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COMPARISON OF STAINING TECHNIQUES FOR SPERM MORPHOLOGICAL EVALUATION IN CANINE SEMEN

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ABSTRACT

The growth of multispecies families in Brazil highlights the need for ethical and high-quality reproductive practices, especially when it comes to breeding dogs. Success in reproduction depends on several factors, and the seminal evaluation, especially the sperm morphology, is extremely valuable, with emphasis on the complete andrological examination to ensure the reproductive performance of the male. Different staining techniques are used with the purpose of evaluating sperm morphology and diagnosing possible alterations that may compromise the fertility of the animal. Among the commonly used colorings, Panopticon Rapid, Giemsa, Eosin-nigrosine, Methylene Blue and Rose Bengal stand out. Each dye has its particularities, highlights, advantages and limitations in terms of sensitivity, practicality and detailing of sperm structures. The choice of the most appropriate stain for sperm evaluation should be based on the main purpose of the exam, which is the identification and characterization of morphological changes in spermatozoa. In addition, the durability time of the stained blade and the quality of the dyes used must be considered, since these factors directly impact the accuracy of the results obtained. These techniques allow better visualization of sperm structures, favoring the distinction between major and minor defects, as well as the differentiation between viable and nonviable spermatozoa. The adoption of effective staining methods is essential for more accurate diagnoses, contributing to decision-making and greater efficiency of assisted reproduction programs.

Keywords: Dog. Sperm. Semen. Colouring. Andrology.

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1 INTRODUCTION

Lately, dogs have come to be considered essential members of the family nucleus, and are often seen as part of the family. Initially domesticated for their hunting and protection skills, dogs began to occupy an affective position in human relationships, contributing to the reduction of stress, loneliness and even the improvement of depressive conditions. This close coexistence has driven the search for more humanized treatments and approaches that can ensure a better quality of life for animals, reflected in advances in the health, nutrition, and animal welfare sectors (Friedmann; Sonnenberg, 2009; Gonçalves *et al.,* 2021; Tesi *et al*., 2018; Souza *et. al.,* 2023).

The growing interest in dogs of high genetic value, selected according to specific behavioral profiles and lifestyles, has driven the use of genetic improvement as a fundamental tool in canine reproduction. However, the increase in the prevalence of hereditary diseases in purebred breeds reinforces the need for more judicious reproductive strategies, based on the health, well-being and genetic sustainability of the canine species. In view of this, andrological tests are essential to assess the reproductive health of dogs, detect possible fertility problems and improve the use of biotechnologies, such as artificial insemination. These analyses enable a more accurate and reliable diagnosis, favoring the choice of sires with a higher probability of reproductive success and contributing to the advancement of the genetic health of the breeds. (Medeiros, 2018; . Monteiro *et. al*., 2020; Souza *et. al.,* 2023).

The genetic selection of dogs, standardized in relation to physical characteristics, ended up disregarding the natural morphology of the animals and developed breeds that have numerous health problems, mostly chronic and that are contrary to animal welfare conditions (Corsi, 2018).

However, the practice of inbreeding, that is, between individuals with common ancestry, has generated significant concerns. Studies indicate that such practices can lead to anatomical malformations and an increase in the prevalence of genetic diseases, compromising the quality of life of animals (Toson *et al.,* 2015; Perissutti *et al*., 2019; Baldassaune, 2021; Silva *et al*., 2024). In addition, inbreeding can reduce genetic variability and increase homozygosis, predisposing to the manifestation of deleterious genes and the birth of offspring with malformations (Silva *et al*., 2024).

Early evaluation of breeding stock is a key step in ensuring positive fertility outcomes. Thus, the choice of animals proven to be fertile contributes significantly to obtaining better rates in reproductive management (Bastos *et al*., 2016; Jasmim *et al*., 2024). The selection of breeding dogs is essential to succeed in artificial insemination. The confirmation of normal spermatogenesis in a young male before starting his reproductive life; to evaluate the

production of semen after disease or drug therapy, and also to investigate the effect of prostatic disease on the quality of the dog's semen, as part of the Artificial Insemination program, have been evaluated by means of andrological examination (Tesi *et al*., 2018; Monteiro *et al*., 2020).

The analysis of the ejaculate is carried out through the spermogram, macro and microscopically. Volume, color, density, pH, appearance and odor should be evaluated, which in the canine species has a viscous appearance and odor classified as suis generis. As for microscopic analysis, sperm motility and vigor, sperm concentration and morphology, membrane and acrosome integrity, sperm chromatin integrity, and sperm capacitation are extremely important and may vary according to age, breed, size and frequency of collection (CBRA, 2013; Santos, 2016; Jasmim *et al*., 2024; Paiva *et al*., 2024).

Sperm motility and vigor are estimated subjectively, and analyzed under light microscopy, with a drop of semen between the lamina and coverslip, and its percentage is estimated visually. However, it is the most used technique in the laboratory routine and continues to have great value, especially to differentiate low and high quality semen (CBRA, 2013; Arruda *et al*., 2010).

In the context of canine reproduction, semen morphological analysis is a fundamental tool in the evaluation of the fertility of sires. The morphological integrity of spermatozoa is directly associated with their fertilizing potential, and structural abnormalities may indicate testicular and epididymal dysfunctions, or even the effects of environmental and nutritional factors. The morphology analysis aims to examine structural abnormalities of the sperm (Vecchiettti et al., 2018; Surmacz *et al*., 2022, Andrade, 2023; Jasmim *et al*., 2024; Paiva *et al*., 2024). A sperm is considered normal if the shape and size of the head, intermediate piece, and flagellum are within the classification adopted for a given species (Banaszewska *et al*., 2015; Czubaszek *et al.,* 2019).

