1-1-2004

Antimicrobial and Spermicidal Activity of Hydrogen Peroxide and Nonoxynol-9 Based Gel Preparations

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Antimicrobial and Spermicidal Activity of

Hydrogen Peroxide and Nonoxynol-9 Based

Gel Preparations

Thesis Submitted to
The Graduate College of
Marshall University

In Partial Fulfillment of
the Requirements for the Degree
Master of Science
Biological Sciences

by

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Marshall University

June 2004

ABSTRACT

Eighty gel formulations were prepared using five different buffer systems (0.01M citrate, 0.02M citrate, 0.01M tartrate, 0.02M tartrate, and 0.01M citrate/0.01M tartrate), four hydrogen peroxide (H_2O_2) concentrations (0%, 0.3%, 0.9%, and 1.2%), and four nonoxynol-9 (N-9) concentrations (0%, 1%, 1.5%, and 2%).

The eighty gel formulations were then tested for physio-chemical properties (viscosity and pH), antimicrobial effectiveness (Minimum Inhibitory Concentration Assay) against *Candida albicans*, *Escherichia coli*, and *Streptococcus agalactiae*, and spermicidal efficacy (Sander Cramer Assay).

Viscosity values were highest in the 0.01M citrate buffer group, ranging from 53,333 centipoise (cP) to 113,000 cP. The mean viscosity of 0.01M citrate buffer gels was 81,975 cP. The lowest viscosity values were obtained from gels prepared with 0.02M tartrate buffer, ranging from 21,467 cP to 52,667 cP. The average viscosity of gels prepared from 0.02M tartrate buffer was 36,460 cP.

All eighty gel formulations had final pH values ranging from 4.02 to 4.20. The mean pH of all buffered gels was 4.13. The average pH of gels made in 0.01M citrate buffer was 4.10. Gels made in 0.02M citrate buffer had an average pH of 4.13. Gels made in 0.01M tartrate buffer had an average pH of 4.11. Gels made in 0.02M tartrate buffer had an average pH of 4.15. The average pH of gels formed from 0.01M citrate and 0.01M tartrate buffers was 4.16.

Minimum Inhibitory Concentrations (MIC) values were greater for samples containing H_2O_2 than without H_2O_2. In general, the greater the concentration of H_2O_2 present in the gel, the

greater the antimicrobial effectiveness. MIC values ranged from <1/2 (least effective) in all 0% H_2O_2 gels to 1/128 (most effective) in many of the gels that contained 1.2% H_2O_2. *Candida albicans* (a yeast) was shown to be more resistant to H_2O_2 than *Escherichia coli* (Gram negative bacterium), which was more resistant to H_2O_2 than *Streptococcus agalactiae* (Gram positive bacterium).

Sander Cramer assays for spermicidal effectiveness yielded values ranging from <1/2 (least effective) in gels with 0% N-9 to 1/256 (most effective) in gels containing 1.5% and 2% N-9. Citrate buffered gels (0.01M) containing 1.5% and 2% N-9 were slightly more spermicidal than other buffer systems of the same concentration of N-9.

The gel formulation that contained the best combination of physio-chemical, antimicrobial, and spermicidal properties was a 0.01M citrate buffered hydrogel, containing 0.9% H_2O_2 and 1.5% N-9. Similar results were also obtained with 0.02M citrate buffered gels containing 0.9% and 1.2% H_2O_2 with 1.5% and 2% N-9. Future clinical trials will be performed on the 0.01M citrate/ 0.9% H_2O_2 / 1.5% N-9 hydrogel at the University of Osteopathic Medicine and Health Sciences in Des Moines, Iowa.

ACKNOWLEDGMENTS

I would like to thank a few people who have guided, motivated, and inspired me over the time-line of this body of work. First, I would like to thank my parents for instilling in me a work ethic that allowed me to persist in completing this thesis. They have provided me with unconditional support and love. I would also like to thank a couple of my Marshall University professors, Dr. Franklin Binder and Dr. Ronald Gain, for all the guidance and instruction over the years. Their knowledge and experience have been invaluable to me and I appreciate all they have brought to me in my personal and professional life. Words will fall short describing their impact on my life. I would also like to thank Dr. Charles Somerville for participating on my thesis committee and for his professional insights offered in his Microbial Ecology class. Finally, I want to thank my wife, Erica, and my children, Cade and Jonathan, for it is their love and support that provides me with my daily motivation.

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INTRODUCTION

The National Institutes of Health recently estimated that 15.3 million new cases of sexually transmitted diseases (STD's) occur each year in the United States (NIH, 1998). In the1990's, five of the top eleven reportable diseases in the United States were transmitted sexually (chlamydial infection, gonorrhea, AIDS, syphilis and hepatitis B) and accounted for more than 80% of the total reported diseases (MMWR, 1996). On a worldwide basis, the World Health Organization (WHO) estimated 333 million new cases of curable STD's occurred in 1997 alone (1998).

Individual numbers for STD's included AIDS-related deaths worldwide numbering approximately 13.9 million, and an estimated 33.4 million people living with AIDS. Worldwide, the World Health Organization estimated 62 million cases of gonorrhea and 89 million cases of chlamydia occurred in 1997. On a smaller, but no less astounding, scale, approximately 750,000 people in the United States were living with sexually acquired hepatitis B infection, and an estimated 70,000 cases of syphilis occurring each year in the United States (NIH, 1998).

In financial terms, the annual cost of healthcare, including treatment and prevention, is approximately $17 billion in the United States alone. In 1994, the total cost of sexually transmitted HIV infection in the United States was approximately $6.7 billion, $2.0 billion for chlamydial infections, $1.1 billion in costs associated with gonorrhea, with the remaining costs divided among costs for genital herpes, hepatitis B, syphilis, and trichomoniasis (Institute of Medicine, 1997).

Women are especially at risk for STD infection, because many STD's are asymptomatic in women. Thus, treatment is not sought and the disease remains unchecked. STD's may adversely affect pregnancy, causing spontaneous abortion, stillbirth, and pre-term delivery. If a woman is

infected, she can pass syphilis, herpes, gonococcal conjuctivitis, and chlamydial pneumonia to her newborn (Alexander, 1996).

Due to the overwhelming problems with STD's in both the United States and worldwide, the National Institutes of Health awarded a grant to Microbiological Consultants, Inc.(Huntington, WV) to address these problems. The financial funding facilitated the production and analysis of an array of gel formulations that, ideally, would have antimicrobial properties (for STD prevention), as well as a spermicidal modality. In addition to these properties, the gel must have a pH similar to the normal vaginal mucosa and a viscous gel consistency that is chemically and physically stable over time. Esthetic properties of the formulations, such as smell and gel texture, would also be factors in narrowing the possible formulations to one specific formula that yields the highest levels of antimicrobial and spermicidal activity.

Eighty gel formulations were to be prepared using five different buffer systems (0.01M citrate, 0.02M citrate, 0.01M tartrate, 0.02M citrate, and 0.01M citrate / 0.01M tartrate). These buffer systems were employed to maintain the gel at a constant pH range of 4.0 - 4.2, an acidic condition that disrupts bacterial cell membranes, inhibits bacterial enzymes and membrane transport proteins, and alters nutrients available to bacterial invaders. Maintaining an acidic condition in the vaginal mucosa is essential to the prevention of bacterial vaginitis, candidiasis and trichomoniasis (Plourd, 1997). In fact, urogenital problems are experienced by one-third of women 50 years and older, possibly due to the pH increase of the vaginal tract from approximately 4.0 to between 6 and 7, which is caused by menopause (Samsioe, 1998).

Although some organisms are inhibited by low pH, organisms such as *Candida albicans* (a yeast) show little inhibition. Shubair et al.(1990) examined the antifungal properties of two commercial spermicidal gels with pH values ranging from 4.0 to 7.0, and each exhibited minimal

inhibition of *C. albicans*. Another source of antimicrobial activity must be added to the gel formulations since low pH conditions do not guarantee inhibition from all pathogens.

The antimicrobial component of the gel formulations is hydrogen peroxide (H_2O_2), which has been recognized as a microbicide for more than a century. Hydrogen peroxide is effective against bacteria and yeasts, including *Candida albicans* (Fitzsimmons and Berry, 1994). Yoshpe-Purer and Eylan (1968) used low concentrations for the sterilization of water. Naguib and Hussein (1972) showed that 0.1% hydrogen peroxide at 54°C for 30 minutes reduced the total bacterial count in raw milk by 99.999% and the coliform, staphylococcal, salmonellae, and clostridial counts by 100%.

Many reports in the literature suggest that the mode of action of H_2O_2 is not due to the molecule itself, but to the production of a powerful oxidant, the hydroxyl free radical ($OH^{\cdot}$). The hydroxyl radical attacks bacterial plasma membrane lipids, DNA, and other essential cell components (Turner, 1998).

An important part of the study was to determine the appropriate concentration of hydrogen peroxide in these gel preparations. In a previous study by Larsen (1996), gels formulated to contain 0.3% hydrogen peroxide provided inhibitory activity against a variety of bacterial pathogens at inoculum levels of approximately 10^4 viable organisms per ml of gel dilution (Table 1). Four concentrations of hydrogen peroxide (0%, 0.3%, 0.9%, and 1.2%) were used in gel formulations, with antimicrobial efficacy of each tested to determine the optimum effective concentration.

Table 1: Antimicrobial efficiency of hydrogen peroxide based gel preparation*

ORGANISM	NUMBER OF STRAINS TESTED	MIC VALUE (GEL DILUTION)
Neisseria gonorrhoeae	1	2^{-3}
Gardnerella vaginalis	1	2^{-7}
Streptococcus agalactiae	9	2^{-1}- 2^{-7}
Candida albicans	6	2^{0} - 2^{-4} (mean $2^{-1.3}$)
Chlamydia trachomatis	1	2^{-3}
Prevotella bivia	2	2^{-5} - 2^{-6}
Escherichia coli	1	2^{-5}
Staphylococcus aureus	1	2^{-6}
Trichomonas vaginalis	2	2^{-6} - 2^{-7}

*Larsen, Bryan. Preventative/Therapeutic Products for Vaginitis and STD's. Revised Application for National Institutes of Health Grant. July, 1996: 16-36.

