

Research Paper

Effect of Adriamycin on $\alpha 1\beta 1$, $\alpha 1\beta 4$, and $\alpha v\beta 3$ Expression in Rat Endometrium in Estrus Phase

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ABSTRACT

Introduction: Although the effect of adriamycin, a chemical drug used to treat cancer, is known to affect gametogenesis and ovarian function, the drug's effect on endometrial receptivity and expression of integrin genes is unclear. To fill this gap, we aimed to investigate the effect of adriamycin on the expression of integrin genes that play a role in endometrial receptivity.

Methods: A total of 32 female rats weighing about 200 g were tested in the present study. The rats were divided into 4 groups: Healthy (sham), control, and experimental, with adriamycin at 4 mg/kg and 8 mg/kg doses for 6 weeks. Two weeks after the injection of adriamycin at an 8 mg/kg dose in the treatment group, all rats were sacrificed, but the treatment group with a 4 mg/kg dose of adriamycin continued the treatment. Then, the estrus phase was determined in the three groups using vaginal smears. The endometrial tissue was removed, and gene expression levels of $\alpha 1\beta 1$, $\alpha 1\beta 4$, and $\alpha v\beta 3$ genes were evaluated using real-time PCR methods in the three groups.

Results: The rats' weight decreased significantly in the treatment group with adriamycin. The process of weight loss was found to be dose-dependent. Integrin gene expression in the adriamycin group in the estrus phase had no significant difference compared with that in the control and sham groups ($P > 0.05$). In the adriamycin group, the expression of integrin $\alpha 1$ (*Intga1*) increased significantly ($P < 0.05$; $P = 0.013$).

Conclusion: The expression of integrin genes did not change, except for integrin $\alpha 1$, during the estrus phase in the endometrium treated with adriamycin drug. Probably, adriamycin did not have any effect on the expression of integrin genes.

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1. Introduction

Chemotherapy is an essential process for treating people with cancer. As awareness of the side effects of breast cancer chemotherapy increases in terms of reproduction, many breast cancer patients are seeking strategies to maintain their fertility [1]. Although aggressive chemotherapy improves the lives of women with cancer during their reproductive years, the effect on the ovaries usually results in infertility. So far, several studies have been carried out on changes in ovarian reserve, endocrine function, and MIS hormones (Mullerian inhibiting factor, LH, follicle-stimulating hormone, estradiol, and menstrual status) in young women with breast cancer under chemotherapy [2]. It has also been demonstrated that women who have had chemotherapy before menopause are more likely to develop amenorrhea and impaired fertility [1]. Other side effects of chemotherapy include the reduction of AMH hormone, anti-mullerian hormone, and inhibin B, degradation of primary follicles, a decrease in antral follicles, ovarian volume, and ovarian atrophy [2]. An effective chemotherapy drug for the treatment of cancer is adriamycin (doxorubicin), one of the strongest antibiotic anthracycline drugs extracted from *Streptomyces peucetius* var, which is used to treat malignant sarcoma, lung cancer, neuroblastoma, and breast cancer. Acute and chronic cardiotoxicity associated with the dose induced by this drug has limited its use. Endometriosis undergoes morphologic changes during implantation, and in this phase, minor changes occur in the nucleus and mitochondria, the rough endoplasmic network, and the vascular system [3]. The implantation of the fetus in the mother's uterus depends on two factors: The quality of the fetus and the quality of the endometrium. Integrins are one of the most important factors that play a vital role during implantation. Up until now, there have been many types of integrin that have remained known, but three types are expressed during implantation: $\alpha1\beta1$, $\alpha4\beta1$, and $\alpha v\beta3$ [2]. Epithelial integrin $\alpha v\beta3$ is expressed at the epithelial endometrial surface on days 19-24 of menstrual cycles. Previous research has found that the amount of this integrin expressed during the luteal phase is reduced in LPD, endometriosis, and infertility due to unspecified and hydrosalpinx [4]. The integrin molecules $\alpha1\beta1$, $\alpha4\beta1$, and $\alpha v\beta3$ are considered to evaluate the potential for endometrial admission in the implantation phase and are all expressed together. The expression of structural integrin $\alpha2$, $\alpha3$, $\alpha6$, and $\beta4$ is expressed uniformly in all healthy endometrium. The subunits $\alpha2$ and $\alpha3$ are expressed in total cells, whereas $\alpha6$ and $\beta4$ are localized at the lateral

