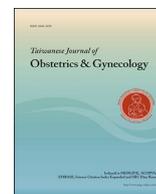




Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Efficacies of vitamin D and omega-3 polyunsaturated fatty acids on experimental endometriosis

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ARTICLE INFO

Article history:

Accepted 26 June 2015

Keywords:

endometriosis
omega-3 polyunsaturated fatty acid
rat
vitamin D

ABSTRACT

Objective: The aim of this study was to investigate the effects of 1,25-dihydroxyvitamin-D3 (vitamin D) and omega-3 polyunsaturated fatty acids (omega-3 PUFA) on experimentally induced endometriosis in a rat model.**Materials and Methods:** A prospective, single-blind, randomized, controlled experimental study was performed on 30 Wistar female rats. Endometriosis was surgically induced by implanting endometrial tissue on the abdominal peritoneum. Four weeks later, a second laparotomy was performed to assess pre-treatment implant volumes and cytokine levels. The rats were randomized into three groups: vitamin D group (42 µg/kg/day), omega-3 PUFA group (450 mg/kg/day), and control group (saline 0.1 mL/rat/day). These treatments were administered for 4 weeks. At the end of treatment, a third laparotomy was performed for the assessment of cytokine levels, implant volumes (post-treatment) and implants were totally excised for histopathologic examination. Pre- and post-treatment volumes, cytokine levels within the groups, as well as stromal and glandular tissues between the groups were compared.**Results:** The mean post-treatment volume was statistically significantly reduced in the omega-3 PUFA group ($p = 0.02$) and the level of the interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF) in the peritoneal fluid were significantly decreased at the end of treatment in the omega-3 PUFA group ($p = 0.02$, $p = 0.03$, and $p = 0.03$, respectively). In the vitamin D group, only IL-6 levels were significantly decreased. In the histopathologic examination, the glandular tissue and stromal tissue scores of the implants were significant lower in the omega-3 PUFA group ($p = 0.03$ and $p = 0.02$).**Conclusion:** Omega-3 PUFA caused significant regression of endometriotic implants. Vitamin D has not been as effective as omega-3 PUFA on endometriosis.Copyright © 2016, Taiwan Association of Obstetrics & Gynecology. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Endometriosis is defined as the presence of functional endometrial gland and stroma outside the uterine cavity and represents one of the most frequent gynecological diseases affecting 10–15% of all women of reproductive age and 30–50% of infertile women [1,2]. In spite of being a very common problem worldwide, the

etiopathogenic mechanisms which lead to development of endometriosis are still unclear. Currently, there is no proven effective treatment for endometriosis, due to the limited understanding of its pathogenesis.

Nowadays, a combination of retrograde menstruation, defective immunological mechanisms preventing the clearance of endometrial cells from the peritoneal cavity, and changes in the peritoneal environment that stimulates cell growth is the most widely accepted explanation for the development of pelvic endometriosis. Disease progression depends on the degree of impaired cell mediated immunity and proliferative effects of cytokines. Immune

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cells in the peritoneal fluid of women with endometriosis also secrete cytokines, as well as growth and angiogenic factors that stimulate implantation and proliferation of misplaced endometrium and local angiogenesis. Various cytokines including tumor necrosis factor- α (TNF- α), vascular endothelial growth factor (VEGF), interleukin 1 (IL-1), IL-6, IL-8, and IL-10 were reported to be increased in the peritoneal fluid of women with endometriosis [3]. Recent studies have reported that those cytokines may be involved in macrophage activation, inflammatory change and enhanced angiogenesis.

Based on these pathogenic mechanisms, many experimental studies have been performed with immune modulators (e.g., rapamycin, pentoxifylline, lipoxin A4) and antiangiogenic agents (e.g., bevacizumab, sorafenib, romidepsin, atorvastatin, endostatin) to contribute to better understanding of the etiopathogenesis and optimal treatment of endometriosis [4]. However, further studies are needed to assess the role of nutritional factors in the prevention and treatment of endometriosis as placebo-controlled experimental studies investigating the effects of nutritional factors on endometriosis are limited.

