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Role of Caspase 3, Caspase 8 and Caspase 9 in Male Infertility and Sperm Cryoinjury: A Review

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ABSTRACT

Apoptosis is an integral part of sperm development, quality control and differentiation. Caspases are proteolytic enzymes that play a key role in apoptosis. The aim of this review article is to analyze the role of caspases 3, 8 and 9 in male infertility and sperm cryopreservation. The review also discusses the effect of cryopreservation on the activation of caspases in sperm, leading to apoptosis and decreased sperm quality. Finally, the article explores the use of caspase inhibitors as a strategy to improve sperm cryopreservation protocols and preserve sperm quality. Caspase 3 is the dominant effector caspase, while caspase 8 and 9 are involved in the extrinsic and intrinsic apoptotic pathways, respectively. Increased activity of these caspases has been associated with various sperm quality issues, such as teratozoospermia, asthenozoospermia, oligozoospermia and reduced cryostability. The review emphasizes the importance of understanding the role of caspases in sperm apoptosis and their potential as diagnostic markers and therapeutic targets for male infertility and sperm cryopreservation.

Key words: Apoptosis, Caspases, Male infertility, Sperm cryoconservation.

Despite the fact that the biological processes of spermatogenesis and sperm function have been well studied in mammals, research in the field of male reproductive health does not lose its relevance every year. These studies have not only a fundamental basis that complements the set of knowledge about the sperm biology, but also an important practical and social significance. The search for new biochemical markers of male fertility disorders can make a significant contribution to understanding the molecular foundations of male infertility. Study of sperm apoptosis can help in regulating the number and quality of germ cells (Asadi *et al.,* 2021; Said *et al.,* 2004; Zalata *et al.,* 2016). In addition, it is important to consider the role of these studies to improve cryopreservation protocols, since this technology has been widely used not only to increase human reproductive capabilities (Said *et al.,* 2010), but also is of great importance for preserving the genetic material of valuable animal breeds (Atroshchenko *et al.,* 2019; Contreras *et al.,* 2023; Upadhyay *et al.,* 2021). Undoubtedly, the reproductive potential of the animals need to be exploited to its maximum to achieve optimum production in a herd (Ahanger *et al.,* 2024; Chandra *et al.,* 2011; Madkar *et al.,* 2022). We also see particular interest in the search for biochemical markers of seminal plasma that can predict the outcome of cryopreservation (Atroschenko *et al.,* 2022, Shitikova *et al.,* 2023, Shitikova *et al*., 2024), while participants in apoptotic events are ideal candidates for this role.

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Apoptosis in spermatozoa

Apoptosis is an integral part of sperm development, quality control and differentiation (Asadi, 2021). In the postnatal period, it is responsible for the death and phagocytosis of premeiotic germ cells in order to bring their number in line with the number of Sertoli cells, as well as for the removal of damaged and abnormal germ cells during active spermatogenesis in the post-puberty period. Genetic and exogenous damages affecting spermatogenesis enhance the endogenous mechanism of germ cell apoptosis and can lead to insufficient or absent sperm production (Murphy *et al.,* 2014). It has been shown that germ cell apoptosis increases after testicular damage, such as exposure to toxic substances, varicocele, testicular torsion, hormonal deprivation and genetic abnormalities, as well as the freeze-thaw reaction (Almeida *et al.,* 2005). Cryopreservation of spermatozoa causes lipid peroxidation, free radical production, formation of intracellular and extracellular ice crystals, dehydration, membrane damage, mitochondrial dysfunction, DNA fragmentation, cytoskeletal disorders which, in turn, can trigger a cascade of apoptotic events in

humans (Said, 2010), bulls (Upadhyay *et al.,* 2021), stallions (Atroshchenko *et al.,* 2019; Contreras *et al.,* 2023). Cryopreservation causes death of spermatozoa using both the external and internal pathways of apoptosis (Savitskaya and Onishchenko, 2016).

