Original Research Article

The effect of Ajuga reptans extract on *in vitro* maturation of rat oocytes

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ABSTRACT

Resumption of nuclear maturation and cytoplasmic changes occur during *in vitro* maturation. *In vitro* maturation of the egg provides a stage called Metaphase II (MII) that is required for *in vitro* fertilization. This study aimed to investigate the effect of replacing Follicle-Stimulating Hormone (FSH) with plant extract that 5 groups were used. The null group, which consisted only of oocytes, the control group, which used Fetal Bovine Serum (FBS) and FSH, the 3 experimental groups, in which different doses of Ajuga L. extract were substituted for FSH. Ajuga extract with appropriate dose increased the prevalence of Germinal Vesicle Degradation (GVBD), growth rate and maturity of immature mouse oocytes to MII stage.

***Keywords*:** Ajuga L, maturation, germinal vesicle degradation

INTRODUCTION

Today, the method of *In Vitro* Fertilization (IVF) and laboratory *In Vitro* Maturation (IVM) is widely used to treat infertility. Infertility is one of the problems that depends on the reproductive system as well as the endocrine system. By affecting the

testes and ovaries by secreting hormones, gonads play an important role in the formation of gametes, their maturation, the health of the reproductive system, and the development of a perfect embryo. Because, to a large extent, reproductive defects are related to the function of stimulating and sexual hormones (FSH, LH); IVM has been able to improve these defects by culturing gametes in the laboratory and maturing them using hormones. Based on the results of recent studies, it can be concluded that more than 80 million couples around the world experience infertility, and in such a situation, one of the most important issues to be discussed is assisted reproductive methods [1]. In the production process of laboratory embryos, the stage of egg maturation is of great importance. During laboratory maturation, in addition to the resumption of nuclear maturation, cytoplasmic changes also occur [2]. Considering the fact that for laboratory reproduction or transfer of nucleus and asexual reproduction, there must be a specific stage of ovulation (metaphase II), it is necessary to perform the stage of egg maturation in the laboratory [3]. In mammalian eggs, meiosis begins in fetal life and stops at birth during the meiotic stage I diploma. After puberty, folliculogenesis begins, where the pre-ovulation stage is associated with LH. The resumption of meiosis from the Germinal Vesicle (GV) stage: following chromosome density, disintegration of the nucleus coating and spindle formation is determined. From the resumption of meiosis to the formation of Metaphase II (MII) stage, it is defined as egg nucleus maturation. Eggs also complete cytoplasmic maturation, such as molecular and structural changes, to produce mature eggs that support the potential for fertilization and early fetal development [4]. Past research shows that antioxidants have been shown to reduce the incidence and prevalence of many diseases [5,6]. Oocytes and sperm that are removed from their natural environment can be exposed to excessive levels of reactive oxygen species (ROS) due to the destruction of antioxidant defense mechanisms, [8] ultimately leading to Oxidative Stress (OS) [9]. Oxidative stress, due to its ability to alter molecules such as lipids, proteins and nucleic acids, has detrimental effects on fertility by disrupting sperm membrane integrity and stimulating structural DNA damage, mitochondrial changes, adenosine triphosphate depletion and apoptosis. [10, 11] Development following oxidative stress is one of the leading causes of defective gametes or poorly growing

embryos in ART (assisted reproductive techniques) [9]. This is because in the IVF process performed in a clinical laboratory, it is not possible to reconstruct the exact conditions under which natural fertilization occurs [12]. One of the important factors in reproductive assistive methods is the strict control of ROS levels, which is maintained at physiological concentration by antioxidants inside the body [13]. In order to maintain physiological ROS levels and prevent the growth of oxidative stress, excess ROS must be continuously neutralized. Antioxidants by preventing the formation of ROS by terminating oxidative chain reactions or by clearing existing ROS, are able to neutralize antioxidants and thus maintain the balance of active oxygen/antioxidant species and thus protect the cell from oxidative damage [14,15]. Components of the human reproductive system contain antioxidants that are endogenously formed or obtained from food sources [16]. In women, natural antioxidants are present in the ovarian fluid, follicles, tubules and peritoneum, and endometrial epithelium [12].

