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The hypo-osmotic swelling test – critical research concerning the clinical applicability in canine reproduction

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Meinem Mann & meiner Familie

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1 INTRODUCTION

Since the first documented artificial insemination in dogs performed by Lazaro Spalanzani in 1788, reproductive technologies are continuing evolving. Still, for dog breeders as well as for veterinarians, the aim of semen evaluation is to give a prognosis about the fertilizing capacity of a semen sample.

For semen evaluation, freshly collected ejaculates are required. Dogs are usually sampled in the presence of a bitch, preferably in heat or by using a vaginal swap from bitches in oestrus to provide stimulation. The ejaculate is obtained by digital manipulation or by using an artificial vagina. Electro ejaculation has been reported but is not necessary in most cases and may lead to urine contamination and low semen quality (Johnston et al. 2001). The canine ejaculate consists of three different fractions: the transparent pre-sperm fraction, the milky-white sperm-rich fraction and the slightly opaque to transparent prostatic fraction. Throughout sampling, these fractions are usually collected into three different pre-warmed (37°C) sterile glass funnels and stored in a water bath (37°C) until further analyses.

Conventional semen analysis includes determination of volume, color, pH, sperm concentration, percentage of progressive motile sperm cells, sperm membrane integrity and percentage of morphologically normal sperm cells of the sperm-rich fraction (Johnston et al. 2001). Semen concentration can be determined cytometrically using a counting chamber. The total number of sperm cells in canine semen varies from 200 to 1200 million, with a concentration of 100 to 700 million sperm cells per milliliter (Farstad 2010). Progressive motility is the percentage of sperm cells with forward movement. It is usually estimated by visual inspection using a light contrast microscope at 100x magnification. In undiluted freshly collected semen values normally range from 75 – 90% (Farstad 2010). For assessment of sperm cell membrane integrity Eosin solution can be used. Sperm cells with damaged plasma membranes are stained by Eosin whereas sperm cells with intact plasma membranes remain unstained (Goericke-Pesch and Failing 2013). Sperm cell morphology can be determined by Spermac® stain (Goericke-Pesch and Failing 2013). After fixation in formalin (4%) the specimen is dyed with three different stains. The various parts of the sperm cell take up the stain differently and facilitate detection of any anomalies of each cell part. Abnormalities are specified as defects in the head, midpiece or tail region of sperm cells. Progressive motility, membrane integrity and morphology may be compromised by cryopreservation, storage or transport under inconsistent temperature or failures during the thawing process (Setyawan et al. 2015).

Besides conventional semen parameters, several additional parameters have been evaluated. One of these additional parameters is the hypo-osmotic swelling test (HOS test). The HOS test is a simple, inexpensive and easily applicable test to assess the functional

integrity of the plasma membrane of sperm cells (Kumi-Diaka 1993, Quintela et al. 2010). The HOS test is based on the semi-permeability of intact cell membranes. According to Petrunkina et al. (2001) functional membranes are closely related to sperm function, as only intact sperm cells are able to adjust to the environment in the female tract. Throughout the period of conducting the HOS assessment sperm cells are exposed to hypo-osmotic solutions. When the intracellular solutes are at a greater ion concentration, an osmotic gradient is established. To compensate this ion gradient, sperm cells with intact membranes swell due to a water influx, resulting in the curling of the tails of sperm cells (Rodriguez-Gil et al. 1994). Under the optical microscope, this phenomenon is easier to observe in the tail than in the head of the sperm cell as the plasma membrane of the tail seems to be less well attached than at the head (Bencharif et al. 2010). The predictive and diagnostic value of the HOS test in correlation with conventional semen parameters has been discussed controversially so far. Kumi-Diaka et al. (1993) and Rodriguez-Gil et al. (1994) observed significant high correlations between HOS test and motility, whereas Rodriguez-Gil et al. also found significant correlations between HOS test and membrane integrity and normal morphology. In contrast to these results, England et al. (1993) found no relationship between HOS test and other semen parameters, such as motility, morphology and membrane integrity.

Therefore, the overall objectives of this study were to analyse relationships between HOS test results and conventional semen parameters before freezing and after thawing and to determine the diagnostic value of the HOS test as a standard parameter for canine semen evaluation.

1.1 Evaluation of published literature in reference to the HOS test

In preparation for the clinical trial, a systematic literature review was conducted in order to evaluate the available literature according to certain quality parameters and test characteristics, such as randomization, blinding, inter- and intraobserver agreement and sample size. A citation map was developed to identify the origin of the quotation of the HOS test.

The results of this systematic review were published in the Journal of Reproduction in Domestic Animals:

S Karger, SP Arlt, P Haimerl and W Heuwieser.

A systematic review of studies performing the hypo-osmotic swelling test to evaluate the quality of canine spermatozoa. Reprod Domest Anim. 2014 Feb; 49 (1): 1-6.

1.2 Evaluation of the prognostic value of the HOS test on canine semen`s post-thaw quality

Relationships between HOS test results and conventional semen parameters before freezing and after thawing were examined and the prognostic value of the HOS test on canine semen`s post-thaw quality was evaluated.

Therefore, semen of 35 dogs was collected and analysed before freezing and after thawing following a 7-day freeze-thaw interval. Conventional semen variables such as sperm cell motility, membrane integrity and morphology were evaluated and the HOS test was conducted. For the prediction of individual cryopreservation capacity, results from assessment of the fresh semen variables of good and poor semen quality were statistically compared.

The results of this study were published online in Animal Reproduction Science:
S Karger, B Geiser, M Grau, O Burfeind, W Heuwieser, and SP Arlt.
Prognostic value of a pre-freeze hypo-osmotic swelling test on the post-thaw quality of canine semen. ANIREP. 2016 Mar; 166: 141-147.

1.3 Evaluation of progressive motility of frozen-thawed canine semen with respect to the HOS test among other conventional semen parameters.

Motility represents only one of the many conventional parameters for canine semen analysis but it is the one most widely used indicator for sperm function. The objective of this experiment was to examine time-dependent changes of motility after thawing cryopreserved canine semen. Semen of 35 dogs was collected. Semen parameters, such as volume, concentration, progressive motility, morphology, membrane integrity and HOS test were evaluated.

The results of this study were recently published in the Journal of Reproduction in Domestic Animals:
S Karger, B Geiser, M Grau, W Heuwieser and SP Arlt.
Short communication: Progressive motility of frozen-thawed canine semen is highest five minutes after thawing. Reprod Domest Anim. 2017 Apr; 52 (2): 350-352.

The three papers are presented in the format outlined in the guide for authors of the respective journal.

2 RESEARCH PAPERS

2.1 A systematic review of studies performing the hypo-osmotic swelling test to evaluate the quality of canine spermatozoa

S. Karger, S.P. Arlt, P. Haimerl and W. Heuwieser. Reprod Domest Anim. 2014 Feb; 49 (1): 1-6.

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A systematic review of studies performing the hypo-osmotic swelling test to evaluate the quality of canine spermatozoa

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CONTENTS

The hypo-osmotic swelling test (HOS test) is a simple and inexpensive test to evaluate the functional integrity of sperm cell membranes. According to the existing literature, its simple applicability has turned it into a valuable additional parameter to standard canine semen analysis. In the recent years, much research has been conducted in this field. The aim of this systematic review was to evaluate the quality of published literature in canine reproduction concerning the HOS test. Using two distinguished databases, 38 articles were detected and analysed subsequently according to various aspects, for example study design, population, semen sampling and implementation concerning the HOS test. Although there are numerous articles available, the diagnostic value of the HOS test remains ambiguous. Until now, neither a recognized test protocol nor reliable reference values have been defined. Most of the trials evaluated show serious methodological flaws and therefore do not permit drawing reliable conclusions. According to our results, approximately half of the studies (n = 20) included a sample size of five or less animals. None of the studies examined the inter- or intraobserver agreement for the HOS test. Further research is warranted including appropriate statistical methods and a sufficient number of animals to establish a standardized test protocol as well as reliable reference values. Most importantly, it is required to clarify a correlation between the HOS test and the fertilizing capacity to determine the diagnostic value of the HOS test.

Keywords: systematic review, dog, semen analysis, citation map

INTRODUCTION

The hypo-osmotic swelling test (HOS test) is a simple, inexpensive and easily applicable test to assess the functional integrity of the plasma membrane of sperm cells (Kumi-Diaka 1993; Quintela et al. 2010). Moreover, the HOS test is considered to be reliable and sensitive (Jeyendran et al. 1984) and to represent a valuable tool for routine semen evaluation (Rodriguez- Gil et al. 1994; Goericke-Pesch and Failing 2013).

The HOS test is based on the semi-permeability of intact cell membranes. According to Petrunkina et al. (2001), functional membranes are closely related to sperm function, as only intact sperm cells are able to adjust in the female tract. The spermatozoon is exposed to major osmotic differences at various stages in its life cycle. During ejaculation, sperm cells are transported from the hyper-osmotic environment of the cauda epididymis with an osmotic pressure of 420 mmol/kg (Yeung et al. 1999) to the almost iso-osmotic fluids of seminal plasma and female genital tract with an osmotic pressure of 330 mmol/kg (Yeung et al. 2000). To withstand these osmotic challenges, intact membranes of sperm cells are able to regulate volume changes by the adjustment of intracellular ion concentration (Jeyendran et al. 1984; Petrunkina et al. 2004a). The functional integrity of sperm cell membranes can be determined by the HOS test (Jeyendran et al. 1984).

When performing the HOS test, spermatozoa are exposed to hypo-osmotic solutions. Under these conditions, spermatozoa swell due to influx of water until equilibrium is reached, resulting in the curling of sperm tails (Rodriguez-Gil et al. 1994). After incubation, replicate slides are examined under a phase-contrast microscope. Afterwards, the number of non-swollen (i.e. damaged membranes) and swollen (i.e. intact membranes) cells can be counted (World Health Organization 2010).

In recent years, many studies have been conducted utilizing the HOS test to evaluate the quality of canine spermatozoa. However, a recognized test protocol or reference values are not available.

