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**COMPARISON BETWEEN SLOW, PROGRAMMABLE FREEZING PROTOCOL AND
VITRIFICATION IN PELLET FORM OF GUINEA FOWL (*Numida meleagris*)
SPERMATOZOA BY USING *IN VITRO* AND *IN VIVO* SPERM EVALUATION ASSAY**

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I. INTRODUCTION AND AIM

Frozen storage of avian semen has been established for approximately more than 60 years. Chickens were the first animals to be produced from frozen spermatozoa within poultry species in 1951 (POLGE, 1951). However, there is little or no commercial use of frozen poultry spermatozoa because of the variable fertilizing ability (DONOGHUE and WISHART, 2000), despite some efforts to demonstrate how such a frozen storage of avian semen could be economically viable in the broiler breeder industry (HAMMERSTEDT, 1995). The significant differences that exist among the poultry species in terms of the viability and functionality of sperm after cryopreservation (WISHART, 1989) is another factor that limits the commercial use of avian frozen-thawed semen. Compared to the other domestic species, the avian species have some unique physiological characteristics which might influence semen preservation. The avian sperm head is cylindrical, contains less cytoplasmic volume and the tail is quite long (THURSON and HESS, 1987), which leads to higher susceptibility to freezing damage.

Recently, the need for semen preservation in poultry is a result of the reduction in genetic variability of domesticated bird livestock and of the increasing risk of line extinction. In 2000, a report of the Food and Agriculture Organization of the United Nations claims that 9% of the global farm animal population is in a critical condition and 39% is threatened with extinction. As many as 30% of poultry breeds is threatened with extinction and 9% has already become extinct. Extinction of species is irreversible and occurring at an accelerated rate due to habitat destruction and breed selection. Thus, assisted reproduction technology such as artificial insemination and cryopreservation of spermatozoa plays an important role in preserving valuable genetic materials and propagate the endangered species which fail to breed in capacity. The *in vitro* cryobanks are necessary for safe gene conservation because several risks of *in vivo* gene banks have been considered. At this moment, semen cryopreservation is the most practical method for the long term storage of the genetic material in poultry species (BLESBOIS et al., 2007).

Several research projects on artificial insemination and cryopreservation of avian semen has been developed. For instance, the European Endangered Programs (EEP) aim to create a sperm cryobank for threatened bird species. In Hungary, the Association of Hungarian Small Animal Breeders (MGE), which was established by the breeders of old Hungarian poultry breeds, with

participation of the Institute for Small Animal Research (KATKI, Gödöllő) is appointed as the only breeding organization of the old Hungarian, indigenous and other local varieties of poultry, including the guinea fowl. Successful application of semen freezing technology has been carried out in chicken, turkey, duck, goose and also guinea fowl, a semi-wild, endangered species in which there is local or national interest and funding. Besides the slow, programmable methods, usage of vitrification method (a very fast freezing in pellet form) looks promising. By this method, the fowl spermatozoa frozen and thawed in pellets gave high fertility rate 84.7-92.7% (TSELUTIN et al., 1995). Since the freezing tolerance of spermatozoa is the lowest among poultry species, due to their characteristic structure of sperm membrane (BLESBOIS et al., 2005), only domestic birds such as chicken, turkey, goose spermatozoa have been successfully frozen by pellet method (DONOGHUE and WISHART, 2000). There was only a single study that described the sperm freezing of guinea fowl so far, in which different intermediate freezing rate, internal cryoprotectants (glycerol, dimethylformamide, dimethylacetamide) and external cryoprotectants such as diluents containing sugars or myoinositol (BHSV and IGGK) were used with straw packaging. This study reported that the highest survival rates of spermatozoa after freezing-thawing (up to 50%) were obtained with semen diluted in IGGK with 6% of the cryoprotectant agent dimethylformamide (DMF), and freezing rate 15°C/min (SEIGNEURIN and BLESBOIS, 2005). However, this method led to a mean of only 20 % fertility.

In 2000, guinea fowls were introduced to Vietnam from KÁTKI (Institute for Small Animal Research, Gödöllő, Hungary). At first, the main purpose of this introduction was to improve agriculture in developing countries. At this time, guinea fowl rearing was considered to be a potential tool for Vietnamese rural people to produce a good quality poultry product for both their own consumption and the Vietnamese market. Twelve years after the first introduction, successfully adapted guinea fowl has become a very popular species in Vietnam. It has helped a lot of people residing in the countryside to free themselves of poverty and has even made one of them, Hai Luc, became a billionaire. Recently, The National Institute of Animal Husbandry has granted a certificate with financial support from the Food and Agriculture Organisation (FAO) for gene pool conservation of black and white guinea fowls to his farm. The gene conservation of guinea fowls in Vietnam has raised more and more attention not only nationally but also internationally, so guinea fowls may be an interesting animal target for research. And, since vitrification of guinea fowl spermatozoa in pellet form has not been described yet, this research aims to characterize this method and to focus on comparing between two protocols: slow, programmable cryopreservation

and pellet formation of guinea fowl spermatozoa by using both *in vitro* and *in vivo* sperm evaluation assay.

II. LITERATURE REVIEW

II.1. Guinea fowl

The term “guinea fowl” is the common name of the seven species of gallinaceous birds of the family *Numididae*. The guinea fowls originated from Africa and were first domesticated by ancient Egyptians (EMBURY, 2001). The popular varieties, which are currently being reared in many parts of the world for their gamey flesh, eggs and ornamental value, are descended from the helmeted guinea fowl (*Numida meleagris*). In Hungary, guinea fowl is considered as an adapted Hungarian poultry species including bluish-grey, white, grey and spotted. The first reports about its breeding in Hungary were published at the beginning of the 20th century and the guinea fowls were kept as a game bird or a semi-domesticated one. The morphological characteristics of a guinea fowl include:

- Body weight: male: 1.30-1.60 kg, female: 1.20-1.40 kg.
- Head: short and wide.
- Beak: short, strong, strongly curved, horn-coloured and red at the end.
- Comb: helmet-like, horny, red, triangular.
- Face: bluish-white, without feather.
- Ear-lob: fit to the face.
- Eye: large, dark brown.
- Wattle: short, thick, red, with a white spot in the middle. The wattles on a male guinea fowls are much larger than on the female.
- Neck: almost no feather under the head, with dark-bluish skin on males (lighter coloured on females). Thin, erected when the bird is disturbed. The lower part of the neck is well-feathered; the upper part is naked, covered with fine hair.
- Trunk: strong. The breast is large, round-shape and full. The back is wide, strongly dome-like and inclines backward.
- Wing: long, wide, fit to the body.
- Tail: short, points downwards. Feathers are longer inside (in the middle) than outside.

- Shank and toes: short. Shank colour is reddish. Males have no heel.

- Plumage: fit to the body with beautiful, bluish-grey basic colour. White spots (pearls) are uniformly distributed all over the plumage. The neck plumage is usually darker-blue without spots. The basic colour of the white variant is pure white, with velvety, bright spots on the plumage.

Male and female guinea fowls differ so little in appearance that it is difficult to distinguish them from each other. Sex may be distinguished by the cry of the birds after they are about 2 months old and by larger, helmet, wattles that have thicker edges. The males always emit one-syllable cries but in the case of females, they are able to emit both one- and two-syllable cries, which sound like “buckwheat, buckwheat” or “put-rock, put-rock”.

The guinea fowls are timid birds, with a more gregarious behaviour than that of the chicken. Darkness and presence of perches may reduce the bird’s timidity where it likes to hide and to remain motionless when afraid. Therefore, animal houses with reduced light intensity allow large numbers of guinea fowls to be raised. The guinea fowls are extremely noisy and cannot be reared near to residential houses. In the wild habitat, plants play an important role in guinea nutrition. However, they can consume all non-conventional feeds that are not able to use in chicken feeding. While feeding they do not scratch with their claws, but use their beaks for tearing with abrupt head movements. This behaviour of guinea fowls may lead to feed wastage when feeding them with mash.

