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Studies on *in vitro* fertilization rates of oocytes from stimulated and non stimulated Sahiwal cows

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Abstract
The aim of the study was to evaluate the effect of FSH stimulation on *in vitro* maturation, *in vitro* fertilization and blastocyst development rates of oocytes collected by ovum pick-up in Sahiwal cows. Sixty four Sahiwal cows aged 3-6 years randomly divided into two groups. Animals in group 1 (FSH stimulated, n=32) were subjected to FSH prestimulation with CIDR before OPU and Animals in group 2 were non stimulated (n=32). A total of 64 OPU sessions were performed in 64 animals with and without FSH prestimulation at random stage of estrous cycle. Performance was evaluated on per OPU session and per cow.

The mean number of follicles available for aspiration (FSH stimulated;18.59± 1.04 and non-stimulated;6.5±0.25) and the mean number of oocytes recovered per session per animal (FSH stimulated; 9.87±0.39 and non-stimulated; 2.56±0.23) was found to be significantly ($P< 0.05$) higher in FSH stimulated group than the non stimulated group. The mean number of Grade A, Grade B, Grade C and Grade D oocytes recovered in non-stimulated group were 0.53±0.13, 0.71±0.11, 0.90±0.10, 0.40±0.10 and in FSH stimulated group were 2.37±0.47, 2.59±0.28, 3.03±0.33, 1.87±0.25, respectively. The mean number of oocytes recovered per session per animal was significantly ($P< 0.05$) higher in FSH stimulated group compared to the non-stimulated group

The significance ($P< 0.05$) difference was observed between the non-stimulated and FSH stimulated group in Cumulus cell expansion rate (57.31% and 68.03%) and First polar body extrusion rate (46.34% and 56.32%).In FSH stimulated group 178 oocytes and in non-stimulated group 82 oocytes were kept for *in vitro* fertilization for 16-18 hrs and further *in vitro* culture for 7 days. The blastocysts development rate was observed on 6th and 7th day of culture. On 6th and 7th day the blastocyst development rate in FSH stimulated and non-stimulated group was 37.64% and 25.60% respectively, the blastocyst development rate was significant ($P< 0.05$) between non-stimulated and FSH stimulated group.

Keywords: Cumulus cell expansion, follicle stimulating hormone, *in vitro* culture, *in vitro* fertilization, *In vitro* maturation, ovum pick-up (OPU), polar body extrusion

1. Introduction
The breeding tract of sahiwal breed is Ferozpur and Amritsar districts of Punjab and Sriganganagar districts of Rajasthan and is known as one of the best dairy breeds in India due to its unique triats such as tick resistance, heat tolerance, high milk production and high resistance to parasites (both internal and external).

Ovum Pick up – *In vitro* Embryo Production (OPU – IVEP) and Embryo Transfer (ET) can be a viable alternative to conserve breeds, increase milk production and at the same time ensure faster multiplication of superior germplasm. Use of OPU in IVEP programmes proved to be a potential alternative to traditional embryo production (Bousquet *et al*., 1999 and Kruip *et al*., 1991) [3, 14]. Repeated aspirations at short time intervals are possible and the oocytes can be recovered from cows irrespective of their reproductive phase (Bungartz *et al*., 1995) [4]. Repeated aspirations can be performed twice weekly for several months without need for hormonal stimulation of donors. Stimulation with FSH prior to OPU increase oocyte recovery rate compare to once in a week non-stimulated OPU procedure (Chaubal *et al*., 2006) [6].

Ovum pick-up (OPU) followed by *In vitro* Embryo Production (OPU-IVEP) is being largely utilized by commercial cattle breeders as the practicality of *In vitro* Embryo Production dictates that oocytes can be collected from living cows with superior genetic value. Use of Ovum Pick Up along with *In vitro* Fertilization allows for the flexibility in which the collected oocytes can be fertilized with semen from different bulls, so that increasing the chance of genetic variability in the resultant embryos. These distinct advantages of Ovum Pick Up-*In vitro* Embryo Production, can increase the selection intensity and shortening the generation interval, suggest that OPU-IVEP is a reliable alternative to MOET in the efficient genetic-improvement of cattle.

