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BLOOD PROFILE OF ZINC AND COPPER AT ESTRUS STAGE AND ITS EFFECT ON SUBSEQUENT PREGNANCIES IN BUFFALOES

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ABSTRACT

The present study aimed to determine the blood profile of copper (Cu) and zinc (Zn) concentrations at estrus and subsequent pregnancy in Nili-Ravi buffaloes. Fifty-one estrus buffaloes (4 to 6 years of age) were selected based on physical signs and rectal palpation and were inseminated on an am-pm basis. The blood samples were collected at the time of artificial insemination (AI) via jugular venipuncture from 51 buffaloes, and 21 buffaloes were further bled at day 16 of AI and pregnancy diagnosis time. The concentrations of Cu and Zn in blood sera were determined using an automatic chemistry analyser. The pregnancy diagnosis was performed through rectal palpation at day sixty post-AI. The pregnancy confirmation was based on the asymmetry of uterine horns with fetal fluids. The results indicate that Zn levels were higher numerically in pregnant than nonpregnant buffaloes. The serum Cu concentration was also similar between pregnant and nonpregnant buffaloes. The conception rate in buffaloes with a standard Zn level was significantly higher than in Zn-deficient buffaloes. There was an increasing tendency of conception in the buffaloes with a standard concentration of Cu as compared to Cu-deficient buffaloes. Post-AI changes in Zn concentration were negligible, but the concentration of Cu decreased with the advancement in pregnancy. In conclusion, a higher Zn level at the time of AI successfully established pregnancy in buffaloes.

Keywords: *Bubalus bubalis*, buffaloes, Cu, Zn, estrus, pregnancy

INTRODUCTION

Changes in blood composition reveal the reproductive health status of the animal. The reproduction rate in animals is directly influenced by many metabolites of the bloodstream (Ashmawy, 2015). Supply of all the bio-elements in an appropriate amount in the form of diet is not only required for the growth and maintenance of the body functions but also for the efficient

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reproduction of the animals. Supply of these constituents below or above the optimal range will influence the reproduction rate (Abraham, 2017). The factors that regulate the onset of puberty in buffaloes include genotype, climate, management of the farms, and nutrition (Gupta *et al*., 2016). The main reason for the delayed maturity is the insufficient supply of nutrients during the growing phase. It can reduce the problem through proper feed management during the growing age of the animal (Qureshi *et al*., 2002).

A balanced diet is essential for the proper functioning of the reproductive system. Vitamins, macro- and micronutrient deficiency result in many reproductive disorders. Late onset of puberty is not an inherent characteristic of the species, and longer age at first calving can be reduced by proper nutritional management at growing age (Michael *et al*., 2020). Nutritional deficiency in buffaloes is one of the leading causes of low productivity. Lack of minerals causes anestrus, repeat breeding, retention of the placenta, and abortion. The deficiency of micronutrients causes ovarian inactivity and, ultimately, significant economic loss due to the low reproduction rate. The buffaloes kept at small farms especially suffer from malnutrition and mismanagement. Mineral deficiencies in the blood may also delay the onset of puberty in cattle. Lack of Cu, Zn, manganese, cobalt and other trace elements may disturb the proper functioning of genital organs. These microelements are the cofactors of many enzymes. Dysfunction of many enzymes due to deficiency of trace elements may lead to infertility in cattle. Production of progesterone and estradiol may affect due to low levels of Cu and Zn (Del Río-Avilés *et al*., 2022). The trace elements directly influence the reproductive efficiency of animals. Micronutrients detoxify free radicals, produce reproductive steroids and other hormones, and metabolise carbohydrates, proteins, and lipids within the cell. The concentration of these elements above or below a particular range may impair spermatogenesis, the development of an embryo, and its survival. Abnormal concentrations of trace elements in the blood may cause repeat breeding and ovarian inactivity.Cu deficiency adversely affects female reproductive development and may lead to abnormal fetus development (Mudgal *et al*., 2014). Cu is also involved in the secretion of LH, FSH, and estrogen. A decrease in serum Cu level may reduce the fertility rate in cattle (Amin *et al*., 2023). Zn is another member of trace element, synthesis of prostaglandins, raising the level of beta-carotene to repair uterine lining, increasing the conception rate and promoting embryo development (Bindari *et al*., 2013). The oestrous cycle and pregnancy rates are affected in Zn-deficient animals because an optimal Zn level is required for the regular activity of FSH and LH, fetal mummification, prolonged labour, abortion, and lower birth weights (Khan *et al*., 2015). Keeping in view the importance of Zn and Cu on reproductive functions, the present study aimed to determine the blood profile of Cu and Zn concentrations at estrus and subsequent pregnancy in *Nili-Ravi* buffaloes.

