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Effects of oocyte source on the developmental competence of in vitro matured goat oocytes fertilized by the intracytoplasmic sperm injection technique

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Abstract: This study evaluated the effects of oocyte source on in vitro maturation (IVM) and embryo developmental competence in goats by the intracytoplasmic sperm injection (ICSI) technique. Oocytes were obtained either by laparoscopic ovum pick-up (LOPU) or by slicing of abattoir ovaries. The donor does used for LOPU were superstimulated and their estrus was synchronized. Recovered oocytes from both sources were cultured in microdrops of IVM medium in the presence of CO_2 (5%) at 38.5 °C in a CO_2 incubator for 27 h. The ICSI was performed with immobilized sperm on the lid of a culture dish (35 mm) under an Olympus inverted microscope fitted with Narishige micromanipulators. Injected oocytes were cultured for <192 h in mSOF medium in the CO_2 incubator. Although oocyte recovery per doe was higher ($P < 0.05$) in abattoir (27.3 ± 3.0) than LOPU (17.6 ± 2.5) source, better quality oocytes were obtained from LOPU. Oocyte maturation rate was higher ($P < 0.01$) in LOPU (73.8 ± 3.0%) than abattoir-derived (54.0 ± 0.7%) oocytes. Fertilization and cleavage rates (60.9 ± 2.1% and 83.1 ± 2.6%, respectively) were also higher ($P < 0.05$) with LOPU than abattoir-derived (50.6 ± 2.3% and 69.3 ± 4.5%) oocytes. A higher ($P < 0.05$) proportion of LOPU-derived embryos (20.0 ± 1.2%) progressed to the morula stage than abattoir (11.3±4.7%) source. In conclusion, better maturation, fertilization, and embryo development rates were obtained with LOPU-derived oocytes than with the abattoir source.

Key words: Goat, LOPU, abattoir, oocytes, IVM, ICSI

Introduction

The intracytoplasmic sperm injection (ICSI) technique is one of the latest in vitro assisted reproductive technologies (ARTs) used worldwide to treat male factor infertility in humans. It is the mechanical insertion of a single sperm directly into the ooplasm of a matured, metaphase II (MII) oocyte using a microscopic needle. Thus, ICSI can bypass many barriers of fertilization including the process of sperm penetration through cumulus-corona cells, zona pellucida, and oolemma. Like humans, domestic animals, including goats, also suffer from infertility or sub-fertility, which lowers their lifetime productivity and reduces the number of offspring that

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could be obtained from a sire. The prevalence of this problem coupled with the desire of people to understand and subsequently control the reproductive processes has led to the application of this technique in livestock production. Therefore, ICSI could be useful not only to alleviate infertility or sub-fertility in valuable bucks but also for gender pre-selection (1) and propagation of useful genes (2). Until now, ICSI studies in goat have been scarce when compared with those in humans or other domestic animals, e.g., cattle. Although many live births by ICSI have been reported in cattle, sheep, and other animals, only one report has been published on the birth of a male kid (2). It has been documented that advances in the production of goat in vitro fertilization (IVF) embryos have been slow when compared to other ruminant species (3). In Malaysia, the main obstacles to produce goat embryos by IVF include poor oocyte quality and limited oocyte sources for in vitro production (IVP). The ICSI technique could be an option to overcome the obstacles and would be a useful tool for studying oocyte competence for embryo development more accurately.

Continuous supply of oocytes is a prerequisite for any IVP program usually accomplished from the abattoir source. Unlike other countries, abattoir-derived ovaries in Malaysia are not readily available due to low slaughtering as a result of acute shortage of does in Malaysia (4). Does that are either very old or culled from breeding programs are generally slaughtered; oocytes from these does are also of poor quality. Therefore, oocyte recovery (OR) from live does would be a good option for the Malaysian situation, where it is usually performed by standard surgical procedures or laparotomy. However, laparotomy has been gradually replaced by the laparoscopic ovum pick-up (LOPU) technique in small ruminants. Snyder and Dukelow (5) first described LOPU in sheep. One of the advantages of LOPU over laparotomy is that the same live donor can be used many times during its reproductive life because the pin-hole surgery is less traumatic and allows faster post-operative recovery. Moreover, LOPU in combination with IVP can improve the number of offspring produced by genetically valuable goats (6). This also enables the production of offspring from prepubertal and aged does that would not be able to reproduce with artificial insemination or multiple ovulation-embryo transfer (6-9). Although there is a variation in individual responses to superovulation, LOPU always results in OR from the donor (7). Previously, most of the ICSI studies in goats used abattoirs as the main source of oocytes and literature related to LOPU is scanty (2). Therefore, we produced and used LOPU-derived ICSI goat embryos in our previous studies (10,11). A search of available literature could not generate any published study on the effects of oocyte source on goat OR, oocyte quality, and their developmental competence. Therefore, this study attempted to compare: (a) the quantity and quality of goat oocytes recovered from live does by LOPU and slicing of abattoir-derived ovaries and (b) developmental competence of these oocytes after in vitro maturation (IVM) and ICSI.

