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Assessment of semen characteristics and in vivo conception rate of preserved buffalo bull semen extended in tris enhanced with *Diospyros kaki*

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Abstract

Background: The freeze-thaw process results in structural and functional damages caused by over accumulation of reactive oxygen species (ROS). Addition of exogenous antioxidants to semen extender is of a great importance to overcome the oxidative damage during the freezing process. The present study aimed to clarify the impact of *Diospyros kaki* on preserved buffalo semen quality. Tris extender enhanced with kaki juice (KEE) at concentrations 0% (control) and 1-10% (v/v). A twenty percent of freshly prepared egg yolk was added to the KEE extender (KEEY), then all tubes were centrifuged to eliminate any debris. Semen was added to the supernatants in other tubes. Semen was evaluated and the conception rate was implemented.

Results: Sperm forward motility was significantly ($P < 0.0001$) kept high after 5 days of chilling for the concentrations 3, 4, and 5% with respect to the control and kept high ($P < 0.01$) at the other concentrations till the 4th day of chilling. Addition of KEE had significantly ($P < 0.03$) ameliorated post freezing sperm motility with all the concentrations of the extender except the concentration (10%). The highest spermatozoal motility was obtained with the concentration of 1% with respect to the control. Alive sperm%, abnormalities%, and % of intact spermatozoa membranes (HOST%) were similar to the control with all concentrations of kaki used. The conception rate was higher when 1-6% KEE were used.

Conclusions: Some concentrations of Diospyros kaki improved buffalo bull semen quality post-chilling, post-freezing, the conception rate, and the concentration 1% gave the best results.

Keywords: Buffalo, Semen, Preservation, *Diospyros kaki*

Background

Sperm cryopreservation and storage are of a great need for maintaining the supergenetic characters of the males and the implementation of artificial reproductive applications as artificial insemination (AI) and in vitro fertilization (IVF) (Medeiros et al. 2002). AI with frozen semen is of a great value for selection schedules participating in increased production of animal species. Semen freezing has many beneficial applications. Several factors (storage temperature, cooling rate, chemical ingredients of the extender, cryopreservative percent, levels of oxygen free radicals, seminal plasma ingredients, and sanitary control) hindered the livability of spermatozoa (Barbas and Mascarenhas 2009). Freezing of buffalo sperm is often accompanied with the over accumulation of free radicals attended with a deficient antioxidant enzymatic system within the sperm which increased the liability of the sperm membrane to oxidative deterioration (El-Sisy et al. 2007) that affect the membrane integrity (Awda et al. 2009). Nowadays, the application of phytochemicals has gained interest among the world population. The fruit antioxidants induced a defensive system that maintains the metabolism and viability of frozen

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bovine spermatozoa (Câmara et al. 2011). Persimmon (*Diospyros kaki*) fruit was one of those plants having high nutritive value and inclosed many biologically active substances including antioxidants, triterpenoids, fiber, and minerals (Gorinstein et al., 2001; Chen et al. 2008; Park et al. 2008; Akter and Eun 2009; Veberic et al. 2010; Zhou et al. 2010; Dembitsky et al. 2011). It had multiple medicinal values, according to the variety of antioxidants involving phenolics, carotenoids, and vitamin C (George and Redpath 2008). Persimmon contained different types of nutritive materials such as polyphenols, organic acid, carbohydrates vitamins, tannins, dietary fiber, carotenoids, and triterpenoids that affected significantly their nutritive and medicinal values (Matsuo and Ito 1978; Ebert and Gross 1985; Gorinstein 1999; Liu and Xie 2001; Ma et al. 2005; Yuan et al. 2006; Celik and Ercisli 2007; Del Bubba et al. 2009). No available literature was found for interpreting the benefit for using those materials in preserving buffalo semen. So, the aim of the study is to clarify the impact of *Diospyros kaki* of preserved buffalo semen quality.

