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Trials for Cryopreservation of Rumen Protozoa in Sheep

[1]H.M. Abdel-Aziz, [2]H.Y. Hassan, [1]Y.M. Abd-El-Raof, [3]Hala A.A. Abou-Zeina and [3]S.A. Galbt

[1]Department of Animal Medicine, Faculty of Veterinary Medicine, Benha University, Egypt
[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Menofia University, Egypt
[3]Department of Parasitology and Animal Diseases, Veterinary Research Division,
National Research Center, Egypt

Abstract: The present study aimed at the long-term storage of rumen ciliate protozoa as living cells in liquid nitrogen for creation of a frozen protozoa bank. Two experiments were carried out, the first; conduct trials for cryopreservation of ciliates using different kinds and concentrations of cryoprotectants for establishment of the most suitable method. The second, trial was carried out to evaluate the activity of the cryopreserved rumen protozoa in sheep.In experiment 1, cryopreservation of rumen ciliates was carried out by the two-step freezing technique using 4, 5 and 6% concentrations of DMSO, glycerol and ethylene glycol as cryoprotectants. In experiment 2, nine ewes were randomly divided into 3 equal groups: group 1 was kept as control, group 2 received single oil drench for defaunation and group 3 was refaunated by intra ruminal injection of cryopreseved protozoa in DMSO 5% (the highest survival). The concentration of protozoa was checked just before inoculation (0 day) and on days 1, 7, 30, 60, 90 and 120 post-inoculation. The obtained results showed that the cryopreservation of rumen protozoa was successful with the 3 used cryoprotectants. However, the viability percentage was significantly different ($P<0.05$) among the 3 kinds of cryoprotectants. The DMSO produced the highest viability. There were no significant changes among the different concentrations of cryoprotectants. The total protozoal count in the defaunated group showed significant reduction ($P<0.05$) compared with control during all times of experiment. In the refaunated group, the protozoal number progressively increased until it reached close to control values after 60 days. The rumen pH was significantly decreased ($P<0.05$) after defaunation as compared with control. The refaunated group significantly regained ($P<0.05$) the rumen pH values to be nearly equivalent to the control values starting from day 30 of experiment. There was a significant decrease ($P<0.05$) in rumen ammonia nitrogen concentration on days 90, 120 in the defaunated group as compared with control and refaunated groups. The refaunation regained the body weight to almost the control values in all time periods whereas, defaunation significantly reduced the live body weight which indicate the importance of rumen protozoa and its effect on digestibility. In conclusion, this work is the first record in Egypt for cryopreservation of rumen protozoa that have made it possible to set up a bank of cryopreserved rumen protozoa. Also, this work showed the successful effect of the refaunation on the performance of experimentally defaunated sheep.

Key words: Cryopreservation · rumen · protozoa · sheep

INTRODUCTION

Rumen Ciliate protozoa are principally of two types; *Ophryoscolecidae and Isotrichidae*, together they represent up to 50% of the total rumen microbial biomass [1]. They are involved in many rumen functions such as digestion of cellulose, starch, proteins and have been estimated to contributed in 1/4 to 1/3 of total fiber digestibility [1-3]. They contribute to the control of the bacterial population [4] and to the formation of the end products of ruminal fermentation [5, 6]. They play an important role in biodegradation of plant toxins and mycotoxins [7] and in the regulation of rumen condition such as pH [6]. Also, they have been shown to eliminate certain pathogens from the digestive tract of ruminants, protecting them from diseases and so improving the food safety of edible animal products [8]. In addition, protozoa might scavenge O2, thus strictly benefiting anaerobic prokaryotes in the rumen [9, 10].

Corresponding Author: Dr. Hala A.A. Abou-Zeina, Department of Parasitology and Animal Diseases, Veterinary Division, National Research Center, P.O. Box 12622, El-Tahrir Str., Dokki, Giza, Egypt

Because of the multifunctional properties of rumen protozoa, a crucial need for their cultivation and preservation was developed. There are two approaches that may be used for preserving rumen ciliates: cryopreservation, [11-13] or culturing in a previously defaunated rumen of an appropriate animal as a mono culture [1]. However, preparing and maintaining of a large number of animals in a monofaunated state for a wide range of ciliate strains is difficult. So, cryopreservation technique 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 [11].

There are various methods of cell freezing. Their effectiveness depends on several variables; 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 [14].

