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Identification of Differentially Expressed Tear Proteins after Corneal Reconstruction with DPSIS Graft in Dogs Suffering from Keratoconjunctivitis Sicca

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ABSTRACT

Differentially expressed tear proteins after corneal reconstruction with decellularized porcine small intestinal sub mucosa (DPSIS) in Keratoconjunctivitis sicca (KCS) affected dogs was explored through Matrix Assisted Laser Desorption/Ionization Mass spectrometry (MALDI-TOF/MS) followed by Peptide mass fingerprinting (PMF). Anterior lamellar keratoplasty followed by DPSIS graft was performed in seven dogs with corneal ulcer (group A) and in nine dogs with corneal ulcer as a complication of KCS (group B). Tear was also collected from 12 healthy dogs with no ocular pathology. PMF based protein identification was performed on tryptic peptides of tear film trapped in major bands of ID-SDS PAGE (in duplicate) using MALDI-TOF/MS. PMF revealed 432 unique tear proteins from major gel bands of DPSIS treated dogs (group A and B) and normal tear from healthy dogs. Based on MOWSE score, relative abundance of proteins in both groups were compared with that in normal tear. Proteins USP37, KRT1, Major allergen Can F1, CCDC39, PRKCD, Serum albumin, TERT and CD163 were found to be significantly ($p<0.001$) down-regulated in group A and group B compared to normal tear from healthy dogs. Protein INVS was found significantly up regulated ($p<0.001$) in Group B post DPSIS graft compared to pre-operative tear. ZNF252 was found significantly up regulated ($p<0.001$) in Group A tear film post-operatively compared to pre-operative tear. Intrinsic cellular stress disrupting DNA replication and cell division, cellular senescence and apoptosis were found as key events underlying corneal pathology in KCS which needs consideration while attempting surgical reconstruction of cornea using DPSIS graft.

HIGHLIGHTS

- Differentially expressed tear proteins post- DPSIS graft in KCS affected dogs were elucidated.
- Intrinsic cellular stress disrupting DNA replication and cell division was the key event underlying corneal pathology in KCS affected dogs.

Keywords: Tear Proteome, MALDI-TOF/MS, DPSIS graft, Keratoconjunctivitis sicca, Dogs

Porcine small intestinal submucosa (PSIS) is a versatile biomaterial extensively used as corneal graft material in canine and feline cases. It is particularly useful for melting corneal ulcer, wherein corneal stroma is destroyed by enzymatic lysis aided by matrix metalloproteases (Andrade *et al.,* 1999). Many authors attribute a high success rate to corneal surface reconstruction with PSIS graft with minimum complications (Goulle, 2012; Singh *et al.,* 2016; Sowbharenya *et al*., 2019). Corneal graft

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acceptance depends largely on the healthy corneal tissue around the graft. Rejection rate of inflamed or immune-compromised graft beds is quite high despite aggressive therapy with immune-suppressants (Featherstone *et al.,* 2001). Keratoconjunctivitis sicca is an immune mediated disease targeted to lacrimal gland, characterised by a pathological reduction in aqueous component of tear film and concomitant pathological changes in ocular surface, keratitis, neovascularisation and corneal epithelial defects (Kim *et al.,* 2009, John *et al,* 2018, Swapna *et al*., 2020, Kumar *et al*., 2020, John *et al.,* 2020). Earlier study with PSIS graft showed pronounced corneal pigmentation in brachycephalic dogs with deep corneal ulcer (Goulle, 2012, John *et al,* 2018, Swapna *et al*., 2020). Detailed investigation into molecular mechanism underlying fate of corneal graft in immune-mediated disease like KCS is rare in literature. Proteomic analysis of tear fluid has proven to be a promising tool to gain information about the pathogenesis of diseases (Farias *et al.,* 2013). The current study was therefore undertaken to identify differentially expressed tear proteins after DPSIS grafting in Keratoconjunctivitis sicca (KCS) affected dogs.

MATERIALS AND METHODS

The study was conducted on sixteen client owned dogs presented to Referral Veterinary Polyclinic cum TVCC, IVRI, Izatnagar, Bareilly, Uttar Pradesh with varying degrees of corneal epithelial defect. Two groups were constituted wherein Group A consisted of seven dogs with corneal defect due to injury / dermoid and Group B consisted of nine dogs having corneal ulcer as complication of KCS. Tear collected from 12 healthy dogs with no ocular pathology were also utilised for this study. Detailed ophthalmic examination protocols were carried out with tonometer (Schiötz C, Riester, Germany) Direct ophthalmoscope (Reister, Germany), indirect ophthalmoscope (Appasamy Associates, Chennai) with a 20D indirect lens (Volk optical Inc,USA), Slit lamp Bio-microscope (Appasamy Associates, Chennai), Goniolens (OptiTech eye care, Tarun enterprises, Allahabad) along with Schirmer tear test strips (STT) (Schirmer tear test ophthalmic strips, (OptiTech eye care, tarun enterprises, Allahabad) and fluorescein staining (OptiTech eye care, Tarun enterprises, Allahabad) to assess the integrity of corneal surface. Due permission to conduct the clinical study was obtained from *Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of environment, Forest and climate change, Government of India (No.F.25/33/2016-CPCSEA dated 16/02/2017).* Written consent of the owner was taken for operative procedures as well as for pre- and post-operative ocular surface epithelial cells collection.