surface of the endometrial glands [5]. Steroid hormones play an important role in the appearance and in regulating the expression of integrins in the secretion phase. $\alpha1\beta1$ is a receptor of laminin and collagen, and progesterone can directly increase its expression in the endometrial secretion phase [5-7]. $\alpha v\beta3$ integrins appear in the endometrial epithelium at 19-20 cycles. Three amino acids of Arg-gly-asp are detected in this integrin, which is effective in the endometrial binding of trophoblast [7]. and its expression in the endometrium depends on endometrial puberty and sexual hormones are capable of affecting this expression [8]. Pregnancy in rodents like mice is based on the Estrous cycle because mating and pregnancy occur only in the estrus phase. Given the importance of the role of $\alpha1\beta1$, $\alpha4\beta1$, and $\alpha v\beta3$ integrins in the implantation, it is expected that the expression of integrins $\alpha1\beta1$, $\alpha4\beta1$, and $\alpha v\beta3$ genes in chemotherapy in the estrous phase be different. The aim of the present study was to determine the effect of adriamycin on the expression of $1\beta1\alpha$, $1\beta4\alpha$, and $v\beta3\alpha$ integrin genes in the endometrial tissue of adult female rats in the estrous phase. Given the effect of adriamycin, the purpose of the present study was to evaluate the effect of adriamycin on the expression of integrin genes.

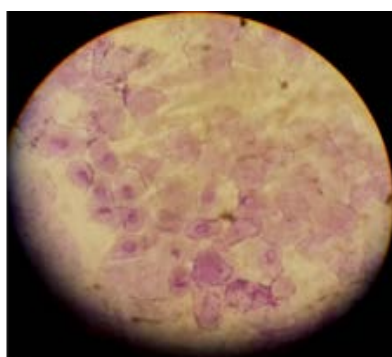
2. Methods

Animals

An experimental study was performed on 32 three-month-old Wistar female rats with a weight of 180–200 g, prepared by the [Pasteur Institute](#) of Tehran. Animals were kept at the central animal house of [Shahid Beheshti University of Medical Sciences](#), Tehran, at 24°C, 12 h of light, and 12 h of darkness, and fed with special rodent food. After adapting the animals to their new environment, they were randomly divided into four groups (sham, control, and experiment with adriamycin at 4 mg/kg and 8 mg/kg doses for 6 weeks), with eight animals in each group.

Adriamycin injection method

The experimental group received 4 and 8 mg/kg of adriamycin (with 10 mg of vial, American Pharmacia Company) via intraperitoneal (i.p.) injection for 4 weeks, each time 0.5 mL (one time per week). The dose is equal to the recommended dose for the treatment of breast cancer in rats [4]. Subsequently, mice were evaluated 2 weeks after injection, and their mortality was calculated.



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Figure 1. Vaginal smear test of the estrous phase in rats (magnification of 40X).

Determining the stage of the estrus cycle

Female rats have a 4–5-day cycle of estrus. The estrous phase was determined for each adult rat using a vaginal smear test. The estrous phase was determined by the proportion of cells in the smear after crystal violet staining. A vaginal smear cytology was performed in one of the four stages of proestrus, metestrus, estrous, and diestrus. A vaginal smear was prepared early morning to ensure the rats had the same cycle. In this method, 50 μ L of distilled water was piped into the animal's vagina and then placed on a slide. After drying, the sample was stained with crystal violet. After being stained, the specimens were detected using an optical microscope with a magnification of 40, and the stages of the estrous cycle were determined based on the classical cellular characteristics of the specimens so that in the estrus stage, surface cells were without nuclei and foamy [9] (Figure 1).

