Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.7/December-2014/18.pdf

RESEARCH ARTICLE
Open Access

Antioxidative capacity of vitamin E, vitamin C and their combination in cryopreserved Bhadavari bull semen

Pawan Kumar Mittal, Mukul Anand, A. K. Madan, S. Yadav and J. Kumar

Department of Physiology, College of Veterinary Sciences and Animal Husbandry, Pt. Deen Dayal Upadhyaya Pashu Chikitsa Vishwavidyalaya Evam Go Anusndhan Sansthan, Mathura, Uttar Pradesh, India.
Corresponding author: Mukul Anand, e-mail: drmukulanandvet@gmail.com, PKM: drpavanmittal111@gmail.com, AKM: akumarmadan@yahoo.com, SY: yadavsarvajeet24@gmail.com, JK: jkvermadr@rediff.com
Received: 18-08-2014, **Revised:** 10-11-2014, **Accepted:** 15-11-2014, **Published online:** 26-12-2014

doi: 10.14202/vetworld.2014.1127-1131. **How to cite this article:** Mittal PK, Anand M, Madan AK, Yadav S, Kumar J (2014) Antioxidative capacity of vitamin E, vitamin C and their combination in cryopreserved Bhadavari bull semen, *Veterinary World 7*(12): 1127-1131.

Abstract

Aim: The aim was to determine the antioxidative capacity of vitamin E, vitamin C and their combination (vitamin E+C) on standard semen parameters i.e., mortality, percent live sperms, percent abnormal sperms and acrosomal integrity in cryopreserved Bhadawari bull semen.

Materials and Methods: Ejaculates collected from four Bhadawari bulls were evaluated and later pooled at 37°C. Pooled semen sample was divided into four equal parts *viz.* T-1,T-2,T-3 and C. The divided samples were diluted with a Tris-based extender containing the antioxidants vitamin E (5 mM) (T-1) vitamin C (5 mM) (T-2), vitamin E+C combination (5 mM+5 mM) (T-3) and extender containing no antioxidants (control, C). The diluted samples with different antioxidant concentration were cooled to 5°C, frozen in 0.25-ml French straws and stored in liquid nitrogen (−196°C). Frozen straws were thawed individually at 37°C for 20 s in a water bath for evaluation.

Results: Semen extender supplementation with vitamin E (5 mM), vitamin C (5 mM) and vitamin E+C combination caused significant ($p<0.01$) increases in the seminal attributes while significant ($p<0.01$) decreases was observed in percent abnormal sperms in comparison to control group indicating that supplementation of antioxidant in form of vitamin and their combination improves the quality of post thaw semen. Significantly higher values of semen parameters were observed in the T-3 followed by T-1 and T-2 indicating the anti-oxidative capacity of different supplements utilized during the study.

Conclusion: The results of present investigation concluded that combination of vitamin E+C has most profound effect in protecting sperms against reactive oxygen species production and cold shock when compared to vitamin E and vitamin C supplemented alone in the extender for semen dilution utilized for cryopreservation.

Keywords: antioxidants, Bhadawari bull, cryopreservation, semen, vitamin E, vitamin C.

Introduction

Buffalo is the backbone of the dairy industry in India producing milk, meat, draft power and hides. In spite this fact, the breeding and reproductive efficiency in buffaloes are poor, and no major breakthrough in augmenting breed improvement and reproduction has yet taken place. Even though, various animal breeding and management programs have been initiated, there are still constraints hampering the growth, which require proper attention by initiating realistic breeding policies for genetic improvement of cattle and buffaloes [1]. Males buffalo, a potent reservoir of superior germplasm are either neglected to die or slaughtered at a very young age, resulting in potential waste of male germplasm. Hence, purebred bull in the country are scarce, and the majority of buffaloes are being covered by genetically inferior nondescript bulls resulting in an increase in nondescript buffalo population [2]. Therefore, maximum utilization of genetically superior bulls through use of assisted reproductive technique, i.e., artificial insemination (AI) is of prime importance.

