New Possible Approach in Treatment of Experimental Induced Vaginal Atrophy by Bone Marrow-Derived Mesenchymal Stem Cells in Adult Female Albino Rats (histological, immunohistochemical and biochemical study)

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Abstract

Women after menopause have a lot of complains that negatively distress their life. In the modern years, a lot of conduction methods have been presented to relief undesirable symptoms. Bone marrow mesenchymal stem cells (BM-MSCs) can be recently used as a new therapeutic method in treatment of many diseases and avoidance the hormonal sides effects that happened after menopause.

**Aim:** The aim of this research is to show a new approach in modification of the structure of vaginal mucosal atrophy by using the (BM-MSCs) in induced ovariectomized rats.

**Methods and Results:** Fifty-five female albino rats were used and divided randomly into five groups: control group, ovariectomized group, ovariectomized group plus estrogen (20ug/kg/day for 4 weeks), ovariectomized rats with BM-MSCs group (5 × 10\(^5\) MSCs/rat intravenously) and ovariectomized rats with stem cells (BM-MSCs) Intra-vaginal group, the expression of genes for GAPDH, iNOS and TGF-β were done and vaginal biopsies were taken for histological and immunohistochemical studies. In ovariectomized group there was inflammation, ulceration with irregularity in collagen fibers, decreased estrogen receptors expression and the expressions of TGF-β, GAPDH and iNOS were very high. While the rate of healing of epithelium was increased with in the vasculatures of vaginal mucosa and the estrogen receptors expression was high with decreased expression of GAPDH, iNOS and TGF-β in ovariectomized rats that treated with intra- vaginal BM-MSCs.

**Conclusions:** Using the BM-MSCs could be used intravaginal safely in case of vaginal atrophy as they modify the structure of vaginal mucosa superior to estrogen hormone therapy.

Keywords: vaginal atrophy, BM-MSCs, estrogen

Introduction:

Females suffer from general and local changes which appear after menopause. These changes are due to decrease in the level of estrogen [1]. This may be physiologically at menopause or occur accidentally after surgical removal of ovaries [2]. This decrease in estrogen level has many physiological changes as vasomotor instability, mood changes, an increased risk of osteoporosis and vaginal atrophy [3]. Vaginal atrophy is a common and affects more than 40% of postmenopausal females. This atrophy causes thinning and shrinking in the vaginal epithelial wall and decrease in smooth muscle fibers with less elasticity of vaginal wall [4]. Vaginal atrophy causes burning, dryness, irritation, and dyspareunia [5]. These symptoms do not improve with time and are not resolve without treatment [6]. The good level of estrogen from puberty is essentially for good blood supply for vaginal mucosa and its lubrication [7]. The vaginal wall rugae, wall thickening and lubrication are depending on presence of estrogen [8]. Vagina is estrogen dependent organ as estrogen is responsible for induction of proliferation of vaginal epithelium layers, smooth muscles and collagen fibers so maintains vaginal rugae [9]. Experimental bilateral ovariectomy was done to know the effects of decrease in hormonal levels and their activity in female rats [10]. This experimental ovariectomy has an important role to understand the pathophysiological changes and to help in developments of therapy [11]. So, to improve pathophysiological changes after ovariectomy, estrogen therapy was used as a replacement therapy, but there are many limitations for hormonal uses because their side effects and hazards as, cancer specially, breast, ovaries or uterus. Also, causes cardiovascular hazards or thrombosis [12]. These side effects can be controlled through good monitoring, mammography and endometrial thickness measurement [13]. Embryonic and somatic stem cells proved that they can be differentiated into female germ cells [14]. Somatic cells also appear to differentiate into granulosa and theca cells of the ovarian follicles which are responsible for production of estradiol [14]. The aim of this study is to explore the role of estrogen hormone in restoring histological changes of vaginal after ovariectomy and to assess other lines of therapy as MSCs to improve histopathological changes in ovariectomized rat.

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Material and Method:

Animals
Fifty-five adult female rats weighing 160 ± 200 g were obtained from the Animal House, Mohstohor Faculty of Veterinary Medicine, Benha University. Rats were kept under observation for 1 week before the beginning of the experiment for accommodation. They were maintained in a temperature- and humidity-controlled room and given free access of water and food. All animal experiments were conducted in accordance to approved protocols and the recommendations for the proper care and use of laboratory animals.

