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Original Article

Embryonic stem cell differentiation to primordial germ cell like cells by Nigella sativa, Brassica Oleracea and Oenothera biennis extracts

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Abstract: Objectives: This study aimed to investigate the induction effects of methanolic extracts of Nigella sativa (NiS), Brassica Oleracea (BrO), and Oenothera biennia (Obi) on transgenic embryonic stem cells (ESCs) and to evaluate the ability of germ cells (GCs) production using these pluripotent cells. Methods: ESCs were amplified using a feeder layer. Embryoid bodies enzymatically dissociated to single cells and induced the extracts in gelatinized plates. Then RNA extraction and cDNA synthesis were performed. In the presence of appropriate primers, the desired genes were quantitatively evaluated by quantitative polymerase chain reaction (qPCR). Results: The copies of all genes in the control group showed a decreasing trend during the first to third weeks. Compared to the control group, the expression level of sex determining region Y-box 2 gene (Sox2) showed the highest level. All four evaluated genes increased in all Obi groups compared to the control group. There is also a slight increase in the Nanog homeobox gene (Nanog). Obi extract in different concentrations has increased the expression of the Sox2 gene. Increased expression of this gene along with octamer-binding transcription factor 4 gene (Oct4) and Nanog indicates a condition close to germ cell-like cells (GCLCs). Conclusions: According to the results of this study, NiS can increase expression of the Oct4, Sox2, Nanog, and stimulated by retinoic acid gene 8 (STRA8) genes and so increase the hope of GCs production. Storage of cells for 21 days in the presence of the extract compared to 14 days has a negative effect on cell growth and differentiation. The effects of meiosis onset and GCs production can be expected in the presence of some herbal extracts. Optimal utilization of these extracts requires further study in the field of different extracts and fractions of each extract to more effectively and purposefully direct the differentiation of stem cells.

Keywords: Mouse embryonic stem cells, differentiation, germ cells, herbal medicine, Nigella sativa, Brassica Oleracea, Oenothera biennia

Introduction

One of the current challenges is how to obtain germ cells (GCs) from stem cells to treat cancer and infertility patients. Human umbilical cord mesenchymal stem cells (hMSCs) have been recently introduced with higher efficacy. To optimize a co-culture method for hMSCs with placental cells to obtain primordial germ cells (PGCs) and to get oocyte-like cells (OLCs) in vitro, an experimental study showed that when hMSCs with placental cells (including amniotic and chorionic cells) were cultured simultaneously, PGC-specific markers, Octamer-binding transcription factor 4 (OCT4) and DEAD box helicase 4 (DDX4), expressed significantly. Still the expression of specific OLC markers such as growth differentiation factor 9 (GDF9) and zona pellucida glycoprotein 3 (ZP3) did not reach statistically significant thresholds [1]. Embryonic stem cells (ESCs) could be obtained from early embryos or early gonads. Due to the ability of pluripotency in ESCs, their differentiation into many types of body cells can be induced in dif-

ferent ways. Consequently, induction of ESCs to differentiate into functional spermatids is feasible. Sarma Urooza C. et al., showed that specific transgenic mouse ESCs (mESCs) could differentiate into OLC structures by tracking the differentiation of these cells in a monolayer culture base [2]. Twenty years ago, ESCs co-cultured with trophoblast cells and other bone morphogenetic protein 4 (BMP4) producing cells; differentiated into PGCs. After PGCs are transplanted into the subcapsular position of the host testis, the sign of tubules and spermatids are appeared after 8 months. These round spermatids have the ability to fertilize the oocytes [3]. Other studies showed that mESCs could produce germ progenitor cells in vitro. Culture medium containing low level of glucose disrupts the formation of GCs from ESCs. The role of STRA8 and protamine 1 (Prm1) promoters to obtain spermatocytes and spermatids from ESCs has been proven by enhanced green fluorescent protein (EGFP) and red fluorescent protein (DsRed) in vitro, especially these spermatids were able to fertilize healthy oocytes [4]. Nayernia and his colleagues showed that ESCs-derived gametes could be fertilized and result in impregnated mice after embryo transfer [5]. It has been shown that in addition to stimulating gene expression using chemical inducers and induction of promoters, co-culture with some cells is also capable of purposefully differentiating ESCs into germ and germ cell-like cells (GCLCs). It has been shown that the co-culture of human ESCs (hESCs) with mitomycin-C inactivated porcine ovarian fibroblasts promote a condition for the differentiation of hESCs into spermatids. The basis of human ESC differentiation into GCs is largely unclear, and effective methods for generating male and female GCs from hESCs need to be further explored.

In addition to binding to their gene promoters, Oct4, Sox2, and Nanog homeobox gene (Nanog) can identify and bind to hundreds of other genes in stem cells [6, 7]. In many cases, stem cell pluripotency is coordinated by binding these three key pluripotency regulators to target genes. These three factors activate and inactivate the transcription of various genes. For example, they activate genes of different transcription factors and chromatin-modifying enzymes. Genes involved in essential signal pathways in ESCs, such as the transforming growth factor beta (TGF-β) and Wnt pathways, are transcriptionally activated. All of these genes are involved in self-replication and maintaining pluripotency. On the other, approximately fifty percent of the genes identified by these three factors are the genes that are inactive in ESC, and their expression causes them to differentiate into a specific cell line. It can be found that the downregulation of Oct4, Sox2, and Nanog leads to the upregulation of genes involved in differentiation. In general, Oct4, Sox2, and Nanog are critical regulators of a transcriptional network involved in ESC pluripotency by increasing the transcription of genes involved in pluripotency and simultaneously reducing transcription of genes involved in differentiation. Given the importance of this self-regulating loop in maintaining stem cell pluripotency, both pathways can be expected to regenerate in reprogrammed somatic cells. Reactivation of these two pathways plays a vital role in induced pluripotent stem cells (IPSCs) induction [8].

Evaluation of the mouse vasa homolog (Mvh), stimulated by retinoic acid gene 8 (Stra8), α6, and β1 integrin genes expression pattern in ESCs and 1-3 day old Embryoid bodies (EBs) showed that all genes, except α6 integrin, were expressed in ESCs [9]. In another study, Cui Ying-Hong et al. (2022) investigated the differentiation potential for iPSCs into male gametes in vitro and in vivo. The cells isolated from iPSC-derived EBs, when injected with testicular cells into the dorsal skin of mice, the histological study confirmed that iPSC-derived cells could regenerate seminiferous tubules, and iPSC-derived GCs could be located in the sub-membranes of regenerated tubules [4].

IPSC can also differentiate into spermatogonial-like and OLCs. In one study, when placed in culture media, the iPSC line spontaneously differentiated into EBs after 3 days of suspension culture and showed specific markers of three germlines. RA and porcine follicular fluid effectively differentiated iPS cells into GCs and OLCs, and iPSCs transplantation could help improve testicular or ovarian function in infertile mice [10].

