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Investigation of stress preconditioning treatment of boar semen using cryopreservation and experiences with its under field conditions

Brief version of PhD thesis

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Introduction

Pork is an important food source. It represents about 40% of red meat consumption worldwide, and is thus an important part of the human diet throughout the world. In the past 20 years pork production has increased from 73 to 98.9 million metric tons, and it is projected that demand will increase to 125 million metric tons by 2020 - most of the increase occurring in the developing countries. The efficiency of pork production has improved as the result of implementing several new housing and feeding-nutrition technologies and the introduction of new breeds. As a result of these advances, the feed efficiency ratio per pound of weight gain has improved, lean meat in the carcass has increased, and the carcass weight produced per sow per year has almost doubled. Several recent biotechnological and genomic advances, if implemented as part of an artificial insemination (AI) program, have the potential to significantly increase global swine populations. The widespread adoption of AI, has resulted from the development of improved extenders, which provide a capability to store semen for up to a week near room temperature. If the semen needs to be stored for a longer period – then deep freezing of the semen is unavoidable. The protection offered by the use of glycerine in the deep freezing of bull semen suggested that it might provide similar protection with boar semen – however this proved not to be the case. It was not until 1970 that the first successful pregnancy with frozen-thawed (FT) boar semen was achieved - to be followed by the first successful impregnations using cervical AI. In 1975 a deep freezing method was developed which significantly lengthened the resultant survival period of boar semen, thus raising the possibility of its use commercially. This method, with various modifications, still forms the basis of all deep freezing methods used today - however its use has remained limited because it is not economical in comparison to the use of fresh semen (FS). Frozen semen requires two to three times more sperm; the litter size is about two to three piglets per litter less; and the farrowing rate is lower by 20-30%. The reason for this is that boar semen suffers from cold shock during cryopreservation, which leads to functional and structural cryodamage, thus lowering post-thaw sperm survival and shortening sperm life span in the female reproductive tract. To compensate for such cold shock losses, and to improve the reproductive outcomes and economic value of using boar FT under field conditions, many modifications of the deep-freezing technology and AI procedure have been tested. These modifications include: using a programmable freezer; using new freezing packages, the FlatPack and MiniFlatPack which contain a complete AI dose and have a more advantageous surface-to-volume ratio; lowering the number of spermatozoa/AI dose; varying the number of AI's and their timing relative to ovulation; reducing the distance the sperm have to travel, by inserting the semen closer to the place of fertilization with the use of intrauterine (UI) or deep

intrauterine insemination (DUI), and hormonally inducing ovulation to shorten the time interval between AI and ovulation. Currently, the latest research reflects those earlier observations, according to which the resistance of boar sperm during incubation - against cold, dilution, cooling, storage and/or deep freezing – is an essential, acquired and changeable property. Response to stress at the cell level is one of detection, evaluation and reaction – and this can lead to a temporary increase in its resistance capacity. The steps in the handling of the semen during the cryopreservation process subject the sperm to a series of stresses – caused by temperature and pH changes, dilution, the centrifugal forces arising under centrifugation, osmotic pressure, the toxic components of the extenders etc. - the effects of the majority of which, on the cell functions, cannot be controlled. To trial if cell resistance capacity could be increased by subjecting it to a controlled stress, a sub-lethal stress was applied to the semen, using hydrostatic pressure (HP) at room temperature, before subjecting it to the cryopreservation process. HP was first used with mouse blastocysts, and then with in vitro produced bovine embryos, bull semen and pig oocytes. The application of HP in the field of biology is one of the most significant scientific discoveries of the past century. The stress induced by the HP application occurs instantaneously and uniformly within the specimen – without penetration problems; its effect is the most accurately controllable of the environmentally caused stresses. On removal of the HP and return of the specimen to atmospheric pressure, the induced stress effects disappear. The aim of this study was to investigate to what extent, under field conditions, such stress preconditioning of boar semen before cryo-preservation, could compensate for the use of a simplified semen preparation and AI procedure (cervical insemination without hormonal induction of ovulation) in terms of improved in vitro parameters and reproductive performance.