In order to optimize gamete quality, prevention is essential to reduce the build-up of any ROS that causes oxidative stress during IVM and IVF. One way to achieve this could be to increase the antioxidant capacity of gametes and fetuses against the harmful oxidation attack. According to reports, the use of plants for laboratory maturation can have different effects on the maturation of gametes as well as fertilization [20,39]. These effects of plants can be due to the presence of flavonoids and phytoestrogens in plants [21,45,39]. Many compounds such as sterols, flavonoids, organic sugars and phenolic compounds are found in plant extracts. [41] Flavonoids are chemical phenyl benzopyrone that are commonly found in all vascular plants. [40] Flavonoids are a class of secondary plant metabolites and are best known for their antioxidant activity in the laboratory [7]. Also, numerous studies have shown that chamomile flavonoids, in addition to having antiviral, anti-allergic, and anti-cancer effects, also have antioxidant effects [48,49]. On the other hand, it has been reported that large amounts of flavonoids can be toxic to eggs. [50] Plants of the Ajuga from the genus Lamiceae and the species austroiraniaca, are appropriate for various activities such as diuretic activity, [22] antioxidants, [23,24,25] anti-arrhythmia [26]. Antiviral against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), [27] antimicrobial, [24] antiseptic, [28] analgesics, [23,29] hypolipidemic [30] and

hypoglycemic [31,32]. This study was performed to determine the effect of Ajuga reptans on the maturation of eggs in IVM, as a hormone and an antioxidant.

MATERIALS AND METHODS

In this experimental study, the eggs of NMRI Syrian mice 6-8 weeks old, which are kept in the pet section of the Royan Research Institute, and the plant extract (Ajuga) were used.

Extract preparation

Ajuga plant was collected from Fars province of Iran. 100 mg of Ajuga plant powder was dissolved in 5 cc of serum-physiology. The resulting solution was kept at room temperature (25 °C) for 2 h. A dry centrifuge tube was weighed and the resulting solution was poured into it and centrifuged at 5000 rpm for 5 min. Then the supernatant was removed completely and the tube with the sediment was placed in the oven for 24 h to dry completely. The centrifuge tube and the dried precipitates were weighed to obtain the weight of the amount of the undissolved material, and the weight of the solute was calculated by subtracting the initial value. Then, by calculating the weight of the dissolved material, the volume of the supernatant increased to a concentration of 10 mg/ml.

Ajuga reptans extract on in vitro maturation

Grouping

In this study, 5 IVM culture medium groups: control group without receiving any extract (Control Group) and the group containing only eggs (null) and three experimental groups of extract with different doses, the extract has replaced FSH; Were used. 1. In the control group, using FSH, Human chorionic gonadotropin (HCG) hormones and FBS, mouse egg development took place in culture medium. 2. In the null, the development of the egg was performed without the use of any substances. 3. In the first experimental group, egg formation was performed using HCG, FBS and 250 ml of plant extract 4. In the second experimental group, egg formation was performed using HCG, FBS and 500 ml of plant extract 5. In the third experimental group, egg formation was performed using HCG, FBS and 750 ml of plant extract.

Study of puberty and premature eggs

Preparation of premature eggs

In dissecting ovarian tissue, female mice sacrificed and their ovaries were removed in sterile conditions and transferred to culture medium droplets: 95 % MEMa and 5 % FBS, 100 IU/ml Streptomycin and 100 IU/ml penicillin, then fat Ovarian detachment was isolated by insulin needles

and dissection of premature eggs occured. At this stage, the eggs were divided by size into two categories: follicles and immature eggs. The immature follicles that contain the germinal vesicle along with the granulosa cells Removed and then the accompanying granulose cells were Removed with a pipette and the eggs that have a clear cytoplasm, a clear and uniform wall of the zona plusida with a suitable yolk space were selected and entered the IVM culture medium.

Immature egg maturation in IVM

During IVM, prematurely were isolated eggs, in culture media: 1. Cultivation environment of control group containing (70 %) MEMa, (10 %) FBS, (10 %) HCG, (10 %) FSH, which is layered covered with mineral Oil 2. Cultivation medium with group (null) that does not contain any material 3. Cultivation medium for experimental groups including (70 %) MEMa, (10 %) FBS, (10 %) HCG, (10 %) Plant extracts (Ajuga), coated with mineral oil, were placed in an incubator for 18 to 24 hours at 37 °C with 100 % humidity and 5 % of CO_2.

Capacity sperm

In the Capacity sperm stage, after sacrificing the male mice their testicles were separated in a sterile environment, next to a flame, and were placed into the sperm culture medium, including (90 %) T6 and (10 %) BSA15, which is coated with a layer of mineral oil. Sperms were removed from the testicles using insulin needles to remove sperm inside them; at the end of this stage, active sperms were isolated and transferred to the IVF culture medium.

In vitro fertilization (IVF)

During IVF, eggs from IVM culture and sperms from sperm culture were transferred to IVF culture. IVF culture: (90 %) T6 and (10 %) BSA15 coated with a layer of mineral oil. After transfer, the ovule was inserted into the sperm droplet to fertilize. Then the egg cell was transferred to the drop of the develop culture medium containing: (90 %) T6 and (10 %) BSA4, which is covered with a layer of mineral oil, and for 18 to 24 h at 37 °C with 100 % humidity and 5 % of CO2 was placed in the incubator.

