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Effect of human umbilical cord stem cells (HUMSC) administration on collagen expression in the anterior vaginal wall in menopausal rats

İnsan göbek kordonu kök hücresi (İGKKH) uygulamasının menopozdaki sıçanlarda vajinal ön duvarın kollajen ekspresyonu üzerine etkisi

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Abstract

Objective: To evaluate the outcome of human umbilical cord stem cells (HUMSC) administration on collagen expression within the frontal vaginal wall of menopausal rats.

Materials and Methods: We conducted an experimental, randomized post-test-only controlled group design. The study samples were 40 healthy female Winstar rat with the age of 8-12 weeks that had been ovariectomized, had never mated, and weighed 18-22 grams. The umbilical cord was obtained from voluntary donors who did not have a history of hepatitis B, hepatitis C, HIV, cytomegalovirus infection, treponema pallidum infection, or a history of other infections transmitted through the blood, placental tract, and genitals. Data collection (frontal vaginal wall of the rat) was carried out in a controlled environment with the consideration that all conditions were maintained equally and could be controlled.

Results: There were 36 samples. A total of 13 menopausal rats (72%) had strong collagen expression and 5 rats had weak-to-moderate collagen expression (28%). On the other hand, 18 menopausal rats (100%) that belonged to the control group had weak-moderate collagen expression, and no menopausal rats appeared to have strong expression (0%). The administration of collagen to the anterior vaginal wall of postmenopausal rats proved to be effective by increasing the strong collagen expression in the damaged anterior vagina of postmenopausal female rats ($p<0.05$).

Conclusion: Administration of HUMSC resulted in an increase in collagen levels in the anterior vaginal tissue of postmenopausal female rats. These results demonstrate significant therapeutic potential for the treatment of pelvic floor dysfunction.

Keywords: Human umbilical cord stem cells, collagen expression, anterior vaginal wall, menopause rats

Öz

Amaç: Bu çalışmanın amacı insan göbek kordonu kök hücresi (İGKKH) uygulamasının menopozdaki sıçanlarda vajinal ön duvarın kolajen ekspresyonu üzerine etkisini belirlemektir.

Gereç ve Yöntemler: Rastgele son test kontrollü grup tasarımıyla deneysel bir çalışma yürütülmüştür. Örnekler, dahil etme kriterlerine uyan; yumurtalıkları alınmış, hiç çiftleşmemiş, 8-12 haftalık ve 18-22 gram ağırlığında 40 sağlıklı dişi Winstar sıçanlarıydı. Göbek kordonu; hepatit B, hepatit C, HIV, sitomegalovirüs enfeksiyonu, treponema pallidum enfeksiyonu veya kan, plasenta yolu ve cinsel yolla bulaşan diğer enfeksiyon geçmişi olmayan gönüllü

PRECIS: This study developed a novelty administration of HUMSC was proven to result in an increase in collagen levels in the anterior vaginal tissue of postmenopausal female rat.

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bir donörden alınmıştır. Veri toplama (sıçanın ön vajinal duvarı) kontrollü bir ortamda, tüm koşulların eşit şekilde sağlanması ve kontrol edilebilmesi durumunda gerçekleştirildi.

Bulgular: Otuz altı örnek vardı. Toplam 13 menopozal sıçanda (%72) güçlü, 5 sıçanda zayıf-orta düzeyde kolajen ekspresyonu (%28) vardı. Buna karşılık, kontrol grubunda 18 menopozal sıçanda (%100) zayıf-orta düzeyde kolajen ekspresyonu vardı ve hiçbir menopozal sıçanda güçlü ekspresyon (%0) görülmedi. Postmenopozal sıçanların ön vajinal duvarına kollajen uygulanması, postmenopozal dişi sıçanların hasarlı ön vajinasındaki güçlü kolajen ekspresyonunu önemli ölçüde artırabilmektedir ($p<0,05$).

Sonuç: İnsan göbek kordonu kök hücresi uygulamasının postmenopozal dişi sıçanın ön vajinal dokusunda kolajen düzeylerinde artışa neden olduğu kanıtlanmıştır. Bu sonuçlar pelvik taban hastalıklarının tedavisinde önemli terapötik potansiyel olduğunu göstermektedir.

