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Seasonal influence on histochemical distribution in testis of non-descript dog (*Canis familiaris*)

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Abstract
The present investigation dealt with seasonal variation in distribution of histochemical moieties in stray dog testis (*Canis familiaris*). The study was conducted on the testes of 18 adult, healthy dogs captured and operated for sterilization at the Veterinary Hospital of Junagadh Municipal Corporation, Ivanagar, Junagadh. Histochemical observations were recorded on the H & E-stained section of the testis of the dog, using Carl Zeiss Zen (Blue edition) microscopic image analysis software. The samples were examined under a light microscope using x100 and then x400 magnification. Presence of neutral mucopolysaccharides, acid mucopolysaccharides, protein, lipids and cholesterol were demonstrated by Periodic Acid Schiff, Alcian Blue, Mercury bromophenol blue, Oil red O stain and Schultz method, respectively. Capsule, trabeculae, basement membrane of seminiferous tubules, interstitial connective tissue and mediastinum revealed an intense positive reaction for PAS in all three seasons. The cytoplasm of Leydig cells showed weak to moderate positive reaction for PAS in summer, while a moderate reaction was observed in the monsoon and winter. Spermatogonia and primary spermatocytes showed a weak reaction in the summer and winter seasons. Whereas, in the monsoon season, the reaction was weak to moderate for PAS. Secondary spermatocytes, spermatids and spermatozoa showed a moderate PAS-positive reaction in monsoon and winter. Weak to moderate reactions for Alcian Blue in all three seasons were observed for the basement membrane of seminiferous tubules. Interstitial connective tissue showed weak to moderate affinity for Alcian Blue in summer and winter, while, during the monsoon, the reaction was moderate. The cytoplasm of Leydig cells was moderately positive in the summer and weak to moderately positive during the monsoon and winter for Alcian Blue. Mercury bromophenol blue positive reaction was observed throughout the testis of dog, indicating the presence of basic protein in the structure of the testis. The concentration of protein was higher in the monsoon and winter seasons as compared to summer. A high protein content was observed in the secondary spermatocytes, spermatids, spermatozoa and Leydig cells during the monsoon and winter seasons in the testes of dog. The content of lipid indicated by moderate reaction to Oil red O and Sudan Black B stains was observed in interstitial tissue and with Oil red O small granules with positive reaction were observed in the seminiferous tubules during the monsoon and winter. The moderate amount of cholesterol was observed in the interstitial tissue during the summer season, as demonstrated by the Schultz technique.

Keywords: Histochemistry, seasons, dog, testis

Introduction
Recent few years have witnessed advancements in our knowledge regarding the processes controlling canine reproduction, particularly the maturation and development of gametes. Moreover, imaging and molecular technologies have been used to generate diagnostic ailments for reproductive abnormalities and to enhance knowledge about reproductive diseases in domestic dogs. More details are needed to comprehend the basic physiology and structure of the dog testicles. Studies on variation in testicular histochemistry during different seasons are essential for determining how the seasons influence testes function. Increased sperm production functionality and internal modifications are shown by changes in testicular histological observations, which are critical and favour canine reproductive success. A few researchers have also shown also their interest in testicular histochemistry of mammals (Pathak *et al.*, 2013; Aslam *et al.*, 2018[a,b] ; Gopikrishna *et al.*, 2017; Patel *et al.*, 2024) [11, 1, 2, 10]. However, there is a dearth of documented literature on studies related to histochemistry of dog testis in relation to seasons. Therefore, distribution of histochemical moieties in testis was studied during different seasons in non-descript dogs.

Materials and Methods

The current study was carried out on the testes of 18 adult and apparently healthy dogs, 06 in each season; Summer: April-May, 2024 (hot-dry weather), Monsoon: September-October, 2024 (hot-humid weather) and Winter: December, 2024-January, 2025 (cold-dry weather), as detailed in Table A. Samples were collected from adult and apparently healthy dogs captured and operated for sterilization-"Neutering"-at the Veterinary Hospital of Junagadh Municipal Corporation, Ivanagar, Junagadh. Tissue samples were collected from the testicles.