RESULTS

Adding Ajuga extract to the mature culture medium of immature mouse eggs increased GVBD levels. The incidence of GVBD increased significantly with the presence of Ajuga extract compared with other

treatment groups and control groups. The amount of eggs left in the GV stage in this group of Ajuga extract is 19 %, the amount of GVBD eggs in this group is 15 % and the amount of eggs that have reached the MII stage is 67 %. Finally, according to the obtained results, it can be said that the percentage of meiosis recurrence (MII + GVBD) in this group is equal to 82 %. According to the results, the use of Ajuga extract with the appropriate dose, increased meiosis restart rate (MII + GVBD) and resulted in high percentage of MII. Concomitant use of Ajuga extract seems to have synergistic effects.

When comparing the groups in terms of cell division progression (Figure 1), the number of cells that became bicellular within 24 h after fertilization, decreased in treatment group 1 compared to the control and without group; also, following this reduction, the rate of four cells in 24 h compared to the control group reached zero. In fact, this dose of the extract had a negative effect in 24 h, but the rate of double cells in 48 h in treatment 1 compared to the control and without has not changed; However, the rate of four-cell and eight-cell growth decreased by 48 compared to controls. Therefore, the greatest effect of extract dose reduction in treatment 1 compared to control occurred during the four-cell and eight-cell division. This dose in treatment 1, compared to control and treatment 2, had a more decreasing effect on the process of cell division, but increased the rate of four-cell formation within 24 h compared to treatment 3 and without. This comparison shows that the extract dose also affects the division process. This dose of extract in treatment 1 had a more positive effect on cell division process than treatment 3 during 48 h. Although the progression of cell division in treatment 3 was higher than treatment 1 in 24 h, but in 48 hours the division process decreased; While this is not the case in treatment 1, and although the dose of treatment 1 has reduced the progression of cell division within 24 h, it has increased the rate of progression in 48 h compared to without, and as a result, this dose with over time can increase the likelihood of cell division progressing, but compared to control it cannot be a replacement hormone.

When comparing treatment 2 with the control group (Figure 1), there was no change in the rate of four-cell division during 24 h and 48 h, but the process of two-cell division during 24 and 48 h was significantly increased compared to other groups; However, it should be noted that this dose of treatment 2 extract, like the

treatment dose 1, only advanced the cell to four-cell division and did not cause eight-cell division. Also, the dose of treatment 2 compared to treatments 1 and 3 generally had a more positive effect on all stages of cell division.

Compared to the treatment 3 and the without group during the two-cell division within 24 h, the presence of the extract didn't affect; But from comparing treatment 3 with control and without group, it can be said that the extract increased the number of cells that have become four cells in 24 h. Also, this amount of extract compared to treatments 1 and 2 during cell division during 24 h in stage four cellular proliferation has increased, but in general, treatment 2 has had a more significant and positive effect on cell progression than other groups (Figure 1).

In the process of the effect of dose on MII adjacent to sperm compared to treatment 1 with control group, the amount of MII oocyte adjacent to sperm decreased and compared to the group without; Contrary to our expectations, the amount of treatment 1 extract had a negative effect on the proximity of the egg to the sperm and the resumption of MII (Figure 2).

By comparing Figure 2 and Figure 1, it can be seen that although in treatment 2 the amount of proximity decreased compared to the control group, the same amount of eggs that were in proximity increased to 2 cells after 24 h of fertilization or 2 cells within 48 h after fertilization; The success rate of fertilization and the rate of cell division show a significant increase in the appropriate amount of extract in treatment 2 compared to other groups. Because in treatment group 2, compared to treatments 1 and 3, the degree of oocyte proximity to sperm and resumption of MII have increased, also compared to without, it can be said that this amount of extract can play the role of hormone in fertilization success to some extent. As a result, the extract in the right dose has a positive effect on the proximity of sperm (Figure 2).

Compared with treatment 3 with control, the dose of extract in treatment 3 reduced the success rate of fertilization compared to control; However, compared to treatment 3 with without, it can be said that the effect of this reduction was not to the extent that this dose of hormone replacement extract had a negative effect, but this amount of proximity to sperm is the same as without (Figure 2).

