Glycine supplementation reduces the severity of chemotherapy-induced oral mucositis in hamsters

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Received 29 June 2013; revised 29 July 2013; accepted 7 August 2013

ABSTRACT

Objective: Oral mucositis (OM) is a devastating toxicity associated with cytotoxic cancer therapy. The OM pathogenesis and the complex interactions occur in response to tissue insult. Application of this evolving model has aided in the development of mechanistically based therapies for the prevention and treatment of mucositis. The present study was to assess the effects of glycine supplementation on chemotherapy-induced oral mucositis. Methods: In a hamster cheek pouch model of chemotherapy-induced oral mucositis, one group of 20 animals received systemic glycine supplementation for 7 days, while another similar control group did not. Clinical mucositis severity and neutrophil infiltrate (on histology) were assessed by blind examiners. Free radical production was measured as malondialdehyde (MDA) levels. Results: As compared to control animals, glycine-treated animals demonstrated a highly significant reduction in clinical severity of oral mucositis, neutrophil infiltrate, and MDA levels (p < 0.001 for all). Conclusions: Glycine supplementation reduces the severity of chemotherapy-induced oral mucositis in an animal model. This effect is at least partly mediated through inhibition of the inflammatory response and reduced production of damaging free radicals.

Keywords: Glycine; Chemotherapy; Oral Mucositis; Neutrophils; Malondialdehyde; Inflammatory

1. INTRODUCTION

Oral mucositis (OM) presents as erythematous and ulcerative lesions of the oral mucosa, secondary to chemotherapy and/or radiation therapy for cancer [1-3]. Clinical manifestations of OM include intense pain, interference with ingestion of food and drink, and impaired communication. Moreover, infection associated with oral mucosal lesions can progress to life threatening sepsis during periods of intense immunosuppression [4]. OM impacts negatively on patients’ survival and quality of life, and is associated with longer hospitalizations and higher costs [5,6]. Perhaps most importantly, dose-reductions in cancer therapy due to mucositis can adversely affect the outcomes of cancer treatment. However, treatment options for OM are very limited and current management strategies are largely palliative [7].

OM is an inflammatory response of the oral mucosa that pathophysiology is complex and multifactorial [8]. Histopathological evaluation of mucositis lesions shows mucosal thinning, caused by apoptosis and depletion of the epithelial basal layer, with subsequent denudation and secondary bacterial infection [9]. Development of oral mucositis involves oxidative stress and the accumulation of reactive oxygen species (ROS) [10]. This oxidative stress can produce lipid peroxidation and inflammation [11]. The inflammatory response in oral mucositis in-
volves the activation of NF-κB and the upregulation of inflammatory cytokines, including TNF-α [12]. Glycine, a simple amino acid, has been shown to have anti-inflammatory, immunomodulatory and cytoprotective effects [13]. Experimental studies have shown its protective effect on inflammatory lesions in various models. For example, glycine is a strong inhibitor of resident liver macrophages and acts via a glycine-gated chloride channel, which subsequently inhibits Kupffer cell (KC) activation by decreasing calcium inflow [14,15]. Moreover, glycine is an essential component of glutathione, which is needed for detoxification processes. In addition, glycine has indirect effects as a free radical scavenger [16].

Glycine inhibits the production of inflammatory mediators, probably by decreasing the activation of NF-κB and TNF-α, reducing the formation of additional free radicals and other toxic mediators, and attenuating further lipid peroxidation and glutathione depletion [17,18]. Recent research by Stoffels et al [19] has demonstrated the ability of glycine to reduce chemotherapy-associated injury. For example, in a clinically relevant in vivo model of chemotherapy-associated liver injury, glycine decreased liver damage (as measured by transaminases after chemotherapy), reduced microvesicular steatosis, and increased hepatic microcirculation [19].

Further, in a rat model of ischemia-reperfusion injury, glycine administration resulted in downregulation of cell apoptosis and the expression of pro-apoptotic genes [20]. Thus, glycine has been demonstrated to have positive effects on many of the pathways involved in the pathogenesis of OM. Therefore, this study was designed to assess the effects of glycine on chemotherapy-induced OM in a hamster model. We examined the effects of systemic glycine supplementation on the clinical severity of OM, degree of inflammatory response (by assessing neutrophil infiltrate histologically), and oxidative stress (by measuring the final product of lipid peroxidation).