MATERIALS AND METHODS

Selection of animals and collection of blood samples

The study area was Dhudial, a small town in the district of Chakwal. Fifty-one buffaloes were chosen for the study. Buffaloes kept by farmers on a small scale in rural areas were studied. The buffaloes showing positive heat signs were considered for the study. The reproductive health of the animals was assessed by observing the genital tract before artificial insemination (AI). Each buffalo understudy was 4 to 6 years old, 1 to 2 lactations with 2 to 4 kg/day milk production, and more than 150 days in milk. None of the selected animals was a repeat breeder. The blood samples of the selected animals (n=51) were collected using a 16-gauge needle through jugular venipuncture during estrus. Among these, 21 buffaloes were further used for blood sampling at day 16 after AI and pregnancy diagnosis time (50 to 60 days after AI). Blood samples were collected in 5ml gel tubes and transported to the laboratory in an icebox to maintain a temperature of 4°C.

Processing of samples

Hemolysed samples were discarded, and the animal was excluded from the study. Each blood sample was centrifuged at 3000 rpm for 6 minutes. The serum was taken out using a micropipette and stored at -20°C in 1.5 ml Eppendorf microtubes for further analysis.

Estimation of Cu and Zn

The serum concentrations of Cu and Zn were determined using an automatic chemistry analyser (Mindray BS-240; China). Commercially available kits (DIALAB, LOT: 8148/CF25181C for Cu and DIALAB, LOT: 8162/ZF01181C for Zn) were used for the determination of serum concentrations of Cu and Zn.

Determination of Cu in the serum

Proteins containing Cu will release Cu in the acidic medium, which reacts with 3-((4-((3,5-dibromo-2-pyridinyl)azo)phenyl) ethylamino)-1-Propanesulfonic acid (DiBr-PAESA) to produce a coloured complex. The absorbance of the coloured complex is directly proportional to the amount of Cu in the sample. Equal volumes of buffer reagent (L1) and colour reagent (L2) were mixed and stored at 2°C before use. One ml of the working mixture was mixed with 0.05 ml of the sample in a clean test tube. The procedure was repeated for each test sample, which was carefully labelled. The samples were then loaded in the automatic chemistry analyser, the Mindray BS-240.

Determination of Zn in the serum

Zn reacts with *Nitro* PAPS in a basic medium to produce purple coloured complex. The intensity of the coloured complex is directly proportional to the concentration of Zn in the sample. A 16 ml buffer reagent was mixed with 4 ml colour reagent to prepare 20 ml of working regent. It was stored at 2°C until use. The working reagent and test sample were mixed in a dry, clean test tube. At room temperature, 1 ml of working reagent was mixed in 0.05 ml of the test sample. The mixture was incubated at 25°C for 5 minutes. All the samples were treated the same way and labelled individually. All the samples were simultaneously loaded in the analyser.

Artificial insemination

After confirmation of estrus by heat signs and examination of the genital tract, the buffaloes were inseminated. The animals were inseminated following the am-pm rule. Frozen thawed semen straws (0.5 ml semen) containing about 30 million sperms were used for AI. A single AI technician inseminated the animals at the insemination centre in district Chakwal. The buffaloes were inseminated during the October-February period of months. The age of each animal was 4 to 6 years, milk production was 2 to 4 kg per day, the lactation period was more than 150 days, and each animal was either at the first or second lactation stage.

