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EFFECT OF SOME ANTIMYCOTIC AGENTS ON VIABILITY OF CHILLED AND FROZEN RAM SEMEN

(With 5 Tables and 5 Figures)

By

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تأثير بعض مضادات الفطريات على حيوية السائل المنوي المبرد والمجمد في الكباش

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تم تجميع عينات سائل منوي من خمس كباش حيث خففت في مخفف الترس الخالي من أو المضاف إليه خمس مضادات للفطريات بتركيزات مختلفة لاميزيل (١٠, ٢٠ أو ٣٠ ميكروجرام / مللي) , دفلوكان (٣٠, ٤٠ أو ٥٠ ميكروجرام/مللي) , جريزفولفين و ميكوناز ونيزورال (٥٠, ٦٠ أو ٧٠ ميكروجرام/مللي). وبعد تبريد وتجميد السائل المنوي بالنظام الفرنسي تم تقييم عينات السائل المنوي من حيث النسبة المئوية بعد التخفيف, وبعد الإسالة, وتشوهات القلنسوة و كذلك حيوية السائل المنوي بعد حفظه في درجة ٥ مئوية, وبعد التجميد لمدة ٧٢ ساعة. كذلك خضع السائل المنوي والمجمد ومخفف الترس للفحص فطريا. أسفرت نتائج العزل أن مجموعة الأسبراجلس كانت هي الأكثر شيوعا في السائل المنوي والسائل المنوي المجمد وكذلك مخفف الترس بينما كان تصنيع السائل المنوي هو المصدر الرئيسي للتلوث بمجموعة الفيوزاريم والميوكر والبنسليم والكلادسبوريم والكنديدا. كما أسفرت النتائج عن الدور الفعال لإضافة اللاميزيل (١٠ ميكروجرام /مللي) والدفلوكان (٥٠ ميكروجرام/مللي) في القضاء على الفطريات داخل السائل المنوي المجمد. لذلك يوصى باستخدام أي منها بأمان كمضادات للفطريات بالإضافة إلى المضادات الحيوية المستخدمة في السائل المنوي المخفف و المجمد للكباش دون أن يكون لها تأثير سلبي على حيوية السائل أو سلامة القلنسوة.

SUMMARY

Semen samples were collected from five rams aged 3- 4 years. They were pooled and diluted in Tris based extender supplemented with or without Lamisil (10, 20 or 30 µg/ ml), Diflucan (30, 40 or 50 µg/ ml),

Griseofulvin, Miconaz and Nizoral (50, 60 or 70 µg/ ml). Cooling and freezing of extended semen were done using IVM system. Percentages of motility after dilution, post thawing and acrosomal integrity as well as viability index of post thawing samples and at 5°C for 72 hours were subjectively assessed. Raw semen, frozen semen and Tris based extender examined mycologically. Mycotic examination revealed that Aspergilli were the most mycotic contaminant of raw semen and frozen semen as well as Tris based extender while the semen processing were the main source of Fusarium sp., Mucor sp., Penicillium sp., Cladosporium and Candida sp. contaminant of frozen semen. The obtained results proved that Lamisil (10 µg/ ml) or Diflucan (50 µg/ ml) were the antimycotics of choice to be used safely as antimycotic agents in combination with antibiotic regime to control the microbial contamination of frozen ram semen as they overcame the fungal infection during semen processing without interference with semen viability and acrosomal integrity.

Key words: *Sheep, semen, antimycotic and preservation.*

INTRODUCTION

The rate of semen contamination with different microorganisms became highly detectable with the widespread application of artificial insemination technology. A vast array of bacteria can grow on semen during processing (Sidhu *et al.*, 1997b). The addition of antibiotics to semen extenders prevent bacterial growth but they have no effect on mould or yeast contaminated semen (Champak *et al.*, 1996).

Yeast and mould are unusual flora of ram semen but they contaminate the semen during processing and their growth is unaffected by the addition of antibiotics (Richard *et al.*, 1976 and Dion, 1979).

Nevertheless, the presence of those microorganisms in semen used for AI remains as a controversial topic in regard to their possible harmful effect on semen quality, fertilization and/or early embryonic development (Mbai *et al.*, 1996) and their capability to infect the inseminated female animals. Fungal infection of the ovine genital tract resulting in infertility have been described by Mbai *et al.*, 1996 and Kadry *et al.*, 2002. Also, several investigators found that the commonest cause of diagnosed abortions in bovine in some years was species of Asperigillus, Fusarium, Mucor, Penicillium and Candida (Sinha *et al.*, 1980; Mishra *et al.*, 1984 and Singh *et al.*, 1991). Therefore, the present study aimed to identify the environmental mycotic contamination of extended and frozen ram semen and to determine the possible

antimycotic agents and its effect on sperm motility, viability and acrosomal integrity.

MATERIALS and METHODS

Animals:

Sexually mature, clinically healthy five rams aged 3 – 4 years were used. They were raised on the experimental farm of Animal Reproduction Research Institute (ARRI), Giza, Egypt.