Anahtar Kelimeler: İnsan göbek kordonu kök hücreleri, kolajen ekspresyonu, ön vajinal duvar, menopoz sıçanları

Introduction

Pelvic floor dysfunction (PFD) refers to various clinical conditions, such as pelvic organ prolapse (POP), stress urinary incontinence (SUI), overactive bladder, fecal incontinence, and sexual dysfunction[1-4]. The rate of POP and SUI increases with age, especially during menopause, affecting women's quality of life. The prevalence report shows that POP affects 30% of middle-aged women in China, 19% in Australia, and over 50% of women aged over 60 years in the United States. By 2050, symptomatic POP is expected to affect at least 46% of the women population in the United States[5]. Although in Denpasar City of Indonesia, POP was reported in approximately 11.38% of cases within 2 years of evaluation[6,7].

The cause of PFD is complex, with many factors involved: Pregnancy, childbirth, and the structure of the pelvic floor's connective tissue[8]. However, despite the pregnancy and childbirth that most women experienced, not all developed PFD. Any disruptions or conditions affecting connective tissue or neuromuscular support can cause increased PFD[9]. Pelvic bones, pelvic floor muscles, and nerves that serve as supporting tissue components of the pelvic floor work together to suitably function. In addition, various cells and extracellular matrix composing pelvic floor muscle play a crucial role in supporting the pelvic organs. The extracellular matrix consists mainly of collagen, glycoproteins, and proteoglycans. Collagen makes up approximately 70% of this matrix and is essential for the development of PFD. In a study, women with uterine prolapse and cystocele had lower collagen levels than those without POP. The study also found increased collagen degradation in patients with POP, and a higher collagen I/III ratio strengthens connective tissue to reduce POP risk[10].

Currently, treatment for PFD relies on managing symptoms once they appear, but alternative options for preventive treatment are needed[1,2]. PFD typically occurs during menopause; therefore, PFD prevention is highly beneficial at that time. Unsuccessful treatment leads to surgery in women. Autologous fascia or tissue repair has a 11% recurrence rate[11]. Common complications after Transvaginal Mesh (TVM) may include constant pain and deterioration of the urethra/bladder. According to the Food and Drug Administration, over 1000 complications were reported from 2005 to 2007 for POP and SUI treatment, increasing from 2008 to 2010[4]. POP repair with TVM does not have significant clinical benefits, as 30% of patients finish off needing additional surgery[12].

In recent years, stem cell use as an alternative method for PFD has attracted much attention from researchers, and many ***in vivo*** tests have been performed to evaluate its use. Furthermore, human umbilical cord stem cell (HUMSC), the stem cell selection in this study, is proven to be easily accessible and can be obtained in large quantities. HUMSC transplantation leads to the formation of organized connective tissue and increased collagen, facilitating the healing process[13]. This suggests that HUMSC may be beneficial and effective in treating POP[14]. HUMSC is expected to alter the collagen content of the extracellular matrix, improving pelvic floor strength and PFD outcome. In other words, the researchers wanted to evaluate the alteration of collagen expression in the anterior vaginal wall of menopausal model rats in the effect of HUMSC administration. This research carried out the potential of being an initial step to be developed in collaboration with other researchers as well, so that the proposed intervention may further be studied in humans and could later be implemented in daily practice.

Materials and Methods

This research is an experimental study with a randomized post-test-only controlled group design in the Animal Lab. Units, Biomedicine, Udayana Faculty of Medicine, Denpasar.

Sampling

The samples were female Wistar rats that had undergone ovariectomy and were in the Animal Lab. Units, Biomedicine, Udayana Faculty of Medicine, Denpasar. The sample size in the study was calculated on the basis of a calculation formula. Added to the possibility of dropping out of 10% of the study during the research, the minimum number of samples per group is 11. Samples were taken using simple random sampling techniques (random number tables in the Microsoft Excel for Windows program). Rat numbers 1-20 were assigned to the treatment group with HUMSC administration, and rat numbers 21-40 were assigned to the control group without HUMSC administration. Sample inclusion criteria were healthy female Wistar rats with the age of 8-12 weeks, who had been ovariectomized, who had never mated, and weighed 18-22 grams. The exclusion criteria were rats that had previously been used in other experiments, and the dropout criteria were sick rats, including rats who died or were lost in the research.

