TITTLE:

Ascorbate-dependent decrease of the mucosal *immune* inflammatory response to gliadin in celiac disease patients

SHORT TITTLE:

Ascorbate inhibition to gliadin.

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KEYWORDS

Coeliac Disease, IL-15, ascorbate, inhibition, therapy

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ABSTRACT

BACKGROUND: Innate immunity, through the IL-15/NF-*κ*B axis, has an important role in Coeliac Disease (CD) that may represent a molecular target for its immunomodulation. Therefore, we studied if ascorbate supplementation (a blocker of the NF-*κ*B activation) to gliadin challenges could inhibit the immune response to gliadin.

METHODS: Duodenal biopsy explants from treated CD patients were gliadin challenged *in vitro* (100μ/ml) with and without 20mM ascorbate. One extra explant basal cultured constituted and internal control. Nitritites (3 hours) and IFN-γ, TNF-α, IFN-α, IL-17, IL-13 and IL-6 (24 hours) secretion were measured on the supernantants. IL-15 was assayed by Western-blot on the whole protein duodenal explant.

RESULTS: The addition of ascorbate to gliadin-challenges blocked the secretion of nitrites (p=0.013), IFNγ (p=0.0207), TNFα (p=0.0099), IFNα (p=0.0375) and IL-6 (p=0.0036) compared to non-ascorbate supplemented gliadin-challenged supernatants. Also, cytokine secretion was downregulated by ascorbate even to lower values than those observed in basal cultures (IFNγ: p=0.0312; TNFα: p=0.0312; IFNα: p=0.0312; and IL-6: p=0.0078). After gliadin-challenge, gliadin induced IL-15 production in treated-CD patients while the addition of ascorbate to culture medium completely inhibited IL-15 production. Moreover, the IL-15 inhibition by ascorbate took place even in the only treated CD-patient who had basal IL-15 production.

CONCLUSIONS:

Ascorbate blocks the immune response to gluten in a biopsy culture model, so It might be revealed as a possible future alternative therapy in CD.

INTRODUCTION

Coeliac Disease (CD) is a gut hypersensitivity to wheat gluten and similar proteins of cereals affecting genetically predisposed individuals (HLA-DQ2/DQ8). The current treatment is a life-long strict gluten-free diet (GFD) (1, 2).

The most accepted model of the CD immunopathogenesis is the two-signal model, which establishes that gliadin has a dual effect on the CD duodenum, first triggering the development of an innate immune response which then facilitates the activation of the adaptive immune response (3) controlled by gluten-reactive T cells with a TH1 cytokine profile characterized by interferon γ (IFNγ) (4). Innate immunity, and specifically IL-15 (5, 6), play a key role in the development of CD through a DQ2-independent mechanism (7). The induction of IL-15 in the CD duodenum is considered to be involved in the initial stages leading to epithelial stress, increase tight-junction permeability, enterocyte apoptosis and dendritic cell (DC) activation (5, 6, 8, 9, 10, 11, 12), facilitating the development of the secondary adaptive response (3). The innate DQ2-independent IL-15 triggering effect of gluten is elicited through a Nuclear Factor (NF)-*κ*B mechanism (13) with a positive feedback due to IL-15, also a potent NF-*κ*B activator (14). Moreover, DCs which are important players in the connexion between the innate and the subsequent adaptive immune response (15) require NF-*k*B for DC development, survival, function and cytokine production (16, 17, 18). Therefore, the IL-15/NF-*κ*B axis is revealed to have an important role in the CD and may therefore represent a molecular target for the modulation of the CD immunopathogenesis (19).

NFkB is a heterogeneous collection of dimeric proteins composed of various combinations of members of the Rel family proteins whose classical pathway is

required for activation of innate immune responses. The NF-kB members are subject to a complex regulatory mechanism (20, 21) including the inhibitor proteins I-κB, bound to NF-κB subunits which became active after dissociation. This dissociation takes place when NF-κB inducers phosphorylate I-κB, which targets the inhibitor protein for degradation by the 26S proteaseome (22) releasing NF-κB dimers to translocate and elicit its function. The I-κB phosphorylation is derived of two I-κB kinases (IKKs), as part of a larger multriprotein complex (23).

It has been recently proposed that vitamin C (ascorbate), may be able to inhibit IKK activation, which therefore inhibits I-κB phosphorylation and as a result NF-κB cannot translocate and bind to its DNA targets (24). Ascorbate has been shown to elicit its inhibitory properties at concentrations of 20mM, which occur intracelluarly *in vivo*. Moreover, at these concentrations ascorbate is not toxic to cells, neither inhibits other inducible factors, like STAT1, nor have any effect on DNA binding of NF-κB, because of the properties of its mechanism (24).

Given the central role of the NF-κB pathway in CD immunopathogenesis, ascorbate is revealed as an early immunity blocker which might be used as an alternative and/or complementary therapy to GFD on CD patients. To address this question we have performed culture of biopsies from CD patients and *in vitro* gliadin challenge with and whithout supplementation of ascorbate. The obtained data confirmed the blocking effect of ascorbate since its addition inhibited the gliadin-induced IL-15 triggering in CD biopsy explants.