Antimycotic agents:

Five antimycotic agents were used in this experiment Lamisil (Terbinafin), Diflucan (Fluconazole), Nizoral (Ketoconazolum), Griseofulvin (Griseofulvin) and Miconaz (Miconazole nitrate).

Semen collection and extension:

Semen samples were collected from each ram using artificial vagina that was set up to proper conditions. Semen ejaculates obtained from all rams were pooled to yield one semen sample in each trial, only semen samples of at least 70% initial motility and $3000x10^6$ sperm/ml were used. Immediately after collection samples were split and diluted (1 part semen + 19 parts extender) in Tris based extender (Evans and Maxwell, 1987), supplemented with or without (control) Lamisil (10, 20 or 30 µg/ml), Diflucan (30, 40 or 50 µg/ml), Griseofulvin, Miconaz and Nizoral (50, 60 or 70 µg/ml).

Processing of ram semen:

For short- term chilled storage:

Immediately after dilution, the extended semen samples were kept in clean, narrow test tubes, then transferred into a beaker containing water at 30 °C and then placed in the refrigerator. The extended semen was stored for 72 hour. Sperm motility was subjectively assessed immediately after dilution as well as after 24, 48 and 72 hours of incubation. The viability index was calculated according to Ahmed *et al.* (1996).

For frozen storage:

Immediately after dilution, the extended semen was cooled to 5°C over a period of 60 minutes in a cold handling cabinet. The cooled semen was loaded into 0.25 ml French straws at 5°C. the straws were arranged horizontally on a cooled (5°C) freezing racks and lowered into liquid nitrogen vapour inside foam box (54 x 35 x 18 cm) for 15 minutes on a hight of 6.5 cm above the level of liquid nitrogen (Khalifa, 2001). Then the straws were immersed in liquid nitrogen and stored.

After one week, frozen ram semen was thawed in a water bath at 40°C for 30 seconds. Sperm motility was subjectively assessed immediately after dilution, after thawing as well as after 1, 2 and 3 hours of thawing. The post thaw viability index was calculated according to Milavanov (1962). The percentage of spermatozoa with abnormal acrosomes was recorded after thawing in smears stained by Fast green FCF according to Wells and Awa (1970).

Mycological examination:

Five samples of raw semen, twenty straws of frozen ram semen (control) and ninety straws of frozen semen supplemented with different concentrations of antimycotic agents as well as five samples of Tris based extender were examined mycologically. These mycological examinations were carried out in mycology lab of ARRI for the presence of different fungi.

Isolation and identification of fungi:

A loopful from each sample was streaked on Sabaroud dextrose agar plus chloramphenicol (0.05 mg/ml). Each sample was cultured separately onto two agar plates. One set of the plates were incubated at 25°C and other at 37°C, then mycotic growth was examined macroscopically and microscopically after 2-5 days of incubation. Identification of mould genus and species was done as described by Al-Doory, 1980 and Barnett *et al.*, 1983.

Antimycotic sensitivity tests:

Isolated strains of yeast and mould were tested for sensitivity to antimycotics using oxid multo disks code 1789E contains: Lamisil 10, 20, 30 µg, Diflucan 30, 40, 50 µg, Nizoral 50, 60, 70 µg, Miconaz 50, 60, 70 µg and Griseofulvin 50, 60, 70 µg.

Plates were incubated at 25 °C and the diameter of the inhibition zone was measured after 5 days of incubation. Inhibition zones were interpreted in accordance with criteria in table 4 which are based on the recommendation of Blair *et al.*, 1970.

Statistical analysis:

All data were statistically analyzed using Costat computer program, version 3.03 copyright (1986) cottort software.

RESULTS

It became clear from the present study that different types of antimycotic agents and different concentrations of them do not reveal any significant effect on motility percentage after dilution, post thaw and

reduction (Table 1) as well as the viability index of diluted semen incubated at 5°C for 72 hours (Table 2).

On the other hand, analysis of variance showed a significant effect of different types of antimycotic agents ($F= 2.65$, $P<0.05$) on the post thawing viability index of ram semen. While, no significant difference between different concentrations of them was detected.

Figures gathered in table (2) indicated that the significant improvement of post thawing viability index was detected with Lamisil, Diflucan and Griseofulvin (102.33± 7.66, 103.00±8.64 and 104.00± 5.72 respectively vs control 94.00± 6.59).

Moreover, the percentage of post thaw acrosomal defects affected by the different types of antimycotic agents. Diflucan, (16.13± 0.98) revealed low percentage of acrosomal defects followed by Lamisil, Griseofulvin and control (19.00±1.30, 20.60± 1.74 and 22.00±3.20 respectively). No detectable significant effect of different concentrations of antimycotic agents on percentage of acrosomal integrity was observed.

The mycotic contamination of raw and extended ram semen is represented in table (3). Mucor sp., Cladosporium sp. and Aspergillus niger were isolated from raw semen (50, 25 and 25% respectively). A. flavus, Fusarium sp., Penicillium sp. and Candida sp. were isolated from Tris diluent (43.33, 13.33, 16.67 and 26.67% respectively). A. flavus, A. achracus, A. niger, Fusarium sp., Penicillium sp., Candida sp., Cladosporium sp. and Mucor sp. were isolated from post thawing extended semen (25.62, 17.36, 16.53, 10.74, 9.92, 6.61, 4.96 and 8.26% respectively).

