CRYOPRESERVATION OF RUMEN PROTOZOA USING THREE DIFFERENT CRYOPROTECTANTS WITH SUCCESSFUL REFAUNATION OF DEFAUNATED SHEEP

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ABSTRACT

Rumen protozoa play an important role in the digestion of cellulose, protein and regulation of rumen pH as well as elimination of pathogen from GIT. Because of their central biological importance, this work aimed to cryopreserve rumen protozoa to establish a rumen protozoal bank followed by using this cryopreserved rumen protozoa in refaunation of experimentally-defaunated sheep. Therefore two experiments were conducted. In the first experiment, the rumen fluids were collected from slaughterhouse and from live sheep by stomach tube and were filtered and mixed with either one of three cryoprotectants: glycerol, ethylene glycol and Dimethyl sulfoxide (DMSO) with different concentration (4, 5, and 6%). The method used was the slow two-step freezing method in which straws containing the mixture of rumen protozoa and cryoprotectant were placed in a cooling water bath at 5°C for 30 minute and then hold in nitrogen vapor for 45 minute (holding time) then directly immersed in liquid nitrogen. Viability was checked monthly till 6 month post-freezing. Results showed that the viability was maximal when using the DMSO (5%) as a cryoprotectant. Consequently, the second experiment was conducted in which 9 female sheep were allocated into 3 equal groups. The first group served as control. The second group was defaunated by single oil drench (cooking oil at 5ml / kg BW). The third group was defaunated by oil drench followed by refaunation by intraruminal inoculation of the content of one straw that contains cryopreserved rumen protoza with DMSO 5% as a cryoprotectant. Result showed that refaunation successfully regained the total protozoal count to near control value, significantly increased the ammonia nitrogen concentration, body weight gain and the rumen pH after being reduced by defaunation. Therefore, it was concluded that successful cryopreservation of rumen protozoa in sheep can be attained by slow two-step freezing method and that cryopreserved protozoal bank could be successfully used for refaunation of sheep that have been defaunated due to acidosis. Consequently, this cryopreserved protozoal bank can be employed in farms to improve digestibility, increase body weight and treat rumen acidosis.

INTRODUCTION

Rumen microbial populations are characterized by a broad diversity of microorganisms, mainly bacteria, ciliate protozoa, and fungi. Protozoa represent up to 50% of the total rumen microbial biomass. They are involved particularly in the rumen digestion of cellulose **(Yoder et al., 1966, Jouany and Senaud, 1979, Coleman, 1983, 1992**), starch (**Veira et al., 1983, Coleman**, **1992**, **and Ivan et. al., 2000)** and proteins (**Shinchi and Abe, 1987**), and they contribute actively to the control of the bacterial population **(Jouany and Ushida, 1999)** and to the formation of the end products of ruminal fermentation (**Jouany et al., 1995, Ushida and Jouany, 1996, and Veira et al., 1983**). They play an important role in the biodegradation of plant toxins and mycotoxins (**Kiessling et al., 1984)** and in the regulation of ruminal conditions such as pH and redox potential **(Blackburn and**

Hobson, 1960, Russell et al., 1979, Mackie et al., 1978, Ushida and Jouany, 1996). They have been shown to eliminate certain pathogens from the digestive tract of ruminants, protecting them from disease and so improving the food safety of edible animal products (**McIntosh et al., 2002).**

Preservation of individual species of rumen protozoa at low temperatures is difficult since the formation of intracellular crystals after freezing causes cell lysis **(Watson 1995**). In the process, the cooling rate, the equilibrium temperature and the cell response to the type of cryoprotectant **(Fiser et al., 1987 and Hubalek, 2003)** are the most critical factors influencing cell viability after freezing.

Although some rumen ciliates can usually be cultivated in vitro, it is difficult to maintain them for a long time, and most species die within a few months to a year **(Michalowski et al., 1986, Onodera and Henderson, 1980 and Nsabimana et al., 2003)**. A cryopreservation technique to preserve rumen ciliate protozoa for several years would thus be most useful. Cryopreservation would also facilitate the transfer of ciliates between laboratories for in vivo or in vitro digestion studies, genomic and biotechnology research, and long-term storage of living cells.

There are various methods of cell freezing. Their effectiveness depends on several variables, including the freezing medium and the type and concentration of cryoprotectant, the equilibration temperature and equilibration time during the contact between the cryoprotectant and cells, the cooling rate, the temperature at which the cells are immersed in liquid nitrogen, and the thawing medium (**Farrant at al., 1977 and Eriksson et al., 2001)**. In the two-step or interrupted slow freezing method (**Liu et al., 2000, Viveiros et al., 2001),** an initial slow freezing period (first step), from the equilibration temperature to the holding temperature (commonly set at -20 °C to -40 °C) **(McGann and Farrant, 1976)**, is followed by maintenance of the cells at that holding temperature (second step) for a given time (holding time). The frozen cells are then immersed in liquid nitrogen and finally stored at -196°C. During the first step, the cooling rates must be strictly controlled; a low cooling rate can damage cells through a solution effect, while fast cooling favors intracellular ice formation, which is often lethal to cells **(Mazur, 1984 and Shirakashi and Tanasawa, 1998).** Consequently, cooling rates must be set to optimize cell dehydration while avoiding intracellular ice formation. To date, the cryopreservation of rumen ciliates has been carried out only on a limited range of species **(Marcin et al., 1989 and Marcin et al., 1992 and Kisidayova, 1995, 1996)**, and in most cases the survival rates have been poor.

In this study, we aimed to investigate the possibility of cryopreservation of ruminal protozoa with addition of three common cryoprotectants, the glycerol, the ethylene glycol and the dimethyl sulfoxide (DMSO) and determine the one that produces the highest viability. Further goal was to use these cryopreserved protozoa in refaunation of experimentally-defaunated sheep.