Surgical intervention and postoperative evaluation

Food and water were withheld for 12 hours prior to anaesthesia. Topical antibiotic (Ofloxacin – Zenfox, Mankind Pharma Ltd, New Delhi)), anti-inflammatory (Flurbiprofen – Flur, Allergen India Pvt. Ltd.) drops were instilled 6 to 24 hours prior to surgery. Surgery was performed under routine general anaesthesia and local instillation of proparacaine eye drops (Propcaine, Cipla ltd, India). Ocular surface of operated eyes was douched with 0.2% povidone iodine solution prior to surgery. Under general anaesthesia, loosely adhered corneal epithelium was removed with fine colibri forceps and keratotome (3.2mm). A corneal trephine (6mm) was used to outline the lamellar keratectomy margins. Anterior lamellar keratectomy was performed with corneal trephine approximately of the same size as that of DPSIS graft. The grafts were secured with simple interrupted sutures using 8/0 polyglactin 910. Subconjunctival injection of Gentamicin and Dexamethasone was given immediately after surgery. Temporary tarsorraphy was done using 2-0 polyamide after DPSIS graft in both groups. Antibiotic (Ceftriaxone sodium, Monodef, Aristo Pharmaceutical Pvt. Ltd., India @ 20 mg/ kg body weight) and anti-inflammatory agent (Meloxicam- M-cam, Unichem Pharmaceuticals, India @ 0.05 mg/kg body weight) was administered intramuscular postoperatively for 5 day. Antibiotic (Ofloxacin – Zenfox, Mankind Pharma Ltd, New Delhi)), anti-inflammatory (Flurbiprofen – Flur, Allergen India Pvt. Ltd.) and topical cyclosporin drops (Hydroeyes 0.05% w/v, Lupin Ltd., Mumbai) were instilled thrice daily for four weeks. Tarsorrhapy sutures were removed on 15th postoperative day. The efficacy of the bio-engineered PSIS for reconstruction of corneal defects was evaluated on 15, 30 and 60 days postoperatively.

ID Electrophoresis and MALDI-TOF MS

Tear samples were collected from dogs of group A and B and were kept in 0.5 ml Eppendorf tubes and were frozen

at -60°C, until tear proteomics study. Tear samples were also collected from healthy dogs using same method. After incubation with 10µl 50nM PBS elution buffer and protease inhibitor cocktail at 4º C for 20h, tear fluid was eluted from STT strips by centrifugation @15000 rpm/ min at 4º C. Total protein concentration in the pooled tear sample of each group was measured by the BCA method (GeNei™, Bangalore). Based on the protein concentration, the remaining pooled tear samples were diluted to final concentration of 30 µg/ 10 µl with sterile distilled water. The tear samples were reduced in DTT, mixed with loading buffer; each sample containing 30 µg proteins was loaded and run in 12% polyacrylamide gel along with popular molecular weight marker (12kDa to 100 kDa). Protein bands were detected by Coomassie Colloidal Blue. Tryptic peptides from at least 4 gels each from group A and Group B (in triplicates) before and after DPSIS graft were subjected to MALDI-TOF/MS and internally calibrated spectra were acquired on a Bruker Daltonics model Ultraflex II Spectrometer. Mass spectra generated were used for PMF search with MASCOT and MS-FIT against Swissprot 2017-06 database with following parameters: global modification of carbamidomethyl (C); possible modification as oxidation (M), Peptide mass tolerance of 300 ppm, Fragment mass tolerance of 0.1 Da and 1 missed cleavage. Proteins were identified based on MOWSE score (>69) and those proteins found common in both groups with respect to normal tear were identified and their relative abundance was calculated based on MOWSE score. Protein ontology classification was performed by importing all identified proteins using software Protein Atlas Through Evolutionary Relationship (PANTHER) classification system (http://www.pantherdb.org/, SRI International, Menlo Park, CA). Data were analyzed by software Statistical Program for Social Sciences (SPSS 20 IBM). Statistical analysis of proteomic data is represented as mean±SE of MOWSE score based on the mass/charge of tryptic peptides. Non-parametric data were analysed using Kruskal-Wallis test, $p<0.05$ was considered to be statistically significant. All the graphs were prepared in Graph Pad Prism (Version 5). Protein ontology analysis was performed by importing all identified proteins using software Protein Atlas Through Evolutionary Relationship (PANTHER) classification system (http://www.pantherdb.org/, SRI International, Menlo Park, CA). Statistical over-representation of proteins identified against reference data of *Canis lupus familiaris* for non random association between the two sets was performed in the same software using Fischer exact test with $P<0.05$.

RESULTS AND DISCUSSION

Demographic data of groups A and B (16 cases) is given in Table 1. Group B corneal ulcer cases with KCS pathology were mostly Pugs where as other breeds like Pomeranian, Spitz and Labrador were included in groups A. Age of

Table 1: Demographic data of Group A and Group B dogs subjected to DPSIS graft

Animal no.	Breed	Age	Gender	OD/OS	Primary lesion	Concurrent anomaly
A-1	Pomeranian	4 years	Female	OD	Deep stromal ulcer, AC not visible	—
A-2	French Mastiff	2 ½ years	Male	OD	Deep stromal ulcer, AC not visible	Entropion
A-3	Mongrel	7 months	Female	OD	Deep stromal ulcer, AC not visible	—
A-4	Labrador	6 ½ years	Male	OS	Deep stromal ulcer, AC not visible	—
A-5	Mongrel	6 months	Male	OS	Deep stromal ulcer	—
A-6	Mongrel	2 years	Male	OD	Deep stromal ulcer	—
A-7	Spitz	4 years	Male	OD	Melting ulcerative keratitis	—
B-1	Pug	6 months	Female	OS	Deep stromal ulcer	KCS
B-2	Pug	2 ½ years	Male	OD	Melting ulcerative keratitis	KCS
B-3	Pug	4 years	Male	OD	Corneal perforation	KCS
B-4	Pug	9 ½ months	Female	OD	Corneal perforation	KCS
B-5	Pug	8 months	Male	OS	Corneal perforation	KCS
B-6	Pug	3 ½ years	Female	OS	Deep stromal ulcer	KCS, CP
B-7	Pug	3 ½ years	Female	OD	Corneal perforation	KCS
B-8	Pug	5 years	Male	OS	Staphyloma	KCS, CP
B-9	Pug	1 ½ years	Female	OS	Deep stromal ulcer	KCS

dogs under this study ranged from seven months to six and a half years in group A (mean ± S.E. value of age 3.75 ± 1.05 years) and from six months to five years in group B (mean ± S.E. value of age 2.44 ± 0.55 years).

