# EFFECT OF SEMEN THAW METHOD ON PREGNANCY RATES IN HOLSTEIN HEIFERS

by

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## REVIEW OF LITERATURE

In the last ten years packaging of frozen semen has changed from the glass ampule to the plastic straw. The increasing popularity of this packaging method can be attributed to: 1. the development of automated systems for packaging semen in plastic straws, 2. reports of higher conception rates for semen stored in straws, 3. more efficient spermatazoal utilization and 4. a smaller storage space per unit of semen (16). As a result of this change in semen packaging new semen handling procedures must be evaluated under field conditions. Rate of thawing is reportedly one of the major factors affecting post-thaw quality of semen. The accepted procedure for thawing semen in glass ampules is an ice water bath (15), but the best method for thawing semen in plastic straws is controversial at present.

### Methods of Semen Thaw

#### Semen thaw and morphological studies

Post-thaw acrosomal retention accounts for approximately 65% of the variation in fertility between bulls, and 30% of this variation in fertility can be attributed to differences in progressive motility (22). Furthermore, a significant correlation exists between post-thaw motility and maintenance of the acrosome. Motility and acrosomal morphology are the standard laboratory analyses performed to evaluate processing and handling procedures for semen frozen in plastic straws. The rate at which acrosomal deterioration occurs is markedly affected by semen handling techniques (21).

In an experiment to determine the effect of freeze rate, thaw rate and glycerol level on maintenance of the acrosome and motility, Saacke et al. (23) found that freeze rate did not have an effect on percent motile sperm but did influence percent intact acrosomes. A highly significant ($P<.01$)

interaction between thaw rate and glycerol concentration existed favoring fast thawing of semen. These workers concluded that optimum recovery of sperm can be achieved by: 1. freezing at the rate of -10 to -80 C in 160 s in nitrogen vapor, 2. a final glycerol concentration of 8.5% and 3. thawing in a 65 C water bath for 7.5 s. In a similar experiment, Rodriguez et al. (19) compared rates of cooling, thawing and glycerol levels to post-thaw motility of semen frozen in straws. Their results indicated that fast freezing (-5 to -130 C in 3.5 m) was superior to moderate (20 m) or slow (40 m) freezing. Optimum levels of glycerol were determined to be between 7 and 11% when averaged over the different rates of freezing and thawing. Thawing rates of 5 C for 3 m, 35 C for 12 s, 55 C for 8 s, 75 C for 7 s and 90 C for 5 s were compared and it was found that post-thaw motility increased significantly ($P<.01$) as thaw rate increased from 5 C for 3 m to 55 C for 8 s. However, furthur increases to 90 C were not beneficial. A faster thawing rate was found to be advantageous by Robbins et al. (17) when rates of cooling, thawing and level of glycerol were compared to acrosomal cap retention. The estimated optima in this experiment were a freeze rate of 11.64 C per m, a glycerol level of 10.22% and a thaw temperature of 92 C. It was determined that rate of thaw had the greatest effect on the acrosomal cap while freeze rate had the least. In a subsequent study, using the same experimental design, these investigators demonstrated effects for glycerol level, freeze rate and thaw rate on acrosomal retention and motility (18). The results of this experiment agreed closely with the work of Saacke et al. (23) indicating that minimum cell injury resulted with a 65 C water thaw, 8.5% glycerol level and a freeze rate of 26.34 C per m.

In a 4 X 4 factorial experiment Almquist and Wiggin (4) compared

the effects of different rates of freezing and thawing on progressive motility. Differences among cooling rates and the interaction of cooling rate X thawing rate were not significant, when thawing rates of 5 C for 2 m, 35 C for 15 s, 75 C for 10 s and 95 C for 7 s were examined over the four cooling rates. Thawing temperatures of 75 C and 95 C yielded greater motility than 35 C. 35 C, 75 C and 95 C were superior ($P<.01$) to 5 C. Contrary to the results indicated in the first investigation by Robbins et al. (17), no significant difference between 75 C and 95 C was found, indicating that sperm survival is not improved by thawing at temperatures greater than 75 C, and an optimum thawing temperature exists somewhere between 35 C and 75 C. When thawing rates of 95 C for 6.5 s, 115 C for 5.5 s and 135 C for 5.0 s were compared Almquist (2) showed that thawing temperatures above 95 C neither improved nor damaged post-thaw motility or acrosomal maintenance. In an earlier investigation using percent unstained spermatoza as an indicator of post-thaw semen quality, Aamdal and Andersen (1) observed that thawing at 4 C or 20 C was inferior to thawing at 35 C and that thawing at 35 C was inferior to thawing at 75 C.

