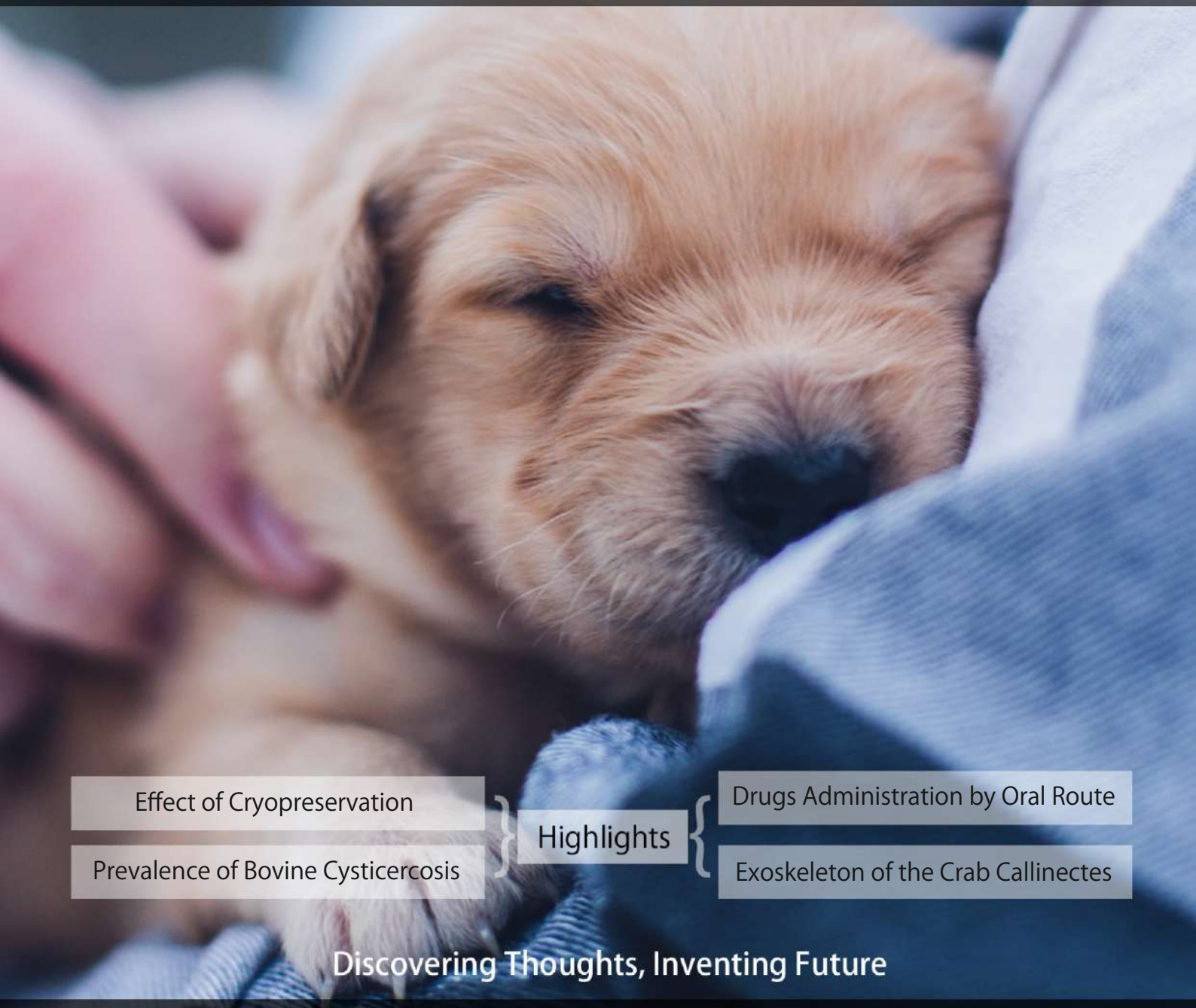
Online ISSN : 2249-4618
Print ISSN : 0975-5888
DOI : 10.17406/GJMRA

GLOBAL JOURNAL

OF MEDICAL RESEARCH: G

Veterinary Science & Veterinary Medicine



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Discovering Thoughts, Inventing Future

VOLUME 21 ISSUE 1 VERSION 1.0

Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine

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Veterinary Science and Veterinary Medicine
Volume 21 Issue 1 (Ver. 1.0)

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 21 Issue 1 Version 1.0 Year 2021
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Is it Possible to Keep the Exoskeleton of the Crab Callinectes Ornatus Soft for Several Days?

By Diogo Barbalho Hungria, Camila Prestes dos Santos Tavares, Ubiratã de Assis Teixeira da Silva, Leandro Ângelo Pereira, Ariana Cella-Ribeiro & Antonio Ostrensky

Federal University of Paraná

Abstract- Soft-shell crab is considered a gastronomic delicacy, reaching high values in the international market. Under normal conditions, the process of hardening of the crab's exoskeleton after moulting takes approximately two days to complete; however, the commercial consistency of soft-shell crab is lost in just 3 hours. The goal of this research was to evaluate the effects of chemical changes of water on the duration of postmoult, specifically at the stage in which they can be marketed as soft-shelled crab. In this research, *Callinectes ornatus* (n=241) underwent two experiments: One group was maintained in tanks with partial daily water renewal (Experiment 1), and other in tanks without water renewal (Experiment 2). In the experiment 1, the chemical characteristics of the water remained unchanged over time ($p > 0.05$), and the median time to hardening of the exoskeleton after moulting was 3 hours.

Keywords: *acidification; ammonification; portunidae; calcium; moult; soft-shell crab.*

GJMR-G Classification: *NLMC Code: QS 124*

Strictly as per the compliance and regulations of:

Is it Possible to Keep the Exoskeleton of the Crab Callinectes Ornatus Soft for Several Days?

Slowing the Hardening of the Crab Exoskeleton

Diogo Barbalho Hungria [α], Camila Prestes dos Santos Tavares [σ], Ubiratã de Assis Teixeira da Silva [ρ], Leandro Ângelo Pereira [ω], Ariana Cella-Ribeiro [¥] & Antonio Ostrensky [§]

Abstract- Soft-shell crab is considered a gastronomic delicacy, reaching high values in the international market. Under normal conditions, the process of hardening of the crab's exoskeleton after moulting takes approximately two days to complete; however, the commercial consistency of soft-shell crab is lost in just 3 hours. The goal of this research was to evaluate the effects of chemical changes of water on the duration of postmoult, specifically at the stage in which they can be marketed as soft-shelled crab. In this research, *Callinectes ornatus* (n=241) underwent two experiments: One group was maintained in tanks with partial daily water renewal (Experiment 1), and other in tanks without water renewal (Experiment 2). In the experiment 1, the chemical characteristics of the water remained unchanged over time ($p > 0.05$), and the median time to hardening of the exoskeleton after moulting was 3 hours. Over the course of experiment 2, there was a reduction ($p < 0.05$) in pH and increases in the ammonia and nitrite concentrations. When moulting occurred in water with a pH below 7.3 and total ammonia concentrations above 6.0 mg/L, the crabs' shells did not harden, and it was possible to keep them soft for up to 5 days.

Keywords: acidification; ammonification; portunidae; calcium; moult; soft-shell crab.

Highlights

1. Total ammonia and pH influence the hardening time of the exoskeleton of C. ornatus;
2. Ammonia above 6 mg/L and pH below 7 keep the crab exoskeleton soft for upto 5 days;
3. C.ornatus survival rate is influenced by pH, nitrite and total ammonia.

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I. Introduction

Callinectes ornatus Ordway, 1863 (Crustacea, Decapoda, Portunidae) is a swimmer crab found from North Carolina (USA) to the Rio Grande do Sul (Brazil). It occurs in areas with sand, mud or shell bottoms and inhabits estuarine to marine areas at a depth of approximately 75 m (Carvalho and Couto 2011, Melo-Filho 1996). Similar to other arthropods, *C. ornatus* grows through a process of periodic exoskeleton changes; each shedding of the exoskeleton is known as ecdysis or moult (Drach 1939, Freeman and Perry 1985, Newcombe, Sandoz et al. 1949). Immediately after shedding its exoskeleton, the crab presents a soft and flexible integument that has a low level of calcification. In this phase, the animals can be commercialised and consumed whole as "soft-shell crab", a delicacy that is appreciated worldwide and that reaches high market values (Hungria, Tavares et al. 2017, Tavares, Silva et al. 2018). According to FAO (2020), soft-shell crab aquaculture is considered a millionaire aquaculture practice in the eastern United States.

Immediately after moult, $CaCO^3$ deposition begins on the protein matrix of the new exoskeleton. This process involves a complex system of absorption of Ca^{2+}, CO_2, and HCO_3^- and the synthesis of $CaCO_3$ and other elements (Greenaway 1985, Perry, Trigg et al. 2001, Wheatly 1999, Zanotto and Wheatly 2002). The initially fragile exoskeleton undergoes rapid hardening, providing rigidity and mechanical protection for the animal. Under natural conditions, the hardening of the exoskeleton takes about two days to complete (Cameron and Wood 1985).

During the hardening process, the exoskeleton can be classified into four sequential levels of consistency: soft, leather, paper and hard (Freeman, Kilgus et al. 1987). Only the first two are valued in the international market of soft-shell crabs (Gaudé and Anderson 2011, Oesterling 1995, Perry, Graham et al. 2010). However, the combined duration of the soft and leathery stages is very short in nature, rarely lasting more than 3 hours(Cameron and Wood 1985), which obliges commercial producers to inspect all of the animals stocked in the premoult phase every 4 hours on average (Oesterling 1995).

Extending the duration in which the crab shells remain at the consistencies of high market value would significantly reduce production costs (Perry, Trigg et al. 2001). Furthermore, it would minimise the damage caused by rapid exoskeleton hardening, providing better quality and uniformity regarding the softness of the product. The goal of this research was to evaluate the effects of chemical changes of water on the duration of postmoult to extend the time during which the animals could be marketed as soft-shell crab.

II. Material and Methods

a) Crab collection and maintenance

Specimens of *C. ornatus* were obtained via trawling by professional fishers at the balneary of Shangri-la, municipality of Pontal do Paraná (25°37'S/48°25'O), Paraná, Brazil. Shrimp trawls 12 m in length and with 20 mm mesh were used. In each sampling campaign, on average, three trawls of approximately 50 minutes each were made. Immediately after crab collection from the net, the crabs were separated and transferred to two polyethylene tanks (70 L volume) with lids, each containing 20 L of seawater. The tanks received continuous aeration supplied via an 18W air compressor. Inside each tank were plastic screens with 2 mm mesh positioned to reduce contact and prevent fights between the animals and minimise injuries and deaths. Thereafter, 100% of the water was renewed every half hour during the campaign.

Immediately after capture, the animals were transported to the Marine Aquaculture and Restocking Center (CAMAR) of the Integrated Group of Aquaculture and Environmental Studies (GIA), Federal University of Paraná (UFPR), at Pontal do Paraná (25°41'29.94"S, 48°27'57.09"W). The time elapsed between animal capture and arrival at the laboratory was consistently less than 4 hours. Animals that were not used were returned to the sea. In the laboratory, the crabs were maintained in 1,000 L tanks containing 100 L of seawater (30 mg/L) supplied with constant aeration for approximately 6 hours. This period was purposely short since a large proportion of the captured individuals were very close to moult. Dead animals were discarded, and the live animals were classified by sex. Then, the crabs were inspected to determine the phase of the moulting cycle.

Those individuals at the premoult phase were selected for the experiments based on macroscopic indicators (visualisation of an inner line along the edges of the fifth pair of pleopods) (Drach 1939, Drach and Tchernigovtzeff 1967, Wehrtmann and Mena-Castañeda 2003). The selected individuals were weighed on an analytical balance (Marte AL 500c, Brazil; accuracy of 0.01 g) and measured (width of the carapace, measured as the distance between the base of the largest lateral spines) with a pachymeter. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered. As it was an invertebrate research project, it was not necessary to submit it for analysis by the Animal Use Ethics Commission of Federal University of Paraná (UFPR). In any case, all experimental procedures used followed current legislation and the rules of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) for the capture and use of native invertebrates.

b) Pilot experiments

Two pilot experiments were carried out. The first experiment tested the influence of fasting on animal survival under laboratory conditions. The animals only began to die after 50 days without access to food. Based on this result and to potential feeding effects on water quality or the process of moult and hardening, the crabs were not fed during the 12 days of each of the main experiments. The second experiment tested the influence of the non-renewal of water on hardening time. The time elapsed between moult and shell hardening was significantly higher under water non-renewal than under the periodic renewal of water. In addition, a higher frequency of moult was observed at night (between 18:00 and 06:00); this information informed the design of the experimental methodology described below.

c) Experimental design

In both experiments, the saltwater had been previously chlorinated and maintained under constant aeration for 24 h. After this period, residual chlorine was neutralised (with 50% sodium thiosulfate), and the water was stored in the dark in 25,000 L tanks. Before use in the experiments, the water was passed through mechanical filters of 5 and 25 μm mesh and a UV filter for disinfection. Two experiments were performed and are represented schematically in FIGURE 1.

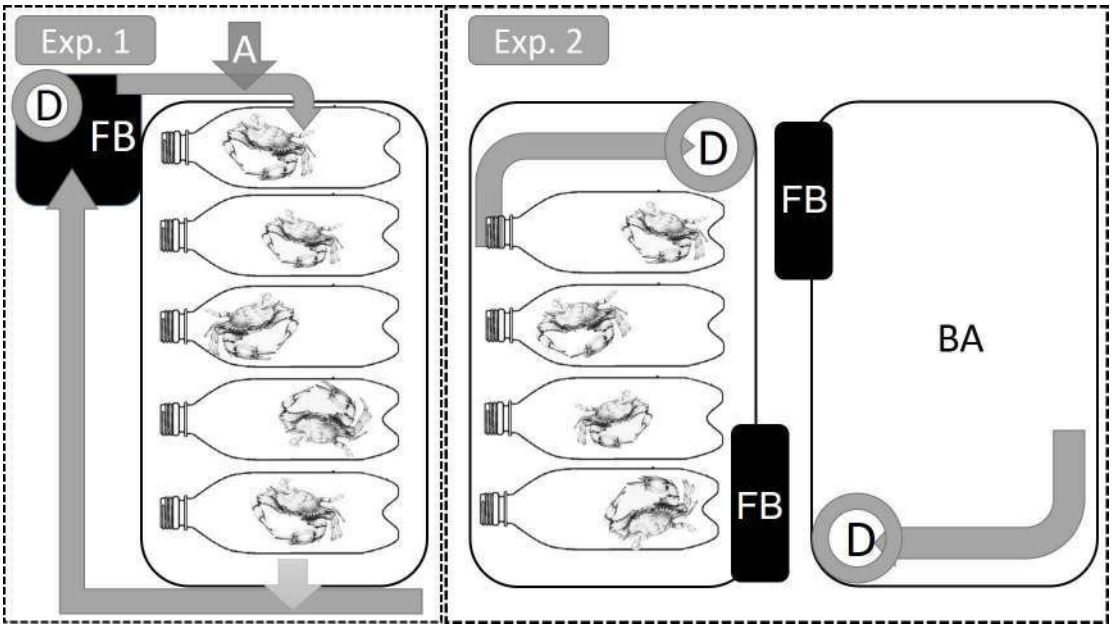

Figure 1: Schematic representations of the experimental systems. Experiment 1 (Exp. 1): crabs maintained in a collective system with filtration and partial daily water renewal. Experiment 2 (Exp. 2): crabs maintained in a collective system with filtration but without water renewal. A = external water supply; D = protein skimmer; FB = mechanical / biological filter; BA = Water control.

i. *Experiment 1: crab maintenance in a collective system with filtration and partial daily water renewal*

C. ornatus crabs (n=66) were individually placed in perforated pet bottles (600 mL) and distributed in a system consisting of 20 polyethylene tanks (71.0 x 35.5 x 35.0 cm, containing 25 L of seawater each). The tanks were interconnected via a skimmer and a mechanical/biological filter system and were under constant aeration, continuous water recirculation and controlled photoperiod (14:10- light: dark).The animals were separated into two groups: A1, premoultcrabs (n = 46), and AC, control crabs (at the intermoult stage) (n = 20). Each day throughout the experimental period (12 days), 1/3 of the total water volume of the system (333 L) was added, promoting mixing with the water already present, and an approximately equivalent amount was removed, keeping the total water volume in the system constant.

ii. *Experiment 2: crab maintenance in a collective system with filtration and without water renewal*

C. ornatus crabs (n = 176) were individually placed in perforated (600 mL) pet bottles and distributed among 12 polyethylene tanks (71.0 x 35.5 x 35.0 cm, containing approximately 30 L of water each). Each tank contained a protein skimmer and a mechanical/biological filter and was subjected to constant aeration, continuous water recirculation and controlled photoperiod (14:10 – light: dark). The animals were subdivided into 3 groups: B1, premoultcrabs (n = 83); B2, premoultcrabs (n = 40); and BC, control intermoult animals (n = 52). In addition, three tanks containing water only were maintained throughout the experimental period (12 days) for comparison of physical and chemical water variables between these tanks and the 3 treatment groups. There was no water renewal during the experiment. The crabs in the B2 group were housed in the same tanks used to house group B1 and maintained in the same water used for group B1.TABLE 1 provides summary information on the subject animals and design of the two experiments.

Table 1: Summary of the general conditions of the experiments performed to evaluate the effects of water quality on the hardening time of the exoskeletonin *Callinectes ornatus* and width and weight data of the animals (mean ± SD). NA: not applicable.

Experiment	Group	Experimental Unit	Stage	N	Width (mm) (±sd)	Weight (g) (±sd)	Water Volume (L)	Water Sampling Frequency
1	A1	Tank	Premoult	46	45.4 (±6.16)	15.04 (±6.11)	0.6	24 h
	AC	Tank	Intermoult	20	61.55 (±10.53)	35.57 (±16.9)	0.6	24 h
2	B1	Tank (Replicate 1)	Premoult	27	50.7 (±7.78)	16.64 (±7.03)	32.5	12 h
		Tank (Replicate 2)		27	3.10 (±7.36)	14.36 (±7.47)	32.5	12 h
		Tank (Replicate 3)		29	47.5 (±5.12)	15.11 (±4.92)	32.5	12 h
	B2	Tank (Replicate 1)		14	48.1 (±8.25)	16.46 (±8.08)	30	12 h
		Tank (Replicate 2)		13	46.4 (±7.02)	15.40 (±6.7)	30	12 h
		Tank (Replicate 3)		13	47.0 (±6.64)	14.75 (±6.3)	30	12 h
	BC	Tank (Replicate 1)	Intermoult	17	56.2 (±2.9)	26.51 (±5.07)	32.5	12 h
		Tank (Replicate 2)		17	59.7 (±3.8)	28.31 (±6.1)	32.5	12 h
		Tank (Replicate 3)		18	56.65 (±4.93)	26.54 (±7.95)	32.5	12 h
	BA	Tank (3 Replicates)	NA	NA	NA	NA	32.5	12 h

d) Experimental procedures

During the experiments, the crabs were monitored every three hours on the first four days, every six hours on the following five days, and every 12 hours on the last three days of experimentation, preferably between 18:00 and 06:00 hours. These times were selected based on the results of the pilot experiments. Monitoring consisted of identifying animals undergoing the moulting process, removing any moulted exoskeletons (to prevent the animals from obtaining calcium by feeding on them), evaluating the consistency of the carapace of those animals that had moulted, and removing any dead animals. Evaluating the consistency of the exoskeleton was performed by pressing the carapace with an index finger. Sufficient pressure was applied to deform the carapace but not injure the animal or break the carapace when rigid. Based on the resistance to pressure and texture of the exoskeleton, its consistency (Co) was classified by the evaluator as follows: hard - before ecdysis (1), soft (2), leather (3), soft paper (4), hard (5) or hard paper - after ecdysis (6). To reduce and standardise the error, a single evaluator performed the consistency assessments in both experiments.

e) Water analysis

In both experiments, salinity (refractometer; Instrutemp, Brazil), temperature (digital thermometer), pH (AZ pH/mV/TDS /Temperature Meter 86505, Taiwan), and dissolved oxygen concentration (Oximeter YSI 550A, US states) were monitored daily in all experimental units. Water samples were collected from the units, labelled and immediately frozen (-20°C) for later evaluation of the physical and chemical variables. For group A1, water collection was performed every 24 hours before the new water was added to the system. For groups B1, B2, BC and BA, 50 mL of water was collected every 12 h.

At the end of the experiments, the frozen water samples were analysed with respect to the following parameters: Na^+, K^+, $Ca^{2+}$and NO_3 (electrodes of the LAQUA twin series, Horiba Scientific®, Japan) and total ammonia (NH_3+NH_4^+) and NO_2- (SpectraMax® m2 spectrophotometer, US states).Measurements were performed following APHA (2005) and Büldt and Karst (1999). The determinations of $Mg_2^+$and Cl^- were performed using colourimetry (Labtest®, Brazil) at a wavelength of 540 nm and 470 nm, respectively (SpectraMax® m2, US states), according to the method described by Clarke (1950).

f) Statistical analyses

The survival of the animals during the experiments was analysed through Kaplan-Meier curves. The data were grouped by treatment (groups), and the normality of the distribution of each variable was tested by using Shapiro-Wilk test. Where the normality hypothesis was rejected, non-parametric Mann-Whitney or Kruskal-Wallis tests were used. Multiple linear regression analysis was performed to model the influences of the physical and chemical variables that determine water quality on exoskeleton hardening time. The assumption of the independence of the physical and chemical variables was upheld, the hypothesis of autocorrelation and collinearity (using Durbin-Watson and the serial error correlation tests) was rejected, and the normality of the error was confirmed.

To limit the number of variables and thereby minimize the complexity of the models without a significant loss of the information offered by the total set of original variables, we select only those variables that: 1) were statistically significant ($p < 0,05$) and; 2) contributed more than 5% to the coefficient of determination (R^2) of the model or that caused the R2value to move into a higher category when it was included in the model, following the classification proposed by Mukaka (2012): very weak:: $R^2 < 0,19$; weak: $0,20 > R^2 < 0,39$; moderate: $0,40 > R^2 < 0,69$; strong: $0,70 > R^2 < 0,89$; very strong: $R^2 > 0,90$.

III. Results

a) Ecdysis

Significant effects of sex on survival rate, ecdysis, or exoskeleton postmoult hardening time were not observed. Therefore, the data from males and females were pooled. In addition, water temperature (27.0 ± 1.1°C), salinity (31.0 ± 2.1 mg/L) and dissolved oxygen concentration (5.0 ± 0.52 mg/L) remained largely stable and did not significantly influence any of the dependent variables. The moulting rate of the animals in premoult at the beginning of the experiments ranged from 40 to 95%. Most moulting events occurred during the night, and 50% of the animals moulted between 52 and 80 hours after the beginning of the experiments. There was a significant effect of moulting on final mortality rate and on survival time after ecdysis (TABLE 2).

Table 2: General results of laboratory experiments to evaluate ecdysis in *Callinectes ornatus.*

Exp.	Group	Stage	n_1	Weight Gain (%)	Ecdysis Performed			Time to Ecdysis (h)			Mortality Rate (%)	Survival Time (h)		
					Period	n_2	%	25%	50%	75%		25%	50%	75%
1	A1	Pre	46	56	day night	14^a 24^b	83	44.5	80	124	24^a	230	-	-
	AC	Inter	20	NA	NA	NA	NA	NA	NA	NA	0^b	-	-	-
2	B1	Pre	83	69	day night	19^a 59^b	94	36	52	78	78^c	107	168	212
	B2	Pre	40	46	day night	3^a 13^b	40	102	-	-	78^c	174	228	413
	BC	Inter	53	NA	NA	NA	NA	NA	NA	NA	26^a	255	-	-

Exp.: experiment number; Pre: premoult; Inter: Intermoult; n_1: number of individuals; Weight Gain: increase in postmoult weight (%); Period: the period in which moult occurred; n_2: number and percentage of crabs that performed ecdysis; Time to Ecdysis: time (h) at which 25, 50 and 75% of the animals had moulted; Mortality rate (%);Survival Time: time (h) at which 25, 50 and 75% of the animals survived after ecdysis; NA: Not Applicable. Different letters indicate significant differences ($p < 0.05$) between the groups according to the Kruskal-Wallis test. Experiment 1 (collective treatment with filtration and partial daily water renovation). A1: premoult organisms; AC (Control): organisms in intermoult. Experiment 2 (collective treatment with filtration but no water renewal). B1: premoult organisms; B2: tanks containing water previously used for group B1, with organisms in premoult; BC (Control): tanks with organisms in intermoult.

b) Physical and chemical water parameters

In Experiment 1, the total ammonia ($NH_3 + NH_4^+$) concentrations remained below the limit of analytical detection, and the median pH was 8.5, with variation between 8.1 and 8.5. The remaining physical and chemical parameters were relatively stable throughout the experimental period (TABLE 3). In Experiment 2, only potassium and sodium concentrations presented differences between the groups B1 and B2. There was a reduction in pH and increases in total ammonia and nitrite concentrations in the experimental treatments (premoult crabs, B1 and B2) in relation to the control (BA, tanks containing water only). The variables monitored in the BC tanks (intermoult crabs) presented intermediate values relative to the other groups (TABLE 4).

Table 3: Median and 1st and 3rd quartiles of the water quality parameters in Experiment 1 (collective treatment with filtration and partial daily water renewal).

Parameter	Median	25-75%
pH	8.50	8.4-8.5
K^+ (mg/L)	380	370-390
Ca^{2+} (mg/L)	350	330-430
Mg^{2+} (mg/L)	589.5	573.3-602.6
Na^+ (mg/L)	11,000	9,900-12,000
Cl^- (mg/L)	16,830	16,059-17,668
TA (mg/L)	0.0	0.0
NH_3 (mg/L)	0.0	0.0
NO_2^- (mg/L)	1.30	1.29-1.30
NO_3 (mg/L)	180	170-200

TA: Total ammonia (NH_3+NH_4^+)

Table 4: Median and 1st and 3rd quartiles of the water parameters in Experiment 2 (collective treatment with filtration but without water renewal).

Parameter	Groups			
	B1	B2	BA	BC
	Median 25-75%	Median 25-75%	Median 25-75%	Median 25-75%
pH	6.7^b (6.3-7)	6.5^b (6.2-7)	8.4^a (8.3-8.4)	7.8^{ab} (6.8-8.1)
K^- (mg/L)	420.00^a (370-540)	280.00^b (230-330)	330.00^{ab} (260-370)	390.00^{ab} (290-420)
Ca^{2+} (mg/L)	430.00^a (400-480)	350.00^a (320-420)	430.00^a (360-490)	450.00^a (390-480)
Mg^{2+} (mg/L)	545.6^b (472.3-585.8)	551.2^b (531.8-622.5)	586.3^{ab} (573.3-597.4)	592.0^a (555.2-607.9)
Na^+ (mg/L)	$12,000^a$ (10,000-14,000)	$7,500^b$ (6,500-9,000)	$11,500^{ab}$ (10,000-13,000)	$12,000^{ab}$ (10,000-14,000)
Cl^- (mg/L)	18279^a (16,507-20,245)	11995^a (9,158-15,782)	15830^a (14,705-17,473)	17602^a (16,122-19,278)
TA (mg/L)	6.8^b (5.7-10.5)	9.5^b (7.4-11.91)	0.0^a 0.0	1.1^{ab} (0.0-3.1)
NH_3 (mg/L)	0.02^b (0.01-0.05)	0.02^b (0.01-0.05)	0.00^a 0	0.01^{ab} (0-0.08)
NO_2^- (mg/L)	4.7^b (2.8-6.5)	5.4^b (2.15-7.8)	1.3^a (1.3-1.4)	5.2^b (4.0-6.1)
NO_3 (mg/L)	230^a (190-310)	260^a (200-340)	210^a (120-260)	230^a (150-280)

B1: crabs in premoult. B2: tanks containing water previously used for group B1 and premoult crabs. BA (Control): tanks containing water only. BC (Control): tanks with crabs in intermoult. Different letters indicate significant differences ($p < 0.05$) between groups according to the Kruskal-Wallis test. TA: Total ammonia (NH_3+$NH4^+$).

c) *Influence of the physical and chemical water parameters on the survival and moulting of C. ornatus*

As expected, crab survival time was influenced by moulting regardless of the experiment. The crabs of group B1 that underwent moult in the first 36 h showed rapid exoskeleton hardening and low mortality rates. Therefore, the B1 data were divided into two categories: M1, animals that moulted within the first 36 h, and M2, those that moulted after 36 h. The survival of M1 animals was strongly influenced by pH and total ammonia and nitrite concentrations, whereas the survival of the M2 animals was moderately influenced by the same variables. TABLE 5 shows the multiple linear regression results. Crab survival rate was significantly influenced by pH, nitrite and total ammonia in all of the experiments. The remaining parameters had no significant influence ($p < 0.05$) on the crab survival time.

Table 5: General results of multiple linear regression analysis of the influences of physical and chemical parameters on crab survival time.