Table A: Weather details in different seasons along with Mean±SE of body weight of dogs whose testes were collected

Particulars	Summer	Monsoon	Winter	p-value
Months	April-May, 2024	September-October, 2024	December 2024-January 2025	-
Weather °C R.H.%	Hot-dry 40.20 °C 47.5%	Hot-humid 33.00 °C 75.9%	Cold-dry 28.45 °C 53.65%	-
Body weight of dog (kg)*	21.39±1.75	18.73±0.53	20.73±0.44	NS (p>0.05)
N	6	6	6	-
Overall body weight: 20.28±0.65 kg *Mean body weight in different seasons did not differ significantly (p>0.05) as per one-way ANOVA & DUNCAN test				

Fixation and Processing of Tissue Sample for Paraffin Sectioning: Immediately after collection, the tissue samples were fixed in 10 percent neutral buffered formalin (10% NBF) for the present histochemical studies. After complete fixation, the tissue samples were processed for paraffin block preparation by the acetone-benzene schedule (Prophet *et al.*, 1992) [14]. The paraffin blocks were prepared and sections of 5-6 µm thickness were obtained on glass slides with the help of a LEICA RM2125 RTS rotary microtome. These paraffin sections were used for various histological staining methods (Table B).

Table B: Histochemical Stains used for paraffin/cryostat sections of the testis of dog for the histochemistry study

Sr. No.	Stain	Purpose	Reference
1	Periodic Acid Schiff	Neutral mucopolysaccharides	Sheehan & Hrapchak (1973) [15]
2	Alcian Bule at pH 1.0 & 2.5	Acid mucopolysaccharides	Prophet *et al.* (1992) [14]
3	Mercury bromophenol blue	Protein	Pears (1968) [12]
4	Oil red O	Lipids	Prophet *et al.* (1992) [14]
5	Sudan black B	Lipids	Chayen *et al.* (1969) [6]
6	Schultz method	Cholesterol	Chayen *et al.* (1969) [6]

Processing of Tissue Sample for Cryostat Sectioning: Fresh unfixed samples of testis were collected and frozen at-20 °C in the deep freezer as soon as possible. These frozen tissues were subjected to cryostat sectioning to obtain 10-12 µm thick sections, which were stained with Oil red O and Sudan Black-B for demonstration of lipids and the Schultz method for cholesterol (Table A). Part of the research work (cryostat sectioning) was carried out in collaboration with the Department of Veterinary Pathology, College of Veterinary Science. & A.H., KU, Junagadh.

Results and Discussion

The findings on demonstration of different histochemical moieties observed and recorded in the capsule, trabeculae and parenchyma of the testis of the dog during different seasons have been deliberated here under.

1. Neutral Mucopolysaccharides: The distribution of neutral mucopolysaccharides (NMPS) in the different structures of the testis of the dog was demonstrated by the Periodic Acid Schiff (PAS) staining technique. The same is detailed in Table 1.

Table 1: Histochemical distribution of neutral mucopolysaccharides (NMPS) and acid mucopolysaccharides (AMPS) in the testis of dog during different seasons*

Structure/Particulars	Summer		Monsoon		Winter	
	NMPS	AMPS	NMPS	AMPS	NMPS	AMPS
Capsule	+++	+	+++	+	+++	+
Trabeculae	+++	+	+++	+	+++	+
Basement membrane of seminiferous tubule	+++	+/++	+++	+/++	+++	+/++
Sertoli cells	+	+	+	+	+	+
Spermatogonia	+	+	+/++	+	+	+
Primary spermatocytes	+	+	+/++	+	+	+
Secondary spermatocyte	NA	NA	++	+	++	+
Spermatid	NA	NA	++	+	++	+
Spermatozoa	NA	NA	++	+	+	+
Interstitial connective tissue	+++	+/++	+++	++	+++	+/++
Leydig cell	+/++	++	++	+/++	++	+/++
Mediastinum	+++	+/++	+++	+	+++	+/++
Rete testis	+	+/++	+	+	+	+

*NA not applicable, +Weak, ++Moderate, +++Strong

The observation for NMPS in the capsule and connective tissue trabeculae demonstrated a strong positive reaction in all three seasons of the year. The basement membrane of the seminiferous tubules also showed a strong PAS positive

reaction. A considerable amount of NMPS was found present in the capsule, connective tissue trabeculae and basement membrane of seminiferous tubules. Sertoli cells revealed weak affinity for the PAS stain, showing a low amount of NMPS in their cytoplasm. Spermatogonia and primary spermatocytes showed weak reaction for PAS in the summer and winter seasons. Whereas, in the monsoon season, the reaction was weak to moderate. Secondary spermatocytes, spermatids and spermatozoa showed moderate reaction in monsoon and winter, respectively (Plate 1).

