



Review Article

Caprine Semen Cryopreservation and the Factors Affecting it: An Overview

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Abstract | Semen cryopreservation is a complex process that which involves balancing many factors in order to obtain satisfactory results. It causes ultrastructural, biochemical and functional damage to the spermatozoa leading to reduced motility, viability, impaired transport and fertility. Caprine sperms are extremely sensitive to cryopreservation compared to other species which is mainly attributed to the compositional variation of the sperm plasma membrane. Different factors affect affects the semen cryopreservation *viz.* species, breed, semen collection technique, collection season, extender composition, type of cryoprotectant used, cooling rate, equilibration time, freezing and thawing rate. All these factors should be given due credit for achieving better post-thaw post thaw quality of semen to be utilized in different advanced reproductive techniques.

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Introduction

Cryopreservation of cells and tissues has high importance in reproductive biotechnology for the preservation of gametes and embryos, implementation of gene banking and breeding programs of rare and endangered species but also for rare breeds of domestic animals. Sperm cryopreservation together with different assisted reproduction techniques (ART's) such as artificial insemination allows us to use the semen from valuable sires, the preservation of endangered species, to solve problems of male infertility and exchange of semen between subpopulations that may become geographically or biologically isolated (Watson and Holt, 2001; Andrabi and Maxwell, 2007). Smith and Polge (1950) did pioneering work in goat semen cryopreservation. Barker (1957) was

the first to suggest that fertility of frozen-thawed goat semen was too low to be of practical value. Since then, different extenders and freezing procedures have been described in different animal species, especially in bull (Martin et al., 2004), goat (Jimenez-Rabadan et al., 2013; Konyali et al., 2013; Sharma, 2018; Sharma et al., 2020) and ram (Aisen et al., 2002; Munyai, 2012) to minimize detrimental effects of cryopreservation on sperm motility, viability, morphology (Gravance et al., 1997), plasma membrane (Parks and Graham, 1992), acrosome (Celeghini et al., 2008) and DNA (Yildiz et al., 2007) integrity.

The cryopreservation of mammalian sperm is an intricate process that involves which involves balancing many factors in order to obtain satisfactory results. To ensure even minimal success, not only

proper diluent, sperm dilution rate, cooling rate and thawing rate are required, but also an intricate knowledge of the sperm physiology for the species is essential to maximize post-thaw recovery of sperm and consequently the fertility.

Sperm cryopreservation produces ultrastructural, biochemical and functional damage to the spermatozoa (Leboeuf et al., 2000) due to dramatic temperature changes, submission to osmotic and toxic stress derived from exposure to cryoprotectants and formation and dissolution of ice in the intracellular and extracellular environment (Medeiros et al., 2002). These effects would result finally in reduced motility, viability, impaired transport and fertility (Leboeuf et al., 2000).

Sperm preservation protocols differ between animal species, due to their inherent abilities to accommodate variations in semen extenders used in the cooling and freezing processes (Barbas and Mascarenhas, 2009). Bailey et al. (2000) proposed compositional differences of the sperm plasma membrane as reason for species variation of sperm cryosensitivity. Hiemstra et al. (2005) observed considerable differences in semen 'freezability' amongst breeds and between individual males of the same species (Hiemstra et al., 2005). In general, the spermatozoa of small ruminants are extremely sensitive to cryopreservation compared to other species (Purdy, 2006; Gangwar et al., 2016).

Semen cryopreservation is distinctly different from processing of cattle or buffalo semen and hence, remains a challenge because of the peculiarity observed in this species. The seminal plasma of goat semen contains Egg Yolk Coagulating enzymes, EYCE (phospholipase A2) and SBUIII from the secretions of the bulbourethral glands that interacts with the egg yolk and skimmed milk (one of essential and economical component of semen extender) thus producing toxic substances that affects sperm cells (Purdy, 2006; Gangwar et al., 2016) and reduces its freezing ability (Leboeuf et al., 2000; Purdy, 2006; Gangwar et al., 2016). It is presumed that EYCE and SBUIII is the same molecule (Leboeuf et al., 2000; Parkinson, 2009). The EYCE was identified as phospholipase A (Iritani and Nishikawa, 1963; Shipley et al., 2007) and SBUIII as a 55–60 kDa glycoprotein lipase from the goat bulbourethral gland BUSgp60 (Pellicer-Rubio et al., 1997). EYCE acts as a catalyst which hydrolyzes egg yolk lecithin into