MATERIALS AND METHODS

Experimental Design

This work included two experiments. The first is the cryopreservation experiment (lasts for 6 months) in which the rumen protozoa in ruminal fluid were cryopreserved by slow two-step freezing method in the presence of three cryoprotectants (Glycerol, ethylene glycol and dimethyl sulfoxide, (DMSO). In the second experiment (lasts for 3 months), nine female sheep with average live weight of

22 kg were randomly divided into 3 equal groups. The first group (n=3) were fed on basal diet and used as control. The second group (n=3) were fed on basal diet and received a single oil drench (cooking oil at 5 ml / kg BW) for defaunation according to **Seng et al. (2001)**. The third group involves 3 sheep that were defaunated by oil drench followed by refaunation by intraruminal inoculation of cryopreserved ruminal protozoa after being thawed.

Sampling and Origin of Ciliates

Approximately 500 ml of ruminal contents were collected from 5 slaughtered sheep at the slaughterhouse and from living animals by using stomach tube before their morning meal. The rumen samples were transported to the lab and kept at 39 °C.

Sedimentation of Protozoa

The sedimentation of rumen protozoa was carried out as previously described (**Nsabimana et al., 2003**). Briefly, upon arrival to the lab, the rumen content was quickly filtered through two-gauze layer. To concentrate the ciliate by sedimentation, 200 ml of filtrate was transferred under Co_2 to a separating funnel held in a water bath at 39 °C under Co_2 for 1-4 hours. Depending on the ciliate species, the small ciliates take longer time to sediment than the larger ones. The white sediment pellet at the bottom of the separating funnel was then collected in several tubes for cryopreservation trials.

Equilibration of Protozoa with the Cryoprotectant Additive

Glycerol, ethylene glycol and dimethyl sulfoxide, (DMSO) were used as cryoprotectants in all trials. One ml of the cryoprotectant (Glycerol, ethylene glycol and dimethyl sulfoxide, (DMSO)) was mixed with the ciliate suspension (with a minimum of 10^4 cells/ml) to obtain final concentrations of 4, 5, and 6%. The mixture was equilibrated at 25°C in a water bath for 5 min (equilibration time), and 0.2 ml of the mixture was placed in 0.5-ml straw before freezing **(Nsabimana et al., 2003).**

Freezing Step

The freezing step was carried out as previously described (**Kisidayova, 1995**). Briefly, ten straws containing the mixture of ciliates and the cryoprotectant (10 straws for each cryoprotectant concentration) were placed in a cooling water bath at 5°C for 30 minute and then hold in nitrogen vapor for 45 minute (holding time) then directly immersed in liquid nitrogen.

Thawing and Evaluation of Survival Rate

Frozen straws were removed from the liquid nitrogen vessel and placed in a water bath at 39°C for 5 min. The thawed protozoa suspension was then diluted under CO_2 in glass tubes containing a thawing medium made up of freshly prepared ruminal fluid or rumen fluid stored for 2 weeks at 4°C. All the media and ciliates suspension were handled under CO_2 (**Nsabimana et al., 2003).**

After thawing, the survival rate was estimated by counting the proportion of motile ciliate under a microscope monthly for 6 month. Motility was stimulated by heating the slide with the thawing suspension of ciliates for 1-2 seconds above a small Bunsen flame. Counts were repeated 5 times per straw and the mean and standard error was calculated (**Nsabimana et al., 2003).**

Method of Defaunation

Three sheep were received a single oil drench (cooking oil at 5ml / kg BW) for defaunation according to **Seng et al. (2001)**.

Inoculation of Thawed Ciliates into the Rumen of Defaunated Sheep (Refuanation)

Trials on *in vivo* inoculation of cryopreserved ciliates were carried out after 6 months of cryopreservation in liquid nitrogen. One cryostraw (with DMSO 5%) was withdrawn from the liquid nitrogen and immediately immersed in water bath at 39°C. After five minutes, the content of the straw was introduced into the rumen through the cannula 2 hours before the morning meal. The concentration of ciliates was checked every day from day 7 post-inoculation (**Nsabimana et al. 2003)**.

Rumen Protozoa Count in Rumen Content

The total protozoal count was conducted according to **Abou El-Naga (1967**). In this method, the usual slide and cover were used for direct microscopic count of protozoa by counting 0.1ml.of the diluted ruminal sample. Two 5 ml duplicate liquors of rumen content were separately taken and diluted five times by addition of 15 ml saline solution and 5 ml of lugol's iodine solution. The iodine solution was used to fix and stain the protozoal cells. Immediately after gentile shaking, one ml liquor was taken up in a one ml wide mouthed graduated pipette. As quickly as possible, exactly 0.1 ml was poured on a dry clean slide which was then carefully covered by a dry clean cover slide with a dimensions of 23 × 51 mm (total area of 1173 mm^2). Counting was carried out using the low power; the field area of that lens was one square millimeters. In each slide, 30 fields were counted which were chosen as representative to the whole area. Calculation was performed as follow: The average count in 30 fields, which represents the protozoal count per one square mm area of the field, was multiplied by 1173 (the area of the cover slide) to give the protozoal count in 0.1 ml of the diluted sample, which represents 0.02 ml of original sample. Therefore, the total protozoal count /1 ml rumen content = average count in 30 field ×1173×50. Each of the two diluted duplicate was counted and average was calculated.

Estimation of Ruminal Ammonia Nitrogen Concentration

The rumen ammonia nitrogen concentration was determined by distillation method according to **Conway (1958**).

Determination of Body Weight and Body Weight Gain

Sheep were weighed in the three groups (control, defaunated and refaunated) on day 0 (just before inoculation) and on days 30, 60, 90 and 120 post-inoculation. Then the live weight gain (BW) per month and per day was calculated as follow:

BW gain per month = BW post-inoculation – BW before inoculation

$$\text{BW gain per day} = \frac{\text{BW post-inoculation} - \text{BW before inoculation}}{30}$$

Determination of Ruminal pH

The pH of the collected rumen juice was determined as soon as possible by using a pH meter according to **Nassar (1971).**

Statistical Analysis

The data were analyzed as previously described by **Nsabimana et al. (2003**). Comparison of viability between the three cryoprotectants was conducted using one-way analysis of variance (ANOVA). The interaction between the cryoprotectant type and concentration (4, 5 and 6%) was analyzed by using two-way ANOVA. In the defaunation experiment, all parameters in the defaunated and refaunated groups were compared to the control group using one-way ANOVA. All data were analyzed by using SigmaStat 3.1, statistical software for data analysis (SPSS Inc., Chicago, IL, USA). Values were represented by means ± standard error (SE). All differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of Cryoprotectants (Type and Conc.) on The Viability of Rumen Protozoa

The results shown in Table (1) and Figure (1) showed that the rumen protozoa viability in the presence of ethylene glycol as a cryoprotectant was significantly higher than glycerol when viability examined after the first month till the sixth month after cryopreservation. Moreover, the viability of protozoa in the presence of DMSO was significantly higher than both ethylene glycol and glycerol when viability examined after the first month till the sixth month after cryopreservation. Regarding the effect of different concentrations of each cryoprotectant, there were no significant changes among these concentrations, although the 5% concentration of each cryoprotectant was numerically, but not significantly higher than the 4% and 6% concentrations.