Sample preparation

After determining the stage of the estrous phase, eight rats were randomly divided into three groups: Control, experiment, and sham. The rats were then sacrificed by dislocation of the cervical vertebrae. A tissue sample of about 2 mL was prepared from 1/3 of the uterus for real-time PCR. Samples were washed with a high volume of buffer. The endometrial tissue was removed and frozen at -20°C in a microtube containing RNA later.

Real-time polymerase chain reaction analysis (RT-PCR)

At first, the samples were crushed. RNA extraction kit was used to extract RNA (Yekta Tajhiz Azma, Iran) according to the manufacturer's protocol. Prime Script™ RT Reagent Kit (Fermentas, Canada) was used to synthesize cDNA. The cDNA synthesis reaction was carried out at 42°C for 60 minutes and stored at -20°C . All

real-time PCR reactions were performed on the Roche machine. The temperature schedule for the device was set in three steps: The first stage causes the denaturation of cDNA molecules (95°C for 10 min). The second stage was 60°C for 30 s of annealing and 30 s at 72°C in 40 consecutive cycles. Finally, the third step was 10 min at 72°C for extension. The primer sequences used for RT-PCR are summarized in Table 1. Relative cartilage gene expression was analyzed using the comparative Ct method ($2^{-\Delta\Delta\text{Ct}}$).

3. Results

Animal weight and mortality rate

Two weeks after the last injection of adriamycin, the survival rate was 100% in the control group, but in the group treated with adriamycin, the highest dose was 8 mg/kg to 0%, and a dose of 4 mg reduced the weight of the rats. The average weight of the animals in the experimental group with a dose of 4 mg/kg (3.12 ± 19.19) was lower than that of the control group (7.27 ± 14.26). There was a significant difference between the control and treatment groups with adriamycin in terms of weight ($P < 0.05$) (Table 2, Figure 2).

Integrin gene expression level

Estrus stage was determined based on the classical cellular characteristics of the specimens two weeks after the last adriamycin injection, and endometrial samples of uterine rats were collected and evaluated for Integrin gene expression level using the real-time PCR technique. Also, the rate of expression of integrin $\alpha 1$ in the treated group was not significantly different from those of the control and sham groups ($P > 0.05$) (Figure 3, Table 3). Moreover, the results showed that there was no significant difference in the expression of integrin genes $\alpha 4$ and $\beta 3$ between the treated groups (adriamycin group) and those of the sham and control groups (Figure 3); however, the level of *intga1* expression in the treated group with adriamycin was significantly increased as compared to the control group ($P < 0.05$, Value=0.027) (Figure 3). The results showed that there was no significant difference in *intgb1* gene expression between the adriamycin group, sham group, and control group ($P > 0.05$) (Figure 4, Table 4). In the current study, injection of adriamycin into rats significantly increased the expression of the *intga1* gene in the endometrium as compared to *intga*, *intgb1*, *intgb3*, and *intga4*.

Table 1. The characteristic of primers used in real-time PCR

Accession No.	Target Gene	Primer Sequence	Product Size
NM_010578	<i>Itgb1</i>	F: 5'-CAATCCAATCCAGGAAACCA-3' R: 5'-ACACCGACCCGAGACCCT-3'	102
NM_016780	<i>Itgb3</i>	F: 5'-CAGGACTCACAGCCAGACAC-3' R: 5'-AGTGGCCGGGACAACCT-3'	169
NM_010576	<i>Itga4</i>	F: 5'-TGTCTTCCACAAGGCTCTC-3' R: 5'-AATGCCTCAGTGGTCAATCC-3'	117
NM_010577	<i>Itga5</i>	F: 5'-TGGTGCACTGGATAGGACTG-3' R: 5'-TTCTCGTGGAGTTTACCG-3'	145
NM_013627	<i>Itga1</i>	F: 5'-GTGGAGGGCAAAGAAACAGT-3' R: 5'-TCTGCCTCAGTGAATCAAGG-3'	127
NM_001001303	<i>GAPDH</i>	F: 5'-TTGATGGCAACAATCTCCAC-3' R: 5'-CGTCCCGTAGACAAAATGGT-3'	110

