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EFFECTS OF MAGNESIUM SUPPLEMENTATION ON ANTI-MULLERIAN HORMONE LEVEL IN PREMENOPAUSAL WOMEN

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Abstract
In human females, fertility is closely linked to anti-müllerian hormone (AMH) levels. As women age, the number of follicles in their ovaries decreases, as a result their AMH levels decline. This decrease in AMH levels is associated with a decrease in fertility, and a higher risk of infertility and pregnancy complications. Magnesium supplementation has been suggested to positively affect the AMH level in women serum. The study aimed to investigate whether oral magnesium supplementation is positively or negatively correlated with the serum levels of AMH among premenopausal females. The study involved 26 females, including 13 normal fertile females (control group) and 13 infertile females (experimental group). The experimental group was further divided into pre-supplement and post-supplement groups, and given magnesium supplementation (Nutrifactor 500 mg) at regular intervals for 4 weeks. The results showed a significant increase in AMH levels in the post-supplement group compared to the pre-supplement group. The findings suggest that magnesium supplementation can enhance AMH levels, which are responsible for follicle recruitment and fertility. This finding is particularly relevant for assisted reproductive techniques, like in-vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI), where AMH levels are critical for success.

Key words: Fertility, anti-müllerian hormone, Follicles, Magnesium, Premenopausal

1.0. Introduction

Anti-Müllerian hormone (AMH) is a glycoprotein that is predominantly produced by ovarian follicles, and it is considered a biomarker for ovarian reserve that plays a significant role in the evaluation of female fertility AMH, unlike other biomarkers, reflects the ovarian reserve more accurately, and its level does not fluctuate during the menstrual cycle. It is considered the best

endocrine marker for assessing age-related ovarian decline in healthy women [1]. AMH is widely used in clinical practice as a tool to determine the ovarian reserve in women going through the assisted reproductive techniques (ART) like in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [2]. The level of AMH is considered a useful predictor of the success rate of IVF and ICSI[3]. In addition to its use in reproductive medicine, AMH also plays an essential role in the control of gonadotropin-releasing hormone (GnRH) secretion and neuron excitability, making it a potential therapeutic target for the treatment of polycystic ovary syndrome (PCOS) [4]. The level of AMH varies with age, with its concentration declining with increasing age. Research shows that a decrease in AMH levels in females is a sign of a decline in ovarian reserve, which indicates the end of reproductive potential. AMH is mainly produced by the granulosa cells of the preantral and small antral follicles. The concentration of AMH is high during early adulthood, reaches its peak at approximately 24.5 years of age, and then begins to decline. The decline of AMH in females can be attributed to the depletion of ovarian follicles as well as the reduced quality of the remaining follicles [5].

The decline of AMH in females has been studied extensively in recent years due to its importance in reproductive medicine. Research shows that the measurement of AMH levels can be used to determine the age of menopause, the chances of conceiving, and the success of IVF. A decrease in AMH levels with age is a natural phenomenon and affects every female differently. It is established that women over 35 years of age had lower AMH levels than women aged less than 35 years of age. Numbers of reports are available for example, a study by [6] examined 17,120 women aged between 21 and 46 years and found that AMH levels decreased steadily with increasing age from 24 years of age. Similar findings were also reported in other mammals like mice where serum AMH levels decreased with increase in age [7]. Moreover, AMH levels have also been found to decrease during certain medical treatments, such as metformin therapy for polycystic ovary syndrome (PCOS). A study by [8] examined the effect of metformin therapy on AMH levels in women with PCOS and suggested that AMH levels decreased during the treatment. Research has also shown that stress can affect AMH levels. It was reported that psychological stress is linked to decrease in serum AMH levels in infertile women [9].

