EFFECT OF IN VITRO ACTIVATION BYGLYCYRRHIZA GLABRA EXTRACT ON CRYOSTORAGE

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Abstract:

The aim of the present study is to investigate the effect of *Glycyrrhiza glabra* extract on certain sperm function parameters using *in vitro* activation technique for cryostorage of buck semen following several days of ejaculation (1,2 and 3 days).

Eight mature indigenous bucks aged (2-3 years) were used. Semen was collected once a week from each male using artificial vagina. The semen diluted by using modified Tris solution (MTS) (Tris 3.03gm+fructose 1.25gm+ citric acid 1.67gm+egg yolk) then stored in refrigerator (4-5) °C for 24, 48, &72 hours and study the effect of adding Gg in modified culture media using simple layer technique. The results of certain sperm function parameters of cryostorage sperm were recorded.

The results showed that Adding 20% Gg in modified culture media and *in vitro* activation for 60 minutes had a significant improvement of sperm motility and grade activity of progressive forward movement following several days of ejaculation. There was a significant decrease in values of sperm concentration after activation compared with that before activation technique. There was no significant ($p>0.05$) differences in the percentage of morphologically normal sperm (MNS) between before and after activation technique. These results were attributed to the constituents of Gg such as vitamins, minerals, estrogenic and anti estrogenic substance, trace element, and polysaccharide which have effect on certain sperm parameters.

The results of this study indicate that using Gg for *in vitro* activation of preserved buck semen improve some sperm function parameters, in which it can be utilized for artificial insemination .

تأثير التنشيط في الزجاج بأستخدام مستخلص عرق السوس للسائل المنوي المحفوظ للمعز المحلي

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الخلاصة :

الهدف من هذه الدراسة هو التحقق من تأثير مستخلص عرق السوس على المعايير الوظيفية المحددة للنطف المنشطة في الزجاج للسائل المنوي المحفوظ للمعز بعد عدة أيام من القذف (1 و2 و3 يوم).تم استعمال ثمانية ذكور ماعز محلية بالغة (2-3). تم جمع السائل المنوي مرة أسبوعيا من كل ذكر باستخدام المهبل الاصطناعي ومن ثم تخفيفه

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بإستخدام محلول الترس المحور (الترس 3.03 غم + الفركتوز 1.25 غم + حامض الستريك 1.67 غم+صفار البيض) وبعد ذلك حفظه في الثلاجة (4-5)°م، بعد 24 و48 و 72 ساعة تم دراسة تأثير أضافة مستخلص عرق السوس للوسط الزرعي بأستخدام طريقة التنشيط في الزجاج (الطباقية البسيطة) وتم تسجيل نتائج المعايير الوظيفية المحددة للنطف المحفوظة.

بينت النتائج إن إضافة 20% عرق السوس للوسط الزرعي المحور و التنشيط في الزجاج لمدة 60 دقيقة أعطت نتائج جيدة في تحسين حركة النطف مع درجة نشاط الحركة التقدمية بعد عدة أيام من القذف،وكذلك حصول إنخفاض معنوي في تركيز النطف بعد التنشيط مقارنة بما قبل التنشيط ، ومع عدم حصول فرق معنوي في نسبة شكلياء النطف السوية قبل وبعد التنشيط.هذه النتائج تعزى الى مكونات عرق السوس مثل الفيتامينات والأملاح والمواد الأستروجينية والمضادة للأستروجين والمعادن المهمة والسكريات المتعددة و التي لها تأثيرعلى المعايير المحددة للنطف.
بينت النتائج ان إضافة عرق السوس لغرض التنشيط في الزجاج للسائل المنوي المحفوظ للمعز يحسن بعض المعايير الوظيفية للنطف و التي يمكن استخدامها لغرض التلقيح الأصطناعي.

Introduction:

Goats are species of animals characterized by many unique biological features such as high fertility, ability to produce twin, triplet and even quadrant pregnancies and have a resistance to different types of diseases (Mohy *et al*,. 1985; Thomson and Thomson, 1987). Goat semen contains coagulating enzymes which convert the lecithin in the egg yolk to lysolecithin. This substance seemed to be lethal to sperms (Pellicer, 1995; Leboeuf *et al*,. 2000).