Therefore, the overall objective of this study was to review studies systematically using the HOS test in dogs. Specifically, we set out to evaluate the available literature according to certain quality parameters and test characteristics, such as randomization, blinding, inter- and intraobserver agreement and sample size.

MATERIAL AND METHODS

A comprehensive literature search was conducted on 28th March 2012 utilizing the databases CAB (http:// ovidsp.tx.ovid.com) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed) to identify the literature related to the analysis of canine semen. The subject heading “dogs + semen analysis” was applied. No limits were set.

All references were imported into EndNote (version X4.0.2; Thomson Reuters EndNote, New York, NY, USA).

Using an automatic function provided by EndNote, duplicates and reviews were identified and excluded. Duplicates and reviews that were not recognized by EndNote were deleted manually. Only studies performing the HOS test on canine semen were included. Additional records were identified through references in the included articles to cover the pertaining literature as wide as possible. All selected articles were reviewed systematically considering preset criteria. Relevant aspects concerning the study design, such as sample size, number of ejaculates, blinding and randomization were assessed according to Sannmann et al. (2012). Furthermore, data concerning the population of the dogs, that is, selection criteria, housing, feeding, age, breed and health status were extracted. Additionally, information regarding semen analysis, such as the applied sperm fraction and whether pooled semen were employed, were collected. Furthermore, a systematic follow-up of citations concerning the HOS test in the eligible articles was conducted to generate a citation map. Every quotation regarding the HOS test was entered into a spreadsheet (Microsoft Excel 2010; Microsoft Deutschland GmbH, Munich, Germany) irrespective of species. Only publications quoted more than three times and related to canine reproduction as well as those which were essential to determine the origin of quotation of the HOS test were included in the citation map. For the development and presentation, an open source mind-mapping software (XMind 2012; XMind Ltd, Hong Kong, China) was used.

To gain an overview of different methodologies for HOS testing, osmolarities, times of incubation, number of examined sperm cells and magnification were documented. Further parameters assigned for semen evaluation, such as motility, concentration, viability and morphology were also considered. All extracted data were entered into a spreadsheet (Microsoft Excel 2010; Microsoft Deutschland GmbH).

RESULTS

Literature search

Using the subject heading ‘dogs + semen analysis’, a total of 354 references were found (CAB: 29, PubMed: 325). After eliminating duplicates (n = 12) and reviews (n = 23), 319 articles remained. Articles regarding other species than dogs (n = 9) and articles not focusing on semen analysis (n = 35) were excluded as well. One article could not be examined as it was written in Korean language. After excluding trials not performing the HOS test (n = 247), 27 articles remained. Additionally, 11 articles were identified through a systematic review of citations in the retrieved papers, leading to a total of 38 articles being eligible for final analysis. A systematic follow-up of citations concerning the HOS test in the

eligible articles was conducted to identify the origin of its quotation (Figure 1).

Of the 38 papers evaluated, 27 were published in the last decade. The articles were published in 13 different journals focusing on animal reproduction, such as Theriogenology (n = 18) and Animal Reproduction Science (n = 5). The majority of these journals (11/13) were peer reviewed; thus, 94.7% of the articles were published in peer-reviewed journals. Seven journals (53.9%) have an impact factor above 1.0.

Study design

All articles were based on prospective studies. None of the 38 included studies was specified as being randomized, and only one study was identified as blinded (Quintela et al. 2010). Reference values for results of the HOS test were defined in one study (Riesenbeck et al. 2001). None of the studies examined the inter- or intraobserver agreement for the HOS test. Only Rota (Rota et al. 2005, 2006) calculated correlations between two observers based on 15 (three ejaculates from five dogs; Rota et al. 2005) and 10 samples from five dogs, respectively (Rota et al. 2006). The correlations vary between $r = 0.83$, $p < 0.001$ (Rota et al. 2005) and $r = 0.844$, $p < 0.001$(Rota et al. 2006). In 21 studies, no information was provided on inclusion or exclusion criteria of the dogs involved.

Population

Of 38 studies, 20 (52.6%) included five or less dogs and seven (18.4%) included six to 10 dogs. Nine trials (23.7%) included more than 10 dogs whereas in two articles the population was not defined. The dogs were aged between 10 months and 8 years, if specified (n = 26). Predominately, beagles were used (12 of 31 studies with species information). For the duration of the studies, the dogs were kept either in indoor or outdoor kennels (n = 15), or lived together with their private owners (n = 3). In 20 articles, housing was not defined. Dogs were commonly fed with commercially balanced diets. In 26 articles, the ration was not specified. Semen was collected by digital manipulation (n = 30), by aspiration after castration (n = 3) or using an artificial vagina (n = 1). Sampling was accomplished at different frequencies, primarily once or twice weekly. In 16 papers, sampling frequency was not specified. In 11 trials, 20 or less ejaculates were included whereas in 16 studies the number of ejaculates was not specified. Only four articles (10.5%) evaluated more than 40 ejaculates. Pooling of semen samples was conducted in six studies. The majority (n = 27) of studies separated the three fractions of the ejaculates (i.e. pre-sperm fraction, sperm-rich fraction, prostatic fraction). In eight articles, the tested fraction was not specified. The second fraction was used for the HOS test in 24 studies. In three cases,

semen samples were taken by aspiration after castration. Consequently, number and fraction of the ejaculates as well as precognition of these dogs could not be determined.

HOS test protocols

To evaluate the functional integrity of human spermatozoa, Jeyendran et al. (1984) used ejaculates from three men divided into 35 aliquots and exposed these to different osmotic solutions. According to the authors, solutions at 150 mOsmol led to optimal and repeatable results.

To appraise functional integrity of canine spermatozoa, two authors introduced the HOS test to veterinary medicine (England and Plummer 1993; Kumi-Diaka 1993). Kumi-Diaka (1993) examined ejaculates of eight dogs. Canine spermatozoa were submitted to solutions of different osmolarities. According to their results, it was recommended to incubate solutions at 60 mOsmol and 37°C for 45 min.

For the method described by England and Plummer (1993), two ejaculates were collected from each of the six dogs. Referring to Jeyendran et al. (1984), canine spermatozoa were exposed to solutions with different osmolarities. Similar to the results postulated by Jeyendran et al. (1984), a solution of 150 mOsmol incubated at 37°C for 30 min generated a high proportion of swollen spermatozoa.

Both authors considered the HOS test to be a reasonable assay to assess membrane integrity. Protocols of various studies (20/35) conducted afterwards are referring to these reports (Table 1).

Additional parameters

In every trial investigated (n = 38), sperm quality was evaluated by combining HOS test with additional parameters. In 36, 34 and 30 cases, the HOS test was combined with motility, morphology and concentration, respectively. The majority of trials (n = 27) combined all four parameters. In 10 studies, correlations between HOS test and motility were calculated ranging from $r = 0.35$, $p < 0.05$ (Rota et al. 2005) to $r = 0.98$, $p < 0.002$ (Kumi-Diaka and Badtram 1994). Also, correlations existed between HOS test and viability in fresh semen [n = 2; $r = 0.68$, $p < 0.05$ (Pinto and Kozink 2008) and $r = 0.6825$, $p \leq 0.005$ (Rodriguez-Gil et al. 1994)] or acrosome reaction in fresh semen [n = 2, $r = 0.83$, $p < 0.05$ (Kumi-Diaka and Badtram 1994) and $r = 0.72$, $p < 0.03$ (Petrunkina et al. 2004a)].

DISCUSSION

In the past, dog breeding has experienced increasing interest and cryopreservation of canine semen appears to be more important than ever (Kim et al. 2010b). Functional sperm cell membranes are crucial for successful freezing and thawing processes. With regard to the HOS test, many researches have been carried out especially in the last decade. It's diagnostic value, however, still remains ambiguous. Until now, neither a standardized test protocol nor reliable reference values for the HOS test have been defined.

When accomplishing a systematic review, it is important to consider the study designs of the articles (Khan et al. 2003). Study designs, that is, procedures under which studies are carried out, should be set up in a way that bias is unlikely to occur. Consequently, studies concerning diagnostic tests should describe the methods of testing accurately to assure reproducibility (Watson and Petrie 2010). The majority of the evaluated articles provided detailed information on equipment and methodological procedures. However, there was only little information provided concerning selection criteria and background of the dogs enrolled.

To establish a new, reliable diagnostic method, it is important to assess the repeatability and reproducibility of the measurement process (Watson and Petrie 2010). Repeatability is defined as an agreement between two measurements on the same samples, whereas reproducibility means that two individuals are using the identical methodology on identical samples (Watson and Petrie 2010). Our findings reveal that except for Rota (Rota et al. 2005, 2006), neither an intra- nor an interobserver agreement for the HOS test was determined. According to our results, 20 (52.6%) of the studies included a sample size of only five or less animals. Merely nine trials (23.7%) included more than ten animals. More important than the absolute number of animals included is the question whether the sample size of each group was adequate relative to the scope of the study (Lenth 2001). In fact, eight studies used semen, which was pooled and therefore did not analyse the individual semen quality in each dog (Batista et al. 2012). We conclude that sample size as one important component of evidence was either not addressed at all or the number of dogs was marginal in more than half of the studies performed. Overall, most of the studies investigated show serious methodological flaws and therefore do not permit drawing reliable conclusions. When examining the implementation protocols for HOS testing, parameters, such as osmolarity, incubation time and incubation temperature varied from study to study. Based on their results, Kumi-Diaka (1993) and England (1995) recommended diverging parameters. Therefore, all following HOS studies either established their protocols on the basis of these two recommendations or generated new parameters. It is unclear, however, if the utilization of different parameters provides comparable results. To minimize potential bias, it is required to develop standardized test protocols (Watson and Petrie 2010).