The interstitial connective tissue showed strong positive reaction for PAS during the summer, monsoon and winter seasons (Plate 1 A, B & C). The cytoplasm of Leydig cells showed weak to moderate positive reaction in summer, while a moderate reaction was observed in the monsoon and winter seasons (Plate 1 A, B & C). The mediastinum testis was intensely positive for the presence of NMPS. The cytoplasm of epithelial cells of the rete testis was weakly positive for the same. In the mediastinum and rete testis, no seasonal differences were noted in the reaction for PAS. These findings suggested that spermatids and spermatozoa contained more neutral mucopolysaccharides in the form of hyaluronidase in the monsoon and winter seasons.

In contrast to the findings of the present study, the study of Gofur *et al.* (2008) [7] on the indigenous bulls, the spermatogonia, primary spermatocytes, and secondary spermatocytes exhibited negative affinity for PAS stain; however, the basement membrane of tubules, spermatids, and spermatozoa displayed positive affinity, which aligned with the present findings.

The results of the current study are supported by Bansal *et al.* (2009) [4], who observed that the Leydig and spermatogenic cells showed a positive but moderate to strong PAS reaction and strong positive results for PAS in the basement membrane of seminiferous tubules. As observed in the present study, Aslam *et al.* (2018[a]) [1] reported that Leydig cells revealed weak PAS-positive activity during summer, which became weak to moderate in autumn and moderate in spring and winter. Sudhakar *et al.* (2010) [16] also found that the cytoplasm of the interstitial cells showed a strong reaction for glycogen, whose intensity did not vary in the different seasons, while in the present study, it varied slightly in intensity from weak to moderate among different seasons.

As found in the present study, Gopikrishna *et al.* (2017) [5] also found that the spermatids and capsule showed a strong positive reaction for PAS, indicating a massive presence of neutral mucopolysaccharides. Similar to the current findings, Patel *et al.* (2024) [10] also reported strong positive reaction for PAS reaction for the tunica albuginea, basement membrane of seminiferous tubules and mediastinal testis.

2. Acid Mucopolysaccharides: The distribution of acid mucopolysaccharides (AMPS) in the different structures of the testis of the dog was demonstrated by the Alcian Blue (AB) staining technique.

As detailed in Table 1, a weak positive reaction was observed for the capsule and trabeculae during all three seasons. A weak to moderate reaction for alcian blue was noted in the basement membrane of seminiferous tubules in all three seasons. Cytoplasm of Sertoli cells, spermatogonia and primary spermatocytes had a weak affinity for the AB in all seasons. The secondary spermatocytes, spermatids and spermatozoa revealed weak alcianophilic reaction in the monsoon and winter seasons. Interstitial connective tissue showed weak to moderate affinity for alcian blue reaction in the summer and winter, while, during the monsoon, the reaction was moderate. The cytoplasm of Leydig cells was moderately positive in the summer and weak to moderately positive during the monsoon and winter, for AB (Plates 2 A to C). The observations on the mediastinum testis showed weak to moderate reaction during the summer and winter, whereas, in the monsoon, the reaction was weak. The epithelial cells of the rete testis were weak to moderately positive for the alcian blue in summer and weakly positive during the monsoon and winter (Plates 3 A, B & C).

The findings of the present study were, in general, endorsed by Bansal *et al.* (2009) [4] who observed a mild alcian blue reaction in the spermatogenic cells. Contrary to the current observations for AMPS, Gopikrishna *et al.* (2017) [5], who found strong positive reactions in the basement membrane of the seminiferous tubules and Aslam *et al.* (2018[b]) [2], who reported weak alcian blue reactions in the tunica fibrosa and vasculosa layers during the spring and summer seasons, which became moderate in the autumn and winter seasons.

3. Basic Protein: The histochemical distribution of protein in the testis of dog was demonstrated by the Mercury bromophenol blue technique. The results presence of basic protein in the testis of dog during different seasons has been presented in Table 2.

In the summer season, the capsule showed a weak positive reaction for bromophenol blue, while in monsoon and winter, the reaction was moderate. In the trabeculae, weak to moderate affinity during summer, moderate reactivity was manifested during the monsoon and winter seasons (Plate 4). In the basement membrane, the weak positive reaction was observed in summer, which became moderate in monsoon and winter seasons. Sertoli cells, spermatogonia and primary spermatocytes revealed weak reaction for the bromophenol blue in summer, which was moderate in the monsoon and winter. Moderate to strong positive reaction was observed in the secondary spermatocytes, spermatids and spermatozoa in monsoon and winter. Interstitial connective tissue and the Leydig cells were weakly positive in summer, while during the monsoon and winter seasons, the reaction was moderate for the bromophenol blue (Plate 5). The weak positive reaction for mediastinum and rete testis was noted without any seasonal differences. The presence of basic proteins indicated that the cell membranes were lipoprotein in nature.