The present study combines cell therapy as a modern and traditional medicine approach to evaluate the effects of three herbal extracts of traditional medicine to induce differentiation into GCs progenitors.

The use of plant oils is widespread in traditional medicine, and they are used for the treatment of physiological diseases and to prevent infection [11]. Black caraway, known as Nigella sativa (NiS), has been a medicinal plant in traditional medicine for over 2000 years. In addition to vitamins, thymoquinone, P-Cymene, carvacrol, longifolene, indazoles, and isoquinolines are some of the compounds in NiS [12]. Evening-primrose with the scientific name of Oenothera Biennis (Obi), the fully ripe and dried seeds, form the medicinal part of this plant, which is brown with irregular shapes, odorless, oily, and bitter taste. This plant has a high potential for producing gamma-linolenic acid. According to the monograph of the Drug Commission of the World Health Organization (WHO), ranked as second therapeutic properties, and the therapeutic effects of this plant have been proven in clinical studies [13].

Broccoli, called Brassica Oleracea (BrO), is the essential cabbage in terms of nutritional value. In addition to vitamins, minerals, carbohydrates, and phytochemicals, it contains such carotenoids as α-Tocopherol, β-Carotene, and Zeaxanthin [14]. In a study, Ebrahimnia et al. (2020) evaluated the effects of chicory leaf extract on the differentiation potential of P19 ESC (an embryonal carcinoma stem cell line) into insulin-producing cells. They concluded that chicory extract as a natural plant extract effectively induced P19 ESCs into cell clusters similar to pancreatic islets with molecular, cellular, and functional characteristics of mature β cells [15].

Researchers studied a co-culture system using human embryonic stem cells (hESCs) to investigate the effects of the drug on hematopoiesis. Their results showed that catechins, an active ingredient in Spatholobus suberectus dunn, could increase the efficiency of hematopoietic precursors and erythroid. Their innovative co-culture system can also effectively screen active compounds in traditional drugs that enhance hematopoiesis and may have clinical and pharmacological applications [16].

This study focused on the effects of some of these compounds on ESC differentiation, with the initial belief that there are many diverse effective and sometimes miraculous compounds in natural plant and animal resources. Cells with a unique ability to produce animal cell types, which are exposed to specific molecules, can open up new perspectives on interdisciplinary sciences.

Materials and methods

Herbal extraction

NiS, Obi, and BrO were evaluated and approved by a botanist. First, it was ensured that the plants were completely healthy and free of any foreign substances, pests, and diseases. Plant samples were washed with plenty of water and rinsed with distilled water. The plants were then dried away from light conditions and powdered using a mill. The length of the drying period varied for each plant. Methanol was added to the powders in a ratio of 1:10. The solvent-powder mixture was shaken for 48 hours at a low speed at 120 rpm in dark conditions. After three consecutive filtering with Whatman filter paper, a rotary evaporator was used for the efficient and gentle removal of solvents from samples by evaporation.

Feeder layer

Direct attachment of ESC to the plate surface triggers the differentiation process. Therefor a growth-arrested monolayer cells called feeder layer is needed to prevent the differentiation and culture of ESCs while maintaining stemness. Mouse Embryonic Fibroblast (MEF) cells were isolated from 13-day-old embryos, and after primary cell culture, the third and fourth passages were used as a feeder layer. Ethical approval for the study was provided by North Research Center Ethics Committee (Project Number 0608204). The MEF is capable of producing and secreting Leukemia inhibitory factor (LIF) and therefore prevents ESC differentiation. Mitomycin C (MMC) (M0503-2MG Sigma) was used to arrest the proliferation of feeder layer cells. MMC is an antibiotic that acts as a double-stranded DNA alkylating agent. It covalently crosslinks DNA, inhibiting DNA synthesis cell proliferation, and Mitotically inactivates MEFs for use as feeder cell layers in ESC co-culture systems [17].

ESC culture

Oct4-green fluorescent protein (OCT4-GFP) transgenic ESCs were used to observe differentiation or maintain potency in this study. Oct4 is

Table 1. Primer pairs, PCR product sizes and the GenBank accession numbers

Gene names	Forward primers (5'-3')	Reverse primers (5'-3')	Product size (bp)	GenBank accession number
Hprt	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT	131	NM_013556.2
OCT4	TGTTCCCGTCACTGCTCTGG	TTGCCTTGGCTCACAGCATC	82	NM_013633.3
Nanog	GAACGGCCAGCCTTGGAAT	GCAACTGTACGTAAGGCTGCAGAA	74	NM_001289828.1
Sox2	TTCGAGGAAAGGGTTCTTGCTG	TCCTTCCTTGTTTGTAACGGTCCT	71	NM_011443.4
STRA8	ACAACCTAAGGAAGGCAGTTTAC	GACCTCCTCTAAGCTGTTGGG	173	NM_009292.2

a pluripotency factor and is expressed in stem cells. When Oct4 is expressed, GFP will also produce a bright green color under a fluorescent microscope that confirms the Oct4 gene expression. This study used knock knock-out (KO)-DMEM (Gibco) culture media to ESC proliferation and differentiation. The cells undergo an initial differentiation up to the stage of EB formation. There are several ways to form EB; hanging drop has been used in the present study. After preparing the differentiation medium, 40,000 single ESCs suspended in one milliliter of the differentiation medium; 20 microliters of these droplets were left on the plate's lid. About 150 drops containing ESC (800 cells/μl) were placed on the lid of a 12 cm sterile plate. 20 μl of sterile distilled water was poured on the bottom of the plate, and the lid with drops was gently placed on the plate. After three days of incubation (37°C, 85% humidity and 5% CO_2), the EBs were collected by adding the medium and then dissociated into single cells using the collagenase IV enzyme and mechanically, finally passed through the mesh filter and differentiated by culture mediums containing inducers.

Culture and differentiation

ESC medium included KO-DMEM, Knockout Serum Replacement (KSR), LIF, non-essential amino acids (NEAA), penicillin and streptomycin (Pen/Strep), 2-Mercaptoethanol (2ME), EB medium contained all of the above except LIF, and EB medium-plus mouse basic fibroblast growth factor (mbFGF) was used for differentiation purposes. All herbal extracts as desired inducers have been added to differentiation culture media. Cnt (control without any inducer), Nis50 (50 μg/ml Nis in differentiation culture media), Nis500, Nis2000, Bro50, Bro200, Bro500, Obi10, Obi50, and Obi200 was the study groups. The differentiation process was investigated by changing the cell morphology and the amount of green fluorescent light emitted from GFP expression. Also, the change in the expression pattern of the studied genes toward the target cells confirmed the differentiation into the PGCLC. For the cells to adhere to the bottom of the differential plate, gelatin was added to the plate surface under sterile conditions. After the desired time and cell differentiation in the presence of inducers, the trypsinized cells were rapidly transferred to the -80 freezer.