Drug sensitivity of mould and yeast isolated from contaminated ram semen is showed in table (4), most of the toxigenic strains, A. flavus, A. achracus, Fusarium sp., Penicillium sp. and Candida sp., were sensitive to Lamisil at low concentration (10μg), Diflucan at high concentration (50μg) and Nizoral at high concentration (70μg), while they were resistant to Miconaz and Griseofulvin.

The figures (1, 2, 3, 4 and 5) represent the sensitivity of Aspergillus flavus strains to different concentrations of antimycotic agents were illustrated.

Table (5) indicate mycological examination of frozen ram semen after addition of different concentrations of antimycotic agents, A. flavus, A. achracus, A. niger, Fusarium sp., Penicillium sp., Candida sp., Cladosporium sp. and Mucor sp. were not isolated with the addition of Lamisil at low concentration (10μg), Diflucan and Nizoral at high concentration (50 μg and 70 μg respectively), except A. niger. On the

other hand Miconaz and Griseofulvin were not effective at any concentrations.

DISCUSSION

The widespread presence of certain microorganisms in the environment can pose a potential reproductive problem when they contaminate ram semen. Although most of these organisms may be non-pathogenic, some are opportunistic pathogens such as Pseudomonas, Coryne bacterium pyogens, Streptococci, Staphylococci, certain anaerobes, mould and yeast (Wierzbowski, 1981; Eaglesome *et al.*, 1992; Mbai *et al.*, 1996 and Kadry *et al.*, 2002).

Semen collection and processing equipments, animal handlers, laboratory personal and semen extenders remain the main sources of semen contamination (Rick *et al.*, 1980 and Sidhu *et al.*, 1997b).

In the present study Mucor sp., Cladosporium sp. And Aspergillus niger were isolated from raw semen. In addition A. flavus, A. achracus, Fusarium sp, Penicillium sp and Candida sp. were isolated from Tris based extender and frozen ram semen (Table 3). These results agree with Sidhu *et al.*, 1997b and Kadry *et al.*, 1999, as they isolated Penicillium sp., Aspergillus sp. Fusarium sp. and Candida sp. from fresh extended and frozen buffalo semen. Aspergillus sp. considered the main fungi responsible for reproductive disorders, clinical mastitis as well as mycotic abortion (5% of all abortion cases) in livestock (Diekman and Grecor 1992 and Kundston and Kirkbride 1992). Moreover, many researchers have been discussed the toxigenic effect of aflatoxin secreted by some species of Aspergillus on semen performance. They recorded such toxin as a main cause of the significant decrease in sperm viability, percentage of live spermatozoa, sperm concentration and the increased sperm abnormalities of ram (Kadry *et al.*, 2002), buffalo (Hafez *et al.*, 1982), boar (Picka *et al.*, 1986), man and rat (Ibeh *et al.*, 1994) and rabbit (Salem *et al.*, 2001). Fusarium sp. was isolated from Tris based extender and frozen semen (13.33 and 10.74% respectively). Zearalenone mycotoxin is the major secretion of Fusarium species. It has a negative effect on animal reproductive performance as the semen become unfit for insemination. The reproductive effects seen with zearalenone were attributable to its estrogenic activity (Vanyi and Szeky, 1980). Regarding the sensitivity of the isolated fungi to the most common antimycotics, it was observable that most of A. flavus, A. achracus, Fusarium, Penicillium, Candida strains were sensitive to Lamisil, Diflucan and Nizoral while they were resistant to Miconaz and Griseofulvin (Table 4). These results relatively agree with the results

obtained by Kadry *et al.*, 1999 in bull semen. The resistance of most of the isolates to Miconaz and Griseofulvin is probably due to the indiscriminate use of these drugs for the treatment of mycotic troubles. (Johnston *et al.*, 1983). So, the presence of these microorganisms in ram semen provides a potential for reproductive problems after artificial insemination. Sidhu *et al.* (1997b) recommended the addition of antimycotic agents to processed semen used for artificial insemination. The present data revealed that the addition of antimycotic Lamisil (10μg/ ml), Diflucan (50 μg/ ml) or Nizoral (70 μg/ ml) was effective while Miconaz and Griseofulvin have no effect on fungal growth in frozen semen (Table 5).

Regardless to the effect of antimycotic agents on fungal growth, Lamisil (10μg/ ml) and Diflucan (50 μg/ ml) have a pronounced positive effect on the viability and frozen activities (post thaw viability index and post thaw acrosomal defects) without unfavorable interaction with other substances present in semen extender (Table 2). These results agree with (Kadry *et al.*, 1999), who reported that addition of Lamisil (10μg/ ml) to buffalo extended semen could control the mycotic contamination of processed buffalo semen without harmful effect on livability and acrosome of spermatozoa. Also, Sidhu *et al.* (1997a) reported that the use of (Nystatin) at 30 μg/ ml as antimycotic drug, prevent the growth of fungi without negative effect on buffalo semen. From the above mentioned results, it could be concluded that Lamisil (10μg/ml) or Diflucan (50 μg/ml) can be used safely as antimycotic agents in combination with antibiotic regime to control the microbial contamination of frozen ram semen.

