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Influence of adding different energy sources to the bull and ram spermatozoa exposed to different refrigerating times

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ABSTARCT

Semen storage changes sperm synthesis and biochemical characteristics and hence hinders fertilizing ability. Mixture of two various sugar molecules appeared an improved positive impact on stored spermatozoa of different species. The current study aimed to investigate the effect of fructose, sucrose and raffinose supplementation to Tris-glucose-egg yolk extender (TGY) beside comparing their effect with the effect of commercial Triladyl® extender on bull' and ram' semen stored at 5 °C for 96 hours. The semen samples were pooled, extended and divided into five portions. TGY or Triladyl® extenders were diluted at rate 1 part of raw semen: 5 parts of the extender. The TGY diluent was split into four diluent parts in sterilized test tubes. TGY and Triladyl® extenders contained one type of sugar, while the other three extended semen samples contained two types of sugar including TGY plus 0.990 g fructose (TGYF), 0.700 g sucrose (TGYS) and 0.700 g raffinose (TGYR) /100ml extender. The extended semen was stored at 5°C for 0, 24, 48, 72 and 96 hours. The obtained data show that spermatozoa parameters significantly ($P < 0.01$) decreased with increasing preservation period of all diluent samples. Meantime, supplementation with fructose, sucrose or raffinose with TGY extended samples improved significantly ($P < 0.01$) sperm properties (motility, livability with acrosomal status and normality, enzymatic actions of AST, ALT, LDH, and ALP, and sperm penetration ability for both bull and ram diluted semen. Concerning the sperm characteristics of the commercial Triladyl® extender, it was better than those of TGY without any supplementation for both bull and ram diluted semen.

In conclusion, the obtained results suggest that obtaining a reasonable and advantageous impact for the preservation of bull and ram spermatozoa could be acquired by using diluents containing a sugar blend. However, as these results depend on *in vitro* assessments, there is a need for further fertility trials to confirm it.

Keywords: *Extended semen, sugars, preservation, and penetration.*

INTRODUCTION

Artificial insemination and semen preservation could turn out to be strong tools for hereditary management of livestock breeding programmers. These reproductive procedures could help semen preservation from genetically precious males, and bypass health problems that prevent some animals from reproducing. Additionally, it may encourage exchange of semen between animals that are biologically or geographically isolated **(Watson and Holt, 2001)**. Preservation of spermatozoa in the liquid state needs adequate energy supplementation in the diluents **(Sansone *et al.*, 2000)**. Therefore, it is required to add an energy source to semen extender in order to improve spermatozoa function. Mammalian spermatozoa need external substrates for a diversity of functions, such as ensuring intracellular energy reserves, cell ingredients, and particularly for support motility **(Salisbury *et al.*, 1978)**. Sperms can gain energy during glycolysis and oxidative phosphorylation of mitochondrial by using glycolysable sugars, like mannose, fructose, glucose, and maltose **(Salisbury and VanDemark, 1961)**.

Many studies illustrated the beneficial influences of sugars on spermatozoa viability when frozen extenders were thawed **(Garde *et al.*, 2008; Tonieto *et al.*, 2010)**. Sugars can be divided into mono-, di- and tri-saccharide types. Each type has a variety of cryoprotective properties

that save spermatozoa through thawing and freezing **(Yildiz *et al.*, 2000; Medeiros *et al.*, 2002)**.

Monosaccharides are found mainly in seminal plasma. They are important enhancers, as energy source, for extended semen which has a protective effect on spermatozoa **(Salamon and Maxwell, 2000)**, while sucrose, as disaccharide, gives viable assurance to phospholipid membrane of sperm head, such as cryoprotective impact. Even so, some investigations recommended that sperm properties post-thawing could enhance by supplementation with a mixture of mono- and disaccharides, such as sucrose, to the frozen extended semen **(Aboagla and Terada, 2003; Farshad and Akhondzadeh, 2008)**. Sugars like fructose (monosaccharide), sucrose (disaccharide) and raffinose (trisaccharide) play a significant role in protecting the viability of sperm after thawing by giving an energy source, boosting the secretion of extracellular water to reduce the formation of ice crystals within the cells, and keeping the osmotic pressure of the diluent. Nevertheless, their defensive impacts are reliant on storage temperature, atomic weight, and the specific buffer utilized in the diluent **(Abdelhakeam *et al.*, 1991; Garde *et al.*, 2008)**. Sugars like Xylose, fructose, glucose, galactose, maltose, sucrose and raffinose are used effectively for cryopreservation of bull semen **(Nagase, 1964)**. The mixture of sugars added to a frozen diluent cause enhance buck sperm capacity, viability and quality after cryopreservation **(Farshad *et al.*, 2009; Naing *et al.*, 2010)**. Few investigations suggested the utilization of a mixture of fructose and trehalose could accomplish a higher level of intact and viable spermatozoa in ram **(Aisen *et al.*, 2002; Matsuoka *et al.*, 2006)**. Fructose is the main energy source naturally occurs in the seminal plasma for metabolic procedures of buffalo-bulls spermatozoa **(Sansone *et al.*, 2000).** Fructose is thought to be the main source of energy for ejaculated spermatozoa **(Nagai *et al.*, 1982)**. In numerous species, fructose and glucose have been investigated for their different effects on gametes which promote the metabolizable energy, and their beneficial effects vary among species **(Williams and Ford, 2001)**.

To accomplish the most viable utilization of stored extended semen, it is essential to consider the impact of various sugars on preserved sperm. Therefore, the present study aimed to determine which of these sugars or their combination in locally manufactured extender could be the most beneficial compared with commercial Triladyl® extender for ram and bull diluted semen and sperm penetration ability through storage at 5 °C for up to 96 hours.

MATERIALS AND METHODS

All semen samples were gathered from Sids Research Station belonging to Animal Production Research Institute (APRI).

Experimental animals and feeding

Six Egyptian bulls and rams (3 of each) were used in this investigation. They were healthy and mature, 2-3 years old with 502±11.09 and 52.0±1.09 kg average body weight, respectively. Animals fed on the pelleted diet, rice straw, and corn silage twice daily. Fresh water and minerals were available *ad libitum* through all daytime during the tested period.

Semen extension:

Two basic types of semen extenders were used; commercial Triladyl® (Minitube, Germany) and locally-manufactured extender (Tris, glucose, citric acid, egg-yolk: TGY) prepared in the laboratory by mixing 3.634 g Tris, 0.800 g glucose, 1.99 g citric acid, 200 µl Vertrocin antibiotic and distilled water to make 100 ml sol as stock. All synthetic substances used for making diluents were acquired from Sigma Trade Company. Upon the day of semen gathering, 20 ml of the conclusive working TGY diluent was set up by adding 20% egg yolk to the stock arrangement that was already prepared. Essentially, 20 ml of the last Trilady® diluent was set up by including one volume of Triladyl® (contains citric acid, Tris,

glycerol, fructose, tylosin, spectinomycin, gentamicin, and lincomycin according to the producers' particulars) to three volumes of distilled water and one volume of egg yolk. Subsequent to blending the fixings both extenders were separated by filter paper (Whatman™, 125 mm Ø x 100 circles, GE Healthcare UK Limited, Amersham Place, China).