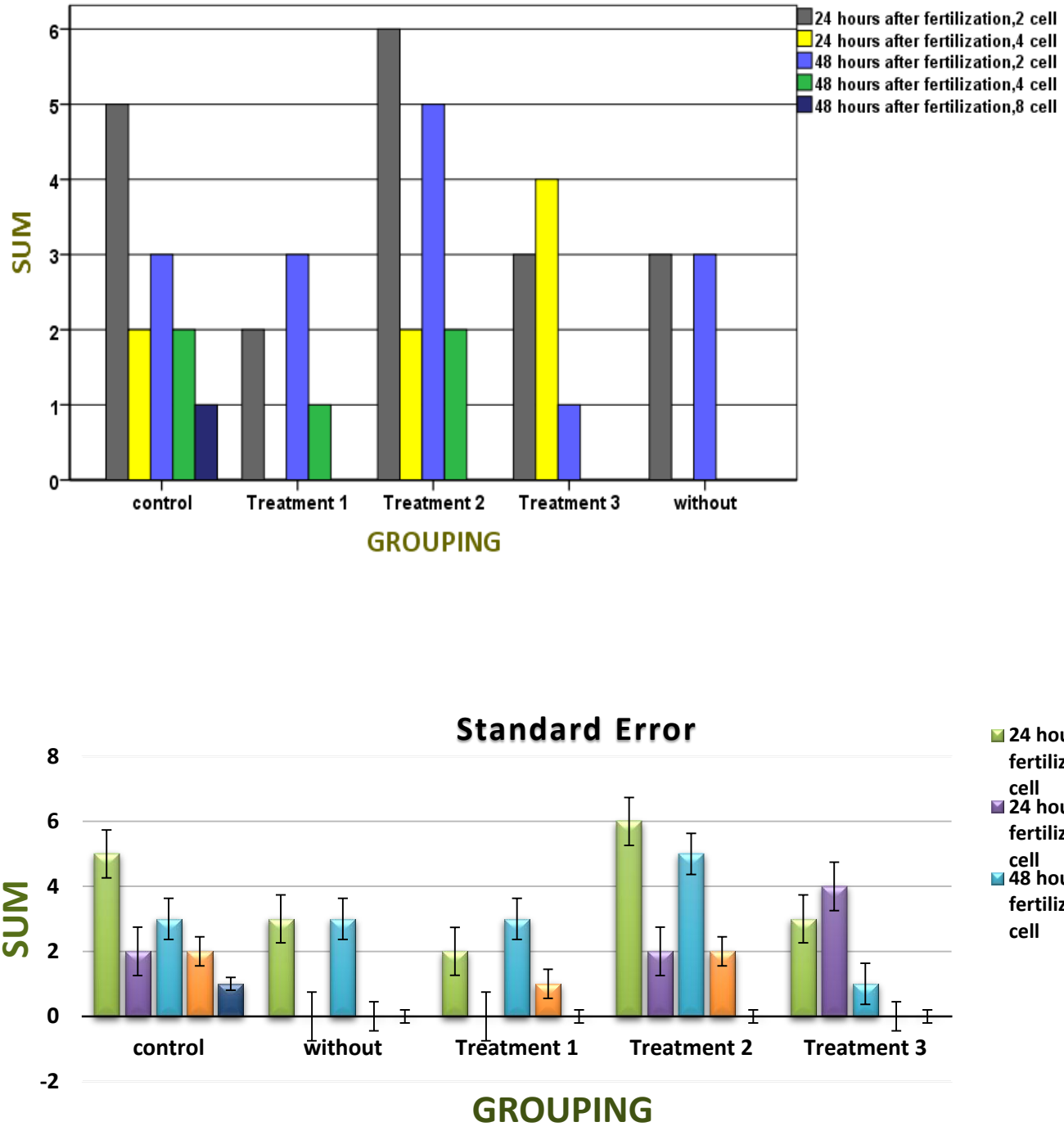
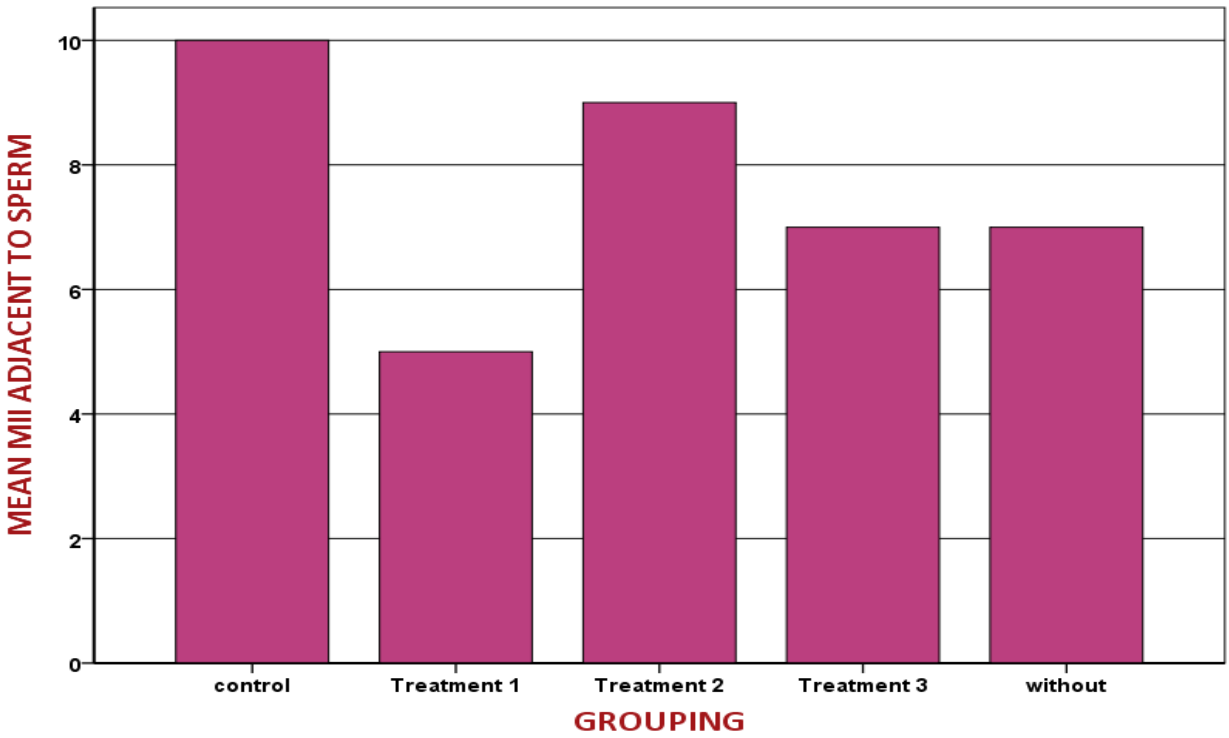