REFERENCES

Ahmad, M.; Khan, A.; Shah, Z.A. and Ahmad, K.M. (1996): Effect of removal of seminal plasma on the survival rate of buffalo bull spermatozoa. Anim. Reprod. Sci., 41: 193.

Al–Doory, Y. (1980): Laboratory Medical Mycology. Lea Febiger Philadelphia, Kimpten publisher, London.

Barnett, J.A.; Payne, R.W. and Yarraw, D. (1983): Yeasts characteristics and Identification. 1st ed. Cambridge Univ. press. New Rochelle Melbourne. Sydney.

Blair, J.E.; Lennette, E.H. and Truant, J.P. (1970): Manual clinical microbiology. American Society for microbiology. Bethesda, Maryland 21202 USA.

Champak- B, Raina- VS, Nagpaul-LK andd Bhakat- C (1996): Effect of antibiotic treatment on bacterial load and preservability of child bovine semen. Ind. J. Anim. Reprod. Mang., 1996, 12: 2, 82.

Costat computer program copyright (1986): version 3.03 copyright software.

Diekman, M.A. and Grecor, M. L. (1992): Mycotoxin and reproduction in domestic livestock. J. Anim. Sci., 70 (5): 1615.

Dion, W.M. (1979): The origin and species of yeasts in commercial preparations of bovine semen. Can. J. Comp. Med. 43: 16.

Eaglesome, M.D.; Gorcia, M.M. and Stewart, R.B. (1992): Microbial agents associated with bovine genital tract infections and semen. Part II. Haemophillus semnus, Mycoplasma species, Ureaplasma species, Chlamydia psittaci pathogens and semen contaminants. Treatment of bull semen with antimicrobial agents. Vet. Bull., 92: 887.

Evans, G. and Maxwell, W.M.C. (1987): Salamon's artificial insemination of sheep and goats. Butterworths pty limited, Australia.

Hafez, A.H.; Megalla, S.E. and Mohamed, A.A. (1982): Effect of dietary aflatoxin on the morphology of buffalo bull spermatozoa. Mycopathologia, 77 (3): 141.

Ibeh, I.M.; Uraih, N.I. and Ogonan, J.I. (1994): Dietary exposure to Aflatoxin in human male infertility in Benin city, Nigeria, Int. J. Fert. Menopausal stud., 39 (4): 208.

Johnston, D.W.; Bruce J. and Hill, J. (1983): Extraction of Aflatoxin in a lactating cow. J. Appl. Bacterial. 54: 77.

Kadry, R.M.; Ismail, T.I.M. and Ahmed, A.A. (1999): A trial to control environmental mycotic contamination of extended buffalo semen. J. Egypt. Vet. Med. ass., 59 (3): 787.

Kadry, R..M.; Riad, R. M. and Osman, R..H. (2002): The effect of dietary aflatoxin on semen characteristics of mature ram. J. Egypt. Vet. Med. ass., 62 (6): 137.

Khalifa, T.A.A. (2001): effect of some antioxidants on viability of preserved buffalo and ram semen. Ph. D. theriogenology, Fac. Vet. Med., Cairo Univ.

Kundtson, W.U. and Kirkbride, C.A. (1992): Fungi assocated with bovine abortion in the northern Plains state (USA). J. Vet. Diag. Invest. 4: 181.

Mbai, K.; Munyua, S.F.M.; Gathumbi, P.K. and Mbiuki, S.M. (1996): Actinobacillus seminis as a cause of ram infertility in Kenya. Small Ruminant Researcl, 21: 3, 227.

Milovanov, V.K. (1962): Biology of reproduction and artificial insemination of farm animals. Monograph. Selkhoz, Lit. J. and Plakatov, Moscow.

Mishra, P.K.; Mishra, A. and Panda, S.N. (1984): Fungal agents associated with abortion and repeat breading in cross bred cattle of Orissa. Ind. J. Anim. Health, 23 (2): 115.

Picha, J.; Cerovsky, J. and Pichova, D. (1986): Fluctuation in the concentration of sex steroids and aflatoxin B_1 in the seminal plasma of boars and its relation to sperm production. Vet. Med. (prala) 31 (6): 347.

Richard, F.L.; Fichtner, R.E. and Pier, A.S. (1976): yeasts in bovine semen. Cornell Vet. 66: 362.

Rick, P.M.; Pickett, B.W. and Creighton, K.A. (1980): Sources of bacterial contamination in frozen bovine semen. A survey proceeding of the English technical conference on Artificial Insemination and Reproduction. Nation. Assoc. Anim. Breed. USA. Pp. 40.

Salem, M.H.; Kamel K.I, Yousef M.I.; Hassan G.A. and El- Nouty, F.D (2001): Protective role of ascorbic acid to enhance semen quality of rabbits treated with sub lethal doses of Aflatoxin B_1. Toxicology, 162 (3): 209.

Sidhu, S.S.; Pangawkar-G.R. and Choudhary, R.K. (1997a): Microbiological assay of nystatin in buffalo bull sperm. Ind. J. Anim. Reprod., 18 (1): 59.