It has been found that there are many medicinal herbs have the capability to improve the reproductive performance of male and female genital system. One of these medicinal herbs is *Glycyrrhiza glabra* (Gg*)*. The plant constitute of different compounds that enhance man sperm motility *in vitro* and increase the reproductive efficiency in human and mice *in vivo* (Al-Dujaily *et al*., 2006; Al-Muala, 2006). However, in our knowledge no studies concerning the effect of adding medicinal herbs to the diluents of livestock semen had been researched.

Therefore, the present study was conducted to find out the effect of adding Gg extract to the modified Tris extender on goat sperm using *in vitr*o activation technique.

Materials &Methods:

Eight Mature local bucks 2-3 years old were housed in semi opened shade with same management and care in farm of College of Veterinary Medicine-University of Baghdad. Semen was collected once weekly from each male by artificial vagina using female in estrus phase or after induction of estrus by injecting estradiol benzoate.

Semen was diluted 20 folds (semen 1:19 diluent) using modified Tris solution prepared by Tris (hydroxymethyl aminomethane) 3.03gm with citric acid 1.67 gm, fructose 1.25gm and completed to 100ml by distilled water. Then removal of 0.5ml from this solution and replaced by 0.5ml of egg yolk. The sample of semen was put in warmed

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water (37°C) for 10 minutes, after that the diluted semen was kept in refrigerator at 4-5°C.

Semen was diluted 20 times as mentioned above and kept in refrigerator (4-5) °C. Seminal fluid analysis include determination of: sperm concentration, sperm motility percentage, morphologically normal sperm percentage, grade activity (grade a: rapid forward progressive liner, grade b: liner but not rapid or no liner but rapid, grade c: circle motility, grade d: immotile sperm) (WHO, 1999). Diluted semen with modified Tris solution was centrifuged for 20 minute at 3000 rpm. Seminal plasma and Tris solution superior layer was discarded to have only the pellets of sperm in the bottom of tube. Simple layer technique was used to separate the motile sperms from immotile sperm and other debris. The simple layer technique was utilized the capacity of motile sperms to migrate (swim-up) from an under layer to upper layer of the medium.

Three plastic conical sterile test tubes were prepared, each tube contains one concentration of the solution as follows (MTG_0, MTG_{10}, MTG_{20}):-

MTG_0 =Tris(50%)+ Medium(HAM's F-10) (50%).

MTG_{10}=Tris(50%)+ Medium(HAM's F-10) (40%)+Glycyrrhiza(10%).

MTG_{20}=Tris(50%)+ Medium(HAM's F-10)(30%)+ Glycyrrhiza(20%).

Then sperm pellets layered equally in each tube underneath of culture medium. Samples were kept in refrigerator (4-5) °C. Certain sperm function parameters were measured again following the activation periods (AL-Dujaily *et al*., 1999).

The completely Randomized Design (CRD) within the SAS (2001) program was used to study the effect of treatment and time (factorial experiment) in certain sperm function parameters. Analysis of variance (ANOVA) was performed and Least Significant Difference (LSD) test was used to comparison between means (Sorlie ,1995).

Results:

Effect of adding *Glycyrrhiza glabra* to the culture media on certain goat's sperm function parameters using *in vitro* activation technique.

Tables 1, 2 & 3 showed a significant (p<0.001) decrease in sperm concentration (million/ml) following 10 minutes of activation using media MTG_0 (0%Gg), MTG_{10} (10%Gg), and MTG_{20} (20%Gg) compared with before activation values. The mean of sperm concentration was higher when MTG_{20} was used than MTG_0 & MTG_{10} were used. The percentage of sperm motility and grade activity after activation showed significant (p<0.05) higher than that before activation specially when used MTG_{20} with activation for 60 minutes, however the percentage of sperm motility and grade activity of progressive movement after activation when MTG_{20} was used it's higher than other media. (p>0.05)Effect of *in vitro* activation periods on certain goat's sperm function parameters following adding *Glycyrrhiza glabra* to the culture media:

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Table (1): Effect of adding *Glycyrrhiza glabra* in culture media and *in vitro* activation periods on certain goat's sperm function parameters following 1 day of ejaculation using *in vitro* activation technique.