Figure 1. The effect of the extract on cell division after 24 and 48 h after fertilization in the treatment groups.



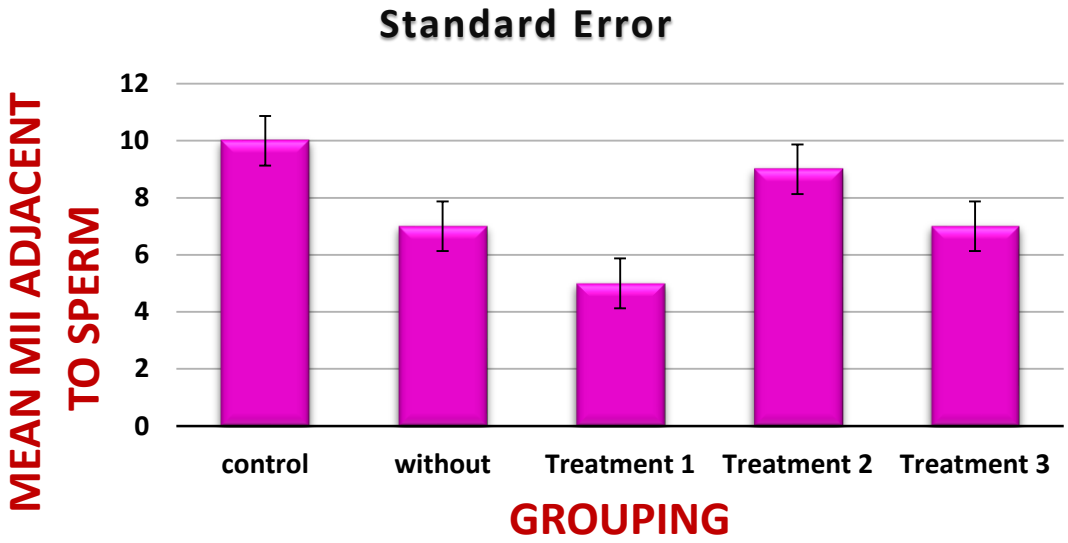


Figure 2. The effect of dose increasing in the treatment groups on changes in the MII adjacent to sperm and its effect on fertilization and comparison with the hormones in the control group**.**

Based on the amount of degenerated fetuses in each group (Figure 3), it can be said that in comparison with treatment 1 and without group, the extract reduces the amount of degeneration, but in comparison with control groups and treatments 2 and 3, it can be seen that the dose of extract is also involved in reducing degeneration. As can be seen in the graph, in the treatment group 3, where the dose of the extract is higher than treatments 1 and 2, so the degeneration rate is zero. Compared to control and without, it can be said that the hormone in the control group cannot reduce the amount of degeneration to the same amount as the replaced hormone extract. In fact, the extract definitely has a more positive effect than the hormone in reducing degeneration because it can act as an antioxidant. It should be noted that although, with increasing the dose of the extract, the degeneration decreases to a greater extent, a large amount of the extract can also have a negative effect on the development of cell division (Figure 3).