Materials and methods

Chemicals, reagents, and media: All the chemicals and media used in this study were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. For all the media, the pH was adjusted to 7.2-7.4 and osmolarity to 280 mOsm/kg.

Animals and samples: Samples were collected from 2 different sources, (a) through LOPU from live does and (b) by slicing of ovaries from the abattoir source. For LOPU, a total of 8 does (16 ovaries) of mixed local breeds were used. In each replication (LOPU session) only 1 doe was used due to the shortage of goats. The does were housed in a farm within the university where they were fed a diet consisting of good quality Napier grass and pellet feed and given water ad libitum. On the other hand, a total of 18 ovaries (from 9 slaughtered does) were collected from a local abattoir.

Estrus synchronization and ovarian superstimulation: Does used for LOPU were subjected to estrus synchronization and superstimulation. Estrus was synchronized with the insertion of a controlled internal drug release device (CIDR, progesterone, 0.3 g; Pharmacia and Upjohn Limited, Auckland, New Zealand) for 14 days combined with a luteolytic treatment of cloprostenol (Estrumate, 125 mg; Schering-Plough, NSW, Australia) 48 h prior to CIDR removal. Ovarian

superstimulation was obtained with gonadotrophin treatment consisting of Ovagen (oFSH, 35 mg; ICPbio Ltd., Auckland, New Zealand) and Ovidrel (hCG, 250 IU; Laboratories Serono, Aubonne, Switzerland) administered intramuscularly 60 h prior to LOPU as recommended by Abdullah et al. (11).

Anesthesia: All the does were off-fed for 24 h prior to LOPU. Anesthesia was induced with intramuscular (i.m.) administration of Xylazine hydrochloride (Ilium Xylazine-20, Troy Laboratories Pty Ltd, NSW, Australia) at the rate of 0.22 mg per kg body weight (bwt). Ketamine hydrochloride (Ketamil, Troy Laboratories Pty Ltd) (22 mg/kg bwt) was administered i.m. as maintenance doses every 20-30 min or as required.

LOPU: The abdominal and inguinal skins were shaven, scrubbed, and cleaned with Hibiscrub. Surgical iodine was applied on the surgical surface prior to the onset of the LOPU procedure. LOPU was performed with a 7 mm Storz laparoscope attached to a video system (Aesculap A. G. & Company, Tuttlingen, Germany). A pneumoperitoneum was created in the anesthetized doe. Small incisions (3-5 mm) were made, 1 on the right and 2 on the left side of the lower abdomen to facilitate insertion of trocars into the abdominal cavity. A pediatric Storz laparoscope connected to a video camera was inserted into the right side of the abdominal cavity through the trocar sheath. A pediatric grasper and the ovum pick-up (OPU) needle (FAS Set C2, Gynetics Medical Product, N.V. Hamont-Achel, Belgium) were inserted into the left side of the abdominal cavity. The ovary was held by the grasper and follicles were individually punctured, flushed and aspirated by the OPU needle connected to the Cook aspiration and flushing system (V-MAR 5100; V-Mar 4000 respectively, Cook Australia, Queensland, Australia). Dulbecco phosphate-buffered saline (DPBS, Dulbecco A, BR0014G, Oxoid Limited, Hampshire, UK) supplemented with gentamicin sulfate (50 mg/mL, G1272, Sigma-Aldrich) and heparin (100 mg/mL, H0777, Sigma-Aldrich) was used as follicle flushing and oocyte aspiration medium.

