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Technique comparison to assess sperm DNA fragmentation in hair ram

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ABSTRACT

Objective: To evaluate 5 DNA fragmentation techniques in thawed sperm samples from hair ram subjected to fragmentation with H_2O_2.

Design/methodology/approach: Samples were 36 straws from 4 Blackbelly rams that were thawed, mixed (pool), diluted in PBS to a concentration of~ 30×10^6 sperm/mL and divided into three treatments: T0: sample without oxidant (considered as 0% damaged DNA), T100: sample incubated with 300 μM H_2O_2 for 24 hours (induction of DNA fragmentation (100%)). Subsequently, half of T0 and T100 were mixed to obtain a proportion of 50% sperm with fragmented DNA (T50). The samples were analyzed with different techniques: Aniline Blue (AB), Toluidine Blue (TB), Acridine Orange (AO), Chromomycin A3 (CMA3) and Sperm Chromatin Dispersion (SCD).

Results: In the linear regression, all the techniques presented a significance level of less than 5%, as well as a significant correlation ($r=0.962$, $P<0.01$). However, between treatments, it was observed that the AO technique (34.82±3.00%) at T50 and the AB technique (89.55±1.45%) at T100 were the least sensitive in detecting DNA damage compared to the other techniques.

Limitations on study/implications: New techniques are increasingly.

Findings/conclusions: The techniques that best evaluate the DFI of sperm in hair ram are CMA3, SCD and TB.

Keywords: Fluorescence, H_2O_2, Semen Quality, Blackbelly.

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INTRODUCTION

Currently, the fertility potential of the ram can be determined based on semen analysis, through a series of assessments, in which motility, morphology, metabolic activity and integrity of the sperm membranes stand out; however, these assessments are inefficient to predict fertility (Kamimura *et al.*, 2010; Alves *et al.*, 2015; Arbaiza-Barnechea and Cabrera-Villanueva, 2021). This is why the study of integrity of the sperm DNA has been considered an important index of fertility, since there is evidence of subfertility in males with normal values in routine parameters and with higher percentages of DNA fragmentation. Therefore, the assessment of the DNA Fragmentation Index (DFI) should be considered an important parameter to include in the traditional assessment in various animal species, since it stands out as a fertility indicator, a good predictor of embryo development and paternal genetic information (Ortega et al., 2010; Andraszek *et al.*, 2014; Czubaszek *et al.*, 2019; Huanca *et al.*, 2020; Ribas-Maynou *et al.*, 2021).

Image by Gerd Altmann at Pixabay

There are diverse methodologies that allow analyzing the sperm DNA fragmentation which allows predicting more exactly the male's fertility through direct methods to evaluate the amount of protamine or to measure the chromatin structure based on the different stains. These methods can be divided into two groups; the first includes the techniques that mark DNA cleavage because they incorporate molecules marked with fluorochromes on the cleavage ends, such as TUNEL (*terminal d-utp nick-end labeling*), SCD (*Sperm Chromatin Dispersion*) and ISNT (*in situ nick translation*), although many of these techniques are laborious and require high equipment accuracy, and the field conditions also do not allow performing complex techniques (Rui *et al.*, 2017). However, the SCD technique can determine sperm DNA fragmentation more accurately, since it is one of the simplest, fastest, most accurate, highly reproducible and inexpensive tests (Fernández *et al.* 2005, Carretero *et al.* 2012). Thus, a second group is found, where the techniques that are based on stains are included: Aniline Blue (AB), Toluidine Blue (TB), Acridine Orange (AO), and Chromomycin A3 (CMA3) (Kazerooni *et al.*, 2009). Therefore, the objective of this study was to evaluate the efficacy of five different techniques in the identification of sperm DNA damage in thawed samples from Blackbelly ram.

MATERIALS AND METHODS

Study area

The experiment was carried out in the Molecular Genetics Laboratory of the Instituto Tecnológico de Conkal, in the municipality of Conkal, Yucatán. Likewise, at the Instituto Nacional de Investigaciones Agrícolas y Pecuarias (INIFAP), located in the experimental field of Mocochá on Kilometer 25 of the former Mérida-Motul highway, located on 21° 06' 18" latitude North and 89° 27' 12" longitude West, with sub-humid tropical climate (Awo) and mean annual temperature of 26.5 °C, with total precipitation of 900 mm and 9 masl (INIFAP 2018).

Ram selection. Four male ram of the Blackbelly breed were used, with an average age of 2.0±0.5 years and average live weight of 42.5±2.9 kg.

