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Various Thawing Methods of Frozen Bull Semen for Quality Parameters and Fertility of Spermatozoa: Current Situation and Future Perspectives

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Abstract | The current review aims to summarize recent advances of post-thaw quality characteristics of frozen bull spermatozoa associated with the different thawing protocols. We will also further discuss the elevance of these procedures to the critical mechanisms of how to minimize the adverse effects during thawing on bull spermatozoa, and further necessary investigations for definite conclusion. Although the conventional thawing methods at around body temperatures are currently recommended for artificial insemination (AI) in cattle husbandry, very slow thawing ways of spermatozoa seem to induce deleterious events during thawing and consequently trigger injury to plasma membrane and/or biological membrane of organelles. Rapid thawing at higher temperatures for shorter durations (RT) or rapid transient thawing (RTT) followed by subsequent stabilization (SS) at around physiological temperatures for a while can appear to overcome these disadvantages, thus improving not only numerous important quality parameters but also fertility due to shorter exposure to temperatures outside the physiological range, despite several contradictory results. Although no clear recommendation for the ideal thawing procedure of frozen bull semen straws can be made, it is quite clear that overall positive effects of RT or RTT followed by SS seem to be predominant than the currently conventional thawing protocols. Our current knowledge of the application possibility of various thawing protocols continues to change since we gain a greater understanding of the critical mechanism for less damage to the organelles and biological membranes in frozen-thawed bull spermatozoa during thawing. The novel insight into critical mechanisms during thawing under various procedures from this review provides a new strategy for enhancing AI effectiveness.

Keywords | Bull spermatozoa, Frozen semen, Thawing methods, Quality, Fertility

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INTRODUCTION

Thawing is an important process in cryopreservation responsible for returning sperm cells from the inactive frozen state to the physiological temperature by which reactivating metabolic processes necessary for normal physiological functions (Athurupana *et al.*, 2015a; Nguyen, 2023). It has been demonstrated that the freezing-thawing

processes could detrimentally alter the status of biological membranes and the expression of important proteins of spermatozoa (Rezaie *et al*., 2021), consequently affecting sperm quality (Chatterjee *et al*., 2001) and fertility (Hezavehei *et al*., 2021). In fact, many spermatozoa can die through the cryopreservation and subsequent significant reduction in post-thawed motility and/or viability (Celeghini *et al*., 2008; Defoin *et al*., 2008), although it provides several valuable advantages for the cattle industry from the technology of frozen-thawed spermatozoa (Khalil *et al*., 2018; Ugur *et al*., 2019; Sharafi *et al*., 2022). Viability and motility of bull spermatozoa are popular crucial factors for evaluation of successful artificial insemination (AI) and fertility (Pace *et al*., 1981; Lyashenko, 2015; Selçuk *et al*., 2020), since a significant correlation between these post-thawed parameters and fertility has been demonstrated (Correa *et al*., 1997; Larson-Cook *et al*., 2003; Rastegarnia *et al*., 2013; Athurupana *et al*., 2015a). In addition to the viability and motility, other important parameters, such as mitochondrial activity and oxidative stress are needed to use for evaluating the fertilizability of frozen-thawed spermatozoa (Nguyen *et al*., 2023a).

In recent decades, fertility issues have been identified as major problems in the cattle industry. Thus, current low conception and pregnancy rates of frozen-thawed spermatozoa following AI (López-Gatius, 2003; Royal *et al*., 2008) appear to be main concerns in cattle production and reproduction (Upadhyay *et al*., 2021). Recently, numerous investigations on various thawing methods with different temperatures and osmotic pressures have been conducted to determine the optimal thawing protocols and to maintain the potential fertility spermatozoa not only in bulls (more details in the following parts) but also in other species, such as buffaloes (Ahmad, 1984; Narasimha *et al*., 1986; Dhami *et al*., 1996; Rastegarnia *et al*., 2013; Rajbari *et al*., 2022), boars (Eriksson and Rodriguez-Martinez, 2000; Córdova-Izquierdo *et al*., 2006; Tomás *et al*., 2014; Athurupana *et al*., 2015a), stallions (Snoeck *et al*., 2012; Pugliesi *et al*., 2013), sheep (Paulenz *et al*., 2004; Nicolae *et al*., 2014), dogs (Peña and Linde-Forsberg, 2000; Kim *et al*., 2011) or cats (Chatdarong *et al*., 2010). Furthermore, it has been reported that appropriate thawing rates for frozen bull spermatozoa are dependent upon other numerous factors in the cryopreservation, including extenders, glycerol concentration, equilibration time, packaging method, and freezing rate (Rodriguez *et al*., 1975; Robbins *et al*., 1976; Gilbert and Almquist, 1978; Li *et al*., 2006). However, the definitive conclusion on the optimum thawing temperature and duration with frozen bull spermatozoa has not been obtained yet due to inconsistent results among the *in vitro* studies and limited assessment from *in vivo* fertility under practical field, as well as there is no comprehensively systematic overview of all existing findings on this issue.

Therefore, we have, in the present systematic review of all available literature, covered and comprehensively discussed the efficacy and application possibility of different thawing procedures on post-thawed quality and fertility of frozen bull spermatozoa. We will further provide insight into the critical mechanisms of how the ultrastructural, biochemical, and functional damages of frozen bull sperm would be suffered during thawing, which may be minimized and/or avoided to overcome currently low quality and fertilizability of frozen-thawed spermatozoa, as well as consequently, bull fertility.

The Freezing-Thawing Procedures for Spermatozoa

Cryopreservation is a widely used technique used to preserve live cells for long-time storage at very low temperatures in liquid nitrogen (Jang *et al*., 2017). This approach is effective and convenient since frozen semen can be transported over far distances, safely quarantined and as a useful genetic bank for desirable traits from good sires (Faezah *et al*., 2012). In bull spermatozoa, it is a complex process with many steps, including a reduction in temperature, dehydration, freezing in subzero temperatures and storage in liquid nitrogen for many years as frozen straws, by which stop all biological metabolism activities (Ugur *et al*., 2019; Upadhyay *et al*., 2021). The freeze-thawing process still induces injury to subcellular organelles associated with the integrity of spermatozoa due to the negative changes in the membrane structures and metabolism (Hammerstedt *et al*., 1990) even though sperm cells are thought to be less susceptible to cryodamage or oxidative stress due to low water content and high membrane fluidity (Ugur *et al*., 2019). Indeed, these alterations during the cryopreservation could inevitably cause ultra-structural, biochemical, and functional damages as well as deleteriously alter the status of plasma and biological membranes of spermatozoa, and consequently may reduce sperm motility and viability (Senger, 1980; Chatterjee *et al*., 2001). During freezing, spermatozoa are very sensitive to the deleterious events of the formation of ice crystals, hyper-osmolarity, volume alterations and protein denaturation (Jang *et al*., 2017). Therefore, the freezing process negatively affects the quality parameters of post-thawed bull spermatozoa such as all motility indicators (Januskauskas *et al*., 2003, 2005; Doležalová *et al*., 2015).

Artificial insemination (AI) using frozen-thawed spermatozoa from the cryopreservation is the popular technique in modern animal reproduction (Pagl *et al*., 2006) first introduced and applied in the 1950s (Vishwanath, 2003), especially in the dairy and beef production. Today, frozen-thawed semen is the majority for AI application in cattle production, about 95% (Chupin and Schuh, 1995; Koch *et al*., 2022), although it is collectively recognized

that post-thawed spermatozoa are more sensitive than fresh spermatozoa due to inevitable damages from the freezing and/or thawing processes (Selçuk *et al*., 2020). At present, bull frozen semen can be kept in liquid nitrogen at -196˚C in the most popular straws of either 0.25 or 0.5 mL (Diskin, 2018) before AI. It has been demonstrated that AI has much more advantages than the direct mating service by males (Lima *et al*., 2010; Lamb and Mercadante, 2016; Mohammed, 2018), contributing to the significant genetic progression with efficient reproduction and profitable improvement for decades in the cattle industry (Rodgers *et al*., 2012; Baruselli *et al*., 2017).