Pregnancy diagnosis

The same AI technician confirmed the pregnancy in the inseminated animals fifty to sixty days post-AI. The pregnancy diagnosis was based on specific criteria like the asymmetry of uterine horns, the presence of fetal fluids, and the membrane slip test. Early pregnancy diagnosis was impossible due to the unavailability of ultrasound and trained personnel. Out of 51 inseminated buffaloes, 30 were diagnosed pregnant and 21 non-pregnant.

Statistical analysis

All the data were analysed using statistical software (SPSS; version 17.0.1 Chicago, IL, USA). The Chi-square test was used to analyse the Cu and Zn deficit data or normal buffaloes against pregnancy status. The Zn and Cu levels data in pregnant and nonpregnant buffaloes against different sampling times were compared through repeated measures ANOVA. The student t-test was applied to compare the overall Cu and Zn levels in pregnant and nonpregnant groups. The p-value was calculated to analyse whether the difference in the mean values was significant at $P<0.05$.

Pregnancy rate in buffaloes having normal and deficient Cu or Zn values relative to the time of AI

The pregnancy status of normal and deficient Cu or Zn buffaloes has been presented in Table 1. The pregnancy rate of buffaloes with normal (68.75%) and deficient (42.1%) Cu values was similar ($P>0.05$). However, there was an increasing tendency ($P=0.062$) in pregnancy for buffaloes having a normal status of Cu at the time of estrus. In contrast, a significantly ($P<0.05$) higher number of buffaloes got pregnant having normal (72.4%) Zn values at the time of AI compared to Zn-deficient (40.9%) buffaloes.

Serum Cu and Zn level at the time of AI, 16 days after AI and time of pregnancy diagnosis in buffaloes

The average serum level of Cu and Zn at the time of AI, 16 days after AI, and the time of pregnancy diagnosis in buffaloes has been presented in Table 2. The mean value for the serum concentration of Cu was significantly different ($P<0.05$) at estrus in pregnant (56.4±2.4 µg/dl) and nonpregnant (37.0±5.3 µg/dl) buffaloes. In contrast, the serum levels of Cu after 16 days of AI and pregnancy diagnosis time were similar ($P>0.05$) among pregnant and nonpregnant buffaloes. The mean level of Cu was the same ($P>0.05$) in nonpregnant buffaloes at each sampling time (at the time of AI, 16 days after AI and time of pregnancy diagnosis); however, there was a decreasing trend ($P<0.05$) in the Cu level of pregnant buffaloes from estrus stage to the time of pregnancy diagnosis. The mean Zn level was significantly higher ($P<0.05$) in pregnant buffaloes compared to nonpregnant buffaloes at the estrus stage (105.6±2.5 vs 80.0±4.6), 16 days after AI (105.3±2.6 vs 75.8±2.9) and time of pregnancy diagnosis (101.5±3.4 vs 84.6±3.7). The mean Zn level did not fluctuate ($P>0.05$) at the time of AI, 16 days after AI, and time of pregnancy diagnosis in pregnant and nonpregnant buffaloes.

Serum Cu level in pregnant and nonpregnant buffaloes

The mean serum Cu concentration comparison between pregnant and nonpregnant buffaloes has been presented in Figure 1. The blood samples were collected from buffaloes for serum levels of Cu. The buffaloes were further examined for declaring pregnant and nonpregnant. The mean serum Cu concentration in pregnant and nonpregnant buffaloes was 51.7±1.3 and 38.6±2.6 µg/dl, respectively. The level of Cu was significantly different ($P<0.05$) between pregnant and nonpregnant buffaloes. The mean observed values for pregnant buffaloes were within the reference value range (50 to 126 µg/dl), whereas the serum profile of nonpregnant buffaloes was deficient for Cu.

Serum concentration of Zn in pregnant and nonpregnant buffaloes

The comparison of mean serum Zn concentration among pregnant and nonpregnant buffaloes has been presented in Figure 4.3. The blood samples of buffaloes were examined for serum Zn level, and later, the buffaloes were examined for pregnancy status. The mean serum Zn was 103.9±1.7 µg/dl and 79.7±2.2 µg/dl in pregnant and nonpregnant buffaloes, respectively. The level of Zn was significantly higher in pregnant compared to nonpregnant buffaloes. The recorded values of pregnant buffaloes matched reference values (90 to 148 µg/dl), and nonpregnant buffaloes showed lower values of Zn than the normal range.