Daily essential nutrients and vitamins can modulate immune and inflammatory processes which are altered in women with endometriosis. For example, besides its well-known effects on calcium-phosphate homeostasis, 1,25-dihydroxyvitamin-D3 (vitamin D) has strong immune modulating potential and effects on cell proliferation and differentiation in normal as well as in malignant cell types. Both endometriosis and vitamin D deficiency have been associated with autoimmune diseases such as rheumatoid arthritis, Crohn disease, and psoriasis [5]. Furthermore, the vitamin D receptor and vitamin D metabolizing enzymes are found in the endometrium and ovaries of women with and without endometriosis, in addition to the different immune cells and it is probable that vitamin D has the capability of acting in an autocrine/paracrine manner in a local immunologic environment [6].

In recent years, antiangiogenic, anti-inflammatory, anti-apoptotic, and antiproliferative effects of omega-3 polyunsaturated fatty acids (omega-3 PUFA) have been demonstrated in several experimental studies [7,8]. In line with these observations, omega-3 PUFAs may be a very suitable treatment option for the pathways in the pathophysiology of endometriosis.

In terms of immunomodulatory, antiangiogenic, and anti-proliferative effects, vitamin D and omega-3 PUFAs are among the most frequently cited nutrients. To optimally reveal these properties of vitamin D and omega 3 PUFA, we performed a single-blind placebo-controlled study. In this study, experimentally induced endometriosis was treated with vitamin D and omega-3 PUFA. Thereafter, macroscopic and histologic changes in the implants as well as the changes of cytokine levels in peritoneal fluid were investigated.

Materials and methods

This prospective, single-blind, randomized, controlled experimental study was performed to evaluate the efficacy of vitamin D and omega-3 PUFA on endometriotic implants in a rat endometriosis model. The experimental procedures were conducted at Firat University Experimental Research Center with the approval of the animal ethics committee of the university. Thirty mature, female Wistar albino rats weighing between 200 and 220 g were used. Animals were fed *ad libitum* and housed in steel cages having a temperature-controlled environment ($21 \pm 2^\circ\text{C}$) with 12-h light/dark cycles. The animals used in this study were handled in accordance with international guidelines for the care and use of laboratory animals.

Each rat underwent three consecutive laparotomies. The rats on estrus cycle detected by vaginal smear were anaesthetized by intramuscular administration of 60 mg/kg ketamine hydrochloric acid (Ketalar, Eczacibasi Warner-Lambert Ilac Sanayi, Istanbul, Turkey) and 7 mg/kg xylazine hydrochloric acid (Rompun, Bayer, Istanbul, Turkey). After preparation for aseptic surgery, the first laparotomy was performed on the rats through a 4-cm vertical abdominal incision. We performed autologous uterine horn transplantations to induce endometriosis. One uterine horn was ligated by 4-0 vicryl (polyglactin 910, Ethicon Ltd, Somerville, NJ, USA) suture at a site between the uterotubal junction and the cervical end and excised. The excised horn was immersed in sterile normal saline. The uterine horn was incised longitudinally and the myometrium removed by scraping with a scalpel blade. This piece of uterine tissue ($\sim 5 \text{ mm} \times 5 \text{ mm}$) was transplanted using a single suture with 5-0 polyglactin onto the inner surface of the anterior abdominal wall with the endometrium facing the peritoneal cavity. The fragment was always fixed over a large blood vessel. The skin incision was closed using 3-0 polyglactin sutures. After the first laparotomy, all rats were observed for 4 weeks, during which they did not receive medication. Four rats died due to complications related to surgery.

Four weeks after the first, a second laparotomy was performed to evaluate the development of the endometriotic implants and whether the implant was transformed into a cystic structure. Firstly, after opening the abdominal cavity, peritoneal fluid samples of the rats were obtained by irrigating the peritoneal cavity with 3 mL of saline. Then, the pre-treatment implant volumes were calculated *in vivo* by measuring their dimensions (length, width, and height in millimeters) and using the ellipsoid volume formula ($\pi/6 \times \text{length} \times \text{width} \times \text{height}$). Lastly, the rats were randomized into three intervention groups: (i) the vitamin D group ($n = 8$), (ii) the omega-3 PUFA group ($n = 9$), and (iii) the control group ($n = 9$).