Stimulation of animals with Follicle Stimulating Hormone prior to Ovum Pick Up will increase the oocyte recovery rate when compare to once a week non stimulated OPU procedure (Chaubal *et al*. 2006) [6]. Breed, age, stage of lactation, nutrition and seasonal conditions also influence the oocyte recovery rate. Ovum Pick Up in Indian cattle was reported by Manik *et al*. (2003) [18].

To improve the *In vitro* Maturation and *In vitro* Fertilization and *In vitro* Embryo production rates in Sahiwal cows the *In vitro* Maturation and *In vitro* Fertilization rates between stimulated and non-stimulated Sahiwal cattle oocytes were selected.

2. Materials and Methods

2.1 Experimental location

The present study was undertaken at Livestock Farm Complex, College of Veterinary Science, Korutla, Jagitial district, Telangana (latitude: 18° 49´36.71"N; longitude: 78° 42´ 50.39"E; altitude: 295.99 m above mean sea level) under the project implementation of ET & IVF Technology under Rashtriya Gokul Mission, Dept. of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Korutla during the period between October 2021 and March 2022. The climate in the region was semi-arid to sub humid with hot summer and cool winter. The decennial average rainfall was 922 mm and the ambient temperature range from a lowest of 13 °C during January to a peak of about 45°C during May/June.

2.2 Experimental animals

Sahiwal cows(n=64) aged about 4-6 years and weighing between 250 and 450 kg body weight were selected as oocyte donors through Ovum Pick-Up (OPU). Among 64 animals, In Group I animals (Stimulated, n = 32), CIDR was inserted at random stage of estrous cycle and FSH stimulation (Follitropin-V, 200 mg i/m in 8 divided doses - 80, 60 and 40 and 20mg) was done after CIDR application. Where as in Group II 32 animals were subjected to ovum pick-up (OPU) at random stage of estrous cycle without prestimulation.

2.3 Ovum pick-up technique (OPU)

The ovaries were manipulated per rectum and either the right or left ovary was positioned between the fingers. After thorough cleaning, the lubricated (Ultrasonic coupling gel) transvaginal probe with glove was advanced into the anterior vagina as far as possible, with the transducer surface in a position either to the left or to the right side of the external os of the cervix. Ultrasound scanning was done by using 7.5 MHz frequency, 64-72 gain and 56% brightness.

The ovary was manipulated gently and positioned against the probe head in order to obtain a clear image of the follicles on the ultra-sonographic monitor. The number of follicles per ovary was recorded and the diameter of the follicles (Fig.1) was measured by freezing the image on the monitor and by using an inbuilt calliper and then the diameters of follicle was calculated by averaging the lengths of long and short axes. Based on the diameter, the follicles were classified as small (<4mm), medium (4 - <8mm) and large (≥8 mm). The presence of CL on the ovary and its diameter was also recorded. When the ovary and targeted follicle were stabilized, the needle fitted with aspiration line was inserted through the plastic probe carrier and advanced to reach the fornix vagina and into the follicle antrum.

Follicular fluid of each follicle was aspirated by using continuous negative pressure of 70-75 mmHg, applied with a foot operated suction pump. During aspiration the needle was gently rotated to curette the follicle and to dislodge the oocyte still attached to its wall or trapped in its folds. Prior to aspiration of next follicle the needle was withdrawn from the ovary, but kept in position exterior to the fornix vagina with the tip still visible on the monitor. Before and after OPU, the needle and aspiration line were thoroughly rinsed with pre heated (37 °C) OPU recovery medium (Catalog no.19982/1281, BoviPlus, Minitube, USA) to prevent blood from clotting or oocytes from sticking to the tubing.

The oocyte recovery rate was calculated as the number of oocytes recovered from the number of follicles aspirated for each cow expressed as a percentage (Goodhand *et al*., 2000) [10].

During each aspiration, all visible follicles of >4mm diameter were aspirated and the contents were collected in a 50ml tube. A successful aspiration was confirmed by the disappearance of individual fluid - filled (non-echogenic and dark) follicle image on-screen display and the process was repeated until all the suitable follicles were aspirated. After aspiration of all follicles on one ovary, the same procedure was repeated in the opposite ovary. A separate needle was used for each donor.

