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Research Article

POSSIBLE ADVERSE EFFECTS OF ONCE-DAILY ORAL THERAPEUTIC DOSE OF EITHER GLUCOSAMINE SULFATE OR GLUCOSAMINE/CHONDROITIN SULFATE ON BLOOD CELLS COUNT IN RATS

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ABSTRACT

This study was designed to investigate the possible adverse effects that may be induced by once-daily therapeutic doses of either glucosamine sulfate or glucosamine/chondroitin sulfate administered orally to rats for 30 days on blood cells (RBCs, WBCs and platelets) counts. Forty three white healthy adult Albino rats of both sexes were selected randomly for this study. They were divided into three groups (I, II, III). Group I received 0.05 ml distilled water, group II received once daily therapeutic dose of glucosamine sulphate and group III received once daily therapeutic dose of glucosamine sulphate/chondroitin sulphate orally. The treatment period was for 30 days. At day 31, the animals were subjected to light ether anaesthesia and blood was withdrawn from the eye by retro-orbital puncture for the estimation of blood cells (RBCs, WBCs and platelets) count. Treatment with single daily therapeutic dose of either GS alone or GS/CS for 30 days on blood cells count in rats produced a non significant change in RBCs counts compared to control and to each other. There were no statistically significant differences in total WBCs count at day 31 in animals administered once daily therapeutic dose of either GS or GS/CS orally compared to control group. In contrast, there was a statistically significant elevation in total WBCs count in GS/CS- treated rats compared to that in the GS-treated rats. The results of this study also showed that there was statistically significant decrease in neutrophils percentage in both drug treatment groups compared to control group. A statistically significant reduction in the percentage of monocytes was observed in GS/CS group compared to the corresponding percentage in animals of control group; while, there were non-significant differences in the percentage of monocytes in GS treated rats compared to that in the control group. There were no significant differences in the percentage of monocytes at day 31 of GS/CS compared to the corresponding percentage in GS- treated rats. There were no statistically significant differences in the percentage of lymphocyte in rats administered once daily therapeutic dose of GS orally at day 31 compared to that in the control group. In contrast, rats orally administered once daily therapeutic dose of GS/CS for 30 days produced a statistically significant elevation in the percent of lymphocytes compared to that in animals of the control group. Moreover, GS/CS produced a statistically significant elevation in the percentage of lymphocytes compared to that in GS- treated rats. Oral administration of once daily therapeutic dose of GS for 30 days produced a statistically significant elevation in platelets count compared to that of controls. In contrast, there were no statistically significant differences in platelets count in rats treated with GS/CS compared to the corresponding count of control animals. Besides, there was a statistically significant elevation in platelets counts in animals administered once daily therapeutic dose of GS at day 31 compared to the corresponding count in GS/CS- treated rats.

According to the data obtained from this study, we can conclude that GS alone or GS/CS produced different effects on blood cell counts; where, the increase in lymphocyte percentage seen in GS/CS- treated rats not in GS alone may reflect that CS component may induce lymphocytosis. While, the decreased of either monocytes or neutrophils percentage may be due to their consumption to combat infection in group of animals treated with GS/CS. Finally, the increased platelets count observed in GS-treated group may explain that GS may possess hyper-coagulable property.

Keywords: glucosamine sulphate, glucosamine sulphate/chondroitin sulphate, RBCs, WBCs, platelets, blood cell counts.