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4. Discussion

Although aggressive chemotherapy improves the lives of women at reproductive age with breast cancer, it frequently results in infertility, ovarian damage, and ovarian cancer. Three chemotherapeutic drugs, adriamycin, taxol, and cyclophosphamide, are used in the literature to treat breast cancer, from which adriamycin was used in the present study [10]. Ottewell and his colleagues (2008) used adriamycin at doses of 2 mg/kg, 4 mg/kg, and 8 mg/kg for breast cancer treatment. Their research showed that the dose of 8 mg/kg was effective in improving cancer patients compared to the other two doses [11]. However, in the present study, this dose was known as a toxic dose due to the high mortality rate at a dose of 8 mg/kg.

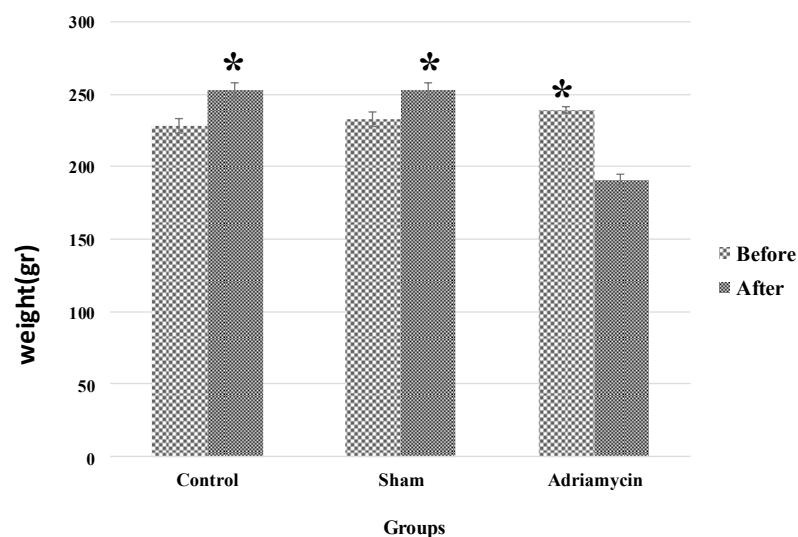
According to recent studies by other researchers, it can be concluded that one of the reasons for weight loss is vomiting and lack of appetite, which can be due to the stimulation of chemical receptors on the bottom of the ventricle located in the medulla. In the present study, in-

traperitoneal administration of adriamycin for 6 weeks at a dose of 4 mg/kg significantly reduced the rats' weight, consistent with the findings reported by Tei et al. (2003) [12]. According to the results of the present study, there was no significant difference in the expression of integrin genes $\beta 1$, $\alpha 4$, α_v , and $\beta 3$ except $\alpha 1$ between the treated groups with adriamycin and the control group. Thomas et al. (2002) investigated the stimulation of gonadotropins on $\alpha 1\beta 1$, $\alpha 4\beta 1$, and $\alpha v\beta 3$ expression using the immunohistochemistry method, which reduced expression in all three integrins in glandular epithelium and decreased $\alpha\beta 3$ expression in luminal epithelium. Regarding the significant effect of the expression of these integrins on the implantation phase and the stimulatory effect of gonadotropins in IVF, a reduction of the expression of these integrins was observed [7]. In another study, immunohistochemistry was used to assess the expression of $\alpha v\beta 3$ integrin during the implantation phase in 10 fertile women and 22 women with infertility of unknown cause. In this study, patients with infertility, due to an unknown reason, had lower levels of $\alpha v\beta 3$ integrin expression [13]. However, in our study, using

Table 2. Mean \pm SD of adriamycin (treatment group), sham, and control groups

Group	Mean \pm SD	
	Before	After
Control*	228.75 \pm 4.98	253.25 \pm 4.87
Treatment (4 mg/kg)**	239.37 \pm 2.57	191.37 \pm 3.6
Sham	233 \pm 5.1	253 \pm 5.3