Average protein concentration was 42.5 µg, 36.5 µg, 31 µg and 16.5 µg in group A and 35 µg, 2 µg, 30 µg and 14 µg in Group B per 10 µl of pooled tear before graft and 15, 30 and 60th day post-DPSIS grafting. ID electrophoresis revealed differential expression of tear proteins trapped in ID gel (30 µg/lane) at different post DPSIS graft intervals (day 15, 30 and 60) compared to pre-op tear (Fig. 1a &1b).

MALDI-TOF/MS revealed 432 unique tear proteins from major gel bands of DPSIS treated dogs (group A and B) and normal tear from healthy dogs. Overlap of common and unique proteins in Group A and Group B before and after DPSIS graft with that in normal tear was pictorially depicted by Venn diagram (Fig. 2). Abundance of proteins for biomarker discovery and elucidation of disease mechanisms is adequately explored through tear film in previous studies (Winiarczyk *et al.,* 2015; Jung *et al.,* 2017). There were 58 proteins common among the two groups (pre and post treatment) and normal tear. MOWSE score for proteins USP 37, KRT1, Major allergen Can F1, CCDC39, PRKCD, Serum albumin, TERT and CD163 were found to be significantly ($p<0.001$) low in group A (pre op) and group B (pre and post -op) compared to normal tear (Fig. 3). Krt10 and its type II pair Krt1 were considered to be keratinisation markers (Toivola *et al.,* 2015) and KRT1 regulates the activity of PKC & SRS kinases via binding to ITB1 and RACK1(Pekny and Lane, 2007) and elicits unfolded protein response (UPR) (Allen *et al.,* 2016). USP37 has been implicated in X-linked retinal disorders in human (Deborah *et al.,* 1996). Genome-wide association studies (GWAS) implicating the role of the collagen and extracellular matrix pathways in the regulation of keratoconus has identified USP37, ZNF469 and many other genes (Gao *et al.,* 2013; Lu *et al.,* 2013). TERT, the catalytic subunit of telomerase, inhibited apoptosis and augmented DNA repair (Colitz, 2006). Telomerase activity was found to protect replicative senescence and extends life span of keratinocyte stem cells and conjunctival cells (Pellegrini *et al.,* 2004). Major allergen Can f1 (Can F1) is an extracellularly secreted protein structurally homologous to LCN1, the human tear lipocalin. Proteomic analysis of tear film in human dry eye syndrome also showed down regulation of LCN1 (Jung *et al.,* 2017; Huang *et al.,* 2018).

Protein Inversin (INVS) score was found significantly high ($p<0.001$) in Group B post DPSIS graft compared to Pre-operative tear (Fig. 3a) and that of ZNF252 was found significantly high ($p<0.001$) in Group A post DPSIS graft compared to Pre-operative tear (Fig. 3b). INVS acts as a molecular switch between different Wnt signalling pathways; it inhibited the canonical Wnt pathway and was found associated with abnormality of retinal pigment epithelium (May-Simera *et al.,* 2018). It also influences ciliary disassembly by regulating Wnt signalling (Simons *et al.,* 2005). Serum albumin was significantly unregulated ($p<0.001$) in group A (pre op) compared to group B (pre-op). Functional enrichment of differentially expressed

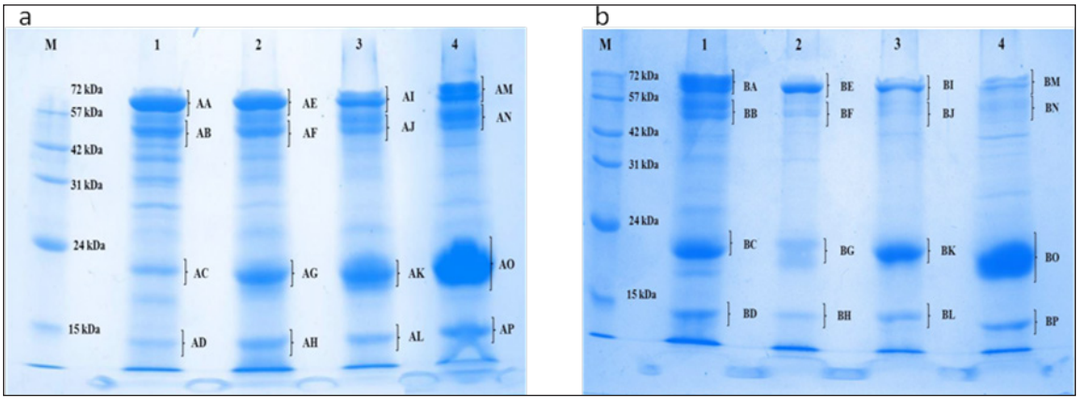
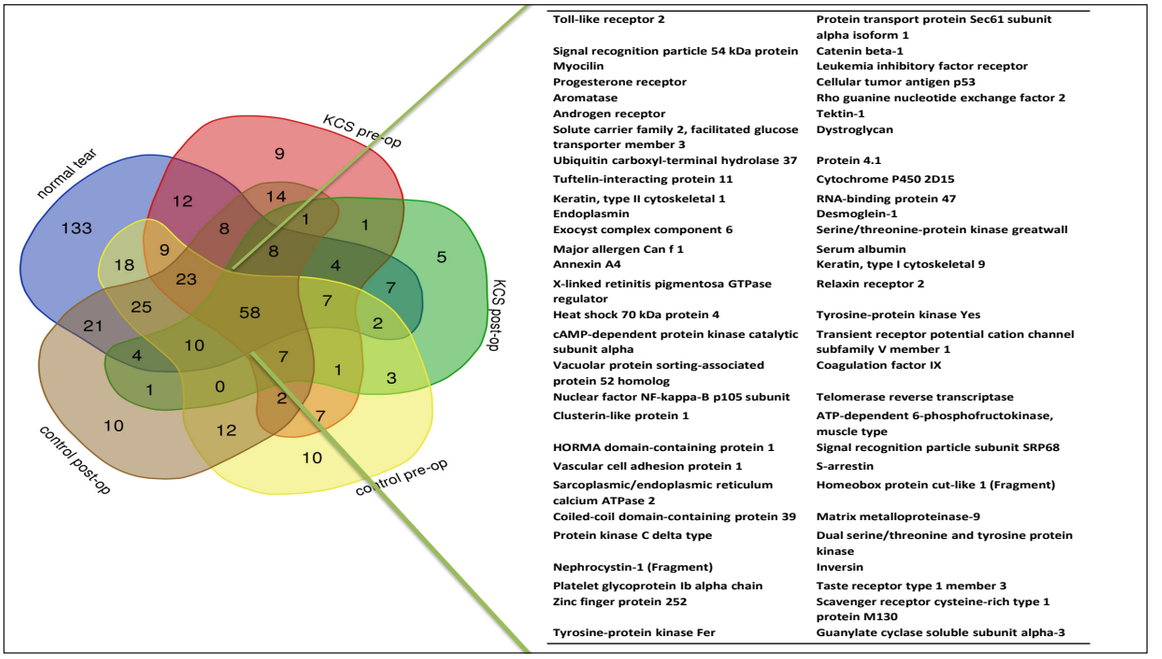