Recently, egg and meat production of guinea fowls raised attention of farmers because of its excellent quality, are rich in vitamins and are low in cholesterol. They are very resistant to heat and most of the common chicken diseases (AITKEN et al., 1977). Moreover, they are more tolerant to mycotoxins such as aflatoxin. Guinea fowl is also a promising genetic resource for evolving a low input and grain saving poultry alternative for production in the developing countries such as Vietnam. The low costs of keeping guinea fowl makes it one of the best poultry species for natural production. The meat of guinea fowls was rated as one of the best poultry products in Vietnam nowadays, with the price being 1.5-2 times more than others. And, more than 30 000 day-old chicks are sold out of Thuy Phuong Poultry Research Centre (POREC) every year (TIEN et al., 2007).

II.2. Avian semen collection

The first non-invasive avian semen collection method was developed successfully in chicken and turkey by Burrows and Quinn in 1937. It was named dorso-abdominal massage technique. The principle of this technique was based on ejaculatory responses of birds when they were stimulated to by massaging of its abdomen between the gizzard and the pelvic bones. It was considered as a two-man technique. One restrained the male, the other one stroked the back from behind the wings towards the tail with firm and rapid strokes. The male responded with erection of the phallus, at which time the handler gently squeezed the cloaca and expressing semen through the external papillae of ductus deferentis and collects the semen into a container. This technique has been sucessfully applied in turkey, goose and pigeon (GEE, 1994). For non-domestic avian species, adaptations were also made for pheasants (SAINT JALME et al., 2003), crane (GEE and TEMPLE, 1978), however, long time training program, additional restraint and modified massage techniques are required. For the crane, the assistant must cradle the bird between the legs with the bird's head protruding behind the assistant's back, and rhythmically massages the bird's legs by grasping the shanks, stroking gently several times in a circular, inward and downward direction. For the pigeon, a brief caudally directed abdominal massage combined with pressure exerted caudally to the cloacal area was applied. The pigeon was held with its chest positioned against the collector's belly, the wings were stretched and while fixing the main feathers (SONTAKKE et al., 2004). Semen collection has been done successfully as well in guinea fowls, a semi-wild bird, with a dorso-abdominal massage technique, adapted from technique applied in chicken. However, it has been noted that efforts to stimulate a male fowl to ejaculate are often attended with some difficulty since the male fowls vary greatly in responsiveness. Male fowls, kept together with hens, usually produced very little or no semen. Separation and individual keeping is suggested to be made at least two days before attempting the first semen collection (BURROW and QUINN, 1937).

II.3. Cryopreservation of avian spermatozoa

Cryopreservation of avian sperm was a natural outgrowth of the development of artificial insemination in poultry. And, the development of cryopreservation techniques has had a significant impact in reproductive biology and genetic management of poultry. The prefix "cryo" comes from the Greek word "kryo" meaning cold or frost. Cryopreservation means maintenance of a viability of excised tissue or organs by storing at very low temperature (BLOOD et al., 2007). Sperm cryopreservation is a process where structurally intact living spermatozoa are preserved by cooling to low sub-zero temperatures, such as -196°C (the boiling point of liquid nitrogen). At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. Successful cryopreservation of avian sperm requires a proper selection of diluents, selection of cryoprotectant, optimal freezing and thawing rates and removal of any materials deleterious to fertility before insemination. An economically useful method must allow recovery of sperm with sufficient fertilization capacity.

II.3.a. Cryoinjury

When the spermatozoa are frozen, they are subjected to stresses, resulting from water-solute interaction that arises through ice crystallization. Crystallization induces the formation of unfrozen pockets of hyper-osmotic solution while cooling to approximately -50°C takes place. This event leads to withdrawal of intra-spermatozoa water, the spermatozoa shrink and there is an influx of ion (MAZUR, 1984). Some of the spermatozoa become excluded from space occupied by ice crystals, thus, they are exposed to hyper-osmotic environment. The hyper-osmotic solution or high salt concentration treatments of spermatozoa have been proven to injure the cell membrane by removing its peripheral proteins. The thawing process involves a reversal of these effects and may cause membrane disruption. The spermatozoa being preserved are often damaged during the approach to low temperatures or warming to room temperature. Freezing injury can be the result of primary damage from ice crystals that pierce or tease apart the spermatozoa, destroying them by direct mechanical action and secondary damage caused by changes in the composition of the liquid phase. In fact, both damaging mechanisms are important, depending on cooling rate, and warming rate. Intracellular ice formation by overly rapid freezing is generally lethal for the spermatozoa. The

optimal cooling rate is considered to be slow enough to prevent lethal effect, but fast enough to minimize the harmful effects of prolonged exposure to hyper-osmotic solution present in unfrozen pocket. That rate differs between spermatozoa of differing size and water permeability.

II.3.b. Cryoprotectants

In 1951, Polge and colleagues published freezing storage protocol of fowl semen in the presence of glycerol. The ability of glycerol to protect cells from freezing injury was discovered accidentally. It acts primarily by increasing the total concentration of all solutes in the system that result in reducing the amount of ice formed at any given subzero temperature. Glycerol is a small, poly-hydroxylated solute with a high solubility in water, and a low toxicity during short-term exposure to living cells. The increasingly high viscosity of glycerol during the cooling process is another property that may also inhibit or decrease ice crystal formation. During the last two decades, a wide range of solutes have been investigated for cryoprotectant activity. Therefore, the current term “cryoprotectants” is the functionally-derived term to describe any additive which can be provided to cells before freezing and yields a higher post-thaw survival rate than can be obtained in its absence. In general, cryoprotectants can be classified according to penetrating ability. An effective penetrating cryoprotectant should be freely permeable across the cell membrane so that it is considered as an internal cryoprotectants. Some common penetrating cryoprotectants are dimethyl sulfoxide (DMSO), propylene glycol, ethylene glycol (EG), glycerol. Non-penetrating cryoprotectants are the substances that act extracellularly. These compounds are generally polymers that form extensive hydrogen bonds with water, help in reducing the water activity such as hydroxy-ethyl-starch (HES), polyvinylpyrrolidone (PVP), polyethylene oxide (PEO).

The range of common internal cryoprotectants used for avian spermatozoa includes glycerol, ethylene glycol (EG), dimethylsulphoxide (DMSO), dimethylacetamide (DMA), polyethylenglycol (PEG), dimethylformamide (DMF). The cryoprotective activity or toxicity of different cryoprotectants varies depending on several factors such as concentration, temperature, freezing rate, freezing method, and post-thaw treatment.

Efficiency and toxicity of cryoprotectant glycerol, EG, DMF, DMA has been studied and compared in many avian species so far including guinea fowl. The fertility rates, reported in the study of guinea fowl, were zero for glycerol and EG. However, it was said to be a better cryoprotectant than EG, DMSO for fowl spermatozoa although the removal of glycerol prior to

artificial insemination was highly suggested (LAKE and STEWART, 1978) due to its susceptible interaction with metabolism of the spermatozoa. It also alters the bi-layer structure of cell membrane (HAMMERSTEDT and GRAHAM, 1992). Another common cryoprotectants used in avian cryopreservation is DMF and DMA. The highest survival rate and fertility has been observed in guinea fowl, using cryoprotectant DMF 6%. DMA is known to be toxic to avian spermatozoa but elimination is not required prior to artificial examination (BLESBOIS and BRILLARD, 2007). Thus, cryoprotectant DMA is simple enough and can be applied to endangered bird species. In the previous study of semen cryopreservation of guinea fowls, the internal cryoprotectants DMA, DMF and the external cryoprotectant IGGK which contains a buffer phosphate significantly enhanced fertility rate of frozen-thawed spermatozoa compared to the external cryoprotectants glycerol, EG and internal cryoprotectant BHSV9.