Caspases

An important role in the process of apoptosis is assigned to cysteine proteases - caspases, calpains and cathepsins. Cathepsins act in the acidic environment of lysosomes and the acrosome of spermatozoa (Moreno and Alvarado, 2006) and perform many different biological functions, which has fueled the interest of researchers in it for many years (Fomina and Kudlaeva, 2016; Fomina *et al.,* 2017). While cathepsins may serve as potential indicators of cryostability in seminal plasma (Shitikova *et al.,* 2023), our current review will concentrate specifically on caspases.

Caspases (cysteine-dependent aspartate specific protease) are proteolytic enzymes that cleave proteins at the locations of the amino acid aspartate (Shalini *et al.,* 2015). These are divided into initiatory, inflammatory and effector (executive) ones. All caspases are initially synthesized as inactive precursors (procaspases) and are activated as needed by initiator caspases due to cleavage of a small portion of the molecule. Initiatory caspases, in turn, are activated in a more complex way - by special protein complexes (apoptosomes, PIDD-osomes, DISC) (Man *et al.,* 2017; Van Opdenbosch and Lamkanfi 2019).

To date, it has been established that caspases 2, 8, 9 and 10 are initiatory, 3, 6 and 7 are effector, 1, 4, 5, 11, 12, 13 are inflammatory (Kesavardhana *et al.,* 2020). When an apoptotic stimulus appears, caspase initiators get activated, triggering the biochemical cascade of apoptosis. Caspase initiator 8 plays the most important role in the extrinsic pathway of apoptosis and activated caspase-9 plays a key role in the intrinsic pathway of apoptosis, cleaves underlying caspases such as caspase-3, -6 and -7, initiating a cascade of caspases. Activation of caspase-3 marks the point of no return in the process of apoptosis and is responsible for the cleavage of key proteins, leading to the final destruction of cells (Chowdhury *et al.,* 2008). Considering the above facts study of caspases 3, 8 and 9 is extremely essential.

Caspase 3

Caspase 3 (CASP3, CPP32/Yama/apopain) is a proteolytic enzyme consisting of 277 amino acid residues, with a molecular weight of 31.608 kDa. Like all caspases, CASP3 is synthesized as an inactive proenzyme, which is proteolytically processed in conservative asparagine residues to form two subunits, large and small, which dimerize to form an active enzyme. The dimerized enzyme cleaves and activates caspases 6 and 7; and the enzyme itself is modified and activated by caspases 8, 9 and 10 (Wyllie, 1997; Crowley and Waterhouse, 2016).

The active center of the enzyme contains a cysteine residue (Cys-163) and a histidine residue (His-121), which stabilize the cleavage of the peptide bond in the protein molecule at the C-terminus at the location of aspartic acid when it is part of a specific 4-amino acid sequence, recognizing the tetrapeptide motif Asp-x-x-Asp (Agniswamy *et al.,* 2007).

Caspase 3 is active in a wide pH range, which is slightly higher (more basic) than many other caspase effectors. This wide range indicates that caspase 3 can be fully active under normal and apoptotic conditions (Stennicke and Salvesen, 1997).

Caspase 3 is activated in the apoptotic cell by both extrinsic and intrinsic pathways (Ghavami *et al.,* 2009). A distinctive feature of the caspase 3 zymogen is the need for strict regulation, because if it is not regulated, excessive caspase activity leads to total cell death (both healthy and pathological cells) (Boatright and Salvesen, 2003). As a caspase effector, the caspase-3 zymogen has practically no activity until it is cleaved by initiator caspase after transmission of a special apoptotic signal (Walters *et al.,* 2009). External activation triggers a caspase cascade characteristic of the apoptotic pathway, in which caspase 3 plays a dominant role. During the internal activation of caspase, cytochrome C molecules from mitochondria work in combination with caspase 9, apoptosis-activating factor 1 (APAF1) and ATP necessary for the enzyme. The development of oxidative distress and mitochondria damage is considered one of the main reasons for the release of cytochrome C from mitochondria (Li *et al.,* 2004; Shalini *et al.,* 2015).

