Percoll Process Can Improve Semen Quality and Fertility in Turkey Breeders

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ABSTRACT : A percoll density gradient technique was developed for producing high quality turkey semen and improving the fertility by removing deleterious cellular components, including spermiophages, bacteria, abnormal or dead spermatozoa, and other cellular debris. The combination of three different percoll densities, 1.05, 1.07, and 1.08, showed the best resolution and was selected to prepare a discontinuous percoll density gradient to obtain healthy spermatozoa from semen samples. Bacteria, spermiophages, and abnormal or dead spermatozoa were detected from the density range from 1.05, 1.05 to 1.07, and 1.07 to 1.08, respectively. Healthy spermatozoa were collected from the density greater than 1.08. Spermatozoa obtained from percoll density gradient centrifugation showed better sperm motility than those from unprocessed pooled semen. Bacteria including *Escherichia coli*, *Staphylococcus aureus*, and *Proteus spp.*, were predominant contaminants in turkey semen, and the numbers of cells were approximately 5×10^5 to 1×10^9 cfu/ml. The overall fertility rates in hens inseminated with processed percoll density gradient were higher than those in hens with unprocessed semen especially for unhealthy sperm. In conclusion, semen quality can be improved by percoll density gradient centrifugation, which augmented the fertility of turkey breeders. *(Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 5 : 702-707)*

Key Words : Turkey, Spermatozoa, Percoll Density Gradient Centrifugation, Spermiophages, Artificial Insemination, Normal or Yellow-Colored Semen

INTRODUCTION

In the turkey industry, there has been a big improvement in fertility since the onset of artificial insemination (AI). AI was applied in the cases of low fertility rate in natural breeding, individual cage housing, a big difference in body size between male and female, or breeding with purpose, etc. However, poor fertility of unknown etiology is often encountered by commercial turkey breeders. Kammerer et al. (1972) reported that differences between individual males in fertility, and the deterioration in semen quality, may also account for infertility in turkeys. In addition, the associations of poor fertility and hatchability with abnormal yellow-colored semen (YS) have been firmly established (Marquez and Ogasawara, 1975; Thurston, 1976). The seminal fluid in the epididymal region of the YS contained an abundance of abnormal or dead spermatozoa, numerous cells including spermiophages, cellular debris, and increased amounts of electron-dense proteinaceous material (Thurston and Biellier, 1972; Thurston et al., 1975). These cellular contaminants in YS may affect fertility directly or indirectly. In addition, spermiophages in turkey semen have been shown to phagocytize normal or abnormal spermatozoa (Marques and Ogasawara, 1975). Because semen is pooled before AI in a commercial setting, good quality semen can be easily contaminated with poor semen containing detrimental cellular components that should be removed before AI.

Several sperm preparation techniques have been used to improve semen quality, including glass wool filtration (Paulson and Polakoski, 1977), ficoll density centrifugation (Kaneko et al., 1980), percoll gradients (Gorus and Pipeleers, 1981; Forster et al., 1983; Check et al., 1993), sperm-rise procedures (Russell and Rogers, 1987), Sephadex G-50 (Check et al., 1993), and Sperm-prepTMII (Check et al., 1993), for intra-uterine insemination or *in vitro* fertilization in humans. Percoll is a non-toxic colloidal silica coated with polyvinyl pyrrolidone and has been extensively used for biological materials. In addition, percoll has been known to significantly increase motile sperm numbers and motility after centrifugation by removing the debris such as lymphocytes found in semen samples (Hyne et al., 1986), and to improve the semen quality in humans (Check et al., 1993). Semen manipulative techniques are not popular in the turkey industry, although good quality semen is essential for maintaining fertility. As mentioned above, due to the use of pooled turkey semen for AI, quality control of turkey semen is more difficult. Therefore, hens are often inadvertantly exposed to contaminated or poor quality semen, which can result in poor fertility. In this study, a percoll density gradient technique was

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Received January 5, 1999; Accepted February 11, 1999

developed to produce high quality turkey semen by removing deleterious cellular components, including spermiophages, contaminating bacteria, lymphocytes, and abnormal or dead spermatozoa resulting in an increase in fertility rate. Furthermore, we studied the relationship between fertility and semen quality, and the possible mechanisms of spermiophages and contaminating bacteria that can affect fertility in turkey breeders.

