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

SOMRAJ CHATTARAJ1, SIDDHARTH BASU2, KALYANI RAY3, SANJOY DATTA4, PRADIP SARKAR5 and DURGADAS MANDAL6

West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata, West Bengal 700 037 India

Received: 28 June 2019; Accepted: 17 July 2019

ABSTRACT

The experiment was conducted to analyze biomarkers like pH, calcium (Ca2+), cholesterol (Ch), estradiol (E2) 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 Ca2+, Ch, E2 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 pH, Ca2+, Ch, E2 and Vit-D3 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.
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 (5°C) 0.15 M phosphate buffered saline (PBS, pH 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 pH of the luminal fluids of each segment were measured using digital pH meter (HI-1093B pH 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 –20°C till experimentimation.

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-Cresolphthalein 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 (E₂) 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-D₃ is the metabolically active form of vitamin-D (Vit-D₃) but the concentration of its precursor, 25-HydroxyVitamin D₃ 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 (Hollick 2009 and Bikle 2010) using 25(OH)D₃ 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. pH, Ca²⁺, Ch, E₂ and Vit-D₃ 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
motility was found absent in caput region (Table 1).

**pH of ELF from the epididymis regions:** The activation of sperm motility occurs in response to changes in the external medium. Among the external factors, pH of ELF and sperm internal pH (pHi) seem to play a pivotal role in sperm physiology in invertebrates, lower vertebrates and to some extent in mammals. The mean (±SE) pH of ELF from caput, corpus and cauda measured and are represented in Table 1 and the pH 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 epididymis regions:** The mean (±SE) concentrations of Ca²⁺ in serum and from ELF and SCF from different epididymis 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, 15.9–73.7 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 epididymis regions:** The mean (±SE) concentration of cholesterol in serum was 49.22±3.51 mg/dl, whereas from different epididymis 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 epididymis regions:** The mean±SE concentration of VD in serum was 45.08±2.14 ng/ml, whereas from different epididymis regions 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 corpus SCF, 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.

| Parameter | Blood serum (Mean±SE) | Epididymal luminal fluid (ELF) (Mean±SE) | Sperm Cytosolic fluid (SCF)(Mean±SE) |
|-----------|-----------------------|------------------------------------------|-------------------------------------|
|           | Caput | Corpus | Cauda | Caput | Corpus | Cauda | Caput | Corpus | Cauda |
| Motility (%) | – | 0 | 15.82±0.24b | 90.95±0.80b | – | – | – | – | – |
| pH | – | 7.16±0.009 | 6.79±0.01 | 6.55±0.01 | – | – | – | – | – |
| Calcium (mg/dl) | 10.54±0.43 | 8.39±0.55 | 5.32±0.42 | 2.45±0.27 | 36.70±2.40 | 24.52±1.39 | 9.11±0.24 |
| Cholesterol (mg/dl) | 49.22±3.51 | 55.54±2.50 | 38.68±1.38 | 22.20±1.99 | 196.2±6.65 | 158.86±5.12 | 89.48±4.23 |
| Vitamin-D₃ (mg/ml) | 45.08±2.14 | 20.47±0.98 | 32.67±0.97 | 47.70±1.73 | 28.65±1.59 | 39.74±1.60 | 51.38±1.31 |
| Estradiol (pg/ml) | 331.73±20.73 | 780.56±44.73 | 521.65±44.43 | 207.73±9.94 | 981.93±54.60 | 621.77±43.47 | 377.21±20.46 |

1) Similar superscripts does not differ significantly (P<0.05).

**Table 2.** Prediction equations to estimate Sperm motility, pH and concentrations of Ca²⁺, Ch, Vit-D and E₂ in caput (Cap), corpus (Cor) and cauda (Cau) ELF from serum (Sr.) levels of Ca, Ch, Vit-D and or E₂