Oocyte recovery (OR)

From abattoir ovaries: The ovaries from slaughtered does were obtained from a local abattoir and transported to the laboratory in DBPS solution supplemented with gentamicin sulfate (50 mg/mL) at 35-38 °C within 1-2 h of slaughter. The ovaries were rinsed in the same solution. Cumulus-oocyte complexes (COCs) were recovered after slicing the ovaries submerged in slicing medium comprised of HEPES-buffered M199 with Earle's salt, sodium bicarbonate, and L-glutamine (M4530, Sigma-Aldrich) supplemented with gentamicin sulfate (50 mg/mL) at 38.5 °C.

From LOPU: During the LOPU procedure, oocytes were aspirated from follicles of 2-3 mm or bigger in diameter; however, this could not be maintained for abattoir-derived ovaries as smaller follicles (<2-3 mm) could not be avoided during slicing. The COCs recovered through LOPU were rinsed in warm DPBS at 38.5 °C.

Oocyte grading: The COCs obtained from both LOPU and the abattoir sources were visually assessed and graded according to the cumulus cell (CC) investment and morphology of the ooplasm as described by Rahman et al. (10) (Table 1). Healthy COCs or cumulus-free oocytes (CFOs) with finely granulated and homogeneous ooplasm were selected for IVM.

IVM: The selected healthy COCs and CFOs were washed 3 times in IVM medium consisting of M199 with Earle's salt, sodium bicarbonate, and L-glutamine (M4530, Sigma-Aldrich) as a base medium supplemented with ovine FSH (oFSH, 10 μg/mL; Ovagen, ICPbio Ltd), hCG (1 mg/mL, Chorulon, Intervet International, B.V., Boxmeer, the Netherlands), 17β-estradiol (1 μg/mL; E2257, Sigma-Aldrich), sodium pyruvate (275 μg/mL; P3662, Sigma-Aldrich), gentamicin sulfate (50 μg/mL), cysteamine (100 μM; M9768, Sigma-Aldrich), and 10% (v/v) heat-inactivated estrus goat serum (EGS). Groups of 10 COCs and CFOs were matured in 100 μL of IVM medium overlaid with light mineral oil (M8410, Sigma-Aldrich) for 27 h at 38.5 °C and 5% CO_2 in air.

Assessment of oocyte maturation: After IVM, the COCs were treated with 100 mL of hyaluronidase (80 IU/mL, H4272, Sigma-Aldrich) in HEPES-buffered modified synthetic oviductal fluid (mSOF) medium (12). The cumulus-corona cells were carefully stripped off the zona pellucida by sequential denuding

Table 1. Grading of the recovered oocytes according to the cumulus cell investment and morphology of the oocyte.

Characteristics of COCs and CFOs	Grades
COCs with more than 5 complete layers of cumulus cells (CCs), finely granulated homogeneous ooplasm and normal morphological features.	Grade A
COCs with 3-5 complete layers of CCs, finely granulated homogeneous ooplasm and normal morphological features.	Grade B
COCs with 1-2 complete layers of CCs or COCs with 3-5 partially invested CC layers, finely granulated homogeneous ooplasm and normal morphological features.	Grade C
CFOs or oocyte with incomplete investment of CCs (1-2 layers), finely granulated homogeneous ooplasm and normal morphological features.	Grade D
Degenerating oocyte or oocyte with abnormal, size, shape and heterogeneous ooplasm, or apoptotic oocytes in jelly-like CC investment or very small oocytes.	Grade E

Source: Rahman et al. (10)

through glass pipettes of 250 mm and 200 mm inner diameters, respectively, under a dissecting microscope. Denuded oocytes were assessed for maturation on an inverted microscope. Oocytes with a clear first polar body (PB-1) were considered as mature (MII) and meiotic competent. These mature oocytes were then cultured in the incubator in the presence of 5% CO_2 at 38.5 °C until ICSI.

