FUNCTIONAL INSIGHTS INTO ENDOCANNABINOID SIGNALLING IN GOAT SPERMATOZOA

Dilip Kumar Swain, Abhishek Kumar Mishra, Sarvajeet Yadav, Brijesh Yadav, and Mukul Anand
Department of Physiology
College of Veterinary Science and Animal Husbandry
DUVASU, Mathura-281001 (UP)

Dr Dilip Kumar Swain (Ph.D)
Assistant Professor
Department of Physiology
COVSc & AH, DUVASU, Mathura- 281001 (Uttar Pradesh)

Background…….

- Endocannabinoids regulate both male and female reproduction through endocannabinoid receptors (CB1 and CB2)
- Has been emerged as the potential reasons behind infertility
- Activation of these receptors have been associated with compromised sperm functions and thereby issues of infertility
- Two naturally found endocannabinoids are- AEA (Anandamide) and 2- Arachidonoylglycerol (2-AG)


GAPS IN KNOWLEDGE

- **No information regarding the localization and distribution of CB1 and CB2 receptors in goat spermatozoa**

- **No studies regarding the functional involvement of CB1 and CB2 receptors in goat spermatozoa**


OBJECTIVES

- **Localization of CB1 and CB2 receptors on buck spermatozoa**

- **To evaluate the functional significance of CB1 and CB2 receptors in regulating spermatozoa function**

METHODOLOGY FOLLOWED FOR THE STUDY

- ✓ **Experimental Animals:** Barbari Bucks (n=4)
- ✓ **Place of Study:** Department of Physiology

- ✓ **Semen collection:** Biweekly from each buck by using Artificial Vagina (as per standard).

- ✓ **Number of ejaculates taken for study**:
    1. Progressive motility= 24
    2. Fluorescent Staining =24
    3. Tyrosine Phosphorylation=12
    4. Agonist study=24
    5. Antagonist Study=24

Drugs used during the study….

- **Met-AEA @ 1µM:** Agonist of CB1 and CB2 receptor
- **SR-141716A @ 10µM:** Specific antagonist of CB1 receptor
- **SR-144528 @ 10µM:** Specific antagonist of CB2 receptor

STATISTICAL MODEL USED

- Mean values were calculated and were compared by using 2-way ANOVA (Post Hoc Tukey Test) by using SPSS 16.1 version, USA).
- Significance was tested at 5% level ($p<0.05$).
- Significance of the difference among the three groups is indicated with superscripts ($p<0.05$).
- Bars represent the standard error of the mean.

RESULTS

Table 1.0: Physical seminal attributes of fresh semen and initial dilution of Barbari bucks (Mean ± SEM, n=24)

PHYSICAL SEMINAL ATTRIBUTES	Mean ± SEM
Ejaculated Volume (ml)	0.65 ± 0.23
Seminal pH	6.59 ± 0.03
Mass Motility (0-5 scale)	3.79 ± 0.09
Sperm Concentration (Million/ml)	3290.05 ± 56.42
Progressive motility (%)	80.50 ± 1.15
Live Spermatozoa (%)	94.65 ± 0.85
HOS response (%)	78.95 ± 0.76

Fig 1: Immunoblot showing **52kDa** protein band corresponding to CB1 receptor on buck spermatozoa. L1: MW marker; L2, L3, L4, L5,L5, L6. L7,L8: Protein samples of six ejaculates.

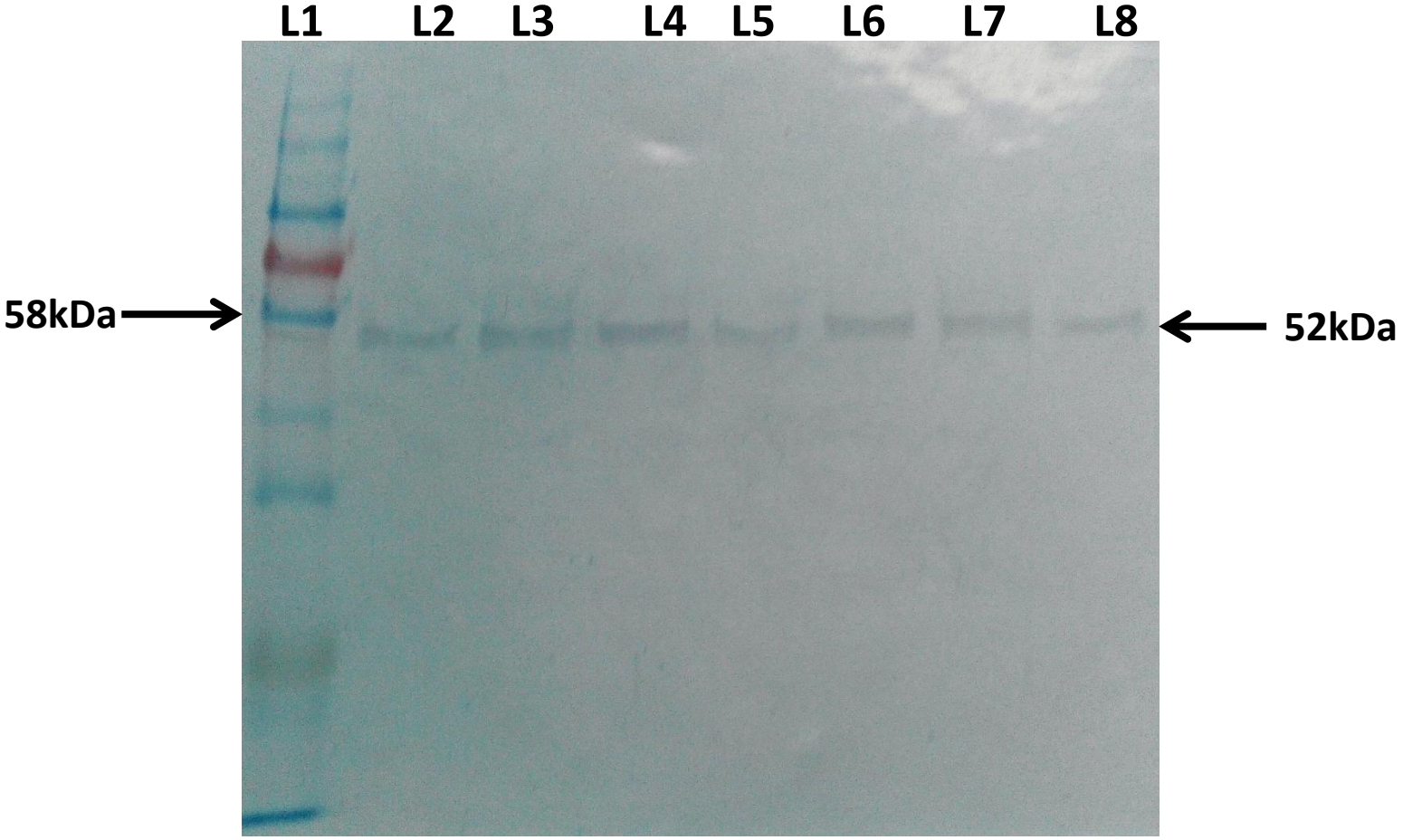
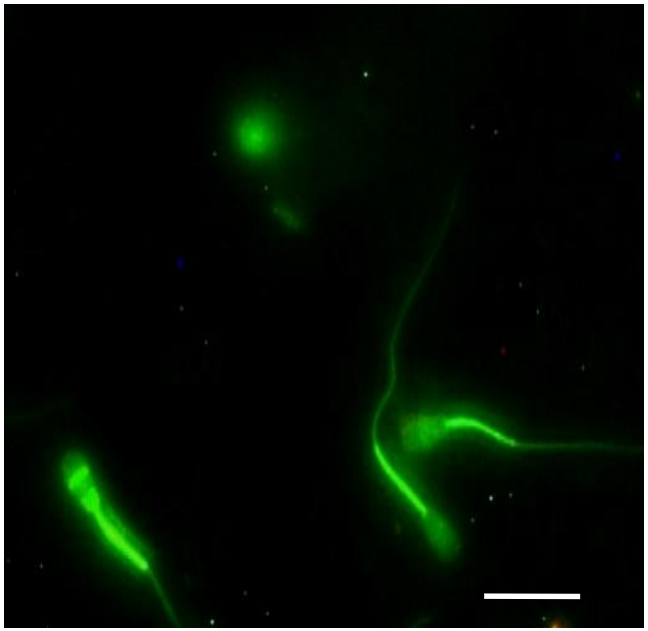


Fig 2: Immunlocalization of CB1 receptors on Buck spermatozoa(40x). Scale bar corresponds to 20µm.