Fig. 1: Coomassie blue-stained SDS-PAGE of tear proteins (M-protein ladder, 1-pre op tear, 2- post DPSIS graft day 15, 3- post DPSIS graft day 30, 4- post DPSIS graft day 60) in Group A (a) and in Group B (b)



Toll-like receptor 2	Protein transport protein Sec61 subunit alpha isoform 1
Signal recognition particle 54 kDa protein	Catenin beta-1
Myocilin	Leukemia inhibitory factor receptor
Progesterone receptor	Cellular tumor antigen p53
Aromatase	Rho guanine nucleotide exchange factor 2
Androgen receptor	Tektin-1
Solute carrier family 2, facilitated glucose transporter member 3	Dystroglycan
Ubiquitin carboxyl-terminal hydrolase 37	Protein 4.1
Tuftelin-interacting protein 11	Cytochrome P450 2D15
Keratin, type II cytoskeletal 1	RNA-binding protein 47
Endoplasmin	Desmoglein-1
Exocyst complex component 6	Serine/threonine-protein kinase greatwall
Major allergen Can f 1	Serum albumin
Annexin A4	Keratin, type I cytoskeletal 9
X-linked retinitis pigmentosa GTPase regulator	Relaxin receptor 2
Heat shock 70 kDa protein 4	Tyrosine-protein kinase Yes
cAMP-dependent protein kinase catalytic subunit alpha	Transient receptor potential cation channel subfamily V member 1
Vacuolar protein sorting-associated protein 52 homolog	Coagulation factor IX
Nuclear factor NF-kappa-B p105 subunit	Telomerase reverse transcriptase
Clusterin-like protein 1	ATP-dependent 6-phosphofructokinase, muscle type
HORMA domain-containing protein 1	Signal recognition particle subunit SRP68
Vascular cell adhesion protein 1	S-arrestin
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	Homeobox protein cut-like 1 (Fragment)
Coiled-coil domain-containing protein 39	Matrix metalloproteinase-9
Protein kinase C delta type	Dual serine/threonine and tyrosine protein kinase
Nephrocystin-1 (Fragment)	Inversin
Platelet glycoprotein Ib alpha chain	Taste receptor type 1 member 3
Zinc finger protein 252	Scavenger receptor cysteine-rich type 1 protein M130
Tyrosine-protein kinase Fer	Guanylate cyclase soluble subunit alpha-3

Fig. 2: Venn diagram showing overlaps of common and unique proteins in 2 technical replicates of tear in ID-SDS PAGE gel: normal tear, control pre and post DPSIS graft (group A), KCS pre and post DPSIS graft (group B)

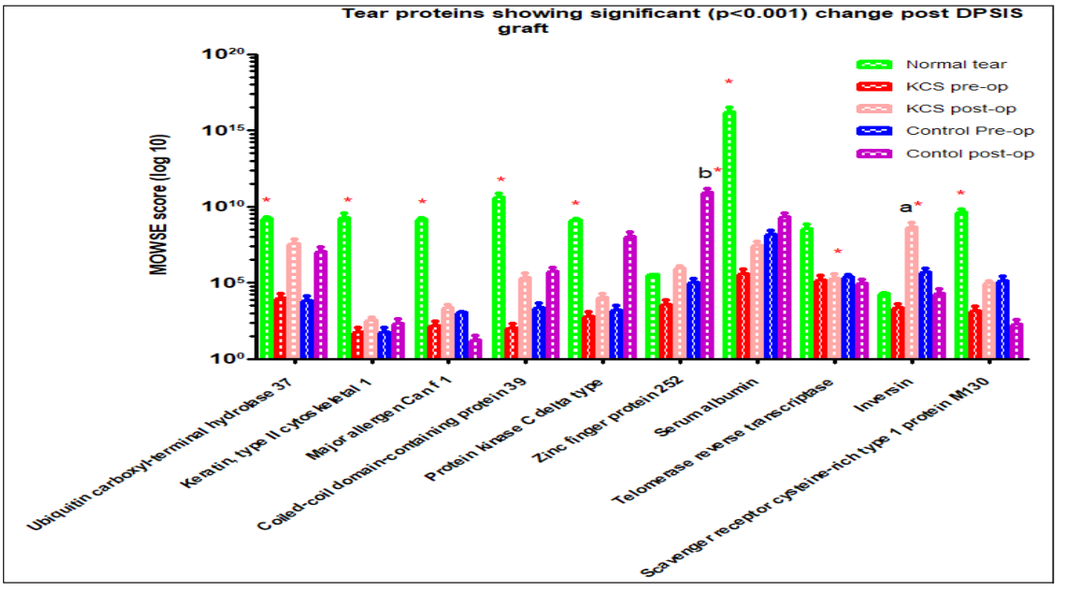


Fig. 3: Differentially expressed tear proteins by MALDI TOF/MS in Group A and Group B before and after DPSIS graft in comparison to tear from healthy dogs; significant difference ($p<0.001$) between groups is depicted by asterisk mark, superscript **a** indicates significant increase in protein score (0.001) of protein INVS between KCS graft post op compared to KCS pre-op **(a)**, superscript **b** indicates significant increase in protein score (0.001) of Zinc finger protein 252 between Control graft post op compared to Control graft pre-op **(b)**

proteins showed cellular process as major Biological process and Binding as the major Molecular function.

