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# Two dimensional SDS PAGE analysis of epididymal tissue proteins of normal and castrated bulls (*Bos taurus*) and identification of an androgen dependant protein

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ABSTRACT

A study was undertaken with an objective of two dimensional SDS-PAGE analysis of protein profile in tissues of all caput, corpus and cauda of epididymis in both castrated and normal bulls and identification of androgen dependent protein/s in the epididymal tissues of bull (Bos taurus). Two dimensional SDS-PAGE analysis for protein spots of different molecular weight (MW) with pI 3.5 to 7.35 and pI > 7.35 to 9.3 between the three regions in the normal and castrated bull as well as between normal and castrated bull of a particular region revealed significant differences. Similarly, comparison between the three regions of the normal and castrated bull epididymis for the number of protein spots irrespective of the MW in pI range of 3.5 to 7.35 and pI range > 7.35 to 9.3 revealed significant differences. The number of protein spots found to be significantly higher in caput, corpus and cauda epididymis of the normal bull when compared to similar region of the castrated bull epididymis. Most of the proteins, which are secreted, are having MW between 20 and 85 kDa. Six proteins, which are known to be highly dependent on androgens, are also of acidic in nature except for one protein having basic pH. A protein spot (pI: 6.55- 6.85, and MW: <20 kDa) which appeared at the same site in all the three regions of the epididymis in the normal bull but was absent at the same site in all the three regions of the castrated bull was subjected for identification of the protein by MALDI-MS. The results revealed that this protein is an interferon-stimulated protein and it could be ISG15/UCRP.

## 1. Introduction

Spermatozoa are produced from germ cells in the seminiferous tubules of the testis. However, spermatozoa acquire motility and fertility in the epididymis[1]. It has been established that the mammalian epididymis provides a luminal microenvironment for the nurture, transport and maturation of sperms besides serving as a reservoir for storage of viable spermatozoa. These functions are thought to be expressed by region specific gene and protein expression patterns by different epididymal segments, *i.e.*, the caput, the corpus and the cauda. All these functions of the epididymis are described to be androgen dependant[2].

Androgens are known to be involved in regulation and synthesis of various proteins by the epididymis. Robaire and Viger postulated that the active regulators responsible for maintaining epididymal structure and other epididymal functions are testosterone and the 5-alpha reductase metabolite dihydrotestosterone[2]. It has also been established that the function of specific proteins in the epididymis of adult rhesus monkeys[3] and rats[4] are androgen dependant. Likewise, *in-vitro* studies have revealed the synthesis and secretion of proteins in different regions of the human epididymis, which are also androgen dependent[5].

There have been several reports characterizing epididymal proteins in different species[6,7]. The bovine secretory

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proteins derived from caput and cauda epididymal epithelial cell cultures are known to be dependent on androgen. It has been observed that a drastic reduction in the level of multiple proteins in various regions of epididymis of orchidectomized rats indicating that the epididymal proteins are necessary for *in-vivo* fertilization and are useful markers to study androgenic action in the epididymis[8].

In order to suggest new criteria that are more accurate and objective in predicting and improving male fertility when compared to conventional semen analysis[9], an insight into development of specific molecular markers is warranted. Hence, the present work is taken with the following objectives: a) to analysis protein profile in tissues of caput, corpus and cauda of epididymis in both castrated and normal bulls by two dimensional SDS-PAGE (2D SDS-PAGE) electrophoresis, and b) to identify an androgen dependant protein in all three regions of epididymis.

## 2. Material and methods

### 2.1. Animals and experimentation

Six healthy castrated indigenous breed cattle bulls (*Bos Taurus*), 2.5 to 3.0 years in age were used in the present study. The testes of these animals were surgically obtained under aseptic condition before slaughter. The castrated animals undergone castration at 1 month old and no spermatozoon was present in the epididymis of the castrated animals. The experiments conducted were MIAPE-compliant as per the minimum information about a proteomics experiment[10]. The gel electrophoresis and mass spectrometry experiments in our studies were according to the guidelines prescribed[11,12].

### 2.2. Tissue processing and storage

The epididymis from all twelve testes were dissected free of the surrounding fat and muscle layers. The caput, corpus and cauda portion of epididymis were separated. The regions of 1.00 cm from the anterior and posterior ends were dissected out as caput and cauda, respectively. The one centimeter region at the center of the epididymis, approximately equidistant from the caput and cauda was considered as corpus. Tissues from each of these three regions were further divided into two parts and they were washed 3 times with phosphate buffer saline (PBS) to remove the sperms and seminal fluids. Tissues from each of these three regions of the first part were subjected to tissue homogenate preparation. Then tissue homogenate was divided into three equal aliquots and stored at-70 °C in labeled micro centrifuge tubes.

### 2.3. Preparation of tissue homogenate

Tissue sample (500 mg) was washed 4 times with PBS (pH 7.4), homogenized and sonicated in presence of 1x protease inhibitor cocktail (Roche Molecular Biochemicals, Germany). The homogenate was centrifuged at 400 *g* for 20 min at 4 °C, to remove cellular debris. The supernatant was stored at -20 °C for assays.

### 2.4. Two-dimensional gel electrophoresis

Two-dimensional electrophoresis was used as the first step for the isolation of proteins for further characterization by mass spectroscopy for accurate protein identification using peptide mass fingerprinting. The samples were subjected to the 2D-PAGE in quadruplicates.

