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Analysis of biomarkers for prediction of idiopathic infertility in Large White Yorkshire boar (*Sus scrofa domesticus*)

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ABSTRACT

The experiment was conducted to analyze biomarkers like *p*H, calcium (Ca^{2+}), cholesterol (Ch), estradiol (E_2) and vitamin D (Vit-D) in the serum for prediction of idiopathic infertility and its early amelioration. pH of epididymal luminal fluids (ELF) and concentrations of Ca^{2+}, Ch, E_2 and Vit-D were measured in serum, as well as in epididymal luminal fluids (ELF) and sperm cytosolic fluids (SCF) from caput, corpus and cauda epididymis of healthy, mature Large Yorkshire breed of boar (n=100). The levels of these bio-molecular components in the serum, in ELF and SCF from different epididymal segments were used statistically to develop a Curve Fit Regression Equations and a prediction equation to predict the quantum of these bio-molecules in the ELF and SCF by estimating their levels in the serum which would have been very difficult otherwise to be estimated in live animals. Further, from the clinical point of view, present findings about the spermatozoa maturation can pave the way for development of specific marker(s) that will assist in the development of new arena for both the prediction and early diagnosis of male infertility and for improving treatment modalities of male infertility, since epididymal dysfunctions are related to cases of idiopathic male infertility.

Key words: Biomolecules, Curve fit regression equations, Epididymal lumen fluid (ELF), Sperm cytosolic fluid (SCF)

The spermatozoa produced at the output of the testes are although morphologically complete but still non-functional and immature. They acquire their physiological functions, i.e. motility and the ability to fertilize an ova upon reaching the end of the epididymis (Robaire *et al.* 2006). This sperm maturation involves morphological and biochemical changes in the sperm surface in response to the epididymal secretions of enzymes, proteins, lipids, glycoproteins, hormones and vitamins which are essential in the process of fertilization (Orgebin-Crist 1967; Robaire *et al.* 2000 and Robaire *et al.* 2006). Among many pivotal factors served by the luminal microenvironment, this study was designed to investigate certain factors essential in initiation of sperm motility like *p*H, Ca^{2+}, Ch, E_2 and Vit-D_3 in serum and the epididymal segments.

Male infertilities are mostly considered idiopathic (Sullivan 2004, Cornwall 2009) that may reflect sperm maturational disorders. These disorders may be due to post-testicular defects where the male gametes are normal in their morphology but with poor motility and fertilizing ability. Thus, lack of optimum levels of these biomolecules in the epididymal lumen fluid (ELF) and sperm cytosolic fluid (SCF) may be the cause of such idiopathic infertility in males. However, as it is very much difficult to estimate the levels of these bio-molecules in ELF and SCF from live animals.

So, the main objective was to establish the relationship(s) statistically between the levels of these bio-molecular components in the serum with that of ELF and SCF from different epididymal segments so as to predict their quantum in the ELF and SCF by estimating their levels in the serum. Thus, these bio-molecular constituents in serum can serve as bio-markers and any deviation in their values from the standard calculated range can be useful to diagnose the cause of male infertility and accordingly, appropriate treatment modalities could be suggested to ameliorate the cause(s) of infertility beforehand.

Besides, as the genome evolutionary distance between porcine and the human being is smaller (Wernersson *et al.* 2005) the present research has been carried out on Large Yorkshire breed of pigs as a model and the findings in pigs can be useful in understanding the interactions of the bio-molecules in human and helpful for developing marker for early detection of male infertility.

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MATERIALS AND METHODS

The blood samples were collected from the ear vein of healthy, reproductively mature Large White Yorkshire (LWY) boar of around 3–4 years of age (n=100) maintained at Pig Farm, Government of West Bengal, Haringhata, Nadia, West Bengal, India, before slaughtering at Meat Plant, Haringhata between April, 2015 to November, 2016. Serum samples were kept in different aliquots, labeled and stored at –20°C.

Both the testis along with epididymis (Fig.1.) were collected from slaughter house from the same pigs and transferred in sterile containers with ice-cold (5ºC) 0.15 M phosphate buffered saline (*PBS, p*H *7.4*). The epididymis from the testis-epididymis complex were separated, three segments, viz. caput, corpus, cauda of epididymis were ligated separately and dissected out (Fig. 1). Further, dissected parts were labeled properly and stored in containers with 0.15 M PBS at 5°C in a refrigerator until processed.