The intraperitoneal route was preferred for drug administration to benefit from the direct action of drugs on peritoneal environment, endometriotic implants and peritoneal fluid. The rats in vitamin D group were given 42 $\mu\text{g}/\text{kg}/\text{day}$, intraperitoneal cholecalciferol (vitamin D3) (Devit-3 ampul, Deva, Istanbul, Turkey). The rats in omega-3 PUFA group were given 450 mg/kg/day intraperitoneal omega-3 fatty acid (Omegaven, Fresenius-Kabi, Istanbul, Turkey). Dosage choices were based on previous studies [9,10]. The rats in control group were given 0.1 mL/day intraperitoneal saline. All rats continued to receive the medications for 4 weeks.

At the end of the treatment period, third laparotomy was performed to evaluate treatment results. Peritoneal fluid samples of the rats were obtained to compare changes in cytokine levels. IL-6, IL-8, VEGF, and TNF- α levels were measured using an enzyme-linked immunosorbent assay kit (rat IL-6 ELISA, Krishgen Biosystems, Mumbai, India; rat IL-8, Eastbiopharm, Hangzhou, China; rat VEGF ELISA kit, Boster Bio. Tech. Co., Ltd., Pleasanton, CA, USA; rat TNF- α ELISA, Krishgen Biosystems) and the sensitivity of the kits was $<31.3 \text{ pg}/\text{mL}$, 2.5 ng/L, $<1 \text{ pg}/\text{mL}$, and $<31.3 \text{ pg}/\text{mL}$, respectively.

The volumes of the cystic endometriotic implants were recalculated (post-treatment volume). The median values of pre- and post-treatment volumes were compared in the same group. During the third surgery, all rats were sacrificed. These implants were totally excised from the peritoneum for histopathological examination. Formalin-fixed endometriotic tissues were embedded in paraffin blocks, sectioned at 5- μm thickness, stained with hematoxylin-eosin and examined under a light microscope. On microscopic examination, glandular tissue (GT) and stromal tissue (ST) contents in the endometriotic foci were histopathologically examined and scored. GT scoring was expressed as the number of secretory glands in 10 high-power fields (hpf). The GT score was 0 for no glands, 1 for one gland, 2 for two to three glands, and 3 for

four or more glands per 10 hpf. ST scoring was expressed as the percentage of areas containing stromal tissue in 10 hpf. The ST score was 0 for no ST, 1 for <25%, 2 for 25–50%, and 3 for >50% of areas containing stromal tissue in 10 hpf. All experimental procedures and histopathological examinations were performed by physicians blinded to the groups.

The statistical analyses were performed using the Statistical Package for the Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA). Because the distributions of both the volume values and the scores of histologic parameters were found to be abnormal, the nonparametric Mann-Whitney U test was used to compare the group differences of GT and ST scores. Also, within each group, the values of pre- and post-treatment volumes were compared by using the Wilcoxon signed-ranks test and p -values <0.05 were considered statistically significant.

Results

Two rats in the vitamin D group, one rat in the omega-3 PUFA group, one rat in the control group died due to surgery-related complications before the end of the study and so they were excluded from the study. In the second laparotomy, it was observed that the endometriotic implants in all the remaining rats had changed into vascularized, cystic structures (Figure 1).

There was no statistically significant difference among the groups in terms of the median volume 1 values during the second laparotomy. At the end of the treatment, the median post-treatment endometriotic foci volumes in groups vitamin D, omega-3 PUFA, and control were 57.5 (39.2–113), 32.9 (16.4–94.1), and 62.8 (47–153.8) mm³, respectively. The median post-treatment volume was statistically significantly reduced compared with volume 1 in the omega-3 PUFA group ($p = 0.02$), while the difference in the vitamin D and control group between volume 1 and volume 2 was not statistically significant (Figure 2).

In the histopathologic examination of the endometriotic implants at the end of the treatment, the mean values of GT and ST scores in the control group were 2 ± 1.1 and 1.8 ± 1.05 , respectively. The same scores in the vitamin D and omega-3 PUFA groups were 1.3 ± 1.06 and 1.2 ± 1.03 , and 0.8 ± 0.7 and 0.7 ± 0.6 , respectively. The GT and ST scores of the implants were significantly lower in omega-3 PUFA group, when compared with the control group. Although the GT and ST scores were lower in vitamin D group compared with the control group, there were no statistically significant differences between these groups.