Examination of the three stages of cell division development and proximity to sperm and the rate of degeneration shows that the dose of the extract, which has a more positive and significant effect in all three stages simultaneously than the other two treatments, is present in treatment 2 (Figure 3).

DISCUSSION

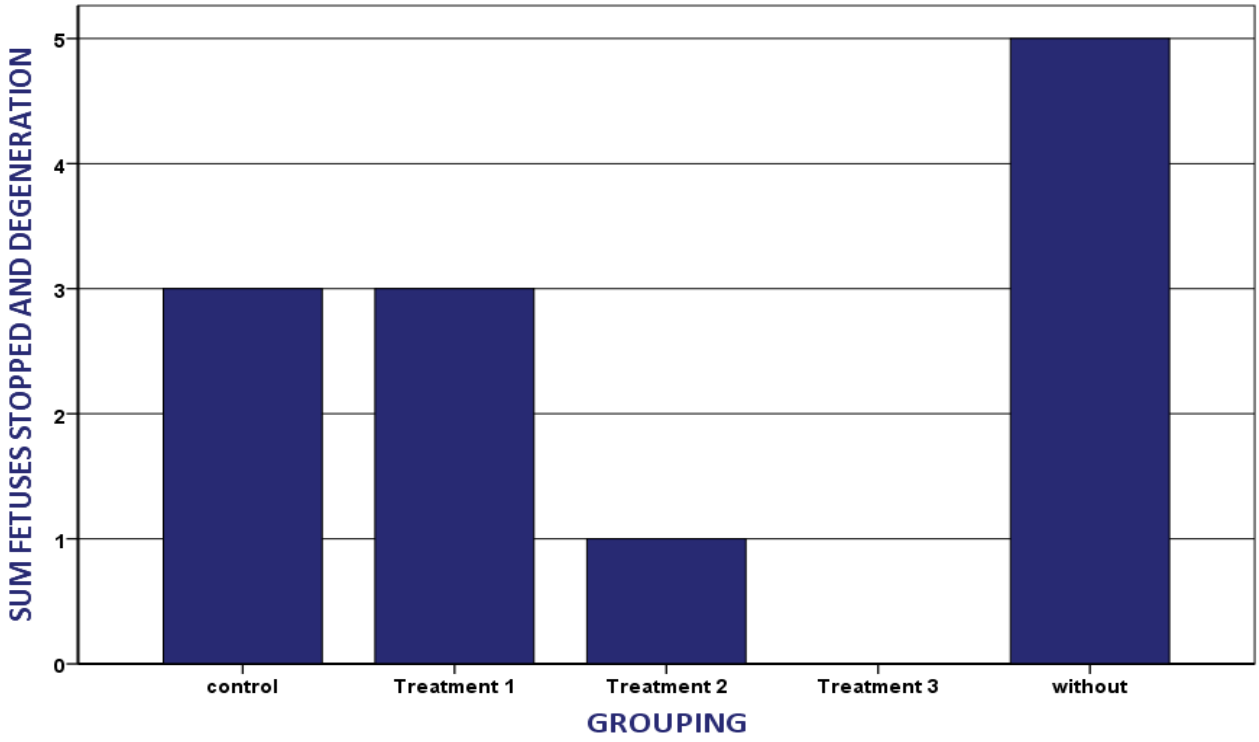
One of the most important factors for successful laboratory fertilization and development is to provide quality eggs that support the next stages of development. [17] One of the challenges that researchers face in laboratory cultivation is the high concentration of oxygen in the *in vitro* environment compared to *in vivo*. [18] The history of ovarian laboratory maturation (IVM) was first described in 1953 by Pincus G. [19] According to reports, the use of plants for laboratory maturation can have different effects on the maturation of gametes as well as fertilization; [20,39] These effects of plants can be due to the presence of flavonoids and phytoestrogens in plants [21,45,39]. Plants of the genus Ajuga are appropriate for various activities such as diuretic activity, [22] antioxidants, [23,24,25] anti-arrhythmia, [26] antiviral against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), [27] antimicrobial, [24] antiseptic, [28] analgesics, [23,29] hypolipidemic [30]and hypoglycemic [31,32]. The results of Ajuga plant phytochemical screening in Tafesse *et al.* study of antidiabetic activities by Ajuga Remota Benth indicate