Epididymal fluids containing the spermatozoa from each segment of epididymis were obtained by mincing method (Frenette *et al.* 2002 and Hori *et al.* 2015) and then *p*H of the luminal fluids of each segment were measured using digital *p*H meter (HI-1093B *p*H electrode, Hanna Instruments).

The ELF from each segment of the epididymis salvaged was centrifuged at 4°C at 700 g for 5 minutes to remove spermatozoa (Frenette *et al.* 2002). The supernatant was again centrifuged at 4°C at 18,000 g for 20 min to remove the remaining cellular debris (Wales *et al.* 1966) and the ELF were stored in properly labeled micro-centrifuge tubes. The spermatozoa thus separated were then sonicated by using an ultrasonicator (Sonics, Vibra-Cell, USA) for separation of SCF (Vijayaraghavan *et al.* 1996). The sonicated materials were further centrifuged at 4°C at 16,000 g for 10 minutes (Vijayaraghavan *et al.* 1996) and supernatant containing the total cell lysate were separated and filtered with membrane filter. SCF were carefully aliquoted in properly labeled micro-centrifuge tubes and were kept at –20ºC till experimentation.

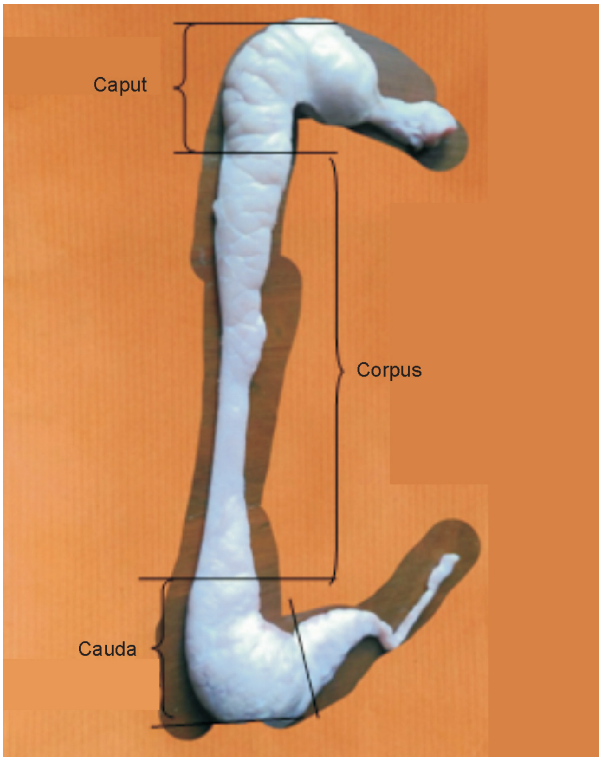

Fig. 1. Parts of boar epididymis

Calcium in the serum and other fluids were estimated using Calcium Assay Kit (Coral clinical systems) through a direct colorimetric assay based on the o-Cresolphtalein Complexone (OCPC) method without deproteinization of the samples (Gitelman 1967 and Baginski 1973).

Cholesterol (Ch) in the serum and other fluids were estimated by using Cholesterol Assay Kit (Coral clinical systems) which was based on CHOD-PAD method (Flegg 1973 and Allain *et al.* 1974).

17 β-Estradiol (E_2) in the fluids were estimated using 17β-Estradiol ELISA Kit (Make-IBL International GmbH) (Hall 1988 and Siiteri *et al.* 1982).

1, 25 dihydroxy-vitamin-D_3 is the metabolically active form of vitamin-D (Vit-D_3) but the concentration of its precursor, 25-HydroxyVitamin D_3 is considered as the best indicator of Vitamin-D status in animals (Arnold *et al.* 2015). Its concentration was measured in serum and ELF, SCF following the protocol (Holick 2009 and Bikle 2010) using 25(OH)D_3 ELISA Kit (Calbiotec, USA).

Analysis of data was done by using SPSS (Windows version 23.0) software. Descriptive statistics and General Linear Model (*GLM*) were used to analyze the data. Pearson's bivariate correlation method was used to find out correlation coefficients. The means were compared using Duncan Multiple Range tests (Duncan 1995). The prediction equations were developed by using curve fit regression equations to establish relationship(s) of the levels of the bio-molecular components, viz. *p*H, Ca^{2+}, Ch, E_2 and Vit-D_3 in the serum with that of ELF and SCF from different epididymal segments of LWY boar.