Effect of Defaunation and Refaunation on Ammonia Nitrogen Concentration

The results shown in Table (2) and Figure (2) revealed a reduction in the ammonia nitrogen concentration starting from the day 30 and 60 after defaunation by cooking oil. This reduction became significant after 90 and 120 days of defaunation. The refaunation by using the thawed cryopreserved protozoa significantly increase the ammonia nitrogen concentration after 90 and 120 days to become nearly close to the control value.

The Total Protozoal Count in Control, Defaunated and Refaunated Groups

The results shown in Table (3) and Figure (3) showed that the total protozoal count was significantly reduced from the first day of defaunation compared to control. This significant reduction continued till day 120. After refaunation, the total protozoal count started to increase after the first day of refaunation compared to defaunated group and this increase became significant starting from day 7. However, the total protozoal number was still significantly lower than the control values till day 60. Starting from day 60 of refaunation, the total protozoal count was nearly equivalent to the control value.

Changes in Body Weight and Body Weight Gain in Sheep.

The results shown in Table (4) and Figure (4) showed that the live body weight (BW) was reduced in defaunated sheep compared to both the control and refaunated group at all time periods (30-120 day). The reduction in live BW became

significant on day 120 of defaunation. Similarly, the body weight gain per month (kg) and the body weight gain per day (gm) were reduced by the defaunation when compared to the control and refaunated groups at all time period. This reduction became also significant on day 120 after defaunation. The refaunation regained the body weight to almost the control values in all time periods.

Effect of Ruminal Defaunation and Refaunation on Ruminal pH in Sheep.

The ruminal pH in sheep was significantly decreased after defaunation compared control (faunated) group when measured just before the meal (0 time) and 2 hours after the morning meal. The refaunation group significantly regained the rumen pH values to be nearly equivalent to the control values starting from day 30 after experiment (Table 5, Figure 5).

Table 1. Viability percentage in the presence of glycerol, ethylene glycol and DMSO as cryoprotectants.

Groups / Month	Glycerol			Ethylene glycol			DMSO		
	4%	5%	6%	4%	5%	6%	4%	5%	6%
First	0.3667 ± 0.03^{a1}	0.4333 ± 0.03^{a1}	0.3333 ± 0.13^{a1}	0.5667 ± 0.03^{b1}	0.6333 ± 0.06^{b1}	0.6333 ± 0.03^{b1}	0.8 ± 0.02^{c1}	0.9167 ± 0.01^{c1}	0.8167 ± 0.03^{c1}
Second	0.3 ± 0.05^{a1}	0.4167 ± 0.08^{a2}	0.3833 ± 0.11^{a3}	0.4667 ± 0.03^{b1}	0.6333 ± 0.03^{b1}	0.6333 ± 0.03^{b2}	0.7667 ± 0.01^{c1}	0.8667 ± 0.06^{c2}	0.8167 ± 0.03^{c2}
Third	0.3667 ± 0.08819^{a1}	0.4167 ± 0.10^{a1}	0.3667 ± 0.03^{a1}	0.5 ± 0.05^{b1}	0.6333 ± 0.06^{b1}	0.6 ± 0.05^{b1}	0.8 ± 0.02^{c1}	0.8667 ± 0.06^{c1}	0.8167 ± 0.03^{c1}
Fourth	0.3333 ± 0.08^{a1}	0.5333 ± 0.03^{a1}	0.4333 ± 0.08^{a1}	0.6 ± 0.05^{b1}	0.5667 ± 0.03^{b1}	0.6333 ± 0.03^{b1}	0.8 ± 0.02^{c1}	0.9333 ± 0.01^{c1}	0.7833 ± 0.03^{c1}
Fifth	0.3667 ± 0.03^{a1}	0.3833 ± 0.07^{a1}	0.35 ± 0.05^{a1}	0.5667 ± 0.08^{b1}	0.6333 ± 0.06^{b1}	0.6 ± 0.05^{b1}	0.8333 ± 0.06^{c1}	0.9 ± 0.02^{c1}	0.8167 ± 0.03^{c1}
Sixth	0.2833 ± 0.06^{a1}	0.5 ± 0.05^{a1}	0.4 ± 0.05^{a1}	0.5333 ± 0.03^{b1}	0.6333 ± 0.03^{b1}	0.6 ± 0.05^{b1}	0.8333 ± 0.06^{c1}	0.8667 ± 0.06^{c1}	0.8 ± 0.02^{c1}

- Different superscript letters of the same raw denote significant difference in cryoprotectant type at $P < 0.05$
- Different superscript numbers of the same raw indicate significant difference in concentration within each cryoprotectant at $P < 0.05$.