MATERIALS AND METHODS

Study Subjects

We studied 8 CD patients treated on a GFD (mean age 41.7 years (yr), range 23-68 yr, 25.0% males) and 3 non-CD healthy controls (mean age 63.6 yr, range 61-68, 0.0% males). The diagnosis of CD was based on compatible symptoms, positive serology (IgA antiendomysial or antitransglutaminase antibodies), genetics (HLA-DQ2/8), and mucosal changes in the duodenal biopsy. At the time of sample collection, CD patients had mucosal recovery (Marsh 0-1) and negative serology for at least one year. Healthy controls were referred to the Gastroenterology Clinics due to other intestinal diseases which were later ruled out, and no mucosal alterations were found in the duodenum. All patients attended to the Adult Gastroenterology Clinics from the “Hospital Clínico Universitario” from Valladolid as part of the routine diagnostic procedures. Informed consent was obtained from patients, and the study protocol was approved by the Ethics Committee from both “Hospital Clínico Universitario” and Faculty of Medicine, University of Valladolid.

Biopsy Culture

Three intestinal biopsy explants were collected from each individual and cultured *in vitro* as previously described (25). Briefly, all biopsies were collected in ice-chilled PBS (Cambrex Iberia Products, Barcelona, Spain) containing 0.1% Gentamicine (Cambrex) and cultured within 1 hour in RPMI 1640 (Cambrex) supplemented with 10% heat-inactivated FBS (Cambrex), penicillin (100 U/mL), streptomycin (100 µg/ml) (Cambrex) and fungizone (0.25 µg/ml) (Cambrex). Each sample cultured in basal medium constituted an internal control. One

explant from each patient was *in vitro* challenged with a gliadin solution (100 µg/ml) (Sigma, St Louis, MO, USA) for only three hours, what is considered normal timing and concentration in the duodenum after a meal, while a second explant was co-cultured both with gliadin (100 µg/ml) and 20mM of ascorbate (Sigma, St Louis, MO, USA), a potent non-toxic NF-*κ*B inhibitor (24). All reagents were checked and discarded for lypopolisaccharide (LPS) contamination with *Lymulus amebocyte* lisate, PYROGENT ® Plus (Cambrex) (Detection limit 0.06 EU/ml). Tissue culture was carried out by immersion in culture dishes placed in a cell incubator with 5% CO_2 at 37ºC. After three hours, biopsy specimens were washed up in PBS containing 0.1% Gentamicine (Cambrex) and later cultured for another 21 hours in new clean culture medium to determine whether gliadin challenge is followed by a secondary response. After 24 hours (3 hours with stimulus + 21 hours with basal medium), tissue was embedded in RNAlater (Ambion) and snap-frozen until protein isolation extraction using the TRIZOL® reagent according to the protocol provided by the manufacturer. Supernantants were collected both at 3 and 24 hours.

Effector molecules on culture supernantants.

Biopsy culture supernatants after 3 hours of culture were assayed for the indirect evaluation of nitric oxide by applying the Griess Reaction which measures the total amount of nitrites, a primary metabolite derived from the instantaneous oxidation of nitric oxide (Molecular Probes, detection limit (D.L.) 1µM) following manufacturer´s instructions. Supernatants at 24 hours were also analyzed by using a multiplex assay (Biorad, Hercules, CA) on a Luminex TM platform (Austin, TX), following manufacturer´s instructions, for the

concentration of interferon γ (IFN-γ) [D.L. 3.38 pg/ml], tumour necrosis factor α (TNF-α) [D.L. 4.331pg/ml], IFN-α [D.L. 89.87 pg/ml], interleukin (IL)-17 [12.98 pg/ml], IL-13 [D.L. 3.49 pg/ml] and IL-6 [0.19 pg/ml].

Western blot analysis

8µg of protein isolated from whole biopsy explants were added per well. They were separated by using a 15% acrilamide/bisacrilamide (37.5:1) gel in a mini-Protean II (BioRad Laboratories Inc, USA), and later transferred onto PVDF membranes of 0,45 Micron (Pierce Biotechnology Inc. IL, USA). Membranes were incubated with primary specific antibodies to human IL-15 (mouse monoclonal MAB247, R&D) at a final dilution of 1/400, performing a second incubation with antibodies to mouse IgG labelled with horseradish peroxidase (Amersham Biosciences Europe, Freiburg, Germany). Chemiluminiscent substrate Lumigen PS-3 (Amersham) and autoradiography film Hyperfilm ECL (Amersham) were used for developing. Recombinant human IL-15 (Peprotech, London, UK) was used as a positive control.

STATISTICAL ANALYSIS

The Friedman test was applied in all cases to compare different culture conditions from the same patient (nonparametric and paired two-tailed test). The secondary Wilcoxon matched paired test between pairs of conditions was only applied in those cases where the Friedman test was statistically significant. The level of significance was fixed at $p < 0.05$.