2. MATERIALS AND METHODS

This study was approved by the Ethics Committee of São Paulo Federal University (UNIFESP) (1916/08). Forty female Golden Syrian hamsters (Mesocricetus auratus), 8 weeks old and weighing approximately 150 g each, were used. The animals were kept in groups of six per plastic container, with food and water available ad libitum.

2.1. OM Induction Protocol

A well-accepted published protocol for chemotherapy-induced oral mucositis in hamsters was used [21]. Briefly, all the animals received 80 mg/kg intraperitoneally of the chemotherapy drug 5-Fluorouracil (5-FU) on day 0, followed by 40 mg/kg 5-FU administered intraperitoneally on day 2. The right cheek pouch of the animals was everted and the mucosa was irritated by superficial scratching with the tip of an 18-gauge needle by the same operator on days 3 and 4.

2.2. Glycine Supplementation

The animals were randomly divided into two groups of 20 animals each. Animals in Group 1 received a 2 mg/g of body weight intraperitoneal injection of Glycine (Ajinomoto, Raleigh, NC), diluted in saline at a concentration of 5%. Treatment with the Glycine, was initiated on day 0, with application once per day (in the morning), for seven days. Animals in Group 2 served as controls and did not receive any glycine supplementation but were treated identically in all other respects.

2.3. Clinical Evaluation of OM

Clinical evaluation of OM was performed by two blinded evaluators. On day 3 and day 7, the right cheek pouch of all animals was turned outward for the clinical evaluation of the severity of the mucositis. Mucositis scores from 0 to 5 was assigned based on the method described by Sonis et al with higher scores indicating greater severity [22] (Table 1).

2.4. Histological Evaluation of OM

All animals were sacrificed on day 7 and the right cheek pouch removed. The cheek pouch samples were labeled, immediately cooled in isopentane for 10 s, and then flash frozen in liquid nitrogen. The fragments were positioned in such a way so as to provide cross-sectional slices during microtomy. Serial slices (10 µm) were obtained in a cryostat at a temperature of −20°C, placed on silanized glass slides, submerged in acetone, and dried at room temperature for 10 min. The serial sections of each sample were stained using hematoxylin-eosin staining and examined under a light microscope by a blinded pathologist. The absence or presence of microscopically visible ulceration and severity of neutrophil infiltrate were each separately scored, using the scale described by Lopes et al [23] (Table 1).

2.5. Measurement of Oxidative Stress: Determination of Malondialdehyde (MDA) Levels

MDA is a final product of lipid peroxidation and a well-established measure of the level of free radicals in intestinal tissue [20,24]. To determine MDA levels, the
Table 1. Scales used for clinical and histological evaluation of oral mucositis.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Pouch completely healthy. No erosion or vasodilatation.</td>
</tr>
<tr>
<td>1</td>
<td>Erythema, but no evidence of mucosal erosion.</td>
</tr>
<tr>
<td>2</td>
<td>Severe erythema, vasodilation and superficial erosion</td>
</tr>
<tr>
<td>3</td>
<td>Formation of ulcers in one or more places, but not affecting more than 25% of the surface area of the pouch.</td>
</tr>
<tr>
<td>4</td>
<td>Severe erythema and vasodilation Cumulative ulcer formation about 50% of pouch surface area.</td>
</tr>
<tr>
<td>5</td>
<td>Virtually complete ulceration of the pouch mucosa. Loss of pliability.</td>
</tr>
</tbody>
</table>

Clinical Assessment

<table>
<thead>
<tr>
<th>Histological Assessment: Neutrophil infiltrate</th>
<th>Grade</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absent or rare neutrophil</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Moderate or severe neutrophil infiltrate</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histological Assessment: Ulceration</th>
<th>Grade</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Ulceration absent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Ulceration present</td>
<td></td>
</tr>
</tbody>
</table>

thiobarbituric acid (TBA) reaction proposed by Kohn and Liversedge [25] was used. Tissue samples were de-frosted, weighed, and a volume equivalent to five times the weight of TRIS 0.01 M/pH 7.4 buffer solution was then added. Tissue samples were homogenized in an ice bath four times, for 30 seconds each, and subsequently centrifuged for 5 minutes at 10,000 rpm, at 4°C. The protein content of the homogenate was determined by the coomassie brilliant blue (CBB) procedure, as described by Kohn and Liversedge [25]. Briefly, the CBB reactant interacts with protein, enabling its quantification by using a standard albumin curve with known concentrations.