Sperm preparation: Motile sperm fractions were selected by swim-up after motility of sperm was evaluated under an inverted microscope. Briefly, 500 mL of frozen buck semen from the Jermasia breed (a breed developed in the University of Malaya) of proven quality was thawed and incubated at 38.5 °C in an humidified atmosphere of 5% CO_2 for 60 min in 2 mL of mSOF medium supplemented with 20% v/v goat serum (GS). After the incubation, the supernatant (600 µL) was removed and centrifuged at 200 × *g* for 10 min. After washing the sperm pellet twice, it was resuspended with an equal volume of mSOF medium containing heparin (50 µg/mL) and incubated for another 15 min in a humidified atmosphere of 5% CO_2 at 38.5 °C.

ICSI: The ICSI was performed on an Olympus IX71 inverted microscope fitted with Narishige hydraulic micromanipulators. The holding pipettes were made from borosilicate glass tubing of 1.0 mm outside diameter (OD) using a Sutter Micropipette Puller (P-97, Sutter Instrument Co., Novato, CA, USA) in combination with a Narishige microforge (Model: MF-9) and Narishige microgrinder (Model: EG-4) (Narishige Scientific Instrument, Tokyo, Japan). The holding pipettes were pulled to an OD of 150 µm and then fire polished to create an inside diameter (ID) of 30-40 µm. The pipette tips were bent to an angle of 35°. Injection needles were purchased from Humagen (Charlottesville, VA, USA) with an ID of 6-7 µm and OD of 8 µm, a sharp spike and a tip bend of 35°.

A 1-2 µL aliquot of washed sperm suspension was added to a 5 µL elongated microdrop of ICSI medium prepared on the left side of the ICSI dish. A motile sperm was aspirated into the injection needle head-first and it was brought into a microdrop containing 10% polyvinylpyrrolidone (PVP) located at the center of the dish. The sperm was immobilized by breaking its tail against the bottom of the dish by the tip of the injection needle. The immobilized sperm was aspirated again, tail-first, into the injection pipette. Each MII oocyte was positioned with its PB-1 at 6 or 12 o'clock on the holding pipette in 1 of the 4 injection microdrop located on the right side of the ICSI dish. The injection pipette was at 3 o'clock position. The sperm was injected, head-first, into the ooplasm of the oocyte. During the injection, ooplasm was aspirated into the injection needle to ensure that the needle is in the ooplasm. The sperm was injected into the ooplasm with a minimum volume (<5 pl) of the medium.

In vitro culture (IVC): Injected oocytes were cultured in vitro in mSOF medium supplemented with 10% heat-inactivated estrus goat serum (EGS, v/v) in 5% CO_2 at 38.5 °C. After 48 h of IVC, presumptive zygotes were cultured in freshly prepared mSOF medium with same proportion of EGS and embryos were cultured for <192 h. Once daily, embryos were assessed and their developmental stage was recorded.

Statistical analyses: The data presented in the present study were mean ± standard error of means (mean ± SEM) and were analyzed using one-way analysis of variance (ANOVA). The effects of oocyte source on OR, IVM, fertilization, cleavage and morula development following ICSI were compared and the significant differences between the means were further analyzed using Duncan's multiple range test (DMRT). The statistical analyses were carried out using the Statistical Packages for the Social Sciences, version 11.5 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

In the present study, oocyte recovery per doe was higher ($P < 0.05$) in the abattoir than LOPU source (Table 2). However, the quality of oocytes was better when retrieved by LOPU than slicing of abattoir ovaries as evidenced by (a) the recovery of higher ($P < 0.001$) proportion of viable or healthy oocytes (Table 2) and (b) higher ($P < 0.05$) proportion of Grade A oocytes (Table 3) in LOPU than the abattoir source. The differences in the recovery of Grades B, C, and D oocytes between the 2 sources were insignificant (Table 3).

Although a higher ($P < 0.001$) number of abattoir-derived oocytes was cultured than LOPU, a lower ($P < 0.01$) proportion of abattoir-derived oocytes survived compared to LOPU (Table 4). On the other hand, the maturation rate was higher ($P < 0.001$) in LOPU-derived oocytes compared to the abattoir source.

