ENHANCED LEUKOPOIESIS IN GHRELIN TREATED MYELOSUPPRESSED RATS

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ABSTRACT

Objective: To determine the role of ghrelin in stimulating leukopoiesis of myelosuppressed rats by stimulating growth hormone release.

Study Design: Randomized controlled trial.

Place and Duration of Study: It was conducted at Department of Physiology, Foundation University Medical College, in collaboration with National Institute of Health, Islamabad, from February 2013 to June 2014.

Material and Methods: Out of 130 Sprague-Dawley rats, 10 were used for baseline sampling and rest was randomly divided into four groups. Group I received ghrelin only while group II and III were administered intraperitoneally with both carboplatin and 5-flurouracil with and without rat ghrelin. Group IV in addition to chemotherapy and ghrelin received growth hormone releasing hormone antagonist (GHRH antagonist). Total leukocyte count, differential leukocyte count and growth hormone levels were measured on day 7 and 14.

Results: The fall in leukocyte count of chemotherapy and ghrelin group on day 7 was less as compared to the chemotherapy-only treated group and chemotherapy, ghrelin and growth hormone releasing hormone antagonist treated group (p<0.05). Further decreases were also prevented in the chemotherapy and ghrelin group. The serum growth hormone levels in chemotherapy and ghrelin treated group were higher as compared to the chemotherapy and chemotherapy, ghrel in, GHRH antagonist treated group (p< 0.05).

Conclusion: Enhanced leukopoiesis in ghrelin treated myelosuppressed rats as compared to the chemotherapy group (p<0.05) suggest role of ghrelin in enhancing leukopoiesis. While the failure of enhanced leukopoiesis and growth hormone level to rise in chemotherapy, ghrelin and GHRH antagonist treated group suggested the possibility of growth hormone as possible mediator of ghrelin in leukopoiesis.

Keywords: Chemotherapy, Ghrelin, Growth hormone, Leukopoiesis, Myelosuppression.

INTRODUCTION

Chemotherapy employs systemically administered drugs that directly damage cellular DNA and RNA[1]. Chemotherapy can improve the therapeutic outcome; however increase in the dose is associated with toxic side effects, most common of which is myelosuppression. Myelosuppression if severe can lead to life threatening infections and hemorrhage. Various treatment modalities are available to overcome myelosuppression like bone marrow transplant, administration of colony stimulating factors and recombinant human growth hormone[2].

Recombinant human growth hormone is a pleiotropic cytokine targeting a variety of hematopoietic and non hematopoietic tissues. It has been shown to stimulate hematopoietic cell recovery and lower the side effects caused by myelosuppression[3]. It increases colony formationby human myeloid and erythroid progenitors in vitro[4,5] and can synergize with other cytokine such as granulocyte monocyte colony stimulating factor in hematopoiesis[6]. In vivo 7 day treatment with recombinant human growth hormone increases the marrow and spleen granulocyte macrophage colony forming units and partially counters the myelosuppressive effects of azidothy-midine[7]. But growth hormone administration is associated with potential side effects like fluid retention, nerve compression and increase risk of diabetes. So recently growth hormone secretagogues are discovered which provide a more physiological route for the release of growth hormone[8]. The endogenous ligand for this receptor is

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ghrelin[9] Ghrelin is a 28 amino acid peptide produced by enteroendocrine cells of stomach in response to negative energy balance. It stimulates hunger, inhibits inflammation, promotes learning and memory and is therefore proposed as a hormone of significant therapeutic benefits[10].

It has been shown that growth hormone response to ghrelin in vivo requires an intact endogenous growth hormone releasing hormone system[11]. The growth hormone production in response to growth hormone secretagogue is suppressed by growth hormone releasing hormone antagonist[11]. Growth hormone releasing hormone antagonist is a synthetic analogue of growth hormone releasing hormone. It acts as a competitive antagonist of growth hormone releasing hormone at its receptor in pituitary and immune cells[12]. It has been shown that nearly 80% of the growth hormone secretagogue mediated growth hormone release is suppressed by prior administration of a growth hormone releasing hormone antagonist in a dose of 400µg/kg[12].

