# Preparation technology for porcine frozen semen

ChangHau propagation station of TLRI

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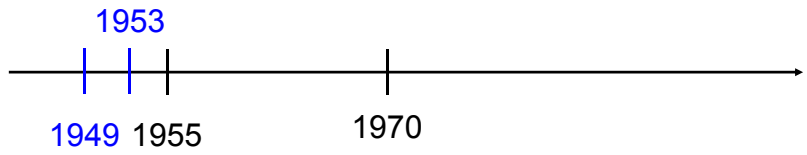
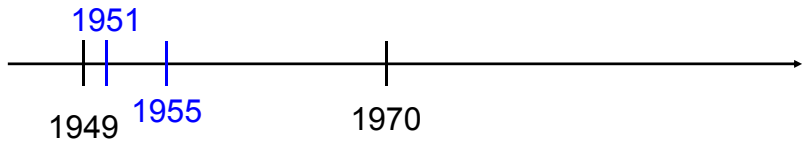
- The development on sperm cryopreservation
- The procedure of boar semen cryopreservation
- To increase the qualities and efficiency

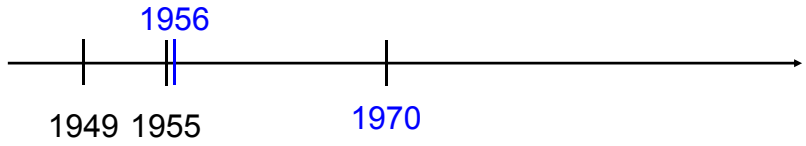
# The development on sperm cryopreservation




- 1949 -- the glycerol provide the foundation for cryopreservation of human sperm (Polge et al.)
- 1953 -- First successful human pregnancy using frozen sperm
  -- Frozen human sperm with 10 percent glycerol on dry ice with a 67% survival rate
  (Sherman and Bunge)




- 1951 --- first calf born using frozen bull sperm
- 1955 --- demonstrated the bull sperm frozen to −79°C on dry ice could still yield high fertility (Bratton et al.)




- 1956 --- first piglet born using frozen boar sperm (Polge et al.)
- 1960 --- Sperm frozen and stored using liquid nitrogen
- 1970 --- the fertility of frozen boar sperm was reassured (Polge et al.)

- ✓ Now, the cryopreservation and artificial insemination (AI) is used on bull industry worldwide
- ✓ And the frozen boar sperm is commercialized

## The procedure of boar semen cryopreservation

- ➢ **The major Composition of semen cooling and freezing extender**
- ➢ **Boar semen preparation**
- ➢ **Boar semen freezing/thawing procedure**

# The procedure of boar semen cryopreservation

- **The major Composition of semen cooling and freezing extender**
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- Carbohydrates
  -- glucose, lactose, fructose, raffinose(棉子糖), saccharose and trehalose (海藻糖 )
- Buffers
  -- TRIS
- Salts
  --- sodium citrate, citric acid
- egg yolk
- antibiotics

- Cryo-protectant agent
  --- glycerol, Orvus ES Paste (OEP) dimethylacetamide (DMA), Dimethyl sulfoxide (DMSO)

## Media components

**common used in freezing sperm**

| Species | Main media components | |
|---|---|---|
| | **Base** | **Cryoprotectant** |
| Bovine | Tris, fructose, citrate | Yolk, glycerol |
| Ovine | Tris, glucose, citrate | Yolk, glycerol |
| Equine | Skim milk, glucose, lactose | Yolk, glycerol, SDS[1] |
| Porcine | Lactose | Yolk, glycerol, SDS[1] |
| Human | HSPM[2] | Glycerol |

[1] sodium dodecyl sulphate
[2] human sperm preservation medium

from Bathgate, 2004

## Optimal glycerol concentrations of different species

| Species | Optimal glycerol concentration (%) |
|---|---|
| Koala (無尾熊) | 15-20 |
| Bovine | 4-8 |
| Primate (靈長類動物 ) | 4-8 |
| Ovine (綿羊) | 3-4 |
| Porcine | <3 |
| Murine | <1.75 |

from Bathgate, 2004; Holt, 2000

## Commercial basal cooling extender/boar

| | Volume (ml/100ml) | | |
|---|---|---|---|
| **Ingredient** | **A** | **B** | **C** |
| 11%(w/v)lactose solution | - | 75 | 80 |
| Boarciphos A solution | 80 | - | - |
| Egg yolk | 20 | 25 | 20 |
| pH (± 0.5) | 6.4 | 5.5 | 5.5 |
| Osmolarity (± 10) | 345 | 370 | 345 |

from Bathgate, 2004

## Commercial basal freezing extender/boar

| | Volume (ml/100ml) | | |
|---|---|---|---|
| **Ingredient** | **A** | **B** | **C** |
| **Cooling extender** | - | 92 | 89.5 |
| Boarciphos B solution | 80 | - | - |
| Egg yolk | 20 | - | - |
| Glycerol | 8.1 | 7.5 | 9 |
| Equex STM | 1.6 | 1.3 | 1.5 |
| pH (± 0.5) | 6.4 | 6.4 | 6.4 |

from Bathgate, 2004

## Generally cooling/thawing rates

In general, on boar semen freezing to get the best sperm survival

- cooling rate of -30 to -50ºC/minute
- thawing rate of 1200 to 1800ºC/minute

(Großfeld et al., 2008)