For MDA measurement, 400 micro liters of the centrifuged homogenate supernatant were collected and added to 1 ml of 20% trichloroacetic acid and 400 ml of 1.6% thiobarbituric acid.

The mixture was incubated for 30 minutes at 95°C. Lipids were extracted by adding n-butanol (1.6 ml) and stirring vigorously. The sample was again centrifuged for 10 minutes at 3000 rpm.

Absorbance of the organic layer was determined through reading at 510, 532, and 560 nm. The following equation, proposed to minimize the interference of both heme pigments and hemoglobin in the measurement of MDA [20,24], was used:

$$\text{MDA}_{532} = 1.22 \left( (A_{532}) - (0.56)(A_{510}) + (0.44)(A_{560}) \right).$$

The calibration curve was drawn with 1, 3, 3 tetramethoxypropane (also known as malondialdehyde bis. MDA levels were calculated and expressed in nmol MDA/mg of protein.

2.6. Statistical Analysis

The Kappa coefficient (k) was calculated to determine inter-examiner agreement for clinical assessments of OM. Qualitative variables (clinical and histological scoring of mucositis severity) were compared using the Pearson’s Chi square test and the Fisher test. Quantitative variables (MDA levels) were compared using the analysis of variance (ANOVA). All statistical analyses were performed with a significance level of 5% ($\alpha = 0.05$).

3. RESULTS

3.1. Clinical Evaluation of OM

There was excellent inter-examiner agreement on the clinical assessment of OM (Kappa = 0.86 for Group 1 (glycine supplementation) and 0.94 for Group 2 (controls). These data demonstrate that there was adequate calibration for evaluation of the clinical characteristics of OM. The mucositis induction protocol consistently caused erythema, hemorrhage and ulceration in the right cheek pouch of all animals. Thus, all animals in both groups were scored as having Grade 3 mucositis on day 3 (Figure 1). However, by day 7, there was a marked reduction in Clinical mucositis severity in most animals in the glycine group, with the majority showing healing of ulcers. In comparison, the clinical mucositis severity in control animals stayed the same or worsened (Table 2). This difference between groups was clinically and statistically significant ($p < 0.001$).

3.2. Histological Evaluation of OM

Histopathological findings in control animals on day 7 were consistent with those previously described for this animal model and mucositis induction protocol [22]. In general, control animals demonstrated an intense cellular infiltration with prevalence of neutrophils, hemorrhagic areas, severe vascular hyperemia, edema, and ulceration. Focal points of surface bacterial colonization and abscesses were seen (Figure 2). In contrast, the glycine group generally exhibited a less intensive histopathological reaction, with discreet vascular hyperemia and slight inflammatory infiltration.

On day 7, 100% of animals in the control group had a
Figure 1. A representative Photographs of the cheek pouch of hamsters at magnification ×400. Glycine group: (A) Day 3 (ulcer present) and (B) Day 7 (re-epithelization of the mucosa). Control group representatives: (C) Day 3 (ulcer present) and (D) Day 7 (persistent ulcer).

Table 2. Clinical evaluation of oral mucositis.

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>Mucositis grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Glycine</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Glycine*</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Control*</td>
<td></td>
</tr>
</tbody>
</table>

*The table represents quantification of data represents number of animals with each grade of oral mucositis at each time-point. *p < 0.001.

3.3. Measurement of Oxidative Stress: Determination of Malondialdehyde (MDA) Levels

At day 7, the mean MDA levels in the cheek pouch of animals in the control group were more than 5-fold the MDA levels in the glycine group (Table 4). Treatment with glycine thus significantly reduced this marker of lipid peroxidation and free radical production (p < 0.001).