Unfortunately there is limited literature available pertaining to role of ghrelin in leukopoiesis. It has been shown that growth hormone secretagogue when administered to young mice in a dose of 5-10 mg/kg orally for 3weeks, increased peripheral lymphocyte count[13]. Ghrelin receptors are shown to be present in various cells and organ systems including immune cells like B lymphocytes, T lymphocytes, monocytes and neutrophils[13]. Ghrelin binds with the growth hormone secretagogue receptor (GHS-R) on the surface of immune cells and stimulates phospholipase C(PLC) which cleavesphosphotidyl inositol biphosphate (PIP2) into dicylglycerol (DAG) and inosital triphosphate (IP3). DAG then stimulates the genetic machinery of immune cell producing growth hormone which when released from immune cells acts on neighbouring immune cells in autocrine or paracrine fashion[14]. The exact mechanism by which ghrelin can effect leukopoiesis is not clear. It is proposed that effect of ghrelin in leukopoiesis may be direct through bone marrow or indirect via growth hormone release[14]. Based on these facts we hypothesize a therapeutic role of ghrelin in improving cell recovery of rats following myelosuppressive chemotherapy by increasing the levels of growth hormone.

MATERIAL AND METHODS

It was a randomized controlled trial conducted at department of Physiology, Foundation University Medical College, Islamabad, in collaboration with National Institute of Health, Islamabad from February 2013 to June 2014. Prior to commencement of the study, permission from Ethical Committee of Foundation University Medical College was obtained.

Animal house facility of National Institute of Health, Islamabad was utilized. One hundred and thirty healthy male Sprague-Dawley rats (3-4 months old and weighing 250-300 g) were used in the present study. Female rats and rats having disease were excluded from the study. Free access to food and water was provided and room was well ventilated with controlled temperature range of 20-22 ^{o}C twelve hours light and dark cycle was maintained.

Out of 130 male rats, intra cardiac blood sampling of 10 rats was done on day 0. Remaining 120 were randomly classified into 4 groups, with 30 rats in each group. Group I: (Ghrelin treated group; n=30) was administered daily with subcutaneous ghrelin (Bioworld USA-Cat No: 21970208) in a dose of 1 nmol/100 µl N/S for 14 days[15]. Group II: (chemotherapeutic drug-treated group; n=30) was given carboplatin intraperitoneally to each rat in a dose of 70 mg/kg on the first day followed by intraperitoneal administration of 5-FU in a dose of 50 mg/kg/day on the second and third days[3]. Group III: (chemotherapeutic and ghrelin treated group; n=30) was treated with both chemotherapeutic drugs for 3 days and ghrelin for 14 days. Group IV: (chemotherapeutic, ghrelin and growth hormone releasing hormone antagonist treated group; n=30) in addition to

chemotherapy and ghrelin, each rat was administered GHRH antagonist (Sigma Aldrich - USA Cat No: SAB1300904) intravenously in a dose of 400 µg/kg daily for 14 days to counter growth hormone releasing effect of ghrelin if any[12].

In addition to baseline blood sampling of 10 rats on day 0, intracardiac sampling of group I-IV was done on day 7 and 14. Ten rats were used for sampling in each group at each time point. Blood (2.5 ml) was transferred into the vacutainers containing ethylenedia minetetracetic acid (EDTA) to prevent clotting of blood while rest of sample (2.5ml) was transferred into pre-cooled plain vacutainers for separation of serum. Total and differential leukocyte count was measured by using automated hematology cell analyser Sysmex-xt 2000i. Enzyme linked immunosorbent assay was used to measure serum growth hormone (ELISA kit, Millipore Inc. USA was used. Cat No: EZRMGH-45K).

Statistical package for social sciences (SPSS) version 17 was utilized for statistical analysis. Descriptive statistics were expressed along with means ± SD. The statistical significance of the difference of various quantitative changes was evaluated by one way analysis of variance (ANOVA) followed by Tukey HSD (Honestly significant difference) post hoc test for multiple comparisons. The difference was regarded as statistically significant if the *p*-value was < 0.05.

RESULTS

The study was done on 130 healthy male Sprague-Dawley rats. The total leucocyte count in

Table-I: **Hematological parameters and serum growth hormone levels of control and group III (day 7 and 14).**

Parameter	Day	Control group Day zero	Group III	*p* value
Total leucocyte count (×109/l)	7	7.33 ± 0.79	5.57 ± 0.38	0.000*
	14		6.1 ± 0.60	0.027*
Neutrophil count (×109/l)	7	3.69 ± 0.407	2.3 ± 0.84	0.002*
	14		3.30 ± 0.80	0.954
Lymphocyte count (×109/l)	7	3.09 ± 0.91	1.71 ± 0.46	0.018*
	14		2.15 ± 0.88	0.284
Serum growth hormone (ng/ml)	7	8.57 ± 4.96	10.24 ± 1.73	0.873
	14		13.09 ± 0.41	0.005*

p-value < 0.05 was considered significant, Tukey HSD (honestly significant difference) Post hoc test used for multiple comparisons, Group III= Chemotherapeutic and ghrelin treated group, All values are expressed as mean ± SD

Table-II: Hematological parameters and serum growth hormone levels of group I and III (day 7 and 14).

