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Oxidant/antioxidant Status and CYP19 Gene Polymorphism in Crossbred Cows in Relation to Ovarian Inactivity

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Abstract: Ovarian inactivity is one of the most important causes of low fertility in farm animals with low calf and milk production and consequently high economic losses. The present study was carried out to investigate the relationship between ovarian inactivity and oxidant/ antioxidant status and polymorphism in CYP19 gene in cows. A Total number of 260 crossbred cows at veterinary clinics of Beni Suef governorate was assigned for the current study. Animals were examined for ovarian cyclicity by rectal palpation and confirmed by ultrasonography and progesterone assy. Blood samples were used to determine plasma levels of Malondialdehyde (MDA), Nitric oxide (NO), Total antioxidant capacity (TAC), Superoxide Dismutase (SOD), Zinc (Zn) and Copper (Cu). The 351bp, containing exon 2 of CYP19 gene was amplified using PCR followed by sequence analysis. Results revealed that MDA ($P<0.01$) and NO ($P<0.05$) increased significantly in cows having inactive ovaries, while, TAC, SOD, Zn and Cu decreased ($P<0.01$), in the same cows as compared to normal cyclic animals. Analyzing the allelic patterns of the PCR product of exon 2 of CYP19 gene indicated that there was no polymorphism in all examined cows and this locus may not influence ovarian activity in Egyptian cows. It was concluded that ovarian inactivity is associated with disturbed oxidant/antioxidant status with no polymorphism in CYP19 gene.

Key words: Oxidant/Antioxidant Status · CYP19 Polymorphism · Ovarian Activity

INTRODUCTION

Cattle are economically important farm animals as a source of meat and milk. Ovarian activity of cows is reflected on the conception rate, annual calf crop and milk yield. Therefore, ovarian inactivity cause great economic losses due to decreased calf crop, milk production and cost of treatment, besides it predispose for infection of the genital system [1]. Nutritional deficiencies are the principle cause for general weakness, stunted growth and infertility [2]. Mal nutrition, Energy imbalance and lack of vitamins, trace elements and antioxidants were associated with low fertility [3].

Balance of oxidant/antioxidant status is required for all body physiological functions including reproduction and in particular folliculogenesis [4].The severity and prevalence of many health and reproductive problems in cows were reported to be related to oxidative stress due to un proper balance between reactive oxygen species (ROS) and antioxidants.The cumulative damage from ROS and oxidative stress was found to be toxic to cells [5] and eventually leads to cell death [6]. Trace elements such as Zn and Cu are necessary for good health in cows because they play many important functions, including metabolism, protein formation, synthesis of connective tissue and immune response [7]. Hypocopperaemia is associated with varieties of clinical manifestations including poor quality coat, anemia, low reproductive performance and over exposure to infectious disease with consequent great economic losses in cows [8]. Zn has important role in immunity, cell replication and proliferation [9].

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Genetic evaluation of animal reproductive performance depends on molecular technology for identifying genes and analysis of their polymorphism whose end products are key enzymes in the metabolic pathways of important physiological functions and are related to phenotypes [10]. Low numbers of genetic markers influencing the reproduction traits were detected, because most of them are characterized by coefficients with small heritability [11]. Reproduction is mainly regulated by estrogens synthesized in the ovaries through the androgens aromatization [12]. The biosynthesis of estrogens from androgen precursors needs enzymatic complex of two proteins, nonspecific microsomal flavoprotein reductase and specific haemoglycoprotein, which is known as cytochrome P450 aromatase [13]. Cytochrome P450 aromatase, the protein product of CYP19 gene is the main enzyme in estrogen biosynthesis. The role of aromatase is the transformation of androgens to estrogens and is important for physiology of reproduction [14].

The present investigation aimed to throw light on the possible relation between ovarian inactivity and oxidant/antioxidant status and polymorphisms of *CYP19* gene in crossbred cows.