Obtaining and processing semen. Through an artificial vagina and with the help of a ewe that served as mannequin, a total of 36 ejaculates were collected (9 ejaculates/ram) that complied with the following criteria: volume >0.5, mass motility >4 (scale 0-5), motility >70%, and sperm concentration >3,000×10^6 spermatozoa/mL. The semen was collected with a frequency of twice per week in the morning hours (8:00).

Sperm dilution. The ejaculates obtained were diluted with OPTIX-cell® (imv, L'Aîgle, France) at a final concentration of 400×10^6 spermatozoa/mL, to later be packaged in French straws (Minitüb®, Tiefenbach, Germany) of 0.25 mL.

Cooling. The straws were placed in a refrigerator at 5 °C for a period of four hours, to be frozen later in liquid nitrogen (LN2).

Semen freezing. Freezing of the samples was carried out by placing the straws four centimeters above the LN2 surface, for 10 minutes. Immediately after, the straws were submerged in LN2 and stored until their assessment.

Semen thawing. The defrosting procedure was carried out through immersion of the straws in a double water bath at 37 °C for 30 seconds.

DNA fragmentation induction with hydrogen peroxide (H_2O_2). The induction of DNA damage was conducted according to the technique proposed by Peris *et al.* (2007). For this purpose, four straws from different males were thawed, mixed (pool), diluted in PBS at a concentration of 30×10^6 spermatozoa/mL and divided into three treatments: T0: sample without oxidant (considered as 0% of damaged DNA); T100: sample incubated with 300 μM of H_2O_2 for 24 hours (induction of DNA fragmentation (100%)). Then, half of the T0 and T100 were mixed to obtain a proportion of 50% of spermatozoa with fragmented DNA (T50).

Technique analysis

Sperm chromatin dispersion (SCD): The SCD technique methodology was carried out based on Fernández *et al.* (2013) with some modification. Suspensions from each T0, T50 and T100 aliquot were taken in the amount of 20 μL from each sample, to mix with 40 μL agarose of low fusion point (Agarose, low gelling temperature, BioReagent, for molecular biology; Sigma-Aldrich®) at 1% in an Eppendorf tube (to obtain a final concentration of 0.7%) at 37 °C. Of the semen-agarose mixture, 30 μL were pipetted on a glass slide covered with standard agarose (Agarose, low EEO, BioReagent, for molecular biology; Sigma-Aldrich®) at 0.65% dry and covered with a clean glass slide cover and introduced in the refrigerator at 4 °C for seven minutes. After that time, the slides were taken out of the refrigerator and the slide covers were removed without altering the subjacent layers; then a lysis solution was used (0.4 M Tris [Sigma-Aldrich®], 50 mM EDTA [Baker®], 0.4 M DTT [Sigma-Aldrich®], 0.3% SDS [Sigma-Aldrich] and 1% Tritón X-100 [Promega®, Molecular biology grade], pH 7.5) adding, according to what was described by Gundogan *et al.* (2010) and Ribas-Maynou *et al.* (2021), 100 μg/mL of proteinase K (Thermo Scientific® 600 U/mL, 20 mg/mL) to the lysis buffer to pipette horizontally to the slides and leave the solution at room temperature for 120 minutes to extract membranes and proteins. After a five-minute wash in abundant distilled water, the preparations were dehydrated in sequential baths of 50, 70 and 100% (v/v) of ethanol (Hycel®) for a two-minute period each, then they were left to dry to later finally stain the cells with 10 μL of propidium iodine (Invitrogen®, Molecular Probes™) for fluorescence microscopy. A minimum of 200 spermatozoa were evaluated per slide. The spermatozoa were classified according to Peris-Frau *et al.* (2019): intact DNA (nuclei with small halos or none) and fragmented DNA (nuclei with large DNA dispersion halos).

Acridine orange (AO): The technique was applied according to what was described by Mohammadi and Soltani (2021) with some modifications. Seven microliters of the sample from each T0, T50 and T100 aliquot were deposited on a glass slide, left to dry in open air, and then fixed in a Carnoy solution (methanol [J.T. Baker®]-acetic acid [Meyer®] 3:1) during 30 minutes. Then, the smears were left to dry for 10 minutes, to later be stained for five minutes with an AO (Meyer®) solution recently prepared in the following way: the mother solution was prepared dissolving 0.05g of AO in 50 mL of distilled water and then storing it at 4 °C without access to light; the stain solution was prepared by mixing 10 mL of the mother solution, 40 mL of 0.1 M citric acid (Reasol®), and 2.5 mL of 0.3 M $Na_2HPO_4{\cdot}7H_2O$ (Wholer®). Then, the smears were submerged in water (10 times quickly

in a container with distilled water), and they were left to dry to be read immediately at 100x with a fluorescence microscope, counting at least 200 spermatozoa, where the identification of spermatozoa with normal DNA structure (green fluorescence) and damaged single-chain DNA (orange fluorescence) was made.