Real-time PCR

After RNA extraction (NucleoSpin RNA Mini kit for RNA purification; MACHEREY-NAGEL, Germany; 740955.50) and cDNA synthesis (High-Capacity cDNA Reverse Transcription Kit; ABI, USA; 4368814); Real-Time PCR (RT-PCR) was performed using StepOnePlus™ RT-PCR system by specific primers (**Table 1**) and desired master mix (SYBR® Green PCR Master Mix; ABI, USA; 4309155). The collected data were analyzed using SPSS V.19 and Excel 2017 software. The results obtained from quantitative RT-PCR (qPCR) as cycle threshold (Ct) were normalized by ΔΔCt method versus HPRT as a housekeeping gene and compared with the mean data of the control as fold change gene expression.

Results

To determine the appropriate dose of the extracts, cell viability was assessed by MTT assay. In the first step, a concentration range of 100 to 5000 micrograms per milliliter was used for all extracts. Then, in the second stage, the target dose was finally evaluated. After ensuring that it was safe for the cell, the final concentrations were determined, and the same concentrations were used for further tests (**Figure 1**). Cells were cultured and amplified on MMC treated MEF feeder layer. Then the pre-differen-

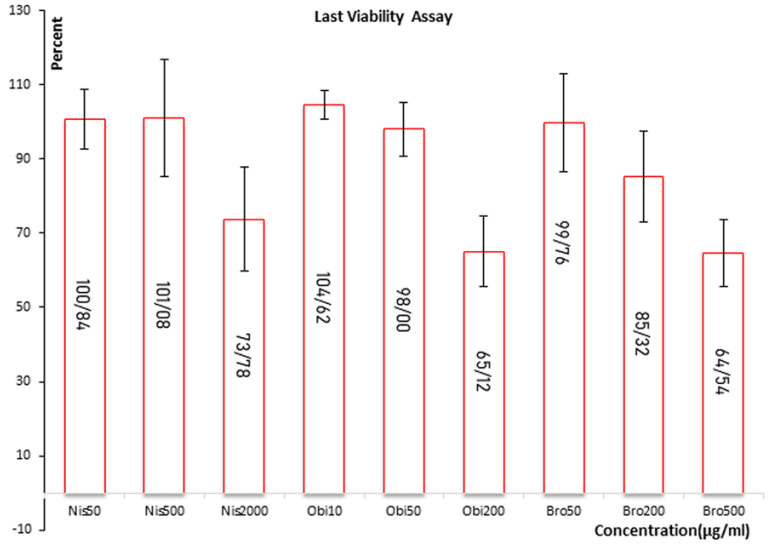

Figure 1. ESC survival rate in the presence of different concentrations of extracts.

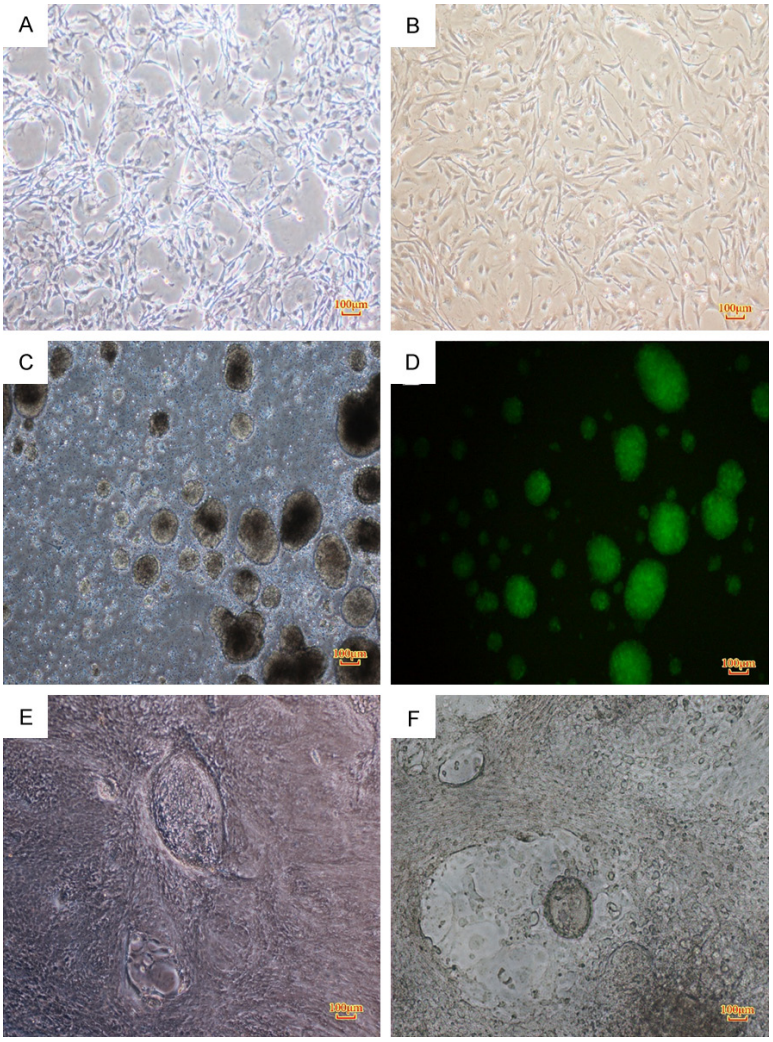

Figure 2. Images of MEF, ESC, EB, and differentiated cells. A. Mitomycin C treated MEF (feeder layer). B. ESC colonies on the feeder layer. C. EBs collected from EB culture media. D. EBs collected under a fluorescent microscope. E, F. A sample of differentiated cells on day-21 of the study.

tiation step was performed by forming EB. Single cells isolated from EB were induced on differentiated gelatinized dishes in the presence of different concentrations of all extracts. **Figure 2** shows images of MEF cells, ESC colonies, and images of differentiated cells.