- Post Acrosomal Region
- Middle Piece
- Tail



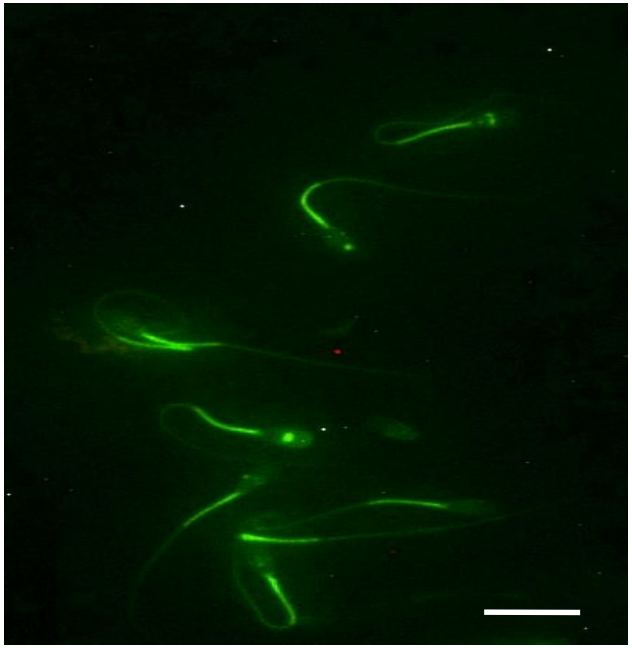


Fig 3: Immunoblot showing CB2 receptor on buck spermatozoa (L2: MW marker; L1, L3, L4, L5, L6, L7, L8 & L 9: Eight ejaculates of four bucks). Immunoblot showing 32 kDa protein corresponding to CB2.

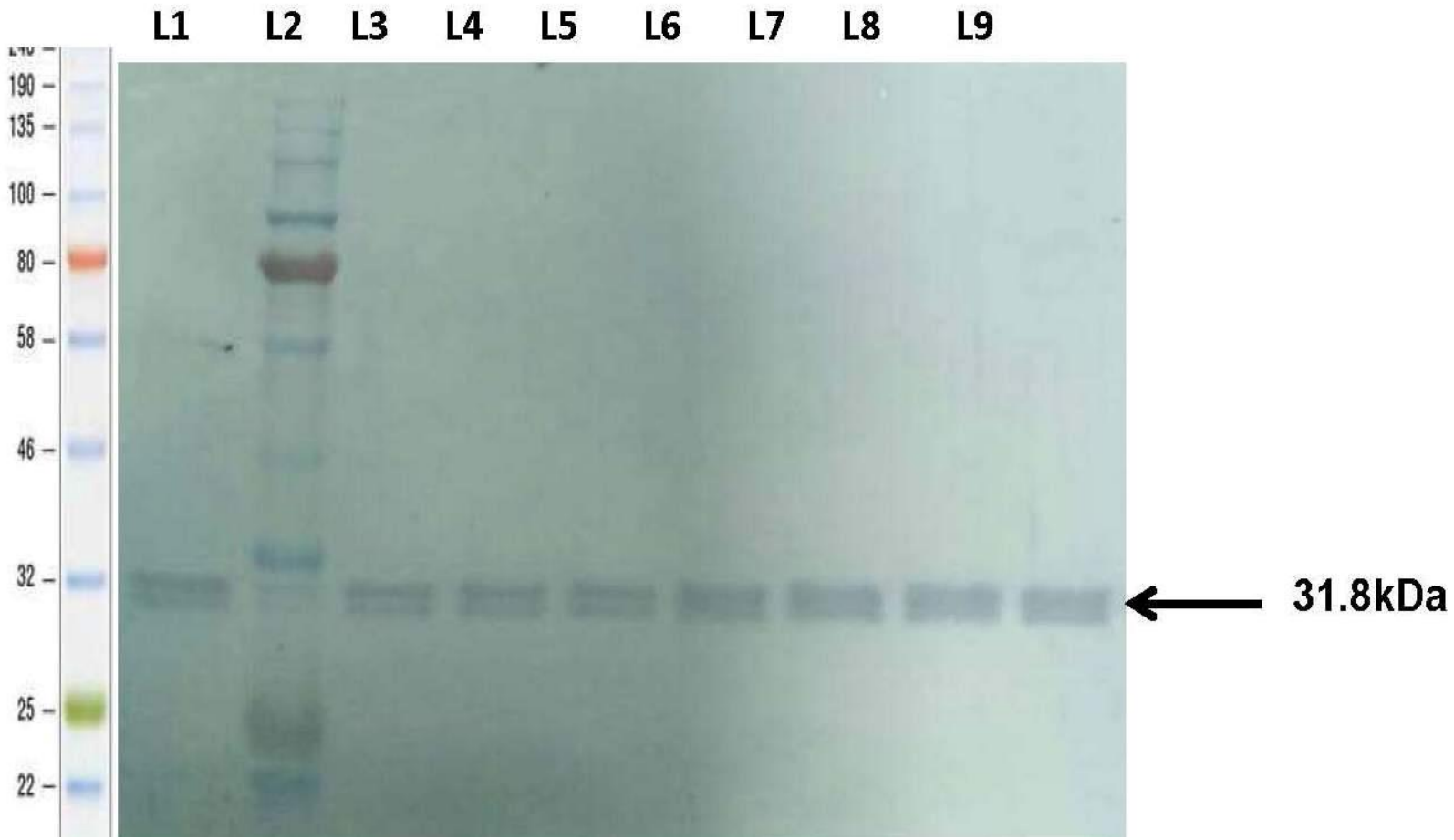
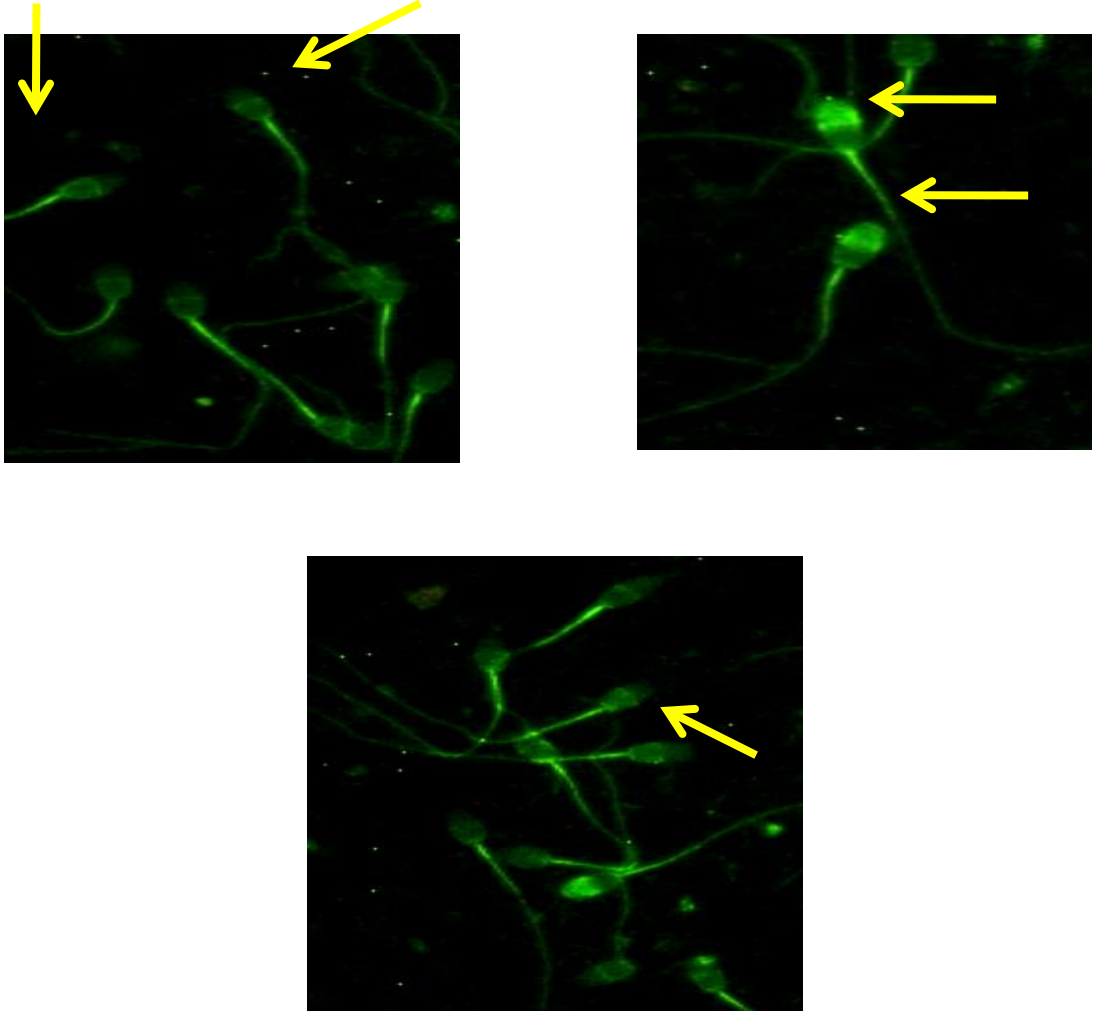


Fig 4 : Photomicrograph showing Immunolocalization of CB1 receptor on buck spermatozoa (40x). Yellow arrow indicate the CB1 localized in spermatozoa head and principal piece .



