Assessment of prenatal ovarian and serum estrogen concentration in Nili-Ravi buffalo fetus

Momen Khan¹, Muhammad Saleem¹, Farmanullah², Sajjad Ahmad², Sarfaraz Ali Fazlani², Ihsan Ullah Kakar², Mohammad Salim³, Saeed Ahmad⁴, Inayat ur Rehman⁵ and Sajid Ali⁶

¹. Directorate General (Extension) Livestock and Dairy Development Department, Bacha Khan Chowk, Peshawar, Khyber Pakhtunkhwa, Pakistan
². Faculty of Veterinary and Animal Sciences, Lasbela University of Agriculture, Water and Marine Science, Uthal, Balochistan, Pakistan
³. Forestry and Wildlife Management Department, University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan
⁴. Institute of Biological Sciences, Sarhad University of Science and Information Technology, Peshawar, Pakistan
⁵. School of Marxism, China University of Geosciences (Wuhan), Hongshan District Region Wuhan, P.R, China
⁶. Department of Agriculture, University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan

*Corresponding author’s email: farman_aup@yahoo.com; farman.vas@luawms.edu.pk

The current study aims to assess the estrogen concentration in both ovaries and serum in the fetus of the Nili Ravi buffalo. For this purpose, 73 female fetuses were collected from Sihala abattoir Islamabad, Pakistan. Both ovaries were obtained from fetuses with age ranging from day 51 to day 290 whereas; serum was available only for the fetuses ranging from the age of 146 days to 290 days. Radio immunoassay was used to measure the estrogen concentration and the data was analyzed through regression analysis of variance. Analysis of the data revealed mean ovarian estrogen concentration as 16.08±1.87 pg/mg of ovaries during the period of 51 to 290 days with peak value of 37.49±10.81pg/mg at 191-210 days. Ovarian estrogen concentration showed significant (P<0.05) increase with the advancing age of the fetus. On the other hand, mean serum estrogen concentration observed was 476.20 pg/ml during 146 to 290 days of fetal life with peak value of 734.11 pg/ml at the age group of 231-250 days. It can be concluded that both fetal ovarian and serum estrogen concentration increases with the advancing age of fetus in Nili-Ravi buffalo.

Keywords: Buffalo; Estrogen concentration; fetus; Ovary; Serum; Nili Ravi

Introduction

Estradiol is involved in regulating follicle formation and activation in bovine fetal ovaries hence affect the size of primordial pool at birth and eventually the female reproductive potential [1, 2]. Fetal ovarian estrogen concentration has been studied in various species like rat [3], mouse [4], rabbit [5], Hamster [6], guinea pig [7], sheep [8], human [9], and bovine [10, 11]. It has been reported that ovarian estradiol production varies considerably over the course of
gestation; increased during early gestation when oogonia are very active mitotically, but its production decline at the beginning of the second trimester at the time of initiation of the follicles formation [1, 12, 13]. In bovine the biosynthesis of estradiol in the fetal ovary has been demonstrated at the time when the sex of embryonic gonads is morphologically distinguishable as early as at 45±3 days with crown to rump length of 3.0 to 3.5 cm [14, 15, 16]. A causal relationship between endogenous estradiol and inhibition of follicle formation and activation has been observed in cattle [2, 17]. However, no such study has been conducted in Nili-Ravi buffalo. Thus this study was designed to assess the estrogen concentration in prenatal ovary and serum of Nili-Ravi buffalo fetus. The results obtained may be used as baseline data in the assisted reproductive biotechnology to enhance the productive and reproductive performance of Nili-Ravi buffalo. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed, and validated for the hormones quantification in cow [18]. The high variation was found in the individual hormonal level of cows. May be this is due to the physiological stage of animal.