The key to the success of AI lies in fertilizing capacity of the diluted or cryopreserved semen, which in turn depends upon the quality of semen and the suitability of the extender to maintain the motile life of spermatozoa for the maximum period at refrigerator temperature (4°C-5°C) as well as at −196°C in liquid nitrogen. However, the cryo-damage during freeze-thawing process is well-established. Cryopreservation generates sublethal sperm injury due to chemical, osmotic, thermal and mechanical stresses, which may result in destruction of acrosomal plasma membrane [3] inactivation of acrosin enzyme and hyaluronidase, prostaglandins diminution, reduction of adenosine triphosphate (ATP) and adenosine di-phosphate synthesis and decrease in acrosomal proteolytic activity [4] and DNA integrity [5]. These deteriorative changes are more profound in buffalo than cattle spermatozoa due to unique physiology of the buffalo spermatozoa and higher polyunsaturated phospholipids levels in the plasma membrane [6] probably affecting the sperm motility, viability, acrosomal and DNA integrity [7]. The most probable reason for poor post-thaw characteristics of buffalo

semen is excessive production of reactive oxygen species (ROS) leading to sperm damage [6]. These ROS molecules cause lipid peroxidation of the bio-membrane system by reducing the antioxidant potential of the cryopreserved semen [8]. As the result spermatozoa suffer molecular lesions during freezing and thawing procedures, such as oxidative damage, and apoptotic, which can lead to negative results after its use in AI [9].

Hence, the semen for its better post thaw quality needs to be supplemented with certain natural antioxidants that can counter excess ROS produced during semen cryopreservation. Vitamin C and E are the major antioxidants naturally present in mammalian semen that regulates ROS, protect the sperm from lipid peroxidation and provides higher integrity to plasma membrane and mitochondria as well as better kinematics for sperm post-cryopreservation [10,11]. Increased ROS production and decreased antioxidant levels are known to occur during sperm cryopreservation and thawing. Hence, diluted semen need to be supplemented externally with natural antioxidants in semen extender for improved post thaw semen quality [12]. Taking into account the role of vitamin C and vitamin E as natural antioxidant, a comparative research was conducted to evaluate role of vitamin E, vitamin C or their combination to overcome oxidative damage during cryopreservation of Bhadavari bull semen.

Materials and Methods

Ethical approval

No ethical approval was necessary to pursue this research work.

Animals and sampling

The present study was conducted on four Bhadawari buffalo bulls aged between 3 and 6 years and weighing between 300 kg and 450 kg body weight, reared at the Instructional Livestock Farm Complex, College of Veterinary Sciences and Animal Husbandry, Uttar Pradesh Pandit Deen Dayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, which is situated in a Semiarid zone of Northern part of India, in the state of Uttar Pradesh. All the experimental bulls were kept in individual pens made up of brick and cement with concrete floor and asbestos roof. The bulls were fed balanced ration as per its availability at farm. Proper vaccination schedule was followed, and animals were vaccinated against important contagious and infectious diseases. Semen collection was made biweekly from each bull with the help of artificial vagina (AV) "Danish model" on dummy animal between 7.00 AM and 8.00 AM during summer and 8.30 AM to 9.00 AM during other seasons using standard protocol. Semen was collected directly into a clean, dry graduated centrifuge tube attached to the latex cone of the AV. Immediately after collection, tube containing semen were marked and placed in the water bath at 37°C. Ejaculates collected from four Bhadavari bulls were evaluated and later pooled. Pooled semen sample was divided into four equal parts *viz.* T-1,T-2,T-3 and C. Each part was diluted separately with a tris-based extender containing the antioxidants vitamin E (5 mM) (T-1), vitamin C (5 mM) (T-2), vitamin E+C combination (5 mM+5 mM) (T-3) and extender containing no antioxidants (control, C) and subjected to process of cyopreservation. The samples were evaluated at different steps during cryopreservation to access the semen quality. The progressive motility of the spermatozoa was observed under high power phase objective (×40) on a thermostatically controlled stage maintained at 37°C [13]. Percent Live sperms were counted using differential staining technique [14]. Live and dead count was also used for enumerating abnormalities. Minimum 200 spermatozoa were counted in different fields and percent abnormal spermatozoa were calculated. Percent sperms with intact acrosome were calculated by staining technique using Geimsa stain [15].