Table 2: Histochemical distribution of basic protein in the testis of dog during different seasons*

Structure/Particulars	Summer	Monsoon	Winter
Capsule	+	++	++
Trabeculae	+/++	++	++
Basement membrane of the S.T.	+	++	++
Sertoli cells	+	++	++
Spermatogonia	+	++	++
Primary spermatocytes	+	++	++
Secondary spermatocyte	NA	++/+++	++/+++
Spermatid	NA	++/+++	++/+++
Spermatozoa	NA	++/+++	++/+++
Interstitial connective tissue	+	++	++
Leydig cell	+	++	++
Mediastinum	+	+	+
Rete testis	+	+	+

*NA not applicable, +Weak, ++Moderate, +++Strong

As observed in the present study, Kishore (2006, as cited in Aslam, 2017) [8] found mild to moderate basic protein reaction in structures of sheep testis and Bansal *et al.* (2009) [4] also found moderate to strong bromophenol blue reactivity in Leydig and spermatogenic cells and the basic proteins had a highly positive luminal concentration in testis of guineapig. On the other hand, Aslam *et al.* (2018[a]) [1] reported that the reaction for basic protein decreased towards the luminal border of the seminiferous tubule in all seasons. The mediastinum testis showed a moderate to high basic protein reaction in the autumn, which weakened in the winter. The findings of Aslam *et al.* (2018[b]) [2] were in contrast with the findings of present study.

4. Lipids: Oil red O and Sudan black B stains were used to demonstrate the presence of lipids in the testis of dog. The observations histochemical distribution of lipids in the dog testis revealed that the interstitial tissue contained lipid**.** The lipid demonstration was weak in summer, whereas moderately positive in monsoon and winter (Plate 6). In the summer season, no lipid granules were found in the seminiferous tubules (Plate 7). During the monsoon and winter seasons, small granules with positive reaction were observed in the seminiferous tubule towards the lumen using the Oil Red O technique (Plate 8).

With the Sudan Black B technique, typical sudanophilic granules were observed in the interstitial tissue. In the summer season, the reaction with the Sudan Black B was weak compared to the other two seasons: monsoon and winter (Plate 9). The amount of sudanophilic lipids present in the Leydig cells of interstitial tissue suggested that these lipids are associated with the process of steroidogenesis in the seasonally active period of breeding, e.g., monsoon and winter.

The current finding revealed the presence of lipid in the interstitial tissue, which was in line with the report of Banks (1993) [3]. The findings of the present study for the monsoon and winter seasons are well supported by Osman *et al.* (1976) and Aslam *et al.* (2018) [1], who revealed that the reaction intensity with Sudanophilic lipids was higher towards the lumen of seminiferous tubules than at the basal portion in camel testis and buffalo bull testis, respectively. In contrast to the results of the current study, Gopikrishna *et al.* (2017) [5] found a strong reaction for lipids in spermatogonia and primary spermatocytes and a moderate reaction in spermatids and Leydig cells in the testis of adult ram.

5. Cholesterol: The presence of cholesterol in the testis of dog during different seasons was demonstrated using the Schultz technique. The positive reaction for cholesterol was observed in the interstitial tissue and seminiferous tubules. The moderate amount of cholesterol was observed in the interstitial tissue during the summer season, while a weak reaction was observed during the other two seasons (Plate 10). The reaction was low to negligible in the seminiferous tubules in all seasons. The lower amount of cholesterol in the monsoon and winter seasons might be due to the conversion of cholesterol into the testosterone hormone resulting in moderate amount in the summer season.

The finding of cholesterol in the interstitium is well in agreement with the results of the studies by Bilaspuri & Guraya (1983, as cited in Gopikrishna *et al.*, 2017) [5] and Pathak *et al.* (2013) [11]. In contrast to the finding of the present study on cholesterol (no or weak reaction of Leydig cells) in the summer season, Sudhakar *et al.* (2010) [16] reported a positive reaction for cholesterol by the Leydig cells, whose intensity was minimum in summer and maximum in autumn. Contrary to the present findings, Perlman (1949) reported a lower amount of cholesterol in the interstitial cells as compared to that in the seminiferous tubules.