Most confident tear proteins were identified by the peptide mass fingerprinting output from 36 major bands through MALDI-TOF/MS analysis and MASCOT (Matrix Science) sequence matching software with Swissprot 2017_06 database (Table 2). TRAM1, WDR46, Major allergen Can f 1 and HGF were the most confident and highly scoring proteins of major protein bands of normal tear. Major allergen Can f 1 was consistently expressed as tear protein of 24kDa gel band in normal and group A tear film whereas there was down regulation of this protein in group B tear before and after DPSIS grafting up to 15th

Table 2: Tear proteins with highest score and hence most confident ones in different gel bands of ID-SDS PAGE identified by MALDI-TOF PMF and MASCOT search

Band ID	Protein band	MASCOT search homology	Molecular weight (Da)	MASCOT Score
NA	~72KDa	Translocating chain-associated membrane protein 1	43304	76
NB	~57KDa	WD repeat-containing protein 46	69116	54
NC	~24KDa	Major allergen Can f 1	19407	45
ND	~15KDa	Hepatocyte growth factor	85625	84
AA	~72KDa	WD repeat-containing protein 46	69116	47
AB	~57KDa	NAD-dependent protein deacylase sirtuin-5, mitochondrial	34547	48
AC	~24KDa	Major allergen Can f 1	19487	42
AD	~15KDa	Vascular cell adhesion protein 1	82501	46
AE	~72KDa	Serum albumin	70556	42
AF	~57KDa	WD repeat-containing protein 46	69116	60
AG	~24KDa	Major allergen Can f 1	19407	51
AH	~15KDa	Arylsulfatase I	64763	46
AI	~72KDa	B-lymphocyte antigen CD20	33319	56
AJ	~57KDa	C-C motif chemokine 17	11273	44
AK	~24KDa	Major allergen Can f 1	19407	139
AL	~15KDa	Tyrosine-protein kinase Yes	60717	57
AM	~72KDa	NAD-dependent protein deacylase sirtuin-5	34547	56
AN	~57KDa	Telomerase reverse transcriptase	126684	53
AO	~24KDa	Major allergen Can f 1	19407	56
AP	~15KDa	Alpha-1B adrenergic receptor (Fragment)	46717	88
BA	~72KDa	Interleukin-6	23216	58
BB	~57KDa	S-arrestin	45376	46
		Protein transport protein Sec61 subunit alpha isoform 1	52661	43
BC	~24KDa	V-type proton ATPase subunit G 1	13641	54
BD	~15KDa	NADH-ubiquinone oxidoreductase chain 3	13058	45
BE	~72KDa	Serum albumin	70556	56
BF	~57KDa	Hepatocyte growth factor	83344	51
BG	~24KDa	Endothelin B receptor	50829	46
BH	~15KDa	Colipase	12597	44
BI	~72KDa	Inversin	120720	65
		S-arrestin	45376	50
BJ	~57KDa	NAD-dependent protein deacylase sirtuin-5, mitochondrial	34547	46
BK	~24KDa	Major allergen Can f 1	19407	47
BL	~15KDa	Cyclic nucleotide-gated cation channel beta-3	89994	42
BM	~72KDa	NAD-dependent protein deacylase sirtuin-5, mitochondrial	34547	60
BN	~57KDa	ATP-dependent 6-phosphofructokinase, muscle type	86362	54
BO	~24KDa	Major allergen Can f 1	19407	57
BP	~15KDa	Vascular cell adhesion protein 1	82501	48

day. SIRT5, VCAM-1 were found as major proteins in group A tear whereas IL-6, SAG, SEC61A1, ATP6V1G1 and MT-ND3 were the major proteins expressed in group B. IL-6 is a potent inducer of acute phase response and plays an essential role in the final differentiation of B-cells into Ig- secreting cells (Tanaka *et al.,* 2014). IL-6 concentration was found significantly high in tear film of patients suffering from Sjogren syndrome (Tishler *et al.,* 1998), in serum and in peripheral circulating lymphocytes of SS patients (Boras *et al.,* 2004), in saliva and labial gland biopsies of SS patients (Tishler *et al.,* 1998; Boras *et al.,* 2004). SAG is a major component of outer segments of photo-pigment rod, its major physiological role being quenching of visual transduction cascade induced by light activation of rhodopsin and may play a role in light dependent degeneration of retinal photoreceptor cells (Granzin *et al.,* 1998). SAG is confined to retina in an immune-privileged location (Craft *et al.,* 1994). It is a potent ocular antigen which can elicit immune response in susceptible hosts (Gery *et al.,* 1986; de Smet *et al.,* 2001). Predominance of IL-6, SAG and down regulation of tear lipocalin Can f 1 was found in corneal ulcer cases with KCS pathology. VCAM-1 was found up-regulated in group A. Pro-inflammatory response induced by cytokines IL-4 and TNF-α can enhance cellular adhesion molecules ICAM -1 and VCAM-1 in corneal fibroblasts (Okada *et al.,* 2005).

Gene ontology analysis of the tear proteins before and after DPSIS graft in group A and Group B was conducted through the Protein Atlas Through Evolutionary Relationship (PANTHER) classification system (http://www.pantherdb.org/, SRI International, Menlo Park, CA). Catalytic activity and Binding were the major molecular function in both groups. Molecular function regulators were up-regulated in group B before and after DPSIS grafting. Transporter activity was found increased in both the groups post DPSIS grafting (Fig. 4a, 4b, 4c & 4d). Cellular process was the most up-regulated biological function in all categories. Biological regulation and