Magnesium is a vital mineral that is essential for human health. It is involved in numerous physiological functions, including energy production, protein synthesis, DNA repair, and the regulation of muscle and nerve function. Moreover, it plays a significant role in reproductive health, especially in ovarian function. Magnesium has been suggested to regulate AMH levels in the body, which makes it a potential target for the management of fertility-related issues in women [10]. According to [11], magnesium supplementation has been found to be beneficial in improving the chances of successful assisted reproductive technology outcomes in women with low AMH levels. The effects of age on magnesium levels in females have also been studied, with some evidence suggesting that serum magnesium levels decrease up to the age of 24 years [12]. Several studies have shown that magnesium deficiency affects the ovarian reserve and reduces AMH levels in the serum of women. It has also been demonstrated that magnesium supplementation increases serum AMH levels in women with PCOS [13]. Similarly, [14] found that magnesium supplementation prevents primordial ovarian follicle lossin mice treated with cyclophosphamide. Despite these findings, the mechanism by which magnesium affects AMH remains unknown. keeping in mind the role of magnesium to regulate AMH level, the present study was designed to investigate the effects of magnesium supplementation on AMH levels in human females by evaluating the levels of AMH before and after magnesium supplementation among infertile females.

2.0. Materials and Methods

2.1. Study Subjects

Current study selected 26 females in the premenopausal age group (35-45) to evaluate their AMH levels before and after magnesium supplementation. All participants voluntarily took part in the research and provided written consent after being fully informed about the study's procedures. The study protocol for clinical trials was approved by the ethical committee of the University of Sialkot.

The participants were randomly assigned the control group (n=13) or the experimental group (n=13).

2.2. Sample Collection

Blood samples were collected before the initiation of supplementation. Supplementation was administered for a period of 28 days. On the 29th day following the completion of supplementation, blood samples were collected by antecubital venipuncture [15] using a blood collection set (IMPROV® company) and a yellow capped gel test tube containing clot activator (IMPROVACUTER®), according to a standardized protocol [16].

2.3. Sample Processing

After collecting the blood sample in a yellow capped gel test tube with clot activator (ready to use) according to a standardized protocol [16] the tube was immediately placed in a refrigerated centrifuge (Kokusan, H-19FmR) [17]. The centrifuge was set at 3000 rounds per minute (RPM) with a temperature of 15°C, and samples were centrifuged for 5-10 minutes [18]. Using gel and clot activator tubes (ready to use) facilitated obtaining pure serum without contamination due to the stable chemical and physical features of the gel after centrifugation [19]. The gel effectively separated the serum from blood cells and prevented any exchange between serum and blood cells [20] so the serum was directly aspirated from the tube without the need to transfer it to another tube.

2.4. Sample Storage

After centrifugation, the sample was allowed to sit in the gel test tube for an appropriate amount of time as insufficient clotting time can result in micro-clotting that may not be visible to the naked eye[21]. Presence of red blood cells (RBCs), fibrin, or suspended matter can lead to inaccurate results. If the serum contained any suspended matter, it was centrifuged again before testing. Prior to testing, the samples were thawed and thoroughly mixed using a vortex-type mixer. Aliquots of the samples were stored in closed primary tubes at +2/+8°C for up to five days [22]. Then samples were placed at room temperature for analysis.

2.5. Sample Analysis

Quantitative measurement of AMH in serum or plasma was performed using the enzyme-linked fluorescent assay (ELFA) technique with the bioMérieux AMH kit on the Vidas® analyzer machine (VD7004262, France) [23]. The assay principle utilized a one-step enzyme-linked immunoassay sandwich method and fluorescent detection. The Vidas® assay had a measurement range of 0.02 to 9.0 ng/mL and was linear between 0.02 and 9.00 ng/mL, as evaluated according to CLSI® EP17-A2 recommendations. The detection limits for Vidas® were as follows: limit of blank (LoB) was 0.00ng/mL, limit of detection (LoD) was 0.01 ng/mL, and limit of quantitation (LoQ) was 0.02 ng/mL. These limits were also determined according to CLSI® EP17-A2 recommendations. The acceptable level of precision (within-lot precision) for the Vidas® AMH assay was fixed at 20% CV (coefficient of variation). Additionally, no Hook effect was found up to 600 ng/mL AMH concentration.The AMH strips (STR) were included in the bioMérieux AMH kit, and were ready to use. The kit was stored in a refrigerator at 2-8°C, as recommended [24].

2.6. Solid Phase Receptacle (SPR)®

The AMH Solid phase receptacle (SPR)® served both as a solid phase and a pipetting device, and was pre-dispensed inside sealed reagent strips. The inside of the SPR® was coated with mouse monoclonal AMH antibodies and each SPR was identified by its unique AMH code. The required number of SPRs was carefully removed from the pouch and the pouch was sealed again to ensure the integrity of the remaining reagents. The kit was prepared according to the protocol outlined by [16] and stored in a refrigerator at 2-8^{o}C, as recommended by [24].