Recently, reproduction has been thought to be one of the most important components and plays a central role in the economic efficiency of cattle husbandry (Anchordoquy *et al*., 2017), besides milk or growth productivity (Trenkle and Willham, 1977). However, the current low conception and pregnancy rates with AI from frozen bull spermatozoa (López-Gatius, 2003; Royal *et al*., 2008) are still the main concerns in cattle production (Anchordoquy *et al*., 2017; Vartia *et al*., 2017). The concerns are originated from many factors and aspects (Kebede, 2018), such as sperm concentration, individual differences (Härtlová *et al*., 2013; Nguyen *et al*., 2023a), glycerol concentration, type of extenders and cryoprotectants (permeable and non-permeable ones) (Moore *et al*., 2006; Athurupana *et al*., 2015b; Städník *et al*., 2015), packaging system (Ansari *et al*., 2011; Diskin, 2018), the duration of equilibration (Andrabi, 2007), the cooling rate (Januskauskas *et al*., 1999), the freezing and thawing rates (Rodriguez *et al*., 1975; Robbins *et al*., 1976; Gilbert and Almquist, 1978; Li *et al*., 2006; Städník *et al*., 2015; Sharafi *et al*., 2022; Nguyen *et al*., 2023a). A large number of investigations have been conducted to determine the effects of these factors on the post-thawed quality parameters and fertility of bull spermatozoa, but there is still a lack of a comprehensive overview from all previous studies under various thawing methods to achieve the final conclusion for practical bull production.

In addition to the freezing process of fresh spermatozoa, thawing process for frozen spermatozoa is also crucial due to its remarkable effect on the post-thawed semen quality and bull fertility (Nur *et al*., 2003; Rastegarnia *et al*., 2013; Lyashenko, 2015; Nguyen *et al*., 2023a), consequently on the reproductive performance of cattle herds (Koch *et al*., 2022). Thawing process is widely applied to bring back the frozen spermatozoa under the inactive status to life and the active state for reactivating their metabolism at the physiological temperature range (Hammerstedt *et al*., 1990; Athurupana *et al*., 2015a). Of course, these changes inevitably lead to negative effects on sperm cells (Hammerstedt *et al*., 1990; Correa *et al*., 1996; Meyers, 2005; Lyashenko, 2015; Gürler *et al*., 2016) and harmfully change the status of plasma and biological membranes (Chatterjee *et al*., 2001) or expression of pivotal proteins (Rezaie *et al*., 2021) of spermatozoa, consequently may decrease sperm quality (such as motility and viability) (Chatterjee *et al*., 2001). Currently, although temperatures around body temperature range have been recommended as the conventional temperatures for thawing the frozen spermatozoa straws of bull semen at AI for decades (Correa *et al*., 1996; Faezah *et al*., 2012), slow thawing methods appear to trigger recrystallization of intracellular ice and rehydration, consequently leading to some injuries to sperm organelles (Hammerstedt *et al*., 1990; Sharafi *et al*., 2022). However, results from various thawing methods under different temperatures and osmotic pressures have indicated that thawing needs to be at maximum speed to minimize the possible damage (Diskin, 2018) even though optimal thawing rate not only depends on temperatures but also on glycerol concentration and equilibration exposure time (Robbins *et al*., 1976; Gilbert and Almquist, 1978; Pace *et al*., 1981). The effectiveness under various thawing procedures at critical mechanisms and field applicability on the multiple quality parameters and fertility of frozen bull spermatozoa will be discussed below in more detail.

Occurrence of Critical Events during Thawing Process

By using a digital recording thermometer to monitor temperature levels inside frozen semen straws every 2 seconds during thawing, it has been indicated that there are two different phases in the temperature change inside the straw before and after 0˚C (the melting point) (Athurupana *et al*., 2015a; Jameel, 2020; Nguyen *et al*., 2023a). Indeed, the temperature alteration has been very fast before the melting point (known as the solid phase), whereas it has been relatively slower after this point (the liquid phase). During thawing, the injurious temperature range for spermatozoa inside the frozen straws often occurs seriously, due to the high risk of the detrimental recrystallization of intracellular ice and rehydration, from -60 to 0˚C (Marshall, 1984; Gao and Critser, 2000). These temperature changes seem to make lipid peroxidation and subsequent membrane damage for the frozen-thawed semen, and negatively affect the survival and motility of spermatozoa (Nur *et al*., 2003; Kadirve *et al*., 2014). It has been demonstrated that lipid membrane peroxidation appears to trigger negative changes in membrane permeability and fluidity, causing irreversible loss of movement, viability, intracellular enzyme leakage and DNA damage of spermatozoa, consequently resulting in subsequent subfertility (defects in oocyte penetrability and oocyte binding capacity) (Sharma and Agarwal, 1996; Zamani *et al*., 2023; Nguyen *et al*., 2025). Furthermore, membrane protein denaturation may occur during the thawing procedure due to mechanical and osmotic stress, leading to disrupted membrane integrity and impaired quality of spermatozoa (Esin *et al*., 2022). In fact, protein denaturation occurs at a temperature range of -20 to 0˚C

during freezing/thawing, which can lead to protease leaks from liposomes in the acrosome and membrane integrity loss of spermatozoa (Öztürk *et al*., 2019). It has also been reported that intracellular ice crystals often appear and can grow to become recrystallized to cause damage to sperm plasma membrane and cell organelles under slow thawing methods (Holt, 2000; Panyaboriban *et al*., 2016). In contrast, rapid passage through the dangerous temperature zone under the optimal thawing speed can directly switch spermatozoa in the frozen semen straw from the glassy to liquid state and ice crystals also do not have enough time to be formed (Marshall, 1984; Lyashenko, 2015; Diskin, 2018) to maintain the membrane integrity, cytoplasm and potential fertility at the highest level possible due to less harmful effects of recrystallization and hydration (Esin *et al*., 2022). It is crucial to switch between these critical temperature intervals as soon as possible during thawing since the main disadvantage of longer duration at the slow thawing method are more osmotic pressure changes and membrane integrity loss (Curry and Watson, 1994) as well as more morphological defects (Esin *et al*., 2022).

Furthermore, low temperatures outside the physiological range, especially from the melting point to below 15°C, are critical to the frozen spermatozoa during thawing and are called the detrimental warm shock temperature zone (Bamba and Cran, 1988; Athurupana *et al*., 2015a). In fact, mammalian spermatozoa (Pursel *et al*., 1973) such as bulls (Nguyen, 2023; Nguyen *et al*., 2023a) and boars (Athurupana *et al*., 2015a) have been demonstrated to be susceptible to dangerous shock temperature ranges (<15°C) (Pursel *et al*., 1973). It has been indicated that the temperature change inside the frozen bull semen straws to 32°C by thawing at warm water (32-35°C) for 30 seconds further improves fertility than the final temperature inside the frozen straws of 0°C after thawing at warm water for only 12 seconds (Almquist *et al*., 1979).

Recently, several investigators have revealed that temperature change and warming rate inside the frozen straws are significant differences among different thawing methods. The temperature change and warming rates inside the frozen bull semen straws are significantly faster under thawing at higher temperatures for shorter durations compared with thawing at around physiological temperatures such as 37°C (Nguyen, 2023) and 39°C (Nguyen *et al*., 2023a) or thawing in the palm of hands of the AI technician (Jameel, 2020), similar to the previous finding in frozen boar semen (Athurupana *et al*., 2015a). It has also been reported that frozen spermatozoa thawed at faster speeds have shorter exposure to concentrated solutes and cryoprotectants (such as glycerol), as well as the recovery of intracellular and extracellular equilibrium occurs more rapidly at faster thawing speeds, as compared with slow thawing rates (Fiser *et al*., 1987; Holt, 2000; Salamon and Maxwell, 2000). Therefore, it is hypothesized that the thawing process must be carried out fast to pass through not only the critically harmful temperature range (-60 to 0°C) but also the detrimental warm shock temperature zone (<15°C) as soon as possible, and consequently minimize the damage to spermatozoa (Athurupana *et al*., 2015a; Lyashenko, 2015; Nguyen *et al*., 2023a). Terminology of rapid thawing appears to reduce the deleterious effects of processes of recrystallization and rehydration, thus preventing injury to the membranes and cytoplasm of spermatozoa.