DISCUSSION

Scanty data focuses on the serum concentration of Cu and Zn at the time of estrus and its relationship to the pregnancy rate in Nili-Ravi buffaloes reared in the arid zone of Punjab, Pakistan. The current findings of this study are preliminary and provide baseline information to estimate the fertility based on the estimation of Cu and Zn serum profiles of buffaloes under field conditions.

The samples were collected via jugular vein puncture in the present study and followed the applied procedures. Previous studies showed that uterine sampling was also considered, but it has disadvantages in improper cervical dilation, small sample size, or side effects of surgical procedures if done. It has also been noted that sample types like milk (Patodkar *et al*., 2018), serum (Akhtar *et al*., 2014), or uterine secretions (Alavi Shoushtari *et al*., 2015) also provide variable estimates of Cu or Zn concentrations in buffaloes. The currently applied sampling method lacked limitations and went smoothly under field conditions where no restraining facility existed. The samples were collected from the same age as the animals by examining teeth growth, and the age of buffaloes varied from 4 to 6 years at the time of sampling.

Moreover, it was ensured that the body condition score (BCS) of animals should be optimum because BCS significantly affects an animal's Cu and Zn profiles (Delfino *et al*., 2018). Given the BCS scale, the sampling was done from only those buffaloes with BCS 2 to 3. Following the standards, the samples were collected from buffaloes of similar age and BCS. Because the same age and BCS overcome the effects of the profile of ration on serum Cu and Zn profile, the feeding regimen was not considered. For samples at estrus, large-size ovarian follicle and uterine changes were the main criteria for being in heat/estrus in buffaloes. Moreover, obtained serum Cu and Zn concentrations were compared with reference values published in earlier reports (AbdEllah *et al*., 2013).The overall Cu level in estrus buffaloes was recorded at 46.98 µg/dl, which is relatively lower than the earlier reports in buffaloes. This variation could be due to feed ingredients and forage growth or the stage of cyclicity of the submitted buffalo group. These obtained values were significantly lower than the Cu content observed in anestrous and cyclic buffaloes reported by Akhtar *et al.* (2009). Moreover, the Cu contents were significantly reduced when Cu analysis was performed from the uterine samples (Shoushtari *et al*., 2015). Earlier studies indicate the physiological status like puberty (Alavi Shoushtari *et al*., 2015), estrus (Alavi Shoushtari *et al*., 2012), anestrous (Mugal *et al*., 2014), lactation (Patodkar *et al*., 2018), and pregnancy (AbdEllah *et al*., 2013) had an impact on the Cu contents in buffaloes; moreover, the pathological conditions like vaginal prolapse (Akhtar *et al*., 2012; Bhatti *et al*., 2006) or repeat breeding (Akhtar *et al*., 2014) of buffaloes also had variable Cu contents. There was also a change in Cu when the buffaloes were synchronised by different progestin-based protocols (Amin *et al*., 2019; Parmer *et al*., 2015), and it has been observed that hormonal treatment affects the Cu contents in buffaloes and the steroids affect the mobility of trace minerals in serum of treated buffaloes. The role of parity and production systems on serum Cu concentration has also been demonstrated in buffaloes (Bhatti *et al*., 2006) as the Cu is a crucial component for the production and release of GnRH, which affects the synthesis and release of LH, which in turn affect fertility and overcome the hormone-related reproductive disorders. Previously, a change in Cu concentration was observed in cyclic, non-cyclic or anestrous buffaloes that were compared (Mugal *et al*., 2014; Akhtar *et al*., 2015). The current study also noted that Cu is high at estrus in pregnant buffaloes, which elaborates on its role in pregnancy establishment. Amin *et al.* (2019) showed that lower levels of Cu following calving in cows and buffaloes influence cyclicity, early embryonic viability, and embryonic and placental development. In the present study, we found that serum Cu levels reduced significantly in advancing pregnancy in buffaloes compared to estrus time. This fashion of Cu level in pregnant buffaloes could be explained by the fact that the pregnancy process also relies on the Cu level, and extra demands of the body for Cu by conception are fulfilled by the dam (Naoman *et al*., 2012).