To our knowledge, no reliable reference values were defined for HOS test in dogs. Only one attempt has been made (Riesenbeck et al. 2001) to determine a reference value, which has not been confirmed yet. Furthermore, it is still not possible to interpret the diagnostic value of the HOS test to predict the fertilizing capacity. Recently, one study compared two different freezing extenders (Bencharif et al. 2010) and investigated standard semen parameters, membrane function and fertility. All of the six bitches were inseminated successfully. A correlation between HOS test and fertilizing capacity, however, was not investigated. Currently, another study (Goericke- Pesch and Failing 2013) analysed retrospectively canine semen evaluations with emphasis on the use of the HOS test in 256 dogs (400 ejaculates). Significant correlations were found between HOS test and motility ($p < 0.0001$), viability ($p < 0.0001$), age ($p < 0.001$), acrosomal status ($p < 0.5$), pathomorphology ($p < 0.0001$) and sperm concentration ($p = 0.011$). A possible correlation between HOS test and fertilizing capacity was assumed by the authors. Our systematic literature assessment demonstrates that a correlation between HOS test response and fertilizing capacity as reported in humans (Jeyendran et al. 1992) has not yet been established in dogs.

Several authors conclude that the HOS test represents an additional tool to conventional evaluation of semen quality despite a dearth of information regarding its diagnostic value. Most of the studies investigated have serious methodological flaws (e.g. limited sample size, no randomization, no inter- or intraobserver agreement) and therefore do not permit drawing reliable conclusions. Until now, neither a standardized test protocol nor reliable reference values have been defined. Further research is warranted including appropriate statistical methods and a sufficient number of animals to establish a standardized test protocol as well as reliable reference values. Most importantly, it is required to clarify a correlation between HOS test and fertilizing capacity to determine the diagnostic value of the HOS test.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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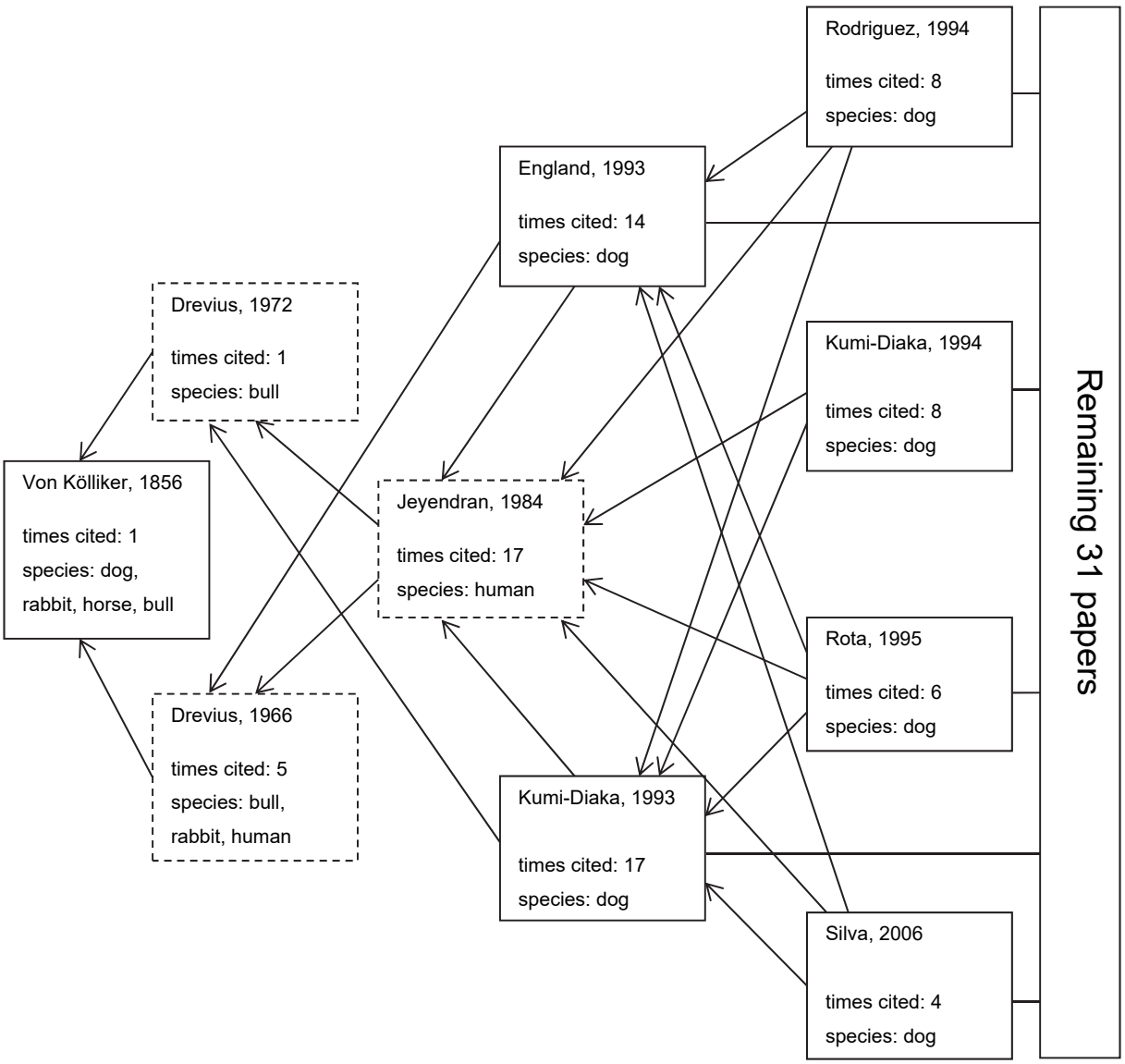
Figure 1. Citation map of studies investigating the origin of the documentation of the HOS test in dogs.



Boxes with dashed lines: papers not being included in the study because of different species. Boxes with solid lines: papers investigated.

Table 1. Methodological parameters applied by the analysed articles concerning the HOS test in dogs.

Author (year)	Osmolarities	Incubation	
	mOsmol/l	°C	min
Mota et al. (2011)	0	38	45
Bencharif et al. (2010)	100	37	60
Kim et al. (2010a)	100	37	45
Kim et al. (2010b)	100	37	45
Futino et al. (2010)	150	37	30
Quintela et al. (2010)	60	37	45
Lopes et al. (2009b)	0	38	45
Corral-Baques et al. (2009)	150; 300	37	15
Michael et al. (2009)	60	Not given	45
Lopes et al. (2009a)	150	37	30
Michael et al. (2008)	60	Not given	45
Pinto et al. (2008)	100	37	1; 60
Michael et al. (2007)	60	Not given	45
De Souza et al. (2007)	150	37	30
Tittarelli et al. (2006)	Not given	Not given	Not given
Oliveira et al. (2006)	60	Not given	Not given
Silva et al. (2006)	150	38	45
Rota et al. (2006)	60	37	45
Rota et al. (2005)	60	37	45
Petrunkina et al. (2005)	180; 300; 450	39	5; 20
Corral-Baques et al. (2005)	150	37	15
Petrunkina et al. (2004a)	180; 300; 450	39	5;20
Nur et al. (2004)	100	37	60
Uysal and Korkmaz (2004)	60	37	0; 5; 30; 60
Hishinuma and Sekine (2004)	0	38,5	5
Petrunkina et al. (2004b)	150; 300	25; 33; 39	5
Hishinuma and Sekine (2003)	0; 150	38,5	5; 60
Riesenbeck et al. (2001)	150	37	30
Saratsis et al. (2000)	60	37	45
Mogas et al. (1998)	100	37	10-120
Rota et al. (1995)	60	37	45
England (1995)	Not given	Not given	Not given
Rodriguez-Gil et al. (1994)	100; 150; 300	37	Not given
Kumi-Diaka and Badtram (1994)	60	Not given	60
Kumi-Diaka and Harris (1994)	60; 100; 150; 200; 300	Not given	Not given
Kumi-Diaka (1993)	60; 100; 150; 200; 300	35; 37	15; 30; 45; 60; 75; 90
England and Plummer (1993)	0; 50; 100; 150; 200; 250; 300	37	30
von Kölliker (1856)	0	Not given	Not given

2.2 Prognostic value of a pre-freeze hypo-osmotic swelling test on the post-thaw quality of canine semen

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Prognostic value of a pre-freeze hypo-osmotic swelling test on the post-thaw quality of canine semen

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CONTENTS

Throughout cryopreservation, sperm are exposed to major osmotic challenges. Only intact membranes of sperm cells are able to regulate these volumetric changes, which can be determined by the hypo-osmotic swelling test (HOS test). Correlations between the HOS test and conventional semen variables are inconsistent. Therefore, the objectives of this study were 1) to examine relationships between HOS test results and standard semen variables before freezing and after thawing and 2) to evaluate the prognostic value of the HOS assessments on post-thaw quality of dog semen. Semen of 35 dogs was collected and analysed before freezing and after thawing following a 7-day freeze-thaw interval. Conventional semen variables such as sperm cell motility, membrane integrity and morphology were evaluated and the HOS test was conducted with results from this test being recorded. In fresh semen, the HOS test was positively correlated with progressive motility of sperm cells: $r = 0.52$, sperm cell membrane integrity: $r = 0.50$ and normal sperm cell morphology: $r = 0.46$ ($P < 0.05$). In frozen-thawed semen, the data obtained with the HOS test were positively correlated with progressive sperm cell motility: $r = 0.67$ and membrane integrity: $r = 0.86$ ($P < 0.05$). The data obtained with the HOS test in fresh semen were positively correlated with sperm cell membrane integrity: $r = 0.50$ normal sperm cell morphology: $r = 0.55$ and data from the HOS test ($r = 0.43$; $P < 0.05$) with frozen-thawed semen. For the prediction of individual cryopreservation capacity, results from assessment of the fresh semen variables of good and poor semen quality were statistically compared. Based on these results, it is not possible to predict the quality of frozen-thawed dog semen using the HOS test.

Keywords: Dog, Semen, Hypo-osmotic swelling test, Cryopreservation, Freezing, Thawing

INTRODUCTION

Freezing dog semen is an extensive, time-consuming procedure consisting of multiple steps, which have to be followed accurately. In general, cryopreservation of dog semen results in few insemination doses in comparison to freezing semen of farm animals. Successful classification of the ejaculates before freezing to assess expected cryopreservation capacity would be thus be a great asset for use of frozen semen for dog breeding (Pena et al., 2006).