When choosing the ideal color, the one that induces the least possible number of changes in the morphological structure of the spermatozoa should be considered, in addition to highlighting the different parts of the cell (Maree *et al*., 2010). Therefore, different staining techniques have been widely used to evaluate sperm morphology in domestic animals and humans. To evaluate the sperm morphology , 200 spermatozoa are counted under an optical microscope, at a magnification of 400x or 1000x (immersion oil). Structural changes in sperm are classified into two categories: major defects (primary), with minor defects (secondary) being considered more severe and less severe. Sperm morphology is classified into major/primary defects (defects of head, acrosome, intermediate pieces, proximal protoplasmic droplet, and tightly coiled tail) and minor/secondary defects (distal protoplasmic

droplet, folded/curled tail, normal isolated head, giant/short/wide, and slender head. Semen with 70.0% or more morphologically normal spermatozoa is considered ideal, with a maximum of 10.0% of total major defects and 20.0% of minor defects being acceptable. Subsequently, the determination of the total defects is carried out, the percentage evaluation of sperm cells is carried out (CBRA, 2013; Andrade, 2023).

The method of staining canine semen slides is a decisive step in the evaluation of fertility, because it allows the identification of abnormal spermatozoa, evaluation of sperm viability and the integrity of the membranes, directly influencing the conclusion regarding the reproductive fitness of the animal, diagnosing possible pathologies. This practice is especially relevant in assisted reproduction programs and in the reproductive performance of sires (Martins *et al*., 2020; Jasmim *et al*., 2024). For a good analysis of the slide to be observed, some prerequisites are necessary regarding coloring, such as: correct formulation of the dye, using good quality material, time of application of the technique adopted and the cost of the methodology used (Streits *et al*., 2004).

The Rapid Panoptic dye (Diff Quik) has been used in the morphological evaluation of spermatozoa, cattle, goats, sheep and canines (CBRA, 2013; Hernandez-corredor *et al*., 2020), presenting an advantage in the speed and practicality of the staining method (Andrade, 2023). This dye allows the evaluation of sperm morphology, which is fundamental for the analysis of semen quality. This aspect is particularly important in artificial insemination procedures, where sperm quality and viability determine the success of the technique (Silva *et al*., 2021; Paiva *et al*., 2024). It is an effective and practical dye, but it has some limitations when compared to other techniques, such as eosin-nigrosine or Giemsa staining. Studies indicate that Panoptic Rapid dye may be less sensitive in detecting more subtle morphological defects in sperm, such as acrosome changes, which can be a critical factor for fertility assessment. It is noteworthy that, due to the composition of the Panoptic Rapido dye, the stained slides do not have a long durability, which may impact the subsequent analysis (Linkgóes *et al.,* 2003; Bastos *et al*., 2016; Farias Júnior, 2018).

However, staining with the Rapid Panopticon has as its main advantages the agility in application, speed and simplicity of the protocol, making it an effective tool for initial evaluations of canine semen, especially in environments with a large volume of samples or that demand a fast process. Especially in laboratories that do not have a greater technological structure, such as the use of phase contrast microscopes or phase interference that enhance sperm analysis, providing greater detail of cell structures and enriching diagnostic criteria (Souza *et al*., 2022).

Another such as Giemsa is especially useful when a more accurate evaluation is desired than that provided by vital dyes such as eosin-nigrosine. It is considered a good alternative in cases where a more refined diagnosis of sperm quality is intended (Oliveira *et al.,* 2018). Studies show that Giemsa can be used on smears fixed with methanol, after washing in phosphate buffer, with staining ranging from 15 to 30 minutes (Lima *et al*., 2019). This technique reveals details such as nuclear vacuoles, irregularities in the contour of the head, thickening of the middle piece, and folds in the tail, which helps classify sperm defects. Furthermore, Giemsa has been used experimentally to assess damage to the acrosome or structures associated with sperm DNA, although this use is more consolidated in species such as horses and humans (Tartaglione *et al*, 2004; Kútvolgyi *et al*., 2006).

The technique of eosin-nigrosin staining is based on the selective permeability of cell membranes: sperm with intact (viable) membranes do not absorb eosin and remain colorless or lightly stained, while those with damaged membranes absorb the dye and appear with intense pink coloration. Nigrosine acts as a background dye, increasing contrast for easier viewing. This staining allows the counting of viable and non-viable cells under common light microscopy and is considered a standard technique in andrological evaluations of dogs (Matos *et al*., 2018; Cardoso *et al*., 2019).

Methylene blue staining is a vital dye widely used in morphological and cytological analyses of canine semen, although less frequent than techniques such as eosin-nigrosine. Its application is based on the ability to penetrate cells with damaged membranes, allowing the differentiated visualization of viable and non-viable cells, as well as intracellular structures such as the nucleus and cytoplasm (Medeiros *et al.,* 2019). In seminal cytology, methylene blue is used to stain fresh or refrigerated semen smears, enabling the identification of sperm, epithelial cells, leukocytes, and bacteria, which contributes to the diagnosis of inflammatory or infectious processes in the reproductive tract (Carneiro *et al*., 2015).

The Rosa Bengala dye has been used in several animal species to evaluate sperm morphology, including dogs, due to its efficiency in highlighting cell structures and the practicality of application**.** However, although Rosa Bengal has practical advantages, it does not completely replace more consolidated techniques for assessing sperm viability, such as eosin-nigrosin staining. It is best used as a complementary or alternative tool when more specific methods are not available (Silva *et al.,* 2010; Cunha *et al.,* 2010; Oliveira *et al*, 2018).

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