RESULTS AND DISCUSSION

The post-gonadal stages of sperm differentiation are set up by successive modifications that occur when the gamete transit to specific parts of the epididymal tubule. At the same time, outside gametes, composition of the luminal epididymal environment also changes sequentially throughout the epididymis.

Spermatozoan motility from the epididymal regions: In boar, motility is an important indication of spermatozoan maturation in the epididymis as described by Dacheux *et al.* (1979), therefore, a subjective evaluation of spermatozoan motility was studied from different segments of epididymis as an indicator for sperm maturation in epididymis. Spermatozoa retrieved from three different regions of the pig epididymis (n= 100) revealed that spermatozoan motility was highest in the cauda segment followed by corpus and

Table 1. Spermatozoan motility (%) and *p*H in ELF; concentration of calcium, cholesterol, vitamin-D and estradiol in serum, ELF and SCF from different regions of pig epididymis

Parameter	Blood serum (Mean±SE)	Epididymal luminal fluid (ELF) (Mean±SE)			Sperm Cytosolic fluid (SCF)(Mean±SE)		
		Caput	Corpus	Cauda	Caput	Corpus	Cauda
Motility (%)	–	0	15.82±0.24^{a}	90.95±0.80^{b}			
*p*H	–	7.16^{a}±0.009	6.79^{b}±0.01	6.55^{c}±0.01	–	–	–
Calcium (mg/dl)	10.54^{c}±0.43	8.39^{cd}±0.55	5.32^{de}±0.42	2.45^{e}±0.27	36.70^{a}±2.40	24.52^{b}±1.39	9.11^{c}±0.24
Cholesterol (mg/dl)	49.22^{ed}±3.51	55.54^{d}±2.50	38.68^{e}±1.38	22.20^{f}±1.99	196.53^{a}±6.65	158.86^{b}±5.12	89.48^{c}±4.23
Vitamin-D_3(ng/ml)	45.08^{b}±2.14	20.47^{e}±0.98	32.67^{d}±0.97	47.70^{ab}±1.73	28.65^{d}±1.59	39.74^{c}±1.60	51.38^{a}±1.31
Estradiol (pg/ml)	331.73^{e}±20.73	780.56^{b}±44.73	521.65^{d}±44.43	207.73^{f}±9.94	981.93^{a}±54.60	621.77^{c}±43.47	377.21^{e}±20.46

$^{a\text{-}f}$Similar superscripts does not differ significantly (P<0.05).

motility was found absent in caput region (Table 1).

p*H of ELF from the epididymal regions:* The activation of sperm motility occurs in response to changes in the external medium. Among the external factors, *p*H of ELF and sperm internal *p*H (pH_i) seem to play a pivotal role in sperm physiology in invertebrates, lower vertebrates and to some extent in mammals. The mean (±SE) *p*H of ELF from caput, corpus and cauda measured and are represented in Table 1 and the *p*H values of ELF ranged between 7.0–7.2 in caput, 6.7–6.9 in corpus and 6.4–6.6 in cauda.

Calcium levels in serum, ELF and SCF from the epididymal regions: The mean (±SE) concentrations of Ca^{2+} in serum and from ELF and SCF from different epididymal regions are mentioned in Table 1, and their concentrations varied between 6.8–14.7 in serum, 3.8–13.8 in caput ELF, 2.0–8.2 in corpus ELF, 0.4–4.6 in cauda ELF, 20.7–56.0 in caput SCF, 15.9–37.6 in corpus SCF and 6.0–10.7 in cauda SCF. The wider range observed was due to the individual variations.

Cholesterol levels in serum, ELF and SCF from the epididymal regions: The mean (±SE) concentration of cholesterol in serum was 49.22±3.51 mg/dl, whereas from different epididymal regions ELF and SCF cholesterol concentrations are represented in Table 1 and their concentrations varied between (14.7–65.9) in serum, 38.7–74.7 in caput ELF, 27.7–51.2 in corpus ELF, 13.3–43.4 in cauda ELF, 132.8–261.8 in caput SCF, 102.7–202.5 in corpus SCF and 51.0–118.9 in cauda SCF. The wider range observed was due to the variations in individual animals.