Along with a microbicide, a spermicide was incorporated into the gel formulations. The spermicide used was nonoxynol-9 (N-9), a nonionic detergent that is commercially used in various spermicidal gels and condoms (Roddy et al., 1998). N-9, even at concentrations too low to kill sperm, would significantly impair sperm motility and motion parameters (Centola, 1998). N-9 has also exhibited various antimicrobial effects. Klebanoff (1992) reported contraceptive preparations containing N-9 were toxic to *Neisseria gonorrhoeae* (cause of gonorrhea), *Treponema pallidum* (cause of syphilis), *Trichomonas vaginalis*, *Chlamydia trachomatis*, herpes simplex virus, and HIV virus. Four concentrations of N-9 (0%, 1%, 1.5%, and 2%) were incorporated into the formula and evaluated in this study.

Along with its antimicrobial effectiveness, N-9 demonstrates adverse effects on the normal microbial flora of the vaginal tract, especially *Lactobacillus acidophilus* (Richardson et al., 1998). Lactobacillus species are the predominant organisms in the vaginal microflora and produce H_2O_2, resulting in antibacterial activity against a wide range of Gram-positive and Gram-negative pathogens (Coconnier et al., 1997), including *Neisseria gonorrhoeae* (Zheng et al., 1994). N-9 applications alone may decrease lactobacilli levels. This could possibly lead to increased colonization by *Escherichia coli*, which is resistant to N-9 (Hooten et al., 1991). *E. coli* vaginal colonization has been linked to recurrent urinary tract infections (UTI's) in women (Gupta et al., 1998) and women without vaginal lactobacilli were more likely to be colonized by *Chlamydia trachomatis* (Hillier et al., 1992).

Because of these negative properties associated with N-9, the addition of H_2O_2 to gel formulas was essential to the prevention of genital tract colonization by enteric organisms. In addition to predisposition to urinary tract infection, *E. coli* colonization may lead to premature births

among obstetrical patients and are associated with low birth-weight infants (Krohn et al., 1997).

Another common problem with N-9 use is its association with inflammation of the genital mucosa, and epithelium disruption. Stafford et al. (1998) reported a 25% (10 out of 40 patients) incidence of genital irritation after one week of a placebo-controlled trial of an N-9 daily use of 100 mg. Conversely, Van Damme et al. (1998), when exposing 179 patients to a spermicide with 52.5 mg of N-9, 178 patients with a gel placebo, and 177 with a negative control for 14 days, reported <2% incidence of epithelial disruption and inflammation. The difference in results may be due to the use of a polymer-based gel matrix in the Van Damme study. In a related study, Gagne et al. (1999) evaluated the capacity of a polyoxypropylene/polyoxyethylene polymer based gel to reduce or eliminate the toxicity of N-9. The incorporation of the N-9 into the gel markedly reduced the N-9 toxicity to the vaginal and cervical mucosa in animal studies. Thus, the use of a gel-forming polymer may be a key to decreasing N-9 irritation and inflammation in the urogenital tract.

The polymer of choice for the gel formulations was an acrylic acid polymer called Carbopol, which, in initial trials, produced gels with physical and chemical stability when a 2% Carbopol (by weight) concentration was used. Although extreme storage conditions (3 months at 45^{o}C) resulted in gel liquefaction, gels stored at 37^{o}C did not lose their gel consistency (Larsen, 1998). Carbopol was chosen after preliminary studies indicated that gels formed from carboxymethyl cellulose were broken down by hydrogen peroxide within days of formulation.

In addition to the above listed components, a 7% concentration of sterilized glycerol was chosen to be part of the gel matrix. Glycerol furnishes the gel formulations with an added quality of lubrication.

All gel formulations used in this study contained a buffered hydrogel, consisting of carbopol, glycerol, water, and one of five buffer systems. The gel formulations also contained four possible

H_2O_2 concentrations as well as four possible N-9 concentrations. The different buffered gel formulations with hydrogen peroxide and nonoxynol-9 are presented in Tables 2-4.

Table 2: Citrate buffered gel formulations with hydrogen peroxide and nonoxynol-9.

	0.01M Citrate				**0.02M Citrate**			
H_2O_2 Concentration	**N-9 Conc. (%)**				**N-9 Conc. (%)**			
	0.0	**1.0**	**1.5**	**2.0**	**0.0**	**1.0**	**1.5**	**2.0**
0.00%	–	–	–	–	–	–	–	–
0.30%	–	–	–	–	–	–	–	–
0.90%	–	–	–	–	–	–	–	–
1.20%	–	–	–	–	–	–	–	–

Table 3: Tartrate buffered gel formulations with hydrogen peroxide and nonoxynol-9.

	0.01M Tartrate				**0.02M Tartrate**			
H_2O_2 Concentration	**N-9 Conc. (%)**				**N-9 Conc. (%)**			
	0.0	**1.0**	**1.5**	**2.0**	**0.0**	**1.0**	**1.5**	**2.0**
0.00%	–	–	–	–	–	–	–	–
0.30%	–	–	–	–	–	–	–	–
0.90%	–	–	–	–	–	–	–	–
1.20%	–	–	–	–	–	–	–	–

Table 4: Citrate/Tartrate buffered gel formulation with hydrogen peroxide and nonoxynol-9.

	0.01M Citrate/0.01M Tartrate			
H_2O_2 Concentration	**N-9 Conc. (%)**			
	0.0	**1.0**	**1.5**	**2.0**
0.00%	–	–	–	–
0.30%	–	–	–	–
0.90%	–	–	–	–
1.20%	–	–	–	–

MATERIALS AND METHODS

Buffer Preparation

Five buffer formulations were prepared: 0.01M citrate buffer, 0.02M citrate buffer, 0.01M tartrate buffer, 0.02M tartrate buffer, and 0.01M citrate/0.01M tartrate buffer. Each buffer was prepared in 2.0 liter quantities.

The 0.01M citrate buffer was prepared by adding 4.20 grams(g) of citric acid monohydrate (Fisher) and 5.88 g of sodium citrate (Fisher) to 2.0 liters distilled water in a 4.0 liter autoclavable bottle. The 0.02M citrate buffer was prepared by adding 8.41 g citric acid monohydrate and 11.76 g sodium citrate to 2.0 liters distilled water in a 4.0 liter autoclavable bottle. The 0.01M tartrate buffer was prepared by adding 3.00 g of tartaric acid (Fisher) and 4.6 g of sodium tartrate (Fisher) to 2.0 liters of distilled water in a 4.0 liter autoclavable bottle. The 0.02M tartrate buffer was prepared by adding 6.00 g tartaric acid and 9.20 g sodium tartrate to 2.0 liters distilled water in a 4.0 liter autoclavable bottle. Finally, the 0.01M citrate / 0.01M tartrate buffer was prepared by adding 4.20 g citric acid monohydrate, 5.88 g sodium citrate, 3.00 g tartaric acid, and 4.60 g sodium tartrate to 2.0 liters distilled water in a 4.0 liter autoclavable bottle.

The pH of all five buffers was measured with an Orion portable pH-meter and autoclaved at 121^{o}C for 20 minutes in a Hirayama Steam Autoclave to minimize the microbial bioburden of the finished gel product. A Buffer Preparation Worksheet is included in Appendix A.

Calibration of pH-Meter

Before samples were formulated, the Orion 250A pH meter was calibrated using two pH buffers, 7.00 and 4.01. First, the pH meter was turned on and the 'mode' key on the keypad was pressed until 'pH' was indicated. The electrode was then placed into the 7.00 pH buffer. The '2nd'

key was pressed, followed by 'CAL'. When the reading was stable, the display read 'READY.' The electrode was then removed, rinsed with distilled water, and then placed into the 4.01 pH buffer. When the reading was stabilized, the 'Yes' key was pressed, and a calibration printout was formulated. The report included the buffers used, the temperature, and the calibration slope. The printout was attached to a Calibration Sheet for Orion pH meter (Appendix B).

Gel Preparation

Each of the eighty gel preparations (100 grams) was made in a biosafety hood in a Class 1000 clean room. Four gel formulations were made per day. Each of the four gels consisted of a constant buffer, a constant hydrogen peroxide concentration (stock 30% H_2O_2, Fisher), and a varying concentration of nonoxynol-9 (0%, 1%, 1.5%, and 2%) for each gel. The four varying nonoxynol-9 (Rhone-Poulenc Igepal CO-630-Special) concentrations represented the four daily concentrations. The weight of hydrogen peroxide (30%) and nonoxynol-9 components had to be determined. Once these were determined, the sum of the weights, along with 7.0 g sterilized glycerol (Fisher) and 2.0 g Carbopol (carboxypolymethylene) (BFGoodrich) were subtracted from the finished product weight of 100 g. The remaining mass was the amount of buffer added to the formulation. The worksheet for these calculations is contained within the Gel Preparation Worksheet (Appendix C).

Using a sterilized graduated cylinder (100 mL), the appropriate mass of sterile buffer was transferred to a 200 ml specimen container. Next, 7.0 g of sterile glycerin was added to the container and the appropriate weight of hydrogen peroxide (30%) was added to the mixture. Finally, the appropriate amounts of nonoxynol-9 (0 g, 1.0 g, 1.5 g, or 2.0 g) were added to the formulation. This process was repeated for each of the four gels. A sterilized magnetic stir-bar was

placed in each of the specimen containers. The containers were then labeled with the contents and placed on a Thermolyne four-position stirrer set at 300 RPM. The formulations were homogenized for approximately 20 minutes.