Research Procedure

Animals were fed pellets (BR 1) and tap water during the acclimatization process for a week before treatment. Body weight was measured, grouping was carried out according to the code with random distribution, and rats that were ready to be ovariectomized for the research process were defined as those whose body weight ranged from 200 to 350 g.

Rat ovariectomy was performed based on the modified method of Ingle DJ and Griffith JQ, 1971. The rat's body weight was measured and then anesthetized with i.m. ketamine at a dose of 40 mg/kgBW. The fur in the abdomen was shaved, the rat was laid on the operating table, sterilized with betadine solution and 70% alcohol, and covered with a sterile drape. A transabdominal incision (1.5-2 cm) is made approximately above the uterus. The incision is made layer by layer until it penetrates the peritoneal wall. The incision wound was pulled to the right and left sides. The fat pads are removed to make it easier to find the oviducts and ovaries. The ovaries look like a bunch of translucent grapes. The ligation is carried out in two places (proximal and distal ovaries) and then continues with the removal of the right and left ovaries. While searching and removing the ovaries, other organs must be kept moist by dripping them with physiological fluids. Before suturing, nebacetin powder is sprinkled in the abdominal cavity. After the wound is stitched, the rat is placed in a cage. Each cage contains only 1 rat. On days 1, 2, and 3 after oophorectomy, a dose of gentamicin i.m. was injected on 5 mg/kg BW/day. During maintenance, adequate drinking and food are provided, with alternating light/dark light for 12 h and at room temperature. Signs of successful oophorectomy can be seen from the appearance of diestrus in the vaginal smear.

Test rats were partitioned into two groups, comprising 18 rats each. The step-by-step procedures included the following: 1) labeling P3-5 HUMSC with DiR dye (XenoLight); 2) subepithelially injecting $3x10^6$ HUMSC into the frontal vaginal wall of test rats, 2 weeks post-ovariectomy; 3) incubating $1 x 10^6$ HUMSC with 1 mL of 80 g/mL DiR at 37 °C for 5 minutes; 4) flushing the HUMSC twice with PBS; 5) euthanizing samples at 12 weeks post-infusion. Following euthanasia, the stomach of the sample rats was dissected to expose the pubic symphysis, while the perineal skin was removed. The frontal vaginal wall was then collected and divided into two fragments: Proximal and distal. The proximal fragment is cut into two parts; one half is for histology and immunohistochemistry use, so it has to be immersed in 10% neutral buffered formalin and embedded in paraffin, while the other half is cryopreserved at 80 °C in OCT compound. In contrast, the distal section is used for mRNA expression analysis; therefore, it must be frozen-stored at 80 °C in liquid nitrogen. Furthermore, before biomechanical testing (4 hours after collecting speciment), the frontal vaginal wall has to be wrapped in a wet gauze and soaked in 0.9% saline.

Preparation of HUMSC from the Infant Umbilical Cord

The research was conducted after obtaining ethical clearance approval from the health research ethics commission of the Faculty of Medicine, Udayana University (approval number: 12704'N14'2'2 vII'l4lLTDo23, date: 15.05.2023). The umbilical cord was obtained from a voluntary donor who signed an informed consent form. Voluntary donors are mothers without a history of hepatitis B, hepatitis C, HIV, cytomegalovirus infection, treponema pallidum infection, or a history of other infections transmitted through the blood, placental tract, and genitals[8].

After the baby is born, the umbilical cord is cut at around 3-5 cm using a sterile knife and stored in a container containing 0.9% normal saline solution, stored at 4 °C and handled aseptically. The surface of the umbilical cord is rinsed with a phosphate salt buffer solution to remove blood adhering to the surface.

Human umbilical cord mesenchymal stem cell extract was prepared using the Quick-DNA Universal Kit, produced by Zymo Research. The sample was human umbilical cord tissue that had been cut negligible and weighed 25 mg.

Statistical Analysis

All data analysis above uses SPSS version 25.0 computer software. Descriptive statistical analysis aimed to describe the results of measuring research variables based on treatment and control groups. The Normality test assesses the distribution of data in each group. The homogeneity of variance test aims to assess whether the data variance between groups is homogeneous or not. The mean comparison test compares the average collagen levels between the groups and assess the treatment of these three levels. The mean comparison is said to be significant if the p-value <0.05.