II.3.c. Cryodiluents

The poultry semen is viscous and highly concentrated, containing 6 to 12 billions spermatozoa/ml and often contaminated with feces, urine, bacteria (DONOGHUE and WISHART, 2000). Therefore, extension of semen by cryodiluents is necessary to reduce sperm concentration, bacterial contamination, and provides energy as well as control pH. It helps to maintain the viability of spermatozoa during transporting, prior to freezing and post-thawing. It also helps to maximize the number of hens that can be inseminated. The differences regarding physiological and metabolic requirement of spermatozoa from avian species make it difficult to establish a common diluent for avian semen. Recently, most of the commercial avian semen diluents were developed mainly based on the biochemical composition of chicken and turkey semen.

Lake and Tselutin extender are two of the most popular cryodiluents in poultry cryopreservation. In 1978, Lake extender was developed for domestic fowls by Lake and Stewart while Tselutin extender was developed for chicken, turkey, gander, drake in 1995 ([Table 1](#)).

Table 1: Composition of semen extenders (g/100 ml)

Composition	*Lake	**Tselutin (chicken)	**Tselutin (turkey)	**Tselutin (gander)	**Tselutin (drake)
Na-glutamate	1.92	1.92	2.4	1.4	2.1
K-acetate	0.5	0.5			0.5
K-citrate				0.14	
Polyvinyl pyrrolidone	0.3	0.3			0.3
D-fructose	0.8	0.8			0.15
Magnesium-acetate	0.08		0.1		
Protaminsulphate		0.032			
Dipotassium hydrogen phosphate			0.35		
Disodium hydrogen phosphate				0.98	
Sodium dihydrogen phosphate				0.21	
Glucose			0.8	0.9	
Inositol			0.2	0.9	
Glycine			0.1		0.2
Glutamic acid			0.14		
pH	7.4	6.85	6.65	7.0	6.85

*Adopted from LAKE and STEWART, 1978

**Adopted from TSELUTIN et al., 1995

II.3.d. Freezing and thawing methods

There are various methods available for avian cryopreservation such as vitrification in pellet form, rotation method, or slow, programmable freezer. Vitrification means the production of an amorphous state without any crystallization, defined by the viscosity. The amorphous state is like a "solid-liquid". The transformation is over a small temperature range described as the glass transition temperature. In simplified terms, it can be described as solidification of a liquid into an amorphous state while maintaining the same molecular orientations that existed in solution before the glass transition. The semen is frozen by rapid cooling through direct plunging of semen droplets into liquid nitrogen to form frozen pellets. The procedure takes a few seconds for cooling so removal of cryoprotectant before artificial insemination is not required. There were several modified pellet

formations which have been described so far. In the study of Tereda, 0.2 ml volumes of semen were dropped onto a depression in solid CO₂, then, transferred into liquid nitrogen (TEREDA et al., 1989). In another study, Tsuletin dropped 0.2 ml volumes of semen onto fluoro-plastic plate held in liquid nitrogen vapour at -70°C, then transferred into liquid nitrogen (TSELUTIN et al, 1995). And, recently, in the study of Iaffaldano, 60 µm and 80 µm volumes of semen were plunged into liquid nitrogen after equilibrating at 4°C for 1 to 5 minutes (IAFFALDANO et al., 2011). In the rotation method described by Kurbarov (1984), 1 ml of samples within a 10ml capacity glass was rotated in liquid nitrogen vapour at -70°C before plunging into liquid nitrogen. Contrary to these fast freezing methods, the slow freezing method using programmable freezers has been described by Lake in 1984, Wishart, Seigneurin and Blesbois in 1995. The samples within glass ampoules or plastic cryovials or straws are cooled from 5°C to -35°C at between 1 to 10°C/min before plunging into liquid nitrogen. Thawing systems involve placing the plunging samples from -196°C into thermal bath or in a thermo-regulated hotplate adjusted to 60°C to 75°C.

II.4. Sperm evaluation

Determination of sperm quality is important both before and after freezing-thawing. It provides an estimate about how good the freezing and thawing protocols are. The avian semen can be evaluated by several characteristic parameters such as volume, colour, concentration, motility, live-dead sperm ratio, morphological abnormalities, plasma membrane integrity, membrane fluidity, acrosomal integrity, mitochondrial function, and chromatin status of spermatozoa. Many of these parameters correlate with fertilizing capacity of spermatozoa. The motility of spermatozoa is reduced after cryopreservation due to changes of the plasma membrane permeability and availability of energy. The integrity of the sperm membrane is needed to maintain the function of spermatozoa during storing in the female reproductive tract. Disruption of this membrane may lead to lipid rearrangement and cellular damage. Determination of acrosome status provides a fundamental importance in the spermatozoa's ability to penetrate the egg envelope and ability to fuse with the egg plasma membrane. The avian sperm DNA is resistant to physical and chemical attack but damaged by cryopreservation. The structure of mitochondria and the integrity of spermatozoa DNA is important for the normal development of the embryo. Volume and concentration can be measured precisely but these parameters seem unable to reflex the true quality

of spermatozoa. Traditional histological, fluorescent stains have been used to determine live-dead ratio, morphological abnormalities and membrane fluidity of spermatozoa. The location of abnormalities can be detected and classified to primary or secondary injuries. The secondary injuries indicate damage of spermatozoa during freezing and thawing. Membrane fluidity may be assessed by measuring fluorescence polarization anisotropy with a fluorescent dye (BLESBOIS et al, 2005). During the last decade, several new *in vitro* techniques such as sperm-egg interaction assays and flow cytometry have been introduced to avian semen assessment. Since the spermatozoa are able to hydrolyze holes in the inner perivitelline layer (IPVL) when fertilization takes place, sperm-egg interaction assay simply involve counting the number of holes per unit area of unstained inner perivitelline layer (IPVL). The changes in sperm function shown in this assay are similar to the changes in the ability of spermatozoa to reach the outer perivitelline layer of eggs *in vivo* (DONOGHUE et al., 1995). The observation of physical characteristics such as cell size, shape, components or function of spermatozoon can be detected by flow cytometry with fluorescently labelled compounds. A large number of spermatozoa can be analyzed at once. This analysis is sensitive, objective and accurate. The most frequently used viability probes for staining sperm is SYBR-14 and propidium iodine (PARTYKA et al., 2010). Another precise, cost-effective method is sperm chromatin structure assay. This assay is based on the susceptibility of chromatin to acid or heat denaturation and metachromic properties of acridine orange. This dye emits fluorescence in green band as it intercalates into the intact double DNA helix and in red band when it is associated with RNA or denatured DNA. Due to the complex nature of male fertility, any *in vitro* evaluating method must include testing of both fertilization and embryonic development, not only individual spermatozoa, but a large, heterogeneous sperm population. Finding an *in vitro* test that can accurately estimates the fertilizing ability of a semen sample is still distant. The *in vitro* assessments can predict potential fertilizing ability of frozen-thawed semen only but not the true fertilizing ability itself. Hence, *in vivo* sperm qualification by artificial insemination, a process in which a true fertility rate can be detected accurately, is essential. Infertile eggs can be identified during incubation by candling method. "Candling" means holding an egg against light source in a darkened room and detect the development of the embryo. The first candling should be done on or after 7 to 10 days. A normal living embryo shows a general healthy appearance with clearly defined blood vessels and no haemorrhagic evidence. The movement of some body should be detected as well when stimulating with candle light. The dead embryo shows a blood ring outlining the inner surface of the egg shell. Eggs with a clear appearance under candling may belong to either the fertile group

without any development yet or the true infertile group. In order to distinguish the true infertile eggs, examination of germinal disc can be done with the help of propidium iodide (PI) staining. PI is a DNA specific, red colour fluorescent dye. If the egg is infertile, there is no nucleus, and the staining shows a dark-red background without lighting points.