In twenty-minute increments, 0.5 g of Carbopol was added to each of the four formulations until a total of 2.0 g of Carbopol was present in each mixture. The samples were left to stir on the four-position stirrer for approximately 20 minutes, or until the entire formulation was homogeneous. Once homogenized, the pH of each mixture was adjusted to a range of 4.0 to 4.2 using 3 N sodium hydroxide. Once the formulation reached this range, the sample formed a uniform gel. After the gel had formed and the pH was stable, the stir-bar was removed and a pH value was printed and attached to the Gel Preparation Worksheet. Each of the gels was assigned a lot number based on the date of preparation and its chemical makeup. After the gels were formulated, chemical and microbiological testing protocols were performed.

Viscosity Measurement

Viscosity measurements were performed on all eighty gel formulations to ensure proper consistency in the finished product. The viscosity, measured in centipoise (cP) or milliPascal-seconds (mP·s), was measured using a Brookfield DV-II+ Programmable Viscometer.

The Viscometer was turned on and allowed to calibrate. A Helipath spindle labeled 'A' was placed on the attachment site and the spindle code (S91) entered into the programmable keypad. The spindle was placed approximately three-quarters of an inch below the surface of the 5000 cP viscosity standard. The timed spindle rotation was set at 10 minutes and the velocity at 1.5 RPM. The value of the standard was expressed on the monitor after 10 minutes. The value of the standard should read between 4800 cP and 5200 cP. The spindle was removed and cleaned, surface

disinfected with isopropyl alcohol and rinsed with distilled water. The spindle was then replaced and the programmed rotation was repeated using the gel samples. The viscosity values were determined, along with the % torque and sample temperature. These values are entered onto the Viscosity Worksheet (Appendix D).

Hydrogen Peroxide Concentration

The concentration of hydrogen peroxide (H_2O_2) in the finished gel product was determined by titration with potassium permanganate. Using an Ohaus GA-110 Analytical Balance, 5.0 g of sample was weighed into a 100 ml volumetric flask. The flask was brought to volume with distilled water. Using a 10 ml sterile pipette, 20 ml sample dilution was transferred from the volumetric flask to a 150 ml beaker. Using a new 10 ml sterile pipette, 20 ml of 2 N sulfuric acid was transferred to the 150 ml beaker. The solution was titrated using a Digitrate digital burette filled with 0.2 N potassium permanganate, until a pink color was formed in the solution. The concentration of H_2O_2 was calculated by multiplying the amount of titrant, 1.701 (a potassium permanganate to hydrogen peroxide equilibrium constant), and 0.2 (normality of potassium permanganate), then dividing the product by the exact weight of the sample (in grams). The 30% hydrogen peroxide bulk was tested weekly to ensure maintenance of proper concentration levels. The 30% H_2O_2 bulk must be between 29% - 32% H_2O_2, according to USP 23, NF 18. The standard operating procedure for the determination of hydrogen peroxide by titration, generated by Microbiological Consultants, Inc., is recorded in Appendix E.

The resulting concentration of H_2O_2 in the product must be within 10% of the predetermined concentration (0%, 0.3%, 0.9%, or 1.2%). If out of range, the product was retested. The calculated

concentration of the sample was entered onto the Hydrogen Peroxide Titration Assay Worksheet, along with the corresponding acceptable ranges. A sample worksheet is attached (Appendix F).

Nonoxynol-9 Concentration

The concentration of Nonoxynol-9 (N-9) in finished gel products was determined by High Performance Liquid Chromatography (HPLC). The HPLC system consisted of a Rainin Solvent Delivery System, a Knauer UV Photometer Detector, set at a wavelength of 254 nm, a Hewlett-Packard HP3394A Integrator, and a Microsorb-MV C18, 15 cm HPLC column. A mobile phase consisting of 90:10 methanol:water, adjusted to a pH of 4.0 with glacial acetic acid, was used as the solvent. The UV photometer range was set at 0.32, the attenuation of the integrator set at 3, and the flow rate of the solvent delivery system was set at 1.5 ml per minute. The solvent flow was begun, and the column was allowed to stabilize for approximately 30 minutes.

Standard preparations of Nonoxynol-9 were prepared at 0.01% and 0.02% of N-9. First, a 1% N-9 stock standard was generated by diluting 1.0 g (weighed on Ohaus analytical balance) to 100 ml volume with methanol in a 100 ml volumetric flask. Next, 1.0 ml of the 1% stock standard was transferred to another 100 ml volumetric flask using a 1 ml Class A volumetric pipette. The 100 ml volumetric flask was then brought to volume with methanol. This solution contained 0.01% N-9. Next, 2.0 ml of the 1% stock standard was transferred to a new 100 ml volumetric flask using a 2 ml Class A volumetric pipette. The volumetric flask was then brought to volume with methanol, and this solution contained the 0.02% N-9 standard.

The 0.01% and 0.02% N-9 standards were each injected (20 µl) into the injection port of the HPLC, and the integrator was started. Nonoxynol-9 peaks appeared at approximately 6 minutes. The

integrator determined the areas of the peaks. Linear values representing the peak were expected to be in relation with the corresponding values of the standard concentration, 0.01% N-9 and 0.02% N-9. If linearity was not achieved, standards were injected again. If standards were linear, sample preparation was performed.

Samples were prepared for injection by weighing 1.0 g into a 100 ml volumetric flask. The flask was then brought to volume with methanol and shaken vigorously to ensure proper dissolution of the gel. Once homogenized, the sample was injected (20 µl) into the injection port and the integrator started. Once the run was complete, the N-9 peak of the sample was expected to be morphologically similar to that of the standards. The integrator determined the peak area value. This value, the sample area, was used to determine the concentration of nonoxynol-9 in the sample.

To calculate the percent N-9, the sample area is divided by the standard area (using the standard concentration closest to the sample's expected concentration), then multiplied by the concentration of the standard, then multiplied by the dilution factor of the sample (100). The calculation process was included in the Nonoxynol-9 Calculation Worksheet (Appendix G). The value determined was expected to be within 10% of the theoretical concentration of the gel in question. If it was not, the sample was prepared again and re-injected into the HPLC. The standard operating procedure for the determination of nonoxynol-9 by High Performance Liquid Chromatography, generated by Microbiological Consultants, Inc., is shown in Appendix H.

Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) tests were performed at four time intervals (10 minutes, 30 minutes, 2 hours, and 18 hours) for each gel formulation to assess their antimicrobial efficacy. The three test organisms used were *Candida albicans* (ATCC 14053), *Escherichia coli*

(ATCC 25922), and *Streptococcus agalactiae* (ATCC 13813), all at concentrations of 10^5 cells/100 µl in the test preparation. Each organism was obtained from a Bactrol disk, which were aseptically transferred to 10 ml of Trypticase Soy Broth (TSB) and incubated overnight at 35°C. After incubation, microbial densities were estimated by plating dilutions of 10^{-4}, 10^{-5}, and 10^{-6} in duplicate onto Trypticase Soy Agar (TSA) plates. Cultures were then incubated for 24 hours at 35°C.

Using all tubes from the serial dilutions of culture, 3 ml was transferred from each into blank disposable cuvets. These sample cuvets were placed in a Spectronic-21 spectrophotometer at a wavelength of 600 nm to measure the optical density (Absorbance) of each dilution. Once the spectrophotometer was zeroed using a water blank, the blank was replaced with each of the samples, and the Absorbance of diluted culture was measured.

After 24 hours at 35°C, colonies were counted on the TSA plates. Counting of the plates revealed that *Candida albicans* overnight culture contained 10^6 colony forming units (cfu's) / 0.1 ml. Therefore, to achieve 10^5 cells/0.1 ml, the overnight culture had to be diluted 10^{-1} in TSB. *Escherichia coli* overnight culture contained 10^8 cfu's / 0.1 ml, requiring a 10^{-3} dilution to achieve the proper concentration for MIC testing. *Streptococcus agalactiae* plate counts yielded a 10^7 concentration of overnight culture, thus requiring a 10^{-2} dilution to reach 10^5 cells / 0.1 ml.

The Absorbance reading of the above dilutions were then compared. The Absorbance of the 10^{-1} dilution in *Candida albicans* overnight culture was 0.053. The Absorbance of the 10^{-3} dilution in *E. coli* was 0.051, and the Absorbance of the 10^{-2} dilution of *S. agalactiae* was 0.050. Using these values, it was then estimated that an Absorbance range of 0.045 - 0.055 in the above mentioned dilutions of cultures is an indicated 10^5 cells / 0.1 ml.

For MIC tests, each sample gel (1.0 g) was diluted 1:64 using 2-fold serial dilutions in 1 ml distilled water (1:2, 1:4, 1:8, 1:16, 1:32, 1:64), using 2 ml deep well assay blocks. These dilutions

are then transferred, 100 µl at a time, to columns 2-6 of an 8-column microtiter plate using an Eppendorf 8-channel pipetter (6 tips). In the first column, 0.1 g of direct gel sample was aseptically transferred, using a sterile syringe. In the last column (#8), a control water blank (100 µl) was added. This process was repeated for nine rows of the microtiter plate. The first three rows were triplicate MIC tests for *C. albicans*, the second three rows were for *E. coli*, and the final three rows for *S. agalactiae*. The appropriate dilutions of each culture were then transferred, in 100 µl quantities, using the 8-channel pipetter to the gel dilutions in the microtiter plate. The first three rows had *C. albicans* added, the second three rows had *E. coli*, and the final three contain *S. agalactiae.* Since equal amounts of culture and gel dilutions were used (100 µl), the final concentration of the gel samples ranged from 1:2 in the first column to 1:128 in the seventh column, along with a control in the eighth column.

Once the organisms were added, the microtiter plate was placed in an incubator at 35°C. A timer was set for 10 minutes, 30 minutes, 2 hours, and 18 hours. At each of these times, a 20 µl subculture of each row was removed from the microtiter plate using the 8-channel pipetter, and streaked down a square TSA plate. The TSA plates, labeled with the time interval of sampling and the culture organism, were then placed in an incubator set at 35°C for 24 hours.