the presence of flavonoids, phenolic compounds and steroids in this plant. [33] These results are in accordance with previous reports [34,35,36]. Mechanism of anti-diabetic effects of A. remota leaf extract [33] may be due to the presence of phytochemicals. It is known to be an antioxidant such as flavonoids, polyphenols and tannins that act as a free radical identifier [37,38]. The hypothesis of the mechanism of action of these antioxidants in Tafesse's research is due to a similar effect of insulin on peripheral tissues by stimulating the process of reduction or release of insulin secretion from the pancreas; which leads to a decrease in blood sugar levels. [37] In the study by SHoorei et al., The hydroalcoholic effect of chamomile (Matricaria chamomilla) on the growth and maturation of laboratory ovarian follicles in mice was investigated. Based on their research results, a significant reduction in the level of reactive oxygen species (ROS) of test group eggs with extract was observed compared to the control group [39]. The most characteristic chamomile compounds are primarily flavonoids, apigenin, quercetin, patulin, luteolin, and glycosides. [40] Many compounds such as sterols, flavonoids, organic sugars and phenolic compounds are found in plant extracts. [41] Flavonoids are chemical phenyl benzopyrone that are commonly found in all vascular plants. [40] One of the compounds in chamomile is flavonoid antioxidants that neutralize reactive oxygen metabolites. By binding to free radicals, antioxidants neutralize their harmful properties, such as the breakdown of body cells and tissues, the fragmentation of DNA, and the peroxidation of membrane lipids. [42] During laboratory follicle culture, free radicals are continuously produced in aerobic cells. Therefore, antioxidants can lead to the elimination of free radicals. [43] Another important compound of chamomile is phytoestrogens. [44] Phytoestrogens are considered to be estrogen-like compounds that have similar effects on the hormone’s estrogen and progesterone. [45] Based on experimental and clinical studies on chamomile, it has been concluded that most of their pharmacological functions are related to its antioxidant activity, which is largely due to its ability to control free radicals or inhibit lipid peroxidation. [46] Oxidative stress can play an important role in follicle by inducing the mechanism of apoptosis. ROS is spontaneously created in the normal metabolism of cells as well as in

physiological phenomena against severe infections that lead to cell death. Flavonoids found in plant drinks, fruits, or plants are powerful natural antioxidants that can protect cells from oxidative stress during cultivation. [47] Another study that has been done in this field; The supplementation of the laboratory culture medium by FSH is to replace the growth of adult follicles and eggs with Justicia insularis plant extract. According to the results of this study, the survival rate of eggs from the culture medium with Justicia insularis extract was higher compared to the FSH culture group. Also, in the plant extract group, metaphase II was higher than in the FSH group. [39] Studies by GoKA et al., in addition to alkaloids, glycosides, polyphenols, also show the presence of flavonoids in the leaves of this plant (Justicia insularis); which act as natural antioxidants and like the hormone FSH. [40] Oxidative stress (OS) is considered to be a prominent mediator in relation to ovarian aging and causes poor fetal growth. [51,52] The quantity and quality of eggs are greatly reduced due to OS induced apoptosis. [53] It is believed that the balance between Reactive Oxygen Species (ROS) and antioxidants in the egg for cell function including chromosome separation, [54,55] mitochondrial activity, [56,57] spindle assembly, [58] surface maintenance Balanced ATP and DNA methylation are very important. [59] Antioxidant supplements have been proven to protect eggs against ROS and OS. [60] Sodium citrate, α-lipoic acid (ALA) and acetyl-L-carnitine (ALCAR) are antioxidants that play an important role in protecting mammalian cells against oxidative stress by inhibiting free radicals. [61,62] Treatment of eggs with ALCAR in laboratory maturity (IVM) can improve egg quality. At the same time, by increasing the ALA supplement to the culture medium *in vitro*, the growth of follicles, mitochondrial activity, gene expression, and fetal growth in older female mice improved [60,63]. In a study by Li-Feng Liang et al., Mouse eggs were kept in an antioxidant-containing medium for 12 to 36 h and then processed in IVM to counteract the effects of antioxidants (sodium citrate, ALA and ALCAR). And other cellular functions, such as mitochondrial activity, spindle formation, chromosome configuration, and DNA integrity. Investigate nuclear maturity. According to their research, in short, a higher percentage of eggs that have been associated with antioxidants have reached metaphase II compared to eggs without