Statistical analysis

Statistical analysis was performed as per the standard procedure [16]. The means were compared using Analysis of Variance, Duncan's multiple range test and presented as mean ± standard error (SE).

Results and Discussion

The results obtained during the experiment are presented in Table-1. The observed mean (±SE) of progressive motile sperms in the control group was 63.44±0.82% just after dilution, 58.13±0.80% after equilibration and 53.22±0.81% after post thaw semen evaluation. The values observed after different antioxidants supplementation (Vitamin E, vitamin C, Vitamin E+C) in semen extender utilized for semen dilution in different trial groups was 70.28±0.97, 66.59±0.99, and 74.72±0.99 just after dilution, 64.88±0.92, 61.31±0.94 and 69.28±0.84 after equilibration and 59.72±0.87, 56.75±0.75 and 64.19±0.77% after post thaw respectively. A significant decrease ($p \leq 0.01$) was observed in progressive motility at different steps of freezing and thawing both within the groups and between the groups. The reason for the difference may be the formation of intracellular ice crystals and ROS production. As a result, concentration of solutes remaining in the unfrozen fraction increases, thereby both depressing the freezing point and increasing the osmotic pressure altering sperm function [17]. The lower values of the progressive motile sperms in control group may be because of excessive generation of ROS by dead, immature and abnormal spermatozoa during sperm processing (e.g., extending, freezing, thawing process), accompanied by low scavenging and antioxidant concentrations in seminal plasma and semen extender inducing oxidative stress [18] while the higher values obtained in the test groups may be attributed to the beneficial

Table-1: Effect of different antioxidants and its combination on semen parameters of Bhadawari bull spermatozoa.

Parameters	Treatment group	Control (C)	Vitamin-E (T-1)	Vitamin-C (T-2)	Vitamin-E+C (T-3)	F value
Progressive motility (%)	Just after dilution	63.44^{aC}±0.82	70.28^{cC}±0.97	66.59^{bC}±0.99	74.72^{dC}±0.99	26.480**
	After equilibration	58.13^{aB}±0.80	64.88^{cB}±0.92	61.31^{bB}±0.94	69.28^{dB}±0.84	29.936**
	Post-Thaw	53.22^{aA}±0.81	59.72^{cA}±0.87	56.75^{bA}±0.75	64.19^{dA}±0.77	33.539**
	F value	39.521**	33.018**	30.135**	36.495**	
Live sperms (%)	Just after dilution	81.03^{aC}±0.41	83.19^{bC}±0.42	82.06^{abC}±0.38	84.72^{cC}±0.43	14.790**
	After equilibration	75.81^{aB}±0.42	78.28^{bB}±0.48	76.75^{aB}±0.46	80.25^{cB}±0.43	18.813**
	Post-Thaw	70.13^{aA}±0.37	72.97^{bA}±0.41	71.25^{aA}±0.46	75.31^{cA}±0.42	29.110**
	F value	183.965**	136.776**	154.272**	120.808**	
Abnormal sperms (%)	Just after dilution	4.91^{cA}±0.14	4.06^{bA}±0.09	4.28^{bA}±0.10	2.97^{aA}±0.09	53.858**
	After equilibration	5.03^{dA}±0.18	3.97^{bA}±0.11	4.44^{cA}±0.16	3.03^{aA}±0.12	33.377**
	Post-Thaw	7.75^{dB}±0.17	6.22^{bB}±0.11	6.84^{cB}±0.12	5.63^{aB}±0.13	46.033**
	F value	91.831**	159.059**	121.452**	173.399**	
Intact acrosomal sperms (%)	Just after dilution	80.16^{aC}±0.50	84.50^{cC}±0.41	81.75^{bC}±0.47	87.03^{dC}±0.62	36.218**
	After equilibration	74.59^{aB}±0.51	79.22^{cB}±0.51	76.91^{bB}±0.51	81.38^{dB}±0.53	31.992**
	Post-Thaw	69.41^{aA}±0.54	74.00^{cA}±0.49	71.59^{bA}±0.48	76.06^{dA}±0.53	31.943**
	F value	108.376**	122.893**	107.796**	95.835**	

Means bearing different superscripts (a, b, c) between columns and (A, B, C) between rows differ significant ($p<0.01$), **Significance level ($p<0.01$)

effects of different antioxidants through its protective action on the sperm cell membrane against ROS and lipid peroxidation during cryopreservation of semen [19,20].