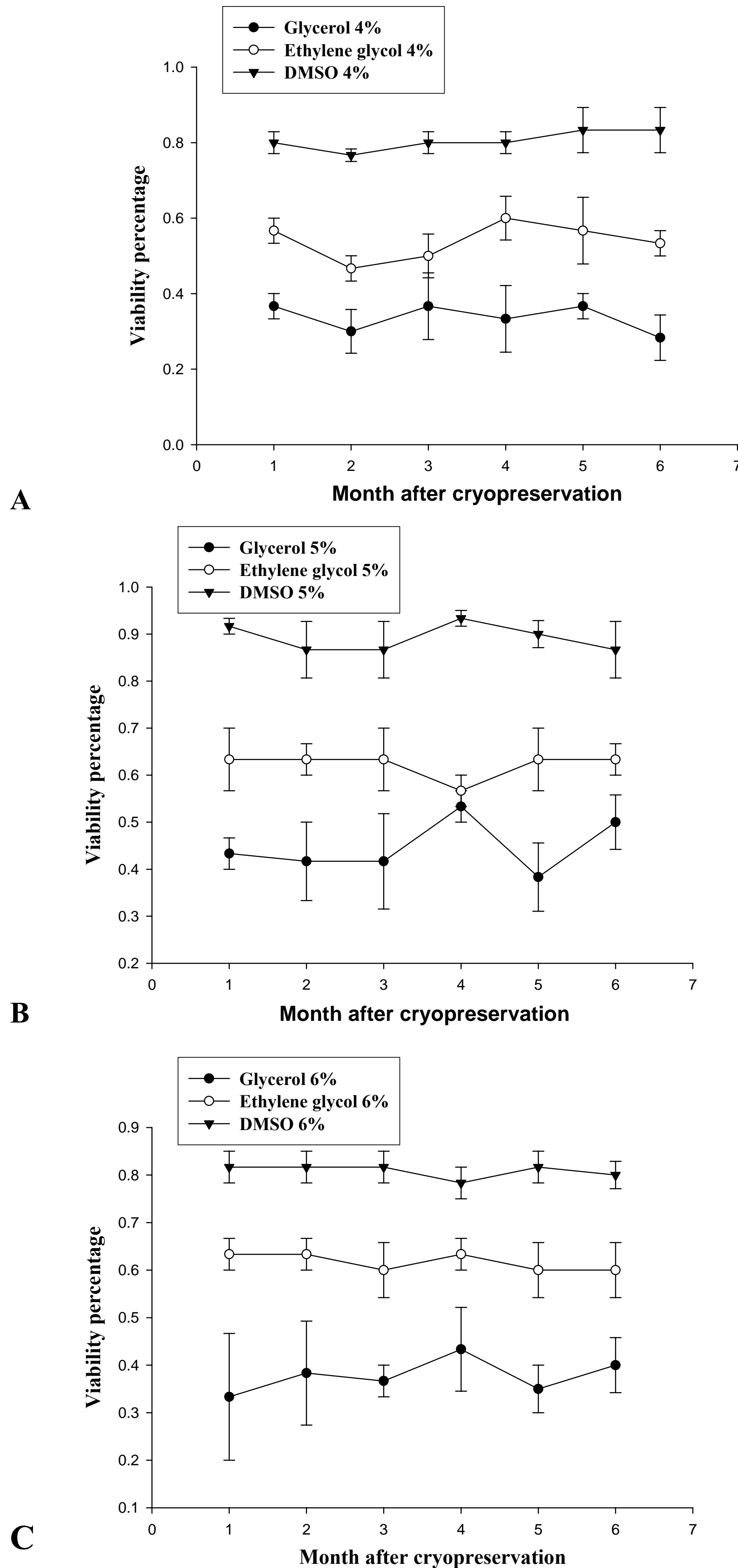

Figure 1. The viability percentage is higher with DMSO as a cryoprotectant than with ethylene glycol and glycerol when used in a concentration of 4% (A), 5% (B) and 6% (C). Viability was measured monthly from the first month to the 6th month after cryopreservation.

Table 2: Ammonia nitrogen concentration in control, defaunated and refaunated groups

Group / Days	Control	Defaunated	Refaunated
0	7.8333 ± 0.40552 [a]	6.8333 ± 0.87433 [a]	9.1000 ± 0.80208 [a]
30	8.6333 ±0 .59255 [a]	7.5333 ± 0.18559 [a]	9.2000± 1.07858 [a]
60	8.3000 ± 0.20817 [a]	7.6333 ± 0.46667 [a]	9.9000 ± 0.88882 [a]
90	9.4000 ± 0.26458[a]	7.2000 ± 0.55076[b]	9.8000 ± 0.60828[a]
120	10.7000 ± 0.5196[a]	7.3667 ± 0.52387[b]	10.2000 ± 0.7211[a]

- The statistical model is one way ANOVA.
- Different superscripts of the same raw indicate significant difference at $P < 0.05$.

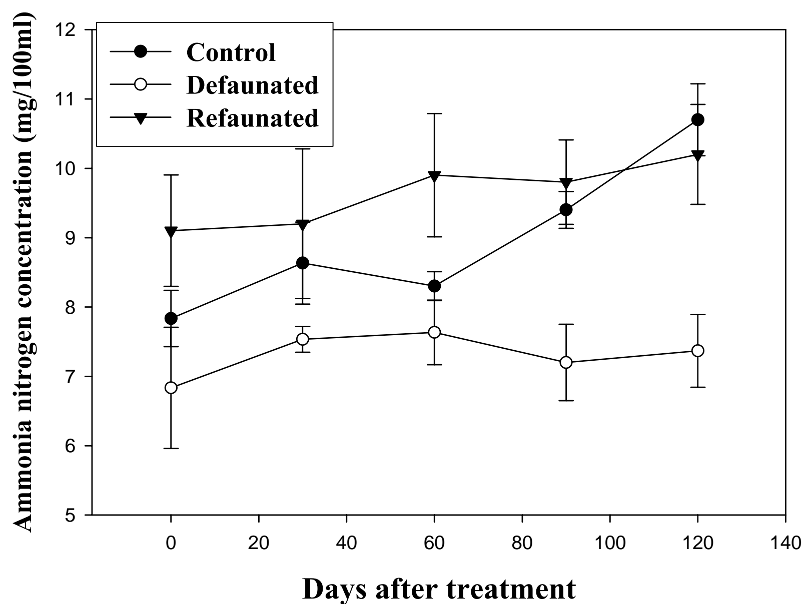

Figure 2. Graphical representation demonstrating the ammonia nitrogen concentration in control, defaunated and refaunated groups. The ammonia concentration was reduced after defaunation but was increased after refaunation.

Table 3: The total protozoal count in control, defaunated and refaunated groups.