| Prediction value of | Prediction Equations | R² |
|---------------------|----------------------|----|
| ELF cap pH | 7.23±0.015 x A + (0.003 x B) - (0.002 x C) | 0.932 |
| ELF cap Ca²⁺ | 9.24±0.11 x A + (0.065 x B) - (0.113 x C) + (0.006 x D) | 0.947 |
| ELF cap Chol | 51.51±0.44 x A + (0.406 x B) - (0.519 x C) - (0.044 x D) | 0.965 |
| ELF cap Vit-D | 3.53±0.029 x A + (0.002 x B) + (0.404 x C) - (0.011 x D) | 0.973 |
| ELF cap E₂ | 1212.31±0.14 x A + (2.39 x B) - (16.91 x C) + (0.32 x D) | 0.956 |
| Sperm Motility (Cor) | 22.67±0.18 x A - (0.022 x B) - (0.032 x C) - (0.007 x D) | 0.949 |
| ELF cor pH | 6.226±0.026 x A + (0.011 x B) + (0.001 x C) | 0.907 |
| ELF cor Ca | -2.84±0.754 x A + (0.49 x B) - (0.01 x C) - (0.005 x D) | 0.933 |
| ELF cor Chol | 47.43±0.665 x A + (0.02 x B) + (0.421 x C) + (0.007 x D) | 0.975 |
| ELF cor Vit-D | 23.08±0.177 x A + (0.138 x B) + (0.366 x C) - (0.001 x D) | 0.967 |
| ELF cor E₂ | 1212.31±0.14 x A + (2.39 x B) - (16.91 x C) + (0.32 x D) | 0.956 |
| Sperm Motility (cau) | 8.257±0.537 x A - (0.047 x B) + (0.284 x C) + (0.012 x D) | 0.930 |
| ELF cau pH | 6.226±0.026 x A + (0.011 x B) + (0.001 x C) | 0.907 |
| ELF cau Ca | 5.94±0.027 x A + (0.026 x B) - (0.10 x C) - (0.002 x D) | 0.985 |
| ELF cau Chol | 51.45±2.190 x A - (0.122 x B) - (0.849 x C) - (0.024 x D) | 0.989 |
| ELF cau Vit-D | 1.81±0.644 x A + (0.236 x B) + (0.936 x C) - (0.003 x D) | 0.955 |
| ELF cau E₂ | 254.97±4.472 x A - (0.103 x B) - (2.799 x C) + (0.111 x D) | 0.945 |

a, constant; b, Regression co-efficient; R², coefficient of determination; A, Sr. Ca; B, Sr. Ch; C, Sr. Vit-D; D, Sr. E₂.
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 + (3.520 \(\times A\)) + (0.268 \(\times B\)) + (0.341 \(\times C\)) + (0.039 \(\times D\)) | 0.960 |
| SCF cap Chol        | 185.541 + (4.096 \(\times A\)) – (0.350 \(\times B\)) – (1.437 \(\times C\)) + (0.150 \(\times D\)) | 0.985 |
| SCF cap Vit-D       | 35.113 – (1.776 \(\times A\)) + (0.001 \(\times B\)) + (0.319 \(\times C\)) – (0.007 \(\times D\)) | 0.967 |
| SCF cap E\(_2\)     | 55.829 + (56.343 \(\times A\)) – (4.241 \(\times B\)) – (2.223 \(\times C\)) + (1.932 \(\times D\)) | 0.973 |
| SCF cor Ca          | 40.500 + (0.903 \(\times A\)) – (0.045 \(\times B\)) – (0.518 \(\times C\)) | 0.959 |
| SCF cor Chol        | 94.278 + (6.417 \(\times A\)) – (0.369 \(\times B\)) – (0.526 \(\times C\)) + (0.117 \(\times D\)) | 0.952 |
| SCF cor Vit-D       | 56.918 + (4.394 \(\times A\)) + (1.010 \(\times B\)) + (0.278 \(\times C\)) + (0.035 \(\times D\)) | 0.962 |
| SCF cor E\(_2\)     | 750.565 + (12.778 \(\times A\)) + (4.72 \(\times B\)) – (10.792 \(\times C\)) – (0.029 \(\times D\)) | 0.975 |
| SCF cau Ca          | 5.215 + (0.137 \(\times A\)) + (0.002 \(\times B\)) – (0.007 \(\times C\)) + (0.008 \(\times D\)) | 0.963 |
| SCF cau Chol        | 40.408 + (1.283 \(\times A\)) + (0.630 \(\times B\)) – (0.257 \(\times C\)) + (0.047 \(\times D\)) | 0.985 |
| SCF cau Vit-D       | 45.140 + (1.456 \(\times A\)) – (0.094 \(\times B\)) + (0.396 \(\times C\)) + (0.025 \(\times D\)) | 0.974 |
| SCF cau E\(_2\)     | 196.660 + (14.615 \(\times A\)) – (3.558 \(\times B\)) – (2.744 \(\times C\)) + (0.980 \(\times D\)) | 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 pH 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. pH, 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 from 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.