Pooled semen samples were extended in TGY or Triladyl® extenders at ratio 1:5 (raw semen: extender). TGY extended samples were divided into four diluent parts in sterilized test tubes. TGY and Triladyl® extenders contain one sugar, while the other three extended semen samples contain two sugars included TGY plus 0.990 g fructose (TGYF), 0.700 g sucrose (TGYS) or 0.700 g raffinose (TGYR) /100ml extender. The extender ingredients of locally-manufactured diluents with various sugar types are presented in Table 1.

Table 1: Synthesis of the locally-manufactured extenders with various sugar types used for the storage process

Extender ingredients	Diluent components /100 ml DW			
	TGY	TGYF	TGYS	TGYR
*Tris (g)	3.634	3.634	3.634	3.634
Citric acid (g)	1.990	1.990	1.990	1.990
Glucose (g)	0.800	0.800	0.800	0.800
Fructose (g)	---	0.990	---	---
Sucrose (g)	---	---	0.700	---
Raffinose (g)	---	---	---	0.700
Vertrocin antibiotic (µl)	200	200	200	200
Egg yolk (ml)	20	20	20	20

***Tris:** Hydroxymethel amino methane; **TGY**, locally-manufactured extender (Tris, glucose, citric acid, egg-yolk); **TGYF**, TGY with fructose; **TGYS**, TGY with sucrose; **TGYR**, TGY with raffinose; **DW**, distilled water.

Diluent samples were put in the water bath of a 500 ml measuring glass containing water at room temperature up to the diluent semen level. At that point, the container was set in the fridge and progressively cooled until came to 5°C inside a time of 1.5-2.0 hours. Diluent samples were stored at 5°C for 0, 24, 48, 72 and 96 hours. The tubes containing diluent samples were covered with dark plastic sheath to avoid the light and any contamination.

Semen collection and evaluation:

Seventy-two ejaculates were collected using an artificial vagina (two ejaculates weekly / up to six weeks / animal) in the presence of an estrous ewe to stimulate mating. Semen characteristics of each ejaculate of a ram were evaluated individually for volume (ml), progressive motility (%), live spermatozoa (%), normal spermatozoa (%), intact acrosome (%) and sperm cell concentration$\times 10^9$ / ml. When ejaculates characterized with semen volume ≥ 0.8, progressive motility ≥75%, live sperm ≥85 %, normal sperm ≥85%, intact acrosome ≥85% and sperm cell concentration ≥2.9×109/ml they pooled, extended and used in experimental procedures. Evaluation of ram spermatozoa characteristics was described by **(Salisbury *et al.*, 1978).**

Acrosomal status was analyzed by the double stain technique according to **Didion *et al.* (1989)**. The accompanying classifications were identified: a- live spermatozoa with intact acrosome (LIA), b- live spermatozoa with reacted acrosome (LRA), c- dead spermatozoa with intact acrosome (DIA), and d- dead spermatozoa with reacted acrosome (DRA), has appeared in Plate 1.

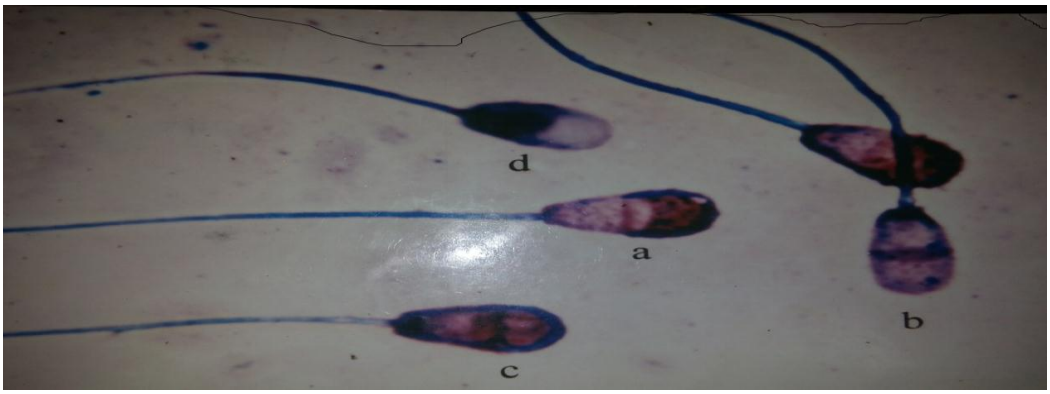

Plate 1: Demonstrates the four classes of spermatozoa created by the double stain technique. a- live spermatozoa with intact acrosome (LIA), b- live spermatozoa with reacted acrosome (LRA), c- dead spermatozoa with intact acrosome (DIA), and d- dead spermatozoa with reacted acrosome (DRA).

Activities of seminal enzymes and penetration ability:

Diluent samples were centrifuged at 3500 rpm for 15 minutes and the supernatant was expelled and utilized for the enzymatic test. Alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactic dehydrogenase (LDH) activities were estimated according to **Graham and Pace (1967)**. Also, sperm penetration into cervical mucus test was determined according to score reported by **Hanson *et al.* (1982)**.

Statistical analysis

The statistical analysis was performed using the SPSS 22.0 program for Windows software **(SPSS, 2013).** ANOVA was used to test the effect of treatment and the differences between means were detected by Duncan's Multiple Range Test **(Duncan, 1955).**

RESULTS

The effects of different energy sources supplemented to locally-manufactured extender compared to commercial Triladyl® extender on bull and ram sperm motility (%) stored at 5 °C for 96 hours are shown in Figure (1). Sperm motility (%) was decreased with increasing preservation time in all extended semen samples. Meanwhile, supplementation of fructose, sucrose or raffinose with TGY extended samples had significantly ($P < 0.01$) higher sperm motility (%) than Triladyl® extender in both bull and ram semen. It was evident from Figure 1 that sperm motility (%) in TGYF extender had the highest values followed by TGYR, TGYS, while TGY recorded the lowest values. Concerning the values of the sperm motility (%) of commercial Triladyl® extender, they were better than those in TGY without any supplementation for both bull and ram semen.

The percentages of live spermatozoa with intact and reacted acrosome were significantly ($P < 0.01$) decreased and the percentages of dead spermatozoa with intact and reacted acrosome were significantly ($P < 0.01$) increased with advance of the storage period in all extended samples of bull and ram diluted semen (Figures 2, 3, 4 & 5). Intact acrosome was significantly ($P < 0.05$) declined with the long storage time in all extended semen samples. TGY extenders which supplemented with different energy sources (TGYF, TGYS or TGYR) had higher percentages of live spermatozoa with intact acrosome than commercial Triladyl® extender, while Triladyl® extender was better than TGY extender without any supplementation for both bull and ram diluted semen. Among rich energy source extenders (TGYF, TGYR, and TGYS), the percentage of live spermatozoa with intact acrosome were

better in both of TGYF and TGYR than TGYS during preservation at 5 °C up to 96 hours.