RESULTS

Ascorbate blocks the increased nitrites secretion induced by gliadin challenge in biopsy samples from CD patients

Statistically significant differences were found in nitrite secretion after three hours of culture when explants from the same patient were compared in basal conditions and after gliadin-challenge, both with and without ascorbate supplementation ($p<0.001$). Gliadin challenged increased the secretion of nitrites, therefore indicating an increase in the secretion of Nitric Oxide, in duodenal explants from treated CD patients (Figure 1) ($p<0.05$) compared to non-challenged explants from the same patient as previously described (26, 27). The addition of ascorbate to gliadin-challenge cultures effectively blocked the induction of nitrite secretion by gliadin at a non-toxic concentration of 20mM ($p< 0.05$, Figure 1). Because ascorbate is also a strong antioxidant, and given that the Griess reaction is based on the determination of nitrites (mainly derived from the oxidation of nitric oxide), these results could reflect an experimental artifact. Therefore, to confirm whether ascorbate really blocks the immune response to gliadin, we have also studied the secretion of cytokines on culture supernantants.

Ascorbate inhibits cytokine secretion induced by gliadin challenge in biopsy samples from CD patients

When studying cytokine secretion at 24 hours (3 hours of gliadin challenge plus 21 hours of basal culture), the Friedman test was statistically significant for IFNγ ($p<0.05$), TNFα ($p<0.01$), IFNα ($p<0.05$) and IL-6 ($p<0.01$) (Figure 2). After gliadin challenge, none of the assayed cytokines was statistically increased

compared to the basal culture. However, the addition of ascorbate to culture medium clearly decreased the secretion of all the cytokines, compared to non-ascorbate supplemented gliadin-challenged supernatants (Figure 2). The decrease was statistically significant for IFNγ ($p<0.05$), TNFα ($p<0.05$), IFNα ($p<0.05$) and IL-6 ($p<0.05$). Moreover, in these cases cytokine secretion was downregulated even to lower values than those observed in basal cultures (IFNγ: $p<0.05$; TNFα: $p<0.05$; IFNα: $p<0.05$; and IL-6: $p<0.01$) (Figure 2), therefore confirming its inhibitory properties.

Ascorbate inhibits duodenal IL-15

Basal IL-15 production in biopsy explants was only detectable in one out of 8 treated-CD patients and was absent in all 3 non-CD controls (Figure3). After gliadin-challenge, as previously described (6, 28, 29), gliadin induced IL-15 production in treated-CD patients, since it was detected in 7 out of 8 cases (Figure 3). Similar results were obtained in non-CD controls, where gliadin was also revealed as an IL-15 inducer in all 3 non-CD cases compared to basal culture, as previously reported by our group (29). Interestingly, gliadin-induced IL-15 had detectable levels even 21 hours after gliadin was removed from culture medium, given that Western blot was performed at the end of the culture (3 hours of gliadin challenge and 21 hours in basal condition). As expected, the addition of ascorbate to culture medium completely inhibited IL-15 production not only in CD biopsy explants but also in those from non-CD controls. Moreover, it is noteworthy that the IL-15 inhibition by ascorbate took place even in the only treated CD-patient who had basal IL-15 production.

DISCUSSION

In this paper we have demonstrated by using a culture model of duodenal explants that the addition of ascorbate to culture medium blocks the immune response to gluten. Moreover, we have blocked the induction of nitrites (and probably reflecting an blocking of the iNOS pathway) and down-regulated the secretion of proimflammatory cytokines (IFNγ, TNFα, IFNα and IL-6) but we have also completely inhibited IL-15 production. Moreover, the IL-15 inhibition took place even a CD patient who had had IL-15 expression in the basal culture.

Although it has been largely reported that gliadin is a potent cytokine inducer in CD patients by using culture models -what is specially true in the cases of IFNγ and TNFα (4, 26)-, there was no statistically significant up-regulation of any of the assayed cytokines in our cultures (Figure 2). The explanation to this discrepancy probably resides in the experimental design, since our gliadin-challenges were only performed for 3 hours (considered to be the normal transit time of dietary antigens through the duodenum) followed by 21 extra hours in basal conditions. Thus, since the challenging time is lower that previous studies in which challenge was performed for 24 hours, it is reasonably to think that the total amount of cytokine released would be smaller than those studies, and therefore the cytokine increase would not be statistically significant.

In this study paper we have confirmed that a non toxic (20mM) supplementation of ascorbate to gliadin challenges inhibited the gliadin-induced nitrite production, probably derived from iNOS induction (30, 31), as well as secretion of IFNγ, TNFα, IFNα and IL-6. In addition, these four cytokines decreased its expression to lower values than those observed in basal cultures (Figure 2).

Moreover, ascorbate also blocked IL-15 induction, considered to be a central cytokine in CD immunopathogenesis given its unique properties of both initiating the innate immune response to gliadin (6, 28) as well as activating dendritic cells (15, 16, 32) and facilitating the development of the secondary adaptive response (3). Future studies addressing whether ascorbate is also capable of inhibiting DCs maturation in the CD duodenum and even its capacity to generate regulatory T cells (33) to gliadin should be carried out.