Sidhu, S.S.; Pangawkar-G.R. and Jaud, S.K. (1997b): Mycological investigations of fresh and frozen bull semen and extenders. Ind. Vet. J., 74: 4, 302.

Sinha, B.K.; Sharma, T.S. and McHrotra, V.K. (1980): Fungi isolated from the genital tract of infertile cows and buffaloes in India. Vet. Rec., 10 6: 17.

Singh, N.P.; Khar, S.K. and Morga, D.P. (1991): Fungal infections in infertile cows and buffaloes. Anim. Reprod., 6- 8 February, 1991.

Vanyi, A. and Szeky, A. (1980): Fusorio toxic cases, b. The effect of F-2 toxin (zearlaenone) on the spermatogenesis of male swine. Magy Allotory Lapja, 35: 242.

Wells, M.E. and Awa, O.A. (1970): New technique for assessing acrosomal characteristics of spermatozoa. J.Dairy Sci., 53: 227.

Wierzbowski, S. (1981): Bull semen opportunistic pathogens and ubiquitary microflora. In FAO. Disease control in semen and embryo. FAO Anim. Reprod. Health., paper 23: 21.

Table (1): Effect of antimycotic agents (μg / ml) on percentages of motility after dilution, post thawing and reduction. Mean ± S.E

	Control	Lamisil				Diflucan				Nizoral				Mucosol				Griseofulvin			
		10	20	30	Over all	30	40	50	Over all	50	60	70	Over all	50	60	70	Over all	50	60	70	Over all
Motility after dilution (%)	80.00 ± 1.58	80.00 ± 2.23	79.00 ± 1.86	80.00 ± 1.57	79.66 ± 1.03	82.00 ± 1.22	82.00 ± 2.11	85.00 ± 1.57	83.00 ± 0.95	84.00 ± 1.86	82.00 ± 1.99	85.00 ± 1.57	83.66 ± 1.03	81.00 ± 1.86	84.00 ± 1.86	82.00 ± 3.38	82.33 ± 1.36	81.00 ± 0.99	81.00 ± 2.44	83.00 ± 2.54	81.66 ± 1.15
Post thaw motility (%)	36.00 ± 2.44	42.00 ± 3.73	36.00 ± 2.44	36.00 ± 2.44	38.00 ± 1.74	42.00 ± 5.82	43.00 ± 3.73	38.00 ± 1.99	41.00 ± 2.29	44.00 ± 5.09	34.00 ± 2.44	30.00 ± 4.47	36.00 ± 2.72	34.00 ± 2.44	40.00 ± 3.16	36.00 ± 5.09	36.66 ± 2.10	42.00 ± 1.99	34.00 ± 3.99	34.00 ± 7.48	36.66 ± 2.87
Motility reduction (%)	54.92 ± 3.24	47.80 ± 3.47	54.42 ± 2.98	54.77 ± 3.67	52.33 ± 2.00	48.82 ± 7.12	47.52 ± 4.67	55.22 ± 2.52	50.51 ± 2.88	47.15 ± 6.88	58.61 ± 2.43	64.78 ± 5.01	56.85 ± 3.35	58.02 ± 2.81	52.00 ± 4.63	55.71 ± 6.53	55.25 ± 2.70	48.08 ± 2.70	57.49 ± 5.77	58.64 ± 9.52	54.74 ± 3.72

Table (2): Effect of antimycotic agents (µg/ml) on viability index during incubation at 5°C for 72 hr, post thaw viability index and percentage of acrosomal defects (Mean ± S. E).

	Control	Lamisil				Diflucan				Nizoral				Mucosol				Griseofulvin			
		10	20	30	Over all	30	40	50	Over all	50	60	70	Over all	50	60	70	Over all	50	60	70	Over all
Viability index during incubation at 5°C	222.00 ± 12.99 a	201.00 ± 6.20	211.00 ± 12.58	224.00 ± 20.14	212.00 ± 7.98 a	246.00 ± 17.40	249.00 ± 15.03	261.00 ± 15.03	252.00 ± 7.50 a	214.00 ± 18.46	234.00 ± 24.81	218.0 ± 22.22	222.0 ± 11.97 a	216.00 ± 13.17	214.0 ± 10.88	209.0 ± 16.22	213.0 ± 7.31 a	208.0 ± 28.12	233.0 ± 14.87	236.00 ± 16.87	225.66 ± 11.50 a
Post thaw viability index	94.00 ± 6.59 ab	135.00 ± 11.72	92.00 ± 3.73	80.00 ± 6.89	102.33 ± 7.66 a	75.00 ± 9.48	131.00 ± 13.17	103.00 ± 11.35	103.00 ± 8.64 a	90.00 ± 9.48	89.00 ± 7.31	72.00 ± 4.89	83.66 ± 4.61 b	78.00 ± 6.43	93.00 ± 5.60	99.00 ± 9.40	90.00 ± 4.57 ab	120 ± 5.24	100 ± 6.51	93.00 ± 13.28	104.00 ± 5.72 a
Acrosomal defects (%)	22.00 ± 3.20 ab	20.40 ± 2.61	17.60 ± 2.01	19.00 ± 2.44	19.00 ± 1.30 ab	16.00 ± 0.70	17.00 ± 2.11	15.40 ± 2.20	16.13 ± 0.98 b	26.00 ± 1.69	18.60 ± 2.72	27.20 ± 5.48	23.93 ± 2.21 a	23.20 ± 4.26	24.60 ± 4.19	25.20 ± 6.18	24.33 ± 2.66 a	22.00 ± 3.93	20.00 ± 3.06	19.80 ± 2.49	20.60 ± 1.74 ab