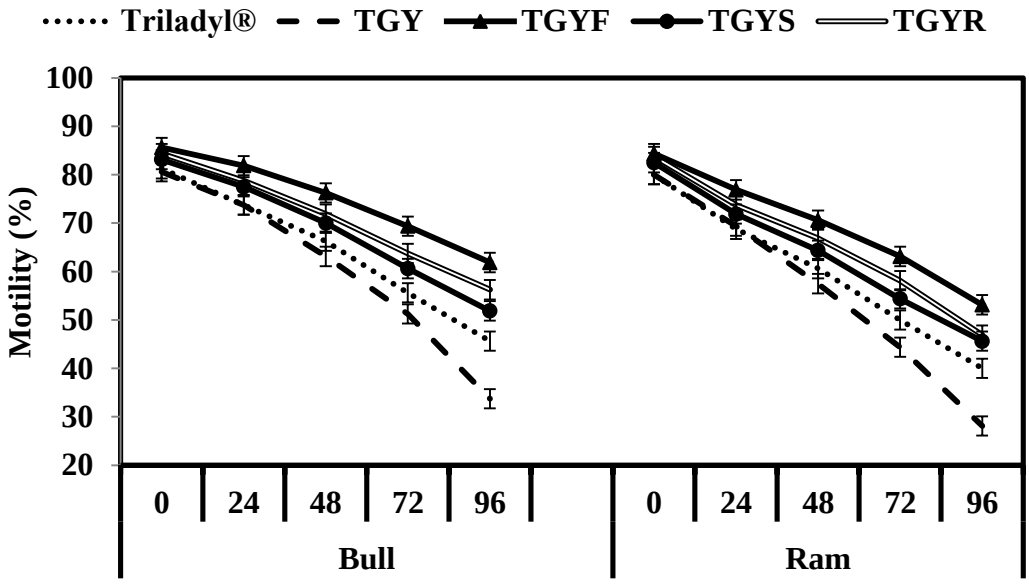

Figure 1: Sperm motility (%) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h

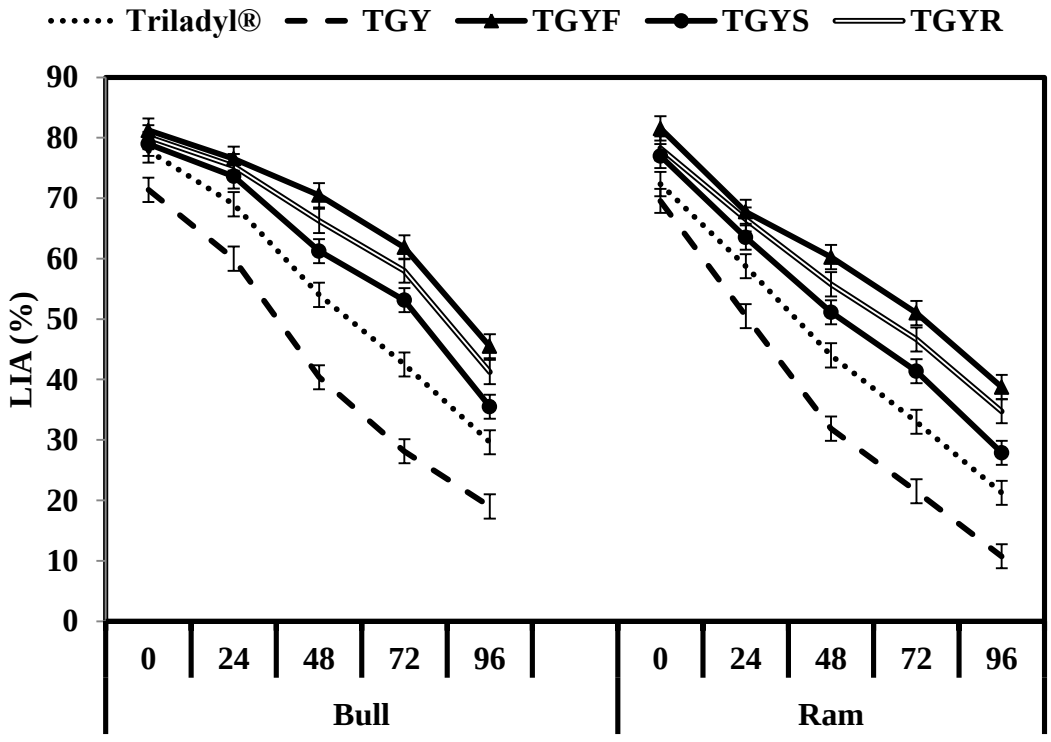

Figure 2: Live spermatozoa with intact acrosome (LIA, %) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h

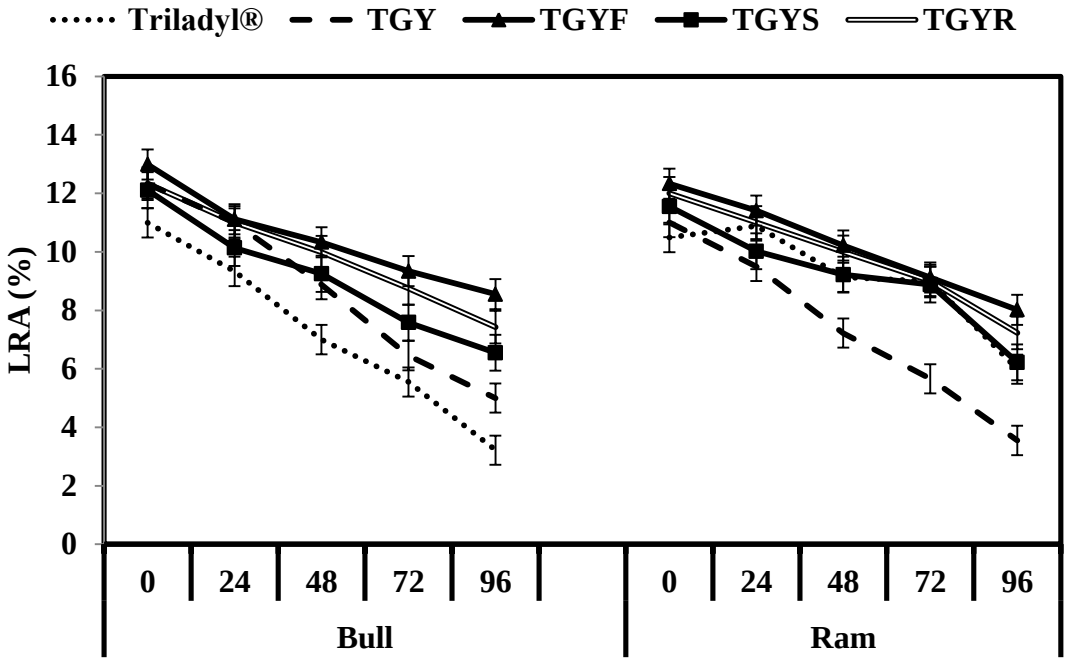

Figure 3: Live spermatozoa with reacted acrosome (LRA, %) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h