FUNCTIONAL STUDY

Fig 5: Effect of different concentrations of Met-AEA on progressive sperm motility (%) of buck spermatozoa (Mean ± SEM, n=24). Data are presented as Mean± SEM. Different superscripts above the bar indicates significance (p< 0.05)

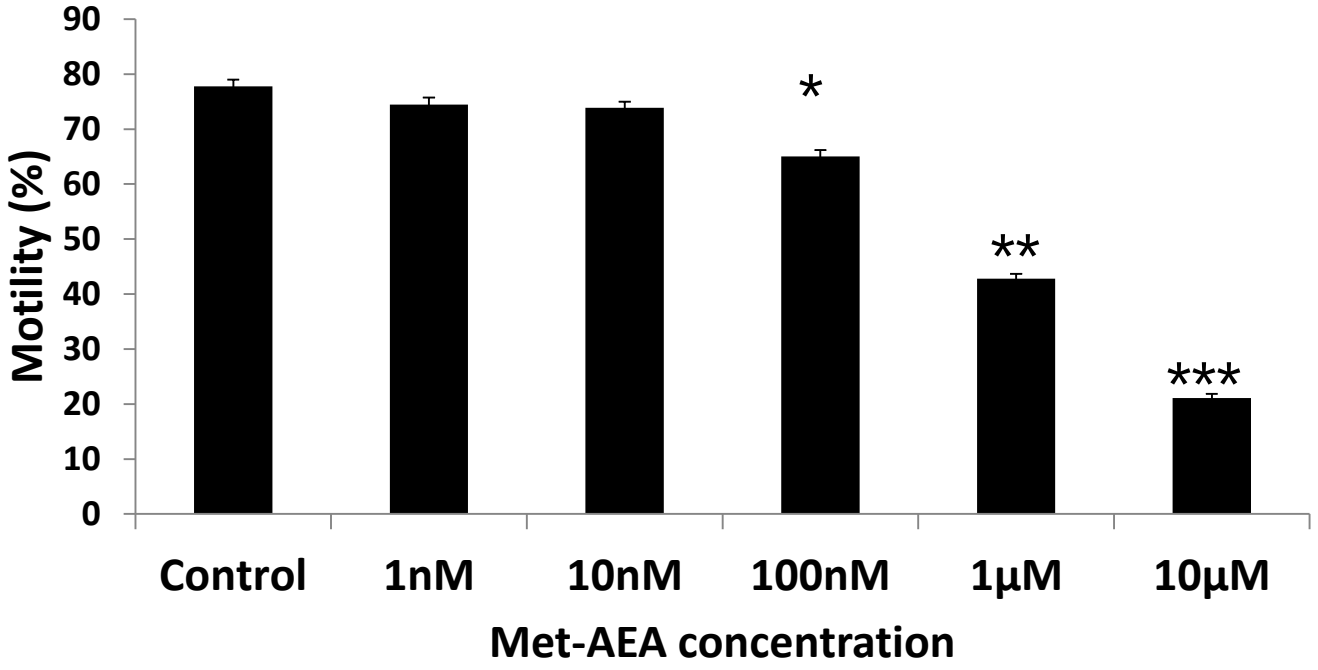


Fig 6 Effect of Met-AEA, SR-141716A (CB1 antagonist), and SR-144528 (CB2 antagonist) alone and in combination on progressive sperm motility (%) of buck spermatozoa (Mean ± SEM, n=24). Data are presented as Mean± SEM. Different superscripts above the bar indicates significance ($p < 0.05$)

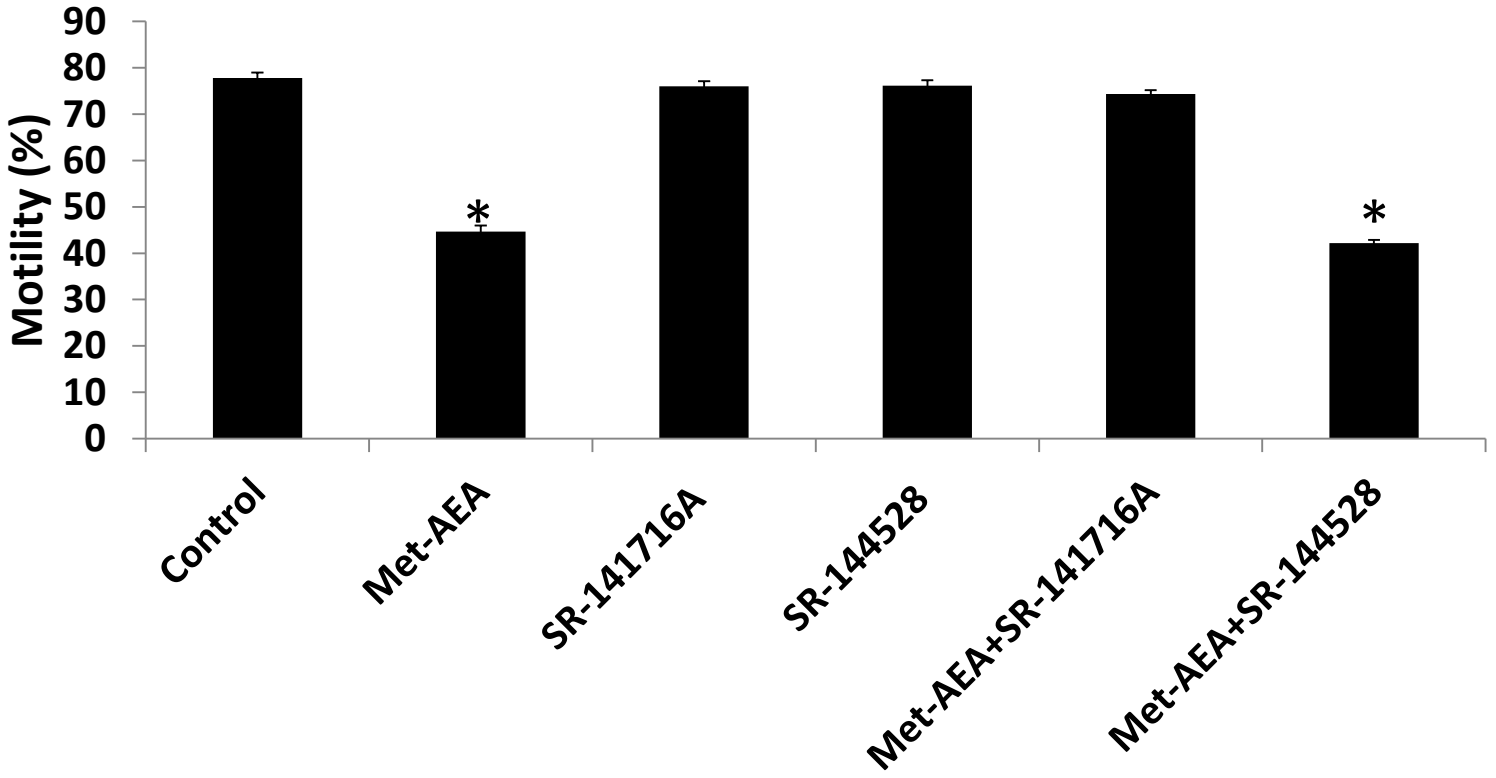


Fig 7 Effect of Met-AEA, SR-141716A (CB1 antagonist), and SR-144528 (CB2 antagonist) alone and in combination on per cent spermatozoa having high mitochondrial transmembrane potential of buck spermatozoa (Mean ± SEM, n=24). Data are presented as Mean± SEM. Different superscripts above the bar indicates significance ($p < 0.05$)