During the early trials, the ciliates were cryopreserved using a slow one-step freezing procedure. However, the viability was extremely low (approximately 5%) [25]. Accordingly, an improved procedure for the cryopreservation of rumen ciliates using two-step or interrupted slow freezing was developed [26].

In the two- step freezing method [13, 15, 16], a slow or rapid cooling step is interrupted by a holding phase during which a constant temperature is maintained. Freezing of the extracellular water occurs in the initial period of cooling before the holding phase. Maintenance of the cells at the holding temperature allows for their adequate dehydration. After maintenance at the holding temperature (usually from -20 to -40°C), the samples are plunged into the liquid nitrogen. 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 the cells [17].

The practical advantages of this method are; simple to carry out, low concentration of additives is effective and inexpensive equipment is required [11].

The present investigation aimed to study the possibility of cryopreservation of dominant species of rumen ciliate protozoa and establishment of the most suitable method for cryopreservation. Also, to conduct trials for using of the cryopreserved rumen ciliates to improve faunation in sheep after experimental defaunation, as a preliminary step for the establishment of a bank for rumen protozoa to be used during emergency digestive troubles, whereas the rumen protozoa are killed.

MATERIALS AND METHODS

The experiments were carried out in Animal Reproduction Institute, EL Haram, Giza, Egypt during a period of one year starting from October 2005.

Experiment 1: Cryopreservation

Sampling: Samples of rumen contents (500 ml) were collected from slaughtered sheep or from living animals by stomach tube before their morning meal and transported to the laboratory at 39°C in anaerobic condition. Tubes and equipments were rinsed with 5% formalin, then saline following each use to prevent artificial inoculation of viable protozoa among animals [13].

Rumen protozoa count in rumen content: Samples of rumen content were filtered through two gauze layers. Two 5ml duplicate liquors of rumen fluid were separately taken and diluted five times by saline solution and lugol's iodine to fix and stain the protozoal cells. 0.1 ml of the diluted ruminal sample was poured on a dry clean slide which was then carefully covered by a dry clean cover slide. The total protozoal count /1 ml rumen content = average count in 30 field ×1173 (area of the cover slid) ×50. Each of the two diluted duplicate was counted and average was calculated [18].

Sedimentation of protozoa: 200 ml of rumen filtrate was transferred to a separating tube held in a water bath at 39°C for 1-4 hrs. The white sediment pellet at the bottom of the separating funnel was then collected in several tubes for cryopreservation trials [13].

Equilibration of protozoa with the cryoprotectant additives: One ml of the cryoprotectant dimethyl sulfoxide (DMSO), glycerol, or ethylene glycol was mixed with the ciliate suspension (with a minimum of 10^4 cells/ml) to obtain final concentrations of 4, 5 and 6% (vol/vol) for each cryoprotectant. 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 [13].

Freezing step: Cryopreservation was carried out by the two-step freezing method according to [11] with little modification. Ten straws containing the mixture of ciliates and the cryoprotectant were placed in a cooling water

bath at 5°C for 30 min. followed by holding the straws in nitrogen vapor (-25°C) for 45 min. (holding time) then direct immersion in liquid nitrogen at -196°C, whereas straws remained there until the thawing step just prior to examination.

Thawing step: 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 rumen fluid or rumen fluid stored for 2 weeks at 4°C. All the media and ciliates suspension were handled under CO_2 [13].

Evaluation of survival rate: After thawing, the survival rate was estimated according to [13], by counting the proportion of motile ciliate under a microscope (Olympus, CX 41-Japan) monthly for 6 months as previously described. Motility was stimulated by heating the slide with the thawing mixture of ciliates and cryoprotectant for 1-2 seconds by small Bunsen flame. Counts were repeated 5 times per straw and the mean was calculated to be considered as individual reading. Three straws per each concentration for each of the cryoprotectants were used in counting and determination of survival rate after cryopreservation trials.

Experiment 2: Evaluation of activity of the cryopreserved rumen protozoa: Nine ewe-lambs with average live weight of 22kg, were randomly divided into 3 equal groups. Group one was used as control, group 2 was defaunated by single oil drench (cooking oil at 5ml/kg BW) according to [19] and group 3 was refaunated-after 3 days of experimental defaunation-by intra ruminal injection of the cryopreserved rumen protozoa. All animals received 2 kg/head/day of basal diet. In addition, each ewe received 0.75 kg/day of the supplementary diet that is composed of; yellow corn 40%, soya bean 22.7%, wheat straw 35%, calcium phosphate 1.5%, salt 0.5% and mineral mixture 0.3%. The feeds were divided into three meals per day.