OCT4

The results of changes in the OCT4 gene expression (**Table 2**) in Day-21 with a higher confidence interval showed a significant difference compared to its concomitant control group. This expression level was 0.161 for the Day-21 control group, which was increased to 0.491 in the NiS50 group and 0.583 in the NiS500 group, but it was 0.222 times higher than ESC in the NiS200 group. These changes were significant in all three NiS groups. In the three groups of BrO, as in Day-14, there were no significant changes in the two BrO50 and BrO200 concentrations ($P > 0.05$). However, the expression value (0.024) was significant in the BrO500 group ($P = 0.005$). Obi10, Obi50, and Obi200 groups also showed changes in Day-21 compared to the control group, which was significant in the first two groups. The expression values of Obi10, Obi50 and Obi200 were 0.779, 0.688 and 0.169, respectively. All three groups

Table 2. Effects of NiS, BrO and Obi extracts in various concentration on relative OCT4 gene expression

	Day-7	Day-14	Day-21
Cnt	0.856±0.051	0.433±0.079†	0.161±0.008‡,†††
NiS 50	2.098±0.503	0.927±0.141*	0.491±0.113†,*
NiS 500	3.675±0.533**	1.348±0.110†,**	0.583±0.150‡,††,*
NiS 2000	0.664±0.101	0.764±0.277	0.022±0.006††,***
BrO 50	0.966±0.153	0.568±0.100	0.301±0.074†
BrO 200	0.576±0.056*	0.376±0.089	0.215±0.054††
BrO 500	0.274±0.033***	0.092±0.015††,*	0.024±0.010‡,††,***
Obi 10	1.057±0.075	2.208±0.371†,**	0.779±0.139‡,*
Obi 50	2.408±0.346*	1.009±0.189†,*	0.688±0.036††,***
Obi 200	0.970±0.185	0.573±0.061	0.169±0.043‡‡

Data were presented as mean ± SEM. NiS: Nigella Sativa; BrO: Brassica Oleracea; Obi: Oenothera Biennis; OCT4: Octamer-binding transcription factor 4. *Significantly different from the control group (P < 0.05); **Significantly different from the control (P < 0.01); ***Significantly different from the control (P < 0.001); †Significantly different from Day-7 in the same group (P < 0.05); ††Significantly different from Day-7 in the same group (P < 0.01); †††Significantly different from Day-7 in the same group (P < 0.001); ‡Significantly different from Day-14 in the same group (P < 0.05); ‡‡Significantly different from Day-14 in the same group (P < 0.01).

Table 3. Effects of NiS, BrO and Obi extracts in various concentration on relative Nanog gene expression

	Day-7	Day-14	Day-21
Cnt	0.636±0.078	0.267±0.073†	0.084±0.026††
NiS 50	1.134±0.229	1.633±0.172**	1.066±0.126**
NiS 500	6.505±1.034**	5.305±1.630*	0.826±0.139††,**
NiS 2000	2.572±0.594*	1.265±0.188**	0.347±0.049‡‡,†,**
BrO 50	0.300±0.117	1.732±0.279††,**	0.081±0.020‡‡
BrO 200	0.352±0.134	0.104±0.050	0.074±0.009
BrO 500	0.993±0.237	0.262±0.033†	0.051±0.016‡‡,†
Obi 10	0.624±0.179	0.641±0.150	0.656±0.119**
Obi 50	0.063±0.017**	0.037±0.012*	0.400±0.101‡,†,*
Obi 200	0.573±0.089	0.092±0.012††	0.240±0.086

Data were presented as mean ± SEM. Nanog: Nanog homeobox. *Significantly different from the control group (P < 0.05); **Significantly different from the control (P < 0.01); †Significantly different from Day-7 in the same group (P < 0.05); ††Significantly different from Day-7 in the same group (P < 0.01); ‡Significantly different from Day-14 in the same group (P < 0.05); ‡‡Significantly different from Day-14 in the same group (P < 0.01).

showed an increase compared to the control group. These values were significant for Obi10 (P = 0.011) and Obi50 (P = 0.00014). However, the expression value in Obi200 was not statistically significant (P = 0.860).

Nanog

The Nanog gene expression in Day-21 showed a significant difference compared to its concomitant control group (**Table 3**). Its expression value was 0.084 in the control group, which increased to 1.066 in the NiS50 group, 0.826 in the Nis500 group, and 0.347 times higher in NiS2000 than ESC. These changes were significant in all three NiS groups. In the three BrO groups, like Day-14, there were no significant changes in the three concentrations of BrO50, BrO200, and BrO500 in terms of the above gene expression (P < 0.05). Obi10, Obi50, and Obi200 groups also showed changes compared to their control group on Day-21, which were statistically significant in the first two groups. The expression values in Obi10, Obi50 and Obi200 groups, were 0.656, 0.400 and 0.240, respectively.

Table 4. Effects of NiS, BrO and Obi extracts in various concentration on relative Sox2 gene expression

	Day-7	Day-14	Day-21
Cnt	0.690±0.147	0.529±0.091	0.214±0.057‡,†
NiS 50	34.444±6.111**	39.099±5.445**	17.569±0.820‡,***
NiS 500	47.650±3.965***	53.892±7.017**	38.584±1.265***
NiS 2000	8.500±1.427**	1.876±0.375†,*	0.026±0.003‡‡,††,*
Br0 50	0.868±0.157	0.546±0.113	0.175±0.030‡,†
Br0 200	1.783±0.427	1.232±0.226*	0.873±0.080**
Br0 500	1.633±0.179*	1.353±0.184*	1.096±0.200*
Obi 10	0.960±0.246	0.838±0.231	0.712±0.080**
Obi 50	2.367±0.248**	2.985±0.157***	1.295±0.180‡‡,†,**
Obi 200	5.117±0.730**	7.217±0.450***	6.571±0.735***

Data were presented as mean ± SEM. Sox2: SRY (sex determining region Y)-box 2. *Significantly different from the control group ($P < 0.05$); **Significantly different from the control ($P < 0.01$); ***Significantly different from the control ($P < 0.001$); †Significantly different from Day-7 in the same group ($P < 0.05$); ††Significantly different from Day-7 in the same group ($P < 0.01$); ‡Significantly different from Day-14 in the same group ($P < 0.05$); ‡‡Significantly different from Day-14 in the same group ($P < 0.01$).

Sox2

Comparison of Sox2 gene expression in different groups and control groups on days 7, 14, and 21 can be seen in **Table 4**. As it is clear from the signs, the studied groups are not significantly different in some cases, and the observed difference is not significant in others. Expression of this gene on day-7 in the control group increased from 0.690 to 34.444, 47.650, and 8.500 in the NiS groups, respectively. These changes were significant in all NiS groups with more than a 99% confidence interval. There was a higher difference in the Sox2 expression changes in Day-21 than in its concurrent control group. The expression value (0.214) recorded for the control group increased to 17.569 in the NiS50 group and 38.584 in the NiS500 group. The expression of the Sox2 gene in NiS200 was associated with a sharp decline, and its value was 0.026 times lower than ESC. These changes were significant in all three NiS groups. The NiS50 and NiS500 groups (99.9% confidence interval) and the NiS2000 group (95% confidence interval) confirmed this change. There were no significant changes in Br050 ($P > 0.05$), but the expression values were significant in Br0200 group (0.873) ($P = 0.03$) and Br0500 group (1.096) ($P = 0.013$). Groups Obi10, Obi50, and Obi200 showed changes compared to their control group on day-21, which were significant in all three groups.