Cromomycin A3 (CMA3): The methodology was used according to what was described by Roodbari *et al.* (2015) and Rahimizadeh *et al.* (2020) with some modifications. Seven microliters of sample from each T0, T50 and T100 aliquot were deposited and then fixed on a Carnoy solution (methanol-acetic acid 3:1) for 30 minutes at 4 °C. Then, the slides were incubated in the dark and a slide cover was placed on them for 20 minutes at room temperature with 100 μL of the CMA3 solution (Sigma-Aldrich®) (0.25 mg/mL in McIlvaine buffer (0.2 M Na_2HPO_4) (Sigma-Aldrich®), 0.1 M citric acid (Reasol®), and 10 mM of $MgCl_2$ (Sigma®), pH 7). A minimum of 200 spermatozoa were counted for each slide with the help of an epifluorescence microscope at 100x magnification, and they were distinguished among positive spermatozoa (spermatozoa stained in brilliant yellow with abnormal condensation of chromatin) and negative spermatozoa (stained in opaque yellow with normal condensation of chromatin).

Aniline blue (AB): Staining was done according to what was described by Oliveira *et al.* (2013) with some modifications. Seven microliters of sample of each T0, T50 and T100 aliquot were deposited on a glass slide, they were left to dry in the air and then fixed in a Carnoy solution (methanol-acetic acid 3:1) for 30 minutes at room temperature. After fixation they were stained for 25 minutes in an AB (Meyer®) aqueous solution at 5% dissolved in acetic acid (Meyer®) at 4% (pH 3.5) and then washed twice with distilled water; then they were left to dry and observed with a clear field microscope at magnification of 100x, and at least 200 cells per smear were evaluated. The spermatozoa that were not stained were considered normal, while those stained with dark blue color were considered as spermatozoa with chromatin faults.

Toluidine blue (TB): Staining was carried out according to what was described by Nava-Trujillo *et al.* (2011) and Carretero *et al.* (2020) with some modifications. Seven microliters of sample from each T0, T50 and T100 aliquot were deposited on a glass slide, left out to dry and then fixed in a Carnoy solution (methanol-acetic acid 3:1) for five minutes at room temperature. The smears were stained with TB (Research organics®) (0.05% in 10 mL of McIlvaine buffer, pH 4.0) for five minutes at room temperature and then they were washed three times with distilled water to later dry. The smears were observed with a clear field microscope with 100x magnification, and at least 200 cells were assessed per smear, which were classified as spermatozoa with normal chromatin, those stained with clear blue color; while those stained with dark or violet blue color were considered cells with damaged chromatin.

Statistical analysis. The techniques assessed on DNA fragmentation were analyzed with a general linear model with procedure (ANOVA) and Tukey's test at $P \leq 0.05$ was used to determine the statistical differences between techniques. In addition, an analysis was carried out with Pearson's correlation coefficient (r^2) to determine the association between techniques and a linear regression analysis with the expected percentages of spermatozoa that exhibited damage on the DNA. The data were subjected to a statistical

analysis system with the Statistical Package for the Social Sciences IBM ® SPSS software, version 25.0.

RESULTS AND DISCUSSION

In the results between treatments, it was observed that the TB, SCD and CMA3 techniques were the ones that presented best sensitivity to detect DNA damage, while the AO technique (34.82±3.00%) in T50 and the AB technique (89.55±1.45%) in T100 were the ones that presented least sensitivity (Table 1).

On the other hand, a linear coefficient regression was observed between the percentages of spermatozoa induced to fragmentation and what is expected for all the techniques (Figure 1). Likewise, the values of the square roots were higher than 0.95 in all the techniques, thus showing a high acuteness and repeatability (Table 2).

The various techniques were compared (Figure 2), where a significant correlation was observed among the five techniques.

Lastly, all the techniques analyzed presented a significant correlation coefficient, higher than 0.962 (Table 3).