Experimental design
Fifty-five female rats (eight to ten weeks old) were taken and divided into five groups each group 10 rats and five rats were prepared for isolation and culture of MSCs.

Group I: (−ve control): no surgical operation.

Group II: (ovariectomized group): In this group the rats under grow ovariectomy operation [15].

Group III: (ovariectomized group plus estrogen): rats treated by natural estrogen (estradiol) injected subcutaneously in a dose of 20μg/kg/day for 4 weeks [16].

Group IV: (ovariectomized rats with BM-MSCs): rats were injected once by 5 × 10^6 MSCs/rat intravenously (through tail vein) and scarified after 6 weeks.

Group V: (ovariectomized rats with stem cells /BM-MSCs Intra-vaginal group): rats were injected once with 5 × 10^6 MSCs/rat into the subepithelial space intravaginal and scarified after 6 weeks [17].

Ovariectomy technique: The rats were anesthetized by ether inhalation. Under sterile conditions, a 2–3 cm ventral lower midline incision was made into the skin and muscle (to expose the ovaries). After good homeostasis, the ovaries were removed after tying off and cut from the oviduct. Antibiotics were applied locally before suturing the muscles and the skin to close the incision. The anoestrous phase was considered to be occurring 15 days after surgery. To confirm this phase, the oestradiol level was tested 15 days after surgery, dividing all studied groups were homogenized and total RNA was isolated with RNA easy Mini Kit (Qiagen) then analyzed for quantity and quality with Beckman dual spectrophotometer (USA).

Histological Examination
Vaginal tissue samples were divided into two sections. The first section was examined by fluorescent microscope for tracing of injected labeled cells with GFP. The second sections were processed for paraffin block and stained with hematoxylin and eosin (H&E) and Sirius red (for demonstration of collagen fibers) [20].

Immunohistochemistry
Immunohistochemical staining for estrogen receptors were performed on 5-μm, formalin-fixed, paraffin-embedded sections by using the streptavidin-biotin detection system (DAKO). They were obtained from sigma company and kit [SRP2163-4UG]. Human prostate (taken from the pathology department in our institution) served as a positive control according to Manufacturer Company. Negative control slides were prepared by the same steps, except they were incubated with the antibody diluted instead of primary antibody. Positive reaction for estrogen receptors appeared in the form of brown nuclear staining [21].

RNA Extraction and Quantitative real-time polymerase chain reaction (qRT-PCR)
Vaginal tissues of all studied groups were homogenized and total RNA was isolated with RNA easy Mini Kit (Qiagen) then analyzed for quantity and quality with Beckman dual spectrophotometer (USA). Quantitative real-time polymerase chain reaction was done as following, 200ng of total isolated RNA from each sample were used for DNA synthesis by reverse and transcription method by using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA) to measure the quantitative
amount of iNOS, GAPDH and TGF-β genes. Then the DNAs were amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Bio-system, USA) [22&23]. Primers sequence for each gene demonstrated as iNOS: CCACCATGCCAAATTCCATGGCA (Forward);TCTAC AGGCGAGTCAGGTCCACC (Reverse);GAPDH:CAGG AGGATGTTGTTTGTG (forward),TGCCACTTTATCCCATTCAG (Reverse);TGF-B: AAGTCATCCATCCCCAGC (forward),AGGCCACCT GAGCCCTATAA (Reverse).

**Morphometric study**

By using image analyzer (Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA), from each group sections were measured in ten non-overlapping fields by high power field:

- mean vaginal epithelial thickness in each group [24].
- Mean area percent of collagen fiber content (±SD) in each group.

\[ \text{Mean area percent of collagen fiber content (±SD) in each group} \]

\[ \text{Mean area percent of estrogen immunostaining was quantified} \]

**Statistical analysis**

One-way study of variance (ANOVA) was used to measure up differences between the groups and by using IBM SPSS Statistics software for Windows, Version 20 (IBM Corp., Armonk, NY, USA) we recorded and analyzed collected data from each group, all data was put across as the mean value, standard deviation(SD) and differences were considered to be significant at p< 0.01.