Experiment	Group		Correlated Parameters	Cases n	p	Adjusted R^2	Correlation
1	A1		pH (max.) TA (min.) $NO2^-$ (min.)	384	0.000 0.000 0.000	0,372	Weak
	AC		NA		NA	NA	NA
3	B1	M1	pH (max.) TA (min.) NO_2^- (max)	233	0.000 0.000 0.000	0,724	Strong
		M2	pH (max.) TA (min.) NO_2^- (med.)	318	0.000 0.000 0.000	0,681	Moderate
	B2		pH (max.) TA (min.) NO_3 (med.)	495	0.000 0.000 0.000	0,430	Moderate
	BC		pH (max.) TA (max.) NO_2^- (max.)	452	0.000 0.000 0.000	0,743	Strong

Experiment 1 (collective treatment with filtration and partial daily water renovation). A1: premoult crabs; AC (Control): intermoult crabs. Experiment 2: Collective treatment with filtration but no water renewal. B1: premoult crabs; B2: tanks containing water previously used for the B1 group, with premoult crabs; BC (Control): tanks with crabs in intermoult; M1: crabs of B1 Group that performed ecdysis within the first 36 hours; M2: crabs that moulted after 36 hours. NA: Not applicable. TA: Total ammonia (NH_3+$NH4^+$).

The results of the multiple linear regression analysis of the effects of the physical and chemical parameters on the time until either the shells fully hardened (reached Co6) or death are presented in TABLE 6. In Experiment 1, only pH had an influence (weak) on the results. In experiment 2, pH, ammonia and nitrite had moderate influences on the results. The duration at which the shell was at consistency 2 (i.e., the consistency with the highest market value) was significantly higher in the M2 animals than in the M1 animals. Furthermore, none of the M2 individuals that moulted after 36 h achieved Co6 (hard) shells, whereas in the M1 group, more than half of the individuals had shells that reached this consistency. In addition, 68% of individuals with shells that hardened remain alive. Among those that did not achieve shell hardening, the survival rate was only 15% (TABLE 7).

Table 6: General results of multiple linear regression analysis of the influences of physical and chemical parameters on the time until shell hardening or death after ecdysis.

Experiment	Group		Correlated parameters	Cases (n)	p	Adjusted R^2	Correlation
1	A1		pH (max.)	68	0.000	0,196	Weak
2	B1	M1	pH (max.)	116	0.000	0,559	Moderate
		M2	pH (max.) AT (min.)	136	0.000 0.000	0,410	Moderate
	B2		AT (min.) NO_2^- (min.)	203	0.000 0.001	0,657	Moderate

Experiment 1 (collective treatment with filtration and partial daily water renovation). A1: crabs in premoult. Experiment 2 (collective treatment with filtration but no water renewal). B1: crabs in premoult; B2: tanks containing water previously used for the B1 group, with crabs also in premoult; M1: crabs that moulted within 36 hours; M2: crabs that moulted after 36 hours. TA: Total ammonia (NH_3+$NH4^+$).

Table 7: Duration of *Callinectes ornatus* at shell consistencies 2 and 3 (Co 2 and Co 3) and associated hardening, ecdysis and mortality data in group B1 (organisms initially in premoult) of Experiment 2 (collective treatment with filtration but no water renewal). Different letters indicate a significant difference ($p < 0.05$) between the groups (within a column) according to the Kruskal-Wallis or Mann-Whitney test.

Group	Co 2/3 (h)(min-max)	n	Hardening(%)		Ecdysis (n (%))	Dead (n (%))
M1	3^a (1-18)	32	Yes No	(54%)[a] (37%)[a]	19 (23%) 13 (16%)	8 (42%)[a] 11 (85%)[b]
M2	61^b (3-129)	46	Yes No	- (100%)[b]	0 46 (55%)	- 43 (93%)[b]

Individuals who moulted before (M1) or after (M2) 36 hours.

Table 8: Water quality parameter measurements and crab survival data registered in the experimental units in which the crabs (*Callinectes ornatus)* moulted.

Exp.	Group	Co	Median (min-max)				Mortality (%)	Survival (h)		
			pH	TA (mg/L)	NO_2^- (mg/L)	Time (h)		25 %	50%	75%
1	A1	1	8.4^{aA} (8.4-8.6)	0^{aA} (0-0.3)	1.31^{aA} (1.28-1.50)	61^{aAD} (2-245)	15	0	0	0
		2-3	8.4^{abA} (8.4-8.5)	0^{aA} 0.00	1.33^{abA} (1.29-1.33)	4^{bA} (1-9)	2			
		4-5	8.5^{abA} (8.3-8.6)	0^{aA} (0-0.3)	1.30^{abA} (1.28-1.50)	26^{cA} (3-137)	4			
		6	8.5^{bA} 8.5-8.6)	0^{aA} (0-0.3)	1.3^{bA} (1.28-1.50)	155^{dA} (17-209)	2			
2	B1	1	8.4^{aA} (7.6-8.4)	0^{aA} (0-4.8)	1.7^{aB} (1.3-2.72)	30^{aC} (0-36)	9	69	150	-
	M1	2-3	7.6^{bA} (6.7-8.4)	4.8^{bA} (0-9.8)	2.7^{bA} (1.3-3.22)	3^{bA} (1-18)	3			
		4-5	7.0^{cD} (5.7-8.3)	6.1^{cD} (1.1-16.5)	3.2^{cC} (1.67-7.85)	42^{cC} (21-234)	31			
		6	6.5^{dB} (5.5-7.7)	8.2^{dB} (4.5-16.5)	5.6^{dB} (2.27-8.02)	183^{cA} (55-240)	20			
	B2	1	7.1^{aB} (5.7-8.4)	6.0^{aB} (0-16.5)	2.8^{aC} (1.3-7.85)	60^{aBD} (36-192)	2	66	91	107
	M2	2-3	6.6^{bC} (5.5-7.5)	7.7^{bD} (3.9-16.5)	5.6^{bB} (2.27-8.02)	61^{aC} (3-129)	52			
		4-5	6.5^{bC} (5.5-7.3)	9.1^{cC} (4.5-16.5)	5.6^{bD} (2.27-8.02)	45^{aAC} (3-222)	35			
	B2	1	6.5^{aC} (5.3-8.1)	9.3^{aC} (3.3-14.7)	5.2^{aD} (1.35-9.40)	177^{aB} (0-186))	38	58	94	144
		2-3	6.5^{aC} (5.8-8.1)	10.1^{abC} (3.4-14.7)	6.3^{aBC} (1.53-9.40)	64^{bBC} (18-126)	20			
		4-5	6.5^{aC} (5.8-7.6)	11.2^{bC} (4.2-14.7)	4.2^{aBC} (1.53-9.40)	80^{bABC} (12-180)	20			

Exp.: Experiment 1 (collective treatment with filtration and partial daily water renovation), Experiment 2 (collective treatment with filtration but no water renewal). A1 and B1: water not used previously; B2: tanks containing water used previously for the group B1; M1: crabs that moulted within the first 36 hours; M2: crabs that moulted after 36 hours. Co: consistency. TA: total ammonia. Time: Length of stay at a given consistency. Different letters indicate significant differences ($p < 0.05$) according to the Kruskal-Wallis test. Lowercase letters indicate differences in carapace consistency within the same group. Uppercase letters indicate differences in carapace consistency among groups.

Table 8 shows the median, minimum and maximum values of the physical and chemical parameters of water quality that most influenced crab survival time and exoskeleton consistency: pH, ammonia and nitrite. The duration at each level of consistency was highly related to the pH and concentration of total ammonia at the time of moulting. When the levels of the physical and chemical parameters favoured hardening, the time until the crabs reached Co4 (soft paper) was short (between two and four hours). In contrast, when the pH was below 7.3 and the total ammonia concentration remained above 6 mg/L, the median time to Co4 was 60 hours.

When water renewal was not performed (Experiment 2), the pH and total ammonia and nitrite concentrations varied significantly (FIGURE 2). As a result, there was an increase in the carapace hardening time and a decrease in the number of individuals reaching Co6. The crabs of Experiment 1 (A1) and the animals that moulted within the first 36 hours of Experiment 2 (M1) spent significantly less time at Co2 and Co 3 than did those that moulted after the first 36 hours (M2) in group B1 and those in group B2.

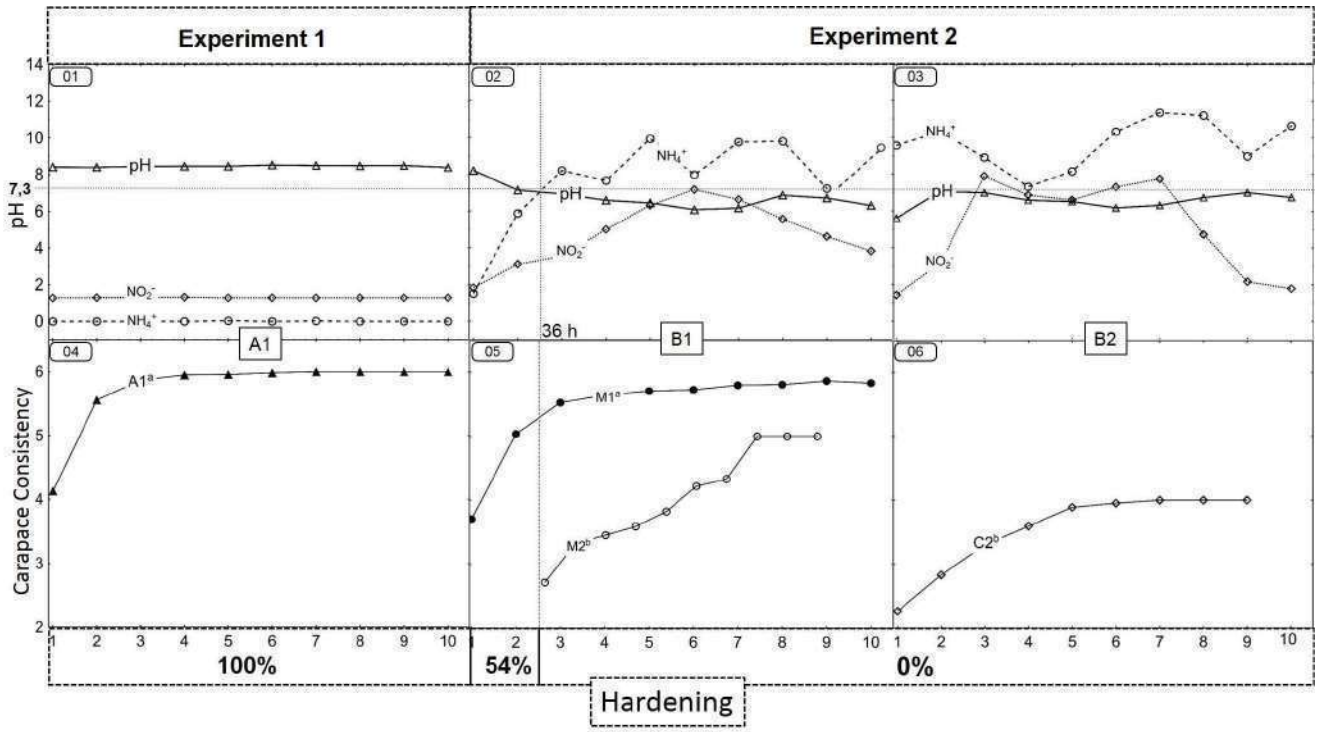

Figure 2: Median pH, total ammonia (mg/L), nitrite (NO_2^-) (mg/L) (01–03) and carapace consistency over time (in days) (04–06). Experiment 1: collective treatment with filtration and partial daily water renewal. Experiment 2: collective treatment with filtration but without water renewal. A1 and B1: previously unused waterand organisms in premoult. B2: organisms in premoult maintained in the reused water of group B1. M1: crabs that moulted within the first 36 hours. M2: crabs that moulted after 36 hours. Different letters indicate significant differences ($p < 0.05$) among groups according to the Kruskal-Wallis test.

IV. Discussion

An issue repeatedly debated among those who investigate the shedding and hardening process in crustaceans is the importance of calcium, the main constituent element of the exoskeleton (Greenaway 1985), in this process (Cameron 1985, Cameron and Wood 1985, Clarke and Wheeler 1922, Freeman and Perry 1985, Granado e Sá, Baptista et al. 2010, Greenaway 1983, Mangum, deFur et al. 1985, Middlemiss, Urbina et al. 2016, Neufeld and Cameron 1992, Pan, Luan et al. 2006, Perry, Trigg et al. 2001, Robertson 1960, Welinder 1974, Wheatly, Zhang et al. 2001, Wheatly 1997, Wheatly 1999, Wheatly, Zanotto et al. 2002, Zanotto and Wheatly 2002). The lack of significant correlations between the concentrations of Ca^{2+} and Mg^{2+} in water and either carapace hardening or *C. ornatus* survival does not indicate that calcium is not important in this process. On the contrary, it indicates that certain processes can directly interfere with the physiology of the absorption and immobilisation of Ca_2^+ in the exoskeleton and thereby significantly increase the time that these animals remain soft after moult.

The organisms of Experiment 1 (subjected to daily water renewal) that underwent ecdysis hardened rapidly, achieving paper consistency (Co 4) a median of 4 hours after moult. This finding is consistent with studies conducted with *C. sapidus* (Cameron and Wood 1985, Freeman, Kilgus et al. 1987). Similar results were observed among the crabs in Experiment 2 that moulted in water with a pH above 7.6 and a total ammonia concentration below 4.8 mg/L, with Co 4 achieved after a median time of 2 to 3 hours. However, among the animals that began moulting in water with a pH below 7.3 and a total ammonia concentration above 6 mg/L, up to 129 hours (median of more than 60 hours) elapsed before either reaching Co4 or death.

To understand this result, it is necessary to understand the chemical processes involved in the calcification of the crab exoskeleton. In a closed system

with water recirculation, it is expected that over time there will be a reduction in the concentration of free Ca_2^+, due mainly to the immobilisation of Ca_2^+ in the form of $CaCO_3$ during exoskeleton hardening (Perry, Trigg et al. 2001). This immobilisationcan be represented by the following equation:

$$Ca^{2+} + HCO_3^- \Leftrightarrow CaCO_3 + H^+ \quad (1)$$

With the increased demands for Ca_2^+ and HCO_3-, crabs begin to consume both metabolic and external CO_2. CO_2 reaches its highest internal concentrations at moulting time (Mangum, deFur et al. 1985), increasing the availability of internal HCO_3- (Cameron and Wood 1985). As soon as moulting occurs, the enzyme carbonic anhydrase (CA), present mainly in the epithelium and the gills, is activated (Mangum, deFur et al. 1985), accelerating the reaction:

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \quad (2)$$

As explained by Detours, Armand et al. (1968) and Zeebe and Wolf-Gladrow (2001), the formed carbonic acid tends to be buffered by the carbonate-bicarbonate system. This process results in an increase in the fraction of CO_3- and acidification of the medium (Greenaway 1974, Mangum, deFur et al. 1985, Wheatly 1997). However, over time, the natural acid neutralisation capacity of the system becomes compromised, and the medium tends to acidify as a result, increasingly compromising the crab's capacity to deposit $CaCO_3$ in its exoskeleton. According to Cameron and Wood (1985), the calcification process can be compromised if the pH outside the body is less than 0.3 to 0.5 above the internal pH.

However, in addition to consuming HCO_3- postmoult, the crab excretes H+ or an equivalent ion such as NH_4^+ (Cameron 1985, Middlemiss, Urbina et al. 2016), which is dissociated into NH_3 and H^+. The rate of H^+/NH_4^+excretion increases after moulting (Cameron and Wood 1985) and may increase further during bacterial denitrification (Rijn, Tal et al. 2005). Under these conditions, the metabolism of excretion also contributes to the acidification of the medium, further reducing the capacity for calcium mobilisation by the crab, as observed in experiment 2. There is evidence that water acidification is more critical for the hardening process of marine crustaceans than for that of freshwater crustaceans. Unlike freshwater crustaceans, marine crustaceans have almost no internal reserves of calcium (gastroliths) and depend exclusively on the environment to supply the demand for Ca^{2+} (Greenaway 1985, Passano 1960, Wheatly 1997).

In a similar manner, acidification might affect the deposition of magnesium in the crustacean exoskeleton. Although magnesium concentrations in water are relatively lower than those of calcium, magnesium also plays an important role in the hardening of the exoskeleton, and it is also obtained through water (Cameron and Wood 1985, Clarke and Wheeler 1922, Welinder 1974)in a process that might be affected by pH (Tao, Zhou et al. 2009).

In addition to Ca^{2+} and Mg^{2+} concentrations, the concentrations of Na^+ and K^+ were monitored in this study. These two ions directly participate in important enzymatic activities that occur postmoult (Towle and Mangum 1985). Studies have shown that if the relative proportions of these two ions are altered, ammonia toxicity can occur due to the retention of ammonia by the organism and potentially compromise the anima's survival (Pan, Luan et al. 2006, Romano and Zeng 2011, Zanotto and Wheatly 1993). However, were observed no significant effects of these ions in our experiments. It is possible that" the factors described above were much more important in influencing exoskeleton hardening and the probability of survival in *C. ornatus*.

There was also a direct relationship between the time to exoskeleton hardening and the mortality rate. However, the mortality rate was only 25% among those crabs that moulted after approximately 60 h. Those that did not moult died or remained alive until the end of the experiment. In addition, in all of the groups except those receiving periodic water renewal, there was an increase in mortality in the postmoult phase. In this case, the analyses again indicated the influences of pH and total ammonia.

It is known that crabs (notably *C. sapidus*, the most studied species of the genus *Callinectes*) can tolerate a pH range of 6.5 to 8.5 (Hochheimer 1988). Nevertheless, in artificial environments, it is recommended that pH be maintained between 7.0 and 8.0 (Oesterling 1995). It is also known that there are behavioural and tolerance differences between young and adult animals in relation to pH (Laughlin, Cripe et al. 1978). In Experiment 2 of the present study, pH values of 5.5 and 5.3 were recorded in groups B1 and B2, respectively. In addition to having a direct effect on the organisms, a reduction of pH causes an increase in the nitrous acid fraction (HNO_2) present in water; HNO_2 is toxic to aquatic organisms (Ary and Poirrier 1989, Lin and Chen 2003, Russo, Thurston et al. 1981, Seneriches-Abiera, Parado-Estepa et al. 2007).

The toxicity of ammonia, in turn, is directly proportional to pH andNH3 concentrations. Romano and Zeng (2007) estimated an LC_{50} for juveniles of *Scylla serrata* of 6.81 mg/L NH_3-N. Koo, Kim et al. (2005) reported that at least 50% of juveniles of *Orithyiasinica* survived for 30 days at approximately 2.33 mg/L NH_3-N.Lakshmi (1984) reported a mortality rate of 20% in *C. sapidus* in premoult at 1.41 mg/L NH_3, which increased to 100% at 2.31 mg/L NH_3. In our experiments, a pH reduction was observed over time, which indicated that the NH_3 concentrations remained sufficiently low as to rule out any toxic effects of ammonia on *C. ornatus*.

Regarding nitrite, there is no consensus regarding the concentrations at which this compound is toxic to crabs. Lakshmi (1984) and Ary and Poirrier (1989) reported that the survival of *C. sapidus* was only affected at NO_2- concentrations above 10 mg/L. According tothose authors, crab mortality reached 100% only after 96 h of exposure to concentrations between 50 and 150 mg/L in water with a pH close to 8.In contrast, Manthe, Malone et al. (1984) found that the moulting efficiency of *C. sapidus* was affected by nitrite concentrations close to 2 mg/L. In the present study, the nitrite concentration reached 7.6 mg/L.Thus, it is possible that the observed mortality might have been influenced by both pH and nitrite levels during the experiments and that they had a cumulative effect. Moreover, a long hardening time, which exposed the animals to unfavourable physiological conditions, appears to have significantly increased the risk of death.

Moulting in *C. ornatus* exhibited strong relationships with the characteristics of the crab's aquatic medium. The crabs drastically altered the physical and chemical characteristics of the water, mainly through processes related to acidification and ammonification. These alterations, in turn, directly interfered with exoskeleton hardening, causing the exoskeletons of the animals to remain at soft or paper consistency for periods of up to 5 days. Commercially, the establishment of such periods would allow crabs to be marketed as soft-shell crabs within a time window more than 20 times longer than that typically observed. If the results observed here can be replicated at the commercial scale, large reductions in workload and operational costs could be obtained, increasing the efficiency and viability of large-scale crab production.

Acknowledgements

This research was supported by a Research Productivity Fellowship from the CNPq (process 302609/2013-0) granted to Dr Antonio Ostrensky and by CNPq financing of the projects associated with this manuscript (processes 381091/2014-7, 473959-2013 and 403705/2013-4) and the project (processes 468251/2014-6) coordinated by Dr Leandro Ângelo Pereira, granting a DTI scholarship to Diogo Barbalho Hungria (Process N.381091/2014-7), granting a doctoral's degree to Camila Tavares (process N.141022/2017-5) and Dr Ariana Cella-Ribeiro receives a scholarship from CAPES, Pró-Amazônia Program (process 1644571).

Conflict of Interest

We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

We recognize that all authors contributed significantly and agree with the content of the manuscript and all individuals listed as authors qualify as authors and have approved the submitted version.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 21 Issue 1 Version 1.0 Year 2021
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Operating Conditioning in Bennett Ualabies (*Macropus Rufogriseus)* Ex *Situ* for Drugs Administration by Oral Route

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Abstract- The objective of this study was the oral administration of trimetoprim sulfamethoxazole as a prophylactic therapy in Bennett's Wallabies (Macropus Rufogriseus) through the use of operant conditioning. In the study participated 15 animals of different gender and age, all of them living at "La Aurora" National Zoo in Guatemala City, Guatemala. The investigation was divided in 2 stages, first the desensitization and conditioning of the animals and second the oral administration of the drug. Results indicated that 76% of the population had a positive response to the desensitization and conditioning, however only 20% consumed the 100% of the dose. In conclusion the operant conditioning is an excellent tool for desensitizing and creating a routine with zoo animals, but further investigation is required to determine the efficiency of the administration of an oral treatment on this species.

Keywords: conditioning, sensitization, stimulus.

GJMR-G Classification: NLMC Code: WA 360

Strictly as per the compliance and regulations of:

Operating Conditioning in Bennett Ualabies (*Macropus Rufogriseus)* Ex *Situ* for Drugs Administration by Oral Route

Condicionamiento Operante En Ualabies De Bennett (*Macropus Rufogriseus) Ex Situ* Para La Administraciónde Fármacos Por Vía Oral

Martínez-Espina, Verónica Isabel [α], Castañeda Díaz-Samayoa, Andrea [σ] & Villatoro-Chacón, Daniela Mariel [ρ]

Resumen- El objetivo de este estudio fue el uso de condicionamiento operante para la administración oral de trimetoprim sulfametoxazol como terapia profiláctica para toxoplasmosis en los Ualabies de Bennett (*Macropus Rufogriseus*). En el estudio participaron 15 ejemplares de diferente sexo y edad que conforman el grupo de animales del Parque Zoológico Nacional La Aurora en la ciudad de Guatemala, Guatemala. La investigación se realizó en 2 etapas, siendo la primera, la desensibilización y condicionamiento de los ejemplares y la segunda la administración oral del medicamento. Los resultados mostraron que el 76% de la población obtuvo un resultado positivo a la desensibilización y condicionamiento, pero sólo el 20% de la población consumió el 100% de la dosis calculada en el estudio. Por tanto, se concluye que el condicionamiento operante es una excelente herramienta para la desensibilización y habituación de los animales a una rutina establecida, pero se requieren estudios más extensos para determinar su eficacia en cuanto a la administración oral de un tratamiento profiláctico en esta especie.

Palabras clave: condicionamiento, sensibilización estímulo.

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I. Introducción

El Parque Zoológico Nacional La Aurora, se encuentra en la ciudad de Guatemala y cuenta con una población de alrededor de 3,000 ejemplares, siendo el único zoológico del país que cuenta con Ualabies de Bennett (*Macropus rufrogriseus)*. Debido a su característica de presa en su hábitat natural, el manejo médico de estos animales es un proceso estresante tanto para el ejemplar como para el personal de los zoológicos. Esto ha obligado al personal encargado de fauna silvestre a implementar métodos que reduzcan el estrés en los animales como lo es el condicionamiento operante (Yin, 2006).

El condicionamiento operante, es una técnica que se ha utilizado en diversas especies como linces (Crowell, 2008), venados (Crowell, 2008), tortugas aldabras (Weiss, 2003), primates del nuevo mundo (Savastano, 2003), iguana cabeza roja(Hellmuth, 2012), cocodrilo del Nilo(Hellmuth, 2012), ñacanina (Hellmuth, 2012), rinocerontes blancos y negros (Holden, 2006), babuinos (Martina, 2020) y pitón de Birmania (Emer, 2015). En Latinoamérica se han realizado estudios en tigres (Gomez, 2015)(Damian, 2016), chimpancés (Hincapié, 2019), león africano (Damian, 2016), león blanco (Damian, 2016), rinoceronte blanco (Molina, 2018)(Hoyos, 2017) jirafas (Uribe, 2019) y jaguar (Jácome, 2012)(Guzmán, 2018).En Centroamérica se ha reportado en zarigüeyas americanas (James, 1937)(Ravizza, 1969) y (Cheney, 1980), sin embargo, (Panini, 1986)menciona que estos estudios realizados en zarigüeyascarecen de bases sólidas para indicar la efectividad del condicionamiento operante en estos animales, ya que las respuestas presentadas por los ejemplares de los estudios pueden ser un acto de sensibilización a los estímulos aberrantes realizados en estas investigaciones.

El presente estudio genera información respecto al condicionamiento operante en *Macropus rufrogriseus* como una herramienta que permite el

manejo médico de la especie para su conservación en cautiverio.

II. Métodos

a) Área de estudio

El Zoológico La Aurora está ubicada en el Boulevard Juan Pablo II, 5ta calle interior Finca "La Aurora", Ciudad de Guatemala, zona 13. Se encuentra frente al aeropuerto nacional La Aurora, entre el boulevard Liberación y el mercado de artesanías. Sus coordenadas geográficas son 14°37´16" latitud norte y 90°31´37" longitud oeste. La ciudad de Guatemala se encuentra a 1,592 metros sobre el nivel del mar. La temperatura oscila entre 12 y 18 grados centígrados. La humedad relativa es de 64% a 84%.(Ordóñez, 2012).

b) Población del estudio

El grupo de Ualabies cuenta con 15 ejemplares, de los cuales se cuenta con 4 machos enteros (de 14 a 17 kg.), 9 hembras enteras (de 10 a 12 kg.) y 2 crías (de 4kg).

c) Hábitat

Los recursos utilizados fueron el recinto de los ualabies de Bennett y canguros rojosque contiene el recinto de exhibición, recinto de cacatúa, patio trasero, dormitorio para canguros y dormitorio para ualabies compuesto de dos áreas (figura 1). Los animales son alimentados con concentrado comercial Leaf eater®, lechuga, zanahoria rallada, manzana, pellets de alfalfa y agua ad libitum.

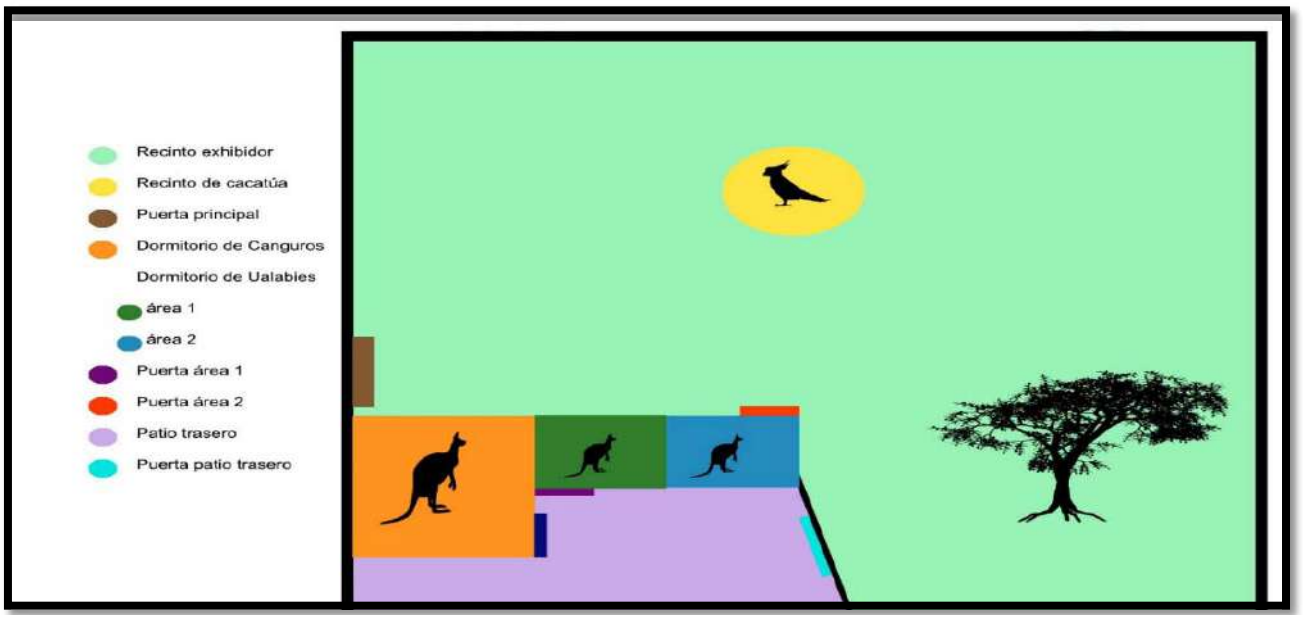


Figura 1: Mapa del recito de los ualabies de Bennett

d) Periodo de desensibilización

El período de desensibilización se realizó en 4 mesesabarcando las etapas de entrenamiento y condicionamiento, y consistió en 2 entrenamientos diarios de 1 hora cada uno aproximadamente, 5 días a la semana (lunes a viernes).