Existing Potential Thawing Methods of Frozen Bull Semen Straws

Thawing technique of frozen bull semen straws for AI plays an important role in the post-thawed quality and potential fertility of spermatozoa, and thus on the reproductive efficiency of cattle industry (Nur *et al*., 2003; Lyashenko, 2015; Koch *et al*., 2022; Nguyen *et al*., 2023a). Until now, numerous different methods for thawing bull semen frozen in straws have been investigated to achieve the general recommendation, such as thawing in the water bath at various temperatures for different periods, in the shirt pocket, in the palms of the hands, in the cow, and etc. For last decades, although the warm-water bath at around body temperatures has been popularly applied for thawing the frozen bull semen on farms, the recommendation for thawing methods has been varied and clear data assessing their effects on the post-thawed conventional quality parameters are contradictory while other crucial quality parameters or the potential fertility under field studies have still been limited so far. Furthermore, the general recommendations from AI organizations vary across different countries (Lyashenko, 2015; Koch *et al*., 2022).

Although there has been a consensus among researchers from previous studies that thawing of frozen sperm should be at the optimal thawing rates for enabling spermatozoa to move faster through the serious critical temperature zones during thawing to minimize the loss of the initial post-thawed quality parameters, and maximum the longer vitality and subsequent fertility, the definitive conclusion of the thawing temperatures and durations for appropriate thawing speed has not been obtained yet and is still under discussion (Figure 1, Table 1 and Table 2). All aspects of the pros and cons of each thawing method will be discussed in more detail in the following parts to have the predominant potential protocols for the practical cattle industry or continue further in-depth investigations for a definitive conclusion.

Thawing in the palm of hand or a shirt pocket: For several decades, thawing the frozen bull semen straws in the palm of the hand of AI technician (termed as hand-thawing method, including just keeping frozen straws in the palm of the hand for a while or rolling straw between

the palms of the hands) or in a shirt pocket (pocket-thawing method) is still popular in practical application on the household cattle farms. Pocket-thawing method is defined as placing the frozen straw into folded paper towel and placing it into the shirt pocket of the AI technician or into a thermally protected pocket for 2-3 minutes before preparing the AI gun (Kaproth *et al*., 2005). These methods can minimize the risk of injurious thermal stress under routine field conditions and avoid the risk of inaccurate thawing temperatures. Moreover, other potential advantages under these thawing methods are the easy applicability, convenience and flexibility under the farm conditions (Kaproth *et al*., 2005). A previous survey conducted at local cattle farms in Afghanistan indicate about 56-68% of the AI technicians applied the hand thawing method for thawing the frozen bull straws (Jameel, 2020).

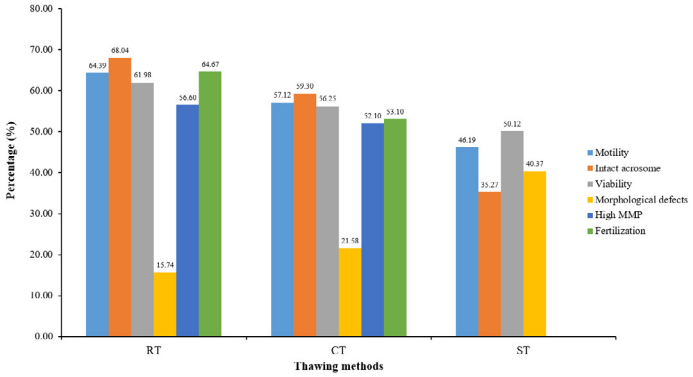

Figure 1: Comparison of acquired data under three popular thawing methods on frozen bull spermatozoa. This figure was analyzed by the meta-analysis of published data from previous papers (Robbins *et al*., 1976; Senger *et al*., 1976; Gilbert and Almquist, 1978; Sender, 1980; Pace *et al*., 1981; Barth and Bowman, 1988; Correa *et al*., 1996; DeJamette *et al*., 2000; Nur *et al*., 2003; Muino *et al*., 2008; Al-Badry, 2012; Faezah *et al*., 2012; Rastegarnia *et al*., 2013; Akmal *et al*., 2015; Lyashenko, 2015; Doležalová *et al*., 2017; Yilmaz *et al*., 2019; Esin *et al*., 2022; Nguyen *et al*., 2023a; Nguyen, 2023). Notes: **RT:** Rapid thawing at 60-70°C; **CT:** Conventional thawing at 35-39°C; **ST:** Slow thawing in iced water at 1-5°C; **MMP:** mitochondrial membrane potential.

However, post-thawed quality of bull semen thawed by rolling straws between the palms of the hands for 50 seconds is significantly lower than that of rapid thawing at 75°C for 7 seconds at 0, 1 and 2 hours of incubation at 38°C after thawing, but similar with that of thawing in iced water for 3 min (Ennen *et al*., 1976) since rolling sperm in straws at the glassy status probably make more injuries to spermatozoa. Furthermore, the post-thawed progressive motility of spermatozoa under thawing by the hand surface temperature is very low (just about 10%) and remarkedly lower than that of other thawing procedures (Chaiprasat *et al*., 2006). The post-thawed quality parameters (motility, viability and acrosome integrity) at 0 and 2 hours of frozen bull semen thawed in a shirt pocket are significantly lower than those of thawing in 35°C water (Barth and Bowman, 1988), while the probability of conception rate of frozen-thawed semen is not different between the pocket and warm-water thaw methods (Kaproth *et al*., 2005). In contrast, the motility of bull semen thawed by using hands is significantly lower than that of thawing at 37°C for 30 seconds (Goshme *et al*., 2021); while the pregnancy rate from frozen bull semen under the pocket thawing method is also significantly lower than that of rapid thawing (Rugg *et al*., 1977). Specialists and researchers in cattle reproduction and AI areas have recognized that it is typically not recommended to thaw the frozen bull semen straws in the pocket (Barth and Bowman, 1988; Looper, 2000; DuPonte, 2007) or in the hand (Ennen *et al*., 1976; Goshme *et al*., 2021), since the thawing speeds are too slow, then significantly decrease the number of viable spermatozoa, and seriously damage the fertility (Barth and Bowman, 1988; Looper, 2000; Krymowski, 2019).

Therefore, it has been concluded that although it is possible to achieve comparable conception rates when sperm dosage is abundant to maintain above threshold sperm numbers, slow thawing rates under these methods usually lead to reduction in sperm motility and viability, of course, are unacceptable in practice (Rugg *et al*., 1977; Barth and Bowman, 1988; DeJarnette *et al*., 2000; DeJarnette and Marshall, 2005; Goshme *et al*., 2021).

Thawing in iced water, low or ambient temperature water: At present, thawing in iced water (1-5°C), low or ambient temperature water is still not recommended to apply in the practical cattle farms (Barth and Bowman, 1988; DeJarnette *et al*., 2000; Al-Badry, 2012) due to the inappropriate thawing rates. Iced water or low-temperature water will not provide as satisfactory a thawing rate as desired, whereas ambient temperature water often varies significantly through the year, and of course, has a noticeable varied effect on the post-thawed sperm quality. Indeed, thawing at ambient temperature above 23°C may not affect the viability, motility and acrosomal change of post-thawed spermatozoa (Hayashi and Isobe, 2005) while the quality parameters are significantly reduced under thawing at less 20°C (Barth and Bowman, 1988; DeJarnette *et al*., 2000; DeJarnette and Marshall, 2005). It is important to note that more osmotic pressure changes are the main problem of slow thawing protocols (Curry and Watson, 1994; Nur *et al*., 2003). A majority of previous studies have indicated that thawing at low temperatures gives significantly lower percentages in post-thawed sperm quality parameters (Almquist and Wiggin, 1973; Pace *et al*., 1981; Barth and Bowman, 1988; Danasouri, 1988; Correa *et al*., 1996; Chaiprasat *et al*., 2006; Al-Badry, 2012) and fertility (Aamdal and Andersen, 1968; Rugg *et al*., 1977; Pace *et al*., 1981; Nur *et al*., 2006), rather than thawing at

Table 1: Summary of all previous existing investigations about potential thawing methods for frozen bull spermatozoa.

