

Evaluation of the effects of *tetrapleura tetraptera* extract and clomiphene citrate to determine influence of reproductive hormones and estrous cycle on leukocyte counts: novel evidence for a potential developmental link between luteinizing hormone and MID cells

Abstract

Estrous cycle influences circulating leukocyte count via reproductive hormones. *Tetrapleura tetraptera* extract has been shown to cause an increase in leukocyte count. Our previous work showed that *Tetrapleura tetraptera* pod extract inhibited LH and estrogen around ovulation stage. Therefore, the present study evaluated the effects of *Tetrapleura tetraptera* extracts and clomiphene citrate on leukocyte counts in order to determine whether the varying reproductive hormones secreted in both extracts and drug, and estrous cycle caused alteration in leukocyte counts.

Twenty eight adult female rats were selectively divided into 4 groups (n=7) according to their estrous phases. The group A served as control. Group B rats were administered 1mg/kg of clomiphene citrate daily. Group C and D rats were administered 50 and 200mg/kg of seed and pod extracts daily. Results showed that total WBC count was significantly reduced ($P<0.05$) in proestrus Group B. Total WBC, and lymphocyte counts remained unchanged. Neutrophil count was significantly reduced ($P<0.05$) in estrus Group C. The MID cell count was significantly reduced in estrus Group C and D. The total WBC counts, lymphocyte count, serum FSH, estrogen and glucocorticoid levels were not statistically significant. Serum LH was significantly high ($P<0.05$) in proestrus Group B. Serum LH was significantly low in estrus Group C and D. Results showed that the significant reduction in the neutrophil in Group D is independent of the hormones and estrous cycle but dependent on dose concentration of *Tetrapleura tetraptera* extract. The significant elevation in MID cell count was associated with significant reduction in LH in Group C and D. However, total leukocyte count and MID cell count were significantly reduced and appeared to be associated with significant elevation in serum LH levels. The present study provides a novel idea of how LH affects MID cell population.

Keywords: neutrophil, MID cell, *tetrapleura tetraptera*, clomiphene citrate, luteinizing hormone, estrogen, white blood cell

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Abbreviations: LH, luteinizing hormone; WBC, white blood cell; FSH, follicle-stimulating hormone

Introduction

The peripheral circulating white blood cell (WBC) or leukocyte count originate in the hematopoietic stem cells and develop along distinct differentiation in response to internal and external cues.¹ The total number of WBC in the circulation is given as the peripheral WBC count and proportion of the total number of WBC represented by differentiated granulocytes (neutrophils, eosinophils and basophils), lymphocytes and monocytes is given as the differential WBC count, and are biomarkers for predicting acute infection, tissue damage, inflammation, diabetes, atherosclerosis and mortality.²⁻⁴

Moreover, *Tetrapleura tetraptera* is a perennial plant that belongs to Fabaceae family generally found in low land forest of tropical

Africa rich. The dried fruit is shiny, glabrous, dark purple brown with unpleasant aroma and rich in flavonoid, alkaloid and hydrogen cyanide.^{5,6} The fruit is used as popular seasoning in Southern Nigeria and has been shown to cause significant reduction in hematological indices in male rabbits⁷ and elevate hematological indices in female rats.⁸ *Tetrapleura tetraptera* has been shown to elevate total and differential WBC counts,⁸ but nothing is known on the mechanism of action of the extract-mediated elevation of WBC counts. However, the total WBC and differential WBC counts have been shown to vary across menstrual cycle with neutrophil increasing around ovulation and steadied in the mid-luteal phase associated with reproductive hormone variation whereas lymphocyte and MID cells remained unchanged during ovulation.⁹ There exists an interaction of reproductive hormones and immune system and this interaction has been attributed to reproductive hormone receptors on the immune cells.¹⁰ Another study also showed that female reproductive hormones

strongly influence production and function of immune system cells and molecules.¹¹ Several decades ago, Reisner demonstrated that estrogen facilitated leukocytosis in the bone marrow.¹² It is well known that estrous cycle is dependent on the sex hormones.