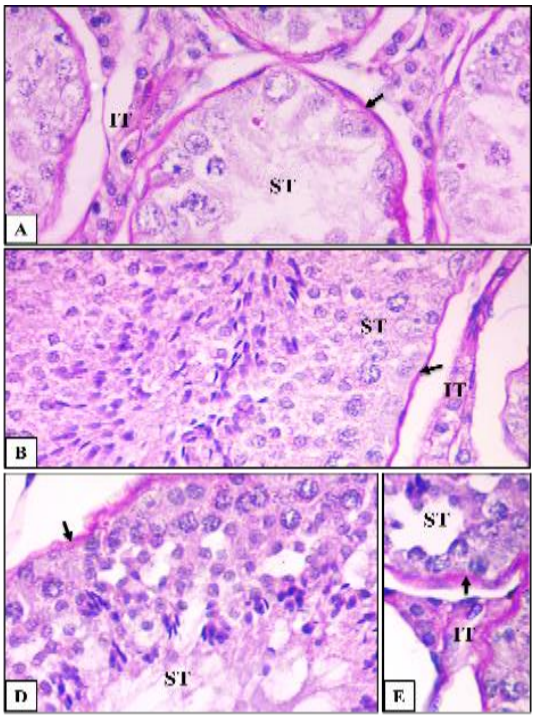

Plate 1: A. Summer, B & C. Monsoon and D & E. Winter seasons. Photomicrograph of dog testis showing histochemical demonstration of MMPS in basement membrane (arrow), seminiferous tubules (ST) and interstitial tissue (IT). PAS × 400.

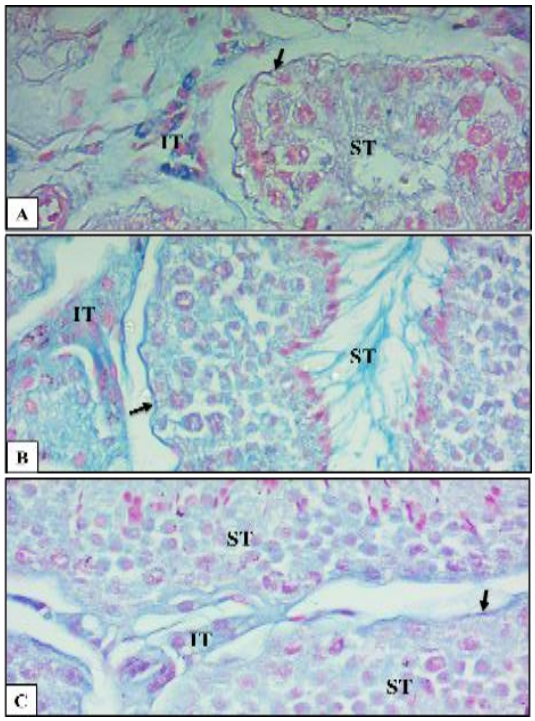

Plate 2: A. Summer, B. Monsoon & C. Winter seasons. Photomicrograph of dog testis showing histochemical demonstration of AMPs in basement membrane (arrow), seminiferous tubules (ST) and Interstitial tissue (IT). AB x 400.

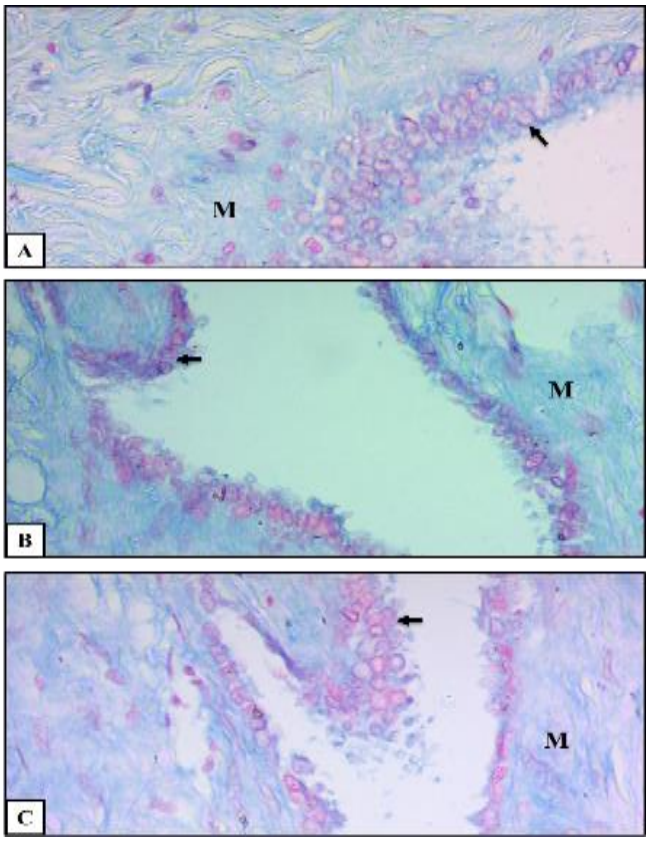

Plate 3: A. Summer, B. Monsoon & C. Winter seasons. Photomicrograph showing histochemical demonstration of AMPS in the mediastinum (M) and epithelium of rete testis (arrow) testis of dog. AB × 400.