2.7. The Reagent Strip

The reagent strip consisted of 10 wells, each covered with a labeled foil seal displaying a bar code indicating the kit lot number, assay code, and expiration date. To introduce the sample, control, and calibrator (with a volume of 200 µm), the foil of the first well was ruptured. The sample was then transferred to the first well containing mouse monoclonal AMH antibodies labeled with alkaline phosphatase conjugate. The Vidas® machine cycled the sample mixture automatically several times, allowing the AMH to bind with the AMH antibodies in the SPR® interior and with the conjugate to form a sandwich. The reagent wells in the center section contained the required assay reagents, while the 10th well contained an irritant agent (6.6% diethanolamine), indicated by the hazard statement "H" and precautionary statement "P". The last well of each strip was a cuvette used for fluorometric reading. Only intact strips were used, and all reagents were checked for their expiration dates, which were indicated on the kit box. To prevent false results, powderless gloves were used, and all reagents were handled carefully to avoid cross-contamination. The Vidas® machine automatically eliminated uncombined substances during the washing steps.

2.8. Detection of AMH levels

In the final detection step, the SPR® was subjected to a cycle of substrate (4-methylumbelliferyl phosphate), which caused the conjugate enzyme to catalyze the hydrolysis of the substrate into the fluorescent product 4-Methylumbelliferone. The fluorescence intensity was measured at 450 nm and was proportional to the concentration of the hormone present in the sample [25]. The fluorescence was measured twice in the reading cuvette of the reagent strip for each sample. The first reading was taken as the background reading of the substrate cuvette before the SPR® was introduced in the substrate. The second reading was taken after incubating the substrate, with the enzyme remaining inside the SPR®. The Relative Fluorescence Value (RFV) was obtained by subtracting the background value from the final value, and the results were automatically calculated by the instrument in relation to the calibration curve stored in the memory of the device. The results were then printed out [26]. All of these steps were carried out automatically by the Vidas® once the samples were mounted.

2.9. Statistical Analysis

The AMH levels of the experimental group before and after magnesium supplementation were compared to the control group using a t-test. The results revealed a significant difference ($P < 0.05$) in the AMH levels after magnesium supplementation compared to pre-supplementation levels. The analysis was performed by SPSS software version 28.0.1.0 (142).

3.0. Results

The study participants randomly divided into a control group (N=13) or an experimental group (N=13). AMH concentrations were measured before and after magnesium supplementation in experimental group using a t-test. The paired t-test of the pre-supplementation and post-supplementation groups resulted in a mean of 22,469 (SD=0.21565, SEM=0.05981), a t-value of 3.757 with 12 degrees of freedom, and a significance level of <0.05. The groups were positively correlated. For the control and pre-supplementation group, the mean was 0.72831 (SD=3.39909, SEM=0.94274), with a t-value of 0.773 and a significance level of >0.05. These results were insignificant. Finally, the control and post-supplementation group had a mean of 0.50362 (SD=3.33077, SEM=0.92379) and a t-value of 0.545, with a significance level of >0.05. (Fig 1 and 2). These results were also non-significant. The concentration of AMH was significantly different in Post-supplementation females (Fig. 3). Conversely, there was no significant difference in AMH concentration between the control group and the pre-supplementation group.