Kinds	General results	Authors and year
Temperatures	Better under rapid thawing at higher temperatures	Aamdal and Andersen, 1968; Almquist and Wiggin, 1973; Rodriguez *et al.*, 1975; Wiggin and Almquist, 1975; Ennen *et al.*, 1976; Robbins *et al.*, 1976; Gilbert and Almquist, 1978; Chandler *et al.*, 1984; Danasouri, 1988; Dhami *et al.*, 1992; Nur *et al* 2003; Muino *et al.*, 2008; Al-Badry, 2012; Lysahenko, 2015; Doležalová *et al.*, 2017; Jameel, 2020; Esin *et al.*, 2022; Nguyen *et al.*, 2023; Nguyen, 2023
	Better under conventional thawing at around body temperatures	Pace *et al.*, 1981; Barth and Bowman, 1988; Correa *et al.*, 1996; DeJarnette and Marshall, 2005; Chaiprasa *et al.*, 2006; Borah *et al.*, 2015
	No significantly overall difference among various thawing temperatures	Rugg *et al.*, 1977; Hayashi and Isobe, 2005; Faezah *et al.*, 2012; Akmal *et al.*, 2015; Yilmaz *et al.*, 2019
Other	Drying thawing device	Selçuk *et al.*, 2020
	In the palm of the hand	Ennen *et al.*, 1976; Chaiprasat *et al.*, 2006; Jameel, 2020
	In a shirt pocket	Rugg *et al.*, 1977; Barth and Bowman, 1988; Kaproth *et al.*, 2005
	Air-thawing method	DeJarnette and Marshall, 2005
	The thaw-in-the-cow method	Rugg *et al.*, 1977; Kaczyk, 2011; Koch *et al.*, 2022

Table 2: Summary of thawing methods and their effects on frozen bull spermatozoa.

Reference	Thawing methods			Findings							
	RT (60-70°C)	CT (35-39°C)	ST (1-5°C)	Motility	Viability	Acrosome integrity	Morphological defects	MMP	ROS	PLCZ1	Conception rate
Robbins *et al.* (1976)	X	X		Better in RT	-	Better in RT	-	-	-	-	-
Senger *et al.* (1976)		X	X	Better in CT	-	-	-	-	-	-	-
Gilbert and Almquist (1978)	X	X		-	-	Better in RT	-	-	-	-	-
Sender (1980)	X	X	X	-	-	Better in RT	-	-	-	-	-
Pace *et al.* (1981)		X	X	Better in CT	-	-	-	-	-	-	-
Barth and Bowman (1988)		X	X	Better in CT	-	Better in CT	-	-	-	-	-
Correa *et al.* (1996)		X	X	Better in CT	-	Better in CT	-	-	-	-	-
DeJamette *et al.* (2000)		X	X	Similar	-	Better in CT	-	-	-	-	-
Nur *et al.* (2003)	X	X		Better in RT	-	Better in RT	Better in RT	-	-	-	-
Muino *et al.* (2008)	X	X		Better in RT	Better in RT	Better in RT	-	-	-	-	-
Al-Badry (2012)	X	X	X	Better in RT	Better in RT	Better in RT	Better in RT	-	-	-	-
Faezah *et al.* (2012)	X	X		Similar	Similar	Similar	-	-	-	-	-
Rastegarnia *et al.*(2013)	X	X		Better in RT	Similar	Better in RT	-	-	-	-	-
Akmal *et al.* (2015)		X	X	-	-	-	-	-	-	-	Similar
Lyashenko (2015)	X	X		Better in RT	Better in RT	-	-	-	-	-	Better in RT
Doležalová *et al.* (2017)	X	X		Better in RT	-	-	-	-	-	-	-
Yilmaz *et al.* (2019)	X	X		Similar	-	Similar	Similar	-	-	-	-
Esin *et al.* (2022)	X	X		Similar	Better in RT	-	Better in RT	-	-	-	-
Nguyen *et al.* (2023)	X	X		Better in RT	Better in RT	Similar	-	Better in RT	Better in RT	Similar	-
Nguyet (2023)	X	X		Better in RT	Better in RT	Similar	-	Better in RT	Better in RT	Similar	-

Note: RT: Rapid thawing; **CT:** Conventional thawing; **ST:** Slow thawing in iced water; **MMP:** mitochondrial membrane potential; **ROS:** reactive oxygen species; **PLCZ1:** phospholipase C zeta1.

around body temperatures or high temperatures (Figure 1 and Table 2). Furthermore, thawing methods at 5 or 20°C have adverse effects on livability of spermatozoa until 2 or 4 hours of incubation at 37°C after thawing (Barth and Bowman, 1988; Al-Badry, 2012).

Therefore, these thawing conditions appear to be unsuitable, since they can cause more damage to the plasma membrane, acrosomal integrity and subcellular organelles and then decrease the viability and motility of frozen spermatozoa, as well as subsequent reduction in potential fertility (Barth and Bowman, 1988; Danasouri, 1988; DeJarnette *et al*., 2000; DeJarnette and Marshall, 2005). In this case, intracellular ice crystals can have adequate time to form, grow and become recrystallized due to too long movement period through the dangerous temperature zone under inappropriate thawing rates (Panyaboriban *et al*., 2016).

Thawing in the genital tract of female cows (the thaw-in-the-cow method): Recently, thawing technique of frozen semen in the reproductive tract of female cows, an abbreviation terminology of the thaw-in-the-cow method, appears in the literature for cattle reproduction (Koch *et al*., 2022). This technique means that frozen semen straw from the liquid nitrogen container is placed into the AI gun and then inserted directly into the cervix of cow for thawing and inseminating in the genital tract. It is considered as an effective and time-saving alternative for the previously traditional thawing procedures (Kaczyk, 2011).

However, the success of this method is also impacted by several potential factors from an uncontrollable big variation of ambient environmental temperatures and duration before insemination (Koch *et al*., 2022), so it has been concluded that frozen bull semen straws should not be thawed in the cow (Rugg *et al*., 1977; Looper, 2000). In fact, the success of AI from frozen bull semen thawed in the cow is not different from that of thawing in 38°C water (Kaczyk, 2011; Koch *et al*., 2022), although the pregnancy rate of cow from frozen bull semen thawed in the cow is significantly lower than that of thawing at 75°C for 7 seconds (Rugg *et al*., 1977). Therefore, despite no clear definitive conclusion from the previous investigations, the thaw-in-the-cow method seems to be unacceptable in the practical conditions for cattle industry.

Thawing in warm water at around body temperatures: For long decades, thawing of the frozen bull semen straws in warm water at around the physiological temperature range for at least 30 seconds has been recommended by most commercial AI breeding organizations. In fact, there have been large variations in around body temperatures applied popularly infield practice or experiments, such as 32-35°C (Almquist *et al*., 1979; DeJarnette *et al*., 2000; DeJarnette and Marshall, 2005; Kaproth *et al*., 2005), 35°C (Senger *et al*., 1976; Gilbert and Almquist, 1978; Barth and Bowman, 1988; Hayashi and Isobe, 2005), 35-37°C (DuPonte, 2007; Borah *et al*., 2015; Krymowski, 2019), 37°C (Pace *et al*., 1981; Chaiprasat *et al*., 2006; Al-Badry, 2012; Faezah *et al*., 2012; Yilmaz *et al*., 2019; Goshme *et al*., 2021), or 38-40°C (Doležalová *et al*., 2017; Koch *et al*., 2022; Zenteno *et al*., 2023). Currently, the recommendations for thawing in AI breeding centers are still varied among different countries, such as the predominance in US from 35-38°C for at minimum 30 seconds and the popularity in Germany from 37.5-38.5°C for 10-12 seconds (Koch *et al*., 2022), or in Ukraine at 35°C for 20 seconds (Lyashenko, 2015). Recently, it has been demonstrated that the post-thawed quality parameters of frozen bull semen are not different among various thawing protocols at around body temperatures, such as between 35°C and 37°C (Borah *et al*., 2015), a range of 36-40°C (Gaillard and Kupferschmied, 1982), between 37 and 39°C (Nguyen, 2023), or 37°C and 40°C (Faezah *et al*., 2012), while significant differences are still found among at 36, 38 and 40°C for 30 seconds (Zenteno *et al*., 2023), or at 35, 37 and 40°C for 30-40 seconds (Goshme *et al*., 2021).