2.4 Oocyte recovery

After completion of the aspiration of the each ovary, the 50mL conical centrifuge tube containing the follicular aspirate was transferred to the laboratory. The follicular aspirate was transferred to a 100 µm oocyte mini filter (25458, WTA, Brazil) and repeatedly washed with OPU recovery medium (Catalog no.19982/1281, BoviPlus, Minitube, USA) in order to make the filtered aspirate free from blood tinge and cloudy follicular fluid. The washed and filtered follicular aspirate was then transferred to 60mm petridish (Catalog no.150360, Thermo scientific, Massachusetts, USA) and examined under zoom stereomicroscope (SMZ - 1270, Nikon, Japan) at 1x magnification to identify the cumulus oocyte complexes (COCs).

The COCs were transferred to a 35mm petridish containing wash medium (BO 01, oocyte and embryo wash media, Vitrogen, Brazil) and examined under stereozoom microscope at 8x magnification for evaluation and grading.

2.5 Evaluation of cumulus oocyte complexes

Generally the morphological evaluation and classification of cumulus oocyte complexes was based on the oocyte integrity, homogeneity of cytoplasm and the quantity of the cumulus cell layer surrounding the oocyte. During evaluation, the cumulus oocyte complexes were classified into four quality grades (grade A,B,C,D) (Chaubal *et al*., 2006) [6].

2.6 *In vitro* maturation (IVM)

The oocytes were washed 4-6 times with Wash medium (BO 01, oocyte and embryo wash media, Vitrogen, Brazil) and then washed twice with IVM medium (BO 02, Vitrogen, Brazil). Cumulus-oocyte complexes were transferred into a over night CO2 equilibrated IVM medium in five well plate (kept in a humidified CO_2 incubator (5% CO_2 in air) at 38.5 °C). After 20-24 hours of *In vitro* Maturation of oocytes, assessment of maturation was done by the degree of expansion of cumulus cell mass (Fig.4) and extrusion of first polar body (Fig.5) into the perivitelline space.

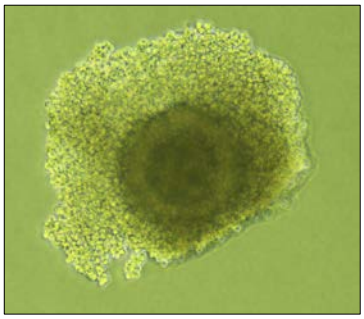

Fig 1: Grade A Oocyte

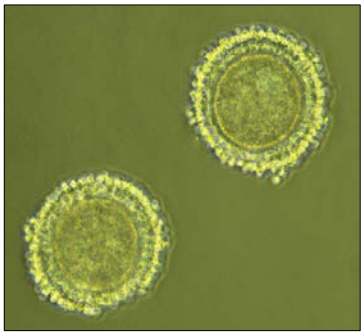

Fig 2: Grade B oocytes

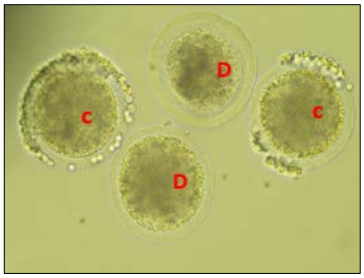

Fig 3: Grade C and Grade D Oocytes

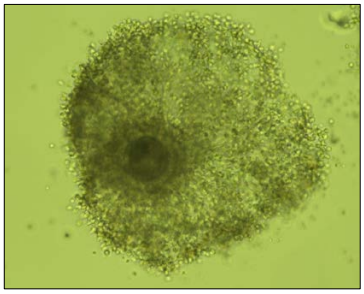

Fig 4: Cumulus cell expansion

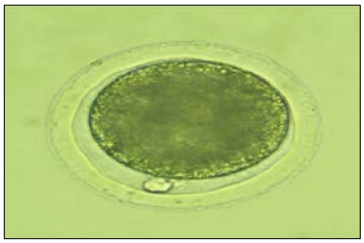

Fig 5: Extrusion of first polarbod

2.7 *In vitro* fertilization (IVF)