### 2.5. Tube gel isoelectric focusing (IEF)

The IEF of the third aliquot of each of the three regions of the epididymis in normal and castrated bull epididymis was performed as described by Berube *et al*[13]. Each sample was subjected for IEF in duplicate (One gel was employed for coomassie stain and other for silver stain). IEF was performed in 11.0 cm×0.2 cm glass rods by employing 12% acrylamide gel solution. The run was performed by employing discontinuous buffer system for 18 h at 400 v in -4 °C enclosure to prevent the heating of the gel. However before application of the sample to the tubes a pre-run focusing was performed for 1 h (300 v for 30 min followed by 400 v for another 30 min) by loading the gel tubes with 20 μL of overlaying solution (9.5% urea, 2% ampholine, 2% NP-40, 5% beta mercaptoetanol). After the run the acidic end of the gel was stained with methyl red dye and stored at -20 °C for subjecting it to SDS-PAGE.

### 2.6. SDS-PAGE of IEF gel

SDS-PAGE was carried out in 1.5 mm thick 16 cm×18 cm slab gels (Protean multiple gel cell). The gel and buffer systems employed were as described by Laemmli[14]. The slab gel was cast between the two glass plates comprised of two wells. The smaller well was employed for loading 7 μL molecular weight marker (Sigma Chemical Co., St Louis, Mo Range: 20-118). The IEF gel was placed on top of the larger well and the length adjusted so as to fit into the well. Subsequently, the larger well, which contains the IEF gel, is overlaid with one percent agarose solution (comprising 1% agarose in sample buffer plus 0.1% bromophenol blue and 1 mol/L urea) such that the IEF gel get sealed and at the same time it stays in contact with the separating gel, and it was left for five min to solidify. Later, the upper and lower tanks were filled with electrode buffer (Electrode buffer: 3.03 g Tris-base, 14.41 g glycine, 1.00 g SDS volume made to one liter with DDW). The electrophoretic run was performed at 100 v until the tracer dye reaches the anode end (approx 5-6 h). The slab gels obtained by running two separate IEF was used for coomassie blue staining and silver staining.

### 2.7. Staining of gel

#### 2.7.1. Silver staining

Silver staining was performed as described by Hochstrasser *et al*[15]. The silver staining was performed for gels subjected for normal SDS-PAGE as well as IEF gels subjected for SDS-PAGE.

#### 2.7.2. Coomassie staining (Coomassie brilliant blue R-250)

Coomassie staining was carried out, as per the method of Bradford as modified by Macart and Gerbaut[16]. Coomassie blue staining, chosen for its property of quantitative staining between different proteins, despite its low-level sensitivity of detection, was used to reveal the major proteins. The coomassie blue staining was performed for both gels subjected for SDS-PAGE (*i.e.*, SDS-PAGE of IEF gel) run gels.

#### 2.7.3. Analysis of the stained gels

The spots obtained on gels stained with coomassie blue were subjected for matrix assisted laser desorption and ionization-mass spectrometry (MALDI-MS) analysis. The gels stained with silver stain were analyzed for comparing each region of epididymis of both castrated and uncastrated bulls.

#### 2.7.4. Analysis of 2D gels

Stained gels were scanned in a wet state with a high-resolution camera (Estaman Kodak, Rochester, NY) and the resulting 2D SDS-PAGE images of each region of epididymis were analyzed for comparing with respective regions of castrated bull. The epididymal proteins with molecular mass between 20 to 118 kDa, and isoelectric point (pI) between 3.5 to 9.3 were catalogued from silver stained gels. All the images were collected on 2D 2920 Master Imager (Amersham Pharmacia Biotech, Piscataway, NJ). The 2D gel images were also carried out in Decyder-DIA software (Amersham Pharmacia Biotech, Piscataway, NJ).

### 2.8. Preparation of coomassie blue stained 2-dimensional gel spots for in-gel trypsin digestion

The coomassie blue stained 2-dimensional gel spot were processed for obtaining peptide sample as described by Umar *et al*[17]. The coomassie stained protein spot of interest were excised and cut into small pieces of one mm with sterile blade and transferred into micro centrifuge tube. Later, the gel was dehydrated in 100 μL of acetonitrile for 5 min and then destained with 100 μL of destaining solution (Destaining solution: 500 μL of acetonitrile and 500 μL of ammonium bicarbonate) for 10 min. The destaining step was repeated three to four times until complete destaining was ensured. Subsequently, the gel that settles as sediment (supernatant being discarded) was treated with 100 μL of acetonitrile for 5-10 min and the gel was dried by speed vacuuming for 10 min. Thereafter, reduction was carried out by addition of 30 μL of 10 mM dithiothreitol (DTT) solution (0.000 7 g DTT in 500 μL ABC) and was kept at 57 °C for 45 min. The supernatant was discarded after centrifugation at 300 r/min for 10 min. Then 30 μL of 55 mmol/L iodoactamide (IAA) was added and vortexed in dark for one hour. The supernatant was discarded. Subsequently the pellets were washed twice with 100 μL solution containing ABC and acetonitrile mixture (500 μL of acetonitrile and 500 μL ABC) and it was dried in vacuum for 5-10 min for purpose of In-gel trypsin digestion.