Results

Characteristics of the Research Subjects

36 samples were divided into 18 control and 18 treatment groups. In this study, the average age of rats when the study began in the treatment group was 76.95±1.31 days, whereas it was 76.45±1.60 days (Table 1). Based on their age, the research subjects from these two groups have passed the reproductive maturity period. The reproductive maturity period for rats begins at the age of 28-42 days, and those aged more than 60 days are classified into the adult stage where the growth and development of their reproductive organs is complete.

Table 1. Demographic characteristics of the treatment and control groups

Characteristics	Treatment group		Control group		p*
	Mean	SD	Mean	SD	
Age (day)	76.95	1.31	76.45	1.60	0.293
Body weight (gram)	211.24	1.73	211.85	1.61	0.275

*: Kolmogorov-Smirnov test, SD: Standard deviation

The results of the Kolmogorov-Smirnov test showed that both groups had normal data distribution (p>0.05), and Levene's test showed that the two groups had homogeneity of variance (p>0.05).

In this study, collagen expression in the frontal wall of menopausal rat vaginas was examined using immunohistochemical techniques. The mean H-score collagen level in the anterior vaginal wall of menopausal rats in the treatment group was 3.2±1.01, whereas it was 1.6±0 in the control group. The mean H-score in the treatment group was higher than that in the control group, and the Shapiro-Wilk test yielded a p-value of <0.05 (Table 2).

Effect of HUMSC Administration on Collagen Expression in a Female Rat Model of Menopause

In the treatment group, five (28%) menopausal rats showed weak-to-moderate collagen expression and 13 (72%) menopausal rats showed high collagen expression. In contrast, within the control group, 18 (100%) menopausal rat showed a weak-moderate collagen expression, and no menopausal rodent showed high collagen expression. With the chi-square test, it was found that the x^2 value=20.3 and the p<0.001, showing a substantial change of collagen expression between the treatment and control groups. Simultaneously, it proves a significant escalation of strong expression of collagen in the damaged anterior vagina of postmenopausal female rats (Figure 1).

Table 2. Distribution of the average H-score for collagen in the treatment and control groups

Variable	Treatment group		Control group		p*
	Mean	SD	Mean	SD	
H-score collagen	3.2	1.01	1.6	0.48	<0.05*

*: Shapiro-Wilk, SD: Standard deviation

Discussion

Due to the limitations of manufactured synthetic mashes for POP, stem cells combined with materials may be a viable therapeutic approach. Achieving therapeutic results *in vivo* is crucial by understanding the interaction between cells and materials and the microenvironment to regulate cell behaviors[15]. However, the limited number of studies evaluating these findings means that recommendations about the effectiveness of evidence-based modalities cannot be established yet. In this study, we evaluated the effect of HUMSC administration on collagen expression in the anterior vaginal wall of menopausal rats.

Table 3. Effect of HUMSC administration on collagen expression in a female rat model of menopause

	Treatment group (n=18)	Control group (n=18)	X^2	p*
Strong-very strong expression Weak-moderate expression	13 5	0 18	20.3	<0.001