Como parte de la desensibilización se inició con el ofrecimiento del concentrado para su consumo desde la mano del cuidador o entrenador. Se ofreció una pieza de concentrado en cada intento, ya que estos animales consumen su alimento a una velocidad moderada, por lo que el ofrecimiento de varias piezas no presentaba ningún beneficio en la mayoría de ejemplares, siendo la excepción, los machos alfa, quienes consumían el concentrado con mayor rapidez. Luego de que esta acción fuera aceptada se continuó con el contacto físico con el animal, este se inició de forma gradual empezando por tocarle la cabeza, luego la espalda y por último la cola. Este proceso fue realizado de forma gradual y con paciencia ya que no todos los ejemplares presentan el mismo grado de confianza debido a diversos factores, incluyendo tratamientos previos, edad, sexo, conducta propia de la especie y personalidad individual de cada espécimen. El tipo de manejo que fue utilizado fue el contacto libre, es decir sin ningún tipo de barrera de restricción, ya que, explica Torres (2004 en Martínez, 2016) es el método comúnmente utilizado con especies que no representan un gran peligro para el entrenador.

e) Entrenamiento y condicionamiento

Para el entrenamiento y condicionamiento se requirió del recurso humano (un entrenador, 2 cuidadores habituales de los animales y 2 sustitutos los días de descanso de los cuidadores principales).

Se realizaron dos entrenamientos:

- El primer entrenamiento se realizó a las 7 horas con los animales dentro de su dormitorio. El entrenador y uno de los cuidadores procedían a colocarse en la salida del dormitorio hacia el patio trasero, cuya

puerta se encontraba abierta. Haciendo uso del alimento comercial, se inició abriendo la puerta corrediza del dormitorio al patio trasero, permitiendo el paso de un animal a la vez. Al momento de salir, al animal se le ofrecía inmediatamente una pieza el concentrado con la finalidad de realizar el refuerzo positivo de la acción de salir y la desensibilización de los individuos a la interacción con el entrenador.

- El segundo entrenamiento se realizó a las 9 horas con los animales en su recinto de exhibición. Con las puertas del dormitorio y el patio trasero cerradas, se procedía a colocar dos comederos dentro del área 1del dormitorio, luego se adicionó una mano del concentrado incluido en la dieta del grupo a cada comedero.

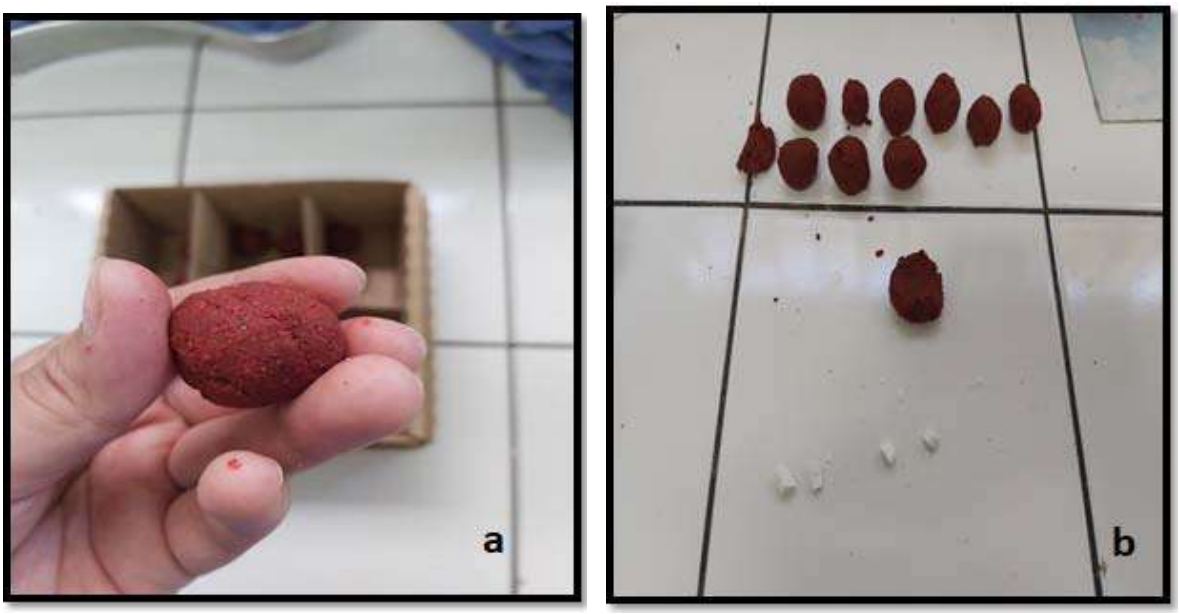
El cuidador se posiciona en la salida del dormitorio al patio trasero, cerrando la puerta corrediza del dormitorio nuevamente, mientras que el entrenador haciendo uso de el silbato y la caja de dieta, llama a los ualabies desde la puerta principal del área 2del dormitorio. Luego de realizar el llamado el entrenador debe retroceder de la puerta para permitir el ingreso de los animales durante 3 minutos. Al terminar el tiempo, el entrenador debe salir por la puerta principal del área 2 del dormitorio y cerrar la puerta. De esta forma los ejemplares quedan de nuevo encerrados en el dormitorio.

Cuando los ualabies se terminan la dieta de los comederos, se repite el entrenamiento de las 7 horas con ayuda del cuidador, permitiéndoles la salida del dormitorio hacia el patio trasero y luego al recinto de exhibición donde se administra la mitad de la dieta asignada para los ejemplares.

f) Administración del tratamiento

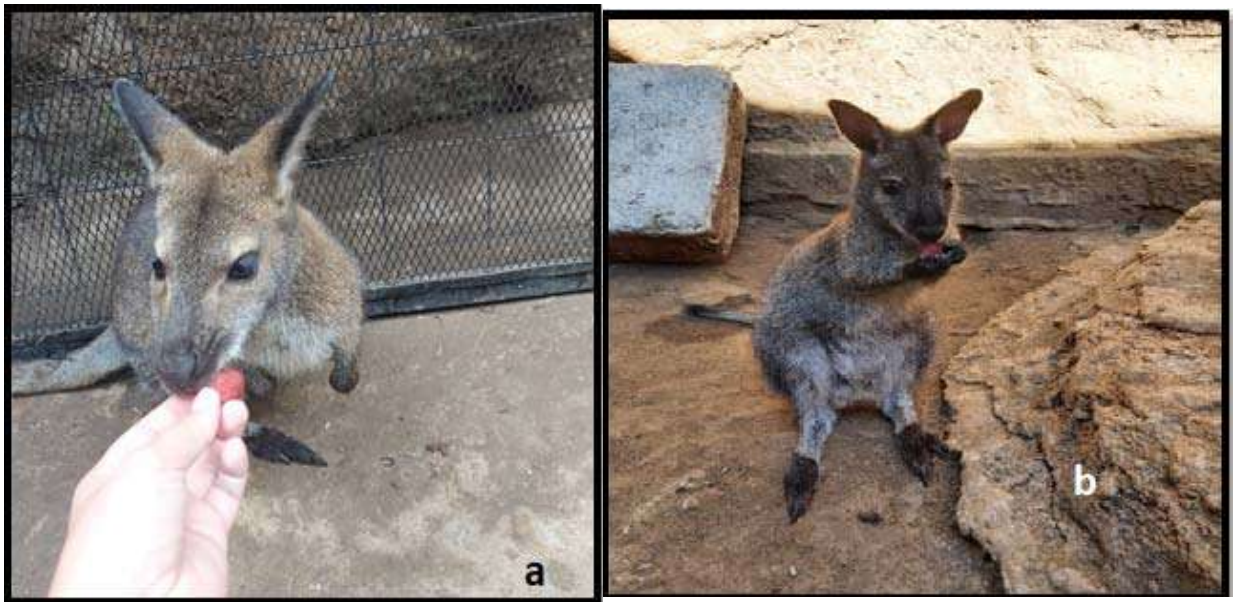
Como parte del cuidado profiláctico de estos animales se tiene establecido la administración de trimetoprim/sulfa para toxoplasmosis. Se utilizaron tabletas de 960 mg de trimetoprima sulfametoxazol. Se utilizó una dosis de 20 mg/kg cada 24 horas por 10 días. Así pues, para los animales de 17kg la dosis fue de 0.4 mg, 14kg a 0.3 mg, 12 kg a 0.3 mg, 10kg a 0.2 mg y 4kg a 0.1mg

El medicamento fue colocado en pequeñas esferas del tamaño del concentrado y fueron pulverizadas y mezcladas con agua para colocarlas en los espacios seleccionados por el peso del animal (figura 2). La administración de las esferas con medicamento se realizó a las 7am, ya que, gracias al ayuno por la noche, eran más propensos a aceptar la medicación fácilmente (figura 3). El cuidador iniciaba sacando a los animales como de costumbre, por la puerta corrediza del área del dormitorio hacia el patio trasero y el entrenador ofrecía el alimento al animal 3 veces.



a. Leaf eater pulverizado mezclado con agua
b. Esferas de concentrado Leaf eater y trozos de pastilla de trimetoprima-sulfametoxazol

Figura 2: Esfera de concentrado Leaf eater pulverizado mezclado con agua



a. Ualabí hembra recibiendo el preparado de concentrado y medicamento
b. Ualabí cría recibiendo concentrado Leaf eater

Figura 3: Ualabí recibiendo concentrado Leaf eater

g) Periodo de evaluación

El periodo de evaluación de la investigación consistió en dos períodos: el período de desensibilización que tuvo una duración de 4 meses y el período de tratamiento que se realizó en 10 días consecutivos. En este último se administró el medicamento oral a los ejemplares. Durante el período de tratamiento se tomaron como válida la administración del medicamento en los primeros 3 ofrecimientos de la mezcla del concentrado medicado. Si el ejemplar no había consumido la dosis, esta se tomaba como un resultado negativo, aunque se le administrara el medicamento después. Se consideró un resultado positivo a la desensibilización a la población que acepto el contacto físico y a la presencia de los cuidadores y entrenador dentro del recinto; siendo negativo en los individuos que no lo aceptaran. Se consideró un positivo en la aceptación del medicamento a los individuos que aceptaran el 100% de la dosis; siendo un resultado negativo los individuos que no consumieran la totalidad de la dosis.

h) Análisis de datos

Los resultados obtenidos fueron resumidos utilizando estadística descriptiva utilizando cuadros para su mejor análisis.

III. Resultados

La desensibilización haciendo uso del condicionamiento operante el 76% de la población tuvo la aceptación de las acciones realizadas, considerándose un resultado positivo. En la tabla 1 se describen las distintas acciones y su aceptación por la población de estudio.

Tabla 1: Periodo de desensibilización de *Macropus rufrogriseus*

Acción	%	n
Aceptar el alimento	100	15
Aceptar alimento sin retirarse	80	12
Contacto físico en la cabeza	80	12
Contacto físico en la espalda	66.7	10
Contacto físico en la cola	53.3	8

Respecto a la aceptación del tratamiento, sólo el 20% de la población total mostró un resultado positivo al ingerir la totalidad de la dosis administrada. En la tabla 2, se describe el porcentaje de aceptación de cada dosis en cada individuo.

Tabla 2: Aceptación de la dosis administrada en *Macropus rufrogriseus*

Aceptación del tratamiento	%	N
Aceptación 100% dosis	20	3
Aceptación 90% dosis	20	3
Aceptación 80% dosis	13.3	2
Aceptación 70% dosis	0	0
Aceptación 60% dosis	6.68	1
Aceptación 50% dosis	13.3	2
Aceptación 40% dosis	6.68	1
Aceptación 30% dosis	6.68	1
Aceptación 20% dosis	6.68	1
Aceptación 10% dosis	0	0
Aceptación 0% dosis	6.68	1

IV. Discusión

En el proceso de desensibilización de los animales*ex situ* indican que el condicionamiento operante es una herramienta útil para crear un nivel de confianza entre el entrenador/cuidador y los ejemplares de una colección (Mattinson, 2012). Sin embargo, es necesario dedicar el tiempo y ser pacientes. El estudio presentado aplicó el condicionamiento operante durante 4 meses, pero los resultados comenzaron a observarse a la 2 semana del experimento, por lo que es factible indicar que, con más tiempo disponible para realizar esta práctica, podrían obtenerse mejores resultados.

El estrés, según (Chapman, 2003), aumenta en un gran porcentaje la susceptibilidad de los marsupiales a las enfermedades y, la necesidad de restringir y manipular a estos animales, puede provocarle al ejemplar lesiones graves e incluso la muerte(Poole, 1971), sin mencionar el peligro que involucra para los cuidadores y médicos veterinarios presentes. Martínez citado en (Damian, 2016) indica que el uso de condicionamiento operante con refuerzo positivo es una técnica con la cual se evita el uso de métodos restrictivos físicos y químicos que alteran el estado de bienestar de los animales, pudiendo ser útil en la detección de enfermedades de forma precoz, para su tratamiento. Por esta razón, la utilización del condicionamiento operante, puede ser una gran herramienta para reducir riesgos en el manejo de cualquier animal en cautiverio.

Respecto al nivel de confianza de los individuos, tres de estos que consumieron el tratamiento, uno es el macho alfa, otro es un macho juvenil y la última es una hembra pequeña de 10 kg. Es importante mencionar que antes del régimen de condicionamiento operante el macho alfa ya se encontraba en un entrenamiento básico con target y de contacto libre. Además, este individuo, según es reportado por los cuidadores, siempre ha demostrado una sensibilidad baja a estímulos humanos, posiblemente por su naturaleza de alfa en el grupo de ejemplares.

En cuanto al macho juvenil y la hembra, los cuidadores reportan que siempre han sido curiosos ante la interacción humana, por lo que, al momento de llegar a la fase adulta, se encontraban bastante desensibilizados. Sin embargo, esto fue un comportamiento natural muy específico en estos animales, ya que, los otros ejemplares del grupo mantenían distancia con los cuidadores y presentaban tendencias de escape ante los movimientos repentinos. Además, como efecto colateral del condicionamiento aplicado a los ejemplares, los cuidadores reportaron una disminución muy marcada del tiempo de ingreso de los Ualabies a su dormitorio. Esta actividad, que anteriormente se realizaba en 1 hora y con la constante persecución de los individuos, fue disminuida a 15 minutos sin la necesidad de perseguir a ninguno.

Una de las limitaciones más grandes del estudio fue la falta de información previa para realizar el plan de condicionamiento, por lo que, este fue planteado en las bases de un condicionamiento más general. También se presentaron factores externos como cambio de cuidadores en ciertos días, ejemplares en celo, ruidos afuera del recinto como animales que se encuentran libres en el parque o el personal de limpieza; estas fueron situaciones que escapaban al control del estudio, pero su importancia en los resultados fue notoria durante el condicionamiento.

Agradecimientos

Al personal del Parque Zoológico Nacional La Aurora, por el apoyo y participación brindad as en elaboración del estudio.

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 21 Issue 1 Version 1.0 Year 2021
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Study on Prevalence of Bovine Cysticercosis and Human Taeniasis in Jigjiga Town, Somali Region, Eastern Ethiopia

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Abstract- A cross-sectional study was conducted from November 2019 to April 2020 on bovine slaughtered in Jigjiga municipal abattoir to determine the prevalence, cyst viability, organ distribution, and public health significance of bovine cysticercosis. Among 340 examined cattle, 11(3.24%) were found to be infected by *Cysticercus bovis*. The comparative higher prevalence was observed in female animals (3.4%) than male (2.9%) but a significant difference was not observed. Age-wise prevalence result showed that significantly ($p<0.05$) higher prevalence was detected in old (3.7%) than adult (2.46%). The body condition of animals had a significant ($p<0.05$) effect on the occurrence of *Cysticercus bovis* infection with higher infection rates observed in animal's poor body condition (3.7%)than animals with medium and good body condition scores. A total of 21 (6.18%) cysts were collected of which 10(2.94%) were viable while others 11(3.23%) have degenerated. Regarding the anatomical distribution of cysticerci were highest in the shoulder muscles 6(1.76%) followed by masseter muscles 5(1.47%), tongue 5(1.47%), heart 4(1.17%), and liver 1(0.29%).

Keywords: *bovine, cysticercosis, cyst viability, jigjiga, organ, prevalence, public health.*

GJMR-G Classification: *NLMC Code: WA 360*

Strictly as per the compliance and regulations of:

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Keywords: bovine, cysticercosis, cyst viability, jigjiga, organ, prevalence, public health.

I. Introduction

The total livestock population in Ethiopia according to 2014 estimation was 56.71 million cattle, 29.33 million sheep, and 29.11 million goats, which places Ethiopia first in Africa and ninth in the world in terms of total stock populations (CSA, 2015). Despite the reported high livestock population of the country, livestock diseases negatively affect public health and impede economic growth by incurring direct (morbidity and mortality) and indirect economic losses (EARO, 2006).

Most parasitic zoonoses are neglected diseases despite causing a considerable global burden of ill health in humans and having a substantial financial burden on livestock industries. The major contributors to the global burden of parasitic zoonoses are toxoplasmosis, foodborne trematode infections, cysticercosis, echinococcosis, leishmaniasis, and zoonotic schistosomiasis (Torgerson and Macpherson, 2011).

Cysticercus bovis is a food-borne parasitic disease caused by the immature form of the human *cestode Taenia saginata* commonly referred to as the beef tapeworm (Karshima *et al.,* 2013). Bovine cysticercosis refers to the infection of cattle with metacestodes of the human tapeworm (Ambachew and Yitagel, 2015). This parasite is universally distributed in developing as well as in developed countries (Gracey and Collins, 2011; Cabaret *et al.,* 2002; Dorny *et al.,* 2009).

Transmission of the parasite occurs most commonly in the environment characterized by poor sanitation, primitive livestock husbandry practice, and inadequate meat inspection, management, and control police (Mann, 2014). Cattle become infected through accidental ingestion of food or water which is contaminated with human feces containing viable T. *saginata* eggs. These eggs can remain viable for several weeks or months in sewage, water, or pasture. After 8-10 weeks the eggs have developed into larvae which establish in bovine skeletal and cardiac muscle and less commonly in fat and visceral organs. They develop into cysticerci (viable cysts), remaining infective for approximately nine months before they eventually die and calcify, and becoming non-infective (non-viable cysts) (Hiepe *et al*., 2005).

Humans become infected after ingestion of raw or undercooked beef containing infective cysticerci (Dorny *et al*., 2010). The disease in humans is called taeniasis which is accompanied by symptoms like nausea, abdominal discomfort, epigastric pain, diarrhea, excessive appetite or loss of appetite, weakness, loss of weight, and intestinal blockage. Sometimes, the mobile gravid segments may make their way to unusual sites

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such as the appendix and biliarytract and may cause serious disorders (WHO, 2013).In cattle, natural infections are normally asymptomatic but they cause financial losses to the cattle industry due to downgrading, condemnation, extra handling, and refrigeration of the infected carcasses.

The main intervention to control bovine cysticercosis is education on hygiene, meat inspection, followed by condemnation or freezing treatment when necessary as prescribed by European legislation, (Laranjo-González, 2000).

The larval stage (Metacestode) of this tapeworm has both public health and economic significance (Taresa *et al.,* 2011) as per an estimate, 50 million cases of such infestations occur worldwide with 50,000 people dying from this problem annually (WHO, 2015). The economic losses resulting from condemnation and downgrading of carcasses and due to treatment of carcasses to make them fit for human consumption (Deressa *et al.,* 2012).

The geographic distribution status of Taeniasis is more serious and less recognized for public health problems in developing countries (Minozzo *et al.,* 2002). Whereas, the distribution of Bovine cysticercosis is international and is very common in Africa. It is highly endemic in areas of Central and East African countries like Ethiopia, Kenya, and Zaire (Acha *et al.,* 2003). According to WHO classification, South American countries are included among the moderate prevalence of *Taenia saginata*. According to Over et al. (2013), *T. saginata* metacestode infections in cattle have been reported with higher prevalence from Senegal (20%), Nigeria (0.2-9%), Cameroon (7.2%), Tanzania (0-27%), and Kenya (38-62%).On the other hand, prevalence is very low in developed countries, such as 0.48-1.08% in Germany (Abuseir *et al*., 2006).

Bovine cysticercosis is widely distributed in Ethiopia and several individuals reported the prevalence of bovine cysticercosis in different parts of the country. According to these reports, a prevalence of 6.4% in Kombolcha Elfora by Alemneh (2015), 9.7% in Gondar by (Dawit, 2012), 21% in Nekemte by Ahmed (2015), 13.85% in Debre Zeit by Getachew (2013), 19.5% in Bahir Dar by (Mulugeta, 2012), and 3.2% in different agro-climatic zones by (Tembo, 2012) was recorded.

The epidemiology of human taeniasis varies from one area to another so control measures appropriate in one area are not necessarily of value in another. Hence, it is essential to have adequate knowledge of the epidemiology of the disease before contemplating control programs, (Teklemariam and Debash, 2015). In Ethiopia, some studies have been conducted on bovine cysticercus at different times. But the studies performed were limited to few parts of the country and there is a scarcity of information on the prevalence of bovine cysticercosis and human taeniasis in and around Jigjiga City. Therefore, the objectives of this study are:

- To estimate the prevalence of bovine cysticercosis and associated risk factors as well as studying the localization/organ distribution and viability/ degeneration of *c. bovis* at jigjiga municipal abattoir.
- To assess the prevalence of human taeniasis (*T. saginata*) and factors associated with its occurrence.

II. Materials and Methods

a) Study area

The study was conducted in Jigjiga municipal abattoir from November 2019 to April 2020. Jigjiga is the capital city of the Somali Regional State (SRS). Jijiga town is found within the Fafan zone and is located 675 km from Addis Ababa. It is astronomically located at 9°30' N latitude and 42°50' E longitude.20Hence, the average annual temperature of the town is 20°C indicating the existence of sub-tropical temperature condition whereas the mean monthly temperature varies from 17.34°C to 21.43°C in December and April, respectively. The hottest month is May while December is the coldest month with an average annual temperature of 20.02°C. The mean annual rainfall of Jijiga is just about 598 mm. The mean monthly amount of rainfall varies between 10.2mm to 102.2 mm in February and April, respectively (Jigjiga meteorological station, 2014).

b) Study Population

The study populations were cattle that are brought to Jigjiga municipal abattoir for slaughter purposes irrespective of their age, sex, body weight, and origin. Accordingly, those animals were subjected as a study population for an active abattoir survey. For the questionnaire survey, respondents were selected based on a systemic random sampling of individuals from Jigjiga city. Accordingly, a certain number of volunteer individuals were interviewed.

c) Study Design

A cross-sectional study was designed to perform from November 2019 to April 2020 to determine the prevalence of bovine cysticercosis and human taeniasis in Jigjiga city.

d) Sample Size Determination

The sample size (n) was determined according to Thursfield (2007) by using the following formula

$$n = \frac{(1.96)^2 \text{Pexp}\,(1 - \text{Pexp})}{\text{d}^2}$$

Where:
n = required sample size
P_{exp} = expected prevalence
d = desired absolute precision = ±5%
$(1.96)^2$ = confidence interval of 95%

There was a previous study with an expected prevalence of 2.25% (Biruk, 2009) in the study area, after the substitution, 34 carcasses were calculated to be sampled. But to increase the precision of the study the sample size was increased to 340 carcasses.

For an outcome scored 0/1 for no/yes, the standard deviation of the outcome scores is given by SD = [p (1-p)/N] $^{1/2}$ where p is the proportion obtaining a score of 1, and N is the sample size. The standard error of estimate SE (the standard deviation of the range of possible p values based on the pilot sample estimate) is given by SE= SD/N^{½}. Thus, SE is at a maximum when p = 0.5. Thus the worst-case scenario occurs when 50% agree, 50% disagree. Therefore, the questionnaire survey sample size was calculated by using the formula:

$$N = 0.25/SE^2 \text{ (Arsham, 2015).}$$

Where: N= sample size, SE (standard error) =5%

The sample size required for the questionnaire survey as per the above formula is 100 individuals.

e) Study Methodology

For the questionnaire survey, 100 volunteers in Jigjiga city were selected randomly based on different ages, sex, and working condition. During the active abattoir survey, individual animals were selected using systematic random sampling. Before ante-mortem inspection was done, each animal ID was assigned for further follow-up during the post-mortem examination and they were recorded according to their age, sex, and body condition.

i. *Ante-Mortem Examination*

The ante-mortem examination was conducted on individual animals' levels, while the animals were in the lairage. Both sides of the animals were inspected at rest and in motion. Moreover, the general behavior of the animals, cleanness, and sign of diseases, and abnormality of any type were recorded according to the standard ante-mortem inspection procedures (FAO, 2006). Additionally, an ID number was given to each animal to identify for the study. Then, data on the origin, age, sex, and body condition score of the animals were recorded. The body condition of cattle was classified as poor (hidebound with obvious bony prominences and deep sunk tail base), medium (ribs and other bony prominences noticeable on visual inspection but have the fair fleshy background on palpation), or good (bony structures notable only on palpation). Animal age was also based on dentition (Alemu *et al.,* 2013).

ii. *Post-Mortem Examination*

During post mortem inspection, palpation of the organs followed by incision of organs was made to examine for the presence of *C. bovis*, according to the guideline by the Ministry of Agriculture (1972), for masseter muscles, the deep linear incision was made according to the guideline by Ministry of Agriculture by making parallel to the mandible; the heart was incised from base to apex to open the pericardium and incision of the cardiac muscle for detail examination. Deep, adjacent, and parallel incisions were made above the pointed elbow in the shoulder muscle. Examination of the kidney, liver, and lung was also being conducted accordingly.

iii. *Cyst evaluation and viability test*

The cyst which was found at meat inspection was removed with the surrounding tissue and taken to the laboratory for viability test. The viability of the cyst was examined by placing them in a normal saline solution with 40% ox-bile and incubated at 37°C for 1 to 2 h. A cyst was regarded as viable if the scolex evaginated during this period (Gracey *et al.,* 2011).Cysts were carefully dissected and numbers and the nature of cysts in each organ were recorded for each animal. The nature of the cyst was recorded as calcified and viable by visual observation of its appearance, as (Ashwani and Gebrehiwot, 2011) dead degenerated or calcified cysticerci from identifiable spots of white and have fibrotic lesions, while the viable cysticerci are pinkish-red in color.

f) Data Management and Analysis

The data collected from ante-mortem, post-mortem, and laboratory findings were entered into an MS Excel spreadsheet and analyzed by using SPSS release 14.0 software (Stata Corp., College Station, Texas). Descriptive statistics were carried out to summarize the prevalence and relative percentage of the disease in each organ. Logistic regression was used to determine the level of significance among different risk factors contributing to the prevalence of bovine cysticercosis and human taeniasis. A level of significance of P $\leq$0.05 was used.

III. Results

In the study period, a total of 340 cattle were inspected in Jigjiga city municipal abattoir from November 2019 to April 2020. From a total of examined cattle, 11(3.24%) were found to be infected by *Cysticercus bovis* as shown in Table 1. comparative higher prevalence was observed 8(3.4%) in females than 3(2.9%) in male. (p = 0.825, (95% CI=0. 142-3.161).Regarding age group prevalence result showed that significantly higher prevalence (p<0.05) was detected in old animals 9(3.7%) than in adult2 (2.4%) (95% CI=0. 303-4.481). The present study also revealed that the body condition of cattle's had a significant effect on the occurrence of Cysticercus infection (p<0.05) with the highest prevalence of 9(3.7%) in Poor followed by medium and good body condition score with a prevalence of 1(1.6%) and 1(1.1%). Concerning origin, the prevalence result showed that the comparative higher prevalence was observed in animals brought from outside the Fafan zone with7(2.7%) than in around

Jigjiga 3(10.0%) and Fafan zone 1(1.9%) as shown in Table 1.

Table 1: Prevalence of Cysticercus bovis and associated risk factors

Risk factors	Categories	No. examined animal	Prevalence (No. positive)	Odds ratio (95% CI)	P-value
Sex	Male	103	3(2.9%)		
	Female	237	8(3.4%)	0.67 (CI=0.142-3.161)	0.825
	Total	**340**	**11**		
Age	Adult	84	2(2.4%)		
	Old	256	9(3.7%)	1.164 (CI=0. 303-4.481)	0.016
	Total	**340**	**11**		
Body condition	Good	62	1(1.6%)		
	Medium	90	1(1.1%)	0.685 (95% CI=.0.042-11.169)	0.003
	Poor	188	9(3.7%)	3.067 (95% CI=.0.381-24.706	
	Total	**340**	**11**		
Origin	Jigjiga	30	3(10.0%)		
	Fafan	100	1(1.9%)	3.968 (CI=0.969.-16.247)	0.086
	Outside Fafan of Zone	210	7(2.7%)	0. .687 (CI=.083-5.702)	
	Total	**340**	**11**		

Regarding the distribution of *C.bovis* in different organs assessed and the result revealed that shoulder muscle was the most frequently infected organ with a prevalence of 6(1.76%) followed by masseter muscles 5(1.47%), tongue 5(1.47%), heart 4(1.17%), (Table 2). Moreover, from the total of 21 cysts, 10(2.94%) were viable the remaining 11(3.23%) were calcified (Table 2).