On the day of IVF, after 20 hours of IVM, matured oocytes were washed 2-3times with prepared IVF media and then transferred into well containing of pre-equilibrated IVF media. The IVF dish containing matured oocytes was placed in the benchtop incubator till the semen was prepared. Semen straw was thawed in thawing machine at 37°C for 30 seconds and was emptied into a 15ml falcon tube containing pre-warmed percoll gradient [400µl of conventional percoll (BO 07, Vitrogen, Brazil) + 400µl of diluted percoll (BO 06, Vitrogen, Brazil)] and centrifuged at 600G for 6min. at 37°C. The supernatant was removed leaving the pellet. To this pellet 400 µl of prepared IVF media was added and centrifuged at 150 G for 3min. at 37 °C. The supernatant was again removed leaving the pellet and semen of approximately 10-20µl from the semen pellet was inseminated into IVF drops containing matured oocytes (Fig.6). The IVF dish was placed in benchtop incubator (Planer incubator BT37, Ref. 026412, IMV Technologies, USA) at 38.8°C, in a humidified atmosphere of 6% CO_2, 5% O_2, and 89% N_2 for 16-18 hrs.

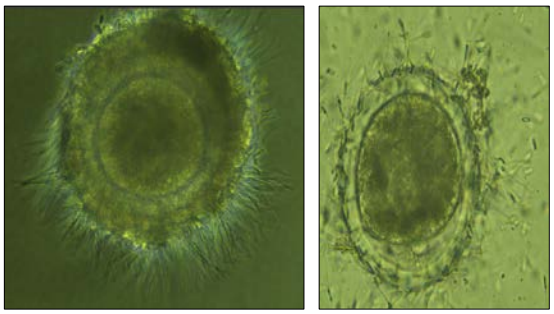

Fig 6: Co-incubation of oocyte and sperm

2.8 *In vitro* culture (IVC)

After 16-18 hrs of co-incubation of gametes, the presumptive zygotes were transferred into the freshly prepared well plate of preheated Wash media. Putative zygotes were mechanically denuded of cumulus cells by repeated pipetting with denudation pipette in Wash media. The denuded zygotes were washed 2-3 times with Wash media and later 2-3 times with equilibrated IVC media (BO 04, Vitrogen, Brazil). Then these zygotes were immediately transferred into equilibrated IVC dish overlaid with mineral oil (Fig.7). *In vitro* culture dish was then kept in benchtop incubator (Planer incubator BT37, Ref. 026412, IMV Technologies, USA) at 38.8°C, in a humidified atmosphere of 6% CO_2, 5% O_2, and 89% N_2 for 6 days.

On 3rd day of culture media was changed and blastocysts development rate were observed on 6th and 7th day of culture.

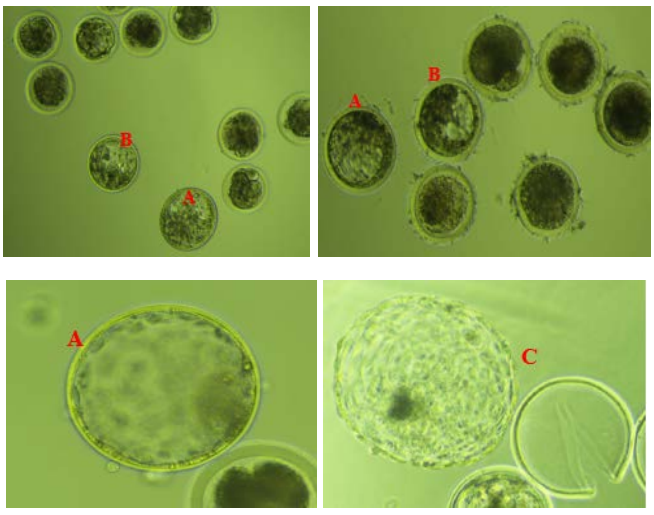

Fig 7: Embryos observed under phase contrast microscope. (A) Blastocyst, (B) Expanded blastocyst and (C) hatched blastocyst

3. Results and Discussion

3.1 Ultrasound guided transvaginal follicular aspiration

Table 1: The data obtained after follicular aspiration, oocyte recovery rate and *in vitro* maturation rate through ovum pick up (OPU) were presented below.

