

Research Article

Evaluation of Milk Yield and Some Lactogenic Hormones in Lactating Wistar Rats after treatment with Ascorbic acid and α -Tocopherol

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Milk yield, Vitamin C
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ABSTRACT

Background: Milk synthesis and ejection is essential for breastfeeding and is influenced by nutritional and non-nutritional factors. Vitamin C (L-ascorbic acid) is an essential nutrient for humans and certain animal species. Vitamin E (α -Tocopherol) is a form of vitamin E that is preferentially absorbed. This study was designed to assess milk yield, serum prolactin and oxytocin hormone in lactating Wistar rats following ascorbic acid and α -Tocopherol supplementation. **Methods:** At parturition, the animals were randomly divided into five groups thus: Group I: (Normal control) was given commercial feed and distilled water, orally (1 ml/kg), Group II: metoclopramide (5 mg/kg), Group III: 100 mg/kg of Vitamin E. Group IV: 100 mg/kg of Vitamin C and Group V was treated with the co-administration of vitamin C and E 100 mg/kg each. Administration was carried out orally from day 3 to day 13 of lactation at 06:00 hours daily. The animals were euthanized on day 14 using ketamine and diazepam at 75 mg/kg and 25 mg/kg given intraperitoneally and thereafter sacrificed. Milk yield 18 hours after gavage as well as serum levels of prolactin and oxytocin were evaluated. **Result:** Statistical analysis was carried out using SPSS version 20 with the aid of one-way analysis of variance (ANOVA) and tukey's post-hoc test. Values of $p \leq 0.05$ were considered significant. There was a statistically significant ($p < 0.05$) increase in milk yield in groups IV and V when compared to control: 3.72 ± 0.37 , 3.60 ± 0.33 vs 2.28 ± 0.08 respectively, and also a significant decrease ($p < 0.05$) in group V compared to metoclopramide-treated group; 9.32 ± 0.57 vs 11.48 ± 0.72 . Serum oxytocin level was significantly increased ($p < 0.05$) in group IV compared to the control group 13.32 ± 1.16 vs 9.46 ± 0.81 and a significant decrease in group V compared to group IV 8.80 ± 0.95 vs 13.32 ± 1.16 was observed. **Conclusion:** This study has shown that Vitamin C possesses more lactogenic activity which increased more milk yield and serum oxytocin level in comparison to vitamin E and metoclopramide.

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INTRODUCTION

Antioxidants are man-made or natural substances that may prevent or delay some type of cell damage (Yadav *et al.*, 2016). Under normal physiological conditions, antioxidants and oxidants are in a dynamic equilibrium with antioxidants scavenging ROS generated in the body. Vitamins C (ascorbic acid) and E (α -tocopherol) are the main natural antioxidants occurring in

biological system. Vitamin C as an extracellular fluid antioxidant reduces excessive ROS generation. Vitamin E is a chain-breaking antioxidant, which exerts its antioxidant effects mainly on cell membrane. It also regenerates the activity of tocopherol by reducing the tocopheroxyl radicals (Abdel-Khalek *et al.*, 2008). Antioxidants are widely used as dietary supplements and numerous studies suggest that supplements of vitamin C and/or vitamin E may contribute to lowering the risk of specific chronic diseases and enhancing the antioxidant activities of breast milk during lactation. Vitamin C is a powerful dietary antioxidant which influences iron absorption and helps fight cell-damaging free radicals. Natural sources of vitamin C

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are fruits and vegetables and they include oranges, grapes, strawberries, red bell peppers, tomatoes, cabbage, spinach green peas just to mention a few while Vitamin E can be found in various foods and oils for instance nuts, seeds, green leafy vegetables and fortified cereals (Moser and Chun, 2016), (Rizvi *et al.*, 2016).

Milk production is dependent on the state of the maternal wellbeing. It has been reported that milk yield can be affected by stressors enhancing ROS production through down regulation of milk synthesis leading to an increase in dopamine and adrenaline levels which in turn inhibit hormones of lactation (Hassioutou, and Geddes, 2012). Insufficient milk let down/supply (hypogalactia) is still being observed as a major and frequently-cited problems associated with lactation leading to early discontinuation of breastfeeding (Odom *et al.*, 2013).

Pregnancy period through lactation has been proposed as one of the central sources of oxidative stress as it is associated with increase in energy metabolism beginning with cost of fetal development, milk production, and energy expenditure from high maternal maintenance and physical activities (Ziomkiewick *et al.*, 2016).