Materials and methods

The semen used in our in vitro research came from thirteen boars (mean age 25.5 ± 5 months) on a pig farm (Felsőbabád Pig Breeding Ltd., Felsőbabád, Hungary), where they were kept in individual pens and fed under standard protocol conditions. The inseminations for our in vivo research were carried out on another commercial pig farm (Győzelem Mezőgazdasági Ltd., Lajoskomárom, Hungary). The same freezing protocol was used in our in vitro and in our in vivo studies - dilution with Ext.I. at body temperature (BT), cooling from BT to room temperature (RT), centrifugation, dilution with Ext.II and Ext.III at RT, cooling to 5°C and equilibration, and finally deep freezing.

In Experiment I. the ejaculates were taken from 8 boars, on 3 occasions. The purpose of this in vitro experiment was to determine the most effective pressure/duration combination of the HP application, on total (TM%) and progressive motility (PM%), by varying the applied pressure level (20/40/80MPa), and the duration of application (40/80/120 min). When each ejaculate had cooled to RT after dilution with Ext.I., it was divided into three groups of four samples each. Each group constituted one of the 40/80/120min application durations. Of the four samples in each group – one was left at ATM throughout, to act as control, the other three were pressurised, before centrifugation, to 20/40/80MPa respectively. Motilities for all twelve samples were measured after HP, after equilibration at 5°C, and after FT. The (40MPa:80min) combination, which yielded the largest increase in post-thaw motility, was utilised throughout the rest of the research.

Experiment II. the ejaculates were taken from 13 boars, on 3 occasions. Our next aim was to investigate, at which of four different stages in the freezing protocol, would the application of the HP (40MPa:80min) produce the most beneficial effect on the post-thawing in vitro parameters (TM%, PM% and membrane integrities - head, acrosome, tail). Each ejaculate was divided into five samples – one control kept at ATM, and four which were pressurised (HP1: after dilution with Ext.I. while still at BT; HP2: after cooling to RT but before centrifugation; HP3: after dilution with Ext.II. and HP4: after dilution with Ext.III.. The HP3 application proved to be the most effective.

In Experiment III. the ejaculates were taken from 4 boars, varying from 9 to 15 occasions. Our final aim was to investigate in vivo, the effect of using the HP treated semen for AI under site conditions on sow fertility, and to examine what, if any, relationship exists between fertility and post-thawed motility. Each ejaculate was divided into a non-pressurized control (C-FT), and a HP-treated (HP-FT) frozen-thawed batch. The HP-FT batches were subjected to the optimum hydrostatic pressure application (40MPa:80min, after dilution of Ext.II. at RT), before dilution with Ext.III. and completion of the freezing protocol. The C-FT batches were kept at RT during this time, then also diluted with Ext.III. at RT, before completion of the freezing protocol. Sows were weaned every week on Thursday, approximately 28 days after farrowing, and placed in individual cages. Routine inspection of the sows for oestrus started on the third-day after weaning - twice daily, using the back pressure test and keeping a breeding boar in front of the sows. To allow two AIs per sow to be performed on the fifth-day after weaning, the breeding sows were selected from those manifesting standing oestrus in the afternoon of the fourth-day. Either 8, 10 or 12 sows were selected in pairs for insemination on ten occasions, and allocated randomly, one each into the C-FT and HP-FT groups – 51 pairs in total. Before using an

ejaculate in AI, the after-thawing TM of its HP-FT batch was determined, and if it was >40% then both batches of that ejaculate were used for AI. From the accepted batches, twelve straws were used for each AI dose yielding $6x10^9$ spermatozoa/dose. The frozen straws were plunged into a 37 °C water bath for 30 sec for thawing, and then emptied into commercial plastic insemination bottles which had also been kept in the water bath. The semen was then diluted by adding 80 ml of Ext. I. Each sow was inseminated with two AI doses: the first in the morning of the fifth-day (between 06:00-08:00hrs) - about 12 h after the sow had first shown standing oestrus; the second about 10 h later, in the evening. All the inseminations were performed by the same two technicians from the pig breeding farm, who did not at any stage know which batch of semen, C-FT or HP-FT, they were using. In each case the semen was deposited into the cervix, using a commercially available, pre-lubricated, cervical insemination foam catheter, with a protecting plastic sheet.