In this case, the temperature range around the body temperatures has been popularly applied to thaw the frozen bull semen in the field conditions due to the easy application, manipulation on the farms and good protection of spermatozoa from excessive harmful heating (Berndtson *et al*., 1976; Gilbert and Almquist, 1978; Pace *et al*., 1981; Faezah *et al*., 2012; Yilmaz *et al*., 2019). The findings from numerous studies have indicated that the quality parameters (motility, viability and acrosomal integrity) and potential fertility of frozen bull semen are significantly superior under conventional thawing at around physiological temperatures (35-39°C) as compared to thawing in ice water, low-temperature water, ambient temperature, the palm of the hand, or the shirt pocket or the reproductive tract of cows (Pace *et al*., 1981; Barth and Bowman, 1988; Danasouri, 1988; DeJarnette and Marshall, 2005; Chaiprasat *et al*., 2006; Faezah *et al*., 2012) (Figure 1 and Table 2) whereas no significant differences are found among various thawing temperatures, such as between at 37-40°C and 60°C (Faezah *et al*., 2012), or 37°C and 23°C (local ambient temperature) (Hayashi and Isobe, 2005). In contrast, not only the sperm motility (Almquist and Wiggin, 1973; Doležalová *et al*., 2017) but also the viability and acrosomal retention (Aamdal and Andersen, 1968; Gilbert and Almquist, 1978) of frozen bull semen are significantly lower under conventional thawing protocols in warm water at 35-38.5°C as compared with other thawing conditions. Intriguingly, thawing at 35-37°C for 30-60 seconds makes lower percentage of acrosome changes and extracellular aspartate aminotransferase release although lower sperm motility and no significant difference in viability compared to rapid thawing at 75°C for 9 seconds (Borah *et al*., 2015).

Furthermore, the post-thawed sperm quality and fertility in the field are significantly superior under thawing at 37˚C than those of very low thawing temperatures (Pace *et al*., 1981), while conception rate is not different between thawing in 38˚C water and in the cow (Koch *et al*., 2022). Livability, motility and abnormality of bull spermatozoa immediately after thawing are significantly better under thawing at 37˚C than those of very slow thawing rates at 5˚C and are similar to those of rapid thawing at 60˚C, however, after 4 hours of incubation at 37˚C, the livability of sperm under thawing at 37˚C is considerably lower than that of rapid thawing but still markedly higher than that of low thawing temperatures (Al-Badry, 2012). It has also been demonstrated that the quality sperm characteristics are more stable and less decline during the incubation until 2 hours at 38˚C after thawing (Doležalová *et al*., 2017).

Moreover, the thawing time is also a crucial factor during thawing. It has been reported that the quality parameters (motility, viability and intact acrosome at 0, 2 and 4 hours after thawing) and fertility of frozen bull semen can significantly improve by longer exposure (30-40 seconds) in warm water from 32-37˚C rather shorter thawing intervals (9-20 seconds) (Almquist *et al*., 1979, 1982; Al-Badry, 2012), while conception rate of cows is not different between 11 and 35 seconds in the same temperature water at 38˚C (Koch *et al*., 2022). Besides, the temperature inside the frozen bull straws just reaches to around 0˚C in 12 seconds or to 32˚C in 30 seconds when frozen semen is thawed at 32-35˚C (Almquist *et al*., 1979, 1982), while the estimated average time for temperature inside the straw to reach from -196 to 15˚C needs at least 13.0-14.6 in the warm water at 37-39˚C (Nguyen, 2023; Nguyen *et al*., 2023a). The frozen-thawed spermatozoa inside the straws are very susceptible to temperatures outside the physiological range, including the critical dangerous temperature zone (<0˚C) and the low shock temperature (<15˚C) (Athurupana *et al*., 2015a; Nguyen *et al*., 2023a). Therefore, the thawing time must be enough for spermatozoa inside the frozen semen to reach to the safe temperature range with normal physiological activities and functions (Senger, 1980; Correa *et al*., 1996; Rastegarnia *et al*., 2013; Selçuk *et al*., 2020). Overall, it tends to indicate that when specific recommendations are not given, or detailed information of the diluent type, extender type, cooling rate and freezing procedures are not taken in account (Barth and Bowman, 1988; DeJarnette *et al*., 2000; Firk *et al*., 2002; DeJarnette and Marshall, 2005; López-Gatius, 2012), thawing the bull semen frozen in straws in the warm water around body temperatures from 33-39˚C for at least 30 seconds is a sufficient thawing rate for attaining the stable post-thawed sperm quality and fertility under farm conditions.

However, it has been demonstrated that slow thawing methods under suboptimal thawing rates seem to promote recrystallization and rehydration, or occurrence of more osmotic pressure changes, consequently induce more severe latent and harmful damages to the organelles in frozen spermatozoa (Hammerstedt *et al*., 1990; Sharafi *et al*., 2022). In contrast, it is believed that thawing rates must be as maximum as possible. Although the application of such higher temperatures is a limiting factor and far from being a practical procedure for thawing the frozen bull straws at the farm fields, it appears to minimize the degree of cellular injury by the shorter exposure to the dangerous temperatures from potential ice crystal formation and osmotic damage (Aamdal and Andersen, 1968; Senger, 1980; Athurupana *et al*., 2015a; Lyashenko, 2015; Nguyen *et al*., 2023a).

Rapid thawing at high temperatures:

Due to the severe deleterious temperature ranges during thawing, especially in traditional slow thawing procedures (Mazur, 1984; Curry and Watson, 1994; Holt, 2000), it is thought that thawing rates should be maximum to prevent the osmotic damage to spermatozoa (Vishwanath and Shannon, 2000; Esin *et al*., 2022). Average temperature changes and warming rates inside the straws are significantly faster when frozen semen is thawed at rapid thawing procedures, so the average time it took for the temperature inside the frozen semen straw to warm up to 15˚C is approximately twice as faster under high-temperature thawing (60-80˚C) as compared with conventional thawing at 37-40˚C (Athurupana *et al*., 2015a; Nguyen, 2023; Nguyen *et al*., 2023a) or with thawing in the palm of the hand (Jameel, 2020).

Rapid thawing of frozen bull spermatozoa by exposure to higher temperatures for shorter duration appears to be effective in reducing the dangerous effects of recrystallization and rehydration, thus resulting in minimizing and/or avoiding the severe latent damages to sperm membrane and cytoplasm (Danasouri, 1988; Watson, 1995; Lyashenko, 2015; Diskin, 2018). Indeed, the fast movement through the critical detrimental temperature range and warm shock zone during thawing is a crucial potential mechanism to prevent the formation of dangerously intracellular ice crystals due to insufficient time (Marshall, 1984; Lyashenko, 2015; Diskin, 2018). Frozen spermatozoa thawed at faster thawing rates also have shorter intervals to the concentrated solute and cryoprotectants, thus the recovery of intracellular and extracellular equilibrium seems to be better than slow thawing (Fiser *et al*., 1987; Holt, 2000; Salamon and Maxwell, 2000).