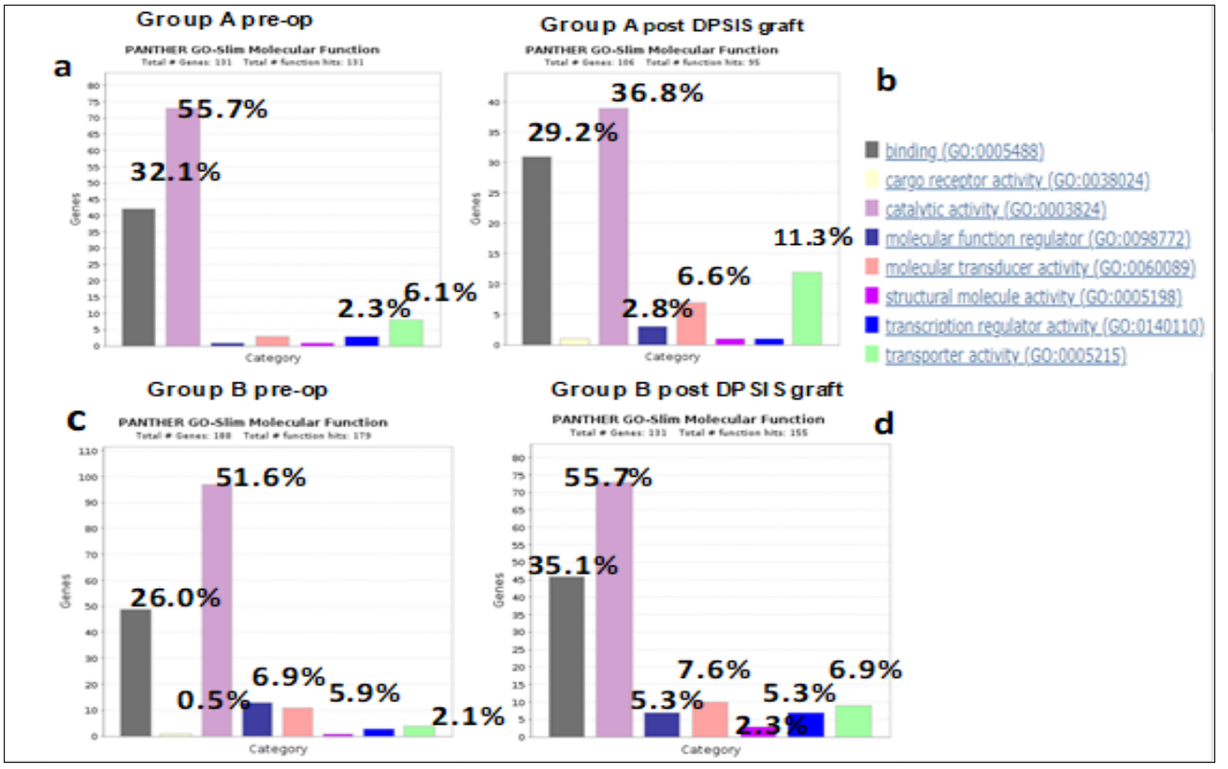


Fig. 4: Gene ontology search on the identified tear proteins for Molecular function in group A before DPSIS graft (a) after DPSIS graft (b) and in Group B before DPSIS graft (c) and after DPSIS graft (d)

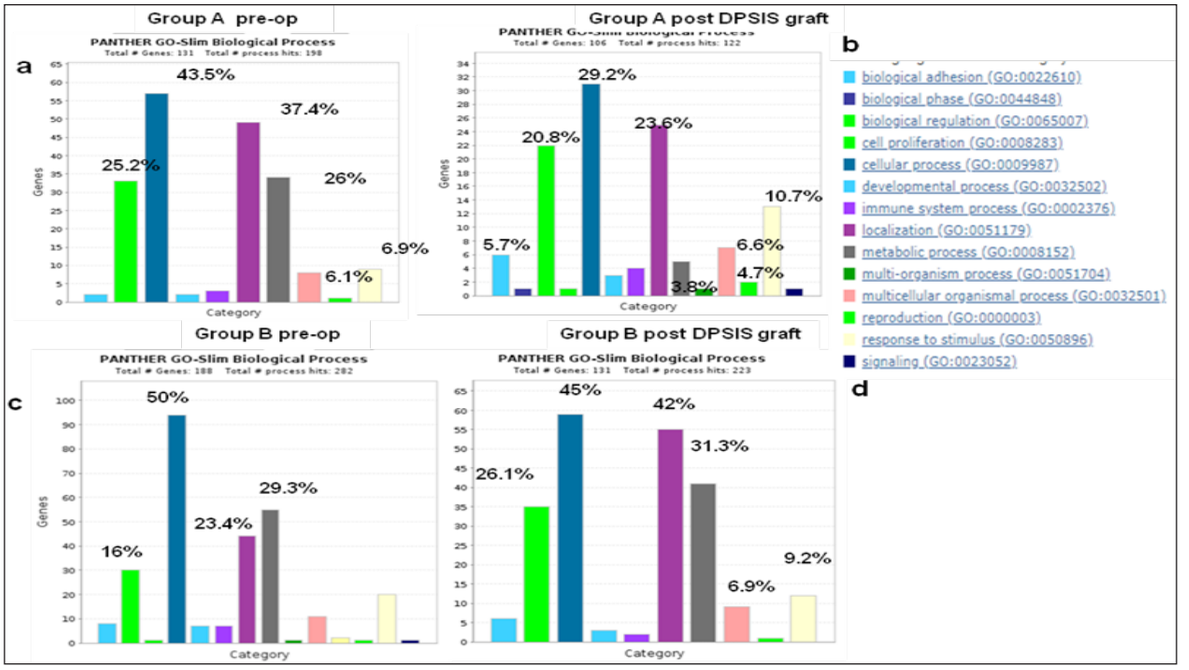


Fig. 5: Gene ontology search on the identified tear proteins for Biological Process in group A before DPSIS graft (a) after DPSIS graft (b) and in Group B before DPSIS graft (c) and after DPSIS graft (d)