MATERIALS AND METHODS

Animals: This investigation was carried on a total number of 260 cows. These animals came to veterinary clinics at Benisuef governorate, Egypt. A full case history and owner complaint of each animal were recorded. The general health condition was examined and the body condition score was recorded. Gynecological examinations were carried out twice for two successive weeks at least to register the reproductive status and/or disorder. Examination was aided by Ultrasonography (Pia Medical Flacse Saote, Netherland) with an endorectal array of 8.6 M Hertz. Animals which did not show estrous signs and have small nonfunctioning ovaries were considered to suffer from ovarian inactivity.

Samples: Blood samples were obtained from the jugular vein in vacutainer tubes containing EDTA. Samples were collected from 24 normal cyclic and 48 cows showing ovarian inactivity. A part of each sample was preserved as whole blood at -20°C for DNA extraction and gene polymorphism analysis. The rest of each sample was centrifuged at 3000 rpm for 15 min. Plasma was separated and kept at -20°C till the time of hormonal and biochemical assay.

Progesterone and Oxidants/Antioxidants Analysis: Plasma samples were examined for the concentration of plasma progesterone using ELIZA micro wells technique, kits from Novotec, Germany [15] and ELIZA reader (Anthos Zenyth 200rt). The kit had a sensitivity of 2.0pg/ml with inter- and intra-run precision coefficient of variations of 2.9 and 4.85, respectively.

Malondialdehyde [16] Nitric oxide [17] Total antioxidant capacity [18] Superoxide Dismutase [19] Zinc [20] and Copper [21] were assayed using kits from Biodiagnostic (Egypt) and Shemizdu UV 240 spectrophotometer.

CYP19 Gene Polymorphism: Blood samples were collected on EDTA and DNA was extracted using G-spin™ total DNA Extraction kit. DNA concentration was determined by using NanoDrop1000 Thermo Scientific spectrophotometer. Primers were synthesized for amplification of exon 2 from *CYP*19 gene [22].

F: 5`GGGCTTGCTTGTTTTGACTC 3`
R: 5`CTGGTATTGAGGATGTGTCC 3`

PCR was done to amplify the desired fragment. The amplified fragments were verified by horizontal electrophoresis on 1.5 % agarose gel stained by ethidium bromide run in 1X TBE buffer. The PCR products were purified by universal DNA purification kit for the sequencing reaction. The sequencing reaction was done on the purified PCR products in (LGC, Germany). Sequence analysis and alignment were performed using online bioinformatics websites NCBI/BLAST/blastn suite and EMBL-EBI/Clustal Omega and bioinformatics program FastPCR 6.1.The nucleotide sequence of the exon 2 from CYP 19 gene in cows was submitted to GenBank (NCBI, BankIt).

Statistical Analysis: Results were computed and statistically analyzed using Student t test [23].

RESULTS

Case history, rectal examination, ultrasonography and progesterone assay confirmed the diagnosis of ovarian inactivity. The level of plasma progesterone was very low or even non detectable in cows suffering from ovarian inactivity if compared to normal cyclic cows (Table 1).

Results revealed imbalance of oxidant/antioxidant status in cows suffering from ovarian inactivity with high plasma MDA ($P<0.01$) and NO ($P<0.05$) and low plasma TAC, Zn and Cu and erythrocytic SOD ($P<0.01$) if compared with the normal cyclic animals (Table 1).

Table 1: Progesterone, Oxidants and Antioxidants Values in Normal cyclic Cows and Cows Suffering from Ovarian Inactivity (Mean ± SE).