Parameter	Day	Group I	Group III	*p* value
Total leucocyte count (×109/l)	7	8.61 ± 0.65	5.57 ± 0.38	0.000*
	14	11.40 ± 1.58	6.1 ± 0.60	0.000*
Neutrophil count (×109/l)	7	3.75 ± 1.24	2.3 ± 0.84	0.001*
	14	3.83 ± 0.89	3.30 ± 0.80	0.780
Lymphocyte count (×109/l)	7	3.88 ± 1.23	1.71 ± 0.46	0.000*
	14	6.74 ± 1.59	2.15 ± 0.88	0.000*
Serum growth hormone (ng/ml)	7	11.28 ± 2.98	10.24 ± 1.73	0.993
	14	12.63 ± 4.08	13.09 ± 0.41	0.986

p-value < 0.05 was considered significant, Tukey HSD (honestly significant difference) Post hoc test used for multiple comparisons, Group I = Ghrelin treated group, Group III= Chemotherapeutic and ghrelin treated group, All values are expressed as mean ± SD.

chemotherapeutic and ghrelin treated group was significantly lower as compared to control and ghrelin treated groups on both days as shown in table-I-II. However, the total leucocyte count of chemotherapeutic and ghrelin treated group was significantly higher than chemotherapeutic ($p< 0.05$) and chemotherapeutic, ghrelin and GHRH antagonist group ($p< 0.05$) on both days as shown in table-III-IV.

Differential leucocyte count (neutrophil and lymphocytes) on other hand were significantly higher in chemotherapeutic and ghrelin treated group as compared to chemotherapeutic group only on day 14 ($p<0.05$) as shown in table-III. In chemotherapeutic, ghrelin and GHRH antagonist group neutrophil count were significantly lower as compared to chemotherapeutic and ghrelin treated group ($p<0.05$) on both days as shown in table-IV.

The serum growth hormone levels were less in the chemotherapy and chemotherapeutic, ghrelin and GHRH antagonist treated group as compared to the chemotherapeutic and ghrelin on both days as shown in table-III and IV. The difference was statistically significant with a p value of 0.000 on both days.

Table-III: Hematological parameters and serum growth hormone levels of group II and III (day 7 and 14).

Parameter	Day	Group II	Group III	*p* value
Total leucocyte count (×109/l)	7	3.69 ± 0.73	5.57 ± 0.38	0.000*
	14	4.42 ± 0.79	6.1 ± 0.60	0.000*
Neutrophil count (×109/l)	7	1.89 ± 0.42	2.3 ± 0.84	0.945
	14	1.87 ± 0.68	3.30 ± 0.80	0.001*
Lymphocyte count (×109/l)	7	1.31 ± 0.51	1.71 ± 0.46	0.980
	14	0.66 ± 0.41	2.15 ± 0.88	0.007*
Serum growth hormone (ng/ml)	7	3.24 ± 0.73	10.24 ± 1.73	0.000*
	14	5.12 ± 0.67	13.09 ± 0.41	0.000*

p-value <0.05 was considered significant, Tukey HSD (honestly significant difference) Post hoc test used for multiple comparisons, Group II = Chemotherapeutic treated group, Group III= Chemotherapeutic and ghrelin treated group.

Table-IV: Hematological parameters and serum growth hormone levels of group III and IV (day 7 and 14).

Parameter	Day	Group III	Group IV	*p* value
Total leucocyte count (×109/l)	7	5.57 ± 0.38	3.38 ± 0.42	0.000*
	14	6.1 ± 0.60	4.47 ± 0.59	0.001*
Neutrophil count(×109/l)	7	2.3 ± 0.84	1.03 ± 0.30	0.006*
	14	3.30 ± 0.80	1.98 ± 0.32	0.003*
Lymphocyte count(×109/l)	7	1.71 ± 0.46	1.32 ± 0.5	0.982
	14	2.15 ± 0.88	0.99 ± 0.41	0.084
Serum growth hormone(ng/ml)	7	10.24 ± 1.73	4.53 ± 0.31	0.000*
	14	13.09 ± 0.41	5.00 ± 1.88	0.000*

* *p*-value < 0.05 was considered significant, Tukey HSD (honestly significant difference) Post hoc test used for multiple

DISCUSSION

Myelosuppression induced by chemotherapy is the leading and potentially lethal adverse effect of cancer treatment. It is, therefore, a frequent cause for limiting intensive chemotherapy in oncology patients[16]. The current study is focused to find out the possible therapeutic role of newly discovered ligand of growth hormone secretagogue receptor, ghrelin

in stimulating leukopoiesis of myelosuppressed rats.