Sperm function parameter	After *In vitro* activation				
	Before activation	Gg concentration	Periods (minutes)		
			10	30	60
Sperm Concentration m/ml	3.148 X10^9 ±0.611 A**	MTG_0	150±5 B**c*c*	325±11.8 B**b*c*	475±12.5 B**a*b*
		MTG_{10}	433±33 B**c*b*	600±10.5 B**b*b*	766±66.6 B**a*a*
		MTG_{20}	600±9.9 B**b*a*	766±18.5 B**a*a*	825±75 B**a*a*
Sperm motility (%)	51.25 ±8.26 B	MTG_0	38.75±14.77 Cb*b*	41.25±13.5 Bb*b*	52.5±11.27 Ba*a*
		MTG_{10}	45±13.7 Cc*b*	58.75±10.07 Ab*a*	71.25±9.21 Aa*a*
		MTG_{20}	55±11.9 Bb*a*	70±10.6 Aa*a*	81.25±5.15 Aa*a*
Grade activity (a.b) %	22.5 ±9.46 B	MTG_0	23.75 ±12.14 Bb*c*	25 ±8.15 Bb*c*	41.25 ±13.6 Aa*c*
		MTG_{10}	31.25±16.37 Ab*b*	47.5±13.75 Aa*b*	52.5±13.15 Aa*b*
		MTG_{20}	41.25±16.87 Ab*a*	62.5±10.3 Aa*a*	68.75±11.5 Aa*a*
Normal Morphology %	85 ±2.04 A	MTG_0	86.45±2.32 Aa*a*	87.2±1.1 Aa*a*	88.5±2.1 Aa*a*
		MTG_{10}	87.1±1.5 Aa*a*	87.4±0.5 Aa*a*	90.4±1.3 Aa*a*
		MTG_{20}	87.8±0.8 Aa*a*	89.2±1.1 Aa*a*	91.32±1.3 Aa*a*

*Values are mean ± SEM

* $P < 0.05$

** $P < 0.001$

*Small letters mean significant results between different periods in the same concentration.

* Italic small letters mean significant results between different concentrations in the same period.

*Capital letters mean significant differences between after and before activation

MTG_0 = media 50% +Tris 50 % , MTG_{10} = media 4 0% + 10% Gg +Tris 50% MTG_{20} = media 30%+Gg 20% +Tris 50%

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Table (2): Effect of adding *Glycyrrhiza glabra* in culture media and *in vitro* activation periods on certain goat's sperm function parameters following 2 days of ejaculation using *in vitro* activation technique.

Sperm function parameter	After *In vitro* activation				
	Before activation	Gg concentration	Periods (minutes)		
			10	30	60
Sperm Concentration m/ml	2.96 X10^9 ±0.53 A**	MTG_0	333.3±33.41 B**b*b*	433.3±33.46 B**a*b*	500±55 B**a*c*
		MTG_{10}	400±60 B**b*b*	500±58 B**b*b*	666.7±12.07 B**a*b*
		MTG_{20}	566.6±67 B**b*a*	666.6±67 B**b*a*	850±76.7 B**a*a*
Sperm motility (%)	58.33 ±1.67 B	MTG_0	30±10 Cb*b*	33.3±8.35 Cb*c*	46.6±8.85 Ca*c*
		MTG_{10}	36.6±12.07 Cb*b*	46.6±11.7 Cb*b*	61.6±10.18 Ba*b*
		MTG_{20}	50±10.45 Cb*a*	59±10.58 Bb*a*	77.33±6.52 Aa*a*
Grade activity (a.b) %	40 ±5 B	MTG_0	20±5.04 Cb*b*	23.6±8.35 Cb*b*	40±11.6 Ba*b*
		MTG_{10}	23.3±8.87 Cb*b*	30±10.04 Cb*b*	43.3±14.59 Ba*b*
		MTG_{20}	36.3±8.0 Bb*a*	43.3±8.87 Bb*a*	60±11.1 Aa*a*
Normal Morphology %	87.3 ±1.45 A	MTG_0	88.4±1.3 Aa*a*	89.8±1.7 Aa*a*	90.3±1.4 Aa*a*
		MTG_{10}	88.8±0.8 Aa*a*	90.5±0.7 Aa*a*	91.6±1.3 Aa*a*
		MTG_{20}	89.6±1.1 Aa*a*	91.4±1.2 Aa*a*	92.5±0.9 Aa*a*