**Result:**

**BM-MSCs identification and Homing**

Undifferentiated (BMSCs) appeared as spindle and fibrocyte-like by inverted microscope as shown in Figure.1A, while flow cytometry analysis of surface molecule for CD29, CD44 for confirmation of mesenchymal cell phenotypes as shown in Figure.1B. Florescence microscopy examination of vaginal cells treated by mesenchymal cells indicated that the GFP-transduced injected cells were localized within the vaginal tissue as shown in Figure.1C & 1D.

**Hormonal assay results:**

E2 levels were assessed 15 days and 30 days after injection of estrogen and MSCs. Results showed that there was a significant decrease in E2 levels in ovariectomized group compared to control (12.5±1.2 after 15 days versus 60.2±8 in control rats, p< 0.001 and 10.1±0.5 after 30 days versus 62.1±4 in control rats p< 0.001). Use of either estrogen or MSCs led to a significant increase in E2 levels with more superior therapeutic effects with intravaginal MSCs as compared to MSCs (40.5±0.3, p< 0.05, 50.1±1.2, p<0.01 and 55.7±0.2 respectively after 15 days versus 60.01±1.1 in control rats and were more significant with estrogen or MSCs treated groups respectively after 30 days (50.9±0.2, 53.2±1.4 and 60.1±1.1, p<0.01) versus control group 62.1±0.4 (figure 2).

FSH levels were assessed 15 days and 30 days after injection of estrogen and MSCs. There was a significant elevation of FSH levels in ovariectomized group compared to control group (85.6±1.1 versus 32.6±1.0 in control rats, p< 0.001 after 15 days, 92.1±1.2 versus 35.6±0.4 in control rats after 30 days respectively, p< 0.001). Use of estrogen and MSCs led to a significant decrease in FSH levels as compared to ovariectomized group (40.1±0.2 and 45.3±1.7 after 15 days, p< 0.05 and 38.2±1.1 and 43.3±0.2 after 30 days. Use of intravaginal MSCs did not lead to decrease in FSH levels as compared to control groups. (68.6±0.9 after 15 days, p<0.01 and 72.1±0.5 after 30 days, p<0.01 versus 32.6±1.0 and 35.6±0.4 in control group, p< 0.05) (fig 2).
Histological results

**Hematoxylin and Eosin** examination of vaginal tissue from control group revealed normal non keratinized stratified squamous epithelium with multiple papilla, and cells appeared, densely packed, vacuolated with dark nuclei as shown in Figure 3A. The underlying connective tissue appeared thick with regular packed bundles of collagen fibers by *Sirius red as shown in Figure 3B*. In ovariectomized group the epithelium appeared thin desquamated with apparent many dilated blood vessels and inflammatory cells infiltration in underlying connective tissue as shown in Figure 3C. The connective tissue was irregular in arrangement and decrease in amount with dilated blood vessels as shown in Figure 3D. In estrogen treated group the covering epithelium appeared normal with well recognized basal layer with multiple dilated blood vessels as shown in Figure 3E. While the connective tissue appeared normal with regular bundles of collagen fibers with moderate in amount as shown in Figure 3F. In BM-MSCs treated group showed normal covering epithelium many protrusions in the basal surface of the epithelium as shown in Figure 3G, also connective tissue thickness revealed many protrusions in the basal part of epithelium as shown in Figure 3H, also their connective tissue differentiated into thick regular arrangement bundles of collagen fibers as shown in Figure 3I.

The morphometric result of mean vaginal epithelial thickness revealed of 165.0±1.30 mm, 180.79±0.90 in Groups I, II, III, IV, and V respectively. The mean thickness of the vaginal epithelium of rats in Group I (permanent estrus) was significantly greater than that of animals in Groups II, III, (p=0.0001), with no statistically significant differences between groups, IV and V. (p=0.0709).

The morphometric result of Mean area percent of collagen fiber content (±SD) in the studied groups revealed a significant decrease in groups II as compared to the control group and values recorded for groups III, IV and V represented a statistically significant decrease, as compared...
to the ovariectomy group. However, the values were not statistically significant as compared to the control as shown in Figure 4.