The entire MIC test was repeated with each gel sample. Therefore, four microtiter plates corresponded to the four gels produced daily. All four plates remained in the incubator until all time interval sampling had occurred.

After the TSA plates had incubated for 24 hours at 35°C, the plates were removed from the incubator and assessed for microbial growth. If colonies were absent at concentrations of 1:2 - 1:128, corresponding to columns 1-7, then it was concluded that the gel inhibited the organism at the corresponding concentration. The MIC was defined as the minimum concentration of sample that

still inhibited the organism. The MIC value for each organism, at each of the four times, was determined for each of the gel samples.

Sander Cramer Spermicidal Activity Test

Samples of the eighty gel formulations were sent to Dr. Bryan Larsen of the University of Osteopathic Medicine and Health Sciences in Des Moines, Iowa for spermicidal activity testing.

The Sander Cramer test performed was similar to the MIC tests performed on the gels, but instead of bacterial cultures, sperm samples from three healthy male donors were used to determine the minimum concentration of gel sample that was spermicidal.

RESULTS

Gel Formulations

Eighty gel preparations were formulated according to specifications, with formulation dates ranged over three months, from 08-12-98 to 11-12-98. Tables of every formulation with lot numbers, and dates of production, are included in Appendix I.

Chemical and Physical Measurements of Gel Formulations

Chemical and physical properties (pH and viscosity) for all gel formulations are presented in Tales 5-9 grouped by buffer system. All gel formulations had final pH values ranging from 4.02 to 4.20. The mean pH of all buffered gels was 4.13. The average pH of gels formed from 0.01M citrate buffer was 4.10. Gels formed from 0.02M citrate buffer had an average pH of 4.13. Gels formed form 0.01M tartrate buffer had an average pH of 4.11, while gels formed from 0.02M tartrate buffer had an average pH of 4.15. The average pH of gels formed from 0.01M citrate/ 0.01M tartrate buffers was 4.16.

The average viscosity of gels formed from 0.01M citrate buffer was 81,975 cP. Gels formed from 0.02M citrate buffer had an average viscosity of 50,417 cP. Gels formed from 0.01M tartrate buffer had an average viscosity of 73,300 cP, while gels formed from 0.02M tartrate buffer had an average viscosity of 36,460 cP. The average viscosity of gels formed from 0.01M citrate / 0.01M tartrate buffers was 49,184 cP.

Minimum Inhibitory Concentration (MIC) Studies

Minimum inhibitory concentration values for all gel formulations inoculated with *Candida*

Tables 5 and 6 : The effect of increasing concentrations of hydrogen peroxide and nonoxynol-9 on pH and viscosity measurements in 0.01M (Table 5) and 0.02M (Table 6) citrate buffered gels.

0.01M Citrate Buffer Gels

	pH				viscosity			
% Hydrogen Peroxide	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**
0% Hydrogen Peroxide	4.02	4.09	4.16	4.15	85,067	71,067	59,600	58,400
0.3% Hydrogen Peroxide	4.06	4.09	4.16	4.12	92,000	92,400	53,333	67,467
0.9% Hydrogen Peroxide	4.04	4.04	4.2	4.14	109,000	68,400	77,200	76,800
1.2 % Hydrogen Peroxide	4.04	4.06	4.07	4.11	98,933	100,000	113,000	88,933

0.02M Citrate Buffer Gels

	pH				viscosity			
% Hydrogen Peroxide	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**
0% Hydrogen Peroxide	4.15	4.17	4.18	4.20	53,333	45,200	52,933	36,800
0.3% Hydrogen Peroxide	4.10	4.16	4.16	4.18	42,400	52,133	37,200	35,867
0.9% Hydrogen Peroxide	4.08	4.13	4.15	4.12	58,933	58,267	49,200	53,067
1.2 % Hydrogen Peroxide	4.11	4.07	4.12	4.06	60,400	54,800	62,133	54,000

Tables 7 and 8 : The effect of increasing concentrations of hydrogen peroxide and nonoxynol-9 on pH and viscosity measurements in 0.01M (Table 5) and 0.02M (Table 6) tartrate buffered gels.

0.01M Tartrate Buffer Gels

	pH				viscosity			
% Hydrogen Peroxide	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**
0% Hydrogen Peroxide	4.09	4.11	4.15	4.18	73,333	72,000	51,600	67,467
0.3% Hydrogen Peroxide	4.08	4.09	4.17	4.06	86,800	66,933	84,933	76,000
0.9% Hydrogen Peroxide	4.04	4.12	4.18	4.10	86,267	78,133	90,667	63,333
1.2 % Hydrogen Peroxide	4.12	4.09	4.09	4.11	102,000	64,667	60,000	48,667

0.02M Tartrate Buffer Gels

	pH				viscosity			
% Hydrogen Peroxide	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**
0% Hydrogen Peroxide	4.12	4.18	4.18	4.20	38,667	30,667	21,467	38,800
0.3% Hydrogen Peroxide	4.09	4.09	4.09	4.17	26,133	26,133	25,332	34,933
0.9% Hydrogen Peroxide	4.09	4.16	4.20	4.20	30,933	40,000	30,800	49,360
1.2 % Hydrogen Peroxide	4.14	4.15	4.15	4.17	52,667	44,800	41,333	51,333

Tables 9 : The effect of increasing concentrations of hydrogen peroxide and nonoxynol-9 on pH and viscosity measurements in 0.01M citrate / 0.01M tartrate buffered gels.

0.01M Tartrate / 0.01M Tartrate Buffer Gels

	pH				viscosity			
% Hydrogen Peroxide	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**	**0% N-9**	**1% N-9**	**1.5% N-9**	**2% N-9**
0% Hydrogen Peroxide	4.09	4.17	4.15	4.19	46677	37200	34000	34000
0.3% Hydrogen Peroxide	4.14	4.09	4.2	4.16	46933	59733	48000	36533
0.9% Hydrogen Peroxide	4.17	4.19	4.18	4.20	62533	46800	47067	56533
1.2 % Hydrogen Peroxide	4.14	4.18	4.15	4.19	54000	51200	72000	53733

albicans, *Escherichia coli*, and *Streptococcus agalactiae* at 10 minutes, 30 minutes, 2 hours, and 18 hours, are presented in Tables 10-24.

All gel formulations that contained 0% hydrogen peroxide had MIC values of <1/2, indicating that no antimicrobial activity was shown is these gels. Gels that contained 1.2% hydrogen peroxide in the sample had MIC values ranging from <1/2 at 10 minutes inoculated with *C. albicans* to 1/128 at 18 hours inoculated with *S. agalactiae*. In general, as inoculation time increased with each organism, the MIC value became greater.

Candida albicans, a yeast, exhibited the greatest overall resistance to gels, yielding MIC values from <1/2 (several gels) to 1/64 (3 samples, all at maximum H_2O_2 concentration at maximum incubation time)(Tables 10-14). When comparing buffer systems to MIC values, 0.02M citrate-buffered gels exhibited the highest MIC values against *C. albicans*. Conversely, the 0.01M citrate/ 0.01M tartrate buffered gels yielded the lowest values, with the highest MIC at 1/8 dilution.

MIC values for *Escherichia coli* revealed the gels have a greater effect against the Gram negative bacterium than the yeast. Gels without H_2O_2 always yielded <1/ 2 MIC values with each buffer system. All buffer systems commonly produced 1/64 and 1/128 MIC values with 1.2% H_2O_2 (Tables 15-19).

Streptococcus agalactiae, a Gram-positive bacteria, exhibited the least resistance to these hydrogels. Several gels had MIC values of 1/128 against *S. agalactiae* were as high as 1/16 at even the earliest incubation time (10 minutes) (Tables 20-24).

Table 10: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Candida albicans* (0.01M citrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	<1/2	<1/2
	30 min.	<1/2	<1/2	<1/2	<1/2
	2 hrs.	<1/2	<1/2	1/4	1/8
	18 hrs.	<1/2	1/4	1/32	1/16
1% N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	<1/2	1/4	1/4
	18 hrs.	<1/2	1/4	1/32	1/8
1.5 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/4	1/4
	2 hrs.	<1/2	<1/2	1/8	1/8
	18 hrs.	<1/2	1/4	1/16	1/16
2 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	<1/2	1/4	1/8
	18 hrs.	<1/2	1/4	1/32	1/16

Table 11: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Candida albicans* (0.02M citrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	1/2	<1/2
	30 min.	<1/2	<1/2	1/2	<1/2
	2 hrs.	<1/2	1/2	1/8	1/4
	18 hrs.	<1/2	1/4	1/16	1/64
1% N-9	**10 min.**	<1/2	<1/2	1/2	<1/2
	30 min.	<1/2	<1/2	1/2	1/4
	2 hrs.	<1/2	1/2	1/4	1/4
	18 hrs.	<1/2	1/4	1/8	1/64
1.5 % N-9	**10 min.**	<1/2	<1/2	1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	1/2	1/8	1/4
	18 hrs.	<1/2	1/4	1/16	1/64
2 % N-9	**10 min.**	<1/2	<1/2	1/2	<1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	1/2	1/4	1/4
	18 hrs.	<1/2	1/4	1/8	1/64

Table 12: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Candida albicans* (0.01M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	1/2	1/4	1/8
	18 hrs.	<1/2	1/8	1/8	1/16
1% N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	1/2	1/4	1/8
	18 hrs.	<1/2	1/8	1/8	1/16
1.5 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	1/2	1/4	1/8
	18 hrs.	<1/2	1/16	1/16	1/16
2 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	1/2	1/2	1/2
	2 hrs.	<1/2	1/2	1/4	1/8
	18 hrs.	<1/2	1/8	1/8	1/32