Pre-treatment and post-treatment levels of cytokines in the peritoneal fluid were evaluated. Among the assayed cytokines, only

IL-6 was significantly decreased in the vitamin D group, while in the omega-3 PUFA group, IL-6, TNF- α , and VEGF levels were significantly decreased when compared with pre-treatment values (Table 1).

Discussion

In this prospective, randomized, single-blind, experimental study, efficacies of vitamin D and omega-3 PUFA against experimentally induced endometriotic implants were evaluated by comparing pre-treatment and post-treatment measurements. The results of this study show that omega-3 PUFA has a regressing effect on endometriotic implants by significantly reducing the volume and histological parameters (GT and ST scores) of experimentally induced endometriotic implants and decreasing the levels of IL-6, TNF- α , and VEGF in the peritoneal fluid. No significant difference in these parameters was observed in the control and vitamin D group.

Recent studies suggest that immunological, inflammatory, angiogenic, and environmental factors may all play an important role in the pathogenesis of endometriosis. Additionally, peritoneal environment in patients with endometriosis is immunologically dynamic and links the reproductive and immune systems. There is some evidence that the local environment of peritoneal fluid surrounding the endometriotic implant has characteristics of pre-inflammatory tissue associated with altered immune response and cytokine production. It has been suggested that these cytokines with autocrine and paracrine effects may impair the intercellular interaction in immune cells and contain the proliferative factors for implantation and development of ectopic tissue [11]. Despite all of these explanations, the pathogenesis of endometriosis is not yet fully understood. Currently, there is no proven effective treatment for endometriosis due to the limited understanding of its pathogenesis. Therefore, there is still a need for further experimental studies investigating the pathophysiology and treatment of endometriosis.

A wide variety of immunologic abnormalities and successful treatment with immunomodulator agents have been reported in women with endometriosis. We wanted to examine the effects of vitamin D, based on their immunomodulatory and anti-inflammatory properties on the endometriotic lesions. Vitamin D has strong immune modulating effects on cell proliferation and differentiation in normal as well as in malignant cell types. Recent studies suggest that there is an association between vitamin D deficiency and the increased risk of systemic lupus erythematosus, rheumatoid arthritis, irritable bowel disease, multiple sclerosis and

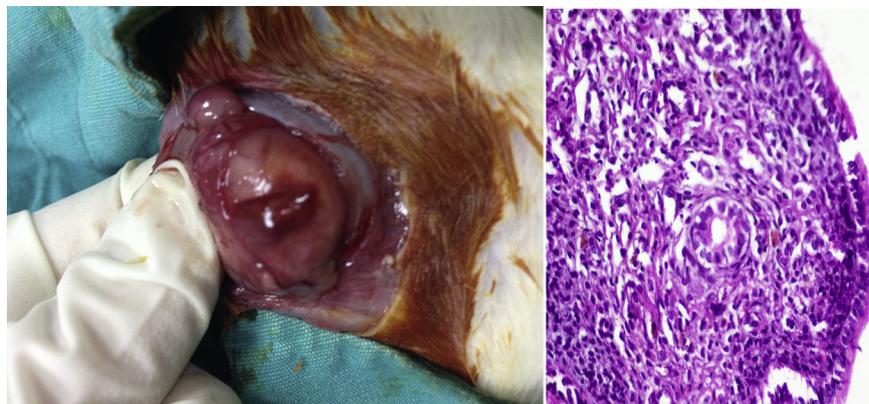


Figure 1. Gross morphological and microscopic appearances of implanted endometrial tissue, 4 weeks after implantation.