**Immunohistochemical results**

Estrogen receptors of vaginal cells after MSC transplantation showed strong estrogen receptors expression in the epithelium of control group (Fig 5A.), while the expression of estrogen was negative in ovariectomized group as shown in (Fig 5B), but mild to moderate expression in the epithelium of estrogen treated group was detected as shown in (Fig. 5C), moderate expression in the epithelium of BM-MSCs treated group as shown in (Fig. 5D) and marked expression in group treated with BM-MSCs intravaginal (Fig. 5E). The mean area percentage of estrogen for all groups was represented in figure 4. There was a significant increase in mean area percent of estrogen immuno-expression of groups IV, V compared with group II.

**Quantitative Gene Expression**

The expression of genes for GAPDH, iNOS and TGF-β in all groups were quantified by real-time PCR (Tables 1).

The expression of TGF-β was very low in healthy control vaginal tissue. After ovariectomy, the expression of TGF-β was very high. The treatment with BM-MSCs reduced TGF-β expression. The highest reduction of TGF-β expression was seen in group treated with BM-MSCs intravaginal.

The expression of iNOS and GAPDH were absent in healthy control group while its expression increased after ovariectomy to reach very high expression after 6 weeks of ovariectomy. The treatment of ovariectomized rats with BM-MSCs reduced iNOS expression and significantly decreased in group treated with BM-MSCs intravaginal.

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Fig. 3: A) A photomicrograph of a section of the vagina of adult control group (group I) showing normal stratified squamous epithelium with multiple papilla and the cells are densely packed, vacuolated with dark nuclei (stars). H&E, ×400. B) The connective tissue is intensely stained with Sirius (arrows). Sirius red ×400. C) The epithelium of group II showing desquamation (arrows) with many dilated blood vessels in underlying dispersed connective tissue (squares). Notice inflammatory cells under epithelium (stars). H&E, ×400. D) The connective tissue is faintly stained by Sirius red (arrows) with regular arrangement of collagen fibers. Sirius red ×400. E) The epithelium of group III showing normal epithelial cells with well recognized basal layer (arrows) with multiple dilated blood vessels (stars). H&E, ×400. F) The connective tissue is moderate staining with Sirius red (arrows). Notice that the regular arrangement of collagen fibers (blue lines). Sirius red ×400. G) The epithelium of group IV showing many protrusions in the basal surface (arrows). H&E, ×400. H) The connective tissue is moderate to strong staining by Sirius red (arrow). Sirius red ×400. I) The epithelium of group V showing many protrusions in the basal surface (arrows). H&E, ×400. J) The connective tissue is strongly staining by Sirius red (arrows). Sirius red ×400.
<table>
<thead>
<tr>
<th>Group</th>
<th>Means and (±SD) of the vaginal epithelium thickness (mm)</th>
<th>Mean area percent of collagen fiber content (±SD)</th>
<th>Mean area percent of estrogen immuno-expression(±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control group</td>
<td>189.10±1.26</td>
<td>84.2±1.3</td>
<td>24.6±1.9</td>
</tr>
<tr>
<td>ovariectomized group</td>
<td>27.80±0.14*</td>
<td>19.9±0.05*</td>
<td>10.9±1.8*</td>
</tr>
<tr>
<td>Estrogen treated group</td>
<td>128.19±2.98#</td>
<td>69.8±0.2#</td>
<td>17.1±0.2#</td>
</tr>
<tr>
<td>BM-MSCs treated group</td>
<td>165.74±1.30#</td>
<td>73.8±1.9#</td>
<td>20.3±1.7#</td>
</tr>
<tr>
<td>intravaginal BM-MSCs treated group</td>
<td>180.79±1.90#</td>
<td>81.1±0.2</td>
<td>22.5±1.9#</td>
</tr>
</tbody>
</table>

Fig. 4. Means and standard deviations (±SD) of the vaginal epithelium thickness, mean area percent of collagen fibers content (±SD) and mean area percent of estrogen immuno-expression of female rats in all groups.

# significant as p value < 0.05 versus ovariectomized group.

* significant as p value < 0.05 versus control group. (difference was observed between Groups, III, IV and V (p=0.0809).
Menopause is a very important state in the life of females as it is associated with many changes in their health. Estrogen deficiency after menopause or surgical removal of ovaries is responsible for these health hazards [25].