**ACKNOWLEDGEMENTS**

The authors are highly thankful to the Hon’ble Vice-Chancellor, WBUAFS and all the members of advisory committee for their valuable contribution and necessary support.

**REFERENCES**

Allain C C, Poon L S, Chan C S G, Richmond W and Fu P C. 1974. Enzymatic Determination of Total Serum Cholesterol. *Clinical Chemistry* **20**(4): 470–75.

Arnold J, Madson D M and Ensley S M. 2015. Survey of serum vitamin D status across stages of swine production and evaluation of supplemental bulk vitamin D premixes used in swine diets. *Journal of Swine Health and Production* **23**(1): 28–34.

Baginski E S. 1973. Direct microdetermination of serum calcium. *Clinica Chimica Acta* **46**(1): 46–54.

Bikle D D. 2010. Vitamin D and the skin. *Journal of Bone and Mineral Metabolism* **28**(2): 117–30.

Cornwall G A. 2009. New insights into epididymal biology and function. *Human Reproduction Update* **15**(2): 213–27.

Dacheux J L, O’Shea T and Paquignon M. 1979. Effects of osmolality, bicarbonate and buffer on the metabolism and motility of testicular, epididymal and ejaculated spermatozoa of boars. *Journal of Reproduction Fertility* **55**: 287–96.

Duncan D B. 1995. Multiple range and multiple F test. *Biometrics* **11**: 1–42.

Flegg H M. 1973. An investigation of the determination of serum cholesterol by an enzymatic method. *Annals of Clinical Biochemistry* **10**(1–6): 79–84.

Frenette G, Lessard C and Sullivan R 2002. Selected Proteins of “Prostasome-Like Particles” from Epididymal Cauda Fluid Are Transferred to Epididymal Caput Spermatozoa in Bull. *Biology of Reproduction* **67**: 308–13.

Gitelman H J. 1967. An improved automatic procedure for the determination of calcium in biologic specimens. *Analytical Biochemistry* **18**: 521–31.

Hall P F. 1988. Testicular steroid synthesis: Organization and
regulation. pp. 975–998. *The Physiology of Reproduction Vol 1.* (Eds) Knobil, E and Neil J D. New York, Raven Press.

Holick M F. 2009. Vitamin D Status: Measurement, Interpretation and Clinical Application. *Annals of Epidemiology* 19(2): 73–78.

Hori T, Atago T, Kobayashi M and Kawakami E. 2015. Influence of different methods of collection from the canine epididymides on post-thaw caudal epididymal sperm quality *Journal of Veterinary Medical Science* 77(5): 625–630.

Orgebin-Crist M C. 1967. Sperm maturation in rabbit epididymis. *Nature* 216(5117): 816–18.

Robaire B, Hinton B T and Orgebin-Crist M C. 2006. The epididymis. *Physiology of reproduction* 3rd Edition. pp 1071–1148.

Robaire B, Syntin P and Jervis K. 2000. *The coming of age of the epididymis*. pp 229–262. Testis, Epididymis and Technologies in the Year 2000. Springer: Hildenberg.

Siiteri P K, Murai J T, Hammond G L, Nisker J A, Raymoure W J and Kuhn R W. 1982. The serum transport of steroid hormones, Recent Progress in Hormone Research 38: 457–510.

Sullivan R. 2004. Male fertility markers, myth or reality. *Animal reproduction science* 82–83: 341–47.

Vijayaraghavan S, Stephens D T, Trautman K, Smith G D, Khatra B, da Cruz e Silva E F and Greengard P. 1996. Sperm Motility Development in the Epididymis Is Associated with Decreased Glycogen Synthase Kinase-3 and Protein Phosphatase 1 Activity. *Biology of Reproduction* 54: 709–18.

Wales R G, Wallace J C and White I G. 1966. Composition of bull epididymal and testicular fluid. *Journal of reproduction and fertility* 12(1): 139–44.

Wemersson R, Schierup M H, Jørgensen F G, Gorodkin J, Panitz F, Staerfeldt H H, Christensen O F, Mailund T, Hornshøj H and Klein A. 2005. Pigs in sequence space: a 0.66X coverage pig genome survey based on shotgun sequencing. *BMC Genomics* 6: 70.