Study the Different Antioxidants Effects on Serum Quality in Dogs

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ABSTRACT
Dogs frequently have subfertility linked to poor semen quality (high percentage of morphologically defective spermatozoa, low rate of gradual mobility and total number of sperm .To examine the anti-oxidative effect of various concentratuons of NAC on cryopreservation in dogs sperm.The animals are divided into three groups: C0, C1 and C2. C0 receives the 0mM NAC, C1 receives 0.35mM and C2 receives 0.85mM. The physical characteristics studied of the spêrm include ejaculated volume, semen color, motility of sperms, percentage of dead sperms and abnormal sperms. After physical evaluation, the samples are diluted with Tri's diluter. The tris diluter is egg-yolk glycerol. The samples are then cryopreserved in liquid nitrogen (-196 C) from October to December. A total of 50 samples are calculated and prepared for freezing in the following way:The motility of sperm is high for 0.35mM dose of NAC, and not very different in the control group and 0.85mM NAC group. The motility is significantly different for 0.35NAC as compared to the other two groups. The p-value (< 0.05) shows the significant difference between the motility of 0.35 NAC and other groups. The sperm viability is highest in the 0.35mM NAC group (30.34). The viability of sperm is highest at 0.85mM. The membrane integrity is highest in 0.35mM NAC, while lowest in the control group(p<0.05).In conclusion, dogs sperm cells can be shielded from oxidative stress by using moderate dosages of NAC supplied in Tris extender without negatively affecting dog semen's ability to freeze.

1. Introduction

Dogs frequently have subfertility linked to poor semen quality (high percentage of morphologically defective spermatozoa, low rate of gradual mobility and total number of sperm (Nizanski et al., 2011). Most of the time, the reason for low-quality semen is still a mystery (Johnston et al., 2001). Numerous factors have been identified as potential causes, including heat, stress, autoimmune illnesses, dietary deficiencies, pollutants, and hormone imbalances. Nutritional deficiencies can have a major effect on the quality of sperm; for example, low consumption of antioxidant-rich nutrients like vitamin E and selenium (Se) can result in poor-quality semen or even infertility (Zubair, 2017). Among the necessary amino acids, arginine has a unique effect on the treatment of numerous developmental and health issues in animals. It is well established that the kidney, intestines, muscles, liver, and testicles are all involved in the interorgan axis, which is where L-arginine metabolism takes place (Wu et al., 2008). Semen cryopreservation is thought to be the most significant assisted reproduction method for preserving endangered breeds. Studies on the cryopreservation and freezability of German shepherd dogs' semen are few. Fertility and infertility are dependent on reactive molecules associated with oxidation. These include OH, ROS, H2O2, and ROOH). This demonstrated that antioxidation defenses are important for the functionality of sperm. Both direct and indirect data suggested that harmful ROS are produced during specific stages of the cryopreservation of semen (Bilodeau et al., 2000., Wang et al., 1997) The seman when ejaculated has sperms and WBC which cause activation of reactive oxygen molecules., according to Griveau and Le Lannou (Griveau and Lannou, 1997). There is evidence that hydrogen peroxide reduces the motile function generated by hydrogen peroxide, Additionally, thiols play an important role in the prevention of oxidation of hydrogen peroxide which causes loss of motility of sperm in animal models including frozen horse semen, and cryopreserved bull semen (Pagl et al., 2006). To guard against oxidative damage to semen during preservation processes, N-acetylcysteine has been added to semen extenders (Bilodeau et al. 2001). The precursors of intracellular glutathione production are cysteine and NAC. On the other hand, no references are currently known on the potential antioxidant protection of different concentrations of N-acetyl cysteine on semen quality in dogs. The current study's objective was to assess the characteristics of dog semen diluted with varying NAC concentrations.

Research Question

What concentration of NAC prevent oxidative stress of sperm after thawing?

Problem statement

Cryopreservation increase the occurrence of ROS intiated oxidative degardation of sperm reducing it mobility, membrane integrity and morphological characteristics. It can leads to infertility. This aim of this study is to evaluate the effect of N acetyl cysteine on sperm in dogs.It is important to discover the antioxtdants which can prevent oxidative damge of the sperms, thus reducig the chanes of infertiity.