Table 2: Frequency distribution of C.bovis in different organs examined

Inspected Organs	No. viable	No. calcified	Total No. cysts
Heart	1(0.29%)	3(0.88%)	4(1.17%)
Tongue	2(0.59%)	3(0.88%)	5(1.47%)
Shoulder muscle	4(1.17%)	2(0.59%)	6(1.76%)
Messer muscle	3(0.88%)	2(0.59%)	5(1.47%)
Liver	0	1(0.29%)	1(0.29%)
Total	10(2.94%)	11(3.23%)	21(6.18%)

The result of the Questionnaire Survey: Of the total 100 voluntary respondents interviewed, 13(13%) of them said they were infected with Taeniasis (*T. saginata*) at least once in their lifetime. There was no statistically significant association ($p>0.05$) was observed in the prevalence of Taeniasis between age, sex, Marital status, educational status, as shown in Table 3. Statistical analysis showed that human taeniasis prevalence is statistically significant $p<0.05$) among the categories of the considered risk factors like raw meat consumption, Religion. In addition, among interviewed respondents 17 had the habit of raw consumption, and 83 had the habit of cooked meat consumption and there is a significant association be out of this 9 (52.94%) and 4 (4.82%) of them were infected respectively as indicated in (Table 3).

Table 3: Prevalence of Human Taeniasis with Risk Factors

Variables	Categories	No of interviewees	No. infected	Prevalence%	P –value
Age	<25 years	27	4	14.81	0.743
	>25 years	73	9	12.33	
Sex	Male	67	10	14.92	0.415
	Female	33	3	9.1	
Religion	Muslim	54	1	1.85	0.000
	Christian	46	12	26.08	
Marital status	Married	60	11	18.33	0.903
	Unmarried	40	2	5	
Habit of raw meat consumption	Consumed	17	9	52.94	0.000
	Not consumed	83	4	4.82	
Educational	Literate	81	12	14.81	0.055
	Illiterate	19	1	5.26	

IV. Discussion

The prevalence of *Cysticercus bovis* in the current study was 3.24% at Jigjiga municipal abattoir. This finding is comparable with the findings of 3% (Bedu, 2011) in Zeway Municipal Abattoir, 3.6% (Nuraddis and Frew, 2012) in Addis Ababa abattoir, 3.65% (Taresa, *et al*., 2011) in Jimma municipal abattoir, 3.11% (Tembo, 2012) in different agro-climatic zones of Ethiopia. However, this prevalence was higher than several studies conducted in different parts of the country such as 1.2% (Bekele *et al.*2017) in Asella municipal abattoir, 2.5% (Dawit Tesfaye *et al.,* 2012) in Wolaita Sodo municipal abattoir, 2.58% (Birhanu, *et al*., 2013) in Bahir Dar Municipal Abattoir, (Abate Worku, 2014) in west Shewa zone, 2.6% (Yacob *et al.,* 2015) in Adama town, 0.2% (Blessing *et al*., 2011) in South Africa and 1.05% (Leonardo *et al*. 2012) in Brazil. Nevertheless lower than the finding of 4.9% (Dawit Saddo, 2004) in Gondar, 4.4% (Bekele *et al.,* 2010) in Jimma municipal abattoir, 4.8% (Karshima *et al*., 2013) in Nigeria, 5.1% (Fetene and Nibret, 2014) in Jimma municipal abattoir, 5.2% (Belay, 2014) in Municipal Abbatoir of Shire, 5.4% (Alula, 2010) in Konbolcha, 5.6% (Lielt Emiru *et al.,* 2015) in Bishoftu, 5.73% Hylegebriel (Tesfay and Alembrehan Assefa, 2014) in Adigrat, 6.4% (Tewodros Alemneh *et al*., 2010) in Kombolcha meat processing factory in the same study area, 12% (Abunna, 2013) in Yirgalem abattoir, 17.5% (Hailu, 2005) in East Shoa, 18.49% (Kebede, 2008) in North West Ethiopia, and 26.3% (Abunna *et al.,* 2008) in Hawassa municipal abattoir. A possible reason for the difference in the prevalence of cysticerci might be due to factors like difference in culture, in environmental conditions, livestock stocking intensity, and livestock movement and social activities in different regions that may contribute to these variations in prevalence (Kebede *et al*., 2009).Moreover, another possible reason for the variation in prevalence rate might be due to the variation of personal and environmental hygiene from area to area (Ngwu *et al.,* 2004).

The result in the prevalence of *c. bovis* between sexes revealed slightly higher in female (3.4%) than male (2.9 %) but significant variation was not observed; this argument is supported by (Wanzala, 2003). who reported that the prevalence of *c. bovis* was slightly higher in female than male cattle. This could be due to the similarity in the socio-economic status and animal husbandry practices of the community in all areas from where animals were bought for slaughter. (Gemmell *et al.,* 2001). In the present study, the age-wise analysis showed that there was a significant difference in prevalence among the age of animals and the highest infection rate was recorded in old the adult. This finding

is similar to the report of (Nuradis and Few, 2012) and (Wondimagegnei and Belete, 2015). This might be due to their longer exposure to infection and to lower immunity to combat infection and these results are concurrent with that of other studies in Ethiopia. (Azlaf and Dakkak, 2006).The present study also showed that there was a strong association between the body condition of cattle and *C. bovis* infection. Significantly poor conditioned cattle were more infected by *C. bovis* than a medium and good one. This is in line with the study of Meseret (Kassaw *et al.,* 2017), this might be due to moderate to severe infection, the parasite may cause retarded performance and growth, reduced quality of meat and milk as well as live weight loss. (Melaku *et al*. 2012).This indicates that body condition loss might be a consequence of infection (Battelli 1997).

During the study period, the most frequently affected organs with the highest prevalence of cysts of *C. bovis* were recorded in shoulder muscles (1.76%) followed by masseter muscles, tongue, heart, and liver. The variations in anatomical distribution depend on several factors, such as blood kinetics and animals" daily activities. Any geographical and environmental factors affecting blood kinetics in the animal affect the distribution of oncospheres as well and hence the predilection sites during meat inspection (Gracey *et al.,* 2011). The finding of the current study is in agreement with the reports of (Bekele *et al.,* 2017) (Opara *et al.,* 2012), (Alemayehu *et al,* 2009) and (Hailu, 2010) who indicated that examination of the shoulder muscles is the most effective means of detection of bovine cysticercosis, while the heart and liver are described as the most frequently infected organ by (Tembo, 2012). Thus, there is no particular "predilection site" which could be acceptable for all cattle. The viability test showed that shoulder muscles had the highest relative frequency proportion of viable cysts 4(1.17%) followed by masseter muscles, tongue, heart, and liver. This observation goes parallel with the findings of (Opara *et al.,* 2012) and (Bekele *et al.,* 2017) who recovered a higher proportion of cysts from shoulder muscles that had the highest proportion of viable cyst. The explanation for this may lie in the fact that muscle activity receives more blood than a muscle at rest, and that the distribution of the cysts is controlled by the volume and intensity of the arterial blood (Gracey *et al.,* 2011).

The prevalence of human taeniasis was recorded based on the questionnaire and indicated an overall of 13% which demonstrates the importance of taeniasis in the study area.

The result of this study was lower when compared to (Mesfin and Nuraddis, 2012) 44% in Hawassa town and (Dawit and Temesgen, 2013) 44.44% in Shire Indasilassie district, (Lielt *et al.,* 2015) 64% in Bishoftu, (Dawit, 2012) 62.5% in Wolaittasoddo, (Fetene and Nibret, 2014) 58%,(Abunna, 2013) 70% in Yirgalem, (Bedu *et al*., 2011)56.7% at Zeway, (Abunna *et al*., 2008) 64.2% in Awassa town and (Megersa *et al*., 2015) 56.7% in Jimma town. The reason for reporting the lower prevalence of human taeniasis in the current study area could be due to the difference in the religious composition of the respondents, and the sample size is taken. Out of the total respondents of the current study, 54% were Muslims that have no traditional habit of consuming raw meat and from the total respondents, only 17% were raw meat consumers. The reason is well known that in the consumption of raw meat the degree of ingesting *C. bovis* with meat is higher (Gajadhar *et al.,* 2012; Garcia *et al.,* 2011). Therefore, as raw meat consumption is low in the area the infection also is low. The other is sample size difference and as sample size increases the precision will also increase. In the present study, the sample size is very low (100) while in the above finding is very high, more than 220. The other point is that some respondents shy to openly tell about taeniasis and this could also end up with the low recovery of positive people in the study area.

Taeniasis prevalence was higher among the Christian community than Muslims in the study area.

Similar to the reports of (Tembo, 2012; Hailu, 2005; Deressa *et al.,* 2012) taeniasis prevalence was higher among the Christian community than Muslims. Because raw meat consumption is not common in Muslims as in Christians and Christians also celebrate several annual festivals with the tradition of raw meat consumption (Teka, 1997). T. saginata infection is highly prevalent in the literate than illiterate respondents and this might be because literate peoples have more chance to occupational status than illiterate which allows them to have the finance to eat raw beef in the butcher"s house than illiterate peoples.

This presentation revealed that males were highly affected than females. This observation is similar to the finding of (Abunna *et al.,* 2007) who reported a higher prevalence of taeniasis among males than females in Awassa town. The difference in the rate of infection between males and females in the study area could be due lead to human feces (Nigatu, 2004). To the fact that males enjoy eating raw beef with local drink ''Tella". The second reason might be males provide and control the finance hence; they can eat raw beef in the butcher house.

T. saginata was observed among old aged people (> 25 years) as compared to young age people (< 25 years). This agrees with (Alemayehu *et al.,* 2009) and (Dejene Bekele *et al.,* 2017). The observation that the older people greater chance of eating raw beef and hence contracting taeniasis. Therefore, the two age groups might be because older people have the finance to eat raw beef in the butcher's house *Taenia saginata* was observed among old aged people as compared to young age people. This agrees with (Tembo, 2001), (Alemayehu, *et al.,* 2009), and (Shimeles, 2004)

observation that older people were greater the chance of eating raw beef and hence contracting taeniasis. Depending on the marital status, married peoples were more infected than unmarried ones. This might be because married peoples have the finance to eat raw beef in the butcher‟s house than unmarried peoples.

The questionnaire survey result showed that the prevalence of taeniasis in the human population is decreasing and it also indicated that there was a strong relationship between the occurrence of *T.saginata* infection and age, sex, the habit of raw meat consumption, Marital status, and educational status of the respondents. Therefore, continues public education should be provided to avoid consumption of raw meat and encourage the use of latrines and improved standards of human hygiene and backyard slaughtering of cattle should be restricted and slaughterhouse which fulfills the necessary facilities and with qualified meat inspector should be constructed.

V. Conclusions

The abattoir survey evidence of the present investigation showed that *C.bovis* is prevalent in cattle slaughtered at Jigjiga municipal abattoir the prevalence of *C.bovis* was affected by different risk factors such as age and body condition score but not affected by the sex and origin of animals. The most frequently affected organ with the highest number of cysts was the shoulder muscles followed by masseter muscles, tongue, heart, and liver, regarding the questionnaire survey *T. seginata* was an important parasitic cattle disease and in terms of its public health implication in the study area. The viability test showed that shoulder muscles had the highest relative frequency proportion of viable cystsshoulder muscle followed by masseter muscles, tongue, heart, and liver. The questionnaire survey finding indicated that the infection rate of taeniasis was higher in the study area and deserves due attention on control and prevention of the disease. Finally, the finding of the present study reflects the zoonotic and economic impact of the disease which needs serious attention by the various stakeholders to safeguard public health.

Acknowledgments

The author would like to acknowledge with great appreciation to Jimma University, College of Agriculture and Veterinary Medicine, School of Veterinary Medicine for providing the financial aid to complete this work. Also highly thankful is given to Dr. Wubit Tafese for her valuable advice, suggestions, experience share during the writing of this paper. Moreover, special thanks are given to Jigjiga University and Somali Regional State Livestock and Rural Development Bureau for their cooperation to use their laboratory materials and equipment while doing sample collection and viability tests.

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 21 Issue 1 Version 1.0 Year 2021
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Protective Effect of Mesenchymal Stem Cells Derived Secretome in an *in Vitro* Pro-Inflammatory Model of Intervertebral Discogenic Pain

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Abstract- *Study design:* This work is an analytical and experimental study. Low back pain has become one of the principal leading cause of work disability in recent decades. Stem cells have emerged as a key element in regenerative medicine therapies, thus providing numerous potential cell therapies in the treating of a range of degenerative diseases and traumatic injuries. Stem cells can secrete potent combinations of trophic factors which modulate the environment of molecular composition to evoke responses from resident cells.

Objectives: The aim of this work is to make an attempt at stopping an irreversible inflammatory cascade at some point using adipose tissue mesenchymal stem cells derived-conditioned medium as therapeutic strategy.

Materials and methods: An *in vitro* model of inflammation of intervertebral disc has been developed in order to evaluate the effectiveness of secretome from the culture of adipose tissue mesenchymal stem cells to produce an immunomodulation of pro-inflammatory cytokines and neurotrophic factors.

Keywords: *discogenic pain, mesenchymal stem cells, secretome, immunomodulation.*

GJMR-G Classification: *NLMC Code: QW 70*

Strictly as per the compliance and regulations of:

Protective Effect of Mesenchymal Stem Cells Derived Secretome in an *in Vitro* Pro-Inflammatory Model of Intervertebral Discogenic Pain

Effect of Secretome in Discogenic Pain

M. Esteban-Blanco [α], ML. González-Fernández [σ] & V. Villar-Suárez [ρ]

Abstract- ***Study design:*** This work is an analytical and experimental study. Low back pain has become one of the principal leading cause of work disability in recent decades. Stem cells have emerged as a key element in regenerative medicine therapies, thus providing numerous potential cell therapies in the treating of a range of degenerative diseases and traumatic injuries. Stem cells can secrete potent combinations of trophic factors which modulate the environment of molecular composition to evoke responses from resident cells.

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Results: Genes analyzed in the *in vitro* model of discogenic pain showed how their expression developed when annulus fibrosus and nucleus pulposus cells had been stimulated with tumor necrosis factor. Also, the secretome was capable of modulating the response of various pro-inflammatory cytokines produced in the pathogenesis of discogenic pain.

Conclusions: Our data showed the immunomodulatory role of mesenchymal stem cells-derived secretome reducing inflammation and these effects could be a key element in inhibiting the activity of the molecular factors involved in the process of tissue degradation and possibly pain control in IVD degeneration pathology.

Keywords: discogenic pain, mesenchymal stem cells, secretome, immunomodulation.

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Abreviations

AF: Annulus Fibrosus
ASCs: Adipose Tissue derived-Mesenchymal Stem Cells
BDNF: Brain-derived Neurotrophic Factor
CM: Conditioned Medium
DMEM: Dulbecco's Modified Eagle's Medium
DRG: Dorsal Root Ganglion
FBS: Fetal Bovine Serum
IFNγ: Interferon Gamma
IVD: Intervertebral Disc
NGF: Nerve Growth Factor
NP: Nucleus pulposus
MSCs: Mesenchymal Stem Cells
PDN: Population Doubling Number
PDT: Population Doubling Time
qPCR: Real Time Quantitative PCR
SD: Standard Deviation

I. Introduction

Studies have suggested that between 60-90% of population is affected by low back pain, a debilitating disorder which decreases productivity and has caused considerable therapeutic expenses [1]. The intervertebral disc (IVD) is an avascular and aneural cartilaginous structure composed of the central gelatinous nucleus pulposus (NP), the surrounding elastic annulus fibrosus (AF), and the cartilaginous end-plates [2]. Degeneration of the intervertebral disc and herniation is associated with back pain [3]. Although their etio-pathogenesis is not fully understood, it is known that a complex interaction between molecular and biomechanical procedures of the spine which may leads to tissue degradation and thus, loss of function and pain in the IVD [4].

Degeneration is thought to be mediated by both the NP and the AF cells as well as macrophages, T cells and neutrophils which induce the abnormal secretion of pro-inflammatory cytokines [5]. These molecules trigger off a scope of pathogenic responses by NP and AF cells which can promote different responses as autophagy, senescence and apoptosis [6, 7]. These secreted pro-inflammatory mediators include Tumour Necrosis Factor

(TNF), Interleukins: IL-1 α/β, IL-6, IL-17, IL-8, IL-2, IL-4, IL-10 and Interferon gamma (IFN-γ). TNF is involved in disc herniation and nerve irritation [8, 9]. In addition to the infiltration of immune cells in the disc, there is also proliferation of nociceptive nerve fibres derived from the dorsal root ganglion (DRG) [2, 10]. At this point, cytokines as well as neurogenic factors particularly Brain-derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (NGF) are released by NP and the infiltrating immune cells [7].

So many treatments are currently being used to treat inflammatory diseases such as IVD disease. However, these treatments are costly, unspecific, and none of them lead to a remission of pain in the absence of medication. Over the last decade, mesenchymal stem cells (MSCs)-based therapy has emerged as a novel treatment and they have been proposed as the most interesting candidate cells when treating IVD degeneration and herniation [11, 12]. Multipotent mesenchymal stromal cells or mesenchymal stem cells are one of the promising cell types for the use in regenerative medicine, particularly in the treatment of musculoskeletal disorders [13].

Initially, therapeutic effects of transplanted MSCs were regarded as being mediated by the homing of MSCs to a damaged site and differentiating in the cells concerned. However, several studies have recently indicated that MSCs exert its therapeutic effects by secreting trophic factors [14]. The stem cell secretome, also referred to as conditioned medium (CM), is a collective term for the paracrine soluble factors produced by stem cells. These factors are thought to control angiogenesis, immune response, protection of the tissue and to accelerate wound healing. Hence, if therapeutic effects based on MSCs are replaced with trophic factor(s) derived from MSCs, there would be great advantages [15].

The aim of this work is to study the protective effect of conditioned medium from adipose tissue mesenchymal stem cells (ASCs) in an *in vitro* pro-inflammatory model of IVD degeneration. For this purpose we analyse if CM is capable of performing an immunoregulation on cytokines and the factors responsible of IVD inflammation.

II. Materials and Methods

a) Biological material

Adipose derived-mesenchymal stem cells ASCs, nucleus pulposus (NP) and annulus fibrosus (AF) human cells were commercially obtained from Innoprot®.

b) ASCs culture and conditioned media obtaining

ASCs were multiplied in monolayer T150 flasks (Corning®) at a density of $1x10^6$ cells/mL using low-glucose DMEMc at 37°C in a humid atmosphere containing 5 % CO_2. CM was obtained from cells every 48 h of culture at passages 0 and 1, centrifuged at 2,000 g and conserved until their use at −80 °C.

c) Characterization of ASCs

In order to characterize ASCs the expression of specific markers was determined using Flow Cytometry and Confocal Microscopy.

d) Flow Cytometry Analysis

With the aim of confirming the identity of ASCs, the expression of different surface markers was determined: anti-CD73, anti-CD90 and anti-CD105 (1:100) (Abcam®). Cells were stained with secondary streptavidin-Alexa 488 antibodies (1:100) (Invitrogen®). In order to conduct cytometry, 1×10^6 cells per sample were used. Three samples and a control (only secondary antibody) were used for each cell type. Cells were acquired in a FACS Cyan ADP (Dako®). About $1x10^4$ events (minimum) were used for fluorescence capture with Summit 4.3 (Cell Quest®) software. All the experiments were developed using three biological and three technical replicates.

e) Confocal Characterization of ASCs

On the 8-well Nunc Lab-Tek Chamber Slide System® ($2x10^3$ cells/well) ASCs were sub-cultured. Cells were fixed with 2% paraformaldehyde for 15 minutes. Then they were incubated with primary mouse anti-CD73, CD-90 and anti-CD105 antibodies (1:100) (Abcam®) overnight at 4°C. Samples were treated with secondary biotinylated anti-mouse antibodies (1:100) (Abcam®). They were then stained with streptavidin-Alexa 488 antibodies (1:100) (Invitrogen®). Finally, Vectashield mounting medium containing DAPI was used. All the experiments were developed using three biological and three technical replicates.

f) Confocal Characterization of AF and NP cells

The AF and NP cells were sub-cultured on 8-well Nunc Lab-Tek Chamber Slide System® (2×10^3 cells/well). Cells were fixed with 2% paraformaldehyde for 15 minutes prior to incubation with anti-decorin (Abcam®) and anti-cytokeratin-19 (Thermo Scientific®) primary antibodies. They were then stained with streptavidin-Alexa 488 and streptavidin-Alexa-568 antibodies (1:100) (Invitrogen®). Finally, chamber slides were mounted using Vectashield mounting medium containing DAPI. All the experiments were developed using three biological and three technical replicates.

g) NP and AF cells growth curve population doubling in numbers (PDN) and time (PDT)

NP and AF cells were seeded into a 24-well culture plate at a density of 6×10^3 cells/well. Growth kinetics were obtained after 7 days. For each passage the number of population doublings (PDN) and the time required by cells for each population doubling (PDT) were determined using the following formulae [16].

$$PDN = \log (N/N_0) x\ 3.31$$

PDT = CT/PDN

Where N is the cell number at the end of cultivation period, N0 is the cell number at culture initiation, and CT is the cell culture time.

All the experiments were developed using three biological and three technical replicates.

h) Conditioned medium (CM) collection

ASCs were maintained in DMEMc (DMEM, Hyclone®) supplemented with 10% (v/v) 22 fetal bovine serum (FBS, Hyclone®) and 1% (v/v) penicillin/ streptomycin (Hyclone®) to 23 approximately 80% confluency (~1 x 10^6 cells) with 2 passages. Supernatants were collected (conditioned medium) 24 h after the cells were supplemented with serum-free DMEM (Hyclone®) and with 1% penicillin/streptomycin (Hyclone®), so as to avoid possible contaminations with factors present in the FBS.

i) In vitro model of discogenic pain

NP, AF and ASCs were seeded in six well plates to perform an *in vitro* model of inflammation. For this purpose, 6x10^6 total cells were seeded in each well and when cell confluence was reached, 5 µL of TNF (10 µg/mL) or TNF and conditioned medium were added and incubated for 12 hours. After 12 hours, cells were collected and analyzed using qPCR. All the experiments were developed using three biological and three technical replicates.

j) qPCR

Total RNA of cultures was extracted using the GeneMATRIX universal RNA purification kit (EURx®) following the manufacturer´s instructions. cDNA was obtained using a high capacity cDNA reverse transcription kit (Applied Biosystems®) following the manufacturer´s indications. 600 ng of total RNA was used for synthesis of cDNA. Gene expression of IL-1α, IL-1β, IL-6, IL-8, NGF, BDNF, IFN-γ and TNF were determined using qPCR. GAPDH was used as an endogenous gene. All primer sequences for qPCR used are listed in Table 1. The qPCR reactions were conducted in a 25µl volume with 350 ng of DNA using Syber Green qPCR master mix (2x), plus ROX solution (EURx®) following the manufacturer´s instructions. Amplification was carried out using a StepOne real-time PCR system (Applied Biosystems®). Target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and was normalized to ACT-β.

k) Statistical analysis

The results in this study were expressed at the mean ± standard deviation (SD). Statistical analysis was performed using IBM® SPSS® Statistics 17. Significant differences among groups were determined using ANOVA followed by Tukey post-hoc analysis. Results with $p \leq 0.05$ were considered statistically significant.

III. Results

a) ASCs Characterization

Human mesenchymal stem cells were isolated from adipose tissue with collagenase type I digestion in Dulbecco's modified eagle medium. When ASCs reached a confluence of 80%, they were analyzed using flow cytometry and confocal microscopy in order to confirm the expression of specific stem cells markers CD73, CD 90 and CD105. Analysis was positive for the expression of all markers. The histograms of positive cell markers and confocal microscopy results are given in Figure. 1. All markers were positive in more than 95% and an intense fluorescence in confocal microscopy was also observed.

b) NP and AF cells morphology, characterization and Growth Kinetic

NP and AF showed adherent, spindle-shaped fibroblast-like cells (Fig. 2A). AF cells reached 80-90% confluency by days 4-6 after culture, though NP cells were confluent within 9 days after seeding. A confocal characterization of NP and AF cells was carried out. NP and AF cells were markedly positive to cytokeratin-19 and decorin respectively (Fig. 2A).

The growth curve for NP and AF cell populations was characterized by an initial lag phase of 48 hours in AF cells and 72 hours in NP cells followed by a log phase, whereas AF cells immediately grew logarithmically until day 6. NP cells reached this maximum at day 7-8 (Fig. 2B). In order to determine the population doubling time of cells was calculated at passages P1, P3 and P5 (Fig. 2B). Our results showed that the population doubling-time of AF cells increased every 1.90 days and 2.0 in NP cells. We confirmed that the proliferation potential was higher in AF cells than in NP cells. The nucleus was stained with DAPI (blue).

c) ASCs derived-CM was able to trigger an immunomodulatory response on NP and AF cells

So as to analyze the immunomodulatory effect of CM on AF and NP cells, inflammation was induced by adding TNF to the culture medium. The concentration of TNF and the incubation time for the optimal response of the *in vitro* inflammation model were determined by previous experiments in our laboratory. Different concentrations and action times were tested, establishing a TNF concentration of 10 µg/mL and hours of incubation as parameters with an optimal inflammatory 12 response.

AF, NP and ASCs cells were seeded both with and without TNF/CM and incubated for 12 hours as previously described. RNA extraction and qPCR were then developed to analyze the relative expression of different genes (TNF, IL1-α, IL1-β, IL-6, IL-8 and IFN-γ) related to inflammation and therefore discogenic pain. These genes were analyzed under the different

conditions to determine the TNF and CM action in this pathology.

The increase in TNF expression in AF and NP cells when they were stimulated with TNF compared to non-stimulated cells (controls) (*p ≤0.05) is given in Figure 4. A decrease was also observed in this expression when we cultured the TNF-stimulated cells with CM (^ ^p≤ 0.01) in both cell types. Surprisingly, an elevated expression of TNF in ASCs where inflammation has been induced was observed and was statistically significant regarding unstimulated ASCs (*p ≤ 0.05), although the values were lower than in AF and NP cells.

We observed a statistically significant high expression (***p ≤ 0 .005) in TNF-3 stimulated AF cells with regard to the control of the IL-1α gene as well as a decrease in this expression when the inflamed cells were cultured with MC, which were also statistically significant (^ ^p ≤ 0.01) (Fig. 3). A similar pattern was shown in NP cells, observing an elevated expression of IL-1α in ASCs, where the inflammation has been induced, though to a lesser extent than AF and NP cells stimulated with TNF. The difference in stimulated ASCs was statistically significant with regard to non-stimulated ASCs (*p ≤ 0.05) (Fig. 3).

When IL-1β expression was analyzed, results showed a statistically significant (**p≤ 0.01) elevated expression in AF and NP cells stimulated with TNF compared to non-stimulated cells with outstanding NP cells with very high expression. We also observed a decrease in this expression when TNF-stimulated cells were cultured with CM (^p ≤0.05) in both cell types. With regard to ASCs, a high expression of IL-1β when inflammation had been induced was observed, and it was statistically significant regarding non-stimulated ASCs (*p ≤ 0.05), these values being lower than in AF and NP cells stimulated with TNF (Fig. 3).

On analyzing IL-6 and IL-8 results, we observed an elevated expression in AF and NP cells stimulated with TNF with respect to the control (** p≤ 0.01). When CM was added, IL-6 and IL-8 expression decreased significantly (^ ^ p≤ 0.001). IL-6 expression in TNF-stimulated NP cells is also high (* p ≤ 0.05), but to a lesser extent than in AF cells. With regard to IL-8 The same tendency was shown in both types of cells, though higher values were obtained in NP cells. The same effect was observed in ASCs with TNF, higher values were obtained, which were statistically significant (*p ≤ 0.05) compared to the non-stimulated ASCs (Fig. 3).

When IFN-γ was studied in AF and NP cells stimulated with TNF a considerable increase with respect to the non-stimulated cells was shown. Once again, IFN-γ levels were reduced significantly when CM was added in both cell types. Conversely, a low expression, almost nil, of IFN-γ was observed in stimulated-ASCs (Fig. 3).

d) ASCs derived-CM down regulate neurotrophic factors in NP and AF cells

With regard to the neurotrophic factors analyzed, the elevated expression of NGF in AF and NP cells stimulated with TNF with respect to non-stimulated cells (*p≤ 0.05) is shown in Figure 4. Thus, we observed a decrease in this expression when CM was added in both cell types. The expression of NGF in ASCs was high in the control group and values were very close to those in the inflamed group.

Finally, analyzing BDNF, cell behavior was equivalent to that observed in response to the other factors. However, the decrease in NP stimulated cells cultured with CM was significantly higher (Fig. 4).

These results are given in Table 2. This table shows the genes analyzed in the *in vitro* model of discogenic pain, how their expression has developed when AF and NP cells have been stimulated with TNF and how CM is capable of modulating the response.

IV. Discusion

Low back pain has become a principal leading cause of work disability in recent decades owing to its high incidence. However, existing treatments are not carried out in a simple enough way. There is therefore an urgent need to establish an effective and simple remedy to treat this disease in the clinic practice [17]. Stem cells have emerged as a key element of regenerative medicine therapies, thus providing numerous potential cell therapies for treating a range of degenerative diseases and traumatic injuries. A recent paradigm shift has come about suggesting that the beneficial effects of stem cells may not be limited to cell restoration alone but may also be due to their transient paracrine actions. Stem cells are capable of secreting potent combinations of trophic factors which modulate the environment of molecular composition to evoke responses from resident cells [18].