HUMSC: Human umbilical cord stem cells

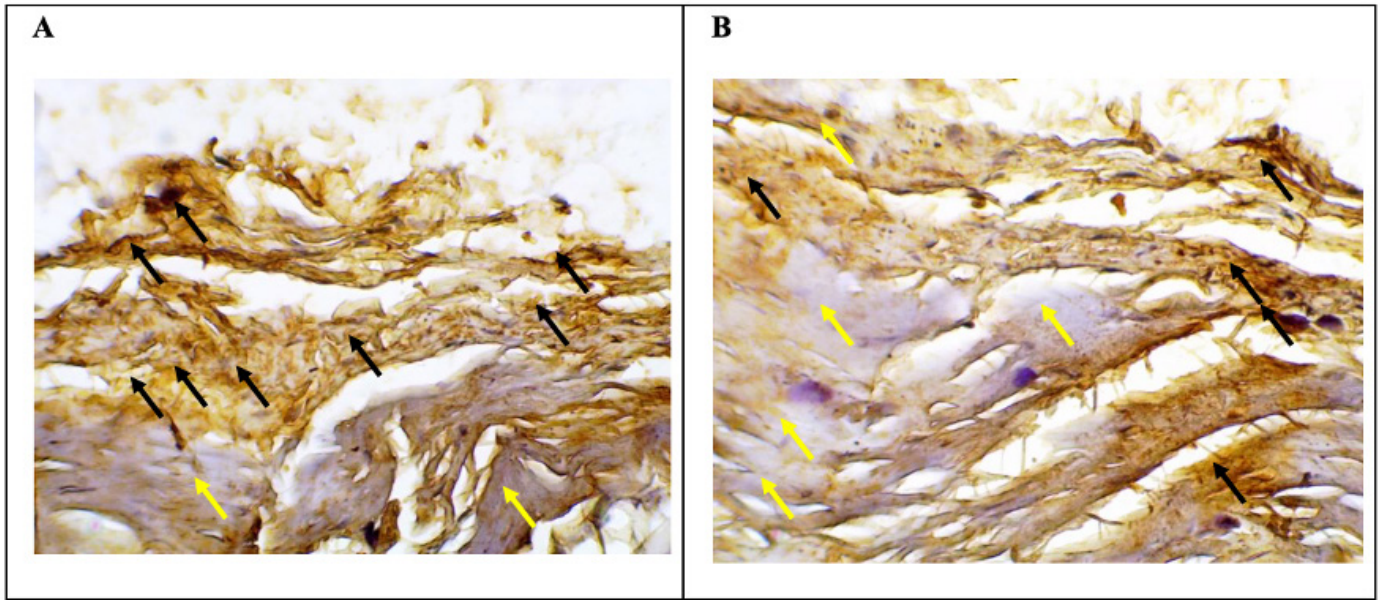

Figure 1. Collagen expression in the treatment group **(A)** and control group **(B)**. **A.** H-score = 4.3. Strong expression. Out of 346 endometrial epithelial cells, 118 epithelial cells were stained with strong intensity; 133 with moderate intensity; 64 with weak intensity; 31 cells were unstained. Magnification 400. Note: intensity 0 = weak, 1 = moderate, 2 = strong, 3 = strong. **(B)** H-score = 1.37. Weak moderate expression. Of the 345 epithelial cells, 7 were stained with strong intensity; 12 with moderate intensity; 82 with weak intensity; 244 unstained. Magnification 400. Note: intensity 0 = weak, 1 = moderate, 2 = strong, 3 = strong

Preclinical research on macaque monkey vaginas showed increased collagen and microvascular thickness in the vaginal lamina propria after transplantation of mesenchymal stem cells from the umbilical cord. In addition, smooth muscle in the vagina also increased. Mesenchymal stem cell transplantation enhances vaginal biomechanics by increasing the elastic modulus of the vagina and making it stiffer[10]. It is still difficult to develop animal models for POP-related studies. Strategies like vaginal extending, ovariotomy, or prolapse in rats/sheep are commonly performed. Insertion is typically found underneath the stomach or vaginal wall. Cell-based tissue plan strategies have improved vaginal repair in rats by obtaining epithelial cell phenotypes and reducing inflammation caused by TVM in sheep vagina[16]. We used female Wistar rats that were ovariectomized, never mated, 8-12 weeks, and weighed 18-22 grams. These rats can be used as a model in this test, and surgical procedures are available.

Research conducted on rat showed that application of HUCMSC to the vaginal walls of ovariectomized Sprague-Dawley rat increased collagen levels, and the collagen I:III ratio of HUMSC is thought to not only increase collagen but also the expression of genes that produce collagen based on research examining gene expression after administration of HUMSC[17].

Smooth muscle plays an important role in supporting the pelvic viscera and vaginal wall. In women with POP, there is a decrease in smooth muscle. Our study showed that increasing collagen in the anterior vaginal wall of postmenopausal female rats can significantly improve smooth muscle expression in the damaged anterior vaginal wall. Matrix metalloproteinases (MMPs) and issue inhibitors of metalloproteases (TIMPs) affect extracellular component homeostasis. Mesenchymal stem cells can create MMPs and TIMPs from the extracellular matrix. The study found a decrease in MMP2, MMP9, and MMP13, which break down collagen types I and II. Mesenchymal stem cells aid in reducing collagen degradation[16].