Galactogogues are substances or medications used in assisting initiation and maintenance or augmentation of milk production (Yahuza *et al.*, 2016). Some of this galactogogues include dopamine antagonists such as Metoclopramide, domperidone, antipsychotics, sulpiride, chlorpromazine; hormone synthetic analogues such as oxytocin, thyroxine and medroxyprogesterone are also included in the synthetic galactogogues list (Zuppa *et al.*, 2010). This study was designed to assess milk yield in lactating Wistar rats following treatment with ascorbic acid and α -tocopherol.

METHODS

Experimental Protocol

A total of 30 female Wistar rats and 15 male Wistar rats, weighing 150-250g were purchased from the experimental animal house of the Department of Human Physiology, Ahmadu Bello University Zaria. Female Wistar rats were randomly assigned into groups of two each (n=2), and then mated alongside one male counterparts in the ratio 2:1 in a stainless steel metal cage. The rats were fed with commercial feed and tap water *ad libitum* and were provided with approximately 12hrdark/light cycle. Ethical approval was obtained from the Ethical Committee of Ahmadu Bello University, Zaria on animal handling, consistent with standard animal welfare guideline. At parturition,

weight of dams and pups were recorded and the number of pups per dam was culled to 4 (Yahuza *et al.*, 2016). Lactating Wistar rats were randomly grouped into five groups of six animals each (n=6).

Experimental Design

The animals were grouped as shown in Table 1. Administration of agents was carried out orally for a period of ten (10) days starting from day 3 to day 13 of lactation (Bako *et al.*, 2015).

Table 1. Experimental groups used in the study

Groups	Treatment
Group I	1 ml/kg normal saline
Group II	5 mg/kg metoclopramide (Jiangsu Pengyao Pharmaceutical Inc. China)
Group III	100 mg/kg Vitamin E (Michelle laboratories LTD)
Group IV	100 mg/kg Vitamin C (Michelle laboratories LTD)
Group V	100mg/kg co-administration of vitamin C and E

Evaluation of milk yield in lactating Wistar rats

Milk yield and body weight of dams and weight of pups were measured each day with an electronic balance (Salter). Milk yield was estimated 18 hours after gavage indirectly from the relationship between weight gain of pups pre and post suckling (Sampson and Jansen, 1984; Ann and Linzell, 2003). Following administration of agents at 06:00 pm, the pups were weighed every day during the study period at 07:00 am the next day and recorded as (W_1) and then isolated from their dams for a period of four (4) hours (Samson and Jensen, 1984). At 11:00 am, the pups were weighed (W_2) and re-united with their dams and allowed to feed for 1 h. At 12:00 am, they were weighed again (W_3). Milk yield 18 hours after gavage was estimated as $W_3 - W_2$ with a correction for weight loss due to metabolic processes in the pups as $(W_2 - W_1)/4$ (Ouedraogo *et al.*, 2004; Bako *et al.*, 2013).

Milk synthesis and ejection 18 hours after gavage = $W_3 - W_2$

Where W_3 = Post suckling weight of pups (12:00 pm)

W_2 = Pre-suckling weight of pups (11:00 am)

*Correction for weight loss due to metabolic processes was calculated as follows:

Weight loss correction 18 hours after gavage = $W_3 - W_2/4$

Where W_2 = pre-suckling weight pups, W_1 = pre-isolation weight of pups (taken four hours before W_2 at 7:00 am (Morag, 1970).

Prolactin Analysis

Blood samples were obtained via cardiac puncture into specimen bottles and allowed to clot and separated by centrifugation at $2,000 \times g$ for 10 minutes using Centrifuge Hettich (Universal) and the sera obtained and used for biochemical assays. The analysis was conducted at the Department of Human Anatomy, Ahmadu Bello University, Zaria. Prolactin analysis was carried out using the specie-specific Prolactin rat ELISA kit which was designed for the quantitative evaluation of rat prolactin according to the manufacturer's manual. The microplate was first coated with monoclonal antibody prolactin. The sera sample containing the antigen was pipetted into an antibody-coated microplate and allowed for two (2) hours during which the prolactin antigen in the sample binds to the antibodies fixed on the inner surface of the wells. Non-reactive components were removed by washing steps. Afterwards a second polyclonal horseradish peroxidase-labelled antibody was added, a sandwich complex which consisted of two antibodies and the rat prolactin was formed during 1 hour of incubation. The excess enzyme conjugate was washed out and a chromogenic substrate, tetra-methyl benzidine (TMB) was added to the wells and was allowed to incubate for 30 minutes, during which the substrate was converted to a coloured end product (blue) by the fixed enzyme. The enzyme reaction was inhibited by adding hydrochloric acid as the stop solution. Absorbance was measured using a microplate reader at 450 nm. A standard curve was obtained by plotting the concentration of standard versus the absorbance from which the prolactin concentration was gotten. The lowest detectable level of prolactin with this test was 0.8 ng/mL (Bako *et al.*, 2015).