Wiggin and Almquist (27) compared glycerol levels of 7, 9, 11, 13, and 15%; equilibration times of 1, 2, 4, 8 and 16 h; and thawing rates of 35 C for 15 s, 50 C for 13 s, 65 C for 11s, 80 C for 9 s and 95 C for 7 s. As predicted by multiple regression, optimal post-thaw motility was observed with a combination of 10.7% glycerol, 2 h equilibration time, and 76 C water thaw. These findings are in agreement with the work of Rodriquez et al. (19). In a 2 X 4 factorial experiment the effects of glycerol equilibration time and thawing rate on acrosomal maintenance and progressive motility were investigated by Wiggin and Almquist (26). Equilibration times of .5 and 2 h were similar. Thawing rates of 35 C for 15 s, 55 C for 12 s, 75

for 9 s and 95 C for 7 s were compared and a significant increase ($P<.01$) in percent intact acrosomes and percent motility for each increase in thawing rate was observed. This is in disagreement with other investigations which indicated no advantage in increasing the thawing temperature above 75 C. The interaction of equilibration time X thaw rate was significant ($P<.01$) for percent intact acrosomes in favor of .5 h but was not significant for motility. In a similar experiment, Gilbert and Almquist (13) compared the effects of cooling time (from 25 C to 5 C in .5 or 3.5 h), equilibration times (0, 3 or 9 h) and thawing method (35 C for 10 s, 65 C for 7.5 s or 95 C for 6 s) on acrosomal retention and motility. Interactions of equilibration time with cooling time and thawing method were significant ($P<.01$). Acrosomal retention and motility were higher ($P<.01$) after cooling 3.5 h if semen was not equilibrated. Thawing at 65 C or 95 C was superior ($P<.01$) to thawing at 35 C. Acrosomal retention and motility were best ($P<.01$) after 9 h of equilibration when semen was thawed at 35 C; or after 3 or 9 h when thawed at 65 or 95 C. These results concur with the findings of Almquist and Wiggin (4), who found no differences in thawing at 75 and 95 C. Ennen et al. (10) utilized a factorial design to determine the effects of cooling rate and glycerol equilibration time on post-thaw motility of semen thawed in ice water for 3 m, by rolling between the palms of the hands for 50 s, or in 75 C water for 7 s. Optimum motility was observed when straws were either cooled to 5 C in 2 h, with a 4 or 10 h equilibration period or when cooled in 4 h with a 2 or 4 h equilibration period. Semen thawed in 75 C water resulted in significantly higher ($P<.01$) motility than semen thawed by the other two methods.

When thawing rates of 35 C for 1 m and 5 C for 3 m were compared by Senger and coworkers (24) with post-thaw treatments of 1, 20 or 37 C water

baths, significant differences ($P<.01$) in both motility and acrosomal maintenance due to thaw rates were found. Also a significant interaction ($P<.01$) existed between thaw rate and post-thaw treatment for both acrosomal maintenance and motility. Semen thawed at 35 C had significantly greater ($P<.01$) acrosomal retention than semen thawed at 5 C following 20 and 37 C post-thaw treatments. Semen thawed at 5 C suffered significant acrosomal damage ($P<.01$) with 20 and 37 C post-thaw treatments when compared to 1 C. These workers concluded that rapid thawing of semen is superior to slow thawing. In a similar experiment the same conclusion was drawn by Almquist (3) when he subjected semen thawed in either 35 C or 5 C water to post-thaw incubations of 37 C and 5 C. He noted that rapid thawing was consistently superior ($P<.01$) to slow thawing and that cold shock was only seen when semen was thawed rapidly in warm water to above 5 C and then subjected to temperatures below 5 C in an ice water bath. Yotts and Wells (28) exposed semen thawed at 0 C for 2 m, 20 C for 1 m, 35 C for 20 s and 95 C for 7 s to incubation periods of 5 m, 1h, 3 h and 5 h at 37 C. Averaged over all incubation periods, the highest percentage of live sperm and of normal cells with intact acrosomes were obtained with a thawing temperature of 35 C. This is in contrast with the findings of many investigators who determined that higher thawing temperatures were superior to 35 C (1,4,13, 17,26). D'Abreu et al. (8) utilized a factorial experiment to study the influence of rate of warming after thawing on semen thawed at different rates. Straws were thawed at 5 C for 2 m, 35 C for 12 s or 75 C for 6 s, warmed to a final temperature of 35 C and incubated at 37 C for 2 h. These workers evaluated semen for motility and percent intact acrosomes and concluded that warming of semen after thawing, such as might occur with exposure to high ambient temperatures in the field, was not detrimental

to semen quality.