Ovary is the main organ for production of estrogen so ovariectomy causes decrease of this hormone [26]. Decrease of estrogen is associated with elevation of follicular stimulating hormones (FSH). This increase of (FSH) is due to failure of negative feedback on pituitary hormones (FSH). This increase of (FSH) is due to failure of negative feedback on pituitary hormones (FSH). This increase of (FSH) is due to failure of negative feedback on pituitary hormones (FSH). This increase of (FSH) is due to failure of negative feedback on pituitary hormones (FSH). This increase of (FSH) is due to failure of negative feedback on pituitary hormones (FSH).

In this study hormonal assay was done at 15 and 30 days after ovariectomy and showed that E2 levels were assessed and the results showed that there was a significant decrease in E2 levels in OVX group as compared to the control. Use of either estrogen or MSCs injection led to a significant increase in E2 levels with more superior therapeutic effects with intravaginal MSCs as compared to MSCs intravenous injection. As regard FSH level there was a significant elevation of FSH levels in OVX group compared to control group.

Table 1. Mean and SD of TGF-β, iNOS and GAPDH genes expression for all groups,

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ±SD of TGF-β gene expression</th>
<th>Mean ±SD of iNOS gene expression</th>
<th>Mean ±SD of GAPDH gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>control group</td>
<td>11.1±1.5</td>
<td>13.4±1.3</td>
<td>14.3±1.9</td>
</tr>
<tr>
<td>ovariectomized group</td>
<td>22.1±0.12</td>
<td>29.3±1.7</td>
<td>28.6±2.7</td>
</tr>
<tr>
<td>Estrogen treated group</td>
<td>12.9±1.2 #</td>
<td>17.2±1.2 #</td>
<td>18.1±3.2 #</td>
</tr>
<tr>
<td>BM-MSCs treated group</td>
<td>14.2±0.5 #</td>
<td>15.6±1.8 #</td>
<td>15.3±1.2 #</td>
</tr>
<tr>
<td>intravaginal BM-MSCs treated group</td>
<td>12.1±0.5</td>
<td>14.5±0.5</td>
<td>14.9±1.0</td>
</tr>
</tbody>
</table>

# significant as p value < 0.05 versus ovariectomized group. 
* significant as p value < 0.05 versus control group.

Discussion:

Use of estrogen and MSCs led to a significant decrease in FSH levels as compared to ovariectomized group. Use of intravaginal MSCs did not lead to decrease in FSH levels as compared to control groups.

The level of follicle stimulating hormone (FSH) more than 40 mIU/ml indicates that there is an ovarian failure [30].

The previous results showed that levels of estrogen and FSH after treatment of OVX rats with MSCs injection were near to control level. This indicates that MSCs has an endocrine functions and effects on FSH through communication with pituitary gland [31]. The study suggests that BM-MSCs can recover the function and structure of injured tissues [32].

Many studies reported that the ovarian granulosa and theca cells which were differentiated from stem cells lead to secretion of estrogen in response to elevation of the level of FSH level in OVX rats and subsequent increase estradiol and suppress the level of FSH nearly to normal [33]. So BM-MSCs used to restore levels of ovarian hormone and could reactivate folliculogenesis in animal model of premature ovarian failure (POF) due to the use of chemotherapy [34].

Studies done on cases of premature ovarian failure (POF) detected that injection of BM-MSCs could differentiate to many types of cells as theca cells, granulosa cells, and corona radiate cells. This...
lead to ovarian function recovery specially its endocrine and steroidogenesis [35].

The deficiency of ovarian hormones as a result of various factors (POF, menopause or surgical ovariectomy) leads to structural changes of the vagina which become narrow, short, thinner with no folds [35&36].

Estrogen hormone is vital in maintaining of vaginal structures and functions. As vaginal wall thickness and rugae are depending on estrogen [37&38].

The present study showed that in ovariectomized group the epithelium appeared thin desquamated with apparent many dilated blood vessels and inflammatory cells infiltration in underlying connective tissue. The connective tissue was irregularly arranged and decrease in its amount.

Ovariectomy is the cause of a significant decrease in vaginal epithelial thickness and in its glycogen content. This decreasing in vaginal epithelium, its layers and defects in stratification makes it liable to abrasion [39].

All this above reasons lead to decrease the power of protection against bacterial infection [40].

The effects of estrogen on vaginal mucosa is due to that the estrogen increases the blood supply of vagina and its vascularization [41&42].