Oxytocin Analysis

Oxytocin analysis was carried out using the oxytocin rat ELISA kit according to the manufacturer's manual. The oxytocin specie-specific enzyme-linked immunosorbent assay (ELISA) kit in microplate was designed for the quantitative evaluation of rat oxytocin. The analysis was conducted at the Department of Human Anatomy, Ahmadu Bello University, Zaria. Oxytocin analysis was carried out using the specie-specific Oxytocin rat ELISA kit which was designed for the quantitative evaluation of rat Oxytocin according to the manufacturer's manual. The microplate is pre coated Oxytocin. The sera sample which containing the antigen was pipetted into an antibody coated microplate, and allowed for two (2) hours, during the reaction, oxytocin in the sample or standard competed with a fixed amount of oxytocin on the solid phase supporter for sites on the Biotinylated

Detection Antibody specific to oxytocin. Excess conjugate and unbound sample or standard were washed from the plate, and HRP-Streptavidin (SABC) was added to each microplate well and incubated. Then TMB substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm.

A standard curve was obtained by plotting the concentration of standard versus the absorbance from which the oxytocin concentration was gotten. The lowest detectable level of oxytocin with this test was 6.3 pg/mL.

Data analysis

All data were expressed as Mean \pm Standard Error of Mean. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Results were considered significant at $p \leq 0.05$. Statistical Package for Social Sciences (SPSS) version 20 was used.

RESULTS

Milk Yield

The result of milk yield in lactating Wistar rats treated with ascorbic acid and α -tocopherol showed a statistically significant ($p < 0.05$) increase in group IV (ascorbic acid (vitamin C-treated group) and also group V (co-administration of both vitamin C and E) when compared to control; 3.72 ± 0.37 and 3.60 ± 0.33 versus 2.28 ± 0.08 respectively (Fig. 1 below).

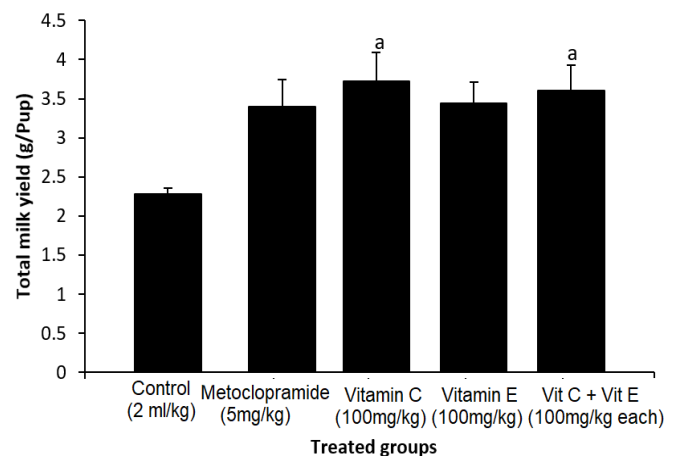


Fig. 1. Mean milk yield in lactating Wistar rats following supplementation with vitamins C, E and their co-administration. Superscripts a indicate statistical significance at $p < 0.05$ compared to control