In addition, an association of Cu and GnRH, FSH, and LH was also determined during *in vitro* experiments suggesting that Cu-GnRH has higher potency to augment the FSH and LH from the pituitary gland compared to natural GnRH activity (Michaluk and Kochman, 2007). It could be elucidated from the experiments that lower fertility has a direct relation to Cu level in animals. However, Alavi Shoushtari *et al.* (2015) reported a decreasing trend in Cu serum concentration during pro-estrus and estrus stages in buffaloes, where higher levels of FSH and LH might decrease the serum Cu content and enhance Cu mobility towards the uterine lumen. It has also been speculated that LH surge in response to elevated estradiol levels influences the Cu concentration in the body. A lower level in serum and increment in uterine Cu contents at the time of estrus and diestrous could be required for embryonic development and neonatal metabolism. Previously, Cu deficiency resulted in pathological alteration in organs like skeletal and cardiac tissues (Michaluk and Kochman, 2007). In this context, Mudgal *et al.* (2014) observed that Cu is essential in maintaining the cyclicity in buffaloes. Cu supplementation induced reproductive cyclicity in hypo cupric anestrous buffaloes through their diet.

Zn in sperm cells and seminal plasma protects the cells from excessive ROS. Likewise, its accumulation in oocytes during maturation is considered necessary for growth and lipoproteins for embryonic development. Moreover, it plays a vital role in completing meiosis-I in oocytes for maturation in ovulating follicles; hence, a sufficient amount is necessary for oocyte maturation around the ovulation period, particularly in ruminants. In addition to mentioned mechanisms, different intracellular functions are governed by Zn in conjunction with female reproductive processes (Murarka *et al*., 2008). Several reports suggest the role of Zn on female reproduction, which has been considered a prime trace element for regulating cell function. In the present study, the concentration of Zn was measured at the time of estrus, after breeding and upon pregnancy confirmation in Nili-Ravi buffaloes. The Zn level in pregnant buffaloes was observed to be higher, and the proportion of buffaloes with a higher level (within reference level) of Zn at the estrus time had a greater conception rate. The level of Zn reported by the previous researcher is 100 to 190 ug/dl.

Moreover, the sample of type, i.e. milk, blood serum or uterine secretions, has also affected the reported values of Zn in experimental animals. The necessity of Zn during pregnancy has primary importance, and Zn deficiency leads to complications at the term of gestation in large animals, such as retained placenta and vaginal or uterine prolapse or uterine infections (Akhtar *et al*., 2015) as the redox mechanism of the animals gets compromised due to low Zn level in the body. It has been observed that Zn-deficient rats have reduced preimplantation loss when fed diets containing Zn during the different stages of gestation. Zn provision as dietary supplementation can reduce the incidence of fetal dysmorphology and postnatal mortality if caused by any atmospheric toxin (Sharkar *et al*., 2011). There are also high chances of early embryonic mortality in dairy animals due to low Zn contents in the blood profile. In addition, raising the animal Zn deficient soiled has infertility issues, whereas the chance of abortion is evident (Anchordoquy *et al*., 2019). The occurrence of infertility issues has been associated with a decline in the Zn due to hemodilution, less Zn binding protein concentration, and hormonal changes during estrus, diestrus, anestrous, or pregnancy, which cause less availability of active transport of general Zn circulation to the reproductive tract (Patodkar *et al*., 2018; Kumar *et al*., 2007).

In contrast, there is also a report of excess Zn effects in rats where the size of fetuses was affected, and retorted sex organs growth was also observed. The same observations were seen when animals were on a low Zn diet. The exact mechanism is unknown how the excess ingestion of Zn affected the pregnancy or developing fetuses (Sharkar *et al*., 2011). In females, Zn deficiency might have impaired FSH and LH production, altered follicular development, irregular oestrous cycle, increased abortion rate, and stillbirths. However, dietary supplementation during the dry period enhances cyclicity; minimises embryonic or fetal loss and parturition complications (Wang *et al*., 2014).