Previous works have shown that *Tetrapleura tetraptera* pod extract inhibited luteinizing hormone (LH) and estrogen during proestrus stage in female rats^{13,14} thereby could affect circulating WBC population. Meanwhile, clomiphene citrate is mainly an antiestrogenic drug used in treating female infertility.¹⁵ We also demonstrate that clomiphene citrate caused significant elevation in LH level.¹⁴ Since *Tetrapleura tetraptera* pod extract caused opposite effects of clomiphene citrate on LH that is central to ovulation induction in females, therefore, the aim of the present study was to evaluate the effects of administration of *Tetrapleura tetraptera* extracts and clomiphene citrate on leukocyte counts in order to determine whether the varying reproductive hormones secreted namely follicle-stimulating hormone (FSH), LH, estrogen and glucocorticoid in both extracts and drug altered counts during ovulation.

Materials and methods

Twenty eight female albino Wistar rats were bought in the Veterinary Department, University of Nigeria, Nsukka and transported to Animal House of Department of Human Physiology, Madonna University Nigeria. The rats were kept in standard cages (Henan, China), received normal rat chow and tap water ad libitum. They were acclimatized for two weeks under 12hours light/12 hours dark cycle in a room temperature of 24±2°C. The rats were selectively divided into 4groups: A-D (n=7) according to their estrous cycles. The control group A rats received only rat chow and tap water.

The experimental Group Brats were administered 1mg/kg (orally) of clomiphene citrate daily according to Boyar et al.¹⁶ The experimental Group C rats were administered 200mg/kg (orally) of *Tetrapleura tetraptera* pod extract daily according to Agbai et al.¹⁴ The experimental Group D rats were administered 50mg/kg (orally) of *Tetrapleura tetraptera* seed extract daily according to Agbai et al.¹⁴ All administration lasted for 14days. *Tetrapleura tetraptera* dried fruits (weighing 950g) were bought from a local market, Afor Ogbé in Ahiazu Mbaise Local Government Area of Imo State. The pods were crushed with a pestle and the seeds were carefully removed from the pod. The pod fragments and seeds were sun-dried and ground into a coarse form respectively for Soxhlet method of extraction. 300ml of methanol (Sigma Aldrich, USA) was poured in Soxhlet containing powdered form of pod. Afterwards the methanol (solvent) was separated from the pod extract using Rotavapor device. The same method of extraction was used for the seed except the volume of methanol was reduced to 150ml.

Three grams tablets of clomiphene citrate (50mg/tablet) was ground into a powdered form, suspended in methanol diluted with

water and sieved with Whatman paper (no. 1) to remove excipients. The filtrate was taken as clomiphene citrate. All the rats received humane care according to the criteria outlined in the Guide for the Care of Laboratory Animals prepared by the National Academy Science.¹⁷ The estrous cycle was determined daily using the method described by Marcondes et al.,¹⁸ and all the rats in proestrus and estrus phases on the 14th day were selected and sacrificed under urethane anesthesia before 12 noon. Blood was collected via cardiac puncture and blood was drawn using 5ml syringe and blood was collected in well-labeled EDTA bottles for evaluation of total white blood cell counts, neutrophil, lymphocyte and MID-cells (total value of mid-size cells such as monocytes, eosinophils and basophils counts) using electronic cell counter (Coulter Electronics, UK) and FSH, LH, estrogen and glucocorticoid levels using Enzyme Linked-Immunesorbent Assay (ELISA) methods.

