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Role of Endoplasmic Reticulum Stress in The Male Reproductive System

Mohsen Rahmani, M.Sc.1, Marziyeh Tavalaee, Ph.D.1*, Joël R Drevet, Ph.D.2*, Mohammad Hossein Nasr-Esfahani, Ph.D.1*

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Abstract

Testicular dysfunction, whether linked to varicocele, obesity, diabetes, aging, inflammation, or lifestyle or environmental issues, is frequently accompanied by an accumulation of unfolded or misfolded proteins, indicating impaired endoplasmic reticulum (ER) function. In this review, we examined the Google Scholar, Scopus and PubMed databases (from 2011 to 2022) to support the association of ER stress with defective spermatogenesis in animal models and humans. ER stress, whether in its pro-survival or pro-apoptotic aspect, appears to be closely linked to each studied situation. Several studies have demonstrated a significant increase in oxidative stress (OS) levels in infertile men compared to fertile individuals, which is associated with poor spermatogenesis quality. OS is likely the result of the interplay between ER stress and spermatogenesis defects. These findings suggest that therapeutic strategies aimed at mitigating both ER stress and OS could be of interest in restoring male reproductive function.

Keywords: Apoptosis, Endoplasmic Reticulum Stress, Spermatogenesis, Varicocele

Introduction

Male infertility exhibits a wide range of origins, encompassing individual genetics and environmentally associated factors (1). Recent evidence suggests a frequent association of male infertility with other conditions such as cancer, cardiovascular disease, and autoimmune responses (2). This implies that male infertility and/or impaired sperm quality could potentially serve as early indicators of underlying dysfunctions, serving as essential biomarkers for an individual’s overall health (3).

The specific nature of these associations remains uncertain, but they likely stem from shared factors such as genetic predisposition and/or lifestyle and environmental influences. There is a general consensus indicating that oxidative stress (OS), whether originating systemically and/or locally due to environmental interactions or inherited imbalances in reactive oxygen species (ROS) generation/recycling inherent to aerobic cellular metabolism, may serve as a common underlying mechanism contributing to these pathological associations (3). At the subcellular level, OS closely correlates with a fundamental cellular dysfunction known as endoplasmic reticulum stress (ER-stress) (4), which has received limited investigation in relation to male infertility. This review aims to enhance our comprehension of ER-stress and male infertility, examining various scenarios, both pathological and non-pathological, where ER-stress has been linked to male fertility. These scenarios encompass varicocele, obesity, diabetes, aging, inflammasome activation, and environmental impacts.

The review study was conducted in the PubMed (https://pubmed.ncbi.nlm.nih.gov/), Google Scholar (https://scholar.google.com/), and Scopus (https://Scopus search.com/) databases from 2017 to 2022. The screening process was performed by two independent investigators to identify eligible publications, focusing on keywords such as male fertility, reproduction, varicocele, spermatogenesis, apoptosis, and ER stress. Excluded from the study were publications without complete data, poster studies, and repeated review papers that shared identical...
scientific content. Based on our initial pool of 898 papers, we have carefully reviewed and selected 79 published papers that align with the main objectives of the current study.

**The endoplasmic reticulum organelle**

The ER is a vast intracellular membrane network where translated nascent proteins mature with the assistance of chaperones. It is within this organelle that protein structures are formed and acquire their three-dimensional organization through complex post-translational modifications. ER protein maturation is influenced by various pathophysiological conditions, leading to an ER-stress response characterized by the accumulation of misfolded or unfolded proteins, also known as the unfolded protein response (UPR). Depending on the intensity of ER-stress, this response can result in either a pro-survival or pro-apoptotic outcome. To promote survival, the ER response operates at multiple levels, including the temporary reduction of translation to decrease the influx of nascent proteins into the ER. Additionally, it enhances ER protein trafficking, such as by increasing chaperone production and expanding the ER organelle network. Ultimately, to handle misfolded proteins, the ER response augments their degradation via the ubiquitin-proteasome system and/or autophagic-lysosomal processes in a pathway known as the ER-associated degradation pathway (ERAD) (5). If these responses fail to restore ER homeostasis, apoptosis is initiated. The BIP/GPR78/HSPA5 protein, a member of the heat shock protein family (6), mediates the orchestration of this pro-survival/pro-apoptotic balance. As depicted in Figure 1, BIP activates the ER response by interacting with ER membrane sensor proteins, including PERK, IRE1, and ATF6 (5).

![Fig.1: Survival and death pathways associated with ER stress. The UPR pathway is modulated by three ER sensors under all circumstances. During survival conditions, PERK phosphorylates eIF2α, resulting in reduced translational activity. PERK also promotes the expression of ATF4, which further triggers the expression of genes involved in ER quality control chaperones, XBP-1, CHOP, and antioxidants. Additionally, activated IRE1 leads to the specific splicing of XBP1 messenger RNA, generating the XBP1s variant that regulates the transcription of ER-resident chaperones, lipogenesis, and the ER-associated degradation pathway (ERAD). Simultaneously, ATF6 translocates to the nucleus, stimulating the expression of ER chaperones (Grp78, Grp94, etc.), XBP1, and antioxidants. Under conditions of chronic stress, these same sensors initiate a death signal. In particular, PERK induces the transcription of CHOP, which leads to the activation of numerous genes involved in cell apoptosis. Activated IRE1 also triggers the apoptotic pathway by recruiting TRAF2 and ASK1 to the ER membrane, activating the IKK/NFkB pathway, MAPK signaling, and RNA degradation within the ER. Furthermore, calcium flux dysregulation contributes to apoptosis. ER; Endoplasmic reticulum and UPR; Unfolded protein response.](image-url)
Endoplasmic reticulum-stress and spermatogenesis

Spermatogenesis is a complex differentiation pathway that occurs in the testis and is regulated by Sertoli and Leydig cells. These two cells tightly control germ-cell mitotic proliferation, meiotic reduction, and spermatozoa cyto-differentiation through their endocrine functions (7). Receptor tyrosine kinases (RTKs) play a crucial role in multiple steps of this pathway, with their activities being negatively modulated by phospho-tyrosine phosphatases (PTPs) (8). Maintaining a balance between RTK and PTP activities is crucial for optimal spermatogenesis and cellular homeostasis. However, under stress, particularly OS in aerobic metabolism, proteins in the ER undergo oxidative modifications, such as defective disulphide bridging processes, protein carbonylation, protein sulfoxidation, and the generation of advanced glycation end-products (AGES), which impairs their maturation and function (9) (Fig.2A, B). Both PTP and RTK are susceptible to these events, as PTP can be oxidized, leading to inactivation, and RTK may lose its ability to bind ligands due to defective sulfoxidation. It is not surprising that oxidative stress-associated conditions, such as Varicocele, are accompanied by ER stress when defective spermatogenesis occurs. For instance, Tisp40, a testis-specific transcript induced during spermiogenesis, has been found to play a critical role in the testis’ ER-stress survival response, thereby participating in sperm chromatin packaging processes (10).

Fig.2: Phosphorylation signals, ROS, and the endoplasmic reticulum roles. A. Phosphorylation signals play a crucial role in initiating protein synthesis by ribosomes, whether they are free or associated with the rough ER. The RTK receptor, existing in inactive non-oligomerized and active oligomerized forms through binding to a growth factor, transmits a significant phosphorylation signal. Depending on the strength of this signal - weak, moderate, or severe - different pathways are activated. In the case of a weak signal (left pathway), the active form of PTP, responsible for preventing excessive responses to stimuli, dephosphorylates the RTK, allowing only weak signals to be transmitted to the nucleus. Under oxidative stress, particularly after NOX activation, PTP undergoes oxidation of a thiol group (Cys-SH to Cys-SOH) at its catalytic site, resulting in temporary inactivation of PTP. This inactivation enables the middle pathway for RTK signaling. In instances of high ROS production and accumulation, intramolecular disulfide bonding occurs within PTP, leading to its inactivation and subsequent transmission of intense RTK phosphorylation signals to the nucleus. B. In addition to the RTK/PTK pathway, ROS also influence the ER-mediated folding of nascent proteins. ROS contribute to the formation of disulfide bonds in maturing proteins within the ER via two main pathways. First, hydrogen peroxide generated by NOX4, ERO1, and PRDX4 facilitates the formation of disulfide bridges in PDI, which then exerts its bridging activity on thiol-containing proteins transiting through the ER. ROS originating from mitochondrial activity directly impact PDI activity, thereby affecting the disulfide bridging processes of nascent proteins transiting the ER. ROS: Reactive oxygen species; ER: Endoplasmic reticulum; NOX: NADPH oxidase; P: Phosphate group; RTK: Receptor tyrosine kinase; PTP: Phosphotyrosine phosphatase; GSH: Glutathione.
Varicocele and endoplasmic reticulum-stress

Varicocele is characterized as an abnormal dilation of the pampiniform venous plexus of the spermatic cord in the scrotum. Clinically, it is the most well-known cause of male infertility, affecting about 15% of the general population and between 19–41% of the infertile population. Varicocele leads to progressive testicular insufficiency due to the malfunctioning of the spermatic vein valves, resulting in slowed blood flow and/or reflux (11). This compromised blood supply to the testis impairs its function by depriving it of systemic factors, including gonadotropic and sex hormones. Moreover, varicocele induces testicular hypoxia and hyperthermia, causing damage to the organ and its sperm-producing function (12). Nutrient deprivation, along with a redox imbalance due to hypoxia and hyperthermia, gradually leads to the development of an inflammatory state, ultimately resulting in the apoptosis of germ cells once the countermeasures of testicular cells are overwhelmed. Increased ROS, accumulation of misfolded/ unfolded proteins and AGEs, as well as evidence of ER-stress and UPR activation, have been extensively reported in the testis affected by varicocele (13). Soni et al. (14) were the first to demonstrate that ROS-mediated ER stress is triggered in the human testis affected by varicocele. In an animal model of varicocele, Karni et al. (15) also established the presence of testicular oxidative stress, ER stress, and mitochondria-mediated apoptosis. Specifically, they observed elevated testicular expression of ER-stress markers, including BIP, as well as activated phosphorylated forms of IRE1α (p-IRE1α) and JNK (p-JNK), alongside upregulation of apoptotic factors, namely cleaved caspase-3 and Bax/Bcl2 ratio. More recently, studies conducted by us and others have reported that the IRE1α pathway, associated with UPR, acts as the primary mediator of germ cell death in a rat model of varicocele, triggering the pro-apoptotic JNK response (16). Interestingly, the early marker of ER-stress response, BIP, was not found to be elevated in the human testis affected by varicocele. However, this discrepancy may be attributed to the different kinetics observed between acute varicocele situations in animal models and the chronic varicocele situation commonly observed in humans (17).

Animal models with experimentally induced varicocele can be considered acute scenarios, while in human varicocele patients, it is typically a chronic condition that may require different management approaches. This difference could explain the contrasting findings when evaluating early markers of ER-stress in the human testis compared to rodent models. Nonetheless, both animal models and human varicocele patients exhibit evidence of ER-stress in the testis, in addition to the early activation of the ER-stress chaperone BIP.

Efforts to mitigate OS in varicocele and alleviate ER stress have been pursued due to the suggested role of excessive ROS. Various approaches have been taken, including supplementation with herbal concoctions possessing antioxidant properties or a single antioxidant like alpha-lipoic acid (ALA), as well as the utilization of iron chelators such as Deferasirox, all of which have been evaluated in animal models of varicocele. These interventions consistently demonstrate a reduction in ROS generation and ER-stress response, along with improvements in sperm production and quality (18, 19). Recently, the translation of these findings into clinical practice has been explored in patients undergoing Varicocelectomy, revealing significant enhancements in sperm functional parameters when accompanied by ALA supplementation (20).

Alongside hypoxia, hyperthermia is recognized as another significant factor contributing to testicular dysfunction in varicocele (12). Given the feasibility of investigating this parameter, extensive research has been conducted to examine the effects of acute and/or chronic heat stress on testicular dysfunction and the induction of ER/UPR stress responses. Furthermore, this line of research has shed light on the impact of repeated exposure to hot baths or saunas on male reproductive capacity. Notably, studies have demonstrated that subjecting mouse testes to a single 15-minute heat stress at 42°C triggers ER/UPR stress as an adaptive pro-survival signal (21). Conversely, repeated heat stress has been found to induce ER-stress-mediated apoptosis in the testis. In the same study, it was observed that BIP, the early initiator of ER stress, exhibited high expression in the mouse testis compared to other tissues, suggesting a specific adaptation of the testis to heat stress-induced cell death—a response expected due to the temperature sensitivity of spermatogenesis in mammals. Interestingly, it was observed that testicular BIP levels decreased after repeated heat stress, leading to ER-stress-mediated apoptosis facilitated by caspase-3 activation (21, 22). Additionally, among the three ER membrane sensors involved in integrating the ER/UPR response, only PERK and ATF6 were found to be activated, while IRE1α was down-regulated through autophagic processes (22). In a separate study, Li et al. (22) proposed that testicular hyperthermia was associated with an increase in serum testosterone levels, suggesting an anticipated response to Leydig cell dysfunction that triggers central compensation through activation of the hypothalamus/pituitary/gonadal (HPG) axis. Leydig cell dysfunction and reduced testosterone synthesis during heat stress, accompanied by the induction of ER stress, have been confirmed in other studies (23). It has been...
suggested that the increased testosterone supply facilitated by HPG axis adjustment may safeguard the testis from oxidative damage by activating Nrf2-dependent antioxidant mechanisms (24). Obesity is another condition in which OS and chronic low-grade inflammation have been linked to ER stress and male infertility, alongside varicocele.

**Obesity, endoplasmic reticulum-stress, and male fertility**

Lipid disorders, including hypercholesterolemia leading to metabolic syndrome and obesity, have been clearly associated with male infertility, yet this connection has not received the attention and public awareness it deserves (25). Particularly in developed and developing countries with unbalanced diets, this issue remains overlooked (26). In both human and animal models of dyslipidemia, elevated levels of saturated free fatty acids (FFA), such as palmitic acid (PA), have been observed in plasma (27). These FFAs have demonstrated a time- and dose-dependent ability to suppress Leydig cell survival and drive them towards apoptosis (28). Consequently, FFA-mediated impairment of Leydig cells has been proposed as a contributing factor to reduced reproductive performance and hypogonadism in obese individuals (29). Moreover, FFAs have been found to induce increased expression of BIP, activation of the ER membrane sensor PERK, and heightened production of the ER-mediated apoptotic signal enhancer, the homologous C/EBP (CHOP) protein (30). Correspondingly, mice fed high-fat diets exhibit elevated expression of BIP and CHOP, as well as phosphorylation of IRE1 and PERK in the testis, indicating that high-fat consumption may induce ER stress in the testis, a condition that can potentially be mitigated by antioxidant supplementation (31). Similarly, in the study conducted by Mu et al. (32), it was demonstrated that a high-fat diet not only increases the expression of BIP and CHOP but also activates other ER-stress sensors, including ATF6 and XBP-1. The detrimental effects of this activation could be alleviated through the administration of sulforaphane, an antioxidant, anti-inflammatory, and anticancer compound found in cruciferous vegetables. Furthermore, ATF6, the ER-stress adaptation factor, has been shown in other research to act as a positive regulator for testis-specific serine/threonine protein kinase 4 (TSSK4), which is involved in sperm maturation and serves as a link between ER-stress responses and spermatogenesis (33). In addition to the direct impact of FFAs on ER stress, lipid disorders are closely linked to chronic systemic oxidative stress, which contributes to pro-inflammatory conditions known to exacerbate the ER/UPR response in various cell types (34). Therefore, the combination of systemic lipid imbalance and OS plays a critical role in the testicular ER-stress response and the development of subfertility/infertility associated with dyslipidemia.

The dysregulation of adipokines, such as leptin, commonly observed in dyslipidemic conditions, represents another mechanism by which lipid disorders can affect testosterone production and, consequently, testicular function (35). This disruption leads to hypogonadism and oligoasthenozoospermia. Additionally, studies have demonstrated that the adipokine Vaspin binds to and activates the ER-stress sensor BIP in the adult rat testis, where it is suspected to regulate steroidogenesis and, incidentally, spermatogenesis (36). Further investigations have unveiled the potential involvement of other endocrine factors, such as irisin, a myokine induced by exercise, in the regulation of obesity-related impairments in spermatogenesis. In both obese men and obese mice, irisin levels were found to be correlated with insulin resistance, severity of NAFLD (non-alcoholic fatty liver disease), and the decline in sperm quality associated with OS (37). Recently, it was reported that supplementing obese mice with irisin could alleviate high-fat diet-induced oxidative stress, ER stress, and testicular apoptosis through the activation of the AMPKα signaling pathway (38).

**Diabetes, endoplasmic reticulum-stress and male fertility**

Diabetes is a disease known to be associated with elevated systemic oxidative stress, inflammation, and ER stress, which contribute to male hypogonadism and impaired testicular function (39). Shi et al. (40) also demonstrated that diabetes reduces testicular autophagy, a cellular process involved in managing the accumulation of misfolded/unfolded proteins while increasing OS and ER stress. Increased expression of insulin receptor substrate-1 (IRS-1) in the testes has been observed in association with diabetes and is considered a compensatory response to tissue dysfunction (41). Specifically, the upregulation of IRS-1 in the diabetic testes has been linked to reduced expression of CHOP and NF-KB, both of which play roles in ER/UPR stress and inflammatory responses (40). Moreover, similar to dyslipidemia, the reduction of systemic OS through antioxidant supplementation in a rat model of diabetes has been shown to decrease NF-KB and P38 MAPK PMK-3 expression, as well as inhibit P-JNK activation, all of which are active components in the IRE-1-mediated ER-stress signaling cascade (42). Consistent with these findings, hyperglycemia has been demonstrated to induce Leydig cell apoptosis through mitochondrial
pathways (Bax/Bcl2/caspase-12) and BIP/CHOP-mediated ER stress, and these effects can be alleviated by antioxidant supplementation, such as melatonin, resveratrol, and fucoidan (43, 44).

**Inflammasome, endoplasmic reticulum-stress and male fertility**

Testicular inflammatory conditions, whether originating from immune responses or other causes, have a significant impact on spermatogenesis and male fertility (45). In a recent review, we highlighted the crucial role of the NLRP3 (nucleotide-binding oligomerization domain-like receptor family Pirin domain containing 3) inflammasome pathway, a Pattern Recognition Receptor (PRR), in testicular dysfunction (46). It has been observed that uncontrolled UPR/ER stress in the testis consistently triggers the activation of the NLRP3 inflammasome, leading to the release of pro-inflammatory cytokines that adversely affect sperm structure and function (46, 47). Furthermore, bacterial or viral unilateral/bilateral orchitis has been shown to activate both the NLRP3 inflammasome and the UPR/ER stress responses (48, 49). OS has emerged as a common underlying factor, as antioxidant supplementation has proven effective in protecting the testis from UPR/ER stress and inflammasome activation (46).

**Aging, endoplasmic reticulum stress, and male fertility**

Human aging is characterized by a decline in testicular and sperm function, which can result in reduced sperm nuclear integrity and have consequences for reproductive success, embryonic development, live birth rates, and the quality of life of offspring (50). In animal models of aging, it has been observed that BIP expression decreases, potentially leading to disengagement from ER/UPR membrane sensors and subsequent activation of the ER-stress response (51). Interestingly, in aging human testes, the three ER sensors (IRE-1, PERK, and ATF6) are also downregulated, limiting the ability to initiate the ER/UPR stress response. This downregulation is considered an adaptive process in aging, preventing excessive activation of the ER-stress response in a less efficient ER metabolic context (51, 52). However, despite the reduced ER capacity to respond, downstream effectors of the ER-stress response, such as CHOP, P-JNK, and Caspase-12, are significantly increased in the aging testes (53), indicating the presence of an active ER-stress response. This observation is somewhat contradictory and requires further investigation. While the activation of p-JNK and caspase-12 in the aging testes may be influenced by other signaling pathways, the activation of CHOP is particularly perplexing and necessitates additional testing for a comprehensive understanding.

**Lifestyle/environmental impacts, endoplasmic reticulum stress, and male fertility**

Testicular function and reproductive performance are increasingly recognized as important indicators of individual fitness and the ability to cope with various chronic and acute stresses. Regardless of the type of stress—whether chemical, physical, or psychological—reports suggest that testicular function can be impaired, and ER stress is observed as a consistent response in the testis (54). Researchers have explored different approaches to mitigate the detrimental effects of chemical components on spermatogenesis and the induction of ER stress. For example, Yin et al. (55) demonstrated that Bisphenol A (BPA) disrupts cell and testis functions, inhibits cell proliferation, increases apoptosis rates, and accumulates testicular ROS. They found that knocking down the PERK/eIF2α/CHOP pathway and using the ROS scavenger NAC (Acetylcysteine) can help restore cellular survival. Ji et al. (56) showed that Melatonin alleviates cadmium-induced cellular stress and germ cell apoptosis in male CD-1 mice by effectively inhibiting ER stress and the UPR in the testes. Zou et al. (57), working with Leydig cells from rat testes, demonstrated that NAC prevents Nickel-induced ROS generation and inhibits apoptosis through mitochondrial and ER stress pathways (GADD153/Caspase 12) in rat Leydig cells. Additionally, Soni et al. (58) investigated the effects of DA-9401 (a commercial antioxidant) in Sprague Dawley rats and found that it reduced ER stress and apoptotic markers while improving fertility, genital organ weight, sperm parameters, and sex hormone levels. To summarize the available information in the literature, Table 1 provides a compilation of environmental toxins/pollutants, specific drugs, and a limited number of bacterial toxins that have been tested for their ability to induce ER/UPR-stress responses either in cultured testicular cells or in animal models. Where available, Table 1 also includes the potential corrective effects obtained with various strategies. Overall, it is evident that ER stress is a common response of the testis to these different exposures, and antioxidant treatment frequently proves effective in reducing the extent of the testicular ER stress response.

**Conclusion**

It is noteworthy that ER stress is a prevalent characteristic in various cases of testicular dysfunction linked to male subfertility/infertility. OS stands out as a key factor in this association, implying that therapeutic interventions targeting antioxidants may hold promise for enhancing reproductive performance.
Table 1: Compilation of environmental toxins, drugs, and bacterial toxins inducing ER/UPR-stress responses in testicular cells and animal models

<table>
<thead>
<tr>
<th>Agent</th>
<th>ER/UPR components monitored</th>
<th>Corrective treatment used</th>
<th>Main findings, model and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibutyl phthalate (DBP)</td>
<td>p-PERK/NRF2/</td>
<td>Melatonin</td>
<td>Melatonin attenuates DBP-induced damage (ROS generation/ER stress and mitochondrial-related damage/decrease in mitochondrial mass, mtDNA copy number, COX IV protein level, and ATP level) and mitochondrial-dependent apoptosis by regulating PERK/Nrf2/ARE the signal antioxidant path in mouse spermatocyte-derived GC-2spd(ts) cell line model (59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Specific inhibitor p-PERK</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>(GSK2606414)</td>
<td></td>
</tr>
<tr>
<td>Di-(2-ethylhexyl) phthalate (DEHP)</td>
<td>p-IRE-1α/ p-JNK/ CHOP/GRP78</td>
<td>-</td>
<td>Pubertal exposure to high doses of DEHP induces germ cell apoptosis and distorted seminiferous tubules by initiating ER stress in testes (five-week-old male mice) (60)</td>
</tr>
<tr>
<td>Lead diacetate</td>
<td>GRP-78 and BAX</td>
<td>Turmeric and vitamin C</td>
<td>Turmeric and vitamin C mitigate testicular atrophy induced by lead diacetate via regulation of GRP-78/17β-HSD pathways and subsequent thr reduction of oxidative injury in male Wistar albino rats model (61)</td>
</tr>
<tr>
<td>Particulate matter (PM2.5)</td>
<td>IRE1, p-JNK, beclin-1, LC3/LC3I</td>
<td>STF083010 (an IRE1 inhibitor)</td>
<td>STF083010 exerted specific protective effects on reproductive injury-related effects in male rats (male Sprague–Dawley rat model) exposed to PM2.5 such as improved sperm quality and serum testosterone level, with effects mediated via ER (IRE1/JNK)/autophagy signaling (62)</td>
</tr>
<tr>
<td>ZnO Nanoparticles</td>
<td>p-IRE1α/XBP1s/BIP/CHOP/p-JNK</td>
<td>Salubrinal, a specific inhibitor of eIF2α phosphatase enzymes and ER stress inhibitor</td>
<td>Salubrinal inhibit or preserve ER stress and apoptotic marker, and also improve sperm parameters, and testis function, molecular and enzymatic testosterone hormone in male Kunming mice model (63)</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>GRP78/p-IRE1α/XBP1s/CHOP/p-eIF2a/p-PERK</td>
<td>-</td>
<td>Long-term 1-NP activated oxidative stress and ER stress and downregulate steroidogenic genes and enzymes, and disrupted T biosynthesis without disturbing testicular spermatogenesis in 6-week-old male ICR mouse model testes (64)</td>
</tr>
<tr>
<td>Nonylphenol (Np)</td>
<td>JNK, MKK4, p53, and p38,</td>
<td>-</td>
<td>NP led to testicular structure disruption and a reduction in testicular size and testosterone levels and induces apoptosis through ROS/JNK signaling in GC-1 spg cell model (65)</td>
</tr>
<tr>
<td>X-Irradiation</td>
<td>GRP78, IRE1α, CHOP and Caspase-12, and Caspase-3</td>
<td>Human amniotic membrane-derived mesenchymal stem cells (hAMSCs) and conditioned medium (hAMSCs-CM)</td>
<td>Transplantation of hAMSCs and hAMSCs-CM into testis mice led to reducing ER stress by suppressing the UPR response as well as a decrease in FSH and LH and an increase in testosterone level which have contributed to the improvement of spermatogenesis (66)</td>
</tr>
<tr>
<td>Dinitramine (DN), a synthetic herbicide</td>
<td>GRP78/IRE-1/CHOP/p-eIF2a/Erk/p-P38/p-Jnk</td>
<td>2-ABP, inhibitors of IP-receptors and TRP channels</td>
<td>BAPTA, a chelate Ca$^{2+}$ 2-ABP and BAPTA prevent anti-proliferative DN effects on testicular cell lines (Leydig and Sertoli cells model) mediated via activating P3k/Akt pathway and inhibiting ER stress-induced calcium dysregulation in the cytosol and mitochondria (67)</td>
</tr>
<tr>
<td>Zeaalenone (ZEA), a non-steroidal mycotoxin</td>
<td>CHOP/BIP</td>
<td>BAPTA-AM, chelator of Ca$^{2+}$ &amp; Mg$^{2+}$</td>
<td>NAC decreases activating of AMPK and autophagy-related protein by scavenging ROS and also 2-ABP and BAPTA-AM prevent ER stress markers by activating CaMKβ and AMPK and decreasing the concentration of Ca$^{2+}$ and autophagy in TM4 cells (mouse Sertoli cell line model) (68)</td>
</tr>
<tr>
<td>PbSe nanoparticles (PbSe-NPs)</td>
<td>GRP78/Caspase-12</td>
<td>-</td>
<td>PbSe-NP administration led to a reduction in the quantity and quality of sperm, which caused a great fertility decrease by endoplasmic reticulum and mitochondria-mediated cell apoptosis in specific-pathogen-free (SPF) Sprague–Dawley (SD) rats (6 to 7 weeks old, 170-200 g) (69)</td>
</tr>
</tbody>
</table>
Table 1: Continued