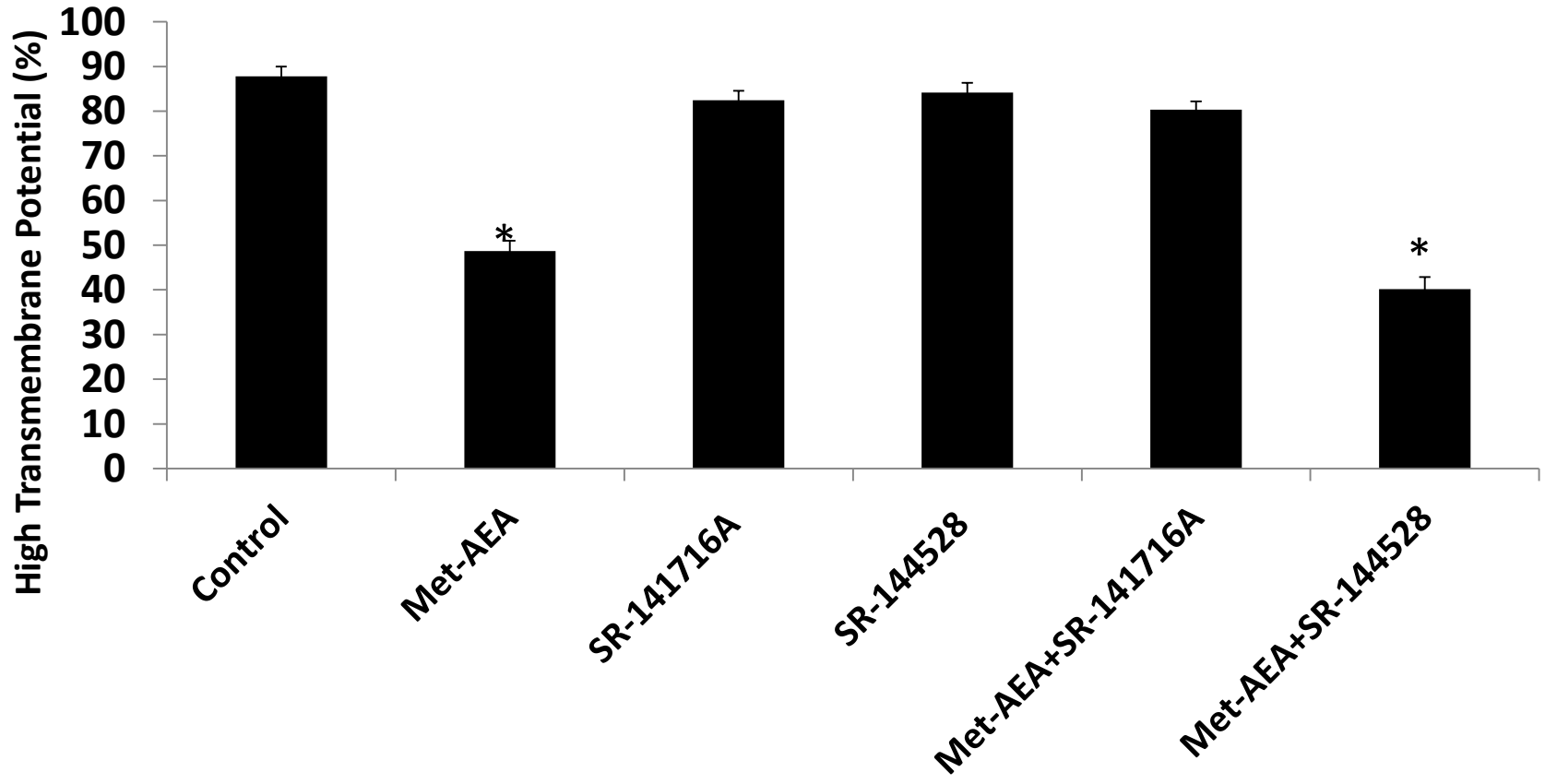
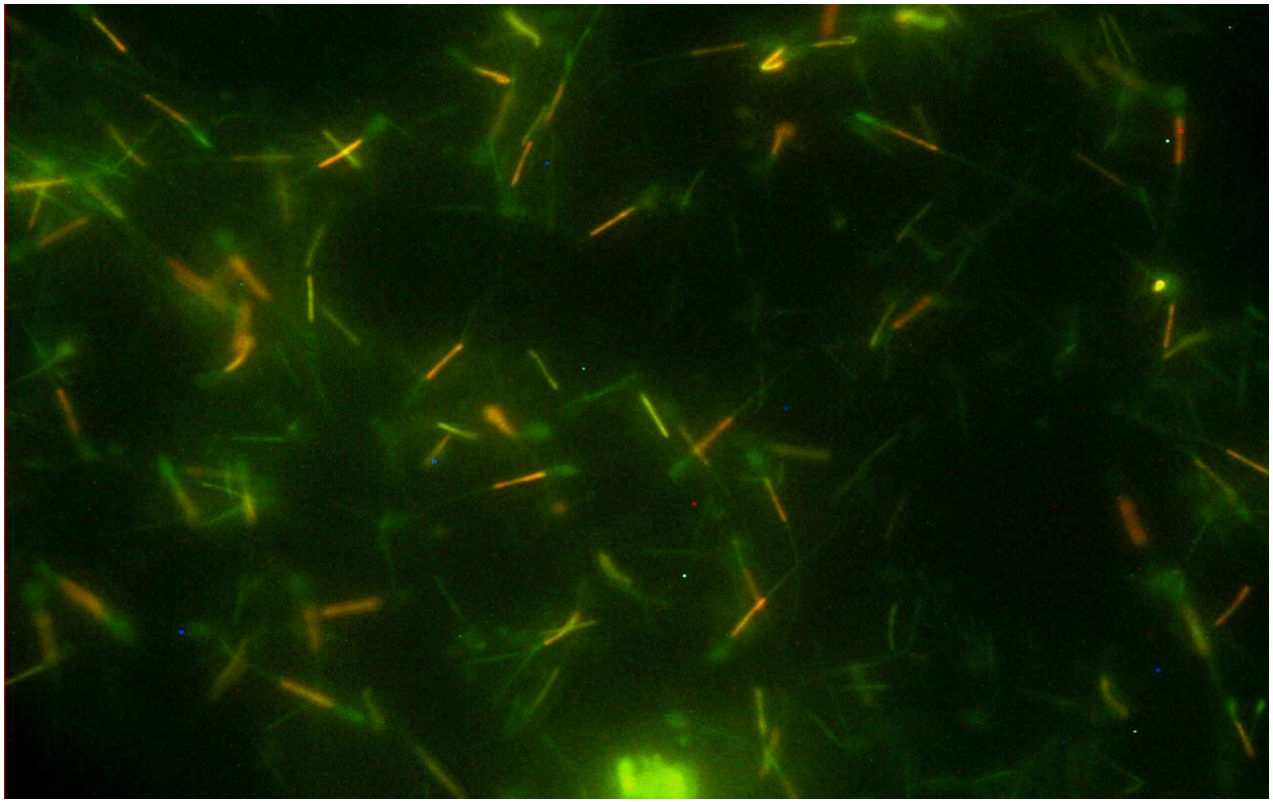


Fig 8: EVALUATION OF MITOCHONDRIA TRANSMEMBRANE POTENTIAL IN SPERMATOZOA USING JC I STAINING (40x)



SPERMATOZOA SHOWING ORGANGE RED FLOURESCENCE ARE HAVING HIGH TRANSMEMBRANE MITOCHONDRIAL POTENTIAL

SPERMATOZOA SHOWING GREEN FLOURESCENCE ARE HAVING LOW TRANSMEMBRANE MITOCHONDRIAL POTENTIAL

Fig 9: Effect of different concentrations of Met-AEA on % B-pattern of buck spermatozoa (Mean ± SEM, n=24). Data are presented as Mean± SEM. Different superscripts above the bar indicates significance ($p < 0.05$)

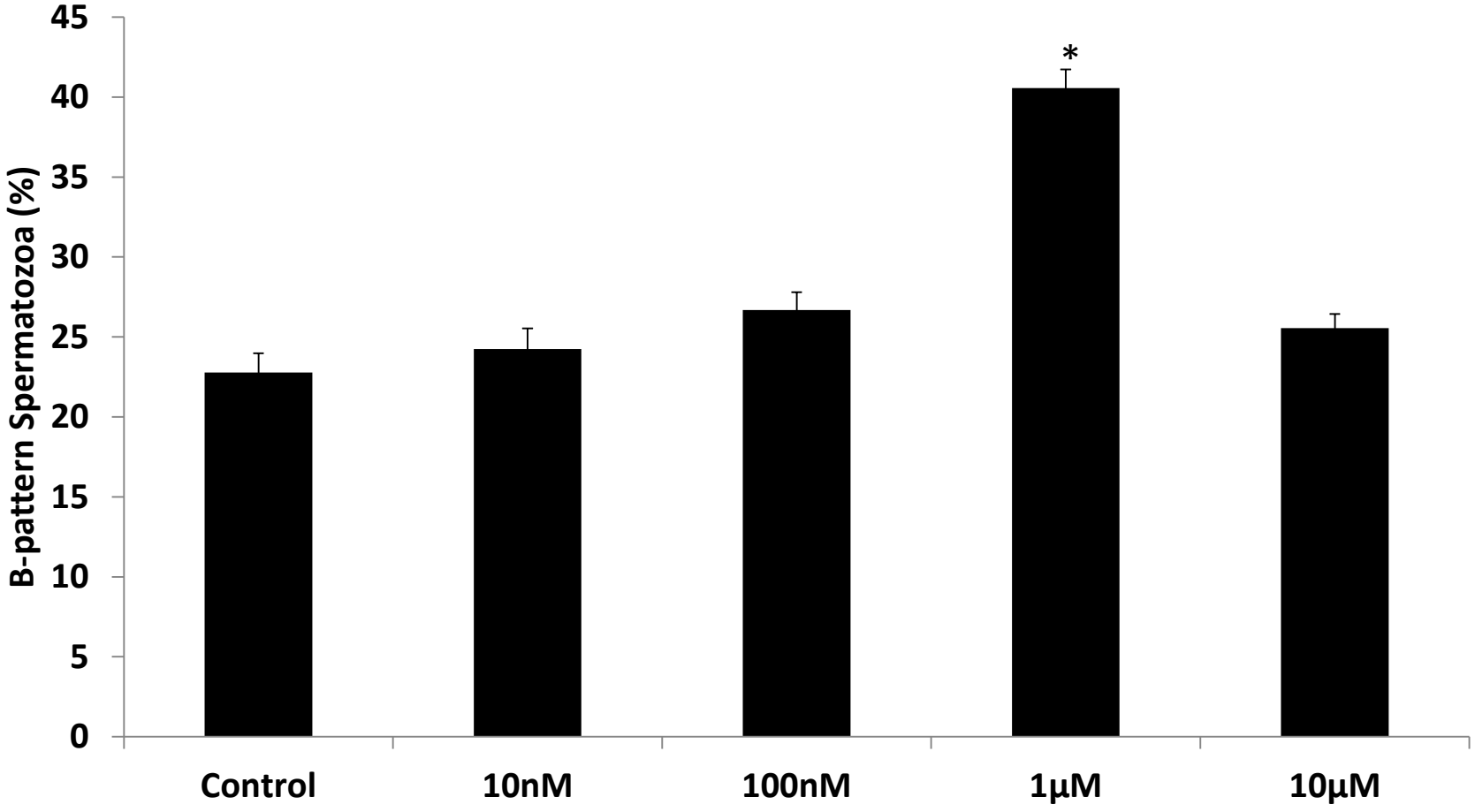
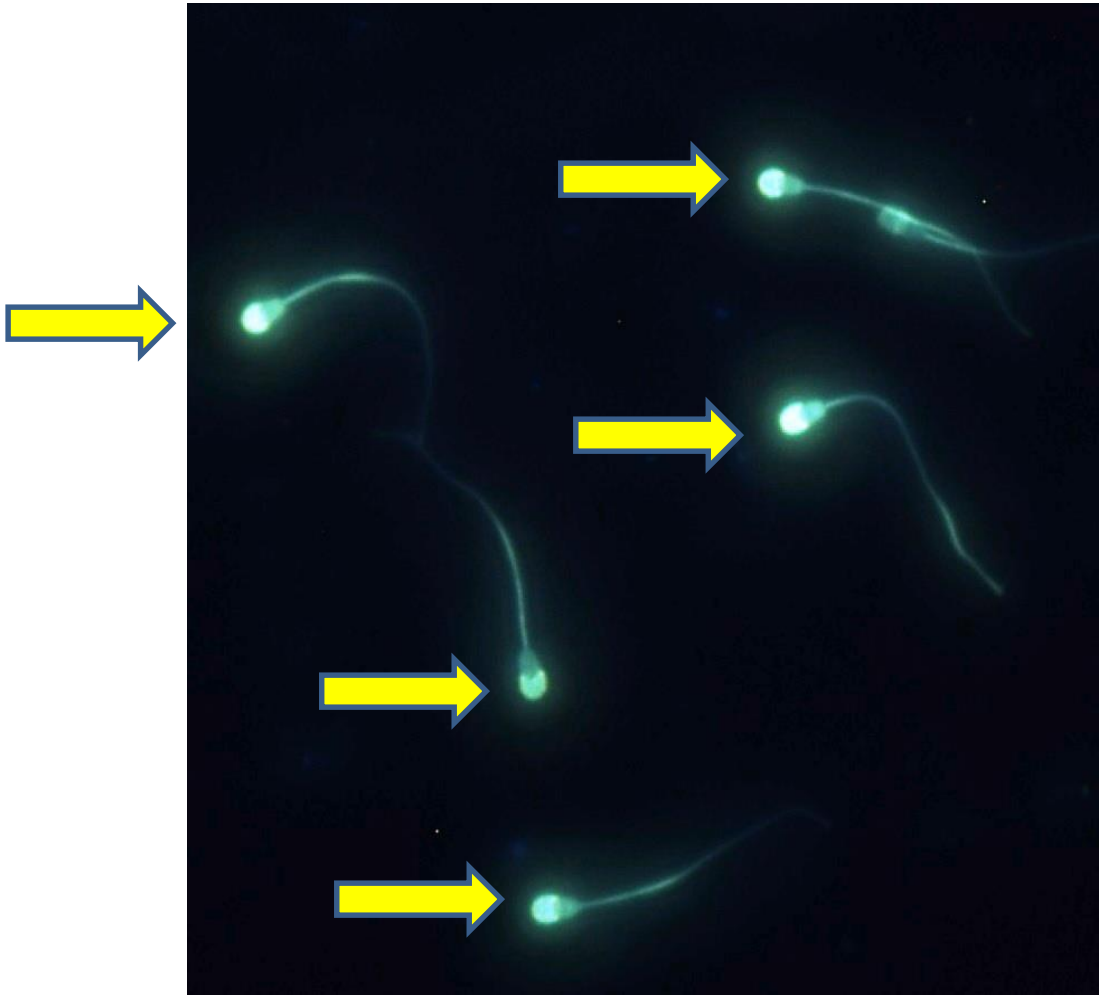