The observed mean (±SE) of a percent live spermatozoa in the control group were 81.03±0.41% just after dilution, 75.81±0.42% after equilibration and 70.13±0.37% after post thaw semen evaluation. The values observed after different antioxidants supplementation (vitamin E, vitamin C, vitamin E+C) in semen extender utilized for semen dilution in different trial groups was 83.19±0.42, 82.06±0.38 and 84.72±0.43 just after dilution, 78.28±0.48, 76.75±0.46 and 80.25±0.43 after equilibration and 72.97±0.41, 71.25±0.46 and 75.31±0.42% after post thaw respectively. A significant difference ($p\leq0.01$) was observed in percent live spermatozoa at different steps of freezing and thawing both within the groups and between the groups. Decreasing proportion of live sperms at each steps of semen cryopreservation within a group can be attributed to ROS production and lipid peroxidation that involves peroxidation of polyunsaturated fatty acids to generate lipid peroxides that have a detrimental effect on spermatozoa, interfere with the mitochondrial electron transport chain and promote cellular generation of superoxide anion, leading to membrane damage, leakage of ATP, disrupting DNA integrity and a rapid loss of sperm motility and viability [21]. A non-significant different ($p\leq0.05$) was observed in the control group and T-2 indicating that vitamin C supplementation did not show a significant effect on percent live sperms at different step involved in semen cryopreservation. The reason for this non-significant difference in the present study may be the concentration of natural antioxidants. The vitamins C concentration that was used during the experiment may have been sub-optimum for preserving buffalo sperm viability, as the effect of may vary with concentration [22].

The observed mean (±SE) of total sperm abnormalities in the control group were 4.91±0.14% just after dilution, 5.03±0.18% after equilibration and 7.75±0.17% after post thaw semen evaluation. The values observed after different antioxidants supplementation (vitamin E, vitamin C, vitamin E+C) in semen extender utilized for semen dilution in different trial groups was 4.06±0.09, 4.28±0.10 and 2.97±0.09 just after dilution, 3.97±0.11, 4.44±0.16 and 3.03±0.12 after equilibration and 6.22±0.11, 6.84±0.12 and 5.63±0.13% after post thaw, respectively. A significant difference ($p\leq0.01$) was observed in total sperm abnormalities at different steps of freezing and thawing both within the groups and between the groups. During cryopreservation, a significant increase was observed in the percent abnormal sperms within the groups at different steps during the cryopreservation in test and control group. The reason for this difference in the test groups compared with control may be weaker plasma membranes more susceptible to lipid peroxidation, that increases with decrease in temperature [23] and reduced ability of mammal's sperm to encountering with peroxidation during the freezing and thawing processes making sperms more vulnerable for lipid peroxidation and increased sperm abnormality [24].

The observed mean (±SE) of intact acrosome in control group was 80.16±0.50% just after dilution, 74.59±0.51% after equilibration and 69.41±0.54% after post thaw semen evaluation. The values observed after different antioxidants supplementation (vitamin E, vitamin C, vitamin E+C) in semen extender utilized for semen dilution in different trial groups was 84.50±0.41, 81.75±0.47 and 87.03±0.62 just after dilution, 79.22±0.51, 76.91±0.51 and 81.38±0.53 after equilibration and 74.00±0.49, 71.59±0.48 and 76.06±0.53% after post thaw respectively. A significant difference ($p\leq0.01$) was observed in acrosomal integrity at different