**Materials and Methods**

**Fetuses**

Total of 73 fetuses of Nili–Ravi buffalo were collected for this study from Sihala Abattoir, Islamabad. After slaughtering of the animals, fetuses were collected immediately and the crown to rump (C-R) length was measured with measuring tap to assess the age of the fetus according to [19] using the formula “y = - 11.27 + 0.3063 x” where y is C-R length “a” is the intercept, “b” is the regression coefficient “x” is the age. y = - 11.27 + 0.3063 x. A “v” shape incision from umbilical cord to the caudal end of the body on ventral side was given to collect the ovaries. They were studied at interval of 20 days and thus arranged in twelve groups. After collection, ovaries were stored at -20°C in deep freezer until analyzed for estrogen. Likewise, a sternal incision was given to expose the heart, posterior and anterior vena cava for collection of blood. The blood was cooled in ice, transported to the laboratory and centrifuged at 3000 rpm for 10 minutes. Serum was collected and kept at –20°C until analysis.

**Estrogen assay**

The radio immunoassay (RIA) for the assessment of ovarian and serum estrogen was carried out according to [20, 21, 22]. For ovarian tissues estrogen RIA, 15 mg of ovarian tissues were homogenized in 1 ml normal saline and the homogenates were extracted in 2.5 ml x 2 reagent grade diethyl ether and dried under air in water bath at 60°C. Ether extracts were reconstituted with 1.5 ml of steroid phosphate buffer (0.1M containing NaCl 0.9%, gelatin 0.1%, sodium aside 0.1%, PH 7.2) and 500 µl of sample was incubated with antibody [3H] estradiol (200 µl) for 18-24 hrs at 4°C. Following incubation 200 µl dextral coated charcoals was added to each tube. The tubes were kept for 30-35 minutes at 4°C and centrifuged at 3000 rpm for 10 minutes. Liquid scintillation counting (LSC) is the standard laboratory method to quantify the radioactivity of low energy radioisotopes, mostly beta-emitting and alpha-emitting isotopes. The sensitive LSC detection method requires specific cocktails to absorb the energy into detectable light pulses. The clear supernatant was decanted into scintillation vials and added 5 ml of scintillation fluid (0.5% perm blend 111-tris-MSB1 Packard international, Zurich Switzerland). Radioactivity was measured in Beckman LS 1801 liquid scintillation
counter. All tubes were measured in duplicates. Results were calculated by WHO immunoassay data processing program. Serum was also processed for RIA in the same manner.

Statistical analysis
The results were analyzed for significance by analysis of variance test (ANOVA).

Results

Ovarian estrogen concentration

The mean ovarian estrogen concentration assessed during fetal life from 51 to 290 days is presented in (Table 1). The overall mean estrogen concentration observed was 16.08±1.87 pg/mg with peak value (37.49±10.8 pg/mg) at the age group of 191-210 days. Regression analysis of variance revealed a significant (P<0.05) increase in fetal ovarian estrogen concentration with advancement of fetal age in this study (Fig. 1).

Table 1. Ovarian estrogen concentration at different age groups in Nili-Ravi buffalo

| Age group (days) | Estrogen (pg/mg) |
|-----------------|-----------------|
| 51-70           | 07.29±0.80      |
| 71-90           | 12.48±3.62      |
| 91-110          | 08.76±2.46      |
| 111-130         | 12.13±3.55      |
| 131-150         | 12.92±4.64      |
| 151-170         | 14.31±0.25      |
| 171-190         | 22.74±6.06      |
| 191-210         | 37.49±10.8      |
| 210-230         | 18.14±0.19      |
| 231-250         | 25.73±0.85      |
| 251-270         | 17.78±3.63      |
| 271-290         | 22.08±5.17      |
| Overall mean    | 16.08±1.87      |

Figure 1. Ovarian estrogens concentration at various age groups of Nili-Ravi buffalo fetus

Serum estrogen concentration
Serum estrogen concentration assessed at different age groups in Nili-Ravi buffalo fetus is presented in (Table 2). The overall mean serum estrogen concentration observed during this investigation was 476.20 pg/ml. A sharp increase has been observed at the age group of 171-190 days of fetal life which is maintained till 231-250 days with slight
fluctuation. Serum estrogen declines at the fetal age of 271-290 days.