Group / Days	Control ($x 10^5$)	Defaunated ($x 10^5$)	Refaunated ($x 10^5$)
0	3.378 ± 0.116	3.37667 ± 0.305	3.16267 ± 0.191
1	3.27500 ± 0.110[a]	0.04800 ± 0.008[b]	0.08000 ± 0.007[b]
7	3.25067 ± 0.219[a]	0.05867 ± 0.0217[b]	1.73167 ± 0.133[c]
30	3.27000 ± 0.221[a]	0.06233 ± 0.004[b]	2.77100 ± 0.225158[c]
60	3.03300 ± 0.263[a]	0.05800 ± 0.009[b]	3.06933 ± .043971[a]
90	3.43400 ± 0.224[a]	0.14533 ± 0.0113[b]	2.99033 ± 0.067[a]
120	3.30100 ± 0.124[a]	0.18500 ± 0.066[b]	3.51967 ± 0.148[a]

- The statistical model is one way ANOVA.
- Different superscripts of the same raw indicate significant difference at $P < 0.05$.

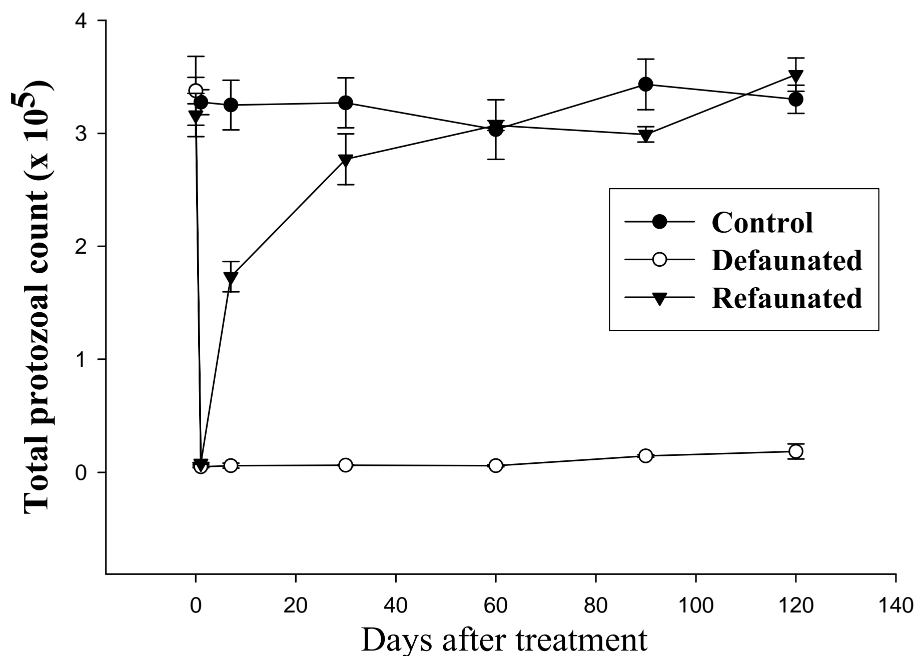

Figure 3. Graphical representation demonstrating the total protozoal count in control, defaunated and refaunated groups. The protozoal count was dramatically reduced after the 7th day of oil treatment while the refaunation gradually elevates the count toward control values.

Table 4: The live body weight (BW) and the BW gain per month and per day in control, defaunated and refaunated groups.

	Day	Control	Defaunated	Refaunated
Live BW (kg)	0	17.0 ± 0.28[a]	16.16 ± 0.44[a]	16.33 ± 0.44[a]
	30	21.0 ± 0.57[a]	20.00 ± 0.57[a]	20.66 ± 0.33[a]
	60	25.0 ± 0.20[ab]	24.25 ± 0.25[a]	25.66 ±0.16[b]
	90	30.66 ± 0.44[a]	28.50 ± 0.28[b]	30.83 ±0.44[a]
	120	36.16 ± 0.16[a]	32.50 ± 0.28[b]	36.0 ±0.28[a]
BW gain/month (kg)	30	4.0 ± 0.50[a]	3.83 ±0.16[a]	4.00 ±0.28[a]
	60	4.25 ± 0.43[a]	4.25 ± 0.75[a]	5.00 ±0.28[a]
	90	5.66 ± 0.16[a]	4.0 ± 0.28[a]	5.16 ±0.44[a]
	120	5.50 ± 0.57[a]	4.00 ± 0.28[b]	5.16 ±0.16[ab]
BW gain/day (gm)	30	133.33 ± 16.6 6[a]	127.75 ± 5.5 4[a]	133.33 ±9.62[a]
	60	141.66 ± 14.43[a]	141.68 ± 24.9 8[a]	166.66 ±9.62[a]
	90	188.88 ± 5.55[a]	133.32 ± 9.62[b]	172.22 ±14.69[ab]
	120	183.33 ± 19.24[a]	133.33 ± 9.61[a]	172.21 ±5.55[a]

- The statistical model is one way ANOVA.
- Different superscripts of the same raw indicate significant difference at $P < 0.05$.