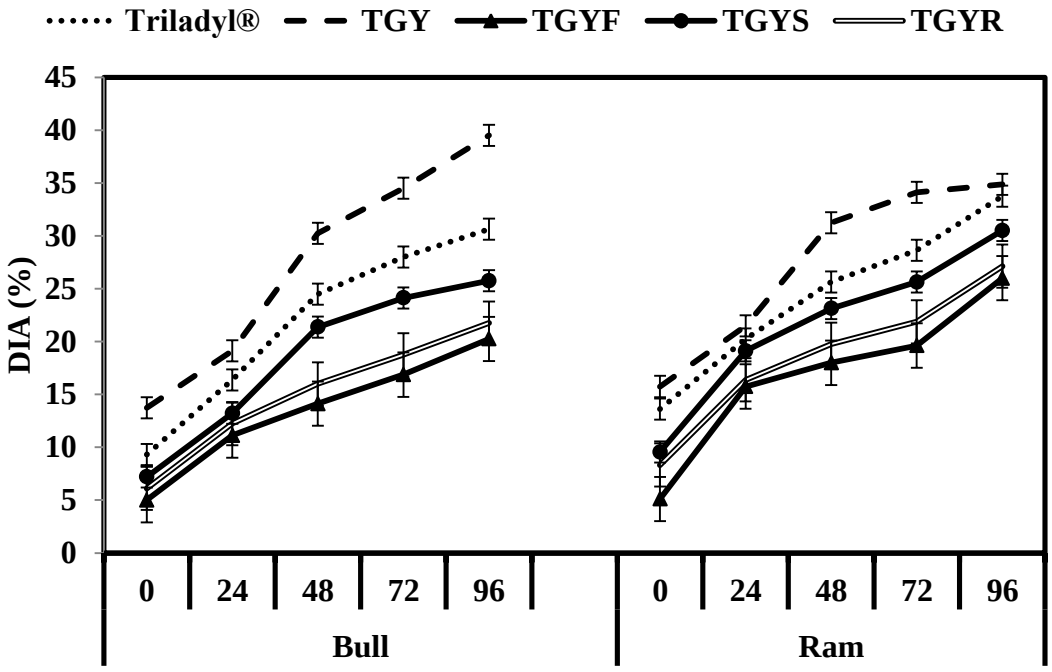

Figure 4: Dead spermatozoa with intact acrosome (DIA, %) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h

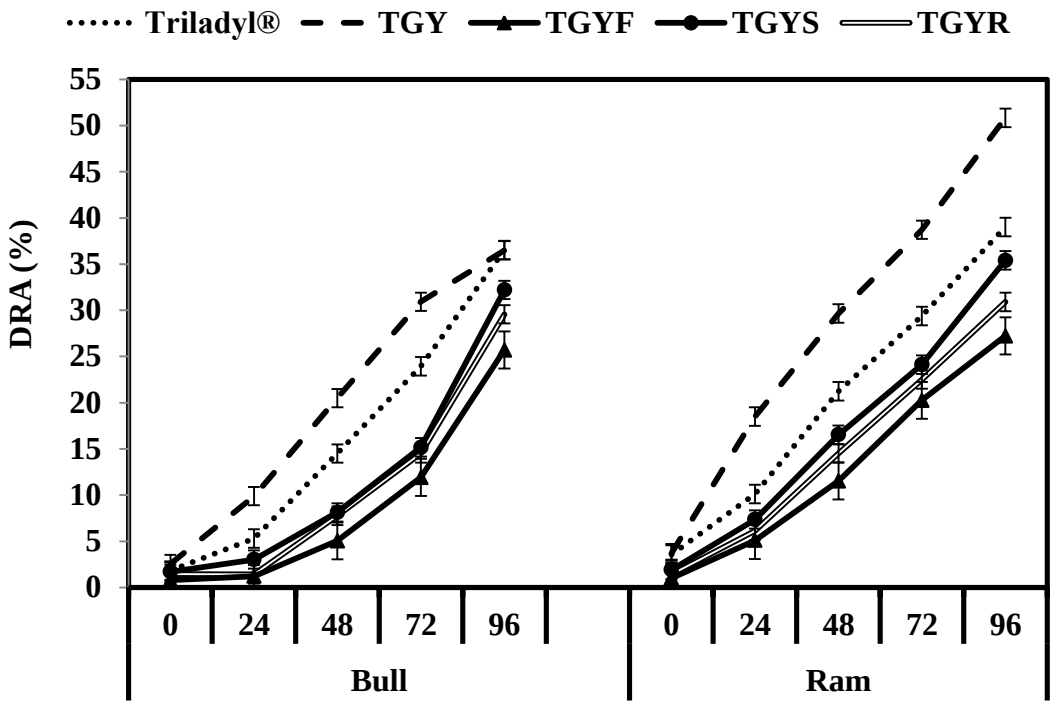

Figure 5: Dead spermatozoa with reacted acrosome (DRA, %) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h

The obtained data in Figure 6 indicated that the percentage of normal sperm morphology significantly ($P < 0.01$) decreased with increasing storage period among different extenders. Normality (%) in TGY extenders that were rich with fructose, sucrose and raffinose were significantly ($P < 0.01$) higher than in Triladyl® and TGY extended semen samples. Concerning Triladyl® extender, it was better than TGY extender for both bull and ram.

In seminal plasma of diluted bull and ram semen, enzyme activities of ALT, AST, ALP, and LDH were significantly ($P < 0.01$) higher for Triladyl®, TGY and TGYS than in TGYF and TGYR during the storage period (Tables 2, 3, 4 and 5). However, the preservation at 5 °C for up to 96 hours increased levels of ALT, AST, ALP, and LDH enzymes released into the extracellular medium.

Figure 7 showed that the penetration ability into female cervical mucus was significantly ($P < 0.05$) better with the extended bull and ram semen for TGYF, TGYS, and TGYR than Triladyl® and TGY during preservation time. However, storage time significantly ($P < 0.01$) decreased the penetration score in all extended samples.

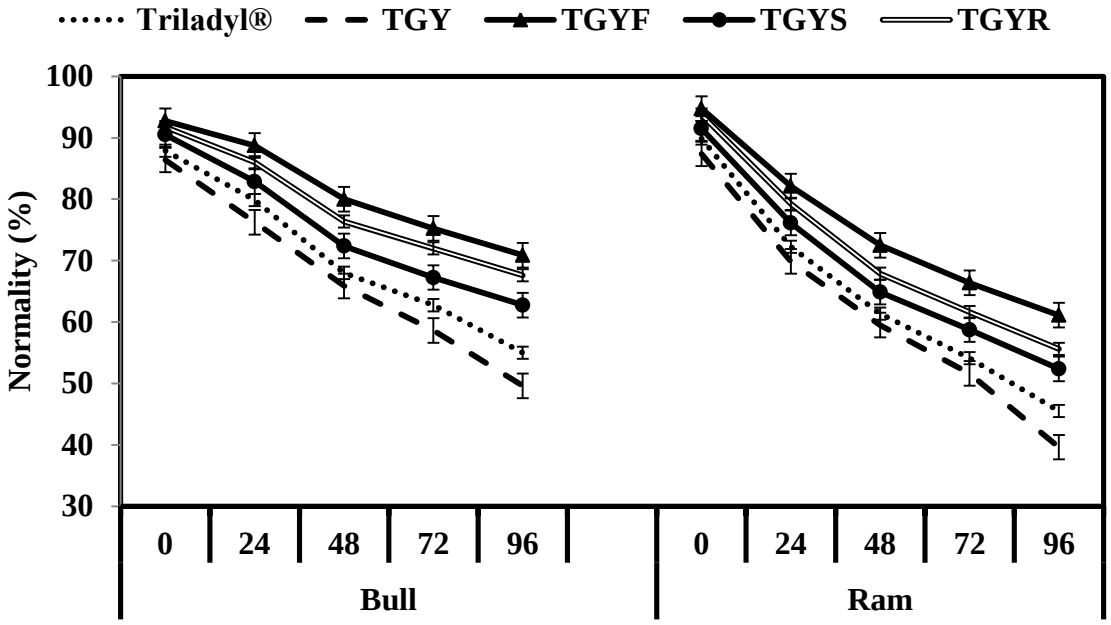

Figure 6: Sperm normality (%) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h.