fatty acids and lysolecithin (Iritani and Nishikawa, 1963; Shipley et al., 2007). This hydrolysis reaction causes the sperm membranes to be more fusogenic thereby inducing the acrosome reaction (Upreti et al., 1999; Purdy, 2006), and chromatin decondensation (Sawyer and Brown, 1995), both of which are toxic to the sperm. BUSgp60 lipase has structural homology to porcine pancreatic lipases (Carriere et al., 1994) and, similar to EYCE, BUSgp60 is responsible for hydrolysis of plasma membrane triglycerides and triglycerides in the skim milk that results result in fatty acid production (oleic acid) which is again toxic to sperm (Pellicer-Rubio et al., 1997; Pellicer-Rubio and Combarnous, 1998). Irrespective of mechanisms of action of either EYCE or BUSgp60, their activation in diluents is detrimental to the quality of the sperm cells during cryopreservation (Pellicer-Rubio et al., 1997; Pellicer-Rubio and Combarnous, 1998).

The conventional method of overcoming the harmful interactions of seminal plasma and egg yolk or milk proteins is to dilute the goat semen sample in a buffered diluent and then separate the seminal plasma from the sperm by centrifugation (Leboeuf et al., 2000; Purdy, 2006; Nuti, 2007; Tabarez et al., 2017; Narwade et al., 2017; Sharma, 2018; Sharma et al., 2018a, b; Sharma and Sood, 2019a, b). Nevertheless, another group of researchers observed that removal of seminal plasma may have a negative effect on sperm quality (Azeredo et al., 2001). Accordingly, there still exists a contradiction on whether to remove seminal plasma or not. Washing semen samples, however, is a time-consuming process that may damage cells if performed improperly but, if performed correctly, can be beneficial. Alternative diluents which minimize the sperm and lipase interactions have been proposed, including adding BUSgp60 lipase inhibitors, using lipid-free cow milk, a triglyceride-free diluent containing the milk protein casein, or using milk from species other than dairy cows where fatty acid and triacylglycerol structure differ so that the enzymatic reactions don't doesn't occur (Pellicer-Rubio and Combarnous, 1998; Purdy, 2006). Kundu et al. (2000, 2001, 2002) demonstrated the usage of egg yolk and milk-free media for goat semen cryopreservation.

Semen cryopreservation involves common steps to any protocol such as collection and extension of semen, addition of cryoprotectant, cooling above 0°C, cooling below 0°C, storage and thawing (Curry, 2007).

Cryopreservation extender and cryoprotectants

Extender: The main purpose of a cryopreservation extender is to supply the sperm cells with source of energy, protect the cells from cold shock and maintain a conducive environment for the spermatozoa to survive temporarily. In general, a goat sperm cryopreservation medium includes a non-penetrating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant (glycerol, ethylene glycol, or dimethyl sulfoxide DMSO), a buffer (Tris or Test), one or more sugars (glucose, lactose, raffinose, saccharose, dextran or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) (Evans and Maxwell, 1987; Sharma, 2018). A non-fat dried skim milk diluent (Corteel, 1974) or a Tris-glucose diluent (Salamon and Ritar, 1982; Beltran et al., 2013; Sharma, 2018; Sharma et al., 2020) are most commonly used for cryopreserving goat sperm. Various researchers suggested Tris-citric acid to be most satisfactory buffer for goat spermatozoa (Mishra et al., 2010; Sharma, 2018; Sharma et al., 2018a, b; Sharma and Sood, 2019a, b; Sharma et al., 2020). Recently, the coconut based extenders (Waidi et al., 2007; Mollineau et al., 2011), soybean based extenders (Roof et al., 2012; Salmani et al., 2013; Vidal et al., 2013; Chelucci et al., 2015; Narwade et al., 2017; Kakati et al., 2019), plant extract extenders (Zanganeh et al., 2013; Zaenuri et al., 2014) have also been used with good results.