A majority of the existing studies have proven that the post-thawed quality parameters (motility, viability and acrosome integrity) of bull spermatozoa increase as the thawing rates increase, especially at 60-80˚C (Aamdal and Andersen, 1968; Almquist and Wiggin, 1973; Rodriguez *et*

al., 1975; Wiggin and Almquist, 1975; Robbins *et al.*, 1976; Ennen *et al.*, 1976; Gilbert and Almquist, 1978; Danasouri, 1988; Dhami *et al.*, 1992; Nur *et al.*, 2003; Al-Badry, 2012; Lyashenko, 2015; Doležalová *et al.*, 2017; Esin *et al.*, 2022) (Table 1), although the limited overall improvements in the post-thawed sperm quality and fertility under rapid thawing procedures are still reported in other ones (Rugg *et al.*, 1977; Faezah *et al.*, 2012; Borah *et al.*, 2015; Yilmaz *et al.*, 2019). These inconsistent findings across recent studies could be due to different interacting factors associated with thawing rate (Doležalová *et al.*, 2017; Koch *et al.*, 2022), including cows (breed, individuality, age) or semen processing (extenders, cryoprotectants, glycerol concentrations, concentration, packaging, equilibration time, freezing rate) (Robbins *et al.*, 1976; Moore *et al.*, 2006; Beran *et al.*, 2011; Doležalová *et al.*, 2016; Koch *et al.*, 2022).

Similarly, better quality parameters are also observed under rapid thawing procedures in other species such as buffaloes (Ahmad, 1984; Dhami *et al.*, 1996; Rastegarnia *et al.*, 2013), pigs (Hernández *et al.*, 2007; Tomás *et al.*, 2014; Athurupana *et al.*, 2015a), horses (Snoeck *et al.*, 2012; Pugliesi *et al.*, 2013), sheep (Paulenz *et al.*, 2004; Nema *et al.*, 2009), cats (Chatdarong *et al.*, 2010) or dogs (Peña and Linde-Forsberg, 2000; Kim *et al.*, 2011). Interestingly, a higher thawing rate at 75°C for 9 seconds results in higher percentages of sperm motility, acrosome changes and extracellular aspartate aminotransferase release, but similar proportion of sperm survival as compared to thawing at 35 or 37°C (Borah *et al.*, 2015), whereas not only motility but also viability, injured acrosome and morphological defects can be noticeably improved under rapid thawing at 70°C for 5 seconds rather than at 37 or 50°C (Nur *et al.*, 2003).

Percentages of viability, membrane integrity, head defect, tail defect, total abnormal morphology, normal chromatin condensation and chromatin decondensation of spermatozoa are also significantly improved after thawing at 70°C for 6 seconds rather than at 37°C for 30 seconds (Esin *et al.*, 2022). In addition to these conventional quality parameters, mitochondrial health of frozen bull semen such as mitochondrial membrane potential (MMP) and intracellular reactive oxygen species (ROS) could be further improved under rapid thawing rather than traditional thawing at around body temperatures (Jameel, 2020; Nguyen, 2023; Nguyen *et al.*, 2023a), which is line with previous one in other species (Athurupana *et al.*, 2015a). As data in Table 3 demonstrated that rapid thawing method may induce less oxidative stress associated with ROS production and less damage of biological membrane related to mitochondrial health in spermatozoa, and consequently higher motility and viability of post-thaw spermatozoa (Nguyen, 2023; Nguyen *et al.*, 2023a). It has been demonstrated that not only more osmotic pressure changes and membrane integrity loss (Curry and Watson, 1994) but also higher levels of oxidative stress in spermatozoa after slow freezing-thawing process (Tran *et al.*, 2018) due to more adverse effects during thawing (Esin *et al.*, 2022; Nguyen, 2023; Nguyen *et al.*, 2023a). Therefore, better improvement in MMP and ROS of spermatozoa after the rapid thawing method (Table 3) probably comes from less oxidative stress during thawing, since a strong correlation of MMP with ROS production and oxidative stress (Kadirvel *et al.*, 2009; Jing *et al.*, 2023; Nguyen *et al.*, 2023b, 2025; Funahashi *et al.*, 2024).

Table 3: Mitochondrial health and oxidative stress of post-thaw bull spermatozoa among different thawing protocols.

Thawing temperature and period	Percentage of spermatozoa after thawing with:			
	High MMP	High ROS level	Motility	Viability
37°C for 46 second and then 39°C for 14 second	44.5 ± 1.2[b]	44.3 ± 2.7[b]	45.1 ± 1.6[b]	61.8 ± 0.5[b]
39°C for 60 second	46.7 ± 0.9[b]	44.0 ± 2.8[b]	45.5 ± 1.6[b]	62.6 ± 1.0[b]
70°C for 8 second and then 39°C for 52 second	51.9 ± 1.8[a]	35.0 ± 2.7[a]	52.5 ± 1.5[a]	67.0 ± 1.7[a]

Note: Different superscripts indicate a significant difference ($p<0.05$) in the parameter within the same column. **MMP:** mitochondrial membrane potential; **ROS:** reactive oxygen species. Data adapted from Nguyen (2023).

Furthermore, rapid thawing at higher temperatures (60-70°C) for shorter durations can lead to better improvement not only in initial post-thawed sperm quality parameters, but also in longer vitality of spermatozoa until 4-5 hours or even longer after thawing as compared to slower thawing procedures (Wiggin and Almquist, 1975; Muiño *et al.*, 2008; Al-Badry, 2012; Lyashenko, 2015; Nguyen *et al.*, 2023a) (Table 2). Interestingly, sperm motility and dynamics of frozen bull semen thawed at higher temperatures are significantly better than slower thawing at physiological temperatures of 38.5°C, but these significant differences are disappeared after 2 hours of incubation at 38°C (Doležalová *et al.*, 2017). In contrast, another study has revealed that no differences in post-thawed quality parameters are observed at just after thawing among three different thawing temperatures, but significantly better quality is found after 2 hours of incubation at 37°C under high thawing rates (Muiño *et al.*, 2008). The motility of post-thawed bull sperm is significantly higher under rapid thawing while the incidence of acrosomal changes and extracellular release of aspartate aminotransferase are significantly lower under thawing in warm water at 35-37°C (Borah *et al.*, 2015). On the contrary, no significant differences in the post-thaw bull sperm motility and membrane integrity, including the plasma and acrosomal membranes at 0 hour after thawing

are found between thawing in 35°C water and rapid thawing temperature at 50 or 70°C, but these quality parameters are significantly better after 2 hours of incubation at 37°C in fast thawing procedures (Muiño *et al*., 2008). Therefore, these temperatures can be conveniently applied as optimal thawing temperatures for bull semen frozen in straws even under field conditions for maximum post-thawed quality characteristics and successful conception rate, while temperature over 80°C is not recommended for routine use due to its difficult control of overheating, especially for technicians AI technicians (Dhami *et al*., 1992).

Findings from one newest study with AI under various thawing procedures have demonstrated that thawing at 65-70°C for 6-7 seconds is the optimum thawing procedure, since motility parameters and viability at 0, 1, 3 and 5 hours of incubation at 38°C after thawing are significantly improved, as well as the fertilization rate of cows after AI is also increased by 11.6% compared to conventional thawing at 35°C for 20 seconds (Lyashenko, 2015), which is coincident with a farm study published a long time ago (Aamdal and Andersen, 1968). In fact, the post-thawed quality parameters and potential fertility of frozen semen are largely affected by not only the thawing process but also other potentially dependent important variables, such as individuality, semen processing and sperm concentration (Robbins *et al*., 1976; Beran *et al*., 2011; Doležalová *et al*., 2016). Recently, results from a comprehensive experimental design with two variables have indicated that rapid thawing provides significant differences in numerous important quality parameters of frozen bull spermatozoa rather than conventional thawing condition although they are also significantly different among bulls (Nguyen *et al*., 2023a). Besides, other potential factors such as diluent type, extender, cryoprotectant, glycerol concentration, semen packing, cooling rate and programmable freezing, are demonstrated to variably interact with thawing protocols for final effectiveness (Rodriguez *et al*., 1975; Robbins *et al*., 1976; Gilbert and Almquist, 1978; Li *et al*., 2006). Different kinds of straw (0.25 or 0.5mL) also have different vulnerabilities to the thawing temperatures due to the different surface-to-volume ratio (Diskin, 2018). In general, the various and inconsistent results from different studies can be explained by these accompanied factors (Rastegarnia *et al*., 2013; Doležalová *et al*., 2017).