Table 2: Alanine aminotransferase enzyme (U/10^9 spermatozoa) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h.

Semen extenders	Bull				
	Storage time (hour)				
	Zero	24	48	72	96
Triladyl®	15.97±0.25^{b}	16.23±0.22^{a}	18.93±0.28^{a}	20.27±0.24^{b}	24.11±0.23^{b}
TGY	16.90±0.14^{a}	16.87±0.07^{a}	19.36±0.19^{a}	21.39±0.57^{a}	26.60±0.90^{a}
TGYF	11.71±0.19^{e}	12.32±0.18^{d}	14.77±0.22^{d}	16.73±0.20^{d}	18.50±0.21^{d}
TGYS	14.52±0.30^{c}	14.53±0.35^{b}	16.70±0.46^{b}	18.81±0.33^{c}	21.51±0.64^{c}
TGYR	13.66±0.42^{d}	13.66±0.28^{c}	15.70±0.18^{c}	17.33±0.14^{d}	19.64±0.12^{d}
	Ram				
	Zero	24	48	72	96
Triladyl®	17.20±0.25^{b}	22.05±0.32^{a}	25.13±0.32^{a}	36.67±0.28^{b}	50.20±0.32^{b}
TGY	18.13±0.13^{a}	22.78±0.08^{a}	25.63±0.22^{a}	37.95±0.65^{a}	53.37±1.29^{a}
TGYF	12.94±0.20^{e}	17.58±0.20^{d}	20.37±0.25^{d}	32.62±0.23^{d}	44.34±0.40^{d}
TGYS	15.75±0.29^{c}	20.10±0.40^{b}	22.59±0.53^{b}	35.00±0.38^{c}	47.36±0.63^{c}
TGYR	14.89±0.41^{d}	19.11±0.32^{c}	21.44±0.20^{c}	33.31±0.16^{d}	45.48±0.21^{cd}

$^{a:e}$, Means with different superscripts, within each column, are significantly different ($P < 0.01$). Triladyl®, commercial extender; TGY, locally-manufactured extender (Tris, glucose, citric acid, egg-yolk); TGYF, TGY with fructose; TGYS, TGY with sucrose; TGYR, TGY with raffinose.

Table 3: Aspartate aminotransferase enzyme (U/10^9 spermatozoa) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h.

Semen extenders	Bull				
	Storage time (hour)				
	Zero	24	48	72	96
Triladyl®	32.17±0.64^{b}	31.26±0.56^{b}	35.03±0.59^{b}	38.37±0.68^{b}	43.00±1.38^{b}
TGY	34.75±0.36^{a}	34.52±0.91^{a}	39.45±1.83^{a}	41.45±1.04^{a}	47.45±0.62^{a}
TGYF	28.07±0.07^{d}	27.17±0.12^{c}	30.33±0.21^{c}	34.00±0.32^{c}	37.00±0.32^{c}
TGYS	29.60±0.50^{c}	28.98±0.50^{c}	32.70±0.61^{bc}	35.77±0.57^{c}	38.60±0.68^{c}
TGYR	29.00±0.32^{cd}	28.29±0.31^{c}	31.80±0.37^{c}	34.60±0.51^{c}	37.87±0.67^{c}
	Ram				
	Zero	24	48	72	96
Triladyl®	34.30±0.65^{b}	37.02±0.67^{b}	41.49±0.70^{b}	60.43±0.81^{b}	72.92±1.63^{b}
TGY	36.88±0.37^{a}	40.87±1.08^{a}	46.71±2.17^{a}	64.08±1.23^{a}	78.19±0.73^{a}
TGYF	30.20±0.10^{d}	32.17±0.13^{c}	35.92±0.25^{c}	55.26±0.37^{c}	65.82±0.37^{c}
TGYS	31.73±0.52^{c}	34.32±0.59^{c}	38.72±0.72^{bc}	57.35±0.67^{c}	67.71±0.80^{c}
TGYR	31.13±0.30^{cd}	33.49±0.37^{c}	37.66±0.44^{c}	55.97±0.60^{c}	66.84±0.80^{c}

$^{a:f}$, Means with different superscripts, within each column, are significantly different ($P < 0.01$). Triladyl®, commercial extender; TGY, locally-manufactured extender (Tris, glucose, citric acid, egg-yolk); TGYF, TGY with fructose; TGYS, TGY with sucrose; TGYR, TGY with raffinose.

Table 4: Alkaline phosphatase enzyme (U/10^9 spermatozoa) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h.

Semen extenders	Bull				
	Storage time (hour)				
	Zero	24	48	72	96
Triladyl®	76.77±0.28^{b}	81.88±0.30^{ab}	86.83±1.01^{b}	99.82±0.79^{a}	106.86±3.11^{b}
TGY	80.09±1.21^{a}	82.33±1.21^{a}	90.03±1.55^{a}	100.20±1.10^{a}	120.17±3.62^{a}
TGYF	72.49±0.64^{d}	76.80±0.31^{c}	79.30±0.38^{d}	87.12±0.46^{c}	95.37±0.95^{c}
TGYS	75.08±0.42^{bc}	80.03±0.44^{b}	83.52±0.59^{c}	91.35±1.84^{b}	99.88±0.57^{bc}
TGYR	73.77±0.11^{cd}	77.34±0.18^{c}	80.00±0.18^{d}	88.16±0.24^{c}	98.37±0.51^{c}
	Ram				
	Zero	24	48	72	96
Triladyl®	78.90±0.22^{b}	84.64±0.28^{ab}	93.77±0.98^{b}	103.79±0.74^{a}	130.61±1.57^{b}
TGY	82.22±1.11a	85.05±1.11^{a}	96.88±1.51^{a}	104.15±1.04^{a}	140.84±2.77^{a}
TGYF	74.62±0.66^{d}	79.96±0.28^{c}	86.43±0.37^{d}	91.76±0.44^{c}	112.29±1.10^{c}
TGYS	77.21±0.49^{bc}	82.93±0.41^{b}	90.54±0.57^{c}	95.76±1.75^{b}	126.84±0.40^{bc}
TGYR	75.90±0.16^{cd}	80.46±0.16^{c}	87.12±0.17^{d}	92.74±0.23^{c}	122.84±1.72^{c}

$^{a:f}$, Means with different superscripts, within each column, are significantly different ($P < 0.01$). Triladyl®, commercial extender; TGY, locally-manufactured extender (Tris, glucose, citric acid, egg-yolk); TGYF, TGY with fructose; TGYS, TGY with sucrose; TGYR, TGY with raffinose.