It has been recently shown that oral supplementation of ascorbate attenuated anaphylactic reaction to soybean glycinin-induced hipersensensitivity on a swine model (34). In humans, oral supplementation has been reported to delay the progression of transplant-associated coronary arteriosclerosis (35), the progression of cardiac transplant-associated arteriosclerosis (36), and even reduced xenobiotic-induced T-cell hyperactivation (37). Ascorbate is also capable of inhibit phytohaemagglutinin and concanavalin A mitogen-stimulated peripheral blood mononuclear cells by suppressing both formation and release of IFNγ (38). Ascorbate is also capable of inhibiting DC activation as well as its TH1 cytokine secretion and immunostimulatory capacity. Moreover, ascorbate-treated DCs were also able to generate regulatory T cells with FoxP3+ expression (33). All together, these results clearly point out the possible therapeutical use of ascorbate in diseases, like CD. However, further studies are needed before this approach would be feasible, including for instance the characterization of the optimal amount of ascorbate to be provided to the patients..

As a final remark, ascorbate is revealed as a possible future alternative therapy to the GFD. This effect should be deeper studied given the relevance of our

findings. Futures studies should confirm these results, and specifically should study the coeliac DCs immunomodulation by ascorbate, given that they are central players in the CD immune response. These obtained results would provide new insights in the possibility of a new cheap and safe alternative and/or complementary treatment in CD.

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DECLARATION OF INTERESTS

Non exist

FIGURE 1

Organ culture secretion of nitrites in 8 treated CD patients after 3 hours of gliadin challenge (100µg/ml) with (Gli+Ascorb) and without (Gli) ascorbate supplementation (20mM), compared to basal culture (all at the 3 hours). Statistically significant differences are shown ($p<0.05$, Wilcoxon matched paired test). Horizontal bars indicate median and whiskers maximum and minimum values. IQR: interquartile range.

FIGURE 2

Organ culture secretion of IFN-γ, TNF-α, IFN-α, IL-17, IL-13 and IL-6 in 8 treated CD patients after gliadin challenge (100µg/ml) with (Gli+Ascorb) and without (Gli) ascorbate supplementation (20mM) (3 hours of challenge and 21 hours of basal culture) compared to the basal culture. Statistically significant differences are shown ($p<0.05$, Wilcoxon matched paired test). Horizontal bars indicate median and whiskers maximum and minimum values. IQR: interquartile range.

FIGURE 3

Representative western-blot analysis using whole protein biopsy explants after 24 hours of basal culture (lanes 1, 4), and after 3 hours of gliadin challenge (100µg/ml) with (lanes 3, 6) and without (lanes 2, 5) ascorbate (20mM) supplementation and 21 hours of basal culture. C: human recombinant IL-15 lane.

Basal IL-15 was only detected in one CD patient. Gliadin induced IL-15 production both in non-CD controls (3 out of 3) and treated CD patients (7 out of 8). Ascorbate inhibited IL-15 production in all the cases, even in one patient who had basal detectable levels of IL-15.

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ABSTRACT

BACKGROUND: the IL-15/NF-*κ*B axis has an important role in Coeliac Disease (CD) and may represent a molecular target for its immunomodulation. Ascorbate (Vitamin C) is known to show inhibitory effects on NF-*κ*B. Therefore, we studied if ascorbate supplementation to gliadin challenges could down-regulate the mucosal immune response to gliadin in CD.

METHODS: Duodenal biopsy explants from treated CD patients were gliadin challenged *in vitro* (100 µg/ml) with and without 20mM ascorbate. An extra tissue explant in basal culture was used as internal control. Secretion levels of nitrites (3 hours), and IFN-γ, TNF-α, IFN-α, IL-17, IL-13 and IL-6 (24 hours) were measured on the supernatants. IL-15 was assayed by Western-blot on whole protein duodenal explants.

RESULTS: The addition of ascorbate to in vitro culture gliadin-challenged biopsies blocked the secretion of nitrites (p=0.013), IFNγ (p=0.0207), TNFα (p=0.0099), IFNα (p=0.0375) and IL-6 (p=0.0036) compared to samples from non-ascorbate supplemented culture. Cytokine secretion was downregulated by ascorbate even to lower values than those observed in basal cultures (IFNγ: p=0.0312; TNFα: p=0.0312; IFNα: p=0.0312; and IL-6: p=0.0078). Gliadin-challenge induced IL-15 production in biopsies from treated CD patients while the addition of ascorbate to culture medium completely inhibited IL-15 production. Moreover, the inhibition of IL-15 by ascorbate took place even in the only treated CD-patient who had basal IL-15 production.

CONCLUSIONS: Ascorbate decrease the mucosal inflammatory response to gluten in an intestinal biopsy culture model, so it might be revealed as a possible future supplementary therapy in CD.

KEYWORDS

Ascorbate. Coeliac Disease. Inhibition. IL-15. Therapy.

INTRODUCTION

Coeliac Disease (CD) is a common gastro-intestinal disorder caused by an hypersensitivity reaction to wheat gliadin and similar proteins from rye and barley, affecting genetically predisposed individuals (HLA-DQ2/DQ8). The current treatment is a life-long strict gluten-free diet (GFD) (1 ,2).