antioxidants. Antioxidants also improve spindle formation and chromosome configuration in the egg. In this study, egg quality was also determined by spindle morphology and chromosome level. Ordinary eggs have a common barrel-shaped spindle and chromosomes that are regularly located on the bipolar equatorial surface. An abnormal egg, on the other hand, has a spindle with irregular microtubule distribution and the presence of chromosomes that do not align at the spatial equatorial level. Antioxidant supplements significantly reduced the number of eggs with abnormal spindle formation in 24 and 36 hours in these groups compared to control (without antioxidant supplements) [64]. An imbalance between ROS and antioxidants, or too much ROS, can lead to various forms of DNA damage. However, especially the double strand DNA break (DSBs) is dangerous for the cell because it can lead to re-regulation of the genome and threaten the normal functioning of the eggs and even their survival. [65] The Li-Feng Liang study used the G-H2AX DSBs marker to study the intensity of fluorescence in eggs, which showed different degrees of DNA damage to different degrees of fluorescence staining. The experiment found that the intensity of fluorescence was low in fresh eggs and increased in eggs that were IVM for 12-36 h. Although fluorescence intensity was present in all IVM groups, it decreased significantly in antioxidant-free groups compared to the lack of antioxidants. [64]

Adding Ajuga extract to the mature culture medium of immature mouse eggs increased GVBD levels. So much so that the highest number of eggs that reached stage MII was related to the group that received the Ajuga extract at the appropriate dose. This group led to an increase in the maturation of immature mice to stage MII, which was significantly different from other treatment groups and control groups ($p \leq 0.05$). When comparing treatment 1 with null, it can be concluded that this amount of extract dose reduces the progression of cell division but amount of extract dose in treatment 2, it can be a good alternative to hormones because not only it has not changed the progression of cell division, but it has increased the progression of cell division.



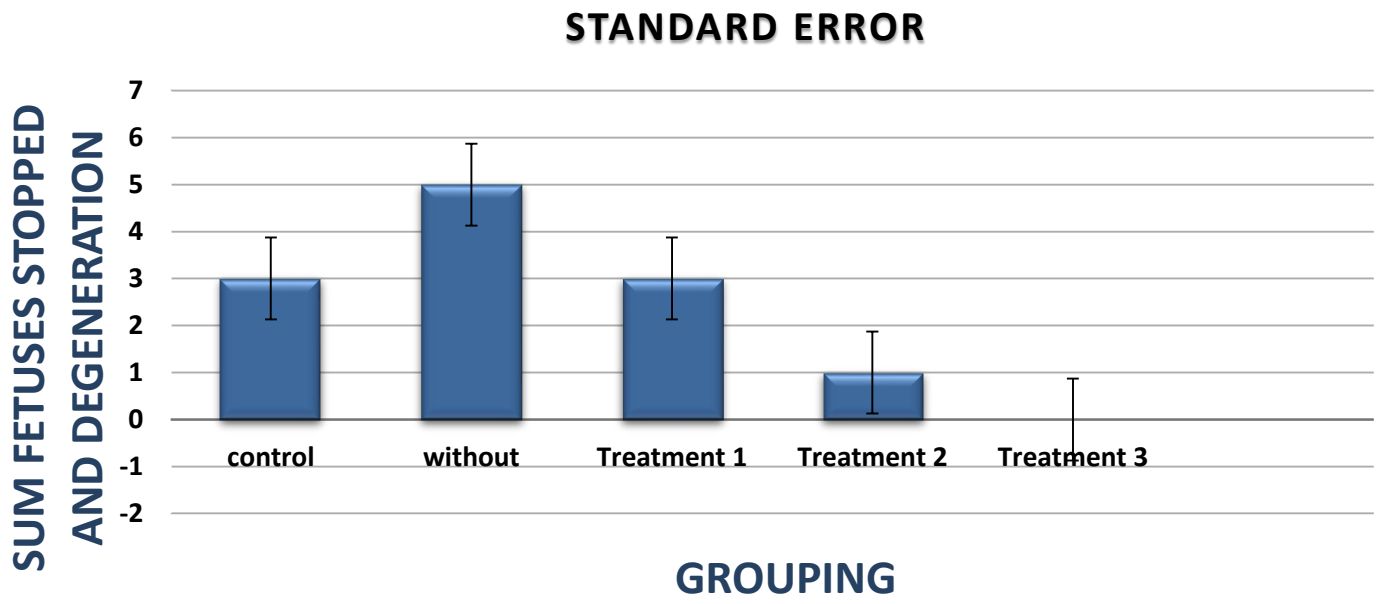


Figure 3. Evaluating the effect of increasing the dose of extract in preventing fetus degenerative agents.

CONCLUSION

According to the results, Addition of Ajuga extract to the culture medium of immature mouse oocytes increased Germinal vesicle breakdown (GVBD) levels. Among the 5 experimental groups, the highest growth rate of eggs that reached Metaphase II (MII) stage belonged to the group containing Ajuga extract with proper dose. Therefore, the use of Ajuga increased the recurrence rate of meiosis (MII + GVBD) and a high percentage of MII.

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