STRA8

STRA8 gene expression in different groups on days 7, 14, and 21 could be seen in **Table 5**. These changes were significant in all three NiS groups on day-21. The NiS500 and NiS2000 groups confirmed the change with a 99.9% confidence interval. The changes in this gene expression were significant in all three Br050, Br0200, and Br0500 ($P < 0.05$). There were changes in the STRA8 gene expression in Obi10, Obi50, and Obi200 groups compared to their control group on Day-21, which were significant in the first two groups.

Figures 3-6 are related to the correlation between the control group and other groups. Each figure shows the line equation and the coefficient of determination (correlation coefficient). As the figure shows, the closer the correlation coefficient is to 1 or -1, the greater the correlation between the two variables.

Discussion

While ESCs can form cell types, adult stem cells are multipotent and produce only a limited number of cells. Considering the pluripotency of ESCs and the unlimited self-renewal capacity of these cells, ESCs-based treatment has been proposed for reconstructive medicine and tissue replacement after tissue injury or disease. New research has focused on differentiating ESCs into various cell types for cell alternative

Table 5. Effects of NiS, BrO and Obi extracts in various concentration on relative STRA8 gene expression

	Day-7	Day-14	Day-21
Cnt	0.076±0.009	0.015±0.005††	0.003±0.001††
NiS 50	0.517±0.061**	0.525±0.113*	0.437±0.120*
NiS 500	1.346±0.089***	0.930±0.131**	2.573±0.265‡‡,†,***
NiS 2000	2.173±0.116***	3.914±0.387†,***	2.569±0.176‡,***
BrO 50	0.374±0.116	0.592±0.061***	2.851±0.205‡‡‡,†††,***
BrO 200	0.262±0.059*	0.461±0.107*	0.199±0.069*
BrO 500	0.391±0.080*	0.162±0.042*	0.742±0.114‡‡,**
Obi 10	0.075±0.008	0.128±0.045	0.012±0.002††,*
Obi 50	0.529±0.097**	0.367±0.045**	0.487±0.093**
Obi 200	0.078±0.006	0.087±0.005***	0.002±0.001‡‡‡,†††

Data were presented as mean ± SEM. STRA8: Stimulated by Retinoic Acid gene 8. *Significantly different from the control group ($P < 0.05$); **Significantly different from the control ($P < 0.01$); ***Significantly different from the control ($P < 0.001$); †Significantly different from Day-7 in the same group ($P < 0.05$); ††Significantly different from Day-7 in the same group ($P < 0.01$); †††Significantly different from Day-7 in the same group ($P < 0.001$); ‡Significantly different from Day-14 in the same group ($P < 0.05$); ‡‡Significantly different from Day-14 in the same group ($P < 0.01$); ‡‡‡Significantly different from Day-14 in the same group ($P < 0.001$).

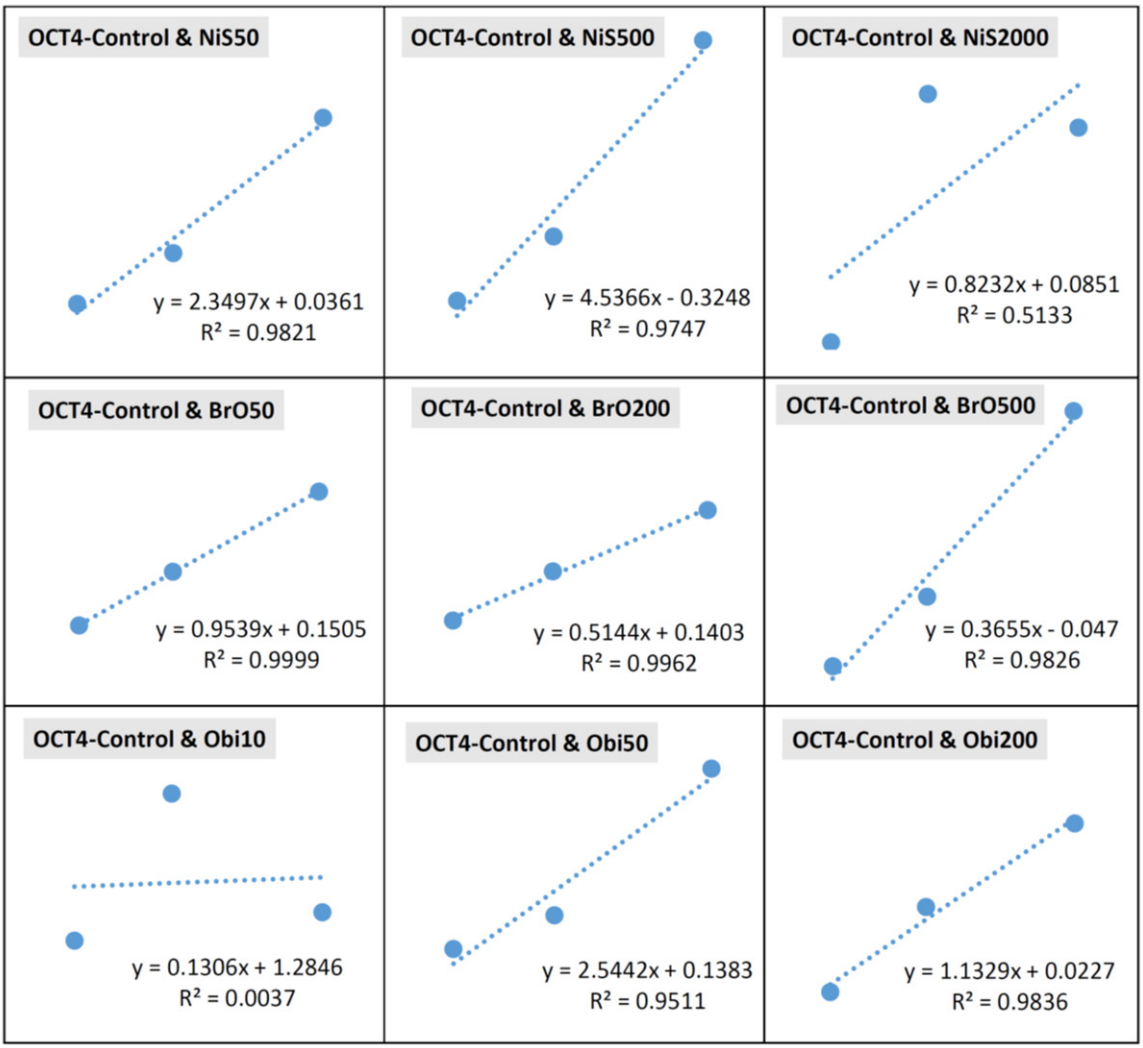

Figure 3. Correlation rate of OCT4 gene expression in different groups with trendline line, equation and coefficient of determination.