Osmolality, pH and buffering systems:

Osmotic stress seems to be an important factor in cryopreservation. Goat spermatozoa survive cryopreservation and remain fertile in media composed of a wide range of constituents (Purdy, 2006). A variety of sugars, salts and buffers, could be added in different molar concentrations in the cryopreservation medium, without damaging the spermatozoa. In brief goat spermatozoa prefer hyperosmotic medium for cryopreservation. Salamon and Ritar (1982) observed that the optimal tonicity of a Tris-glucose-citric acid goat sperm medium to be 948 kPa (540 mOsm) whereas, Bowen et al. (1988) reported minimum damage to goat sperms with extender having osmolalities between 425 and 525 mOsm, in comparison to either 325 or 625 mOsm.

Significant changes in semen pH can result in cellular and sub-cellular damage, infertility, or sperm mortality. So, in order to sustain the viability and fertilizing ability of spermatozoa it is essential to maintain a

proper environment by controlling the pH alterations in the cryopreservation media (Purdy, 2006; Gangwar et al., 2016). Generally, buffering solution for goat sperm media should have a pKa of 7.0 (pH range of 6.0–8.0), be water-soluble, water soluble and membrane-impermeable, have minimal interactions with salts, be minimally affected by buffer concentration, temperature and ionic contents on the dissociation of the buffer, and be able to withstand enzymatic and non-enzymatic degradation (Graham et al., 1972). However, the pH of egg yolk or milk-based goat sperm diluents should normally be between 6.75 and 6.8 (Tuli and Holtz, 1992).

Different theories determining the action of the buffers have been proposed, but it is generally assumed that buffers aid in the cellular dehydration process by creating an osmotic environment thereby increasing the physical stability of the sperm cell plasma membrane and neutralizing acids generated during *in vitro* storage (Molinia et al., 1994). Tris seems to be the buffer of choice for use with buck sperm, but it is not the case in other species where negative effects of the buffer, such as increased capacitation and acrosome reaction rates and swelling of the apical ridge of the sperm cell have been reported (Molinia et al., 1996).

Sugars: Sugars play an important role in sperm respiration and provide osmotic balance and cryoprotection in diluents. The semen diluents contain a variety of sugars, with a wide range of osmolalities (6–375 mM) (Salamon and Ritar, 1982; Evans and Maxwell, 1987). The choice of sugar to be included in a diluent may be based on the functionality of the chemical. Fructose is a primary substrate for glycolysis in goat seminal plasma, so, it is logical to include this sugar in the medium (Pellicer-Rubio et al., 1997). Similarly, glucose is an excellent substrate in goat sperm metabolism and is essential for providing energy so that the sperm cells can function in a normal physiological manner (Corteel, 1974; Purdy, 2006; Gangwar et al., 2016).

Both fructose and glucose are low-molecular weight low-molecular weight sugars that can pass through the plasma membrane of a sperm. Various effects can be observed when sugars that are not capable of diffusing across a plasma membrane, such as lactose, sucrose, raffinose, trehalose or dextrans (Aboagla

and Terada, 2003; Kundu et al., 2002; Beltran et al., 2013; Narwade et al., 2017) are added to the diluent. These sugars create an osmotic pressure, inducing cellular dehydration and therefore, a lower incidence of intracellular ice formation. These sugars also interact with the plasma membrane phospholipids, reorganizing the membrane which results in sperm which is able to survive the cryopreservation process (Molinia et al., 1994; Aisen et al., 2002). Unlike the simple sugars glucose and fructose, these disaccharides act primarily as cryoprotectants. Trehalose has also been included in goat sperm cryopreservation extenders. It penetrates the plasma membrane of the spermatozoa and formed hydrogen bonds with the polar head groups of the phospholipids (Liu et al., 1998; Aboagla and Terada, 2003). The addition of trehalose into the membrane limits the amount of dehydration and consequently the physical damage due to cell volume changes associated with freezing and thawing (Liu et al., 1998). Trehalose also causes an increase in membrane fluidity due to protein and phospholipid reorganization, suppression of the injurious effects of the membrane lipid phase transition (Aboagla and Terada, 2003). In brief, cellular damage is minimized due to less intracellular ice formation leading to increased viability following cryopreservation (Aboagla and Terada, 2003; Yodmingkwan et al., 2016; Narwade et al., 2017).