# The procedure of boar semen cryopreservation

- 
- **Boar semen preparation**
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## The guiding principle for boar semen preparation

- In general,
  ---- a higher semen quality is needed to produce higher quality frozen boar semen

## Cooling procedure

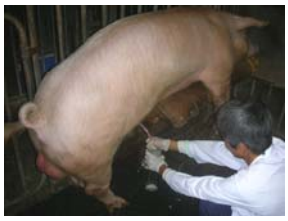
- Semen collection and quality determination
- BTS dilution, cooling to 15℃( 3 h）
- 3000rpm (800×g)/10 min centrifugation
- Cooling extender re-suspension at 15℃（3 h）
- Add freezing extender (4℃)
- Loading into 5ml straw

## Freezing procedure

- Cooling to-5℃( -10℃/min）
- Freezing to -80℃( -40℃/min）
- Deceasing to -120℃( -20℃/min)
- Plunge into liquid nitrogen

## Boar semen collection

- Most of boar semen is collected with hand glove technique



## Semen quality evaluation

---to determine the sperm concentration, motility, morphology using spectrophotometer, microscopy, or commercial boar semen quality determine machine

---and we also microscopy to make double check for semen qualities

## Semen extension

- The collected sperm-rich boar semen is diluted with 1-2 times extender (BTS, Beltsville thawing solution )

## Cooling

- The semen was cooled to room temperature (25ºC) for 2 hours, and then to 16ºC over 2 hours

## Shipping

The samples should be packaged inside a container that contains cool gel packs at 16°C in order to keep the right temperature of the semen samples

## Shipping container


# The procedure of boar semen cryopreservation

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- **Boar semen freezing/thawing procedure**

## Centrifugation

- The samples are centrifuged for 10 minutes at 3000 rpm (800xg), at 16ºC.
- The supernatant is removed and the sperm pellets re-suspended

## Semen pellet resuspending

- The samples are first diluted to half of the final volume with the cooling extender at 16ºC. And then the resuspended samples cooled to 4ºC/3 hours.
- The second dilution is the freezing extender (with glycerol) which also half final volume is used, at 4ºC.

- After 10 minutes
- The samples are loading into 5ml straws and then freezing it, using the freezing machine


A kind of freezing machine

- Using the freezing machine with the following curve:
  5ºC to -5ºC at -10ºC per minute,
  -5ºC to -120ºC at -40ºC per minute.
  The samples are then plunged in liquid nitrogen for storage

## Thawing Process

- the straws in water at 50℃ for 45 sec, and then at 30℃ /10min
- After thawing, the semen was diluted (1:8) with an extender (BTS, 30℃)
- The thawed semen is need insemination immediately, in general no more than 30 minutes

# Fertilizing capacity of frozen boar semen

## Fertilizing capacity of frozen boar semen*

| | Number |
|---|---|
| Gilts inseminated | 13 |
| Gilts with fertilized | 11 (85%) |
| Ova recovered | 125 |
| Normal fertilized ova | 104 (83%) |

* Using pellet frozen semen

Pursel and Johnson 1975

## The fertility and litter size on frozen and fresh semen

| Type of semen used | Fertility | Litter size | |
|---|---|---|---|
| | (%) | Total piglets | Live piglets |
| Frozen-thawed* | 72.3±3.8** | 11.3±0.3 | 10.1±0.3 |
| Liquid fresh semen | 78.8±3.6 | 11.6±0.3 | 10.2±0.3 |

* Using FlatPack container
** LS means±S.E.M

Eriksson et al., 2002

## Our results

## Fertilizing capacity of frozen boar semen*

| | July | August | Total |
|---|---|---|---|
| Swine inseminated | 5 | 7 | 12 |
| Fertilized swine | 3 (60%) | 6 (85%) | 9 (75%) |

* Using 5ml straw frozen semen

## The piglet in this study

Litter size: 10.5 piglets

## To increase the qualities and efficiency on boar semen cryopreservation

- Although cryopreservation of boar semen for AI was developed 40 years ago
- In general believe that AI with frozen-thawed sperm were still can not achieve the fertility levels similar to using fresh semen. Especially at field farm
- And it still limited in pig industry

The use of frozen boar sperm in the industry is less than 1%, using frozen-thawed boar semen

(Johnson et al., 2000)

The major reasons to limit on pig industry using frozen-thawed semen:

- higher cost per dose
- The lower survival rates of frozen-thawed boar sperm, --- resulting lower fertility rates, compared to fresh liquid semen

- But frozen boar semen is still useful and valuable for breeding

## The lower survival rates of frozen-thawed boar sperm

- Boar sperm are more sensitive to stress by changing
  ---osmotic balance
  ---low-temperature exposure
  ---cryoprotectant intoxication
  ---oxidative stress