The most accepted model of the CD immunopathogenesis is the two-signal model, which establishes that gliadin has a dual effect on the CD duodenum, triggering the development of an innate immune response in the epithelium, and activating an adaptive immune response controlled by gluten-reactive T cells with a Th1 cytokine profile (3, 4). Innate immunity, and specifically interleukin (IL)-15 (5, 6), plays a key role in the development of CD through a DQ2-independent mechanism (7). The induction of IL-15 seems to be involved in the initial stages of the disease leading to epithelial stress, increase tight-junction permeability, enterocyte apoptosis and dendritic cell (DC) activation (5, 6, 8, 9, 10, 11, 12), facilitating the development of the secondary adaptive response (3). Moreover, the gliadin amplifies the production of inflammatory cytokines through the Nuclear Factor (NF)-κB (13) with a positive feedback by IL-15, which is also a potent NF-κB activator (14). Moreover, DCs are important players in the connection between the innate and the subsequent adaptive immune response (15), and require NF-κB for its development, survival, function and cytokine production (16, 17, 18). Thus, the IL-15/NF-κB axis is revealed to have an important role in the pathogenesis of CD and may represent a molecular target for strategies of immunomodulation (19).

NF-κB is a heterogeneous collection of dimeric proteins subjected to a complex regulatory mechanism (20, 21), involving the inhibitory proteins I-κB that bind to

NF-κB subunits which became active after dissociation. This takes place when NF-κB inducers promote I-κB phosphorylation mediated by two I-κB kinases (IKKs), and targets I-κB for its degradation by the 26S proteaseome (22, 23), followed by translocation of NF-κB dimmers to the nucleus and elicit their function. It has been recently proposed that ascorbate, may be able to inhibit IKK activation and, therefore, by blocking I-*κ*B phosphorylation, NF-*κ*B cannot translocate and bind to its DNA targets (24). These inhibitory properties of ascorbate can be elicited at concentrations of 20mM, intracellularly *in vivo*, without showing toxic effects to cells, or inhibiting other inducible factors.

The nitric oxid (NO) is involved in the histological changes produced in coeliac disease. In the mouse monocyte/macrophage cell line RAW 264.7, pre-challenged with IFNγ, the gliadin is able to enhance the NF-κB activity and iNOS protein expression and therefore NO production. Both effects were reduced by NF-κB activation inhibitors, thereby indicating that gliadin should modulate iNOS gen expression through NFKB activation (25).

Given the role of the NF-κB pathway in the pathogenesis of CD, we wondered whether ascorbate has an effect on the inhibition of the early/innate immune response triggered by gluten and, therefore, can be used as a supplementary therapeutical strategy to GFD on CD patients. To address this question we have cultured biopsies from treated CD patients stimulated *in vitro* with gliadin, with and whithout supplementation of ascorbate. Our data confirm that ascorbate inhibits the gliadin-induced expression of IL-15 in CD biopsy explants.

MATERIALS AND METHODS

Study Subjects

We studied 8 CD patients treated on a GFD for a minimum of 6 months (mean age 41.7 years, range 23-68 yr, 25.0% males) and 3 non-CD healthy controls (mean age 63.6 yr, range 61-68, 0.0% males). The diagnosis of CD was based on compatible symptoms, positive serology (IgA antiendomysial or antitransglutaminase antibodies), positive genetic markers (HLA-DQ2/8), and mucosal changes in the duodenum. At the time of sample collection, CD patients had mucosal recovery (Marsh 0-1) and negative serology for at least one year. Healthy controls were referred to the Gastroenterology Clinics due to other intestinal diseases which were later ruled out, and no mucosal alterations were found in the duodenum. All patients were attended to the Adult Gastroenterology Clinics from the “Hospital Clínico Universitario”, Valladolid, as part of the routine diagnostic procedures. Informed consent was obtained from patients, and the study protocol was approved by the Ethics Committee from both “Hospital Clínico Universitario” and Faculty of Medicine, University of Valladolid.

Biopsy Culture

Three intestinal biopsy explants were collected from each individual and cultured *in vitro* as previously described (26). Briefly, all biopsies were collected in ice-chilled PBS containing 0.1% Gentamicine and cultured within 1 hour in RPMI 1640 supplemented with 10% heat-inactivated FBS, Penicillin (100 U/ml), Streptomycin (100 µg/ml) and Fungizone (0.25 µg/ml) (all from Cambrex Iberia Products, Barcelona, Spain). One sample from each individual was cultured in

basal medium was used as an internal control. One explant from each patient was challenged *in vitro* with a gliadin solution (100 µg/ml) (Sigma, St Louis, MO, USA) for only three hours, which is considered normal exposure and concentration in the duodenum after a meal. A second explant was co-cultured both with gliadin (100 µg/ml) and 20mM of ascorbate (Sigma), a potent non-toxic NF-κB inhibitor (24). After three hours, biopsy explants specimens were washed up in PBS containing 0.1% Gentamicine and later cultured for another 21 hours in new clean culture medium to determine whether gliadin challenge is followed by a secondary response. Tissue culture was carried out *in vitro* by immersion in culture dishes placed in a cell incubator with 5% CO_2 at 37ºC. After 24 hours (3 hours with stimulus + 21 hours with basal medium), tissue was embedded in RNAlater (Ambion, Applied Bisystems, Austin, TX, USA) and snap-frozen until protein extraction using the TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s protocol. Supernatants were collected both at 3 and 24 hours. All reagents were checked and discarded for lypopolisaccharide (LPS) contamination with *Lymulus amebocyte* lisate, PYROGENT ® Plus (Cambrex) (Detection limit 0.06 EU/ml).