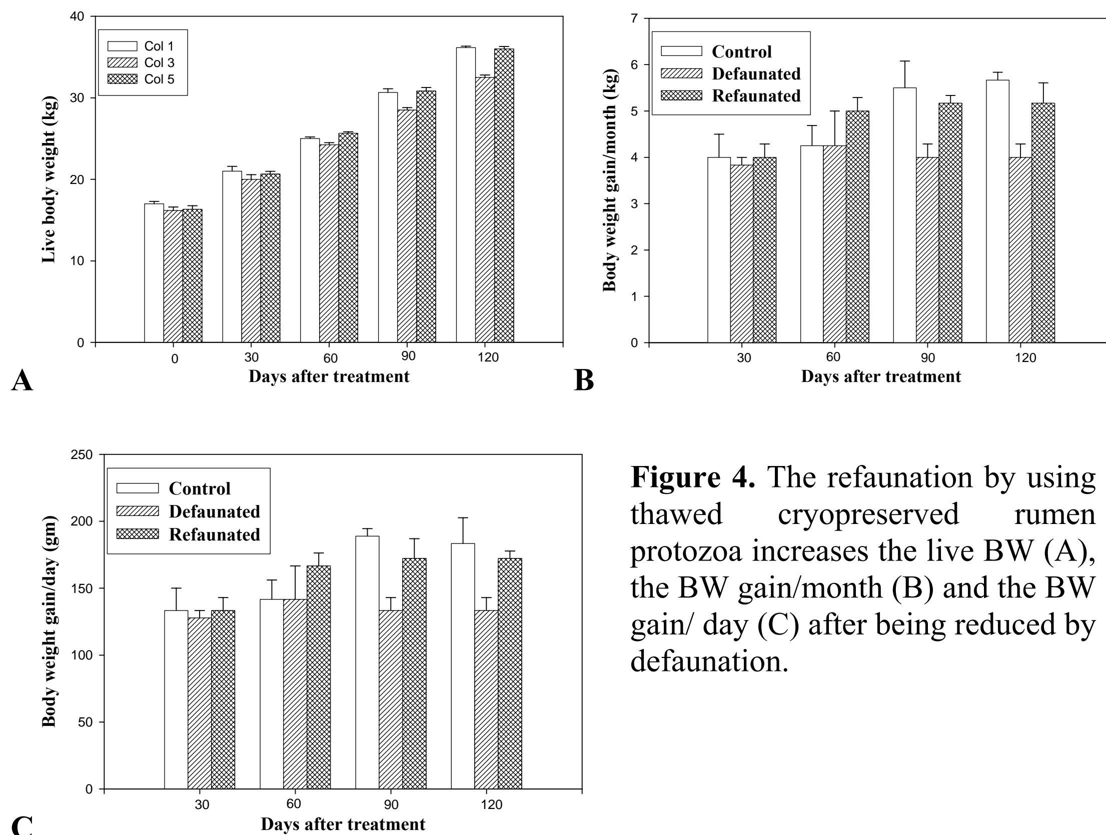

Figure 4. The refaunation by using thawed cryopreserved rumen protozoa increases the live BW (A), the BW gain/month (B) and the BW gain/ day (C) after being reduced by defaunation.

Table 5: The value of pH in control, defaunated and refaunated sheep as measured at 0 time (just before meal) and 2 h after meal.

Day	Time (h)	Control	Defaunated	Refaunated
0	0	6.23 ± 0.03[a]	5.83 ± 0.08[b]	6.13 ± 0.12[a]
	2	6.46 ± 0.12[a]	5.76 ± 0.08[b]	5.9 ± 0.1[b]
30	0	6.23 ± 0.06[a]	5.9 ± 0.05[a]	6.16 ± 0.23[a]
	2	6.53 ± 0.08 [a]	5.76 ± 0.12 [a]	6.36 ± 0.34 [a]
60	0	6.5 ± 0.25[ab]	6.06 ± 0.06[a]	6.9 ± 0.05[b]
	2	6.43 ± 0.14[a]	5.83 ± 0.12[b]	6.53 ± 0.21[a]
90	0	6.5 ± 0.11[a]	5.7667 ± 0.06[b]	6.9 ± 0.15[a]
	2	6.66 ± 0.16[a]	5.8 ± 0.11[b]	7.13 ± 0.06[b]
120	0	6.56 ± 0.23[ab]	5.93 ± 0.14[a]	6.76 ± 0.03[b]
	2	6.56 ± 0.13[a]	5.96 ± 0.15[b]	6.83 ± 0.08[a]

- The statistical model is one way ANOVA
- Different superscripts of the same raw denote a significant difference at $P < 0.05$.

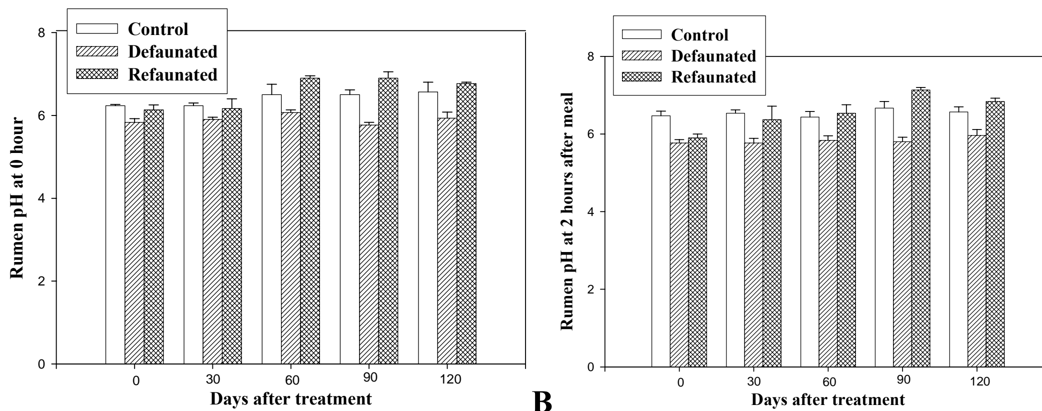

Figure 5. The Rumen pH measured just before the morning meal (0 time) (A) and 2 h after the morning meal (B) is reduced in defaunated sheep compared to control sheep. The refaunation increases the rumen pH to be equivalent to the control values.

DISCUSSION

Rumen ciliates need specific environmental conditions to survive. For example, oxygen must be absent and the temperature must be maintained at 39°C. Therefore, it is so difficult to preserve the ciliates in vitro cultures and in deep-freeze conditions (**Williams and Coleman, 1992**). Although some rumen ciliates can usually be cultivated *in vitro*, it is difficult to maintain them for a long time, and most species die within a few months to a year **(Broad and Dawson, 1976)**. Therefore, a new technique was developed to preserve the rumen protozoa by cryopreservation.