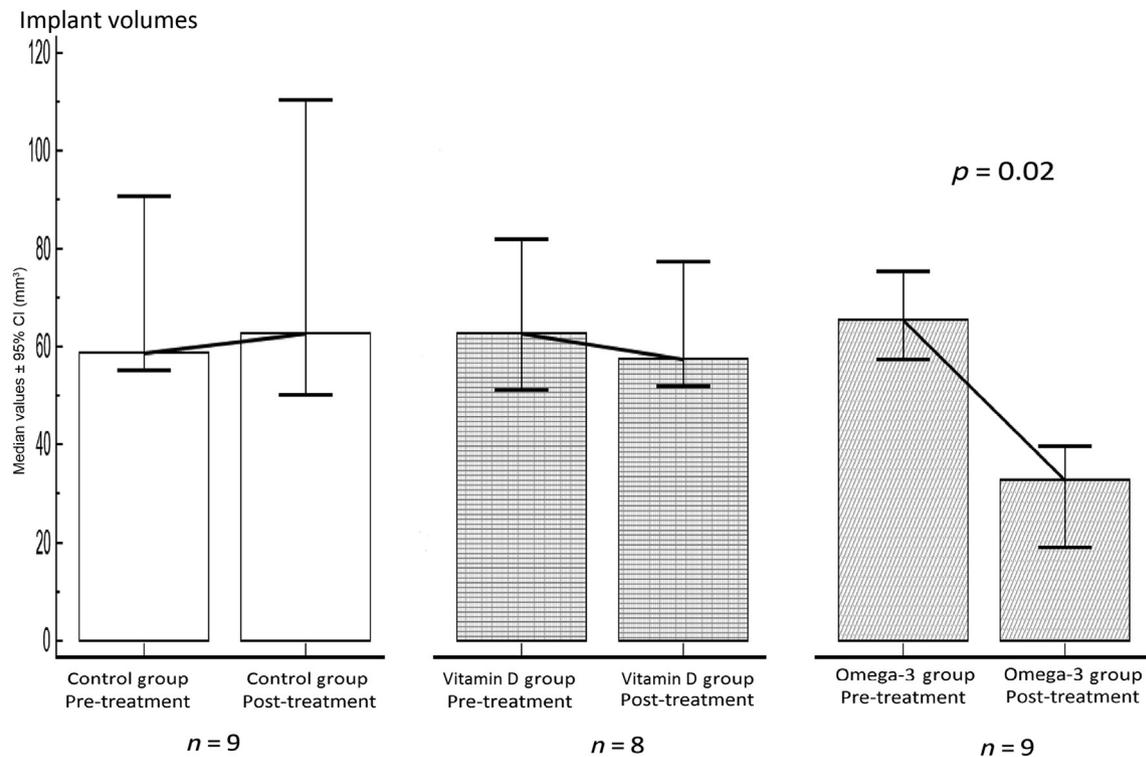


Figure 2. Comparison of the pre- and post-treatment volumes of the endometriotic implants in the study groups.

type 1 diabetes, all known to have an autoimmune component [12]. The vitamin D receptor and vitamin D metabolizing enzymes are found in the endometrium and ovaries of women with and without endometriosis in addition to the different immune cells. Furthermore, there is a dysregulation of some vitamin D enzymes and receptors in endometriosis and it is probable that vitamin D has the capability of acting in an autocrine/paracrine manner in a local immunologic environment [6].

A recent prospective cohort study reports that greater predicted plasma 25(OH) D levels and higher intake of dairy foods are associated with a decreased risk of endometriosis [13]. In another study, the selective vitamin D receptor agonist, elocalcitol, was demonstrated as reducing endometriosis development in a mouse model by inhibiting peritoneal inflammation [14]. Abbas et al investigated the effects of vitamin D intraperitoneally on experimental endometriosis in rats [9]. They reported that vitamin D reduced significantly the cross sectional area of endometriotic implants. In another prospective cross sectional study, it was found that endometriosis was associated with higher serum levels of vitamin D

[15]. In the present study, although the decreased rates of the post-treatment volume versus the median pre-treatment volume values in the vitamin D group were found to be 8.5% (62.8–57.5 mm³), there were no statistically significant differences between these volumes ($p > 0.05$). However, the cyst volume in the vitamin D group was significantly lower than in the control group. To the best of our knowledge, this is the second study to evaluate the efficacy of vitamin D in an experimental model of endometriosis in rats. Consequently, further animal studies are needed to clarify this subject. Studies have shown that vitamin D supplementation decreases pro-inflammatory cytokines IL-6, interferon- γ , IL-2, and TNF- α and increases anti-inflammatory cytokines (transforming growth factor beta-1, IL-4), suggesting that vitamin D may help to improve some chronic inflammatory and autoimmune diseases such as inflammatory bowel disease, insulin-dependent diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [16,17]. When the pre- and post-treatment levels of cytokines in the peritoneal fluid were compared, of all the cytokines measured, only IL-6 levels were significantly lower in the vitamin D group.