INTRODUCTION

Adverse events come in many different forms, and all drugs, even placebos, have adverse events associated with their use[1, 2]. The vital functions that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication, makes the hematopoietic system unique as a target organ[3,4,5]. Red blood Cells (RBCs) are anucleate cells; they are packed with the O2—carrying protein hemoglobin[5]; their half life is 120 days in humans and 45-68 days in rodents; the normal red blood cells count is 7000 cell/µl[6] in humans and 7.8 × 10^6 cells/µl in rats[7]. They function only within the blood stream to bind oxygen for delivery to the tissues, bind carbon dioxide for removal from the tissues[8]. White blood cells (WBCs) are spherical-shaped cells while suspended in blood plasma, but some of their types become amoeboid after leaving blood vessels to combat foreign materials. They are divided into two groups according to the type of granules in their cytoplasm and the shape of their nuclei: granulocytes (polymorph nuclear leukocytes) and agranulocytes (mononuclear leukocytes)[8]. The most frequent granulocytes[9, 10] are neutrophils (constitute about 60-70 % of total blood leukocytes)[8] that are constantly generated in a high number in bone marrow and circulate with the blood stream until activation in response to infections[10]. Additionally, they are the most abundant cells of the immune system next to macrophages[9]. In contrast, eosinophils (2-4% of total leucocytes)[8] are resident in various organs such as the GIT, mammary glands as well as BM and may contribute to tissue and immune homeostasis[10]. The least common granulocytes in the circulation are basophils (less than 1% of total blood leucocytes)[8]. These cells are not only one of the main sources of histamine in allergic reactions, but also associated with the differentiation of naïve T cells towards TH2 cells during allergic and anti-parasitic immune responses via antigen presentation and IL-4 production[11]. Agranulocytes include lymphocytes and monocytes. The former cells are the most common agranulocytes and account for about 30% of the total blood leukocytes. Most lymphocytes found in blood or lymph as they represent re-circulating immune-competent cells. The latter cells are the largest of the WBCs in a blood smear. They travel from the BM to the body tissues, where they differentiate into the various phagocytes of the mononuclear phagocytic system, *i.e.,* connective tissue macrophages (histiocytes), osteoclasts, alveolar macrophages, perisinusoidal macrophages in the liver (Kupffer cells) and macrophages of lymph nodes , spleen, and BM. Monocytes remain in the blood for only about 3 days[11]. Platelets are small anucleate cells circulating in the blood. They are the second most abundant cells, after RBCs, in the blood circulation with a normal concentration in

humans is of 150-400 × 10^9/L [6]. They are produced from their precursor megakaryocytes in the BM. The major physiological role of platelets is to accumulate at sites of damaged blood vessel endothelium and initiate the blood clotting process[12]. There are a large number of drugs that can cause varying degrees of blood dyscrasias, ranging from mild thrombocytopenia to severe pancytopenia [3] [13]. The mechanism(s) by which these drugs cause dyscrasias varies from agent to agent[1].