steps of freezing and thawing both within the groups and between the groups. A significantly higher value of acrosomal integrity was observed just after dilution in all the three trial groups when compared with the control group. A significant difference was also observed between the trial groups with highest values observed in the T-3 followed by T-1 and T-2 in all the three steps followed for freezing-thawing process. A significant difference was observed within the control and different trial groups with highest observed mean (±SE) of acrosomal integrity just after dilution followed by equilibration and post thaw evaluation. The reason for this decrease in the acrosomal integrity at various steps may be due to loss of lipid component of the plasma membrane, loss of plasmalemma over the entire acrosome, extensive vesiculation and disruption of plasmalemma and outer acrosomal membranes [25] during freeze-thawing. A significant difference ($p \leq 0.01$) was observed between the control and the test groups at different steps during cryopreservation. Cryopreservation affects osmotic tolerance and ion homeostasis of bovine sperm enhances generation rate of O_2^- and H_2O_2 or in the intracellular concentration of free calcium ions (Ca^{2+}) [26] resulting in premature acrosomal reaction, altered mitochondrial function, reduction of motility and oxidative DNA damage [27], all of which damage the acrosome and influence the fertility of the sperm cells [28]. The difference observed may be attributed to the difference in the anti-oxidative capacities of the antioxidant and their combination supplemented in semen extender during the experiment. The highest values of intact acrosome was observed in the T-3 followed by T-1 and T-2, indicating that the combination of two antioxidants plays better role to overcome the effect of ROS and temperature variation during cryopreservation in comparison to vitamin E and C.

Conclusion

From the findings of the present study, it can be concluded that the combination of vitamin E+C has most profound effect in protecting sperms against ROS production and cold shock when compared to vitamin E and vitamin C supplemented alone in the extender for semen dilution utilized for cryopreservation.

Authors' Contributions

SV and AKS designed the study. PKM and MA conducted the study and analyzed the data. JK contributed in sample collection. MA and JK drafted and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors are thankful to Vice Chancellor, Pt. Deen Dayal Upadhaya Pashuchikitisha Vigyan Vishwa Vidyalay Evam Go Anusandhan Sansthan and Dean, College of Veterinary Sciences and Animal Husbandry for providing funds and facilities to pursue this research work.

Computing Interest

The authors declare that they have no competing interests.

References

1. Sreenivas, D. (2013) Breeding policy strategies for genetic improvement of cattle and buffaloes in India. *Vet. World,* 6(7): 455-460.
2. Singh, C.V. and Barawal, R.S. (2010) Buffalo breeding research and improvement strategies in India. In: Proceedings of 9th World Buffalo Congress, Argentina. p1024-1031.
3. Bucak, M.N., Ates, S., Ahin, A., Varisli, O., Yuce, A., Tekin, N. and Akcay, E. (2007) The influence of trehalose, taurine, cysteamine and hyaluronanon ram semen, microscopic and oxidative stress parameters after freeze–Thawing process. *Theriogenology,* 67: 1060-1067.
4. Barbas, J.P. and Mascarenhas, R.D. (2009) Cryopreservation of domestic animal sperm cells. *Cell Tissue Bank,* 10(1): 49-62.
5. Yu, D., Simon, L. and Lewis, S.E.M. (2011) The impact of sperm processing and cryopreservation on sperm DNA integrity. Sperm Chromatin. Springer, New York. p397-409.
6. Sndrabi, S.M.H. (2009) Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod. Domest. Anim.,* 44(3): 552-569.
7. Nair, S.J., Brar, A.S., Ahuja, C.S., Sangha, S.P. and Chaudhary, K.C. (2006) A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim. Reprod. Sci.,* 96(1-2): 21-29.
8. Kumar, R., Jagan, G., Mohanarao, A. and Atreja, S.K. (2011) Freeze-thaw induced genotoxicity in buffalo (*Bubalus bubalis*) spermatozoa in relation to total antioxidant status. *Mol. Biol. Rep.,* 38(3): 1499-1506.
9. Pen˜a, F.J., Macías García, B., Samper, J.C., Aparicio, I.M., Tapia, J.A., Ortega Ferrusola, C. and (2011). Dissecting the molecular damage to stallion spermatozoa: The way to improve current cryopreservation protocols? *Theriogenology*, 76(7): 1177-1186.
10. Akhter, S., Rakha, B.A., Ansari, M.S., Andrabi, S.M.H. and Ullah, N. (2011) Storage of Nili-Ravi buffalo (*Bubalus bubalis*) semen in skim milk extender supplemented with ascorbic acid and α tocopherol. *Pak. J. Zool.,* 43: 273-277.
11. Silva S.V., Soares, A.T., Batista A.M., Almeida F.C., Nunes, J.F., Peixoto, C.A. and Guerra, M.M.P. (2013) Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. *Anim. Reprod. Sci.,* 137(1-2): 37-44.
12. Maia, M.S., Bicudo, S.D., Sicherle, C.C., Rodello, L. and Gallego, I.C.S. (2010) Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. *Anim. Reprod. Sci.,* 122(1-2): 118-123.
13. Ahmad, N. (1994) Clinical and experimental studies of reproductive functions in the ram and male goat with special reference to the use of diagnostic ultrasound. Ph.D Thesis. Department of Large Animal Med. & Surgery, Royal Veterinary College. University of London, London.
14. Bloom, E. (1950) A minute live-dead sperm stain by means of eosin-nigrosin. *Fertil. Steril.,* 1: 176-177.
15. Campbell, R.C., Dott, H.M. and Glover, T.D. (1956) Nigrosin-eosin as a stain for differentiating live and dead spermatozoa. *J. Agric. Sci.,* 48: 1-8.
16. Snedecor, G.W. and Cochran, W.G. (1994) Statistical Methods. 8th ed. Iowa State University Press, Ames, Iowa.