### 2.9. In-gel trypsin digestion

In-gel trypsin digestion was performed as per the method of KOC *et al*[18]. A 12 μL of trypsin (0.05 mg of trypsin/mL of double distilled water) was added, and incubated at 37 °C for 12 h. After 30 min, 50 μL of solution containing ABC and acetonitrile (Mixture 1:1) was added before 12 h of incubation. Then extraction was done by adding 5 μL of mixture containing 60% acetonitrile, 35% DDW and 5% trifluroacetic acid). The contents were sonicated for 5 min, supernatant was used for MALDI-MS.

### 2.10. Mass spectrometry

Peptides were eluted from the gel with 30% (v/v) ACN/0.1% (v/v) trifluoric acid, and 0.5μL peptide solution was mixed with four volumes of matrix solution (2 mg cyano hydroxycinnamic acid in 100% acetonitrile). It was left to dry at ambient temperature. Later it was spotted on to the target plate. Mass spectra were generated on a Biflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-MS) (Bruker Daltonik) and peptide fingerprints were analyzed using MASCOT database (Matrix Science, London, UK).

### 2.11. Statistical analysis

The results obtained were subjected for statistical analysis (ANOVA and *t*-test). The computer assisted statistical software package (Graph Pad Prism, San Deigo, USA) was used for analyzing the data. The logarithm of the relative protein content was used as the dependent variable. Statistical analysis was carried out using the General Linear Model procedures. Significance or non-significance of differences between mean values was determined at 5% level of significance ($P<0.05$).

## 3. Results

### 3.1. 2D SDS-PAGE analysis of three different regions of epididymis

The 2D SDS-PAGE analysis was done to identify the number of protein spots with molecular weight <20, 20-26, 26-36, 36-47, 47-85, 85-118 and >118 kDa in caput, corpus and cauda epididymal region of the normal and castrated

bulls. The 2D SDS-PAGE analysis for all the three regions of the epididymis in both normal and castrated bull were done using ampholine with pI ranging from 3.50 to 9.30. The number of protein spots for different molecular weights in the caput, corpus and cauda region of the normal and castrated bulls with pI 3.50 to pI 7.35 are presented in Table 1 and the same with pI>7.35 to pI 9.30 are presented in Table 2.

The mean number of the protein spots when compared between the three different regions of epididymis (<20, 20-26, 26-36, 36-47, 47-85, 85-118 and >118 kDa molecular weight) with pI 3.50 to 7.35 in both normal and castrated bull revealed significant difference ($P$<0.001). However, no significant difference was observed in the number of protein spots of 20-26, 26-36 and >118 kDa molecular weight with pI 3.50 to 7.35 when compared between corpus and cauda region of the normal bull epididymis. Similarly, no difference was observed in the number of protein spots of <20, and >118 kDa molecular weight with pI 3.50 to 7.35 when compared between corpus and cauda region of the castrated bull epididymis.

Likewise, significant difference ($P$<0.001) was observed in the mean value of the protein spots when observed within a region (caput or corpus or cauda) between normal and castrated bull epididymis of <20, 26-36, 36-47, 47-85, 85-118 and >118 kDa molecular weight with pI 3.50 to 7.35. However, no significant difference was observed in the mean value of the number of protein spots when observed between normal and castrated bull epididymis in all three regions of 20-26 kDa molecular weight with pI 3.50 to 7.35.

The mean number of the protein spots when compared between the three different regions of epididymis of <20, 20-26, 26-36, 36-47, 47-85, 85-118 and >118 kDa molecular weight with pI 7.35 to 9.30 in both normal and castrated bull revealed significant difference ($P$<0.001). However, the number of protein spots (pI 7.35 to 9.30) of 36-47 kDa, 85-118 and >118 kDa molecular weight were found to be statistically similar when compared between caput and cauda, between caput and corpus and between all three regions respectively in the normal bull epididymis. So also, the number of protein spots (pI 7.35 to 9.30) of <20, 36-47, and 85-118 kDa were found to be statistically similar when compared between caput and cauda epididymis of the castrated bull, while the same of >118 kDa molecular weight was found to be similar in all three regions of castrated bull epididymis.

Significant difference ($P$<0.001) was observed in the mean value of the protein spots when observed within a region (caput or corpus or cauda) between normal and castrated bull epididymis of <20, 26 -36, 36-47, 47-85, 85-118 and >118 kDa molecular weight with pI 7.35 to 9.3 . However, no significant difference was observed in the mean number of protein spots when observed between normal and castrated bull epididymis in corpus region of 20-26 kDa molecular weight as well as in all the three regions of >118 kDa molecular weight.