This study shows that the TB, SCD and CMA3 techniques were the ones that showed the best sensitivity to detect damage in the Blackbelly ram sperm DNA, compared to the AO and AB techniques. These results are like those reported by Chohan *et al.* (2006), who observed differences in the high levels of DNA damage of human spermatozoa, both fertile and infertile, evaluated with the SCD technique while no differences were observed with the AO technique. Likewise, Rahiminia *et al.* (2018) obtained similar results with the TB,

Table 1. Percentage of spermatozoa with DNA damage in the different treatments.

Treatments	TB	AO	CMA3	AB	SCD
T0	3.01±0.75 $^{a, C}$	2.68±0.62 $^{a, C}$	2.11±0.41 $^{a, C}$	3.36±0.65 $^{a, C}$	4.29±0.72 $^{a, C}$
T50	52.93±1.86 $^{a, B}$	34.82±3.00 $^{b, B}$	48.06±1.73 $^{a, B}$	49.64±1.43 $^{a, B}$	47.06±2.47 $^{a, B}$
T100	98.51±0.55 $^{a, A}$	99.55±0.25 $^{a, A}$	95.58±1.50 $^{a, A}$	89.55±1.45 $^{b, A}$	97.97±1.03 $^{a, A}$

T0: Sample without oxidant (considered as 0% damaged DNA); T100: sample incubated with 300 μM H_2O_2 for 24 hours (induction of DNA fragmentation (100%)). Subsequently, half of T0 and T100 were mixed to obtain a proportion of 50% sperm with fragmented DNA (T50). Toluidine Blue (TB); Acridine Orange (AO); Chromomycin A3 (CMA3); Aniline Blue (AB); Sperm Chromatin Dispersion (SCD). (^{ab}) Different letters in the same row show significant differences between techniques by groups. (^{AB}) Different letters in the same column show significant differences in the technique within the group. Level of significance for both letters ($P<0.001$ ***).

Table 2. Square roots and linear regression equation of validation to evaluate the integrity of DNA through Aniline Blue (AB); Toluidine Blue (TB); Acridine Orange (AO); Chromomycin A3 (CMA3) and Sperm Chromatin Dispersion (SCD). All the linear regressions presented a level of significance lower than 5%.

Test	Square root	Linear regression
Aniline Blue	R^2=0.98	y=4.42+0.68*x
Toluidine Blue	R^2=0.95	y=12.77+0.82*x
Acridine Orange	R^2=0.99	y= 3.73+0.96*x
Chromomycin A3	R^2=1.00	y=1.85+0.93*x
Sperm Chromatin Dispersion	R^2=0.99	y=2.93+0.94*x

