Effect of an asynchrony between ovulation and insemination on the results obtained after insemination with fresh or frozen sperm in rabbits

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Abstract

The effects of the introduction of an 8-h asynchrony between ovulation and insemination on litter size components from rabbits were assessed. A total of 202 females belonging to a maternal line were used. Fresh and frozen sperm were used to perform the inseminations. Sperm was frozen with an extender composed of 1.75 M DMSO and 0.05 M sucrose. Four experimental groups were obtained depending on the type of sperm used (fresh or frozen) and on the moment that ovulation had been induced relative to the insemination (at the same time as insemination (t₀) or 8 h before insemination (t₈)). Laparoscopy was performed on 12th day of pregnancy in pregnant females, and the ovulation rate, normal and total implanted embryos were noted. At kindling, total and live-born rabbits were noted. Results showed that better results were obtained after insemination with fresh semen than with frozen sperm (for females in the group t₀: 79% versus 61% fertility rate, 10.2 versus 6.4 normal implanted embryos and 8.1 versus 5.2 total number born, for fresh and frozen sperm, respectively). On the other hand, after the introduction of an 8-h asynchrony between ovulation and insemination, results were lower for both fresh (50% fertility rate, 7.5 normal implanted embryos and 5.7 total number born for the group of the asynchrony) and frozen sperm (31% fertility rate, 4.6 normal implanted embryos and 3.4 total number born for the group of the asynchrony). Although an approach between the moment of insemination and ovulation is justified when sperm survival could be compromised, results observed after the induction of an 8-h asynchrony were not those expected, perhaps due to the ageing of the oocytes before being fertilised, leading to both lack of fertilisation or early embryonic mortality.

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1. Introduction

Insemination in rabbit farms has increased in recent years, obtaining results when working with fresh semen comparable to those obtained by natural mating (79% fertility rate and 6.8 live-born rabbits/female for both, natural mating and artificial insemination, Alabiso et al., 1996). However, when frozen sperm was used to perform inseminations in commercial farms, results in both fertility and prolificacy were lower than those obtained with fresh semen (39 and 50% fertility rate and 5.4 and 8.0 total born reported by Theau-Clément and Roustan, 1982 and Mocé et al., 2002, respectively). Although several diluents and protocols have been developed to freeze rabbit sperm, obtaining good results for fertility rate with some of them (between 70 and 80% reported by O’Shea and Wales, 1969; Weitze et al., 1976), none of them seem to give such good results as fresh sperm for litter size, obtaining almost 1.5 less born rabbits with frozen semen. Only Viudes-de-Castro and Vicente (1996) reported similar results when working with frozen sperm as when fresh semen had been used (88% versus 85% fertility rate and 8.1 versus 8 total born for fresh and frozen semen, respectively).

Some authors have observed that freezing provokes changes in ram sperm which are similar to those produced during the capacitation process (Pérez et al., 1996). The alteration of the membranes during the freezing protocol provokes a reduction in the lifespan of the spermatozoa, which could explain in part the worse results obtained after insemination with frozen sperm (Parks and Graham, 1992; Watson, 2000). Mocé and Vicente (2002), observed

![Diagram of work protocol]

Inseminate females from:
2. Treated group (18): ovulation induced 8 hours before the insemination was performed.

Fig. 1. Work protocol.
an increase in the number of oocytes (not fertilised) recovered after insemination of rabbit females when frozen sperm was used (0.5 versus 2.4 oocytes and 7.8 versus 3.8 normal embryos for fresh and frozen sperm, respectively).

Ovulation in rabbits is not spontaneous, so it is easy to induce controlled asynchronies between ovulation and insemination. Asynchronies could be a useful tool to approach insemination and ovulation in cases in which sperm survival could be compromised, since sperm is found in the oviducts 1 h after insemination is performed (El-Banna and Hafez, 1970), and only $10^3$ or $10^4$ fresh sperm inseminated in the oviduct are required to reach high fertility levels (Kanayama et al., 1987).