VD levels in serum, ELF and SCF from the epididymal regions: The mean±SE concentration of VD in serum was 45.08±2.14 ng/ml, whereas from different epididymal regions in ELF and SCF VD values are represented in Table 1 and their concentrations varied between 23.5– 57.8 in serum, 10.8–27.3 in caput ELF, 20.5–37.8 in corpus ELF, 26.2–55.3 in cauda ELF, 14.7–41.8 in caput SCF, 22.2–46.3 in corpus SCF and 39.7–59.1 cauda SCF. The wider range observed was due to the individual variations in animals.

Table 2. Prediction equations to estimate Sperm motility, pH and concentrations of Ca^{2+}, Ch, Vit-D and E_2 in caput (Cap), corpus (Cor) and cauda (Cau) ELF from serum (Sr.) levels of Ca, Ch, Vit-D and or E_2

Prediction value of	Prediction Equations	R^2
ELF cap *p*H	$7.23^{a} – (0.015^{b} \times A) + (0.003^{b} \times B) – (0.002^{b} \times C)$	0.932
ELF cap Ca^{2+}	$9.2^{a} – (0.1^{b} \times A) + (0.065^{b} \times B) – (0.113^{b} \times C) + (0.006^{b} \times D)$	0.947
ELF cap Chol	$51.51^{a} + (2.079^{b} \times A) + (0.406^{b} \times B) – (0.519^{b} \times C) – (0.044^{b} \times D)$	0.965
ELF cap Vit-D	$3.53^{a} + (0.202^{b} \times A) + (0.002^{b} \times B) + (0.404^{b} \times C) – (0.011^{b} \times D)$	0.973
ELF cap E_2	$1212.31^{a} – (14.406^{b} \times A) + (2.39^{b} \times B) – (16.91^{b} \times C) + (0.32^{b} \times D)$	0.956
Sperm Motility (Cor)	$22.673^{a} – (0.188^{b} \times A) – (0.022^{b} \times B) – (0.032^{b} \times C) – (0.007^{b} \times D)$.	0.949
ELF cor *p*H	$6.226^{a} + (0.026^{b} \times A) + (0.001^{b} \times B) + (0.001^{b} \times C)$	0.907
ELF cor Ca	$–2.845^{a} + (0.745^{b} \times A) + (0.049^{b} \times B) – (0.010^{b} \times C) – (0.005^{b} \times D)$.	0.933
ELF cor Chol	$47.439^{a} + (0.665^{b} \times A) + (0.020^{b}$ x B) – $(0.421^{b} \times C) + (0.007^{b} \times D)$.	0.975
ELF cor Vit-D	$23.084^{a} – (1.270^{b} \times A) + (0.138^{b} \times B) + (0.366^{b} \times C) – (0.001^{b} \times D)$	0.967
ELF cor E_2	$1212.31^{a} – (14.406^{b} \times A) + (2.393^{b} \times B) – (16.919^{b} \times C) + (0.32^{b} \times D)$	0.956
Sperm Motility (cau)	$82.572^{a} – (0.573^{b} \times A) – (0.047^{b} \times B) + (0.284^{b} \times C) + (0.012^{b} \times D)$	0.930
ELF cau *p*H	$6.226^{a} + (0.026^{b} \times A) + (0.001^{b} \times B) + (0.001^{b} \times C)$	0.907
ELF cau Ca	$5.947^{a} + (0.027^{b} \times A) + (0.026^{b} \times B) – (0.100^{b} \times C) – (0.002 \times D)$	0.985
ELF cau Chol	$51.451^{a} + (2.190^{b} \times A) – (0.122^{b} \times B) – (0.849^{b} \times C) – (0.024^{b} \times D)$	0.989
ELF cau Vit-D	$1.811^{a} – (0.644^{b} \times A) + (0.236^{b} \times B) + (0.936^{b} \times C) – (0.003^{b} \times D)$	0.955
ELF cau E_2	$254.975^{a} + (4.472^{b} \times A) – (0.103^{b} \times B) – (2.799^{b} \times C) + (0.111^{b} \times D)$	0.945

a, constant; b, Regression co-efficient; R^2, coefficient of determination; A, Sr. Ca; B, Sr. Ch; C, Sr. Vit-D; D, Sr. E_2.