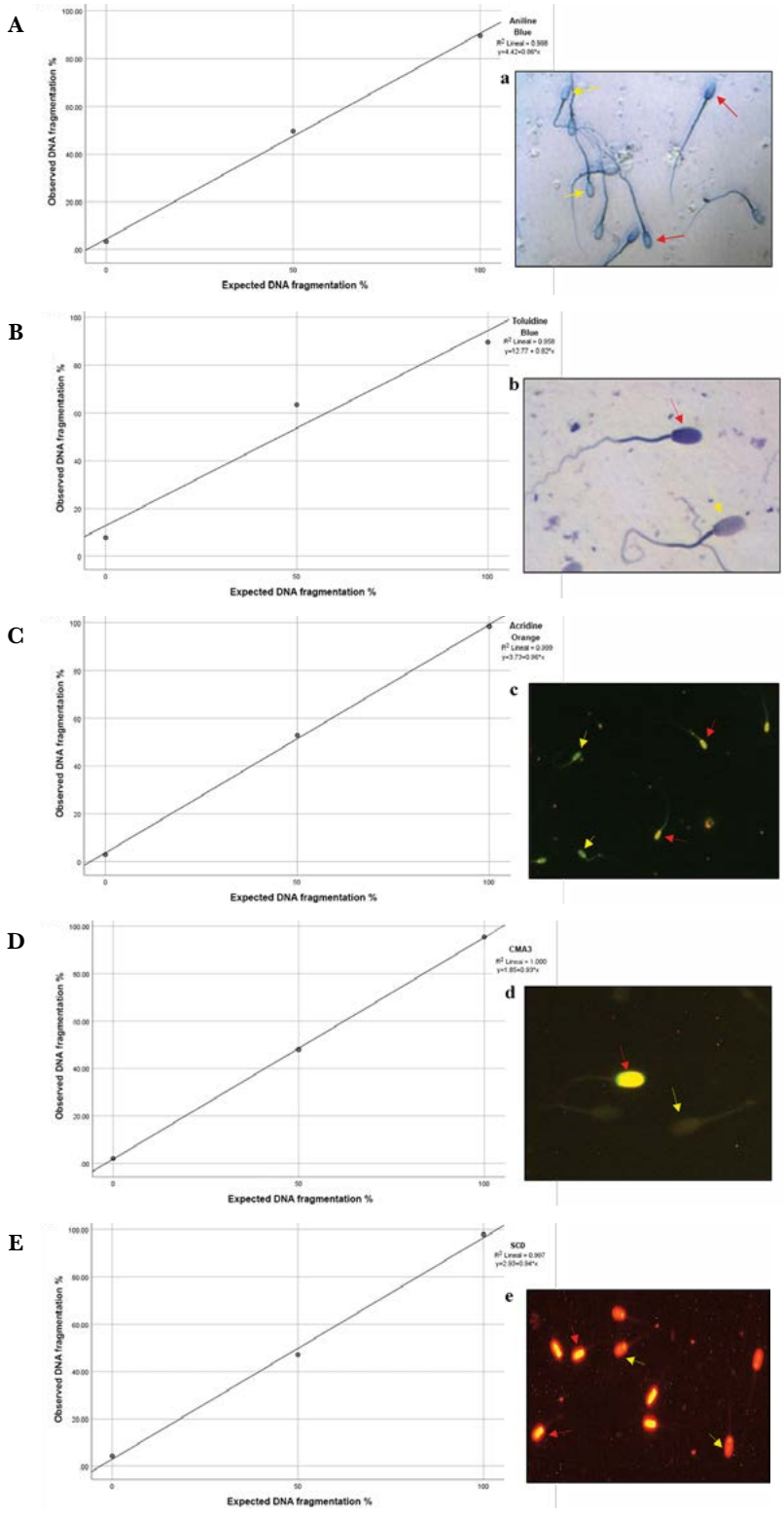

Figure 1. Linear regression analysis (A, B, C, D, E) and images (a, b, c, d, e) of DNA damaged spermatozoa among different treatments (T0, T50 and T100). The presence of DNA damaged spermatozoa was detected with (A, a) Aniline Blue; (B, b) Toluidine Blue; (C, c) Acridine Orange; (D, d) Chromomycin A3 and (E, e) Sperm Chromatin Dispersion. DNA damaged spermatozoa are marked with "b" and intact DNA spermatozoa with "a" in all images.