Cryoprotectants: A cryoprotectant is included in a cryopreservation medium to minimize the physical and chemical stresses resulting from the cooling, freezing and thawing of sperm cells. Cryoprotectants are classified as either penetrating or non-penetrating. A penetrating cryoprotectant is membrane permeable and acts both intra as well as extracellularly. Penetrating cryoprotectants are solutes, which cause dehydration of spermatozoa due to the osmotically driven flow of water, which varies according to compound. After a short period of time, the cryoprotectant and water equilibrate and result in similar intracellular and extracellular concentrations (Amann, 1999; Sathe and Shipley, 2015). The penetrating cryoprotectants also cause membrane lipid and protein rearrangement, which results in increased membrane fluidity, greater dehydration at lower temperatures and therefore, an increased ability to survive cryopreservation (Holt, 2000). Additionally, penetrating cryoprotectants are solvents that dissolve sugars and salts in the cryopreservation medium (Purdy, 2006; Gangwar et al., 2016).

Many membrane-permeable cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol), and their combinations, have been tested with buck sperm by various researchers (Tuli and Holtz, 1994; Ritar et al., 1990; Singh et al., 1995; Kundu et al., 2000; Leboeuf et al., 2000; Purdy, 2006; Gangwar et al., 2016; Sharma, 2018; Sharma and Sood, 2019a), but the most frequently used penetrating cryoprotectant is glycerol. The addition of glycerol may be performed in a 1, 2 or 3-step technique at either 37 °C or 5 °C (Tuli and Holtz, 1994; Leboeuf et al., 2000; Beltran et al., 2013; Kulaksiz et al., 2013). Glycerol, dimethyl sulfoxide (DMSO) and ethylene glycol are generally used in a range of 1–8% per cent, but the greatest recovery of sperm post-thawing has been achieved with glycerol (Tuli and Holtz, 1994; Singh et al., 1995; Kundu et al., 2000; Kulaksiz et al., 2013; Narwade et al., 2017; Sharma, 2018; Sharma and Sood, 2019a; Sharma et al., 2020). Combinations of cryoprotectants, such as glycerol and DMSO, have also been tried and yielded synergistic positive results (Kundu et al., 2001).

Non-penetrating cryoprotectant cryoprotectants cannot cross the sperm plasma membrane and therefore, only acts extracellularly (Amann, 1999; Sathe and Shipley, 2015). Therefore, a non-penetrating cryoprotectant may modify the plasma membrane of a cell, or act as a solute and lower the freezing temperature of the medium (Amann, 1999). The most common non-penetrating cryoprotectants used are egg yolk (2–20%) (Tuli and Holtz, 1994; Beltran et al., 2013; Gangwar et al., 2016; Sharma, 2018; Sharma et al., 2018b) and non-fat skim milk (10%) (Purdy, 2006; Kulaksiz et al., 2013; Gangwar et al., 2016). Ferreiral *et al.* (2014) suggested higher egg yolk extender (10%) provide better viability to Sannen goat semen cryopreserved at –196 °C and seminal plasma removal does not improve the seminal parameters. Similar results were observed by Sharma et al. (2018a) in Gaddi goats and Priyadharsini et al. (2011) in Jakhrana goat semen. Whereas, Beltran et al. (2013) recommended Tris-Citric acid-Raffinose-Glycerol extender supplemented with 5% per cent egg yolk, 2.5% per cent goat serum or 30 mg BSA for the cryopreservation of Anglo-Nubian, Boer and Saanen buck semen on basis of sperm motility, percentage of intact acrosome and percent abnormalities at post-thawpost thaw.

Apart from above, certain membrane-impermeable membrane impermeable chemicals may be used as

cryoprotectants. [Kundu et al. \(2001\)](#) demonstrated that amino acids (l-proline, l-alanine, glycine or glutamine, 100–150 mM) could be used as cryoprotectants in goat sperm cryopreservation media with better post-thaw results. He also hypothesized the synergistic effect of combination of glycerol, DMSO and dextran (10–2000 kDa) during cryopreservation which is both intracellular and extracellular ([Kundu et al., 2002](#)).