It is universally acknowledged that the major problem of slow thawing protocols comes from formation of dangerous recrystallization and occurrence of more osmotic pressure changes and subsequent injury to subcellular organelles, but too high thawing rates can lead to unequal rates of egress of the cryoprotective agents (such as glycerol) from and influx of water into sperm cells (Hammerstedt *et al*., 1990; Curry and Watson, 1994; Sharafi *et al*., 2022). Although rapid thawing seems to be a good protocol for overcoming the disadvantages under previously traditional thawing procedures, it should be borne in mind that exposure time of frozen straws to such high temperatures is very crucial since it is very little room for error unlike the lower thawing conditions. Indeed, if frozen straws are left in the high water for longer intervals, it may negatively lead to pH fluctuation and protein denaturation as temperatures inside the straws over 41°C, consequently induce sperm death and reproductive failure (Senger, 1980; Mortimer, 2000). However, few farm studies have been demonstrated that the conception rate of cows following AI under rapid thawing at 65-70°C for 6-7 seconds or even 75°C is better and considered as the optimal thawing temperature and exposure for the practical conditions (Aamdal and Andersen, 1968; Lyashenko, 2015), even although several researchers think these techniques seem to be not suitable on the routine farm practice due to its danger of overheating (Robbins *et al*., 1976; Al-Badry, 2012; Faezah *et al*., 2012). Probably, it can overcome the concerns of the overheating under high thawing temperatures by carefulness or high skill from AI technicians during thawing. Based on the existing data, however, it needs to further evaluate the comprehensive effectiveness of rapid thawing at higher temperatures (60-70°C for 5-8 seconds) on the practical conditions with the large scale of animal population to achieve the definitive conclusion about optimal thawing procedure for bull semen frozen in straws. Of course, a small water bath to control the temperature during thawing and a timer to manage the thawing duration, which are convenient and portable for on-farm use, should be prepared to avoid the risk of overheating.

Rapid transient temperature followed by subsequent stabilization: Recently, it has been mentioned that the subsequent stabilization at around physiological temperatures for a while immediately after the rapid transient thawing at higher temperatures for shorter intervals could be an ideal condition to support the recovery of sperm functions, such as MMP, ROS production and penetrability (Athurupana *et al*., 2015a; Nguyen, 2023). In fact, a subsequent stabilization at 39°C following the rapid transient thawing at 60-70°C for 7-8 seconds can result in better mitochondrial health besides the conventional parameters at just after thawing or until several hours (Jameel, 2020; Nguyen, 2023; Nguyen *et al*., 2023a) although no considerable differences in acrosomal integrity and distribution of phospholipase C zeta1 (PLCZ1) are found among various thawing procedures (Nguyen, 2023; Nguyen *et al*., 2023a). There have been many studies indicating that faster thawing at 60-80°C does maintain better membrane and acrosome integrity, which contains many key proteins significantly associated with the function and fertility of spermatozoa (Rodriguez *et al*., 1975; Nur *et al*., 2003; Hernández *et al*., 2007; Esin *et al*., 2022). PLCZ1 is one of the PLC family members (Cooney *et al*., 2010;

Torra-Massana *et al*., 2019), universally recognized as a strong potential candidate responsible for resumption of the meiosis of oocytes, through inducing intracellular free calcium ($[Ca^{2+}]_i$) oscillations at fertilization (Cooney *et al*., 2010; Bedford-Guaus *et al*., 2011; Gonzalez-Castro *et al*., 2019). It has been demonstrated that cryopreservation appears to affect the presence and distribution of PLCZ1 significantly (Heytens *et al*., 2009; Kashir *et al*., 2011; Moreau *et al*., 2019) and consequently the fertility in humans (Heytens *et al*., 2009; Kashir *et al*., 2011; Dai *et al*., 2019; Moreau *et al*., 2019; Wang *et al*., 2020). However, the thawing rates seem to have no considerable effect on PLCZ1 (Nguyen, 2023; Nguyen *et al*., 2023a).

Similarly, rapid thawing at 60-80°C just significantly improves the viability, motility and acrosome integrity of frozen boar spermatozoa without a difference in MMP, while a subsequent stabilization at 39°C after rapid thawing can remarkedly improve the high MMP and penetrability besides these parameters as compared to the conventional thawing at 39°C (Athurupana *et al*., 2015a). Therefore, it seems that greater thawing rates could enable spermatozoa inside the straw to move faster through critically dangerous zones during thawing, while immediately subsequent stabilization process may give optimal chances to restore sperm functions after thawing, thus taking together leading noticeable improvements in multiple post-thawed quality characteristics. However, it also needs to reconfirm the efficacy of the combined method of rapid transient thawing at higher temperatures and subsequent stabilization for a while in the practical studies to have a complete evaluation of AI results using frozen-thawed bull semen.

Other thawing methods: Recently, it has been found that a dry thawing system is a newly developed device, an effective alternative and has some potential advantages compared to the warm-water thawing bath (Selçuk *et al*., 2020). Thawing by a dry device seems to be more practical than in a water bath due to less risk of mixing sperm with water or less difficulty of stable water temperature control in cold ambient weather. The dry thawing system also appears to be more convenient due to its portability and easy use at anywhere in the farms as well as no necessity for wiping water outside of frozen-thawed semen straws. In contrast, the main disadvantage of the drying thawing device may come from the high cost of initial investment and maintenance of the heating tools during use. However, no significant differences in post-thawed sperm motility and viability are found between thawing in a warm-water bath and dry thawing system although some sperm dynamics (such as straightness and amplitude of lateral head displacement), acrosomal defects and total abnormal rates are remarkably better under the drying device (Selçuk *et al*., 2020). Similarly, there was a unclear improved trend in some parameters (such as total motility, progressive motility, straightness, linearity, wobbling) of buffalo spermatozoa after thawing in watery system, as compared to the dry thawing system (Akal *et al*., 2021). Therefore, it seems to conclude that thawing in a drying device is probably an effective alternative to the warm-water bath and useful for cattle farms with frozen-thawed bull semen (Selçuk *et al*., 2020; Akal *et al*., 2021). However, there is still a limitation with the results from the *in vivo* evaluation for the complete conclusion of conception rates in cattle because of no farmed-animal studies so far, so it needs further *in vivo* studies to evaluate the true efficiency of this method under farm conditions.

On the other hand, an air-thawing method has been tried for frozen bull semen straws as a convenient alternative as compared to the warm-water thawing bath (DeJarnette and Marshall, 2005; Kaproth *et al*., 2005). Although the air-thawing technique is initially thought to be a potential and convenient alternative for the warm-water thawing bath, a previous study has indicated that not only lesser post-thawed sperm quality parameters (such as motility or viability) but also the reduced conception rate of cows are associated with the air-thawing method, rather than the warm-water thawing bath (DeJarnette and Marshall, 2005). Therefore, this method seems to be unsuitable for the practice of cattle husbandry.

Bull Fertility Associated with Various Thawing Methods

Bull fertility is a critically important factor in cattle husbandry and a great crucial economic value in the dairy and beef industry (Trenkle and Willham, 1977; Abdollahi-Arpanahi *et al*., 2017; Anchordoquy *et al*., 2017). It has been demonstrated that good quality parameters, such as viability, motility and acrosome integrity, of frozen-thawed bull semen are much important factors for high conception and pregnancy rates following AI, since sperm quality characteristics are found to have significant correlations with bull fertility (Correa *et al*., 1997; Dogan *et al*., 2015; Sellem *et al*., 2015; Kumaresan *et al*., 2017). There has been a consensus about the significant effects of different thawing methods on the post-thawed sperm quality parameters and subsequent fertilization ability (Nur *et al*., 2003; Rastegarnia *et al*., 2013; Lyashenko, 2015; Nguyen *et al*., 2023a).