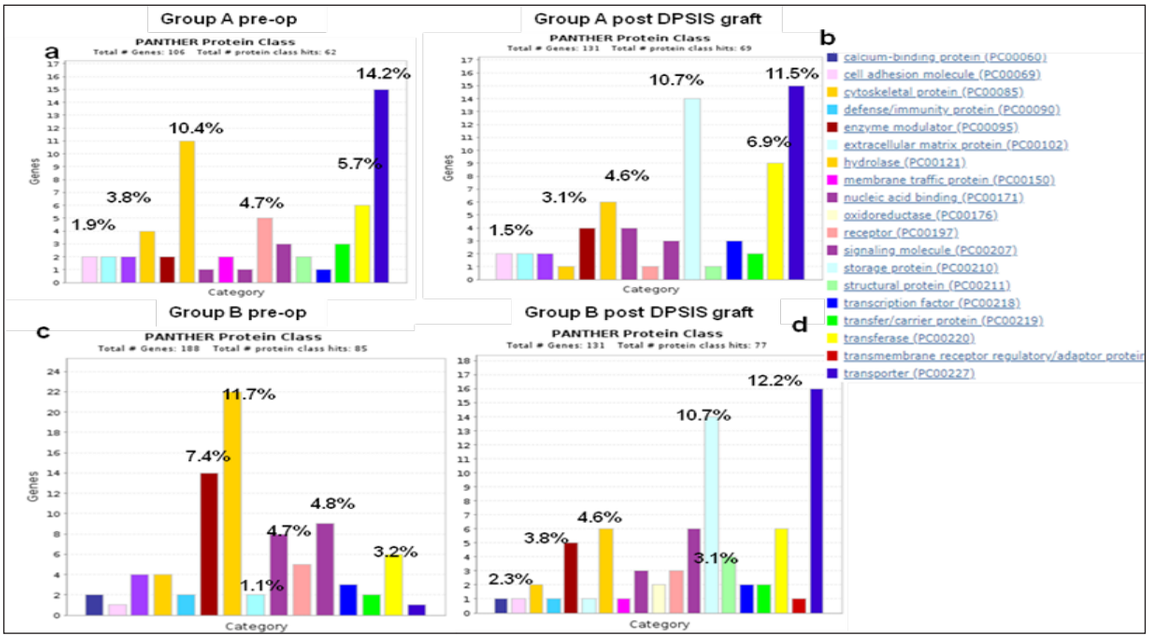
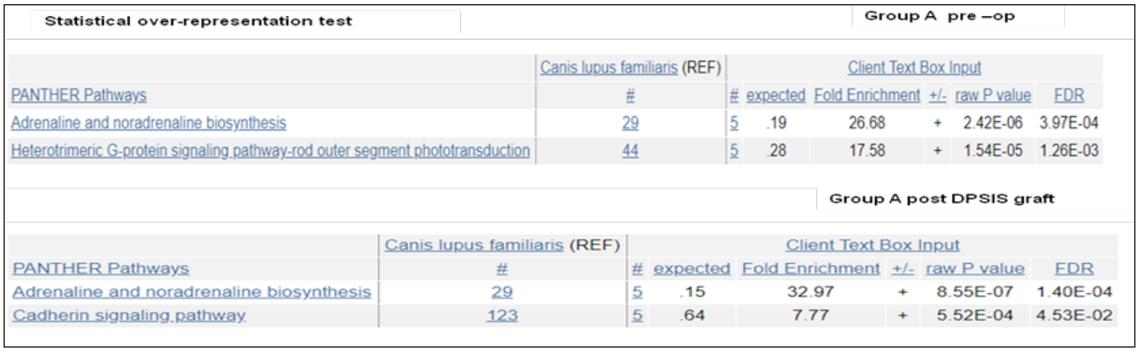


Fig. 6: Gene ontology search on the identified tear proteins for Protein Class in group A before DPSIS graft (a) after DPSIS graft (b) and in Group B before DPSIS graft (c) and after DPSIS graft (d)



Statistical over-representation test

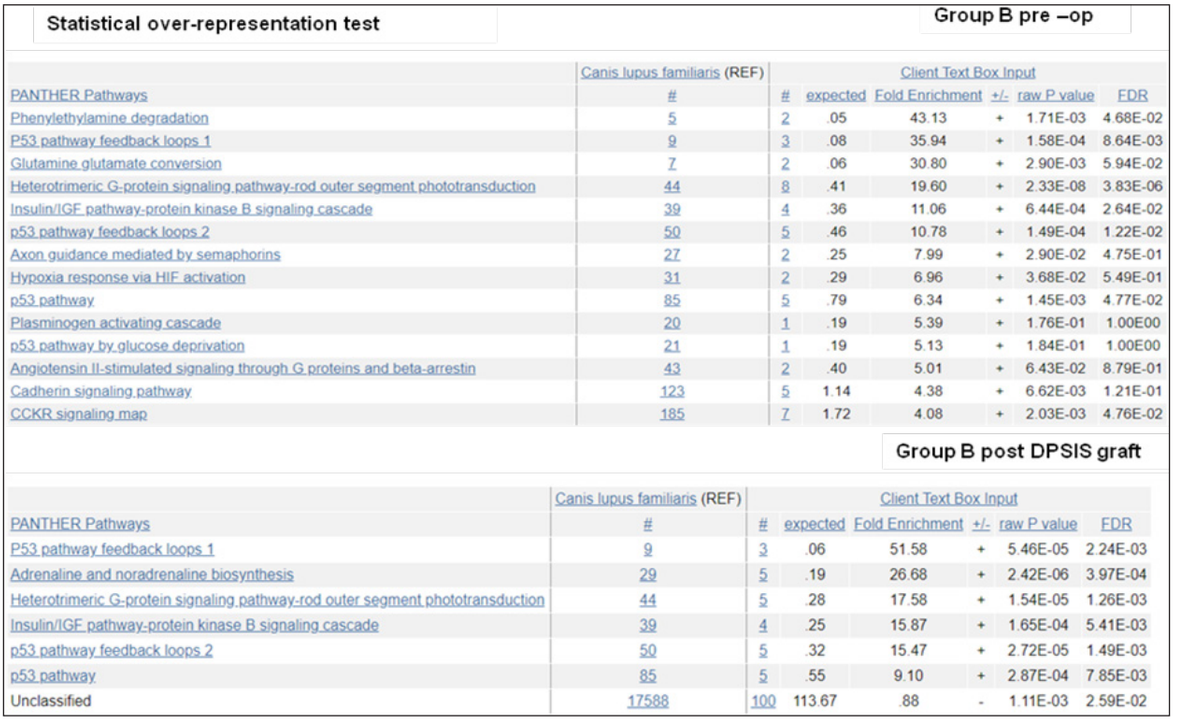
Group A pre –op

PANTHER Pathways	Canis lupus familiaris (REF) #	Client Text Box Input #	expected	Fold Enrichment	+/-	raw P value	FDR
Adrenaline and noradrenaline biosynthesis	29	5	.19	26.68	+	2.42E-06	3.97E-04
Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction	44	5	.28	17.58	+	1.54E-05	1.26E-03