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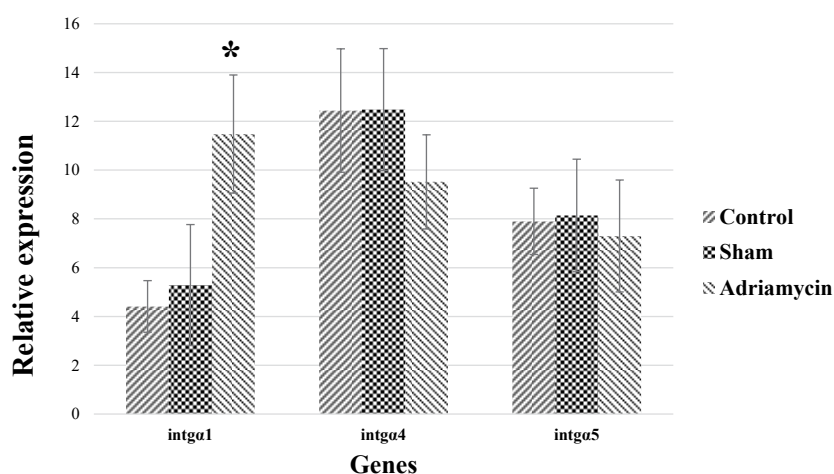
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Figure 2. Comparison of the mean weight of rats in the treatment group with adriamycin and control and sham

*There is a significant difference between the control group ($P=0.04$), **Significant difference between the treatment group (adriamycin) ($P=0.001$), *Significant difference between the sham group is $P=0.03$.

Real Time-PCR, the gene expressions of the treatment group with adriamycin, $\beta 1$, $\beta 3$, $\alpha 5$, and $\alpha 4$ were found to be different compared with those of the control group. In addition, the expression of $\alpha 1$ was upregulated in the adriamycin group. Peyghambari et al. conducted a study on the expression changes of $\beta 3$, αv , $\beta 1$, and $\alpha 4$ and osteopontin ligands in the phases of the sexual cycle in 15 rat rats. Because binding of integrins to the extracellular matrix requires a specific ligand such as collagen 4, fibronectin, laminin, and osteopontin, immunohistochemistry results showed that expression of $\beta 3$, αv , $\beta 1$, $\alpha 4$,

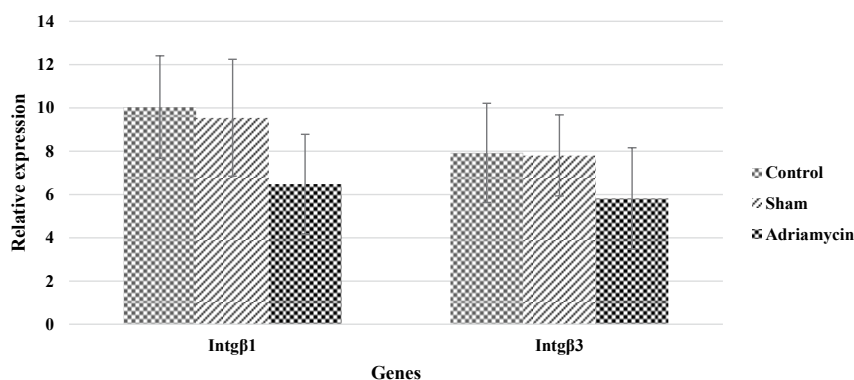
and $\beta 4$ was only in the met estrus stage. Among these Specific ligands, osteopontin is one of the extracellular matrix molecules found in most organisms secreted in the osteopontin genital system by endometrial secretion cells, invasive trophoblasts. This study also determined that osteopontin is secreted by progesterone from the uterine gland and that its ligand is $\alpha v \beta 3$ [14]. In the study mentioned above, the expression of integrin genes is considered to be in the met estrus stage, while in our study, the expression of integrin $\alpha 1$ gene with real-time PCR was positive only in the estrus stage as a result of



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Figure 3. Comparison of *intga1* expression in control, adriamycin, and sham groups with real-time PCR technique

*The level of *intga1* expression in the treated group with adriamycin was significantly increased compared to the control group ($P<0.05$, value=0.027). The expression level of *intga5* in the treated group was not significantly different from that of the treated group ($P>0.05$). The rate of *intga4* in the treated group was not significantly related to the control group ($P>0.05$).