There are various methods of cell freezing. Their effectiveness depends on several variables, including the freezing medium and the type and concentration of

cryoprotectant, the equilibration temperature and equilibration time during the contact between the cryoprotectant and cells, the cooling rate, the temperature at which the cells are immersed in liquid nitrogen, and the thawing medium (**Eriksson et al., 2001**). During the early trials, the ciliates were cryopreserved using a slow one-step freezing procedure. However, the viability was extremely low (approximately 5 %) **(Marcin et al., 1989)**. Accordingly, an improved procedure for the cryopreservation of rumen ciliates using a two-step or interrupted slow freezing was developed **(Kisidayova and Gyulai, 1991).**

In this study, the first experiment included cryopreservation trials of sheep ruminal protozoa using the slow interrupted freezing technique that was successful with the three cryoprotectants [glycerol, ethylene glycol, and dimethyl sulphoxide (DMSO)] (Table 1, Figure 1). However, the viability percentage was significantly different among the three kinds of cryoprotectants. The DMSO in concentration of 5% produced the highest viability, a result that coincided with that reported by **Eriksson et al. (2001).** On the other hand, the lowest viability was recorded with glycerol and intermediate viability was attained by ethylene glycol. This result was consistent with that of **Habib et al. (2004)** who demonstrated that DMSO was superior to glycerol by slow freezing during cryopreservation of Trichomonas vaginalis. Moreover, **Miyake et al. (2004)** demonstrated that of six cryoprotectants examined, dimethyl sulfoxide and glycerol showed the strongest cryoprotective effects. The difference between DMSO and glycerol is the speed at which they permeate the cell. Glycerol permeates more slowly than DMSO. Therefore, it has been reported that equilibration is necessary when glycerol is used as a cryoprotectant. It was also reported that glycerol and DMSO have different nature and rate of penetration. The viability of cryopreserved Diploplastron affine differs according to the cryopreservant (4.7% glycerol and 5.0% DMSO) **(de la Fuente et al., 2006).**

The mechanism by which the cryoprotectant maintains the viability of protozoa during freezing was explained in a number of studies. The cryoprotectant acts at the cell membrane level by limiting the effect of cell dehydration during freezing **(Tanasawa, 1998)**, lowering the freezing point of extra- and intracellular biological liquids **(Cassidy-Hanley et al., 1995)**, and promoting vitrification rather than intracellular ice crystal formation **(Nsabimana et al., 2003).** Therefore the addition of cryoprotectant is very important step during cryopreservation of rumen protozoa which coincided with **de la Fuente et al. (2006)** who demonstrated that adding the cryopreservant (either glycerol or DMSO) at 25°C (equilibration temperature) improves viability of Diploplastron affine after thawing. The higher efficacy of DMSO as a cryoprotectant might be attributed to its rapid penetration of the cells **(Hubalek, 2003)** and consequently interacts with the intracellular water during rapid cooling, resulting in the majority of the intracellular solution becoming vitrified **(Liu et al., 2000).**

In the second experiment, the investigation was directed to study the effectiveness of cryopreserved rumen protozoa in refaunation of oil-induced defaunation in sheep. The parameters to be assessed were ammonia nitrogen concentration, total protozoal count, body weight, and rumen pH.

The results represented in Table (2) showed a significant increase in rumen ammonia nitrogen concentration at 60, 90 and 120 day in control and refaunated groups than defaunated group. Similar result was obtained by **Seng et al. (2001)** who

demonstrated a highly significant reduction of rumen ammonia nitrogen concentration after a single drench of cooking oil. Moreover**, Hristov et al., (2001)** showed that completely eliminated protozoa reduced ammonia concentration, compared with untreated control by 60%. In addition, **Eugene et al. (2004)** demonstrated that faunated animals consistently have higher ruminal ammonia concentrations. The defaunation or reduction in the protozoa population leads to an increase in the bacterial population, which uses ammonia as the source of nitrogen for cell synthesis. Thus more ammonia is being used when the bacterial population is increased. The reduction in ammonia concentration could thus be due to high rate of ammonia assimilation by bacteria, as well as reduced sources of ammonia entering the pool when protozoa are absent or present in small numbers **(Seng et al., 2001).** Protozoa have high a capacity for proteolytic and deaminase activities (**Ushida et al., 1984)**; and there is an increase in the rumen outflow of protein from bacteria and fungi in the absence of protozoa **(Newbold and Hillman, 1990).**

With regard to the effect of oil drench on the total protozoal count, results represented in Table (3) and Figure (3) showed a highly significant decrease in the total number of rumen protozoa in defaunated animals when compared to control and this significant reduction continued till day 120. This result coincides with that observed by **Seng et al. (2001).** It has been demonstrated that feeding fats to ruminants is commonly associated with decreased protozoal numbers in the rumen (**Doreau and Ferlay, 1995)**. The toxic effect could be due to increasing acidity, resulting from the free fatty acids liberated from the oil. Protozoa are more sensitive to pH than bacteria **(Newbold et al., 1986a)**. Although the oil drench significantly reduced the number of protozoa, the protozoa did not completely disappear. This may be explained by the resistant of some protozoal species to defaunation by oils, such as Entodinium species **(Hristov et al., 2001 and Ivan et al., 2001).**

The refaunation by injection of the rumen protozoa cryopreserved with the DMSO 5% (highest viability) increased the protozoal number progressively until the number was equivalent to the control values after 60 days (Table 3, Figure 3). The growth of ciliates in the rumen of defaunated sheep after cryopreservation evidenced their capacity to totally recover their basic metabolic functions for ATP production **(Nsabimana et al., 2003)**. This result indicated that cryopreservation by this method was a successful procedure to refaunate animals that have been defaunated due to different ruminal conditions such as lactic acidosis and impaction. Therefore, the cryopreserved straw could be used as a therapy in these affections that are accompanied by death or reduction of ruminal protozoa.