<table>
<thead>
<tr>
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<th>Corrective treatment used</th>
<th>Main findings, model and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorocholine chloride (CCC)</td>
<td>GPR78, CHOP</td>
<td>4-phenyl butyric acid (4-PBA), an ER stress inhibitor</td>
<td>4-PBA rescued the testosterone secretion disorders and alleviated CCC-induced increase in the ER stress-related protein levels in Sprague-Dawley rats Leydig cells model (70)</td>
</tr>
<tr>
<td>Aluminum (AlCl₃)</td>
<td>CHOP, Bcl-2, Bax, and XBP1</td>
<td>Taurine</td>
<td>Taurine leads to increased gene expression of vimentin, Bcl-2, and BNCA accompanied by decreased CHOP, Bax, and XBP1 gene expression. In other words, Taurine amends both ER stress and mitochondrial impairment in the testes and epididymis induced by AlCl3 in forty-eight adult male albino rats model (71)</td>
</tr>
<tr>
<td>Drug toxicant</td>
<td></td>
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</tr>
<tr>
<td>Cisplatin</td>
<td>GPR78, CHOP, IRE1α, XBP1</td>
<td>Grape seed proanthocyanidin (GSPE)</td>
<td>GSPE relieved endoplasmic reticulum stress-mediated apoptosis via PREK/eIF2α and IRE1α/XBP-1/caspase-12 pathways as well as PI3K/Akt/mTOR and Bad/CytC/caspase-9/caspase-3 pathways in Forty-eight male Wistar rats model (72)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>CHOP/NRF2/PERK/p-IRE1(Ser724)/</td>
<td>LCZ696 (sacubitril/valsartan), a receptor neprilysin inhibitor and a Ca²⁺ sequestration inside the ER</td>
<td>LCZ696 reduce apoptotic, oxidative, and ER stress markers LCZ696 and improve Testicular atrophy, spermatogenic function, and antioxidant defenses, and cause increased lncRNA TUG1 in adult male 8-week-old Wistar rats model (200 ± 20 g) (73)</td>
</tr>
<tr>
<td>Sodium fluoride (NaF)</td>
<td>GRP78/PERK/p-eIF2α/CHOP/</td>
<td>N-acetylcysteine (NAC)</td>
<td>NAC effectively blocked the damage of Sertoli cells through the activation of ER stress, suggesting that NaF-induced ROS is an early event that triggers ER stress in Sertoli cells from male Sprague-Dawley rats (18-day-old) (in vitro) model (74)</td>
</tr>
<tr>
<td>Busulfan</td>
<td>Caspase-12/CHOP/GRP78/ATF4/p-IRE1/XBP1</td>
<td>Melatonin</td>
<td>Melatonin blocks or decreases ER stress markers and related apoptosis proteins, therefore, reducing the extent of damage to mouse testes and improving the survival rates of busulfan-treated mice in Mouse testes (in vivo) and the C18-4 cell line (type A spermatogonia stem cell, in vitro model (75)</td>
</tr>
<tr>
<td>Midazolam (MDZ)</td>
<td>p-eIF2α/ATF4/ATF3/CHOP/JNK/</td>
<td>-</td>
<td>Midazolam could activate caspase, MAPKs, and ER stress pathways and impede Akt pathway and cell cycle to induce apoptosis in TM3 mouse Leydig progenitor cells model (76)</td>
</tr>
<tr>
<td>Triptolide (TP), a diterpenoid epoxide</td>
<td>PERK/CHOP/JNK/NRF2/p-JNK/ATF4/peIF2α/peIF2α</td>
<td>Aucubin (AU) Nrf2siRNA</td>
<td>AU prevented apoptosis through an effective inhibition of PERK/CHOP and JNK-dependent apoptosis pathway, as well as improved testicular weight, and sperm morphology and protected the integrity of BTB by corresponding up-regulating genes in male adult mice (25-27 g) (in vivo) (77)</td>
</tr>
<tr>
<td>High hCG, Human chorionic gonadotropin</td>
<td>Grp78/Chop/ATF4/Xbp1/p-IRE1</td>
<td>Knockdown of melatonin receptors (MT1 and MT2)</td>
<td>Inhibition of melatonin receptors increased hCG-induced expression of Grp78, Chop, and ATF4, but not Xbp1 and IRE1, suggesting that hCG may modulate IRE1 signaling pathways in a melatonin receptor-dependent manner in male Kunming White outbred strain mice (in vivo) and the murine Leydig tumor cell line (in vitro) model (78)</td>
</tr>
<tr>
<td>Bacterial toxin</td>
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<tr>
<td>Microcystins-LR (MCLR)</td>
<td>GRP78/caspase-12/CHOP/PERK/p-eIF2α/peIF2α/XBP1/p-p38/p-JNK/JNK/p38/p-Erk1/2</td>
<td>Bapta-AM, a Ca²⁺ chelator</td>
<td>Bapta-AM pretreatment attenuated partially MCLR-stimulated such as elevated intracellular Ca²⁺, p-CaMKII, and mitochondrial dysfunction in mouse TM4 Sertoli cells model (79)</td>
</tr>
</tbody>
</table>

HCG; Human chorionic gonadotropin, ER; Endoplasmic reticulum, ROS; Reactive oxygen species, and mtDNA; Mitochondrial DNA.
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Mouse Embryonic Fibroblasts-Derived Extracellular Matrix Facilitates Expansion of Inner Ear-Derived Cells

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Abstract

Objective: Previous reports showed that mouse embryonic fibroblasts (MEFs) could support pluripotent stem cell self-renewal and maintain their pluripotency. The goal of this study was to reveal whether the decellularized extracellular matrix derived from MEFs (MEF-ECM) is beneficial to promote the proliferation of inner ear-derived cells.

Materials and Methods: In this experimental study, we prepared a cell-free MEF-ECM through decellularization. Scanning electron microscope (SEM) and immunofluorescent staining were conducted for phenotype characterization. Organs of Corti were dissected from postnatal day 2 and the inner ear-derived cells were obtained. The identification of inner ear-derived cells was conducted by using reverse transcription-polymerase chain reaction (RT-PCR). Cell counting kit-8 (CCK-8) was used to evaluate the proliferation capability of inner ear-derived cells cultured on the MEF-ECM and tissue culture plate (TCP).

Results: The MEF-ECM was clearly observed after decellularization via SEM, and the immunofluorescence staining results revealed that MEF-ECM was composed of three proteins, including collagen I, fibronectin and laminin. Most importantly, the results of CCK-8 showed that compared with TCP, MEF-ECM could effectively facilitate the proliferation of inner ear-derived cells.

Conclusion: The discovery of the potential of MEF-ECM in promoting inner ear-derived cell proliferation indicates that the decellularized matrix microenvironment may play a vital role in keeping proliferation ability of these cells. Our findings indicate that the use of MEF-ECM may serve as a novel approach for expanding inner ear-derived cells and potentially facilitating the clinical application of inner ear-derived cells for hearing loss in the future.

Keywords: Decellularized Extracellular Matrix, Fibroblasts, Hearing Loss, Organ of Corti

Introduction

Compared to non-mammals, hair cells (HCs) and spiral ganglion neurons (SGNs) don’t have a regenerative ability in the mammalian cochlea following loss (1, 2). Recent studies have demonstrated that stem cells derived from organ of Corti, nominated inner ear-derived cells (2), possess the capability to proliferate and differentiate into new HCs and SGNs, indicating that inner ear-derived cells are promising cell source for HC regeneration (3). However, as one ages, the number of inner ear-derived cells decreases, thus rendering them unable to fully replenish lost HCs and SGNs (4). In addition, the limited proliferation ability of inner ear-derived cells currently hinders their potential application in the treatment of sensorineural hearing loss (SNHL) (5). Currently, there has been no successful method developed to efficiently increase the number of inner ear-derived cells (6). Strategies aimed at enhancing the proliferation of inner ear-derived cells are likely to be advantageous for the treatment of SNHL.

It is currently understood that the cultivation of pluripotent stem cells, such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSCs), necessitates a distinct microenvironment or niche (7). At the present time, mouse embryonic fibroblasts (MEFs) have been utilized as feeder cells for expanding pluripotent stem cells due to its capacity to sustain self-renewal and retain pluripotency (8). The use of MEFs was motivated by their potential to generate and release various biomolecules including but not limited to extracellular matrix (ECM), leukemia inhibitory factor (LIF), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) (9). In addition, ECM has a central role in establishing an environment that is conducive to tissue-specific cell functions and in the case of stem cells, this environment is the stem cell niche, where ECM signals participate in cell fate decisions (10, 11). It is possible that a lack of suitable ECM could be a contributing factor to the decline in the multipotency of stem cells observed in the course of cultivating inner ear-derived cells in a typical tissue culture environment (12).
In this study, a cell-free MEF-ECM through decellularization was prepared. The characterization of MEF-ECM was conducted by using scanning electron microscope (SEM) and immunofluorescent staining. Cell counting kit-8 (CCK-8) was used to evaluate proliferation capability of inner ear-derived cells cultured on the MEF-ECM and tissue culture plate (TCP). The objective of this research was to evaluate the feasibility of obtaining MEF-ECM method, and determine the influences of MEF-ECM on the proliferation of inner ear-derived cells. This exploration holds significant promise in creating a favorable microenvironment to facilitate the expansion of inner ear-derived cells.

Materials and Methods

Otosphere harvest

In this experimental study, the sensory cochlear sensory epithelia were extracted from postnatal day 2 Institution of Cancer Research (ICR) mice (Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China). The tissue samples underwent a 15-minute exposure to 0.125% trypsin (Invitrogen, USA) at 37°C, followed by blocking with a trypsin inhibitor and DNase I solution (Invitrogen, USA). The pellets were suspended in Dulbecco’s modified Eagle medium/F12 (1:1) (DMEM/F12, Invitrogen, USA) supplemented with N2 and B27 (Sigma, USA), 10 ng/mL basic FGF (bFGF, Wako, Japan), 20 ng/mL epidermal growth factor (EGF, Sigma, USA) and 50 ng/mL ampicillin (Sigma, USA). The suspension was filtered using a 70 μm cell strainer (BD Bioscience, San jose, CA) and subsequently transferred into a 6-well cell culture plate. Following a 2-day culturing period, the cell suspension was transferred into new cell culture plate that allowed to expand. Subsequently, the otospheres were identified after 5-6 days of culturing. Before visual inspection under an inverted microscope (Nikon Eclipse 80i, Japan), the otospheres were cultured in 6-well cell culture plates with DMEM/F12 containing N2, B27, 10 ng/mL bFGF, 20 ng/mL EGF, 50 ng/mL ampicillin and 10% fetal bovine serum (FBS, Gibco BRL, Life technologies, USA) for 5 days. Animal experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Ethics Committee of Donghua University (DHUEC-NSFC-2019-01).

Reverse transcription and polymerase chain reaction analyses

Total RNA derived from adherent inner ear-derived cells at passages (P) 1, 2, 3 and 4 were extracted with Trizol reagent (TaKaRa, Japan). The integrity of the RNA samples was evaluated by the A260/A280 ratio. Four μg of RNA was successfully transcribed into cDNA utilizing the Prime Script RT reagent kit (TaKaRa, Japan) followed by PCR using ProFlex™ PCR (Thermo) to detect the gene expression. The expression of stem cell markers (Sox2 and Nestin), early inner ear stem cell markers (Bmp7 and P27kip1) and mature HC markers (Espin and Myosin VIIA) was detected. The organ of Corti-derived atmosphere’s expression from postnatal day 2 mice were utilized as a positive control. The primer sequences presented in Table 1 have been designed and synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

Preparation of the extracellular matrix derived from mouse embryonic fibroblasts

MEFs were isolated and cultured according to previous research (13). In brief, embryos were harvested from female ICR mice 10.5 days after the appearance of the copulation plug. MEFs were extracted from embryos and seeded in TCP with DMEM (Gibco, USA) supplemented with 10% FBS, 1% penicillin-streptomycin (Gibco, USA) and 2 mM L-glutamine (Gibco, USA). The MEF-ECM was obtained by the method previously described with minor modifications (14). Briefly, the MEFs at P3 were subjected to a 6-day culture. Subsequently, the culture medium was removed, and the cells were exposed to a solution containing 0.25% Triton X-100 (Sigma, USA) and 20 mM NH₄OH (Sigma, USA) at 37°C for a duration of 5 minutes. Finally, the MEF-ECM underwent incubation with 100 U/mL DNase I (Invitrogen, USA) for 10 minutes. Subsequently, rinsing with phosphate buffered saline (PBS) was carried out thrice, following which the sample was stored at 4°C to facilitate further study.

Morphology of the extracellular matrix derived from mouse embryonic fibroblasts

The morphology of MEF-ECM was observed by the method previously described with minor modifications.
(15). The specimens were fixed with 2.5% glutaraldehyde (MP Biomedicals, Irvine, CA, USA) for 30 minutes at 4°C. After that, all samples were dehydrated with 60, 70, 80, 90 and 100% absolute ethanol for 20 minutes each time and air dried. The dried samples were fixed on the sample stage of the SEM using conductive adhesive and subsequently sputter-coated in gold for 90 s. Imaging was obtained by using a SEM (S-520; Hitachi, Japan) with a voltage of 10-15 kV.

Immunofluorescent staining

Immunofluorescent staining was conducted to evaluate the component of MEF-ECM. The MEFs and MEF-ECM were firmly fixed with a 4% paraformaldehyde solution for 15 minutes at room temperature. To facilitate permeability, the specimens were treated with a 0.1% Triton X-100 solution for 10 minutes. Subsequently, the specimens were blocked using a 1% bovine serum albumin (BSA, Sigma, USA) solution for the next 30 minutes. Next, primary antibodies including rabbit anti-laminin (1:100, Invitrogen, USA), collagen I (1:300, BD Biosciences, USA) and fibronectin (1:200, BD Biosciences, USA) were utilized to incubate MEF-ECM samples overnight at 4°C. The MEF-ECM specimens underwent incubation with Alexa Fluor-488 conjugated goat anti-rabbit (1:300, Invitrogen, USA) in a dimly lit environment for an hour at ambient temperature. The MEF samples were incubated with Alexa Fluor-568 conjugated phalloidin (1:1000, Cell Signaling Technology, USA) for 30 min at dark. Subsequent to the completion of the staining process, 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) was utilized to counterstain all samples within 10 minutes, after which images were obtained under an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Proliferation assays and cell propagation

After culturing on the MEF-ECM and TCP for 1, 4, and 7 days, the morphology of inner ear-derived cells was observed by an inverted phase contrast microscope (Nikon, Japan). For proliferation assays, the CCK-8 (Sigma, USA) was employed to detect the proliferation of inner ear-derived cells at P4 and P8 cultivated on the MEF-ECM and TCP. The inner ear-derived cells at a density of 1×10⁴ cells per well were seeded on the MEF-ECM and TCP with DMEM/F12 supplemented with 10% FBS and 50 ng/mL ampicillin at 37°C under 5% CO₂ atmosphere. After culturing for 1, 4 and 7 days, CCK-8 working solution was introduced into the wells. After incubation for a duration of 2 hours at 37°C, optical density (OD) values were taken at 450 nm by using a plate reader (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

The data were presented as mean values ± standard deviation (SD) with the number of independent experiments (n=3). The statistical significance between groups was analyzed by One-way ANOVA followed by Tukey’s test. The analysis was performed through the utilization of the SPSS 13.0 software (SPSS Inc., Chicago, IL). P<0.05 was considered statistically significant.

Results

Morphology of mouse embryonic fibroblasts and extracellular matrix derived from mouse embryonic fibroblasts

In this study, MEF-derived ECM was obtained through decellularization. As shown in Figure 1A, mouse embryonic fibroblasts in a polygonal shape were cultured in TCP. After decellularization, the cellular structure was removed entirely (Fig.1A’). SEM images showed the micromorphology of the MEFs before decellularization and the MEF-ECM after decellularization (Fig.1B’). As shown in Figure 1B, the MEFs exhibited a polygonal morphology demonstrating strong adhesion. Notably, the SEM images revealed the presence of a three-dimensional (3D) cellular structure. Following decellularization, the MEF-ECM exhibited robust binding to the TCP substrate and maintained its 3D structure.

Immunofluorescence staining of MEF-ECM

In this study, the MEF-ECM was obtained by using modified method of decellularization. The morphology of MEFs cultured on TCP before decellularization (Fig.2A) and after decellularization (Fig.2B) identified by cytoskeleton/nuclear staining. The MEFs appeared polygonal shape and attached well before decellularization and cytoskeleton of MEFs was removed entirely after decellularization. The results indicated that various significant protein constituents, including laminin (Fig.2C, D), fibronectin (Fig.2E, F) and collagen I (Fig.2G, H), were found to be present in the cell-deposited matrix before decellularization and after decellularization. Significantly, the observed high fluorescence intensity of laminin and collagen I indicated a high abundance of these proteins within the MEF-ECM. However, fibronectin was found to be expressed at a comparatively lower level.

Identification of inner ear-derived cells

As shown in Figure 3A and B, otospheres were produced from isolating cochlear sensory epithelia and transited to an adherent condition to promote attachment. To determine whether the adherent otospheres exhibit properties of stem cells, an examination was conducted to evaluate gene expression of various markers, including stem cell markers (Nestin, Sox2), inner ear progenitor makers (Bmp7, P27⁰¹⁰) and HC markers (Myosin VIIA, Espin). As shown in Figure 3C, the expression of Nestin and Sox2 was observed in adherent cells. Nevertheless, the expressions of Bmp7 and P27⁰¹⁰ were detected in the otospheres group and no Bmp7 expression was detected throughout the propagation process, while Myosin VIIA expression was detected during subsequent adherent culture stages.
Expansion of Inner Ear-Derived Cells

**Fig. 1:** Morphology of MEF-ECM. Phase contrast microscope images of MEFs A. Before and A'. After decellularization. SEM images of the MEFs B. Before decellularization and B'. SEM images of MEF-ECM after decellularization (scale bar: A, A': 500 μm; B, B': 20 μm). MEF-ECM; Extracellular matrix derived from mouse embryonic fibroblasts and SEM; Scanning electron microscope.

**Fig. 2:** Immunofluorescent staining of MEF-ECM. The MEF-derived ECM was examined before (upper panels) and after (lower panels) decellularization. Phalloidin/DAPI staining of MEF-ECM A. Before decellularization and B. After decellularization. Immunofluorescence staining of matrix proteins: C, D. Laminin, E, F. Fibronectin, G, H. Collagen I in MEFs and MEF-ECM (scale bar: 100 μm). MEF-ECM; extracellular matrix derived from mouse embryonic fibroblasts.
The MEF-ECM promotes proliferation of inner ear-derived cells

To assess the impact of MEF-ECM on the proliferation of inner ear-derived cells, cells at passages 4 and 8 were cultured on the MEF-ECM and cells at passage 4 cultured on the TCP. The proliferation ability of inner ear-derived cells was determined by CCK-8 assay. As shown in Figure 4A, a consistent increase in the number of inner ear-derived cells was observed throughout the in vitro cultivation period of 1 to 7 days. Of noteworthy significance is the observed augmentation of cell density cultivated on MEF-ECM, as a result of cell-matrix interactions. In addition, inner ear-derived cells showed proliferation in all substances during the initial seven days of observation. On the 7th day, the proliferation of P4 cells cultured on the MEF-ECM was significant difference compared with TCP group and P8-MEF-ECM group. Overall, the proliferation ability of P4 cells cultured on the MEF-ECM was stronger compared with P8 cells (Fig.4B).

**Fig.4:** Proliferation of inner ear-derived cells were influenced by the substrates. **A.** Phase contrast microscope images of P4 inner ear-derived cells cultured on uncoated TCP and MEF-ECM. **B.** The CCK-8 assay for evaluating the viability of inner ear-derived cells cultured on different substrates. The results showed MEF-ECM obviously promoted the cell proliferation compared with TCP. P4-MEF-ECM: P4-adherent cells cultured on the MEF-ECM; P8-MEF-ECM: P8-adherent cells cultured on the MEF-ECM; TCP: Tissue culture plate, and MEF-ECM: Extracellular matrix derived from mouse embryonic fibroblasts.
Discussion

Cochleae that are formed during earlier stages of development have the potential to contain multipotent stem cells with the ability to self-renew and differentiate into various cell types (16, 17). Previous study has revealed round spherical mass of cells that can be extracted from the basilar membrane of postnatal mice, and such cells have been nominated as inner ear-derived cells (18). Under suspension culture condition, these stem cells transform into spherical structures which possess the capability of proliferating and differentiating into HCs (19). Meanwhile, the number of cochlear stem cells decreases dramatically within the first postnatal days (20) severely hindering endogenous stem cell application in treating hearing loss. So far, there have been no reports of effective proliferation in vitro with inner ear-derived cells (21). In our previous study, we have conducted successful investigations into the manipulation of inner ear-derived cells behavior through the use of adherent culture techniques for otospheres (19). In this study, we have successfully produced an in vitro model of adherent otospheres by using the adherent culture. The expression of stem cell markers (Sox2 and Nestin) was observed in the cells derived from adherent otospheres and no expression of HC marker (Espin) observed, which proved that the cells derived from adherent otospheres were the inner ear-derived cells (22). However, the expression of Myosin VIIA was observed in the cultured cells after subsequent passages. This might have been due to partial differentiation of cells derived from adherent otospheres after subsequent passages and further studies will be needed to analyze these phenomena. Our RT-PCR analysis of Espin as a HC marker for actin filament cross-links in stereocilia is in full accordance with previous studies (23, 24). However, the existence of heterogeneous cell population may result in a decline in purity of stem cells affecting the experiment result directly.

Previous studies have demonstrated that ECM has the potential to serve as an efficient expansion system for the production of great amount of the cells with high quality cells for cartilage tissue engineering and regeneration (25). Recently, several investigators have demonstrated that the molecular components of the cochlear bio-matrix significantly influence the modulation of cell proliferation, survival, and migration (26, 27). These findings indicate that effective ECM could simulate stem cell microenvironment while simultaneously resulting in a substantial yield of high quality adult stem cells in vitro (26). ESCs and iPSCs are usually cultured with feeder cells to preserve their pluripotent differentiation and proliferation capabilities (28). So far, MEFs are commonly used as feeder cells to facilitate the establishment and preservation of pluripotent/multipotent stem cells (29). Essential growth factors and cytokines secreted by MEFs have been identified as crucial elements in sustaining the pluripotent state of stem cells (30).

In this study, the application of optimized decellularization treatments yielded an ECM derived from MEF providing an important microenvironment for promoting the proliferation of inner ear-derived cells. Our results indicated that the main constituents of the MEF-ECM, including collagen I, fibronectin, and laminin, were successfully detected following the decellularization, which supported the previous report (31). The high fluorescence intensity of laminin and collagen I indicated a high abundance of these proteins within the MEF-ECM. However, it was observed that the fluorescence intensity of fibronectin was relatively low. These findings also supported previous report (32). In this study, the adherent atmospheres in P4 and P8 had a better cellular growth behavior than the adherent atmospheres in P1 after the culture of otospheres. Therefore, we chose the adherent otospheres in P4 and P8 with a more stable and uniform state through observation. We found that MEF-ECM obviously promoted the inner ear stem cell proliferation, supported the hypothesis that the bioactive components of MEF-ECM provided an important microenvironment to promote cell proliferation. In addition, the adherent cells in P4 cultured on the MEF-ECM had a higher proliferative ability compared with the adherent cells in P8. This is probably due to younger cells in P4 and the viability of P4 adherent cells is higher compared with the P8 adherent cells. Recent researches showed that ECM influenced the fate and promoted the expansion of inner ear-derived cells (33). It has been suggested that the MEF-ECM could effectively promote proliferation and adhesion of human umbilical vein endothelial cells (HUVEC) (34). Similar outcomes have been observed in the cultivation of inner ear-derived cells. However, the lack of characterization of expanded cells results in a direction of uncertainty of the differentiation of the expanded cells. Further studies would be needed to find if expanded cells could differentiate into HCs or other auditory cells. In addition, the underlying mechanisms of how MEF-ECM contributes to the modulation of the biological function of inner ear-derived cells remains a topic of debate. In mammals, ECM is composed of approximately 300 proteins, including collagen subunits, proteoglycans, and glycoproteins (14). It is challenging to predict the potential impact on inner ear-derived cells due to the complex interplay among ECM constituents. Several studies have shown that ECM plays a crucial role in determining cell fate during the early stages of embryonic development, that is also instrumental in upholding the pluripotency of inner cell mass cells, as occurs in the case of TGF-β (35, 36). It has been determined that bFGF is a crucial
growth factor that is naturally produced by feeder cells utilized in the cultivation of hESC (37, 38). Besides, proteins or short peptide sequences extracted from intact MEF-ECM, exhibiting distinct interactions with cell receptors, have the potential to promote inner ear stem cell proliferation (39). Thus, combination of the growth factors and/or proteins within MEF-ECM could be useful substrates to enhance the capability of ex vivo expansion of inner ear-derived cells and to advance their effectiveness in regenerating HCs. However, additional research is required for the elucidation of the processes by which the MEF-ECM modulates the proliferation of stem cells.

Conclusion

The MEF-ECM containing multiple bioactive proteins was extracted successfully using decellularization. Furthermore, our research highlighted the capacity of utilizing MEF-ECM for the expansion of inner ear-derived cells in vitro, which significantly augmented the proliferative rate of inner ear-derived cells. Future research should prioritize the illustration and characterization of the mechanism of action of specific bioactive components in MEF-ECM. The MEF-ECM could promote the proliferation of inner ear-derived cells expected to achieve auditory cell regeneration and hearing restoration in the future.

Acknowledgements

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Authors’ Contributions

X.L., J.W.; Contributed to the conception and design of the experiments. J.Z., L.L.; Sample preparation, analysed the experiments. J.Z., L.L.; Carried out the experiments, statistical data, edited and revised the manuscript. All authors read and approved the final manuscript.

References

Expansion of Inner Ear-Derived Cells


The Effect of The Conditioned Medium from Human Embryonic Stem Cells on Mouse Oocytes In Vitro Maturation


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Abstract

Objective: Some reports have indicated that conditioned medium from growing mouse embryonic stem cells (ESCs) provides a supportive condition for small follicles growing, oocyte maturation, and following embryo growth. The aim of this study is assessing in vitro maturation (IVM) and consequent in vitro fertilization (IVF) outcome of immature mouse oocytes using human embryonic stem cells conditioned medium (HESCM).

Materials and Methods: In this experimental study, 240 germinal vesicle (GV) oocytes were took from NMRI female mice, aged 4-6 weeks, 48 hours before injection of 5 IU pregnant mare serum gonadotropin (PMSG). 120 GV oocytes without cumulus cells were cultured in each of the groups. 120 GV were cultured in HESCM as control group and also 120 GV cultured in human embryonic stem cells medium (HESM) as test group. After evaluating the metaphase II (MII) oocyte maturation rate at 8, 16, and 24 hours, the MII oocytes subsequently were fertilized in vitro and the two-cell embryo development rate was recorded at days 1, 2, and 3. Statistical analysis was performed by using the generalized estimating equations (GEE) method that calculated their rate ratio.

Results: Our data indicated there are significant differences between the maturation rates in HESCM and HESM (P=0.004), also the two-cell embryo development was significant between two culture media (P=0.00).

Conclusion: Similar to some other studies, the secretome of the HESCM showed a significant impact on the IVM outcomes in mice.

Keywords: Assisted Reproductive Techniques, Conditioned Medium, Germinal Vesicle, Human Embryonic Stem Cells, In Vitro Maturation

Introduction

Since the first attempts for in vitro maturation (IVM) of mammalian oocytes by Pincus and Enzmann in 1935, on immature rabbit oocytes (1), studies are carrying on to improve this technology. IVM is one of the assisted reproductive techniques (ART) inducing the meiotic to improve this technology. IVM is one of the assisted reproductive techniques (ART) inducing the meiotic...
subsequently provide an medium culture with mitogenic factors, growth factors, cytokines, and chemokines (11, 12) which might be useful to improve IVM outcomes.

In this study, the aim was to determine whether IVM of mouse GV oocytes can be improved by using human embryonic stem cells conditioned medium (HESCM) or not.