Fig 10: CTC ASSAY SHOWING B- PATTERN OF CAPACITATED BUCK SPERMATOZOA (40x)



INTACT ACROSOME- FLOURESCENCE AT THE TIP- CAPACITATED

Fig 11: Effect of Met-AEA, SR-141716A (CB1 antagonist), and SR-144528 (CB2 antagonist) alone and in combination on per cent capacitated spermatozoa (B- pattern) of buck spermatozoa (Mean ± SEM, n=24). Data are presented as Mean± SEM. Different superscripts above the bar indicates significance ($p < 0.05$)

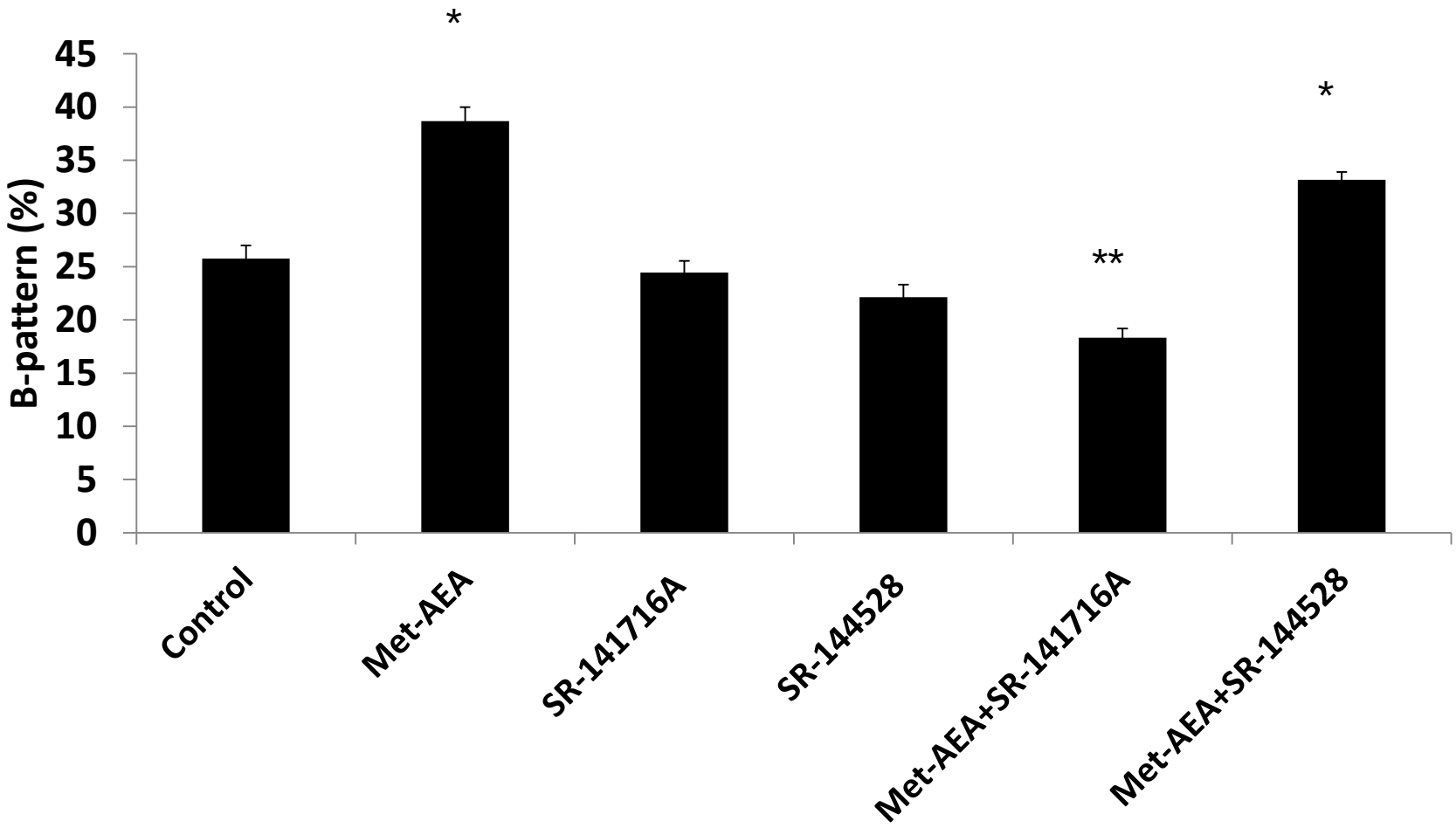


Fig 12: Immunoblot showing Tyrosine Phosphorylated proteins in sperm lysates treated with Met-AEA.