**Table 1**

2D SDS-PAGE protein spots of different molecular weight with pI value between 3.50 and 7.35 of epididymal tissue from three epididymal regions of normal and castrated bulls.

| Epididymal | <20 kDa | | 20-26 kDa | | 26-36 kDa | | 36-47 kDa | | 47-85 kDa | | 85-118 kDa | | >118 kDa | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| tissue | N | C | N | C | N | C | N | C | N | C | N | C | N | C |
| Caput | 5.00±0.57$^{a1}$ (4-6) | 1.00±0.25$^{a2}$ (0-1) | 2.00±0.30$^{a1}$ (1-3) | 2.00±0.21$^{a1}$ (1-3) | 17.00±0.57$^{a1}$ (16-18) | 15.00±0.57$^{a2}$ (14-17) | 15.00±0.36$^{a1}$ (14-17) | 13.00±0.36$^{a2}$ (12-14) | 21.00±0.57$^{a1}$ (20-22) | 15.00±0.57$^{a2}$ (14-17) | 3.00±0.57$^{a1}$ (2-4) | 6.00±0.57$^{a2}$ (5-7) | 1.00±0.25$^{a1}$ (0-2) | 3.00±0.25$^{a2}$ -(2-4) |
| Corpus | 7.00±0.57$^{b1}$ (5-8) | 3.00±0.25$^{b2}$ (2-4) | 2.00±0.36$^{a1}$ (1-3) | 1.00±0.25$^{b1}$ (0-2) | 21.00±0.57$^{b1}$ (20-22) | 14.00±0.25$^{b2}$ (12-15) | 36.00±0.57$^{b1}$ (35-37) | 24.00±0.57$^{b2}$ (22-25) | 16.00±0.57$^{b1}$ (15-17) | 22.00±0.57$^{b2}$ (20-23) | 1.00±0.25$^{b1}$ (0-1) | 12.00±0.57$^{b2}$ (10-13) | 1.00±0.25$^{a1}$ (0-1) | 4.00±0.25$^{b2}$ (3-5) |
| Cauda | 10.00±0.57$^{c1}$ (8-12) | 3.00±0.25$^{b2}$ (2-4) | 6.00±0.57$^{b1}$ (4-7) | 5.00±0.36$^{c1}$ (3-7) | 21.00±0.57$^{b1}$ (20-22) | 23.00±0.57$^{c2}$ (21-25) | 32.00±0.57$^{c1}$ (30-34) | 21.00±0.36$^{c2}$ (20-22) | 34.00±0.57$^{c1}$ (32-36) | 27.00±0.57$^{c2}$ (25-28) | 13.00±0.57$^{c1}$ (12-14) | 10.00±0.51$^{c2}$ (8-12) | 7.00±0.57$^{b1}$ (6-8) | 4.00±0.25$^{b2}$ (3-5) |

N = normal bull, C = castrated bull.

Superscript bearing different small letters within a column differs significantly ($P$<0.05).

Superscript bearing different numbers within a row for particular molecular weight between normal and castrated bull epididymis differs significantly

Values in parenthesis indicate range.

**Table 2**

2D SDS-PAGE protein spots of different molecular weight with pI value between more than 7.35 and 9.30 of epididymal tissue from three epididymal regions of normal and castrated bulls .

| Epididymal | <20 kDa | | 20-26 kDa | | 26-36 kDa | | 36-47 kDa | | 47-85 kDa | | 85-118 kD | | >118 kDa | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| tissue | N | C | N | C | N | C | N | C | N | C | N | C | N | C |
| Caput | 4.00±0.36$^{a1}$ (3-5) | 0.16±0.16$^{a2}$ (0-1) | 2.00±0.36$^{a1}$ (1-3) | 1.00±0.25$^{a2}$ (0-2) | 7.00±0.57$^{a1}$ (5-8) | 3.00±0.25$^{a2}$ (2-4) | 7.00±0.57$^{a1}$ (5-9) | 2.00±0.36$^{a2}$ (1-4) | 11.00±0.36$^{a1}$ (9-13) | 0.16±0.16$^{a2}$ (0-1) | 11.00±0.36$^{a1}$ (9-13) | 0.16±0.16$^{a2}$ (0-1) | 0.00±0.00$^{a1}$ | 0.00±0.00$^{a1}$ |
| Corpus | 3.00±0.25$^{b1}$ (2-4) | 0.83±0.16$^{b2}$ (0-2) | 0.00±0.00$^{b1}$ | 0.00±0.00$^{b1}$ | 10.00±0.57$^{b1}$ (9-12) | 0.16±0.16$^{b2}$ (0-1) | 10.00±0.57$^{b1}$ (9-11) | 0.83±0.16$^{b2}$ (0-2) | 4.00±0.25$^{b1}$ (3-5) | 0.83±0.16$^{b2}$ (0-2) | 4.00±0.25$^{b1}$ (3-5) | 0.83±0.16$^{b2}$ (0-2) | 0.00±0.00$^{a1}$ | 0.00±0.00$^{a1}$ |
| Cauda | 2.00±0.25$^{c1}$ (1-3) | 0.16±0.16$^{a2}$ (0-1) | 4.00±0.36$^{c1}$ (3-5) | 3.00±0.25$^{c2}$ (2-4) | 6.00±0.25$^{c1}$ (4-7) | 1.10±0.16$^{c2}$ (0-2) | 7.00±0.36$^{a1}$ (6-8) | 2.00±0.25$^{a2}$ (1-3) | 6.00±0.25$^{c1}$ (5-7) | 2.00±0.25$^{c2}$ (1-3) | 6.00±0.25$^{c1}$ (5-7) | 2.00±0.25$^{c2}$ (1-3) | 0.00±0.00$^{a1}$ | 0.00±0.00$^{a1}$ |

N = normal bull, C = castrated bull.

Superscript bearing different small letters within a column differs significantly ($P$<0.05).

Superscript bearing different numbers within a row for particular molecular weight between normal and castrated bull epididymis differs significantly ($P$<0.05).

Values in parenthesis indicate range.