Materials and Methods

Ethical consideration

All animals were preserved according to the ethical guidelines provided by the Yazd Reproductive Sciences Institute Ethical Committee for animal studies (IR.SSU.REC.1397.087).

Materials

Our study was an experimental study. Chemicals and reagents were purchased from Sigma Aldrich Co. (UK). Culture media and supplements were purchased from Invitrogen Co. (UK) unless otherwise stated.

Preparation of HESM and HESCM

The human ESC growing medium was provided from the stem cell biology research center, Yazd reproductive sciences institute as explained elsewhere (10). HESCM was obtained from a HESCs (Fig.1A, B) cultured in a microdrop system (Fig.1C). After sufficient growth of undifferentiated colonies within a microdrop and before passage, the culture medium around the colony was collected and used as a conditioned medium (HESCM) for mouse oocytes IVM.

Animals

The animals used for the experiments were the Naval Medical Research Institute (NMRI) female mice, aged 4-6 weeks. Similar to our previous reports (7, 8), these mice were housed and bred in the animal house at the Biotechnology Unit of the Yazd Reproductive Sciences Institute. The mice were kept on a 12 hours’ light dark cycle, a temperature range of 22 to 25°C, humidity 40 to 60% and enough nutrients.

Immature oocytes collection and their in vitro maturation

The immature oocyte collection and IVM process was done as explained elsewhere (7, 8). But the test and control medium are HESCM and HESM in the current study.

In this way, twenty 4-6 weeks old female mice received an injection of 5 IU pregnant mare serum gonadotropin (PMSG). Forty-eight hours after injection, immature GV oocytes from the ovaries were taken out. The GV oocyte retrieval was done by scratching the ovaries with a sterile 28-gauge needle under a stereomicroscope. GV oocytes (Fig.2A) were individually cultured in micro drops (Fig.1C) of HESCM (test group) and of HESM (control group). In the present study, 240 GV oocytes (120 per group) were included and incubated at 37°C in a 5% CO₂ incubator for 24 hours. At 8, 16, 24 hours Oocyte maturation was evaluated by using stereo microscopy, only those oocytes that showed the first polar body in the perivitelline space were selected as metaphase II (MII) for IVF.

![Fig.1: HESCM preparation. A, B. HESM from growing Yazd2 hESC line was collected and C. Microdrops of HESCM prepared for IVM of mouse oocytes (scale bars of A: 500 µm and B: 100 µm). HESCM; Human embryonic stem cell conditioned medium and IVM; In vitro maturation.](image-url)
In vitro fertilization and embryo development

Following IVM, the two-cell embryo development potential rate of MII oocytes was evaluated by IVF. Male mice spermatozoa were obtained from the cauda epididymis that capacitated for 1 hour at 37°C. MII oocytes were incubated with sperms in the GIVF medium (Vitrolife, Sweden) for 4 hours. For the removal of additional spermatozoa, oocytes were washed and then cultured in a G1-plus droplet (Vitrolife, Sweden; 5-6 oocytes in one drop) and in 37°C and 5% CO2 in air for 3 days. The embryo development rate was checked and evaluated everyday by using a stereomicroscope.

Statistical analysis

The trend of changes in oocyte maturation and embryo development rate in mice oocytes were evaluated at each stage and compared between test (HESCM) and control (HESM) groups. Generalized estimating equations (GEE) method was used for comparing the trend change of each outcome variable through different time. Chi-square and Fisher exact-test were used for comparison of the frequencies of each outcome variable between HESM and HESCM groups. A significant level was considered as P≤0.05.

Results

In vitro maturation outcomes using human embryonic stem cells conditioned medium

Mice GV oocytes (Fig.2A) were cultured in HESCM (test group) and HESM (control group) for further maturation in vitro. IVM of mice oocytes was assessed during 24 hours. Resumption of meiosis from GV (Fig.2A) to the MI (Fig.2B) and MII (Fig.2C) stage was considered to be oocyte IVM. However, our data shows that HESCM provides more supportive conditions for maturation of mouse immature oocytes to the MII stage after IVM (P=0.004).

In two groups some of the GV oocytes were developed to MI stage after 8 hours (Table 1). The number of MI oocytes in HESCM group after 8 hours was 21 (17.5%) from 120 oocytes and in control group was 27 (22.5%) from 120. The number of the MII oocytes in HESCM was 3 (2.5%) from 120 and in control group was 1 (0.8%) from 120 (Table 1). The number of MI and MII oocytes in HESCM after 16 hours [respectively 49 (40.83%) and 11(9.16%) from 120] was higher than control group [respectively 41 (34.16%) and 8 (6.66%) from 120] (Table 1). In test group after 24 hours 17 (14.16%) of oocytes developed to MI and 32 (26.66%) oocytes reached to MII, whereas in control group only 30 (25%) from 120 oocytes developed further to MI stage and 28 (23.33%) of them developed to MII stage (Table 1). Significant difference was existed between the MII maturation stage rates in HESCM and HESM (P=0.004). Two groups support the further development of the GV oocytes to MI stage which this progress was insignificant in both groups. MI formation rate in HESCM and HESM has increasing and then decreasing trend that is because of the maturity of the oocytes to MII after 24 hours (Table 1). It is notable that the GV oocyte maturation to MII increased after 24 hours that shows the positive effect of the time on oocyte in HESCM group (Table 1). Similar to development of oocytes to MI stage in control group.

In sum, HESCM support the maturation of GV oocytes to MII stage. This data shows that there are factors in human ESCs which support IVM process in mouse.

Fig.2: Different stages of mouse oocyte during in vitro maturation in our study. Each experiment was repeated for 120 oocytes. A. GV oocyte, B. MI oocyte, C. MII oocyte (scale bar: 15 μm). GV; Germinal vesicle, MI; Meiosis I; and MII; Meiosis II.
Table 1: IVM rate at 8, 16, and 24 hours in the HESM and HESCM groups

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Data are presented as n (%). MI, MII, and developmental rate at different times at test and control medium at 8, 16 and 24 hours. GEE method was used for change trend in MI and MII. P value of Chi-square test for MI and MII was 0.52 and 0.004 respectively (statistical test: Chi-square). HESM; human embryonic stem cell medium, HESCM; Human embryonic stem cell conditioned medium, MI; Meiosis I, MII; Meiosis II, GEE; Generalized estimating equations. Each experiment was repeated for 120 oocytes.

Embryo development following in vitro fertilization of in vitro maturation oocytes

The developing competence of IVM oocytes was evaluated by subsequent IVF and embryo culture to the 2-cell cleavage stage (Fig.3).

Fig.3: Embryo development following IVF of MII oocytes. Picture of two cells embryos after 48 hours and 72 hours (scale bar: 15 µm). Each experiment was repeated for 32 MII oocytes obtained from HESCM medium and for 28 MII oocytes obtained from HESM medium. IVF; In vitro fertilization, MII; Meiosis II, and HESM; Human embryonic stem cell medium.

26.66% of oocytes that matured in HESCM were fertilized in GIVF medium. The IVF success rate was determined according to the number of embryos that reached the 2-cell stage at 24 hours after IVF. Number and Percentage of two-cell embryo development from HESCM after 1, 2 and 3 days were respectively 0 (0%), 7 (21.87%), and 7 (21.87%). Table 2 shows results for the percentage of IVF success at 1, 2, and 3 days in the HESCM. Table 2 indicates the two-cell embryo development rate increased until the second day after IVF, and then remained at this stage on the third day. 23.33% of oocytes (28 from 120 GV) that matured in HESM were fertilized in GIVF medium. Percentage of two-cell embryo development rate from HESM at 3 days was 3 (10.71%, Table 2).

Table 2: Two-cell embryo development following IVF of MII oocytes

<table>
<thead>
<tr>
<th>IVF</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>HESM</td>
<td>0 (0)</td>
<td>3 (10.71)</td>
<td>3 (10.71)</td>
</tr>
<tr>
<td>HESCM</td>
<td>0 (0)</td>
<td>7 (21.87)</td>
<td>7 (21.87)</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.000</td>
<td></td>
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</tbody>
</table>

Data are presented as n (%). Each experiment was repeated for 32 MII oocytes obtained from HESCM medium and for 28 MII oocytes obtained from HESM medium. P value=0.000 (statistical test: Chi-square). IVF; In vitro fertilization, MII; Meiosis II, HESM; Human embryonic stem cell medium, and HESCM; Human embryonic stem cell conditioned medium.

Discussion

Since many factors are involved in the maturation and development of oocytes, much research has been done based on the selection of more appropriate culture medium containing these factors (11-13). For example, factors such as EGFs have been shown to increase the rate of oocyte maturation (13). Some studies have shown that the addition of a series of substances secreted from oocytes to the IVM culture medium of mice (14) and bovine (15, 16) significantly increases the maturity of oocytes.

There are some factors secreted by ESCs (EGF, LIF, TGF-β, IGF) in the culture medium which supports IVM of oocytes (12). The effect of these factors on IVM, have been verified in several species (17, 18). On the other hand, Giuffrida and colleagues have shown that human ESCs (hESCs) secrete soluble factors that inhibit cancer cells growth (19). Therefore, in this study, we prepared a conditioned medium from ESCs culture to be containing these factors.
In this study, the maturation and two-cell embryo development rate of mouse immature oocytes in HESCM, and HESM were evaluated. Our data indicated that HESCM to be more supportive than HESM in IVM 32(26.66%) MII from 120(100%) GV vs. 28(23.33%) MII from 120(100%) GV and two-cell embryo development 7(21.87%) from 32(26.66%) MII oocytes vs. 3(10.71%) embryos from 28(23.33%) MII oocytes in HESCM, and HESM groups respectively, and this difference was statistically significant.

Previously, we have shown that conditioned media from human testicular cells (7) and human cumulus cells (8) support IVM in mice. In compare with our previous studies after 24 hours human testicular cells conditioned medium (hTCCM) is the most supportive and then HESCM and at last is human cumulus cell conditioned medium (hCCCM) with 31.67% (7) vs. 26.66% vs. 24.16% (8) IVM rate respectively. On the other hand, interestingly HESM seems to be more supportive than DMEM+20%FBS as the basal medium in previous study, to support IVM in mice (23.33% vs. 0%).

Chian and Tan (20) showed that immature human oocytes without cumulus cells are also able to support the early stages of embryonic development following IVM in a suitable culture medium. In our study, in order to investigate the effect of HESCM on IVM in mouse GV oocytes, immature oocytes without cumulus cells were used for IVM in both test and control groups.

It has also been reported in some studies that the rate of maturation in GV resulting from stimulation cycles (21) is higher than that without stimulation cycles in the laboratory (22). In our study; 5 IU of PMSG was used to stimulate the ovaries 48 hours before taking an immature oocyte.

In other studies, cross species experiments have shown the conditioned medium from different species support IVM of GV oocytes from other species (7, 8, 23). For instance, IVM of canine oocytes was evaluated by using conditioned medium from mouse (23) or bovine (24) sources. In our study, the effect of human source of ESCs was investigated on the IVM of mouse oocytes.

In sum, our data supports the previous report of the supportive effect of mouse ESCCM on mouse IVM (12), however, this supportive effect in our results was less than the study, which might be due to the human source of the conditioned medium used in our work. Moreover, from our experiences at the same laboratory (7, 8) HESM as a basal medium has a better impact on IVM in mice in comparison with DMEM+20% FBS. The other issue is that we have used a single culture for each individual GV oocyte whereas in some of the previous studies GV oocytes (12) were cultured in groups which may have an impact on the result. Additionally, in some reports GV oocytes were not completely denuded from granulosa cells which also might cause some variations between different studies. In addition, several studies have reported beneficial effects of conditioned media for enrichment IVM culture medium (25).

Conclusion
This study demonstrated the advantageous effect of the HESCM on the IVM outcomes in mice providing a framework for further investigation.

Acknowledgements
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Authors’ Contributions
R.R.R.S., B.A.; Conceptualization, Methodology, and Software. R.R.R.S.; Data curation, Writing, and Supervision. J.G., F.M., M.A.; Visualization and Investigation. M.I.; Software and Validation. B.A., M.D.A., S.M.S.; Were responsible for overall supervision, study design, and data analysis. R.R.R.S.; Drafted the manuscript, which was revised by B.A. All authors read and approved the final manuscript.

References


The Effect of Training Type on The Signaling Pathway of Ceramide-Dependent Insulin Resistance in The Flexor Hallucis Longus Muscle of Streptozotocin-Induced Diabetic Rats

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Abstract

Objective: This study aimed to compare the effect of different physical training on the mechanism of ceramide-dependent insulin resistance in the flexor hallucis longus (FHL) muscle of diabetic rats.

Materials and Methods: In this experimental study, 7 healthy as a healthy control (HC) group, and 21 diabetics (55 mg/kg Streptozotocin) Wistar rats (200-220 g; 8-10 weeks old) divided into the diabetic control (DC), moderate continuous training (MCT), and moderate intensity interval training (MIIT) groups. Both MCT (55-70% of maximal oxygen uptake (VO₂max), and MIIT (85% VO₂max) groups trained for 10-25 minutes at a speed of 10-20 m/minutes. The changes in the expression of blood glucose, insulin, insulin resistance, lipid profile and total ceramide were measured as well as ceramide synthase-1, Glucose transporter type 4 (GLUT4), Protein kinase B known as Akt, phosphorylated protein kinase B known as pAkt, protein kinase C (PKC), and tumour necrosis factor α (TNFα).

Results: Blood glucose, triglyceride (TG) and ceramide synthase-1 (CS1) expression levels in the MCT group decreased in comparison with the DC group. FHL protein expression of GLUT4 in the MCT group was higher than the DC group. FHL expression of GLUT4, pAKT, AKT/pAKT, PKC, CS1 and total ceramide in the MIIT group were higher than the DC group. Cholesterol, low-density lipoprotein (LDL), TG, and TNF-α protein expression in the MIIT group were lower than the DC group. GLUT4, PKC, pAKT, AKT/pAKT in the MIIT group were higher, and total ceramide and TNF-α were lower in the MIIT group than the MCT group.

Conclusion: It seems that both training plan MIIT and MCT have favorable effects on the metabolism of glucose, insulin, lipids, and the decrease of TNFα level in the diabetes, but in connection with the improvement of the ceramides mechanism, it seems that the MIIT training plan is more optimal than MCT training plan.

Keywords: Ceramides, Diabetes Mellitus, Exercise, Insulin Resistance, Muscle

Introduction

Diabetes mellitus is recognized as a major global health problem that affected 463 million people worldwide to date (1). This disease includes 2 main types. In the type 1 diabetes, the body’s immune system attacks and destroys the cells that produce insulin, and the body does not produce enough insulin. Type 2 diabetes leads to the compensatory hyperinsulinemia response following an insulin sensitivity reduction and/or an increase in insulin resistance (2, 3). An Insulin dysfunction in both type 1 and 2 diabetes in the long term can lead to cellular redox disorders, increased inflammatory factors, and mitochondrial dysfunction (1, 4). In the other words, a disorder in the metabolism of lipids disrupts the metabolism of sphingolipids.

Sphingolipids are a class of complex lipids which is composed of a long-chain amino alcohol, and ceramide as their core structure. The ceramide disorders lead to increase insulin resistance, impair transport of fatty acids into the cell and the accumulation of ceramides (1). Also, ceramides activate protein kinase C (PKCζ), Akt/protein kinase B (PKB) inhibitor and cytosolic protein phosphatase 2 A (PP2A) activator, disrupts glucose transporter pathway (GLUT) (1, 5, 6). Increased levels of ceramid16:0 (C16:0), decrease ceramide synthase 6 (CerS6) level, impairs sphingomyelin production and sphingomyelinase enzyme function (as enzymes involved in the metabolism of cell membrane lipids) lead to decreased insulin sensitivity (5, 7).

On the other hand, increasing progress of diabetes,
the need for non-invasive methods to prevent and treat this disease has been examined, so that the role of regular physical activity in reducing cardiovascular risks, hypoglycemia, and improved insulin in diabetic patients reported by researchers (8). Exercise can reduce the accumulation of ceramides in muscle cells by the mechanism of increasing mitochondrial biogenesis, increasing GLUT4 expression, increasing lipid metabolism, and improving sphingolipid synthase 1 and 2 (SGMS1/2) enzyme function. Also, it has been reported that 12 weeks of combined aerobic and resistance training, increased the insulin sensitivity, diacyl glycerol, ceramide, and SGMS1 in obese women, nevertheless, SGMS2 and Glut4 protein levels did not change (9). A three-hour acute training session in trained and untrained individuals was associated with increased sphingomyelin levels after exercise, nevertheless, sphingomyelinase and ceramide levels did not change significantly (7). 12 weeks of high intensity interval training (HIIT), reduced ceramide synthase 1 (Cers1), interleukin 6 (IL6), Nuclear factor kappa-light-chain-enhancer of activated B -1 (NFKB1) and tumor necrosis factor alpha (TNF-α) gene expression in skeletal muscle (10). Although, the response of ceramides to exercise depends on the duration (11), type (9), intensity of the exercise (12), the mechanism of effect of interval and continuous exercise on ceramide-dependent insulin resistance in skeletal muscle tissue is not yet fully understood.

In addition, the review of previous studies has mostly been in the field of comparative high and low intensity exercises, few studies have compared moderate continuous training (MCT) and moderate intensity interval training (MIIT). Therefore, considering the importance of the mechanism of ceramides in insulin resistance and lipid metabolism, it seems that studying the type of training on this mechanism will help researchers to obtain more information in this field. According to the published studies, it seems that the comparison of two types of MICT and MIIT exercises on the mechanism of glucose, insulin and the changes of inflammatory factors related to ceramides is one of the innovations of the current research and can provide more comprehensive information in this field to the researchers in the field of exercise and health. Therefore, the present study aimed to compare the effect of MIIT and MCT on the mechanism of ceramide-dependent insulin resistance in the flexor hallucis longus (FHL) muscle of diabetic rats.

**Material and Methods**

All steps of working with laboratory animals in this project were taken according to the Helsinki agreement and under the supervision of the Ethics Committee of Islamic Azad University of Marvdasht Branch, Shiraz, Iran (IR.IAU.M.REC.1400.037).

**Maintenance and grouping of rats**

Twenty eight Wistar rats with age range 8-10 weeks and an average weight of 200-220 g were purchased from the Laboratory Animal Breeding and Reproduction Center of Baqiyatallah University (Tehran, Iran) and transferred to the medical science laboratory of Kurdistan University (Kurdistan, Iran). The animals were kept in standard polycarbonate cages with autoclave capability, with free access to food and water as well as standard conditions (12:12 hour light-dark cycle, relative humidity of 55- 60% and a temperature of 22-24°C). Then, after 14 hours of fasting and under anesthesia, 21 rats received peritoneally 55 mg/kg streptozotocin (572201, Sigma Aldrich, USA), dissolved in citrate buffer (P4809, Sigma Aldrich, USA) (2). After four days, blood glucose levels in rats were measured using a glucometer (DA12-B1, Delta, Taiwan), and ear punching procedure. Based on blood glucose, diabetic rats (blood glucose above 250 mg/dL) were divided into 3 groups of 7 rats as 1) diabetic control (DC), 2) MCT and MIIT as well as 7 healthy rats selected as healthy control (HC) group to review the effects of diabetes induction.

**Continuous and interval training**

To perform adaptation training, rats trained every day with a speed of 10 m/minutes and slope of zero for 15 minutes for one week. Then, the MCT group members were trained 10-25 minutes with speed of 10-20 m/minutes, equivalent to 55-70% of the maximum oxygen consumption, for 3 sessions per week. The MIIT group members were trained at a speed of 10-15 meters per minute, with 2-3 repetitions of 5-8 minutes at a high intensity equivalent to 85% maximum oxygen consumption and low intensity repetitions at a low intensity of 50-55% maximum oxygen consumption on a 0 to +5 slope for 3 sessions per week (Table 1).

**Sampling**

The rats sacrificed by injection of xylazin (10 mg/kg) (CAS 7361-61-7. Santacruz, USA) and ketamine (90 mg/kg, CAS 1867-66-9. Santacruz, USA) after 14 hours of fasting. After laboratory experts’ complete ensuring of anesthesia by gently squeezing the animal’s foot and testing the sensation of pain, 5 cc blood of the heart left ventricle was taken. Then the serum samples of taken bloods in this study were used to measure glycemic indices (blood glucose, insulin, insulin resistance) and lipid profile (cholesterol, triglycerides, low-density lipoproteins and high-density lipoproteins). After that the FHL muscle was carefully extracted under sterile conditions by making an incision on the dorso lateral region of the lower limb and cutting the tendon. Then, it was immediately stored at -80°C until transfer to the laboratory.
Table 1: Continuous and interval training protocol in the present study

<table>
<thead>
<tr>
<th>Groups</th>
<th>One</th>
<th>Two</th>
<th>Three</th>
<th>Four</th>
<th>Five</th>
<th>Six</th>
<th>Seven</th>
<th>Eight</th>
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<tbody>
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<td>12</td>
<td>14</td>
<td>16</td>
<td>18</td>
<td>20</td>
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</tr>
<tr>
<td>Speed (m/minutes)</td>
<td>25</td>
<td>25</td>
<td>22</td>
<td>20</td>
<td>18</td>
<td>15</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Duration (minutes)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Slope (percentage)</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>MIIT</td>
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<td>10</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>14</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Speed (m/minutes)</td>
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<td>3*8</td>
<td>3*7</td>
<td>2*9</td>
<td>3*7</td>
<td>3*8</td>
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<td>Duration (minutes)</td>
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<td>+5</td>
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<tr>
<td>Slope (percentage)</td>
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<td>+2</td>
<td>+3</td>
<td>+4</td>
<td>+5</td>
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</tr>
</tbody>
</table>

MCT; Moderate continuous training and MIIT; Moderate intensity interval training.

Measurement of lipid profile, blood glucose, and insulin resistance

In this study, insulin resistance and blood glucose were measured using HOMA-IR method (13). Also, serum levels of high-density lipoprotein (HDL), cholesterol (Chol), low-density lipoprotein (LDL), and triglyceride (TG) were measured with an immunohistochemistry based method (Pars Azmoon Co., Tehran, Iran).

Western blotting Measurement

Using a lysing buffer (E-CK-A105, Elabscience, Houston, Texas, USA), protein samples were separated by page gel made of polyacrylamide and the addition of ammonium persulfate (APS) and tetramethyethylene diamine (TEMED). After the electrophoresis step, the gel proteins on PVDF paper were shaken in methanol and washed for 1 minute with distilled water and then placed in a transfer buffer. After transferring the proteins to the surface of PVDF paper, the paper was shaken with blotting solution for one hour and 15 minutes at room temperature. The paper was then exposed to the initial β-actin antibody overnight at 4°C and the next day the paper was washed 3 times each time for 15 minutes with TBS-T buffer. The paper was then incubated with Anti Rabbit secondary antibody for one hour. After incubation, the paper was washed three times with TBS-T buffer for 15 minutes each time. For exposure, the desired protein band was exposed with the ECL advanced reagent kit (GERPN2236, Sigma Aldrich, USA) and using radiography films. The blots were then washed in the stripping buffer and β-actin antibody was added to the paper and incubated again with the secondary antibody. Thus, the control β-actin was also exposed in the radiology film, and finally the exposed bands went through densiometry using Imaje J software (NIH, USA) (14).

Measurement of total ceramide and insulin levels

In this study, total ceramide levels were measured in FHL muscle tissue with a sensitivity of 1 ng/ml, and serum insulin levels were measured by ELISA kit (MBS7255105., Mybiosurse Co., Canada) with a sensitivity of 200 pg/ml.

Statistical data analysis tests

Normal distribution of data examined by Shapiro-Wilk test. Considering the normality of data distribution, one-ANOVA and Tukey’s post-hoc tests were used to review the difference between groups. And statistical significance was defined as P<0.05. To do the statistical data analysis, SPSS software version 19 was used (IBM CO, Chicago, USA). In addition, to draw the graphs, Graphpad Prism 8.3.2 software (Dotmatic company, San Diego, California, USA) was used.

Results

Changes in the levels of serum blood glucose, insulin, insulin resistance and glucose transporter (Glut4) proteins

Our results of blood glucose serum levels showed that there was a significant difference between the DC group in comparison with the HC group (P<0.0001), nevertheless in the MCT group a significant decrease was observed in comparison with the DC group (P=0.0258, Fig.1A).

Regarding serum insulin levels, the results indicated that. Insulin levels in the DC group showed a significant...
Different Exercise Training and Ceramide-Dependent Insulin Resistance
decrease in comparison with the HC group (P=0.0134) and also, it showed a significant increase in the MCT group in comparison with the MIIT group (P = 0.0237, Fig.1B).

There was no significant difference between the groups in the index of insulin resistance (Fig.1C).

There was a significant difference in the Glut4 protein concentrations in the FHL muscle of the DC group showed a significant decrease in comparison with the HC group (P<0.0001) and increased in the MCT (P=0.0343) and MIIT (P<0.0001) groups compared to DC group as well as in MIIT group increased significantly compared to MCT group (P<0.0001, Fig.1D).

**Lipid profile**
The results showed high-density lipoprotein (HDL) levels decreased in the DC group in comparison with the HC group (P<0.0001) and increased in the MCT group in comparison with the MIIT group (P=0.0023, Fig.2A). Also, cholesterol levels increased significantly in the DC group in comparison with the HC group (P<0.0001), nevertheless in the MIIT group decreased in comparison with the DC group (P=0.0242, Fig.2B).

LDL levels increased in the DC group in comparison with the HC group (P<0.0001) and in the MIIT group decreased in comparison with the DC group (P=0.0419, Fig.2C).

TG levels in the DC group increased in comparison with the HC group (P<0.0001) and decreased in the MCT (P=0.0005) and MIIT (P=0.0005) groups in comparison with the DC group (Fig.2D).

**Total changes in ceramide and ceramide synthase-1**
The results showed that there is a significant difference between total ceramide levels in the DC in comparison with the HC group (P<0.0001), while it was decreased in the MIIT group in comparison with the CD (P<0.0001) and MCT (P<0.0001) groups (Fig.3A).

The CS1 level in the DC group increased significantly in comparison with the HC group (P=0.0002), and also its level was decreased in the MCT (P=0.0060) and MIIT (P=0.0004) groups in comparison with the DC group (Fig.3B).

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**Fig.1:** Glycemic indices of rats.  
A. Glucose, B. Insulin, C. Insulin resistance, and D. GLUT4 protein concentration in FHL muscle.  
HC; Healthy control, DC; Diabetic control, MCT; Moderate continuous training, MIIT; Moderate intensity interval training (n=7), and FHL; Flexor hallucis longus.

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Fig. 2: Lipid profile levels of rats. A. HDL levels, B. Total cholesterol, C. LDL levels, and D. TG levels. HC; Healthy control, DC; Diabetic control, MCT; Moderate continuous training, MIIT; Moderate intensity interval training (n=7), HDL; High-density lipoprotein, LDL; Low-density lipoprotein, and TG; Triglyceride.

Fig. 3: Total ceramide levels and ceramide synthase-1 concentrations in the FHL muscle tissue of rats. A. Total ceramide and B. Ceramide synthase concentration-1. HC; Healthy control, DC; Diabetic control, MCT; Moderate continuous training, and MIIT; Moderate intensity interval training (n=7).
Changes in insulin resistance signaling pathway proteins (PKC, pAKT, AKT and TNF-α) in the FHL muscle

Regarding Akt, although no significant difference was observed among groups (Fig.4A), its phosphorylated form (pAkt) decreased in the DC group in comparison with the HC group (P<0.0001). Also, the pAkt protein concentration in the MIIT group increased significantly in comparison with the DC (P<0.0001) group (Fig.4B). Also, the Akt / pAkt ratio in the DC group decreased significantly in comparison with the HC group (P<0.0001), while we observed a significant increase in the MIIT group in comparison with the DC (P<0.0008), while in the MIIT group increased in comparison with MCT (P=0.0004) groups (Fig.4C).