Materials and methods

Preparation of semen extender: kaki-enhanced extender [KEE]

Five milliliters of blended Persimmon (*Diospyros kaki*) flesh was supplemented to 45 ml tris-citrate-fructose (TCF) to obtain 10% stock solution. KEE was set by supplementing variable concentrations 0.0 ml/5.0 ml (control, 0%), 0.5 ml/4.5 ml (1%), 1 ml/4 ml (2%), 1.5 ml/3.5 ml (3%), 2.0 ml/3.0 ml (4%), 2.5 ml/2.5 ml (5%), 3.0 ml/2.0 ml (6%), 3.5 ml/1.5 ml (7%), 4.0 ml/1.0 ml (8%), 4.5 ml/0.5 ml (9%), and 5.0 ml/0.0 ml (10%) to TCF to obtain a final volume of 5 ml. Twenty percent of whole egg yolk was enclosed in each tube to get KEE with 20% egg yolk (KEEY), all tubes were centrifuged to eliminate the debris. Supernatants were got into other tubes and then stored at −20 °C till used. A preliminary study on the employ of 10 to 50% had given bad results, so the former concentrations were introduced as the persimmon contained high amount of astringent tannin (Akagi et al. 2010) which might be the cause of those poor results

Semen collection and primary assessment

Semen was collected from three mature buffalo bulls kept at Semen Freezing Center, General Organization for Veterinary Services Ministry of Agriculture, Abbasia, Egypt. Ejaculates were collected using an artificial vagina at weekly intervals for 4.5 months. Collected semen was assessed for forward sperm motility and sperm concentration. Ejaculates fulfilling forward sperm motility (70%) were used and pooled to get enough semen for a replicate and to avoid the individual bull variation. Semen was kept for 10 min at 37 °C in the water bath before the extension.

Semen processing

Collected semen was extended with TCF extender and kept as a control and other portions of pooled semen were extended with TCF extenders containing the different concentrations of kaki to achieve a concentration of 60 million sperm/ml. Diluted semen was refrigerated slowly (approximately for 2 h) to 5 °C and equilibrated for 2 h. Semen was filled into a 0.25 ml polyvinyl French straws. After equilibrium periods, the straws were horizontally placed on a rack and exposed to vapor 4 cm above liquid nitrogen for 10 min and were then plunged in liquid nitrogen. A portion of extended semen from control and each concentration of the additives were refrigerated at 5 °C for 7-10 days (chilling) and sperm forward motility was daily assessed.

Assessment of semen quality parameters

The evaluation was implemented on after freeze-thawed buffalo bull spermatozoa. Also, sperm forward motility was evaluated for raw semen, 2 h post cooling and chilled semen daily up to 7-10 days. Frozen straws were thawed at 37 °C for 1 min. The parameters done were motility, alive, abnormality, and sperm membrane integrity (HOST) % (Salisbury et al. 1978).

In vivo fertility rate (CR)

One hundred and forty-five buffalo cows were inseminated with straws representing the different concentrations of KEEY post-thawed semen (one straw for each buffalo in estrus). Each straw contained 15×10^6 forward motile sperm necessary for a desirable fertility level (Mohanty et al. 2018). Ten buffalo cows were inseminated with the post-thawed semen extended in TCFY (control group). Pregnancy was detected by rectal palpation after 2 months from insemination (non-return rate). The inseminated buffalo cows were used via the cooperation in Beni-Suef Governorate. CR was computed according to the equation:

$$\mathrm{CR} = \frac{no.of\ conceived\ cows}{total\ no.of\ inseminated\ cows} \times 100$$

Statistical analysis

Statistical analysis data were computed with SPSS (2005) computerized program v. 14.0 to calculate the one-way analysis of variance (ANOVA) for the different parameters between control and the different concentrations within column either for the cooling in each day (without considering the time factor) or for the after freeze-thawing procedures. Significant difference among means was calculated using Duncan multiple range test at $P < 0.05$.

Results

Effect of tris extender enhanced with *Diospyros kaki* on forward sperm motility of chilled buffalo bull semen

Concerning the time effect on sperm motility, the sperm motility gradually and normally decline with the progress of time ($P < 0.0001$) (Table 1).

Sperm forward motility was significantly ($P < 0.0001$) kept high after chilling for 5 days with the concentrations 3, 4, and 5% (41.67 ± 1.67, 41.67 ± 1.67, and 41.67 ± 1.67, respectively) when compared to the control (20.00 ± 2.89) and also significantly ($P < 0.01$) kept high at the other concentrations till 4 days of chilling (Table 1).