Glucosamine (2-amino-2-deoxy-D-glucose) is an amino monosaccharide[14] that is an essential component of mucopolysaccharides and chitin[15]. It is found in almost all human tissues but is highest in concentration in the liver, kidney and cartilage. It is the most fundamental building block required for the biosynthesis of various compounds including glycolipids, glycoprotein (GP), glycosaminoglucans (GAGs), hyaluronate and proteoglycans (PGs), which are all intimately involved with joint structure and function[16]. It was found that GA takes about 1 month to exert its full effect[17]. The usual oral dose of GS per day is 1500 mg in divided doses[14, 18]. Glucosamine (GA) has many favorable effects on cartilage. *First*, it has shown an anabolic stimulating effect on cartilage synthesis. *Second*, it inhibits by means of several anti-inflammatory and antioxidant mechanisms, the catabolic cartilage degenerating reactions observed in OA. This can delay cartilage degeneration in OA which leads to a reduction in pain[19] and swelling as well as to increased mobility of the affected joint[20, 21]. Glucosamine sulphate (GS) significantly reduced resorptive activity[22]. It has been confirmed by authors that people with a shellfish allergy may be more susceptible to allergic skin reactions (erythematous rash, angioedema, urticaria, rash and pruritus) when taking GA sourced from seafood[23]. Currently, the British National Formulary (BNF) advises caution in patients with impaired glucose tolerance and recommends monitoring blood glucose concentration before treatment and periodically thereafter[24]. However, it must be stressed that since GA is not a medically licensed product, content and quality control of the product remain a source of concern[25]. Thus, other side effects of GA and its various formulations need to be investigated. Chondroitin sulfate (CS) is a glycosaminoglycan, a major component of the extracellular matrix (ECM) of many connective tissues including: cartilage, bone, skin, ligaments and tendons. It has been classified as a symptomatic slow-acting drug in OA (SYSADOA) and as a structure/disease modifying anti-OA drug (S/DMOAD)[26, 27]. Commercial CS is mainly derived from trachea, nasal septa, chicken keel shark cartilage and fish[28]. It is taken orally in capsules and tablets[22]. The usual dosage of CS is 1200 mg/day in three divided doses[18]. The authors observed a latency period of about a month before an advantage of chondroitin with respect to pain relief and function was seen, as well as an effect lasting beyond the cessation of treatment[22, 29]. Chondroitin sulfate (CS) increases the hyaluronan production by human synovial cells, which has a beneficial effect on maintaining viscosity in the synovial fluid. Moreover, it has been shown that CS stimulates the chondrocyte metabolism, leading to the synthesis of collagen and proteoglycans, the basic components of new cartilage [20, 29]. Furthermore, it influences the symptoms of OA such as pain and inflammation, but also acts as a structure-modifying drug (SMOAD) in OA. The ability of CS to slow down the development of OA has been demonstrated in several clinical trials[22,30]; while, other authors reported that CS had no significant effect on patients with severe OA [22]. Although no serious adverse events were found to be related to CS [31] as it is well tolerated and has few significant side effects mainly gastrointestinal discomfort[18]. In the clinical setting, it was demonstrated that although the addition of CS to subjects already receiving GS did not appear to provide significant improvements; but, it has been found that the combination of CS and GS is more efficacious than CS alone in improving several of the disease markers examined[32]. It has been reported that chondroitin sulfate proteoglycans (CSPGs) may facilitate melanoma progression through its interaction with metalloproteinase type three (MMP3)[33]. Moreover, biochemical studies show that tissue concentration of CS is increased in benign prostatic hyperplasia (BPH), which suggests that CSPGs are associated with the proliferative responses that occur in this disorder[34].

MATERIALS AND METHODS

Drugs: Glucosamine sulphate (500 mg) tablets. Principle Health Care International (Limited), United Kingdom and glucosamine sulphate and chondroitin sulphate (900mg) tablets, AG Adrien Gagnon, Canada

Animals: Forty three white healthy adult Albino rats of both sexes, weighing 150-250 g were used in this study. They were purchased from The Animal House of College of Veterinary Medicine, University of Mosul and maintained under conditions of controlled temperature, ventilation and 12 hr day light 12 hr darkness. The animals were fed standard pellets ad libitum and had free access to tap water.

Study Design: The animals were selected randomly and allocated as follows:

Group I (control): fifteen rats were administered 0.05 ml distilled water (DW) by oral gavage once daily for 30 days. investigation

Group II: By utilizing 500mg tablet of GS, 14 rats were administered a therapeutic dose of the drug (20 mg/kg B.wt) (35); where a 500 mg tablet of GS was dissolved in 5 ml DW to obtain a concentration of the drug (100mg/ml), then according to B.wt of each animal, an appropriate volume of the drug was administered by oral gavage once daily for 30 days.

Group III: By utilizing 900mg tablet containing GS/ CS, 14 rats were administered a therapeutic dose of the drug (20 mg/kg B. wt. of GS and 17 mg/kg B.wt of CS[17], where 900 mg tablet was dissolved in 5 ml DW to obtain a concentration of the drug (180mg/ml), then according to B. wt of each animal, an appropriate volume of the drug was administered by oral gavage once daily for 30 days.

At day 31 of either DW or drugs administration, rats were subjected to light ether anaesthesia and blood was collected into potassium ethylene diamine tetraacetic acid (EDTA) tubes after puncturing the ocular vein (retroorbital plexus) with a fine sterilized glass capillary tube[35] and the blood was utilized for the determination of haematologic parameters (RBCs count, WBCs count, differential WBCs and platelets count) using automated haematology analyzer (Abbott, USA).