III. MATERIALS AND METHODS

The experiments for this research were carried out at the Institute for Small Animal Research (KATKI, Gödöllő, Hungary). All the samples have been collected routinely and examined at the reproductive biological laboratory. The first experiment in which fresh, frozen-thawed semen was evaluated by *in vitro* assay was carried out from September to November in 2010. The second experiment in which fresh, frozen-thawed semen was evaluated by *in vivo* assay was carried out in September and October of 2011.

III.1. Animals and keeping method

30 one-year old rural Hungarian guinea cocks and 30 guinea hens were used as sperm donors and sperm receivers. They were placed in individual cages at an isolated unit of the researching animal house of KÁTKI in Gödöllő, Hungary. They were fed with commercial food *ad libitum*. The animals were kept under a 16 hour lighting program.

III.2. Semen collection

The semen was collected by a non-invasive method, the dorso-abdominal massage technique, adapted from chicken semen collecting method (BURROWS and QUINN, 1937) individually. Two people were required. Guinea fowl is known as an avian species that is difficult to handle because of their aggressiveness. One was trained to catch the guinea cock quickly to minimize the effect of stress on semen collection and restrained the guinea cock by keeping the legs down and the wing up. The operator stimulated the cock by the right hand, stroked the back from behind the wings towards the tail with firm strokes, and gently pressed the cloaca at the end of each stroke. The guinea cock responded with its tail rising up if the massage technique was correct. At which time, the operator placed the right hand behind the tail while pressing the cloaca with the right thumb and

index finger. The guinea cock responded with erection and protruding of the phallus. Gently squeezing the cloaca made semen express through the external papillae of ductus deferentis. The semen was whitish, cloudy, milk-like. Firstly, the operator wiped the cloaca by clean tissue paper to remove dust and droppings (waste products) of the cock, and minimize the contamination of semen samples. It was possible to make 2 or 3 „milking” at one time depending upon the guinea cock condition. Then, semen was collected into an Eppendorf tube. The tube was kept warm by grasping it inside the operator’s palm during the whole collecting process and transporting from animal house to the laboratory. It took about 15 minutes to collect semen of 30 guinea cocks. The guinea cock fresh semen loses fertility quickly within 48 hour storage, thus evaluation and freezing was done right after the collection. Two-week training of guinea cocks was carried out prior to 8-week semen collection program. The operator who collected the semen was the same person during the whole collection period. The pool of semen collected from all 30 guinea cocks on each occasion was considered as one sample. For *in vitro* test in 2010, the semen was collected twice a week for 4 weeks. Each week, one occasion was used for slow, programmable freezing method, and the other for vitrification in pellet form. In 2011, semen samples were also collected for artificial insemination in the same way.

III.3. Freezing protocols

Two freezing protocols were used in this study, the slow, programmable and vitrification with pellet formation. The comparison of two protocols was demonstrated by [Table 2](#).

Table 2: Freezing protocols

Protocol	Slow, programmable	Pellet formation
Type of cryocontainer	Cryovial (200 µL)	Pellet (25 µL)
Diluents	Lake 's diluent (1986)	Tselutin's diluent (1995)
Dilution rate	1:3	1:1
Equilibration time	25 mins at 3 °C	20 mins at 2°C
Freezing rate	-1°C/min until -30°C -30°C/min until -60°C	Dropped directly into liquid nitrogen
CPs	10% EG	6% DMA

Slow, programmable protocol

After collecting, pooled semen was diluted 1:3 with Lake's extender (LAKE, 1986) at room temperature. Then, 10% ethylene glycol (EG) was added into the diluted semen. 200 µl from the samples were measured and put into cryovials. These cryovials were placed into the programmable freezing machine (Planer KRYO10). Cooling was started from 20°C with freezing rate -3°C/min. 25 minutes of equilibration time followed when the temperature reached 3°C. Then, freezing rate -1°C/min was applied to cool the cryovials down to -30°C, and later freezing rate -30°C/min from -30°C to -60°C. Finally, cryovials were removed from the freezing machine and plunged into liquid nitrogen. Composition of Lake's extender is demonstrated in [Table 3](#).

Vitrification in pellet form

After collecting, pooled semen was diluted 1:1 with Tselutin's extender (TSELUTIN et al, 1995) at room temperature. Then, the diluted semen was equilibrated for 20 minutes at 2°C and treated with cryoprotectant dimethyl-acetamide (DMA) 6%. Treated semen was directly dropped into the liquid nitrogen (25 µl / pellet) with a pipette. The pellets were collected into cryovials and stored in liquid nitrogen. Composition of Tsuletin's extender is demonstrated in [Table 3](#).

Table 3: Composition of Lake and Tsuletin extender (g/100ml)

Composition	*Lake	**Tselutin
Na-glutamate	1.92	1.92
K-acetate	0.5	0.5
Polyvinyl pyrrolidone	0.3	0.3
D-fructose	0.8	0.8
Magnesium-acetate	0.08	
Protaminsulphate		0.032
pH	7.4	6.85

*Adopted from LAKE and STEWART, 1978

**Adopted from TSELUTIN et al., 1995

III.4. Thawing procedure

The samples frozen by slow, programmable freezing were thawed in cooling cabin at 5°C. The samples frozen by pellet method were thawed in automatic machine at 70°C.

III.5. Semen evaluation

III.5.a. *In vitro* sperm evaluation

The *in vitro* assay was carried out before freezing and after thawing on each occasion except motility and concentration parameter. Motility was examined only before freezing by subjective scoring from 0 to 5 based on the percentage of motile sperms and direction of sperm movement. One drop of fresh semen was needed to prepare a smear and examined under light microscope with 40x magnification.

- Score 0: indicates no motility
- Score 1: indicates non-progressive motility (5-20% of spermatozoa move)
- Score 2: indicates slow, progressive motility (20%-40% of spermatozoa move)
- Score 3: indicates side to side movement accompanied by slow, progressive motility (50% - 70% of spermatozoa move)
- Score 4: indicates fast progressive motility (75%-85% of spermatozoa move)
- Score 5: indicates very fast progressive motility (85%-95% of spermatozoa move)

Sperm concentration was measured by spectrophotometer (Accucell IMV, France) in million/ μ l unit. Accucell IMV is a tool developed for an accurate measurement of the concentration of all types of ejaculate. And, the concentration of frozen-thawed semen was expected to be the same as the concentration of fresh semen used for freezing. Morphological abnormalities and live/dead spermatozoa ratio of both fresh semen and frozen-thawed semen were determined by aniline blue-eosin staining. The dye contains 0.2 g of eosin and 0.8 g of aniline, dissolved in 10 ml of extender (Table 4).