Table 13: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Candida albicans* (0.02M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	<1/2	1/4	1/4
	18 hrs.	<1/2	1/4	1/8	1/32
1% N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	1/2	1/4	1/4
	18 hrs.	<1/2	1/8	1/8	1/32
1.5 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	<1/2	1/4	1/4
	18 hrs.	<1/2	1/4	1/8	1/32
2 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/2
	2 hrs.	<1/2	<1/2	1/4	1/4
	18 hrs.	<1/2	1/4	1/8	1/32

Table 14: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Candida albicans* (0.01M citrate / 0.01M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/4
	2 hrs.	<1/2	<1/2	1/4	1/4
	18 hrs.	<1/2	1/2	1/4	1/8
1% N-9	**10 min.**	<1/2	<1/2	1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/4
	2 hrs.	<1/2	<1/2	1/4	1/4
	18 hrs.	<1/2	1/2	1/4	1/8
1.5 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/4
	2 hrs.	<1/2	<1/2	1/2	1/4
	18 hrs.	<1/2	1/2	1/4	1/8
2 % N-9	**10 min.**	<1/2	<1/2	<1/2	1/2
	30 min.	<1/2	<1/2	1/2	1/4
	2 hrs.	<1/2	<1/2	1/4	1/4
	18 hrs.	<1/2	1/2	1/8	1/8

Table 15: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Escherichia coli* (0.01M citrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	<1/2	1/8
	30 min.	<1/2	1/4	1/8	1/16
	2 hrs.	<1/2	1/4	1/16	1/32
	18 hrs.	<1/2	1/4	1/16	1/64
1% N-9	**10 min.**	<1/2	<1/2	1/8	1/16
	30 min.	<1/2	<1/2	1/8	1/16
	2 hrs.	<1/2	<1/2	1/16	1/16
	18 hrs.	<1/2	1/4	1/16	1/32
1.5 % N-9	**10 min.**	<1/2	1/4	1/8	1/8
	30 min.	<1/2	1/8	1/8	1/16
	2 hrs.	<1/2	1/8	1/16	1/32
	18 hrs.	<1/2	1/16	1/16	1/64
2 % N-9	**10 min.**	<1/2	<1/2	1/4	1/16
	30 min.	<1/2	<1/2	1/8	1/32
	2 hrs.	<1/2	<1/2	1/8	1/32
	18 hrs.	<1/2	1/4	1/32	1/64

Table 16: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Escherichia coli* (0.02M citrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	1/4	1/8
	30 min.	<1/2	1/4	1/8	1/16
	2 hrs.	<1/2	1/16	1/16	1/16
	18 hrs.	<1/2	1/16	1/32	1/64
1% N-9	**10 min.**	<1/2	<1/2	1/8	1/8
	30 min.	<1/2	1/4	1/16	1/8
	2 hrs.	<1/2	1/8	1/32	1/8
	18 hrs.	<1/2	1/16	1/64	1/64
1.5 % N-9	**10 min.**	<1/2	<1/2	1/16	1/16
	30 min.	<1/2	1/4	1/16	1/16
	2 hrs.	<1/2	1/8	1/32	1/32
	18 hrs.	<1/2	1/16	1/32	1/64
2 % N-9	**10 min.**	<1/2	<1/2	1/8	1/16
	30 min.	<1/2	1/2	1/16	1/16
	2hrs.	<1/2	1/8	1/64	1/32
	18 hrs.	<1/2	1/16	1/64	1/128

Table 17: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Escherichia coli* (0.01M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	1/2	1/8	1/8
	30 min.	<1/2	1/2	1/16	1/16
	2 hrs.	<1/2	1/8	1/64	1/64
	18 hrs.	<1/2	1/32	1/64	1/64
1% N-9	**10 min.**	<1/2	1/2	1/8	1/16
	30 min.	<1/2	1/2	1/16	1/16
	2 hrs.	<1/2	1/8	1/64	1/64
	18 hrs.	<1/2	1/32	1/128	1/128
1.5 % N-9	**10 min.**	<1/2	1/2	1/8	1/16
	30 min.	<1/2	1/4	1/16	1/16
	2 hrs.	<1/2	1/4	1/64	1/32
	18 hrs.	<1/2	1/64	1/128	1/64
2 % N-9	**10 min.**	<1/2	1/2	1/8	1/16
	30 min.	<1/2	1/2	1/16	1/16
	2 hrs.	<1/2	1/8	1/64	1/32
	18 hrs.	<1/2	1/64	1/128	1/64

Table 18: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Escherichia coli* (0.02M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	1/4	1/8	1/16
	30 min.	<1/2	1/4	1/16	1/32
	2 hrs.	<1/2	1/8	1/32	1/64
	18 hrs.	<1/2	1/32	1/64	1/128
1% N-9	**10 min.**	<1/2	1/4	1/8	1/8
	30 min.	<1/2	1/4	1/16	1/16
	2 hrs.	<1/2	1/8	1/32	1/32
	18 hrs.	<1/2	1/16	1/64	1/128
1.5 % N-9	**10 min.**	<1/2	1/4	1/8	1/16
	30 min.	<1/2	1/4	1/16	1/32
	2 hrs.	<1/2	1/8	1/32	1/64
	18 hrs.	<1/2	1/32	1/64	1/128
2 % N-9	**10 min.**	<1/2	1/4	1/8	1/16
	30 min.	<1/2	1/4	1/16	1/16
	2 hrs.	<1/2	1/8	1/32	1/64
	18 hrs.	<1/2	1/16	1/64	1/128

Table 19: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Escherichia coli* (0.01M citrate / 0.01M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	1/4	1/8
	30 min.	<1/2	1/4	1/4	1/16
	2 hrs.	<1/2	1/16	1/16	1/32
	18 hrs.	<1/2	1/32	1/32	1/64
1% N-9	**10 min.**	<1/2	1/4	1/8	1/4
	30 min.	<1/2	1/4	1/16	1/8
	2 hrs.	<1/2	1/16	1/16	1/16
	18 hrs.	<1/2	1/32	1/64	1/64
1.5 % N-9	**10 min.**	<1/2	1/2	1/4	1/8
	30 min.	<1/2	1/4	1/8	1/16
	2 hrs.	<1/2	1/8	1/16	1/32
	18 hrs.	<1/2	1/32	1/32	1/64
2 % N-9	**10 min.**	<1/2	1/2	1/8	1/8
	30 min.	<1/2	1/4	1/16	1/16
	2 hrs.	<1/2	1/8	1/32	1/32
	18 hrs.	<1/2	1/16	1/32	1/64

Table 20: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Streptoccoccus agalactiae* (0.01M citrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	<1/2	1/8
	30 min.	<1/2	<1/2	1/8	1/16
	2 hrs.	<1/2	<1/2	1/16	1/32
	18 hrs.	<1/2	1/4	1/128	1/128
1% N-9	**10 min.**	<1/2	1/8	1/16	1/16
	30 min.	<1/2	1/16	1/16	1/32
	2 hrs.	<1/2	1/16	1/32	1/32
	18 hrs.	<1/2	1/16	1/128	1/128
1.5 % N-9	**10 min.**	<1/2	1/8	1/32	1/16
	30 min.	<1/2	1/8	1/32	1/32
	2 hrs.	<1/2	1/8	1/64	1/64
	18 hrs.	<1/2	1/32	1/128	1/128
2 % N-9	**10 min.**	<1/2	1/8	1/8	1/16
	30 min.	<1/2	1/8	1/16	1/32
	2 hrs.	<1/2	1/8	1/32	1/32
	18 hrs.	<1/2	1/64	1/128	1/128

Table 21: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Streptoccoccus agalactiae* (0.02M citrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	1/8	1/4
	30 min.	<1/2	1/4	1/8	1/8
	2 hrs.	<1/2	1/16	1/16	1/8
	18 hrs.	<1/2	1/32	1/64	1/128
1% N-9	**10 min.**	<1/2	1/2	1/8	1/8
	30 min.	<1/2	1/8	1/8	1/8
	2 hrs.	<1/2	1/16	1/32	1/16
	18 hrs.	<1/2	1/16	1/64	1/64
1.5 % N-9	**10 min.**	<1/2	<1/2	1/16	1/16
	30 min.	<1/2	1/8	1/16	1/16
	2 hrs.	<1/2	1/16	1/64	1/16
	18 hrs.	<1/2	1/16	1/64	1/64
2 % N-9	**10 min.**	<1/2	1/2	1/16	1/16
	30 min.	<1/2	1/8	1/32	1/16
	2 hrs.	<1/2	1/16	1/64	1/32
	18 hrs.	<1/2	1/32	1/64	1/128

Table 22: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Streptoccoccus agalactiae* (0.01M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	<1/2	1/8	1/8
	30 min.	<1/2	1/2	1/8	1/16
	2 hrs.	<1/2	1/4	1/64	1/32
	18 hrs.	<1/2	1/64	1/128	1/64
1% N-9	**10 min.**	<1/2	1/2	1/8	1/16
	30 min.	<1/2	1/4	1/8	1/16
	2 hrs.	<1/2	1/16	1/64	1/32
	18 hrs.	<1/2	1/64	1/128	1/64
1.5 % N-9	**10 min.**	<1/2	1/4	1/8	1/16
	30 min.	<1/2	1/8	1/16	1/16
	2 hrs.	<1/2	1/16	1/64	1/32
	18 hrs.	<1/2	1/128	1/128	1/64
2 % N-9	**10 min.**	<1/2	1/4	1/8	1/16
	30 min.	<1/2	1/8	1/16	1/16
	2 hrs.	<1/2	1/16	1/64	1/64
	18 hrs.	<1/2	1/128	1/128	1/128