Parameter	Normal Cyclic (n=24)	Inactive ovary (n=48)
Progesterone Level (ng/ml)	0.17±0.009	0.08±0.01**
Malondialdehyde Level (mmol/L)	2.18± 0.30	4.26±0.63**
Nitric Oxide Level (µmol/L)	31.72±5.62	48.69±4.80*
Total Antioxidant Capacity Level (mmol/L)	1.04±0.026	0.61±0.09**
Superoxide Dismutase Level (U/ml)	1629.63±15.32	1537.83±27.72**
Zinc Level (µg/dl)	55.17± 2.94	36.49±2.87**
Copper Level (µg/dl)	123.58±12.53	71.50±7.81**

*p<0.05** p< 0.01

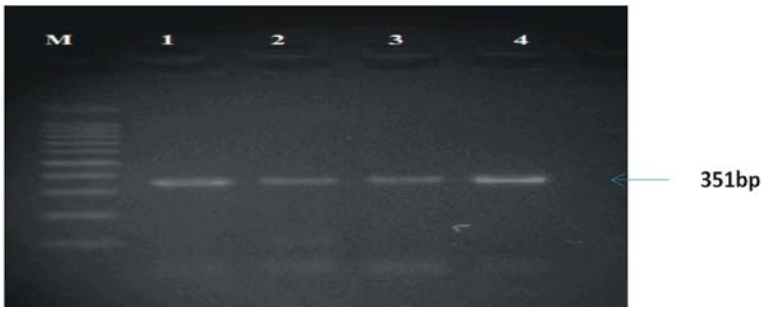

Fig. 1: Showing an agarose gel stained with ethidium bromide showing the PCR product of *Cyp*19 gene in cow. M: 100-bp ladder. Lanes 1-4: 351bp PCR product of *Cyp* 19 gene amplified from DNA.

Fig. 2: The sequence alignment of *Cyp*19 gene in two cows during cyclicity and Ovarian Inactivity (Alignment by ClustalW2)

The PCR product with the expected size (351bp) was shown by 1.5% agarose gel Electrophoresis (Fig. 1). Sequence analysis of the studied fragment revealed that there is no difference in the genetic architecture between normal cyclic and ovarian inactive animals (Fig. 2). The sequences of this *CYP19* locus in Egyptian cows (Submitted to GenBank with accession number KX944483) showed no polymorphism.

DISCUSSION

Reproductive potential of Egyptian cow is alarmingly not high, with consequent great economic losses. Ovarian inactivity is still one of the most prevalent reproductive problems in this species [24, 25]. Malnutrition as well as deficiencies in minerals, trace elements or vitamins in association with other factors are mainly responsible for the occurrence of subfertility in cows [26, 27].

In the present study, animals showed ovarian inactivity revealed high MDA and low TAC values as compared to the normal cyclic animals. That means the affected animals suffered from oxidative stress. Under proper physiological conditions, bodies usually have sufficient antioxidant reserves to cope with the production of free radicals [28] which are output during metabolism and may increase as a result of any pathological condition and other circumstances [29]. When ROS generation exceeds the body's antioxidant

production capacity, oxidative stress develops. In dairy cows, the transitional period including the peripartum and lactation, is especially critical and constitutes considerable metabolic stressors that may contribute to the onset of the oxidative stress and other disorders. This is due to complex metabolic adaptation to low energy balance in such period [30].

This study revealed significant decrease in some trace elements (Zn and Cu) in plasma of animals having low fertility. It was reported that hypocopperaemia is associated with many clinical disorders such as anemia, reduced fertility, increase exposure to infectious disease and generalized poor health causing great economic losses [31]. Following copper supplementation, a high proportion of sub fertile animals have been responded. [32, 33]. Moreover, it was reported that reduced conception rates, anovulation and anestrum and decreased LH levels were positively correlated with Cu, Fe, Zn and Se deficiency, especially if the animal in phase of growth, reproduction or lactation [34-36]. Animals having Zn deficiency and hypocopperaemia are usually suffering from stunted growth, general body weakness and become more susceptible to infection and infertility [37]. Ovaries in such animals showed reduced size and low response to FSH. Moreover, it was reported that hypocopperaemia has a central effect through the hypothalamus- pituitary axis on LH secretion with subsequence reduced ovarian estradiol secretion and absence of estrus [38].