Table 5: Lactic dehydrogenase enzyme (U/10^9 spermatozoa) of the extended bull and ram semen with various types of sugars during storage at 5 °C for 96 h.

Semen extenders	Bull				
	Storage time (hour)				
	Zero	24	48	72	96
Triladyl®	188.32±0.28^{b}	193.88±0.30^{ab}	211.83±1.01^{b}	244.82±0.79^{a}	266.86±3.11^{b}
TGY	191.64±1.21^{a}	194.33±1.21^{a}	215.03±1.55^{a}	245.20±1.10^{a}	280.17±3.62^{a}
TGYF	184.04±0.64^{d}	188.80±0.31^{c}	204.30±0.38^{d}	232.12±0.46^{c}	255.37±0.95^{d}
TGYS	186.63±0.42^{bc}	192.03±0.44^{b}	208.52±0.59^{c}	236.35±1.84^{b}	259.88±0.57^{bc}
TGYR	185.32±0.11^{cd}	189.34±0.18^{c}	205.00±0.18^{d}	233.16±0.24^{c}	258.37±0.57^{c}
	Ram				
	Zero	**24**	**48**	**72**	**96**
Triladyl®	190.45±0.25^{b}	209.64±0.28^{ab}	238.77±0.98^{b}	308.79±0.74^{a}	390.61±1.57^{b}
TGY	193.77±1.15^{a}	210.05±1.11^{a}	241.88±1.51^{a}	309.15±1.04^{a}	400.84±2.77^{a}
TGYF	186.17±0.66^{d}	204.96±0.28^{c}	231.43±0.37^{d}	296.76±0.44^{c}	372.29±1.09^{d}
TGYS	188.76±0.45^{bc}	207.93±0.41^{b}	235.54±0.57^{c}	300.76±1.75^{b}	386.84±0.41^{bc}
TGYR	187.45±0.13^{cd}	205.46±0.16^{c}	232.12±0.17^{d}	297.74±0.23^{c}	382.84±1.72^{c}

$^{a:f}$, Means with different superscripts, within each column, are significantly different ($P < 0.01$).
Triladyl®, commercial extender; TGY, locally-manufactured extender (Tris, glucose, citric acid, egg-yolk); TGYF, TGY with fructose; TGYS, TGY with sucrose; TGYR, TGY with raffinose.

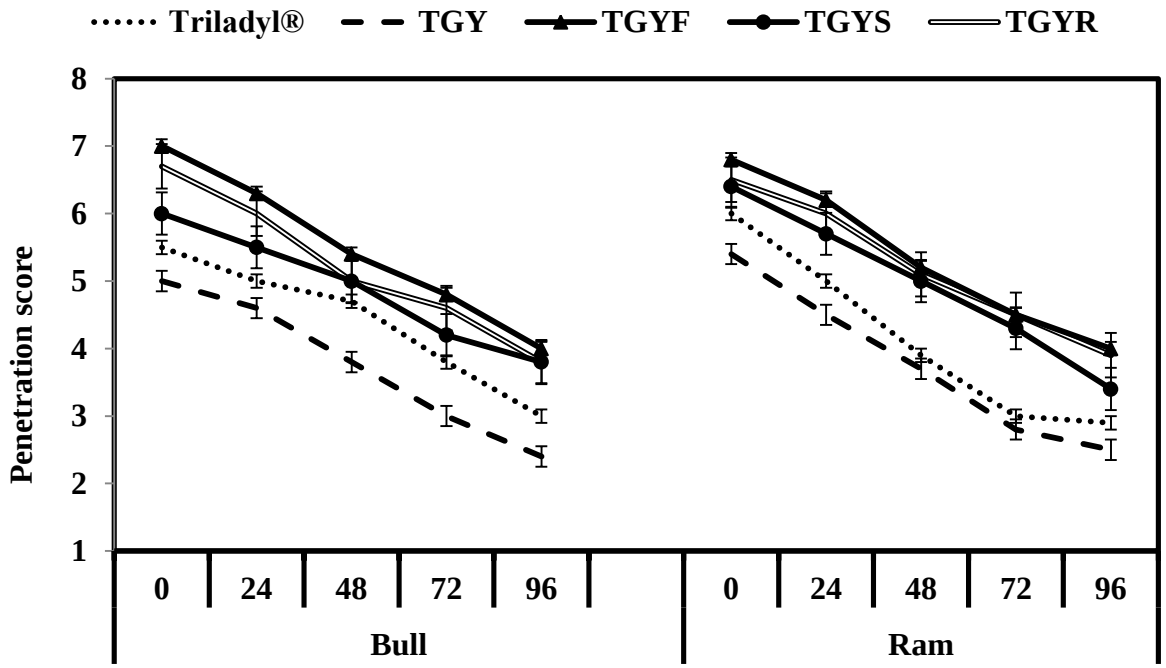

Figure 7: Sperm penetration ability of the extended bull and ram semen with various types of sugars into female cervical mucus during storage at 5 °C for 96 h.

Triladyl®, commercial extender; TGY, locally-manufactured extender (Tris, glucose, citric acid, egg-yolk); TGYF, TGY with fructose; TGYS, TGY with sucrose; TGYR, TGY with raffinose;

DISCUSSION

Current study was designed as a comparative study between commercial Triladyl® and locally-manufactured extenders supplemented with different energy sources on the sperm quality (motility, livability with acrosome integrity and normality) of bull and ram diluted semen stored at 5 ºC for up to 96 hours and its effects on sperm penetration ability.

In the present study, motility, livability with acrosome integrity and normality of bull and ram spermatozoa in the first 24 hours of storage remained stable in all tested diluents. While, sperm motility and live spermatozoa with intact acrosome in 48, 72 and 96 hours of storage were better ($P < 0.01$) for extenders supplemented by various energy sources as compared to Triladyl® and TGY (Figures 1 & 2). Motility is viewed as the most important property related to the fertilizing ability of spermatozoa and as an exhibition of their viability and acrosomal integrity **(Kathiravan *et al.*, 2011)**. The evaluation of spermatozoa morphology (acrosome, head, mid piece and tail) evaluation is viewed as the most critical strategy for separating between high and low semen capacity **(Ball & Poters, 2004)**.

It is trusted that acrosomal uprightness appraisal can be a viable instrument to anticipate the fertilizing capacity of wild bull spermatozoa. A positive relationship has been built up between the fertilizing ability of stored semen and abnormalities **(Soderquist *et al.*, 1991)**. In the current study, percentages of live spermatozoa with normal morphology and intact acrosomes among tested extenders of bulls and rams diluted semen were significantly different and were within the physiological range. It is all around perceived that sperm motility is influenced by the structure of diluting media **(Andrabi, 2009)**. In previous studies, **Akhter *et al.* (2010)** reported that elevation of motility percentage of buffalo bull spermatozoa post lactose and fructose addition was higher than glucose addition in Tris diluent. Also, enhancement of motility and % livability of buffalo spermatozoa was shown in liquid and frozen state post fructose, sucrose and raffinose supplementation to milk and Tris diluent **(Kumar *et al.*, 1994 a&b)**. Furthermore, 1% fructose and 2% glucose gave better motility with Tris diluent of buffalo semen at 5°C **(Kumar *et al.*, 1992)**.