Previously it has been reported that low Cu and Zn levels affected the cyclicity in buffaloes and resulted in a higher incidence of anestrous in buffaloes (Akhtar *et al*., 2012). Proper blood profile of Zn or Cu estimation, dietary or soil Cu and Zn estimation, and supplementation of Cu and Zn could minimise the incidence of cyclicity in buffaloes. The same observations have been observed in beef cows, and beneficial effects of Cu and Zn were obtained in terms of pregnancy after dietary supplementation (Kumar *et al*., 2007; AbdEllah *et al*., 2013). Additionally, dietary provisions minimise post-calving uterine infections and embryonic loss in the subsequent cycle. Compared to non-supplemented animals, an

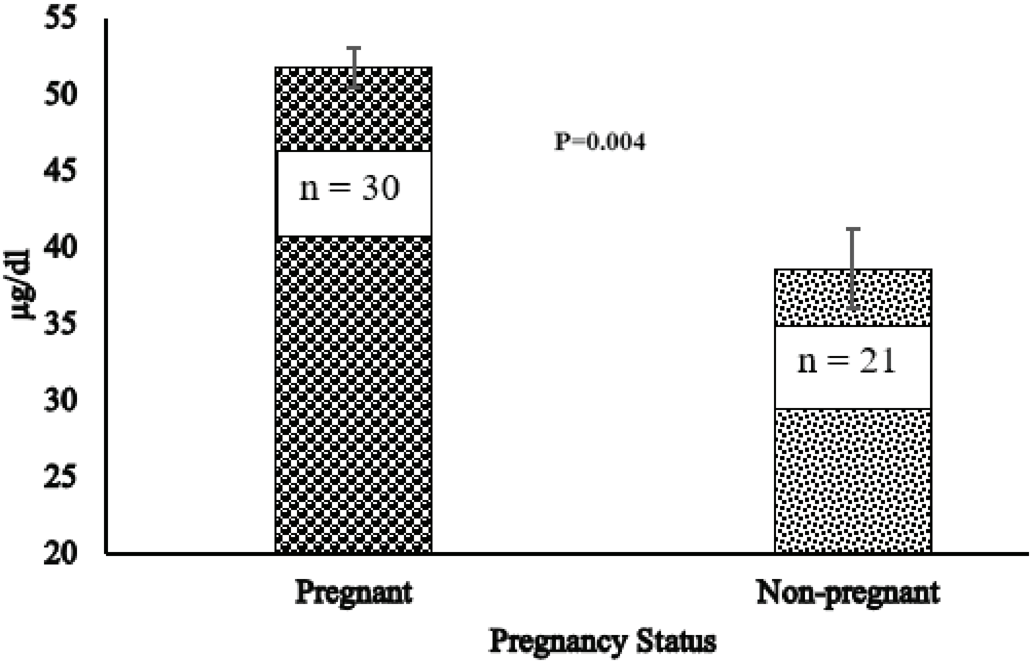

Figure 1. Serum Cu level in pregnant and non-pregnant buffaloes.