Healthy IVM oocytes with finely granulated homogeneous ooplasm with extrusion of the PB-1 were selected for ICSI with frozen-thawed buck semen. These oocytes were evaluated for their capacity to support fertilization and subsequent embryo development. There were no significant differences in the survival rates of injected oocytes within 16-18 h of ICSI procedure between the 2 sources (Table 5). LOPU-derived oocytes yielded a higher ($P < 0.05$) proportion of fertilization and cleavage than the abattoir source. It is also evident that a higher ($P < 0.05$) proportion of cleaved embryos from LOPU source progressed up to the morula stage compared to abattoir oocytes (Table 5). Percentage of morulae obtained from LOPU was nearly double that of the abattoir source.

Discussion

In the present study, LOPU source yielded 17.6 oocytes per doe, which was higher than the author's earlier OR rate of 6.7 (10) as well as the findings of other authors reporting an OR rate of 13.4 per adult doe and 15.7 per aged doe of >7 years old (13,14). The

Table 2. Goat oocyte recovery (mean ± SEM) from LOPU and the abattoir sources.

Oocyte source	Number of replicates	Number of does (ovaries) used	Number of oocytes retrieved	% nonviable oocytes	% viable oocytes
LOPU	8	8 (16)	17.6 ± 2.5^{a} ($n = 141$)	6.6 ± 3.5^{a} ($n = 12$)	93.4 ± 3.5^{b} ($n = 129$)
Abattoir	5	9 (18)	27.3 ± 3.0^{b} ($n = 246$)	36.2 ± 4.5^{b} ($n = 83$)	63.8 ± 4.5^{a} ($n = 163$)

Number of oocytes was based on per doe (2 ovaries); n = Number of oocytes used.
a, b, Superscripts in the same rows indicate significant differences [$P < 0.05$ for Number of oocytes retrieved and $P < 0.001$ for % nonviable (unhealthy) and viable (healthy) oocytes].

Table 3. Percentage of viable oocytes (mean ± SEM) retrieved from 2 different sources according to grades.

Oocyte source	Grades of viable oocytes (%)			
	Grade A	Grade B	Grade C	Grade D
LOPU	32.6 ± 3.8[b] (*n* = 44)	28.5 ± 3.3[a] (*n* = 36)	23.0 ± 2.8[a] (*n* = 28)	16.0 ± 1.1[a] (*n* = 21)
Abattoir	19.0 ± 3.6[a] (*n* = 30)	26.2 ± 2.9[a] (*n* = 41)	33.5 ± 5.5[a] (*n* = 53)	21.2 ± 4.0[a] (*n* = 39)

n = Number of oocytes used.
a, b, Superscripts in the same rows indicate significant differences (P < 0.05).

Table 4. Oocyte survival and the maturation rates (mean ± SEM) in relation to the oocyte source.

Oocyte source	Number of replicates	Number of Oocyte cultured	Number of oocytes died during IVM	Oocyte survival rate (%)	Oocyte maturation rate (%)
LOPU	8	16.1 ± 1.9[a] (*n* = 129)	8	93.8 ± 2.1[b] (*n* = 121)	73.8 ± 3.0[b] (*n* = 88)
Abattoir	5	32.6 ± 2.1[b] (*n* = 163)	30	81.9 ± 0.8[a] (*n* = 133)	54.0 ± 0.7[a] (*n* = 72)

Number of oocytes was based on per replication; *n* = Number of oocytes used.
% immature and mature oocytes were calculated from number of oocytes that survived after IVM.
a, b, Superscripts in the same rows indicate significant differences (P < 0.01).

Table 5. Embryo development (mean ± SEM) of in vitro matured oocytes microinjected with frozen-thawed spermatozoa in goats.

Oocyte source	Number of IVM oocytes injected	Survival rate (%)	Fertilization rate (%)	Cleavage rate (%)	Morula (%)
LOPU	88	93.4 ± 2.3[a] (*n* = 82)	60.9 ± 2.1[b] (*n* = 50)	83.1 ± 2.6[b] (*n* = 41)	20.0 ± 1.2[b] (*n* = 10)
Abattoir	72	88.8 ± 1.8[a] (*n* = 64)	50.6 ± 2.3[a] (*n* = 30)	69.3 ± 4.5[a] (*n* = 20)	11.3 ± 4.7[a] (*n* = 4)

Number injected oocytes and embryos were based on per replication (8 replicates in LOPU and 5 replicates in the abattoir source); *n* = Number of oocytes or embryos used.