MATERIALS AND METHODS

Experimental animals and semen sample preparation

Nicholas breeder toms at 38 to 42 wk of age from Willmar Poultry Company (Willmar, MN 56201) were used for semen collection. Yellow, normal and premilking semen were collected by abdominal massage from turkeys and pooled in each semen sample.

Each pooled normal, yellow, or premilked semen sample was divided into two groups. One group was not treated (unprocessed) and the other group was treated (processed) with percoll (Pharmacia Biotech Inc., Piscataway, NJ 08855-1327) density gradients (PDG) centrifugation. Discontinuous layers of various percoll densities were used to separate healthy spermatozoa from other cellular components of pooled turkey semen. Briefly, multiple layers of PDG were prepared with the densities of 1.05, 1.07 and 1.08 using 1.5 M NaCl as a dilution buffer. Using 15 ml conical centrifuge tubes, volumes of 3 ml percoll solution with each dilution were carefully layered one over another using an 18 gauge spinal needle (Sherwood Medical, St. Louis, MO). Semen samples were diluted with Minnesota Turkey Grower Association (MTGA) semen extender without antibiotic at a 1:2 dilution. Three milliliters of diluted semen was layered on top of the PDG in 15 ml conical tubes. The tubes were centrifuged at $1,500\times g$ for 20 min at room temperature, and each fraction was collected and checked by light microscopy.

Microscopic assessment of sperm motility

Sperm motility of PDG unprocessed or processed pooled normal semen was assessed at three different temperatures (4, 25, and 41℃) with various storage times (0, 1, 3, 6, and 24 h). The motility was evaluated using a modified method of Leffler and Walters (1996). Briefly, samples from different conditions were diluted with or without PBS (pH 7.2, 10mM), and were brought up to room temperature. Twenty microliters of each sample was dropped onto a standard microscope slide and the average value from three replicates was taken as the number of motile spermatozoa per 100 spermatozoa counted under a phase contrast microscope.

Isolation of spermiophages

Spermiophages from normal and yellow-colored semen samples were isolated at a density of 1.05 to 1.07 by PDG centrifugation. A 100 ml of sample from the fractions containing spermiophages was mixed with PBS (pH 7.2, 0.01M) and 100 ml of sample was cytospined on a gelatin coated slide at 1,500 STET for 10 min. Cells were stained with LeukostatTM stain (Fisher Scientific Co., Itaca, IL) according to the manufacturer's recommendation, and examined by an inverted light microscope.

AI and fertility test

Pooled turkey semen collections characterized as normal, yellow, and premilked were diluted with MTGA semen extender (1:1), and used for the insemination as unprocessed semen samples. The fractions containing healthy spermatozoa obtained from percoll-processed pooled normal, yellow, or premilked turkey semen were diluted with MTGA semen extender to make up the original volume, and inseminated as a percoll processed semen sample. To collect premilked semen, semen was milked from toms prior to onset of semen collection for AI. The number of spermatozoa for insemination was 0.1 to 0.15×10^9 per hen. The fertilities were checked by candling each egg and all visible dead embryos were classified as infertile.

Isolation and identification of bacteria isolated from turkey semen

The fraction containing bacteria obtained from yellow, and normal semen by PDG was directly cultured on sheep blood agar plates. The type of bacteria was identified using Gram staining and biochemical testing. The EMB agar, oxidase test, and quick-stop latex agglutination tests were also performed. For counting the number of bacteria, a 100 ml of sample from the fraction was serially diluted with sterile PBS (0.01 M, pH 7.2), and cultured in triplicate on tryptic soy agar for standard plate counts.

Statitical procedure

Statitiscal analysis of the data was carried out by Student's paired t-test, and $p<0.05$ was defined as representing a significant difference.