2. Literature Review

Sperm cryopreservation is a practical and successful treatment for several illnesses that may impair sperm quality, including chemotherapy, donor semen preservation, and infertility surgery (Dillon and Fiester, 2012). Effective sperm cryopreservation influences the outcomes of treatments for infertility (Agarwal, & Majziub, 2017). Cryopreservation of semen can lead to the production of ice crystals, which can negatively impact sperm viability, even if it is an efficient method for maintaining sperm functional characteristics (Tomás et al., 2019). The vitrification method has been suggested as a means of preventing ice crystals from forming during the cryopreservation procedure (Arav et al., 2018). The prepared samples are directly placed into a container filled with liquid nitrogen in the vitrification technique (Isachenko et al, 2018). This technology is not only less expensive and takes less time to adopt than traditional sperm cryopreservation techniques, but it also lessens the harm caused by the process. It has been discovered that the freeze-thaw process generates reactive oxygen species (ROS). Sperm are particularly vulnerable to damage from free radicals because of their distinct features, which include a high number of mitochondria and low antioxidant levels (Partyka et al.,2018). When spermatozoa are cryopreserved, their antioxidant defenses against damage are diminished. In fresh semen, the antioxidant system partially shields the sperm plasma membrane from damage (Surai et al., 2001). There are numerous methods to strengthen sperm resilience to cryopreservation cold shock using amino acids. Among the several antioxidants in the thiol class are glutathione (GSH), N-acetylcysteine (NAC), and cysteine. NAC is a precursor to the formation of intracellular GSH. Nitric oxide synthase is an enzyme that converts L-arginine into nitric oxide, commonly referred to as nitrogen monoxide. An increase in nitric oxide concentration improves sperm features, and nitric oxide synthase is a crucial enzyme regulating nitric oxide production (Ren et al., 2015). Over the past ten years, nitric oxide, a highly reactive free radical, has come to play a significant functional role in several biological pathways, including the reproductive system (Oyeyipo, Raji, and Bolarinwa, 2015). The biosynthesis of intracellular glutathione is mediated by both cysteine and NAC. Strong antioxidant qualities are exhibited by the thiol-containing molecule N-acetyl cysteine (NAC). L-cysteine, which interacts with ROS to neutralize free radicals, is a product of NAC. NAC is used as an antioxidant to make up for the decreased glutathione content during oxidative stress. When sperm are cryopreserved, NAC stops membrane proteins from sedimenting and enhances their concentration following cold shock (Kumar & Balagnar, 2019) This amino acid protects against ROS activity by acting as an antioxidant. The impact of NAC in cryopreservation media has been shown in numerous research, which has improved sperm functional characteristics (Koohestanidehaghi et al., 2021).

3. Materials and Methods

Six healthy, reproductively mature dogs were employed in this study: German Shepherd dogs, weighing between 24 and 56 kg, and with a track record of reproducing after natural mating. The dogs were all between the ages of 2 and 7. Before the study, the dogs had received training in semen collection and were frequently utilized as donors of semen. Every animal was given a full series of vaccinations, routine antiparasitic treatments, natural lighting in both inside and outside areas, commercial dry dog food once a day, and unlimited access to water. From October to January, when the lowest (minimum) and highest (maximum) temperatures varied between 23 –35°C and 14–28°C, respectively, the experiment was carried out during the high breeding season. Every dog received a full

history evaluation and a male breeding soundness exam, which included an ultrasound and clinical examination of the reproductive organs. Every dog was in good health.

Study Location

This study was conducted at the Artificial insemination department, Baghdad, Abou-Gharib.

Semen collection

The semen is collected early morning, once a day each week. the sperm was collected by stimulating the bulbous glands of the dogs. The dogs were stimulated by massage, and sperm was collected in the bags designed for sperm collection. A total of fifty ejaculations were evaluated during the study. The samples were collected in plastic bags. The bags were sent immediately to the laboratory for inspection to check the physical characteristics of the sperm. The sample was kept in a water bath at 37-38C. The physical characteristics studied of the spêrm include ejaculated volume, semen color, motility of sperms, percentage of dead sperms and abnormal sperms,

Physical Properties

Ejaculated Volume
The ejaculated volume is determined by a graduated measuring plastic tube (Freshman,2001).

Semen Colour

The semen colour is pearly white.

PH Level Of the Semen

The pH of semen is analysed by using PH indicator strips**.**

Experimental Design and Cryopreservation of Sample

Early morning semen samples are calculated every day from the German Shepherd dogs. After physical evaluation, the samples are diluted with Tris diluter. The tris diluter is egg-yolk glycerol. The samples are then cryopreserved in liquid nitrogen (-196 C) from October to December. A total of 50 samples are calculated and prepared for freezing in the following way:

Physical evaluation of Semen
The volume of ejaculation, color, and consistency of semen, sperm concentration 103/ml, percentage of progressive motility, percentage of non-viable sperms, percentage of irregular sperm, hypo-osmotic sperm test (HOST).