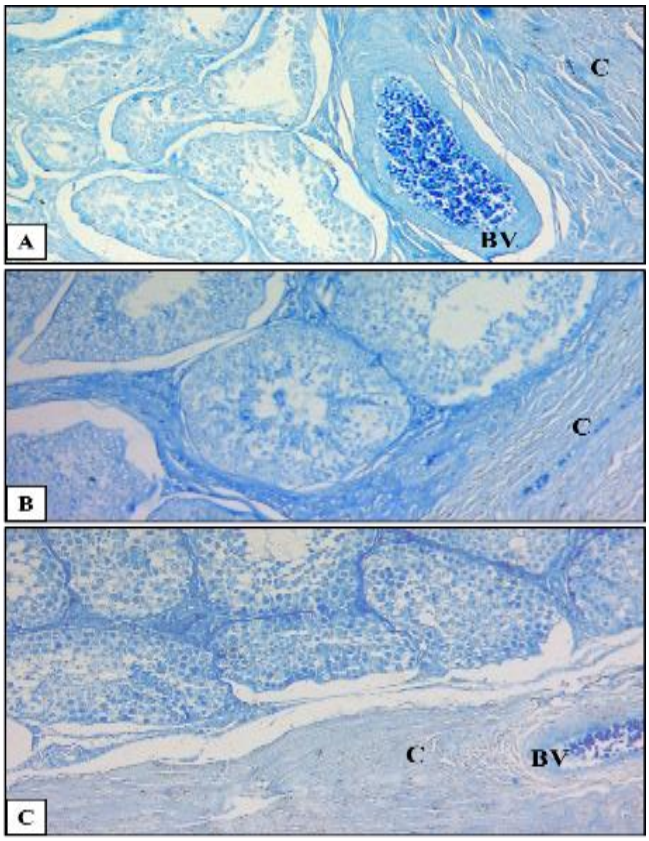

Plate 4: A. Summer, B. Monsoon & C. Winter seasons. Photomicrograph showing histochemical demonstration of protein in the capsule (C), blood vessel (BV) and the parenchyma of dog testis. AB × 100.

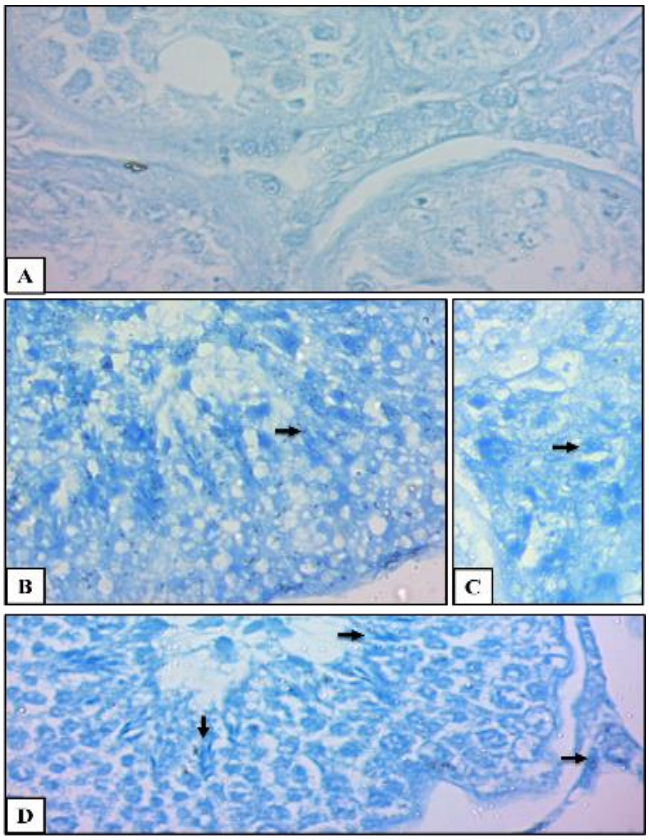

Plate 5: A. Summer, B & C. Monsoon, D Winter seasons. Photomicrograph of dog testis showing histochemical distribution of protein (arrow). Mercury-bromphenol blue × 400.