One of the ways to inhibit CASP3 is to use the IAP family of proteins (apoptosis inhibitors), which includes c-IAP1, c-IAP2, XIAP and ML-IAP (Lavrik *et al.,* 2005; Poreba *et al.,* 2013).

Caspase 3 in sperm

It has been established that caspases-3, 8, 9 are present in both sperm and seminal plasma. The level of caspase-3 and caspase-8 in human seminal plasma is significantly higher than in sperm, so it makes sense to investigate the activity of caspases both in the spermatozoa themselves and in the seminal plasma. *In vitro* sperm processing methods (centrifugal force during mechanical processing, magnetic field strength during MAC separation, cooling during deep hypothermia, centrifugation in a density gradient) have a certain effect on spermatozoa, therefore it is difficult to determine whether caspase is activated before or after ejaculation or simultaneously. Provided that caspase is present in the seminal plasma, the effect due to *in vitro* treatment can be ignored and the state of sperm apoptosis before ejaculation can be reflected directly. Caspases isolated from spermatozoa may reflect apoptosis after ejaculation (Wei *et al.,* 2015).

Almeida *et al.* (2005) found that the activity of caspase-3 of sperm cells in humans is apparently associated with teratozoospermia and asthenozoospermia. This is due to the fact that nuclear, mitochondrial and cytoskeletal abnormalities cause activation of caspase-3 during

spermiogenesis or maturation of spermatozoa (Almeida *et al.,* 2005). Xu *et al.* (2020) found that miR-210-3p (microRNA) induces apoptosis of spermatogenic cells, contributing to the activation of caspase-3. The activity of caspase-3 was detected in sperm midpiece region and it was shown that it is largely associated with low sperm motility (Weng *et al.,* 2002) or with reduced normal sperm concentration, motility and morphology (Wang *et al.,* 2003). Ejaculate with a large number of caspases positive spermatozoa negatively correlates with the rate of fertilization after *in vitro* fertilization (Marchetti *et al.,* 2004). It has been experimentally established that thermal exposure to the scrotum, especially for a long time, increases the activity of caspase 3 (Zhang *et al.,* 2015). Increased activity of caspase-3 was found in Sertoli cells of patients with Sertoli cell-only syndrome suggesting that apoptosis might be an active mechanism in this syndrome. Hypospermatogenesis and oligozoospermia are also associated with an increase in caspase 3 activity (Almeida *et al.,* 2013).

Pichardo *et al.* (2010) revealed that the swim-up procedure in sheep sperm significantly reduces the percentage of spermatozoa expressing caspases 3 and 7, which is accompanied by an increase in chromatin abnormalities in samples with low motility. They identified that active caspases were located in the implantation fossa region.

Cryopreservation activates the activity of caspases in the sperm of stallions (Ortega-Ferrusola *et al.,* 2008). Caspase-3-positive spermatozoa are immature spermatozoa that appear in the ejaculate. Moreover, higher the proportion of such spermatozoa in the stallion's ejaculate, poorer the cryostability of the samples. Conversely, the lower the activity of caspase 3 and the higher the thiol content, the more cryoprotective the sperm are (Muñoz *et al.,* 2016). Da Silva *et al.* (2023) proposed to use a multiparametric assessment of stallion sperm using flow cytometry, including the detection of 4-HNE, caspase 3 and 7 activity, live/dead spermatozoa and for these purposes it is proposed to investigate live, dead, caspase 3, ROS, mitochondrial membrane potential in stallion semen (Becerro-Rey *et al.,* 2024).

Protein kinase B maintains the integrity of the membranes of ejaculated stallion spermatozoa by inhibiting caspases 3 and 7, preventing the development of apoptosis (Bolaños *et al.,* 2014). At the same time, incubation with rosiglitazone (a drug that preserves the glycolytic activity of spermatozoa) supports the phosphorylation of protein kinase B and reduces the activation of caspase 3, also improves the functioning of the mitochondria of stallion sperm after cryopreservation (Ortiz-Rodriguez *et al.,* 2019); Caselles *et al.* (2014) demonstrated for the first time the presence of apoptotic bodies in horse semen. The number of apoptotic bodies varied greatly in stallions and positively correlated with caspase 3 activity in fresh samples and negatively with sperm viability and motility after cryopreservation.