Table 2. Serum estrogen concentration at different age groups in Nili-Ravi buffalo

| Age group (days) | Estrogen (pg/ml) |
|-----------------|-----------------|
| 151-170         | 80.30           |
| 171-190         | 505.65          |
| 191-210         | 682.94          |
| 210-230         | 427.59          |
| 231-250         | 734.11          |
| 271-290         | 426.62          |
| Overall mean    | 476.20          |

**Discussion**

Ovarian estradiol production varies considerably over the course of gestation. It has been observed that its production increased during early gestation when oogonia are very active mitotically, but decline at the beginning of the second trimester at the time of initiation of the follicles formation and remained low till the first growing follicles appear [1, 12, 13]. Contrary to these researches, both ovarian and serum estrogen concentration significantly increased with the advancement of fetal age in Nili-Ravi buffalo. Estradiol negatively regulates follicle formation in mammals including cattle [2, 13]. The higher concentration of estrogen in fetal ovaries and serum observed in this study seems to be a cause of the relatively small size of primordial follicles reserve in buffalo [23, 24]. However, in-depth investigation is needed to probe this relationship. The estrogen concentration determined in fetal serum from 146 to 290 days indicates maximum concentration on 8th month of fetal age. But in case of cow it’s reported that in mixed umbilical cord blood fetal estrogen concentration is maximum on 6th month of fetal age. However these levels are much higher than reported in buffalo heifers from birth and puberty on 27-30 months of age. No information is available regarding the prenatal ovarian cholesterol in Nili-Ravi buffalo. The cholesterol concentration are low during the early fetal period observed which increase significantly (P≤0.001) with increase in fetal life reaching maximum on 271-290 days. Limited information is available regarding the fetal serum cholesterol. In Nili-Ravi buffalo fetus the mean serum cholesterol is 71.57 mg/100 ml from 130-290 days of intrauterine life. The highest values are 108.10 mg/100 ml and 99.96 mg/100 ml which are obtained during 5 to 6 month of fetal life. The serum cholesterol from the 6th month onward shows a gradual decrease and the minimum value (42.26 mg/100 ml) is observed at 290 days of fetal life. No information is available on estrogen production by buffalo fetal ovary, however, the bovine fetal ovary is steroidogenically active in vivo have been reported by someone, and this study also indicates that the buffalo ovary is active in producing steroid hormone during pregnancy as is indicated by the estrogen hormone concentration determined during various gestational periods. During the early fetal age (51-70) days ovarian estrogen concentration is low, which increase significantly (P≤0.05) with advance in fetal age reaching maximum concentration on 210 days (7 month) of fetal life.

**Conclusion**

It can be concluded from this study that fetal ovarian and serum estrogen concentration significantly increased with the advancing
fetal age, a phenomenon contrary to other mammals.

**Authors’ contributions**
Conceived and designed the experiments: M Khan & M Saleem, Performed the experiments: M Khan, M Saleem & S Ahmad, Analyzed the data: Farmanullah, M Salim, I Rehman & S Ali, Contributed materials/analysis/tools: S Ahmad, SA Fazlani, IU Kakar, Wrote the paper: M Khan, M Sleem & Farmanullah

**Acknowledgements**
I express sincere thanks to our Research Team/Group for their kind collaboration and assistance. Special thanks to Professor Dr. Samina Jalali for their supervision and guidance. I am also very appreciative to the co-authors for their critical and technical improvement of our manuscript.