RESULTS

Standardization of percoll density gradient

Various percoll densities were prepared ranging from 1.04 to 1.09, and evaluated for their ability to separate cellular components from normal spermatozoa. The combination of three different percoll densities, 1.05, 1.07, and 1.08, gave the best resolution. These three gradients were selected and used throughout the

experimental trial. Figure 1 is a schematic diagram of the cellular components in each fraction obtained by PDG centrifugation. Spermatozoa from different fractions obtained from PDG centrifugation are shown in figure 2 A, B, and C. Yellow semen had more dead and abnormal spermatozoa characterized as coiling, crooked-necks, and swollen, than normal semen.

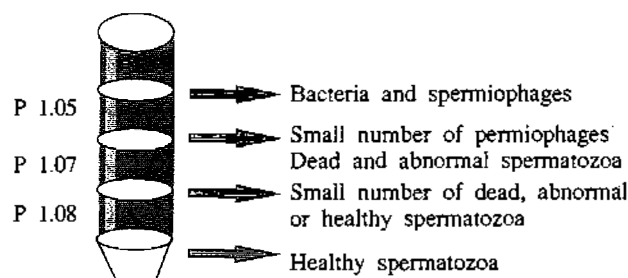
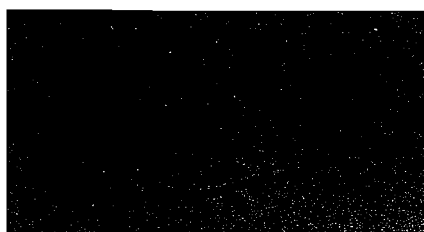


Figure 1. Cellular components in fractions from percoll-processed turkey serum. Used percoll densities were 1.05, 1.07 and 1.08.

A

B



C

Figure 2. Turkey spermatozoa isolated from fractions by percoll density gradient centrifugation. A; normal spermatozoa from normal turkey semen, B; coiled and crooked-neck spermatozoa from yellow-colored turkey semen, C; swollen spermatozoa from yellow-colored turkey semen.

Microscopic assessment of sperm motility

Sperm motility of unprocessed or percoll processed semen samples were assessed at different temperatures (5, 25, and 41℃) and storage time (0, 1, 3, 6, and 24 h). As shown in table 1, percoll processed semen kept at 5℃ showed over 90% sperm motility for up to 3 h of storage, but the sperm motility of unprocessed semen dropped to 75% at 5℃ for 1 h storage. When semen was kept at 25℃, processed semen showed 85 to 95% sperm motility for up to 3 h storage, but only 50% sperm motility in unprocessed semen after 3 h storage. The sperm motility by percoll processed or unprocessed semen kept at 41℃ showed less than 25% after 3 h of storage. No motile spermatozoa were detected after 24 h of storage at any temperature whether they were percoll processed or not.

Table 1. Assessment of the motility of spermatozoa obtained from unprocessed and percoll-processed normal semen during 24 storage

Time (h)	Motility of spermatozoa					
	Un-processed			Processed		
	5℃	25℃	41℃	5℃	25℃	41℃
0	100	100	100	100	100	100
1	75	50	50	95	95	99
3	75	50	25	90	85	25
6	70	50	0	85	50	0
24	0	0	0	0	0	0

Spermiophages from turkey semen

Spermiophages were detected in turkey semen samples at a percoll density between 1.05 and 1.07. Spermiophages obtained from the fraction after PDG centrifugation were examined by cytospin followed by quick stain (figure 3).

Figure 3. Spermiophage detected in turkey semen. Fraction having spermiophages obtained from turkey semen separated by percoll density gradient centrifugation was cytospinned followed by LeukostatTM stain. The morphology of spermiophage (arrow) was round and granulated.

The number of spermiophages in turkey semen varied among individual turkeys (data not shown).