Statistical analysis

Statistical Package for the Social Sciences [SPSS] (Version 13.0) software was used for analysis. The significance of differences between the mean values among drug-treated animals of same group with controls and between drug

treated groups was calculated using Duncan test. The results were expressed as mean ± SEM. P-values of 0.05 or less were considered significant for all data presented in the results of this work.

RESULTS

The data obtained from table 1 showed that at day 31 of oral administration of once daily therapeutic doses of either GS (group II rats) or GS/CS (group III rats) for 30 days showed no statistically significant (P> 0.05) differences in RBCs count compared to the corresponding count in animals of the controls (group I). Similarly, there were no statistically significant (P> 0.05) differences in RBCs count at day 31 in GS/CS- treated rats as compared to that in GS- treated rats.

Table 1: Effects of Once Daily Therapeutic Doses of either Glucosamine Sulfate (GS) or Glucosamine/Chondroitin Sulfate (GS/CS) on Red Blood Cells Count at day 31 of each Drug treatment in Rats

	RBCs count ($\times 10^6$ cell/µl)
Group I – Control N=15	6.3323± 0.18 NS
Group II GS- treated N=14	6.3500± 0.206 NS
Treated Group III - GS/CS- N=14	6.832± 0.105 NS

Each value represents Mean ± SEM; NS: None significant differences among groups; N= number of animals.

The results of table 2 showed that there were no statistically significant (P> 0.05) differences in total WBCs count at day 31 in animals administered once daily therapeutic dose of either GS or GS/CS orally as compared to those counts in animals of control group. In contrast, there was a statistically significant (P< 0.05) elevation in total WBCs count in GS/CS- treated rats compared to that in the GS- treated rats.

Concerning the effect of once daily oral administration of therapeutic doses of either GS (group II) or GS/CS (group III) on the percentage of neutrophils, table 2 showed that there was a statistically significant (P< 0.05) reduction in the percentage of neutrophils in either treated groups compared to the intended percentage in control (group I). The neutrophils percentages were 1.5, 1.67 and 2.64, respectively.

Besides, the results of table 2 showed that there were no statistically significant (P> 0.05) differences in the percentage of neutrophils when a comparison was made between the effects of once daily therapeutic doses of either GS or GS/CS administered to rats at day 31 of each drug treatment.

With respect to the effect of once daily therapeutic dose of either GS or GS/CS administration for 30 days on the percent of eosinophils, there were no statistically significant (P> 0.05) differences in the percentage of eosinophils at day 31of each drug treatment compared to control group. Furthermore, a comparison between the effects of once daily therapeutic dose of either GS or GS/CS administered to rats was performed. There were no statistically significant (P> 0.05) differences in the percent of eosinophils at day 31of each drug treatment. Moreover, the results of table 2 showed that there was no statistically significant (P> 0.05) differences in the percentage of basophils in groups of rats treated with either GS or GS/CS at day 31 compared to that percentage in animals of control group. Additionally, there were no statistically significant (P> 0.05) differences in the percentage of basophils in GS/CS- treated rats at day 31 as compared to the corresponding percentage in GS- treated rats.

Besides, the results of table 2 showed that at day 31 of oral administration of once daily therapeutic dose of GS/CS produced a statistically significant (P< 0.05) reduction in the percentage of monocytes as compared to the corresponding percentage in animals of control group. While, there were non-significant (P> 0.05) differences in the percentage of monocytes in GS treated rats compared to that in the control group.

In comparison between the effects of either GS or GS/CS treatment, the data obtained from table 2 showed that there were no significant (P> 0.05) differences in the percentage of monocytes at day 31 of GS/CS compared to the corresponding percentage in GS- treated rats.

With the regard to the effects on the percentage of lymphocytes, table 2 showed that there were no statistically significant (P> 0.05) differences in the percentage of lymphocyte in rats administered once daily therapeutic dose of GS orally at day 31 compared to that in the control group. In contrast, rats orally administered once daily therapeutic dose of GS/CS for 30 days produced a statistically significant (P< 0.05) elevation in the percent of lymphocytes as compared to that in animals of the control group. Table 1-2. Moreover, the results of table 2 showed that, oral administration of once daily therapeutic dose of GS/CS for 30 days produced a statistically significant (P< 0.05) elevation in the percentage of lymphocytes compared to that in GS- treated rats.