Stranzinger et al. (1971) obtained better fertility for fresh and frozen sperm (15% more fertility rate) when ovulation had been induced 5 h before insemination, but they did not test any other asynchrony. Asynchronies of 5 or 10 h between ovulation and insemination with frozen sperm have been tested (Chen et al., 1989a,b), obtaining similar results for prolificity for 5 h and no asynchrony, and worse results for fertility and prolificity after an asynchrony of 10 h; however, no works have been done to test intermediate asynchronies (between 5 and 10 h).

The aim of this study was to evaluate the litter size components in rabbits after insemination of females with fresh and frozen sperm that had been induced to ovulate at the same time or 8 h before insemination (Fig. 1).

2. Material and methods

2.1. Animals

Females used in this study belonged to three different strains of rabbits selected for maternal characteristics (lines A, V and H). Selection methodologies were described by Estany et al. (1989) and Cifre (1997). In a previous study, no differences were found in results obtained after insemination with frozen sperm between these lines (Mocé et al., 2002). All of them were multiparous non-lactating females.

Sperm from six males was used to perform the inseminations. All males used in the experiment belonged to one of the lines selected for maternal characteristics (line V).

2.2. Semen collection and evaluation

Two ejaculates were collected per male and week, with an interval between the collection of the two successive ejaculates of 30 min (Mocé et al., 2000). The following measurements were taken from fresh sperm.

- **Volume**: was measured in a graduated conical tube.
- **Motility**: to measure the motility rate subjectively, a dilution 1:50 was made with a tris–citric acid–glucose extender. A drop of 10 µl was laid over a slide and it was covered with a coverslip (20 mm × 20 mm). Motility was examined at 37 °C under a microscope with phase contrast optics, at 125× and connected to a monitor through a camera. Only those ejaculates with more than 70% of motile sperm were used to freeze.
Concentration: one sample of sperm was fixed in a solution of glutaraldehyde (2%) and concentration was measured using a Thoma–Zeiss cell counting chamber.

Morphological examination: percentage of spermatozoa with normal apical ridge (NAR), cytoplasmic droplets or abnormal spermatozoa were measured in a sample fixed with glutaraldehyde (2%), at a magnification of 400× with a differential interference contrast microscope (Nomarski contrast).

In the frozen sperm, motility and acrosomal integrity estimations were performed in the same way as for fresh semen.

2.3. Freezing protocol

The extender to dilute the fresh sperm (extender 1) was composed of 0.25 M of tris[hydroxymethyl]aminomethane (Sigma), 88 mM of citric acid anhydrous (Sigma), and 47 mM of D (+) glucose (Sigma), (Viudes-de-Castro et al., 1999).

The freezing extender (extender 2) was composed of the extender 1, and 3.5 M of dimethyl sulfoxide (DMSO, Sigma) and 0.1 M of sucrose were added as cryoprotectants (Vicente and Viudes-de-Castro, 1996).

Once the production (volume × concentration) was known, sperm was diluted to obtain 120 million sperm/ml with the extender 1; only those males which produced more than 250 million sperm were used for the experiment. After dilution of the sperm to 120 million sperm/ml, 0.5 ml (or 60 million sperm) were taken to perform the inseminations with fresh sperm; it was diluted 1:1 with the extender 1 to have a concentration of 60 million sperm/ml. The rest of the sperm was diluted 1:1 with the freezing extender (extender 2), so the final concentration of the cryoprotectants was 1.75 M of DMSO and 0.05 M of sucrose. Sperm was packaged in 0.5 ml plastic straws (IMV, France) and they were sealed with modelling paste. The freezing protocol consisted of two phases: 45 min at 5°C followed by a stage in a freezer at −30°C for 30 min, and later the straws were plunged into liquid nitrogen (Fig. 1). To thaw the straws, a water bath at 50°C was used, and they were shacked during 10 or 12 s.

2.4. Experimental design

Only receptive females (red colour of vulvar lips) were inseminated. The females from the asynchrony group were induced to ovulate 8 h before insemination was performed, using a synthetic analogue of GnRH (1 μg of busereline acetate, Hoechst) injected intramuscularly. Females from the control group (without asynchrony) were induced to ovulate at the same time as insemination was performed. Thirty million total sperm/female were inseminated (0.5 ml of semen/doe), using a glass curved pipette. Two females were inseminated each day per male with fresh semen (one of them for the control and the other for the asynchrony group), and four does were inseminated per male each day with frozen sperm (two for the control and two for the asynchrony group).