Regarding PKC concentrations in the FHL muscle, our findings showed that the amount of this signaling protein in the DC group decreased significantly in comparison with the HC group (P<0.0001), while in the MIIT group increased in comparison with the DC (P<0.0001) and MCT (P<0.0001) groups (Fig.4D).

The amount of TNF-α inflammatory index increased in the DC group rather than the HC group (P<0.0001). However, interval training (MIIT group) caused the levels of this inflammatory protein to be lower than the DC (P=0.0004) and MCT (P=0.0041) groups (Fig.4E).

Fig.4: Concentrations of insulin resistance signaling pathway proteins in the FHL muscle tissue of rats. A. Akt concentration, B. pAkt concentration, C. Akt / pAkt ratio, D. PKC concentration, and E. TNF-α concentration. HC; Healthy control, DC; Diabetic control, MCT; Moderate continuous training, MIIT; Moderate intensity interval training (n=7).
Discussion

The present study showed that MCT decreased the level of blood glucose as well as MIIT, and MIIT increased muscle GLUT4 protein concentration. The MIIT had more favorable effects on the increase of muscle GLUT4 protein concentration and decrease of insulin level in comparison with the MCT. Consistent with the present study, eight weeks of swimming and high-intensity swimming training increased the tissue GLUT4 protein and decreased insulin level in the type 2 diabetes rats, but high-intensity training had a more favorable effect on these indices such as an aerobic capacity (15). However, it seems that the effect of intensity of exercise on the dominant fuel for the type of exercise is a factor for not having a significant effect of increasing GLUT4 and decreasing blood glucose levels. In the present study, low intensity training also improved the lipid profile, decreased TG, and increased HDL. Also, in an eight-week of MICT with 50% VO2max and HIIT with 90% VO2max increased the protein level of the GLUT4 in the skeletal muscle of obese rats, so that both trainings had the same effect (16). According to the above studies, it can be concluded that the acute exercise with intensities above 55% VO2max leads to an increased glucose uptake from non-insulin-dependent pathways, increases membrane levels of glucose carriers, and also by increasing pathways to hypersensitivity, increases energy demands, rises intracellular Ca++ levels and activates phosphatidylinositol 3-kinase (PI3K). In addition, the intensity of a long-term training to select the dominant muscle fuel supply leads to the adaptation of pathways of a glucose uptake increase or lipolysis pathways (17).

In the present study, MCT decreased TG as well as MIIT decreased levels of TG, LDL and total cholesterol; also MIIT had more significant effects on the HDL level increase in comparison with the MCT. A study showed that the exercise increases diacylglycerol (DAG), and phosphoinositide 3-kinases (PI3K) (5). It has been reported that 6-8 months of endurance training and resistance training caused an increase in the levels of lipoprotein, insulin resistance index (18); therefore, the role of training duration in improving lipid and glucose metabolism. However, Mendham et al. (9) showed that 12 weeks of combined training along with resistance training improved the lipid profile and increased DAG in the skeletal muscle tissue, but had no significant effect on serum DAG levels in women with obesity. In addition, it has been shown that LDL and very-low-density lipoprotein (VLDL) levels decreased in overweight girls; however, cholesterol, C-reactive protein (CRP), and HDL levels did not change significantly after six weeks of HIIT (11). Given the consistency of the present study with Ross et al. (17), Sokolowska et al. (5) and Mendham et al. (9) researches, long-term training at moderate and high intensities seems to have a significant effect on lipid profile (5, 9, 18).

In the present study, MCT and MIIT decreased ceramide synthase-1 (CS1) levels and total ceramide levels in the MIIT group were lower than DC and MCT groups. Mandal et al. (1) have shown that increased ceramides are associated with cardiovascular disease and insulin resistance. Consistent with this study, a review study showed that same exercise reduces ceramides in the skeletal muscle (5). In the study of Kasumov et al. (19), it was suggested that 12 weeks of training with an intensity of 80-85% maximal heart rate, increases insulin sensitivity, and decreases plasma levels of C14:0, C16:0, C18:1 and C24:0 ceramide in obese women with type 2 diabetes. Also, 12 weeks of combined training along with weight resistance training, increased mitochondrial respiratory capacity and ceramide 18 (C18) levels, but it had no significant effect on the levels of total ceramide, insulin sensitivity and GLUT4 protein expression in obese women (9). Also, an acute training session for 90 minutes of exercise with an intensity of 50% VO2max increased serum levels of ceramide and glycosylceramide (20). It appears that a wide range of ceramides and their function are also affected by exercise activity. In addition, the mechanism of anti-inflammatory effect of high-intensity exercise, especially in disease conditions, is still not well known. Therefore, differences in the intensity and length of a training period can be the reasons for a discrepancy in the results.

In our study, the levels of pAkt, Akt/pAkt ratio, PKC in the MIIT group increased in comparison with the DC and MCT groups. Also, the amount of TNF-α inflammatory index in the MIIT group decreased in comparison with the DC and MCT groups. In line with the present study, it has been reported that following 16 hours of an acute training, no significant changes in the levels of blood insulin and glucose were observed, however seven days of training was associated with an increase in the concentrations of PI3K, pAkt, AS160 proteins and an improvement of guanosine triphosphatase (GTPase) function as an important protein in the transport of GLUT4 to the muscle cell membrane (21). Also, eight weeks of endurance training with different intensities reduced inflammatory factors such as IL-4, IL-6, TNF-α, IL-1β and declined oxidative stress in diabetic rats. Furthermore, a MCT and MIIT exercise had a more favorable effect on reducing inflammatory factors in diabetic rats (22). Given the differences in the intensity between the present study and Kim et al. (22) study, it appears that the intensity and frequency of training are important factors in modulating or altering the balance of oxidative stress and activating inflammatory factors in diabetic conditions. However, according to the results of Mendham et al. (9), O’Gorman et al. (21), Gerosa-Neto et al. (23) and Martinez-Huenchullan et al. (24), it seems that high intensity interval training has a significant effect on inflammatory factors than moderate-intensity continuous training.

In general, lipid metabolic disorders such as disorders of glucose and insulin metabolism are caused by cytokine changes that bring an increase the serum levels of LDL, cholesterol, VLDL, TG. This increase consequently rises inflammatory and pro-inflammatory factors such as TNF-α, IL-1β, CRP and additionally disrupts the pathway...
of AMP-activated protein kinase (AMPK), PI3K/Akt, that may result in an insulin sensitivity impairment and an insulin resistance increase in the skeletal muscle tissue (25, 26). In addition, disruption of the metabolism of sphingolipids, which contain ceramide nuclei, from the TLR-4 receptor-like pathway leads to an increase in inflammatory cytokines and ceramides. While, in the next step, ceramides reduce Akt/PKB ratio, decrease GLUT4 expression level, and increase insulin resistance by increasing PKCε levels. This ceramides level increase also coincides with an oxidative stress increase, which leads to increased levels of caspases, PKCε and inflammatory factors, that results in the cell apoptosis in pancreatic beta cells and impairs the insulin secretion procedure in these cells (1). In this regard, studies have shown that an increase in the ceramides levels is associated with the incidence of cardiovascular disease (1); also a ceramide/sphingolipid catalysis increase is also associated with the insulin resistance (18). A study found that although C16-ceramide in healthy individuals is directly related to the insulin signaling pathway improvement, C16-ceramide levels increase in individuals with metabolic disease and lead to the insulin resistance increase (27).

On the other hand, an exercise with the mechanism of improving lipid metabolism, increasing GLUT4 protein expression and increasing glucose uptake from non-insulin-dependent pathways during exercise, improves the lipid metabolism and mitochondrial biogenesis. Exercise also increases the Sphingomyelin Synthase 1 & 2 (SGMS1 and SGMS2) expression by increasing the levels of sphingomyelin synthase in the Golgi apparatus, and increases the production of the DAG in the endoplasmic reticulum and the Golgi apparatus to produce phospholipid in the cell. Therefore, the increase of some ceramides (not total ceramide) along with the increase of DAG is associated with an increase in the mitochondrial biogenesis (9). Studies have shown that exercise not only decreases the level of skeletal muscle ceramide but also increases insulin sensitivity, levels of DAG, PI3K, anabolic enzymes of the Akt/PKB pathway, PP2A inhibitor, and inflammatory factors, as well as protects skeletal muscle against cell damage and death (5). However, changes in glucose and lipid metabolism pathway in skeletal muscle following exercise can be depending on duration, intensity and type of it. For example, 12 weeks of combined training improves mitochondrial biogenesis and lipid profile reduces inflammatory markers and ceramide levels (9). However, an acute training session increased serum ceramide and glycosylceramide levels and did not significantly change the levels of sphingosine-1-phosphate and the sphingomyelin, but after 30 minutes of recovery, their levels decreased significantly (20).

For example, 12 weeks of combined training improves mitochondrial biogenesis and lipid profile and reduces inflammatory markers and ceramide levels (9). Acute training session increased serum ceramide and glycosylceramide levels (20).

It seems that long-term training is preferred over short-term training, but adaptation to long-term training depends on its intensity and involvement of lipid-dependent metabolic pathways and lipid catabolism threshold in ceramide changes. For example, 12 weeks of training increase insulin sensitivity and decreases ceramide plasma levels, it was a useful plan to manage obese women with type 2 diabetic inflammatory response. The intensity of the MIIT protocol in our research and Kasumov et al. (19) study was similar. Here, we suggest that performing high-intensity interval training, especially during rest, maybe more helpful than low-intensity training.

Therefore, a possible advantage of this study is that MIIT compared to MICT has a superior effect by affecting ceramide metabolism. Considering the role of sphingolipids, sphingomyelin, TLR-4, sphingomyelin synthase, and DAG in the mechanism of ceramide-dependent insulin resistance, it seems that the lack of measurement of these variables is one of the limitations of our present study. Hence, these variables evaluations are suggested for future research.

In summary, future studies should examine the intensity and duration of exercise in type 1 and type 2 diabetes in animal models and humans.

**Conclusion**

According to the results of the present study on improving the metabolism of glucose, lipids and improving inflammatory factors in the muscle tissue following two types of MICT and MIIT exercises. It seems that both of the mentioned training methods in the condition of diabetes with the improvement of energy substrates decrease of the muscle tissue inflammatory factors. However, the effect of MIIT on ceramides is more optimal than MICT.

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**Authors’ Contributions**

P.R., D.Sh.-V., M.R.M.; Investigation and Writing. D.Sh.-V.; Project administration, Supervision, Writing, and Editing. S.Gh.; Conceptualization, Methodology, and Editing. M.R.M.; Resources, Data Curation, Formal analysis, and Validation. P.R.; Visualization and Software. All authors read and approved the final manuscript.

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ERMP1 Facilitates The Malignant Characteristics of Colorectal Cancer Cells through Modulating PI3K/AKT/β-Catenin Pathway and Localization of GRP78

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Abstract

Objective: Endoplasmic reticulum-metallopeptidase 1 (ERMP1) is involved in cellular response to oxidative stress. However, its functional role in proliferation and progression of cancer cells remains unknown. The focus of this study was to investigate the molecular-mechanisms in which ERMP1 modulates the proliferation and progression of colorectal cancer (CRC) cells under normal and environment stress conditions.

Materials and Methods: In this experimental study, ERMP1 expression was evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in CRC cells. ERMP1 was knocked down using lentiviral transduction of ERMP1-specific shRNA into HCT116 cells. ERMP1 was also upregulated using lipofectamine transfection of ERMP1-overexpressing vector into SW48 cells. To evaluate the role of ERMP1 in the cellular and environmental stress conditions, ERMP1-downregulated cells were exposed to stressful conditions including starvation, serum free medium, and treatment with redox or chemotherapy agents for 72 hours. The expression of AKT, p-AKT, phospho-mammalian target of rapamycin (p-mTOR), β-catenin, p-β-catenin, E-cadherin, and Glucose-regulating protein 78 (GRP78) proteins was evaluated by western blotting. The expression of ERMP1, CYCLIN D, and c-MYC was evaluated by RT-qPCR. The cell surface localization of GRP78, cell cycle distribution, and apoptosis were determined by Flow cytometry.

Results: ERMP1 knock-down reduced the cellular proliferation, inactivated the PI3K/AKT pathway, prompted the

Conclusion: ERMP1 functioned as an oncogene in CRC cells by promoting malignant characteristics. The phosphoinositide 3-kinases (PI3K)/AKT/β-catenin pathway and localization of GRP78 were closely related to the effects of ERMP1. Consequently, ERMP1 might be regarded as a promising target in therapeutic strategies related to CRC.

Keywords: β-catenin, Colorectal Cancer, ERMP1, GRP78, PI3K/AKT

Introduction

Endoplasmic reticulum-metallopeptidase 1 (ERMP1) is a member of the M28 Zn-peptidase family with nine transmembrane-domains typically incorporated in the endoplasmic reticulum (ER) (1). ERMP1 gene is located at chromosome 9p24 and encodes an approximately 100 kDa protein. It was first recognized as a new, hypothetical gene named Felix-in-a, expressed in the granulosa cells of ovarian follicles in rats. Its role in follicular organization was attributed to its proteolytic function on precursor proteins which is essential for intra-ovarian cell to cell communication (2). Primarily, ERMP1 was proposed as a candidate oncogene due to its localization on amplified chromosome in cancers (3). In 2016, it was identified as a chief player in the unfolded protein responses (UPR) and defense against oxidative stress in cancer cells. This identification was based on the findings, showing that ERMP1 expression is extremely affected by thapsigargin-induced ER stress and other oxidative stresses. The overexpression of ERMP1 is verified in multiple cancers including breast, ovary, colon and lung; meanwhile the maximum expression level is attributed to CRC (1).

Colorectal cancer (CRC) is regarded as one of the most common causes of morbidity and mortality from cancer and public health problem worldwide. Although CRC can occur at any age, it is mostly categorized as an old-age disease with the most occurrence in the fifth decade of human life. Unfortunately, it is highly metastatic and resistant to anticancer therapies (4). Despite progression in cancer therapy, survival is less than 5 years in most of the patients, which necessitates...
the exploration of molecular mechanisms and cancer-specific-cellular targets in CRC for novel therapeutic strategies (5). ERMP1 is suggested as a new target for anti-cancer therapies because of its effect on GRP78. Accordingly, ERMP1 mediates the ER stress-promoted activation of GRP78 and loss of ERMP1 suppresses the GRP78 expression (1). GRP78 was initially recognized by glucose deprivation led to the overexpression of this 78 kDa protein in chick embryo fibroblasts; therefore, it is called glucose-regulated protein 78 (GRP78) (6). It was routinely regarded as an ER luminal protein because of a retention KDEL motif in carboxyl domain supposed to exert its anti-apoptotic effects in cancer just by regulating the activation of UPR. Later, a sub-fraction of this protein was detected at the cell surface of cancer cells as a multifunctional receptor that mediates the signaling pathways, a finding that unwrapped novel mechanisms wherein GRP78 exerts its anti-apoptotic and pro-proliferative effects in cancer cells. GRP78 upregulation is attributed to various stressful parameters such as nutrient deprivation, hypoxia, low pH condition, chemotherapies and, radiation (7).

In 2018, miR-148b was defined as a tumor suppressor because of its negative impact on ERMP1 expression in human endometrial cancer. Following the ERMP1 suppression, the proliferation of endometrial cells was suppressed (8). Recently, the correlation between ERMP1 and phosphatidylinositol-3-kinase (PI3K)/AKT pathway was distinguished. This association was ascribed to miR-328-3p suppressing the PI3K/AKT pathway via targeting ERMP1 in hepatocellular carcinoma and reduces the cancer cells proliferation and invasion (9).

The PI3K/AKT signaling pathway is implicated in the cellular proliferation and survival at both physiological and pathological conditions. Because cancer cells are under cellular and environment stresses, the PI3K/AKT pathway plays a critical role in cancer progression. Multiple targets of AKT included in glucose metabolism, protein synthesis, and cell cycle control comprise glycogen synthase kinase-3β (GSK-3β), insulin receptor substrate-1 (IRS-1), mammalian target of rapamycin (mTOR), cyclin-dependent kinase (CDK) inhibitors like p21 and p27, and so on. As to β-catenin, AKT/ GSK-3β/β-catenin transduction pathway activates an axis that positively mediates the cell cycle progression from G1 to S phase via inactivating GSK-3ββ, upregulating CYCLIN D, and suppressing some transcription factors which ultimately cause p27 depletion (10).

AKT is overactivated in most human cancers that causes the uncontrollable cell growth, metastasis, and angiogenesis of cancer cells by activating the PI3K/ AKT/mTOR pathway (11). The overactivation of proliferative pathways in cancer cells and application of chemotherapeutic agents induce stressful condition in tumor microenvironment (12) known as cellular and environment stresses and considered as a barrier for effective treatments (13).

Considering the important role of the AKT/GSK-3β/β-catenin pathways and GRP78 in cancers, we made an attempt to determine the functional role of ERMP1 in proliferation and progression of CRC cells and related pathways under normal or environment stress conditions.

Materials and Methods

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1400.279).

Materials

In this experimental study, RPMI-1640, fetal bovine serum (FBS), and DMEM (Dulbecco’s Modified Eagle Medium) were acquired from Pan biotech (Germany). The pLKO.1, pCMV6-AN-GFP, and pCDNA3.1 (+)-ERMP1 vectors were prepared from Addgene (USA), OriGene (USA), and Biomatik (Canada) companies, respectively. Lipofectamine 2000 were obtained from Thermo Fisher (USA). The ERMP1 and scramble oligo sequences were purchased from metabion (Germany). Super ECL reagent, and BCA assay kit were purchased from Abcam (UK), and Thermo Scientific (USA), respectively. Annexin-V apoptosis detection kit, puromycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were obtained from MabTag (Germany), Santa Cruz (USA), and Sigma-Aldrich (USA), respectively. Antibodies for AKT (Cat. #4691), p-AKT, S473 (Cat. #9271), and p-β-Catenin (Cat. #9554) were purchased from cell signaling (USA). Also, antibodies for GAPDH (Cat. #sc-47724), E-Cadherin (Cat. #sc-71009), and β-Catenin (Cat. #sc-7963) were obtained from Santa Cruz Biotechnology (USA). p-mTOR S2448 (Cat. #610301) antibody from BioLegend (USA), anti-rabbit IgG, and anti-mouse IgG peroxidase-conjugated secondary antibodies were acquired from Sigma-Aldrich (USA), and Alexa-conjugated secondary antibody was also purchased from Razi BioTech (IRAN). The designed shRNA against ERMP1 and scramble shRNA were prepared through Metabion Company (Germany).

Cell culture

Seven human CRC cell lines including HT29, HCT116, LS180, SW1116, SW742, SW480, SW48, and HEK293T cell line, as a human-embryonic-kidney cell line, were obtained from National cell bank of Iran for this experimental study. All cells were cultured in RPMI-1640 medium except LS180 and HEK293T cell lines cultured in DMEM, containing 10% FBS and 5% CO2 at 37˚C humidified incubator. Whenever the cells reached 70-80% confluency, they were trypsinized and seeded in an appropriate plate for each experiment. Each experiment was performed 3 times independently, and at least the mean of 3 independent assays was represented.

RNA extraction and reverse transcription-quantitative polymerase chain reaction

Total RNA was elicted from the cells using the RNA extraction Kit (SinaClon, Iran), according to the manufacturer’s guidelines. Subsequently, cDNA was
synthetized employing cDNA synthesis kit (SinaClon, Iran), according to the kit procedure. The mRNA expression analysis was performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) on QuanStudio™ 5 Real-Time PCR system. First, the mRNA expression level of ERMP1 was determined in 7 CRC cell lines (SW48, SW480, SW742, SW1116, LS180, HT29, and HCT116). Then, the cell line with the highest (HCT116) and lowest (SW48) expression of ERMP1 were selected for ERMP1 knock-down and overexpression, respectively. The primers that were used to detect each gene product are listed in Table S1 (See supplementary Online Information at www.celljournal). GAPDH was employed as a control gene to normalize all expression data. The relative expression levels (fold changes) were quantified by utilizing the 2^{-ΔΔCT} method.

Transduction with shRNA-encoding lentiviruses

Lentiviral-based transduction of short hairpin (sh) RNA was used in stable knock-down of the ERMP1 gene. Initially, ERMP1-specific shRNA oligos (shERMP1) or shRNA against scramble sequence (shscramble) (negative control) were cloned into pLKO.1 vector at Agel and EcoRI restriction sites and, then, amplified by transformation to the competent DH5α E.coli. Lentiviral particles expressing shERMP1 or shscramble were produced in 6-well plate containing HEK293T cells by co-transfecting 2 µg pLKO.1 plasmid (pLKO.1-shERMP1, or PLKO.1-shscramble) plus 0.5 µg enveloping-plasmid (pMD2.G) and 1.5 µg packaging-plasmid (pSPAX2) (which encode the viral capsid and transcriptional machinery).

HCT116 cells were seeded in the 6-well plate (1.0×10^5 cells/ well). After 24 hours, lentiviruses expressing shERMP1 or shscramble (negative control) were added with 8 µg/ml polybrene. Following transfection, the medium was replaced with fresh and complete medium. Then, puromycin selection with optimal dose (1 µg/ml) was carried out 72 hours post-transduction for two weeks. Finally, the efficacy of ERMP1 knock-down was assessed by RT-qPCR.

The upregulation of ERMP1 gene using overexpression vector

SW48 cells were seeded in 24-well plates to reach 70-80% confluency on the day of transfection. After replacing the medium, transfection mixture was added to the cells (2.5 µl of Lipofectamine-2000 for each µg of pCDNA3.1 (+)ERMP1, pCDNA3.1 or pCMV6-AN-GFP) and incubated for 4 hours at 37°C with 5% CO₂. To reduce cytotoxicity, we replaced the transfection medium with fresh one, and the cells were incubated for 48 hours. The efficiency of transfection was assessed by fluorescence microscopy using the pCMV6-AN-GFP vector as a control. Finally, the cells were collected for next steps.

MTT proliferation assay

Cells were plated in 96-well in a medium containing 10% FBS with about 5,000 and 7,000 cell/well for HCT116 and SW48, respectively. Seventy two hours after treatment or 48 hours post-transfection, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 hours at 37°C to quantify the cell viability. After 4 hours, the medium was removed and the resulting MTT formazans solubilized in 100 µl of dimethyl sulfoxide (DMSO) to check each solution spectrophotometrically at 570 nm.

Cell treatment

The ERMP1 stably knocked down HCT116 cell line or HCT116 scramble cell line were seeded in appropriate plates in 5 or 6 groups. The next day, the medium was changed in all groups. In the first group, the cells were exposed to the normal condition (medium was changed whenever it was necessary). The second group was exposed to the starvation condition (medium was not changed for 72 hours to induce starvation). The remaining groups were also exposed to the serum free medium (0.25% FBS) and medium containing Dithiothreitol [(DTT) 10 µM], 5-Fluorouracil [(5-FU) 8 µM], and cisplatin (6 µM) for 72 hours, respectively.

Colony forming assay

The cells (200 cell/well) were seeded in 6-well plate and cultured for 2-3 weeks at 37°C in a 5% CO₂ incubator. The medium was replaced every 7 days. After the colonies became visible (50 cell/colony), 95% methanol was used to fix them. Then, the colonies were stained with crystal violet followed by PBS washing to be viewed and counted by microscope (×40 magnification). Afterwards, 10% acetic acid was used to dissolve the crystal violet for quantification of the stained colonies. The solutions were added to a 96-well plate to determine the absorbance at 590 nm using the spectrophotometer (14).

Immunoblotting analysis

Western Blot assay was performed for protein analysis. Briefly, the cells were harvested and lysed to be centrifuged for 8 minutes with 10.000 g at 4°C. The supernatant was ready to be used for loading on dodecyl sulfate (SDS)-polyacrylamide gel. Therefore, proteins were separated based on their molecular weight by electrophoresis in an SDS-polyacrylamide gel under denaturing conditions; then, they transferred to a nitrocellulose membrane using the wet transfer apparatus at 100 V or 400 mA. After blockage of nitrocellulose membrane with PBS-T + 5% skim milk at RT, proteins were ready for incubation with primary antibodies (anti-AKT, p-AKT, GAPDH, E-Cadherin, β-catenin, p-β-catenin and p-mTOR) for overnight with agitation. The membrane was then incubated with relative secondary antibody at RT. Finally, proteins were identified through a chemiluminescence imaging system (Bio-Rad Chemidoc XRS, USA).

Evaluation of the cell cycle by Flow Cytometry

The respective cells were cultured in 6-well plates, and
after being detached by 0.25% Trypsin-EDTA solution on the day of experiment, the cells centrifuged at 1500 g for 5 minutes at 4°C to be washed in PBS and re-suspended in a hypotonic propidium iodide (PI) lysis buffer (0.1% Triton X-100, 1% sodium citrate, 0.5 μg/ml RNase A, 40 analyzed by flow cytometry (BD FACS Calibrator, Biosiences Company, USA).

**Evaluation of the apoptosis by Flow Cytometry**

Cells were cultured in 6-well plates and on the day of experiment, they were detached, washed with PBS, and re-suspended in 500 μl of annexin V binding buffer. Then, 5 μl of annexin V conjugate and 5 μl of PI solution were added to the samples to be incubated in the dark for 20 minutes. After centrifuging at 400 g for 5 minutes and re-suspending in 200 μl annexin V binding buffer, we analyzed each sample by flow cytometry.

**Detection of the cell surface GRP78**

The cells were harvested and re-suspended in ice cold PBS. Primary antibody (10 μg/ml) was added to 100 μl of the cell suspension and incubated for at least 30 minutes at 4°C. Then, the cells were washed 3 times with PBS and centrifuged for 5 minutes at 400 g. Alexa-conjugated secondary antibody dilution was added, and the cells were re-suspended in this solution to be incubated in the dark for 20-30 minutes at 4°C. After being washed 3 times with PBS and centrifugation, the cells were ready to be analyzed with flow cytometry.

**Statistical analysis**

All data are reported as means ± SD from at least 3 independent assays. Statistical data analysis was conducted by using the GraphPad Prism 7.0 for Windows (GraphPad Software, Inc, USA). T test and the 1- or 2-way ANOVA tests were used for comparison of two and multiple groups, respectively. * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001 were considered as statistically significant.

**Results**

**ERMP1 is variably expressed in colorectal cancer cells**

The total RNA was elicited from 7 CRC cell lines to assess the expression level of ERMP1 by RT- qPCR (Fig.1A). We found a relatively high expression level of ERMP1 in HCT116 cells (P<0.0001) and a low expression in SW48 cells. Accordingly, to explore the biological function of ERMP1 in CRC, we selected HCT116 and SW48 cell lines to down- and up-regulate ERMP1, respectively (Fig.1B, C).

Transduction of cells through lentiviral-based shRNA approach provided a quick and stable method to successfully diminish the ERMP1 expression (Fig.1B). To overexpress ERMP1 gene, we designed and ordered pCDNA3.1 (+)- ERMP1 vector. Later, pCDNA3.1 (+)-ERMP1 vector was transformed into the E.coli and amplified. Then, it was extracted and confirmed via digestion with Pmel restriction enzyme. SW48 cells were transiently transfected with pCDNA3.1 (+)-ERMP1 and empty vector(pCDNA3.1). The transfection efficiency was checked 48 hours later as illustrated in Figure 1C.

**ERMP1 mediates the proliferation of colorectal cancer cells**

ERMP1 has a remarkable expression in various cancer types including CRC cancer prompted us to verify the cancer-related function of ERMP1. As shown in Figure 2A, MTT assay was conducted to evaluate the proliferation of ERMP1 shRNA-mediated knock-down HCT116 cells (-ERMP1 cells) compared to HCT116 control cells (transduced with scramble shRNA). The results of the proliferation assay in the SW48 cells transfected with pCDNA3.1 (+)-ERMP1 vector (+ERMP1 cells) compared to the SW48 control cells (transfected with empty vector) are also shown in Figure 2B. The current results indicated that ERMP1 overexpression promoted the proliferation rate in SW48 representing the probable role of the ERMP1 in proliferation pathways of CRC cells.