Regarding the effect of defaunation and refaunation on live body weight and body weight gain, the results represented in Table (4) and Figure (4) showed that the mean of live body weight and body weight gain were significantly reduced on day 120 of defaunation compared to control and refaunated groups. The refaunation regained the body weight to almost the control values in all time periods. Altogether, these results indicate the importance of rumen protozoa and its effect on digestibility. The effect of defaunation and refaunation on live body weight is controversial. Some authors demonstrated that defaunation increases the body weight while others demonstrated an opposite effect and a third group demonstrated no significant difference. **Usuelli and Fiorini, (1938)** reported that faunated sheep grow slightly better than defaunated. It was also reported that faunated animal usually exhibited a smoother coat than defaunated **(Abou Akkada and El- Shazly, 1956** and **1964).** They found that the relatively large size and abundance of the protozoa make them an

important element in the rumen. Moreover**, Christiansen et al. (1965) and Borhami et al. (1967)** reported that there could be an increase of up to 30% in rate of weight gain of faunated animals compared with protozoa-free animals. On the other hand, some authors found that defaunation reduces body weight gain (**Bird and Leng 1978)** which may be attributed to an increase in the rumen outflow of protein from bacteria and fungi in the absence of protozoa (**Newbold and Hillman, 1990)** or defaunation can consistently decrease fiber digestibility (**Eugene et al., 2004)**. Other findings showed no significant difference in the body weight gain between defaunated and faunated animals (**Eadie and Gill, 1971 and Williams and Dinusson, 1973).** The differences could be due to the nature and level of protein in the diets **(Seng et al., 2001)**. This was explained in the results of **Bird and Leng (1978)** who demonstrated that defaunation had no effect on the growth rate of animals fed molasses/straw but defaunation increased both the rate of growth (43%) and the feed conversion ration (39%) when protein was also fed.

With regard to the effect of defaunation and refaunation on ruminal pH (Table 5, Figure 5), the ruminal pH in sheep was significantly decreased after defaunation compared with control group when measured just before the meal (0 time) and 2 hours after the morning meal. This result coincided with that recorded by **Nguyen Thi Hong Nhan et al. (2001)** and **Santra and Karim (2002).** The reduction of rumen pH after defaunation could be attributed to the major role of protozoa in slowing down the fermentation by ingesting starch grains and taking up soluble sugars and converting them to storage polysaccharides **(Schwartz and Gilchrist, 1975; Williams and Withers, 1993 and Williams and Coleman, 1997)**. Moreover, the total volatile fatty acid concentration was slightly elevated in defaunated sheep (**Mendoza et al., 1993**) that could contribute to lowering rumen pH. In contrast, **Newbold et al. (1986b)** found that defaunation or refaunation had no effect on rumen pH. The method of sampling rumen fluid by stomach tube, with variable degrees of contamination by saliva, could be the explanation of the failure to observe differences after defaunation in the other studies **(Seng et al., 2001).** The refaunation significantly regained the rumen pH values to be nearly equivalent to the control values starting from day 30 after the experiment (Table 8, Figure 8). This confirmed the role of rumen protozoa in maintaining the ruminal pH around the normal values. This observation agreed with **Russell et al. (1979)** who demonstrated that ruminal ciliates play an important role in regulation of ruminal conditions, such as pH.

In conclusion, this work demonstrated a successful method for cryopreservation of rumen protozoa and generation of rumen protozoal bank. The using of cryopreserved rumen protozoa in refaunation of oil-induced defaunation in lambs was also successful in regaining the normal protozoal count, the rumen ammonia nitrogen concentration, the normal ruminal pH and the live body weight. Therefore this cryopreserved protozoal bank could be employed in farms to improve digestibility, increase body weight and treat rumen acidosis.

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حفظ وتجميد بروتوزوا الكرش باستخدام ثلاثة مواد حافظة مع نجاح زراعة هذة البروتوزوا فى كرش الاغنام بعد اخلائها من البروتوزوا

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تلعب بروتوزوا الكرش دورا رئيسيا فى هضم السليلوز والبروتين كما تقوم بتنظيم درجة حامضية الكرش وتخلص القناة الهضمية من الميكروبات المرضية. ونظرا للدور المحورى البيولوجى لهذة الكائنات استهدفت هذه الدراسة حفظ وتجميد بروتوزوا الكرش لانشاء بنك للبروتوزوا وزراعتها فى كرش اغنام مخلاة من البروتوزوا. ولذلك تم اجراء تجربتين حيث تضمنت التجربة الاولى تجميع سائل الكرش من المجزر ومن الاغنام الحية باستخدام انبوبة اللى المعدى وتم ترشيح هذا السائل لازالة الشوائب ثم تم خلطها مع ثلاثة من المواد الحافظة وهى الجليسرول والاثيلين جليكول والداي ميثيل سلفوكسيد كل على حدة بتركيزات 4% و 5% و 6% ثم الحفظ بالتجميد البطىء على خطوتين والتى فيها وضعت الانابيب فى ماء بارد بحمام مائى درجة حرارتة 5 درجة مئوية لمدة 30 دقيقة يليها تعريض الانابيب لابخرة النيتروجين لمدة 45 دقيقة يليها غمس مباشر لهذه النابيب فى النيتروجين السائل. تم فحص حيوية البروتوزوا شهريا لمدة 6 شهور بعد الحفظ والتجميد. اظهرت النتائج ان اكبر حيوية للبروتوزا ظهرت مع استخدام الداي ميثيل سلفوكسيد كمادة حافظة بتركيز 5%. وبالتالى تم استخدام محتوى هذة الانابيب فى زرع البروتوزوا فى اكراش اغنام تم اخلائها من البروتوزا. ولذلك تم اجراء التجربة الثانية والتى استخدم فيها 9 نعاج قسمت الى ثلاثة مجموعات متساوية. استخدمت المجموعة الاولى كضابطة والثانية تم اخلاء بروتوزوا كرشها بتجريعها زيت طعام (5 مللى/كجم) والثالثة تم اخلاء بروتوزوا كرشها بتجريعها زيت طعام (5 مللى/كجم) ثم تم زراعة البروتوزوا المجمدة بعد ازابتها مباشرة فى الكرش. اظهرت النتائج ان اعادة زراعة البروتوزوا فى الكرش اعادت عدد البروتوزوا الى الطبيعى وأدى ذلك إلى زيادة تركيز الامونيا وكذلك وزن الجسم واعادت درجة حموضة الكرش الى مستواها الطبيعى. وخلصت الدراسة الى ان عملية حفظ وتجميد البروتوزوا قد تم بنجاح بالتجميد البطىء على خطوتين ونجحت فى اعادة نمو البروتوزوا فى الاغنام التى تم اخلائها من البروتوزوا. وبالتالى يمكن استخدام هذا البنك فى المزارع لتحسين عمليات الهضم وزيادة وزن الحيوانات وعلاج حامضية الكرش.