Table 4: Aniline-eosin extender

Composition	Unit	Amount
Sodium-glutamate (monohydrate)	gram	1.92
Magnesium-acetate (tatrahydrate)	gram	0.08
Tripotassium-citrate (monohydrate)	gram	0.13
Sodium-acetate (amhydrosus)	gram	0.51
Glucose	gram	0.6
Distilled water	ml	100

10 μ l of semen and 20 μ l of Eosin-Aniline blue solution were pipetted into Eppendorf tube. Then the tube was shaken gently for a second to mix thoroughly. 10 μ l of mixture was spread on slide and dried up. The slide was covered with one drop of immersion oil and examined under light microscope with 100x magnification. 200 spermatozoa were counted totally from each slide. The number of dead spermatozoa; live, intact spermatozoa and live, abnormal spermatozoa were determined from this population of 200 spermatozoa. The normal smear, stained with Eosin-Aniline, was seen with a bluish background. Normal and intact spermatozoa had an empty-appearance. When the sperms were injured, eosin dye could get into the intracellular space of the

sperm cell, thus it was pinkish or reddish stained. Abnormality of sperm was classified by location of injury. Damage of spermatozoa might take place at their acrosome, head, mid-piece or tail.

The parameters of morphological abnormalities and live/dead spermatozoa ratio were calculated based on formulas mentioned below.

$$\% \text{ live spermatozoa} = (\text{live, intact} + \text{live, abnormal}) \times 100 / 200$$

$$\% \text{ dead spermatozoa} = (\text{dead} \times 100) / 200$$

$$\% \text{ live, intact} = (\text{live, intact} \times 100) / 200$$

$$\% \text{ live, abnormal spermatozoa} = (\text{live, abnormal} \times 100) / 200$$

Survival rate of live and intact spermatozoa after freezing was calculated by the following formula.

$$\text{Survival rate (\%)} = (\% \text{ live, intact of frozen-thawed semen} \times 100) / \% \text{ live, intact of fresh semen}$$

III.5.b. *In vivo* sperm evaluation

❖ Artificial insemination

30 guinea hens were divided into 3 groups of 10 animals. Ten hens, used as controls, were inseminated with fresh, semen two-fold diluted in Lake's extender while the remaining of guinea hens were inseminated with frozen-thawed semen, 10 hens with frozen-thawed semen produced by slow, programmable freezing and other 10 hens by vitrification in pellet form. Artificial insemination was made 8 times per group totally, for 3 weeks, with average frequency 2 times/week and average inseminating dose 290 ± 52 million spermatozoa/hen.

❖ Detection of infertile eggs and eggs with embryonic death

Collection of eggs started 2 days after the first insemination. Then, eggs were incubated by an incubator and checked by candling on day 7 or day 10 of incubation. All eggs from the incubator were removed and placed in egg cartons. One egg was held at a time, against a candling torch. Under the candling lamp, eggs with a healthy embryo appeared as a dark shadow with well-defined blood vessels and responded to the light by moving. Eggs with embryonic death during incubation had broken blood vessels. They appeared as a dark ring around the egg. Eggs without an embryo had a clear appearance. Viable eggs were returned to the incubator. Eggs without an embryo were checked further by examination of germinal disc by staining with PI. The germinal discs of these eggs were removed from the vitelline membrane, put into 0.9% NaCl solution, and stained on slide

with 5µl 0.005mg/ml PI. The eggs with embryonic death in oviduct showed a stained nucleus under fluorescence microscope while the infertile eggs without a nucleus showed dark-red background without lighting points.

The total number of eggs, the number of infertile eggs, eggs with normal development or early embryonic death in oviduct and during incubation produced in 3 weeks of each group was documented. Finally, the *in vivo* evaluation of semen was expressed by four parameters calculated below (LIPTÓI et al., 2004).

$\% \text{ eggs with normal development} = (\text{normal development} \times 100) / \text{total eggs}$

$\% \text{ eggs with embryonic death in oviduct} = (\text{death in oviduct} \times 100) / \text{total eggs}$

$\% \text{ eggs with embryonic death during incubation} = (\text{death during incubation} \times 100) / \text{total eggs}$

$\text{Fertility rate (\%)} = (\text{total eggs} - \text{infertile}) \times 100 / \text{total eggs}$

III.6. Statistical analyses

For statistical analysis, Mann-Whitney U test and Kruskal Wallis ANOVA test of STATISTICA version 10 were used (STATSOFT INC., 2011). Mann-Whitney U test was applied for interpreting the *in vitro* evaluation parameters of semen. It is a nonparametric alternative to the t-test for independent samples. The data was arranged in the same way as for the t-test for two independent samples. The data-file contained a coding variable (independent variable) with two distinct codes that uniquely identify the group membership of each case in the data file. The variable under consideration was measured on an ordinal scale. The interpretation of the Mann-Whitney U test was computed based on rank sums. This test is the most sensitive nonparametric alternative to the t-test for independent samples. For such a small sized sample used in this study (8 samples in total, 4 samples of slow, programmable freezing method and other 4 samples of vitrification in pellet form protocol), STATISTICA computed an exact probability associated with the respective U statistic. This probability was based on the enumeration of all possible values of U, given the number of observations in the two samples.

The Kruskal-Wallis test is a non-parametric alternative to one-way (between-groups) ANOVA. The interpretation of the Kruskal-Wallis test is basically similar to that of the parametric one-way ANOVA, except that it is based on ranks rather than means. It was used to compare three groups of samples from *in vivo* evaluation of semen in this study. It tested the null hypothesis that

the different samples in the comparison were drawn from the same distribution or from distributions with the same median. And, the multiple comparison of mean ranks were used for all groups.

IV. RESULT AND DISCUSSION

IV.1. Results on *in vitro* evaluation

The statistical analysis ([Table 5](#)) showed that motility, concentration and morphological abnormalities of fresh semen collected for two freezing protocols are not significantly different ($p>0.05$). However, after freezing and thawing, compared with vitrification protocol, the slow, programmable freezing produced a significantly lower percentage of live, intact spermatozoa (17.99% vs. 21.35%, $p=0.030$) but a very significantly higher percentage of live, abnormal spermatozoa (23.06% vs. 10.02%, $p<0.001$). Comparison of quality of frozen-thawed semen produced from two freezing protocols is demonstrated in [Figure 1](#). The survival rate of live, intact spermatozoa was significantly higher in the pellet formation than in the slow protocol (28.59% vs. 23.53%, $p=0.022$). Interestingly, only 59.86% of spermatozoa was found dead in the frozen-thawed semen from slow, programmable protocol, while significantly higher percentage of dead spermatozoa was detected in the case of vitrification (68.63%, $p<0.001$).