Defaunation: It was conducted according to [19]. The experimental animals were adapted to the diets for 2 weeks before giving the oil directly into the rumen using a stomach tube. On the day before treatment, animals were fasted but with free access to water for 18 hrs.

Refaunation: The refaunation was conducted according to [13], by using the content of one straw that contains mixture of the rumen protozoa and 5% DMSO as cryoprotectant. One cryostraw with the 5% DMSO (for each sheep of group 3) was withdrawn from the liquid nitrogen and immediately immersed in water bath at 39°C. Five min. later, the content of the straw was inoculated into the rumen of previously defaunated sheep using the syringe and needle, 2hrs before the morning meal. The concentration of ciliates was checked just before inoculation (0 day) and on days 1, 7, 30, 60, 90 and 120 post-inoculation.

Rumen juice examination: The pH and ammonia nitrogen concentration of the collected rumen juice from the 3 groups of experimental animals were determined according to [20] and [21], respectively.

Determination of live body weight and body weight gain: Ewes were weighed in the three groups on day 0 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:

$$\text{BW gain per month} = \text{BW post-inoculation} - \text{BW before inoculation}$$

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

Data were statistically analyzed y using one-way and two- way analysis of variance (ANOVA) [22].

RESULTS

Experiment 1: Rumen protozoa are successfully cryopreserved following treatment with different concentrations (4, 5 and 6%) of glycerol, ethylene glycol and DMSO as shown in Table 1. DMSO produced the highest viability ($P<0.05$).

Experiment 2:

- Total protozoal count in sheep ranged between $3.03\text{-}3.43\times10^5$/ml rumen content. This number significantly ($P< 0.05$) reduced following defaunation, while following refaunation, the total number of protozoa increased and became close to the control value (Table 2).
- Rumen pH significantly ($P<0.05$) decreased following defaunation and became close to control value following refaunation (Table 3).

Table 1: The viability percentage of rumen protozoa in the presence of glycerol, ethylene glycol and DMSO as cryoprotectants (Mean±SE)

	Groups								
	Glycerol			Ethylene glycol			DMSO		
Month	4%	5%	6%	4%	5%	6%	4%	5%	6%
First	36.67±3.30^{a1}	43.33±3.10^{a1}	33.33±13.00^{a1}	56.67±3.12^{b1}	63.33±6.01^{b1}	63.33±3.31^{b1}	80.01±2.21^{c1}	91.67±1.08^{c1}	81.67±3.22^{c1}
Second	30.00±5.33^{a1}	41.67±8.12^{a1}	38.33±11.02^{a1}	46.67±3.12^{b1}	63.33±3.11^{b2}	63.33±3.841^{b2}	76.67±1.10^{c1}	86.67±6.21^{c1}	81.67±3.20^{c1}
Third	36.67±8. 82^{a1}	41.67±10.01^{a1}	36.67±3.15^{a1}	50.00±5.05^{b1}	63.33±6.00^{b1}	60.11±5.04^{b1}	80.03±2.15^{c1}	86.67±6.05^{c1}	81.67±3.01^{c1}
Fourth	33.33±8.42^{a1}	53.33±3.22^{a1}	43.33±8.13^{a1}	60.00±5.01^{b1}	56.67±3.14^{b1}	63.33±3.11^{b1}	80.04±2.05^{c1}	93.33±1.15^{c2}	78.33±3.00^{c1}
Fifth	36.67±3.21^{a1}	38.33±7.02^{a1}	35.35±5.03^{a1}	56.67±8.01^{b1}	63.33±6.04^{b1}	60.73±5.03^{b1}	83.33±6.01^{c1}	90.56±2.03^{c1}	81.67±3.11^{c1}
Sixth	28.33±6.01^{a1}	50.00±5.01^{a2}	40.023±5.22^{a1,2}	53.33±3.11^{b1}	63.33±3.14^{b1}	60.423±5.11^{b1}	83.33±6.11^{c1}	86.67±6.01^{c1}	80.56±2.14^{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

Table 2: The total protozoal count (10^5/ml) in control, defaunated and refaunated sheep (Mean±SE)