Dilution of sample

Tris diluent is used to dilute the sample in a water bath at 37C.

Addition of anti-oxidant

The extender used for dilution of semen is Tris. The samples are designed in the following manner.

C1 is the control group with Tris extender only 0mM NAC
C2 is 0. 35mM NAC + Tris
C3 is 0.85mM NAC+ Tris
These test tubes are placed in a water bath at 33C.

Cooling at 5C

The water bath consists of a big beaker holding test tubes with the semen, diluent, and different concentrations of anti-oxidants added to the test tubes. The semen samples with diluent and anti-oxidant are placed in a water bath at the temperature of 5C for 1 to 1.5 hours. Ice cubes are added to the water bath if the temperature falls below 5C. A sensitive thermometer is used to measure the temperature of the water bath.

Cryopreservation of Sperm

The semen diluted with tris extender is equilibrated with different concentrations (0mM, 0.35mM, 0.85mM) at the temperature of 5C. A dosage of 100 X 104 spermatozoa per 0.25 ml French straw was created by loading diluted semen into the straws. We sealed plastic straws with powdered polyvinyl alcohol. These straws are placed horizontally on a rack prior to thawing for accliatization, housed on the cryogenic nitrogen in an insulated container, the straws were given time to acclimatize. The nitrogen vapor caused the temperature within the straws to drop to -120°C in about 15 minutes. The straws were then swiftly transferred to LN2 tanks that were -196°C. The straws were maintained in LN2 until the inspection.

Plasma Membrane Integrity

The membrane integrity is assess by diluting the seman sample of 50 µL with 450 µL of 100 mM hypotonic solution (made up of 4.9 g sodium citrate and 9 g fructose per liter of distilled water) to assess membrane integrity. Bright-field microscopy is the technique used and the hydro-osmotic swelling test (HOST), a smear was generated after one hour and assessed based on the percentage of sperm tail curling. Spermatozoa with curved tails were deemed to be membrane intact (Azerêdo, Esper, and Resende, 2001). Following a 45-minute incubation period of 0.05 ml semen in 0.5 ml of 60 mOsm fructose solution, the proportion of 200 spermatozoa with enlarged tails was calculated for the HOST (Rota et al.,. 1995).

Motility Essay of Sperms

According to Axe et al., the viability of sperm is studied with different concentration of NAC after thawingis determine by eosin staining. The Eosin staining is composed of 1.67 grams of Eosin-Y, and 2.9 grams of sodium citrate in distiled water volume 100 ml. On a heated slide, one drop of semen was combined with two drops of stain, and the stain was quickly spread using a second slide to create the sperm smears. 200 sperm cells were counted using bright-field microscopy (400x) to determine the viability (Olympus CX21, Olympus Optical Co. Ltd., Japan). Eosin and nigrosin are used to differentiate between live and dead sperms. The dead sperms stain pink, while alive sperms do not take any color. (Memon et al. 2012). The procedure used to prepare the stain is the active ingredient of the stain, 1.67 grams of eyosin-y, 2-10 gm of nigrosine, and 3gm of sodium citrate mixed in 100 ml of double distilled water. It is followed by boiling, mixing, and filtration. The eosin-nigrosine stain was prewarmed at 37C. Sperm exhibiting full or partial coloration were regarded as dead or non-viable. Sperm were only deemed to be alive if they strictly excluded the stain (Hafez and Hafez, 2000).

Effect Of Cryopreservation On Semen Analysis

The cocentartion of NAC used are 0mn, 0.35mM, nad 0.85mM and two straws are placed in each group. These straws are placed in a warm bath (37°C) following two months of storage. Following a minute, the straw's contents were studied under a microscope, as detailed below. Six samples in all were examined to determine the effect of NAC on the cryopreservation of dog semen. Following thawing, a three µL sample tube placed on heated slide at 37°C, protected with a cover slip, and examined under a Nikon Eclipse phase-contrast microscope at a 400x magnification. Four or five different fields were inspected for each sample, and the percentage of motile sperm cells was recorded. Without being aware of the experimental groups, two technicians assessed each sample during the experiment, and a percentage of the mean values was noted. 1 mL of Hancock's solution, was used to neutralise one drop sample from each group (Schäfer and Holzmann, 2000). After that, the sample was studied using phase-contrast microscopy (E400, Nikon Corp., Japan) while immersed under a coverslips.