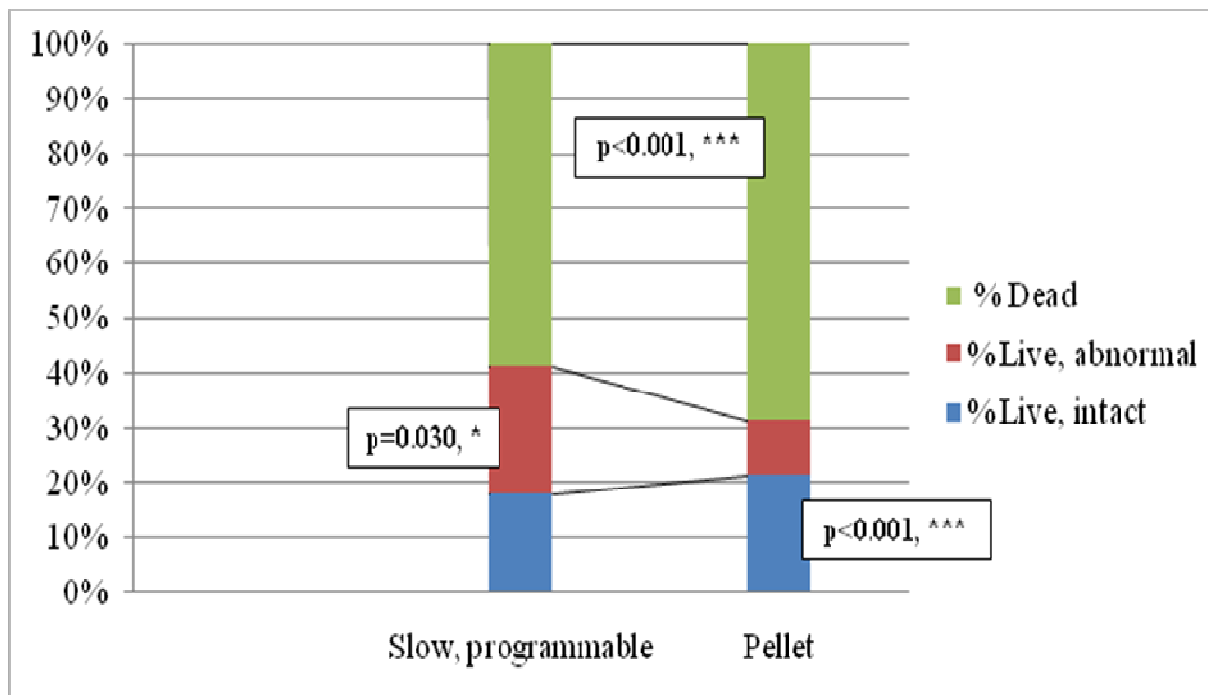


Figure 1: Comparison of quality of frozen-thawed semen produced from slow, programmable protocol and vitrification in pellet form.

Table 5: In vitro evaluation of fresh, frozen-thawed semen (Mann Whitney U test)

Characteristics		n	Mean	Standard deviation	Median
Fresh semen	Motility, score, p=0.388, NS				
	Slow, programmable	4	3.75	0.957	3.5
	Pellet	4	4.50	1.000	5.0
	Total	8	4.13	0.991	4.5
	Concentration, million/ μ l, p=0.061, NS				
	Slow, programmable	4	3.04	0.670	2.70
	Pellet	4	4.52	0.857	3.93
	Total	8	3.78	1.068	3.81
	Live, intact spermatozoa, %, p=1.000, NS				
	Slow, programmable	4	76.4	6.29	73.75
	Pellet	4	74.5	2.27	75.00
	Total	8	75.5	4.50	74.00
	Live, abnormal spermatozoa, %, p=1.000, NS				
	Slow, programmable	4	12.65	7.35	15.30
	Pellet	4	13.63	4.77	14.25
	Total	8	13.14	5.76	14.80
	Dead spermatozoa, %, p=1.000				
	Slow, programmable	4	10.03	2.69	10.85
	Pellet	4	11.88	4.59	10.50
	Total	8	11.40	3.52	10.50
Frozen-thawed semen	Live, intact spermatozoa, %, p=0.030, *				
	Slow, programmable	37	17.99 ^a	5.49	18.00
	Pellet	12	21.35 ^b	5.35	22.00
	Total	49	18.81	5.60	19.00
	Live, abnormal spermatozoa, %, p<0.001, ***				
	Slow, programmable	37	23.06 ^b	4.61	23.50
	Pellet	12	10.02 ^a	2.44	10.10
	Total	49	19.86	7.03	22.00
	Dead spermatozoa, %, p<0.001, ***				
	Slow, programmable	37	58.96 ^a	8.22	58.50
	Pellet	12	68.63 ^b	4.90	68.00
	Total	49	61.33	8.59	62.50
	Survival rate, %, p=0.022, *				
	Slow, programmable	37	23.53 ^a	8.35	23.48
	Pellet	12	28.59 ^b	7.36	28.95
	Total	49	34.77	7.60	24.49

NS: p>0.05

*: p<0.05

**: p<0.01

***: p<0.001

a,b: different superscript letters show significant differences (p<0.05) between groups

Table 6: In vivo evaluation of fresh, frozen-thawed semen (Kruskal Wallis-ANOVA test)

Characteristics		n	Mean	Standard deviation	Median
Semen used for AI	Concentration, million/ μ l, $p<0.001$, ***				
	Slow, programmable	8	1.40 ^a	0.148	1.41
	Pellet	8	2.73 ^b	0.307	2.76
	Control	8	2.88 ^b	0.576	3.12
	Total	24	2.34	0.774	2.45
	Live, intact spermatozoa, %, $p<0.001$, ***				
	Slow, programmable	8	23.08 ^a	10.79	25.00
	Pellet	8	20.21 ^a	6.80	23.75
	Control	8	76.58 ^b	5.89	75.75
	Total	24	39.95	27.59	27.50
	Live, abnormal spermatozoa, %, $p=0.034$, *				
	Slow, programmable	8	12.33 ^{ab}	5.41	12.90
	Pellet	8	8.18 ^a	2.75	8.25
	Control	8	13.93 ^b	4.94	16.00
	Total	24	11.48	4.98	11.25
	Dead spermatozoa, %, $p<0.001$, ***				
	Slow, programmable	8	64.6 ^b	15.64	63.00
	Pellet	8	71.74 ^b	8.93	69.15
	Control	8	9.54 ^a	2.97	8.95
	Total	24	48.63	30.12	61.25
Embryonic evaluation of incubated eggs	Infertile eggs, %, $p=0.027$, *				
	Slow, programmable	3	75.5 ^b	6.28	72.9
	Pellet	3	49.8 ^{ab}	11.63	56.3
	Control	3	15.2 ^a	6.36	16.2
	Total	9	46.8	27.21	56.3
	Embryonic death in oviduct, %, $p=0.128$, NS				
	Slow, programmable	3	25.41	8.10	23.53
	Pellet	3	11.79	14.18	4.55
	Control	3	3.23	3.52	2.70
	Total	9	13.48	12.79	6.98
	Embryonic death during incubation, %, $p=0.067$, NS				
	Slow, programmable	3	1.83	1.59	2.63
	Pellet	3	10.05	5.86	9.38
	Control	3	11.16	2.62	11.63
	Total	9	7.68	5.52	8.33
	Normal development, %, $p=0.038$, *				
	Slow, programmable	3	5.88 ^a	10.19	0.00
	Pellet	3	28.37 ^{ab}	24.40	6.25
	Control	3	70.46 ^b	11.70	60.47
	Total	9	34.90	31.86	6.25
	Fertility rate, %, $p=0.027$, *				
	Slow, programmable	3	25.5 ^a	6.28	17.39
	Pellet	3	50.2 ^{ab}	11.63	43.24
	Control	3	84.8 ^b	6.36	79.07
	Total	9	53.2	27.21	29.17

IV.2. Results on *in vivo* evaluation

The statistical analysis is shown in [Table 6](#). Insemination with fresh semen yielded 84.8%, while slow protocol and vitrification yielded 25.5% and 50.2% fertility rates, respectively. By increasing the number of inseminations the fertility increased and reached the peak level to the end of the third week in all groups. Inseminations with slow, programmable frozen-thawed semen produced a significantly higher percentage of infertile eggs compared to insemination with fresh semen (75.5% vs. 15.2%, $p=0.027$). The percentage of infertile eggs in case of insemination with frozen-thawed semen in pellet form was lower than in case of insemination with slow, programmable frozen-thawed semen (49.8% vs. 75.5%) and higher when compared to the control group (49.8% vs. 15.2%). The percentage of eggs with embryonic death was not significantly different between three groups. However, the percentage of eggs with very early embryonic deaths in oviduct were very high in the slow, programmable freezing group during the whole experimental period (week 1: 18.42%, week 2: 34.29%, week 3: 23.53%) while in the pellet freezing and control group, decreasing tendency was observed (from 28.1% and 6.98% in the 1st week to 4.5% and to 0% in 3rd week). Moreover, the percentage of eggs containing normal development in the slow, programmable group was extremely low compared with pellet and control group (5.88% vs. 28.37% vs. 70.46%, $p=0.038$). Fertility and embryo mortality in the three experimental groups following the artificial inseminations is demonstrated in [Figure 2](#).