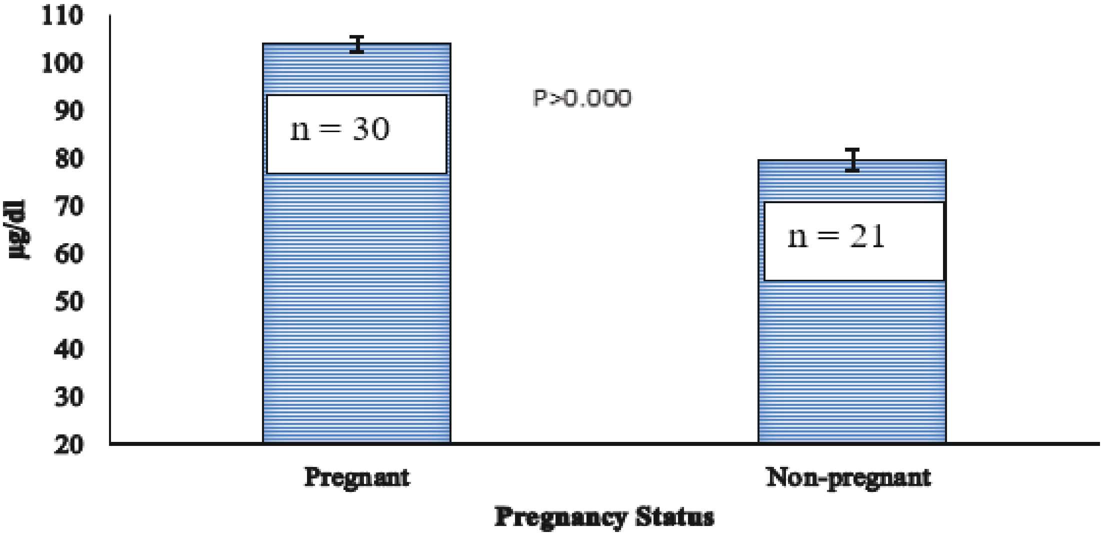

Figure 2. Serum Zn level in pregnant and non-pregnant buffaloes.

Table 1. Comparison of pregnancy outcomes in Cu or Zn deficient and normal buffaloes.

Status	Cu (n=51)	Zn (n=51)
Normal (%) [a]	22/32 (68.75)	21/29 (72.4)
Deficient (%) [b]	8/19 (42.1)	9/22 (40.9)
Chi-square value	3.494	5.126
P-value	0.062	0.043

P-value <0.05 indicates the difference between groups in column

[a]indicate normal value for Zn (90 to 148 µg/dl) and Cu (50 to 126 µg/dl)

[b]indicate the values below reference value for Zn (<90 µg/dl) and Cu (<50 µg/dl).

Table 2. Serum Cu and Zn concentrations at the time of AI, 16 days post-AI, and at pregnancy diagnosis in buffaloes.

Time of sampling	Cu (µg/dl)		Zn (µg/dl)	
	Pregnant (n=12)	Nonpregnant (n=09)	Pregnant (n=12)	Nonpregnant (n=09)
At the time of AI	$56.4 \pm 2.4^{a,x}$	37.0 ± 5.3^{b}	105.6 ± 2.5^{a}	80.0 ± 4.6^{b}
16 days after AI	50.5 ± 2.7^{xy}	40.7±4.7	105.3 ± 2.6^{a}	75.8 ± 2.9^{b}
Pregnancy diagnosis time	48.2 ± 2.3^{y}	38.2±3.8	101.5 ± 3.4^{a}	84.6 ± 3.7^{b}

Superscripts (a,b) show difference in rows for Zn or Cu between pregnant and nonpregnant buffaloes.

Superscripts (x,y) indicate the difference in column between different blood sampling times.

increased fertility rate was observed in Cu and Zn-supplemented animals. (Anchordoquy *et al*., 2019). There are also few *in vitro* reports where Cu and Zn influence on the musculature of myometrium has been reported. It has been concluded that Cu and Zn have opposite effects, where Zn ions act as suppressive, and Cu ions have stimulatory effects on uterine musculature. There is also a report where low-level Cu-Zn dependent superoxide dismutase resulted in lower fertility and affected pregnancy loss, or delivered fetuses had smaller sizes than animals with normal haematological Cu and Zn levels (Essawi *et al*., 2021). Previous studies showed that the Cu and Zn concentrations are higher in cyclic buffaloes compared to pre-pubertal stages. This variation is linked with hormonal fluctuations and other alterations due to puberty increasing the serum Cu and Zn contents (Akhtar *et al*., 2012, 2007). It has also been noted that Zn contents increased in uterine secretions than serum levels. Its level was at its lowest at the stage of estrus in uterine secretions. This lowest level is due to the effects of estrogen dominance, which changes the uterine secretions. The higher uterine secretions dilute the Zn level; hence lower level was seen. In contrast, Zn contents affected the level during diestrous in bovine (Alavi-Shoushtari *et al*., 2012).

In conclusion, a higher Zn level at the time of AI successfully established pregnancy in buffaloes. The Cu level decreased in pregnancy relative to the levels of Cu at the time of AI or estrus onset. Zn levels remained unchanged through the different physiological stages of blood sampling in buffalo. Moreover, estimating Zn or Cu during estrus could be a good indicator for estimating future fertility in buffaloes, as lower Zn or Cu levels could reduce conception rates.

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