The present results showed that commercial Triladyl® extender had greater ($P < 0.01$) percentage of spermatozoa that were motile, viable and intact integrity than TGY diluent during preservation time at 5 °C for up to 96 hours (Figures 1 & 2). The gainful impacts of Triladyl® diluent in semen have been broadly detailed for ram **(Hegedusová *et al.*, 2012; Rekha *et al.*, 2016)** and bull **(Raseona *et al.*, 2017)** what's more, buffalo semen contrasted and specially designed Tris-citric acid extenders (**Hussain *et al.*, 2011**). In contrary to the current results, **Ollero *et al.* (1998)** reported that Tris-based diluent had positive influences on the motility, livability and functional integrity of ram semen compared to Triladyl. Our data showed significant ($P < 0.01$) differences in the percentage of live spermatozoa with intact acrosome and normal spermatozoa morphology between the two diluents. This result is in agreement with **Kulaksiz *et al.* (2012)** who found a different effect of extender on the percentage of abnormal ram spermatozoa. Despite the fact that the essential parts of Triladyl® and TGY extenders are comparative (Tris-based) and gave an identical buffering system during conservation, they came about indifferent sperm quality, may be due to the quantitative diverse structure of Triladyl® ingredients. This indicated that Triladyl® extender was able to supply the spermatozoa with more nutrients as energy source than the TGY.

Previous studies have shown that preservation with raising amounts of sugars could brought better support to sperm motility and movement modality. Also spermatozoa motility depends enormously on the available energy source in an extender or seminal plasma, as ATP, which is created from metabolism, and in this manner requires a steady supply for cell capacity and survival **(Miki, 2007; Wattimena *et al.*, 2009)**. The influences of sugar addition to the diluent on the preserved semen fluctuate as per types of sugar in view of their distinctive usefulness of chemical and atomic weight. Low atomic weight particles can pass through the plasma membrane of spermatozoa and provide energy to function on metabolism in ordinary physiological way. High atomic weight sugars are not fit for diffusion through plasma membrane and make an osmotic pressure causing cell dehydration. Hence it

caused lower frequency of intracellular ice arrangement and gave the more noteworthy survival of spermatozoa **(Nagase *et al.*, 1964; Purdy, 2006; Naing *et al.*, 2010)**.

Sugars have been closely used in animal semen preservation, particularly the non-permanent saccharides, which can add to cell lack hydration before freezing. Disaccharides and trisaccharide might be more successful than monosaccharide at expanding this osmotic dehydration **(Salamon, 1968)**. In the current investigation, the influence of mono-, di- and trisaccharides supplemented to TGY diluent, generally, lead to better preservation than glucose, which was the standard sugar incorporated into this diluent. Inclusion of sucrose with glucose resulted in similar preservation; however, the incorporation of fructose or raffinose prompted better protection for motility. Boosting the idea that, an interaction between sugar and buffering system may happen. The positive effect of the trisaccharide raffinose to preserve sperm is in agreement with earlier results **(Salamon, 1968; Salamon and Maxwell, 2000; Garde *et al.*, 2008)**.

On the contrary, previous studies reported that monosaccharide was more influential than disaccharide for cryopreserving ram sperm **(Molinia *et al.*, 1994),** and trisaccharide had a low influence than monosaccharide for bull spermatozoa **(Garcia and Graham, 1989)**. On account of the distinctive cryoprotective instruments found in different kinds of sugars, it might be guessed that the combined utilization of monosaccharide and disaccharide in appropriate focuses could furnish preferred protection than that achieved with the utilization of monosaccharide or disaccharide alone **(Yildiz *et al.*, 2000)**. The present results demonstrated that the combined utilization of monosaccharide, disaccharide and trisaccharide could give better assurance contrasted with the utilization of monosaccharide alone (TGYF, TGYS and TGYR vs. Triladyl® and TGY), glucose combined with fructose or raffinose could give better protection than sucrose combined with glucose (TGYF and TGYR vs. TGYS).

Contrasts among mono-, di- or trisaccharides were not recognized; motility following cryopreservation was improved in diluents supplemented with fructose and glucose. It appears that monosaccharides were more effective than disaccharides in this investigation for maintaining sperm motility in Tris-based diluents. The useful impact of sugar addition on sperm viability differed among species due to differences in spermatozoa characteristics **(Purdy, 2006).**

The observed variation in semen properties may be attributed to many agents like species **(Aisen *et al.*, 2002; Garde *et al.*, 2008)**, preservation temperature **(Lapwood and Martin, 1966)**, buffer type **(Abdelhakeam *et al.*, 1991)**, freezing period **(Hay *et al.*, 1997)**, or components of diluent **(Chen *et al.*, 1993; Kozdrowski, 2009)**. The current data revealed a better protective agent for mono- (fructose) and tri- (raffinose) when compared with di- (sucrose) saccharides. Raffinose has been utilized in numerous procedures to prevent formation of extracellular ice, it gives hypertonicity and improves the formation of a metastable glass or microcrystalline state **(Storey *et al.*, 1998)**. As mitochondria produce ATP, they contribute greatly on support of motility **(Medeiros *et al.*, 2002)**. Results showed that three sugars supplementation played an important effective role to enhance the sperm characteristics. Besides, fructose and raffinose, especially, caused a stronger protective influence than sucrose in preserving bull and ram diluted semen. Moreover, using extender supplemented with energy source improved sperm characteristics compared with the traditional one. The best value was recorded with TGYF and the lowest one was with TGY. Also, the supplementation of more sugar types in extender as energy source displayed the most capacity to support sperm viability. This could be ascribed for the capacity of these sugars to provide protection and nutrients offered to spermatozoa cells, in addition to the restrain of microbial development and improve the physiologic condition.

Subsequently, the decrease in sperm characteristics with longer preservation time could be attributed to the continuous utilization of nutrients needed for sperm metabolism during storage. Increasing of storage period led to a decrease in sperm characteristics among diluent types. **Olurode and Ajala (2016)** reported that the sharp down in sperm properties could due to the gradual decrease of nutrients, like Na, K, and

serum protein needed for high metabolic requests of sperm. Moreover, the impact of peroxidation that originates from polyunsaturated fatty acids in sperm cytoplasm membrane of ram lead to an absence of cytoplasm membrane diminished sperm motility and restrains fructolysis and breathes **(Albiaty *et al.*, 2016)**. Additionally, **Acharya *et al.* (2016)** illustrated that the decline of sperm properties might related to useless products of ROS and that free radical (O^-_2, H_2O_2 and OH^-) might be contributed to harm sperm membrane. Increasing of storage periods for semen cause ultra-constitutional, biochemical and functional deteriorate of spermatozoa resulting in lowering of sperm motility, live spermatozoa impairment and reduced fertility.

Evaluation of biochemical components of seminal plasma are needed for semen estimation, as physical semen characteristics alone are not perfectly enough for semen assessment **(Mann and Lutwak-Mann, 1981)**. For instance, ALT, AST, ALP, and LDH are fundamental for metabolic procedures that give energy for sperm motility, viability and fertility **(Sirat *et al.*, 1996)**. Accordingly, these enzymes are evaluated in seminal plasma as markers of sperm quality, as they show sperm damage **(Sirat *et al.*, 1996)**. Therewith, there are few studies focused on the existence of these enzymes in ruminant semen. **Okab (2007)** showed that the spillage of LDH and ALP in rabbit seminal plasma could reveal a positive relationship between enzyme release and sperm cell integrity and acrosomal harm.