A study by Saacke et al.(23) compared rate of thaw from -20 to 0 C for thawing temperatures of 5 C for 2 m, 20 C for 1 m, 35 C for 30 s, 50 C for 15 s and 65 C for 7.5 s. Rates of thaw (degrees C/s) were 0.4, 1.3, 2.5, 3.9 and 7.0 respectively.

Thaw method and fertility

Few studies have been conducted comparing methods of semen thaw and fertility. According to laboratory investigations, fast thawing of semen is superior to all other methods. However, conflicting results make recommendations for practical use difficult.

In a study comparing semen thawed either before insemination (temperature unknown), or thawed "in cow", Davidovic and coworkers (9) achieved conception rates of 74.05% and 71.94%, respectively. In another study Rugg et al.(20) compared "in cow" thaw with 75 C for 7 s thaw. In their first trial 218 cows were inseminated and a pregnancy rate of 38.6% vs 23.0% ($P<.05$) in favor of hot water thaw was observed. In a second trial 73 cows were inseminated with pregnancy rates of 29.7% vs 22.2% in favor of hot water thaw. This difference was not statistically significant. The authors noted that overall pregnancy rates were low because the cattle were synchronized to estrus and that greater differences in pregnancy rates between thaw methods were not seen due to the high concentration of the semen they were using, which possibly masked the effects of thaw method on fertility. In contrast to these studies Jondet (14) found thawing "in vivo" superior to thawing at 35 C for 7 s. In his experiment a mean advantage of 3.45 percentage units in conception rate in favor of "in vivo" thaw was obtained. In another study using "in cow" thaw technique with 23 services, Essich (11) obtained a 30-60 day non return rate of 65.2% but no other thaw methods

were used for comparison. In another fertility study, Stewart (25) compared thawing semen at 35 C for 15 s, ambient temperature water (4 to 24 C), or by thawing "in the gun" (similar to "in cow" thaw). Nonreturn rates of 61.8, 63.1 and 63.8% for the three methods, respectively, showed no significant differences due to thaw rate but it is interesting to note that highest nonreturn rate was achieved with "in the gun" thaw while lowest nonreturn rate was seen with warm water thaw. These results are in agreement with those of Jondet (14).

Bean (6) compared ice water thaw (4 C), air thaw (temperature unknown) and warm water thaw (35 C) and obtained 60-90 day nonreturn rates of 71.1, 72.8 and 72.2% respectively, indicating no differences between air thaw and warm water thaw which were both superior to ice water thaw. Another investigation by Forde and Gravir (12) comparing cold water (10 C), warm water (35 C for 30 s) and warm water (35 C for 12 to 15 s) thawing methods showed that nonreturn rate was lower for cold water thaw than for the two warm water thaws, which were not significantly different from each other. In an extensive field trial, Almquist (5) compared thawing rates of 35 C for 12 s and 35 C for 30 s. In contrast with the results of Forde and Gravir, significantly higher non return rates were observed with semen thawed for 30 s (72.0%) than for semen thawed for 12 s (70.1%)

In two other trials conducted by Rugg and coworkers (20) comparing ice water thaw (3 C for 2 m) with 75 C for 7 s thaw, and 75 C for 7 s thaw with 35 C for 12 s thaw pregnancy rates of 45.9 vs 49.1% and 55.3 vs 56.0% respectively were obtained. No significant differences existed between thaw methods contrary to the findings of many laboratory studies.

Further fertility investigations will be necessary to determine the optimum method for thawing semen in the field. Laboratory findings are not

in total agreement with field trials, and the advantages of rapid thawing may be small. Berndtson et al. (7) concluded that, on the basis of present laboratory and fertility trials, rapid thawing of semen in warm water appears to be conducive to maximum reproductive efficiency, and that the differences in fertility due to thawing method may be minimized by the use of sufficient numbers of spermatazoa per insemination, when factors such as bull fertility and inseminator competance are optimal.