As shown in Figure 2C, down-regulation of ERMP1 along with the DTT treatment, obviously (P=0.009) reduced the proliferation rate in -ERMP1 cells compared to the scramble control. Interestingly, 5-FU and cisplatin treatment elevated the proliferation rate in -ERMP1 cells compared to the scramble ones (P=0.03).

Additionally, as shown in Figure 2D, the colony formation assay was performed in the -ERMP1 cells to further determine the ERMP1 effects in CRC cells.

**ERMP1 facilitates the AKT expression and subsequently promotes the PI3K/AKT pathway in colorectal cancer cells**

The PI3K/AKT pathway, as a crucial intracellular signal transduction pathway, contributes to survival, proliferation, and invasion of cancer cells in response to extracellular stimuli via mediating phosphorylation cascade. Therefore, the PI3K/AKT pathway was evaluated in -ERMP1 and +ERMP1 CRC cells. As illustrated in Figure S1A (See supplementary Online Information at www.celljournal) and Figure 3A, ERMP1 knock-down in HCT116 cells considerably reduced the expression of AKT at both mRNA (P=0.007) and protein (P<0.0001) levels compared to scramble ones under normal condition. Moreover, the p-AKT protein level was also checked to distinguish; whether ERMP1 down-regulation affected the phosphorylation of AKT. Related results showed that the p-AKT level in -ERMP1 cells was decreased (P=0.0005).
ERMP1 Accelerates The Proliferation of CRC Cells

**Fig. 1:** ERMP1 relative expressions are normalized to GAPDH and determined by RT-qPCR. 

A. ERMP1 expression level in 7 CRC cell lines (SW48, SW480, SW742, SW1116, LS180, HT29 and HCT116), SW48 is applied as a reference set to 1.0, which exhibited the lowest mRNA expression level. 

B. The RT-qPCR results of the ERMP1 gene in puromycin-resistant colonies of HCT116 transduced cells with ERMP1 and scramble shRNAs. The ERMP1 expression was significantly inhibited in HCT116 cells following transduction with lentivirus expressing ERMP1 shRNA, compared with HCT116 cells transduced with scramble lentivirus (P=0.0002). 

C. Transfection efficiency in SW48 cells is determined using pCMV6-AN-GFP vector as a control and RT-qPCR, which showed that pCDNA3.1 (+)-ERMP1 vector transfection obviously enhanced ERMP1 expression in SW48 cells (P=0.0095) (scale bar: 200 µm). Bars represent the mean ± SD. **; P<0.01, ***; P<0.001, ****; P<0.0001 vs. scramble control or empty vector. One-way ANOVA A. Multiple comparison and B, C. Unpaired t tests were utilized. RT-qPCR; Reverse transcription-quantitative polymerase chain reaction and CRC; Colorectal cancer.

**Fig. 2:** The MTT and colony formation assays for proliferation analysis. 

A. Knock-down of ERMP1 non-significantly reduced the proliferation rate in HCT116 cells compared with scramble ones. 

B. Overexpression of ERMP1 significantly increased the proliferation rate in SW48 cells compared to the control empty vector ones (P<0.02). 

C. Exposure to various stressful conditions as a model of environmental stress, such as Dithiothreitol (DTT), 5-FU and cisplatin, differently affected the proliferation rate of ERMP1 cells. 

D. Colony formation ability was reduced considerably by ERMP1 knock-down (P<0.03) and DTT treatment (P<0.0001). Data are reported as means ± SD. *; P<0.05, **; P<0.01, and ****; P<0.0001 compared with each scramble control or empty vector. A, B. Unpaired t test and C, D. two-way ANOVA multiple comparison tests were used.
In SW48 cells, it was demonstrated that ERMP1 overexpression led to an obvious increase in AKT expression at both mRNA (P=0.002) and protein (P=0.0002) level, compared to empty vector (Fig.S1B, See supplementary Online Information at www.celljournal, Fig.3B). In addition, p-AKT was also enhanced by ERMP1 overexpression (P=0.004). These results demonstrated that ERMP1 regulated the PI3K/AKT pathway by affecting the expression and phosphorylation of AKT in CRC cells.

Interestingly, a reduction in AKT expression was also observed in -ERMP1 cells, which were exposed to serum free, and DTT treatment circumstances compared to their control scramble cells (Fig.S1C, D, See supplementary Online Information at www.celljournal, Fig.3C, D). Since the results of starvation with serum free conditions were in the same line, we continued our experiments with one of them (serum free).

The p-AKT protein level was also checked, and the results were different in each group. As displayed in Figure 3C, D, p-AKT was reduced in DTT-exposed -ERMP1 cells (P=0.03). These data illustrate that ERMP1 knock-down and DTT exert synergistic effects on AKT and p-AKT expression in CRC cells.

The AKT mRNA expression was measured in 5-FU and cisplatin exposed -ERMP cells, which showed an obvious increase in AKT expression at both mRNA (P=0.004) and protein (P<0.0001) levels compared to scramble treated cells for 5-FU-treated cells (Fig.S1E, See supplementary Online Information at www.celljournal, and Fig.3E) and at mRNA level for cisplatin-treated cells (P=0.003) (Fig.S1D, See supplementary Online Information at www.celljournal). Consequently, our results showed that ERMP1 promoted the expression of AKT and its phosphorylation, however this effect can alter subject to different circumstances in tumor microenvironment.

Additionally, we assessed the p-mTOR expression to determine whether the accelerating effects of ERMP1 on PI3K/AKT pathway can influence p-mTOR expression as downstream target of AKT.

As displayed in Figure 3A, the p-mTOR protein expression level was reduced remarkably by ERMP1 knock-down compared with the scramble control (P=0.002); however, by serum free and DTT exposure, p-mTOR expression did not modify in -ERMP1 cells (Fig.3C, D).

In SW48 cells, overexpression of ERMP1 obviously increased (P=0.01) the p-mTOR expression compared with empty vector control (Fig.3B). These results illustrate that ERMP1 facilitates the expression of p-mTOR. Moreover, in the field of p-mTOR, DTT did not exhibit synergistic effect with ERMP1 knock-down; conversely, DTT inhibited the effects of ERMP1 knock-down.

Fig.3: Evaluation of the ERMP1 effect on multiple proteins using Western blotting experiment. A. ERMP1 knock-down effects on PI3K/AKT/β-catenin pathway proteins as well as E-cadherin and GRP78. B. ERMP1 overexpression effects on PI3K/AKT/β-catenin pathway proteins. C. The impact of serum free stress on PI3K/AKT/β-catenin pathway proteins as well as E-cadherin and GRP78 in -ERMP1 cells. D. The effect of Dithiothreitol (DTT) exposure on PI3K/AKT/β-catenin pathway proteins as well as E-cadherin and GRP78 in -ERMP1 cells. E. AKT and GRP78 protein levels in 5-FU treated -ERMP1 cells. All details are explained in results. Data are presented as the mean ± SD. *; P<0.05, **; P<0.01, ***; P<0.001, and ****; P<0.0001 vs. each scramble control or empty vector, and unpaired t test is applied.
ERMP1 induces the stability of β-catenin by facilitating AKT expression in colorectal cancer cells

Since phosphorylated AKT has the potency to regulate the function of multiple substrates involved in cellular survival such as GSK-3β becoming inactivated by p-AKT to trigger the stabilization of β-catenin for nucleus translocation, free β-catenin was measured in this study by western blotting to further investigate the ERMP1 effects on CRC cell lines. The results revealed that compared with the scramble control, the p-β-catenin/β-catenin relative ratio was significantly increased (P<0.0001) in -ERMP1 cells (Fig.3A) indicating a reduction in free β-catenin level by downregulation of ERMP1 in normal condition. In ERMP1 overexpressed cells, the p-β-catenin/β-catenin relative ratio was reduced (P=0.0008) in +ERMP1 cells (Fig.3B). Therefore, it appears that ERMP1 accounts for AKT-mediated GSK-3β/β-catenin pathway through stabilizing free β-catenin.

Thereafter, we examined the p-β-catenin/β-catenin relative ratio in -ERMP1 cells under serum free, and DTT treatment and a significant enhancement (P=0.008) was found in DTT-exposed cells (Fig.3C, D).

Stabilized β-catenin can translocate to the nucleus and incorporate with lymphoid enhancer factor/T cell factor (Lef/Tcf) co-transcription factors to trigger transcription of target genes such as C-MYC and the CYCLIN D, which compromise in survival, proliferation and invasion of cancer cells. Hence, we evaluated C-MYC and CYCLIN D expression at mRNA level to verify the possible role of ERMP1 in regulation of the transcriptional activity of β-catenin. However, the C-MYC expression did not show any change in -ERMP1 cells with an exception for DTT-exposed cells, in which C-MYC expression was reduced (P=0.0004) compared to the related control (Fig.S1A-D, See supplementary Online Information at www.celljournal). In addition, we assessed the C-MYC expression in 5-FU and cisplatin-treated -ERMP1 cells show an enhancement in the proliferation rate compared with the related controls. Relative results indicated that C-MYC expression was upregulated by 5-FU (P<0.0001) and cisplatin (P=0.002) treatment in -ERMP1 cells compared to scramble cells (Fig.S1E, F, See supplementary Online Information at www.celljournal).

As revealed in Figure S1A, B (See supplementary Online Information at www.celljournal) a lower expression level of CYCLIN D (P=0.002) was detected in -ERMP1 cells; however, in SW48 cells, CYCLIN D expression exhibited an obvious upregulation by ERMP1 overexpression. It was revealed that CYCLIN D expression was affected by downstream impact of ERMP1 on free β-catenin, unlike C-MYC. Because the outcomes in -ERMP1 an +ERMP1 cells were opposite, the next procedures were just carried out with -ERMP1 cells to overcome the limitations.

ERMP1 knock-down under serum free and DTT conditions enhances the E-cadherin expression in HCT116 cells

Phosphorylated AKT is implicated in downregulation of E-cadherin expression at mRNA level; thus, the E-cadherin expression was evaluated with western blotting to examine whether E-cadherin contributes to ERMP1 mediated β-catenin stabilization. As displayed in Figure 3A, E-cadherin protein expression was not changed through ERMP1 downregulation in HCT116 cells. To further verify the ERMP1 correlation with E-cadherin, we also measured the expression of E-cadherin in -ERMP1 cells which were exposed to serum free and DTT (Fig.3C, D). The results revealed that serum free condition (P=0.0001) or DTT treatment (P=0.001), upregulated the E-cadherin expression in -ERMP1 cells compared to their scramble controls. These data may reflect the possibility that ERMP1 knock-down is not the solitary agent in downregulation of E-cadherin expression and ERMP1 silencing under stressful condition such as serum free and DTT treatment that can compact the membrane-bounded E-cadherin/β-catenin complex and inhibit a relocation of β-catenin into the nucleus, where β-catenin activates the transcription of the genes associated with enhancing cellular proliferation and progression in CRC cells.

ERMP1 knock-down reduces the GRP78 localization on the HCT116 cell surface

It is believed that ERMP1 has a role in upstream or within UPR and GRP78 overexpression. Therefore, we measured the GRP78 protein level in -ERMP1 cells. Based on our results, the expression of GRP78 at protein level was not changed by ERMP1 downregulation (Fig.3A). However, DTT treatment (P=0.04) and 5-FU exposition (P=0.01) led to the GRP78 overexpression in -ERMP1 cells compared to each scramble controls (Fig.3D, E).

Because the biological effect of ERMP1 on GRP78 and UPRs pathways was not exactly determined, we aimed to elucidate the possible role of ERMP1 in localization of GRP78. The result showed that ERMP1 knock-down diminished (P=0.002) the localization of GRP78 at the cell surface (Fig.4A). GRP78 aggregates at the cell surface, initiates the multiple signals and, thereby, regulates the cell malignancy. Subsequently, to investigate; whether enhancement in the GRP78 protein level under DTT and 5-FU treatment increases the localization of GRP78 at the cell surface, we assessed csGRP78 under stressful circumstances in -ERMP1 cells. As revealed in Figure 4B, exposure to DTT (P=0.002) and 5-FU (P=0.003), not only substantially they did not enhance csGRP78, but also reduced its cell surface content compared with normal condition, which may suggest GRP78 has been trapped in ER lumen to alleviate ER stress. However, cisplatin treatment did not change csGRP78 compared with -ERMP1 cells in normal condition.

Knock-down of ERMP1 promotes apoptosis with Dithiothreitol treatment

MTT results showed that ERMP1 overexpression induced CRC cells proliferation, and its knock-down reduced the proliferation. In addition, we exposed -ERMP1 cells under stressful and DTT, 5-FU, and cisplatin conditions to identify the role of ERMP1...
on proliferation under these circumstances. We found that, in response to tumor microenvironment, ERMP1 differently manipulated the cellular proliferation, suggesting that ERMP1 can differentially act in tumor microenvironment (its function in some conditions may be compensated with other proteins). Meanwhile, we examined cellular apoptosis assay to evaluate the effect of ERMP1 downregulation on apoptosis of -ERMP1 cells in normal condition and presence of environment stresses. The Figure 5 displays live, early/late apoptotic and necrotic cells in the quadrants of flow cytometry (left part) and the comparison of total apoptotic and live cells in the curves (right part). According to the results, there was not any difference in apoptosis of -ERMP1 cells with its scramble control cells (Fig.5A). In the presence of stressful circumstances, just DTT exposure (P=0.04) triggered apoptosis in -ERMP1 cells, suggesting that ERMP1 is not the only factor in promotion of apoptosis in CRC cells. These results show that, in the absence of ERMP1, exposure to DTT induce apoptosis in HCT116 cells.

Analysis of cell apoptosis result of 5-FU treated -ERMP1 cells, revealed a reduction (P=0.001) in the percentage of apoptotic cells compared with that of scramble control, which suggests that ERMP1 downregulation alleviates the desired effects of 5-FU in CRC cells.

ERMP1 knock-down manipulates the cell cycle by promoting the G1 arrest

As cellular apoptotic results revealed that reduction in the proliferation rate of -ERMP1 cells was not the consequence of apoptosis, we assumed that ERMP1 exerts its effects on cell cycle progression. Thus, -ERMP1 cells were evaluated by flow cytometry to verify the cells distribution in various phases of the cell cycle. As illustrated in Figure 6A, -ERMP1 cells showed an obvious reduction in S phase (P<0.0001) of cell distribution as well as G2 (P=0.002) and a remarkable increase in G1 distribution (P=0.001) was also identified in starved -ERMP1 cells with lower/miner intense (Fig.6B). When -ERMP1 cells were under serum free circumstance, cells distribution in S phase decreased (P=0.004), which in turn accumulated the cells in G1 (P=0.0007) (Fig. 6C). Following DTT treatment for 72 hours, S phase of cell cycle reduced (0.008) which led to a drop in G2 (P=0.004), and then blocked the cells in the G1 phase (P=0.0001) compared with its scramble control (Fig.6D). These results suggested that ERMP1 downregulation induced cell cycle arrest in G1. Accumulation of cells in G1 phase could probably be correlated to CYCLIN D expression downregulated with ERMP1 knock-down, and all of these modifications ultimately; led to a decline in cellular proliferation.

Fig.4: Effect of ERMP1 knock-down on cell surface localization of GRP78 using Flowcytometry assay. A. Knock-down of ERMP1 significantly reduced the transportation of GRP78 on the cell surface [P<0.01]. B. Following exposure to starvation, Dithiothreitol (DTT) and 5-FU, csGRP78 was decreased in -ERMP1 cells. Values are stated as the mean ± SD. **; P<0.01, and ****; P<0.0001 vs. each scramble control. A. Unpaired t test and B. One-way ANOVA multiple comparison tests were employed.
In order to explore the cause of enhancement in the proliferation rate of 5-FU and cisplatin-treated -ERMP1 cells, we measured modifications in the cell cycle of these -ERMP1-treated cells. The results revealed that exposure to 5-FU and cisplatin for 72 hours elevated S phase distribution by reducing the cell numbers in G1 following 5-FU treatment (P<0.0001), compared with scramble control (Fig.6E, F).

In SW48 cells, overexpression of ERMP1 noticeably reduced the cell number in the G1 phase (P=0.0013). Therefore, it seems that ERMP1 contributes to cell cycle progression.

Taken together, these data demonstrate that -ERMP1-mediated proliferation suppression is due to cell cycle intervention.

**Discussion**

Knock-down and overexpression of ERMP1 in CRC cells revealed that ERMP1 could possess a role in proliferation of CRC cells under normal, stressful, and therapeutic condition detectable in tumor microenvironment because of high proliferation rate of cancer cells and therapy goals. In the same line with our study, a considerable loss (2-fold reduction) in cellular proliferation of MCF7 and SK-BR-3 cells has been observed subsequent to transfection with ERMP1 siRNAs (1). This loss in proliferation of
breast cancer cells has been attributed to the ERMP1 function in UPR and oxidative stress. In another study, Qu et al. (8) indicated that miR-148b repressed the cellular proliferation and oxidative stress responses in human endometrial cancer RL95-2 cells by targeting ERMP1. In a recent observation, it has also been found that miR-328-3p suppresses the proliferation and colony formation rate of Huh-7 hepatocellular carcinoma cells through binding to the 3’-UTR end of the ERMP1 gene (9).

Cancer cells in tumor microenvironment are exposed to stressful conditions due to intrinsic (high proliferation rate and nutrient deficiency) (15) or external (tumor microenvironment) factors (16). Accordingly, ERMP1 differently regulated the cellular proliferation in response to stressful conditions and chemotherapeutic agents, suggesting that ERMP1 can possess dual effects in response to tumor microenvironment.

DTT, as a redox agent, exacerbated the ERMP1 knock-down effects, suggesting DTT as an efficient therapy in ERMP1 knock-down CRC cells. Our findings are in agreement with studies reported DTT as an inducer of cell death in multiple cancer cells (17-19).

It was also found that the effect of ERMP1 knock-down in HCT116 cells became reversed with 5-FU/cisplatin treatment, which suggests that more attention should be paid in deciding which drug or therapeutic manipulation is likely to function in ERMP1 knock-down CRCs. Besides, knock-down of ERMP1 can be considered as a stress accelerating resistance to anti-cancer drugs, as it has been previously been declared that tumor microenvironment stress alleviates drug efficiency by modulating molecular- and biochemical-mechanisms (13). However, more studies are needed to explore the underlying mechanisms. In agreement with our outcomes, it has been found that applying drug treatment, with the aim of enhancing tumor microenvironment stress, increases the intratumor heterogeneity and contributes to chemotherapy resistance (20). Additionally, the consequence of exposition to harsh tumor microenvironment is proposed to develop invasive and aggressive features of cancer cells in vitro (21).

The molecular mechanisms of ERMP1 function, as an extremely expressed protein in CRC was elucidated. Although studies documented that ERMP1 was implicated in UPR and oxidative stress responses of cancer cells, whether ERMP1 exerts its functional effects via PI3K/ AKT/mTOR pathway that has not yet been studied. Our results indicated that one of the unidentified mechanisms through which ERMP1 elicits its proliferative and progressive effects on CRC cells, is PI3K/AKT pathway. ERMP1 facilitated the AKT expression at both mRNA and protein levels, which subsequently promoted the PI3K/AKT pathway in CRC cells by enhancing the phosphorylation of AKT, which in turn augmented the phosphorylation of mTOR as well. PI3K/AKT/mTOR pathway has a significant role in modulating the survival, proliferation, progression, and invasion of CRC cells (24). AKT, as a key regulator of this pathway controls the function of multiple signaling pathways, including cell growth or proliferation, and cell cycle progression or apoptosis (11). Therefore, our results for the first time revealed that ERMP1 exerted its proliferative and colony forming impacts on CRC cells through PI3K/AKT pathway. In agreement with our study, Lu et al. (9) reported that the miR-328-3p overexpression suppressed the AKT phosphorylation by targeting ERMP1 and reduced the p-mTOR/mTOR ratio. Consequently, it decreases the malignant proliferation and invasion in liver cancer.

Phosphorylated AKT can turn drive epithelial-mesenchymal transition (EMT) in human breast cancer cells (29). In CRC, it has been verified that PIK3CD overexpression obviously stabilized β-catenin to be free for activating the AKT-mediated GSK-3β/β-catenin pathway, and then prompted the transcription of CYCLIN D, which is demonstrated to cooperate in survival, proliferation, and invasion of cancer cells (27). Our results verified that the CYCLIN D expression was enhanced in +ERMP1 cells; however, it was reversed in -ERMP1 cells. The CYCLIN D down-regulation led to the cell cycle arrest of -ERMP1 cells at G1 phase. One mechanism in which ERMP1 can enhance the free β-catenin is through AKT phosphorylation. The p-AKT phosphorylates and inactivates GSK-3β, which causes a reduction in β-catenin degradation and eventually leads to the activation of Wnt/β-catenin signaling that ultimately trigger the CYCLIN D promoter activity (28). The role of CYCLIN D in G1 arrest has been illustrated in an observation that revealed adiponectin reduced the transcription of CYCLIN D via restraining AKT-induced phosphorylation of GSK-3β and attenuating free β-catenin in breast cancer cells (29). In CRC, it has been verified that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), as a regulator of mitochondrial function, modulates SW620 and SW480 cells proliferation and invasion through AKT/GSK-3β/β-catenin pathway (30). In addition, another study has reported that CYCLIN D, as a downstream target of activated AKT/GSK-3β/β-catenin signaling, is induced by β-catenin as a consequence of PIK3CD overexpression in CRC cells in a way that, via blockage of AKT signaling or depletion of β-catenin, PIK3CD-regulated cell growth, and invasion becomes reversed (31). As p-AKT has the potency to regulate the function of...
multiple substrates involved in cellular survival such as GSK-3β becomes inactivated by p-AKT to trigger the stabilization of β-catenin for nucleus translocation (32) and it was revealed for the first time in this study that downregulation of ERMP1 could reduce intracellular accumulation and nuclear activity of β-catenin, which then decreased the expression of CYCLIN D and elevated the G1 arrest. It appears that AKT is a determining marker in mediating ERMP1 effects, and G1 is the dominant phase in CRC cell cycle affected by ERMP1.

In response to ERMP1 knock-down, the protein level of E-cadherin did not change in normal condition and this may reflect the possibility that ERMP1 is not the solitary agent implicated in regulating E-cadherin expression; this is in accordance with the concept that various factors can be involved in inhibition of E-cadherin (26, 33) as applying starvation or exposure to serum free and DTT circumstances in our study reduced the expression of E-cadherin. Since E-cadherin and β-catenin cohesion, regularly found in the adherent junction, suppresses the release of β-catenin to nucleus and stabilizes the complex formation for cellular adhesion with restoring β-catenin membrane bounded, these results indicate that ERMP1 reduces the complex formation between E-cadherin and β-catenin that further facilitates the release of β-catenin to act as a transcription factor. Therefore, co-exerting serum free or DTT conditions following ERMP1 knock-down could inhibit the AKT-mediated EMT through restoring E-cadherin expression in CRC cell. Research on oral squamous carcinoma cells has revealed that AKT inhibition could bring back the E-cadherin expression, and thereby the mesenchymal-to-epithelial reverting transition (34).

According to our data, knock-down of ERMP1 did not promote apoptosis, possibly due to inability in affecting C-MYC expression with an exception to DTT-treated cells which further caused cell apoptosis and reduced C-MYC expression in ERMP1 downregulated cells. C-MYC promotes the progression through cell cycle phases not only by inducing cell-cycle regulators such as cyclins and cyclin-dependent kinases, but also via repressing the activity of a set of cell cycle inhibitors (35). It has been demonstrated that C-MYC expression is regulated through not only Wnt/β-catenin and PI3K/AKT/GSK-3 pathways, but also many other important signaling pathways (36); possibly because of that result, ERMP1 could not influence C-MYC expression, and ERMP1 was not the only factor to induce apoptosis in CRC cells.

All together, we provided evidence that ERMP1 through activating its downstream signaling could possibly modulate the G1 phase progression. G1 arrest found by ERMP1 downregulation may be attributed to the reduction of CYCLIN D expression, attained in -ERMP1 cells. Since G1 phase and G1 to S phase transition in cell cycle specifically regulates the cellular proliferation and differentiation, the proliferation of CRC cells was affected by ERMP1.

Our data also showed that GRP78 expression was not affected by ERMP1 knock-down, whereas reduction in csGRP78 was considerably explored in -ERMP1 cells. Since ERMP1 is an ER membrane protein (1), GRP78 has probably become trapped in ER membrane to initiate ER stress through ERMP1 downregulation. Nonetheless, in -ERMP1 cells co-existing with starvation, DTT and 5-FU circumstances, both expression and cell surface localization of GRP78 were decreased. The results of DTT are consistent with evidence documented that DTT by reducing GRP78 expression prompted ER stress-promoted HeLa cells apoptosis (17). Although it is proved that ERMP1 contributes to ER stress, whether it acts in upstream or within ER-related UPRs remains unclear, these data suggest that ERMP1 plays an important role in localization of GRP78 and exert its several biological effects via GRP78. GRP78 is upregulated in numerous cancers, and following translocation to the cell surface it acts as a receptor for various ligands that induce cell malignancy by promoting multiple responses such as cell proliferation, migration, invasion, stenness, and chemo-resistance (7, 37). In the case of CRC, csGRP78 also aggregate in the cell membrane, act as a biomarker for prognosis, and promote signaling pathways involved in survival and proliferation of CRCs such as PI3K/AKT pathway and Wnt/β-catenin through binding to α2-macroglobulin, complexing with PI3K, and promoting PI [3, 4, 5] P3 production and co-localization (38, 39). Besides, it has been suggested that GRP78 can indirectly suppress the interaction between β-catenin and E-cadherin through prevention of the E-cadherin expression in CRC (40), and co-existence of starvation or DTT in -ERMP1 cells seems to downregulate the expression of GRP78 and subsequently accelerate its preventive effects on E-cadherin. As a consequence, it seems that ERMP1 exerts its effects on proliferation of CRC cells through not only AKT expression, but also translocation of GRP78 to the cell surface.

In summary, in an attempt to explore the mechanisms whereby ERMP1 enhances the proliferation of CRC cells, we found that ERMP1 played a role in accelerating AKT expression. As a consequence, the augmented p-AKT significantly increased free β-catenin, an affect that elevated the expression of CYCLIN D but not C-MYC; this suggests that activation of p-AKT plays a role in ERMP1-mediated stabilization of β-catenin and depletion of complex formation between E-cadherin and β-catenin. Thus, through enhancing the expression of AKT and facilitating cell surface translocation of GRP78, ERMP1 could promote the AKT/mTOR phosphorylation, free β-catenin stabilization, and subsequently prompt the cell cycle progression of CRC cells into the S phase.

The limitation of the present study was lacking animal experiments. Our next plan is to assess the frequency of the ERMP1 overexpression in CRC patients and
explore the clinical and pathological impact of ERMP1 overexpression in affected patients.

Conclusion

Our in vitro research suggests that ERMP1 acts as an oncogene possibly through accelerating the malignant characteristics of CRC cells via affecting the PI3K/AKT/mTOR/β-catenin signaling pathways and localizing GRP78; therefore, ERMP1 may be regarded as a promising point in therapeutic strategies related to CRC. Additionally, this evidence helps better explore the ERMP1 employed molecular mechanisms underlying CRC proliferation which may further suggest the effectiveness of ERMP1 suppression in combination with DTT for CRC treatment. Further in vivo studies are required to confirm these results.