Cryopreservation implies osmotic stress on sperm as does the formation or reshaping of intracellular ice (Dorado et al., 2011). When the temperature decreases below the freezing point, ice crystals form. Extracellular water crystallizes, thus leaving the remaining solutes at a greater ion concentration (Pena et al., 2006). A major osmotic gradient across the sperm cell membrane is generated (Petrunkina et al., 2004) resulting in an efflux of water from the sperm. Sperm cells shrink because of dehydration. To withstand these osmotic challenges functional membranes of sperm cells are able to regulate volume changes through modulation of intracellular ion concentrations (Jeyendran et al., 1984).

Throughout the period of conducting the HOS assessment sperm cells are exposed to hypo-osmotic solutions. When the intracellular solutes are at a greater concentration, an osmotic gradient is established. To compensate this ion gradient, sperm cells with intact membranes swell due to a water influx, resulting in the curling of the tails of sperm cells (Rodriguez-Gil et al., 1994). Under the optical microscope, this phenomenon is easier to observe in the tail than in the head of the sperm cell as the plasma membrane of the tail seems to be less well attached than at the head (Bencharif et al., 2010). Essential changes for maintaining sperm cell viability take place with major osmotic gradients across membranes occurring during cryopreservation. Therefore, the responsiveness of sperm cells to osmotic challenges as well as the capacity of the cells to regulate cell volume is a characteristic closely related to cryopreservation capacity (Petrunkina et al., 2004). The HOS test has been used to assess the quality of both fresh (England and Plummer, 1993) and frozen-thawed dog semen (Kumi-Diaka, 1993). However, there have been no publications on a possible prognostic value of the HOS test to evaluate the freezing capacity of semen in dogs. Furthermore, correlations between data from the HOS assessment and conventional semen variables are inconsistent (Goericke-Pesch and Failing, 2013).

Therefore, the objectives of this study were 1) to examine relationships between HOS test results and conventional semen variables before freezing and after thawing and 2) to evaluate the prognostic value of the HOS test on the post-thaw quality of dog semen.

MATERIAL AND METHODS

Animals

From September 2012 to October 2013, semen of 46 client owned male dogs was collected. The dogs were selected irrespective of breed (22 different breeds, one mixed breed), age or fertility status (convenience sample). Clients had registered the dogs for the study in advance and a written consent to conduct this research was obtained. The animals were kept in kennels or in houses by the private owners. They were fed individually. All animals were clinically and andrologically healthy as confirmed by a thorough examination of each animal immediately before semen collection including ultrasonic assessments of the prostate and testes. The dogs were between 1 and 11 years ($\tilde{x}$ = 3) and weighed between 2.6 and 65 kg ($\tilde{x}$ = 19.8). One ejaculate was collected from each dog after a sexual rest of at least 7 days.

Semen collection and evaluation

A teaser bitch in oestrus or stocked vaginal swaps from bitches in oestrus were used for sexual stimulation before the time of semen collection. One ejaculate from each dog was obtained by digital manipulation. Throughout sampling, three semen fractions were collected into three different warmed (37°C) sterile glass funnels (Ludwig Bertram GmbH, Germany) using the previously reported procedures of Riesenbeck et al. (2001) and Goericke-Pesch et al. (2013). These consisted of the transparent pre-sperm, milky-white sperm-rich, and slightly opaque to transparent prostatic fractions and these fractions were stored in a water bath (Julabo 5A, Julabo GmbH, Germany) at 37°C. Only the sperm-rich fraction was used for further analysis and it was separated into two equivalent aliquots. The first aliquot was evaluated immediately after semen sampling. The second aliquot was prepared for the freezing process. Semen variables such as sperm motility, morphology, and membrane integrity as well as results from the HOS test were evaluated in fresh and frozen-thawed semen samples. Each ejaculate was tested individually and the results were documented on evaluation forms. Semen volume, color and viscosity were determined using standard techniques. The pH was measured immediately after semen collection from the second fraction using pH-indicator strips (Merck KGaA, Germany; Goericke-Pesch and Failing, 2013).

Immediately after semen collection, sperm motility was assessed at 37°C in a Makler chamber (Sefi-Medical Instruments, Haifa, Israel) under a phase contrast microscope (Olympus BH-2, Olympus Corporation, Tokyo, Japan) at X 200 (Rota et al., 1995). Sperm cells (n = 200) were examined and the percentage of progressive motile (forward

movement), local motile (circular movement) and immotile (no movement) were ascertained.

Concentration of sperm cells was determined cytometrically using an improved Neubauer haemocytometer counting chamber (LO - Laboroptik GmbH, Germany; England and Plummer, 1993). The numbers of sperm cells within 10 squares were counted under a light microscope at X 200 and multiplied by a factor of 5000 to obtain the number of sperm/µL.

For assessment of sperm cell membrane integrity Eosin Y solution 1% in water (Carl Roth GmbH & Co. KG, Germany) was used. Immediately after mixing two drops of Eosin with one drop of the ejaculate, a smear was prepared and air dried. Sperm with damaged plasma membranes are stained by Eosin whereas sperm with intact plasma membranes remain unstained (Goericke-Pesch and Failing, 2013). Sperm cells (n = 200) were evaluated for cell membrane integrity under a light microscope at X 400.

For sperm cell morphology assessment, a smear of ejaculate was prepared and assessed using Spermac™ stain (Fertipro NV, Beernem, Belgium) using the manufacturer's instructions (Goericke-Pesch and Failing, 2013). After fixation in formalin (4%) the specimen was dyed with three different stains and allowed to air dry before examination. The various parts of the sperm cell take up the stain differently, thus these fixation and staining techniques were used to facilitate detection of any anomalies of each cell part. As a result of morphological assessments, sperm cells (n = 200) were classified either as normal or abnormal under a light microscope (Olympus BH-2, Olympus Corporation, Tokyo, Japan) at X 1000 using oil immersion. Abnormalities were specified as defects in the head, midpiece or tail region of sperm cells.

For each HOS test, 1 mL of a pre-warmed (37°C) commercial hypo-osmotic solution (166 mosmol; Fertipro NV, Beernem, Belgium) was mixed with 0.1 mL of ejaculate and incubated at 37°C for 30 minutes. One drop of the specimen was applied on a warmed (37°C) slide with a cover slip being applied and examined under a phase contrast microscope at X 400. Under hypo-osmotic conditions sperm cells with intact membrane function swell due to the influx of water until equilibrium is reached, resulting in the curling of sperm tails (see Figure 1). Sperm with non-functional membranes remain unchanged. Sperm cells (n = 200) were examined and the percentage of cells with curled tails was assessed.

Semen processing

All reagents used in this process were obtained from Minitueb Abfuell- und Labortechnik GmbH & Co. KG (Tiefenbach, Germany) by now MOFA - Minitube of America (Verona, WI, USA).

Freezing

For cryopreservation, two extenders (CaniPRO® Freeze A&B) consisting of purified water, sodium citrate, TRIS, glycerol (B only), glucose, proprietary factors and gentamycin were used and freshly prepared on the day of semen analysis following the manufacturer's instructions (MOFA - Minitube of America (Verona, WI, USA). In addition, a supplementation of 20% fresh egg yolk to both media was required. Eggs used for this purpose were layed on the same day by SPF (specific pathogen free) birds of one stock and were obtained by the Institute of Poultry Diseases (Freie Universität Berlin, Germany). Extenders were freshly prepared before freezing. Egg yolks were neither pooled nor stored. The second fraction from the semen collection was initially extended slowly by adding one part of semen to one part of CaniPRO® A at 37°C. After cooling, the extended semen was stored at 4°C for 2 hours. The CaniPRO® B (4°C) was then added slowly in the same amount as the undiluted semen volume. Following this process, the samples were immediately inserted into pre-cooled, labelled plastic straws (4°C), which were sealed with a metal ball and stored at 4°C for 20 minutes. For cryopreservation, the straws were placed horizontally in an isothermal Styrofoam box for 20 minutes, positioned 4 to 5 cm above the liquid nitrogen level before the samples were plunged into liquid nitrogen for storage for 7 days (Kumi-Diaka, 1993).

Thawing

After a 7-day period in the frozen state, semen was thawed according to the manufacturer's instructions (MOFA - Minitube of America (Verona, WI, USA). Cryopreserved straws were immersed in a water bath at 37°C for 1 minute. Thawed semen was then decanted into a pre-warmed, sterile glass funnel (Ludwig Bertram GmbH, Germany) which was kept in the water bath (37°C) for further analysis. Before evaluation, the semen was slowly diluted by adding one part of CaniPRO® culture medium to one part of the semen sample. The sample was then evaluated for sperm cell motility, membrane integrity, and morphology, as well as for the HOS-response, as reported.

Semen classification

To evaluate the prognostic value of the HOS test for dog semen on post-thawing semen quality, semen was classified according using the methods of Thomasson et al. (2006). Frozen-thawed semen having ≥ 50% progressive motility was classified as "good" and < 50% progressive motility as "poor" quality. If the percentage of morphologically abnormal sperm was > 20%, semen quality was classified as "poor", regardless of sperm cell motility.

Statistical analyses

Statistical analyses of data were performed by IBM® SPSS® Statistics software (version 22 for Windows). The Shapiro-Wilk test was used for normality analysis of semen variables. Non-parametric tests were used for further statistical calculations. The Wilcoxon test was used to evaluate the differences between fresh and frozen-thawed semen. The statistical significance was set at $P < 0.05$. Spearman´s rank correlation was used to analyse the relationship between semen variables at the different times of semen evaluation. To compare the cryopreservation capacity of semen of good or poor quality, the Mann-Whitney-U-test for independent samples was used.

RESULTS

Animals

Of 46 dogs, 11 could not be included in the study because of refusal of the procedure for semen sampling (n = 4), contamination with foreign cells (n = 2) or the absence of sperm in the semen plasma (n = 5). One ejaculate sample was collected from each dog. A total of 35 ejaculates were included in the study.