Results

On the 14th day the control group A and Group B rats exhibited proestrus phase while Group C and D exhibited estrus phase of the estrous cycle. Results showed that pod and seed extract did not cause statistically significant difference ($P>0.05$) in the total WBC count of Group C and D rats ($10,600\pm406.4\text{mm}^3$ and $9,800\pm202.6\text{mm}^3$) compared with control group A rats ($10,900\pm190.9\text{mm}^3$). However, clomiphene citrate caused significant reduction in total WBC ($P<0.05$) of Group B rats ($7,400\pm60.7\text{mm}^3$) compared to control group, Group C and D. There was statistically significant reduction ($P<0.05$) in neutrophil count of Group C rats ($25.0\pm1.0\%$) compared to control group A ($38.0\pm2.0\%$), Group B ($33.0\pm1.0\%$), and Group D ($34.0\pm2.0\%$). The level of lymphocytes was not statistically significant ($P>0.05$) in pod and extract-treated Group C ($70.0\pm1.00\%$) and D ($61.0\pm5.00\%$) rats compared to control group A ($59.0\pm6.00\%$) and clomiphene citrate-treated Group B ($63.0\pm5.00\%$) rats.

There was also statistically significant elevation ($P<0.05$) in MID cells of Group C ($5.0\pm0.50\%$) and Group D ($5.0\pm0.50\%$) rats compared to control group A (3.0 ± 0.50) and Group B ($2.0\pm0.50\%$) rats. There was no statistically significant difference ($P>0.05$) in FSH levels between control group A ($0.17\pm0.01\text{mIU/ml}$) and experimental Group B, C and D ($0.19\pm0.02\text{mIU/ml}$, 0.21 ± 0.02 and $0.19\pm0.02\text{mIU/ml}$). The pod extract caused significant reduction ($P<0.05$) in serum LH level of Group C ($0.17\pm0.30\text{mIU/ml}$) and D ($0.13\pm0.06\text{mIU/ml}$) rats compared to control group A ($0.34\pm0.8\text{mIU/ml}$) and Group B ($0.75\pm0.5\text{mIU/ml}$) rats. The pod extract also caused significant reduction ($P<0.05$) in serum estrogen level of Group C rats ($58.49\pm10.04\text{ pg/ml}$) compared to control group A ($80.07\pm4.68\text{pg/ml}$), Group B ($79.57\pm1.28\text{pg/ml}$) and Group D ($70.00\pm5.46\text{pg/ml}$) rats. There was no statistically significant difference ($P>0.05$) in serum glucocorticoid level of Group B ($5.46\pm0.91\mu\text{g/dl}$), Group C ($5.68\pm2.30\mu\text{g/dl}$) and Group D ($4.49\pm1.27\mu\text{g/dl}$) rats compared with control group A ($7.68\pm0.18\mu\text{g/dl}$) rats (Table 1 & 2).

Table 1 The effects of clomiphene citrate and *Tetrapleura tetraptera* on some hematological indices

	Total WBC count (cubic mm)	Neutrophil count (%)	Lymphocyte count (%)	MID cell count (%)
Control	10,900±190.9	38.0±2.0	59.0±6.0	3.0±0.50
Group B	7,400±60.7*	33.0±1.0	63.0±5.0	2.0±0.50
Group C	10,600±406.4	25.0±1.0*	70.0±1.0	5.0±0.50*
Group D	9,800±202.6	34.0±2.0	61.0±5.0	5.0±0.50*

Values=mean±SEM

*P value significant<0.05

Table 2 The effects of clomiphene citrate and *Tetrapleura tetraptera* on some reproductive hormones

	Follicle-stimulating hormone (mIU/ml)	Luteinizing hormone (mIU/ml)	Estrogen (pg/ml)	Glucocorticoid (µg/dl)
Control	0.17±0.01	0.34±0.8	80.07±46.8	7.68±0.18
Group B	0.19±0.02	0.75±0.5	79.57±1.2	5.46±0.91
Group C	0.21±0.02	0.17±0.30*	58.49±10.04	5.68±2.30
Group D	0.19±0.02	0.13±0.06*	70.00±5.46	4.49±1.27