Table 23: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Streptoccoccus agalactiae*(0.02M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	1/2	1/4	1/8
	30 min.	<1/2	1/2	1/8	1/16
	2 hrs.	<1/2	1/4	1/32	1/64
	18 hrs.	<1/2	1/16	1/64	1/128
1% N-9	**10 min.**	<1/2	1/4	1/8	1/8
	30 min.	<1/2	1/8	1/16	1/16
	2 hrs.	<1/2	1/16	1/32	1/32
	18 hrs.	<1/2	1/32	1/128	1/128
1.5 % N-9	**10 min.**	<1/2	1/8	1/8	1/16
	30 min.	<1/2	1/8	1/16	1/16
	2 hrs.	<1/2	1/16	1/32	1/64
	18 hrs.	<1/2	1/32	1/64	1/128
2 % N-9	**10 min.**	<1/2	1/8	1/16	1/16
	30 min.	<1/2	1/16	1/16	1/32
	2 hrs.	<1/2	1/16	1/32	1/64
	18 hrs.	<1/2	1/32	1/64	1/128

Table 24: Minimum Inhibitory Concentrations, the lowest concentration of the sample that inhibits *Streptoccoccus agalactiae* (0.01M citrate / 0.01M tartrate buffer)

		MIC Values			
% N-9	**time**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**10 min.**	<1/2	1/2	1/4	1/4
	30 min.	<1/2	1/4	1/4	1/16
	2 hrs.	<1/2	1/16	1/16	1/32
	18 hrs.	<1/2	1/32	1/32	1/64
1% N-9	**10 min.**	<1/2	1/2	1/4	1/8
	30 min.	<1/2	1/4	1/8	1/8
	2 hrs.	<1/2	1/8	1/16	1/32
	18 hrs.	<1/2	1/32	1/32	1/64
1.5 % N-9	**10 min.**	<1/2	1/2	1/8	1/4
	30 min.	<1/2	1/4	1/8	1/16
	2 hrs.	<1/2	1/16	1/16	1/32
	18 hrs.	<1/2	1/32	1/64	1/128
2 % N-9	**10 min.**	<1/2	1/2	1/4	1/8
	30 min.	<1/2	1/4	1/16	1/16
	2 hrs.	<1/2	1/8	1/32	1/32
	18 hrs.	<1/2	1/32	1/64	1/128

Sander Cramer Test

The spermicidal activity for each gel preparation was evaluated from three male donors using the Sander Cramer Test.

Gels that contained no nonoxynol-9 in the formula were negative for spermicidal activity as well as the ability to immobilize sperm. Most of these gels have values reported at <1/4, which is the dilution of the sample, for all three sperm samples.

Gel samples containing the maximum 2.0 g N-9 in the formulation produced values of up to 1/256 in some gels with 0.01M citrate buffers and 0.02M citrate buffers. Gels produced with 0.01M tartrate buffer, 0.02M tartrate buffer, and 0.01M citrate/0.01M tartrate buffers produced dilution values of up to 1/64 that exhibited spermicidal activity against the three sperm samples (Tables 25-29).

Choosing a Single Gel

When trying to decide on one specific formulation to use in clinical trials, the antimicrobial, spermicidal, and viscosity factors of the gels were evaluated. After reviewing all the data, the gel sample with the best results in these three categories was the formulation that contained 0.9% hydrogen peroxide, 1.5% N-9, and a 0.01M citrate buffer base gave the greatest results when taking antimicrobial, spermicidal, and viscosity values into consideration. In addition, the viscosity value determined for this gel was 77,200 cP. This formulation was then chosen to be the template for future patient trials at University of Osteopathic Medicine and Health Sciences in Des Moines, Iowa.

Table 25: Sander Cramer Test results for minimum concentrations (expressed as dilutions) of gel formulas that are spermicidal (0.01M Citrate Buffer Gels)

*AGM refers to Average Geometric Mean, which is 1 divided by 2 to the xth power, x = AGM.

		Lowest Spermicidal Dilution			
% N-9	**Donors**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**Donor 1**	<1/4	<1/4	<1/4	1/32
	Donor 2	<1/4	<1/4	<1/4	<1/4
	Donor 3	<1/4	<1/4	<1/4	<1/4
	AGM*	<2	<2	<2	3
1% N-9	**Donor 1**	1/32	1/64	1/128	1/64
	Donor 2	1/32	1/64	1/64	1/64
	Donor 3	1/32	1/32	1/64	1/32
	AGM*	5	5.67	6.33	5.67
1.5 % N-9	**Donor 1**	1/64	1/64	1/256	1/64
	Donor 2	1/64	1/64	1/128	1/64
	Donor 3	1/32	1/32	1/128	1/64
	AGM*	5.67	5.67	7.33	6
2 % N-9	**Donor 1**	1/256	1/128	1/128	1/64
	Donor 2	1/128	1/128	1/128	1/64
	Donor 3	1/64	1/64	1/64	1/32
	AGM*	7	6.67	6.67	6

Table 26: Sander Cramer Test results for minimum concentrations (expressed as dilutions) of gel formulas that are spermicidal (0.02M Citrate Buffer Gels)

*AGM refers to Average Geometric Mean, which is 1 divided by 2 to the xth power, x = AGM.

		Lowest Spermicidal Dilution			
% N-9	**Donors**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**Donor 1**	<1/4	1/8	1/16	1/32
	Donor 2	<1/4	<1/4	<1/4	<1/4
	Donor 3	<1/4	<1/4	<1/4	<1/4
	AGM*	<2	2.33	2.67	3
1% N-9	**Donor 1**	1/64	1/64	1/64	1/128
	Donor 2	1/64	1/64	1/64	1/64
	Donor 3	1/32	1/32	1/32	1/64
	AGM*	5.67	5.67	5.67	6.33
1.5 % N-9	**Donor 1**	1/128	1/64	1/128	1/128
	Donor 2	1/128	1/64	1/128	1/64
	Donor 3	1/32	1/32	1/64	1/64
	AGM*	6.33	5.67	6.67	6.33
2 % N-9	**Donor 1**	1/64	1/64	1/256	1/128
	Donor 2	1/64	1/64	1/128	1/128
	Donor 3	1/32	1/64	1/64	1/128
	AGM*	5.67	6	7	7

Table 27: Sander Cramer Test results for minimum concentrations (expressed as dilutions) of gel formulas that are spermicidal (0.01M Tartrate Buffer Gels)

*AGM refers to Average Geometric Mean, which is 1 divided by 2 to the xth power, x = AGM.

		Lowest Spermicidal Dilution			
% N-9	**Donors**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**Donor 1**	<1/4	<1/2	<1/2	<1/2
	Donor 2	<1/4	<1/2	<1/2	<1/2
	Donor 3	<1/4	<1/2	<1/2	<1/2
	AGM*	<2	<1	<1	<1
1% N-9	**Donor 1**	1/32	1/32	1/32	1/16
	Donor 2	1/32	1/16	1/32	1/16
	Donor 3	1/16	1/32	1/32	1/32
	AGM*	4.67	4.67	5	4.33
1.5 % N-9	**Donor 1**	1/128	1/16	1/32	1/32
	Donor 2	1/64	1/16	1/32	1/64
	Donor 3	1/32	1/32	1/32	1/32
	AGM*	6	4.33	5	5.33
2 % N-9	**Donor 1**	1/256	1/32	1/32	1/32
	Donor 2	1/256	1/16	1/32	1/64
	Donor 3	1/128	1/32	1/32	1/32
	AGM*	7.67	4.67	5	5.33

Table 28: Sander Cramer Test results for minimum concentrations (expressed as dilutions) of gel formulas that are spermicidal (0.02M Tartrate Buffer Gels)

*AGM refers to Average Geometric Mean, which is 1 divided by 2 to the xth power, x = AGM.

		Lowest Spermicidal Dilution			
% N-9	**Donors**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**Donor 1**	<1/2	<1/2	<1/2	<1/2
	Donor 2	<1/2	<1/2	<1/2	<1/2
	Donor 3	<1/2	<1/2	<1/2	<1/2
	AGM*	<1	<1	<1	<1
1% N-9	**Donor 1**	1/16	1/32	1/32	1/16
	Donor 2	1/16	1/32	1/16	1/16
	Donor 3	1/32	1/32	1/32	1/16
	AGM*	4.33	5	4.67	4
1.5 % N-9	**Donor 1**	1/32	1/16	1/32	1/32
	Donor 2	1/16	1/32	1/16	1/32
	Donor 3	1/16	1/16	1/32	1/32
	AGM*	4.33	4.33	4.67	5
2 % N-9	**Donor 1**	1/32	1/16	1/32	1/32
	Donor 2	1/16	1/32	1/16	1/16
	Donor 3	1/32	1/32	1/32	1/32
	AGM*	4.67	4.67	4.67	4.67

Table 29: Sander Cramer Test results for minimum concentrations (expressed as dilutions) of gel formulas that are spermicidal (0.01M Citrate / 0.01M Tartrate Buffer Gels)

*AGM refers to Average Geometric Mean, which is 1 divided by 2 to the xth power, x = AGM.

		Lowest Spermicidal Dilution			
% N-9	**Donors**	**0% H_2O_2**	**0.3% H_2O_2**	**0.9% H_2O_2**	**1.2% H_2O_2**
0% N-9	**Donor 1**	<1/2	<1/2	<1/2	<1/2
	Donor 2	<1/2	<1/2	<1/2	<1/2
	Donor 3	<1/2	<1/2	<1/2	<1/2
	AGM*	<1	<1	<1	<1
1% N-9	**Donor 1**	1/16	1/32	1/16	1/32
	Donor 2	1/16	1/16	1/16	1/16
	Donor 3	1/32	1/32	1/32	1/32
	AGM*	4.33	4.67	4.33	4.67
1.5 % N-9	**Donor 1**	1/32	1/32	1/32	1/64
	Donor 2	1/64	1/32	1/16	1/64
	Donor 3	1/32	1/32	1/16	1/32
	AGM*	5.33	5	4.33	5.67
2 % N-9	**Donor 1**	1/64	1/64	1/32	1/32
	Donor 2	1/32	1/32	1/32	1/16
	Donor 3	1/32	1/32	1/64	1/32
	AGM*	5.33	5.33	5.33	4.67

DISCUSSION

A gel formulation containing 0.9% H_2O_2 and 1.5% N-9 in a 0.01M citrate buffered gel matrix produced favorable results in MIC and Sander-Cramer tests, along with having a stable viscosity at room temperature over several months. The concentrations of H_2O_2 and N-9 in the formula were not the highest possible concentrations. Even though the antimicrobial value of the gel was not as high as the 1.2% H_2O_2 concentration of the same N-9 concentration and buffer system, the spermicidal value was more favorable than the 1.2% H_2O_2 (Tables 20-24).