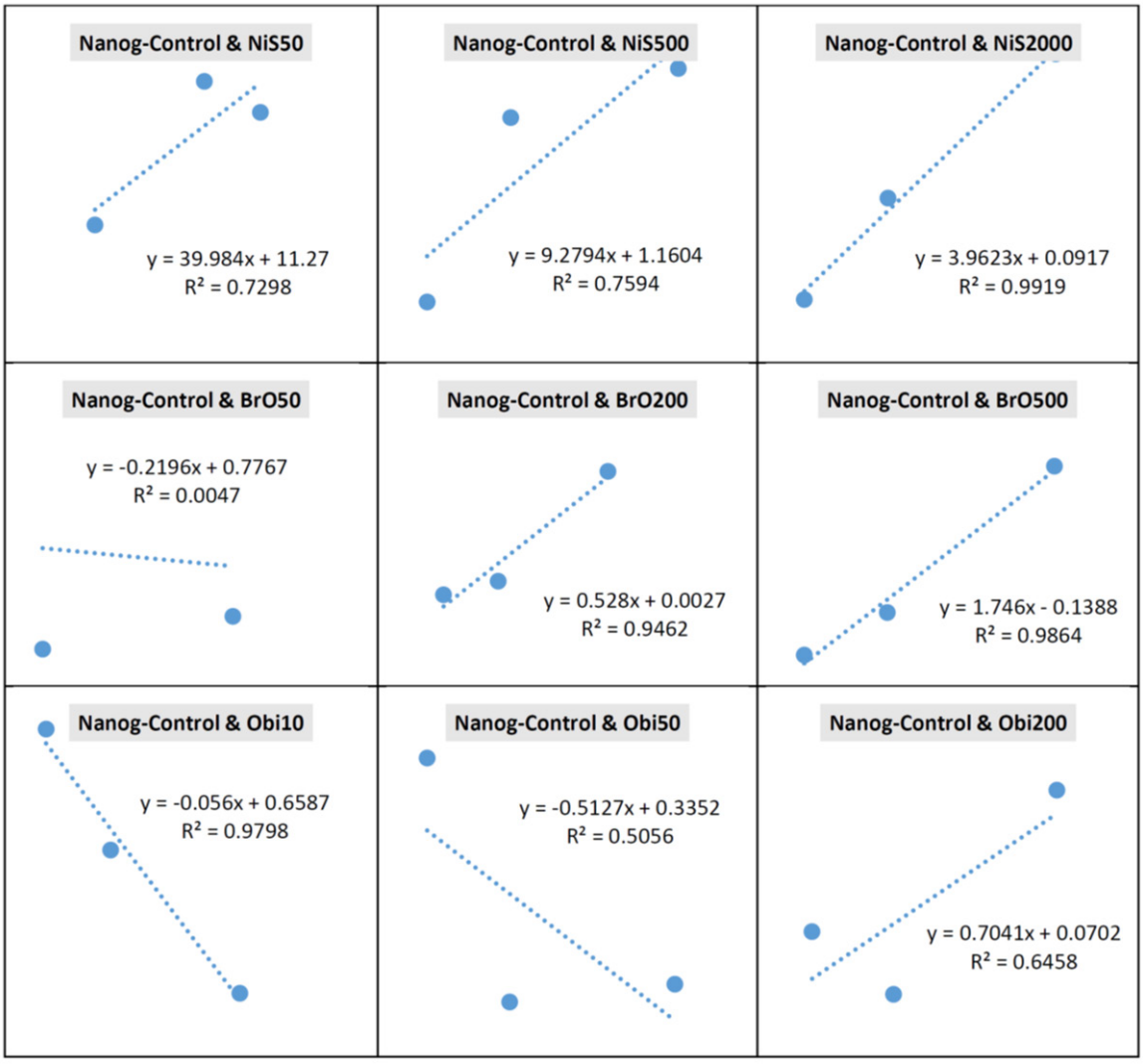

Figure 4. Correlation rate of Nanog gene expression in different groups with trendline line, equation and coefficient of determination.

therapies. According to a 2002 article in the proceedings of the national academy of sciences of the United States of America, hESCs can differentiate into different types of cells. They may, therefore, be helpful as a cellular source for transplantation or tissue engineering. The main concern about ESC transplantation to patients is their ability to form tumors, such as teratoma. The potential key to enhancing ESCs immunity in clinical applications is to differentiate cells into specific cell types (e.g., hepatocytes). At the same time, reduce or eliminate the ability of tumorigenesis.

The WHO announced that the prevalence of infertility was 15% on average. This statistic is not the same in all regions. Therefore, it varies between 8 to 35% in different countries. The lowest and highest prevalence of infertility has been reported in Australia and Africa, respectively [18, 19]. Infertility will affect up to 20% of Iranian couples in some areas. Previous domestic studies showed that six couples who have children would fail. Although this statistic is higher than the average announced by WHO (19.9% in urban and 22% in rural areas), it needs further research to determine the exact