Figures with different subscripts a, b,.... within columns are significantly different at least $p<0.05$

Table (3): Isolation rate of environmental mycotic contamination of raw, extended ram semen and Tris based extender.

Sample	No. exam.	Identification frequency		
Raw semen	5	Mucor	2	50%
		Cladosporium	1	25%
		A. niger	1	25%
Tris based extender	5	A. flavus	13	43.33%
		Fusarium species	4	13.33%
		Penicillium species	5	16.67%
		Candida species	8	26.67%
Post thaw semen (control)	20	A. flavus	31	25.62%
		A. achracus	21	17.36%
		A. niger	20	16.53%
		Fusarium sp.	13	10.74%
		Penicillium sp.	12	9.92%
		Candida sp.	8	6.61%
		Cladosporium	6	4.96%
		Mucor sp.	10	8.26%

Table (4): Drug sensitivity of mould and yeast isolated from contaminated ram semen (58 of 121 strains).

Fungi isolated	Total No. of isolates	Lamisil µg / ml			Diflucan µg / ml			Nizoral µg / ml			Miconaz µg / ml			Griseofulvin µg / ml		
		10	20	30	30	40	50	50	60	70	50	60	70	50	60	70
		S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R	S/R
A. flavus	32	32/4	32/2	32/0	32/8	32/5	32/2	32/12	32/6	32/4	32/18	32/13	32/12	32/20	32/16	32/16
A. achracus	12	12/2	12/0	12/0	12/5	12/3	12/2	12/6	12/2	12/2	12/7	12/5	12/3	12/7	12/5	12/4
Fusarium sp.	4	4/0	4/0	4/0	4/1	4/1	4/0	4/2	4/1	4/1	4/3	4/1	4/1	4/3	4/1	4/1
Penicillium sp.	6	6/1	6/0	6/0	6/2	6/0	6/0	6/3	6/1	6/1	6/4	6/2	6/2	6/3	6/2	6/2
Candida sp.	4	4/0	4/0	4/0	4/1	4/0	4/0	4/1	4/0	4/0	4/2	4/2	4/1	4/2	4/1	4/1

S / R= sensitive / resistant

Table (5): Mycological examination of frozen ram semen after addition of different concentrations of antimycotic agents.

	Lamisil (µg/ml)			Diflucan (µg/ml)			Nizoral (µg/ml)			Miconaz (µg/ml)			Griseofulvin (µg/ml)		
	10	20	30	30	40	50	50	60	70	50	60	70	50	60	70
A. flavus	-	-	-	+++	+	-	++	+	-	+++	+++	++	++++	+++	+++
A. achracus	-	-	-	++	+	-	+	+	-	+++	++	++	+++	+++	++
A. niger	-	-	-	+++++	++	+	+++	++	-	++++	+++	++	+++	++	++
Fusarium	-	-	-	+	+	-	+	-	-	+	+	+	++	+	+
Penicillium sp.	-	-	-	++	-	-	+	-	-	+	+	+	++	++	+
Candida sp.	-	-	-	+++	+	-	++	-	-	+++	++	++	++++	+++	++
Cladosporium	-	-	-	++	+	-	+	+	-	++++	++	++	+++	++	++
Mucor sp.	-	-	-	+++	++	-	++	+	-	+++++	+++	+++	++++	++	+++

+, ++, ...= Scores of fungal colonies appearance after addition of antimycotic agents.