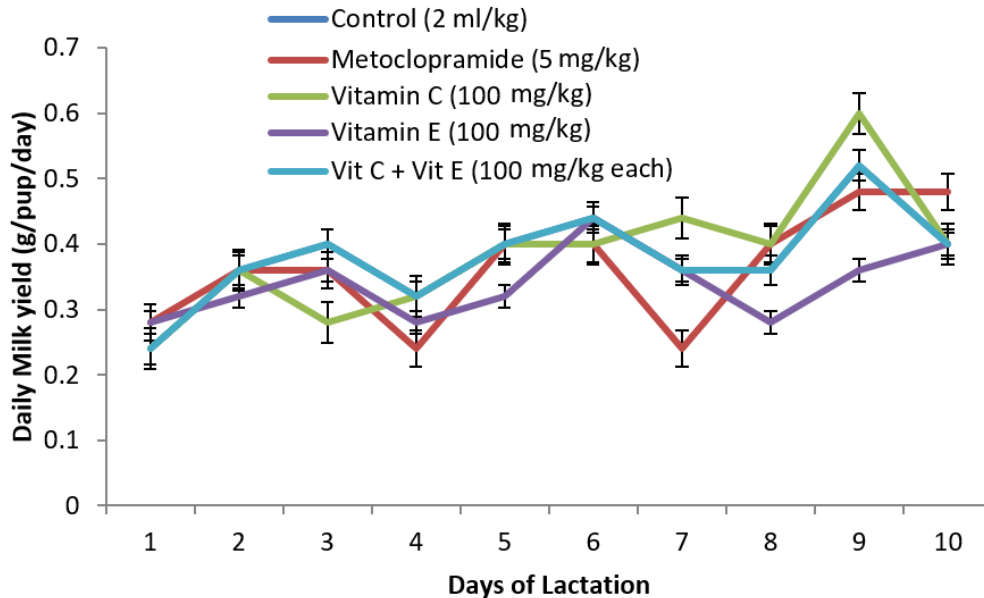


Fig 2. Daily milk yield 18h after gavage in lactating Wistar rats treated with ascorbic acid, α -tocopherol, vitamins C and E combined and metoclopramide are statistically significant at $p < 0.05$.

There was a significant increase in milk yield ($p < 0.05$) from day 3 to day 4 in the vitamin C and E combined treated group, and also a significant increase in the vitamin C-treated group from day 7 to day 9 when compared to the Control group. The highest milk yield recorded was on day 9 of lactation with its lowest being day 7 in group II (metoclopramide-treated group). Although there was an increase in the milk yield in metoclopramide-treated group and α -tocopherol (vitamin E) treated group from day 7 to day 10 it, was however not statistically significant.

Serum Prolactin

There was a statistically significant increase ($p < 0.05$) in group II (metoclopramide treated group) compared to control; 11.48 ± 0.72 versus 9.12 ± 0.29 , and also a significant decrease ($p < 0.05$) in group V (vitamin C and E co-administration group) compared to metoclopramide treated group; 9.32 ± 0.57 versus 11.48 ± 0.72 figure III.

Serum oxytocin

Serum oxytocin level in lactating Wistar rats in the experiment was significantly higher ($p < 0.05$) in group IV (vitamin C-treated group) 13.32 ± 1.16 compared to the control group (9.46 ± 0.81). A significant decrease was also observed in group V (combination treated group) 8.80 ± 0.95 compared to vitamin C-treated group (13.32 ± 1.16). Although there was an increase in the

vitamin E- treated group, it was not statistically significant.

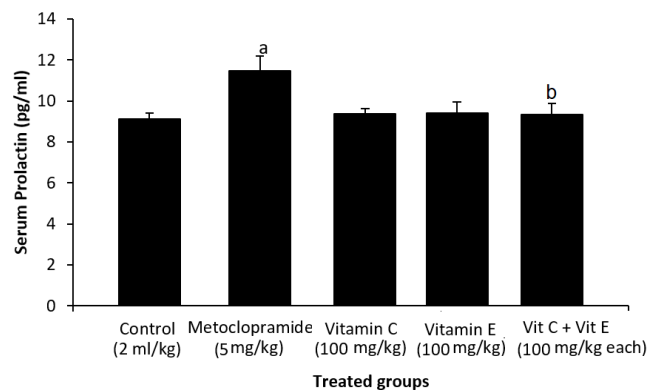


Fig. 3. Effect of ascorbic acid and α -tocopherol on serum prolactin level in lactating Wistar rats. Different superscripts a,b indicate statistical significance at $p < 0.05$ compared to control and metoclopramide respectively.

DISCUSSION

The increase in the milk yield in the vitamin C and their combination could be attributed to the increase in the serum oxytocin level. Reports from studies have shown that water soluble vitamins increase milk yield as they serve as cofactors for the enzymes responsible for the synthesis of amino acids involved in milk production. Vitamin C as a water-soluble vitamin act as a cofactor in a number of reactions and among these is its role as a cofactor to peptidyl-glycine alpha-amidating monooxygenase (PAM) enzyme in enhancing the

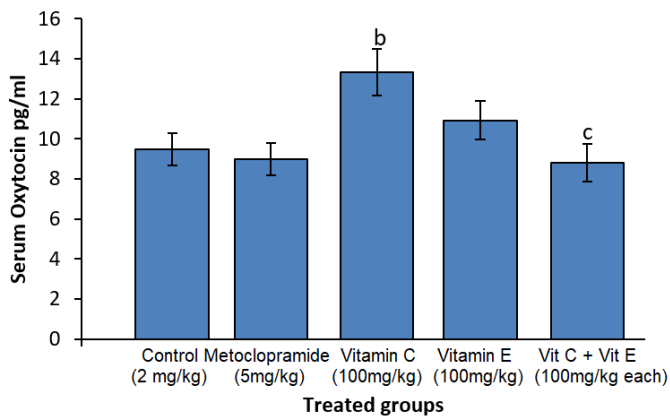


Fig. 4. Effect of ascorbic acid and α -tocopherol on serum oxytocin level in lactating Wistar rats. Different superscripts a,b,c indicate statistical significance at $p < 0.05$ compared to control, metoclopramide and vitamin c-treated group respectively.