Estrogen is a hormone with important endocrine, paracrine and autocrine properties involved in the regulation of female reproduction [39]. A low level of ovarian estrogens is one of the physiological reasons for true anestrum [40]. Estrogen synthesis begins with the mitochondrial cholesterol and androgen substrates, like androstenedione, that are turned into estrogens by the enzyme aromatase. Aromatase cytochrome P450 enzyme, the CYP19 gene protein product is responsible for aromatization or transformation of androgens to estrogens that play a crucial function in physiology of reproduction, growth of cells and differentiation [41]. CYP 19 gene is one of cytochrome *P450* genes which are the part of the multigene super family. The gene has 10 exons and exons II–X are considered as the coding region with translation start site in exon II. [42]. several authors previously reported polymorphic variants of CYP19 gene in cows with reproductive traits. One homozygous genotype CYP19/Pvu II AA (0.84780) in Polish Holstein-Friesian cows [43].Also, Jêdrzejczak*et al*. [44] studied this polymorphism*CYP19-PvuII* in Black-and-White and Jersey cattle. They reported frequencies of 0.8985 for AA, 0.0977 for AB, 0.0038 for BB and 0.9474 for A and 0.0526 for B genotypes and alleles in the Black-and-White cows. In the Jersey, all cows were genotyped as *CYP19AA*. They concluded that there is no relation between *CYP19-PvuII* polymorphism and milk production traits in the studied cows. The present study showed no significant difference in genotyping patterns in the tested animals either normal cyclic or those showed ovarian inactivity suggesting that CYP19 might not stand behind the low fertility of Egyptian cows. Also, we recommend to do further study on larger scale on this point of research to confirm the present result. However, there was a previous study on Egyptian buffalo which revealed genetic variation and polymorphism in *CYP 19* gene in animals showed ovarian inactivity. [45].

CONCLUSION

Ovarian inactivity in local animals is associated with disturbed oxidant/antioxidant status with no variation in the polymorphism of *CYP 19* gene. Further studies on a large scale are required to confirm this point.

REFERENCES

1. Ahmed, W.M., E.M. Hanafi and M.M. Zaabal, 2012. A trial to ameliorate the reproductive performance of native Egyptian cows suffering from reduced fertility. Global Veterinaria, 8(2): 174-178.
2. El Khadrawy, H.H., M.M. Zaabal, W.M. Ahmed and S.I. Shalaby, 2010. Genetic constitutions affecting ovarian activity in Egyptian cattle and buffaloes. Glob. J. Mol. Sci., 5(2): 53-57.
3. Abba, M., J.C. De Luca, G. Mattioli, E. Zaccardi, E. Beutler, O. Duron and M.B. Kelly, 2000. Clastogenic effect of copper deficiency in cattle. Mutat. Res., 46(1): 51.
4. Sharma, R.K., N. Reynolds, M. Rakhit and A. Agarwal, 2013. Methods for detection of ROS in the female reproductive system. In: Studies on Women's Health, Oxidative Stress in Applied Basic Research and Clinical Practice, A. Agarwal, N. Aziz and B. Rizk (Ed.), Springer Science and Business Media, New York, pp: 33-60.
5. Ahmed, W.M., G.M. Nabile, H.H. El Khadrawy, E.M. Hanafi, S.I. Abd El- Moez and A.R. Abd El Hameed, 2010. The relationship between oxidant/antioxidants imbalance and fertility in buffalo-cows. Inter. J. Acad Res., 2(1): 1-9.