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Figure 4. Comparison of the expression of *intgβ1* in three three-group experiments: Adriamycin, sham, and control using real-time PCR

The rate of *intgβ1* in the treated group was not significantly related to the control group ($P>0.05$). The rate of *intgβ3* in the treated group was not significantly related to the control group ($P>0.05$).

injection of adriamycin. According to a study by Yang et al. (1999), who examined the expression of osteopontin mRNA with different concentrations of doxorubicin in the breast cancer cell model using Western blotting and RT PCR, it can be concluded that the osteopontin ligand can be effective in increasing the expression of integrin $\alpha 1$ [15]. In the present study, the increased expression of $\alpha 1$ inhibition in the estrus phase was higher than that of other integrins, which may be one of the reasons for its increase in the expression of the osteopontin ligand.

Vernaev et al. (2007) investigated donated ovum implantation in women who had a history of developing a variety of cancers, including breast cancer and Hodgkin's lymphoma sarcoma, who were treated with chemo-

therapy, and compared the results with those without any history of the disease. The results showed that chemotherapy during the implantation of donated oocytes had no effect on people who had previously been co-treated with chemotherapy [15]. While in our study, the expression of integrin genes that play an important role in endometrial receptors was evaluated, no significant difference was found in the expression of integrin genes of $\alpha 4$, αv , $\beta 3$, and $\beta 1$ in the control and experimental groups.

The results of this study corresponded to those reported by Vernaev et al. (2007), who used adriamycin chemotherapy in the implantation phase. Due to the complications of chemotherapy and infertility, many fertility patients who are planning to have a pregnancy have fertility

Table 3. Mean \pm SD in the expression of adriamycin group gene, sham group, and control

Group (<i>intgα1</i>)	Mean \pm SD
Control	4.41 \pm 1.06
Adriamycin	11.48 \pm 2.53
Sham	5.29 \pm 1.36

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Table 4. Mean \pm SD in adriamycin group gene expression, control group, and sham

Group	Mean \pm SD			
	<i>intgα4</i>	<i>Intgα5</i>	<i>Intgβ1</i>	<i>Intgβ3</i>
Control	12.44 \pm 2.48	7.9 \pm 2.42	10.04 \pm 2.36	7.92 \pm 2.29
Adriamycin	9.52 \pm 2.5	7.30 \pm 1.93	6.48 \pm 2.7	5.82 \pm 1.87
Sham	12.48 \pm 2.31	8.14 \pm 2.29	9.54 \pm 2.3	7.80 \pm 2.34

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assistance options, such as egg cultures, donated eggs, or even fetal storage, until after the completion of chemotherapy and return of the menstrual cycle, when pregnancy occurs through IVF or other ways. But the point to consider is whether endometrial admission is maintained after the end of the chemotherapy period. According to a study by Vernaev et al. (2007) on the effect of chemotherapy on endometrial receptors after oocyte donation in cancer patients, chemotherapy had no effect on oocyte susceptibility, and there was no significant difference in endometrial receptor in chemotherapy patients and in the control group that had oocyte donation [15].

5. Conclusions

Chemotherapy with adriamycin has no effect on integrin $\alpha 4$, $\alpha 5$, $\beta 3$, and $\beta 1$ receptors and can even increase the expression of the $\alpha 1$ endometrial receptor gene. Consequently, for young women who have cancer at fertility age, there is a hope that after the completion of chemotherapy and returning to a menstrual cycle, even in the absence of ovarian reserve, they can experience pregnancy and childbirth with the help of assisted reproductive techniques.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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