Effect of tris extender enhanced with *Diospyros kaki* on sperm criteria of frozen buffalo bull semen

Addition of KEE had significantly ($P < 0.03$) improved post thawing sperm motility% with all the concentrations except the concentrations 10% the highest sperm forward motility was obtained with the concentration 1% (50.00 ± 5.77%) (Table 2).

Effect of tris extender enhanced with *Diospyros kaki* on conception rate of frozen buffalo bull semen

Conception rate was higher in buffalo with concentrations 1-6% kaki (66.6%, 50.0%, 50.0%, 50.0%, 52.9%, and 57.14%) (Table 3).

Discussion

Semen freezing protocol was a vital request (Medeiros et al. 2002). Cryopreservation caused wide-ranging chemical, physical, and mechanical injures to sperm membranes of all mammalian species (Watson 2000), which were related to changes in temperature and in the conversion from the lipid phase, production of oxygen free radicals and osmotic stress (Câmara et al. 2011; Ortega Ferrusola et al. 2009). Moreover, the excessive ROS caused a state of oxidative damage that involved structural damage of sperm membranes, fall of intracellular ATP levels causing a decrease in the vitality of frozen sperms (Baumber et al. 2000; Agarwal et al. 2005). To alleviate the hazardous effects of ROS, seminal

Table 1 Effect of *Diospyros kaki*-enhanced extender on buffalo bull sperm motility during chilling

Treatment	Time (2 h)	Days							*F* value	P<
		1	2	3	4	5	6	7		
Control	90.00^{aA} ± 0.00	80.00^{bAB} ± 0.00	73.33^{cdBC} ± 3.33	60.00^{dC} ± 5.77	45.00^{cD} ± 10.41	20.00^{dE} ± 2.89	1.67^{cF} ± 1.67	0.00^{cF} ± 0.00	61.32	0.0001
1%	90.00^{aA} ± 0.00	80.00^{bB} ± 0.00	70.00^{dC} ± 0.00	68.33^{bcC} ± 1.67	56.67^{abcD} ± 1.67	33.33^{abE} ± 1.67	8.33^{bcF} ± 1.67	0.00^{cG} ± 0.00	383.64	0.0001
2%	90.00^{aA} ± 0.00	85.00^{aA} ± 0.00	70.00^{dB} ± 0.00	66.67^{cdB} ± 3.33	55.00^{abcC} ± 2.89	30.00^{bcD} ± 2.89	8.33^{bcE} ± 1.67	0.00^{cE} ± 0.00	243.38	0.0001
3%	91.67^{aA} ± 1.67	85.00^{aB} ± 0.00	81.67^{abBC} ± 1.67	78.33^{aC} ± 1.67	66.67^{aD} ± 1.67	41.67^{aE} ± 1.67	23.33^{aF} ± 1.67	6.67^{abG} ± 3.33	492.19	0.0001
4%	88.33^{aA} ± 1.67	85.00^{aAB} ± 0.00	81.67^{abB} ± 1.67	76.67^{abC} ± 1.67	63.33^{aD} ± 1.67	41.67^{aE} ± 1.67	25.00^{aF} ± 2.89	10.00^{aG} ± 2.89	375.70	0.0001
5%	90.00^{aA} ± 2.89	85.00^{aB} ± 0.00	81.67^{abB} ± 1.67	76.67^{abC} ± 1.67	63.33^{aD} ± 1.67	41.67^{aE} ± 1.67	20.00^{aF} ± 0.00	3.33^{bcG} ± 1.67	456.14	0.0001
6%	88.33^{aA} ± 1.67	85.00^{aA} ± 0.00	81.67^{abAB} ± 1.67	76.67^{abB} ± 1.67	55.00^{abcC} ± 2.89	30.00^{bcD} ± 2.89	11.67^{bE} ± 3.33	0.00^{cF} ± 0.00	275.42	0.0001
7%	88.33^{aA} ± 1.67	85.00^{aA} ± 0.00	85.00^{aA} ± 0.00	81.67^{aA} ± 1.67	60.00^{abB} ± 2.89	33.33^{abC} ± 4.41	10.00^{bD} ± 2.89	0.00^{cE} ± 0.00	251.39	0.0001
8%	88.33^{aA} ± 1.67	85.00^{aA} ± 0.00	76.67^{bcB} ± 1.67	73.33^{abcB} ± 1.67	48.33^{bcC} ± 1.67	25.00^{bcdD} ± 2.89	10.00^{bE} ± 2.89	0.00^{cF} ± 0.00	356.80	0.0001
9%	88.33^{aA} ± 1.67	85.00^{aAB} ± 0.00	80.00^{abB} ± 0.00	73.33^{abcC} ± 1.67	48.33^{bcD} ± 1.67	23.33^{cdE} ± 1.67	6.67^{bcF} ± 1.67	0.00^{cF} ± 0.00	386.03	0.0001
10%	88.33^{aA} ± 1.67	85.00^{aAB} ± 0.00	80.00^{abBC} ± 0.00	73.33^{abcC} ± 1.67	55.00^{abcD} ± 5.00	25.00^{bcdE} ± 2.89	8.33^{bcF} ± 3.33	0.00^{cG} ± 0.00	205.64	0.0001
F value	0.52	Infty	11.49	5.94	3.01	8.87	9.81	5.90		
P<	0.8578	0.0001	0.0001	0.0002	0.0149	0.0001	0.0001	0.0003		