In this study, human ASCs were obtained from the infrapatellar fat of patients. Immunofluorescence characterization and culture behavior of ASCs confirmed the characteristics of MSCs, with the expression of all markers being very prominent [19]. These results agree with other authors who had previously characterized ASCs for their easy collection and isolation and for their applications in humans [20]. Furthermore, some authors suggested that ASCs could be a more suitable cell type than bone marrow mesenchymal stem cells for IVD regeneration [21]. The AF and NP cells culture posed no difficulty since they were obtained from stable cell lines and their specific cell markers and growth kinetics were tested. As have been published our results confirmed a higher PDT in AF cells than in NP cells [22]. Confocal microscopy analysis was positive for specific markers. Decorin fluorescence was higher in AF cells and cytokeratin-19 in NP cells [23].

a) Immunomodulatory effects of MC on TNF-stimulated AF and NP cells

Discogenic pain entails multifactorial changes occurring with late IVD degeneration which interact with the peripheral nervous system and the central nervous system which cause the pain. Pain may be the result of biomechanical instability, damage to the endplate, inflammation of the nerves or sensitization [24]. Not all degenerated IVDs reveals discogenic pain. However, IVD degeneration is undoubtedly one of the most important keys [25]. In general, there are a vascular response and cell recruitment and activation caused by various cell types. A variety of cytokines have been found in human IVD in varying amounts, depending on whether the IVD is healthy, degenerated or herniated such as TNF, IL-1β, IL-6, IL8, MMPs, among others [26, 27].

The aim of this work is to try and detain an irreversible inflammatory cascade at somepoint using ASCs derived-conditioned medium as a therapeutic strategy.

Since TNF is an important pro-inflammatory cytokine in the discogenic pathology, inflammation was induced in all our culture conditions with TNF, although other authors showed equivalent inflammatory effects with INF-γ and LPS [28]. When we analyzed the expression of pro-inflammatory genes, we observed a high expression of all of them under inflammatory environment conditions produced by TNF, as we know that it is a key mediator in disc degeneration and low back pain [6]. TNF and both isoforms of IL-1 (IL-1α and IL-1β) showed high expression when stimulating IVD cells with TNF, decreasing this expression when the CM was added to the cultures. These results were similar to those obtained in the study developed by Bertolo and collaborators in which they state that MSCs can modulate the inflammatory state of IVD [29].

IL-6 is one of the cytokines responsible for causing pain in osteoarticular disease and is a major inducer of inflammation together with IL-8 [26, 30]. In our study, we observed that in TNF-stimulated ASCs, the expression of these two cytokines is not as low as would be expected because of their immunomodulatory effect. In the case of IL-6 this may be because, although it has been shown to be one of the main interleukins which induce inflammation, its role is currently being debated due to evidence that it may have an anti-inflammatory role [31].

With regard to IL-8, we know that it could be associated with the development of root pain and be activated in acute or subacute herniated discs. Constitutively, MSCs secrete hundreds of factors, among them IL-8 with pro-angiogenic effect [32]. Therefore, as an *in vitro* model of acute inflammation the immunomodulatory capacity of the CM produced by ASCs has been observed in AF and NP cells [33].

IFN-γ, an important macrophage activator and an inducer of the expression of Class II major histocompatibility complex (MHC) molecule, is a cytokine which is critical in both innate and adaptive immunity in humans [34]. Its expression increased when IVD cells were cultured with TNF and decreased when CM was added to culture medium. Kim et al. conclude that IFN-γ levels are significantly reduced when activated T lymphocytes are co-cultured with MSCs, which indicates an immunomodulatory effect of these cells and, consequently, of the CM tested in our studies [35].

Previous studies have shown that, with degeneration, there is also an upregulation of vascular endothelial growth factor (VEGF) and NGF that can promote neurovascular in growth inside the IVD [36, 37]. While the healthy IVD is avascular and aneural, degeneration is thought to induce structural and biochemical changes that contribute to angiogenesis and subsequent disk innervation, which effectively sensitize IVD and resulting in low back pain [3]. Among the molecules presumably involved in hyper-innervation of IVD are growth factors which are members of the neurotrophin (NT) family and are known to be neurotrophic factors. In addition to their role during the development of the nervous system, NTs also play an important role in inflammatory responses and pain transmission [38]. In fact, NTs, in particular NGF, is a peripheral pain mediator, particularly in inflammatory pain conditions. Furthermore, in the sensory innervation of IVD, it has been shown that under normal conditions NGF regulates the expression in nociceptors of a second neurotrophin, a brain-derived neurotrophic factor (BDNF). BDNF is released when nociceptors are switched on, and it acts as a central pain modulator [39]. NGF is regulated under a wide range of inflammatory conditions, and NGF neutralizing molecules should be effective analgesics in many models of persistent pain. Our results confirmed the high expression of these NTs in TNF-stimulated IVD cells, and how CM was capable of decreasing this expression so CM could have the capacity of being a powerful therapy in the research of discogenic pain treatment.

The immunomodulatory role of MSCs is widely described, but taking into account the CM obtained from ASCs studies are scarce [7, 40]. Our data showed the immunomodulatory role of ASCs-derived CM reducing inflammation and these effects could be a key element in inhibiting the activity of the molecular factors involved in the process of tissue degradation and possibly pain control in IVD degeneration pathology.

Declarations

Ethics approval and consent to participate
Not aplicable.

Consent for publication
Not applicable

Availability of data and materials
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no conflict of interest.

Funding Source
This study was financially supported by the Fundación Leonesa Proneurociencias. No separate funding was used specifically for this study. The study sponsor did not have any involvement in the study design, collection, analysis and interpretation of data, writing of the manuscript or in the decision to submit the manuscript for publication.

Authors' contributions
V.V., M.E and M.G. conceived and planned the experiments. M.E. and M.G. carried out the experiments. V.V., M.E and M.G. contributed to the interpretation of the results. V.V. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and supervised the manuscript.

Acknowledgements

The authors are grateful to Elías Rodríguez-Olivera for the design of primers.

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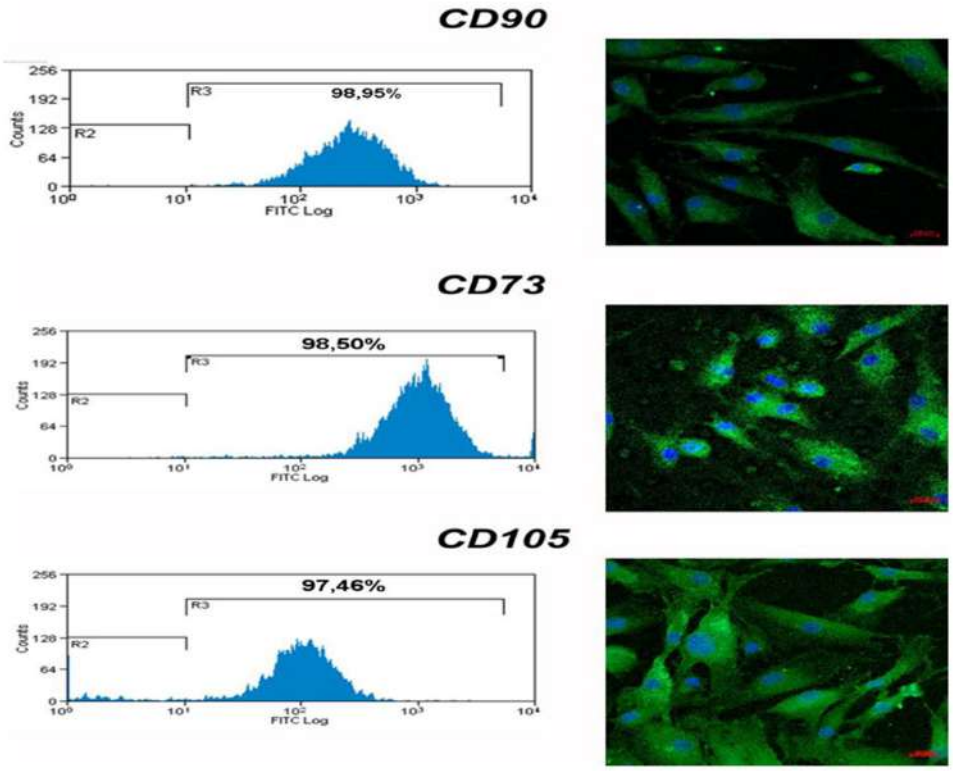

Figure 1: Characterization of human adipose tissue-derived stem cells. Specific stem cells markers such as CD-73, CD-90 and CD-105 were analyzed using flow cytometry and confocal microscopy. Histograms showed the average percentage of positive cells, higher than 95% in all CD-markers. CD-73, CD-90 and CD-105 positive expression was observed on the cell surface (green). The nucleus was stained with DAPI (blue) (scale bar=10 μm).

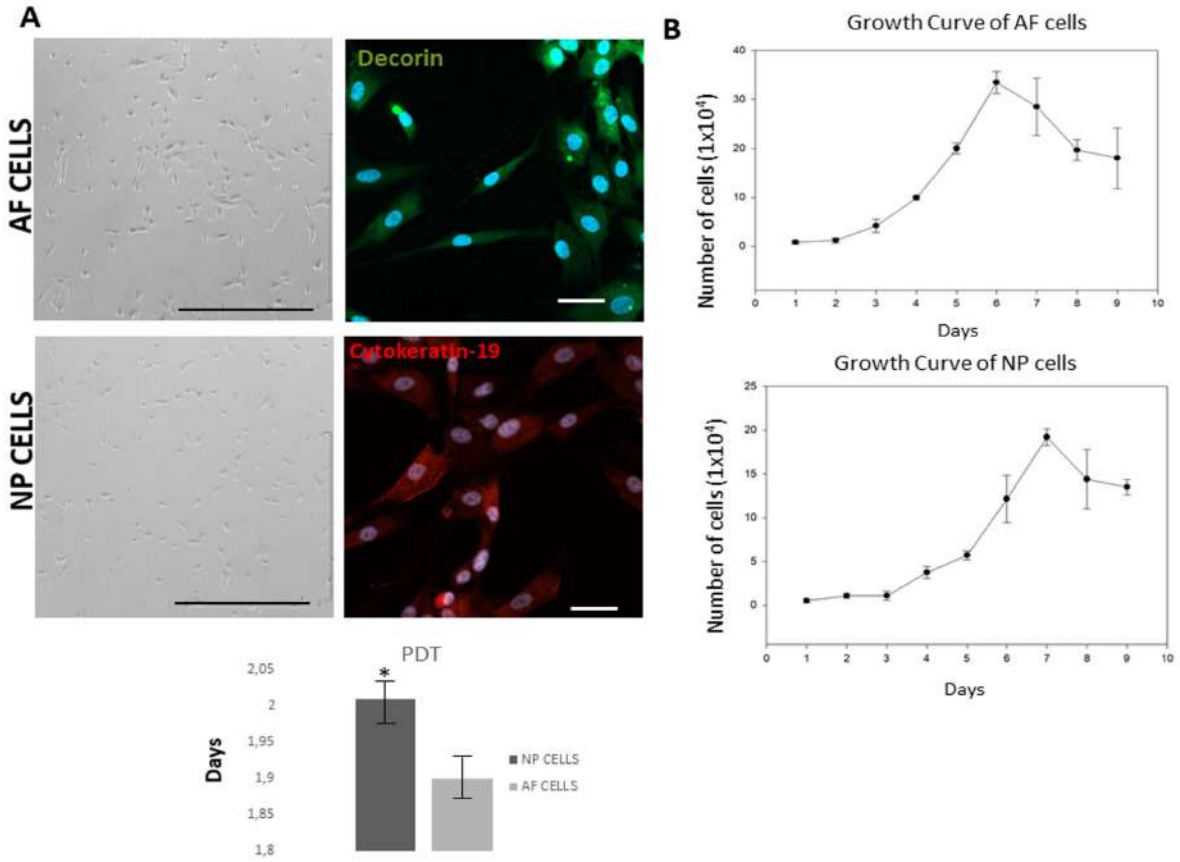

Figure 2: Nucleus pulposus (NP) and annulus fibrosus (AF). (A) Primary AF and NP cells morphology after 5 days of culture (scale bar=1000 μm). Immunophenotypic characterization. Positive AF cells to decorine (green) and positive NP cells to cytokeratin -19 (red). The nucleus was stained with DAPI (blue) (scale bar= 10 μm). (B) Growth Kinetics and population doubling time of AF and NP cells. (A) Growth curves of AF and NP cells (passage 3–5). AF and NP cells were counted every 24 hours. Population doubling time (PDT). The graph shows the cell average with regard to culture time. The highest growth of AF cells was observed 6 days after culture. In NP cells this maximum was reached on day 7 of culture (* $p \leq 0.5$).

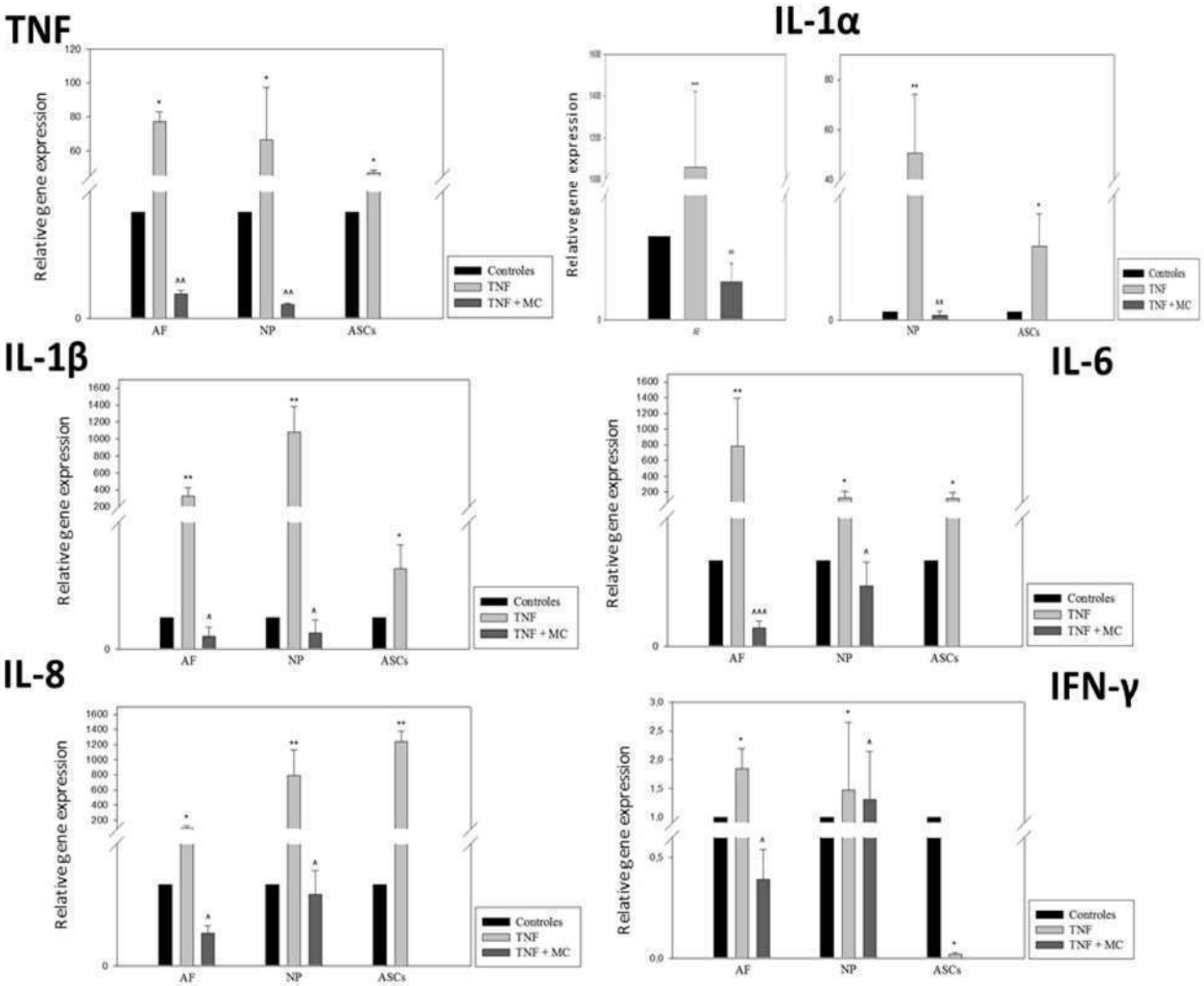

Figure 3: Relative expression of TNF, IL-1α. IL-1β, IL-6, IL-8 and IFN-γ in AF, NP cells and ASCs in an *in vitro* model of inflammation. Inflammation was induced using TNF for 12 h and CM was added to the cultures. * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq$ 0,005) with regard to cells non-stimulated with TNF. ^ ($p \leq 0.05$), ^ ^ ($p \leq 0.01$), ^ ^ ^ ($p \leq 0.005$) with regard to cells-stimulated with TNF.

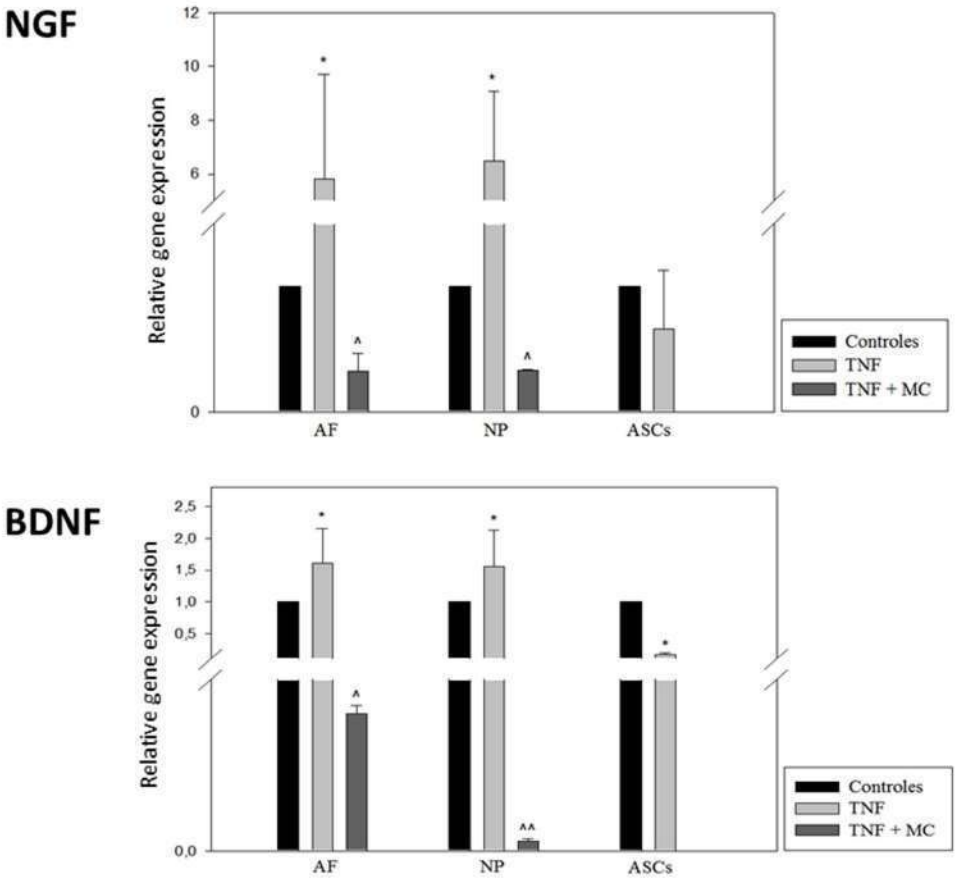

Figure 4: Relative expression of NGF and BDNF in AF, NP cells and ASCs in an *in vitro* model of inflammation. Inflammation was induced using TNF for 12 h and CM was added to the cultures. * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.005$) with regard to cells non-stimulated with TNF. ^ ($p \leq 0.05$), ^ ^ ($p \leq 0.01$), ^ ^ ^ ($p \leq 0.005$) with regard to cells-stimulated with TNF.

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 21 Issue 1 Version 1.0 Year 2021
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Microalgae Cultivation on Poultry Droppings Extract for Biodiesel Production

By Golub Natalia & Levtun Igor
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Abstract- *Background:* Variation of microalgae cultivation conditions affects biosynthetic processes, which leads to a change in the content and qualitative composition of lipids - the raw material for the biodiesel production. The cultivation of microalgae biomass requires mineral nutrition, to provide it natural sources of salts and chemically synthesized compounds are used. The use of agricultural waste will reduce the anthropogenic burden on the environment, reduce the biofuels cost and will help to solve the problems of alternative energy from renewable raw materials production. In this case, in addition to microalgae, it is possible to obtain biologically active substances that can be used as a feedstock for animals and poultry.

Objective: The purpose was to study the Chlorella vulgaris microalgae cultivation while using the extract from poultry droppings as a nutrient medium and its effect on lipid fraction content in the cells. The subject of the study is Chlorella vulgaris development and lipid accumulation in the cells.

Keywords: *cultivation, microalgae, lipids, biomass, Chlorella vulgaris, biodiesel.*

GJMR-G Classification: *NLMC Code: WA 707*

Strictly as per the compliance and regulations of:

Microalgae Cultivation on Poultry Droppings Extract for Biodiesel Production

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Method: Cultivation was carried out in photoreactors with an airlift system for mixing and feeding bubble air with additional CO_2. Observations and purity control of *Chlorella vulgaris* culture was performed by light microscopy, biomass growth – spectrophotometry, lipid yield – by chemical method, microalgae and lipids mass–gravimetric, lipid composition – by chromatographic method.

Results: It has been shown that using the poultry droppings extract as a nutrient medium for microalgae cultivation, the *Chlorella vulgaris* biomass yield increases up to 7 times in relation to cultivation on the Gromov medium. This increases the lipids content up 2 times. In relation to rapeseed oil, the concentrations of myristic, palmitoleic, stearic and linolenic acids are higher.

Keywords: *cultivation, microalgae, lipids, biomass, Chlorella vulgaris, biodiesel.*

I. Introduction

Increase in the diversity of energy sources creates conditions for countries to become less dependent on fossil energy resources. The greatest attention is attracted to the energy sources from renewable raw materials. One such source is microalgae, the high lipids content in which allows them to be used as raw material for biodiesel production. To cultivate microalgae, the salts of nutrient elements and CO_2 as a source of carbon are widely used. In industrial production, the use of mineral salts is significant, which leads to depletion of minerals and increase in the price of the final product. Depending on the nutrients form, the change in microalgae metabolism takes place, always in the direction of certain substances biosynthesis and that includes lipids. Therefore, the study of renewable raw materials (agricultural waste) usage as a source of microalgal nutrition, which also leads to increased lipid content in algae cells, is an urgent problem.

Such raw material is poultry waste (droppings), which contain all the necessary components for algae development. The lipids content and their qualitative composition is influenced by the nitrogen form introduced into the culture medium. Poultry waste contains a mixture of various nitrogen compounds - urea, amines, amino acids, nitrates and ammonia, the ratio of which depends on the storage conditions.

In paper [1], it has been shown that the addition of human urine or urea to standard Tamia medium increases the growth *Chlorella vulgaris* biomass by 20-30%. With the use of ammonium ions, it is necessary to double the amount of salt in relation to urea, as the consumption rate of such nitrogen form decreases. The highest growth rate of Scenedesmus sp. LX1 is characteristic for the higher ammonium ions in the nutrient medium. The increase in biomass decreases when ammonia is replaced by urea and nitrate [2]. In this case, the consumption of ammonium ions leads to a decrease in pH value and microalgae development inhibition.

In other works [3, 4], the results *Chlorella vulgaris* cultivation research for the use of poultry droppings extract for the further production of biodiesel are shown. The use of the extract increases the growth rate and lipid fraction content. Also, as a nutrient medium, extracts from various types of poultry wastes were used to grow *S. obliquus* HTB1. It has been shown that biomass growth depends on preliminary treatment of the droppings. In this case, the growth increases or decreases by 20% in relation to cultivation on the standard medium BG11 [5,6]

In work [7], extract concentrations from 5 to 20% of human urine, bird droppings, cow manure and urine were used as a nutrient source for microalgae *Chlorella singularis*, *Micractiniumpusillum* and *Chlorella sorokiniana* was used. It has been shown that *Chlorella sorokiniana* can grow in all types of medium. The maximum growth rate reached 140 ± 3.1 mg / L / day

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and the lipids yield (45.5 ± 2.3 mg / L / day) was obtained using 20% bird droppings extract.

Among various waste-based mediums, the largest biomass yield was observed with algae cultivation on bird droppings extract [8].

The increased *Chlorella vulgaris* biomass growth observed with the use of poultry droppings extract can be explained by appearance of myxotrophic cultivation – droppings provide organic nutrients, inorganic carbon is introduced as a CO_2 [3,9,10]. In addition, as an inorganic carbon, gas emissions from production can be used [11,12].

This work is devoted to determine the influence of substances concentration extracted from poultry droppings.

II. Materials and Methods

Chlorella vulgaris ACKU 531-06 was provided from the collection of T. Shevchenko Kiev National University, Ukraine. Poultry droppings without litter were provided by the «Авангард» poultry farm, Ukraine.

Microalgae cultivation was conducted in photoreactors with volume of 1.3 dm^3 with illumination by light emitting diodes (color ratio of red / blue / green 2: 1: 1) for 12 hours light and 12 hours darkness during the day, with the temperature 20 ± 2 °C. Photoreactors contain an airlift system for environment mixing with air bubbling provided using compressor Resunair-pump AC-9601 (China). The bubbling speed is 100 dm^3 / h. As a source of carbon, in addition to the substances contained in poultry droppings, CO_2 was used from the gas cylinder, which was fed through the reducer system on daily basis (0.25 dm^3 CO_2/dm^3 of bubbling air).

As the control medium the Gromov medium № 6 was chosen [13].

Poultry dropping extract was prepared as follows. Weighed droppings (on a dry substance) were dissolved in 1 dm^3 of distilled water and infused 24 hours with stirring by magnetic stirrer MM-5 (Russia). The resulting solution was filtered and boiled for 1 hour. The extract was autoclaved for 1 ÷ 1.5 h. at a temperature of 120 ÷ 135°C with a pressure of 250 kPa.

The dry matter content in droppings was determined according to the standard method [14]. Humidity of the droppings fluctuated within 72 ± 2.2%. An aliquot of the solution was injected into a photoreactor.

The pH value, ammonium and nitrate ion content were determined using анИ-160 МИ (Russia) ionmeter.

Observation and control of *Chlorella vulgaris* culture purity was performed using the microscope TM XSP-139TP (Ulab, China) (increase from 40x to 1000x for visual observation).

The biomaterial concentration was measured on a spectrophotometer ULAB 102 (China) with a wavelength of 450 nm (D450) [15].

Allocation of algal cells from culture medium was carried out using a centrifuge ЦЛК-1 (Russia) at 2500 rpm. The precipitate was dried in a drying cabinet СШ-151 (Russia) at a temperature of 105°C to constant weight.

The lipids isolation was carried out according to Blay and Dyer's method [16].

The amount of biomass and lipids was determined using the analytical scales Ohaus Scout-Pro SPU 123 (China).

III. Results and Discussion

Droppings have a variable constituent's content. To find out the effect of the soluble compounds concentration in the droppings extract on biomass growth and the lipids accumulation in algae, as a standard the nitrogen content in nitrate ion and ammonium ion was used. It was presumed that the concentrations of other components varied according to changes in nitrogen concentrations. The content of nitrate and ammonium ions in the culture medium on a chicken droppings extract at the beginning of cultivation is shown in Table. 1. The samples given in the table correspond to the contents of the dry matter substance № 1 - 8 g/dm^3, № 2 - 5 g/dm^3, № 3 - 3 g/dm^3, №4 - 2 g/dm^3.

Table 1: Concentration of nitrate and ammonium ions in the culture medium in droppings extract

	Sample			
Ion	№1	№2	№3	№4
NO_3^-, mg/dm^3	157,9	101,1	54,3	36,7
NH_4^+, mg/dm^3	105,3	70,2	38,6	28,7

It is known [17] that microalgae biomass growth depends on the rational ratio of nutrients. Accumulation of lipids occurs under stress conditions, one of which is the reduction of nitrogen content in the medium. In addition to inorganic nitrogen, the nitrogen in the droppings contains urea, amino acids and amines, which can also be consumed by microalgae. Due to these compounds, the total nitrogen content in the solution increases by 8 ± 1%, which is about 11 mg/dm^3 of nitrogen.

Using a more concentrated extract solution is not rational, since the color of the culture medium becomes brown, which may affect the passing of light energy specially in the middle area of the photoreactor and, accordingly, this will negatively affect photosynthetic processes.

The culture was pre-cultivated on the Gromov medium № 6, which contains nitrate nitrogen. When *Chlorella vulgaris* culture is transferred to the medium with droppings extract, lag phase can be observed, in which the consumption of nitrate and ammonium nitrogen occurs. On the medium with droppings extract there is an increase in the exponential growth phase up to 8 days in relation to the control sample (Fig. 1).