Semen evaluation

Median volume of the sperm rich fraction of the ejaculate was 1.20 mL (IQR 0.8 - 1.5). The characteristics of the second fraction varied from a clear-opalescent to white-opalescent colour with a liquid to milky viscosity that was related to sperm concentration. Analysis of pH revealed a median of 6.3 (IQR 5.8 – 6.7). Evaluation of semen concentration from the second fraction using a Neubauer improved counting chamber provided a median number of 0.66×10^9/mL (IQR 0.31 - 0.99).

The results for sperm cell motility, membrane integrity, and morphology as well as for the HOS test in fresh and frozen-thawed semen are included in Table 1. The cryopreservation processing resulted in a decrease in all semen variables. The median value for progressive motility decreased in frozen-thawed semen compared to that of fresh semen ($P < 0.01$). The percentage of local (circular movement) and immotile sperm increased after freezing and thawing ($P < 0.01$). After freezing and thawing, the percentage of membrane intact sperm decreased ($P < 0.01$) and the number of morphological normal sperm also decreased ($P < 0.01$). The median number of morphologically abnormal sperm increased ($P < 0.01$) after freezing and thawing. For evaluation of sperm cell membrane function, the HOS

test was used. Cryopreservation processing caused a decrease in the percentage of swollen sperm cells ($P < 0.01$).

Correlations

Fresh semen

In fresh semen, the HOS test was positively correlated with progressive sperm cell motility ($r = 0.52$; $P < 0.05$), sperm cell membrane integrity ($r = 0.5$; $P < 0.05$) and normal cell morphology ($r = 0.46$; $P < 0.05$). Negative correlations were detected between data resulting from the HOS test and local (circular movement) sperm ($r = - 0.53$; $P < 0.05$) and immotile sperm ($r = - 0.52$; $P < 0.05$) as well as morphologically abnormal sperm ($r = - 0.46$; $P < 0.05$).

Frozen-thawed semen

With frozen-thawed semen, the results for the HOS test were positively correlated with progressive sperm motility ($r = 0.67$; $P < 0.05$) and sperm cell membrane integrity ($r = 0.86$; $P < 0.05$). Correlations between the HOS test results and normal sperm cell morphology were ($r = 0.39$; $P < 0.05$).

Fresh and frozen-thawed semen

Correlation values between semen variables for fresh and frozen-thawed samples are included in Table 2. The HOS test results with fresh semen were positively correlated with sperm cell membrane integrity ($r = 0.50$; $P < 0.05$), normal sperm cell morphology $r = 0.55$; $P < 0.05$) and results for the HOS test ($r = 0.43$; $P < 0.05$) with frozen-thawed semen.

Prediction of individual cryopreservation capacity

To evaluate the prognostic value of the HOS test on dog semen post-thawing quality, semen samples were classified as previously described. Of 35 frozen-thawed ejaculates, 14 fulfilled the inclusion criteria for good semen quality, having at least 50% progressively motile sperm cells and less or equal to 20% abnormal morphology. To test if fresh semen variables can be used to predict individual cryopreservation capacity, progressive sperm cell motility, and membrane integrity as well as results from the HOS test were grouped by post thawing results (i.e., good and poor post-thaw seminal quality). The HOS test results are depicted in Figure 2. In the present study, statistical analyses revealed identical pre-freezing results with

regard to good and poor post-thawing semen quality. Results from the HOS assessment for fresh semen did not differ between the groups. Based on these results, it is not possible to predict the quality of frozen-thawed dog semen using the HOS test.

DISCUSSION

The first objective of the present study was to examine the relationships between the HOS test results and conventional semen variables before freezing and after thawing. Semen of 35 dogs was collected and evaluated to address this objective.

Fresh semen had normal physical and morphological characteristics. Mean percentages of conventional variables, such as progressive sperm motility and normal morphology in fresh and frozen-thawed semen, were considered to be normal and within the reference ranges (Farstad, 2010, Riesenbeck et al., 2001). The response of dog sperm to hypo-osmotic conditions was similar to that reported by others (England and Plummer, 1993, Goericke-Pesch and Failing, 2013, Kumi-Diaka, 1993, Riesenbeck et al., 2001, Rodriguez-Gil et al., 1994). Mean percentage response to the HOS test in fresh sperm was 91% ($P < 0.01$), and after a 7-day freeze-thaw period was 57% ($P < 0.01$), which is in agreement with results of Kumi-Diaka et al. (1993).

With fresh semen in the present study, there were significant correlations between the response data with the HOS test and progressive sperm cell motility ($r = 0.52$), membrane integrity ($r = 0.50$), and normal morphology ($r = 0.46$), ($P < 0.05$). According to Mukaka et al. (2012) these correlations can be classified as low to moderate. Similar results were obtained by Goericke-Pesch et al. (2013). Kumi-Diaka et al. (1993) and Rodriguez-Gil et al. (1994) observed significant high correlations between HOS test data and sperm cell motility data. Rodriguez-Gil et al. (1994) also found significant correlations between HOS test results and sperm membrane integrity and normal morphology, similar to results from the present study. In contrast to these results, England et al. (1993) found no relationship between HOS test data and data for other seminal variables, such as sperm cell motility, morphology and membrane integrity. These inconsistent results may be due to use of different test solutions or different test procedures (Karger et al., 2014).

To identify the prognostic value of the HOS test on the quality of frozen-thawed dog semen, there were classifications of semen retrospectively, based on post-thaw results using the procedures of Thomassen et al. (2006). Thomassen et al. (2006) reviewed the results of artificial insemination with frozen-thawed dog semen retrospectively from data collected over a 10 year period. A total of 526 bitches were inseminated at conventional times during 685 oestrous cycles. Based on these previous results poor post-thaw semen quality ($n = 93$) resulted in a lesser pregnancy rate ($P < 0.01$) than when semen was used that was classified

as being of good quality (n = 566, 61.3% compared with 77.4%). In the present study, there were positive correlations between the data resulting from the HOS test when using fresh semen and conventional variables with use of frozen-thawed semen ($P < 0.05$). Statistical analyses, however, revealed that the HOS test results do not allow for a reliable prediction if post thaw semen quality is good (n = 14) or poor (n = 21). The HOS test results when using fresh semen did not differ between the two groups which means that assessment of poor quality semen can result in the same post thaw HOS test results as when good quality semen is used. Based on these results, it is not possible to predict the quality of frozen-thawed dog semen using the HOS test.

Studies investigating the fertilizing capacity of dog semen are few. It is generally accepted that conventional semen variables are poorly correlated with the fertilization capacity of dog semen (Kim et al., 2010, Silva et al., 2006). The mechanism underlying the reduction in fertility of preserved semen is not completely understood. In addition to differences among males and cryopreservation protocols, there is also the impact of the female variable to consider with regard to fertilizing capacity of semen samples (Eilts, 2005). In that regard, there are several variables and influencing factors, such as the inherent fertility of the female, variability of each estrous cycle, method of insemination (vaginal, trans cervical), timing of insemination and number of inseminations. In vitro sperm evaluation, if it is to be of predictive value for in vivo fertility should, therefore, include the assessment of as many variables as possible (Eilts, 2005) and findings in the present study substantiate this previous conclusion.

There are some limitations regarding materials and methods used in the present study. In this study client owned dogs were used. The use of laboratory animals would have resulted in a less heterogeneous breeding of experimental animals. Studies with client owned dogs, however, provide for the heterogeneity of breeding typically incurred in veterinary practice, thus, providing for breed backgrounds that are similar to what would be found in dog artificial insemination practices. Recently, a retrospective analysis of dog sperm by Goericke-Pesch et al. (2013) examined correlations between body weight and age and semen variables, such as sperm cell concentration, motility, morphology, and membrane integrity as well as results from the HOS test. In total, 400 semen samples from 250 dogs were evaluated. The results of sperm analysis after grouping the dogs according to body weight were either identical or very similar in all body weight groups, but sperm concentration was obviously different between the groups (<10 kg - 270 x 10^6/ml; 10-30kg - 300 x 10^6/ml; >30 kg - 250 x 10^6/ml). Age was negatively correlated with sperm cell concentration, motility and membrane integrity.

For sperm cell motility assessment, the conventional light microscopic techniques were used in the present study, although CASA (computer assisted sperm analysis) systems

are known to offer an accurate, rapid and simultaneous assessment. Several studies, however, described high correlations between computer-calculated sperm motility and the conventional light microscopic evaluation (Gunzel-Apel et al., 1993, Iguer-ouada and Verstegen, 2001, Rijsselaere et al., 2003). Furthermore, there is still a need for standardization and validation with CASA assessments due to the lack of uniformity among users using different instruments, diluents and analysis temperature in the counting chamber (Rijsselaere et al., 2005).

In the present study, a commercial hypo-osmotic solution (166 mosmol) from Fertipro NV, Beernem, Belgium was used and incubations of the semen from the collections occurred at 37°C for 30 minutes. It is possible that the HOS test kit used in the present study had an effect on the results of this study. A systematic review of studies in which the HOS test was conducted to evaluate the quality of dog sperm revealed that there are no standardized implementations of the HOS test for this purpose (Karger et al., 2014). Osmolarities, used in these previous studies (n = 38) varied from 0 to 450 mosmol, incubation times varied from 0 to 90 min, and incubation temperatures from 25 to 39°C. It is unclear, however, if the utilization of different conditions leads to varying results.

It is generally accepted, that cryopreservation procedures currently used induce a series of osmotic, chemical and mechanical stresses on sperm cells (Martinez, 2004). For freezing and thawing, a commercial kit (CaniPRO®) was used in the present study consisting of two different freezing extenders including glycerol and one thawing medium. Glycerol is the cryoprotectant which is most frequently used for sperm cryopreservation in several species, including dogs (Lopes et al., 2009, Rota et al., 2005, Schafer-Somi et al., 2006). However, glycerol can induce changes in the lipid packing structure of the sperm membrane, thereby altering sperm stability and water permeability (Lopes et al., 2009).