*Values are mean ± SEM

* $P < 0.05$

** $P < 0.001$

*Small letters mean significant results between different periods in the same concentration.

* Italic small letters mean significant results between different concentrations in the same period.

*Capital letters mean significant differences between after and before activation

MTG_0 = media 50% +Tris 50 % , MTG_{10} = media 4 0% + 10% Gg +Tris 50% MTG_{20} = media 30%+Gg 20% +Tris 50%

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Table (3): Effect of adding *Glycyrrhiza glabra* in culture media and *in vitro* activation periods on certain goat's sperm function parameters following 3 days of ejaculation using *in vitro* activation technique.

Sperm function parameter	Before activation	Gg concentration	After *In vitro* activation: Periods (minutes) 10	30	60
Sperm Concentration m/ml	2.45 X10^9 ±0.45 A**	MTG_0	200±8.6 B**c*c*	475±38.3 B**b*b*	625±64.2 B**a*b*
		MTG_{10}	300±26.4 B**c*b*	533±39.8 B**b*b*	750±93.2 B**a*ab*
		MTG_{20}	400±42.7 B**c*a*	625±58.1 B**b*a*	833±79.6 B**a*a*
Sperm motility (%)	40 ±3.4 B	MTG_0	19.0±7 Cb*c*	47.5±10.2 Aa*b*	55.83±7.8 Aa*b*
		MTG_{10}	33.3±11.2 Cc*b*	55.83±13.3 Ab*b*	67.3±9.2 Aa*ab*
		MTG_{20}	47.5±8.9 Ab*a*	73.32±10.1 Aa*a*	79.16±7.2 Aa*a*
Grade activity (a.b) %	21.66 ±4.8 B	MTG_0	6.2±1.6 Cc*c*	21.16±3.4 Bb*c*	40±7.8 Aa*b*
		MTG_{10}	24.16±6.2 Bc*b*	42.5±9.2 Ab*b*	53.32±7.2 Aa*a*
		MTG_{20}	37.5±7.4 Ab*a*	55.83±6.8 Aa*a*	58±6.9 Aa*a*
Normal Morphology %	83.3 ±3.2 A	MTG_0	85.1±1.7 Aa*a*	87.2±1.4 Aa*a*	87.9±0.4 Aa*a*
		MTG_{10}	86.1±1.2 Aa*a*	87.±1.8 Aa*a*	89.2±1.9 Aa*a*
		MTG_{20}	86.5±0.6 Aa*a*	88.8±1.6 Aa*a*	91.1±2.1 Aa*a*

*Values are mean ± SEM

* $P < 0.05$

** $P < 0.001$

*Small letters mean significant results between different periods in the same concentration.

* Italic small letters mean significant results between different concentrations in the same period.

*Capital letters mean significant differences between after and before activation

MTG_0 = media 50% +Tris 50 % , MTG_{10} = media 4 0% + 10% Gg +Tris 50% MTG_{20} = media 30%+Gg 20% +Tris 50%

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The result in tables 1,2&3 showed that the mean of sperm concentration before activation is highly significant (p<0.001) than that of after activation periods using different media (MTG_0, MTG_{10}, and MTG_{20}).However the values of sperm concentration was increase proportionally with the increase the periods of activation to reach its highest after 60 minutes using different media. The percentage of sperm motility and the values of grade activity showed the best results after 60 minutes of activation when different media were used. There were no significant differences in the percentage of MNS between before and after activation periods using MTG_0 or MTG_{10} or MTG_{20} .