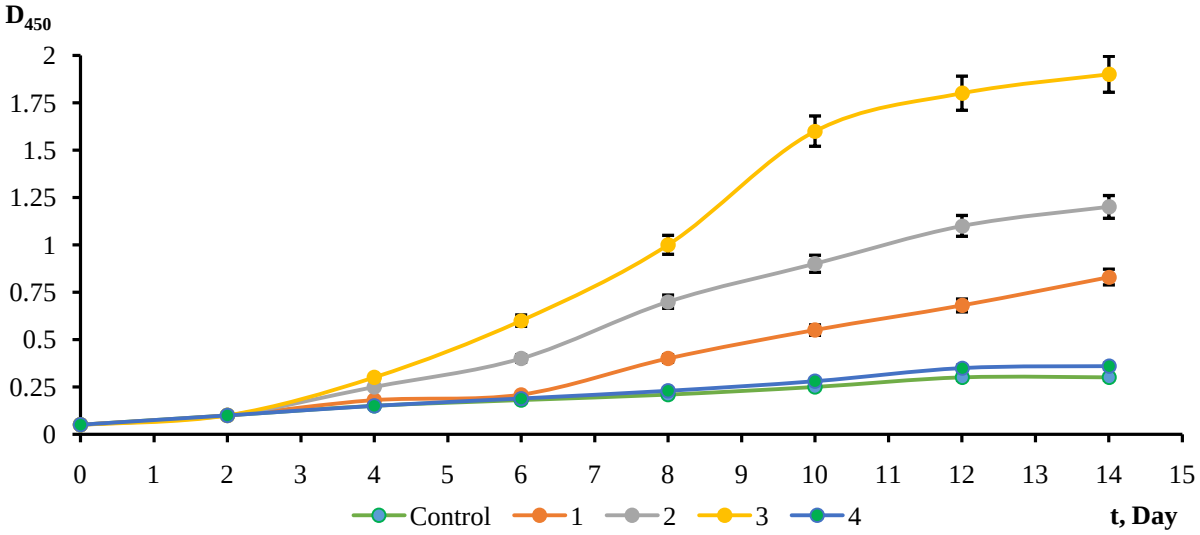

Fig.1: Changes in the optical density (D) of *Chlorella vulgaris* culture during cultivation (t) depending on the droppings content: 1 – 105 mg/dm^3, 2 – 70 mg/dm^3, 3 – 38 mg/dm^3, 4 – 28 mg/dm^3

In the exponential growth phase, the ammonium ion is primarily consumed (Fig. 2 from Table 4, for each sample), which confirms the data in the work [18]. NH_4^+ is the final product of nitrate recovery and functions as a reverse inhibitor that represses nitrate absorption. In the processes of metabolism, NH_4^+ is transformed into organic forms without altering the oxidation degree. Nitrate and urea require ATP energy to restore nitrogen. A slight increase in ammonium ions concentration in the nutrient medium observed during 4-5 day can be explained by the release of excess ammonium ions from the cells formed during urea assimilation from solution [19].

$$ATP + Urea + HCO_3^- \rightarrow ADP + P_i + \text{Urea-1-carboxylate} \quad (1)$$

$$\text{Urea-1-carboxylate} + H_2O \rightleftarrows 2\,CO_2 + 2NH_3, \quad (2)$$

since most species of *Chlorella* lack urease.

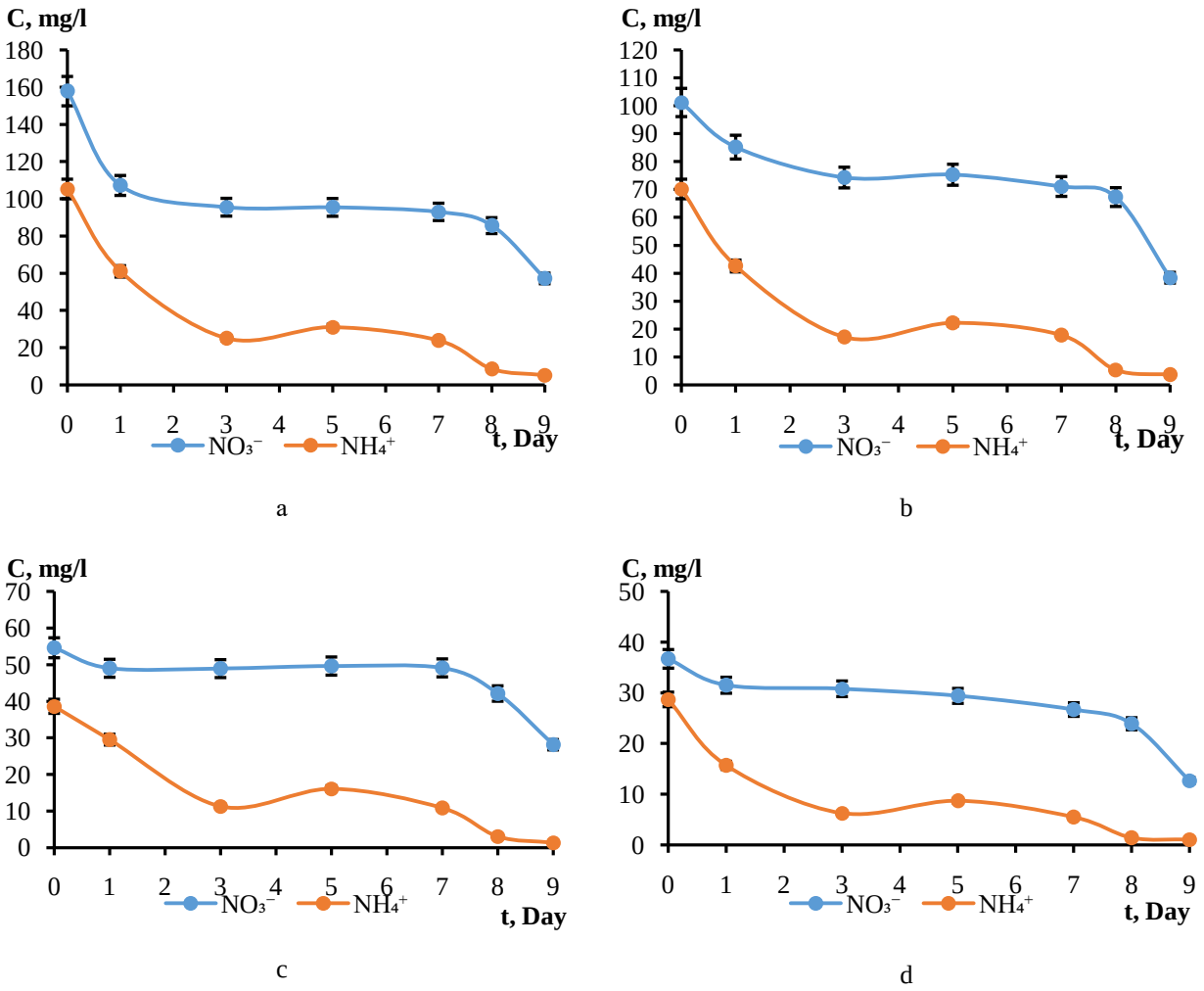

Fig. 2: Change in the nitrogen and ammonium content (C) ions during cultivation (t), depending on the droppings concentration on dry matter: a) 8 g/dm^3 b) 5 g/dm^3 c) 3 g/dm^3 d) 2 g/dm^3.

The initial pH value of the medium during the extract concentration in the culture medium also decreases from 8.3 to 7.4. For the Gromovmedium №6, the initial pH value is 7.2. During bubbling with CO_2 there is a decrease in pH value by 0,3 - 0,4 units. The pH value returns to the initial level within one cultivation day. In case of nitrate ions use as a nitrogen source (control), the pH value is increased up to 7.9 ± 0.2. With the use of the droppings extract, the pH value remains neutral for low concentrations (sample 3.4) and reduces down to 6.5 ± 0.3 with an increase of extract content (sample 1.2). Such change in pH value can be explained by acidification of the medium due to ammonium ions consumption, since during such biochemical process, the proton is released into the culture medium [20]. That is, for the use high extract concentrations, it is necessary to adjust the pH value of the medium.

Fig. 3 shows the biomass growth at different extract concentrations in the photoreactor. Microalgae growth in culture medium containing extract with the initial ammonium ions content of 54 mg/dm^3 exceeds the control 7 times (sample 3). Increase or decrease inextract content leads to a decrease in the production of microalgal biomass. This can be explained by a decrease in the light energy inflow due to an increase in the color intensity of culture medium when the extract content increases, and the presence of substances that at high concentrations inhibit the microalgae development. With a decrease in extract content the lack of nutrients decreases biomass growth.

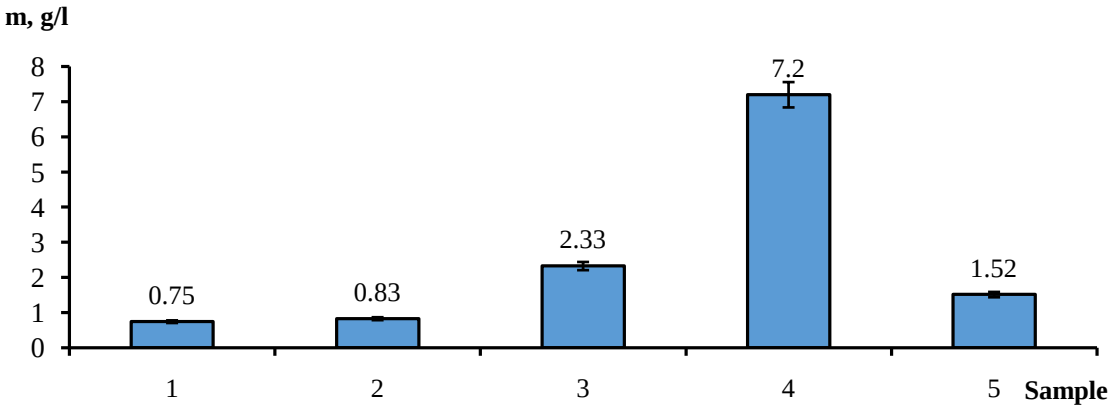

Fig. 3: Growth of *Chlorella vulgaris* biomass (m) at different concentrations of droppings dry matter: 1– control, 2 – 8 g/dm^3, 3 – 5 g/dm^3, 4 –3 g/dm^3, 5 –2 g/dm^3.

The time of *Chlorella vulgaris* culture cells generation is calculated according to the formulas:

$$g_k = \ln 2/\mu \quad (3)$$

$$\mu_{max} = \ln(X_t/X_0)/(t - t_0),$$

where X_0 and X_t are the biomass concentration at the beginning and at the end of the time t, respectively, μ is the exponential growth rate constant (specific growth rate), g is the time of cells generation.

For control it is 2.97 days, for specimens cultivated on the droppings extract - 2.67, 0.95, 0.31 and 1.48 days, respectively. That is, the use of the extract as a nutrient medium increases the cell division rate and at maximum biomass growth (sample№3) the cell doubling rate is 7.44 hours.

The increase in biomass growth rate in sample №3 in relation to control can be explained by a change in the cultivation conditions from autotrophic to myxotrophic. At the same time, there is a decrease in the energy consumption in the cells used on the cells constituents' biosynthesis due to the intake of both inorganic and organic components from the nutrient medium. It is known [18] that during the dark period glucose contained in the droppings, can induce the expression of two transport systems for the amino acids transfer into the cell. This provides cells with an additional source of nitrogen that does not require significant energy usage. The organic compounds contained in the droppings are digested throughout the whole cycle, both in the period of illumination and in the dark [18]. Therefore, the presence in the culture medium of organic and inorganic compounds contained in the droppings will contribute to increase of biomass biosynthesis rate.

Also, the presence of organic substances increases the digestibility of CO_2during the light period, which leads to increase in biosynthetic processes.

The use of an extract as a nutrient medium has the advantage in biomass production in relation to the Gromov № 6. The increase in biomass for samples goes as follows№ 2 – 5 by 11.4%, 208.4%, 860%, 103%, respectively.

The lipids content in dry biomass of *Chlorella vulgaris* at different extract concentrations is shown on Fig. 4. As can be seen from Fig. 4, the cultivation of *Chlorella vulgaris* in the extract containing medium leads to an increase in the lipid content up to three times in all samples in relation to the control. When the extract concentration decreases 4 times, the lipid content decreases by 11%. This can be explained by the influence of inhibitors, the ammonium nitrogen concentration, and change in pH value. The droppings extract contains organic substances that are used by the *Chlorella vulgaris* microalgae cells as a source of carbon in the mixotrophic cultivation, leading to a change in the metabolic pathways and carbon assimilation towards the lipid fraction accumulation [21].

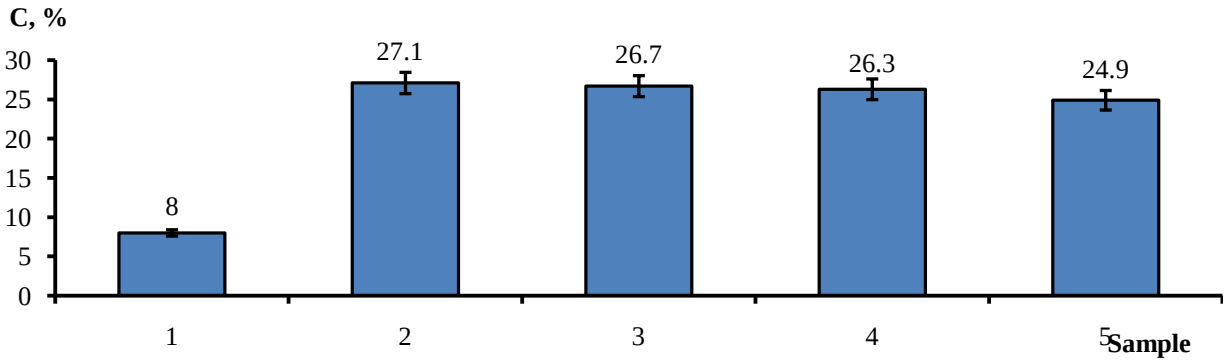

Fig. 4: The lipid fraction content (C) in dry biomass during microalgae cultivation with extract, at a different concentration of dry matter, g/dm^3:1 - control; 2 – 8; 3 – 5; 4 – 3; 5 – 2.

Based on the obtained data, it is rational to obtain biodiesel fuel from microalgae using the droppings extract obtained from 3 g/dm^3 of dry matter. Under these conditions, the lipids yield is 1.89 ± 0.09 g/dm^3 per week of cultivation. When decreasing or increasing the extract content, the lipid output per unit volume is reduced because the reduction of biomass growth.

Table 2 according to the chromatographic analysis shows a comparative description of lipid fraction composition obtained under optimum conditions from the *Chlorella vulgaris* biomass during cultivation on droppings extract. The use of the droppings extract increases the content of saturated fatty acids in the lipid fraction compared to rapeseed oil, which positively affects the production of biodiesel fuel.

Table 2: The content of fatty acids in the lipid fraction of Chlorella vulgaris microalgae cultivated on bird droppings extract

№	Acid	Content in lipids, %	
		Chicken droppings extract	Rapeseed oil [22]
1	Tridecanoic (13: 0)	7,34	-
2	Palmitic (16: 0)	42,2	5,2
3	Palmiotolein (16: 1ω9)	10,8	-
4	Stearic (18: 0)	11,57	0,5
5	Oleic (18: 1)	5,8	58
6	Linoleic (18: 2)	7,4	22,3
7	Linolenic (18: 3)	12,3	13,2

Thus, for the *Chlorella vulgaris* cultivation, it is possible to use bird droppings as a nutrient medium. Under these conditions, the biomass growth and the lipids content in the cells increases. An increase in the growth rate occurs due to mixotrophic cultivation conditions in the presence of organic carbon and nitrogen sources, which are digested by the cell. During the dark period, the amount of biomass that was formed during the illumination period is not reducing. It enables the possibility to use a periodic lighting regime, which will reduce energy costs and reduce the cost of final product. Increase in the content of saturated fatty acids in the lipid fraction makes it possible to use the resulting raw material for the biodiesel production for the application in standard technologies.

IV. Conclusions

The possibility of chicken droppings extract application for Chlorella vulgaris cultivation is shown. It has been established that the maximum biomass increase occurs while using 3 g/dm^3 of droppings dry matter. Under such conditions, the biomass yield increases up to 7 times in relation to culture grown on Gromov medium №6, and the lipids content is also higher up to 3 times.

It has been established that the application of droppings for cultivation increases the saturated fatty acids yield relative to rapeseed oil, which makes it possible to use the resulting oil for the biodiesel production.

Ethics Approval and Consent to Participate

Not applicable.

Human and Animal Rights

No Animals/Humans were used for studies that are basis of this research.

Consent for Publication

Not applicable.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Global Journal of Medical Research: G
Veterinary Science and Veterinary Medicine
Volume 21 Issue 1 Version 1.0 Year 2021
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Effect of Cryopreservation on Biological Markers of Sperm Function and their Correlation in Different Dog Breeds

By Pathania A, Ranjna S Cheema & Sharma A
Guru Angad Dev Veterinary and Animal Sciences University

Abstract- In the present study, we compared motility, viability, plasma membrane integrity (PMI), acrosome integrity (AI), inner mitochondrial membrane potential (IMMP), ATP and LDHC in freshly extended (FE,) and frozen-thaw (FT) semen among the dogs and breeds. Freshly extended semen in Tris-citric acid-fructose-egg yolk plasma-glycerol extenderwas also subjected to in vitro capacitation and acrosome reaction. Pearson correlation coefficient among the sperm attributes of FE and FT semen has been evaluated.Sperm attributes in FE and FT semen vary significantly ($P<0.05$) among the dogs. There was a significant ($P\leq0.05$) decline in motility, viability PMI, AI and high IMMP (HIMMP)during freezing-thawing process, in contrast, in ATP, LDHC and medium IMMP(MIMMP)decline was non-significant ($P\geq0.05$). There was a loss of 21.7-36.7%, 20.9-36.6%, 20.3-53.6%, 18.9-43.9%, 16.1-36% in motility, viability, PMI, AI and HIMMP during freezing-thawing process. A loss in ATP and LDHC content was also observed during freezing-thawing process. Incubation of washed spermatozoa of 13 dogs in canine TALP medium for 6 hrs resulted in a decline in motility, viability, MIMMP and an increase in HIMMP and percentage of the altered acrosome.

Keywords: biological markers, correlation, cryopreservation, semen, dogs.

GJMR-G Classification: NLMC Code: QW 70

Strictly as per the compliance and regulations of:

Effect of Cryopreservation on Biological Markers of Sperm Function and their Correlation in Different Dog Breeds

Cryopreservation of Canine Semen

Pathania A [α], Ranjna S Cheema [σ] & Sharma A [ρ]

Abstract- In the present study, we compared motility, viability, plasma membrane integrity (PMI), acrosome integrity (AI), inner mitochondrial membrane potential (IMMP), ATP and LDHC in freshly extended (FE,) and frozen-thaw (FT) semen among the dogs and breeds. Freshly extended semen in Tris-citric acid-fructose-egg yolk plasma-glycerol extenderwas also subjected to *in vitro* capacitation and acrosome reaction. Pearson correlation coefficient among the sperm attributes of FE and FT semen has been evaluated.Sperm attributes in FE and FT semen vary significantly ($P<0.05$)among the dogs. There was a significant($P\leq0.05$) decline in motility, viability PMI, AI and high IMMP (HIMMP)during freezing-thawing process, in contrast, in ATP, LDHC and medium IMMP(MIMMP)decline was non-significant ($P\geq0.05$). There was a loss of 21.7-36.7%, 20.9-36.6%, 20.3-53.6%, 18.9-43.9%, 16.1-36% in motility, viability, PMI, AI and HIMMP during freezing-thawing process. A loss in ATP and LDHC content was also observed during freezing-thawing process. Incubation of washed spermatozoa of 13 dogs in canine TALP medium for 6 hrs resulted in a decline in motility, viability, MIMMP and an increase in HIMMP and percentage of the altered acrosome. Percentage of average induced acrosome reaction (IAR) was 46.1 ± 2.7%, 37.3 ± 2.3%, 55.9 ± 7.2% and 48.3 ± 2.1% after six hrs of incubation. Positive correlation between motility, viability and AI/HIMMP/IAR in FE and FT semen was observed. A positive correlation between motility x viability; motility x HIMMP; motility x IAR and viability x HIMMP; viability x IAR and HIMMP x IAR was also detected during IAR. It is suggested to perform multiple functional tests of FE/FT semen before selecting the dogs for breeding. Freezing of semen of individual dogs rather than pooled semen to achieve higher fertility rate is advised. Further, an induced acrosome reaction may be an indicator of semen freezability.

Keywords: biological markers, correlation, cryopreservation, semen, dogs.

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I. Introduction

In mammals, the success of fertilization mainly depends on gamete fertilization potential and subsequently upon sperm and oocyte quality. Sperm's contribution to fertilization is generally estimated through the evaluation of sperm attributes. A loss of fertility potential is associated with manipulation and preservation techniques. In dogs, the success of artificial insemination depends on optimal insemination period and the use of high-quality semen, as low-quality semen can significantly decrease the effectiveness of insemination (Niżański et al. 2004). Semen quality is generally assessed by evaluating motility, sperm concentration and morphology. But, motility, concentration and morphology are insufficient to predict fertility and to detect sub- fertility in males. Sometimes sperm function tests viz, hypo-osmotic swelling test (HOST), viability, acrosome integrity (AI) and Hemi-zona assays are also evaluated. Some of these parameters are correlated with fertility though it does not predict male fertility (Petrunkina et al. 2007; Sutovsky and Lovercamp 2010; Dyck et al. 2011). Concentration, morphology, motility and the acrosome status analyzed under light microscopy are considered to be important in evaluating the fertilizing ability of spermatozoa. However, functional assays, such as the zona pellucidabinding assay, the Hemi-zona essay or the hypoosmotic swelling test, are better correlated with the artificial insemination outcome than the results of conventional semen evaluation (Petrunkina et al. 2007; Sutovsky and Lovercamp 2010).

Inability of the *in vitro* assessment methods to accurately predict spermatozoa fertility may be credited to the complexity and multifactorial nature of male fertility. Attempts had been made to escape these limits, which led to the introduction of some advanced tests. These tests included the use of fluorescent markers to assess the acrosomal status, the use of vital staining for mitochondrial activity, the use of particular fluorochromes to detect altered sperm chromatin or DNA integrity along, several molecular regulators of thermal and oxidative stress. Understanding the main factors involved in sperm fertility and how fertility

changes or are influenced by sperm manipulation e.g., cryopreservation and sperm-sorting, would allow to 1) improve the extenders, 2) accurately estimate sperm fertility and 3) predict sperm survival after processing. It is well - known and accepted that cryopreservation damages the sperm, with many of cells losing their fertility potential after freezing/thawing. Further, individual variations also exist on sperm resistance to cell damage during these procedures, justifying that some males are "better freezers" than others, even if no differences are found in fresh semen quality assessment[46,32]. Therefore, in the present study, functional sperm attributes in freshly extended (FE) and frozen-thaw (FT) semen of different dogs among the breeds were compared. At the same time, correlation among the sperm attributes of FE and FT semen have been evaluated.

II. Materials and Methods

a) Maintenance of dogs and semen collection

All the procedures were approved by the CPCSEA, New Delhi, vide F. No 25-19-2018-CPCSEA, dated 22/11/2018. Semen was collected from Labrador Retriever (6), Pug (4), Pomeranian (1), Golden Retriever (1), German shepherd (2) and Shih Tzudogs (2).Six Labrador Retriever and Pug dogs were maintained in individual pens in the university dog house. Labrador (500 g) and Pug (200 g) dogs were fed daily cooked feed supplemented with vitamins and trace minerals twice daily and water provided ad libitum. Dogs were given regular exercise of walking / running for one hour daily in the morning and evening. Deworming of dogs was done and they were vaccinated for rabies, CDV, CAV2, CPV, CPI and CAV1. Pomeranian, Golden Retriever, German shepherd and Shih Tzudogs were pet dogs within a three km of the university. Semen was collected by digital stimulation method at an interval of 3-5 days. Only sperm - rich fraction was used for evaluation and semen freezing.

b) Experimental design

Three ejaculates per dog (n=13 dogs) were evaluated and cryopreserved individually in Tris citric acid-fructose-egg yolk plasma-glycerol (TCFEYP) extender[5,19]). Freshly extended (FE) and frozen-thaw (FT) semen was evaluated for motility, viability, plasma membrane integrity (PMI), AI, inner mitochondrial membrane potential (IMMP), ATP and lactate dehydrogenase (LDHC) concentration, freshly extended semen was also evaluated for rate of induced *in vitro* capacitation/ acrosome reaction.

c) Chemicals and Reagents

All the chemicals were procured from Sigma Aldrich, Thermo scientific, Himedia and BR Biochem. All the reagents were prepared in water filtered through RO-Synergy- Millipore water purification system. Kits for LDHC and ATP assay were procured from Sigma Aldrich and My BioSource, respectively.

d) Analysis of sperm attributes in freshly ejaculated and frozen-thaw semen

i. *Motility and Viability*

Motility was noted by the wet mount method. A drop of semen on a slide, covered with a coverslip was observed microscopically using CCTV. A total of 200 motile and non-motile sperms were observed on the monitor and the percent of motile spermatozoa was calculated. For viability, a drop of semen was added to a drop of 0.5 % aqueous eosin in normal saline, mixed for 60 sec followed by adding a drop of 10 % nigrosin on a slide. Mixed for another 60 sec, and a smear was prepared, dried and observed under a binocular microscope (Olympus) at 1000x. About 200 sperms stained white (live) and pink (dead) were counted in different fields and percentage of live spermatozoa was calculated (Fig 1A).

e) Plasma membrane integrity (PMI)

HOST was performed to analyze the integrity of sperm membrane. The hypo-osmotic solution (300mOsm/L) was prepared by dissolving 735 mg sodium citrate and 1.351 g fructose in 100 ml DDW. Semen (10 µl) was mixed with 100 µl of 60 mOsmol (20 ml of 300 mOsmol and 80 ml DW) HOS solution and incubated at 37°C for 30 min. One drop of incubated semen was placed on a slide, covered with coverslip and examined under bright - field microscope at 400 x for coiled tailed spermatozoa. A total of 200 spermatozoa with coiled and uncoiled tails were observed under different fields and percentage of coiled tailed spermatozoa was calculated. Similarly, 10µl of semen was incubated in phosphate buffer saline (PBS) under similar conditions and percentage of coiled tailed spermatozoa was calculated (Fig 1B). The number of coiled tailed spermatozoa in PBS was deducted from the number in hypo-osmotic solution and the resultant figure was taken as the HOS-reactive spermatozoa.

f) Acrosome integrity(AI, Feng et al., 2007)

AI of spermatozoa was assessed using Coomassie brilliant blue stain(CBB, R-250). Fixative was prepared by dissolving 5gm of Paraformaldehyde in 80ml PBS, pH 7.4 with continuous stirring at 60°C. Added 1N NaOH dropwise till the solution becomes transparent and pH reaches to 7.4. Added 500 µl Triton X-100 and final volume was made up to 100ml with distilled water. Fixative was stored in aliquots at -20°C for further use. Coomassie brilliant blue stain was prepared by dissolving 0.25 g of CBB powder in 10% acetic acid and methanol. A smear of washed semen was prepared on a clean a glass slide and air – dried and fixed the smear in fixative for 15 mins. Washed the slide with PBS, pH 7.0, incubated the smear in CBB for 5 min and again washed with DW. Observed the air-

dried the slide under bright- field microscope (Olympus) at 1000X. About 200 spermatozoa with intact (blue) and damaged (unstained) spermatozoa were counted in different fields and the percentage of spermatozoa with intact acrosomes was calculated. Results are expressed as the percentage of spermatozoa with intact acrosomes.

g) Inner Mitochondrial membrane Potential

It was measured using a fluorescent carbocyanine dye, JC-1 (JC-1 stain kit, Sigma –Aldrich), Semen (10µl.) was mixed with 1µl of JC-1 dye and incubated at37°C for 20 min. After incubation a drop of 10µl semen was placed on glass slide and covered with coverslip. Slide was observed under a fluorescent then the semen is observed under fluorescent microscope (Olympus CX-24) at 400X. About 200 spermatozoa with high IMMP (HIMMP, red / orange), medium (MIMMP) and low (LIMMP) were counted in different fields and the percentage of spermatozoa with HIMMP and MIMMP was calculated (Fig 1C).

h) In vitro Capacitation and acrosome reaction

Basic TALP was prepared by dissolving NaCl (488 mg), KCl (35.6 mg), $CaCl_2$ (17.49 mg), Na_2CO_3 (316 mg), KH_2PO_4 (16.3 mg), Sodium pyruvate (2.75 mg), Sodium lactate (241.5 mg) in 80 ml DW. Adjusted the pH to 7.2 and the final volume was made up to 100 ml with DW. Energy TALP was prepared by dissolving bovine serum albumin (40 mg), glucose (5 mg) and heparin (10 µl of 1mg/ml stock solution) in 10 ml basic TALP just before use. Mixed gently 0.5 ml semen (>60% motility) with 0.5 ml TALP and centrifuged at 1000 rpm for 3 min. Discarded the supernatant and dissolved the loose pellet in energy TALP to get a final sperm concentration 150 x 10^6 / ml. Sperm suspension was incubated at 37°C for 6 hrs and observations were taken for motility, viability, acrosome reaction and IMMP at 2, 4 and 6 hrs as per the protocols explained above.

i) Preparation of sperm extract for ATP and LDHC concentration

Spermatozoa (500 x10^6) were washed twice with PBS, pH 7.4 at 3000 rpm for 5 min. Sperm pellet was suspended in 1.0 ml of 2 % SDS in 62.5 mM Tris-HCl buffer containing 10 µl protease inhibitors (cocktail, BWR). Sperm suspension was sonicated at 20 watts for 3 x 20 secs, centrifuged at 3000 rpm for 15 min. Pellet was discarded and the supernatant was passed through 3 kDa protein concentrators by centrifugation at 10000 rpm for 30 min. Both filtrate (containing ATP) and concentrate (containing proteins) were stored at -20°C.

j) ATP assay (ATP Colorimetric/Fluorometric Assay Kit, Biovision)

For the standard curve, diluted 10 µl of the ATP Standard with 90 µl of DW to generate 1 mMATP standard, mixed well. Added 0, 2, 4, 6, 8, 10 µl into a series of wells and adjusted volume to 50 µl with ATP Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of ATP. Added 50µl each of the standard and sample to the wells. Prepared a reaction mixture by thoroughly mixing 44 µl ATP assay buffer, 2 µl each of ATP converter, probe and developer for per well. Mix well. Add 50 µl reaction mixture to each well containing the ATP Standard and test samples. Samples were incubated at room temperature for 30 min., protected from light. Measured absorbance at OD 570 nm.ATP concentration in samples was calculated from the standard curve plotted OD vs ATP concentration of standards (Fig2 A)

k) Enzyme - linked immuno-absorbent assay for LDHC (Canine ELISA kit, G Bioscience)

Prepared all reagents, standard solutions and samples as per manufacturer's instructions. Added 50 µl standard to standard wells. Added 40 µl sample and 10 µl anti-LDHC to sample wells. A control without sample was also run. Then added 50 µl streptavidin-HRP to all wells except control. Covered the plate with a sealer and incubated for 60 min at 37°C. Removed the sealer and washed the plate with 300 µl wash buffer. Blotted the plate on absorbent paper and added 50 µl each of substrate A and B to each well. Covered the plate and incubated for 10 min at 37°C in the dark. Added 50 µl stop solution and absorbance was read at 450 nm in an ELISA reader (TECAN). LDHC concentration in samples was calculated from the standard curve plotted OD vs LDHC concentration of standards (Fig 2 B).

l) Cryopreservation of semen

Tris-citric acid-fructose buffer (TCF, Tris, 3.08 g; citric acid, 1.78 g; fructose, 1.25 g/100 ml, pH 7.2, gentamycin, 5mg) was prepared, filtered through 0.2-micron membrane filter and supplemented with 15% egg yolk plasma (EYP) and 7 % glycerol (G). TCFEYPG Extender was always prepared fresh and again filtered through 0.45-micron membrane filter. Semen of each dog exhibiting >70% motility was mixed with an extender in the ratio of 1:1 and equilibrated at 37°C for 10 min. The semen suspension was centrifuged at 1000 rpm for 3min and the loose pellet was suspended in the extender to get a final concentration of 200 x10^6 spermatozoa/ml. Extended semen was equilibrated at 37°C for 15 min. During this period, it was analysed for motility, viability, membrane/AI, IMMP, ATP, LDHC and *in vitro* capacitation/acrosome reaction. Then, filling and sealing of straws was done manually at room temperature (25 °C). Straws were kept at 4°C in cold handling cabinet for equilibration. Straws racked in the floating rack, equilibrated for 4 hrs were kept in LN2 vapours in a manual freezer for 10 min before plunging into liquid nitrogen. Semen was thawed after 24 hrs at 37 °C for 30 sec and analyzed for motility, viability, membrane/AI, IMMP, ATP and LDHC.