Smooth muscle morphology changes are crucial in pelvic organ prolapse. The increase in smooth muscle cells is due to MSCs differentiating into them. However, studies have shown that MSCs can also control smooth muscle cells through paracrine effects. The paracrine impact is due to the release of exosome and various active substances from stem cells through exosome or microvesicles, including proteins, RNA, hormones, and chemicals[16].

Changes in vaginal biomechanics may occur with an increase in the elastic modulus during MSC transplantation. This aligns with research on POP patients, which reduces type I collagen but simultaneously increases type III collagen. The changes occur due to changes in the extracellular matrix and smooth muscle regeneration of vaginal walls[16].

Advanced glycation end (AGE) are non-enzymatic products of the glycation and oxidation of proteins and lipids. AGE affects POP by increasing cross-linking in the prolapsed tissue. This is because AGE affects collagen metabolism through AGE receptors without affecting expression or structure. AGE activates the P-P38 pathway, along with MAPK and NF-kB-p-p65, to regulate collagen metabolism[18].

In vivo studies have shown that human stem cells can affect AGE. The ability of HUMSCs to release anti-inflammatory cytokines (such as IL-4, IL-6, and IL-10) might protect against the toxic effects of AGE-induced fibroblasts. The use of MSC treatment for POP has been studied in preclinical studies, with possible mechanisms being to activate the P13K/AKT/PTEN pathway to protect against AGEs' cytotoxic effects[18]. MSCs transplantation in preclinical studies with intravenous or urethral administration also reported the potential to treat urinary and fecal incontinence by increasing the smooth muscle cells, vascular thickness, and connective tissue in the periurethra[10]. Tissue engineering combines cells with materials or mesh to provide support to pelvic tissue, immune modulating, and anti-inflammatory capacities[19].

The spectrum of trophic components created by MSCs consists of exosomes, cytokines, and chemokine, or secretomes. Recent studies have focused on the secretory function rather than the differentiation ability of these cells. This is called acellular therapy, which does not require live cell transplants. This decreases transportation and simplifies standardizing the work. *In vivo* research revealed the effects of exosomes on increasing type I collagen and collagen degradation in vaginal fibroblasts[19].

Study Limitations

This study has some limitations: It did not investigate the fate of HUMSCs after transplantation *in vivo*; it had a small sample size of animal groups due to ethical concerns; and the ovariectomized model used in this study lacked symptoms of prolapse. In addition, further evaluation of the possible interaction of pro- and anti-inflammatory factors on the effectiveness of HUMSC in treating POP is needed, and a longer postoperative follow-up assessment is also necessary to monitor the efficacy of this procedure. Furthermore, because of the small size of the rat vagina, evaluating postoperative complications and biomechanical properties is challenging. We recommend that future research should focus on building and validating POP models with clinical phenotypes, and more studies in larger animal models or human patients and proteomic analysis are needed.

Conclusion

Administration of HUMSC to postmenopausal female rats resulted in an escalation toward collagen levels in the anterior vaginal wall. These results demonstrate significant therapeutic potential for treating PFD cases (possibly referring to a condition or disease). The therapeutic implications of the increase in collagen levels could be an important basis for developing more effective therapeutic strategies for PFD cases (if referring to a medical condition).

Ethics

Ethics Committee Approval: The research was conducted after obtaining ethical clearance approval from the Health Research Ethics Commission of the Faculty of Medicine, Udayana University (approval number: 12704'N14'2'2 vII'l4lLTDo23, date: 15.05.2023).

Informed Consent: The umbilical cord was obtained from a voluntary donor who signed an informed consent form.

Authorship Contributions

Surgical and Medical Practices: K.F.M., I.W.P.S.Y., A.J.K., I.N.M.A., Concept: K.F.M., I.W.P.S.Y., A.J.K., I.N.M.A., Design: K.F.M., I.W.P.S.Y., A.J.K., I.N.M.A., Data Collection or Processing: K.F.M., I.W.P.S.Y., A.J.K., I.N.M.A., Analysis or Interpretation: K.F.M., I.W.P.S.Y., A.J.K., I.N.M.A., Literature Search: K.F.M., I.W.P.S.Y., A.J.K., I.N.M.A., Writing: K.F.M., I.W.P.S.Y., A.J.K., I.N.M.A.

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