Further, the results of the 2D SDS-PAGE for the total number of protein spots irrespective of the molecular weight in caput, corpus and cauda region of epididymis in the normal and castrated bulls for pI 3.50 to 7.35 and pI>7.35 to 9.30 is shown in Table 3 and Table 4 respectively. The results revealed a significant difference ($P$<0.001) in the total number of protein spots (pI 3.50 to 7.35 as well as pI 7.35 to 9.30) when compared between three different regions of epididymis in normal as well as castrated bull. Similarly, significantly higher number of protein spots (pI 3.50 to 7.35 and pI 7.35 to 9.30) was observed in caput, corpus and cauda epididymis of the normal bull when compared to similar region of the castrated bull epididymis.

**Table 3**

2D SDS-PAGE protein spots of epididymal tissue from three different regions of normal and castrated bulls epididymis irrespective of molecular weight in pI range 3.50-7.35.

| Epididymal tissue | Normal bull epididymis | Castrated bull epididymis |
|---|---|---|
| Caput | 64.00±0.89$^{a1}$ (62-67) | 55.00±1.29$^{a2}$ (53-57) |
| Corpus | 84.00±1.46$^{b1}$ (83-85) | 88.00±0.89$^{b2}$ (85-89) |
| Cauda | 123.0 0± 0.52$^{c1}$ (120-125) | 93.00±0.52$^{c2}$ (91-94) |

Superscript bearing different small letters within a column differs significantly ($P$<0.05).
Superscript bearing different numbers within a row for normal and castrated bull differs significantly ($P$<0.05).
Values in parenthesis indicate range.

**Table 4**

2D SDS-PAGE protein spots of epididymal tissue from three different regions of normal and castrated bulls irrespective of molecular weight in pI range more than 7.35-9.30.

| Epididymal tissue | Normal bull epididymis | Castrated bull epididymis |
|---|---|---|
| Caput | 32.00 ± 0.52$^{a1}$ (30-34) | 6.00 ± 0.26$^{a2}$ (4-7) |
| Corpus | 28.00 ± 0.52$^{b1}$ (26-29) | 3.00 ± 0.26$^{b2}$ (1-5) |
| Cauda | 25.00 ± 0.52$^{c1}$ (23-26) | 8.83 ± 0.48$^{c2}$ (6-9) |

Superscript bearing different small letters within a column differs significantly ($P$<0.05).
Superscript bearing different numbers within a row for normal and castrated bull differs significantly ($P$<0.05).
Values in parenthesis indicate range.

The results of the 2D SDS-PAGE for the total number of protein spots irrespective of the molecular weight and pI range of 3.50-9.30 in caput, corpus and cauda region of the normal and castrated bulls is shown in Table 5. The results revealed a significant difference ($P$<0.001) in the total number of protein spots when compared between three different regions of epididymis in normal as well as castrated bull. The number of protein spots was significantly higher in the cauda region, followed by corpus and caput region in both normal and castrated bull epididymis. Further, the number of protein spots found to be significantly higher ($P$< 0.001) in caput, corpus and cauda epididymis of the normal bull when compared to similar region of the castrated bull epididymis.

**Table 5**

2D SDS-PAGE protein spots of epididymal tissue from three different regions of normal and castrated bulls epididymis irrespective of molecular weight in pI range 3.50-9.30.

| Epididymal tissue | Normal bull epididymis | Castrated bull epididymis |
|---|---|---|
| Caput | 96.00±0.52$^{a1}$ (92-99) | 52.00±0.52$^{a2}$ (50-54) |
| Corpus | 112.00±0.52$^{b1}$ (111-115) | 83.00±0.77$^{b2}$ (80-84) |
| Cauda | 148.00±0.52$^{c1}$ (145-150) | 102.00±0.52$^{c2}$ (100-103) |

Superscript bearing different small letters within a column differs significantly ($P$<0.05).
Superscript bearing different numbers within a row for normal and castrated bull differs significantly ($P$<0.05).
Values in parenthesis indicate range.

### *3.2. Identification of androgen dependent proteins in three different regions of epididymis*

The protein spots observed on the gel of the three different regions of epididymis of normal bull and the castrated bull revealed the presence of six additional protein spots in the normal bull epididymis, while the same were not present in the castrated bull. The protein spots that were present in the normal bull with pI value and molecular weight are shown in Figure 1 (caput region), Figure 2 (corpus region), and Figure 3 (cauda region). The molecular weight of all these six spots was less than 20 kDa. The pI of these six spots was in the range of 6.55 to 6.85, 3.50 to 4.55, 5.20 to 5.85, 5.20 to 5.85, 5.85 to 6.55 and 7.35 to 8.15. These spots have been marked as P1, P2, P3, P4, P5 and P6 respectively in Figure 1 (caput region), Figure 2 (corpus region) and Figure 3 (cauda region) and in Table 6.