Isolation and identification of microorganisms in semen

Bacterial contamination was a common characteristic in normal and yellow semen samples. PDG centrifugation separated bacteria from other cellular components. Bacteria were mostly detected from the percoll density 1.05. The numbers of contaminating bacteria in normal and yellow semen were about 5×10^5 to 1×10^9 cfu/ml estimated by standard plate count. The numbers of contaminating bacteria were similar in both normal and yellow semen samples. Three types of bacteria, *Escherichia coli*, *Staphylococcus aureus*, and *Proteus spp*, were predominant in normal and yellow semen determined by biochemical test and Gram staining.

Fertility

The fertility rates in hens inseminated with PDG unprocessed or processed yellow-colored semen (YS) were evaluated. Yellow-colored semen collections from 20 toms exhibiting a dark to light yellow color and having abnormal or dead spermatozoa were pooled. As shown in table 2, the fertility rates in hens inseminated with PDG unprocessed semen were around 46.2 to 66.8%.

Table 2. Fertility in turkey hens inseminated with unprocessed or percoll-processed pooled yellow semen

	Yellow-colored semen			
	Unprocessed		Processed	
	1wk	2wk	1wk	2wk
Total no. of eggs	39.0	50.0	37.0	54.0
Fertility (%)[1]	46.2	66.8	78.4	84.2

[1] Fertility was assessed 1 or 2 weeks after artificial insemination by candle method.

In contrast, the fertility rates in hens inseminated with PDG processed semen was 78.4 to 84.2%. In comparison, the fertility rates were increased by 15 to 30% in PDG-processed semen. The fertility rates were compared between unprocessed and processed percoll premilking semen. The pooled premilked semen was light in color, had low viscosity, fewer numbers of healthy spermatozoa, and greater numbers of immature spermatozoa. The fertility rates in hens inseminated with unprocessed premilked semen were 52.1 to 64.0%. However, the fertility rates in hens inseminated with percoll-processed semen showed a range between 66.0 and 74.0%. The fertility rates were around 14% higher with percoll processed semen than with unprocessed semen (table 3).

Table 3. Fertility in turkey hens inseminated with unprocessed or percoll processed pooled premilked semen

	Premilked semen			
	Unprocessed		Processed	
	1wk	2wk	1wk	2wk
Total no. of eggs	37	40	80	84
Fertility (%)[1]	52.1	64.0	66.0	74.0

[1] Fertility was assessed 1 or 2 weeks after artificial insemination by candle method.

Untreated pooled normal semen or percoll-processed normal semen was inseminated into turkey hens. As shown in table 4, the fertility rates in hens inseminated with PDG processed normal semen were 80.6 to 86.3%. In contrast, hens inseminated with PDG processed semen was 82.3 to 87.9%. In general, hens inseminated with PDG processed semen had higher fertility rates than those with unprocessed semen, especially for unhealthy semen ($p<0.05$).

Table 4. Fertility in turkey hens inseminated with unprocessed or percoll-processed pooled normal semen

	Premilked semen			
	Unprocessed		Processed	
	1wk	2wk	1wk	2wk
Total no. of eggs	367	145	389	173
Fertility (%)[1]	80.6	86.3	82.3	87.9

[1] Fertility was assessed 1 or 2 weeks after artificial insemination by candle method.

DISCUSSION

Several factors influence a fertility, including age, temperature, duration of light, nutrients, and genetics in turkeys (Lorenz et al., 1959; Harper and Arscott, 1969). Turkey breeders pay too little attention to the relationship between semen quality and fertility. Semen quality can be evaluated by the number of motile spermatozoa, and contaminating components such as spermiophages, abnormal or dead spermatozoa, bacteria, and cell debris, that can all be factors in infertility (Thurston and Biellier, 1972; Thurston, 1976).

The sperm morphology test is one assay used to assess gamete viability (Marquez and Ogasawara, 1975). We found that YS contained more abnormal or dead spermatozoa than those from normal semen, which agreed with the results of Marquez and Ogasawara (1975). Thurston (1976) reported that turkey hens inseminated with YS showed a lower fertility rate as compared as with normal semen. Many

other researchers reported that YS or poor quality semen results in poor fertility (Saeki and Brown, 1962; Marquez and Ogasawara, 1975). In our experiment, hens inseminated with YS showed a lower range of fertility from 46 to 67% as compared to over 80% from hens inseminated with pooled normal semen.