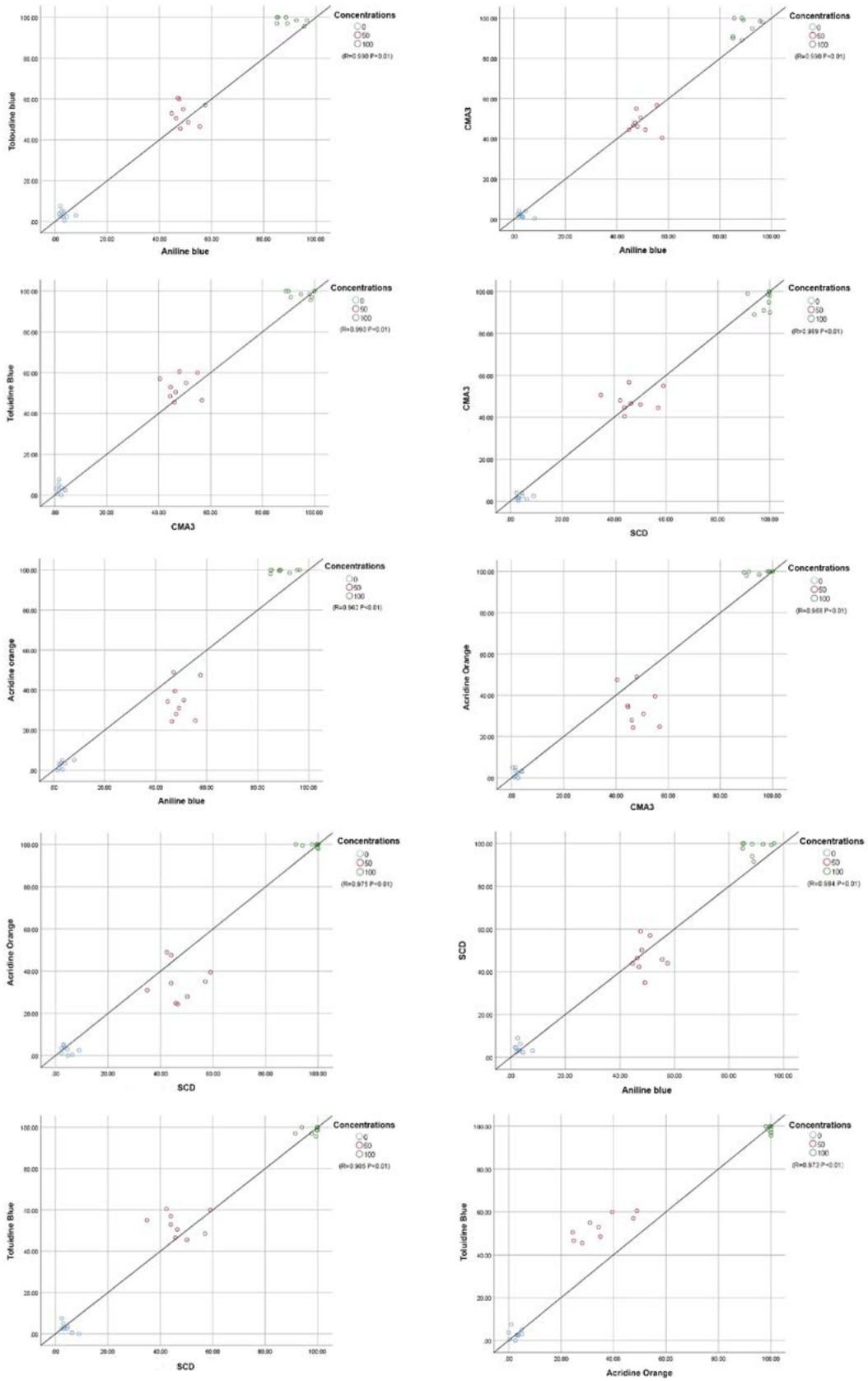

Figure 2. Relationships between percentages of ram spermatozoa with damaged DNA obtained with the different techniques.

Table 3. Correlation matrix of the techniques to detect sperm DNA fragmentation.

	TB	AO	CMA3	AB	SCD
TB	1				
AO	0.973**	1			
CMA3	0.991**	0.968**	1		
AB	0.990**	0.962**	0.990**	1	
SCD	0.986**	0.975**	0.989**	0.984**	1

Toluidine Blue (TB); Acridine Orange (AO); Chromomycin A3 (CMA3); Aniline Blue (AB); Sperm Chromatin Dispersion (SCD). **P≤0.01.

AO and CMA3 techniques in sperm samples from humans with oligoasthenoteratospermia and with normozoospermia. However, in contrast with this study's result, the AB technique was less sensitive than the TB and CMA. Likewise, it was seen that the incubation of Blackbelly ram spermatozoa with 300 μM of H_2O_2 for 24 hours of incubation at room temperature induces damage in the sperm DNA (T100). This result is similar to that reported by Peris *et al.* (2007) in thawed semen samples of Dorset breed ram, incubated with 300 μM of H_2O_2 during 24 hours. Similarly, Aitken *et al.* (1998) observed a significant increase in the DNA damage of human spermatozoa incubated with 200 μM of H_2O_2 during 2.5 hours, reaching 90% of fragmentation. It is well known that hydrogen peroxide (H_2O_2) has a negative impact on the chromatin of the spermatozoon, inducing double-chain breakdown (Kodoma *et al.*, 1997). The result presented in Table 2.3 is similar to what was found by Czubaszek *et al.* (2019), who obtained a correlation coefficient of 0.96 between the techniques CMA3-AB, although the techniques CMA3-AO (0.48) and AB-AO (0.45) differed from what was found in this study. However, Karimura *et al.* (2009) observed a correlation coefficient in ram of 0.89 between the techniques AB-TB, while in goats it was 0.35 respectively. These results are similar to those obtained in this study in ram. These differences can be because the chromatin of ram is the same than that of cattle and pigs, which presented a higher degree of DNA condensation. This is a result of the condensation around protamine 1 and 2, although in the species mentioned only protamine 1 is present in its chromatin, so although there is less efficiency of condensation in their DNA, there are stronger protamine-protamine unions from the high number of cysteine residues that allow stronger unions and, therefore, it is more difficult to achieve the decondensation of chromatin.

CONCLUSIONS

The techniques that best assess sperm DNA fragmentation of hair ram are CMA3, SCD and TB; however, the TB technique is more efficient and inexpensive since it does not require staining with fluorescence or sofisticated equipment for assessment.

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