Statistical Analysis

Table 1. Microscopic And Macroscopic Characteristics Of Fresh Sperm

Indicators	**Characteristics**
Color	Pearly White
Consistency	Thick

ordur	Typical
pH	6.4-6.8
Volume	3ml-5ml
Conc in mm^3	3×10^6 - 4×10^6
Motility %	55-70
Viability	25.87±20.75
Integrity of Sperm cell membrane	24.31± 10.41

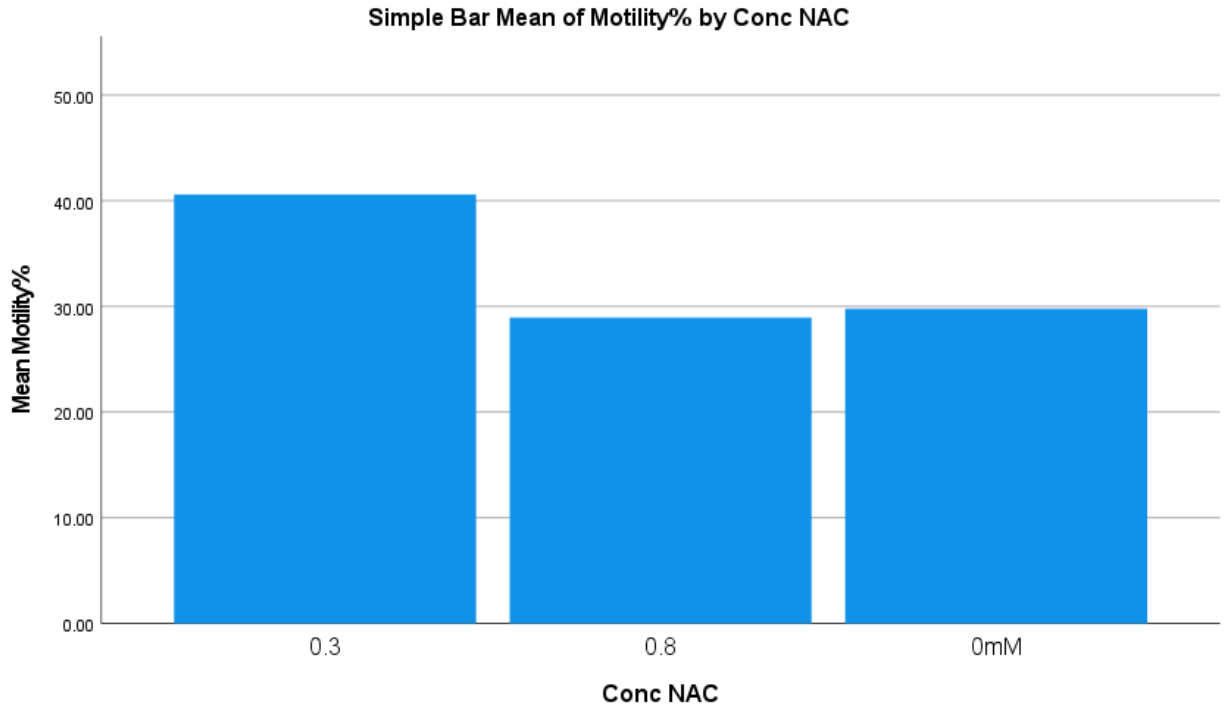
The parameters studied in this paper include effects of thawing on sperm motility, spermatozoa (defunct and irregular) and hypoosmotic swelling test (curling tail spermatozoa). The six test were studied for determine the above mentioned parameters. The software used is SPSS.

Conc. of N-acetyl cysteine(N=6)	**Percentage motility**	**Percentage viability**	**Percentage of abnormality**	**Abnormal Acrosome**	**Membrane Integrity (%)**
0mM	29.75 ±4.1	28.17 ±1.95	42.45 ±4.63	28.71 ±1.37	24.81±2.47
0.35mM	40.59 ±5.73	30.34 ±6.18	25.61 ±3.9	21.5 ±0.76	38.29 ±2.33
0.85mM	28.94 ±3.6	34.99±2.12	42.39 ±1.93	44.94 ±1.9	35.59 ±1.39

Table. 2 German Shepard semen post-thaw parameters using Tris extender and supplemented with varying dosages of NAC (Mean ± Standard Error)

Conc Of NAC (n=6)	**Morphologically Normal Spermatozoa**	**Sperm with intact acrosomes**	**HOST swollen spermatozoa**
0mM	76.16 ±1.72	23.5 ± 1.04	60.1 ±1.21
0.35mM	74±1.58	24.88±2.88	62.8±1.92
0.85mM	70.83±2.31	24±2.61	61.5±1.87