**References**
1. Yang MY & Fortune JE (2008). The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during midgestation and is associated with meiotic arrest of oocytes. *Biol of Rep* 78: 1153-1161.
2. Allen JJ, Herrick SL & Fortune JE (2016). Regulation of steroidogenesis in fetal Bovine ovaries: differential effects of LH and FSH. *J of Mol Endocrinol* 57: 275-286.
3. Weniger JP & Zeis A (1988). Action de 1 AMPc et de FSM Sur la production d, oestrogènes par less gonads foetals de rat en culture in vitro. *CR Acad Sci Paris* 306: 257-260.
4. Terada N, Kuroda H, Namiki M, Kitamura Y & Matsumoto K (1984). Augmentation of aromatize activity by FSH in ovaries of fetal and neonatal mice in organ culture. *J Steroid Biochem* 20: 741-745.
5. George FW, Simpso ER, Milewich L & Wilson JD (1979). Studies on the Regulation of the onset of steroid hormone biosynthesis in fetal rabbit gonads. *Endo* 105: 1100-1106.
6. Vomachka AJ & DiMario KB (1987). Prenatal hamster gonadal androgen and oestradiol production in vitro and response to hCG. *Biol Neon* 52: 42-47.
7. Sholl SA & Goy RW (1978). Androgen and oestrogen synthesis in fetal guinea pig-gonad. *Biol of Rep* 18: 160-169.
8. Mauleon P, Bezard J & Terqui M (1977). Very early and transient 17-Oestradiol secretion by fetal sheep ovary. In vitro study. *Ann Biol Amin Bioch Biop* 17: 399-401.
9. Geroge FW & Wilson JD (1978). Conversion of androgen to oestrogen by human fetal ovary *J of Clin Endocrinol Metab* 47: 550-555.
10. Roberts JD & Warren JC (1964). Steroid biosynthesis in the fetal ovary. *Endo* 74: 846-852.
11. Juarez OMA, Alvarez FG, Lope V, Kawa S. & pedernera E (1993). Steroid Metabolism in the cortex and the medulla of the early fetal bovine ovary. *The J of Exp Zool* 266: 102-107.
12. Nilsson EE & Skinner MK (2009). Progesterone regulation of primordial follicle Assembly in bovine fetal ovaries. *Mol and Cell Endo* 313: 9-16.
13. Fortune JE, Yang MY, Allen JJ & Herrick SL (2013). Triennial Reproduction Symposium: The ovarian follicular reserve in cattle: What regulates its formation and size. *J of Anim Sci* 91(7): 3041-3050.
14. Shemesh M, Ailnberg M, Millaquir F, Ayalon, N & Hansel W (1978). Hormone secretion by cultured bovine pre and post implantation gonads. *Biol Rep* 19: 761-767.
15. Shemesh M, & Hansel W (1983). Hormone productive by the early bovine embryo. *J Steroid Biochem* 19: 979-983.
16. Shemesh M (1980). Estrodiol-17. Biosynthesis by the early bovine fetal
ovary during the Active and refractive phases. *Biol Repr* 23: 577-582.

17. Liu X & Shi H (2015). Regulation of estrogen receptor α expression in the hypothalamus by sex steroids: implication in the regulation of energy homeostasis. *Intern J of Endo* PMCID: PMC4600542

18. Alexandre G, Julia ZC, Karin K, Christoph H, Bing S & Pascal M (2016). Determination of steroid hormones in bovine milk by LC-MS/MS and their levels in Swiss Holstein cow milk. *Food Additives & Contam: Part A* 33(5): 804-816.

19. Pirzada WH, Anwar M, & Mehmood A (1988). Possible role, embryo transfer Technology can play in improvement of sheep and goats in Pakistan. *J of Ani Heal of Pak* 8(1-4).

20. Arslan M, Zaidi P, Lobo J, Zaidi AA & Qazi MH (1978). Steroid levels in preovulatory and gravid lizards (Uromastix hardwicki). *General and comp Endo* 34(3): 300-303.

21. Jalali & Haider G (1985). Seasonal variation in testicular androgen and histology of fish. *Barilius Vag Ham Biol* 31: 129-146.

22. Diagnostic Products Corporation 5700 West 96th Street Los Angeles, CA 90045, Package Insert: C 863, March 13, 1991.

23. Manik RS, Palta P, Singla SK & Sharma V (2002). Folliculogenesis in buffalo (Bubalus bubalis); a review. *Repro Fer & Dev* 14(5): 315-325.

24. Santos SSD, Ferreira MAP, Lima MYS, Sampaio RV, Cordeiro MS, Silva TVG, Costa NN, Miranda MS & Ohashi OM (2011). Quantification, Morphology and Ultrastructure of Preantral Follicles of Buffalo (Bubalus bubalis) Foetuses. Reproduction in Dome Anim.