Factors affecting semen cryopreservation

Various factors affecting the sperm cryopreservation *viz.* species and individuals, sperm collection method, collection season, extender composition, cooling rate, equilibration time, freezing rate and thawing rate ([Purdy, 2006](#); [Gangwar et al., 2016](#)).

Species and individuals: Knowledge of sperm biology for the species is essential to maximize post-thaw recovery of spermatozoa and consequently fertility ([Purdy, 2006](#)). Sperm cryopreservation protocols vary between species due to differences in cell shape and volume as well as organelles size and membrane lipid composition ([Holt, 2000](#); [Medeiros et al., 2002](#)). Boar semen is highly cryosensitive, whereas ram, bull, human, rabbit and rooster sperms are cryoresistant ([Holt, 2000](#)). In small ruminants, particularly goat, the specific differences with regards to sperm susceptibility to freezing methods, has been observed ([Barbas and Mascarenhas, 2009](#)). Also, inter-male or even inter-ejaculates variability has been recognized recognised as a source of variation during the cryopreservation procedure ([Soler et al., 2003](#); [Furstoss et al., 2010](#); [Medrano et al., 2010](#); [Ramon et al., 2013](#)). Hence, it is imperative to identify these species differences in spermatozoan sensitivity to cold shock and apply handling protocols to avoid cellular damage during cryopreservation.

Semen extension and concentration: It is essential for a semen sample to be extended properly so that there are sufficient number of sperms for insemination to obtain optimum fertility rates using the least number of inseminations and the lowest number of sperms per insemination ([Purdy, 2006](#); [Gangwar et al., 2016](#)). Historically, semen samples in farm animals have been extended by adding specific volumes of extender or by specific spermatozoa concentration. Extension rates of 1:1–1:23 (v/v, semen to extender) have been used successfully ([Evans and Maxwell, 1987](#); [Ritar et al., 1990](#); [Sharma, 2018](#)). However, a better method of extension is based on sperm concentration. Successful

freezing of sperms and reasonable fertility in goats have been achieved, with samples ranging from 80 to 600 x10⁶ cells/ml ([Mara et al., 2007](#); [Faigl et al., 2012](#); [Jimenez-Rabadan et al., 2013](#); [Gangwar et al., 2016](#); [Sharma, 2018](#); [Sharma et al., 2018a, b](#); [Sharma and Sood, 2019a, b](#); [Sharma et al., 2020](#)).

Equilibration: Equilibration is the total time during which, spermatozoa remain in contact with glycerol before freezing. However, equilibration process applies not only to glycerol, but also to the other osmotically active extender components. Therefore, the equilibration process can interact with the type of extender used (buffer and cryoprotectant) and could easily interact with other cryogenic procedures ([Vishwanath and Shannon, 2000](#); [Muino et al., 2007](#)). Different equilibration periods ranging from 1 to 5 h with variable post-thaw seminal quality have been used in bucks ([Sivaselvam et al., 2000](#); [Sundararaman and Edwin, 2005, 2008](#); [Narwade et al., 2017](#); [Sharma, 2018](#)). [Bittencourt \(2006\)](#) and [Ranjan et al. \(2015\)](#) observed better post-thaw semen quality with equilibration period of 4 hr. Contrarily, [Baruah et al. \(2003\)](#) did not observe any significant differences in sperm motility and acrosomal integrity in the semen samples equilibrated for 0.5, 1 and 1.5 h.

Freezing of semen: Diluted buck semen cooled to 4–5 °C over 1.5–4 h was then frozen either in pellets or straws ([Leboeuf et al., 2000](#); [Mollineau et al., 2011](#); [Gangwar et al., 2016](#); [Sharma, 2018](#)). Freezing of sperm in pellets is rapid and inexpensive, but inventory management is problematic, as the actual semen samples cannot be labelled. Once the semen sample is cooled, semen aliquots of 0.1–0.3 ml are dispensed into indentations on a block of dry ice (solid carbon dioxide, -79 °C) and frozen for 2–4 min. The pellets are then plunged into liquid nitrogen for storage ([Chemineau et al., 1991](#); [Mollineau et al., 2011](#)). The freezing of sperm in straws is more expensive and laborious than the pellet technique, but each sample can be labeled for accurate inventory management. After extending and cooling the semen samples, the sperms are loaded into 0.25 or 0.5 ml straws, placed on a rack, and frozen in liquid nitrogen vapor, the temperature of which varies by the height above the liquid nitrogen. A styrofoam box containing liquid nitrogen or a programmable freezer that controls the cooling rate had have been successfully used to cryopreserve the goat sperm ([Nutti, 2007](#); [Gangwar et al., 2016](#), [Sharma, 2018](#);