Group A post DPSIS graft

PANTHER Pathways	Canis lupus familiaris (REF) #	Client Text Box Input #	expected	Fold Enrichment	+/-	raw P value	FDR
Adrenaline and noradrenaline biosynthesis	29	5	.15	32.97	+	8.55E-07	1.40E-04
Cadherin signaling pathway	123	5	.64	7.77	+	5.52E-04	4.53E-02

Fig. 7: Statistical overrepresentation of PANTHER pathways in relation to *Canis lupus familiaris* reference genome in Group A before and after DPSIS graft



Statistical over-representation test

Group B pre –op

PANTHER Pathways	Canis lupus familiaris (REF) #	Client Text Box Input #	expected	Fold Enrichment	+/-	raw P value	FDR
Phenylethylamine degradation	5	2	.05	43.13	+	1.71E-03	4.68E-02
P53 pathway feedback loops 1	9	3	.08	35.94	+	1.58E-04	8.64E-03
Glutamine glutamate conversion	7	2	.06	30.80	+	2.90E-03	5.94E-02
Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction	44	8	.41	19.60	+	2.33E-08	3.83E-06
Insulin/IGF pathway-protein kinase B signaling cascade	39	4	.36	11.06	+	6.44E-04	2.64E-02
p53 pathway feedback loops 2	50	5	.46	10.78	+	1.49E-04	1.22E-02
Axon guidance mediated by semaphorins	27	2	.25	7.99	+	2.90E-02	4.75E-01
Hypoxia response via HIF activation	31	2	.29	6.96	+	3.68E-02	5.49E-01
p53 pathway	85	5	.79	6.34	+	1.45E-03	4.77E-02
Plasminogen activating cascade	20	1	.19	5.39	+	1.76E-01	1.00E00
p53 pathway by glucose deprivation	21	1	.19	5.13	+	1.84E-01	1.00E00
Angiotensin II-stimulated signaling through G proteins and beta-arrestin	43	2	.40	5.01	+	6.43E-02	8.79E-01
Cadherin signaling pathway	123	5	1.14	4.38	+	6.62E-03	1.21E-01
CCKR signaling map	185	7	1.72	4.08	+	2.03E-03	4.76E-02

Group B post DPSIS graft

PANTHER Pathways	Canis lupus familiaris (REF) #	Client Text Box Input #	expected	Fold Enrichment	+/-	raw P value	FDR
P53 pathway feedback loops 1	9	3	.06	51.58	+	5.46E-05	2.24E-03
Adrenaline and noradrenaline biosynthesis	29	5	.19	26.68	+	2.42E-06	3.97E-04
Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction	44	5	.28	17.58	+	1.54E-05	1.26E-03
Insulin/IGF pathway-protein kinase B signaling cascade	39	4	.25	15.87	+	1.65E-04	5.41E-03
p53 pathway feedback loops 2	50	5	.32	15.47	+	2.72E-05	1.49E-03
p53 pathway	85	5	.55	9.10	+	2.87E-04	7.85E-03
Unclassified	17588	100	113.67	.88	-	1.11E-03	2.59E-02

Fig. 8: Statistical overrepresentation of PANTHER pathways in relation to *Canis lupus familiaris* reference genome in Group B before and after DPSIS graft

localization increased post DPSIS graft in group B whereas cellular process decreased percentagewise. Cell proliferation and signalling proteins increased in Group A post DPSIS graft (Fig. 5a, 5b, 5c & 5d). Transporters and hydrolases were the major protein class in group A pre op tear whereas hydrolases and enzyme modulators were the major protein class in group B pre op tear. Transporters were found down regulated in group B pre op tear compared to group A pre op tear. Storage protein and transporters were the major protein class in both groups after DPSIS grafting (Fig. 6a-6d).

Statistical overrepresentation of PANTHER pathways in relation to *Canis lupus familiaris* reference genome was

performed through the Protein Atlas Through Evolutionary Relationship (PANTHER) classification system (http://www.pantherdb.org/, SRI International, Menlo Park, CA) (Fisher exact test with FDR P<0.05). A number of pathways were found significantly over represented in Group B before and after DPSIS graft compared to group A which included p53 pathway, p53 pathway feedback loop 1 and 2 (Fig. 8) whereas Cadherin signalling pathway was found over represented in group A post DPSIS graft (Fig. 7). Cellular stress is a major inducer of p53, a tumor suppressor, which under stressors like DNA damage and oncogene activation, gets activated, stabilized and accumulated within cell (Chipuk and Green, 2006; Levine and Oren, 2009).

CONCLUSION

MALDI-TOF/MS is promising tool to analyse unique protein profile of tear film and hence was employed to understand the molecular mechanism of DSPIS grafting in KCS cases of dogs. Proteomic study of tear film collected from DPSIS graft to repair corneal epithelial defects with KCS as underlying pathology was suggestive of molecular events such as cellular senescence and apoptosis as underlying mechanisms of KCS pathology and successful graft uptake warranted strategies to face these. Large number of proteins identified through tear film proteomics and key proteins like USP37, KRT1, Major allergen Can F1, CCDC39, PRKCD, Serum albumin, TERT and CD163 were found down regulated in KCS. Protein INVS was found significantly up regulated in group B whereas ZNF252 was found significantly up regulated in group A post DPSIS graft. Clinically DPSIS was well integrated in both KCS and control groups having corneal ulcer, identification of differentially expressed tear proteins helped to highlight disease pathways specific for KCS, hence useful for management of surgical diseases with underlying immune deviation as in corneal ulcer due to KCS.

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