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Authors’ Contributions

P.M., M.Z.; Conceptualization, Resources, Project administration, Writing, Reviewing, and Editing. N.-R.-K., M.Z.; Methodology. N.-R.-K.; Investigation, Software, Formal analysis, Visualization, and Writing. P.M., N.R., M.Z.; Data curation. P.M.; Supervision, Funding acquisition. All authors read and approved the final manuscript.

References

ERMP1 Accelerates The Proliferation of CRC Cells


Green Synthesized Magnesium Oxide Nanoparticles Reinforce Osteogenesis Properties of Bacterial Cellulose Scaffolds for Bone Tissue Engineering Applications: An In Vitro Assessment

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Abstract

Objective: The use of biocompatible scaffolds with appropriate characteristics to treat large bone defects has attracted significant attention. The main objective of the current study is to fabricate a 3D nanocomposite structure that contains green synthesized magnesium oxide nanoparticles (MgONPs) and bacterial cellulose (BC) nanofibres, as a bioscaffold for bone regeneration.

Materials and Methods: In this experimental study, Camellia sinensis extract was used as the green method to synthesize MgONPs. The synthesized hydrogels were evaluated for their porosity, morphology, degradation rate, mechanical features, cell attachment, and cytocompatibility. Osteogenic differentiation was assessed by alkaline phosphatase (ALP) activity, real-time reverse transcription-polymerase chain reaction (RT-PCR), and alizarin red staining.

Results: MgONPs significantly increased both mechanical strength (P=0.009) and porosity (P=0.01) of the BC hydrogels. Human MG-63 osteoblast proliferation significantly increased in the MgONP-BC group compared to the pure BC group (P=0.003). Expression rates of both the ALP (P=0.001) and osteocalcin (OCN) genes were significantly enhanced in cells seeded on the MgONP-incorporated BC. MG-63 cells had significantly greater calcium deposition and ALP activity (P=0.002) on the MgONP-BC scaffold compared to the BC at day 21.

Conclusion: The MgONP-BC scaffold can promote the osteogenic activity of osteoblast-like cells, which indicates its therapeutic potential for bone tissue regeneration.

Keywords: Bacterial Cellulose, Magnesium Oxide, Nanoparticles Osteogenesis

Introduction

Bone tissue can repair itself; however, self-healing may not adequately repair the injured tissue in large bone defects (>3 cm) that occur with surgical removal and in congenital defects. Though various types of bone graft are available to restore function and improve defects (1), autograft bone remains the gold standard for defect reconstruction as it provides osteoinductive growth factors, an osteoconductive scaffold, and osteogenic cells, all of which are essential for new bone growth. Disadvantages of autografts include pain and scarring at the donor site, antigenicity, and the transmission of infectious diseases. Xenografts and allografts carry the risk of immune rejection and disease transmission upon transplantation. Synthetic bone grafts are promising substitutes for treatment of bone defects because of the limitations associated with bone autografts, xenografts, and allografts (2).

Advantages of synthetic bone substitutes include adequate availability and lack of disease transmission. Tissue engineering techniques to generate artificial bone grafts that mimic natural bone tissue while overcoming traditional graft disadvantages have emerged (3). Bacterial cellulose (BC) has attracted considerable interest in biomedicine because of its intrinsic three-dimensionality, high chemical purity, renewable nature, lightweight nature, and high biocompatibility properties compared to other types of cellulose (4, 5). The incorporation of different bioactive materials like gold nanoparticles into the network of a BC hydrogel can improve its...
Physicochemical properties and stimulate osteogenic activity of the implanted scaffold (6). BC scaffolds in their natural state, however, are not bioactive materials; thus, their application in bone tissue engineering (BTE) is inevitably limited. To address this issue, scaffolds that incorporate nanoparticles such as hydroxyapatite have been proposed (7).

Magnesium oxide nanoparticles (MgONPs) are bioactive materials that can accelerate bone growth (8), yet few studies have investigated the effect of MgO in direct contact with osteogenic cells either in vitro or in vivo. One previous study reported that MgO powder implanted into the marrow cavity of rat tibia caused a 25% increase in bone thickness. Results of studies have shown an increase in alkaline phosphatase (ALP) gene expression of osteoblasts and proliferation when cultured on polymer/MgONP composites, which makes this family of composites more attractive for further research (9, 10).

There are a limited number of publications on green synthesized MgONP-BC as a biomaterial for bone regeneration. Thus, the current study explores the effects of MgONP-incorporated BC scaffolds on ALP activity, viability, cell morphology, and osteogenesis-related gene expression, of ALP, osteocalcin (OCN), and runt-related transcription factor 2 (Runx2).

Materials and Methods
This research was approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (IR.TBZMED.REC.1400.376). No human or animal subjects were used in this research.

Nanocomposite scaffold preparation
Colonies of Acetobacter xylinum ATCC 53582 were cultured in Hestrin & Shramm broth [H&S; 0.5% (w/v) yeast extract, 2% (w/v) glucose, 0.5% (w/v) peptone, 0.15% (w/v) citric acid, and 0.27% (w/v) Na₂HPO₄, pH=5.0]. After the cells were cultured under static conditions at 30°C for two days, an aliquot of these cells was transferred to 50 ml H&S broth (1:10 culture-to-H&S broth ratio) and incubated at 30°C for five days. BC pellets formed at the air/water interface during growth. The BC pellets were rinsed with 1% NaOH distilled water and incubated at 90°C for 15 minutes to eliminate the bacteria. The BC layers were rinsed multiple times with distilled water to ensure that the NaOH was totally removed (7). Then, hydrated BCs were stored at -20°C for one day and lyophilized for 48 hours in a freeze dryer (FD10 Freeze Dryer, Tabriz, Iran). The dried BC were soaked in aqueous MgONPs solution (3% w/v) at ambient temperature under magnetic stirring for 72 hours to enable a reaction with the BC fibrils (7, 11). Afterwards, all scaffolds were sterilized by UV light exposure for two hours prior to cell culture.

Synthesis of magnesium oxide nanoparticles
Green synthesis of magnesium oxide nanoparticles using Camellia sinensis extracts and chemical synthesis of nanoparticles
We mixed 10 g of dried Camellia sinensis herb with 100 ml of double-distilled deionized water; the mixture was boiled at 70°C for 30 minutes with continuous stirring. The prepared solution was filtered through Whatman’s No. 1 paper. The obtained extract was mixed with magnesium chloride hexahydrate (MgCl₂·6H₂O) in a 90:10 v/w ratio and continuously stirred for 4 hours at 70°C. Suspensions were centrifuged (10 000 rpm/10 minutes). Finally, the MgONP pellets were collected, washed with deionized water, and dried at 40°C for two hours. The prepared powders were then calcined for three hours at 500°C. Subsequently, the MgONPs were subjected to several assays in order to confirm nanoparticle formation (8). The chemical synthesized MgONPs were prepared according to a previously published technique (10).

Characterisation of magnesium oxide nanoparticles
UV-visible spectroscopy analysis
MgO nanopowders were resuspended to produce a dilute suspension, and their synthesis was examined by UV-visible (UV-vis) spectroscopy (Shimadzu, Japan) at the range of 200-800 nm.

Dynamic light scattering and zeta potential measurements
The stability and size of green synthesized MgONPs were evaluated by zeta potential (ZP) and a dynamic light scattering (DLS) analyser (Malvern Zetasizer nano-ZS90, UK), respectively.

Antibacterial effects of magnesium oxide nanoparticles
The antibacterial effects of the MgONPs against Staphylococcus aureus (S. aureus), methicillin-resistant S. aureus, Pseudomonas aeruginosa (P. aeruginosa), and methicillin-resistant P. aeruginosa were assessed by disk diffusion. S. aureus, methicillin-resistant S. aureus, P. aeruginosa, and methicillin-resistant P. aeruginosa were prepared with a suspension equivalent to 1.5×10⁶ CFU/mL McFarland and cultured with a sterile swab on the surfaces of Müller-Hinton agar plates. Sterile paper discs (5 mm) were impregnated with different concentrations of MgO-NP suspension (1 and 5%) and placed on the surface of the agar medium by using sterile forceps. The plates were incubated at 37°C for 24 hours, after which the clear halo of stunting was measured. The findings were described according to the diameter of the clear zone (inhibition zone) around each disk (12, 13). Tests were carried out in triplicate.
Characterisation of scaffolds and magnesium oxide nanoparticles

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectroscopy (Nicolet 8700 spectrometer; Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the functional group of the dried MgONPs and scaffolds (14). Spectra data were recorded over a wavenumber range of 400-4000/cm.

Morphological analysis of scaffolds and magnesium oxide nanoparticles

The morphology and size of the MgONPs were determined by field emission scanning electron microscopy (SEM, Carl Zeiss, Germany). Specimens (scaffolds and MgONPs) were covered with gold and observed at an accelerating voltage of 15 kV. The composition of MgONPs and Camellia sinensis leaf extract was analysed by SEM-energy-dispersive X-ray (SEM-EDX) spectroscopy (14).

Mechanical characterisation of scaffolds

The mechanical strength of the scaffolds was evaluated using a Universal Testing Machine (Instron 4505) equipped with a 1 kN load cell at room temperature. The tested specimens were cut into “round disk” shapes with dimensions of 5 mm height and 1 cm diameter. The cross-head speed was adjusted to 2 mm/minute, and the load was set to a 70% reduction in specimen height. The stress-strain curve was plotted, and the slope of the first linear segment of the curve (n=4) was used to determine Young’s modulus.

Porosity study

The porosity of various scaffolds was measured using the liquid displacement procedure. Briefly, the scaffold (1 cm diameter and 5 mm thickness) was soaked for 30 minutes in volume V1 of ethanol. The total volume of the ethanol and scaffold after complete immersion was V2, and the residual volume after the ethanol-impregnated scaffold was removed was V3. The following formula was used to calculate the porosity of scaffolds:

\[ P(\%) = \frac{(V1-V3)}{(V2-V3)} \]

The test was performed four times to obtain the average porosity value (15).

Degradation rate and Mg\(^{2+}\) release of scaffolds

The scaffold degradation rate was determined by immersing the specimens (10-mm diameter and 2-mm thickness) in a phosphate-buffered saline (PBS) solution of pH=7.4 at 37°C for 21 days. After incubation at various time periods (7, 13, and 21 days), the scaffolds were removed from the degradation medium, rinsed with distilled water, and dried in a vacuum oven.

Degradation rate = \( \frac{(WO-WT)}{(WO)} \times 100 \)

W0 and WT represent, respectively, the initial weight of the scaffolds before and after immersion in PBS up to day T (8). The Mg\(^{2+}\) release assay was carried out using the protocol of Suryavanshi et al. (10).

In vitro swelling evaluation

Swelling potential was evaluated by soaking the scaffolds in PBS buffer solution with pH=7.4 for 2, 6, 24, 48, and 72 hours (n=4). The percentage of increase in water uptake was calculated using the following equation:

Swelling ratio (\%) = \( \frac{(Wt - W0)}{W0} \times 100 \)

Where: Wt is the weight of wet scaffolds at time t and W0 is the weight of dry scaffolds (15).

In vitro biological evaluation

Viability/cytotoxicity assay

The MTT assay was used to assess viability of cells seeded (density: 3×10\(^3\) cells) on the scaffolds on days 7, 14, and 21. Briefly, 150 µL of the 0.5 mg/mL MTT solution was added to the scaffolds/cells, which were then incubated for three hours at 37°C and 5% CO\(_2\), and then removed from the solution. After incubation, 50 µL DMSO was added to dissolve the formazan crystals, and the plate was read by an ELISA microplate reader at 570 nm (Biorad, Hercules, CA, USA).

Percentage of cell viability was calculated using the following equation:

\[ \text{Percentage cell viability} = \frac{\text{OD}_{\text{Sample}}}{\text{OD}_{\text{Control}}} \times 100 \]

\[ \text{OD}_{t} = \text{The OD of cells/scaffold; OD}_{c} = \text{the OD of control cell} \] (15).

Cell seeding on scaffolds and culture conditions

Human MG-63 osteoblast-like cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) low glucose supplemented with 1% (v/v) penicillin/ streptomycin solution, 50 mg/L ascorbic acid, 10 mmol/L β-glycerophosphate, 10 nmol/L dexamethasone, and 10% (v/v) foetal bovine serum, and incubated at 37°C with 5% CO\(_2\) and 95% relative humidity. During the 21-day study period, the medium was renewed every three days. When the MG-63 cells reached 70-80% confluency, they were detached using a 0.25% trypsin-EDTA solution (Gibco). Thereafter, 50 µl of cell suspension that contained 2×10\(^5\) cells was slowly dropped onto the surface of the UV-sterilized scaffolds (15).

Cell distribution

We applied 4’,6-diamidino-2-phenylindole (DAPI)
staining to assay MG-63 cell proliferation on the scaffolds. After washing with PBS, the samples were fixed with 3.7% paraformaldehyde for three hours, stained with DAPI, and observed under a fluorescence microscope (Nikon Eclipse Ti2, Japan).

**Measurement of oxidative stress in scaffolds**

The total antioxidant capacity (TAC) and nitric oxide (NO) level of the collected supernatants of the cells seeded on the scaffolds were determined using ferric reducing power (FRAP) and the NO assay, respectively (17, 18).

**Osteogenic measurements**

**Alkaline phosphatase activity determination**

The ALP activity of the cells cultured on different scaffolds was determined after incubation for 7, 14, and 21 days by an ALP assay kit (Pars Azmoon, Iran) according to the manufacturer’s instructions. Finally, ALP activities were normalized versus the total protein content (19).

**Alizarin red staining for mineralisation assay**

The mineralisation of MG63 cells after incubation for 21 days was quantitatively measured using the alizarin red staining assay (19). The alizarin red staining concentration was evaluated by absorbance measurement using a spectrophotometer (Pharmacia, Novaspec II, Biochrom, England) at a wavelength of 405 nm.

**Bone-associated gene expression assay**

The real-time reverse transcription-polymerase chain reaction (RT-PCR) method was performed to evaluate the expression levels of ALP, OCN, and runt-related transcription factor 2 (Runx2) genes after 21 days of cell culture. Briefly, total RNA was extracted from the cell-seeded scaffolds by TRizol/chloroform based on the manufacturer’s protocol (Life Biolab, Heidelberg, Germany). Then, cDNA was synthesized from total RNA by using an AddScript cDNA synthesis kit (AddBio, South Korea) and oligo dT20 and random hexamers. RT-PCR was performed by Ampliqon SYBR Green Master Mix (RealQ Plus 2x Master Mix, Ampliqon, Denmark) and then carried out in a Step One Plus™ system (Applied Biosystems, USA). The expression levels of the Runx2, OCN, and ALP genes were calculated by RT-PCR and normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. The relative changes in intended gene expression were assessed using the 2-\((\Delta\DeltaCT)\) method. The designed primers used in this experimental study are listed below:

**ALP- Human**

F: 5’-GCTGGGAAATCTGTGGGC-3’
R: 5’-CCATGATCACGTCAATGTCCCT-3’

**OCN- Human**

F: 5’-CAGCCACCGAGACACCATGA-3’
R: 5’-CTTGGACACAAAGGCTGCA-3’

**GAPDH- Human**

F: 5’-AAGGTGAAGGTCGGAGTCA-3’
R: 5’-GGGGTCATTGATGGCAACAA-3’

**Statistical analysis**

All quantitative data are reported as mean ± standard deviation (n=4). Statistical significance was determined as a P<0.05 using one-way analysis of variance (ANOVA) and Tukey’s post hoc test in GraphPad Prism, version 8.4.3 (San Diego, CA, USA).

**Results**

**Characterisation of magnesium oxide nanoparticles and scaffolds**

**UV-visible spectroscopy assay**

Figure 1A and B show UV-vis spectroscopy images of the synthesized MgONPs. The MgONPs prepared by chemical synthesis had a maximum peak of 325 nm (Fig.1A), whereas MgONPs prepared by green synthesis showed a maximum peak of 285 nm (Fig.1B).

**Dynamic light scattering and zeta potential of magnesium oxide nanoparticles**

We conducted DLS analysis to determine the dispersion and size of MgONPs in the colloidal solution through the reaction of light beams with biosynthesized MgONPs. The green synthesized MgONPs were observed at 56.03 to 88.33 nm with a polydispersed index value of 0.484. The average size of the MgONPs was 72.18 nm (Fig.1C).

The ZP, a key indicator of the stability of the colloidal dispersions and surface charge of nanoparticles, was -19.1 ± 5.86 mV in the biosynthesized MgONP sample, which indicated that the colloidal solution was stable (Fig.1D) and that the MgONPs had a negative surface charge based on their area.

**Morphology observation by scanning electron microscopy**

Figure 1E shows the morphology and size of biosynthesized MgONPs as observed by SEM. The MgONPs had compact agglomerates. These images also showed that the biosynthesized MgONPs had spherical morphologies with high aggregation, which confirmed polydispersity in the DLS analysis (Fig.1E). EDX analysis of the extract (Fig.1F) and green synthetic MgONPs (Fig.1G) showed the purity of the nanoparticles and confirmed formation of these nanoparticles with strong signals in the O and Mg regions. Quantitative analysis results indicated that the weight percentages of O and Mg ions in the sample were 54.1 and 20.6%, respectively, while the atomic ratio was 50.3 and 17.1%, respectively (Fig.1F).
Fourier-transform infrared spectroscopy characterisation

FTIR spectra of the MgO nano-powder and green synthesized MgONPs are presented in Figure 2A. The stretching bond at 470.63 cm$^{-1}$ clearly confirmed the formation of green synthesized MgONPs. The existence of several bioactive metabolites such as anthocyanins, tannins, and flavonoids was attributed to the strong band observed near 3441.01 cm$^{-1}$ for the O–H bond vibration of the hydroxy group. The stretching vibration of C=C was responsible for the band at 1633-1651 cm$^{-1}$, whereas the stretching vibration of C–C and the bending of the N–H bond were responsible for the band at 1462-1539 cm$^{-1}$. The stretching vibration of the C–O bond was attributed to the band at 1089.78 cm$^{-1}$ (Fig.2A).

Antibacterial activity of magnesium oxide nanoparticles

The data did not show any significant difference related to antibacterial activity of the green and chemically synthesized MgONPs (1 and 5% w/v) for *S. aureus*, *P. aeruginosa*, and methicillin-resistant *P. aeruginosa* (P=0.08) (Fig.2B, D-G). The antibacterial activity of green synthesized MgONPs at the 1% and 5% w/v suspensions significantly inhibited the growth of methicillin-resistant *S. aureus* compared to the *Camellia sinensis* extract (P=0.003, Fig.2F). The green synthesized MgONPs at the 5% concentration (Fig.2C, F) had the largest inhibition zones (P=0.001, Fig.2F).
MgONPs Improve Osteogenesis

Fig. 2: Fourier transform infrared spectroscopy (FTIR) study and antibacterial effects of magnesium oxide nanoparticles (MgONPs). A. FTIR spectra of green and chemically synthesized MgONPs. Results of disc diffusion tests to determine the antibacterial effects of green and chemically synthesized MgONPs (1 and 5%). B. Staphylococcus aureus (S. aureus), C. methicillin-resistant S. aureus, D. Pseudomonas aeruginosa (P. aeruginosa), E. Methicillin-resistant P. aeruginosa. F. Growth inhibition zone of S. aureus and methicillin-resistant S. aureus, and G. growth inhibition zone of P. aeruginosa and methicillin-resistant P. aeruginosa. One-way analysis of variance (ANOVA) test results for antibacterial activity of green and chemically synthesized MgONPs in the different groups. *; P \leq 0.05 growth inhibition zone decreased in green synthesized MgONPs (1, 5%) groups in comparison to the chemical synthesized MgONPs group.

Scanning electron microscopy and Fourier-transform infrared spectroscopy assay of the scaffolds

SEM images showed that a large volume of the BC scaffolds were occupied by pore interconnectivity and interwoven nanofibres (Fig.3A). Examination of the scaffolds demonstrated that MgONP-BC had a highly porous microstructure compared to the pure BC membrane (Fig.3B).

The absorption peak of the BC in FTIR was 1317-1161 cm\(^{-1}\): CO stretching, 1651.7 cm\(^{-1}\): CH\(_2\) symmetric stretching, 2895.15 cm\(^{-1}\): asymmetric CH\(_2\) stretching, and 3361.9 cm\(^{-1}\): OH stretching. The major characteristic peaks of BC (3338.5 cm\(^{-1}\) to H–O, 1561.05 cm\(^{-1}\) to C–H, and 1073.14 cm\(^{-1}\) to C-O) and MgONP (590.63 cm\(^{-1}\)) were shown in the FTIR spectra of the MgONP-BC groups. The coexistence of characteristic peaks of MgONP and BC in the spectra of the MgONP-BC hydrogels confirmed successful incorporation of MgONP into the BC network (Fig.3C).

Porosity, degradation rate, cumulative Mg\(^{2+}\) release and swelling ratio measurement of the scaffolds

Porosity investigations revealed that the scaffolds are highly porous (Fig.3D). The MgONPs-BC had a higher porosity (89.2 ± 3.45%) than pure BC (62.9 ± 3.49%). The results showed no significant difference in swelling ratio between the MgONP-BC group and the BC groups (P>0.05) at 2, 6, 24, 48, and 72 hours. The swelling ratio was 75% for the BC groups and 78% for MgONP-BC after
24 hours, which increased to 79 and 88%, respectively, after 72 hours (P=0.01, Fig.3E).

Figure 3F shows the scaffold degradation rates in PBS (both pH=7.4) after incubation on days 1, 7, 14, and 21. There was no significant difference observed between pure BC and MgONPs-BC on days 7, 14, and 21 (P=0.07). The release curves of Mg$^{2+}$ from the BC and MgONP-BC scaffolds are shown in Figure 3G. In the first week, the MgONP-BC scaffolds exhibited a relative burst release; by day 14, the composite membranes released 70-80% of the nanoparticles in a sustained manner. These findings revealed that the MgONP-BC scaffolds continuously released nanoparticles into the external medium.

**Viability/cytotoxicity assay of the scaffolds**

The MTT assay was used to determine cell viability on the scaffolds (Fig.3H). Both the MgONP-BC and BC scaffolds had a positive effect on cell viability on days 7, 14, and 21. A comparison of those scaffolds showed that cell viability was significantly higher in the MgONP-BC scaffold compared to pure BC after 21 days of cell culture (P=0.003).
Mechanical properties of the scaffolds

Both mechanical strength and Young’s modulus were higher for MgONPs-BC scaffolds compared to the pure BC scaffold (P=0.009). Nonetheless, there were considerable differences in Young’s moduli among all groups (Table 1). The initial linear slope of stress-strain curves was used to determine the stiffness of the scaffolds, and was calculated as Young’s modulus. Young’s modulus was 146.36 ± 4.24 MPa for BC and 191.19 ± 15.10 MPa for MgONP-BC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Young’s modulus (MPa)</th>
<th>Compressive strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure BC</td>
<td>146.36 ± 4.24</td>
<td>36.19 ± 3.18</td>
</tr>
<tr>
<td>MgONPs-BC</td>
<td>191.19 ± 15.10*</td>
<td>53.09 ± 5.02##</td>
</tr>
</tbody>
</table>

Table 1: Young’s modulus and compressive strength of various scaffolds

One-way analysis of variance (ANOVA) test results for Young’s modulus and compressive strength in the research groups. *; P≤0.01 Young’s modulus increased in the magnesium oxide nanoparticle-bacterial cellulose (MgONPs-BC) groups in comparison to the BC group and ##; P≤0.01 increased compressive strength in the MgONPs-BC group compared to the BC group.

Assessment of total antioxidant capacity and nitric oxide levels

Figure 4A shows the TAC levels. After 14 days, the MgONP-BC scaffolds had higher TAC than the BC scaffolds (P=0.004). After 21 days of incubation, however, TAC levels in the MgONP-BC scaffolds did not show any significant difference compared with pure BC scaffolds (P=0.05). According to Figure 4B, there was a significant difference in NO level for the MgONP-BC scaffolds. NO levels significantly decreased (P=0.001) after 21 days in the MgONP-BC scaffolds compared with the pure BC scaffolds.

Cell distribution

DAPI staining was used to evaluate cell proliferation on the scaffolds. After seven days of culture, the number of cells in the biosynthesized MgONPs-BC scaffold (Fig.5A) increased in comparison with the pure BC scaffold (Fig.5B). Overall, MgONPs appeared to improve cell proliferation on the BC scaffolds; therefore, they were used in this study. MG-63 cells were cultured on a plate with the same cell culture conditions on the scaffold and considered to be a two-dimensional (2D) group (Fig.5C).

Osteogenic measurements

Alizarin red staining for mineralisation

The green synthesized MgONP significantly enhanced mineralisation levels of cells on BC scaffolds at 21 days of incubation (P=0.004). These findings showed that the presence of MgONPs in the BC scaffolds could accelerate MG-63 cell mineralisation activity (Fig.5D).
Osteogenesis-related gene expression

The MgONP-incorporated BC scaffolds exhibited increased \( ALP \) (\( P=0.001 \)) and \( OCN \) (\( P=0.004 \)) expressions compared with pure BC scaffolds at 21 days. No significant difference in human \( \text{Runx2} \) expression was observed between the MgONP-BC and pure BC scaffolds (Fig.5E).

Measurement of alkaline phosphatase activity

The MG-63 cells on BC showed lower ALP activity compared to the MgONP-BC scaffolds after 14 (\( P=0.005 \)) and 21 (\( P=0.002 \)) days of cell culture (Fig.5F). ALP activity levels did not show any significant change for cells on the BC and MgONP-BC scaffolds at seven days.

Fig.5: The analysis of 4',6-diamidino-2-phenylindole (DAPI) staining, alizarin red s concentration and gene expressions of \( ALP \), \( OCN \), and \( \text{Runx2} \) in the different groups. A. DAPI staining of MG-63 cells on magnesium oxide nanoparticle-bacterial cellulose (MgONP-BC), B. BC scaffolds, and C. A well plate at seven days after cell seeding (scale bar: 100 \( \mu \)m). D. One-way analysis of variance (ANOVA) test results for alizarin red s staining concentration in the different groups. *; \( P\leq0.05 \) Alizarin red s staining increased in the MgONPs-BC group in comparison to the BC group (scale bar: 100 \( \mu \)m). E. ANOVA results for gene expressions of \( ALP \), \( OCN \), and \( \text{Runx2} \) in the different groups. *; \( P\leq0.05 \) \( ALP \), \( OCN \), and \( \text{Runx2} \) expressions increased in the MgONPs-BC group in comparison to the BC group. F. ALP activity in cells cultured on BC and MgONP-BC scaffolds. *; \( P\leq0.05 \) ALP activity increased in the MgONPs-BC groups in comparison to the BC group.
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Discussion

Green synthesized MgONPs incorporated into BC scaffolds improved their physicochemical properties. In the current study, we proved that these MgONP-incorporated BC scaffolds increased porosity, compressive strength, cell proliferation, and mineralisation. To the best of our knowledge, this study was the first to report that MgONP-BC scaffolds could promote the osteogenic activity of MG-63 cells. UV-spectrophotometry analysis indicated a maximum absorbance peak at 322 nm, which showed the formation of small-sized green synthesized MgONPs (20). The results of this study supported those of previous studies (20), although there was a sharp absorbance peak of green synthesized MgONPs at 285 nm.

The present study revealed that the green synthesized MgONP of *Camellia sinensis* significantly inhibited bacterial growth of methicillin-resistant *S. aureus* in a dose-dependent manner. In line with this data, Ravoor et al. (21) reported that MgONPs inhibited the growth of methicillin-resistant *S. aureus*. In the current study, chemical synthesized MgONPs exhibited no antibacterial effects against *S. aureus*, methicillin-resistant *S. aureus*, methicillin-resistant *P. aeruginosa*, or *P. aeruginosa*. In the case of methicillin-resistant *S. aureus*, the highest antibacterial activity was observed at 5% w/v green synthesized MgONP. Notably, the 1% w/v and 5% w/v concentrations of green synthesized MgONP had no antibacterial activity against *S. aureus*, *P. aeruginosa*, or methicillin-resistant *P. aeruginosa*. Because *Camellia sinensis* extract had an antibacterial effect on methicillin-resistant *S. aureus*, we hypothesized that the antibacterial effect of green synthesized MgONP nanoparticles could be related to the biomolecules of the *Camellia sinensis* extract that attached to the nanoparticle surfaces.