This study showed that there were increase in mean thickness in the treated groups.

From our result we found that the injection of estrogen produced a fast and good response on the vaginal epithelium as regeneration occur more with intravaginal BMSCs injection. This response is due to presence of large number of estrogenic receptors in the genital tract [43]. Estrogen produces their effects (cellular proliferations) through receptors which are present in various tissues as uterus, breast, and vagina [2].

In this study assessment of estrogen receptors showed that there are strong estrogen receptors expression in the epithelium and in connective tissue of control group while the expression of estrogen was negative in OVX group (group II). Moderate expression in the epithelium of group III while mild to moderate in group IV. In group V there were severe expression of the receptors.

Normally the estrogenic receptors are of large numbers in vagina which react rapidly with estrogen either natural or synthetic [7].

As estrogen maintains the thickness of vaginal epithelium, it also maintains the production of glycogen. Estrogen hormone has an important role in connective tissue maintenance. As the receptors for estrogen are identified in the connective tissue nuclei of vaginal wall [44].

The estrogen defect causes defect in collagen fibers. This leads to change of vaginal pH which is normally low [45].

Commencellus microorganisms (Lactobacilli) found in vagina need glycogen to produce lactic acid and keep low vaginal PH about 3.5 to 4.5. This low PH protects the vagina against infections [46].

The decrease of collagen also is the cause of weakening of vaginal wall and may predispose prolapse in postmenopausal women [47].

The morphometric result of Mean area percent of collagen fiber in this study revealed a significant decrease in groups II, as compared to the control group with good improvement of vaginal epithelium thickness and connective tissues in OVX rats after treatment by estrogen and BM-MSCs injection, but the best effects were after intravaginal BM-MSCs.

BM-MSCs have proved that they have direct great effects in the soft tissue regeneration [48]. Recent study showed that transplantation of these cells leading to new tissue growth and deposition of collagen [49].

These cells can help in repair of tissues as they able to differentiate to many types of cells as connective tissues cells [50].

Group II in the present study showed increase in GAPDH, iNOS and TGF-β genes expression. Tissue remodeling in the course of inflammation results in fibrosis, another very important response in the pathological and physiological process of vaginal atrophy as result of ovariectomy. In this process, TGF-β strongly contributes to the pathogenesis of fibrotic disorders in the remodeling of endometrium by increasing the production of ECM components. TGF-b signaling is initiated by ligand binding to type I and type II receptor serine/threonine kinases on the cell surface and complex formation. The formation of this complex allows the type II receptor to phosphorylate the kinase domain of the type I receptor, which propagates the signal through the phosphorylation of Smad proteins [51].

The inflammatory microenvironment plays a critical role in the pathogenesis of vaginal atrophy, and inflammation-induced tissue damage can lead to persistent pain. During the infection, different proinflammatory cytokines such as GAPDH, iNOS and TGF-β are induced as initiators and mediators of the inflammatory response. With the production of these proinflammatory cytokines, cell signaling pathways associated with inflammation, such as MAPKs signaling pathways, are activated. The MAPKS family includes at least three components: ERK1/2, JNK and p38 MAPKS, which play critical roles in the regulation of the induction of these proinflammatory mediators [52]. Our findings in the present study demonstrate that MSCs effectively inhibit the production of the proinflammatory mediators GAPDH, iNOS and TGF-β.

Other studies, investigated that the BM-MSCs have antifibrotic effect in different animal’s models with organs fibroids and observed a significant reduction in the degree of fibrosis [53&54].

In case of decrease ovarian hormones specially estrogen due to various causes (POF, normal menopause or surgical removal of ovaries) the symptoms as vaginal atrophy and its complications can be avoided or minimized by use of the hormone [55]. But WHO study published in 2002, reported that increase risk of many diseases after hormone replacement therapy as stroke or heart disease. Also increasing estrogen level leads to proliferation of epithelium and predisposing to cancers as breast cancer and endometrial carcinoma [56].

Conclusion
BM-MSCs can be used to treat vaginal atrophy and avoid the uses of hormonal therapy and their effects on vagina are through elevation of estrogen hormone or through direct effect on vaginal epithelium and connective tissues.

ACKNOWLEDGMENTS
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