Pregnancy was checked on 12th day of gestation by abdominal palpation; in those females that were pregnant a laparoscopy was performed. This technique was described by Santacreu et al. (1990). Ovulation rate (OR), normal implanted embryos (NIE), dead embryos (DE) and total implanted embryos (TIE, normal embryos + dead embryos) were noted. The
following ratios were calculated: normal implanted embryos compared with total implanted embryos (NIE/TIE), normal implanted embryos compared with ovulation rate (NIE/OR), total implanted embryos compared with ovulation rate (TIE/OR).

Females were allowed to kindle. After kindling, number of total and live-born were noted, and at weaning, number of males and females were noted.

2.5. Statistical analysis

A $\chi^2$-test (STATGRAPHICS PLUS, 1994–1999) was performed to study if there were differences between treatment and types of sperm in fertility and kindling rate.

When male and genetic origin of females were included in the model, no significant differences were obtained between them, so these factors were excluded from the statistical model. To study if there were differences between all the experimental groups (four in total), for all the parameters studied, a General Linear Model (GLM, SAS Institute, 1997) was performed, including the treatment group as fixed effect and the ovulation rate was included as a covariate for the analysis of normal implanted embryos, dead embryos and total implanted embryos.

For the analysis of total and live-born, a GLM (SAS Institute, 1997) was performed including the treatment group as fixed effect (4 levels).

3. Results

Results obtained for fresh and frozen sperm for the different treatments (with or without asynchrony between induction of ovulation and insemination) can be seen in Table 1. No significant differences were found between fresh and frozen sperm in any of the groups studied. However, for both types of sperm, significant differences were found in fertility and kindling rates between treatments (with or without asynchrony), obtaining better results when no asynchrony had been induced between ovulation and insemination (approximately 30% more fertility and kindling rates for fresh and frozen sperm when no asynchrony had been induced than when an asynchrony of 8 h had been provoked).

Results obtained after laparoscopy of females are summarised in Table 2. Significant differences were obtained between groups for total and normal implanted embryos, the fresh semen group with no asynchrony obtaining the best results. As can be seen, when an asynchrony of 8 h between ovulation and insemination had been induced, results for both

<table>
<thead>
<tr>
<th>Type of sperm</th>
<th>Percent fertility rate (number)</th>
<th>Percent kindling rate (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No asynchrony</td>
<td>Asynchrony (8 h)</td>
</tr>
<tr>
<td>Fresh</td>
<td>79 a (27/34)</td>
<td>50 b (22/44)</td>
</tr>
<tr>
<td>Frozen</td>
<td>61 a (39/64)</td>
<td>31 b (22/70)</td>
</tr>
</tbody>
</table>

Values in the same row with different letters are statistically different ($P < 0.01$).
Table 2
Results obtained after laparoscopy of females

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Number of females</th>
<th>Ovulation rate (L.S.M. ± S.E)</th>
<th>Total implanted embryos (L.S.M. ± S.E)</th>
<th>Normal implanted embryos (L.S.M. ± S.E)</th>
<th>Dead embryos (L.S.M. ± S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh semen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No asynchrony</td>
<td>24</td>
<td>12.2 ± 0.44 d</td>
<td>10.3 ± 0.57 a</td>
<td>10.2 ± 0.56 a</td>
<td>0.2 ± 0.08</td>
</tr>
<tr>
<td>Asynchrony after 8 h</td>
<td>17</td>
<td>11.1 ± 0.52 d,e</td>
<td>7.6 ± 0.66 b</td>
<td>7.5 ± 0.65 b</td>
<td>0.1 ± 0.09</td>
</tr>
<tr>
<td>Frozen semen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No asynchrony</td>
<td>35</td>
<td>10.6 ± 0.36 e</td>
<td>6.4 ± 0.47 b</td>
<td>6.4 ± 0.46 b</td>
<td>0.02 ± 0.07</td>
</tr>
<tr>
<td>Asynchrony after 8 h</td>
<td>22</td>
<td>11.2 ± 0.46 d,e</td>
<td>4.7 ± 0.58 c</td>
<td>4.6 ± 0.57 c</td>
<td>0.1 ± 0.08</td>
</tr>
</tbody>
</table>

Values in the same column with letters a, b, c are statistically different (P < 0.01) and with letters d, e are statistically different (P = 0.05). L.S.M.: least square means. S.E: standard error.