III. Results and Discussion

a) *Analysis of motility, viability, plasma membrane and acrosome integrity in freshly extended semen*

Values for motility, viability, PMI and AI varied from 66.7±4.4 – 83.3±1.7%, 72.9±1.6 – 89.5±1.5%, 60.6±1.8 – 87.6±1.6%, 66.5±3.2 – 90.0±1.4%, respectively among the dogs of four breeds (Table 1). All sperm attributes vary significantly ($P<0.05$) among the dogs irrespective of breed. Although number of dogs was not similar in all breeds, but still, significant ($P<0.05$) difference was observed among four breeds. Motility in normal canine semen is between 70-90% (Johnston et al. 2001; Ignee Ouada and verstegen, 2001). It has also been proposed that fertile dog should have at least 70% total sperm motility (Larson, 1980). It is also stated that good - quality canine semen should have at least 80% morphologically normal and viable spermatozoa (Johnston et al. 2001). Based on motility and viability, semen of 10 dogs out of 13 was of good - quality (Table 1).

HOST is considered as a suitable assay to test male fertility (Stanger et al. 2010; Baiee et al. 2017). PMI is essential for sperm fertilizing capacity (Rigsselaere et al., 2005). Sperm without a functionally intact membrane is defined as deteriorated and is not capable of fertilizing an ovum (Graham and Moce, 2005). Semen evaluation conducted on pooled fresh semen of 20 dogs of different breeds revealed average values of motility, viability and mitochondrial potential as 76.3%, 69% and 67.4% respectively (Abedin et al.2020). Contrary to their observation viability was higher in all the breeds, whereas PMI was higher in Labrador, Pug and lower in Pomeranian and German Shepherd breeds in the present study. However, values of motility in four breeds correspond to the observation of Abedin et al. 2020). The mean rates of progressive motility (87.2±5.0%) and viability (82.66±2.8%) observed in pooled semen samples of two German Shepherd, two Golden Retrievers and two Labrador dogs were similar to our observations on four separate breeds (Cheuqueman et al., 2012).

Earlier acrosome reaction make sperm infertile and hence, evaluation of AI in fresh as well as frozen-thaw semen before assisted reproduction procedure (Silva and Gadella, 2006) is essential. AI observed in Labrador (82.6%), Pug (75.8%) and German shepherd breeds (84.9%) separately was slightly lower than observed in pooled samples of different breeds (Veznik et al. 2003).

b) *Inner mitochondrial membrane potential, ATP and LDHC in freshly extended semen*

HIMMP, MIMMP, ATP and LDHC were in the range of 38.7±4.4-77.1±1.5%, 20.2±7.2-57.7±8.1, 6.6±1.4 -36.5±3.3 nM /10^9 spermatozoa and 5.2±0.8-30.9±1.3 μg/ 10^9 spermatozoa among the dogs of four breeds (Table 2). Mitochondrial membrane potential is another important indicator of sperm functionality. It is an indicator of sperm functionality that can be assessed using specific fluorescent markers (Volpe et al. 2009). IMMP showed marked variation (53-87%) in ejaculates of ten dogs (Volpe et al. 2009). We evaluated HIMMP and MIMMP separately in four breeds, a total of IMMP was 93.6%, 89.9%, 76.4 % and 95.6% in Labrador, Pug, Pomeranian and German Shepherd breeds, respectively. High inner mitochondrial membrane potential (80.9±17%) was also detected in pooled fresh spermatozoa of three breeds[6].

ATP content (nmol/10^8 spermatozoa) was in the range of 3.09±0.42-3.70±0.31 nmol/ 10^8 spermatozoa) in 30 ejaculates of 5 crossbreed dogs during four seasons of a year. However, ATP content measured separately in dogs of four breeds ranged from 0.66 nM-3.65 nM/ 10^9 spermatozoa (Chequema et al. 2011).

c) *Analysis of motility, viability and PMI and AI in post-thaw semen*

Values for motility, viability, PMI and AI ranged from 40.7±10.4-56.7±1.7%, 45.8±10.1-63.6±.8%, 17.6±7.3-60.9±1.6% and 32.9±11.0-68.3±1.9% in frozen thaw semen of 13 dogs irrespective of the breeds (Table 1). There was a significant ($P\leq0.05$) difference in motility, viability, PMI and AI among the dogs. Comparison of sperm attributes among the breeds also revealed a significant ($P\leq0.05$) difference though the number of dogs was not similar in four breeds. Motility, AI and PMI were 34.8±7.4% 49.7±4.7% in frozen thaw semen of two Shar-pei, one Labrador-Retriever, one Barret-Hound and one cross breed dogs cryopreserved in Tris-citric acid-fructose-egg yolk-Ethylene glycol extender (Oliviera et al., 2006). Pena et al. (1998) also observed post - thaw motility, viability and AI as 60.0±8.2%, 57.0±12.8% and 69.0±16% in pooled semen, cryopreserved in Tris-citric acid-fructose buffer-20 % egg yolk and 8% glycerol of different dog breeds. The difference in post - thaw sperm attributes in our study and previous studies may be due to the difference in extender used. Moreover, our study was focused on semen cryopreservation of individual dogs rather than pooled semen of different dog breeds.

d) *Analysis of inner mitochondrial membrane potential, ATP and LDHC in post - thaw semen*

HIMMP, MIMMP, ATP and LDHC were in the range of 17.8±8.3% – 45.0±1.6%) 30.4±54-52.2±3.3%, 2.9-32.5 nM/ 10^9 spermatozoa and 6.0±0.1-30.1±2.4 μg/10^9 spermatozoa in post – thaw semen of 13 dogs irrespective of the breed (Table 2). Difference in HIMMP, MIMMP, ATP and LD|HC were significant ($P<0.05$) among the dogs. A significant ($P<0.05$) difference was also evident among the breeds.

e) Effect of cryopreservation on sperm attributes

It has been indicated that even with optimized protocols, 40-50% of the sperm do not survive after cryopreservation due to irreversible damage[46]. Significant ($P<0.05$) difference was observed in motility, viability PMI, AI and HIMMP between FE and FT semen in all dogs, whereas in ATP, LDHC and MIMMP difference was non-significant ($P>0.05$). There was a loss of 21.7-36.7%, 20.9-36.6%, 20.3-53.6%, 18.9-43.9%, 16.1-36% in motility, viability, PMI, AI and HIMMP in post-thaw semen of 13 dogs (Table 1 & 2). Loss in PMI and AI was more in Pug breed than Labrador, Pomeranian and German Shepherd breeds. Percentage of spermatozoa that did not survive after freezing-thawing was <30% in post - thaw semen of Labrador dog breed (Cheema et al. 2020). A loss in ATP (0.6-35.2 nM/10^9 spermatozoa) and LDHC (0.3 – 16.8 μg/10^9 spermatozoa) content was also observed in post - thaw semen. A decline in HIMMP, motility and increase in MIMMP may be due to loss of ATP in the frozen thaw semen. Sperm motility is dependent on intracellular ATP content (Ford, 2006). Impairment of mitochondrial function is also associated with reduced sperm motility (Thomas et al. 1998 and Fraser et al.2002).

Recently Sicherle et al. (2020) observed the effect of cryopreservation on semen of five dogs of different breeds and concluded that total and progressive motility, PMI and IMMP suffered from the deleterious effects caused by cryopreservation.

f) In vitro capacitation and acrosome reaction in freshly extended semen

Three stages of acrosome reaction were observed i.e., swollen, vesiculated, partially sheded and completely sheded acrosome during incubation in canine TALP from zero to six hrs of incubation (Fig 3). Most of the altered spermatozoa were with swollen heads after two hrs of incubation. Spermatozoa with vesiculated, partially and completely sheded acrosome were observed only after 4 hrs of incubation. Hyperactivation (spermatozoa with progressive movement) started after 2 hrs of incubation. There was a significant percentage of cells with progressive movement at 2 - 4 hrs of incubation. Incubation of washed spermatozoa of 13 dogs in canine TALP medium for 6 hrs induced agradual average decline in motility, viability and MIMMP of Labrador, Pug, Pomeranian and German Shepherd breeds, respectively (Table 3 &4, Fig 4). Contrary to MIMMP, there was an increase in the percentage of HIMMP from 0 to 6 hrs incubation in Labrador, Pug, Pomeranian and German Shepherd breeds (Table 4, Fig5). The change in motility, viability, MIMMP and HIMMP were accompanied by an increase in the percentage of altered acrosome from 15.6/ 24.4/ 16.7/ 15.1% to 61.7/ 62.6/ 72.6/ 63.4% after 6 hrs of incubation in Labrador, Pug, Pomeranian and German shepherd breeds, respectively (Table 5, Fig6). But the percentage of average induced acrosome reaction (IAR) was 46.1 ± 2.7%, 37.3 ± 2.3%, 55.9 ± 7.2% and 48.3 ± 2.1% after six hrs of incubation (Fig 7). Significant ($P<0.05$) variation in change in motility, viability, HIMMP, MIMMP and IAR during incubation of spermatozoa at 37°C for 6 hrs was observed among the dogs and breeds. Similar to our observations, a decline in viability and increase in altered acrosome during incubation of dog spermatozoa in I-CCM medium for 4 hrs was also observed (Albracin et al. 2004). Percentage of acrosome reacted spermatozoa was higher at 6 hrs of incubation than 4 hrs. Significant increase in the percentage of acrosome reacted spermatozoa was induced by incubation with solubilized zona pellucida (Kawakami et al. 1993). They also believed that the percentage of acrosome reacted spermatozoa was higher at 7 hrs of incubation than 4 hrs. It suggested that more sperm become capacitated and could respond to induction of acrosome reaction as the incubation time increases. Since alteration in acrosome and motility pattern was observed at 2 hrs of incubation, therefore, it indicated that capacitation starts at this time in some of the spermatozoa. Increase in HIMMP and decline in MIMMP observed during the present study may be related to the hyperactivity of spermatozoa during incubation. Mitochondrial membrane potential has been described as one of the most sensitive parameters for evaluating sperm function. Its reduction indicated an imminent loss of sperm motility, fertility and survival in the female reproductive tract (Kasai et al. 2002 and Grunurald et al. 2008). The loss of potential is well known marker in somatic cells and related to an uncoupling of the electron transport chain for ATP synthesis and an increased generation of reactive oxygen species (ROS, Rajender et al. 2010). Spermatozoa themselves produce a small amount of ROS, which are essential to many physiological processes i.e., capacitation, hyperactivation and oocyte fusion. Low levels of ROS are also required for fertilization. Therefore, gradual increase in HIMMP during induction of acrosome reaction may be related to the maintenance of an adequate level of ROS.

g) Pearson correlation among the sperm attributes of freshly extended and frozen - thaw semen

Pearson's correlation coefficient among the sperm attributes of FE and FT semen is given in Tables 6. Positive and negative correlations were observed among the functional sperm attributes. Negative correlation is used to measure the amount that changes in one variable can affect an opposite change in another variable. A positive correlation is a relationship between two variables in which both variables move in the same direction. Therefore, positive correlation between motility, viability and AI/HIMMP/IAR in FE as well as FT demonstrated semen with a higher motility will have higher percentage of viable spermatozoa and

spermatozoa with intact acrosome, high membrane potential and induced *in vitro* acrosome reaction. A correlation of motility is also reported with acrosome reaction in dog (Cheuquema et al. 2011). PMI and good motility are also known to be highly correlated (Schafer Somi and Aurich, 2007). Several reports had also suggested that sperm mitochondrial function of sperm may also be a means to assess sperm motility and changes in IMM could be a good indicator of a functional status of spermatozoa (Volpe et al. 2009). A highly significant correlation between HIMM and motility is described in stallion (Love et al. 2003) and humans[25]. A negative correlation between motility/viability and MIMMP indicated that motility and viability of spermatozoa are more dependent of HIMMP. A strong correlation between PMI and HIMMP in FE and FT is supported by the finding of Volpe et al. 2009) Higher the percentage of combined HIMMP and MIMMP than motility and viability in FE and FT spermatozoa (Table 1,2), indicated that even immotile and dead spermatozoa have shown a HIMMP or MIMMP. These results are in accordance with the findings of Volpe et al. 2009). Contrary to our observations, there was no correlation between mitochondrial potential and motility in dog[27]. They used a DiCO2 probe to measure potential and suggested inhibition of ATP production would not be sufficient to reduce sperm motility.

A negative correlation between LDHC/ATP concentration and motility/viability/PMI/AI/HIMMP revealed that variation in LDHC/ATP concentration in the spermatozoa of different dogs has impacts on sperm function. Laudat et al. (1997) did not observe a correlation between seminal LDHC-4 concentration and motility. It is suggested that mature spermatozoa could oxidize lactate for energy source through LDHC-4 (Laudat et al. 1997).Energy produced by spermatozoa is necessary for motility. It may be perceived from this that although seminal LDHC-4 is not an indicator[22], but sperm LDHC4 may be a good indicator of sperm function. However, positive correlation between LDHC-4 and IAR revealed its relationship with capacitation. This observation is supported by the fact that disruption of LDHC gene results in sperm which cannot reach the capacitation site (Gavella et al. 1985). Negative correlation between ATP content and motility, viability, PMI, AI and HIMMP in FE and FT semen may be due to variation in sperm function among the dogs. Motility, viability, PMI, AI in post - thaw semen were> 45% in most of the dogs. Although, freezing-thawing process resulted in a significant ($P<0.05$) decline in sperm attributes, but decline in ATP content was non-significant ($P>0.05$). It may be due to the statement given by Calemera et al. (2010) that almost consistent level of ATP in FT semen represents the balance between biosynthesis and use of ATP. Although they thaw human semen at 40°C but thawing of dog semen was done at 37°C due to its more sensitivity for> 37°C temperature. ATP content of FE and FT spermatozoa is positively correlated with LDHC and IAR. It may be explained as only motile sperm may undergo capacitation and ATP is required to sustain the motility. A fully active glycolytic pathway is required for multiple steps in the fertilization cascade including capacitation-dependent tyrosine phosphorylation, hyperactivated motility and oocyte penetration (Travis et al. 2001, Goodson et al.2012; Odet et al., 2013; Tang et al., 2013). It is also established that hyperactive sperm (a distinct pattern of capacitated sperm) display an increase in energy demand requiring ATP ((Mujica et al., 1994). LDHC-4 is localized in the sperm principal piece, involved in sperm capacitation and facilitates the reversible conversion of pyruvate to lactate in glycolysis (Duan and Goldberg, 2003).

A positive correlation between motility x viability (0.96, $P\leq0.01$); motility x HIMMP (0.35); motility x IAR (0.045) and viability x HIMMP (0.22); viability x IAR (0.80) and HIMMP x IAR (0.080) was also detected during induced acrosome reaction in canine TALP medium. However, there was a negative correlation between motility x MIMMP (-0.287); viability x MIMMP (-0.265); HIMMP x MIMMP (-0.652, $P\leq0.05$) and MIMMP x IAR (-0.208) during induction of acrosome reaction. It indicated that rate of capacitation/ AR is associated to motility, viability and mitochondrial potential of spermatozoa. This study concluded that TCFEYPG extender was suitable for semen freezing of Labrador, Pug, German Shephard and Pomeranian dog breeds. It is suggested to perform multiple functional tests of FE/FT semen before selecting the dogs for breeding. Freezing of semen of individual dogs rather than pooled semen to achieve higher fertility rate is advised. Further, induced acrosome reaction may be an indicator of semen freezability.

Acknowledgements

Authors are thankful to Department of Biotechnology, Ministry of Science and Technology, New Delhi for providing the grant [SAN No.102/IFD/SAN/5331/2017–2018].

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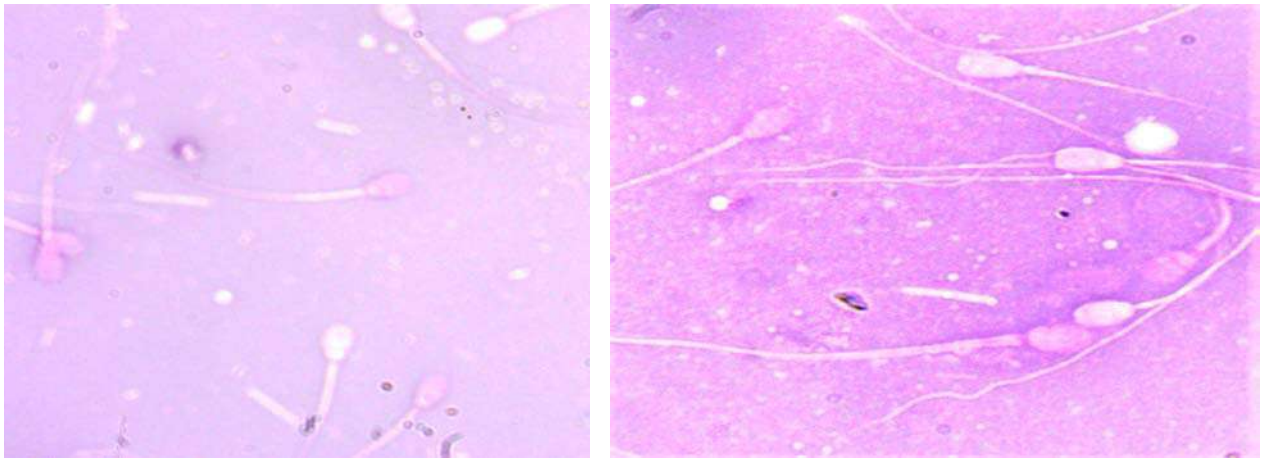
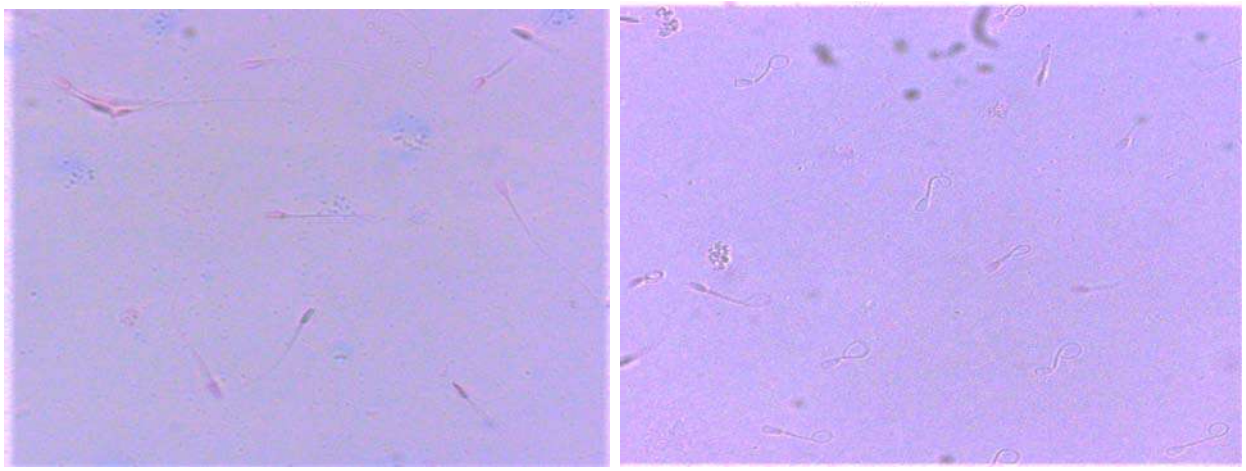
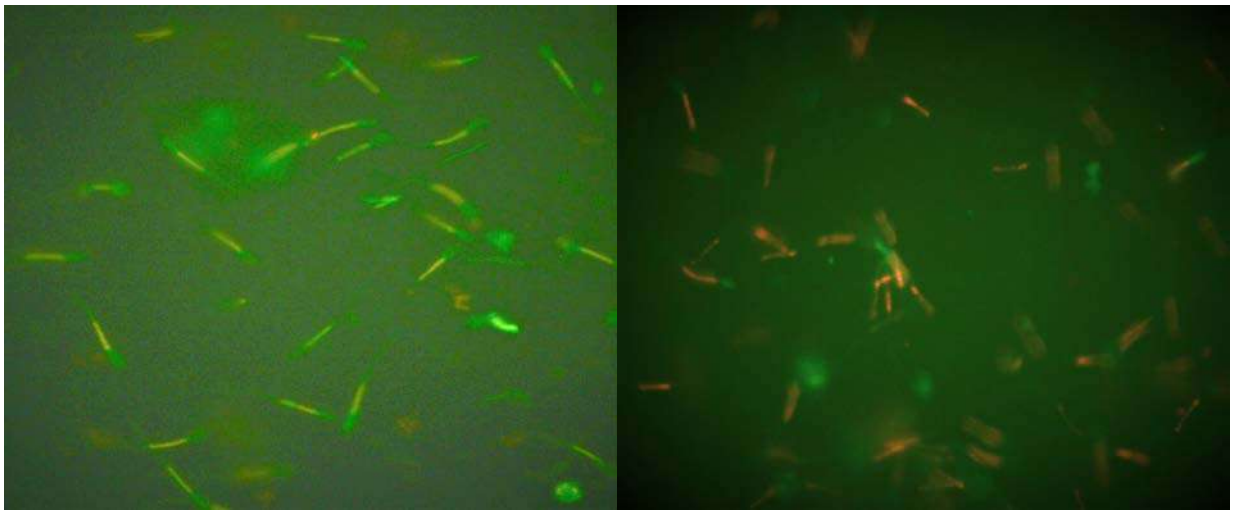


(A) Live (white) and Dead (pink) spermatozoa

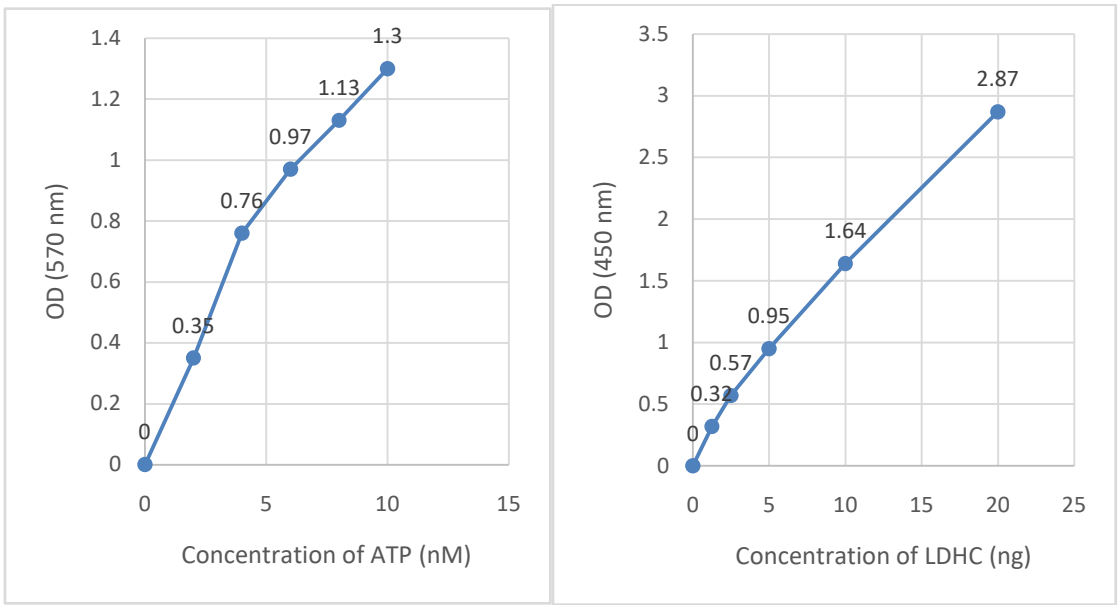


(B)Spermatozoa with straight tail (a) andcoiled tail (b)



(C) Green, yellow, orange-red fluorescence on mid piece indicate low, medium and high inner mitochondrial membrane potential.

Fig. 1: Viability: Eosin-nigrosine staining (A), plasma membrane integrity: Hypo-osmotic swelling test (B)and Inner mitochondrial potential: JC-1 (C) in canine spermatozoa.



(A) (B)

Fig. 2: Standard curve for estimation of ATP (A) and LDHC (B)

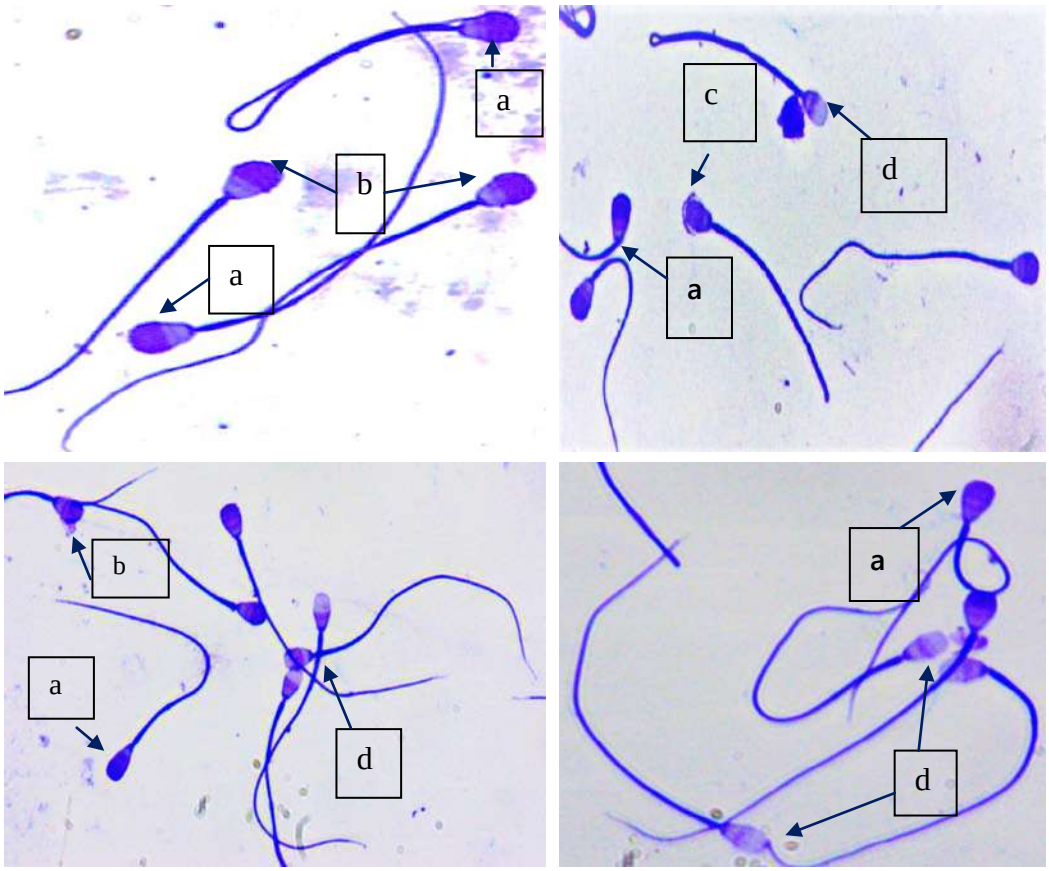


Fig. 3: Different stages of acrosome reaction in canine spermatozoa during incubation at 37°C in canine – TALP. a) Normal sperm, b) swollen and vesiculated acrosome, c) partially sheded acrosome, d) completely sheded acrosome.