Results

In Experiment I. the motility parameters (TM%, PM%) measured after the HP treatment did not vary significantly between the three duration groups - either for the control samples, or at the 20, 40, or 80MPa pressure levels. However - compared to the controls within each duration group - the impact of the 80MPa treatment proved to be significantly greater (TM%: $P<0.001$, PM%: $P<0.05$) than that of the 20 and 40 MPa applications. Measurements of motility following the 5ºC cold-acclimatisation, once again showed no significant variation between the three duration groups. However, relative to the post HP values, the TM% of the three control samples showed significant ($P<0.05$) reductions; changes in the pressurised samples were not significant ($P>0.05$). After thawing, all values of both motility parameters were significantly lower than the post pressure application values. In this case the results were influenced by both the applied pressure level ($P<0.001$) and its duration ($P<0.05$). The absolute values for the 120min duration group samples remained higher than those for the 40min and 80min duration groups; and, within each group, the results for the 20MPa and 40MPa treatments remained significantly higher ($P<0.05$) than those for the control/ATM or 80MPa treatments. The largest relative improvement in the TM% due to a pressure application - compared to the control sample - occurred with the 40MPa:80min combination; for the PM% this occurred with the 40MPa:120min combination – however the improvement was only slightly, but not significantly ($P>0.05$) higher than for the 40MPa:80min combination.

In Experiment II. after thawing, the HP3 treatment values of the in vitro parameters - TM%, PM% and membrane integrities (acrosome, head and tail) showed the largest improvements compared to the control/ATM results. The improvement in the TM% was significant.

In the in vivo experiment a statistically significant differences ($P<0.05$) were found between the two experimental groups for the non-return rates, and for the pregnancy rates as determined by ultrasonography - with the HP-FT group values being higher in each case than those of the C-FT group. There was also a numerical improvement in the farrowing rates (58.8 vs. 78.4%), but this was not statistically significant ($P>0.05$). The odds of the sows in the HP-FT group becoming non-returned to oestrus, becoming pregnant, or farrowing – were 3.3, 2.9 and 2.5 times respectively greater, than those for the sows in the C-FT group (Fisher-test, odds ratio). The total number of piglets/litter born to the sows in the HP group was significantly higher ($P<0.05$) than those born to the sows in the C-FT group. This difference was also evident ($P<0.05$) in the number of live piglets/litter born in the two groups. Both groups suffered a similar rate of attrition in terms of live piglets/litter – about 15% – from farrowing to the time of weaning, and even up to two weeks later; however, the differences between the numbers/litter in the two groups remained significant at each stage. Body-weight/piglet at farrowing, and at subsequent stages was not significantly different between the two experimental groups. Both the total and progressive motilities of the HP-FT semen were found to be significantly higher ($P<0.001$) than the corresponding values for the C-FT group. No significant differences ($P>0.05$) in these parameters were found, in either group, between pregnant and non-pregnant sows. No significant correlation (Pearson correlation, $r=0.11–0.20$, $P>0.05$) was found between total piglets/litter and the motility measurements (TM% or PM%), in either group.

Discussion

Trials with varying laboratory procedures in terms of semen deep-freezing protocol, sample packaging, extenders and insemination procedures – number of spermatozoa/dose, number of doses used, place of semen deposition (UI, DUI) and hormonal treatments – have resulted in fertility parameters approaching those of using FS. However, the achievement of an adequate FT semen quality, requires laboratories equipped with additional facilities, interventions, and significantly greater skill, patience and consistency on the part of the staff – particularly in the field – which involves additional training. Under the standard freezing protocol, storage and handling of the semen – prior to the actual freezing – are performed at 15-17°C; this can take as little as (2-4h), or as long as (12-15h). After cooling to 4-5°C, and equilibrating at this

