative hemoglobin (g/dl)	10.3±1.5		10.1±1.3		0.100
Drop in hemoglobin (g/dl)	-0.7±1.2		-0.9±1.0		0.157
Febrile morbidity (n, %)	19	16.9%	15	10.6%	0.478
Endometritis (n, %)	3	2.1%	1	0.7%	0.311
Wound infection (n, %)	10	7.0%	6	4.2%	0.297
UTI (n, %)	5	3.5%	6	4.2%	0.479
Blood loss (ml)	561.5		565.5		0.465
Endometrial cavity thickness (mm)	7.8±4.1		6.8±3.8		***0.044***
VAS	5.4±3.0		4.6±2.8		***0.023***

SD: Standard deviation; UTI: Urinary tract infection; VAS: Visual analogue scale

In the study population, 123 and 119 women had a previous CS in each group. The other indications for cesarean delivery in women were: Cephalopelvic disproportion (2.1%, 2.8%), breech presentation (7.7%,7.7%), poor obstetric history (1.4%,2.1%), hypertensive disorders (1.4%,2.8%), placenta previa (0.7%,0.00%) and multiple gestation (0.7%,0.7%) (Table 3).

Table 3. Indications for elective cesarean sections

		Cervical dilatation (n = 143),(n,%)		**No Cervical dilatation (n = 142),(n,%)**	
Indication	Repeated CS	123	86.0%	119	83.8%
	Cephalopelvic disruption	3	2.1%	4	2.8%
	Breech presentation	11	7.7%	11	7.7%
	Poor obstetric history	2	1.4%	3	2.1%
	Hypertensive disorders	2	1.4%	4	2.8%
	Placenta previa	1	0.7%	0	0.0%
	Multiple gestation	1	0.7%	1	0.7%
CS, caesarean section; CPD, cephalopelvic disproportion;					

Discussion

Several CS methods to minimize surgery-related morbidity have been investigated. However, the advantages and disadvantages of cervical dilation during CS are still controversial. Dewhurst pointed to the pros and cons of the cervical dilation still being questionable, and thus requiring further investigation (10). The presence of dilatation during C-section has been examined by several authors over the years and various ideas have been put forth (11, 12). Nevertheless, there are only a few randomized studies on the relevant topic in the literature (13-16).

Bollapragada et al. emphasized the need for ultrasonography in the absence of lochia in uterine, which may be caused by intrauterine collection after CS, and hence cervical dilatation must be performed in the patients undergoing elective CS (17). We observed in this study that the endometrial thickness was significantly reduced in the dilatation cases; however, we have doubts about the clinical significance of these results. Our study also indicates that the thickness of endometrium evaluated at postoperative 24th hour was not associated with

postoperative pain. There are studies suggesting an ascending bacterial infection to cause endometritis, peritonitis and wound infections in the setting of cervical dilatation (18, 19). However, in this study, none of the cases developed peritonitis.

In the randomized controlled study on 131 patients by Ahmet et al., no difference was detected between patients with dilation and without dilation during C-section regarding febrile morbidity and endometritis, and none of these patients developed wound site infection. Even though we obtained similar results in this study, the wound site infection was less common in patients who had dilatation, but the difference was not statistically significant. Güngördük et al. did not observe any significant difference in febrile morbidity and infection between the patients; however, they reported the duration of the operation to be significantly longer in cases with cervical dilation (15). In the present study, no difference in the duration of the operation was detected.

As a result of their study, Tosun et al. argued that routine dilation is not necessary during CS. However, researchers that have examined the endometrial thickness after cesarean section have reported the endometrium to be significantly thinner in the dilation group (13). Although similar results were seen in our study, we do not believe it to have any clinical significance. None of our patients with increased endometrial thickness and fluid accumulation in the intrauterine cavity developed a hemorrhagic complication. We did not detect any difference in VAS scores of patients with vaginal labor history; however, the heterogeneous nature of the study population is a weakness of the study.

On the other hand, the investigation of the patients' pain symptoms using the VAS on the 7th postoperative day to identify the association of cervical dilation in CS with postoperative pain can be considered as one of the strengths of the study. We measured significantly lower VAS scores for the patients that had cervical dilation. Moreover, single center randomized study with and the performance of operations by the same surgeons are the strengths of the study.

A limitation of the current study was that the differences between the patients with normal vaginal labor history and those who had primary cesarean section could not be evaluated due to limited number of cases. Additionally, the study population was not homogeneous with regards to the number of previous CS operations. Furthermore, the influence of prior CS operation counts on VAS score could not be assessed. Nevertheless, this is one of the very few studies in the literature on the relevant topic.

In conclusion, cervical dilatation during cesarean section does not increase the incidence of postoperative endometritis or wound site infection, but it may decrease postoperative pain. However, larger studies on homogenous patient groups are required for generalization of these results.