The presence of spermiophages can be detected in both normal and YS (Phadke, 1975; Thurston, 1976; Holstein, 1978; Haidl, 1990), and those in the reproductive organs can cause reproductive failure, including macrophage-mediated spermatozoa phagocytosis (Holstein, 1978; Muscato et al., 1982; Ball et al., 1984), oocyte phagocytosis (Weissman et al., 1978), preimplantation embryo destruction (Hurst et al., 1977), alteration of progesterone production and secretion (Halme et al., 1985), and abnormal prostaglandin or interleukin-1 production (Fakih et al., 1987). Because birds have a sperm storage gland whereas mammalians do not, the presence of spermiophages in the turkey semen samples might be more detrimental than in mammals. AI in hens with semen having spermiophages can result in hypospermatozoa in the sperm storage gland by spermiophage related phagocytosis. In addition, although oocyte phagocytosis is not known in turkeys, it has been reported for other species (Weissman et al., 1978). Therefore, spermiophages in the reproductive organs in hens may possibly be deleterious to the oocytes. We speculate that the presence of spermiophages in male or female turkey reproductive organs can affect the fertility in several ways.

Beside the presence of spermiophages, bacteria were also detected from both normal and YS. Three predominant bacteria, *E. coli*, *S. aureus*, and *Proteus spp.* in normal and abnormal semen might originate from the milking process, because these bacteria are not normally present in reproductive organs in turkeys. Harry (1962) reported that a large number of *E. coli* in the vagina in inseminated hens originated from AI. Bacterial contamination has not been seriously considered by turkey breeders. Although bacterial contamination did not indicate a pathological condition, it will trigger an immune response in the reproductive organs of immunosuppressed as well as healthy turkeys. We speculate that transferring bacteria into hens by AI might account for the decline in fertility with frequent insemination. Moreover, spermiophages can produce oxygen radicals incorporated with contaminated bacteria, and lipopolysaccharide (LPS) by oxygen burst (unpublished data). Oxygen radicals can lead to membrane protein denaturation and lipid peroxidation of spermatozoa (Mazzilli and Rossel, 1992). In addition, spermiophages by reacting with contaminated bacteria or LPS, may secrete cytokines and free oxygen radicals. Fedder and Ellermann-Eriksen (1995) reported that interferon-g and reactive oxygen species can inhibit sperm motility. Although there is lack of information on the influence of fertility by each cellular contaminant in turkey semen, we speculate that contaminated spermiophages and bacteria as well as dead spermatozoa in semen may all contribute to infertility. We also surmise that this orchestrated process by semen contaminants may lead to hypospermia or azoospermia in hens as well as toms.

Percoll is not considered to be harmful to biological substances, therefore it has been applied preferentially to isolate spermatozoa (Check et al., 1993). A single PDG centrifugation has been used to isolate spermiophages in turkey semen (Perez et al., 1994). We developed a multiple PDG centrifugation for the manipulation of turkey semen. We found that healthy spermatozoa can be obtained from turkey semen by fractionating other components out by PDG centrifugation. Hens inseminated with healthy spermatozoa harvested from semen samples by PDG centrifugation showed higher fertility than those inseminated with unprocessed pooled semen (table 3, 4, and 5). Furthermore, the sperm motility of percoll processed semen was better than that of unprocessed semen. Sperm motility is one of the critical factors for fertility. Based on this experiment, we suggest that several cellular contaminants in turkey semen affect fertility and we recommend the use of PDG centrifugation to improve turkey breeders' fertility.

ACKNOWLEDGMENTS

We thank the Willmar Poultry Company (Willmar, MN, 55201, USA) for providing the animals and animal facility. This study has been funded by the Midwest Poultry Consortium, Inc. Project No. 9605069.

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