Fresh and frozen sperm were worse than when ovulation was induced at the same time as insemination was performed (2.8 and 1.7 less normal embryos for fresh and frozen sperm, respectively when an asynchrony had been induced). Numbers of dead embryos were not different between groups, which indicates that the lower number of embryos obtained when the asynchrony had been induced is due either to a problem in the fertilisation of the oocytes or to an early embryo mortality (before implantation is produced).

Results for the ratios obtained in the laparoscopies are summarised in Table 3. Less normal implanted embryos at day 12 of pregnancy were found when females had been inseminated with frozen sperm than when they had been inseminated with fresh semen; on the other hand, for both fresh and frozen sperm, when an asynchrony had been induced between ovulation and insemination, less normal implanted embryos compared with ovulation rate were observed.

Results obtained at kindling, are summarised in Table 4. It can be seen again that results obtained after insemination with fresh sperm were better than when insemination had been performed with frozen sperm. When females had been induced to ovulate 8 h before insemination was performed, results were worse for both fresh and frozen sperm, but the
Table 4
Results obtained at kindling

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Number of females</th>
<th>Total number born/kindling female</th>
<th>Number alive born/kindling female</th>
<th>Total number born/normal implanted embryos at day 12 (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh semen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No asynchrony</td>
<td>25</td>
<td>8.1 ± 0.55 a</td>
<td>7.8 ± 0.55 a</td>
<td>81</td>
</tr>
<tr>
<td>Asynchrony after 8 h</td>
<td>18</td>
<td>5.7 ± 0.65 b</td>
<td>5.4 ± 0.65 b</td>
<td>90</td>
</tr>
<tr>
<td>Frozen semen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No asynchrony</td>
<td>35</td>
<td>5.2 ± 0.47 b</td>
<td>4.8 ± 0.47 b</td>
<td>87</td>
</tr>
<tr>
<td>Asynchrony after 8 h</td>
<td>20</td>
<td>3.4 ± 0.62 c</td>
<td>3.3 ± 0.62 c</td>
<td>75</td>
</tr>
</tbody>
</table>

Values in the same column with different letters (a, b) are statistically different (P < 0.01).

<sup>a</sup> Results in this column are represented without an angular transformation of the data.

mortality of embryos between 12 day of pregnancy and the end of the pregnancy was not different between groups, so the differences between groups in number of total born were produced before the implantation period.

When the distribution of sex between the born rabbits was checked at weaning, no differences were obtained between percentage of males and females in any of the groups, the percentage of females at weaning being between 47 and 62% in all the groups.

4. Discussion

Better results were obtained for both fertility and kindling rate after insemination with fresh semen than with frozen sperm (79% versus 61% for fresh and frozen sperm, respectively, Table 1); this is in agreement with other authors (Stranzinger et al., 1971; Battaglini et al., 1988).

When an asynchrony was produced between ovulation and insemination, fertility and kindling rate decreased for both types of sperm (approximately 30% less fertility rate for fresh and frozen sperm, Table 1). Several asynchronies between ovulation induction and insemination have been tested, and reports between works have usually been contradictory. Working with fresh semen, similar results were observed in fertility rate for 0 and 8 h of asynchrony between ovulation and insemination (More O’Ferrall, 1973), 5 h (Parrish and Foote, 1986; Chen et al., 1989b) or 10 h of asynchrony (Chen et al., 1989a). However, in other works, worse results were observed for females with an asynchrony of 10 h (More O’Ferrall, 1973), and better results were observed by Stranzinger et al. (1971) after asynchronies of 5 h (15% more fertility rate) than when no asynchrony had been induced.