Sharma et al., 2020). When using a styrofoam box, the rack containing the samples is placed into the liquid nitrogen vapor at a height of 3–4 cm above the liquid nitrogen for 7–8 min and the straws are then plunged in liquid nitrogen for storage (Nutti, 2007; Gangwar et al., 2016; Sharma, 2018). Alternatively, Chemineau et al. (1991) reported straw size should determine the freezing height above the liquid nitrogen. He suggested that 0.5 ml straws should be frozen 4 cm above liquid nitrogen for 5 min, and then, plunged into the liquid nitrogen, while 0.25 ml straws should be placed 16 cm above the liquid nitrogen for 2 min, lowered to 4 cm for 3 min, and plunged into the liquid nitrogen for storage. However, researchers (Gravence et al., 1997; Leboeuf et al., 2000; Beltran et al., 2013; Kucuk et al., 2014; Yodminkwan et al., 2016; Narwade et al., 2017; Sharma, 2018) have used variable freeze height and time combinations (4–5 cm above liquid nitrogen for 5–7 min) with acceptable results. Programmable freezers are convenient for the freezing of large quantities of semen straws and for controlling the rate of freezing. These freezers may be used to replicate pellet freezing by placing the semen straws in a freezer at -80°C for 7–15 min and then plunging the semen straws into liquid nitrogen (Blash et al., 2000; Batista et al., 2009). Ritar et al. (1990) observed sperm frozen in pellets to yield superior motility following thawing (39%) compared to sperm frozen in straws. However, there was no variation in post-thaw motility (PTM) in terms of type of straw used (33% PTM, 0.25 ml; and 34% PTM, 0.5 ml straw). Differences in PTM, viability and fertility may be attributed to the different cooling rates produced by the pellet and straw techniques (Ritar, 1993).

Thawing of goat semen: The thawing of semen samples is determined by the method used to freeze the sperm. Sperm pellets of goat semen are thawed in a dry test tube at 37°C while the thawing of goat semen straws may be performed using various methods (Evans and Maxwell, 1987). Traditionally, a straw is thawed by placing it in a 37°C water bath for 12–30s (Deka and Rao, 1987; Sariozkan et al., 2010; Narwade et al., 2017; Sharma, 2018; Sharma et al., 2018a, b; Sharma and Sood, 2019a, b). This method is superior to a slow thawing technique (36.1 *versus* 18.9% motility), where the semen straw is placed in a 5°C water bath for 2 min (Deka and Rao, 1987). Increasing the thawing temperature to 70°C and thawing the semen straw for only 7s resulted in significantly higher progressive motility (36.9%) and

plasma membrane integrity (39.8%) compared to thawing straws at 37°C for 2 min (31.5%, 33.7%, progressive motility and plasma membrane integrity, respectively), or 40°C for 20s (32.4, 33.5%, progressive motility and plasma membrane integrity, respectively) ($P < 0.05$, Tuli et al., 1991). Similarly, Penitente-Filho et al. (2014) observed improved sperm membrane functional integrity ($P < 0.05$) by thawing the straws at 60°C for 7s. Ramchandran et al. (2015) used thawing rates at 40°C for 45s with good post thaw results (31.47%, progressive motility) in Jamunapari bucks.

Conclusion and Recommendations

Sperm cryopreservation is an intricate process leading to multidimensional damages to the sperm, interns reducing their fertilizing capacity. Species-specific variations in plasma membrane and other components occur in bucks posing huge problems with regards to cryopreservation. All the factors responsible for affecting the cryopreservation results should be given due importance. Keeping interbreed variations in mind, researchers should now focus on standardization of extender and its different components for a particular breed.

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Authors Contribution

All authors worked in collaboration for writing the review.

Conflict of interest

The authors have declared no conflict of interest.

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