synthesis of oxytocin hormone (Epipper *et al.*, 1994). Increase synthesis of oxytocin enhances the release of oxytocin upon stimulation of the nipple during sucking. The increase in milk yield in the combination group could be due to a synergistic effect of vitamin C in addition to the vitamin E, the high milk yield in the vitamin E group even though not significant can be attributed to the increase in oxytocin. Literatures have shown that most traditional galactogogues like *Hibiscus sabdariffa* (Okasha *et al.*, 2008) exhibit their lactogenic activity through stimulation of prolactin production, however in this study antioxidants (vitamin C and E) increased milk yield without stimulating prolactin hormone compared to the standard drug metoclopramide which caused a significant increase in serum prolactin hormone level. It is therefore apparent that vitamin C and E do not have any effect on prolactin hormone, this agrees with reports of Simelane *et al.*, (2012); Yahuza *et al.*, (2016) and is suggestive that most galactogogues with antioxidant properties stimulate milk production and milk yield without stimulating prolactin hormone and therefore have less effect on this lactation hormone. Metoclopramide as a standard drug has been known for its ability to increase prolactin level through its antidopaminergic effect on the dopaminergic cells leading to a subsequent suppression of dopamine release resulting in increased prolactin release (Bako *et al.*, 2013). Prolactin is known for its numerous roles with its major effect seen on the mammary gland ranging from development of the mammary gland to milk synthesis and maintenance of milk secretion (Freeman *et al.*, 2006). After parturition, prolactin induces lactation by stimulating the synthesis of milk in the epithelial cells and also causes proliferation of secretory cells (Yahuza *et al.*, 2016).

Oxytocin aside its other roles is known for its role in milk ejection brought about by neuroendocrine reflexes. In this study vitamin C could have increased oxytocin level by stimulating oxytocin release while inhibiting prolactin release causing oxytocin to have more effect on the myoepithelial cells of the mammary gland, enhancing more milk yield through the milk ejection reflex. This effect on oxytocin level could be responsible for the increase in the milk yield observed in the vitamin C-treated group in this study. The effect of these supplements agrees with Abdel khalek *et al* (2008) who observed a better performance in lactating doe rabbits following treatment with supplements of vitamins C. This is suggestive that vitamin C promotes milk letdown via an inhibitory mechanism on the dopaminergic cells along the hypothalamo-hypophyseal axis (Esmaeilpour-Bezenjani, and Abbasnejad, 2013) thereby inducing an increase in oxytocin synthesis in the lactotrophs cells on the adenophysis. This occurs through inhibition of D_2 receptors on dopaminergic cells resulting in potassium (K^+) channel opening which in turn increases its intracellular concentration while reducing calcium Ca^{2+} entry and its intracellular concentration. As a result of decreased calcium concentration within these cells, there is a corresponding decrease in dopamine release (Kauppila *et al.*, 2001; Gupta and Gupta 2005). Vitamin C could also have increased oxytocin level by enhancing activities of peptidylglycine alpha amino amidating mono oxygenase (PAM) enzyme during oxytocin synthesis leading to an increase in the serum oxytocin level (Epipper *et al.*, 1994). These supplements could have also elicited the observed activity through activation of phospholipase C (PLC) and protein kinase C (PKC) thereby increasing Ca^{2+} intracellular concentrations and mobilizations from the endoplasmic reticulum within the lactotrophic cells thereby increasing formation of vesicular oxytocin and subsequent release (Rizvi *et al.*, 2015). Suckling triggers milk ejection reflex through neuroendocrine reflexes which causes release of oxytocin from the hypothalamo hypophyseal axis leading to milk ejection (Yahuza *et al.*, 2016). Vitamin C from this study with higher oxytocin level has been found to increase milk yield/milk production in lactating Wistar rats through the milk ejection mechanism however the receptors to which vitamin C act upon to enhance oxytocin secretion remains unknown.

CONCLUSION

The study has shown that Vitamin C supplementation in lactating Wistar rats improved milk yield by enhancing milk ejection due to its effect on serum

oxytocin hormone better than vitamin E and the co-administration of vitamin C and E.

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