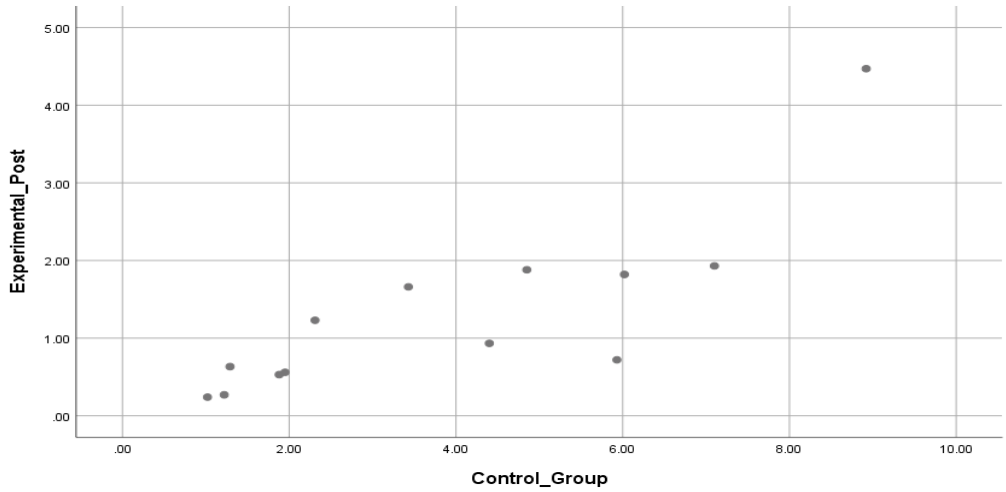

Fig. 1. Magnesium level in control group

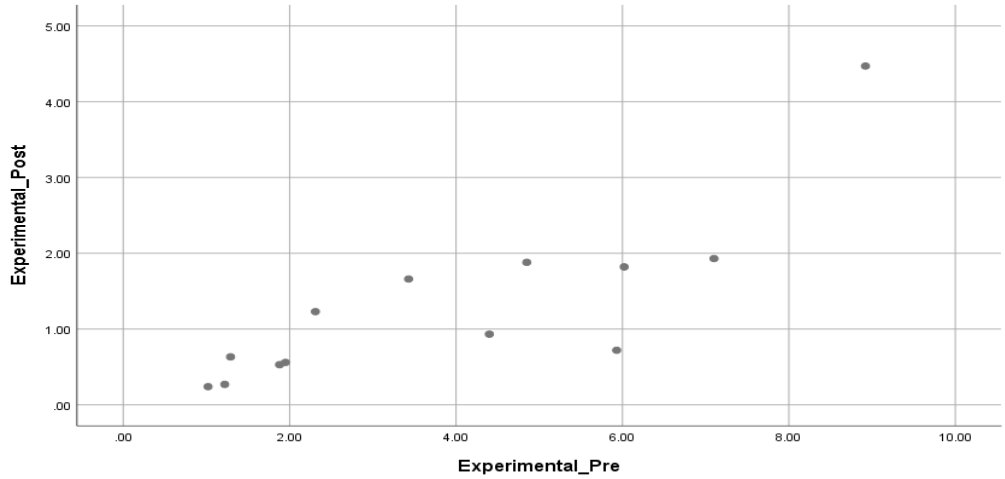

Fig. 2. Effect of Magnesium after supplementation

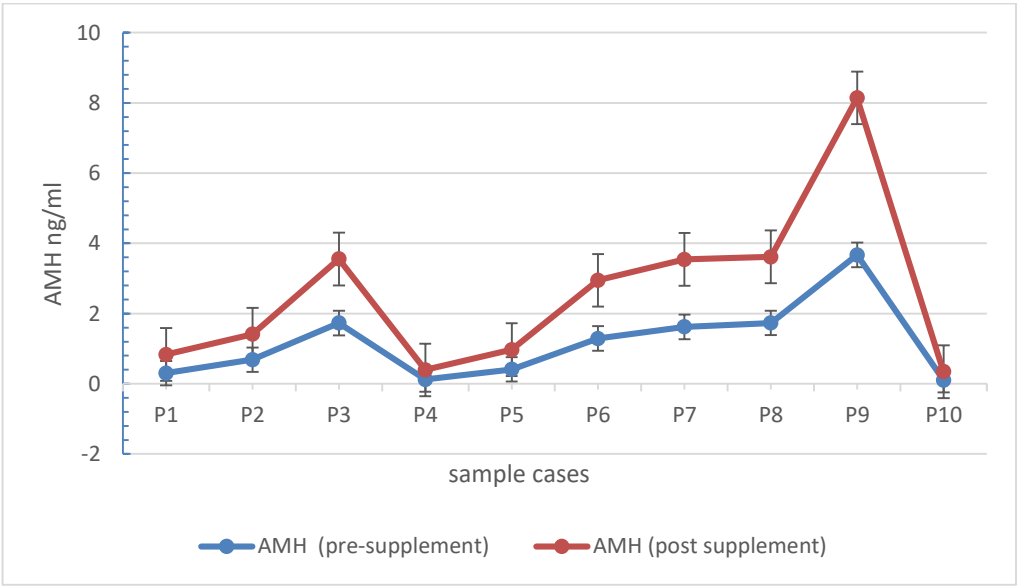

Fig.3. Comparison between pre and post magnesium supplementation group

The samples were divided into three age wise treatment groups, Group 1 (30-<40, Group 2 (>40-45) and group 3 (>45). The percent increase in AMH values was compared among these group to predict the age related impact of magnesium supplementation normalized with control group. The