Attribute	FSH stimulated group (Group1)	Non-stimulated group (Group2)
Number of Aspirations.	32	32
No.of follicles available for aspiration.	595	208
Mean No.of follicles aspirated /cow/session.	18.59±1.04 [a]	6.5±0.25 [b]
Total No.of COCs recovered.	316	82
Recovery rate% (COCs /aspirated follicles).	53.10	39.42
Mean No.of COCs recovered / cow / session.	9.87±0.39 [a]	2.56±0.23 [b]
No of oocytes kept for maturation.	316	82
No. of oocytes matured.	215	47
Cumulus cell expansion rate (%).	68.03	57.31
1st polar body extrusion rate (%).	56.32	46.34

Values bearing superscripts (a,b) in a row differ significantly ($P< 0.05$)

3.1.2 Effect of stimulation on follicular population in sahiwal cows

The number of follicles aspirated were 208 in non-stimulated group and 595 in FSH stimulated group. The mean number of follicles available for aspiration were found to be significantly ($P< 0.05$) higher in FSH stimulated group; 18.59±1.04 when compared to the non-stimulated group; 6.5±0.25 (Table 1).

The present findings were in agreement with Srimannarayana (2019) [22] and Goodhand *et al.* (2000) [10]. However, the present findings were in contrary to the study of Chaubal *et al.* (2006) [6], could be due to individual donor animal variation as they used the angus breed cows for FSH stimulation and they removed the dominant follicles before stimulation and the administration of FSH hormone was subcutaneously in donor animals and the follicular dynamics variation of the donor animal, vacuum pressure and needle diameter used during the process of aspiration. Operator experience also effects the follicular aspiration.

3.1.3 Effect of stimulation on oocyte yield per cow per session and recovery rate

The total number of oocytes recovered were 82 and 316 respectively in non-stimulated and FSH stimulated group with a recovery rate of 39.42 per cent and 53.10 per cent. The mean number of oocytes recovered per session per animal was significantly ($P< 0.05$) higher in FSH stimulated group; 9.87 ± 0.39 when compared to the non-stimulated group;2.56±0.23 (Table 1).

The present findings were in agreement with the study of Jeyakumar (2004) [12], Aller *et al.* (2010) [1], Vieira *et al.* (2016) [24] and Srimannarayana *et al* (2019) [22]. However, the present findings were in contrary with the study of Vieira *et al.* (2014) [23] and Silva (2017) [20] may be due to the multiple factors that influence the oocyte recovery rate include vacuum pressure, needle diameter, operator experience and the follicular population size of the donor animal, breed of the donor animal, hormonal pre-stimulation of the animal, timing and frequency of OPU sessions and follicular wave synchronization of the animal.

3.1.4 Effect of stimulation on oocyte maturation

3.1.4.1 Cumulus cell expansion rate and Extrusion of 1st polar body

A total number of 82 oocytes were collected from non-stimulated group and subjected to *in vitro* maturation. Among 82 oocytes the cumulus cell expansion was observed in 47 oocytes with an expansion rate of 57.31%. A total number of 316 oocytes were collected from FSH stimulated group and subjected to *in vitro* maturation of which cumulus cell expansion was observed in 215 oocytes with an expansion rate of 68.03%. The significant difference ($P< 0.05$) was observed on cumulus expansion between FSH treated and non treated groups (Table 1). In non-stimulated animals among 82 oocytes, in 38 oocytes extrusion of 1st polar body into perivitelline space was observed with an extrusion rate of 46.34%. In FSH stimulated group among 316 oocytes, in 178 oocytes extrusion of 1st polar body into perivitelline space was observed with an extrusion rate of 56.32% (Table 1).

The present findings were in agreement with results of Donnay *et al.* (1997) [8]. However the present findings were not in collaboration with the studies of Manik *et al.* (2003) [18], Looney *et al.* (1994) [17], Hasler *et al.* (1995) [11], Goodhand *et al.* (1999) [9] could be due to the breed of the animal, age of the animal, cyclicity of the animal, follicle wave emergence at the time of OPU.

In this study the *in vitro* maturation rates was associated with many factors, like animal genotype, size and quality of follicles, oocyte damages due to pressure applied during follicular aspiration, quality selection of oocytes and the medium provided for *in vitro* maturation. The first major and crucial factor for successful *in vitro* maturation is the medium that is provided. Hunter and Moor (1987) concluded that the degree of cumulus cell expansion is stimulated by gonadotropins and growth factors that are produced and secreted hyaluronic acid, which disperses the cells, a process known as expansion.