Caspase 8

Caspase 8 (CASP8) is a proteolytic enzyme effector caspase, encoded by the CASP8 gene. The length of the polypeptide chain of the protein is 479 amino acid residues and the molecular weight is 55.391 kDa (Newton *et al.,* 2019).

It is necessary for extrinsic cell death pathways initiated by members of the TNF family. Death receptors (*e.g*. Fas receptor (CD-95)) recruit DISC after binding of specific TNF family ligands and trimerization. Procaspase-8 is incorporated into this complex by binding through its N-terminal prodomain consisting of two death effector domains to the FADD adapter protein. N-terminal prodomain is joined using a linker of 50 residues to a catalytic domain of 270 amino acids, which contains two sites of cleavage by caspase-8 for autoproteolytic processing. Within the catalytic domain, there are two additional cleavage sites that allow it to be cleaved into two different subunits, called α or p18 and β or p11. Dimerized procaspase-8 molecules in DISC are activated by reverse cleavage. The activated caspase-8 then initiates a subsequent apoptotic cascade cleaving caspase-3, caspase-7, or Bid. The Bid protein is a link between the external and internal pathways of apoptotic signal transmission (Ghavami *et al.,* 2009; Dyatlova *et al.,* 2018; Keller *et al.,* 2009).

In addition to regulating cell death, caspase-8 also acts as a key mediator of inflammatory cytokine production. After activation of the death receptor or TLR, caspase-8 can stimulate the production of cytokines mediated by NF-kB (TNF-α and IL-6). Caspase-8 can also promote inflammation by regulating the signaling of the NLRP3 inflammasome. This mediates the involvement of caspase 8 in the development of various inflammatory and neurodegenerative diseases (Kumar *et al.,* 2023).

Caspase 8 in sperm

Caspases 3, 8 and 9 are present in sperm and seminal plasma and are responsible for germ cell apoptosis (Said *et al.,* 2004; Grunewald *et al.,* 2009; Wei *et al.,* 2015; Asadi *et al.,* 2021). In humans, active caspases 8 are localized mainly in the postacrosomal region (Paasch *et al.,* 2004a). At the same time, it was found that the activity of caspases 8 and 9 in sperm and seminal plasma strongly negatively correlates with sperm concentration and motility (Wei *et al.,* 2015). In oligozoospermia, along with an increase in the activity of caspase 3, there is an increase in the activity of caspase 8, since these enzymes are links of the same signaling pathway. It can be assumed that this condition is associated not only with a violation of meiosis, but also with damage to membranes, which triggers an apoptotic cascade (Almeida *et al.,* 2013).

The possibility of activating the receptor mechanism of apoptosis under the action of cryopreservation is indicated that, after thawing of stallion spermatozoa, exposure of TNR1 and TNFR2 death receptors, as well as

the presence of the TNF ligand, was revealed on the plasma membrane. These data were obtained by immunocytochemical staining methods (Bolaños *et al.,* 2014). In addition, there is an increase in the expression of IL-1β, TGF-β and corresponding receptors (IL-1R, ACVR1C) in cryopreserved human embryonic stem cells. Activation of the MAP kinase pathway is also detected, which causes activation of caspases. Activation of death receptors may be the result of hyperosmotic shock caused by high concentrations of DMSO, which is used as a cryoprotector (Xu *et al.,* 2010; Ichikawa *et al.,* 2012).