Table 2: Effects of once Daily Therapeutic Doses of either GS or GS/CS on Total White Blood Cells (WBCs) Count and Differential WBCs Percentage at Day 31 of each drug treatment in Rats.

	Group I Control N=15	Group II GS- treated N=14	Group III GS/CS- treated N=14
Total WBCs ($\times 10^3$ cell/µl)	14.3877±1.899	10.587±1.297[a]	17.98±1.767[b]
Differential WBCs percentage (%)			
Neutrophils	2.6365±0.346	1.5001±0.255[a*]	1.6657±0.239[a*]
Eosinophils	0.4727±0.079	0.4091±0.055[a]	0.4567±0.071[a]
Basophils	0.5259±0.134	0.3545±0.13[a]	0.3015±0.139[a]
Monocytes	0.7263±0.197	0.524±0.269[a]	0.0746±0.025[a*]
Lymphocytes	10.03±1.751	7.796±1.219[a]	15.466±1.582[b*]

Each value represents Mean ± SEM; *: p < 0.05, significant difference in comparison with control group; Values with non- identical superscripts (a, b) within each parameter are significantly different; N= number of animals.

Oral administration of once daily therapeutic dose of GS produced a statistically significant (P< 0.05) elevation in platelets count at day 31 of drug treatment compared to that of controls. In contrast, there was no statistically significant (P> 0.05) differences in platelets count in rats treated with GS/CS at day 31 compared to the corresponding count of control animals. Table 3

Besides, there was a statistically significant (P< 0.05) elevation in platelets counts in animals administered once daily therapeutic dose of GS at day 31 as compared to the corresponding count in GS/CS-treated rats. Table 1-3

Table 3: Effects of Once Daily Therapeutic Dose of GS or GS/CS on Platelet Count at Day 31 of each drug treatment in Rats

Group	Platelet count (× 10^3 cell/µl)
Group I Control N=15	597.77±30.276
Group II GS- treated N=14	723.6±38.733[a*]
Group III GS/CS- treated N=14	620.00±47.143[b]

Each value represents Mean ± SEM; *: P < 0.05: significant difference in comparison with control group; Values with non- identical superscripts (a, b) within each parameter are significantly different; N= number of animals.

DISCUSSION

The results of this study showed no differences in RBCs count in both drug treatment groups. This is consistent with the results performed by others; where, the laboratory tests including haematological parameters (RBCs, WBCs, differential WBCs, hemoglobin Hb and erythrocyte sedimentation rate ESR) were not different from the control. Similarly no adverse events on hematological measurements resulting from oral chondroitin sulfate were observed[36, 37, 38, 39].

In the current work, there was a statistically significant (P< 0.05) elevation in total WBCs count in GS/CS- treated rats (mainly due to increased lymphocytes) compared to that in the GS-treated rats as shown in table 2.

Concerning the effect of once daily oral administration of therapeutic doses of either GS (group II) or GS/CS (group III) for 30 days on the percentage of neutrophils, table 2 showed that there was a statistically significant (P< 0.05) reduction in the percentage of neutrophils in either treated groups compared to the intended percentages in the control (group I). No changes in WBCs and platelets count were observed in the clinical study by Daniel, U. et. al (2004); where, CS was administered to patients with knee OA at a dose of 400mg once daily for two periods of 3 months during one year [29].

Glucosamine has been shown to possess anti-inflammatory action; where, it acted on the arthritic process in joint by suppressing: iNOS expression, neutrophil functions, both the activation of T-lymphoblasts and dendritic cells in collagen-induced osteoarthritic (CIOA) mice [17].

In the study by Muller, I. et.al (2010) in which CS was administered to 129 patients with concomitant knee OA and psoriasis, common cold was the most significant adverse event and no clinically significant changes in laboratory tests including complete blood count was observed [40].