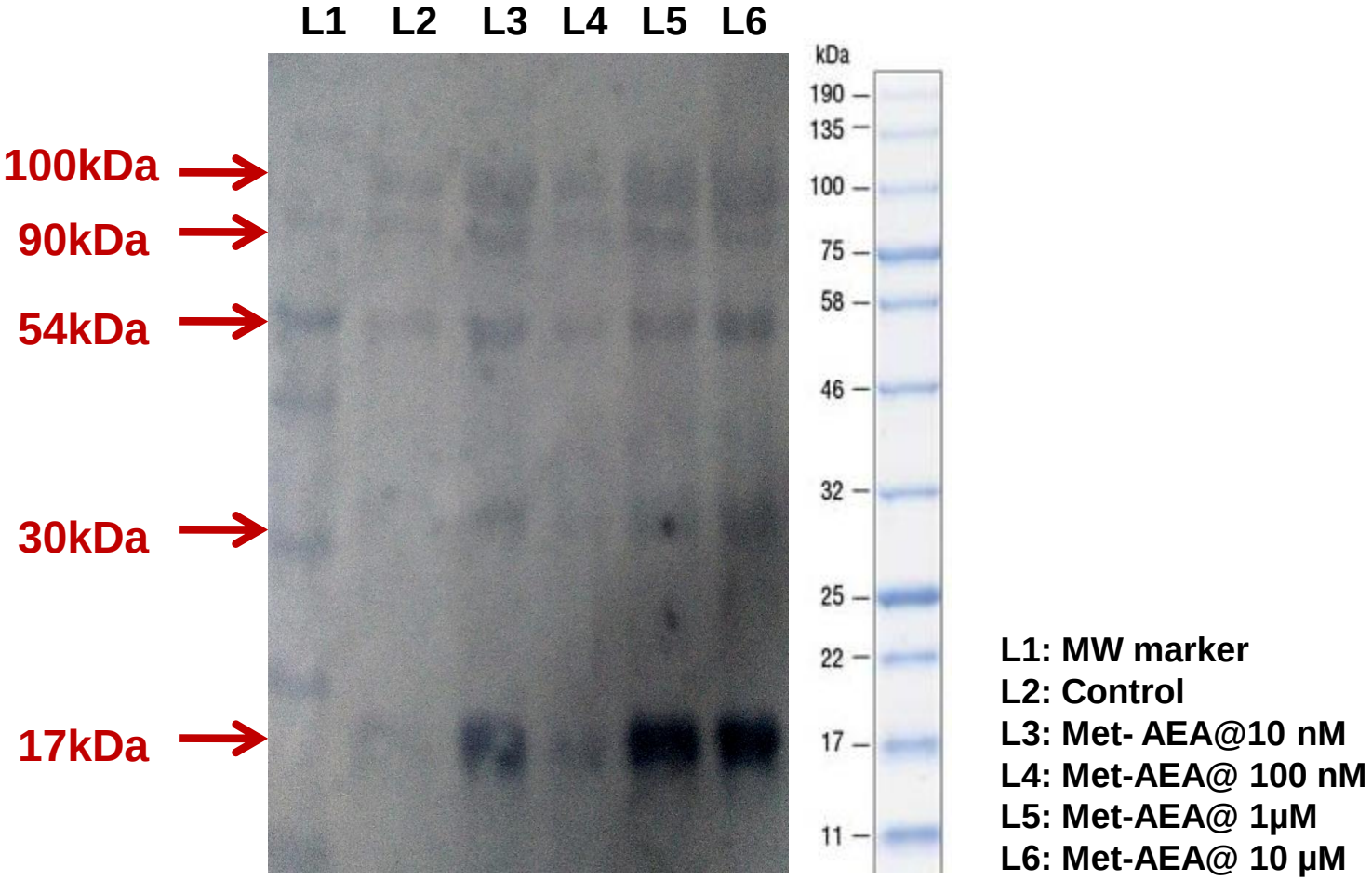


Fig 13: Immunolocalization of tyrosine phosphorylated proteins in buck spermatozoa treated with Met AEA (40x). Yellow arrow indicate the localisation of tyrosine phosphorylated proteins at the middle piece of spermatozoa.

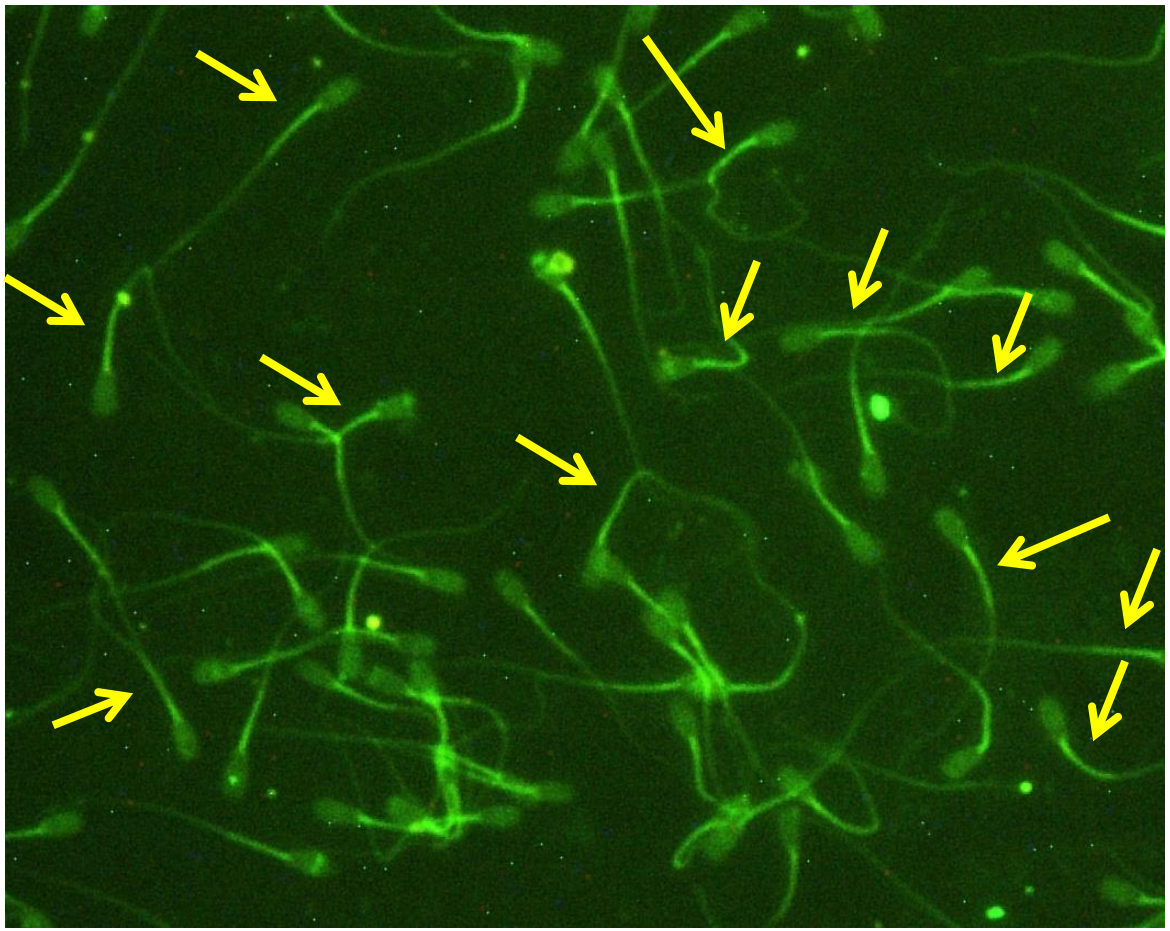


Fig 13: Immunoblot Showing Tyrosine Phosphorylated Protein in sperm lysate treated with soluble Adenyl Cyclase inhibitor (KH7)

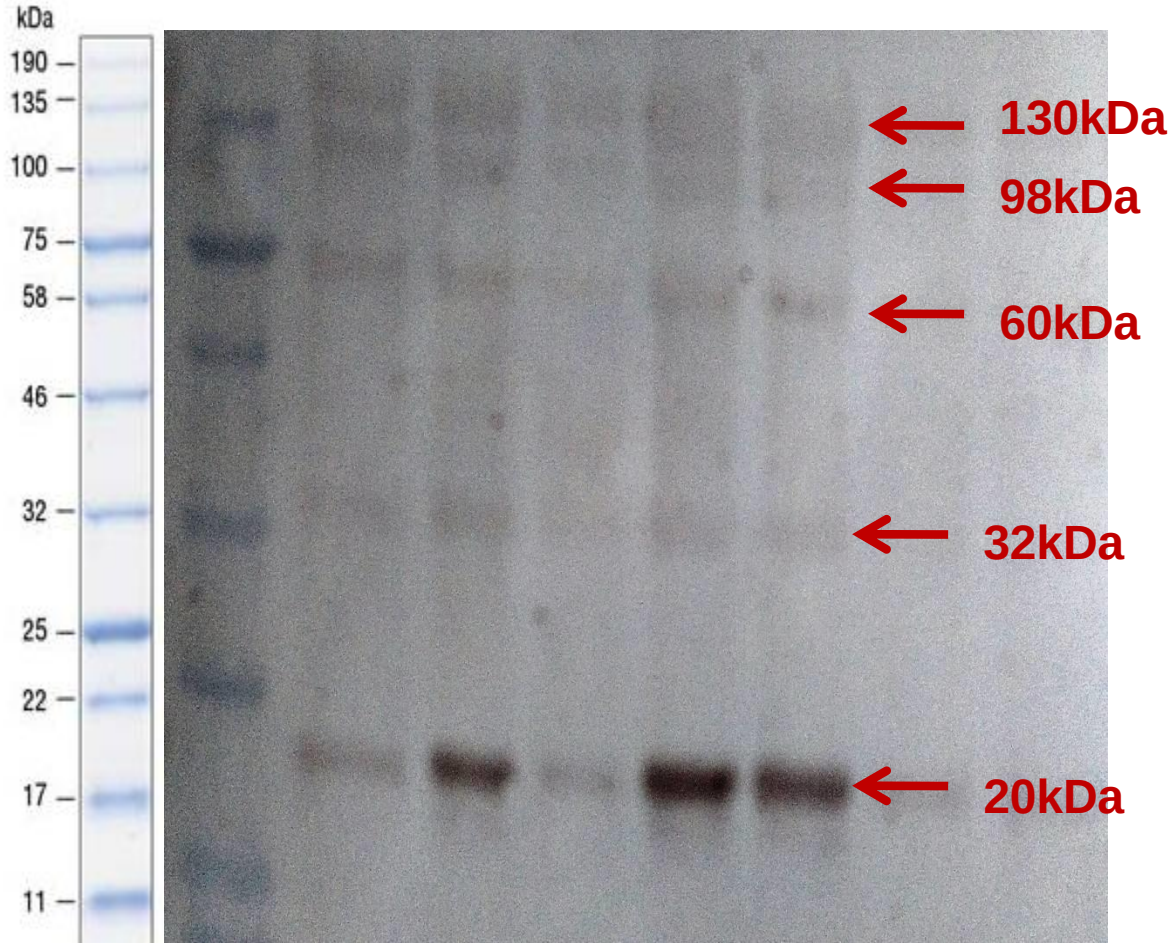


Fig 14 : Immunoblot Showing Tyrosine Phosphorylated Protein in sperm lysate treated with soluble Protein Kinase A inhibitor (P9115)