Egg yolk was required for preparation of freezing extenders. Use of egg yolk is known to protect cell membranes against cold shock and prevent or restore the loss of phospholipids from the sperm cell membrane. Egg yolk is not a defined entity, but a complex biological compound containing proteins, phospholipids, vitamins, glucose and antioxidants. Nevertheless, egg yolk can be a deleterious compound when used in semen extenders if there is large variation in yolk content or if disease agents are a component of the egg yolk (Farstad, 2009). To minimize heterogeneity and to reduce the risk of contamination in the present study, eggs from one stock that were laid on the same day were obtained by the Institute of Poultry Diseases (Freie Universität Berlin, Germany). These eggs were from specific pathogen free (SPF) animals, which means, the eggs were free from viruses, fungi and bacteria. Freezing extenders were prepared on the same day of semen analysis.

A variety of freezing regimens, extenders and thawing protocols have been described in literature (Eilts, 2005, Stanescu and Birtoiu, 2010). The components differ significantly and

also the process of the addition: one-step dilution compared with two-step dilution (Stanescu and Birtoiu, 2010). However, an aim was to use methods that are commercially available and well described to represent relevant procedures used in dog artificial insemination practices in Europe. It remains unknown if other freezing procedures or extender use would lead to different results.

CONCLUSIONS

In conclusion, only low to moderate correlations between the HOS test and conventional semen variables were detected in the present study. Based on statistical analyses, the HOS test had little prognostic value on the post-thaw quality of dog semen. To improve the assessment of the fertilizing capacity of a semen sample, a combination of tests measuring different aspects of sperm function and morphological variables provides for more reliable results than one test alone. It is recommended that each semen sample be evaluated before freezing with awareness that a prediction of the post-thaw quality is not possible unless the fresh semen is of poor quality.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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Figure 1. Dog semen processing, arrow: curled sperm tail indicating an intact cell membrane under hypo-osmotic conditions.

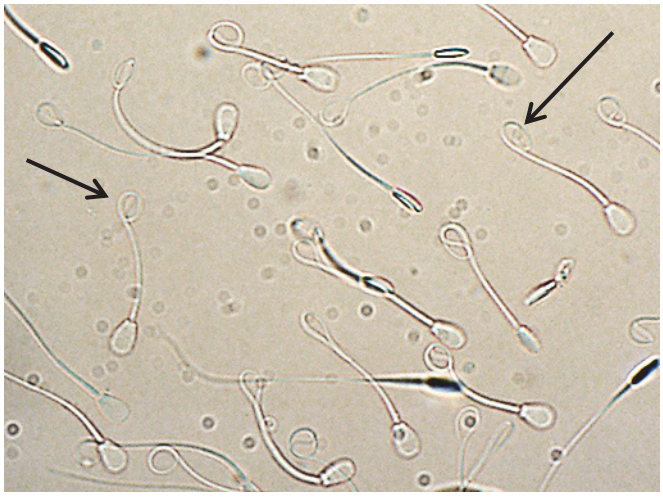


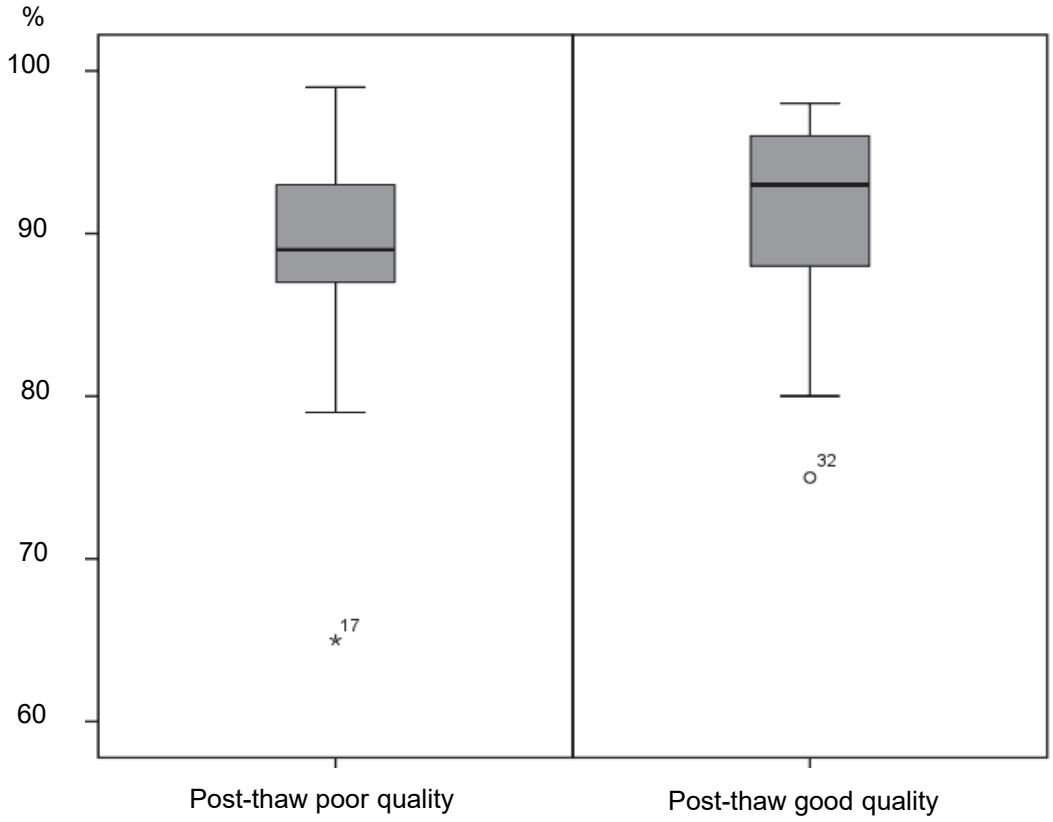
Table 1. Median ($\tilde{x}$) results and interquartile range (IQR) in % of ejaculate analysis of the sperm-rich fraction in fresh and frozen-thawed dog semen. P-value < 0.01.

Variable	Median % (IQR)	
	Fresh semen	Frozen-thawed semen
Motility		
Progressive	83 (74-90)	52 (31-62)
Local	8 (5-13)	14 (11-19)
Immotile	7 (4-11)	35 (22-46)
Membrane integrity	86 (81-90)	58 (48-69)
Morphology		
Normal	88 (82-91)	82 (77-87)
Head abnormalities	5 (4-7)	7 (5-9)
Midpiece abnormalities	5 (3-8)	6 (4-9)
Tail abnormalities	2 (1-4)	3 (2-5)
HOS test	91 (87-96)	57 (47-66)

Table 2. Results of correlation analysis between sperm variables in fresh and frozen-thawed dog semen. P-value < 0.05.

Fresh semen	Frozen-thawed semen			
	Progressive Motility	Membrane Integrity	Normal Morphology	HOS test
Progressive Motility	0.22	0.29	0.27	0.34
Membrane Integrity	0.33	0.45	0.23	0.37
Normal Morphology	0.19	0.26	0.92	0.27
HOS test	0.35	0.50	0.55	0.43

Figure 2. Boxplot showing the percentage of HOS test* results in fresh semen grouped by post-thaw poor and good quality semen. Frozen-thawed semen showing ≥ 50% progressive motility was classified as “good”, and < 50% progressive motility as “poor” semen quality. If the percentage of morphologically abnormal sperm was > 20%, semen quality was classified as “poor”, regardless of sperm motility. Statistical analysis revealed similar pre-freeze results with regard to good and poor post-thaw semen quality. P-value < 0.05.



* HOS test: hypo-osmotic swelling test. Under hypo-osmotic conditions sperm with intact membrane function swell due to the influx of water until equilibrium is reached, resulting in the curling of sperm tails. Sperm with non-functional membranes remain unchanged.

2.3 Short communication: Progressive motility of frozen-thawed canine semen is highest five minutes after thawing

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Progressive motility of frozen-thawed canine semen is highest five minutes after thawing

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3 DISCUSSION

For dog breeders as well as for veterinarians the aim of a semen evaluation is to give a prognosis about the fertilizing capacity of a semen sample. The quality of diagnostic tests has thus a tremendous impact on the diagnosis and on reproductive success. This may affect decisions such as the selection of a stud dog, rejection of other stud dogs, assignments for semen cryopreservation and after all the reproductive success.

A diagnostic test should show the highest possible sensitivity and specificity (Robinson et al. 2015). Sensitivity represents the proportion of truly diseased animals in a screened population that are identified as being diseased by the test. It is a measure of the probability of correctly diagnosing a condition. Specificity is the proportion of truly non-diseased animals that are identified as healthy by the same diagnostic test. It is a measure of the probability of correctly identifying a non-diseased animal (Akobeng 2007).

In small animal reproduction only few diagnostic tests are available which are defined as gold standards (Enoe et al. 2000). Gold standards are methods having established accuracy for determining a diagnosis that provides a standard to which a diagnostic test can be compared. The deficiency for these gold standards is based on the fact, that only few studies have been conducted so far investigating the quality of diagnostic methods (Baadsgaard and Jorgensen 2003).

The overall objective of this study was to determine the diagnostic value of the HOS test as a standard parameter for canine semen evaluation. Specifically, the objectives were 1) to evaluate the quality of published literature in canine reproduction concerning the HOS test, 2) to examine relationships between HOS test results and conventional semen parameters before freezing and after thawing and 3) to evaluate the prognostic value of the HOS test on canine semen`s post-thaw quality.

The results of the first study indicate that although there are numerous articles available, the diagnostic value of the HOS test remains unclear. Until now, neither a recognized standard operating procedure nor reliable reference ranges have been defined. Most of the studies investigated, have serious methodological flaws (e.g. limited sample size, no randomization, no determination of inter- or intraobserver agreement) and therefore do not permit reliable conclusions. According to our results, about half of the studies (n = 20) included a sample size of five or less animals. None of the authors mentioned a calculation of sample size for the study. In fact, eight studies used semen which was pooled and therefore did not analyse the individual semen quality in each dog (Batista et al. 2012). We conclude that sample size as one important component of evidence was either not addressed at all or the number of dogs was marginal in more than half of the studies performed. To establish a new, reliable diagnostic method, it is important to assess the

repeatability and reproducibility of the measurement process (Watson and Petrie 2010) but except for two studies by Rota et al. (2005, 2006) neither an intra- nor an interobserver agreement for the HOS test was determined. When examining the implementation protocols for HOS-testing, parameters, such as osmolarity, incubation time and incubation temperature varied from study to study. It is unclear, however, if the utilization of different parameters provides comparable results. To minimize potential bias, it is required to develop standardized implementations (Watson and Petrie 2010). Further research is warranted to establish a standardized implementation as well as reliable reference ranges. Most importantly, it is required to clarify a correlation between HOS test and fertility to determine the diagnostic value of the HOS test.