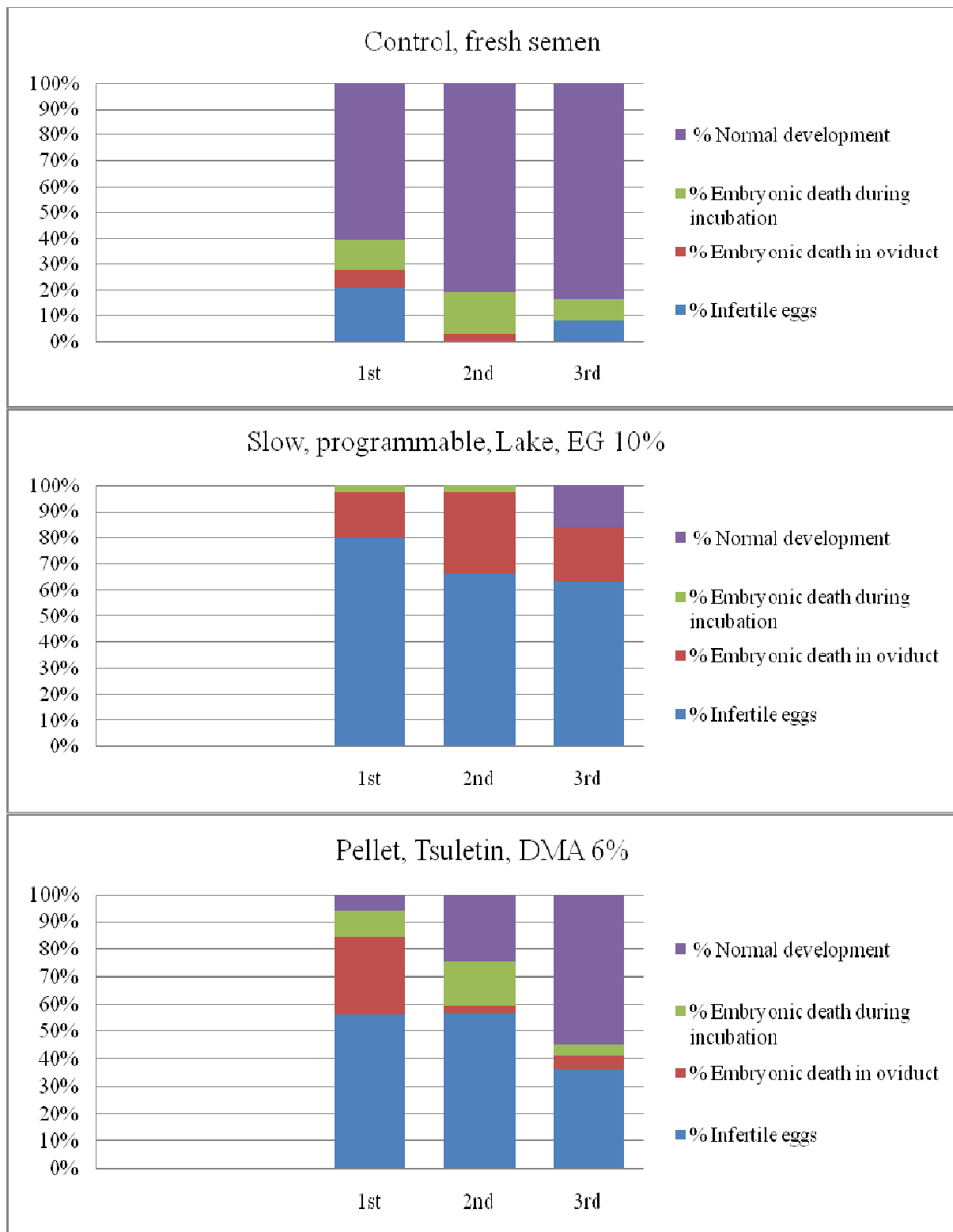


Figure 2: Fertility and embryo mortality in the three experimental groups following the artificial inseminations.

IV.3. Discussion

In the present study, fresh semen of guinea fowls were successfully collected by adapted dorso-abdominal massage technique from chicken semen collecting method and successfully frozen by both slow, programmable protocol with 10% EG and vitrification in pellet form with DMA 6%. They produced an acceptable average sperm survival rate of live and intact spermatozoa (23.53% and 28.59%). The highest sperm survival rate of live and intact spermatozoa, achieved in this study was 44.2% in case of the slow freezing and 36.49% in the pellet method. This result was absolute lower than the highest sperm survival rate in previous study (higher than 60%). Significantly higher percentage of live, abnormal spermatozoa was produced by slow, programmable freezing than produced by vitrification (23.06% vs. 10.02%, $p < 0.001$). However, the number of dead spermatozoa in pellet form was significantly higher than freezing by slow, programmable protocols (68.63% vs. 58.96%, $p < 0.001$). Although the live, abnormal spermatozoa are able to fertilize after AI (VÉGI et al., 2005), the high rate of early embryonic death (a mean of 25.41%) was observed in week 1 (18.42%), 2 (34.29%) and 3 (23.53%) while in case of pellet group, high rate of early embryonic death was observed only in the first week (28.13%), then it dropped dramatically in the second week (2.7%) and third week (4.5%) . This observation showed that percentage of live, abnormal spermatozoa in frozen-thawed semen might give an effective estimate of eggs with embryonic death produced after AI. Even though, not only *in vitro* but also *in vivo* tests were required for reliable testing of the effectiveness of freezing protocols applied in guinea fowl because the simple *in vitro* examinations used in this study were not able to detect injuries such as DNA damage of spermatozoa during the freezing and thawing process. The *in vitro* tests were necessary to predict the survival rate of spermatozoa and fertility rate in general and the *in vivo* tests were inevitable to demonstrate the true quality of frozen-thawed guinea fowl semen. According to fertility results of the slow protocol, 75.5% of eggs were infertile, despite the seemingly acceptable sperm survival rate. Contrary to the slow freezing protocol, frozen-thawed semen produced by pellet method produced 50.2% fertility rate after AI. Fertility rates of both slow, programmable and pellet group achieved in this study were higher than the mean fertility rate obtained in the first study (20%). Apart from quality of AI semen expressed by the number of intact, viable spermatozoa remaining in post-thawing semen, it was observed that the increasing of number of spermatozoa stored in the guinea hens oviduct by the 3rd weeks of AI might be important in determining the

fertility rate. The choice of cryoprotectant might be another factor that affected the fertilizing ability of frozen-thawed spermatozoa. Since, the fertility rates, 0% for EG and 8% for DMA, were reported in the first study, and 25.5% for EG, 50.2% for DMA, the cryoprotectant DMA seemed to be less deleterious to sperm cells than EG.

V. CONCLUSION AND RECOMMENDATION

In this study, vitrification in pellet form with Tselutin extender and cryoprotectant DMA 6% was proven to be another promising method for long term sperm storage of guinea fowl with 28.59% survival rate and 50.2% fertility. Although, the spermatozoa of guinea fowl are known to be very difficult to freeze, the highest survival rate 40.2%, produced by slow freezing protocol and highest fertility rate, 63.64%, produced by vitrification has been observed from this research. It is difficult to compare results reported in the first literature to the result obtained in this study due to variations in experimental designs such as differences in semen collecting schedule, semen dose, storage interval of semen, methods of freezing, methods of evaluating frozen/thawed semen. Therefore, the concurrent comparison of semen freezing systems in guinea fowls should be carried out in further researches studies. In order to establish an effective freezing protocol for guinea fowl semen with improved viability of spermatozoa during cryopreservation and fertilizing ability of frozen-thawed spermatozoa, the development of new sensitive *in vitro* assays to evaluate sperm function may be valuable assessments.