Many researcher tried

**to increase the qualities of frozen-thawed boar sperm**

## 1、Cryopreservation packaging

- Different container (package) have been developed such as
  ---pellets(1971)
  ---0.5ml straw
  ---maxi-straw (5 mL) (1975),
  ---plastic film bag (many shapes and sizes, 1975-1995)

## 2、Add antioxidants or chelating agents

- Because, boar sperm exposed to lipid peroxidation during freezing and thawing, ----which causes damage to the sperm membranes and reduce energy metabolism
- The added antioxidants or chelating agents Ex. catalase, vitamin E, glutathione, butylated or superoxide dismutase

(Großfeld et al., 2008)

## 3、Individual differences on boar

- --- The range of sperm freezability among individuals is large difference

## 4、develop convenient frozen procedure

The freezing procedure usually takes between 8 and 9 hours from collection to storage in liquid nitrogen, being still inconvenient.

## AI strategies is important for efficiency

- Because of the low sperm survival on frozen-thawed boar semen
- Artificial insemination strategies to improve the fertility become very important

## Estimating oestrus/ovulation

- Owing to shorter life span of frozen-thawed boar semen, the semen require an AI-to-ovulation interval not longer than 4-6 h

## Timing of insemination

- Estimation the duration of oestrus is important to establish appropriate AI-schedules
- Swine ovulation most often occurs when two-thirds of oestrus passed

## OPTIMUM TIME TO INSEMINATE WITH FROZEN SEMEN

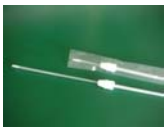
| | Single Insemination | Double Insemination |
|---|---|---|
| **Gilts** | 29-32 h | 1st 24-29 h |
| | | 2nd 30-34 h |
| **Sows** | 33-36 h | 1st 29-32 h |
| | | 2nd 34-38 h |

The times are after the sow or gilt exhibits standing estrus

From SGI, LTD.

## Selecting AI catheter


- **Foam tip AI catheter**



- **Deep foam catheter for deep insemination**


- **Deep intra uterine insemination catheter**

from http://www.sound-ai.com/

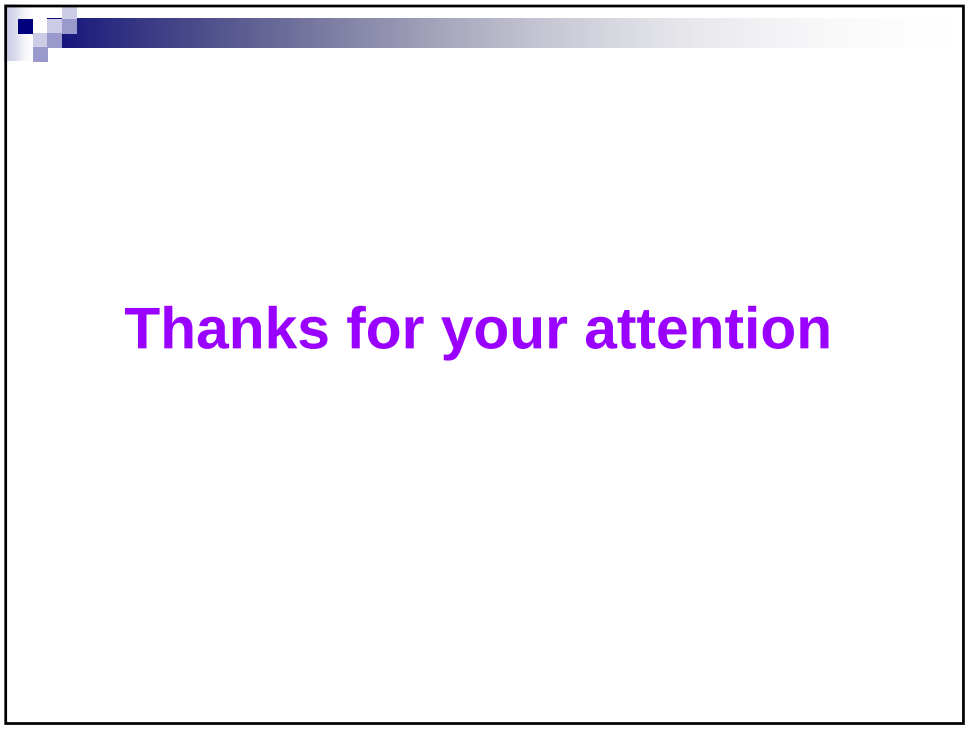
## Using deep (uterine) insemination

- which allows placement of a lower semen dose
- Fresh boar semen with deep intra-uterine insemination, the sperm per dose has been decreased from 6 to 1 billion ($10^9$) sperm without decreasing litter size

(Großfeld et al., 2008)

## Conclusion

- Frozen boar semen is important and useful for breeding
- No method has been found that it drastically improved frozen-thawed quality on boar semen


Thanks for your attention