Therefore, the objectives of the second study were 1) to examine relationships between HOS test results and conventional semen variables before freezing and after thawing and 2) to evaluate the prognostic value of the HOS test on the post-thaw quality of dog semen. In fresh semen, we found significant correlations between the HOS test results and progressive sperm cell motility, membrane integrity and normal morphology. According to Mukaka et al. (2012) these correlations can be classified as low to moderate. Statistical analyses revealed that the HOS test results do not allow for a reliable prediction if post-haw semen quality is good (n = 14) or poor (n = 21). The HOS test results when using fresh semen did not differ between the two groups which means that assessment of poor quality semen can result in the same post thaw HOS test results as when good quality semen is used. Based on these results, it is not possible to predict the quality of frozen-thawed dog semen using the HOS test.

The third study revealed significant positive correlations between progressive motility and conventional semen parameters, such as progressive motility, morphology, membrane integrity and HOS test, which were highest five minutes after thawing, although sperm motility is usually examined immediately after thawing. Once more, this study shows the need for standardization of diagnostic tests to obtain comparable results.

Studies investigating the fertilizing capacity of dog semen have rarely been published. It is generally accepted that conventional semen variables are poorly correlated with the fertilization capacity of dog semen so far (Kim et al. 2010, Silva et al. 2006). Furthermore, the mechanisms underlying the reduction in fertility of preserved semen are not completely understood. In addition to differences among males and cryopreservation protocols, there is also the impact of the female variable to consider with regard to fertilizing capacity of semen samples (Eilts 2005). In that regard, there are several variables and influencing factors, such as the inherent fertility of the female, variability of each oestrus cycle, method of insemination (vaginal, trans-cervical), timing of insemination and number and technique of inseminations.

A variety of freezing protocols, extenders and thawing protocols have been described in literature (Eilts 2005, Stanescu and Birtoiu 2010) to prevent cryoinjury and to consistently achieve a high quality i.e. high survival rates of sperm cells. Canine semen has in general a low resistance to freezing (Alhaider and Watson 2009, Rota et al. 2005), resulting in a post-thaw decrease of semen parameters, especially of membrane integrity and motility (Eulenberger et al. 2009, Kim et al. 2010, Monteiro et al. 2009). The type of semen extender and the specific freezing-method are important factors influencing the post-thaw sperm quality (Setyawan et al. 2015, Belala et al. 2016), which may have an impact on the success rate of artificial insemination. This means that it remains open if our results would have been different if we had used a different extender.

For freezing and thawing we used a commercial kit (CaniPRO®) distributed by Minitube of America (MOFA), Verona, WI, USA. MOFA is an important provider of assisted reproduction technologies for porcine, bovine, equine and canine. Current annual data for market share, however, have not been published. In comparison to non-commercial self-made solutions, commercial freezing extenders are likely to offer a standardized, high, assured quality. All reagents we used in our studies are provided by internationally acknowledged manufacturers for assisted reproduction technologies. This may improve the comparability of our semen evaluation results with those from other studies. In the second study, a commercial hypo-osmotic solution (166 mosmol) from Fertipro NV, Beernem, Belgium was used and incubations of the semen from the collections occurred at 37°C for 30 minutes according to manufacturer's instructions. It is possible that the HOS test kit used in the study had an effect on our results. Our first study revealed that there are no standardized implementations of the HOS test for this purpose. Osmolarities, used in the analyzed studies (n = 38) varied from 0 to 450 mosmol, incubation times varied from 0 to 90 min, and incubation temperatures from 25 to 39°C.The test we selected uses parameters that can be considered to be close to the averages of the parameters of the analyzed studies. In addition, in most studies temperatures of 38°C or 39°C were used. In that regard, we tried to find a test that is most likely to be comparable to other HOS approaches. Nevertheless, the standardization of HOS-test protocols will be an essential part in future studies.

Although we found significant correlations between the HOS test and conventional semen parameters, we could not confirm a prognostic value regarding the quality of frozen-thawed canine semen. In conclusion, the HOS test can be used as an additional parameter for completion of conventional canine semen analysis to get additional information about membrane integrity and function. However, the prognostic value for fertility remains unclear. So far, there is no sole test available to predict the quality of frozen-thawed canine semen. To improve the assessment of the fertilizing capacity of a semen sample, further research is necessary. Assumedly, a combination of standardized tests measuring different aspects of

sperm function and morphological variables provides for more reliable results than one test alone. Each semen sample should be evaluated before freezing with awareness that a prediction of the post-thaw quality is not possible yet unless the fresh semen is of poor quality.

In general, also other semen evaluation parameters should be assessed critically in regard to standard operating procedures and interpretation of test results. The presented three studies underline the necessity for critically scrutinizing generally accepted diagnostic tests. This is an essential prerequisite to warrant the comparability of information about semen quality. In times of Evidence-based Veterinary Medicine, further research analysing the accuracy of diagnostic tests to standardize implementations is necessary. The scientific instruments to do this are already available (Eusebi 2013).

4 SUMMARY

Sandra Karger: The hypo-osmotic swelling test – critical research concerning the applicability in canine reproduction

For dog breeders as well as for veterinarians the aim of a semen evaluation is to give a prognosis about the fertilizing capacity of a semen sample. Conventional semen analysis includes determination of volume, color, sperm concentration, progressive sperm motility, sperm membrane integrity and sperm morphology. Besides, several additional parameters have been evaluated like the hypo-osmotic swelling test (HOS test). The HOS test is an easily applicable test to assess the functional integrity of the plasma membrane of sperm cells.

The overall objectives of this thesis were to analyse relationships between HOS test results and conventional semen parameters before freezing and after thawing and to determine the diagnostic value of the HOS test as a standard procedure for canine semen evaluation.

In preparation for the clinical trial, a systematic literature review was conducted in order to evaluate the quality of published literature concerning the HOS test. Using two databases, 354 articles were found. Out of these, 38 articles were eligible for further analyses according to specific quality parameters such as randomization, blinding, inter- and intraobserver agreement and sample size. Also test characteristics, such as study design, population, semen sampling and implementation concerning the HOS test were investigated. A citation map was developed to identify the origin of the quotation of the HOS test. Although there were numerous articles available, the diagnostic value of the HOS test remained unclear. Until then, neither a recognized test implementation nor reliable reference values have been defined. Most of the trials evaluated showed serious methodological flaws and therefore did not permit drawing reliable conclusions. According to our results, approximately half of the studies (n = 20) included a sample size of five or less animals. None of the studies examined the inter- or intraobserver agreement for the HOS test. Overall, the results of the study indicated the need for standardization of test implementations as well as for reliable reference values. Furthermore, a possible correlation between the HOS test results and the fertilizing capacity will have to be clarified to determine the diagnostic value of the HOS test.

The objectives of the second study were 1) to examine relationships between HOS test results and conventional semen parameters before freezing and after thawing and 2) to evaluate the prognostic value of the HOS test results on the post-thaw quality of canine semen. Semen of 35 dogs was collected and analysed before freezing and after thawing

following a 7-day freeze-thaw interval. Conventional semen variables such as sperm cell motility, membrane integrity and morphology were evaluated and also the HOS test was conducted. For the prediction of individual cryopreservation capacity, semen was classified into good and poor quality according to certain semen characteristics. Results from the assessment of fresh semen parameters of good and poor semen quality were statistically compared. Based on these results, it is not possible to predict the quality of frozen-thawed dog semen using the HOS test.

The objective of the third experiment was to examine time-dependent changes of motility after thawing cryopreserved canine semen. Semen motility is expressed as the percentage of total motile or progressive motile sperm cells. It is usually estimated by visual inspection using a light contrast microscope at X 100 at 37°C immediately after semen collection or immediately after thawing frozen semen and should be one of the first steps of a semen analysis. Standard operating procedures, however, have never been established for this test. For this experiment, semen of 35 dogs was collected and volume, concentration, progressive motility, morphology, membrane integrity and HOS test were evaluated. For cryopreservation CaniPRO® Freeze A&B was used. Semen was thawed and diluted using CaniPRO® Culture Medium. After thawing, semen was evaluated as before. In addition, every sample was tested for progressive motile sperm cells 0, 5, 20 and 60 minutes after thawing. Overall, the study revealed significant positive correlations between progressive motility and conventional semen parameters, which were highest five minutes after thawing. To obtain comparable results for motility assessment of cryopreserved canine semen, the development of standard operating procedures was recommended.

The presented three studies indicate the need for standardization of test implementations as well as for reliable reference values to warrant the comparability of information about semen quality. Although significant correlations were found between the HOS test results and conventional semen parameters, the HOS test is not suitable to give a prognosis about the quality of frozen-thawed canine semen.

5 ZUSAMMENFASSUNG

Sandra Karger: Der Hypo-osmotische Schwelltest – eine kritische Untersuchung zur klinischen Anwendbarkeit in der Reproduktionsmedizin des Hundes

Sowohl für Hundezüchter als auch für Tierärzte ist es wünschenswert, im Rahmen einer Samenanalyse die Befruchtungsfähigkeit prognostizieren zu können. Eine Samenanalyse beinhaltet in der Regel die Bestimmung von Volumen, Farbe, Spermienkonzentration, Spermienbeweglichkeit, Membranintegrität sowie Spermienmorphologie. Darüber hinaus gibt es zahlreiche zusätzliche Untersuchungsmöglichkeiten, wie beispielsweise den Hypo-osmotischen Schwelltest (HOS-Test). Der HOS-Test ist ein einfach zu handhabener Test, um die funktionelle Integrität der Plasmamembranen von Spermienzellen zu bestimmen.