4. DISCUSSION

OM is a complex process involving not only direct cell injury caused by chemotherapy or radiation, but also a complex cascade of biological events [10]. The process begins with clonogenic cell death and the release of reactive oxygen species (ROS), progressing through a series of steps in which multiple biological pathways are activated and amplified, culminating in ulcer development, and finally healing [4]. Investigations into the patho-
**Table 4.** Measurement of oxidative stress: Determination of malondialdehyde (MDA) levels (Day 7).

<table>
<thead>
<tr>
<th>Group</th>
<th>Snmol MDA/mg protein</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.185*</td>
<td>0.118</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.085*</td>
<td>0.225</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.001.

 genesis of OM show the importance of the inflammatory response, which includes the involvement of many different inflammatory mediators including nuclear factor kappa B (NF-κB) [12,26,27], cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and platelet activating factor (PAF) [1,22,28,29], as well as the cyclooxygenase pathway [1, 23,26,30].

Therefore, there has been significant interest in the evaluation of anti-inflammatory strategies for the amelioration of oral mucositis [31]. Glycine is a simple non-essential amino acid that acts as an inhibitory neurotransmitter in the central nervous system (CNS), via a glycine-gated chloride channel (GlyR) [32]. Outside of the CNS, glycine had been presumed to be independently biologically neutral for a long time, functioning only as a building block for proteins. More recently, however, evidence has accumulated indicating that glycine possesses anti-inflammatory properties [19]. Our current results support this anti-inflammatory role for glycine. In a well-accepted animal model of chemotherapy-induced OM, we found that glycine supplementation significantly reduced the severity of clinical mucositis. The attenuated clinical severity was accompanied by a marked reduction in neutrophil infiltrate, which suggests that glycine suppressed the inflammatory response associated with mucositis. Furthermore, glycine also reduced the production of damaging free radicals, as measured by MDA levels. MDA is a final product of lipid peroxidation and a well-established measure of the level of free radicals in intestinal tissue [20,24].

The anti-inflammatory effects of glycine are believed to be mediated, at least in part, due to its mechanism of action in the cell membrane where it activates the chloride channel that stabilizes or hyperpolarizes the membrane potential [33]. Glycine blocks the increase of intracellular calcium which stimulates the formation of the cytokine cascade, inhibiting cells that activate the inflammatory process, probably by blocking activation of NF-κB and TNF-α [34], decreasing the formation of free radicals and other toxic mediators [35]. The free radical nitric oxide and/or its derivatives can cause lipid peroxidation [36], oxidation of protein sulfhydryls [37] and nitration of tyrosine residues on a variety of proteins, including inactivation of enzymes and/or receptors [38-40]. These effects result in tissue injury, which can lead to an excessive local amplification of the inflammatory response.

Mikalauskas et al. demonstrated that glycine decreased chemotherapy-induced liver injury, accompanied by a significant reduction in inducible nitric oxide synthase [19]. Our finding of reduced MDA levels and neutrophil infiltrates in glycine-treated animals is consistent with a role for glycine in reducing lipid peroxidation, free radical formation, and the subsequent tissue injury and inflammatory response. In a rat model of rheumatoid arthritis, glycine supplementation reduced joint swelling, accompanied by a reduction in TNF-α, inflammatory cell infiltrate and edema [41]. Furthermore, in a rat model of ischemia-reperfusion injury, glycine administration resulted in increased mucosal viability and thickness, likely mediated via a down-regulation of cellular apoptosis [20,42]. Another possible mechanism is derived from the fact that glycine participates in the formation of a third of the structure of collagen. Thus, glycine supplementation may result in increased basement membrane stability [43]. Collectively, these studies point to several mechanisms whereby glycine may be beneficial in ameliorating chemotherapy-induced mucosal injury.

5. CONCLUSION

In conclusion, glycine supplementation significantly reduced chemotherapy-induced oral, mucosal injury, neutrophil infiltrate and free radical production in an animal model.

REFERENCES


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[38] Beckman, J.S. (1996) Oxidative damage and tyrosine nitration from peroxynitrite. Chemical Research in Toxicology, 9, 836-844. doi:10.1021/tr950144g