Effector molecules on culture supernantants.

Biopsy culture supernatants after 3 hours of culture were assayed for the concentration of oxidative stress by applying the Griess Reaction following manufacturer´s instructions (Molecular Probes, Invitrogen) (detection limit (D.L.) 1µM). The nitric oxid (NO) is very unstable therefore the Griess Reaction measures the total amount of nitrites, which are primary metabolites derived from the instantaneous oxidation of nitric oxide. Supernatants at 24 hours of

culture were also analyzed by using a multiplex assay on a Luminex TM platform (BioRad, Hercules, CA, USA), for the concentration of IFNγ [D.L. 3.38 pg/ml], tumour necrosis factor α (TNFα) [D.L= 4.331pg/ml], IFNα [D.L= 89.87 pg/ml], IL-17 [D.L= 12.98 pg/ml], IL-13 [D.L= 3.49 pg/ml] and IL-6 [D.= 0.19 pg/ml].

Western blot analysis

From whole biopsy explants, 8µg of protein isolated were added per well. They were separated by using a 15% acrilamide/bisacrilamide (37.5:1) gel in a mini-Protean II (BioRad), and later transferred onto PVDF membranes of 0.45 Micron (Pierce Biotechnology Inc. IL, USA). Membranes were incubated with primary specific antibodies to human IL-15 (mouse monoclonal MAB247) (R&D, Minneapolis, MN, USA) at a final dilution of 1/400, performing a second incubation with antibodies to mouse IgG labelled with horseradish peroxidase (Amersham Biosciences Europe, Freiburg, Germany). Chemiluminiscent substrate Lumigen PS-3 (Amersham) and autoradiography film Hyperfilm ECL (Amersham) were used for developing. Recombinant human IL-15 (Peprotech, London, UK) was used as a positive control.

Statistical analysis

The Friedman test was applied in all cases to compare different culture conditions from the same patient (nonparametric and paired two-tailed test). The secondary Wilcoxon matched paired test between pairs of conditions was only applied in those cases where the Friedman test was statistically significant. The level of significance was fixed at $p < 0.05$.

RESULTS

Ascorbate blocks the secretion of nitrites induced by gliadin challenge in biopsy samples from CD patients

Statistically significant differences were found in nitrites secretion after three hours of culture when explants from the same patient were compared in basal conditions and after gliadin-challenge, both with and without ascorbate supplementation ($p<0.001$). Gliadin challenge increased the secretion of nitrites, therefore indicating an increase in the production of nitric oxide (NO) in duodenal explants from treated CD patients ($p<0.05$) (Figure 1) compared to non-challenged explants from the same patient as previously described (27, 28). The addition of ascorbate, at a non-toxic concentration of 20mM, to gliadin-challenged cultures effectively blocked the induction of nitrite secretion by gliadin ($p< 0.05$) (Figure 1). Because ascorbate is also a strong antioxidant, and given that the Griess reaction is based on the determination of nitrites (mainly derived from the oxidation of NO), these results could reflect an experimental artifact. To confirm that ascorbate really blocks the immune response to gliadin, we have also studied the secretion of cytokines on culture supernantants.

Ascorbate inhibited the secretion of cytokines induced by gliadin challenge in biopsy samples from CD patients

Our findings showed that in the supernatants at 24 hours (3 hours of challenge with gliadin plus 21 hours of basal culture) none of the assayed cytokines were statistically increased compared to the basal culture. However, the addition of ascorbate to culture medium, for those 3 hours of challenge, clearly decreased the secretion of all cytokines, compared to non-ascorbate supplemented gliadin-

challenged supernatants (Figure 2). The decrease was statistically significant for IFNγ (p=0.0156), TNFα (p=0.0312), IFNα (p=0.0469) and IL-6 (p=0.0312). Moreover, in these cases cytokine secretion was downregulated even to lower values than those observed in basal cultures (IFNγ: p=0.0312; TNFα: p=0.0312; IFNα: p=0.0312; and IL-6: p=0.0078) (Figure 2), thereby confirming its inhibitory properties.

Ascorbate inhibited duodenal IL-15

Basal IL-15 production in biopsy explants was only detectable in one out of 8 treated-CD patients and was absent in all 3 non-CD controls (Figure3). After gliadin-challenge, as previously described (6, 29, 30), gliadin induced IL-15 production in treated-CD patients. In this assay it was detected in 7 out of 8 cases (Figure 3). Similar results were obtained in non-CD controls, where gliadin was also revealed as an IL-15 inducer in all 3 non-CD cases compared to basal culture, as previously reported by our group (30). Gliadin-induced IL-15 had detectable levels even 21 hours after gliadin was removed from culture medium, given that Western blot was performed at the end of the culture (3 hours of gliadin challenge and 21 hours in basal condition). As expected, the addition of ascorbate to culture medium completely inhibited IL-15 production not only by CD biopsy explants but also by those from non-CD controls. Moreover, it is noteworthy that the IL-15 inhibition by ascorbate took place even in the only treated CD-patient who had basal IL-15 production.