Table 3: After varying amounts of NAC were given to seminal fluid diluent, the proportions of sperm cells with normal structure, undamaged acrosomes, and HOST enlarged spermatozoa in dog semen that was extended for 72 hours are displayed as mean (±SEM). Table 3 shows the effect of different concentrations of NAC on the morphology of sperm, acrosomal integrity, and HOST. Table 2 shows the results. The motility of sperm is high for 0.35mM dose of NAC, and not very different in the control group and 0.85mM NAC group. The motility is significantly different for 0.35NAC as compared to the other two groups. The p-value (< 0.05) shows the significant difference between the motility of 0.35 NAC and other groups. The sperm viability is highest in the 0.35mM NAC group (30.34). The viability of sperm is highest at 0.85mM. The membrane integrity is highest in 0.35mM NAC, while lowest is among the C0 (control group) ($p<0.05$). Table 3 shows that different concentrations of NAC have not changed the morphology, acrosomal integrity, and HOST.



Graph 1 : The above graph shows that motility was maximum at 0.35mM NA

In the current investigation, a high dose of NAC (0.35 mM) specifically reduced post-thawing motility, whereas a lesser dose of NAC did not influence sperm characteristics when contrast to the control group after thawing. Certain cryopreservation procedures have been shown to entail the production of hazardous reactive oxygen molecules, which harm sperm mobility and viability. Anti-oxidants like vitamin E, selenium, and folic acid increase quality and infertility of sperm quality. (Alonge et al., 2019). In another study, the highest concentration of N-acetyl cysteine (5nM) is demonstrated to be harmful to sperm motility and has detrimental effects on acrosomal integrity. It also causes premature canine spermatozoa hyperactivation. It is not anticipated that the cold canine semen combined with NAC will considerably increase conception rates (Michael et al., 2010). NAC is a well-known anti-oxidant that is used in the preservation of the sperm. It is a thiol agent and is water-soluble (Cocco et al. 2005). The HOST percentages and viability were unchanged, indicating that the occurence of NAC does not affect whatsoever the effective integrity of the sperm membrane. While other thiols have been employed, there are no prior reports on the use of NAC in the prolonged semen of other domestic animals. In a prior study, spermatozoa's vitality and in vitro infiltartion during an oocyte penetration test were markedly enhanced by the inclusion of cysteine and glutathione during the liquid preservation of pig semen (Funahashi and Sano 2005). Thiols were previously utilized to stop sperm motility loss in dogs (Michael et al. 2007) and bulls (Bilodeau et al. 2001) that had their semen cryopreserved. High doses (10 mM) of antioxidants like glutathione and NAC shielded human spermatozoa from the detrimental effects of leucocyte-derived ROS on sperm mobility; these antioxidants may have therapeutic applications in assisted reproductive techniques (Baker et al. 1996). Given the antioxidant characteristics associated with thiol usage in semen extenders, a stronger antioxidant shield from NAC is anticipated in the current study. Previously, conducted studies have studied the effect of NAC on the cryopreservation of sperms. Seyedasagri and his co-workers studied the impact of varying N-acetyl cysteine concentrations on the fertility, quality, and liquefaction of frozen semen in camals, and concluded that the liquefaction of dromedary semen is aided by adding NAC to the semen extender at concentrations of 6 mM, without sacrificing sperm quality during subsequent storage in cold condition (F. Seyedasgari, Asadi and Kim, 2024). Many studies have supported the use of NAC in different concentrations on cryopreservation of animals. While there hasn't been much research on adding NAC to a skim milk-based extender for ram semen, Pagl et al., added 0.2 mM NAC to a specific milk protein fraction extender for equine semen stored at 5°C. When they compared the 0.2 mM NAC group to the

control group, they found that neither group's sperm motility was affected negatively nor favourably. However, the results of our current investigation showed that sperm survival, morphology, acrosome, and membrane integrity were somewhat improved by moderate doses of NAC (0.35 and 0.85 mM). Furthermore, neither a negative nor a positive effect on sperm motility was observed with modest dosages of NAC. The limitations of the present study is only one breed of dogs is studied in the experiment, so the results cannot be generalized to larger extent.The strength of the study is different parameters of the seman sample is analysed. The physical pararmeters are studied, membrane integrity is evaluated.

4. Conclusion

In conclusion, dogs sperm cells can be shielded from oxidative stress by using moderate dosages of NAC supplied in Tris extender without negatively affecting dog semen's ability to freeze. The moderate doses used in the study are 0.35 and 0.85. In cryopreservation media, NAC and LC can reduce the level of oxidative stress. Further recommendation is to study these effect in humantrials to etablish a dose-response relationship and prevent oxidation of human sperms.Further, there is need to find out optimal dose which can enhance the effect of NAC in reducing the oxidative stress.

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