The results of table 2 showed that oral administration of once daily therapeutic dose of GS/CS for 30 days produced a statistically significant (P< 0.05) reduction in the percentage of monocytes as compared to the corresponding percentage in animals of control group.

Peripheral blood monocytes are physiological precursors of tissue-resident mononuclear cells. Upon different stimuli during inflammatory response, monocytes migrate into the tissues and differentiate into either macrophages or immature dendritic cells. There is a body of information showing that CS is a modulator of innate immunological reactions. Chondroitin-4-sulphate activates monocytes to secrete monokines [41]. Santangelo et al (2001) reported that the BM is driven to monocytopoiesis following thermal injury and infection in mice. In our work, the reduction in monocyte percentage in GS/CS-treated group may be due to their consumption to combat infection. Traumatic injury and infection have been shown to be associated with an increase in myeloid progenitor cells in the circulation and increased production of granulocyte/macrophage colony-forming units [42]. From this work, we can conclude that CS administration in group III animals was responsible for the decreased percentage (%) of monocytes in peripheral blood.

Rats orally administered once daily therapeutic dose of GS/CS for 30 days produced a statistically significant (P<0.05) elevation in the percent of lymphocytes as compared to that in animals of the control group. Moreover, the results of table 3-5 showed that, oral administration of once daily therapeutic dose of GS/CS for 30 days produced a statistically significant (P< 0.05) elevation in the percentage of lymphocytes compared to that in GS- treated rats. Reactive lymhocytosis is primarily due to physiologic or patho-physiologic response to infection, toxins, cytokines or unknown factors [43, 44]. It has been demonstrated that the typical morphology of the lymphocytes i.e. large with abundant blue cytoplasm having enlarged nuclei with fine chromatin and prominent nucleoli with an increased lymphocyte count are typical for viral infections [43, 45].

Chondroitin sulfate (CS) has been found to induce allergic response, increase of basophils percentage, and increase lymphocyte % [46]; but, in the present study, CS produced no statistically significant differences in basophil % (i.e increased basophils required for allergic reactions). Table 2

The results of table 3 showed that oral administration of once daily therapeutic dose of GS produced a statistically significant (P< 0.05) elevation in platelets count at day 31 of drug treatment compared to that of controls.

In contrast, there was no statistically significant (P> 0.05) differences in platelets count in rats treated with GS/CS at day 31 compared to the corresponding count of control animals. Besides, there was a statistically significant (P< 0.05) elevation in platelets counts in animals administered once daily therapeutic dose of GS at day 31 as compared to the corresponding count in GS/CS- treated rats. Table 3. Thus, the results of our study showed that GS provoked an increase in platelets count and this may account for its hypercoagulable property that may possess. Thus, the results

of the present study are inconsistent with that observed by others where, it has been demonstrated that glucosamine interfere with platelets, and in turn, the process of blood clotting, thus, it may increase the risk of bleeding. [47]

Glucosamine was suggested to possess anti-inflammatory and immunsuppressive effects. For instance, several studies have been shown that this substance inhibits the synthesis of proinflammatory mediators in human chondrocytes in vitro, suppresses the activation of NF-κB and modulates the bioactivity of IL-1β in rat chondrocytes, and suppresses unprimed T-cell responses by interfering with antigen-presenting cell functions proliferation of murine T cells. However, experimental and by a direct inhibitory effect on anti-CD3-induced studies suggest that glucosamine functions as an immunomodulator rather than as an immunosuppressant in vivo. Thus, it is conceivable that depending on the type of the disease, the use of glucosamine can cause either beneficial or deleterious immune mediated effects. Although the exact underlying mechanism by which glucosamine induces anti inflammatory effects are not fully understood, the results from several studies suggests that glucosamine by acting on the innate immune cells particularly macrophages down regulates the expression of nuclear factor kappa B (NF-kB) and mitogen-activated protein kinases (MAPK), the key transcription factors that are required for the activation of inflammatory genes (e.g., TNF-α, IFN-γ and IL-1β). It is possible that treatment with glucosamine activates the cells of the adaptive immune system, but suppresses the activity of innate immune cells [48].

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