SEM images in the current study indicated a mean diameter of 74 nm and a spherical shape for green synthesized MgONPs. DLS analysis also confirmed the ~72 nm diameter size distribution. We observed a ZP of approximately -19.1 mV, which indicated good stability of the synthesized nanoparticles.

The small size (<100 nm) of MgO offers valuable advantages for bone regeneration. First, it effectively promotes osteoblast adhesion and osteogenic activity of the nanocomposite scaffolds. Second, it stimulates the angiogenic response and reduces inflammation (22, 23). Our RT-PCR evaluation showed that MgONPs-loaded BC scaffolds had greater expression of the osteogenesis-specific marker genes ALP and OCN compared to the BC scaffolds.

ALP activity levels are important for bone mineralisation and are measured as an osteogenic marker. Based on the data, ALP levels exhibited no significant difference for the cells seeded on the scaffolds at seven days. In contrast, increased ALP activity was seen in cells seeded on MgONP-BC scaffolds on days 14 to 21 compared to day 7. Other study results have shown that higher ALP activity is integral for ECM regeneration before mineralisation in the presence of osteoblasts (24).

The EDX result of MgONPs synthesized from the aqueous extract of *Pterocarpus marsupium* revealed two peaks for O and Mg ions with weight percentages of 69.3 and 30.6%, respectively (25). The FTIR spectrum of green synthesized MgO nanoparticles showed sharp peaks at 653.51-825.42 cm⁻¹, which corresponded to the Mg–O–Mg groups as well as at 3447.11 cm⁻¹ that was attributed to the O–H bending of the hydroxy group, and was related to the presence of several bioactive metabolites (anthocyanins, tannins, and flavonoids). An absorption peak found at 1590.22 cm⁻¹ corresponded to the carbonyl group of flavonoids (26). Similarly, EDX analysis of this study revealed that the green synthesized MgONPs were extremely pure, and contained both Mg and O ions.

In line with previous studies, the current study showed that the peak intensity of O–H in the FTIR spectrum of the green synthesized MgO nanoparticles increased, and the existence of broad peaks of MgO and *Camellia senses* extract in the FTIR spectra of green synthesized MgONPs indicated successful synthesis of the MgONPs of the plant extract; this was consistent with the EDX data.

In the present study, SEM imaging revealed high porosity in the MgONP-BC scaffolds compared to BC scaffolds. BC contains many free hydroxyl groups that react with water molecules, and this causes a reduction in pore size and porosity (27). The interaction of water molecules with the free hydrophilic functional groups of the scaffolds reduces the pores (28). Our porosity results were similar to those of Khan et al. (29), who reported that the enhancement of pore size and porosity in BC-based scaffolds incorporated with nanoparticles was related to an increase in the amount of retained water, which was most likely predominantly unbound water. Unbound water is defined as water that freezes and cause pores.

The results of other studies have shown that the magnetic nanoparticles loaded in the scaffolds acted as a backbone and improved the mechanical strength and high porosity in the nanocomposite scaffolds (30). The presence of graphene oxide (GO) nanoparticles increased the porosity and interparticle space of composite scaffolds by increasing their ability to retain water. Despite increased porosity, GO increased the mechanical properties of the scaffold because of the interactions between the fillers and the polymer matrix and improved cell attachment and proliferation (31). Chitosan-collagen scaffolds incorporated with zinc oxide nanoparticles showed higher porosity than chitosan-collagen scaffolds (32). We hypothesized that the increased water absorption in scaffolds that
contain MgONP and the interaction of nanoparticles with the free hydroxyl group of the BC scaffolds could increase the number of bound water molecules in the scaffolds. After freezing and the freeze-drying process, larger pores form in these scaffolds.

MgONP-incorporated sodium alginate can increase mechanical strength. The results of mechanical property evaluations showed that Young’s modulus of the MgONPs (4 wt%)-sodium alginate scaffolds enhanced about 44% compared with sodium alginate scaffolds. Nasri-Nasrabadi et al. (9) reported that the increase in Young’s modulus at 4% MgONP could be attributed to the mobility limitations of the polymer chain and the formation of strong intra-molecular hydrogen bonds between the hydroxyl groups of the scaffold and nanoparticles. The current results showed that MgONP (3%)-BC had good porosity and mechanical strength. We hypothesized that the hydrogen/covalent bonding that occurred between the OH groups of the BC scaffolds and MgONPs enhanced the toughness and reduced the chains’ mobility, and enhanced the mechanical strength of the MgONPs-BC. Numata et al. (27) demonstrated that a slow-release system that combined a BC hydrogel and nanoparticles could be effectively used in the biomedical field. In the current study, MgONPs were released in a burst from the nanofibres within 14 days with about 70-80% release for MgONP-BC.

SEM images of the BC scaffolds in the current study showed a smooth and uniform surface. After incorporation of MgONPs into the BC scaffold, the nanocomposite surface exhibited highly porous structures. Therefore, the current findings supported the results where, in the absence of silver nanoparticles, BC scaffolds formed dense structures with irregularly shaped and highly interconnected pores (33). In the present study, SEM analysis of the MgONPs-BC scaffolds showed spherical particles entrapped between the BC nanofibrils. Aggregates of nanoparticles were also observed on the nanofibres.

It is crucial to evaluate the liquid uptake ability of scaffolds when they are considered for use in tissue engineering. Improvement in the swelling ratio of scaffolds is a prerequisite for waste diffusion, facilitated nutrients and an enhanced cell attachment, and cellular interactions which enhance tissue regeneration. Although an enhancement in the swelling capacity of the scaffold seems to be significant, continuous swelling leads to a decrease in the compressive strength of the scaffold and its disintegration (34). In the current study, the swelling ability for BC and MgONP-BC scaffolds was found to be incremental at 2, 6, 24, 48, and 72 hours and this showed their ability to absorb fluids. Therefore, the presence of MgONPs did not affect the ability of the scaffolds to absorb liquids.

FTIR analysis indicated BC had typical peaks of 3361.9 cm\(^{-1}\) (O-H stretching) and 2895.15 cm\(^{-1}\) (C-H stretching), and MgONP-incorporated BC had peaks of 480.5 cm\(^{-1}\). In the FTIR spectra of alginate/polymer (vinyl alcohol) scaffolds, the Mg-O stretching vibrational band at 540 cm\(^{-1}\) could not be isolated because those peaks overlapped significantly with the intense peaks of the scaffold (35). According to our results, a wavelength of 400-650 cm\(^{-1}\) can be used to determine the presence of MgONPs in the BC scaffold. Therefore, this study showed that MgONPs were successfully incorporated in the BC scaffold.

Previous studies have reported that green synthesized MgONPs have an antioxidant effect (36) and can stimulate some bone regeneration mechanisms (37). The differentiation of pre-osteoclasts into osteoclasts is activated by oxidative stress and enhances bone resorption. Antioxidant compounds promote osteoblast growth and bone production while inhibiting the differentiation and activity of osteoclasts. High levels of ROS have opposing effects; they inhibit osteoblast differentiation and decrease reduce mineralisation and osteogenesis activity (38).

NO has dose-dependent effects on osteoclast and osteoblast activity. Therefore, the balance of NO levels can be important to the appropriate regeneration of critical-size bone defects. Low NO levels lead to increased osteoblast proliferation and bone formation, while high NO levels stimulate osteoblast apoptosis (39). These obtained results agree with the current results, in which NO levels were reduced in MgONP-BC compared to BC scaffolds, while TAC levels were significantly higher for MgONP-BC scaffolds than for pure BC scaffolds.

DAPI-stained images on day 7 of the study revealed an enhanced number of cells in the MgONP-BC compared to the other groups. MTT analysis also exposed an increased cell survival rate in cells on the MgONP-BC scaffolds.

The current results introduce MgONP-BC nanocomposite as an advisable high-performance scaffold for BTE applications. Some limitations in areas of this study that need further attention include the lack of investigation into the osteogenic effects of MgONP-BC \textit{in vivo}. This evaluation can provide valuable data about the behaviour of scaffolds in healing of bone defects. These MgONP-BC scaffolds elevate the osteogenic differentiation of MG-63 cells and display good cytocompatibility, which indicates their feasibility as a biomaterial for bone regeneration.

**Conclusion**

Green synthesized MgONPs showed antioxidant and antibacterial effects. After loading, the BC scaffold exhibited good biological characteristics for cell attachment and proliferation. MgONP-BC scaffolds could overcome the limitations of cell adhesion and
mineralisation during the bone regeneration process in vitro. The MgONP-BC hydrogel could be an efficient system for bone regeneration, and MgONPs can be suggested as a bioactive factor in bone repair; therefore, in vivo experiments on the use of these additive materials are recommended.

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Authors’ Contributions

E.Gh., B.N., M.Kh., A.M., A.B.K.; Participated in study design, Methodology, Data collection, Analysis, and Interpretation. E.Gh., M.Kh., A.B.K.; Wrote, Reviewed, and Edited the manuscript. All authors read and approved the final manuscript.

References


Synergistic Effects of Capsaicin and Quercetin Improved Induced Premature Ovarian Failure in Rat

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Abstract
Objective: Premature ovarian failure (POF) is a heterogeneous disorder. POF is defined as hypergonadotropic hypoestrogenism in women under 40 years. There is no effective treatment to cure POF patients. Antioxidants prevent ovarian damage by reducing the lipid peroxidation cascades affecting folliculogenesis, meiosis and ovulation. Hence; the aim of present study was to investigate the effects of Capsaicin (CAP) and Quercetin (QUR) on cyclophosphamide (CYC)-induced POF in rat model.

Materials and Methods: In this experimental study, POF was induced by intraperitoneal injection of 200 mg/kg CYC on first day and then 8 mg/kg/day for the following 3 days. After 4 days of CYC administration, rats were randomly divided into five groups (n=6/group) as follows: POF, dimethyl sulfoxide (DMSO), CAP (0.5 mg/kg/day), QUR (100 mg/kg/day) and CAP+QUR. Biochemical, hormonal, gene expression, and histological evaluations were performed on blood serum and tissue samples after 14 days of treatment with the CAP and QUR.

Results: CAP, QUR and CAP+QUR groups showed signs of restored ovarian function in the form of a significant increase in serum total antioxidant capacity (TAC), estrogen, progesterone and anti-mullerian hormone (AMH) levels versus POF and DMSO groups and a significant improvement in histological parameters and follicle numbers in treatment groups compared to POF and DMSO groups. Polymerase chain reaction (PCR) analysis demonstrated that CAP and QUR upregulate the expression of BAX gene and decreased the expression of apoptosis inducing genes (BCL-2 and P53).

Conclusion: CAP and QUR treatment of CYC-induced POF rats showed a positive effect on reducing ovarian damage by improving TAC levels, expression of apoptotic genes, levels of ovarian reserve markers, and histological parameters. Our results suggest that treatment with CAP or QUR may be a conservative treatment approach for CYC-induced POF.

Keywords: Capsaicin, Cyclophosphamide, Premature Ovarian Failure, Quercetin, Total Antioxidant Capacity

Introduction

Decreased ovarian activity with increased levels of follicle-stimulating hormone (FSH) and decreased levels of estradiol (E2) in women under 40 years of age is defined as premature ovarian failure (POF) (1). These patients usually experience oligomenorrhea, amenorrhea, infertility, osteoporosis, cardiovascular and mental diseases showing long-term effects of hypoestrogenism (2). POF is one of the growing diseases affecting the life of 1% of women at the age of 40; although genetic, autoimmune, metabolic and infectious factors are related to the occurrence of POF, iatrogenic factors including chemotherapy is one of the most predictable reasons (3).

Recent studies have shown that the number of patients with POF caused by chemotherapy has been increased in recent years, and its incidence has reached 70-100% of people undergoing chemotherapy (4). Currently there is no effective treatment for POF, but hormone replacement therapy (HRT) including the use of both estrogen and progesterone, is used to reduce the symptoms of POF and preventing osteoporosis in these patients; new studies have clarified the relationship of this treatment method with the increased possibility of cardiovascular diseases (5). Cyclophosphamide (CYC), one of the most successful and widely used antineoplastic drugs, can increase the production of ROS via some signaling pathways such as the PI3K/Akt/mTOR pathway and some molecular complexes including the NADPH complex (NADPH/NADP⁺) and the mitochondrial electron respiratory chain (6).

Duo to fewer side effects, recent studies focus on the use of natural phytochemicals and dietary supplements, including flavonoids and carotenoids (7, 8). One of these compounds is capsaicin (CAP), which is abundantly found...
in red pepper. CAP (8-methyl-N-vanillyl-6-nonanamide) is a phenolic compound reducing inflammation and oxidative stress because of its antioxidant, anti-inflammatory and anti-cancer properties that limits the negative effects of proteases and lysosomal enzymes (9, 10). CAP corrects the function of macrophages by reducing inflammatory mediators (11). Pretreatment with CAP before radiotherapy protects the primordial follicle reserve preventing ovarian that can be a plausible way to prevent radiation-induced POF (12).

Quercetin (QUR) is a natural product abundant in vegetables, fruits, tea, and olive oil that has antioxidant, anti-inflammatory, and anti-cancer properties (13). QUR (3, 3’, 4’, 5, 7-pentahydroxyflavanone) is one of the flavonoid compounds that play a role in reducing inflammation, oxidative stress, proteases, and lysosomal enzymes (9, 14) as well as in preventing and treating many chronic cardiac, nervous diseases and cancer (15). QUR’s effect in preventing ovarian, breast, prostate, liver and lung cancers has been reported (16). In cases of ovarian cancer, it shows anticancer activity by controlling the cell cycle, preventing tumor growth and angiogenesis, inducing apoptosis (17) by activating Nrf2-ARE pathway that prevents the expression level of anti-inflammatory enzymes. Phase II oxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH) and thioredoxin inhibit oxidative stress (18, 19) and its toxicity. Izaguirry et al. (20) used QUR as a natural antioxidant against the gonadotoxic effects of cadmium on the ovaries of rats and mice by scavenging free radicals and improving the activities of other antioxidants such as catalase, SOD, and GPx. Therefore, it improved ovarian health by increasing the secretion of E2, FSH, and luteinizing hormone (LH). Wang et al. (21) examined the effects of QUR on ovarian antioxidant capacity in menopausal mice showing no significant change in serum levels of total antioxidant capacity colorimetric (T-AOC), SOD, GSH, GPX-PX, and glutathione S-transferase (GST), but an increase in mRNA and protein expression of oxidative stress-related genes, including SOD -1, CAT, and glutathione synthetase (GSS). QUR prevents oxidative stress and histopathological changes in the ovaries at IR injury (ovarian ischemia-reperfusion injury) (22). There is evidence that QUR can decrease caspase 3 and TUNEL-positive cells in the ovaries, as well as the level of ischemia-modified albumin (IMA) in the serum of IR -injured rats. On the other hand, QUR also has pro-oxidative effects leading to genotoxicity and antiproliferative activity (23).

Endogenous antioxidant defense systems in humans are incomplete without exogenous reducing compounds such as ascorbic acid, tocopherols, carotenoids, phenolics- flavonoids, nonflavonoids, and polyphenols, which play an essential role in many antioxidant mechanisms in living organisms. Therefore, there is a constant need for exogenous antioxidants to prevent oxidative stress, representing a redox imbalance in favor of oxidation. Antioxidants can positively influence each other, such as the synergy of synthetic phenolic antioxidants or the regeneration of tocopherol from its oxidized form, the tocopheroxyl radical, by reduced coenzyme Q (24).

Therefore, considering the importance of POF in women of reproductive age and its high prevalence in women who are undergoing chemotherapy or radiation therapy and the side effects that chemotherapy drugs have as one of the inducing factors of this disease, the objective of current study was to investigate the synergistic effects of CAP and QUR on CYC-induced POF.

Materials and Methods

This in vivo animal experiment was performed according to the animal ethics guidelines of the Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1400.080). 36 healthy adult female Wistar albino rats (age: 3 to 4 months) (weight: 210 ± 10) obtained from central animal house and were housed in clean polypropylene rat cages under a 12/12-h light/dark cycle at an ambient temperature of 21-23°C. Food and water were provided ad libitum. Rats were given 3 days to adapt to the environmental conditions.

In the first phase of this study, the rats were randomly divided into POF (n=30) and control (n=6) groups. In order to establish the chemotherapy-induced POF model in rats after testing POF induction methods based on previous studies (25) and according to our pilots study, POF was induced by intraperitoneal injection of 200 mg/kg CYC (Endoxan -N, Batch No: BUX1035, India) on day 1 and then 8 mg/kg/day for the following 3 days.

In the second phase of the study, POF rats were randomly divided into five groups (n=6): POF, DMSO, CAP (0.5 mg/kg), QUR (100 mg/kg), and CAP+QUR. Rats received intraperitoneal injections for 14 consecutive days. The control group received no treatment. The doses of CAP and QUR were chosen based on previous studies (26). The weight of the rats was measured at four-day intervals and at the time of dissection of each rat. Twenty-four hours after the last injection of CAP / QUR or CAP+QUR, the animals were euthanized painlessly after deep anesthesia with 90 mg/kg Ketamine (Batch No: 1402053-01) and 10 mg/kg Xylazine (Batch No: 087238-4), and blood samples were collected for biochemical and hormonal studies. The weight of the ovaries was measured and used for histopathological and molecular examination.

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Biochemical and hormonal assays

After clot formation, samples were centrifuged at 2500 g for 15 minutes to separate the serum (Froilabo SW14, France). Serum anti-Mullerian hormone (AMH) was determined by the enzyme-linked immunosorbent assay (ELISA) method, and progesterone (P) and E2 were determined by the chemiluminescence immunoassay (CLIA) method. The concentration of these hormones was determined by measuring the absorbance at 450 nm using a spectrophotometer (Jenway 3620D, England).

Ferric reducing antioxidant power assay

The total antioxidant capacity (TAC) of the serum was evaluated by the Ferric Reducing Antioxidant Power Assay method (FRAP). Briefly, 150 μl of serum was mixed with 1.5 ml of fresh FRAP reagent (10 mM 2, 4, 6-tripyridyl-s-triazine, 20 mM FeCl₃, 6H₂O solution, and 300 mM acetate buffer pH=3.6) and incubated at 37°C. The incubation lasted for 10 minutes. Subsequently, the absorbance was measured at 593 nm using a spectrophotometer (Jenway 3620D, England) and compared to a standard curve obtained with known concentrations of FeSO₄ 7H₂O (27).

Histological examinations

Ovarian tissue was fixed in 10% formalin and embedded in paraffin wax, they were cut into 5 μm thick serial sections, mounted on slides, and stained with hematoxylin and eosin (H-E). Five larger and complete sections for each ovary were examined and the mean value of the number of primary, preantral, antral follicles and corpora lutea was determined using an optical light microscope (ZEISS Primostar 3, Germany).

Gene expression

The effects of CAP and QUR on the expression levels of BAX, BCL-2 and p53 genes were measured using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). In this method, Master Mix 2x SYBR Green I, High Rox (BioFACT, South Korea Cat. No. DQ385-40h) was used. Total mRNA was extracted from ovarian samples using Trizol reagent (Life Biolab, Germany, CatNO.LB38055) according to the manufacturer’s instructions and then the quality and quantity of RNA was determined using a NanoDrop spectrophotometer (2000c, Thermo Scientific, Grand Island, New York, USA). cDNA was immediately synthesized using Two-step cDNA synthesis kit (BioFACT, South Korea) according to the instructions provided by the manufacturer. qRT-PCR was performed using a qRT-PCR system (Real-time PCR Applied Biosystems ™ Real-Time PCR, USA). The PCR primers are listed in the supplementary Table 1. GAPDH housekeeping gene was used as internal reference. The target gene expression was normalized to GAPDH and calculated using the comparative quantification method (2⁻ΔΔCT). Cycle conditions were as follows: 95°C for 15 minutes (denaturation) and was followed by 40 cycles (95°C for 15 seconds and 60°C for 60 seconds).

Statistical analysis

Results of this experiment were analyzed to identify the significant levels using One-way ANOVA in GraphPad Prism software (GraphPad Prism 9, Software Inc., USA) and presented as the mean ± SEM. P<0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>Primer sequence (5′-3′)</th>
<th>Amplicon length (bp)</th>
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<tr>
<td>BAX</td>
<td>24887</td>
<td>F: CCTGTGCACCAAGGTGCCGAAC&lt;br&gt;R: CCACCCTGGTCTTGAGATCCAGCC</td>
<td>99</td>
</tr>
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<td>24224</td>
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<tr>
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<td>24842</td>
<td>F: TAACAGTTCTGCATGGGGCCG&lt;br&gt;R: AGGACAGGCACAACACGCACC</td>
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<td>24383</td>
<td>F: GTCTCTCTAGCTTCAACAGCG&lt;br&gt;R: ACCACCTGGTGCTGTAGCCAA</td>
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</tr>
</tbody>
</table>
Results

Body and ovarian weight

CYC significantly decreased body weight in POF and dimethyl sulfoxide (DMSO) groups (187.5 vs. initial weight 218.75, 189.32 vs. initial weight 213.47, respectively) (P<0.0001). Treatment with CAP and QUR increase body weight in all groups, and there was a significant (P<0.01, P<0.001) increase body weight in QUR and CAP+QUR groups compared to POF group (Fig.1A). Furthermore, there was a non-significant decrease in ovarian weights of POF and DMSO groups compared to the control group. Ovarian weight of QUR and CAP+QUR was significantly increased after 14 days of treatment (Fig.1B).

FRAP assay

TAC levels were significantly decreased in POF and DMSO groups compared to the control group (P<0.0001). There was a significant increase in TAC level in CAP, QUR and CAP+QUR groups after receiving the treatment compared to the POF group (Fig.2A).

Hormonal assessment

CYC-induced POF decreased AMH level and treatment with CAP and QUR increased it. AMH level in CAP, QUR and CAP+QUR groups showed a significant improvement in comparison with POF and DMSO groups (Fig.2B). Estradiol and Progesterone level were also decreased in POF and DMSO groups. E2 level was significantly increased in QUR and CAP+QUR group (Fig.2C). Progesterone level was increased in CAP, QUR and CAP+QUR, while the biggest impact was observed in the CAP group (P<0.0001, Fig.2D).

Molecular assessment

The qRT-PCR results showed a significant increase of BAX and P53 genes expression in POF group compared to the control group (P<0.001, P<0.0001, respectively). BAX gene expression in CAP, QUR and CAP+QUR groups were downregulated after 14 days of treatment. Expression level of P53 gene was decreased significantly in all the treatment groups (P<0.0001); However, there were no significant differences between treated groups regarding the BAX and P53 genes expression. Our result for BCL-2 gene expression showed a significant increase in all the treatment groups (P<0.0001, Fig.3A-C).

Histological results

Histological parameters were significantly improved after administration of CAP and QUR. Our results showed a significant increase in the number of primary follicles in the CAP, QUR and CAP+QUR groups compared to the POF and DMSO groups (P<0.0001). There were no significant differences between CAP and QUR groups and a significant increase in the number of primary follicles was observed in CAP+QUR group compared to CAP and QUR groups (P<0.001). The number of preantral and antral follicles in the CAP, QUR and CAP+QUR groups was significantly increased compared to the POF and DMSO groups (P<0.05), and this increase was significantly higher in the CAP+QUR group than in the other treatment groups (P<0.001). Similarly, a significant increase in the number of Copora lutea was observed in the CAP and QUR groups compared to the POF and DMSO groups. An increase was observed in the CAP+QUR group, but it was not significant (Figs.4A-D, 5).

Fig.1: Changes in body and ovarian weight in CAP and/or QUR-treated POF rats compared to POF and DMSO groups. A. Body and B. Ovarian weight in different treatment groups (P<0.05). Data were represented as mean ± SEM (n=6). Different letters mean significant differences between groups. POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.
**Fig. 2:** Changes in TAC level in CAP and/or QUR-treated POF rats compared to POF and DMSO groups. A. Changes in TAC level in CAP and/or QUR-treated POF rats compared to POF and DMSO groups (P<0.05). TAC increased in the groups treated with CAP and QUR (0.5, 100 mg/kg) compared to the POF and DMSO groups. B. AMH level was assessed in different groups by the enzyme-linked immunosorbent assay (ELISA). Data is expressed as AMH ng/ml of serum. C, D. CAP and QUR increased estradiol and progesterone concentrations in treatment groups by CLIA. Data are expressed as estradiol and progesterone pg/ml of serum. Data were represented as mean ± SEM (n=6). Different letters mean significant differences between groups. TAC; Total Antioxidant Capacity, POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin, AMH; Anti-Müllerian Hormone, and CLIA; Chemiluminescence immunoassay.

**Fig. 3:** Genes expression levels in CAP and/or QUR-treated POF rats compared to POF and DMSO groups. A, B. BAX and PS3 expression significantly decreased in CAP and/or QUR-treated POF rats compared to POF group (P<0.05). C. BCL-2 expression significantly increased in CAP and/or QUR-treated POF rats compared to POF Group (P<0.05). Data were represented as mean ± SEM (n=6). Different letters indicate significant differences between groups. POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.
Fig. 4: Comparison of the number of follicles in different groups. A. Primary follicles, B. Preantral follicle, C. Antral follicle and D. Corpora lutea. Different letters indicate significant differences between groups. Data are presented as mean ± SEM (P<0.05, n=6). Different letters mean significant differences between groups.

POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.

Fig. 5: Photomicrograph of ovarian tissue (H&E staining; 10, scale bar: 200 μm). Primary follicles (yellow arrows), preantral follicles (green arrows), graafian follicles (red arrows), antral follicle (blue arrow), and corpora lutea (black arrows). POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.
Discussion

In this study, the ovarian protective effects of CAP and QUR were investigated in a rat model of CYC-induced POF.

Chemotherapy played a causative role in ovarian reserve damage, which was associated with increased tissue oxidative stress, impaired hormone secretion and gene expression, and increased histological damage. In contrast, tissue oxidative stress parameters, ovarian reserve markers, and histopathological changes were significantly improved in rats receiving CAP and QUR alone or in combination after chemotherapy.

To the best of our knowledge, this is the first report to investigate the effects of CAP and QUR on the prevention of chemotherapy-induced ovarian damage. The results suggest that these two agents have a protective effect against CYC-induced ovarian failure. Ovarian weight of rats exposed to CYC was significantly reduced compared to the control group, which may be attributed to the ovarian toxicity of alkylating agents such as CYC. These agents generate DNA cross-links, which in turn induce DNA breaks and ultimately trigger apoptosis (28). In all treatment groups after being treated with CAP and QUR ovarian weight was significantly increased.

Roness et al investigated agents that prevent chemotherapy-induced ovarian damage and found that AS -101, AMH, imatinib, sphingosine-1-phosphate, granulocyte colony-stimulating factor, bortezomib, and multidrug resistance gene-1 play key roles in preventing chemotherapy-induced ovarian damage. They found several mechanisms of action associated with each protective agent, including prevention of follicle activation, anti-apoptosis effects, vascular effects, and upregulation of genes (29). CAP has been demonstrated to exert anti-apoptotic activity by over-activating TRPV1 (30). Tsuji and Aono (31) demonstrated that SA13353 (1-[2-((1-adamantyl)ethyl]-1-pentyl-3-[3-(4-pyridyl) propyl]urea), a novel TRPV1 agonist, inhibited tumor necrosis factor-α production by activating capsaicin-sensitive afferent neurons and reduced symptom severity in renal injury, lung inflammation, arthritis, and encephalomyelitis. These results suggest that TRPV1 agonists may act as anti-inflammatory in vivo in certain inflammatory and autoimmune diseases. In addition, Leonelli et al. (32) have shown that TRPV1 channels are involved in the control of early apoptosis during retinal development and that mitogen-activated protein kinase signaling may be involved in this process. Arzuman et al. (33) reported the beneficial effects of CAP and curcumin with monofunctional platinum (II) complex in platinum-resistant ovarian cancer cell lines.