% oocytes fertilized were calculated from number of oocytes that survived the injection procedure and % embryos of different stages were calculated from number of oocytes fertilized.

a, b, Superscripts in the same rows indicate significant differences (P < 0.05).

present finding was much higher than the minimum OR rate of >5 per doe (7,15) and previous findings of 3.7-6.7 per doe in our laboratory (16-18). However, compared with the abattoir source (27.3 per doe), OR rate was significantly lower in LOPU in the present study. This is due to the fact that, in LOPU, only follicles bigger than 2-3 mm were aspirated, whereas irrespective of size all the follicles were sliced from abattoir ovaries including those of smaller follicles (<2 mm). Although OR by LOPU was lower than the abattoir source, the OR rate in the present study was very good as this was from a live donor, which could be used a few more times. Moreover, oocyte quality was better in LOPU compared to the abattoir source.

From the IVM data, it is clear that LOPU provided a higher oocyte survival and maturation rates than the abattoir source. During IVM higher numbers of abattoir-derived oocytes died than LOPU source, which definitely affected the maturation of abattoir-derived oocytes. On the other hand, abattoir-derived oocytes were highly heterogeneous in nature. This heterogeneity came from different grades of growth and atresia of the oocytes obtained, which might have contributed to the lower maturity in some of these oocytes. On the other hand, in LOPU as does were stimulated with hormones, and only follicles >2-3 mm were punctured, chances of survivability and maturity were better than with abattoir oocytes. In the present study, all the viable oocytes of heterogeneous grades were cultured including COCs with <1 complete CC layer or CFOs due to a shortage of oocytes in both sources. However, other authors cultured good grades of oocytes with at least 4 (9,19,20), 2 and above (21,22), or 1-2 (2,23) layers of CC. In the present study, although better maturation rates were obtained from LOPU source, the reason for lower maturity of abattoir oocytes might be their lower quality. The higher maturation rates (>70% to >80%) of abattoir oocytes reported by other authors (22,24-26) were mainly due to the quality or grades of those oocytes and different IVM media. In contrast, the maturation rate of abattoir oocytes in the present study was consistent with some other authors (27-29) reporting 35%-65% maturation rates. The maturation rates of LOPU oocytes in the present study were higher than those published by Phua (17) and Amir (18), who reported 31.0%-67.8% maturation rates. Although not with LOPU, the current maturation rate was lower than that reported by Gall et al. (30) and Samaké et al. (31), who obtained 96% to 100% maturity, respectively, from hormonally treated and estrus synchronized slaughtered does.

In this study, it was noteworthy that the fertilization, cleavage, and morula development rates were significantly higher in LOPU compared to abattoir oocytes. Wang et al. (2) obtained a similar cleavage rate (71%-90%) using better quality LOPU-derived oocytes (>1-2 CC layers); however, they used a highly sophisticated Piezo-ICSI and a better IVC medium (mTALP-mKOSM). Using a slightly modified SOF medium, Keskintepe et al. (21), and Jiménez-Macedo et al. (9,19,20) obtained 73.4%, 67.0%, 60.3%-75.0%, and 51%-68.2% cleavage rates, respectively, with abattoir oocytes. These results are consistent with the present study. It is to be noted here that these authors used good quality oocytes with at least 2 layers of CC and they activated oocytes or sperm with chemicals. In the current study, the majority of the oocytes from LOPU and the abattoir sources acquired competence for development up to the 8- to 16-cell stage, but few gained the ability to form morula. However, morula development rate was significantly higher in LOPU compared to abattoir-derived oocytes. Although morula development rate in the present study is lower than other groups, the result is much better than the authors' previous study (10). In this study, we found that there was a lower morula development rate in abattoir oocytes compared to LOPU and that other groups might be directly related to the quality of the oocytes used. The ICSI technique and IVC media may also contribute to better results in other groups (2,9,19-21).

In conclusion, LOPU provided higher quality oocytes, and better maturation and subsequent embryo development rates compared to the abattoir source in the present study. To the best of our knowledge, this is the first report of IVM and ICSI in goats according to oocyte source. As there is an acute shortage of abattoir-derived goat ovaries in Malaysia, it may be suggested that LOPU could be a better and alternative OR method for the production of goat embryos using IVP techniques like IVF or ICSI in Malaysia.

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