Since the extrinsic pathway of apoptosis can switch to the intrinsic one under certain conditions (Savitskaya and Onishchenko, 2016; Dyatlova *et al.,* 2018). Most researchers detect a simultaneous increase in the activity of caspases in the studied biomaterial. Thus, cryopreservation of sperm is accompanied by a significant increase in the activity of caspases 3, 8 and 9 in human sperm (Paasch *et al.,* 2004b; Said *et al.,* 2010). When cryopreserved sperm is thawed from infertile men, the activity of caspases 8 and 9 increases significantly more than in healthy men, indicating lower cryostability (Grunewald *et al.,* 2009). Similar conclusions about the increased activity of caspases 8 and 9 during cryopreservation were reached by Wundrich *et al.* (2006). In addition, the researchers found that there is a dependence on the concentration of glycerin used during freezing. In the samples where glycerin with a concentration of 14% was used, the increase in caspase activity was more significant than in the samples with 7% glycerin. This is probably due to the toxic effect of glycerol on sperm mitochondria (Wundrich *et al.,* 2006).

Some scientists believe that caspase 8 serves as a switch between apoptosis and necroptosis. Active caspase 8 inhibits the phosphorylation of RIPK1 and RIPK3, which leads to apoptosis. However, when caspase 8 is inactivated, RIPK1 and RIPK3 mutually activate their phosphorylation and subsequently activate the underlying MLKL, causing necroptosis (Li *et al.,* 2021; Hai *et al.,* 2024).

Caspase 9

The mitochondrial (intrinsic) pathway of apoptosis is triggered by pro-apoptotic BH3 proteins, which can be activated by harmful stimuli, including ROS, gamma radiation and DNA damage. These proteins inhibit the anti-apoptotic Bcl-2, Bcl-xL, thereby weakening the inhibition of the pro-apoptotic factors BAX and BAK. The BAX/BAK-mediated increase in mitochondrial permeability leads to the loss of mitochondrial membrane potential (ΔψM) and the release of Cytochrome c from the intermembrane space into the cytosol. The release of cytochrome c leads to the activation of caspase 9 through the apoptosome. Activated caspase 9 eventually activates caspase 3, which promotes the cleavage of cytoskeletal proteins and activation of DNases (Chowdhury *et al.,* 2008; Castellini *et al.,* 2021; Dyatlova *et al.,* 2018; Asadi *et al.,* 2021).

The active form of caspase 9 has a dimeric form. Allosteric inhibition of the enzyme is possible due to phosphorylation and conformational changes. Like other caspases, caspase-9 has three domains: an N-terminal prodomain, a large subunit and a small subunit. The N-terminal prodomain is also called the long prodomain and it contains a caspase activation domain (CARD) motif. The prodomain is linked to the catalytic domain by a linker loop. The linker loop connects the pro domain and the catalytic domain, which consists of large and small subunits. The caspase-9 is activated by dimerization rather than cleavage, although caspase-9 showed full activity in its non-cleaved form, probably due to the long linker loop between the subunits. It is assumed that due to its length and the function of connecting large and small subunits, as well as due to its recruitment and dimerization in the apoptosome, the linker loop moves and gets access to the active site without splitting. Dimerization of caspase-9 leads to rapid autocatalytic cleavage, which results in the formation of caspase-9 (Li *et al.,* 2017; Srinivasula *et al.,* 1996; Renatus *et al*., 2001).

The non-apoptotic role of caspase-9 includes the regulation of necroptosis, cellular differentiation, innate immune response, maturation of sensory neurons, mitochondrial homeostasis and organization of the cortical-spinal chain. Increased activity of caspase-9 is associated with the progression of amyotrophic lateral sclerosis, retinal detachment, as well as various other neurological, autoimmune and cardiovascular diseases (Avrutsky and Troy, 2021). Caspase 9 is found in all cells and tissues. Caspase 9 in the cell is located in the nucleus, cytoplasm and mitochondria (Zhivotovsky *et al.,* 1999). Its greatest representation is in the tissues of the brain and nervous system, skeletal muscles, liver, pancreas (Srinivasula *et al.,* 1996; Han *et al.,* 2006).