The viscosity of this gel was also slightly higher than other comparable products. This may suggest that the concentrations of N-9 and hydrogen peroxide used may be ideal for the addition to the 0.01M citrate buffer, which yielded higher viscosity and spermicidal activity than other systems (Table 5).

When comparing concentrations of N-9 with antimicrobial and spermicidal effectiveness, the 1.5% N-9 yielded higher values than 2% N-9 gels of the same 0.9% H_2O_2 concentration. This may be a favorable feature since N-9 has been linked to irritation and inflammation in the urogenital tract. Also, the activity of N-9 against normal vaginal flora may suggest that a smaller dose of N-9, still exhibiting spermicidal activity, may lessen *Lactobacillus* destruction. If the normal *Lactobacillus acidophilus* population could be maintained, the prevention of pathogenic activity from opportunists may result. *Lactobacillus acidophilus* contains a surfactant called surlactin. Surlactin has been shown to inhibit the adhesion of various uropathogenic bacteria, along with a few yeast strains (Velraeds et al., 1998). Surlactin was particularly effective against *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus epidermidis*.

Testing of this gel for its effects against *Lactobacillus* may later be performed to ensure the gel is not too potent. All patient testing will be performed at the University of Osteopathic Medicine and Health Sciences, in Des Moines, Iowa. Bryan Larsen, Ph.D., will oversee all clinical patient testing. Testing will be performed using at least 10 vaginal isolates of *Lactobacillus* obtained from women in clinical trials (Larsen, 1998).

The normal flora of the vaginal tract also maintains the pH by producing lactic acid. Lactic acid is produced by *Lactobacillus* from a natural glucose polymer called glycogen, produced by the vaginal mucosa. Glycogen is produced by the mucosa during the reproductively active years of females, when estrogen is present. Once a woman reaches menopause and estrogen production stops, pH levels return to a neutral level, similar to the skin. Due to this occurrence, urogenital pathogens are a problem with post-menopausal females. Therefore, a spermicidal gel that can maintain a pH level of 4.0 - 4.2 may be useful to post-menopausal females in the prevention of bacterial vaginitis, candidiasis and trichomoniasis.

Another important characteristic of the gel may be its activity against the HIV virus. The National Institutes of Health will test the efficacy of the gel against HIV. The potential of the gel should be favorable, considering the gel contains an active concentration of N-9, which is toxic to HIV, as well as hydrogen peroxide, which has been shown to significantly suppress HIV type 1 virus infection (Ranjbar and Holmes, 1996). Four gel products consisting of the buffered hydrogel, the gel plus the 0.9% H_2O_2, the gel plus the 1.5% N-9, and a gel with both N-9 and H_2O_2, will be tested.

Once the gel formulation was selected based on its physical and chemical properties, the N-9 and H_2O_2 concentrations need to be evaluated in a patient trial consisting of 40 volunteers. Ten will

use the buffered hydrogel base, ten will use the gel with 0.9% H_2O_2, ten will use the gel with 1.5% N-9, and ten will use the full formulation.

A product dosage of 5 g per day will be used for one week. Patient vaginal flora will be studied at pre-dosage and at post-dosage times to compare effects of the gels. Also, possible patient complaints of irritation and inflammation of the vaginal tract may be noted, thus allowing for changes in gel formulas to alleviate these side-effects. Neither the physician-investigator nor the patient will know the composition of the product assigned.

In the future, post-coital testing involving the same four gel samples may be performed on couples who consent to participate. Women participating in the study must be unable to conceive and must have previously undergone a successful tubal ligation more than six months before the study. Initial exams will be performed, to rule out initial cases of vaginitis or infection. The female partner will insert the product intravaginally within 30 minutes of intercourse. Following this, she will return to the clinic where cervical mucus will be sampled and evaluated for the presence of viable sperm (Larsen, 1998).

The goal of pursuing the proposed grant was to create a potential product that allows women to protect their own health. Male condoms, when used correctly, are the standard by which other barriers are compared. However, many women may not be able to negotiate condom use. These women need a barrier that they can use to protect themselves and their partners. The use of the gel formula in question should require little or no cooperation from a male partner.

Also, the low cost of the components makes it a reasonable product for use in developing and third world countries, including those in which STD's are substantial, and which cause an asymmetrical threat to females and their offspring. The relatively low component cost should

facilitate a cost effective STD barrier to a wide socio-economic gambit of females, and could be financially available in poorer countries.

Ideally, the future may hold vaccines or other effective methods of STD prevention, but for now, new female-controlled topical microbicides/spermicides are an urgent priority. A small change in STD trends in worldwide populations resulting from a new STD barrier could have an important impact on world health, especially in women. Therefore, the importance of a viable STD barrier for women should be a global concern.

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APPENDIX

Appendix A: Procedure for the preparation of 0.01M Citrate Buffer Solution

0.01M CITRATE BUFFER

BUFFER PREPARATION

1. Using a 1000 mL graduated cylinder, fill a 4 L bottle with 2.0 L distilled water.

2. Using an analytical balance, weigh 4.20 g of citric acid monohydrate, and transfer to the 4 L bottle.

3. Using an analytical balance, weigh 5.88 g of sodium citrate, and transfer to 4 L bottle.

4. Mix thoroughly using the Digitrate Stirrer set at 200 RPM.

5. Measure pH of buffer using Orion portable pH meter. pH reading_____

6. Label bottle with name and pH.

Signature of technician_____ Date_____

Appendix A(cont.): Procedure for the preparation of 0.02M Citrate Buffer Solution

0.02M CITRATE BUFFER

BUFFER PREPARATION

1. Using a 1000 mL graduated cylinder, fill a 4 L bottle with 2.0 L distilled water.

2. Using an analytical balance, weigh 8.41 g of citric acid monohydrate, and transfer to the 4 L bottle.

3. Using an analytical balance, weigh 11.76 g of sodium citrate, and transfer to 4 L bottle.

4. Mix thoroughly using the Digitrate Stirrer set at 200 RPM.

5. Measure pH of buffer using Orion portable pH meter. pH reading_____

6. Label bottle with name and pH.

Signature of technician_____ Date_____

Appendix A(cont.): Procedure for the preparation of 0.01M Tartrate Buffer Solution

0.01M TARTRATE BUFFER

BUFFER PREPARATION

1. Using a 1000 mL graduated cylinder, fill a 4 L bottle with 2.0 L distilled water.
2. Using an analytical balance, weigh 3.00 g of tartaric acid, and transfer to the 4 L bottle.
3. Using an analytical balance, weigh 4.60 g of sodium tartrate, and transfer to 4 L bottle.
4. Mix thoroughly using the Digitrate Stirrer set at 200 RPM.
5. Measure pH of buffer using Orion portable pH meter. pH reading_____
6. Label bottle with name and pH.

Signature of technician_____ Date_____

Appendix A(cont.): Procedure for the preparation of 0.02M Tartrate Buffer Solution

0.02M TARTRATE BUFFER

BUFFER PREPARATION

1. Using a 1000 mL graduated cylinder, fill a 4 L bottle with 2.0 L distilled water.
2. Using an analytical balance, weigh 6.00 g of tartaric acid, and transfer to the 4 L bottle.
3. Using an analytical balance, weigh 9.20 g of sodium tartrate, and transfer to 4 L bottle.
4. Mix thoroughly using the Digitrate Stirrer set at 200 RPM.
5. Measure pH of buffer using Orion portable pH meter. pH reading_____
6. Label bottle with name and pH.

Signature of technician_____ Date_____

Appendix A(cont.): Procedure for the preparation of 0.01M Citrate/ 0.01M Tartrate Buffer Solution

0.01M CITRATE/ 0.01M TARTRATE BUFFER

BUFFER PREPARATION

1. Using a 1000 mL graduated cylinder, fill a 4 L bottle with 2.0 L distilled water.
2. Using an analytical balance, weigh 4.20 g of citric acid monohydrate, and transfer to the 4 L bottle.
3. Using an analytical balance, weigh 5.88 g of sodium citrate, and transfer to 4 L bottle.
4. Using an analytical balance, weigh 3.00 g of tartaric acid, and transfer to the 4 L bottle.
5. Using an analytical balance, weigh 4.60 g of sodium tartrate, and transfer to 4 L bottle.
6. Mix thoroughly using the Digitrate Stirrer set at 200 RPM.
7. Measure pH of buffer using Orion portable pH meter. pH reading_____
8. Label bottle with name and pH.

Signature of technician_____ Date_____

Appendix B: Orion 250A pH Meter Calibration Sheet

CALIBRATION SHEET
ORION 250A PORTABLE pH METER

1. Turn on pH meter by pressing the **POWER** button.

2. Switch Orion 900A printer to **ON** position.

3. Press **MODE** key until pH mode is indicated.

4. Place electrode into 7.00 pH buffer.

5. Press the **2nd** key, then the **CAL** key. "P1" will be displayed in the lower field. When the electrode is stable, "READY" will be displayed and the temperature-corrected value for the buffer is displayed. "P2" will then be displayed in the lower field.

6. Rinse electrode and place into 4.01 buffer. Wait for the stable pH reading and then press **YES**.

7. A calibration printout is then generated. Attach printout to worksheet.

Buffer 1:_____ Buffer 2:_____

Temperature:_____ Temperature:_____

Slope:_____

Signature of technician_____ Date_____

Appendix C: Worksheet for the preparation of gels.