Table 3. Prediction equations to estimate concentrations of Ca^{2+} Ch, Vit-D and E_2 in caput (Cap), corpus (Cor) and cauda (Cau) SCF from serum (Sr.) levels of Ca, Ch, Vit-D and or E_2

Prediction value of	Prediction Equations	R^2
SCF cap Ca	$14.501^a + (3.520^b \times A) + (0.268^b \times B) - (0.341^b \times C) - (0.039^b \times D)$	0.960
SCF cap Chol	$185.541^a + (4.090^b \times A) - (0.350^b \times B) - (1.437^b \times C) + (0.150^b \times D)$	0.985
SCF cap Vit-D	$35.113^a - (1.776^b \times A) + (0.001^b \times B) + (0.319^b \times C) - (0.007^b \times D)$	0.967
SCF cap E_2	$55.829^a + (56.343^b \times A) - (4.241^b \times B) - (2.223^b \times C) + (1.932^b \times D)$	0.973
SCF cor Ca	$40.500^a + (0.903^b \times A) - (0.045^b \times B) - (0.518^b \times C)$	0.959
SCF cor Chol	$94.278^a + (6.417^b \times A) - (0.369^b \times B) - (0.526^b \times C) + (0.117^b \times D)$	0.952
SCF cor Vit-D	$56.918^a - (4.394^b \times A) + (0.102^b \times B) + (0.278^b \times C) + (0.035^b \times D)$	0.962
SCF cor E_2	$750.565^a + (12.778^b \times A) + (4.72^b \times B) - (10.792^b \times C) - (0.029^b \times D)$	0.975
SCF cau Ca	$5.215^a + (0.137^b \times A) + (0.002^b \times B) - (0.007^b \times C) + (0.008^b \times D)$	0.963
SCF cau Chol	$40.408^a + (1.283^b \times A) + (0.639^b \times B) - (0.257^b \times C) + (0.047^b \times D)$	0.985
SCF cau Vit-D	$45.140^a - (1.456^b \times A) - (0.094^b \times B) + (0.396^b \times C) + (0.025^b \times D)$	0.974
SCF cau E_2	$196.660^a + (14.615^b \times A) - (3.558^b \times B) - (2.744^b \times C) + (0.980^b \times D)$	0.975

a, constant; b, Regression co-efficients; R^2, coefficient of determination; A, Sr. Ca; B, Sr. Ch; C, Sr. Vit-D; D, Sr. E_2.

Estradiol (E_2) levels in serum, ELF and SCF from the epididymal regions: The mean (±SE) concentration of E_2 in serum was 331.73±20.73 pg/ml, whereas, in ELF and SCF from different epididymal regions are also given in Table 1 and their concentrations varied between 87.9–480.4 in serum, 380.8–1085.9 in caput ELF, 221.8–960.9 in corpus ELF, 131.0–294.9 in cauda ELF, 433.5–1484.3 in caput SCF, 286.1–960.9 in corpus SCF and 155.6–605.4 cauda SCF. The wider range observed was due to the variations in individual animals.

Estimation of p*H of ELF and concentrations of Ca^{2+}, Ch, Vit-D and E_2 in ELF and SCF in three epididymal regions calculated from their levels in serum:* The estimated values of the biomolecules are standard values in their normal physiological conditions in the breed of boar. In any boar where the infertility is idiopathic due to variation of these biomolecules, remains undiagnosed as collection of samples and estimation of values of these biomolecules in ECF and SCF in not possible in live animals. Whereas collection of serum being easy and thus estimating the values of these biomolecules in serum is possible. If the values in serum could be used to predict their values in ECF and SCF, the variations could be easily be detected thus idiopathic infertility could be detected at the earliest.

The relationship(s) of the levels of the bio-molecular components, viz. *p*H, Ca^{2+}, Ch, E_2 and Vit-D in the serum with that of ELF and SCF from different epididymal segments of LWY boar have been established and their levels in the ELF and SCF have been predicted from levels in the serum through "prediction equations" developed by using "Curve Fit Regression" (Table 2 and 3).

Hence, it is concluded that the levels of these bio-molecular constituents in serum may serve as bio-markers, i.e. standards and any deviation in their values from the standard calculated range, could help us to assess the variation in the value in ECF and SCF, thus can diagnose the condition quickly whether the male is infertile due to variations in their concentrations of these bio-molecules. An appropriate treatment modalities could therefore be suggested/possible to ameliorate the cause(s) of infertility beforehand.

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