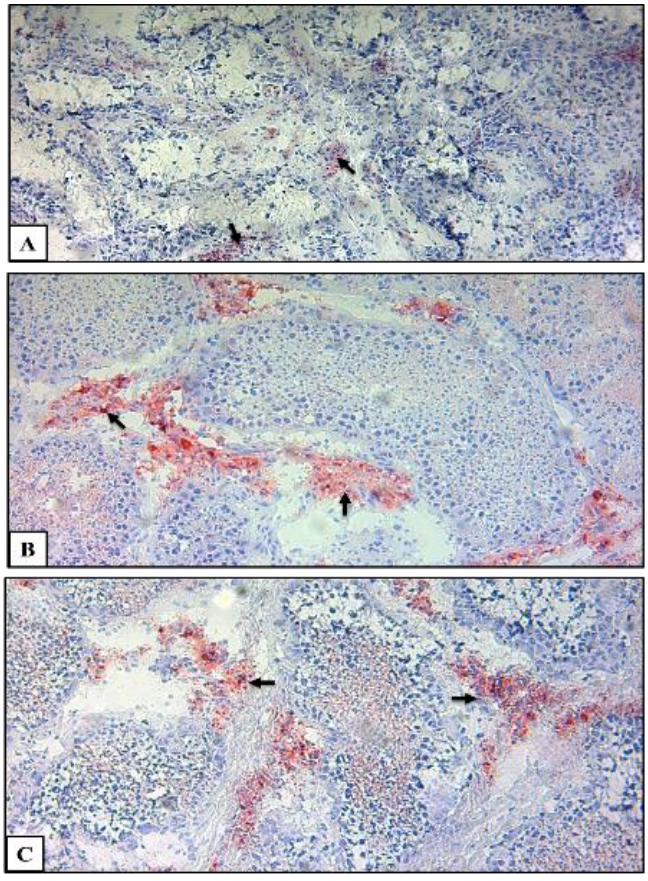

Plate 6: A. Summer, B. Monsoon & C. Winter seasons. Photomicrograph showing the histochemical distribution of lipids in the interstitial tissue (arrow). Oil red O. A x 40, B & C × 100.

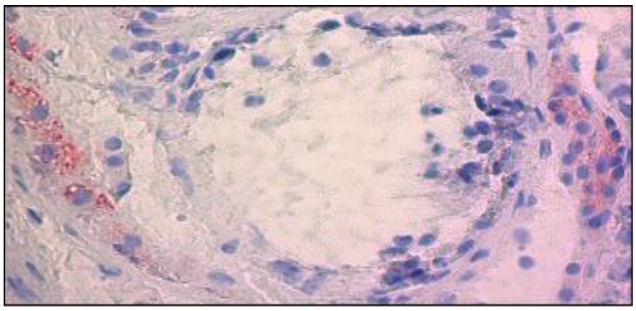

Plate 7: Summer season. Photomicrograph showing no histochemical distribution of lipid in seminiferous tubules. Oil red O × 400

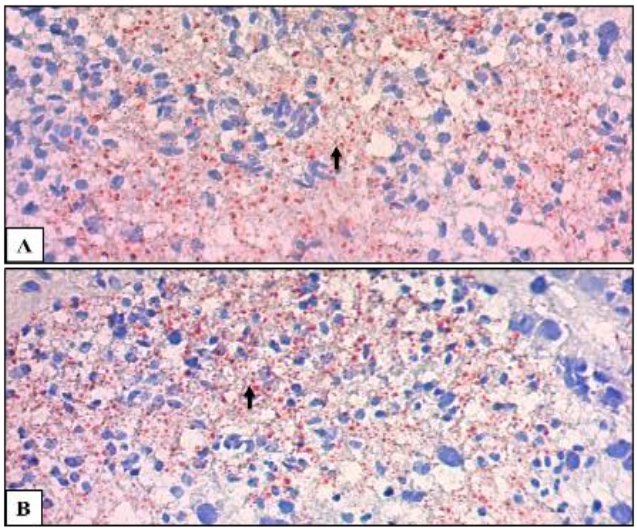

Plate 8: A. Monsoon & B. Winter seasons. Photomicrograph showing histochemical distribution of lipid granules (arrow) in seminiferous tubules. Oil red O × 400.