**Table 6**

Androgen dependent protein spots in three regions of epididymis in normal bulls.

| Spot number | Molecular weight in range | Isoelectric point in range |
|---|---|---|
| P1 | <20 kDa | 6.55-6.85 |
| P2 | <20 kDa | 3.50-4.55 |
| P3 | <20 kDa | 5.20-5.85 |
| P4 | <20 kDa | 5.20-5.85 |
| P5 | <20 kDa | 5.85-6.55 |
| P6 | <20 kDa | 7.35- 8.15 |

### *3.3. MALDI-MS for identification of androgen dependent protein in the cauda epididymis*

A protein spot (pI: between 6.55 and 6.85, and MW: <20 kDa) which appeared at the same site in all the three regions of the epididymis in the normal bull but was absent at the same site in all the three regions of the castrated bull was subjected for identification of the protein by MALDI-MS. The protein spot selected is represented as P1 in Figure 1, 2 and 3. The results of the MALDI-MS revealed that this protein (17.4 kDa) is an interferon-stimulated protein.

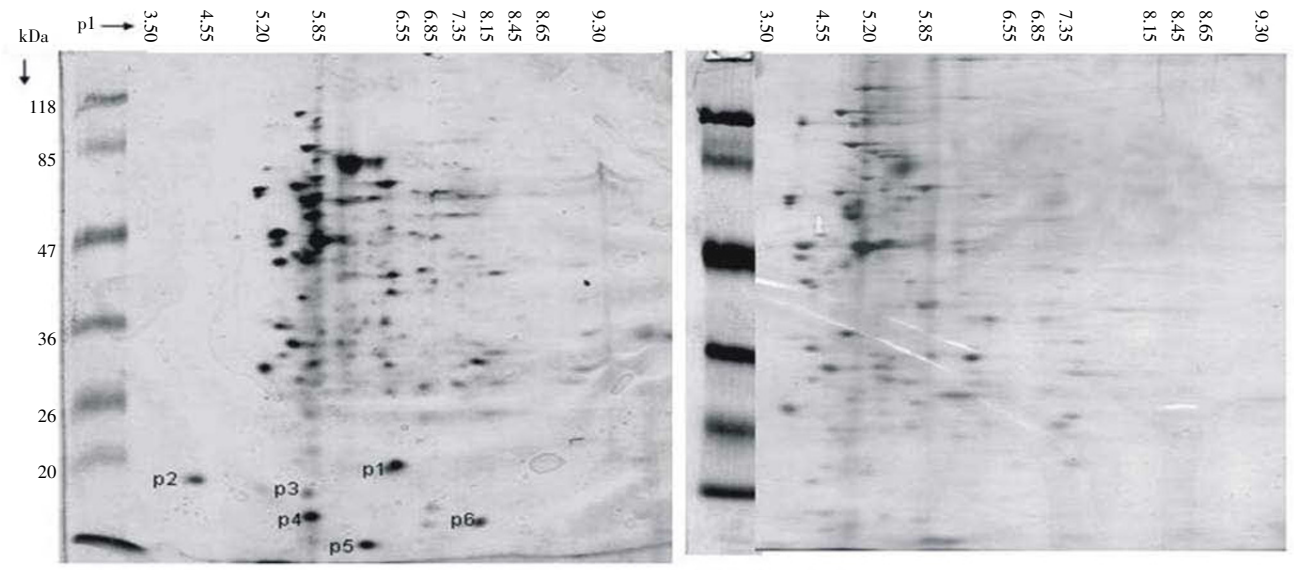


**Figure 1.** 2D SDS-PAGE of caput epididymis of normal (elft) and castrated bull (right).

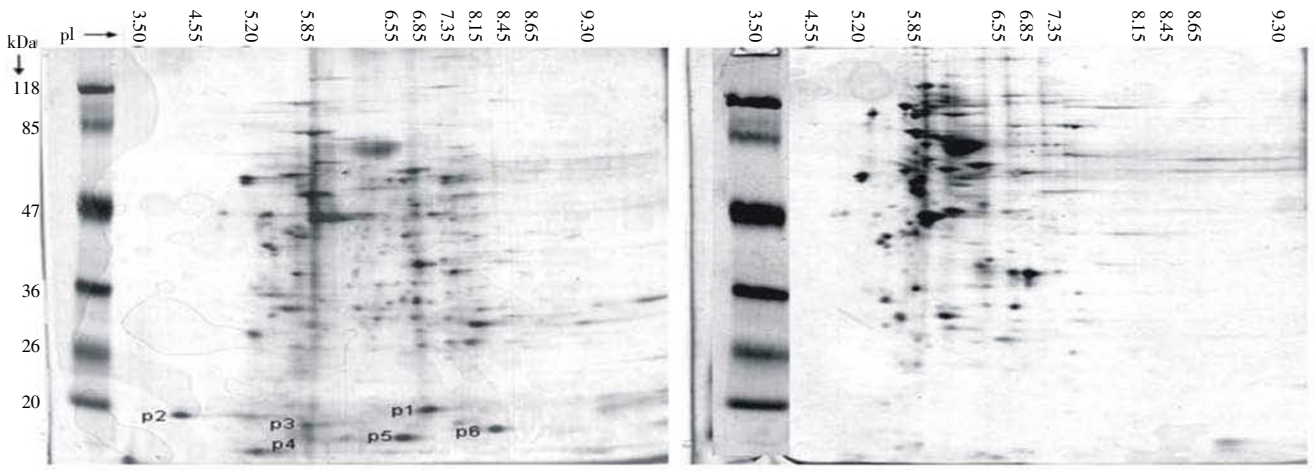


**Figure 2.** 2D SDS-PAGE of corpus epididymis of mormal (left) and castrated bull (right).

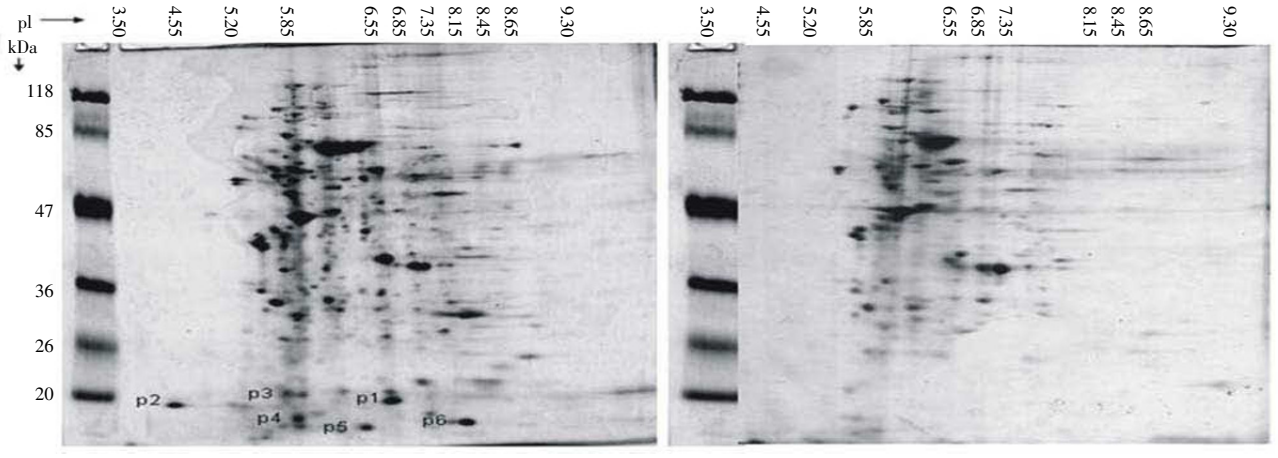


**Figure 3.** 2D SDS-PAGE of cauda epididymis of mormal (left) and castrated bull (right).

## 4. Discussion

The present study is the first report on 2D SDS-PAGE analysis of proteins in the caput, corpus and cauda region tissue of normal and castrated bull epididymis. Two dimensional SDS-PAGE analysis of the cauda epididymal fluid[7], protein secretions of the caput and cauda epithelial monolayer[19] and bovine seminal plasma[20-23] have been established. Arlindo *et al*[7] and Ingrid *et al*[19] have observed 118 and 47 spots respectively with the objective of relating these proteins to fertility. However, they had not either specified the molecular weight of each of the spots or established relationship of each of these spots to fertility and/or castration. Likewise, Killian *et al.* reported the relationship of only six protein spots of the bovine seminal plasma with fertility, although he was able to identify many more spots by 2D SDS-PAGE analysis[22]. Similarly Van *et al.*[23], Esch *et al.*[20] and Miller and Winer[21] established the relationship of only three protein spots, three protein spots and group of 15-17 kDa proteins respectively of the bovine seminal plasma to fertility although many spots were observed by 2D SDS-PAGE.