Ziel dieser Arbeit war es, die Beziehung zwischen den Ergebnissen des HOS-Tests und herkömmlichen Spermienparametern vor dem Einfrieren und nach dem Auftauen zu untersuchen, um die diagnostische Wertigkeit des HOS-Tests als Standardparameter der caninen Samenanalyse zu bestimmen.

In Vorbereitung auf eine klinische Studie wurde zunächst eine systematische Literaturübersicht angefertigt, um die Qualität von Publikationen in Bezug auf den HOS-Test zu bewerten. Mit Hilfe zweier Datenbanken wurden 354 Artikel ausfindig gemacht, von denen im Ergebnis 38 Artikel den Anforderungen entsprachen und anschließend analysiert wurden. Die Qualität der Studien wurde nach spezifischen Parametern, wie der Durchführung von Randomisierung, Verblindung, Inter- und Intraobservervariabilität und der Probandengröße systematisch bewertet. Zusätzlich wurden weitere Testcharakteristika, wie das Studiendesign, die Population, das Verfahren der Samengewinnung und die Spezifika der Anwendung des HOS-Tests erhoben. Um den Ursprung der literarischen Zitate in Bezug auf den HOS-Test zu ermitteln, wurde eine Citation Map angefertigt. Obwohl zahlreiche Studien zu diesem Thema publiziert wurden, konnte die diagnostische Wertigkeit des HOS-Tests nicht abschließend definiert werden. In keiner der untersuchten Studien wurden standardisierte Testparameter oder Referenzwerte für den HOS-Test festgelegt. Die meisten Studien wiesen erhebliche methodologische Mängel auf und ließen deshalb keine verlässlichen Schlussfolgerungen zu. Nach unseren Ergebnissen wurden ca. die Hälfte der untersuchten Studien (n=20) mit einer Population von fünf oder gar weniger Tieren durchgeführt. In keiner der Studien wurde die Inter- oder Intraoberservervariabilität für den HOS-Test untersucht. Insgesamt wiesen die Ergebnisse der Studie auf die Notwendigkeit hin, Testverfahren zu standardisieren und verlässliche Referenzwerte festzulegen. Um die

diagnostische Wertigkeit des HOS-Tests zu bestimmen, muss in künftigen Studien die Beziehung zwischen dem HOS-Test und der Befruchtungsfähigkeit geklärt werden.

Ziel der zweiten Studie war es, die Voraussagbarkeit der Qualität von aufgetautem Tiefgefriersamen von Hunden anhand der Ergebnisse des HOS-Tests im Frischsamen zu bestimmen. Dazu wurden zunächst die statistischen Beziehungen zwischen den Ergebnissen des HOS-Tests zu herkömmlichen Spermienparametern vor dem Einfrieren und nach dem Auftauen untersucht. Hierfür wurde Samen von 35 Hunden gewonnen. Der Samen wurde vor dem Einfrieren und nach einem siebentägigen Einfrier-Auftau-Intervall untersucht. Neben der Bestimmung herkömmlicher Spermienparameter, wie Spermienbeweglichkeit, Membranintegrität und Morphologie wurde der HOS-Test durchgeführt. Zur Bestimmung der individuellen Einfrierbarkeit einer Spermienprobe wurden diese anhand der Untersuchungsergebnisse nach dem Auftauen im Nachhinein unterteilt in Proben, welche den mindestanforderungen entsprachen, und solche, deren Werte darunter lagen. Die Ergebnisse der Frischsamenuntersuchung der so eingeteilten Proben wurden statistisch miteinander verglichen. Demnach zeigte sich, dass es nicht möglich ist, die Qualität von aufgetautem Tiefgefriersamen mit Hilfe der Ergebnise des HOS-Tests von Frischsamen vorauszusagen.

Ziel der dritten Studie war es, im aufgetauten Tiefgefriersamen von Hunden zeitlich abhängige Änderungen in der Spermienbeweglichkeit zu untersuchen. Die Vorwärtsbeweglichkeit wird als prozentualer Anteil angegeben. Für gewöhnlich wird die Beweglichkeit mit Hilfe eines Kontrastmikroskops mit einer 100-fachen Vergrößerung durch visuelle Inspektion bei einer Temperatur von 37°C ermittelt. Die Beweglichkeit sollte als erster Schritt der Samenuntersuchung entweder unmittelbar nach der Samenkollektion oder unmittelbar nach dem Auftauen von Tiefgefriersamen untersucht werden. Standardisierte Verfahren für diesen Test sind bisher jedoch nicht veröffentlicht worden. Für dieses Experiment wurde der Samen von 35 Hunden gewonnen und hinsichtlich Volumen, Spermienkonzentration, Vorwärtsbeweglichkeit, Spermienmorphologie, Membranintegrität untersucht. Zusätzlich wurde der HOS-Test durchgeführt. Für die Kryokonservierung wurde CaniPRO® Freeze A&B verwendet. Der Samen wurde aufgetaut und verdünnt mit Hilfe von CaniPRO® Culture Medium. Nach dem Auftauen wurde der Samen wie zuvor analysiert. Zusätzlich wurde jede Probe auf Vorwärtsbeweglichkeit 0 (T0), 5 (T5), 20 (T20) und 60 (T60) Minuten nach dem Auftauprozess untersucht. Die Ergebnisse zeigten positive Korrelationen zwischen der Vorwärtsbeweglichkeit und anderen herkömmlichen Spermienparametern auf. Diese waren fünf Minuten nach dem Auftauen der Probe am höchsten. Daraus lässt sich ableiten, dass standardisierte Testverfahren entwickelt werden sollten, um vergleichbare Ergebnisse für die Bestimmung der Spermienbeweglichkeit von aufgetautem Tiefgefriersamen zu erhalten.

Die vorliegenden drei Studien heben die Notwendigkeit hervor, Testverfahren zu standardisieren und valide Referenzwerte zu ermitteln, um die Vergleichbarkeit von Informationenen über die Qualität einer Samenprobe zu gewährleisten. Obwohl signifikante Korrelationen zwischen dem HOS-Test und herkömmlichen Spermienparametern in den Studien nachgewiesen werden konnten, wird der HOS-Test als ungeeignet eingeschätzt, eine Prognose über die Qualität von aufgetautem Tiefgefriersamen von Hunden abzugeben.

6 REFERENCES FOR INTRODUCTION AND DISCUSSION

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7 PUBLICATIONS

Research articles

S. Karger, S.P. Arlt, P. Haimerl and W. Heuwieser. (2014):

Review article: A Systematic Review of Studies Performing the Hypo-Osmotic Swelling Test to Evaluate the Quality of Canine Spermatozoa.

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S. Karger, B. Geiser, M. Grau, O. Burfeind, W. Heuwieser and S.P. Arlt. (2016):

Prognostic value of a pre-freeze hypo-osmotic swelling test on the post-thaw quality of canine semen.

Animal reproduction science. 2016 Mar; 166: 141-147.

S. Karger, B. Geiser, M. Grau, W. Heuwieser and S.P. Arlt.:

Short communication: Progressive motility of frozen-thawed canine semen is highest five minutes after thawing.

Reproduction in domestic animals = Zuchthygiene, 2017 Apr; 52 (2): 350-352

Oral presentations

S. Galle, S. Arlt, P. Haimerl, W. Heuwieser

The hypo-osmotic swelling test – a valuable parameter for canine semen evaluation?

16th EVSSAR Congress 2013, Toulouse (France), 05.07-06.07.2013.

S. Galle, S. Arlt, P. Haimerl, W. Heuwieser

Der Hypoosmotische Schwelltest - ein wertvoller Parameter?

8th PhD-Symposium – Bringing Future Scientists Together & DRS Presentation Seminar 2013, Berlin (Germany), 15.07.2013.

Poster presentations

S. Karger, S. Arlt, B. Geiser, M. Grau and W. Heuwieser

Motility of cryopreserved canine semen should be evaluated 5 minutes after thawing.

47. Jahrestagung Physiologie und Pathologie der Fortpflanzung, Gießen (Germany), 27.02.-28.02.2014.

In: Reproduction in domestic animals = Zuchthygiene; 49 (Suppl. 1), S. 26.

S. Karger, S. Arlt, B. Geiser, M. Grau and W. Heuwieser

Motility of cryopreserved canine semen should be evaluated 5 minutes after thawing.

18th ESDAR Conference, Helsinki (Finland), 11.09.-13.09.2014.

In: Reproduction in domestic animals = Zuchthygiene; 49 (Suppl. 3), S. 74-75.

S. Karger, B. Geiser, M. Grau, O. Burfeind, W. Heuwieser and S. Arlt

Is the hypo-osmotic swelling test (HOS test) a suitable diagnostic tool to predict canine semen freezability?

17th EVSSAR Conference, Wroclaw (Poland), 26.09.2014.

In: 17th EVSSAR Congress. Reproduction and Pediatrics in Dogs, Cats and Exotics S. 187
ISBN: 978-83-63026-96-7.

S. Karger, P. Haimerl, W. Heuwieser and S. Arlt

The hypo-osmotic swelling test: a systematic review of test characteristics.

1st International Evidence-Based Veterinary Medicine Network Conference Windsor (UK), 23.10.-24.10.2014.

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9 DECLARATION OF INDEPENDENCE

Hiermit erkläre ich, dass ich alle Studien selbstständig durchgeführt und die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

This is to declare that I conducted all of the studies described herein myself and the manuscripts were produced independently. I confirm that I have used only the specified resources and tools to complete this thesis. My personal contributions to the research projects presented under this cumulative doctoral thesis are summarized in the following table.

Own Contribution	Research project 1	Research project 2	Research project 3
Study design	++[1]	++	++
Data collection	+++	+++	+++
Data analyses	+++	+++	+++
Manuscript writing	+++	+++	+++
Manuscript editing	++	++	++

[1] Score: + = < 50%; ++ = 50% to 70%; +++ = > 70%

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