GEL PREPARATION WORKSHEET

_____BUFFER SYSTEM **7.0 g GLYCEROL**

_____% HYDROGEN PEROXIDE **2.0 g CARBOPOL**

_____% NONOXYNOL-9

1. Fill in appropriate concentration for buffer system, hydrogen peroxide, and nonoxynol-9 in the blanks above.

2. Determine amount of each component to formulate a 100 g sample.

   _____% x 100mL / 30% = _____g hydrogen

   _____% x 100mL = _____g nonoxynol-9

   7.0 g glycerol
   2.0 g carbopol

   Above Total (in grams) = _____g

   Amount buffer (100g - above total) = _____g

3. Add determined amount of buffer to 140 mL sterile specimen cup.

4. Add determined amount of glycerol to 140 mL sterile specimen cup.

5. Add determined amount of hydrogen peroxide to 140 mL sterile specimen cup.

6. Add determined amount of nonoxynol-9 to 140 mL sterile specimen cup.

7. Mix on Thermolyne stirrer at 300 RPM until homogenized (approximately 20 minutes.)

8. In 20 minute increments, add 0.5 g of carbopol to mixture at each increment until determined amount of carbopol has been added. Continue to stir at 300 RPM.

9. Once mixture has been homogenized, adjust pH to 4.0-4.2 with 3.0 N sodium hydroxide.

10. Final pH reading of sample_____

Signature of technician_____ Date_____

Appendix D: Gel Viscosity Worksheet

VISCOSITY WORKSHEET

SAMPLE NAME_____ Tracking #_____

_____

1. Power **ON** Helipath Stand.
2. Power **ON** Brookfield Viscometer.
3. Remove Spindle. Press any key.
4. Attach Helipath Spindle 'A' and set spindle code at **S91**.
5. Place spindle approximately 3/4 inch below the surface of 5000 viscosity standard.
6. Insert temperature probe, turn motor **ON**, and set speed at 1.5 RPM.
7. Determine value for viscosity standard 5000.

   Reading of Standard_____ Certified Value_____
8. Turn motor off, remove temperature probe.
9. Rinse spindle with water, and replace standard with sample gel.
10. Insert spindle, insert temperature probe, and turn motor **ON**.
11. Set measuring time for 10 minutes.
12. Determine viscosity value for samples.

_____cP _____RPM

_____% Torque _____Temp.

Signature of technician_____ Date_____

Appendix E: Procedure for the Determination of Hydrogen Peroxide in Gels by Titration

MICROBIOLOGICAL CONSULTANTS, INC.
605 Ninth Street, Suite 101
Huntington, West Virginia 25701
(304) 522-6438 – Fax (304) 522-3396

R. E. Gain, Ph.D. F. L. Binder, Ph.D.

STANDARD OPERATING PROCEDURE

Date Issued: *08-12-98 (Original Issue)* **Supercedes:** *N/A*	**Page** *1 of 2*
Title: *Determination of Hydrogen Peroxide (H_2O_2) by Titration*	
Products: *Hydrogen Peroxide, 30%* *Gels containing 0.3% H_2O_2* *Gels containing 0.9% H_2O_2* *Gels containing 1.2% H_2O_2*	Analyte Range: *29 -32 %* *0.25 - 0.35 %* *0.81 - 0.99 %* *1.10 - 1.30 %*
Originator: Paul E. Grimmett	**Approval Signature:**

1.0 SCOPE

This method is used to determine percent hydrogen peroxide in 30% hydrogen peroxide bulk and spermicidal gels (finished product.)

2.0 REAGENTS

2.1 Potassium permanganate, 0.2 N
2.2 Sulfuric Acid, 2.0 N
2.3 Distilled water

3.0 EQUIPMENT AND MATERIALS

3.1 Digitrate Digital Titrator
3.2 1400 mL titration bottle
3.3 100 mL volumetric flask

Date Issued: *08-12-98 (Original Issue)* **Supercedes:** *N/A*	**Page** *2 of 2*
Title: *Determination of Hydrogen Peroxide (H_2O_2) by Titration*	

3.4 150 mL beaker
3.5 1 mL and 10 mL graduated pipettes

4.0 PROCEDURE

4.1 Sample Weight

4.1.1 30% H_2O_2 bulk - 0.5 g into 100 mL volumetric flask.
4.1.2 Gel samples - 5.0 g into 100 mL volumetric flask.

4.2 Bring to volume with distilled water. Mix well.

4.3 Transfer 20mL sample dilution to 150 mL beaker.

4.4 Add 20 mL 2.0N sulfuric acid to 150 mL beaker.

4.5 Titrate using Digitrate titrator and 0.2 N potassium permanganate as the titrant.

4.6 Titrate until a permanent light pink color forms.

5.0 CALCULATIONS

$$\%H_2O_2 = \frac{\text{amount titrant x 0.2N x 0.01701 x 100}}{\text{weight of sample}}$$

6.0 REFERENCES

6.1 USP 23, NF 18, 1995.

Appendix F: Hydrogen Peroxide Titration Worksheet

HYDROGEN PEROXIDE TITRATION WORKSHEET

TRACKING #_____

1. Weight of sample _____g

2. Volume of titrant (potassium permanganate)used _____mL

3. Formula for hydrogen peroxide (H_2O_2) concentration:

$$\frac{\text{Titrant used} \times 0.2\ \text{N} \times 1.701}{\text{weight of sample}} = \%\ H_2O_2$$

4. Results:

(_____mL KMnO4 × 0.2 × 1.701) ÷ _____g sample = _____%H_2O_2

5. Sample concentration _____% H_2O_2

   Specification range _____% H_2O_2

   Sample within range _____yes _____no

Signature of technician_____ Date_____

Appendix G: Nonoxynol-9 Calculation Worksheet

NONOXYNOL-9 CALCULATION WORKSHEET

Tracking #_____

0.01% Standard value peak area(from HPLC)_____

0.02% Standard value peak area(from HPLC)_____

Are values linear through zero? _____Yes _____No

CONCENTRATION NONOXYNOL-9 =

VALUE SAMPLE / VALUE STANDARD × STANDARD CONC. × DILUTION FACTOR

_____/ _____ × _____ × 100 =

_____% N-9

Actual Conc. N-9 _____%

Specification range _____

Sample within spec? _____Yes _____No

Signature of technician_____ Date_____

Appendix H: Procedure for the Determination of Nonoxynol-9 in Gels by HPLC

MICROBIOLOGICAL CONSULTANTS, INC.
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R. E. Gain, Ph.D. F. L. Binder, Ph.D.

STANDARD OPERATING PROCEDURE

Date Issued: *08-12-98 (Original Issue)* **Supercedes:** *N/A*	**Page** *1 of 4*
Title: *Determination of Nonoxynol-9 (N-9) by HPLC*	
Products: *1% N-9 Gels* *1.5% N-9 Gels* *2.0% N-9 Gels*	Analyte Range: *0.90 - 1.10 %* *1.35 - 1.65 %* *1.80 - 2.20 %*
Originator: Paul E. Grimmett	**Approval Signature:**

1.0 SCOPE

This method is used to determine percent of Nonoxynol-9 in finished gel products.

2.0 REAGENTS

2.1 Nonoxynol-9, standard grade
2.2 Methanol, HPLC grade
2.3 Distilled water

3.0 EQUIPMENT AND MATERIALS

3.1 HPLC - Rainin Solvent Delivery System and Monitor
Knauer Photometer Detector (254 nm λ)
Hewlett Packard HP3394A Integrator
C18, 15cm Microsorb-MV HPLC Column

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Title: *Determination of Nonoxynol-9 (N-9) by HPLC*	

3.2 Glassware
100 mL volumetric flasks
1 mL and 10 mL graduated pipettes
17 mL sample vials

4.0 PROCEDURE

4.1 STANDARD PREPARATION

4.1.1 Prepare 0.01% and 0.02% N-9 standards..

1% N-9 stock standard
Dilute 1.00 g of N-9 stock standard to 100 mL with MeOH

0.01% N-9 standard
Solution 1: Dilute 1.0 mL of 1% N-9 to 10 mL with MeOH.

Solution 2: Dilute 1.0 mL of Solution 1 to 10 mL with MeOH This solution contains 0.01% N-9.

0.02% N-9 standard
Solution 1: Dilute 2.0 mL of 1% N-9 to 10 mL with MeOH

Solution 2: Dilute 1.0 mL of Solution 1 to 10 mL with MeOH This solution contains 0.02% N-9.

4.1.2 Perform system suitability test before analysis of samples.

4.2 SYSTEM SUITABILITY TEST

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Title: *Determination of Nonoxynol-9 (N-9) by HPLC*	

4.2.1 Inject all standards and samples using the following conditions:

Mobile Phase: 90:10 MeOH:H_2O Injection volume: 20 µL
Mobile Phase pH: 4.0 Attenuation: 3
Flow Rate: 1.5 mL/min. Range: 0.32

4.2.2 Determine stable baseline and baseline resolution of all peaks. Adjust flow, attenuation, and/or range to achieve a suitable chromatogram.

4.2.3 Determine linearity of the calibration standards 0.01% and 0.02% N-9 using the measured peak areas (and a zero blank) from the HP integrator.

4.3 SAMPLE PREPARATION

4.3.1 Sample Solution 1: Dilute 1.0 g sample (measure to nearest 0.001 g) to 10 mL volume with MeOH.

Sample Solution 2: Dilute 1 mL Sample Solution 1 to 10 mL volume with MeOH. Filter, and inject into HPLC.

4.3.2 Analyze the samples using the same chromatographic conditions determined for the system suitability tests in section 4.2.

5.0 CALCULATIONS

5.1 Single standard calibration method:

% N-9 = [sample area ÷ standard area] × standard conc.(%) × 100

Date Issued: *08-12-98 (Original Issue)* **Supercedes:** *N/A*	**Page** *4 of 4*
Title: *Determination of Nonoxynol-9 (N-9) by HPLC*	

6.0 REFERENCES

6.1 Microbiological Consultants, Inc., developed method.