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Review of ER:YAG Laser-Assisted Pulpotomy in Primary Dentition

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Abstract

The endodontic infection of primary teeth is the infection of the root canal system and can involve the pulp as well as the periapical tissues surrounding the apex of the tooth root. The main goal is to maintain the integrity and health of the primary tooth until their physiological exfoliation. Pulpotomy is the most commonly used method for endodontic treatment of deciduous dentition. Knowledge of the alternative pulpotomy techniques is a useful advantage of dentists to meet the functional problems associated with endodontic infection in very young and anxious patients. The aim of this article is to systematically review the contemporary scientific literature concerning the effect of Er:YAG laser technology in the pulpotomy of primary teeth. A critical evaluation of the various parameters for laser ablation and unconvincing results has been made. The expository analysis summarizes the succession of this therapeutic approach. Future studies should also seek and compare the long-term effects of the use of traditional and alternative pulpotomy techniques.

Keywords: *Pulpotomy, Er:YAG laser, Primary dentition, Pediatric dentistry*

Introduction

The endodontic infection of primary teeth is the infection of the root canal system and can involve the pulp as well as the periapical tissues surrounding the apex of the tooth root. In these cases, there are two treatment options – pulpotomy and pulpectomy. Pulpotomy is a treatment method including removal of the coronal

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pulp (vital or mortal amputation) and preservation of the radicular pulp treated with a pulpotomy medicament to maintain its vitality or to transform it into an aseptic bundle, which is like plastic (1). The other treatment method, pulpectomy, represents a complete removal of necrotic and irreversibly infected pulp tissue of the pulp chamber and root canals of a tooth affected by extensive caries lesions, traumatic injuries or other causes (2,3). The main goal of endodontic treatment of primary teeth is to maintain the integrity and health of the primary tooth until their physiological exfoliation (4). As the tooth remains asymptomatic, in anatomical and functional conditions, it performs an extremely important role in the masticatory process and has phonetic, aesthetic and morphological functions (2,4).

Pulpotomy is the most commonly used method for endodontic treatment of deciduous dentition (1). Several important and essential elements in pulpotomy as a pulp therapy are required – adequate debridement of the infected coronal pulp tissue and antimicrobial activity of the irrigants and medicaments, sufficient hemostasis and no thermal affection of the remaining pulp and surrounding tissues. Owing to the properties of Er:YAG lasers, they can be used as an alternative to the `gold standard`, i.e., formocresol, for pulpotomy in primary teeth. This laser family has hemostatic and antimicrobial effect as well as causes no or slight thermal alterations of the surrounding tissues and the underlying radicular pulp (5,6,7,8).

Few studies described the results of Er:YAG laser-assisted pulpotomy in primary teeth. Due to the numerous advantages of Er:YAG lasers, modern pediatric dentistry involves their use in routine daily practice (9,10,11). In addition, pulpal diseases in primary dentition are a common dental problem (1). Thus, the aim of this article is to systematically review the contemporary scientific literature concerning the effect of Er:YAG laser technology in the pulpotomy of primary teeth. A critical evaluation of the various parameters for laser ablation and unconvincing results has been made. The expository analysis summarizes the succession of this therapeutic approach (Table 1).

Table 1. Overview of the Er:YAG pulpotomy studies

Authors	Year of publication	Country	Type of article	Average pulse-energy	Hz	irra-dia-tion time	Water spray	Material used after Er:YAG laser pulpotomy
Jayawardena et al.	2001	Japan	Experimental animal study	150 mJ/ pulse	10	n/a	+	Sterile saline solution
Kimura et al.	2003	Japan	Experimental animal study	34mJ/pulse	n/a	15 s	n/a	n/a
Huth KC et al.	2005	Germany	Clinical trial	180mJ/ pulse	2	n/a	-	Formocresol-moistened pellets for 5 minutes
Olivi et al	2007	Italy	Clinical trial	75 -100 mJ/ pulse	3	60 s	+	Immediately filled with adhesive systems: a flowable composite
Kotlow et al.	2008	USA	Clinical case	55mJ/pulse	30	15s, 3 times	+	Zinc oxide eugenol cement and the appropriate restoration
Hasheminia et al.	2010	Iran	Experimental animal study	200mJ/ pulse	3	15s	-	MTA
Huth KC et al.	2011	Germany	Clinical trial	120mJ/ pulse	2	n/a	-	Ferric sulphate-wetted pellets for 15s

*n/a - not applicable

In an experimental animal study, Kimura et al. found that the effects of the Er:YAG laser irradiation on pulp tissues during a pulpotomy are minimal. The authors reported no inflammation or resorption in the investigated rat molars when 34mJ/pulse for 15 seconds was used. As a result of the laser irradiation, there was no bleeding after coronal pulp removal (6). As a result of other animal studies, the researchers concluded that the investigated parameters for laser irradiation were not recommended for the treatment of human teeth in clinical settings (7,12). However, other authors stated that if appropriate parameters were used, the application of Er:YAG laser is a treatment option for pulpotomy in primary teeth (6).