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# EFFECT OF SEMEN THAW METHOD ON PREGNANCY RATES IN HOLSTEIN HEIFERS


## ABSTRACT

Two procedures for thawing semen were compared in a fertility experiment involving 118 Holstein heifers over a 9 month period. Semen packaged in .5 ml French straws was thawed either by hot water (65 C for 7 s) or by "in cow" technique. Pregnancy rate to all services was significantly higher ($P<.005$) for heifers bred by hot water thaw than by "in cow" thaw (84.0% vs 61.8%). First service pregnancy rates favored ($P<.06$) hot water thaw (76.9% vs 60.3%).


## INTRODUCTION

Plastic straws for storage of frozen bull semen have become increasingly popular in recent years due to several advantages over glass ampules (9). Rate of thawing is one of the major factors affecting post-thaw quality of semen and considerable work has been done to determine the best method of thawing semen packaged in plastic straws. On the basis of post-thaw acrosomal maintenance, progressive motility or percent unstained sperm researchers have determined optimum thawing temperatures between 65 C and 76 C (1,3,10,12,15). There is concern that temperature shock might occur after semen is thawed rapidly in hot water during periods of cold ambient temperatures. However, reseachers investigating the effects of post-thaw treatments on acrosomal maintenance and motility concluded that rapid thawing of semen was consistently superior to slow thawing and cold shock was detrimental only when the temperature of semen was raised above 5 C during rapid thawing and lowered suddenly to 5 C by exposure to an ice water bath (2,13).

Fertility can be greatly affected by semen handling techniques, but few fertility trials have been conducted to substantiate the advantages of rapid thawing found in the laboratory. The objective of this study was to compare the effects of hot water thawing of semen to thawing "in cow" on pregnancy rates.

## EXPERIMENTAL PROCEDURE

This experiment was conducted from October 1978 to June 1979 with heifers in the Kansas State University Dairy Research herd. Semen was obtained from routine collections of three Holstein bulls housed at the Kansas Artificial Breeding Service Unit. Semen was extended in a Na citrate-egg yolk medium containing antibiotics and cooled to 5 C in 2.75 h. The final extended semen, packaged in French straws, had a 6% glycerol level and a minimum post-thaw concentration of $17 \times 10^6$ motile sperm/ml. Heifers, as they came into estrus, were bred to predetermined sires, alternately by "in cow" or hot water (65 C for 7 s) thaw technique. Repeat breeders were handled in the same manner of alternate thaw technique regardless of method used on previous service(s), but service sire was not changed. Heifers entered the breeding herd when a minimum weight of 341 Kg and age of 13 mo was attained. Heifers were observed for estrus twice daily and inseminated approximately 12 hours after detection.

Semen for "in cow" thaw technique was removed from the liquid nitrogen (LN) unit, and the end of the straw to be clipped was rolled between the fingers until semen at that end was thawed. That end was then clipped, the straw was loaded into the sheath, and insemination performed immediately. For hot water thaw, water in a clear glass jar was heated by an electric immersion heater and stirred frequently with a stainless steel

dial thermometer to assure even water temperature. The jar was illuminated by a flashlight and when water temperature reached 65 C the heater was unplugged. The semen straw was then removed from the LN unit by forceps and submerged in the water bath. By careful observation it could be determined when semen had thawed by noting a thin ribbon of frozen semen form and dissipate within the straw. At 7 seconds the ribbon formed and the straw was removed from the water bath, dried with tissue paper, and the end clipped. A semen carrying device, consisting of two stainless steel tubes containing sheath and French gun fixed to a piece of plastic, was placed under the technician's clothing during preparations. When the straw was thawed it was loaded into the sheath contained in the semen carrier, carried to the breeding chute, and inseminated immediately. The semen carrier was used for all hot water services, regardless of climatic conditions, throughout the experiment.

All inseminations were performed by one technician. Pregnancy was determined by palpation per rectum between 40 and 60 days from the last breeding.

Statistical analysis was performed by use of multidimensional chi-square analysis. Three factors were used: M(Method of thaw), S(Sire) and C(Conception, a yes or no dichotomous response). All effects were tested using the methods described by Brown (5). To evaluate the effects of climatic conditions on the effectiveness of the two methods of thawing, analysis of covariance was used, using ambient temperature as a covariate.

## RESULTS AND DISCUSSION

The chi-square analysis showed a highly significant ($P<.005$) conception by method interaction effect for total services performed. Table 1 shows

that the pregnancy rate was higher for the hot water thaw (84.0% vs 61.8%). For first service pregnancy rate the conception by method interaction effect approached significance ($P<.06$), again showing higher pregnancy rate for the hot water thaw (Table 2). No differences in conception due to sire was found for either the first service or total services results.