	Group		
Days	Control	Defaunated	Refaunated
0	3.38±0.12	3.38±0.31	3.16±0.19
1	3.28±0.11^{a}	0.05±0.01^{b}	0.08±0.01^{b}
7	3.25±0.22^{a}	0.06±0.022^{b}	1.73±0.13^{c}
30	3.27±0.22^{a}	0.06±0.01^{b}	2.17±0.23^{c}
60	3.03±0.26^{a}	0.06±0.01^{b}	3.07±0.045^{a}
90	3.43±0.22^{a}	0.15±0.011^{b}	2.99±0.07^{a}
120	3.30±0.12^{a}	0.18±0.07^{b}	3.52±0.15^{a}

Different superscripts of the same raw indicate significant difference at P<0.05

Table 3: The value of rumen pH in control, defaunated and refaunated sheep as measure at 0 time (just before meal) and 2 hrs after meal (Mean±SE)

Day	Time (h)	Control	Defaunated	Refaunated
0	0	6.23±0.03^{a}	5.83±0.08^{b}	6.03±0.02^{c}
	2	6.46±0.12^{a}	5.76±0.08^{b}	5.91±0.10^{b}
30	0	6.23±0.06^{a}	5.90±0.05^{b}	6.16±0.23^{ab}
	2	6.53±0.08^{a}	5.76±0.12^{b}	6.36±0.34^{ab}
60	0	6.5±0.25^{ab}	6.06±0.06^{a}	6.90±0.05^{b}
	2	6.43±0.14^{a}	5.83±0.12^{b}	6.53±0.21^{a}
90	0	6.52±0.11^{a}	5.76±0.06^{b}	6.90±0.15^{c}
	2	6.66±0.16^{a}	5.80±0.11^{b}	7.13±0.06^{c}
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}

Different superscripts letters of the same raw denote a significant difference at P<0.05

Table 4: Rumen ammonia nitrogen concentration in control, defaunated and refaunated sheep (mg %; Mean±SE)

	Group		
Days	Control	Defaunated	Refaunated
0	7.83±0.41^{a}	6.83±0.87^{a}	8.10±0.80^{a}
30	8.63±0.59^{a}	7.53±0.18^{a}	9.20±1.08^{a}
60	8.30±0.21^{a}	7.63±0.47^{a}	9.90±0.89^{a}
90	9.40±0.26^{a}	7.200±0.55^{b}	9.80±0.61^{a}
120	10.70±0.52^{a}	7.3667±0.52^{b}	10.20±0.72^{a}

Different superscripts of the same raw indicate significant difference at P<0.05

Table 5: The live body weight (BW) and the BW gain per month and per day in control, defaunated and refaunated sheep (Mean±SE)

	Day	Control	Defaunated	Refaunated
Live BW (kg)	0	17.00±0.28^{a}	16.16±0.44^{a}	16.33±0.44^{a}
	30	21.00±0.57^{a}	20.00±0.57^{a}	20.66±0.33^{a}
	60	25.00±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	30	4.0±0.50^{a}	3.83±0.16^{a}	4.00±0.28^{a}
gain/month (kg)	60	4.25±0.43^{a}	4.25±0.75^{a}	5.00±0.28^{a}
	90	5.66±0.16^{a}	4.02±0.28^{b}	5.16±0.44^{ab}
	120	5.50±0.57^{a}	4.00±0.28^{b}	5.16±0.16^{a}
BW	30	133.33±16.66^{a}	127.75±5.54^{a}	133.33±9.62^{a}
gain/day(gm)	60	141.66±14.43^{a}	141.68±24.98^{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^{b}	172.21±5.55^{a}

Different superscripts letters of the same raw indicate significant difference at P<0.05

- Rumen ammonia nitrogen concentration significantly (P<0.05) decreased following dafaunation and reached close to the control value after the refaunation (Table 4).
- The mean live body weights of experimental sheep were significantly (P<0.05) decreased following defaunation and regained following refaunation (Table 5).

DISCUSSION

Rumen protozoa 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 [1]. 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 [23, 24]. Therefore, a new technique was developed to preserve the rumen protozoa by cryopreservation.