As to the effect of Er:YAG laser on the pulp tissue bleeding, Olivi et al. pointed out as a disadvantage of Er:YAG laser the lack of complete hemostasis due to the minimal thermal effect of the surrounding tissues (13). However, the authors reported an easier control of bleeding after laser irradiation compared to the conventional treatment procedures (13). In 2015, Nazemisalman et al. confirmed the data obtained by Olivi et al. They found favorable results after 2 years of follow-up investigating the pulp coagulation effect of Er:YAG laser (14). Keller et al. reported a 3-6 minutes duration about the reduction of pulp microcirculation after Er:YAG laser irradiation as well as no hyperemic actions (8,15).

In a long-term study, Huth et al. investigated the effectiveness of Er:YAG laser in pulpotomy of primary teeth (8,15). After 36 months, the authors reported 73% success rates which are comparable to those of the application of `gold standard` (formocresol) – 72%, and ferric sulfate - 76%. The follow-up included clinical and radiograph examinations. The parameter settings used were wavelength 2940 nm, 2 Hz and 180 mJ per pulse without water cooling, 31.5±5.9 mean number of laser pulses per tooth. No significant differences in total success rates between the different pulpotomy techniques were found (8,15).

Kotlow et al. presented a protocol for performing Er:YAG laser-assisted pulpotomy in deciduous teeth. The authors described a series of clinical cases of endodontic treatment in primary molars using laser technology (16). The successful results, clinical and paraclinical, confirmed the effectiveness of Er:YAG laser in pulp therapy of primary teeth as an alternative to the chemical agents or electrosurgery. The teeth from the presented clinical cases were irradiated with Er:YAG laser (HOYA ConBio`s DELight) emission wavelength 2940 nm, three times for 15 seconds. The parameter settings used were 30 Hz and 55mJ, 20cc per minute water spray, average power 1.65 watts. In the study, the sapphire tip size used was 600-μm in a 90-degree handpiece. In contrast, Huth et al. described the use of a special handpiece (KEY Laser 1242; handpiece 2051, KaVo, Biberach, Germany), (15).

In a systematic review, Coster et al. analyzed seven articles and indicated the need for future studies about laser-assisted pulpotomy in order to formulate general recommendations for the clinical use of Er:YAG laser in primary teeth (17). The obtained data of another systematic review and meta-analysis are in line with the results obtained by Coster et al. who found a lack of convincing results and the necessity of further investigations (18).

Conclusion

There is not enough information describing the level of effectiveness of the use of the Er:YAG laser for pulpotomy despite its importance in the pulp therapy of deciduous teeth. In few publications, the success rate reported was the same as that of formocresol and ferric sulfate. Thus, this technology can be used successfully as a safe alternative to conventional pulp therapy agents. However, if Er:YAG laser is not part of the routine clinical setup and dental equipment, it would be difficult for general dental practitioners to implement this technology for endodontic treatment of primary teeth.

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Manuscript Preparation

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The upper limit of plagiarism determined by the editorial board for the journal is 20 percent. The filtering options in the plagiarism detection program are set to neglect references, quotes, and text sections of less than five words.

If there is an institution that supports the study, the last word of the article title should have an asterisk (*) and the information on the same page should be given as a footnote.

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A separate title page should be submitted and this page should include:

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- Grant information and detailed information on the other sources of support,
- Nameandaddress,phone(includingthemobilephonenumber) number and email address of the corresponding author,
- Acknowledgment of the individuals who contributed to the preparation of the manuscript without fufilling the authorship criteria.

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constructed with subheadings (Objective, Methods, Results, and Conclusion). All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition. Please refer to Table 1 below for word count specifications.

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All manuscripts except Brief reports and Letters to the Editor must be accompanied by a minimum of three to a maximum of six keywords at the end of the abstract. Keywords should be selected from Medical Subject Headings (MeSH) of Index Medicus (https://www.nlm.nih.gov/mesh/MBrowser.html) Keywords will be used for subject indexing.

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Article Type	Text Words	Abstract Words	Keywords	References	Tables	Figures/ Images
Original Article	7.500	300	5	30	10	10
Review Article	10.000	250	5	50	10	20
Case Report	1.500	200	3	20	1	10
Brief Report	2.000	200	3	20	1	10
Letter to the Editor	1.000	No abstract	No keywords	10	1	No figures/ images

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Information on statistical analyses should be supplied in a separate subheading under the Materials and Methods section and the statistical software that was used during the process must be included. Units should be prepared in accordance with the International System of Units (SI).
Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

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Font: Times New Roman style and 11 pt. font size are used for the whole text. All article should be justified. Single line spacing should be used throughout the main text and between the paragraphs.

Title: Bold capital letters in 14-pt must be used fort he main title. Subtitles should be written in bold and 11 pt,. After the title, author names, author ORCID numbers and e-mail addresses should be stated in 11 pt font size, with two lines of space.

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