SUMMARY

Semen cryopreservation is a practical method for banking germplasm from valuable indigenous poultry species which have an increasing risk of extinction. Many scientific publications have described different protocols of avian sperm cryopreservation for both domesticated and non-domesticated avian species, involving cryoprotectant type and packaging method, as well as freezing and thawing rates.

However, there was only a single study (slow, programmable method) that described the sperm freezing of guinea fowl so far. In this research, a comparative approach was used to evaluate two freezing protocols for guinea fowls: the modified slow programmable method using 10% ethylene glycol (EG), and the newly applied fast freezing method (pellet formation) with 6% dimethylacetamide (DMA). The efficiency of two protocols is measured by both *in vitro* sperm evaluation assay (determination of sperm concentration and motility, morphological and live/dead sperm analysis) and *in vivo* sperm evaluation assay (artificial insemination, determination of fertility rate and embryonic death). For fertility determination candling of incubated eggs was used, extended by checking of the ratio of early embryonic mortality. *In vitro* qualification showed that the survival rate of live and intact spermatozoa was significantly higher after pelletation than after slow protocol (28.59% vs. 23.53%, $p=0.022$, Mann-Whitney U test). *In vivo* qualification; artificial insemination of frozen-thawed semen yielded 50.2% fertility rate with the pellet, while only 25.5% with the slow programmable method (while it was 84.8% in control, $p=0.027$, Kruskal-Wallis ANOVA test). The simple *in vitro* examinations used in this research were not able to detect injuries which caused embryonic death during freezing procedure, therefore, despite the seemingly acceptable sperm surviving rate, the ratio of eggs containing a normal embryo in the slow, programmable frozen-thawed semen artificial insemination case was significantly lower than in case of fresh semen artificial insemination case (5.88% and 70.46%, while it was 28.37% in case of pellets, $p=0.038$, Kruskal Wallis ANOVA test).

Key words: poultry, guinea fowl, sperm freezing, cryobank

ÖSSZEFOGLALÁS

A nagy genetikai értéket képviselő őshonos baromfifajok génmegőrzését elősegítő génbankok kialakítására az ondó mélyhűtéses tárolása a leggyakorlatiasabb megoldás. Számos tudományos közleményt találunk az egyes baromfifajok és vadmadarak ondómélyhűtésével kapcsolatban, melyek fagyasztási protokolljaikban különböző krioprotektánsokat, hűtési- és felolvasztási sebességeket, valamint tárolási módokat alkalmaznak.

A gyöngytyúk ondómélyhűtésére vonatkozóan a szakirodalomban pillanatnyilag egyetlen (lassú programozott eljárás) adatot találhatunk. Kísérletünkben kétféle mélyhűtési protokollt hasonlítottunk össze: a módosított lassú programozott eljárást 10% etilén-glikolt használva krioprotektánsként, valamint az újonnan alkalmazott pellet-módszert 6% dimetil-acetamid (DMA) hozzáadásával. A mélyhűtési protokollok hatékonyságát *in vitro* (spermium-koncentráció, motilitás, morfológiai rendellenességek és élő-holt sejtarány meghatározása) és *in vivo* (mesterséges termékenyítés sikeressége) vizsgálatokkal is ellenőriztük. A termékenység meghatározásakor a lámpázás során kiesett tojásokban vizsgáltuk a korai embrióelhalások mértékét is. *In vitro* vizsgálataink azt mutatják, hogy a pellet-módszer esetében szignifikánsan jobb volt az élő, normális morfológiájú sejtek túlélési aránya, mint a lassú, programozott protokollnál (28,59% vs 23,53%, $p = 0,022$, Mann-Whitney U teszt). *In vivo* eredményeink szerint a mesterséges termékenyítési periódus végére a fagyasztott-felolvasztott ondóval való termékenyítés a pellet-módszernél 50,2%-os, míg a lassú programozott módszernél csak 25,5%-os termékenységet eredményezett (míg ez az érték a kontroll csoportban 84,8%, $p=0,027$, Kruskal-Wallis ANOVA teszt). *In vitro* vizsgálataink feltehetőleg nem mutatták ki, a korai embrióelhaláshoz vezető, a mélyhűtési eljárás során bekövetkezett sérüléseket, ennek köszönhető, hogy a lassú, programozott eljárás esetében az elfogadható túlélés ellenére a normális fejlődésű embriót tartalmazó tojások aránya nagyon alacsony maradt (5,88% és 70,46%, míg ez az érték a pellet-módszer során 28,37% volt, $p = 0,038$, Kruskal-Wallis ANOVA teszt).

Kulcsszavak: baromfi, gyöngytyúk, ondómélyhűtés, kriobank.

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APPENDICES

Appendix 1: Photo of guinea fowls keeping in individual cages (KÁTKI, 2010)



Appendix 2: Photo of semen collection (KÁTKI, 2010)



Appendix 3: Photo of guinea fowl AI (KÁTKI, 2011)



Appendix 4: Photo of cooler cabin for equilibration (KÁTKI, 2010)



Appendix 5: Photo of Planer CRYO 10 freezing machine (KÁTKI, 2010)



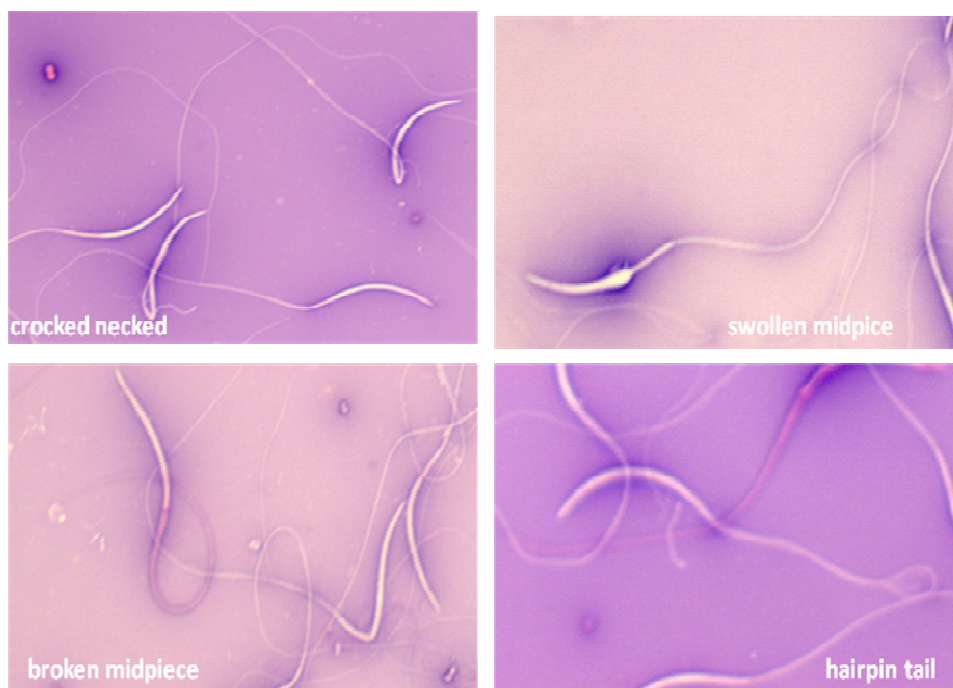
Appendix 6: Photo of pellet formation (KÁTKI, 2010)



Appendix 7: Photo of Accucell, IMV, France (KÁTKI, 2010)



Appendix 8: Photo of morphological abnormalities (KÁTKI, 2010)



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