In the current trials all rumen ciliates treated with 4, 5 and 6% concentrations of glycerol, ethylene glycol and DMSO were successfully cryopreserved. The choice of these three cryoprotectants was based upon previous data recorded by [27], who demonstrated that glycerol, ethylene glycol and DMSO are the most common cryoprotectants used in cryomicrobiology. However, the viability percentage of ciliates was significantly differ among the three kinds of cryoprotectants. DMSO produced significantly higher viability ($P<0.05$) than both glycerol and ethylene glycol during all time of experiment after cryopreservation [28]. Moreover, the rumen protozoa viability in the presence of ethylene glycol was obviously higher than glycerol. There were no significant changes among the different concentrations of cryoprotectants although the 5% concentration of each cryoprotectant was numerically, but not significantly higher than the 4 and 6% concentrations. This result was consistent with that of [29], who demonstrated that DMSO was superior to glycerol by slow freezing during cryopreservation of *Trichomonas vaginalis*. Moreover, [30] demonstrated that of six cryoprotectants examined, DMSO showed the strongest cryoprotective effect. It has been demonstrated that the optimal concentrations of the cryopreservatives used for cryopreservation of *Theileria parva* were 7.5% for glycerol, 5% for DMSO and ethylene glycol [31]. The higher efficacy of DMSO as a cryoprotectant might be attributed to its rapid penetration of the cells [27, 28] and consequently interacts with the intracellular water during rapid cooling, resulting in the majority of the intracellular solution becoming vitrifie. The mechanism by which the cryoprotectant maintains the viability of protozoa during freezing is well described. The cryoprotectant acts at the cell membrane level by limiting the effect of cell dehydration during freezing [32], lowering the freezing point of extra- and intracellular biological liquids [33] and promoting vitrification rather than intracellular ice crystal formation [13]. With the liquid nitrogen vapor, it is possible to control the conditions of cooling during the first step of cryopreservation. It was noted that the cooling rate during the period following extracellular ice formation until the holding temperature (-25°C), strongly influenced the survival rates of the rumen ciliates [13, 17]. Because rumen juice is the natural medium for ciliates, this can explain why its use in the freezing and thawing steps improves the viability of cells [13].

In the second experiment, the investigation was directed to study the activity of frozen protozoa inculcated into rumen of previously defaunated sheep. With regard to the effect of oil drench on the total protozoal count, the obtained results showed a highly significant decrease in the total number of rumen protozoa from the first day of defaunation as compared to control and this significant reduction continued till day 120. This result coincides with that observed by [19]. It has been demonstrated that feeding fats to ruminants is commonly associated with decreased protozoal numbers in the rumen [34]. The toxic effect of lipids to protozoa could be due to increasing acidity, resulting from the free fatty acids liberated from the oil [35]. Although the oil drench significantly reduced the number of protozoa, but 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 [36, 37].

Refaunation by injection with straw of the rumen protozoa cryopreserved with the DMSO 5% progressively increased the protozoal number until it reached close to control values after 60 days of experiment. 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 [13]. These results have made it possible to use the cryopreserved straw as a therapy in digestive disorders that are accompanied by death or reduction of rumen protozoa.

With regard to the effect of defaunation and refaunation on rumen pH, the obtained results agree with [38, 39]. 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 [2, 40]. Also it could be contributed to elevatión of the total volatile fatty acid concentration in defaunated sheep [41]. In contrast [42] 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 [19]. The refaunated group significantly regained the rumen pH values to be nearly equivalent to the control values starting from day 30 after the experiment. This confirmed the role of rumen protozoa in maintaining the rumen pH around the normal values [43].

In this experiment the significant depression in rumen ammonia nitrogen concentrations on days 90 and 120 observed in the defaunated group compared with control and refaunated groups, was similar with [19], who demonstrated a highly significant reduction of rumen ammonia nitrogen concentration after a single drench of cooking oil. Moreover [36] showed that completely eliminated protozoa reduced ammonia concentration, compared with untreated control by 60%. In addition [3] demonstrated that faunated animals consistently have higher ruminal ammonia concentrations. The defaunation leads to an increase in the bacterial population, which uses ammonia as the source of nitrogen for cell synthesis. 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 [19].

The mean live body weight and body weight gain were significantly reduced ($P<0.05$) 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, a result that indicate the importance of rumen protozoa and its effect on digestibility. The obtained results agree with [46], who found that defaunation reduces body weight gain due to increased in rumen outflow of protein from bacteria and fungi in the absence of protozoa [47] or that defaunation can consistently decrease fiber digestibility [3]. Moreover [44, 45] reported an increase of up to 30% in rate of weight gain of faunated animals compared with protozoa-free animals. On the other hand, other findings reported no significant difference in the body weight gain between defaunated and faunated animals [48, 49]. These controversial results could be due to the nature and level of protein in the diets [19].

In conclusion, this work demonstrated for the first time in Egypt that rumen protozoa could be successfully cryopreserved and the establishment of protozoal bank is possible. The cryopreserved protozoa could be used as a therapy in digestive disorders that are accompanied with death or reduction of protozoa.

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