Caspase 9 in sperm

Caspase 9 is present in human spermatozoa and the mitochondrial fraction of the enzyme is located mainly in the middle piece of the sperm. At the same time, activation of this initiatory caspase most often occurs in sperm endoplasmic reticulum (Paasch *et al.,* 2004a). In immature human spermatozoa, the activity of caspase 9 is initially slightly higher (Zalata *et al.,* 2016). The level of caspase-9 in semen was significantly increased in infertile men compared with healthy fertile subjects from the control group. The activity of casapse-9 spermatozoa has a significant negative correlation with the number of spermatozoa, sperm linearity index, sperm motility, sperm velocity, sperm linear velocity and normal morphology (Zalata *et al.,* 2011).

Many researchers have confirmed an increase in the activity of caspases, including 9, with the development of oxidative stress, an increase in the concentration of reactive oxygen species. These factors trigger a cascade of proapoptotic events, which negatively affects the

parameters of the spermogram (Lysiak *et al.,* 2007; Mostafa *et al.,* 2016), including in horses (Ball, 2008).

The negative effect of excessive CASP9 activation on spermatogenesis is also confirmed by the fact that the use of its inhibitors improves sperm quality in rats: sperm concentration and motility increase (Zhang *et al.,* 2018).

Cryopreservation/thawing causes activation of caspase 9 in human spermatozoa (Paasch *et al.,* 2004b) and boar spermatozoa (Zeng *et al.,* 2014). The predominance of the mitochondrial pathway of apoptosis is also evidenced by a decrease in the level of caspase activity in stallion spermatozoa under the action of bongkrekic acid, a mitochondrial permeability transition pore inhibitor (Ortega *et al.,* 2010), a significant decrease in the level of the mitochondrial membrane potential (ΔψM) in bull spermatozoa (Martin *et al.,* 2004).

Table 1: The use of caspase 3, 8, 9 inhibitors in sperm cryopreservation.

Inhibitor	Target	Concentration	Effects	Animal used	Reference
Z-VAD-FMK	Caspases 3, 8 and 9	100 μM	Before freezing - increase in motility and ΔψM, decrease in sperm DNA fragmentation After freezing- increase in sperm membrane integrity, decrease in DNA fragmentation	Bull spermatozoa	[Pagano *et al.,* 2020]
		100 μM	No change in acrosome and plasma membrane integrity	Stallion spermatozoa	[Peter *et al.,* 2005]
Z-IETD-FMK	Caspase 8	20 μM	Increase in ΔψM, increase in % sperms with low phospholipase activity	Buffalo bull spermatozoa	[Sharma *et al.,* 2015]
Z-LEHD-FMK	Caspase 9	2 μM	Increase in % of individual motility, increase in ΔøM		
Z-DEVD-FMK	Caspase 3	20 μM	Increase in ΔψM, increase in % sperms with low phospholipase activity		
Y27632	ROCK (Rho-associated protein kinase)	30 μM	Decrease in expression levels of BAX and CASP-9 genes, increase in expression of Bcl-2, decrease in ROS level	Bull spermatozoa	[Behnam *et al.,* 2023]