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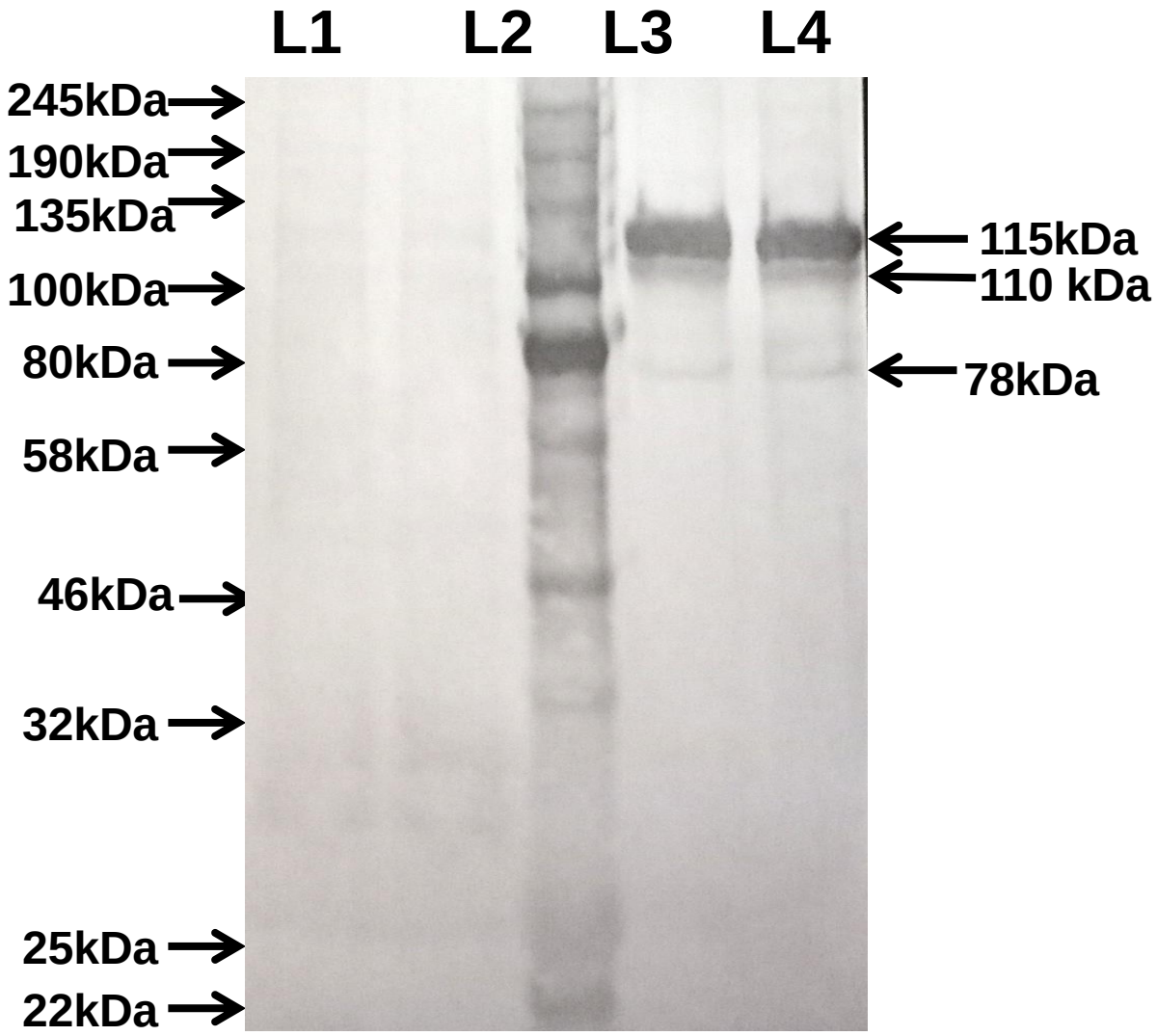


Fig 15: Immunoblot Showing Tyrosine Phosphorylated Protein in sperm lysate treated with Verapamil (Calcium channel opener)

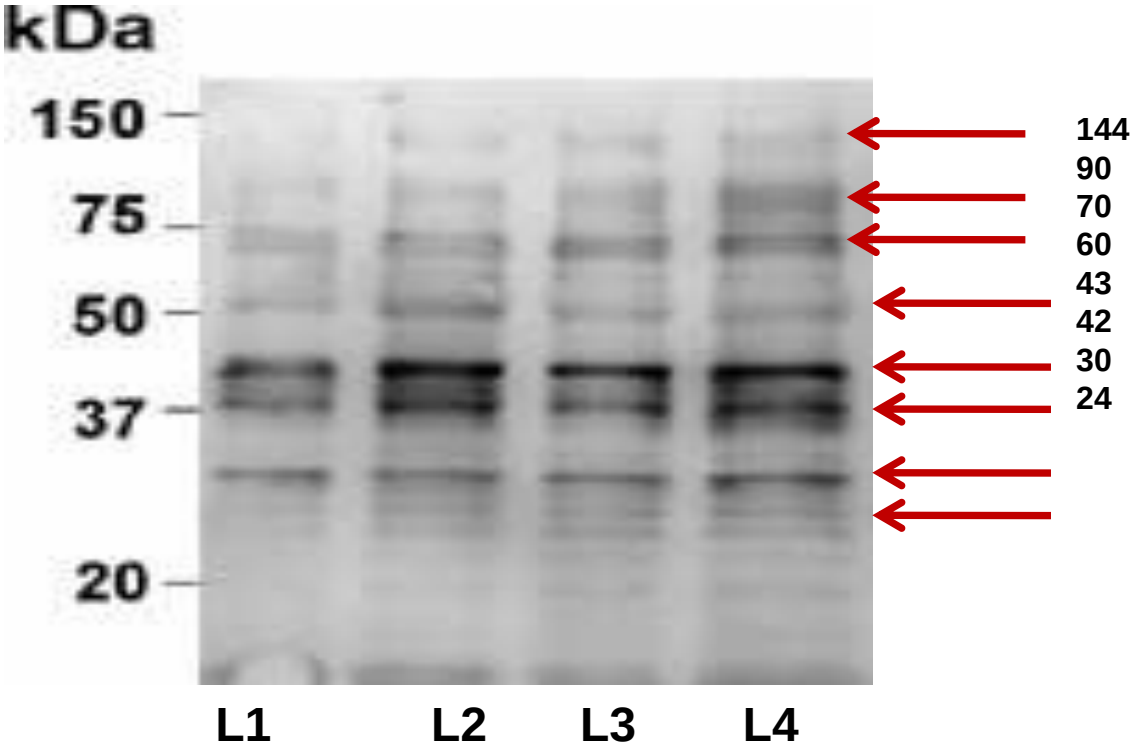
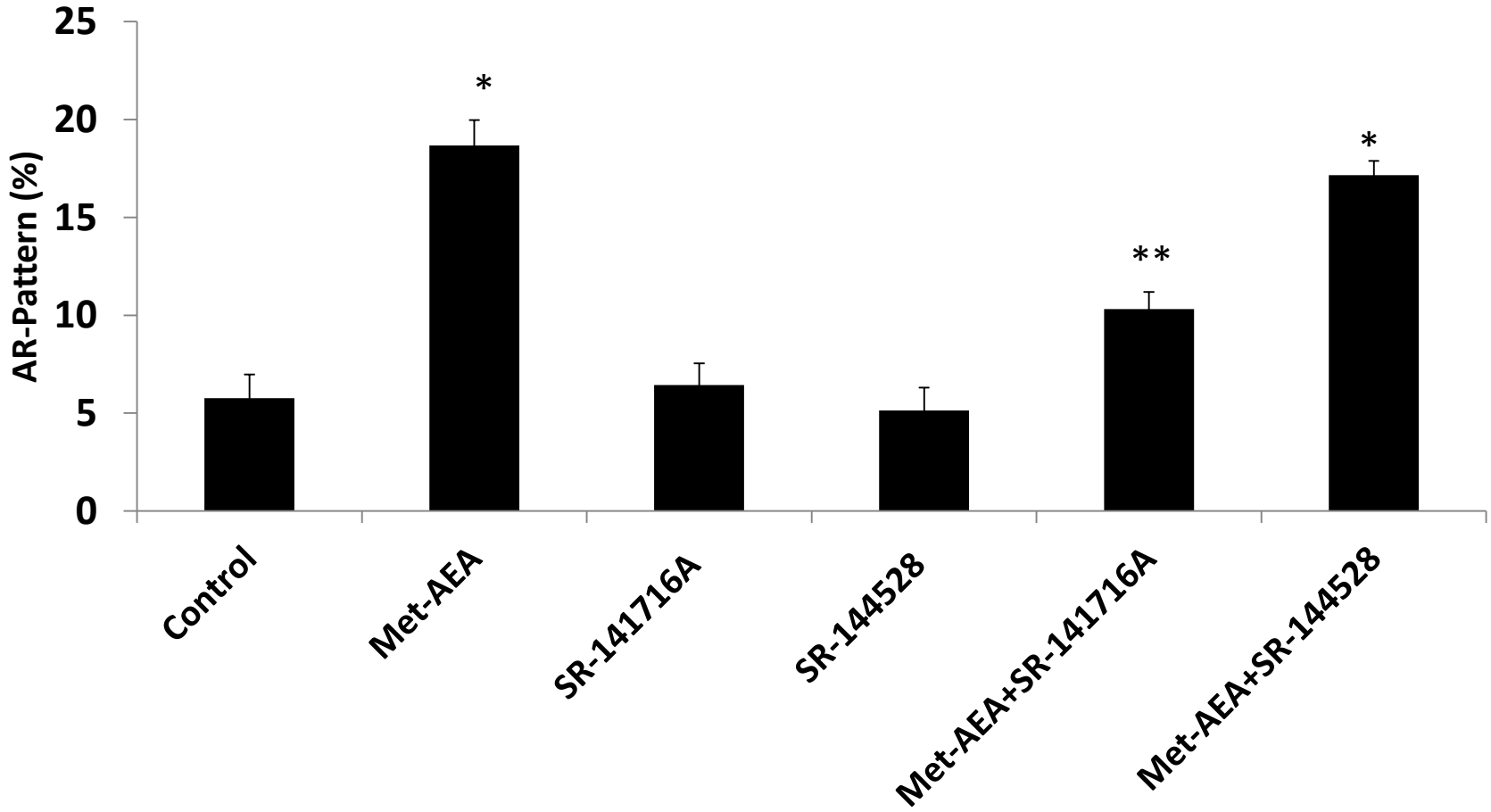