In current experiment we demonstrated that CAP and QUR downregulate the expression of pro-apoptotic genes, P53 and BAX, and upregulate BCL-2 gene expression. Melekoglu et al. (34) showed that treatment with CAP and Curcumin can improve tissue oxidative stress markers. Odunisi et al. (35) reported that curcumin can improve ovarian function recovery after chemotherapy exposure. In this study, we demonstrated that treatments with CAP and QUR improved histological parameters in ovarian tissue subjected to treatment with CYC. The results of our study confirmed the protective effects of CAP and QUR against CYC-induced ovarian failure in rat ovaries by demonstrating the antioxidant effects of CAP and QUR, including increased antioxidant activity and improved histological parameters. We demonstrated a significant improvement in TAC by CAP and QUR after chemotherapy-induced ovarian damage. Similarly, Chaudhary et al. investigated the protective effect of CAP against oxidative stress. They found that CAP had a significant protective effect against oxidative stress by increasing FRAP, GSH level and PMRS activity, and improving ROS, MDA, PCO and AOPP (36). In agreement with this study, Park et al. (37) demonstrated a protective effect of CAP against testicular injury induced by scrotal hyperthermia. They showed that pretreatment with CAP significantly suppressed oxidative stress malondialdehyde (MDA) level, phospholipid hydroperoxide GPx, heat shock 70-kDa protein 1, and manganese SOD) and heat stress-induced apoptosis in testes. The results of this study also showed that CAP and QUR improved the markers of ovarian reserve after CYC-induced ovarian failure. Significant increase in AMH, E2 and progesterone levels were observed in POF+CAP, POF+QUR and POF+CAP+QUR groups compared to POF group.

Few experimental studies have examined the effects of chemotherapy-induced ovarian damage and antioxidants on markers of ovarian reserve. Özcan et al. (38) investigated the effect of resveratrol against cisplatin-induced oxidative damage to ovarian reserve in rats. They found that resveratrol significantly increased AMH levels compared to the control group. The improvements observed in ovarian reserve markers in their study suggest that resveratrol, a phenolic compound, has a beneficial effect on ovarian function recovery after chemotherapy exposure. In the present study, we demonstrated that treatments with CAP and QUR improved histological parameters in ovarian tissue subjected to treatment with CYC. The number of primary follicles, preantral follicles, antral follicles and corpus luteum was significantly reduced in POF rats compared to the control group. Similarly, Elkady et al. (39) and Melekoglu et al. (34) in separate studies showed a decrease in primordial follicles and an increase in atretic follicles in the POF model. The mechanism by which CAP and QUR protect ovarian tissue...
may be related to reduced exposure to oxidative damage and reduced stimulation of TRPV receptors, which are thought to have antioxidant and anti-inflammatory activities (31).

Nowadays, using combination therapies is one of the most common strategies to achieve better clinical outcomes. Previous studies have reported the combined effects of natural compounds on altering the amount of apoptotic genes (27); therefore, due to resistance, toxicity and side effect of chemotherapeutic agents, the use of natural compounds as complementary therapies can be a promising alternative for current ineffective treatments.

Conclusion

Our results indicated that CAP and QUR show anti-apoptotic and anti-cancer effects, we confirmed that CAP and QUR alone, and in combination improved the TAC, hormone secretion, apoptosis regulatory genes expression and the number of follicles; CAP and QUR as natural occurrence antioxidants can be used to reduce and even prevent the adverse effects of chemotherapy on the ovaries in cancer patients.

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Authors’ Contributions

Z.R., M.Kh.; Contributed to conception and study design. S.M., Z.R.; Contributed to all experimental work, data collection and evaluation, drafting and statistical analysis. All authors performed editing and approving the final version of this manuscript for submission, participated in the finalization of the manuscript and approved the final draft.

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Oleuropein as An Effective Suppressor of Inflammation and MicroRNA-146a Expression in Patients with Rheumatoid Arthritis

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Abstract

Objective: Rheumatoid arthritis (RA) is a common progressive autoimmune disorder that causes chronic inflammation of the joints and damage to other organs. Previous studies have reported the important role of miRNA-146a in the pathogenesis of RA. In addition, the anti-inflammatory and modulatory effects of oleuropein (OLEU) on the expression pattern of microRNAs (miRNAs) have been shown in different diseases. Therefore, this study aimed to evaluate both the sensitivity and specificity of miRNA-146a and determine the potential effects of OLEU on the expression levels of miRNA-146a and tumour necrosis factor-alpha (TNF-α) in RA patients.

Materials and Methods: The participants in this experimental study were divided into 2 groups: RA (n=45) and healthy controls (n=30). The isolated peripheral blood mononuclear cells (PBMCs) were treated with different concentrations of OLEU, and the level of TNF-α expression, anti-citrullinated protein, and miRNA-146a were determined using enzyme-linked immunoassay and real-time polymerase chain reaction, respectively. In addition, the receiver operating characteristic (ROC) curve analysis evaluated the sensitivity and specificity of miRNA-146a in RA patients.

Results: Results revealed a positive correlation between the levels of miRNA-146a expression with the serum levels of C-reactive protein (CRP) and rheumatoid factor (RF) in RA patients. In addition, OLEU treatment decreased the levels of TNF-α and miRNA-146a expression in treated PBMCs samples compared with untreated cells. The ROC curve analysis showed an 85% sensitivity and 100% specificity of miRNA-146a in RA patients.

Conclusion: Therefore, miRNA-146a can be used as a useful biomarker for RA diagnosis, particularly for early detection. In addition, OLEU could suppress inflammation in RA patients through the regulation of miRNA-146a.

Keywords: Anti-Citrullinated Protein, miRNA-146a, Oleuropein, Rheumatoid Arthritis, Tumour Necrosis Factor-Alph

Introduction

Rheumatoid arthritis (RA) is a progressive chronic autoimmune disease (1). Chronic inflammatory reactions at the cellular and molecular levels are major causes of bone and cartilage degeneration in this disease (2). Deposition of immunoglobulin (Ig) IgM-IgG complexes in joints, infiltration of inflammatory cells, and production of proinflammatory cytokines and chemokines have been proposed as the most important causes of inflammation (3). In addition, during the inflammation process, an autoantibody called anti-citrullinated protein (anti-CCP) antibody is produced after enzymatically converting arginine amino acid residues into citrulline in both rheumatoid factor (RF)-positive and RF-negative RA patients. Studies have shown that tissue degradation and the development of anti-CCP antibodies are caused by the local abnormal citrullination of various proteins, such as fibrins, in RA joints. In the very early phases of RA disease, these antibodies were identified with 95% specificity and >70% sensitivity (4).

According to previous studies, microRNAs (miRNAs) may play a significant role in the etiology of several disorders, including autoimmune diseases (5, 6). miRNAs are a type of short noncoding RNA molecule that regulate the posttranscriptional expression of proteins in different physiological and pathophysiological conditions (7, 8). It is well established that miRNAs are able to regulate different immune responses by suppressing the expression of many molecules, such as transcriptional factors, cytokines, chemokines, and key signaling proteins at the posttranscriptional level (7, 9, 10). However, several
studies reported the association between the expression level of different miRNAs with various autoimmune disorders such as RA (7, 11-13). Numerous studies have also examined the function of miRNA-146a in the regulation of the immune system, particularly inflammatory responses (14-16). Taganov et al. (17) study revealed that treatment of THP-1 cells, a human leukemia monocytic cell line, with microbial lipopolysaccharide (LPS), interleukin (IL)-1β, and tumour necrosis factor-alpha (TNF-α) increased the expression levels of mature miR-146a via activation of nuclear transcriptional factor (NF)-κB. Besides, Pauley et al. (9) reported the association between the upregulation of miR-146a with suppression of IL-1 receptor-associated kinase 1 (IRAK-1) and TNF receptor-associated factor 6 (TRAF6)-2 key molecules in the induction of proinflammatory cytokines IL-1 and TNF-α.

In addition, a previous study investigated that the level of miR-146a expression was related to a decrease in TNF-α production and disease activity of patients with RA (18). It has been also found that miRNA-146a stimulates the differentiation of Th17 cells by regulation of IRAK-1 and suppressor of cytokine signaling 1 in RA patients (19). Overexpression of miR-146a regulates FAS-associated factor 1 and inhibition of CD4+ T-cells apoptosis in patients with RA (20). Inhibition of apoptosis may be leading to inflammation progress and increased severity of RA. As a result, alteration in the expression levels of miR-146a could be related to inflammatory immune responses and the pathogenesis of RA. On the other hand, it was revealed that diverse natural/herbal products are a growing source of new medications that exhibit effective and therapeutic effects against a variety of disorders by controlling the expression of miRNAs.

There is a growing interest in the pharmacological and medical uses of oleuropein (OLEU), a nontoxic polyphenolic chemical found in olive tree leaves and olive oil because of its anti-inflammatory and antioxidant properties (21, 22). The results of our previous study revealed the positive effects of OLEU in shifting CD4+ T cells to CD4+CD25+FoxP3 regulatory T cells (Tregs) in RA patients in a dose-dependent manner (23). Moreover, various studies revealed the modulation effects of OLEU on the pattern of miRNAs expression in several disorders such as cancer (24, 25). These findings suggest the important impacts of OLEU on the expression level of miR-146a in RA patients. This study aimed to evaluate the sensitivity and specificity of miRNA-146a in RA patients. In addition, the possible relationship between the expressions of miRNA-146a with TNFα was evaluated. Finally, the effects of OLEU on the expression pattern of these factors were investigated.

Material and Methods

Participants

This experimental study was conducted at the Shahroud University of Medical Sciences, Shahroud, Iran, from November 2020 to July 2021. The minimum sample size of 41 was considered for the study group and 25 cases for the control using statistical tools. In this study, the medical histories of 45 RA cases referred to the rheumatology clinic were screened. The Disease Activity Score 28 (DAS-28) for these patients with RA was finalized based on clinical symptoms and the C-reactive protein (CRP) value.

Inclusion criteria

The study included RA patients who were over 18 and below 60 years of age at the time of diagnosis with active disease, had a disease duration of more than a year, and fell under the 1987 Revised Criteria for Disease Classification of the American Rheumatism Association.

Exclusion criteria

Participants under 18 years with a disease duration of <1 year who were also taking common immunosuppressants, such as infectious illnesses, immunodeficiency, uncontrolled blood pressure, and hepatic or renal disorders, were excluded from the study. A control group of 30 healthy people with no signs of disease or a family history of autoimmune disorders was used in this study. Inflammatory conditions of any kind, RF positivity, and increased CRP were all considered exclusion criteria for the control group. The pregnant participants who had a special diet or were breastfeeding were also excluded from the study.

Blood sampling and peripheral blood mononuclear cells isolation

Blood samples were taken from both types of patients with RA and HCs, preserved in 2 serum-separating tubes and dipotassium (K2) ethylene diamine tetraacetic acid (EDTA) tubes to separate serum samples and peripheral blood mononuclear cells (PBMCs), respectively. After centrifuging, the serum sample was separated and maintained at -20°C until evaluation. PBMCs were also separated from whole blood samples using the standard Density Gradient Centrifugation (DGC) technique and Ficoll-Paque (GE Healthcare®, Buckinghamshire, UK) (26). The percentage of live cells was assessed using a trypan blue exclusion assay. PBMCs were suspended in an RPMI-1640 (Gibco, USA) medium supplemented with 100 ug/ml penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (Gibco, USA). 5×10^5 cells of isolated PBMCs were transferred to each well of 24-well tissue culture plates and incubated for 72 hours at 95% humidity and 37°C after stimulation with appropriate concentrations of LPS (Gibco, USA) to stimulate cell proliferation and enhance the total number of cells. The results were compared with the cell culture of the control group, both with and without LPS stimulation.

Anti-citrullinated protein measurement

The serum level of anti-CCP was measured...
using enzyme-linked immunoassay (ELISA) kits (ichromx Anti-CCP Plus, Boditech, South Korea, Lot No: ACREA15E) for all HCs and RA patients according to manufacturer’s protocol. In brief, 100 µL of reference controls, Calibrators, and each sample were transferred into appropriate wells and incubated for 60 ± 10 minutes at 18°C to 25°C. Each well was washed 4 times with 300 µL of wash buffer. In addition, 100 µL of conjugated antibody was added to each well and incubated at 18°C to 25°C for 30 ± 5 minutes. The plates were washed 4 times as described previously. Then, 100 µL of substrate solution was added to each well and the plates were incubated at 18°C to 25°C for 30 ± 5 minutes. Then, 100 µL of stop solution was added to wells to stop the reactions. The optical density (OD) of each sample was determined at 450 nm using an ELISA reader.

**MiRNA-146a extraction**

Extraction of miRNA146a from serum or isolated PBMCs was performed using a TBioFACT kit (Korea, Cat No: Rp101-050/RP101-100) according to manufactures instructions. Briefly, 10 µL of 2ME buffer, 350 µL of RB buffer, and 5 µL of protein kinase K were added to the 250 µL of serum, or the cells list was vortexed for 30 seconds and incubated at 56ºC for 10 minutes. The samples were centrifuged at 1400 rpm for 3 minutes at 4°C, followed by transferring supernatants to a new Microtube. In addition, 250 µL of ethanol (100%) was added to each Microtube and vortexed for 30 seconds at RT. The prepared solution was pipetted to the spin columns that were placed into collection tubes and centrifuged at 1400 rpm for 30 seconds at 4°C. The columns were transferred to a new collection tube, and 500 µL of RNA washing solution (RW buffer) was added to each spin column and centrifuged for 30 seconds at 1400 rpm at 4°C. This step was repeated twice. The columns were transferred to an RNase and DNase free Microtube, and 100 µL of RNase-free water was added to each column and incubated for 1 minute at RT, followed by centrifugation for 1 minute at 1400 rpm and 4°C. Then, fluids containing RNA were collected. Finally, the concentration of RNAs was determined using nanodrop and maintained at -80°C until use.

**cDNA synthesis**

The cDNA synthesis was conducted using the Ana micro–RNA Detection kit (Norgen Biotek, Canada, Cat No: MI001) according to the manufacturer’s protocols. Two Microtubes were prepared for each sample. Moreover, 1 µL of extracting mine was transferred into each Microtube. Appropriate concentrations of RT stem-loop housekeeping and RT stem-loop miRNA146a primers were added into tubes. The prepared final volume of each solution was at 14.5 µL using RNase-free distilled water. Microtubes were incubated at 70°C for 5 minutes and then spun quickly. Each Microtube was placed in the cool box; and 4 ml of the 5X RT reaction buffer, 1 µL of dNTP mix (10 mm), and 0.5 µL of RT enzyme were added at a final volume of 20 µL. The tubes were placed in a thermocycler, and the synthesis of cDNA was performed according to the following protocol: 60 minutes at 37°C and 5 minutes at 70°C.

**Real-time quantitative polymerase chain reaction**

Real-time quantitative polymerase chain reaction (PCR) was performed according to previous studies(27). The procedure of this method is defined in a Table 1. Briefly, 2 µL of miRNA146a and the housekeeping gene were added to separate the strip and then 10 µL of Sybr Green was pipetted to the strips. Besides, 0.8 µL of forward and reverse primers were added to the strips, adjusting the final volume to 20 µL with distilled water. Finally, the strips were lidded, and a real-time PCR reaction was carried out using the BioRad instrument according to the manufacturer’s protocol. The cycle threshold of the samples was calculated using the 2−∆∆CT formula.

**OLEU preparation**

To prepare a stock solution of OLEU, Cayman Chemical Company’s powdered olive leaf extract (Sigma Aldrich, USA, Cat: 12247-10MG) was dissolved in dimethyl sulfoxide (DMSO), which was then stored at -20°C or -80°C until use.

<table>
<thead>
<tr>
<th>Table 1: Real-time polymerase chain reaction procedure</th>
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<tr>
<td>Material</td>
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<tr>
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</tr>
<tr>
<td>cDNA</td>
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<tr>
<td>2X QPCR Master mix (Sybr Green)</td>
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<tr>
<td>miRNA146a-specific forward primer (10 µm)</td>
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<td>HK-specific forward primer (10 µm)</td>
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<td>Revers primer (10 µm)</td>
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<td>dH2O</td>
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<td>Final volume</td>
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**Investigation of the cytotoxic effects of OLEU on PBMCs**

The cytotoxic effects of various concentrations of OLEU on PBMCs were assessed using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test based on the previous studies (23). The wells with only OLEU solvent (DMSO) and LPS were selected as controls. The results were determined as a percentage of OD in both treated and control cells. Using linear regression, the half-maximal inhibitory concentration (IC₅₀) was evaluated. A suitable concentration (100 µg/ml) of OLEU was established to continue the study.
Tumour necrosis factor-alpha measurement

The concentration of TNF-α was measured in the serum of patients with RA and HCs as well as cell culture supernatants from both untreated and treated PBMCs with 100 μg/ml OLEU (optimal dose after MTT assay). The serum samples and supernatants were collected, spun down at 1500 rpm for 5 minutes, and cell-free samples were stored at -80°C until use. The immunoassays technique was performed in duplicate using human TNF-α commercial ELISA kits (Bioassay Technology Laboratory, China) according to the kit instructions. The OD of serum samples and supernatants of each well was determined by the ELISA reader at 450 nm and the concentration of TNF-α was calculated using a standard curve and dilution factor.

Statistical analysis

The serum levels of RF and CRP in RA patients compared with healthy individuals presented as mean ± SEM. A t test was used to analyze serum levels of TNF-α and anti-CCP between patients and the control group. The expression of miRNA-146a was analyzed as the 2^-ΔΔCt method. P<0.05 was considered statistically significant. The correlation between anti-CCP with miRNA-146a was determined using linear regression. The sensitivity and specificity of anti-CCP and miRNA-146a were determined by receiver operating characteristic (ROC) curve analysis.

Ethics approval and consent to participate

The study protocol was approved by the local Ethics Committee (IR.SHMU.REC.1398.100) of Shahroud University of Medical Sciences, Shahroud, Iran. All the experimental methods followed the guidelines of the Declaration of Helsinki (2008), as stated in the guidelines of Iran’s Medical Ethics Committee, Ministry of Health. The samples were collected after receiving informed consent from every participant.

Results

Demographic, clinical, and laboratory information

Table S1 (See Supplementary Online Information at www.celljournal.org) shows the demographic, clinical, and laboratory parameters of RA patients and healthy controls (HCs). All patients were under treatment with sulfasalazine, hydroxychloroquine, leflunomide, methotrexate, and azathioprine either alone or in combination with prednisone. The duration of RA disease in all patients was more than 1 year, and the disease activities were performed according to the DAS28 score. The DAS–28 <2.6: remission; the DAS–28 ≥2.6 and ≤3.2: low disease activity; the DAS–28 >3.2 and ≤5.1: moderate disease activity; and the DAS–28 >5.1: high disease activity. All HCs were RF- and CRP-negative. Blood samples were collected from each RA patient to analyze the main diagnostic factors, including RF, CRP, and anti-CCP.

Cytotoxicity effects of OLEU

Based on the IC_{50} value, treatment of PBMCs with various concentrations of OLEU (50-200 μg/mL) for 24, 48, and 72 hours did not reveal a significant effect on the percentage of cell viability at 50 and 100 μg/mL in all times of the treatment. Therefore, treatment of PBMCs with 100 μg/mL of OLEU for 24 hours was selected to continue the study (Fig.1).

Fig.1: Effects of various concentrations of OLEU on the cell viability (%) at time intervals. A. 24 hours, B. 48 hours, and C. 72 hours. The IC{subscript}_{50} value showed that OLEU did not have cytotoxicity effects on the percentage of cell viability in different concentrations of 25, 50, and 100μg/ml at three examined time points. OLEU; Oleuropein, IC{subscript}_{50}; Half-maximal inhibitory concentration, LPS; Lipopolysaccharides, DMSO; Dimethyl sulfoxide, and h; Hours,
Expression levels of tumour necrosis factor-alpha

Higher levels of TNF-α were detected in the serum of RA patients compared with HCs (P<0.0001, Fig.2). Treatment of cultured PBMCs with an optimal concentration of OLEU significantly decreased the expression levels of this proinflammatory cytokine in supernatants of treated cells compared with untreated cells after 24 hours of incubation (P<0.0001, Fig.2A, B).

Fig.2: The levels of TNF-α. A. The serum levels of TNF-α were significantly higher in RA patients than in healthy controls. B. Whereas, a significant reduction in the amount of TNF-α was observed in treated PBMC samples with OLEU compared to untreated samples. TNF-α; Tumor necrosis factor alpha, RA; Rheumatoid Arthritis, PBMCs; Peripheral blood mononuclear cells, OLEU; Oleuropein, **; The significance level, and ****; P<0.0001.

Relative expression of miRNA-146a

The relative expression of miRNA-146a in the serum of patients with RA and HCs as well as treated PBMCs with 100 μg/mL of OLEU is depicted in Figure 2. The results of data analysis revealed higher levels of miRNA-146a in patients with RA compared with HCs (P<0.0001, Fig.3A). However, the expression level of miRNA-146a was significantly lower in treated PBMCs than untreated samples (P<0.006, Fig.3B).

Fig.3: The levels of miRNA146a. A. The results of qRT-PCR revealed higher levels of miRNA-146a in RA patients compared to the healthy controls. B. Significantly lower expression of miRNA-146a was identified in treated PBMCs samples with OLEU than in untreated samples. qRT-PCR; Quantitative real-time reverse transcription-polymerase chain reaction, PBMCs; Peripheral blood mononuclear cells, OLEU; Oleuropein, TNF-α; Tumor necrosis factor alpha, and ****; P<0.0001.

Association between CRP, RF, and anti-CCP levels with miRNA-146a

The linear regression analysis showed a positive correlation between different concentrations of CRP and RF with the expression levels of miRNA-146a (Fig.4A, B). However,
a negative association was identified between the levels of miRNA-146a expression with anti-CCP in RA patients (Fig.4C).

**Sensitivity and Specificity of anti-CCP and miRNA-146a**

The ROC analysis was performed to determine the sensitivity and specificity of anti-CCP and miRNA-146a in patients with RA. The sensitivity and specificity of anti-CCP were determined at 76 and 96%, respectively (area under the ROC curve [AUC]: 0.9733 at a cutoff value of >28 U/mL) (Fig.5A). In addition, the results showed an 85% sensitivity and 100% specificity of miRNA-146a in these patients (AUC: 0.9958 at a cutoff value of >2.19) (Fig.5B).

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**Fig.4:** Correlation between CRP, RF, and anti-CCP with miRNA-146a. A positive association were identified between different concentrations of A. CRP, and B. RF with miRNA-146a. C. While anti-CCP showed a negative association with expression levels of miRNA-146a. CRP; C-reactive protein, RF; Rheumatoid factor, and anti-CCP; Anti-citrullinated protein antibody.

**Fig.5:** The Sensitivity and specificity of anti-CCP and miRNA-146a. A. The sensitivity and specificity of anti-CCP were 76 and 100% respectively whereas, B. Shows a high sensitivity (85%) and specificity (100%) of miRNA-146a in patients with RA. anti-CCP; Anti-citrullinated protein antibody and RA; Rheumatoid arthritis.
Discussion

In this study, the expression levels of TNF-α and miRNA-146a were evaluated in patients with RA compared with HCs. The potential role of miRNA-146a in the regulation of immune responses and different biological activities has been reported by multiple studies (14, 16, 28). MiRNA-146a is involved in controlling inflammatory responses through different mechanisms such as the regulation of anti-inflammatory cytokine IL-1β (28, 29). This miRNA, together with miRNA-155, has a critical role in the gene expression profiling of Th1/Th2 lymphocytes through various mechanisms such as targeting critical inflammatory cytokines and different signaling pathway proteins (30, 31). In addition, miRNA-146a modulates the regulation of signal transducer and activator transcription 1 activator and promotes lymphocyte responses to Th1 cells (32).

A positive association was also reported between the increased expression of miRNA-146a with various inflammatory disorders of the central nervous system, such as Alzheimer disease (33). Kriegsmann et al. (34) detected higher levels of miRNA-146a in synovial fluid of RA patients compared with patients with osteoarthritis and introducing miRNA-146a as a beneficial biomarker for diagnosis of Sjögren syndrome.

A potential correlation between the relative expression levels of this miRNA-146a and various concentrations of CRP, RF, and anti-CCP was also examined in reference to studies that have shown a substantial correlation between the levels of expression of this miRNA and the severity of the RA.

In the present study, data analysis indicated a significant positive correlation between the relative expression levels of miRNA-146a with RF and CRP. However, the results showed that miRNA-146a is inversely associated with anti-CCP concentration, which may be related to an increase in the frequency of T follicular regulatory cells in inactive or stable-remission patients (35). T follicular regulatory cells are a subset of T cells that control immunological responses in the germinal center (GC) by interacting with T follicular helper and preventing the generation of antibodies such as anti-CCP (36).

These data could be suggested a positive association between miRNA-146a and the pathogenesis of RA. In contrast to these findings, other studies have revealed contradictory data on the relationship between miRNA-146a and the pathophysiology of several autoimmune disorders, including RA. Churov et al. (37) reported a limited association between the levels of miRNA-146a expression and RA disease severity in a systematic review study. Fan et al. (38) have reported a decreased expression of miRNA-146a in the serum of patients with systemic lupus erythematosus (SLE), which was associated with a reduction in CRP in these patients. However, Sun et al. (39) showed no association between the levels of miRNA-146a and the risk of SLE.

Another meta-analysis study conducted by Fan et al. (38) on the association between miRNA-146a and SLE showed a significant association between the expression levels of miRNA-146 with susceptibility to SLE in European and Asian populations. This discrepancy may be due to the use of a limited sample size in previous research.

On the other hand, we evaluated the potential effects of OLEU, as a natural anti-inflammatory and anti-oxidative product, on the expression levels of TNF-α and miRNA-146a. Data showed that OLEU significantly decreased the production of TNF-α in the culture media of treated PBMCs compared with untreated cells, which is inconsistent with prior findings. It also has the ability to reduce the level of miRNA-146a in treated PBMCs. Several studies have revealed the potential effects of OLEU on the expression pattern of different miRNAs in various disorders. Besides, they proposed OLEU as an important anti-inflammatory product (21, 40). However, the small sample size is the main limitation of the present study. In addition, the potential effects of OLEU on other RA pathogenesis-related parameters have not been studied. Future research is necessary to introduce OLEU as a beneficial natural medicine for RA treatment.

Conclusion

The present study revealed a significant correlation between miRNA-146a expression levels and key RA pathogenesis-related parameters, including RF and anti-CCP. This suggested that miRNA-146a might play an important role in RA susceptibility as well as other RA-related autoimmune diseases including SLE. Consequently, miRNA-146a may be a useful and valuable biomarker for the diagnosis or early detection of RA. In addition, it seems that OLEU could modulate immune responses by decreasing the levels of main proinflammatory factors such as TNF-α. However, future studies with larger sample sizes are necessary to introduce OLEU as a beneficial natural medicine for RA treatment.

Acknowledgments

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Authors’ Contributions

H.M., Z.Y., F.T., M.Y., M.J.S., R.J.; Conceptualization, Investigation, Writing - Original Draft, and Methodology. Z.Y., R.J.; Review and Editing. M-H.S., N.S.; Data analysis. The authors read and approved the final manuscript.

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Oleuropein Regulates Inflammation and miRNA-146a Expression in RA Patients


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