Similarly 2D SDS-PAGE analysis of stallion epididymal luminal fluid, epididymal tissue of all three regions of adult boar have been reported by Sophie *et al.*[6] and Patrick *et al.*[24] respectively. Sophie *et al.* reported presence of 186, 220 and 150 protein spots in the caput, corpus and cauda region of epididymis, respectively[6]. Patrick *et al.* also reported presence of 92, 34 and 22 protein spots in the tissue of caput, corpus and cauda region of epididymis by 2D analysis[24]. However, both Sophie *et al*[6] and Patrick *et al*[24] isolated and identified only few protein spots and had described only these protein spots. Similarly, 2D analysis of caput, corpus and cauda region of epididymis of rabbit[25], murine epididymis[26], luminal fluid of all three regions of the rat epididymis[27] and protein secretions of epididymal tissue minces of rat[28] have revealed many protein spots, however not all these spots have been described individually.

In view of the results and description of 2D analysis of either epididymal tissue or protein secretions of epididymal epithelial monolayer as described above by the previous workers, explanation for the links between their expression and relationship with androgens is still a matter of hypothesis and also the precise identity and function of each of these proteins in sperm maturation and storage processes remains to be established. Hence, the results of the 2D SDS-PAGE proteins spots were presented for explanation as protein spots in three different regions of normal and castrated bull epididymis irrespective of molecular weight in pI range 3.50 to 7.35 and pI 7.35 to 9.30. This eventually also would establish the number of spots in three different regions of epididymis in both normal and castrated bulls that were below the physiological pH of 7.35 and above the physiological pH of 7.35.

No published information is available on the total number of protein spots either in the epididymis or in the different regions of epididymis of normal and castrated bull. However, Arlindo *et al.* reported for 118 protein spots in the cauda epididymal fluid of the bulls[7] and Ingrid *et al*[19] reported 47 and 50 protein spots in the secretion of caput and cauda epithelial monolayer respectively. In the present study, the total number of protein spots in the normal bull epididymis of pI range 3.50 to 7.35 was 123, 84 and 64, while the same in the pI range from 7.35 to 9.30 was 25, 28, and 32 in the caput, corpus and cauda region respectively. The values also indicate that the number of acidic proteins (pI<7.35) is higher in all the three regions of normal bull epididymis when compared to number of proteins spots whose pI value is >7.35. These results are in agreement with the findings of Ingrid *et al.* who reported the increase in the number of acidic proteins (pI range of 4.0 to 6.5) in the caput and cauda region of the bull[19].