There have been a number of studies concerning the effects of omega-3 PUFA on the pathophysiological processes. However, there has not been a randomized, placebo-controlled, experimental study investigating the effects of omega-3 PUFA on endometriosis. Recently, in vivo and in vitro studies have shown that omega-3 PUFA has potential anti-inflammatory, antiapoptotic, anti-proliferative, and antiangiogenic effects. Besides, omega-3 PUFA inhibits the activation of NF- κ B and decreases the formation of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1. Therefore, the hypothesis has been raised that these nutritional elements could have a role in the treatment of various disorders including systemic lupus erythematosus, inflammatory bowel disease, rheumatoid arthritis, psoriasis, and even malignancies [18]. Since the pathogenesis of endometriosis often resembles systemic inflammatory

Table 1
Pre- and post-treatment cytokine levels in the peritoneal fluid.

		Vitamin D group	Omega 3 PUFA group	Control group
IL-6 (pg/mL)	Pre-treatment	65.2 ± 12.7	70.7 ± 10.8	67.3 ± 6.5
	Post-treatment	59.9 ± 11.2 ^a	63.1 ± 7.4 ^a	62.2 ± 7.0
IL-8 (pg/mL)	Pre-treatment	35.7 ± 5.3	38.6 ± 11.5	35.9 ± 12.1
	Post-treatment	31.8 ± 6.6	32.4 ± 8.5	35.2 ± 10.5
TNF- α (pg/mL)	Pre-treatment	17.9 ± 5.3	15.4 ± 4.5	19.9 ± 5.4
	Post-treatment	15.5 ± 5.7	10.5 ± 2.2 ^a	18.5 ± 4.3
VEGF (pg/mL)	Pre-treatment	16.3 ± 4.1	19.3 ± 3.7	17.4 ± 5.1
	Post-treatment	17.1 ± 3.0	13.7 ± 4.7 ^a	16.4 ± 4.5

^a Significantly different from values measured before the medication in the same group ($p < 0.05$).

disease and malignancies in terms of cytokine production, immune dysfunction, and angiogenesis, it is logical that omega-3 PUFA would have beneficial effects in a variety of inflammatory conditions as well as in endometriosis. Tomio et al demonstrated that the endogenous production of omega-3 PUFAs and exogenous eicosapentaenoic acid (EPA) provide protection against the development of peritoneal endometriotic lesions [19]. In that study, unlike ours, they used homologous or heterologous endometria obtained from congenic animal or human specimens as inoculant in the animal peritoneum. They found that EPA may be protective against the development of endometriosis through their anti-inflammatory effects and, in particular, the 12/15-lipoxygenase pathway products of EPA may be key mediators suppressing endometriosis [19].

In conclusion, the results of the present study show that omega-3 PUFA regressed significantly, both the size and histological parameters of experimentally induced endometriotic implants in rats. These findings were supported by a significant decrease of peritoneal level of IL-6, TNF- α , and VEGF. When compared with the omega-3 PUFA group, it was seen that vitamin D was not as effective as omega-3 PUFAs in the treatment of endometriosis. The median cyst volume in the vitamin D group was lower than that in the control group but no significant difference was observed between the pre- and post-treatment volume in the vitamin D group. Detailed and long-term studies may reveal more clearly the association between vitamin D and endometriosis. While other studies have focused on the effect of pharmacological agents on the treatment of endometriosis, this study was directed toward the role of some nutritional factors. When considered from a public health perspective, further experimental investigations are needed to clarify the beneficial effects of some nutritional elements on endometriosis.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgement

This work was supported by Scientific Research Projects Coordination Unit of Firat University. Project number TF1416.

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