DISCUSSION

In this study we have shown, by using a culture model of duodenal explants, that the addition of ascorbate to culture medium decrease the secretion of inflammatory mediators in response to gluten in CD patients. Supplementation of ascorbate, of a non toxic concentration of 20mM, to gliadin challenged biopsy culture not only inhibits the gliadin-induced production of nitrites, but also down-regulates the secretion of proinflammatory cytokines (IFNγ, TNFα, IFNα and IL-6), and completely inhibits the of IL15.

Although it has been largely reported that gliadin is a potent cytokine inducer in CD patients by using culture models - especially in the cases of IFNγ and TNFα (4, 27), no statistically significant up-regulation of cytokine expression was observed in any of the culture supernatants (Figure 2). The explanation to this discrepancy probably resides in the experimental design, where the time of challenge in culture (3 hours, which is considered a normal exposure and concentration in the duodenum after a meal, followed by 21 extra hours in basal conditions) was lower than previous studies in which challenge was performed for 24 hours. In this situation, is reasonable to think that the total level of cytokine released should be smaller than previous studies.

Gliadin is capable of increasing the production of NO and this is related to the expression of iNOS. In addition, gliadin increased the binding activity of NF-κB/DNA, the degradation of IκBα and the nuclear translocation of p50 and p65 subunits (25).

With these findings we have confirmed that by using a non toxic (20mM) supplementation of ascorbate to biopsy culture challenged with gliadin, the gliadin-induced production of nitrites is inhibited. The effects of gliadin are

probably mediated by iNOS and ascorbate acts blocking its pathway (25, 31). The expression levels of the cytokines IFNγ, TNFα, IFNα and IL-6 are decreased below to those levels observed in basal cultures (Figure 2). This decrease could be the result of the inhibition of the NF-κB pathway induced by ascorbate.

Moreover, ascorbate also affects the IL-15 pathway. This property of ascorbate is very interesting because IL15 is considered to be a central cytokine in CD immunopathogenesis given its capacity to initiate the innate immune response to gliadin (6, 29) and to activate dendritic cells (15, 16, 32), therefore facilitating the development of the secondary adaptive response (3).

Ascorbate is capable of inhibiting DC activation, blocking cytokine secretion and the immunostimulatory properties. Moreover, ascorbate-treated DCs are able to generate regulatory T cells with FoxP3+ expression (33). Future studies should address whether ascorbate is also capable of inhibiting DCs maturation in the duodenum of CD patients and even its capacity to generate gliadin specific regulatory T cells (33).

It has been recently shown that oral supplementation of ascorbate attenuate several anaphylactic reactions to soybean glycinin-induced hipersensensitivity on a swine model (34). In humans, oral supplementation has been reported to delay the progression of transplant-associated coronary arteriosclerosis (35), and of cardiac transplant-associated arteriosclerosis (36), and even reduces xenobiotic-induced T-cell hyperactivation (37). Ascorbate is also capable of inhibit phytohaemagglutinin and concanavalin A mitogen-stimulated peripheral blood mononuclear cells by suppressing both formation and release of IFNγ (38). All together, these results clearly point out to the possible therapeutical

use of ascorbate in diseases, like CD. However, further studies are needed to confirm this, including double-blind placebo-controlled trails aiming to characterize not only the optimal dosis *in vivo* of ascorbate, but also the safety amount of gluten intake tolerated by patients on an ascorbate trial.

As a final remark, this property of ascorbate of modulating the immunologic response to gluten could be used as a suplement to the GFD. Considering the residual amount of gluten that some “free gluten products” still have, ascorbate could be a necessary supplement to the dietary treatment of CD. Given the relevance of our findings, this effect should further studied in order to confirm these results, and specifically to analyze how ascorbate exerts its immunomodulatory effects on DCs, given that they are central players in the CD immune response.

DECLARATION OF INTERESTS

Non exist.

FIGURE 1

Biopsy culture secretion of nitrites in 8 treated CD patients after 3 hours of gliadin challenge (100µg/ml) with (Gli+Ascorb) and without (Gli) ascorbate supplementation (20mM), compared to basal culture (all at the 3 hours). Statistically significant differences are shown ($p<0.05$, Wilcoxon matched paired test). Horizontal bars indicate median and whiskers maximum and minimum values. IQR: interquartile range.

FIGURE 2

Supernatants Biopsy culture secretion of IFN-γ, TNF-α, IFN-α, IL-17, IL-13 and IL-6 in 8 treated CD patients after gliadin challenge (100µg/ml) with (Gli+Ascorb) and without (Gli) ascorbate supplementation (20mM) (3 hours of challenge and 21 hours of basal culture) compared to the basal culture. Statistically significant differences are shown ($p<0.05$, Wilcoxon matched paired test). Horizontal bars indicate median and whiskers maximum and minimum values. IQR: interquartile range.

FIGURE 3

Representative western-blot analysis using whole protein biopsy explants of non-CD controls and treated-CD patients, after 24 hours of basal culture (Basal), and after 3 hours of gliadin challenge (100µg/ml) with (Gli+Asc) and without (Gli) ascorbate supplementation (20mM) and 21 hours of basal culture. C: human recombinant IL-15 lane.