3.2 Effect of stimulation on oocyte quality

The mean number of Grade A, Grade B, Grade C and Grade D oocytes recovered in non-stimulated group were 0.53±0.13, 0.71±0.11, 0.90±0.10, 0.40±0.10 and in FSH stimulated group were 2.37±0.47, 2.59±0.28, 3.03±0.33, 1.87±0.25, respectively. The mean number of oocytes recovered per session per animal was significantly ($P< 0.05$) higher in FSH stimulated group compared to the non-stimulated group (Table 2).

The present findings were in agreement with the studies of Srimannarayana *et al* (2019) [22], Aller *et al.* (2010) [1], Jeyakumar (2004) [12], Perez *et al.* (2000) [19] and Looney *et al.* (1994) [17] reported that FSH stimulation prior to OPU improve more number of good quality oocytes.

The present findings were not in collaboration with the studies of Silva *et al.* (2017) [20] could be due to influence of individual donor, breed, stage of lactation, physiological

stresses and the effect of environmental factors like humidity, hot or cold climate, season and follicular populations.

Table 2: Oocyte quality in non-stimulated and stimulated group

Attribute	Mean number of oocytes (Stimulated group)	Mean number of oocytes (Non-stimulated group)
Grade A	2.37±0.47	0.53±0.13
Grade B	2.59±0.28	0.71±0.11
Grade C	3.03±0.33	0.90±0.10
Grade D	1.87±0.25	0.40±0.10

The Values in a row differ significantly ($p< 0.05$)

3.3 Effect of stimulation on *in vitro* fertilization, embryonic development

Table 3: Effect of stimulation on *In vitro* Fertilization, embryonic development

Attribute	FSH Stimulated Group Animals	Non-stimulated Group Animals
Number of oocytes kept for *In vitro* Fertilization and Culture.	178	82
Cleavage Rate (%).	64.04	46.56
Blastocyst Rate (%).	37.64	25.60

The Values in a row differ significantly ($p< 0.05$)

In FSH stimulated group 178 oocytes and in non-stimulated group 82 were kept for *in vitro* fertilization for 16 to 18 hours and the presumptive zygotes kept for *in vitro* culture for 7 days. The blastocysts development rate observed on 6th and 7th day of culture (Table 3).

The cleavage rates observed in present experiment were 64.04 percent and 46.56per cent for zygote of FSH stimulated group and non-stimulated donor cows. The cleavage rate was significant ($p< 0.05$) between non-stimulated and FSH stimulated group. On 6th and 7th day the development of blastocyst stage was observed and the blastocyst development rate in FSH stimulated and non-stimulated group was 37.64 per cent and 25.60 per cent respectively. The blastocyst rate was significant ($p< 0.05$) between non-stimulated and FSH stimulated group (Table 3).

The present findings were better than that of Holm *et al*. (1999), Looney *et al*. (1994) [17] and Manik *et al*. (2003) [18], Lonergan *et al*. (2003) [16] might be due to the media used for the process of *in vitro* culture and the animals selected for their study.

The present results were better than the studies reported by scientists like Kruip *et al*. (1994) [14], Krisher *et al* (1999) [13], Ali and Sirard (2002) [2], Lonergan *et al*. (2003),) [16] may be due to influence of the culture media used in the present study and quality of oocytes selected, damages due to the pressure applied during the process of follicular aspiration, variation in environmental conditions and the medium provided for the process of *in vitro* maturation.

5. Conclusion

The breed, age, cyclicity, and nutrition of the animal, as well as the technical parameters of vacuum pressure, aspiration needle diameter, and operator skill, all have an impact on the success of OPU- IVM- IVF- ET. Furthermore the inherent ability of individual animal that supports the growth of the healthy follicles and the growth of the follicles in response to external hormones also effect on the outcome of the OPU. In conclusion, 200mg of FSH stimulation at decreasing doses before OPU enhanced the number of medium and large follicles available for aspiration as well as the follicular population. FSH pre-treatment also improved the mean number of oocyte recovery and maturation rate including cumulus cell expansion and 1st polar body extrusion and *in vitro* fertilization rate and blastocyst development rates. However further studies are needed to improve the oocyte yield and oocyte maturation rate through OPU and IVM-IVF technique in native cattle breeds.

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