Values = mean±SEM

*P value significant<0.05

Discussion

The results showed that the pod and seed extract of *Tetrapleura tetraptera* did not cause significant change in the total WBC and lymphocyte but significantly reduced neutrophil count and elevated MID cells. In spite of the changes in the differential cells that sum up the total WBC count, the total WBC remained unchanged. Moreover, results in figure 1 showed that pod extract of *Tetrapleura tetraptera* caused significant reduction in neutrophil count. Study has shown that *Tetrapleura tetraptera* dried fruit extract elevate hematological indices in female rats.⁸ The current study does not support previous research in this area. However, the marked reduction in neutrophil count usually results from events that decrease the normal rate of production of these cells in the marrow or from processes that accelerate neutrophil destruction, sequestration, or egress from the circulation as well as apoptosis. Fleischer and colleagues have noted that noted some important flavonoid contents in *Tetrapleura tetraptera* namely 2', 4,4'-trihydroxychalcone, 2',3,4,4'-tetrahydroxychalcone (butein) and 4',5,7-trihydroxyflavanone (apigenin).¹⁹ Studies have shown that high intake of these flavonoid compounds in *Tetrapleura tetraptera* can prevent many chronic diseases including allergy, and cancer.^{20,21} Because *Tetrapleura tetraptera* prevent allergy and cancer meant that immune system can be affected. More so, it well established that hematopoietic stem cell differentiation pathway into WBCs is dependent on granulocyte-colony stimulating factor and granulocyte/monocyte-colony stimulating factor.²² Therefore, alteration in secretion of these colony stimulating factors impair proliferation and differentiation of leukocytes. Because studies have shown that flavonoids inhibit IL-1β-stimulated granulocyte-colony stimulating factor expression and subsequent neutrophil differentiation as well as suppressing the expression of granulocyte/monocyte-colony stimulating factor^{23,24} could suggest that *Tetrapleura tetraptera* pod extract inhibited proliferation and differentiation processes required to necessitate neutrophil production resulting in the reduction in the peripheral neutrophil count.

Besides inhibition of granulocyte-colony stimulating factor and granulocyte/monocyte-colony stimulating factor, apigenin and chalcones have been shown to inhibit interleukins-6 and blocked tumor necrosis factor-α.^{25,26} Moore and colleagues have demonstrated that tumor necrosis factor-α injection caused activation of neutrophil.²⁷ Since flavonoids inhibited tumor necrosis factor-α implied that activation of neutrophil will be inhibited therefore lends support to the idea that pod extract of *Tetrapleura tetraptera* significantly reduced circulating neutrophil count. In addition, interleukin-6 has been shown to affect proliferation by targeting high proliferative potential-colony forming cells that share to a certain extent the characteristics of stem cell that produce leukocytes.²⁸ To further support the reduction in neutrophil count by pod extract of *Tetrapleura tetraptera*, chalcone

derivatives have been shown to inhibit neutrophil chemotaxis²⁹ thereby inhibiting the recruitment and migration of neutrophils in the circulation thereby causing reduction in the circulating neutrophil.

Interestingly, the seed extract of *Tetrapleura tetraptera* did not cause significant change in the circulating neutrophil count. Since the seed extract is rich in flavonoid compounds such as apigenin, chalcones and butein, therefore, the same fall in neutrophil count in pod extract-treated Group C is expected in the Group D rats administered seed extract of *Tetrapleura tetraptera* suggesting the action of *Tetrapleura tetraptera* extract could be dose dependent because high concentration of flavonoids has been shown to inhibit proliferation of rat bone marrow stromal cells.³⁰

On the other hand, there was no significant difference between extracts, clomiphene citrate and control group as far as lymphocyte count is concerned. Moreover, lymphocytes are responsible for specificity of adaptive immune responses. The T cells and B cells populate the lymphocyte pool and are lodged in lymphoid compartments with only tiny fraction of total lymphocyte population that continuously circulates between lymph and blood waiting for encounter with their antigen.³¹

The MID cell count was statistically elevated in the pod and seed extract-treated Group C and D rats. Previous studies have demonstrated that chalcones and apigenins exhibited inhibition of basophil degranulation and down-regulated high-affinity IgE receptor FcεRI expression on basophils.^{32,33} These report substantiate the fact that *Tetrapleura tetraptera* extracts caused increase in MID cell count because basophil counts contribute to MID cell count. Several studies have shown that flavonoid compounds inhibited eosinophil count in ovalbumin-induced asthma as well as inhibiting the release and gene expression of monocyte-chemoattractant protein-1,^{34,35} therefore, suggesting that eosinophils and monocytes might not be central in the elevation of MID cell count in this present study.