For inseminations with frozen sperm, beneficial effects after induction of asynchronies of 5 h were observed by several authors (15 and 25% more fertility rate observed by Stranzinger et al., and Parrish and Foote, 1986), but Chen et al. (1989b), did not observe differences between females induced to ovulate 5 h before or at the same time than insemination was performed (67% fertility rate in both cases). When asynchronies of 10 h have been tested, results obtained with frozen sperm were always lower than those reported for females induced to ovulate at the same time as insemination was performed (67 and 100% versus
50 and 42% fertility rate when no asynchrony and asynchrony of 10 h had been induced, Chen at al., 1989b,a).

With respect to the results observed on day 12 of pregnancy for females inseminated with fresh semen and with no asynchrony, 89% of the ovulated oocytes implanted. This result is in agreement with the observations made by other authors (11 and 14% of pre-implantation mortalities observed by Adams, 1960 and Santacreu et al., 1992, respectively). Post-implantation mortalities observed by several authors have been estimated between 18% and 22% (Adams, 1960 and Santacreu et al., 1992), which is similar to the results obtained in the present work for all the groups (between 10 and 25% loss of embryos after implantation, Table 3).

When comparing fresh and frozen sperm, more normal implanted embryos were obtained after insemination with fresh semen than with frozen sperm. Robson and Shaver (1979) used frozen rabbit sperm and an asynchrony of 5 h, and observed 55% of blastocysts related to the ovulation rate, which is similar to the result observed in this work in the group inseminated with frozen sperm without asynchrony (58%, Table 3), but they did not perform inseminations with no asynchrony to compare.

After the induction of an 8-h asynchrony between ovulation and insemination, not only fertility and kindling rate were compromised, but number of normal implanted embryos decreased also (2.7 and 1.7 less normal implanted embryos obtained for fresh and frozen sperm after induction of an asynchrony of 8 h, Table 2). Since dead embryos were similar in all the groups, it can be concluded that the difference in embryos between them was produced before the implantation period. With respect to the differences observed between fresh and frozen sperm in total and normal implanted embryos, Maurer et al. (1976) working with 5 h of asynchrony recovered less embryos at day 6 of pregnancy when inseminating with frozen sperm due to either lack of oocyte fertilisation or higher early embryonic mortality in this group. Defective sperm can initiate embryonic development, but only those oocytes fertilised with non-defective spermatozoa generally develop to the foetal stage; Robson and Shaver (1979) observed 15% of blastocysts at day 6th of pregnancy with chromosome abnormalities in females which had been inseminated 5 h after ovulation induction using frozen sperm, whereas only 6% of the blastocysts recovered from females inseminated with fresh semen presented chromosome abnormalities. In the present work, the worst results were obtained in the group of females with asynchrony and inseminated with frozen sperm and it was statistically significant (Tables 3 and 4). Mocé and Vicente (2002), working with sperm from males belonging to the same genetic origin and frozen by the same protocol, observed that when frozen sperm had been used to perform the inseminations (no asynchrony had been induced), the number of fertilised oocytes decreased when compared to the fresh sperm, so it seems that the decrease in the number of recovered embryos when working with frozen semen is due to a lack of fertilisation of oocytes. However, the problem of the decrease in normal implanted embryos for the asynchrony group (inseminated with fresh or frozen sperm) would probably be provoked by the fact that aged ova are not fertilised, as well as to anomalies in early stages of development and increased embryonic mortality.