Figure (1) showed the percentage of sperm motility and grade activity progress forward movement after 24, 48, 72 hrs of ejaculation and activation *in vitro* for 60 minutes using MTG_{20}. The results showed there was no significant differences in the percentage of sperm motility (81.25, 77.33, 79.16) % respectively and grade activity progress forward movement (68.75, 60, 58) % respectively between 24, 48, 72 hrs.

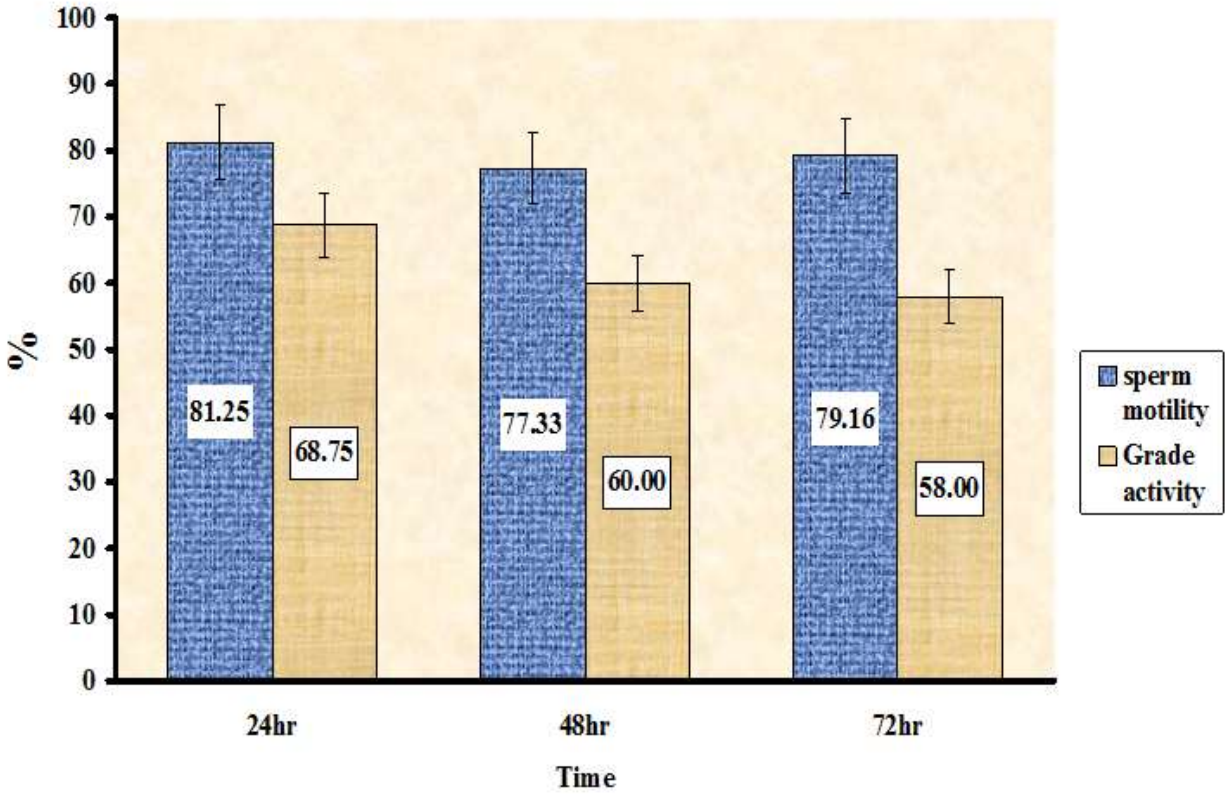

Figure 1: The goat sperm motility and grade activity (a-b) percentage following *in vitro* activation for 60 minutes after 24,48,72 hrs of ejaculation and cryostorage at 4-5°C .

Discussion

The results of the present study indicated that adding Gg to the culture media enhances different sperm function following 30 and 60 minutes of activation mainly

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sperm motility and grade activity of forward progressive movement. This improvement may be resulting from the constituents of Gg.