Mean ± S.E.

Different small letter superscripts indicate a significant difference between means within column (for treatment) using the multiple range Duncan's test at $P < 0.05$

Different capital letter superscripts indicate a significant difference between means within row (for time) using the multiple range Duncan's test at $P < 0.05$

Table 2 Effect of *Diospyros kaki*-enhanced extender on post thawing buffalo bull sperm characteristics

Treatment	Parameter			
	Motile %	HOST %	Alive %	Abnormality %
Control	$26.67^{b} \pm 3.33$	$73.33^{a} \pm 8.25$	$80.00^{a} \pm 1.73$	$18.67^{a} \pm 2.40$
1%	$50.00^{a} \pm 5.77$	$83.00^{a} \pm 1.15$	$78.33^{a} \pm 1.33$	$22.67^{a} \pm 2.19$
2%	$43.33^{a} \pm 6.67$	$78.00^{a} \pm 1.53$	$74.00^{a} \pm 2.52$	$23.67^{a} \pm 2.33$
3%	$45.00^{a} \pm 5.00$	$79.67^{a} \pm 0.88$	$81.00^{a} \pm 0.58$	$23.00^{a} \pm 4.73$
4%	$40.00^{ab} \pm 0.00$	$84.67^{a} \pm 0.88$	$84.00^{a} \pm 4.93$	$22.33^{a} \pm 6.84$
5%	$46.67^{a} \pm 8.82$	$78.67^{a} \pm 1.86$	$79.00^{a} \pm 1.00$	$21.33^{a} \pm 5.36$
6%	$41.67^{ab} \pm 1.67$	$84.67^{a} \pm 3.18$	$80.33^{a} \pm 5.46$	$17.67^{a} \pm 3.18$
7%	$41.67^{ab} \pm 1.67$	$74.67^{a} \pm 0.88$	$74.33^{a} \pm 1.76$	$22.00^{a} \pm 2.52$
8%	$40.00^{ab} \pm 5.00$	$79.67^{a} \pm 0.88$	$70.67^{a} \pm 1.76$	$25.67^{a} \pm 4.26$
9%	$36.67^{ab} \pm 3.33$	$81.00^{a} \pm 1.00$	$71.33^{a} \pm 2.96$	$24.00^{a} \pm 3.06$
10%	$26.67^{b} \pm 3.33$	$78.00^{a} \pm 3.06$	$79.00^{a} \pm 4.93$	$21.33^{a} \pm 6.33$
F value	2.49	1.50	1.88	0.29
P<	0.0360	0.2059	0.1045	0.9759