age specific serum AMH values showed <25-30% increase in the sample group 2 while least 15-20% approximate increases was observed in women of 45 years and above (Figure 4).

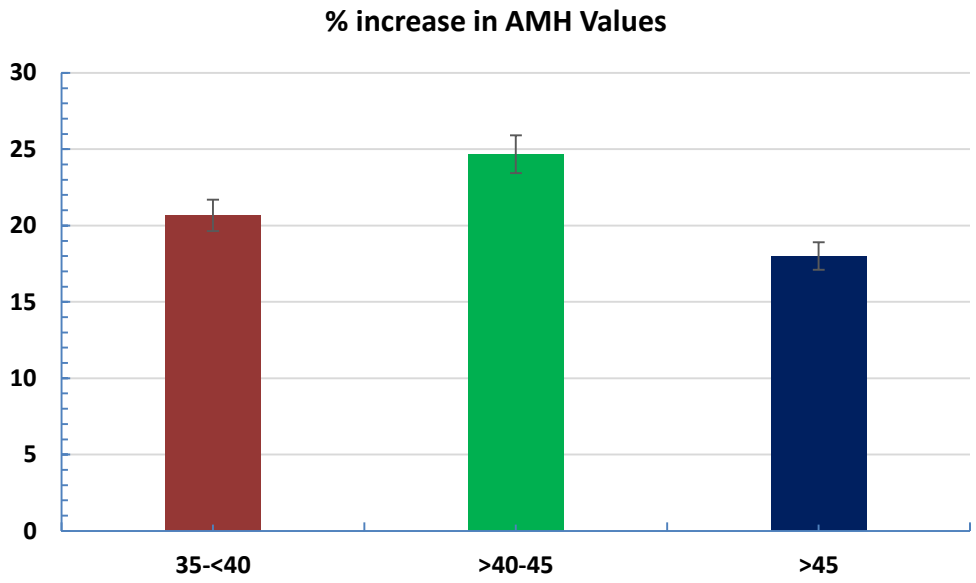

Fig.4. Comparison between different age wise grouping of women for magnesium supplementation

4.0. Discussion

The levels of magnesium are critical for female fertility because they influence the production of reproductive hormones and contribute to the development of follicles in the ovaries. On the other hand, AMH is produced by the cells in the developing follicles in the ovaries and is an important marker of ovarian reserve, indicating the remaining follicles that are capable of developing into mature eggs. As a result, the levels of magnesium and AMH are significant predictors of female fertility, and this essay aims to provide a discussion of the relationship between these two factors and human female fertility.

The current study demonstrated that serum AMH levels are positively correlated with magnesium among premenopausal females. In this study where an experimental group was given magnesium supplementation, pre and post supplementation tests were conducted to evaluate the levels of AMH. Results showed that there was a significant difference in AMH levels between pre and post supplementation blood samples. Infertile females who are magnesium supplemented showed high level of post supplementation AMH.

Several studies have shown that magnesium levels are significantly associated with AMH levels in women. There are reports saying that women with higher levels of magnesium had higher levels of AMH, and therefore, better ovarian reserve. A study conducted by [27] found that women with PCOS had lower levels of magnesium and AMH than healthy controls. The authors concluded that low magnesium levels may contribute to the pathogenesis of PCOS and its associated infertility by affecting AMH levels. One possible mechanism by which magnesium may affect AMH levels is by acting on the granulosa cells in the ovarian follicles. AMH is predominantly produced by the granulosa cells of preantral and small antral follicles [28]. These granulosa cells are highly sensitive to the changes in the extracellular environment, including the levels of various ions and nutrients. Magnesium is known to affect the activity of several enzymes involved in the production and metabolism of hormones, including AMH [29]. Therefore, it is possible that magnesium affects AMH production and secretion by modulating the activity of enzymes involved in its biosynthesis and metabolism.