It has been found that Gg has an estrogenic activity due to the presence of glabridin which is known to be a phytoestrogenic and has the ability to bind to human estrogen receptors (Tamir *et al*., 2000).Estrogens are known to improve sperm characteristics including sperm motility and grade activity in addition to induction of hyperactive motility (Chan *et al*., 1985; Al-Jarah, 2000).

Both 10% and 20% of Gg added to the modified Tris solution showed an improvement of sperm motility percentage. Further increment of Gg concentration to the medium was not done because of antiestogenic activity of Gg extract (Tamir *et al*., 2000). Increasing Gg concentration could increase antiestrgenic activity over its estrogen activity, since estrogen found in Gg is basically estriol (Griffin, 2000).

The sperm concentration was significantly reduced following *in vitro* activation technique. The decrease in sperm concentration may be attributed due to elimination of dead and non motile sperm (AL-Dujaily, 1996).Simple layer technique is efficient to improve the sperm motility and grade activity of forward movement obtain from infertile semen of men (Check *et al* ., 1993) and epididymal sperm mice (AL-Dujaily, 1996).

Cohen, *et al*. (1985) has reported that during layering technique more active sperms selectively accumulate in the upper layer of the medium and sperm progressive movement accordingly being improved is the final preparation.

The sperm motility percentage and grade activity of progressive movement significantly decreased following 10 minutes of activation when MTG_0 and MTG_{10} were used. This may be due to the constituent of modified Tris solution.

Adding of egg yolk to the preparation of Tris solution at a concentration of 0.5% may decrease the accumulation of Ca^{++} extracellular to prevent the shock from cooling and gradual freezing (Ritar, 1993).This action may prevent the sperm acrosome reaction that act following hypermotility (Ritar, 1991).

It has been found that the Gg contain Ca^{++}, glucose, fructose, vitamin E, vitamin C and many other substances e.g.: Zn^{++}, sucrose, amino acid (Grieve, 1995).All these substances stimulate sperm motility and the grade activity of forward movement (Al-Dujaily *et al*., 2006).

The certain sperm function parameters after storage for 48 and 72 hrs at 4°C and activated *in vitro* for 60 minutes revealed an improvement in sperm motility and grade activity (a+b) by using MTG_{20}.This finding may be resulted from washing of semen with physiological solution having positive effect on goat's sperm motility and prolong sperm's vitality (Corteel *et al*., 1988; Ali, 2005). Gg contains vitamins such as vitamin E and vitamin C in addition to vitamin B and carotene, they all play important role in preventing the changes caused by free radicals to unsaturated fatty acids of the sperm cell membrane leading to maintaining its integrity and viability (Aitken *et al* ., 1989 ;Dawson *et al* ., 1991). Al-Fahdawi (2003) showed that adding of vit C (0.2gm/100ml Tris)

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increase percentage of sperm motility and decrease of abnormal morphological sperm. Verma and Kanwar (1999) observed improvement of motility and viability following vit E supplemented to culture media of human's sperm.

Moreover, Al-Dujaily, *et al.* (2006) reported that using of Gg for sperm activated *in vitro* of asthenospermic patient will decreased the leukocyte count which may lead to decrease the free radicals. These free radicals have interfering effect of sperm motility and grade activity of progressive forward movement (Balen *et al*., 1997). Furthermore it has been found that Gg also contain sugar like glucose, fructose, sucrose and maltose (Trease and Evans, 2002). These sugars considered as source of energy for sperm motility. In addition to that, Gg contain protein and amino acids (Langer, 1998), which sustain and maintain sperm osmorality and in turn integrity of sperm cell membrane. Consequently the protein and amino acids that found in Gg and culture media have colloidal effect in sperm membrane resulted in increase in sperm motility and viability.

Moreover, the Gg contain Ca^{++}. It has been known that Ca^{++} found in Gg prevent the degradation of cAMP by inhibition phosphate diestrase enzyme (Gamick *et al*., 1982), therefore increase Ca^{++} in sperm medium through Gg and culture media may increase sperm motility and hyperactivation (Fakih *et al*., 1986; Nassar *et al*., 1998).

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