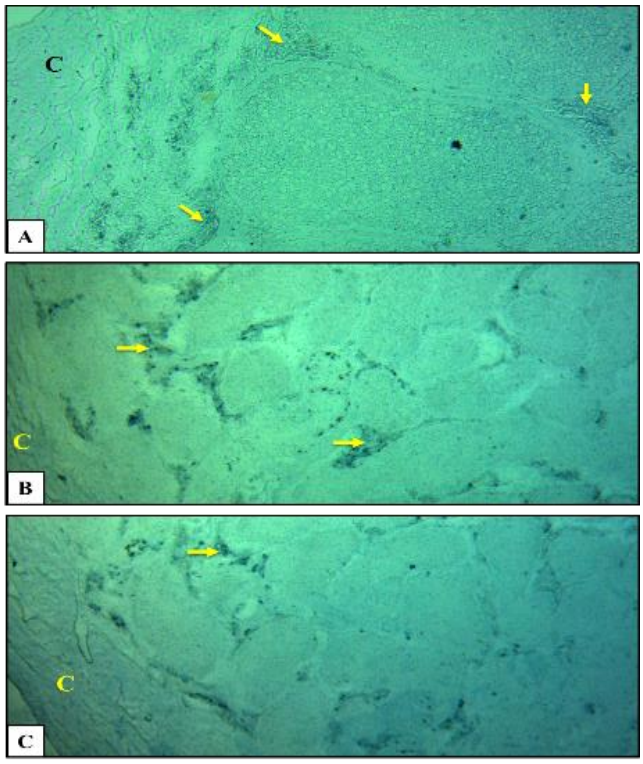

Plate 9: Summer, B. Monsoon & C. Winter seasons. Photomicrograph showing the histochemical distribution of lipids (arrow) in the interstitial tissue. Sudan Black B. A x 100, B & C x 40.

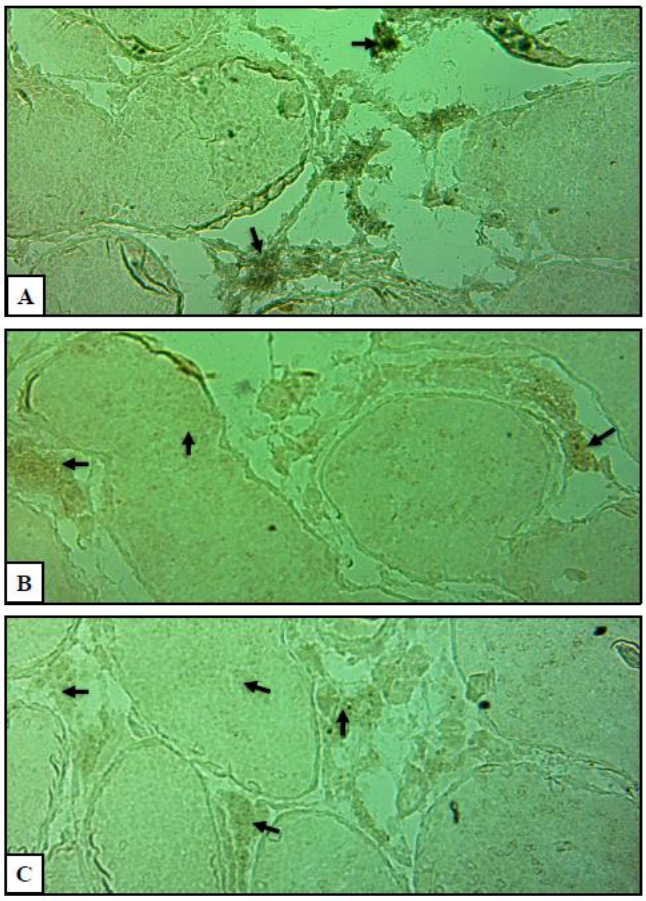

Plate 10: A. Summer, B. Monsoon & C. Winter seasons. Photomicrograph of dog testis showing the presence of cholesterol (arrow) in the interstitial tissue and seminiferous tubules. Schultz x100.

Conclusion

The overall results of the histochemical study in dog testis tended to indicate that the cytoplasm of Leydig cells showed weak to moderate positive reaction for PAS in summer, while a moderate reaction was observed in the monsoon and winter. Spermatogonia and primary spermatocytes showed a weak reaction in the summer and winter seasons. Whereas, in the monsoon season, the reaction was weak to moderate for PAS. Secondary spermatocytes, spermatids and spermatozoa showed a moderate PAS-positive reaction in monsoon and winter. The cytoplasm of Leydig cells was moderately positive in the summer and weak to moderately positive during the monsoon and winter for Alcian Blue. Mercury bromophenol blue positive reaction was observed throughout the testis of dog, indicating the presence of basic protein in the structure of the testis. The concentration of protein was higher in the monsoon and winter seasons as compared to summer. The content of lipid indicated by moderate reaction to Oil red O and Sudan Black B stains was observed in interstitial tissue and with Oil red O small granules with positive reaction were observed in the seminiferous tubules during the monsoon and winter. The moderate amount of cholesterol was observed in the interstitial tissue during the summer season, as demonstrated by the Schultz technique.

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Conflict of Interest

All authors declare no conflict of interest.

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