There were significant ($P < 0.01$) differences in activities of ALT, AST, ALP and LDH enzymes at all times in all tested extenders. Their levels were significantly ($P < 0.01$) higher in ascending order of TGY and Triladyl® as compared to TGYF, TGYS and TGYR extenders during the storage period. Also, there were significant ($P < 0.01$) increases in the spillage of alanine transaminase, aspartate transaminase, alkaline phosphatase and lactic dehydrogenase enzymes into the extracellular medium through preservation time. These finding are similar to those obtained by **Feng *et al.* (2015), Patel *et al.* (2016) and Mohamed (2017)**.

The obtained data indicated a negative correlation between enzymes activity and content of extenders of sugars in seminal plasma of both bull and ram diluted semen. The continued elevation in spillage of alanine transaminase, aspartate transaminase, alkaline phosphatase and lactic dehydrogenase enzymes in the extracellular medium through preservation may due to the destruction of sperm cellular membrane through preservation **(Zeidan *et al.*, 2004)**. The spoilage of ALP enzyme from sperm cells into the seminal plasma, because of cold shock was elevated significantly **(White *et al.*, 1954)**. Such increment in the enzymes activity post storage might be an indication of expanding the cell harm, which happened with the preservation process **(Zeidan, 1994)**. In the present study, the release of LDH enzyme into the extracellular medium was significantly elevated during the storage period, which might be attributed to degradation of cytoplasmic droplets and plasma membrane when spermatozoa exposed to cold or osmotic shock. Similar results were found by **Dhami *et al.* (1995)** in bulls. LDH plays significant role in viability during the preservation, freezing and metabolic activity of spermatozoa **(Stalloup and Hayden, 1960)**. So, LDH enzyme has been implicated in fertilizing the sperm's ability and viability **(Smith *et al.*, 1957)**. The ALP is the most dynamic dephosphorylation enzyme in semen that may be directly related to fertility. Its concentration mirrors the functional condition of the accessories sex glands and sperm metabolic activity **(Veerabramhaiah, 2011)**.

The main results of this study demonstrate that supplementation of fructose and raffinose to locally-manufactured extended bull and ram semen stored at 5°C improve sperm penetration (SP) ability compared with other extenders.

SP test incorporates an acrosome response test that surveys how well a sperm can complete the fertilization process. Sperm that is unfit to appropriately go during the acrosome response won't most likely fertilize an egg **(Miyazaki *et al.*, 1990)**. The present results regarding SP (Figure 7) showed that the permeation ability into cervical mucus was significantly ($P < 0.05$) better in the diluted bull and ram semen with TGYF, TGYS and TGYR than Triladyl® or TGY during preservation time. These results may be related to the presence of seminal plasma in rich extended semen with different energy sources cause elevate in the liberation of amino acid oxidase, an

enzyme responsible for lowering sperm motility and viability **(Martinus *et al.*, 1991)**. **Aitken and Kelly (1985)** discovered a positive relationship between the development of spermatozoa in human semen and their permeation capability into cervical mucus. Likewise, **Murase *et al.* (1990)** indicated that time of sperm motility and SP distance in the mucus positively correlated to fertility and pregnancy.

It conclude from the present study that fructose and raffinose supplementation have better protective effects than sucrose for preserving sperm, which improves motility and averts acrosomal harm and morphological damage of both bull and ram sperm preserved at 5 ºC for 96 h. Supplementation with these sugars preceding the liquid storage might be prescribed to improve the sperm preservation strategies for the livestock industry.

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تأثير إضافة مصادر مختلفة من الطاقة للحيوانات المنوية للثيران والكباش المعرضة لفترات التبريد المختلفة

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تخزين السائل المنوي يغير من تركيب الحيوانات المنوية والخصائص الحيوية لها، وبالتالي يُعيق قدرتها على الإخصاب، ووجد أن مزج نوعين من السكر مختلفين في الوزن الجزيئى يعزز ويرفع كفاءة الحيوانات المنوية المخزنة للأنواع الحيوانية المختلفة. لذا هدفت هذه الدراسة إلى دراسة تأثير إضافة كلا من سكر الفركتوز أو السكروز أو الرافينوز على مخفف الترس- جلوكوز ومقارنته بالمخفف التجارى (التراى لاديل) وتأثيرهم على السائل المنوي للثيران والكباش المخزنين على درجة حرارة 5 مئوية لمدة 96 ساعة. تم جمع عينات السائل المنوى من ثلاثة ثيران وثلاثة كباش ناضجة مرتين في الأسبوع لمدة ثلاثة أسابيع باستخدام المهبل الاصطناعي. تم تجميع السائل المنوي وتخفيفه وتقسيمه إلى خمسة أجزاء. تم تخفيف عينات السائل المنوي المجمع بواسطة مخففات الترس- جلوكوز و التراى لاديل بنسبة جزء واحد من السائل المنوي الخام: 5 أجزاء من المخفف. تم تقسيم كل عينة من السائل المنوي المخفف بالترس- جلوكوز إلى أربعة أجزاء في أنابيب اختبار معقمة، تحتوي كلا من الترس- جلوكوز والتراى لاديل على نوع سكر واحد ، بينما تحتوي الثلاثة عينات الأخرى على نوعين من السكر والتى تشتمل على: مخفف الترس-جلوكوز مع 0.999 جم من الفركتوز أو 0.700 جم سكروز أو 0.700 جم من رافينوز / 100 مل من المخفف. تم تخزين السائل المنوي على 5 درجات مئوية لمدة 0 ، 24 ، 48 ، 72 و 96 ساعة. أظهرت النتائج المتحصل عليها انخفاض كل قيم خصائص الحيوانات المنوية بشكل ملحوظ مع زيادة فترة التخزين في جميع العينات المخففة. كما تحسنت بشكل ملحوظ قيم خصائص الحيوانات المنوية للعينات المضاف لها سكريات الفركتوز والسكروز والرافينوز مع مخفف الترس- جلوكوز (مثل حركة الحيوانات المنوية، الحيوية وعدد الحيوانات المنوية الطبيعية، والنشاط الأنزيمي لكل من ALT، AST، ALP و LDH، والقدرة على الاختراق في كل من عينات السائل المنوى المخففة للثيران أو الكباش). فيما يتعلق بقيم خصائص الحيوانات المنوية في مخفف التراى لاديل التجاري، فقد كانت أفضل من تلك الموجودة لمخفف الترس- جلوكوز بدون أي إضافات سكرية في كل من السائل المنوي المخفف من الثيران والكباش. الاستنتاج النهائى، تشير النتائج التي توصلنا إليها إلى أن التأثير الملحوظ والمفيد في الحفاظ على السائل المنوي للثور والكبش يمكن الحصول عليه باستخدام مخفف يحتوي على مزيج من أنواع مختلفة من السكريات. ومع ذلك، فإن مؤشرات هذه النتائج تعتمد على القياسات المعملية، لذا فإن هناك حاجة إلى مزيد من تجارب الخصوبة التطبيقية.