Several researchers have associated estrous cycle with variation in the WBC count.^{9,36,37} These researchers provided further evidences that secretory phase or ovulation of the menstrual cycle cause increases in total WBC count and neutrophil count.^{9,36} More so, the ovarian estrogen has been demonstrated to promote the release of neutrophils from the bone marrow.³⁷

In the present study, the hormonal analysis did not confirm any significant differences between *Tetrapleura tetraptera* extracts, clomiphene citrate and control in terms of serum estrogen, glucocorticoid and FSH levels during proestrus and estrus phases of the estrous cycle. Moreover, it is worth mentioning that the estrous cycle corresponds to the secretory phase in menstruation. Previous studies demonstrated that *Tetrapleura tetraptera* extract inhibited LH and estrogen secretion during proestrus phase of the estrous cycle.^{11,13}

Our current study contrast with what was previously thought that neutrophil reduction could have corresponded with low estrogen level. The marked observation to emerge from the data comparison was the reduction in serum LH level. However, data have shown that leuprolide acetate, a luteinizing hormone-releasing hormone agonist increases the myeloid progenitor cells in the bone marrow.³⁸ The first studies on LH and hematopoietic stem/progenitor cell found that hematopoietic stem/progenitor cell express functional FSH and LH receptors that proliferate in vivo and in vitro in response to FSH and LH.³⁹ Abdelbaset-Ismail and colleagues further affirmed that LH stimulates growth of human hematopoietic/progenitor cells and mesenchymal cells *in vivo* and *in vitro*.⁴⁰ These did not concur well with the reduction of LH by the *Tetrapleura tetraptera* pod and seed extracts association with changes in the neutrophil count, result is a confirmation of direct effect of *Tetrapleura tetraptera* on leukocyte production that is dose dependent. There appears also a non-alignment in the *Tetrapleura tetraptera* pod and seed extract-mediated elevation of MID cell count and low level of LH based on evidences that showed high level of LH facilitates hematopoietic stem/progenitor cell proliferation, however, the present result is a confirmation of the sparing effect of *Tetrapleura tetraptera* flavonoid content on basophil degranulation although this finding that low LH level is associated with high MID cell count could become a novel study.

To further analyze whether drug that induce ovulation via elevated LH can cause changes in the leukocyte count is a proven fact. Results indicated that clomiphene citrate caused significant reduction in total WBC count and MID cell count whereas neutrophil and lymphocyte counts were not affected. It is evident the decrease in the total WBC count emanated from the reduction in MID cells in the clomiphene citrate-treated Group B rats. This present result suggests that high level of LH inhibits MID cell production while low level of LH enhances MID cell production. This has provided further evidence that LH strongly affects MID cell count. Moreover, it has been reported that clomiphene citrate elevated neutrophil and basophil counts in cocks treated with ≥ 20 mg/kg of clomiphene citrate while lymphocyte, eosinophil and monocyte counts were not statistically significant⁴¹ suggesting that clomiphene citrate effect on leukocyte count is dose dependent.

Conclusion

The results of this study suggest that *Tetrapleura tetraptera* extract caused dose-related significant reduction in the neutrophil count independent of the estrous cycle and reproductive hormones. The extract further caused significant elevation in MID count associated with significantly low level of serum LH while clomiphene citrate caused significant reduction in MID cell count associated with significantly high level of serum LH. We have found a novel report that varying levels of serum LH affect MID cell count adding to a growing body of literature on LH effect on hematological indices. Our work clearly has some limitations on identifying the individual leukocyte cells in MID cells. Nevertheless we believe our work could be the basis for further work on the correlation between LH and monocytes, basophils and eosinophils.

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Conflict of interest

The author declares no conflict of interest.

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