However, until now, there has not been a final conclusion on optimum thawing rate and duration due to the inconsistent results of thawing methods on post-thawed semen quality and limited evaluations on the farm conditions even though it is universally acknowledged that thawing of frozen bull semen should be at maximum speed. The current opposite findings can come from numerous potential factors (Doležalová *et al*., 2017; Koch *et al*., 2022), such as from cows (breed, individuality, age) or from the semen processing (extenders, cryoprotectants, glycerol con-

centrations, sperm concentration, packaging, equilibration time, freezing rate, and interactions between equilibration time and freezing rate) (Robbins *et al*., 1976; Moore *et al*., 2006; Beran *et al*., 2011; Doležalová *et al*., 2016; Koch *et al*., 2022). Results from a majority of *in vitro* studies have been demonstrated that thawing at higher temperatures for shorter durations can overcome a low quality of post-thaw spermatozoa including not only the motility, dynamics and viability but also MMP, ROS production or acrosome integrity (Rodriguez *et al*., 1975; Senger, 1980; Chandler *et al*., 1984; Dhami *et al*., 1992; Nur *et al*., 2003; Al-Badry, 2012; Lyashenko, 2015; Nguyen *et al*., 2023a), due to the remarkable decrease in morphological, biological and functional damages, rather other current thawing protocols (Table 2).

Furthermore, longer vitality of the frozen-thawed bull spermatozoa can also achieved under rapid thawing (Muiño *et al*., 2008; Al-Badry, 2012; Lyashenko, 2015; Nguyen *et al*., 2023a). In fact, damage of the plasma and biological membranes or organelles of spermatozoa has been demonstrated to easily occur during slow thawing (Panyaboriban *et al*., 2016). In contrast, there have still been reports that rapid thawing could not significantly improve the quality of frozen bull semen (Rugg *et al*., 1977; Faezah *et al*., 2012; Yilmaz *et al*., 2019). Besides, only few field studies of the success rate following AI has been reported that bull fertility can be higher under rapid thawing (Aamdal and Andersen, 1968; Lyashenko, 2015).

Interestingly, the findings in Figure 1 by the meta-analysis from published data in all scientific papers associated with three popular thawing methods on frozen bull spermatozoa indicated that rapid thawing at 60-70°C seems to be the most effective method due to better not only the *in intro* quality parameters but also the *in vivo* fertilization, as compared with conventional thawing at 35-39°C or slow thawing in iced water at 1-5°C. In particular, the percentages of frozen bull spermatozoa after thawing with motility, intact acrosome, viability, high MMP and also fertilization were higher under the rapid thawing at 60-70°C rather than the conventional thawing at 35-39°C or slow thawing in iced water at 1-5°C, while there was an opposite trend in the level of morphological defects (Figure 1).

In general, all existing data on thawing procedures indicate that the effectiveness of rapid thawing at higher temperatures for shorter durations or a combination of rapid transient thawing followed by subsequent stabilization are such predominant than the other thawing methods, resulting in better initial post-thawed quality parameters and longer vitality until several hours in numerous laboratory investigations or even higher fertility rate in only few farm studies. In the last 2 decades, especially, frozen bull spermatozoa thawed at 60-70°C for 5-8 seconds (Nur *et al*., 2003; Muiño *et al*., 2008; Al-Badry, 2012; Lyashenko, 2015; Jameel, 2020), or at 70°C for 8 seconds followed by subsequent stabilization at 39°C for 52 seconds (Nguyen, 2023; Nguyen *et al*., 2023a), seems to be the optimal thawing temperatures and exposures for higher quality and fertilizability of frozen bull spermatozoa, probably due to less susceptible to critical dangerous zone and osmotic shock during thawing, similar with results in frozen semen of other mammalian species (Snoeck *et al*., 2012; Rastegarnia *et al*., 2013; Tomás *et al*., 2014; Athurupana *et al*., 2015a).

On the other hand, it has been demonstrated that the freezing-thawing process could alter the expression of crucial proteins of spermatozoa (Rezaie *et al*., 2021), in addition to the conventional quality parameters, consequently affect sperm fertility (Hezavehei *et al*., 2021). In fact, it could induce the substantial alteration of proteomes associated with key structural and functional roles for sperm fertilizability (Westfalewicz *et al*., 2015; Pini *et al*., 2018; Perez-Patiño *et al*., 2019; Peris-Frau *et al*., 2019) at any stages of cryopreservation (Bogle *et al*., 2016). Especially, membrane lipids of frozen spermatozoa are damaged, leading to instability of the membrane and large reduction of multiple proteins during the freezing-thawing process (Hezavehei *et al*., 2021). Only one recent study, however, provides the data of protein expression, PLCZ1, under different thawing protocols (Nguyen *et al*., 2023a), so it is needed to evaluate the effectiveness of different thawing protocols on the expression and activity of numerous important proteins associated with semen quality and sperm fertilizability.

Therefore, not only the frozen bull semen quality but also the subsequent fertility of spermatozoa can further improve under the optimal thawing techniques with appropriate thawing temperature and duration.

CONCLUSIONS AND RECOMMENDATIONS

The review summarizes major advances made in several previous decades and unresolved questions about the efficacy and application possibility of different thawing procedures on post-thawed quality and fertility of frozen bull spermatozoa. This review also further provides insight into the critical mechanisms of how the ultrastructural, biochemical and functional damages of frozen bull sperm would be suffered during thawing, which may be minimized and/or avoided to overcome the low quality and fertilizability of frozen spermatozoa and consequently conception rate following AI.

Based on the existing data from multiple previous studies, although no clear recommendation for the ideal thawing procedure of the frozen bull semen straws can be made,

rapid thawing at higher temperatures for shorter exposure or a combination of rapid transient thawing followed by subsequent stabilization at around physiological temperatures appear to be further improvements of numerous important post-thawed sperm quality parameters and bull fertility due to shorter exposure to temperatures outside the physiological range. However, there is still the limitation of their clear effectiveness under *in vivo* studies in the field, so further investigations of the effects of thawing protocols (rapid thawing at 60-70°C for 5-8 seconds or a combined method of this rapid transient thawing followed by subsequent stabilization at 39°C for 52-55 seconds; Figure 2) on the success rate of AI with conception and pregnancy rates under the practical conditions for the comprehensively definitive conclusion. It is also needed to evaluate the expression of important proteins under *in vitro* conditions and their related mechanisms for better semen quality and sperm fertilizability in next studies.

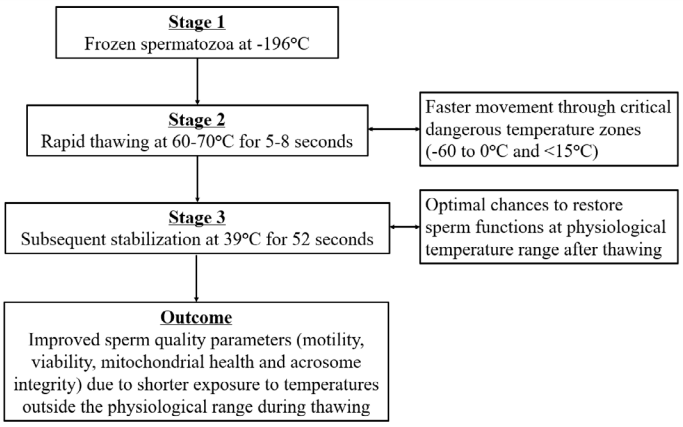

Figure 2: Schematic diagram of thawing process.

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NOVELTY STATEMENT

Systematic review under comprehensive discussion within all aspects and mechanisms associated with of how to minimize the adverse effects during thawing on bull spermatozoa.

AUTHOR'S CONTRIBUTIONS

Loan Hong Thuy Vu Nguyen and Hai Thanh Nguyen: responsible for the concept, manuscript preparation and edition.

Anh Ngoc Thi Dang, Thuong Thi Nguyen and Phong Ngoc Van: contributed to improvement and brushing up on the quality of this review.

All authors have read and approved the finally completed version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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