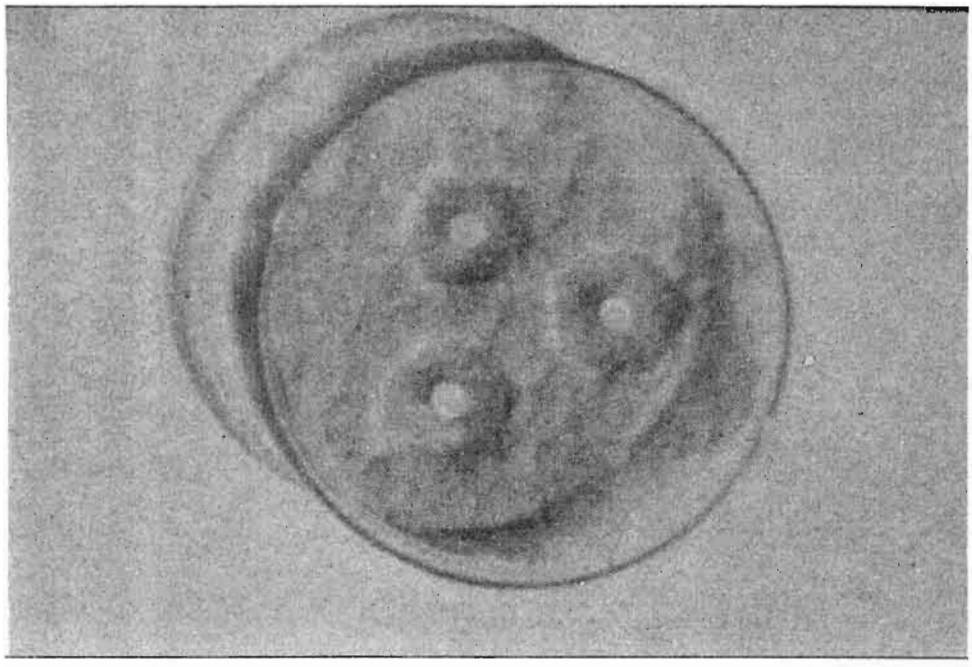
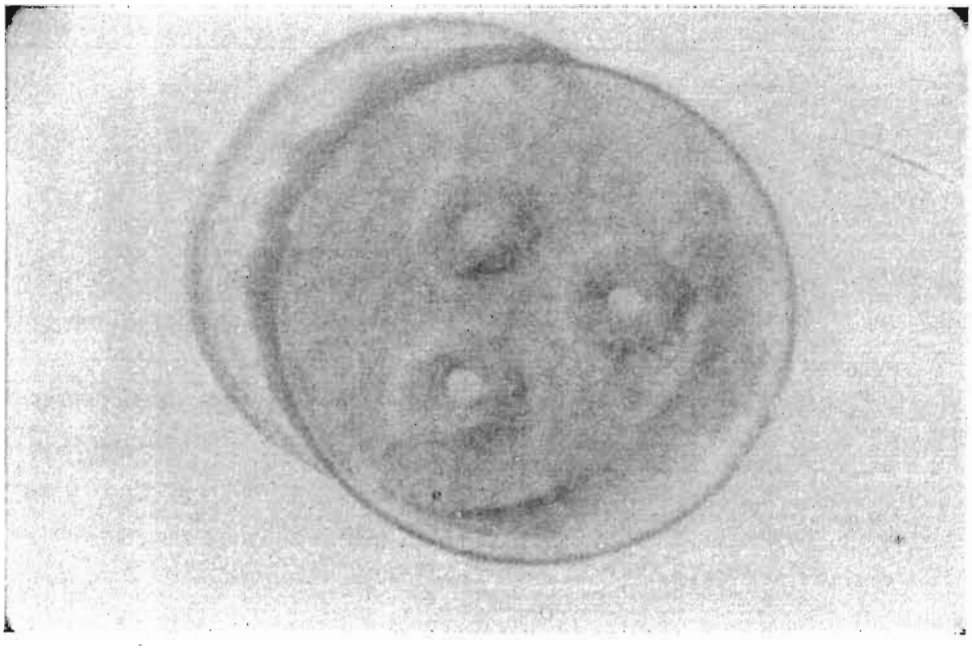


Fig.1: The plate represent Aspergillus flavus strain with a wide clear zone of inhibition with Lamisil discs 10,20,30 ug.



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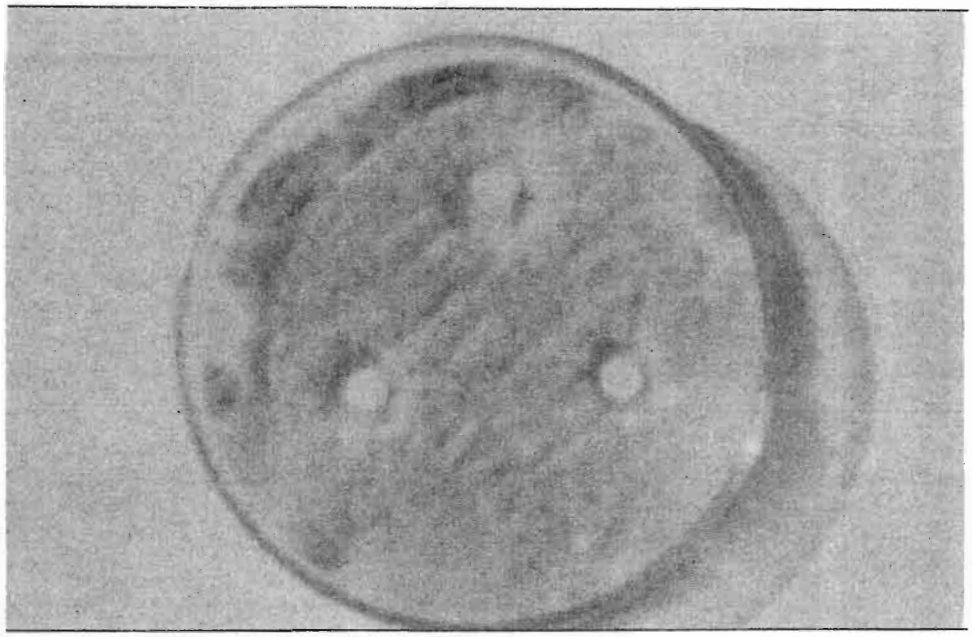

Fig.3: The plate represent Aspergillus flavus strain with moderate zone of inhibition with Nizoral discs 50,60,70 ug.