The earlier return of total and differential leukocyte towards normal in ghrelin treated myelosuppressed rats as compared to chemotherapeutic and chemotherapeutic, ghrelin and GHRH antagonist group suggest valuable role of ghrelin in stimulating hematopoiesis. It has been reported in previous studies that differentiation and proliferation of leukocytes is affected by variety of endocrine hormones including glucocorticoids, prolactin, testosterone, estradiol, and androgen and growth hormone.3It has been proposed now that ghrelin, as a novel member of endocrine system with hematopoietic effects, may have substantial effects on leukocyte count. In a study done in Iran, similar increase in total and differential leukocyte count was observed in newly hatched chicks following in ovo administration of ghrelin. It was observed that in ovo administration of ghrelin in 100ng dosage at day 10 resulted in an increase in total leukocyte count, neutrophil, eosinophil and basophil but decrease in lymphocyte count[17].

Another study was done on Sprague-Dawley rats to see the effects of ghrelin administration on hematological parameters. Rats in the experimental group were administered ghrelin in an amount of 10nmol/kg/day subcutaneously, for 5 days. There was a significant rise in lymphocyte count (p<0.05) while erythrocyte count and hemoglobin levels were not affected significantly[18]. The lymphopoietic role of rodent ghrelin is same as observed in the current study while failure of erythrocyte count and hemoglobin levels to rise may be due to short duration of study.

The beneficial effect of ghrelin on thymus and lymphocyte development has also been observed in another study in which ghrelin administration lead to increase T cell production from an old thymus. Ghrelin produced this effect by enhancing migration of lymphoid precursors from the bone marrow by activating GHS-R–specific pathway[19], suggesting role of ghrelin in stimulation of hematopoiesis.

The failure of ghrelin to stimulate leukopoiesis in chemotherapeutic, ghrelin and GHRH antagonist treated group clearly shows that useful effect of ghrelin in leukopoiesis is mediated mainly through growth hormone release. It has already been observed that ghrelin strongly stimulates growth hormone release both in vitro and in vivo. It increases growth hormone secretion per cell by depolarizing somatotroph membrane and by stimulating receptors present on immune cells[20]. The growth hormone released in the bone marrow microenvironment in response to ghrelin stimulation[14] might be responsible for enhanced hematopoietic recovery of ghrelin treated myelosuppressed rats.

The role of growth hormone is, however, well established in stimulating hematopoiesis. It has been observed in a number of studies that growth hormone plays a crucial role in immune restoration of certain immunosuppressed individuals and may also assist in multilineage renewal of hematopoietic deficiencies[21]. Studies have shown that this age related loss of marrow hemopoietic cells and decline in thymic function is reversed by growth hormone. Similar effects have also been observed with ghrelin. It has been observed that signalling of ghrelin through GHS-R plays a crucial part in production of naive T cells and age-related thymic deterioration. These findings clearly demonstrate an innovative role of ghrelin and its receptor in hematopoiesis and recommend a probable beneficial effect of utilizing this path in the restoration of haematopoietic function in immune-compromised individuals[21].

In general, it is believed that ghrelin, like GHRH analogs, stimulates a more physiological profile of growth hormone levels in plasma than subcutaneously delivered recombinant growth hormone. Due to its unique pharmacokinetic properties and high bioavailability following oral or parenteral administration, it is preferred over growth hormone. Although natural peptide like

ghrelin is potentially less toxic and has fewer side effects. This is due to its unique alkylation at the 3rd serine residue, which acts both as a powerful guard against protease degradation and as a suitable integrated transport system but ghrelin being a natural peptide is costly[22].So, recent research is focused to develop novel ghrelin analogs which can be modified peptides and non-peptide analogs. These analogs can substitute ghrelin in a cost effective way and are more prone to clinical applications[22]. Due to paucity of funds and research facilities, we were unable to perform bone marrow examination to observe the morphological changes in blood cells and measure cell proliferation. Further studies are recommended to observe the effect of ghrelin on cell proliferation in bone marrow by measuring proliferating cell nuclear antigen (PCNA). More studies are recommended to demonstrate the cellular mechanism of ghrelin in stimulating hematopoiesis and its effect on bone marrow microenvoirnment.

CONCLUSION

The current study confirms strong association between ghrelin and stimulation ofleukopoiesis in myelosuppressed rats. While the failure of hemopoietic recovery and growth hormone level to rise in chemotherapeutic, ghrelin and GHRH antagonist treated group suggest the possibility of growth hormone as possible mediator of ghrelin in leukopoiesis.

CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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