temperature over (1.5-2.5h), the semen is further diluted with the freezing medium, placed into straws, and frozen. As our laboratory was not equipped with a cooling centrifuge and a programmable freezer – currently widely accepted as necessary for using the standard freezing protocol – we had to modify this protocol. To minimise the number of temperature changes to which the samples would be subjected in carrying out all the pre-freezing operations i.e. centrifugation, dilutions, HP treatment, re-dilution with the freezing medium and transfer into straws – all these operations were performed at RT. The straws were then cooled in two stages over a period of 1h to 15°C, then over 2h to 5°C before being frozen. The application of the HP treatment was intended to counter this modification of the standard freezing protocol and the use of a simple AI procedure. Based on our Experiments I and II, we observed that the spermatozoa exhibited a flexible response to the application of a HP, with the 40MPa/80min treatment, applied after dilution with Ext.II. at RT, producing the largest improvement in the in-vitro motilities. For AI, we only used the C-FT and H-FT batches of those ejaculates which yielded a TM>40% for the H-FT batch; this is in line with TM values used with FT sperm by others in the field. In our case, 85.7% (42/49) of the HP-FT ejaculates, and 53.1% (26/49) of the C-FT ejaculates met the TM>40% criterion. The result of 85.7% acceptable ejaculates for the HP-FT samples indicates that their pressurization before freezing has had a positive effect on sperm motilities. Whether this positive effect has only partially, fully, or over-compensated for the negative effect of modifying the freezing protocol, could only have been determined if a third sample from the ejaculates had been processed following the standard freezing protocol without the pressurization. Sperm motility has been shown to be possibly a useful indicator for boar sperm fertility; however, a direct relationship between boar sperm motility parameters and fertility outcomes, either with FS or FT sperm, has still not been unequivocally established. In particular, several studies have not found any significant correlation between post-thaw TM% and the fertility outcomes; thus, it appears, that other boar semen related parameters than motility, may be more useful factors in selecting boars for AI purposes. In our study the post-thawing total and progressive motility percentages of the HP-FT semen samples were significantly higher than those of the C-FT semen. The fertility parameters of the sows in the HP-FT group – such as non-return, pregnancy and farrowing rates, and the total number of piglets/litter – were in each case also significantly higher than those of the C-FT group. At the same time our results showed no significant differences in motilities between those ejaculates which resulted in pregnancy, and those which did not – within either the C-FT or the H-FT group of sows. There was also a lack of correlation between motilities and total number of piglet/litter in both groups. Thus, though the HP treatment has on average improved both sperm motility

and the fertility parameters, there is no direct correlation between the two improvements. No negative effects due to the HP treatment were detected either on the survival, or on the growth rate of the piglets from the HP-FT group. This is shown by the body-weight/piglet not being significantly different between the two groups at farrowing, or at subsequent growth stages and by the attrition of the number of piglets/litter in the two groups occurring at the same rate over these stages. Considering these findings and the previous observations it is possible, that beside its effect on sperm motility, the HP treatment may also have affected other functions of the sperm – which may have resulted in the improved fertility parameters. This possibility requires further investigation, but is in line with reports in previous publications where sub-lethal stress treatment of cells has resulted in increased cryosurvival and better retained function. The majority of field trials describing the use of frozen boar semen indicate that acceptable fertility could be achieved by the use of programmable freezing, ovulation synchronisation, UI, DIU; or their combination. In our study, despite using only routine cervical insemination, the HP-FT group's farrowing rate of 78.4% and total number of piglet/litter of 10.8, compare favourably with those obtained using the above more elaborate procedures (farrowing rate ranging from 51-78%, total number of piglets/litter ranging from 8.0-12.5). As we did not have daily experience with FT semen, and bearing in mind the possibility of back flow and the damaging effect of cryopreservation on the sperm, we used the highest number of spermatozoa/dose that we found to have been used with FT semen in a previous field study, under nearly similar conditions ($6x10^9$ sp/dose, cervical insemination with a 100ml AI dose). Further experiments are needed to reduce this number of spermatozoa /dose, as it is more than twice the number used with FS, and in order to minimize any putative uterine immune reactions against the spermatozoa. In conclusion, the use of frozen-thawed boar semen in commercial practice has undoubted merits. The present report shows that preconditioning the sperm through the HP treatment, before subjecting it to the stress caused by cryopreservation, can compensate for using a modified, simpler freezing protocol and a standard cervical AI protocol. Further trials are required to more fully understand the effect of the HP treatment on the other in vitro sperm parameters, and also to determine how the AI doses can be varied while still providing acceptable fertility results for farmers.

New scientific results

We were the first to investigate the effect of the sub-lethal stress caused by the application of HP, in improving the in-vitro performance of spermatozoa. We optimised the variable features of the application (pressure/time/temperature) and incorporated these in the deep freezing protocol for boar semen.

With this new protocol - in contrast to the “standard” deep freezing protocol - handling of the sperm (including the addition of the cryo-protector) takes place at room temperature. Results with this modified protocol have achieved similar success rates to those using the “standard” protocol.

We were the first to conduct large scale on-site AI using HP treated deep frozen sperm which proved that even without cervical insemination or hormonally induced oestrus this procedure can achieve improvements in the sows’ fertility parameters (pregnancy %, farrowing %, total piglets/litter, total live piglets/litter).

On the basis of these results, we can claim to have developed a new and effective technology, to assist with the deep freezing protocol used by the pig breeding industry.

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