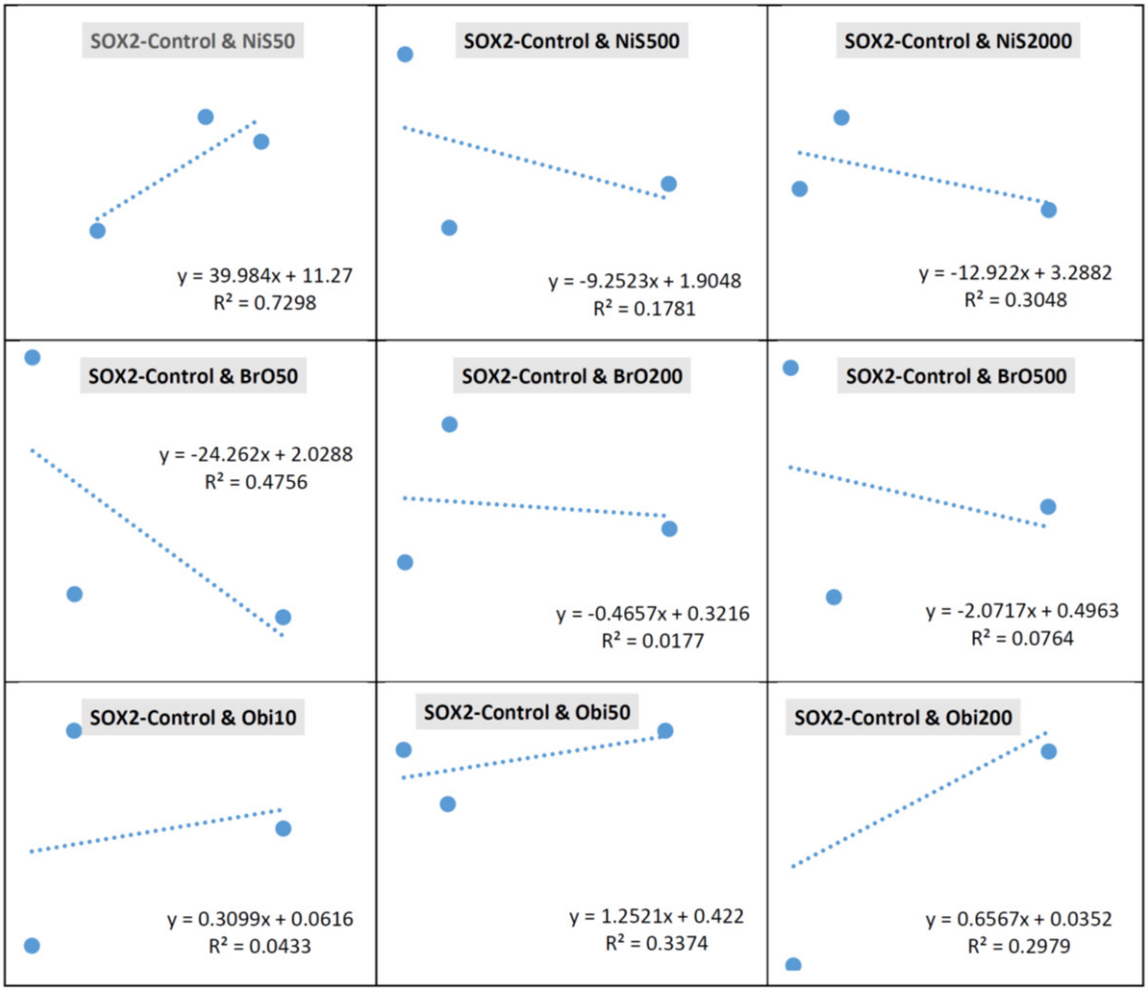

Figure 5. Correlation rate of Sox2 gene expression in different groups with trendline line, equation and coefficient of determination.

national statistics by the Ministry of Health. Previous studies have mentioned the prevalence of infertility in other parts of Iran [20, 21].

In addition to assisted reproductive techniques (ART), each of which, in some way, is used to treat different types of infertility, using stem cells and differentiating to GCs is one of the new laboratory and experimental methods that infertility specialists are trying to achieve. In vitro reconstruction of meiotic divisions is the most critical barrier to directing stem cells to form cell lines and ultimately male or female gametes [22].

Recent studies have shown that mouse embryonic stem cells (mESCs) and iPSCs can differentiate into primordial germ cells (PGCs) and eventually move to early gametes. Follicular fluid has stimulated mesenchymal stem cells proliferation at low concentrations and differentiation at high concentrations [23]. It is possible for cells driven toward male or female gametes; to reach the fertilization stage after undergoing their evolutionary process under certain conditions [16, 24]. However, gamete production's success from stem cells is early. It should be noted that many of the details of cell differentiation and their transport toward GCs are not exactly clear [25].

The present study investigated two indices of concentration and time and the inducers. Although each inducer has been involved in the Oct4 expression, the duration of stem cell exposure to the inducer (21, 14, 7 days) can provide valuable information. Also, the inducer dose plays an influential role in the study result.

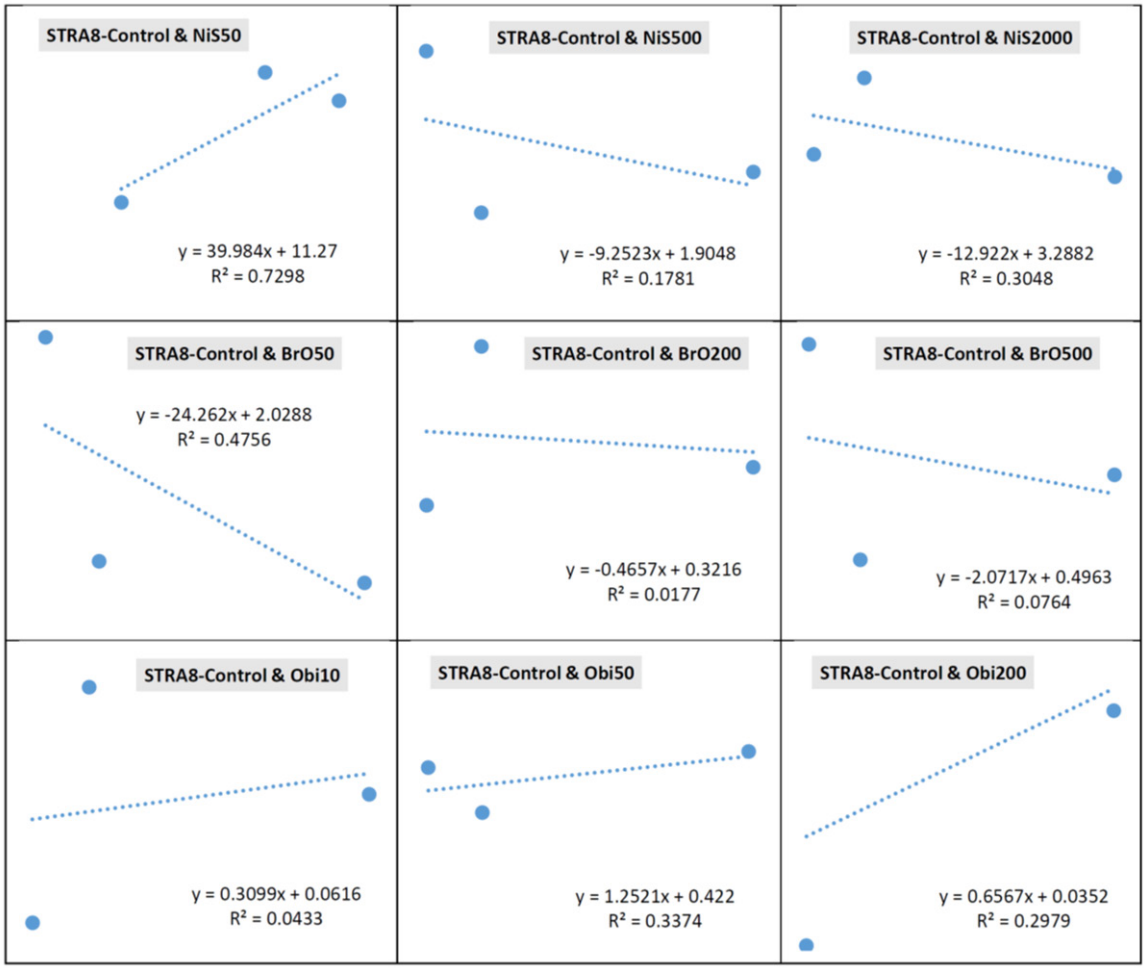

Figure 6. Correlation rate of STRA8 gene expression in different groups with trendline line, equation and coefficient of determination.