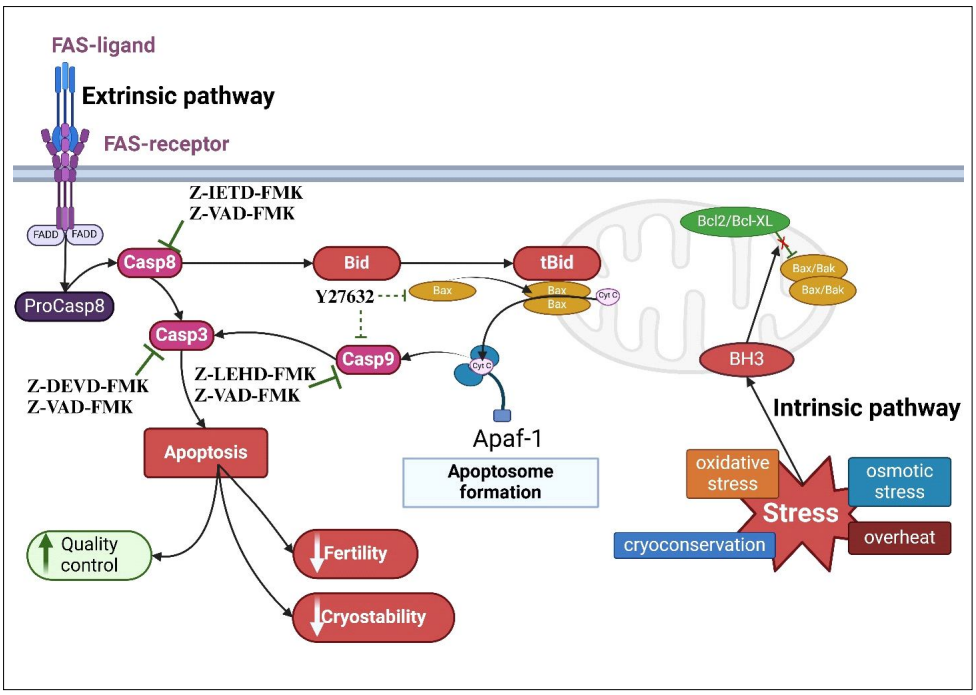


Fig 1: Caspases 3, 8, 9 in sperm apoptosis as application points for inhibitors (Created in BioRender. BioRender.com/y82s086. Agreement number: TT27DL2SEK.).

The use of caspase inhibitors to improve sperm cryopre-servation protocols

The caspase family has been actively studied for many years in the development of medicines (Aly *et al.,* 2020; El-Sheref *et al.,* 2020; Poreba *et al.,* 2013). Dhani *et al.* (2021) described in detail the classification of caspase inhibitors and the possibilities of their use for the treatment of inflammatory, neurodegenerative, metabolic and tumor diseases. Table 1 shows examples of the use of caspase inhibitors 3, 8 and 9 to improve sperm cryopreservation protocols.

To optimize cryopreservation protocols, substances that inhibit caspase activity, specifically caspases 3, 8 and 9, are utilized not only for their direct effect on these enzymes but also for their ability to indirectly suppress apoptosis. For example, the selective ROCK (Rho-associated protein kinase) inhibitor Y27632 influences the apoptosis cascade by targeting upstream regulatory mechanisms, thereby enhancing cell survival (Table 1). We would also like to focus on the study by Peter *et al.* (2005), which didn't find a positive effect when the pancaspase inhibitor Z-VAD-FMK was added to the stallion sperm in a cryopreservation medium. In this work, only one concentration of Z-VAD-FMK was studied and the sample of the studied stallions was limited to three animals, which indicates the relevance of further studies on the use of caspase inhibitors in cryopreservation of stallion sperm.

A summarizing scheme of caspases 3, 8, 9 participations in sperm apoptosis as application points for caspase inhibitors is shown in Fig 1.

CONCLUSION

Apoptosis is one of the well-known mechanisms of sperm quality control. However, increased levels of apoptosis can have an adverse effect on sperm production, ultimately endangering male fertility. Therefore, controlling the rate of apoptosis for male fertility is of particular importance. It follows from the literature data that a higher frequency of apoptosis is observed in the sperm of subfertile men and an assessment of caspase activity is proposed as a diagnostic marker of sperm quality, which provides a more complete sperm analysis for andrological examinations. This aspect is also insufficiently studied in animal husbandry and is quite perspective. It is essential to investigate the activity of caspases both in spermatozoa and in seminal plasma.

In addition, it is relevant to study the types and mechanisms of cell death, as well as the role of caspases in the implementation of these mechanisms in the process of cryopreservation of spermatozoa. This can help in choosing the right cryoprotector, as well as choosing substances that can inhibit apoptosis at different stages, including suppressing the activity of caspases 3,8,9.

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Disclaimers

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Conflict of interest

The authors declare that there is no conflict of interest.

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