Another possible mechanism by which magnesium may affect AMH is by acting on the signaling pathways that regulate AMH production and secretion. The receptor for AMH is expressed on the surface of Leydig cells, which are responsible for testosterone production in the testes [28]. AMH signaling is mediated by the type II receptor (AMHRII) and the downstream Smad signaling pathway [30]. Magnesium is known to affect the activity of several kinases and phosphatases involved in the regulation of intracellular signaling pathways, including the Smad pathway [31]. Therefore, it is possible that magnesium affects AMH production and secretion by modulating the activity of the AMH receptor or the downstream signaling pathways.

However, the relationship between magnesium levels and female fertility is not straightforward. Some studies have found no significant association between magnesium levels and fertility outcomes. For instance, a study by [32] found no significant differences in magnesium levels between fertile and infertile women. Similarly, a study by [33] found no significant association between magnesium levels and the success of IVF treatment. However, age specific AMH levels have been used as biomarkers for ovarian reserve categories. Consent upon this agreement the study also demonstrated age related response to magnesium supplementation and in this regard, age-specific AMH levels may be a more accurate biomarker for categorizing women in different ovarian reserve categories. The results suggested the magnesium supplementation has potential to increase the serum AMH level by 30% during the reproductive age of >30 and <45 years in Pakistani females' sample groups. It could be established that previous reports of ethnic and geographical aspects of ovarian aging showed differences in Asian, African and middle eastern counties. (35,36). Nonetheless, there is lack of enough evidence to support that age and magnesium deficiency negatively correlated with AMH value and ovarian reserves. It could be deduced from current study that despite of considerable variation in ovarian aging, AMH values and age of individuals, magnesium supplementation was most effective for women of age 35 and above. The reason for these dispersive results may be due to the fact that magnesium levels are influenced by several factors, including diet, lifestyle, and genetics, and these factors may differ across populations. For instance, magnesium deficiency is more common in certain populations, such as pregnant women, elderly individuals, and those with certain medical conditions, such as diabetes and kidney disease [34].

In conclusion, while the relationship between magnesium levels and female fertility is not fully understood, several studies suggest that magnesium plays a role in the regulation of AMH levels, which is an important marker of ovarian reserve. However, further studies are required to evaluate the optimal levels of magnesium for fertility and the mechanisms by which it affects fertility outcomes. In addition, magnesium supplementation may be beneficial for women with low magnesium levels, but further studies are needed to confirm its efficacy and safety in improving fertility outcomes.

5.0. Conclusion

In conclusion, this study has shown that magnesium supplementation may be an effective way to enhance AMH levels in premenopausal women. As women age, their levels of AMH and other sex hormones decline, and in developing countries, this decline may occur even earlier due to various factors including genetics, environment, and nutrition. Magnesium is a crucial nutrient for proper metabolism and a deficiency in magnesium can lead to chronic heart disease, hypertension, coronary blockage, diabetes, and hormonal imbalances resulting in the degeneration of oocytes and spontaneous abortions. Therefore, by increasing AMH levels, magnesium supplementation may potentially help to mitigate the negative effects of hormonal imbalances and related health issues in premenopausal women. This study has demonstrated a positive correlation between magnesium supplementation and AMH levels in premenopausal females, with a notable increase in AMH levels after only 4 weeks. Based on these results, it is recommended that premenopausal females consider taking magnesium supplements on a regular basis to enhance their AMH levels, especially those living in developing countries where the onset of premenopausal age is early due to various factors,

including nutritional deficiencies. It is important to note that the long term effects of magnesium supplementation on AMH levels remain unclear, and further research is necessary to fully understand the potential benefits and risks.

Author Contribution

Rabia Sundas, Asma Ul Husna and Rukshanda Saleem were involved in the conception and design of the study. Rabia Sundas was involved in acquisition of data. Saima Qadeer, Rabea Ejaz, Asima Azam, Wajeeha Saeed and Saleha Ashfaq were involved in interpretation of data and drafting the article. Asma Ul Husna were involved in final approval of the version to be submitted.

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