6. Circu, M.L. and T.Y. Aw, 2010. Reactive oxygen species, cellular redox systems and apoptosis. Free Radic. Biol. Med., 48: 749-762.
7. Griffiths, L.M., S.H. Loeffler, M.T. Socha, D.J. Tomlinson and A.B. Johnson, 2007. Effects of supplementing complexed zinc, manganese, copper and cobalt on lactation and reproductive performance of intensively grazed lactating dairy cattle on the South Island of New Zealand. Anim Feed Sci. Technol, 137: 69-83.
8. Sharma, M.C., C. Joshi, N.N. Pathak and H. Kaur, 2005. Copper status and enzyme, hormone, vitamin and immune function in heifers. Res. Vet. Sci., 79(2): 113-123.
9. Weiss, W.P. and J.W. Spears, 2006. Vitamin and trace mineral effects on immune function of ruminants. In: Sejrsen, K., Hvelplund, T., Nielsen, M.O. (Eds.), Ruminant Physiology. Wageningen Academic Publishers, Utrecht, the Netherlands, pp: 473-496.
10. Beuzen, N.D., M.J. Stear and K.C. Chang, 2000. Molecular markers and their use in animal breeding. The Vet J., 160(1): 42-52.
11. Szatkowska, I., W. Grzesiak, M. Jêdrzejczak, A. Dybus, D. Zaborski and D. Jankowiak, 2011. An analysis of CYP19, CYP21 and ER genotypes in Polish Holstein-Friesian cows with regard to the selected reproductive traits. Acta Veterinaria Brno, 80: 65-71.
12. Kowalewska-luczak, I., 2010. Polymorphism of the CYP19 gene and milk production traits of dairy cattle. Turkish Journal of Veterinary and Animal Sciences, 34 (6): 493-496.
13. Kowalewska-luczak, I., E. Michniewicz and H. Kulig, 2013. Effect of CYP19 SNPs on milk production traits of jersey cows. Acta Scientiarum Polonorum, 12(1): 33-40.
14. Jêdrzejczak, M., W. Grzesiak, I. Szatkowska, A. Dybus, M. Muszyñska and D. Zaborski, 2011. Association between polymorphisms of CYP19, CYP21 and ER1 genes and milk production traits in Black and White cattle. Turk. J. Vet. Anim. Sci., 35(1): 41-49.
15. Wisdom, G.B., 1976. Competitive immunoenzymatic colorimetric method for quantitive determination of progesterone in serum or plasma. Clin. Chem., 22(8): 1243-1255.
16. Ohkawa, H., W. Ohishi and K. Yagi, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., 95: 351-358.
17. Montgomery, H.A.C and J.F. Dymock, 1961. Determination of nitric oxide. Analysts, 86: 4-14.
18. Koracevic, D. and G. Koracevic, 2001. Method for the measurement of antioxidant activity in human fluids. J. Clin. Pathol, 54: 356-361
19. Nishikimi, M., A. Rao and K. Yagi, 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. Biochem. Biophys. Res. Commun., 46: 849-854.
20. Hayakawa, R. and J. Jap, 1961. Determination of Zinc. Toxic Environ. Health, 8: 14-18.
21. Ventura, S.and E.J. King, 1951. Determination of copper and zinc in blood serum. Biochem. J., 48(5): Ixi-Ixii
22. Kumar, O.S., D. Sharma, D. Singh and M.K. Sharma, 2009. CYP19 (cytochrome P450 aromatase) gene polymorphism in murrah buffalo heifers of different fertility performance. Research in Veterinary Science, 86: 427-437.
23. Snedecor, G.W. and W.G. Cochran, 1980. Statistical methods. 7ED, Iowa State Univ. Press, Ames, Iowa, U.S.A.
24. Ahmed, W.M., 2007. Overview on some factors negatively affecting ovarian activity in large farm animals. Glob. Vet, 1:53 -66.
25. Nanda, A.S., P.S. Brar and S. Prabhakar, 2003. Enhancing reproductive performance in dairy buffalo: major constraints and achievements. Reproduction Supplement, 61: 27-36.
26. Perera, B.M., 2008. Reproduction in domestic buffalo. Reproduction of Domestic Animals, 43 Supplement, 2: 200.
27. Oswin Perera, B.M., 1999. Reproduction in water buffalo: Comparative aspects and implications for management. J. Reproduction and Fertility (Supplement), 54: 157-168.
28. Castillo, C., J.L. Benedito, M. Lopez-Alonso, M. Miranda and J. Hernandez, 2001. Importancia del estr_esoxidativo en Ganado vacuno: en relaci_on con el estadofisiol_ogico (pre~nez y parto) y la nutrici_on. Archivos de MedicinaVeterinaria, 33: 5-20.
29. Roth, E., 2000. Oxygen free radicals and their clinical implications. Acta Chirurgica Hungarica, 36: 302-305.
30. Mohamed G.E., E. Abd- elnaser and H.K. El Sayed, 2015. Preliminary study on lipid profile with relation to total antioxidant capacity and some hematological and biochemical changes of pre-post-partum buffalo heifers at Assiut city. Assiut Vet. Med. J. Vol., 61: 144.
31. Sharma, M.C., C. Joshi, N.N. Pathak and H. Kaur, 2005. Copper status and enzyme, hormone, vitamin and immune function in heifers. Res. Vet. Sci., 79(2): 113-123.