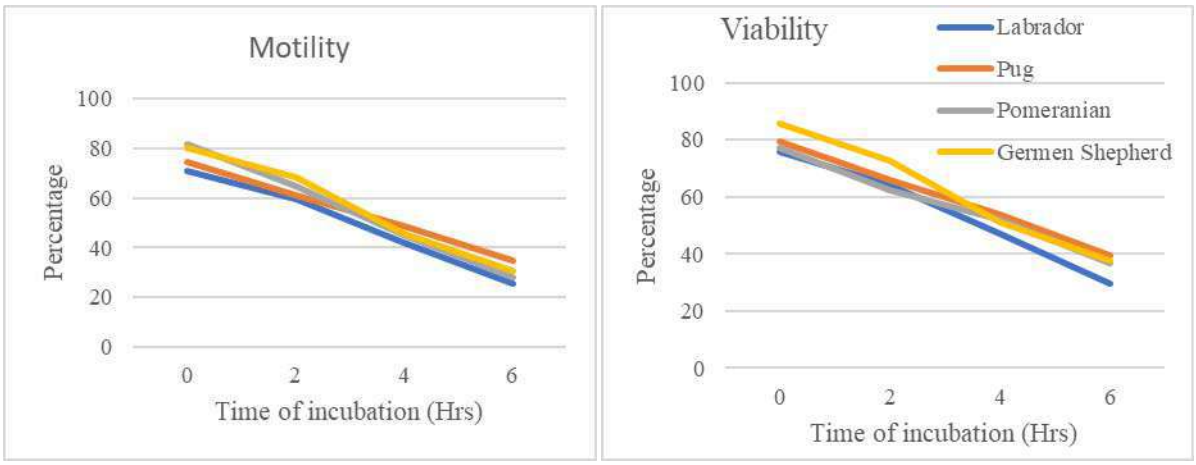


Fig. 5: Effect of incubation of spermatozoa in canine TALP medium on motility and viability.

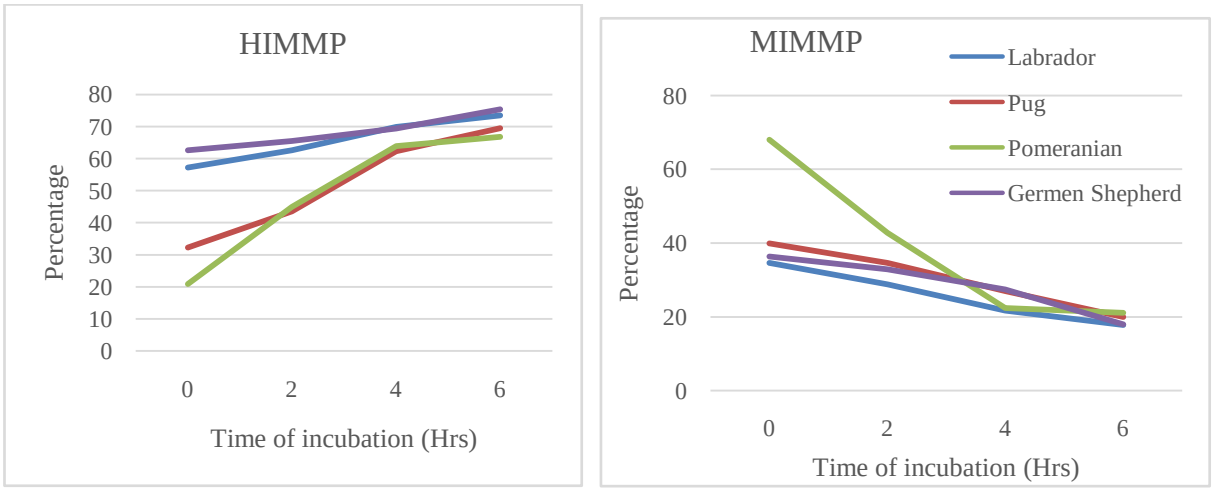


Fig. 6: Inner membrane mitochondrial membrane potential (IMMP). high IMMP (HIMMP) and medium IMMP (MIMMP).

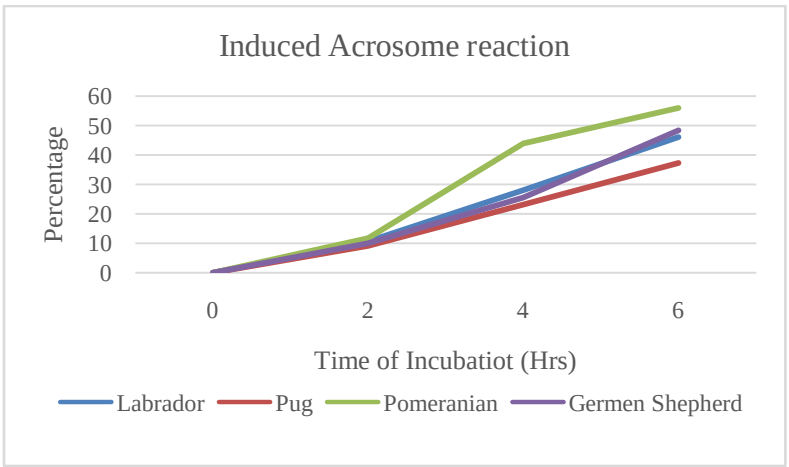


Fig. 7: Effect of incubation of spermatozoa in canine TALP medium on induced *in vitro* acrosome reaction.

Table 1: Motility, Viability, Plasma membrane- and Acrosomal integrity (Mean ± SE) in freshly extended and frozen-thaw semen of different dog breeds.

Dog breed/ No	Motility (%)		Viability (%)		Plasma Membrane Integrity (%)		Acrosome Integrity (%)	
	FE^{A}	FT^{B}	FE^{A}	FT^{B}	FE^{A}	FT^{B}	FE^{A}	FT^{B}
Labrador Retriever								
1	78.3^{ab} ±1.7	45.0^{b} ±5.0	83.6^{ab} ±2.4	50.4^{b} ±4.3	85.7^{a} ±0.5	53.0^{b} ±5.1	81.8^{ab} ±2.3	57.4^{c} ±2.6
2	78.3^{ab} ±1.7	41.7^{c} ±4.4	84.5^{ab} ±1.6	47.9^{ab} ±4.1	86.4^{a} ±1.7	47.2^{b} ±2.6	83.5^{ab} ±1.0	56.1^{c} ±3.3
3	68.3^{b} ±2.9	46.7^{b} ±1.7	72.9^{b} ±1.6	48.9^{ab} ±0.8	62.0 ±4.4	41.7^{c} ±1.6	66.5^{c} ±3.2	43.7 ±1.0
4	83.3^{a} ±2.9	45.0^{b} ±2.9	88.7^{a} ±1.1	50.2^{b} ±3.7	85.8^{a} ±0.7	51.3^{b} ±1.7	88.2^{a} ±1.0	61.8^{b} ±4.6
5	83.3^{a} ±1.7	56.7^{a} ±1.7	89.5^{a} ±1.5	63.6^{a} ±0.8	87.3^{a} ±1.8	60.9^{a} ±1.9	87.2^{a} ±2.2	68.3^{a} ±1.9
6	83.3^{a} ±1.7	55.0^{a} ±2.9	87.0^{a} ±1.5	60.0^{a} ±1.8	87.6^{a} ±1.6	59.3^{a} ±0.7	88.4^{a} ±4.2	64.8^{ab} ±2.7
Average	79.1^{C} ±2.1	48.3^{CD} ±2.6	84.3^{C} ±1.6	53.5^{CD} ±2.3	82.5^{C} ±1.8	52.2^{C} ±2.1	82.6^{C} ±2.3	58.6^{C} ±2.7
Pug								
1	78.3^{ab} ±1.7	53.3^{a} ±1.7	83.5^{ab} ±3.4	57.2^{ab} ±1.2	80.3^{ab} ±1.4	26.7^{e} ±2.1	81.3^{ab} ±4.1	53.8^{c} ±1.0
2	80.0^{a} ±5.0	43.3^{bc} ±1.7	82.2^{ab} ±6.0	47.9^{bc} ±1.0	73.9^{ab} ±1.7	21.3^{f} ±4.6	75.8^{b} ±4.5	40.4^{d} ±1.6
3	75.0^{ab} ±2.9	43.3^{bc} ±1.7	79.6^{ab} ±3.2	49.9^{bc} ±1.9	72.3^{ab} ±4.5	29.4^{e} ±6.5	76.8^{b} ±6.3	32.9^{e} ±11.0
4	66.7^{b} ±4.4	40.0^{c} ±10.4	76.9^{b} ±3.2	45.8^{c} ±10.1	77.6^{ab} ±5.3	17.6^{f} ±7.3	69.3^{c} ±3.8	45.3^{d} ±7.7
Average	75^{D} ±3.5	45.0^{D} ±4.5	80.5^{CD} ±3.9	50.2^{bD} ±3.5	76.0^{D} ±3.2	23.7^{E} ±5.1	75.8^{D} ±4.7	43.1^{D} ±5.3
Pomeranian								
1	72.9^{abD} ±4.3	46.7^{bD} ±1.7	73.4^{bD} ±5.9	49.2^{bcD} ±1.1	69.6^{bE} ±1.1	34.6^{dD} ±4.3	83.3^{abC} ±1.1	62.8^{bC} ±1.9
Germen Shepherd								
1	80.0^{a} ±2.9	53.3^{a} ±7.3	85.6^{ab} ±2.6	60.2^{ab} ±7.7	60.6^{c} ±1.8	35.7^{d} ±8.2	90.0^{a} ±1.4	60.6^{b} ±4.0
2	76.7^{ab} ±8.8	55.0^{a} ±2.9	81.0^{ab} ±8.4	60.1^{ab} ±3.1	65.6^{b} ±3.0	28.8^{e} ±4.4	79.8^{ab} ±3.9	65.2^{ab} ±6.0
Average	78.3^{C} ±5.8	54.1^{C} ±5.1	83.3^{CD} ±5.5	60.1^{C} ±5.4	63.1^{F} ±2.4	32.2^{D} ±6,.3	84.9^{C} ±2.6	62.9^{C} ±5.0

FE: Freshly extended semen and FT: Frozen thawed semen
Superscripts A and B indicate significant difference in sperm attributes in FE and FT semen
Superscripts C, D and E indicate significant difference among the breeds
Superscripts a, b, c, d, e and f indicate significant difference among the dogs irrespective of breed

Table 2: Inner mitochondrial membrane potential, ATP and LDHC concentration (Mean ± SE) in freshly extended and frozen-thaw semen of different dog breeds.

Dog breed/ No	HIMMP (%)		MIMMP (%)		ATP (nM/10^9 spermatozoa)		LDHC (ng/10^9 spwermatozoa)	
	FE ^{A}	FT^B	FE^A	FT^A	FE ^{A}	FT^A	FE ^{A}	FT^A
Labrador Retriever								
1	69.1^a ±1.9	42.8^{ab} ±1.8	24.1^e ±1.5	36.9^c ±2.5	26.8^b ±6.0	23.2^c ±2.5	13.2^c ±1.2	5.2^e ±0.8
2	72.2^a ±3.4	41.4^{ab} ±3.8	22.3^e ±4.6	34.3^c ±5.6	25.3^{bc} ±5.2	18.1^c ±4.6	14.3^c ±1.8	9.8^{de} ±0.2
3	55.3 ±2.1	32.8^{bc} ±1.4	28.3^{cd} ±2.0	34.9^c ±0.8	14.6^c ±1.3	13.6^d ±1.0	22.1^b ±0.4	6.2^e ±1.1
4	55.5 ±2.6	39.4^{ab} ±1.6	38.2^b ±2.8	35.1^c ±3.5	8.3^{de} ±0.6	7.7^d ±3.7	10.9^{de} ±0.6	6.9^d ±0.4
5	77.1^a ±1.5	45.0^a ±1.6	25.0^{de} ±1.9	33.6c ±2.3	13.8^d ±0.3	12.9 ±3.1	9.5^{cd} ±0.1	7.5^e ±0.5
6	74.6^a ±6.1	42.8^{ab} ±1.3	20.2^e ±7.2	34.1^c ±1.3	11.0^d ±2.3	2.9^e ±0.3	16.2^{bc} ±0.5	6.0^d ±0.1
Average	67.3^C ±2.9	40.6^C ±1.6	26.3^E ±3.3	34.8^D ±2.7	16.6^D ±2.6	13.0^D ±2.5	14.3^D ±0.8	6.9^B ±0.6
Pug								
1	71.1^a ±1.3	35.1^b ±3.4	22.0^e ±0.7	52.2^a ±3.3	11.3^c ±0.6	6.6^e ±1.4	7.3^e ±0.8	7.0^d ±0.1
2	48.7 ±6.1	28.1^c ±4.6	42.8^b ±7.3	43.0^b ±10.4	12.6^c ±0.8	11.7^d ±1.5	20.8^b ±0.4	18.7^{bc} ±1.4
3	60.1^b ±7.8	30.2^{cd} ±4.9	29.1^{cd} ±6.2	34.9^c ±2.0	25.9^b ±1.0	21.0^b ±1.0	21.6^b ±1.4	8.7^{cd} ±2.1
4	46.5^d ±7.1	25.9^d ±4.6	39.5^b ±5.1	30.4^c ±5.4	16.7^c ±1.0	12.5^d ±0.6	30.9^a ±1.3	15.1^c ±0.5
Average	56.6^D ±5.6	29.8^D ±4.3	33.3^{DE} ±4.8	40.1^C ±5.3	16.4^D ±0.8	12.9^D ±1.1	20.1^C ±1.0	12.4^B ±0.4
Pomeranian	18.7^{eE} ±4.4	17.8^{eE} ±8.3	57.7^{aC} ±8.1	41.2^{bC} ±4.7	29.4^{bC} ±3.0	24.3^{bCD} ±1.1	16.5^{cB} ±1.7	14.9^{cCD} ±1.3
Germen Shephard								
1	56.5^c ±1.4	31.3^{cd} ±1.1	41.5^b ±1.2	37.7^{bc} ±0.7	28.1^b ±4.6	24.8^b ±1.5	30.1^a ±2.4	14.4^c ±1.3
2	56.9^c ±2.7	39.0^{ab} ±1.0	36.4^c ±1.6	36.3^{bc} ±2.7	36.5^a ±3.3	32.5^a ±0.9	26.5^{ab} ±2.3	19.7^{bc} ±0.7
Average	56.7^D ±2.0	35.1^C ±1.0	38.9^D ±1.4	37.0^{CD} ±1.7	32.3^C ±3.9	28.6^C ±1.2	17.0^{CD} ±1.0	28.3^A ±2.3

FE: Freshly extended semen and FT: Frozen thawed semen
HIMMP: High inner mitochondrial membrane potential, MIMMP: Medium inner mitochondrial membrane potential, ATP: Adenosine triphosphate, LDHC: Lactate dehydrogenase C
Superscripts A and B indicate significant difference in sperm attributes in FE and FT semen
Superscripts C, D and E indicate significant difference among the breeds
Superscripts a, b, c, d, e and f indicate significant difference among the dogs irrespective of breed

Table 3: Effect of incubation time on motility, viability (Mean ± SE) of ejaculated spermatozoa incubated in canine-TALP medium of different dog breeds.

Dog No and Breed/ Time of incubation	Motility (%)				Viability (%)			
	0 H	2 H	4 H	6H	0 H	2 H	4 H	6H
Labrador								
1	78.3^{ab} ±1.7	68.3^{ab} ±1.3	45.0^{cd} ±2.9	30.0^{bc} ±2.9	83.6^{ab} ±2.4	73.9^{ab} ±2.1	51.2^{cd} ±3.1	34.1^{bc} ±3.3
2	63.3^{c} ±1.7	53.3^{c} ±1.3	30.0^{e} ±2.9	20.0^{d} ±2.9	68.5^{d} ±0.7	56.4^{c} ±2.1	34.8^{e} ±3.0	16.5^{e} ±6.6
3	66.7^{c} ±1.7	53.3^{c} ±2.7	38.3^{de} ±3.3	21.7^{d} ±6.7	70.9^{cd} ±1.3	58.7^{c} ±7.5	42.3^{d} ±3.0	25.0^{cd} ±6.9
4	68.3^{c} ±1.7	60.0^{bc} ±2.3	43.3^{cd} ±7.3	21.7^{d} ±7.3	71.9^{cd} ±1.3	64.7^{b} ±2.1	45.4^{d} ±5.0	27.6^{c} ±8.4
5	83.3^{a} ±2.9	73.3^{a} ±1.3	63.3^{a} ±1.7	43.3^{a} ±1.7	89.5^{a} ±1.5	78.9^{a} ±1.5	70.3^{a} ±2.7	49.9^{a} ±2.0
6	66.7^{c} ±1.7	50.0^{c} ±2.3	33.3^{e} ±1.7	18.3^{d} ±3.3	71.7^{cd} ±2.7	53.1^{c} ±2.7	38.0^{e} ±1.1	23.5^{d} ±3.2
Average	71.1^{A} ±1.9	59.7^{B} ±1.9	42.2^{C} ±4.1	25.8^{D} ±4.1	75.9^{A} ±1.4	64.3^{B} ±3.0	47.0^{C} ±3.0	29.4^{D} ±5.1
Pug								
1	78.3^{ab} ±1.7	68.3^{ab} ±4.4	56.7^{b} ±6.0	38.3^{ab} ±7.3	83.4^{ab} ±3.1	73.1^{ab} ±5.3	62.8^{b} ±6.8	45.4^{a} ±7.6
2	73.3^{b} ±4.4	61.7^{bc} ±3.3	48.3^{c} ±1.7	40.0^{ab} ±2.9	79.8^{ab} ±4.7	65.8^{b} ±3.7	53.6^{c} ±1.9	43.2^{ab} ±2.7
3	75.0^{b} ±2.9	53.3^{c} ±6.7	43.3^{cd} ±4.4	26.7^{c} ±6.0	79.6^{ab} ±3.1	60.1^{bc} ±6.7	48.1^{d} ±4.8	30.3^{c} ±6.2
4	71.7^{bc} ±4.4	61.7^{bc} ±4.4	46.6^{c} ±3.3	33.3^{b} ±6.7	75.7^{c} ±3.8	65.8^{b} ±3.6	51.5^{cd} ±3.7	39.6^{b} ±6.6
Average	74.6^{A} ±2.6	61.2^{B} ±4.7	48.7^{C} ±3.8	34.6^{D} ±5.7	79.6^{A} ±2.5	66.2^{B} ±4.8	54.0^{C} ±4.3	39.6^{D} ±5.8
Pomeranian	81.7^{aA} ±1.7	65.0^{bB} ±2.9	45.0^{cC} ±2.9	28.3^{cD} ±1.7	77.1^{cA} ±1.3	62.6^{bB} ±5.6	52.0^{cC} ±3.4	37.0^{bD} ±3.2
Germen Shephard								
1	80.0^{a} ±2.9	66.7^{ab} ±1.7	38.3^{d} ±4.4	28.3^{c} ±6.7	85.5^{ab} ±2.6	70.5^{b} ±1.6	46.9^{d} ±2.8	34.6^{bc} ±5.7
2	80.0^{a} ±5.0	70.0^{ab} ±2.9	53.3^{bc} ±4.4	33.3^{b} ±6.0	86.0^{ab} ±4.3	74.8^{ab} ±1.6	55.2^{c} ±1.5	40.4^{ab} ±5.3
Average	80.0^{A} ±3.9	68.3^{B} ±2.3	45.8^{C} ±4.4	30.8^{D} ±6.3	85.7^{A} ±3.4	72.6^{B} ±1.6	51.0^{C} ±2.1	37.5^{D} ±5.5

Superscripts A, B, C and D indicate significant difference in motility and viability among incubation periods
Superscripts a, b, c, d and e indicate significant difference among the dogs irrespective of breed

Table 4: Effect of incubation time on Inner mitochondrial membrane potential (Mean ± SE) of ejaculated spermatozoa incubated in canine-TALP medium of different dog breeds.

Dog No and Breed/ Time of incubation	HIMMP (%)				MIMMP (%)			
	0 H	2 H	4 H	6H	0 H	2 H	4 H	6H
Labrador Retriever								
1	69.1^{b} ±1.9	72.9^{b} ±2.3	79.2^{ab} ±0.9	81.4^{ab} ±0.8	24.1^{e} ±1.5	18.0^{c} ±1.2	12.8^{d} ±1.2	10.4^{c} ±1.4
2	60.9^{bc} ±3.0	64.8^{c} ±2.3	70.0^{b} ±1.7	72.3^{b} ±1.2	30.8^{d} ±3.8	29.2^{b} ±2.7	27.4^{ab} ±3.9	21.0^{ab} ±0.8
3	56.2^{cd} ±1.4	60.0^{c} ±1.1	65.7^{bc} ±1.6	70.2^{b} ±1.4	25.2^{e} ±1.8	22.1^{bc} ±1.3	19.0^{cd} ±1.0	16.0^{b} ±1.4
4	37.6^{e} ±5.7	41.5^{e} ±5.0	54.7^{d} ±4.5	62.6^{c} ±2.9	46.9^{c} ±3.6	40.3^{a} ±3.7	29.9^{ab} ±4.1	25.3^{a} ±3.2
5	77.1^{a} ±1.5	83.4^{a} ±1.2	85.3^{a} ±1.5	87.5^{a} ±1.0	26.2^{d} ±2.0	22.4^{bc} ±1.3	16.8^{cd} ±1.0	13.9^{bc} ±1.0
6	42.2^{d} ±2.9	53.0^{d} ±2.0	64.8^{bc} ±4.7	67.0^{bc} ±3.3	54.3^{b} ±2.9	40.9^{a} ±3.6	24.4^{b} ±8.0	20.5^{ab} ±5.4
Average	57.2^{D} ±2.7	62.6^{C} ±2.3	$69.^{B}$ ±2.5	73.5^{A} ±1.8	34.6^{A} ±2.6	28.8^{B} ±2.3	21.7^{C} ±3.2	17.8^{C} ±2.2
Pug								
1	57.9^{c} ±9.8	61.3^{c} ±9.4	68.5^{bc} ±7.0	73.6^{b} ±6.9	32.2^{d} ±9.3	28.2^{b} ±8.9	22.5^{b} ±6.2	15.7^{b} ±4.6
2	45.5^{d} ±3.1	52.5^{d} ±4.2	61.0^{c} ±3.9	68.4^{bc} ±3.2	46.1^{c} ±6.4	38.3a ±7.3	29.8^{ab} ±4.5	23.6^{ab} ±6.1
3	49.7^{d} ±9.0	56.1^{d} ±7.7	64.9^{bc} ±4.7	71.8^{b} ±9.7	41.7^{cd} ±9.0	35.8^{ab} ±8.6	28.2^{ab} ±4.9	18.1^{ab} ±4.2
4	40.8^{e} ±2.2	48.7^{e} ±3.6	54.8^{d} ±2.9	64.1^{c} ±3.7	39.8^{cd} ±0.6	36.3^{ab} ±0.7	27.7^{ab} ±2.6	22.7^{ab} ±1.7
Average	48.4^{D} ±6.0	54.6^{C} ±6.2	62.3^{B} ±4.6	69.5^{A} ±4.1	39.9^{A} ±6.3	34.6^{B} ±6.4	27.0^{C} ±4.5	20.0^{D} ±4.1
Pomeranian	20.9^{fC} ±4.5	44.9^{eB} ±8.4	63.9^{bcA} ±2.2	66.8^{bcA} ±2.4	68.0^{aA} ±6.5	42.8^{aB} ±10.0	22.4^{bC} ±7.0	21.1^{abC} ±6.6
Germen Shephard								
1	58.7^{c} ±2.6	61.9^{c} ±1.4	65.3^{bc} ±3.2	74.5^{b} ±2.8	39.1^{cd} ±2.7	36.2^{ab} ±1.4	32.6^{a} ±3.7	20.2^{ab} ±3.1
2	66.5^{b} ±2.9	69.1^{a} ±2.5	73.6^{b} ±3.6	76.3^{b} ±2.9	33.7^{e} ±1.9	29.6^{b} ±2.3	22.4^{b} ±3.2	15.9^{b} ±1.8
Average	62.6^{A} ±2.7	65.5^{A} ±1.9	69.4^{B} ±3.4	75.4^{C} ±2.8	36.4^{A} ±2.3	32.9^{A} ±1.8	27.5^{B} ±3.4	18.0^{C} ±2.4

Superscripts A, B, C and D indicate significant difference in high inner mitochondrial membrane potential (HIMMP) and medium IMMP (MIMMP)among incubation periods.
Superscripts a, b, c, d and e indicate significant difference among the dogs irrespective of breed

Table 5: Effect of incubation time on induced acrosome reaction (Mean ± SE) of ejaculated spermatozoa incubated in canine-TALP medium of different dog breeds.

Dog No/ Breed	Time of incubation (Hrs)			
	Abnormal acrosome (%)	Induced Acrosome reaction (%) (Total abnormal acrosomes-Abnormal acrosomes at zero hour)		
	0	2	4	6
[ab] Labrador Retriever				
1	$18.2^{bc}\pm2.3$	$9.2^{b}\pm3.6$	$35.1^{b}\pm2.7$	$47.7^{b}\pm1.0$
2	$16.5^{bc}\pm1.0$	$8.0^{b}\pm0.3$	$34.8^{b}\pm5.1$	$51.0^{a}\pm2.4$
3	$34.5^{a}\pm3.2$	$9.4^{b}\pm0.7$	$24.3^{cd}\pm5.2$	$39.9^{c}\pm4.5$
4	$11.8^{c}\pm1.0$	$11.6^{ab}\pm2.1$	$28.5^{c}\pm6.7$	$38.6^{c}\pm4.1$
5	$12.8^{c}\pm2.2$	$11.8^{ab}\pm2.0$	$45.2^{a}\pm1.7$	$55.5^{a}\pm0.6$
6	$11.6^{c}\pm4.2$	$14.2^{a}\pm3.0$	$33.1^{b}\pm4.2$	$43.8^{bc}\pm3.4$
Average	15.6 ± 2.3	$10.7^{C}\pm1.9$	$28.0^{B}\pm4.3$	$46.1^{A}\pm2.7$
Pug				
1	$19.7^{bc}\pm4.1$	$10.8^{ab}\pm3.5$	$20.2^{d}\pm3.0$	$34.9^{cd}\pm2.3$
2	$24.2^{b}\pm4.5$	$11.0^{ab}\pm0.4$	$28.6^{c}\pm1.9$	$44.0^{bc}\pm3.0$
3	$23.2^{b}\pm6.3$	$8.5^{b}\pm2.2$	$23.9^{cd}\pm1.5$	$39.5^{c}\pm2.2$
4	$30.7^{ab}\pm3.8$	$6.2^{c}\pm0.5$	$20.2^{a}\pm2.3$	$30.7^{d}\pm1.8$
Average	24.4 ± 4.7	$9.1^{C}\pm1.6$	$23.2^{B}\pm2.2$	$37.3^{A}\pm2.3$
Pomeranian	$16.7^{bc}\pm1.1$	$11.7^{ab}\pm3.1$	$43.8^{a}\pm3.8$	$55.9^{a}\pm7.2$
Germen Shepherd				
1	$10.0^{c}\pm1.4$	$15.6^{a}\pm6.1$	$24.4^{cd}\pm3.8$	$51.5^{a}\pm1.7$
2	$20.2^{bc}\pm3.9$	$6.5^{c}\pm0.8$	$26.6^{c}\pm7.1$	$45.1^{b}\pm2.5$
Average	15.1 ± 2.6	$10.0^{C}\pm3.4$	$25.5^{B}\pm5.4$	$48.3^{A}\pm2.1$

Superscripts A, B, C and D indicate significant difference in acrosome reaction among incubation periods
Superscripts a, b, c and dindicate significant difference among the dogs irrespective of breed

Table 6: Correlation coefficient among different sperm quality traits of freshly extended and frozen-thaw semen.

Sperm attribute	Mot	Via	PMI	AI	HIMMP	MIMMP	ATP	LDHC	IAR
Mot	1								
Via	0.91 (0.97**)	1							
PMI	0.51 (0.35)	0.62** (0.38)	1						
AI	0.84** (0.66*)	0.77** (0.65*)	.38 (0.60*)	1					
HIMMP	0.52 (0.41)	0.69** (0.50)	0.54 (0.73*)	-0.28 (0.45)	1				
MIMMP	-0.29 (0.22)	-0.44 (0.10)	-0.53 (0.35)	-0.06 (-004)	-.938** (-0.24)	1			
ATP	-0.11 (0.11)	-.193 (0.11)	-0.31 (0.22)	-.001(0.26)	-.05 (-0.16)	).002 (0.05)	1		
LDHC	-0.55 (0.03)	-0.46 (0.01)	-0.71** (0.64)	-0.43 (0.02)	-0.41 (-0.50)	.43 (0.05)	0.001(0.452)	1	
IAR	0.39 (0.33)	0.20 (0.34)	-.010 (0.48)	0.56 (0.55)	-.05 (0.12)	.185 -0.05)	0.02 (0.46)	0.21 (0.15)	1

Figures in parentheses are of frozen thaw semen
**. Correlation is significant at the 0.05 level (2-tailed).*
***. Correlation is significant at the 0.01 level (2-tailed).*
ATP: Adenosine triphosphate, LDHC: Lactate dehydrogenase C, IAR: Induced acrosome reaction (Freshly extended semen), Mot: Motility, Via: Viability, PMI: Plasma membrane integrity, AI: Acrosome integrity, HIMMP: High mitochondrial membrane potential, MIMMP: Medium mitochondrial membrane potential

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Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.

6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

Criterion for Grading a Research Paper (Compilation) by Global Journals

Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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Global Journal of Medical Research

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ISSN 9755896