17. Alvarez, J.G. (2012) Male Infertility: Loss of intracellular antioxidative enzymes activity during sperm cryopreservation: Effect of sperm function after thawing. Springer, New York. p237-244.
18. Sikka, S.C. (2004) Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J. Androl.,* 24: 5-18.
19. Tuncer, P.B., Bucak, M.N., Sariozkan, S., Sakin, F., Yeni, D., Cigerci, I.H., Ates, S., Ahin, A., Avdatek, F., Gundogan, M. and Buyukleblebici, O. (2010) The effect of raffinose and methionine on frozen/thawed angora buck (*Capra hircus ancryrensis*) semen quality, lipid peroxidation and antioxidant enzyme activities. *Cryobiology,* 61: 89-93.
20. Naijian, H.R., Kohram, H., Shahneh, A.Z., Sharafi, M. and Bucak, M.N. (2013) Effects of different concentrations of BHT on microscopic and oxidative parameters of Mahabadi goat semen following the freeze-thaw process. *Cryobiology,* 66(2): 151-155.20.
21. Alvarez, J.G. and Aitken, J.R. (2012) Lipid peroxidation in human spermatozoa. Studies on Mans Health and Fertility. Springer, New York. p119-130.
22. Medeiros, C.M., O Forell, F., Oliveria, A.T.D. and Rodrigues, J.L. (2002) Current status of sperm cryopreservation, why is n't it better? *Theriogenology,* 57(1): 327-344.
23. Correa, J.R and Zavos, P.M. (1994) The hypo-osmotic swelling test, its employment as an assay to evaluate the functional integrity of the frozen thawed bovine spermatozoa. *Theriogenology,* 42(2): 351-360.
24. Alvarez, J.G. and Storey, B. (2005) Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol. Reprod. Dev.,* 42(3): 334-346.
25. Krogenaes, A., Berg, K.A. and Hafne, A.L. (1994) Membrane alterations in bull spermatozoa after freezing and thawing and after *in vitro* fertilization. *Acta Vet. Scand.,* 35(1): 17-26.
26. Blasse, A.K., Oldenhof, H., Hundrieser, M.E., Wolkers, W.F., Sieme, H. and Bollwein, H. (2012) Osmotic tolerance and intracellular ion concentration of bovine sperms are affected by cryopreservation. *Theriogenology,* 78(6): 1312-1320.
27. Paoli, D., Lombardo, F., Lenzi, A. and Gandini, L. (2014) Sperm cryopreservation: Effect on chromatin structure. *Adv. Exp. Med. Biol*., 791: 137-150.
28. Ahmad, M., Nasrullah, R., Riaz, H., Sattar, A. and Ahmad, N. (2014) Changes in motility, morphology, plasma membrane and acrosome integrity during stages of cryopreservation of buck sperm. *J. S. Afr. Vet. Assoc.,* 85(1): 972-976.

****