32. Black, D.H. and N.P. French, 2000. Copper supplementation and bovine pregnancy rates: Three types of supplementation compared in commercial dairy herds. Irish Vet. J., 53: 213-222.
33. Kendall, N.R., D.V. Illingworth and S.B. Telfer, 2001. Copper responsive infertility in British cattle: The use of a blood ceruloplasmin to copper ratio in determining a requirement for copper supplementation. In: Fertility in the High-Producing Dairy Cow, Occasional Publication. MG Diskin. Edinburgh (Ed.), UK: British Society of Animal Science, 26(2): 429-432.
34. Phillippo, M., W.R. Humphries, T. Atkinson, G.D. Henderson and P.H. Garthwaite, 1987. The effect of dietary molybdenum and iron on copper status, puberty, fertility and oestrous cycles in cattle. J. Agril. Sci., 109: 321-336.
35. Pederson, C.H., 1989. Infertility and disease surveillance using a milk recording scheme in the Sahiwal district of Pakistan. Tropical Animal Health Production, 22: 60.
36. Graham, T.W., 1991. Trace element deficiencies in cattle. Veterinary, 7(1): 153-215.
37. Shuttle, N.F., 1986. Copper deficiency in ruminants, recent developments. Veterinary Record, 119: 519-522.
38. Kendall, N.R., P. Marsters, L. Guo, R.J. Scaramuzzi and B.K. Campbell, 2006. Effect of copper and thiomolybdates on bovine theca cell differentiation *in vitro*J. Endocrinol., 189: 455-463.
39. Heine, P.A., J.A. Taylor, G.A. Iwamoto, D.B. Lubahn and P.S. Cooke, 2000. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. Proc. Natl. Acad. Sci. USA, 97: 12729-12734.
40. Hafez, E.S.E. and B. Hafez, 2000. Reproduction in farm animals. Seventh Ed, Lippincott Williams and Wilkins publications, PP: 261-263.
41. Conley, A.J. and M. Hinshelwood, 2001. Mammalian aromatases. Reproduction, 121: 685-695.
42. Simpson, E.R. and S.R. Davis, 2001. Minireview: aromatase and the regulation of estrogen biosynthesis-some new perspectives. Endocrinol, 142(11): 4589-4594.
43. Szatkowska, I., W. Grzesiak, M. Jêdrzejczak, A. Dybus, D. Zaborski and D. Jankowiak, 2011. An analysis of CYP19, CYP21 and ER genotypes in Polish Holstein-Friesian cows with regard to the selected reproductive traits. Acta Veterinaria Brno, 80: 65-71.
44. Jêdrzejczak, M., I. Szatkowska, S. Zych, W. Grzesiak, E. Czerniawska-Pi¹tkowska and A. Dybus, 2006. Evaluation of associations of the polymorphism in the placenta-specific promoter 1.1 of the CYP19 gene in Black-and-White and Jersey cattle with milk production traits. Arch. Tierz., 49: 311-314.
45. Othman, E.O., W.M. Ahmed, E.A. Balabel, M.M. Zaabal, H.H. El Khadrawy and E.M. Hanafy, 2014. Genetic polymorphism of Cyp19 Gene and its association with ovarian activity in Egyptian buffaloes. Global Veterinaria, 12(6): 768-773.