Analysis of covariance failed to show any effect of ambient temperature on conception rate for either method of thawing semen. We feel that the semen carrying device minimized the possibility of cold shock for the thawed semen.

Only those heifers diagnosed pregnant by the termination date of the experiment were included in the statistical analysis. One heifer was deleted from the study due to a closed cervix.

Our findings agree with those of Davidovic et al.(5), who achieved conception rates of 74.0% for 166 inseminations with semen thawed prior to insemination (temperature unknown) compared to 71.9% for 392 inseminations with semen thawed "in cow". Rugg et al.(11) conducted two trials comparing hot water thaw (75 C for 7 s) with "in cow" thaw. Pregnancy rates of 38.6% for 110 cows and 23.0% for 108 cows in favor ($P<.05$) of hot water thaw were observed. In a second trial pregnancy rates of 29.7% for hot water thaw and 22.2% for "in cow" thaw were not different statistically. Overall low pregnancy rates in this experiment were probably due to an estrus synchronization treatment. These workers suggested that the lack of significance in the second trial was due to the high concentration of spermatazoa prior to freezing ($30x10^6$/inseminate) which would tend to mask any real effect of thawing method. However, differences in pregnancy rate were detected in our study using semen with a minimum concentration of $27x10^6$ motile spermatazoa per inseminate prior to freezing. In another fertility trial

Stewart (14) compared thawing semen at 35 C for 15 s, ambient temperature water (4-24 C), and by thawing "in the gun" (similar to "in cow" thaw). Nonreturn rates of 61.8, 63.1 and 63.8% were similar for the three methods. In an extensive field trial Jondet (7) achieved a mean advantage of 3.45 percentage units in nonreturn rate in favor of "in vivo" thaw over warm water thaw (35 C for 7 s). This disagreement with our data may be due to technician effects, because in his experiment one technician bred by "in vivo" thaw technique compared with 98 technicians using warm water thaw.

Most of the fertility problems associated with the use of frozen semen are due to improper handling or deposition of the semen by the technician (8). Furthermore, according to Graham (6) the largest variation in any experiment involving artificial insemination in fertility field testing is due to technician variation, attitude and bias. Our experiment was designed to eliminate this variation by having only one technician perform all inseminations, alternately using the two semen thaw methods as heifers came into estrus. Heifers were used in this study, rather than cows, in an effort to eliminate possible variation due to postpartum reproductive disorders and stress associated with lactation.

Our data demonstrate an advantage in pregnancy rate for hot water thawing of semen over "in cow" thaw. Although we do not feel that hot water thaw technique will make up for improper handling of semen or poor insemination technique, we conclude it can be used very successfully in herds with good reproductive management systems.

Table 1. Effect of thawing technique on pregnancy rates for all services.

| Thawing method | Inseminations | Pregnancy rate (%)[a] |
|---|---|---|
| 65 C for 7 s | 75 | 84.0 |
| "in cow" | 76 | 61.8 |

[a]Pregnancy rates differ significantly (P<.005).

Table 2. Effect of thawing technique on first service pregnancy rates.

| Thawing method | Inseminations | Pregnancy rate (%)[a] |
|---|---|---|
| 65 C for 7 s | 52 | 76.9 |
| "in cow" | 58 | 60.3 |

[a]Pregnancy rates approach statistical difference (P<.06).

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ABSTRACT

Two procedures for thawing semen were compared in a fertility experiment involving 118 Holstein heifers over a 9 month period. Semen packaged in .5 ml French straws was thawed either by hot water (65 C for 7 s) or by "in cow" technique. Heifers entered the breeding herd when a minimum weight of 341 Kg and age of 13 mo was attained. Heifers were bred to three predetermined sires alternately by the two thawing methods, as they came into estrus. Repeat services were handled in the same manner of alternate breeding regardless of method of thaw used on previous service(s), but service sire was not changed. Semen was obtained from routine collections of Holstein bulls housed at the Kansas Artificial Breeding Service Unit. The semen contained 6% glycerol with a minimum concentration of $17 \times 10^6$ motile spermatazoa per inseminate post-thaw. Statistical analysis was performed by use of a multidimensional chi-square design. Based on pregnancy rates to all services, fertility was significantly higher ($P<.005$) for heifers bred by hot water thaw than by "in cow" thaw (84.0% vs 61.8%). First service pregnancy rates approached significance ($P<.06$) in favor of hot water thaw (76.9% vs 60.3%). No conception by sire interaction was observed. Analysis of covariance revealed no significant effects of ambient temperature on pregnancy rates for the two thaw methods.