Mean ± S.E.
Different letter superscripts indicate a significant difference between means within column using the multiple range Duncan's test at $P < 0.05$

plasma had a potent source of oxygen free radicals, scavengers that offered protection for sperm, including superoxide dismutase, glutathione peroxidase, catalase enzymes, vit. C, and α-tocopherol (Aitken and Baker 2004; Sikka 2004). Including cryoprotectants in the semen diluent during refrigeration, freeze-thawing of sperm cells reduced the spermatozoal damage and consequently improved livability and consequent fertilizing capacity (Gadea et al. 2007; Uysal and Bucak 2007; Bucak et al. 2008). The natural additives contained antioxidants to antagonize the hazardous action of the oxygen-free radicals. Concerning the buffalo semen, semen was chilled to ensure till which day of chilling it could be used in artificial insemination where the sperm motility must not be less than 50%. The decline of sperm motility in chilled semen in all concentrations of kaki extract as well as in the control was used as a good indicator to inform us on which day we do not use it in in vivo fertility. Sperm forward motility was kept high after chilling for 5 days at the concentrations 3, 4, and 5% KEEY while it was kept high up to chilling for 4 days for the other concentrations. Conversely, KEEY had improved the post freezing spermatozoal motility with all concentrations (1-9%) except the 10%. Higher conception rate in buffalo with concentrations 1-6% kaki coincided with the good post-thawing sperm parameters with these concentrations. The used concentrations (1-10%) had maintained alive%, abnormalities%, and % of intact spermatozoal membranes (HOST%). Freezing of buffalo semen exerted an additional source of reactive oxygen (ROS) attack and lipid peroxidation (El-Sisy et al. 2007) which affected the membrane permeability (Awda et al. 2009). The increased ROS level was attributed to the reduced levels of antioxidant enzymes. Natural antioxidants maintained the metabolism and livability of frozen buffalo spermatozoa (Câmara et al. 2011).

Table 3 Effect of *Diospyros kaki* enriched extender on a field conception rate test in buffalo

Treatment	In vivo fertility rate (CR, %)
Control	45.50
KEE 1%	66.6%
KEE 2%	50%
KEE 3%	50%
KEE 4%	50%
KEE 5%	52.9%
KEE 6%	57.14%
KEE 7%	33.33%
KEE 8%	33.33%
KEE 9%	25%
KEE 10%	50.00%

Diospyros kaki contained potent antioxidant ingredients as represented by high levels of carotenoids (Zhou et al. 2010; Zaghdoudi et al. 2015), flavonoids, and polyphenols (Denev et al. 2013; Sun et al. 2014; Xie et al. 2015), organic acids, and vitamins (Zhou et al. 2010; Karakasova et al. 2013), minerals (Marqués et al. 2015), and carbohydrates (Baltacioglu and Artik 2013; Zhou et al. 2010). Olayemi et al. (2011) and Aljady et al. (2000) recorded that flavonoids and phenolics compounds had efficient antioxidant potential and superior viability of preserved spermatozoa. Flavonoids and phenolics were strong eliminators of the oxygen free radicals and the number of hydroxyl group on the phenyl ring improved the antioxidant capability of polyphenolic molecule (Wettasinghe and Shahidi 2000; LeBlanc et al. 2009). Kaki contained large amounts of flavonoids that possessed high antioxidant capacity and decreased the release of nitric oxide and malondialdehyde and minimized the apoptotic damage (Sun et al. 2014). Flavonoids, vitamins, and organic acids found in pollen grains and honey are antioxidants that improve the preservability of bull semen (El-Sheshtawy et al. 2014a, b).

Conclusion

It could be concluded that some concentrations of *Diospyros kaki* improved buffalo bull semen quality post-chilling and post-freezing, also upregulated the conception rate and the concentration 1% gave the best results.

Abbreviations

KEE: Kaki-enhanced extender; KEEY: Kaki-enhanced extender egg yolk; HOST: Hypo-osmotic swelling test/sperm membrane integrity test;

AI: Artificial insemination; IVF: In vitro fertilization; TCF: Tris citrate fructose extender; TCFY: Tris citrate fructose egg yolk extender; CR: Conception rate; ANOVA: Analysis of variance

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Consent of publication
Not applicable.

Authors' contributions
Both authors RES and WEN had shared all the items of the experimental design, the collection of semen, the extraction, the diluting concentrations, the preparation of the manuscript, and Dr. WEN had performed the statistical analysis and tabulation.

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Availability of data and materials
The authors claimed the availability of data and materials.

Ethics approval and consent to participate
The experimental design on buffalo bulls and their ejaculates has been approved by the Medical Research Ethics Committee at the National Research Centre, Egypt under the license no. 17/155.

Competing interests
The authors declare that they have no competing interests.

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