Table 1 shows the results of the Oct4 expression changes in all groups and during different periods. As concluded in the first impression, NiS and Obi increase the Oct4 expression, and BrO decreases its expression compared to the control group. Increased Oct4 expression increases the ability to produce cells with the potential to become stem cells that have the ability of folliculogenesis and be converted into OLCs in vivo [26]. The increase of Oct4 gene transcriptional activity and Sox2 in the presence of inducers has been demonstrated in the present study and some other studies [27]. Continuous cell exposure to Oct4 in a reprogramming medium allows the adult cells to produce iPSCs capable of competing with ESCs and generate three embryonic germinal layers. Obviously, it can produce GCs in the embryonic stage and therefore, produce GCLCs. An increase of Oct4 in Obi10 media and in the second week of culture can be evaluated as the best condition to this result achievement [28].

According to the results of RNA microarray, the Oct4 expression in PGC is higher than in EB, while the ESC expression of this gene is close and similar to PGC cells. Therefore, some inducers can increase the expression of the desired gene and bring it closer to ESC expression. The present study results are consistent with a study by Lopez et al. [29]. Transfer of induced cells to the ovary of neonatal severe combined immunodeficiency disease (SCID) mice leads to the formation of a mass of cells in vivo. Therefore, the presence and role of Oct4 as one of the worthwhile achievements of some studies [30] in the production of GCs confirms the results obtained in this study especially on the use of Obi10 on day 14.

The Nanog gene reduced expression in the differential groups compared to the EB cells derived from it in the Cnt group. The results of this study indicated that the decreasing trend was very low in the first week but reached less than 0.1 during the second and third weeks. The highest increase in Nanog expression in the first week of the study occurred in the NiS500 and then NiS2000 groups. This means that NiS plays a better role in expressing this protein during the first week of treatment. This role is also appropriately observed in the BrO500.

Nanog is even capable alone differentiating epiblast-like cells into PGC-like cells. The role of Nanog has not been fully elucidated, but it is considered a pluripotent factor and is expressed in ICM blastocysts. Interestingly, this molecule is also expressed in the unipotent cells of PGC of mice. The presence of Nanog can be a sign of stem cell differentiation toward GCLCs [31]. In Nanog-positive cells, the expression of Nanog, Oct3/4, Sox2, and Klf4 genes is significantly increased. Induction of Nanog expression in human amniotic stem cells and reprogramming purposes differentiate the stem cells into cardiomyocyte-like cells [32]. The present study increased the Nanog expression in a few days by using inducers and producing cells with suitable targeted differentiation. Nanog inhibition and a decrease in its expression increase genes' expression in the endoderm and trophectoderm pathways [33]. Consistent with other studies, the present study confirms the role of Nanog in the differentiation process.

The presence of Nanog and OCT4 in the absence of Sox2 could be a sign to distinguish seminoma-like cells from ESCs. According to the present study, the Sox2 expression showed a slight decrease in the control group compared to EB on day 7. This slight decrease continued with a decrease in the second and third weeks. A decreasing trend in the expression of this gene, similar to the Nanog gene, can be observed in different groups during three weeks. This decreasing trend, in turn, can be interpreted as a sign of differentiation into specialized non-gamete cells or a decrease in the number of cells over time. But this reduction trend has not disrupted the research process for any reason. At first glance, we can observe the positive effect of inducers on Sox2 expression in all groups compared to the control group during the first week of the study. The highest and the lowest expression is observed in NiS and BrO groups, respectively.

Sox2 can be expressed in Sertoli cells of seminiferous tubules with carcinoma in situ (CIS), whereas it is not expressed without CIS [34].

Granulosa cells can reprogram into iPSC cells using minimal transcription factors such as Sox2 and Oct4. Not only do these cells endogenously express the c-Myc and Klf4 molecules, but LaminA is not expressed in their nuclei, which is an effective aid to efficient reprogramming to be converted to iPSC. Therefore, it can be expected that the role of cell pluripotency is to preserve cells' stemness or differentiate them into PGCLCs. Therefore, NiS extract is expected to be more effective in logical differentiation, especially at 50 and 500 concentrations [35]. The results of a study by Weber et al. on NiS and Obi on the Sox2 expression showed that the interaction between Artd1 and Sox2 is critical for the first stage of reprogramming, and the role of Sox2 in iPSC production and cell pluripotency can be confirmed [36].

As shown in **Table 4**, STRA8 gene expression significantly decreased in control groups and reached about one-third of this gene expression in ESC at the end of the third week. During the first week, STRA8 expression increased significantly in the NiS-treated groups compared to the control group, twice the expression of this gene in ESC. The highest increase occurred in NiS2000, and this incrementation trend continued in the second week, but there is a slight decrease compared to the second week in the third week. BrO also had a good effect on increasing the STRA8 expression compared to the control group. But, the STRA8 expression was less than the basal cell (ESC) in all BrO groups, except for the BrO50 group in the third week; this alteration was not significant but more and mainly significant than the corresponding control group. Spermatogonial and sperm-like cells could be generated using retinoic acid (RA) and thus increasing the STRA8 expression in myoblast transgenic cells. These cells are a good model for studying the differentiation of unipotent cells [37].

Increased STRA8 expression in NiS groups and some other groups can indicate the differentiation of cells into GCs and GCLCs. RA-based

induction of ESCs can increase the STRA8 expression, and increasing the expression of these genes will activate the differentiation pathway towards GCs. Upon the RA induction, Creb-binding protein and P300 play a role in the STRA8 expression [38]. The presence of compounds similar to RA and terpene molecules in plant extracts is not unexpected. Obviously, one of the primary reasons for the increased STRA8 expression in all three types of extracts could be a result of the presence of similar molecules.

In contrast, other compounds detected in plants have inhibitory effects on the STRA8 expression. Nevertheless, the black seed extract significantly increases the expression of this gene and thus, has a more favorable effect than expected. Researchers have developed a new vector with two GCs promoters simultaneously (STRA8 and c-Kit). This vector has two reporter genes, and its efficiency has been investigated using STRA8 and Dimethyl sulfoxide (DMSO). RA-like compounds can differentiate pluripotent stem cells into GCLCs [39]. Shanmugam et al. (2018) isolated thymoquinone from black seed extract. They claimed that this compound could control some oncogenic transcription factors using thymoquinone [40]. So it is possible that the observed effects in the NiS groups were due to the presence of thymoquinone.

This study could not perform further tests to confirm the differentiation into target cells due to lack of adequate funding and financial support. The authors hope to conduct tests such as flow cytometry, Western blotting, immunocytochemistry, and gene expression on a larger scale and to study other plant extracts in the future.

Conclusion

The conclusion of the present study, beyond any good or bad role, is this new perspective that we can open an account on stimulating the differentiation of ESCs on known or unknown plant extracts. Herbal extracts with a world of wonders and a wide range of compounds can have beneficial or detrimental effects on specific cellular activity.

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Disclosure of conflict of interest

None.

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