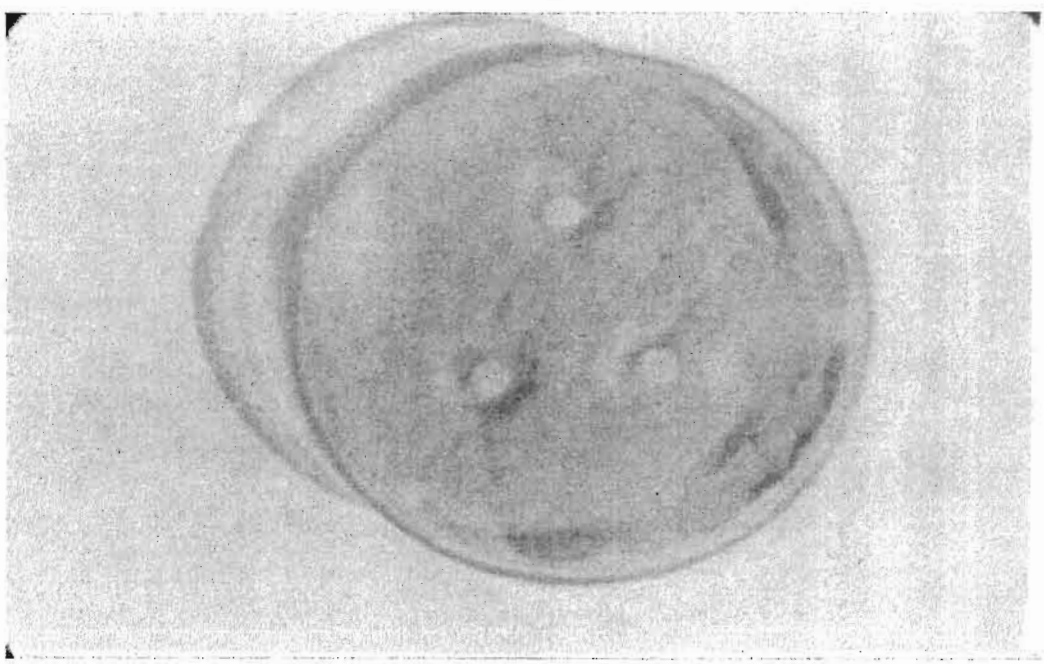

Fig.4: The plate represent Aspergillus flavus strain with very narrow zone of inhibition with Miconaz discs 50,60,70 ug.

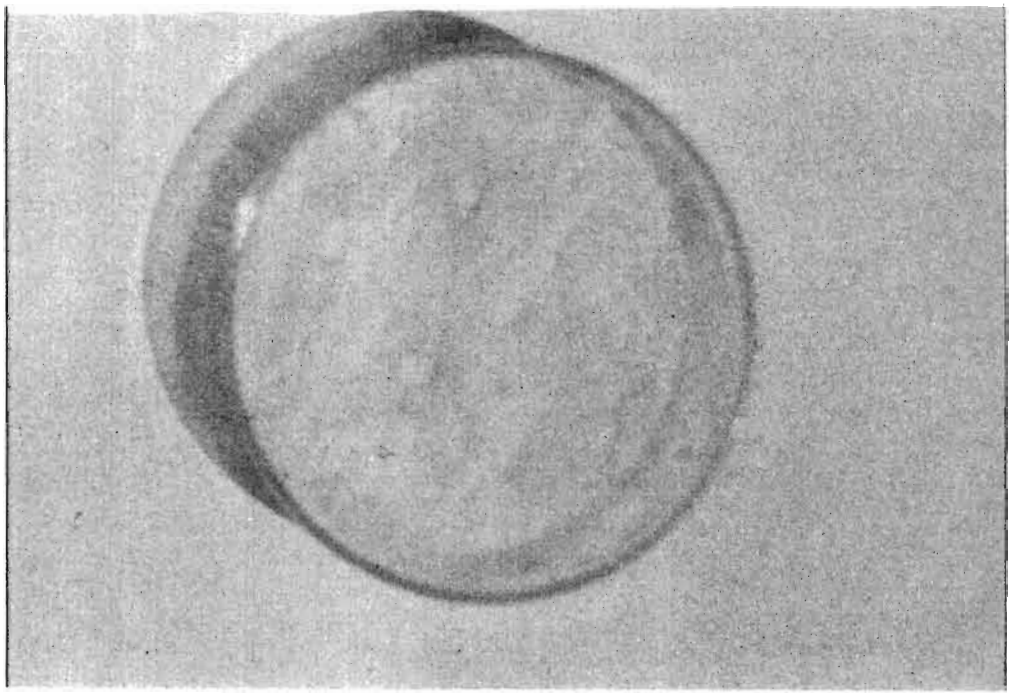

Fig.5: The plate represent Aspergillus flavus strain with no zone of inhibition with Griseofulvin 50,60,70 ug.