When fresh rabbit semen is used, effects of asynchronies between ovulation induction and insemination are variable between works. Shaver and Carr (1967), working with mated females, observed that when no asynchrony had been induced, 82% of the ovulation rate were blastocysts (and 88% were normal embryos in the present work), and when an asyn-
chrony of 8 h had been induced, 74% were blastocysts (and 68% in this work, Table 3); they observed a decrease in embryo recovery when asynchronies were longer than 6 h, and the decrease was very pronounced after delays of 10 h or more. For asynchronies of 5 or 6 h between ovulation and insemination, some authors reported better results when an asynchrony had been induced (7.4 and 5.6 versus 5.6 and 5.0 total number born for asynchrony and no asynchrony, Stranzinger et al. (1971) and More O’Ferrall (1973), respectively), whereas Chen et al. (1989b) observed a decrease in the total number born, although they did not observe differences in fertility rate (5.4 versus 6.3 total number born when asynchrony and no asynchrony were used, respectively). Capacitation time for fresh rabbit semen has been estimated at 6 h (Dziuk, 1965) and ovulation in rabbits occurs 10 h after injection of LH (Harper, 1963), so the decrease in the number of normal embryos recovered after asynchronies between ovulation and insemination of more than 6 h could be due to the ageing of the oocytes (losing their ability to be fertilised) before the sperm had completed the capacitation process. In rabbits, the majority of the sperm which take part in the fertilisation process reach to the site of fertilisation during the peri-ovulation period, when insemination is performed with fresh semen and ovulation is induced at the same time as insemination (Overstreet and Cooper, 1978); however, when ovulation is induced 8 or 12 h before insemination, there is an important transport of spermatozoa to the caudal oviduct during the peri-ovulation period, but they remain in the isthmus and do not reach the ampulla, so they do not associate with the ova (Overstreet and Cooper, 1979). Working with fresh rabbit semen, Chang (1952) observed that oocytes could be fertilised up to 8 h after being ovulated, but their fertilisability decreased a lot after 4 h, so this could explain the worse results observed after an asymmetry of 8 h.

After using frozen rabbit sperm and asynchronies of 5 h, a decrease in litter size was observed in several works (4.5 and 4.7 versus 6.0 and 5.6 total number born when an asynchrony and no asynchrony had been induced, observed by Stranzinger et al., 1971 and Chen et al., 1989b, respectively). Murdoch and O’Shea (1973), observed less supplementary spermatozoa attached to the oocytes when frozen sperm (stored at $-79^\circ$C) had been used and ovulation had been induced at the same time as insemination was performed, and they suggested that frozen sperm could remain intact long enough to fertilise ova but then deteriorate rapidly so that contact of further sperm with the fertilised oocyte was reduced. Parrish and Foote (1986) observed that functional sperm transport was altered for frozen-thawed sperm if compared with cooled sperm, observing less sperm attached to the oocytes when frozen sperm rather than cooled sperm had been used; however, when ovulation had been induced 5 h before insemination, the same number of sperm attached to the oocytes was observed as when no asynchrony had been induced, which indicated that asynchronies did not improve or increase the sperm transport. Nevertheless, fertility rate improved after an asymmetry of 5 h, and it was assumed that this was due to the fact that frozen sperm is partially capacitated, so although less frozen sperm than cooled semen was present in the oviducts, they could fertilise at the same level as cooled semen did.

It is known that freezing provokes changes in the membranes of the sperm, and some authors have reported for frozen sperm from several species similar changes to those observed in capacitated spermatozoa (Watson, 1995; Pérez et al., 1996; Thundathil et al., 1999; Bailey et al., 2000). Garde et al. (1993) observed that frozen ram sperm could penetrate zona free hamster eggs in vitro immediately after thawing, without any preparatory incubation period,
meanwhile fresh sperm needed several h of incubation to attain the highest penetration rate. Asynchronies between ovulation induction and insemination are then justified when survival sperm is compromised and when sperm is partially capacitated, and this is the case when working with frozen semen. Robson and Shaver (1979) did not recover blastocysts when frozen sperm had been used to inseminate females with no asynchrony, and they concluded that frozen sperm were not capable of fertilising oocytes for much longer than the 6 h required for sperm capacitation, and they had to use 5 h of asynchrony. However, after induction of an asynchrony of 8 h, results were those expected when inseminating with frozen semen, perhaps due to the ageing of the oocytes before being fertilised, leading to a higher early embryonic mortality (between 15 and 20% less embryos had implanted in the females with an asynchrony of 8 h, Table 3).

Further studies are needed to introduce changes in the insemination technique (intravaginal inseminations or double inseminations) or in the freezing protocol in order to improve the results obtained after artificial insemination with frozen sperm in commercial farms, to make it possible to disseminate those males with high genetic value or to create sperm banks with semen from races or animals in danger of extinction.

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