Further, analysis of the results revealed a significant increase in the number of protein spots of pI range 3.50 to 7.35 in the cauda followed by corpus and caput. While the analysis of the same for pI range from 7.35 to 9.30 revealed a reversed situation, *i.e.*, the protein spots were found to be significantly highest in the caput region followed by corpus and cauda region. Arlindo *et al.* reported an increase in presence of alpha-L fucosidase (pI 6.6), cathepsin (pI 6.8) apolipoprotein A-1 (pI 6.0) in high fertility bulls and decrease in presence of prostaglandin D-synthase (pI 6.3) in the low fertility bulls[7]. On the other hand, Fouchecourt *et al.* identified prostaglandin D-synthase in the murine epididymal fluid and put forth that this protein is highly polymorphic protein with pI range 6.5 to 8.8 and it is present all along the length of epididymis[29]. Likewise, it is also established that the capacity of the sperm to acquire motility and to fertilize ovum occurs in cauda[30]. Hence, an increase in the number of protein spots that are acidic in nature in the cauda, as well as a decrease in the number of protein spots that are alkaline in nature in the same region when compared to other regions of the normal bull, could probably be because of increase in number of acidic proteins having a role in fertility[7] enabling the cauda region to have an edge over the other regions with respect to its physiological role on fertility as put forth by Amman and Griel[30].

Analysis of 2D SDS-PAGE results on the number of protein spots in three different regions of castrated bull epididymis irrespective of molecular weight in pI range of 3.50 to 7.35 revealed an increased number in the cauda when compared to other two regions, followed by corpus and caput regions. On the other hand, the same in pI range of 7.35-9.30 revealed an increased concentration in cauda followed by caput and corpus. The reasons for these variations are yet to be established.

The results of the number of protein spots of pI range 3.50 to 7.35 in all three regions of the normal bull epididymis was found to be higher when compared to a particular region in the castrated bull epididymis. Toney and Danzo reported

a 17 kDa protein spot (pI 5.1) in all three regions of rabbit epididymis to be dependent on testosterone[25]. Hence, an increase in number of protein spots of pI range 3.50 to 7.35 in all three regions of normal bull epididymis when compared to castrated bull epididymis could be due to the influence of androgen. Similarly, an increase in the number of protein spots of pI range from 7.35 to 9.30 in all three regions of the normal bull epididymis when compared to a particular region in the castrated bull epididymis could also be due to the influence of androgen. Jimnez *et al.* reported two different polypeptides of pI 8.4 and 8.8 to be under the control of androgens[26].

Above all, an increase in the total number of protein spots in the cauda region of epididymis of normal bull irrespective of molecular weight and pI could be because of more number of protein spots being acidic in nature appearing in this region thereby reflecting the major role of cauda region in sperm maturation when compared to other two regions. Likewise, an increase in the number of protein spots in all three regions of the normal bull epididymis irrespective of molecular weight and pI when compared to a particular region in castrated bull epididymis reflects the role of androgens as explained above.

It has been confirmed that castration leads to cessation of blood supply to the testicles thereby leading to absence of action of gonadotrophic hormones on the testicular tissue which in turn leads to absence of production gonadal hormones[31]. Hence, the presence of these spots in all three regions of the normal bull epididymis and the absence of the same in castrated bull indicates that these protein spots could be highly dependent on androgens and/or they may be collectively referred to as androgen dependant proteins. On the other hand, the reason for presence of protein spots in all three regions of castrated bull epididymis and that not been found in similar regions of normal bull epididymis is not known.

Further, these findings being observed and put forth for first time in bull, it is of the opinion that there is need for further study on this aspect. However, one protein spot from among the androgen dependent protein in the cauda region was employed for further study by MALDI-MS. Each of the six protein spots identified as androgen dependant proteins in the normal bull epididymis were found to have almost similar molecular weight and pI in all the three regions of the epididymis. Hence, a protein spot (P1) from cauda was employed for the identification of protein by MALDI-MS. This particular protein spot was identified as interferon stimulated protein.

Interferons were produced and liberated by animal cells following exposure to inducing agents. Among the various proteins that are stimulated by interferon are interferon-stimulated gene 15 (ISG 15) also referred to as ubiquitin cross-reactive protein (UCRP)[32], plasma membrane protein[33], inducible proteins (PI 10)[34], Fas proteins[35] and enzymes[36]. Among these proteins, ISG 15/UCRP is a 15 kDa ubiquitin like protein identified as a product of an interferon stimulated gene in humans[32].

It was observed that ISG 15 had a role in defense against virus as well as stimulation of immune response[37]. Further, it was also postulated that HE2 protein[38] and EPPIN[39,40] of molecular weight 4-8 kDa and 27 kDa respectively were androgen dependent proteins having antimicrobial activity as well. Hence the protein spot (P1) identified as interferon stimulated protein in the present study as per the mass spectrometric analysis could be ISG 15/UCRP, similar to that of HE2[38] and EPPIN[39] which had also been concluded to be androgen dependant proteins with antimicrobial activity.

## Conflict of interest statement

We declare that we have no conflict of interest.

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