Fig 14 Effect of Met-AEA, SR-141716A (CB1 antagonist), and SR-144528 (CB2 antagonist) alone and in combination on per cent acrosome reacted spermatozoa (AR- pattern) of buck spermatozoa (Mean ± SEM, n=24). Data are presented as Mean± SEM. Different superscripts above the bar indicates significance ($p < 0.05$)



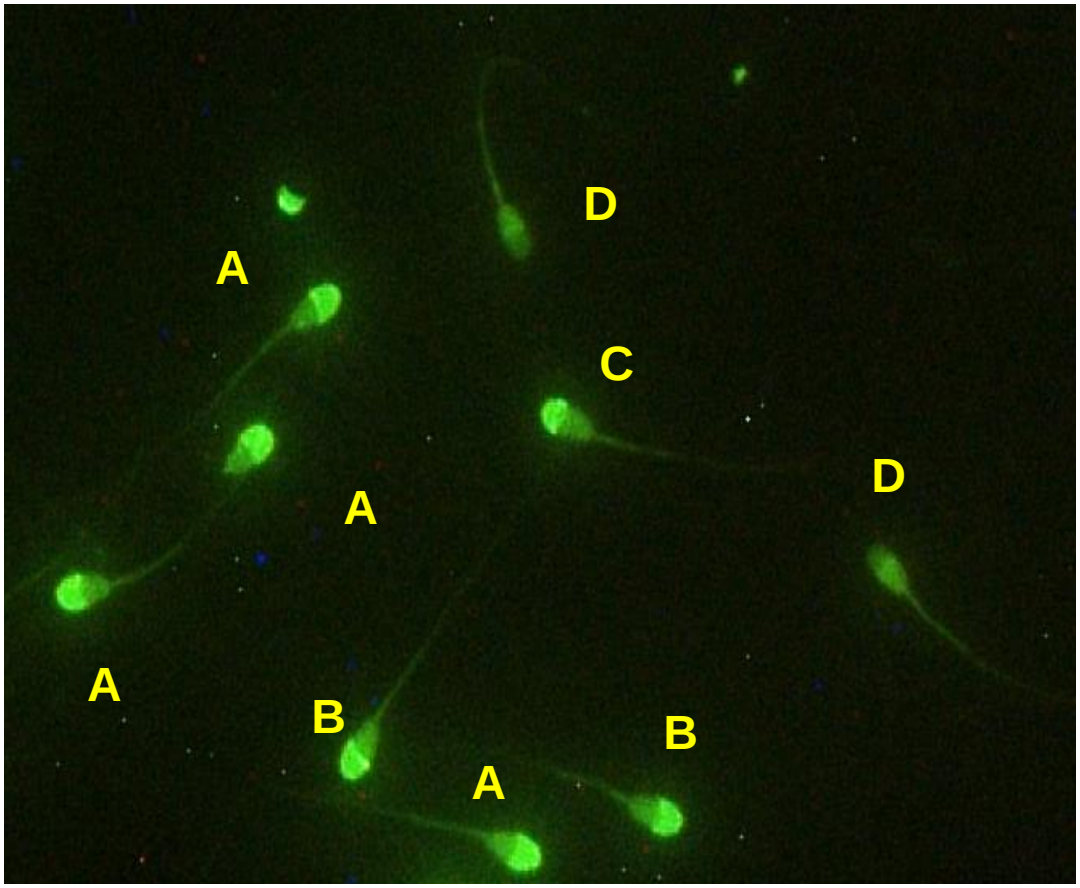


Fig 15: FITC PSA labeling of sperm acrosome

A------NORMAL SPERMATOZOA WITH INTACT ACROSOME
B-------PRIMARY DAMAGE
C-------SECONDARY ACROSOME DAMAGE
D-------TERTIARY ACROSOME DAMAGE- AR PATTREN

Conclusion

- CB1 and CB2 receptors are found to be present on buck spermatozoa
- Functionally these receptors are involved in sperm motility, capacitation and acrosome reaction
- CB1 is predominant and lowers the mitochondrial transmembrane potential
- Regulate capacitation through phosphorylation of Tyrosine containing proteins

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Functional and molecular characterization of voltage gated sodium channel Na_v 1.8 in bull spermatozoa

Dharmendra Singh Chauhan [a], Dilip Kumar Swain [e,*], Nadeem Shah [b], Hanuman Prasad Yadav [b], Udayraj P. Nakade [c], Vijay Kumar Singh [b], Rajesh Nigam [d], Sarvajeet Yadav [e], Satish Kumar Garg [c]

[a] College of Biotechnology, UP Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, 281001, Uttar Pradesh, India
[b] Department of Veterinary Gynecology & Obstetrics, College of Veterinary Science & Animal Husbandry, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, 281001, Uttar Pradesh, India
[c] Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science & Animal Husbandry, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, 281001, Uttar Pradesh, India
[d] Department of Veterinary Biochemistry, College
Vishwavidyalaya Evam Go Anusandhan Sansthan,
[e] Department of Veterinary Physiology, College of
Vishwavidyalaya Evam Go Anusandhan Sansthan,

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Effect of four different *in vitro* incubation temperatures on functional dynamics, process of capacitation and apoptosis in goat spermatozoa

Dilip Kumar Swain [a,*], Sarvajeet Yadav [a], Shanker K. Singh [b]

[a] Department of Veterinary Physiology, College of Veterinary Science & Animal Husbandry, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura 281001, Uttar Pradesh, India
[b] Department of Veterinary Medicine, College of Veterinary Science & Animal Husbandry, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura 281001, Uttar Pradesh, India

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Modulation of voltage-gated sodium channels induces capacitation in bull spermatozoa through phosphorylation of tyrosine containing proteins

Dharmendra Singh Chauhan [a], Dilip Kumar Swain [d, *], Nadeem Shah [b], Hanuman Prasad Yadav [b], Abhishek Sharma [c], Brijesh Yadav [d], Sarvajeet Yadav [d], Rajesh Nigam [e], Satish Kumar Garg [c]

[a] College of Biotechnology, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, 281001, Uttar Pradesh, India

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REVIEW ARTICLE
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Insights into pH regulatory mechanisms in mediating spermatozoa functions

Abhishek Kumar Mishra[1], Akshay Kumar[2], Dilip Kumar Swain[3], Sarvajeet Yadav[3] and Rajesh Nigam[1]

1. College of Biotechnology, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura - 281 001, Uttar Pradesh, India; 2. Department of Gynaecology and Obstetrics, College of Veterinary Sciences and Animal Husbandry, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura - 281 001, Uttar Pradesh, India; 3. Department of Physiology, College of Veterinary Sciences and Animal Husbandry, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura - 281 001, Uttar Pradesh, India.
Corresponding author: Dilip Kumar Swain, e-mail: dilip_swain@yahoo.com
Co-authors: AKM: mishraakvet@gmail.com, AK: akshay.rajawat@gmail.com, SY: yadavsarvajeet24@gmail.com, RN: dr_rajesh2005@rediffmail.com
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