Basal IL-15 was only detected in one CD patient. Gliadin induced IL-15 production both in non-CD controls (3 out of 3) and treated CD patients (7 out of

8). Ascorbate inhibited IL-15 production in all cases, even in a patient who had detectable basal levels of IL-15.

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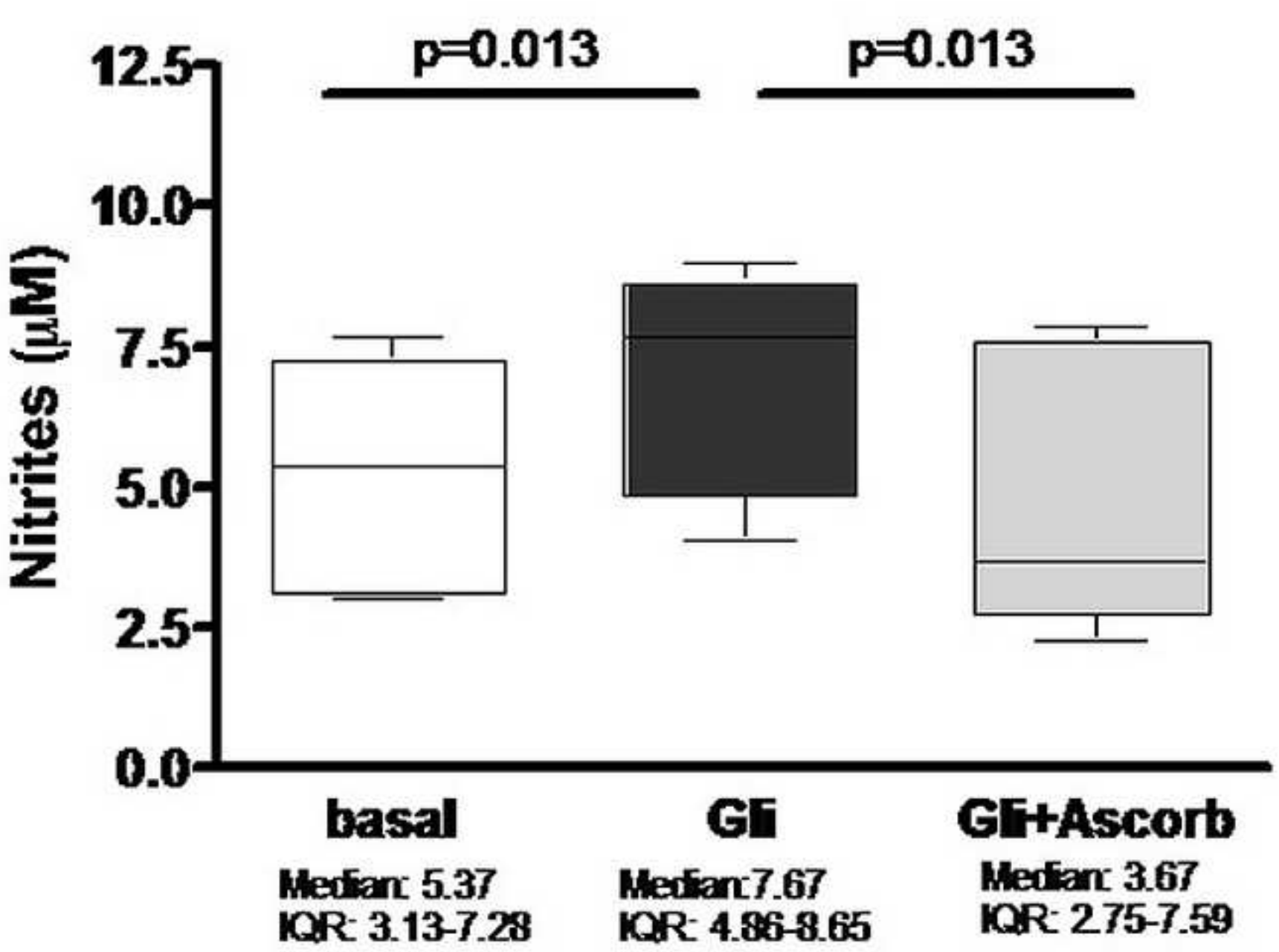

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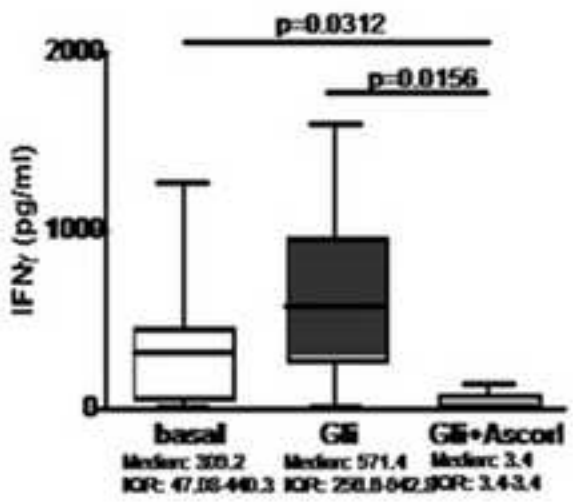

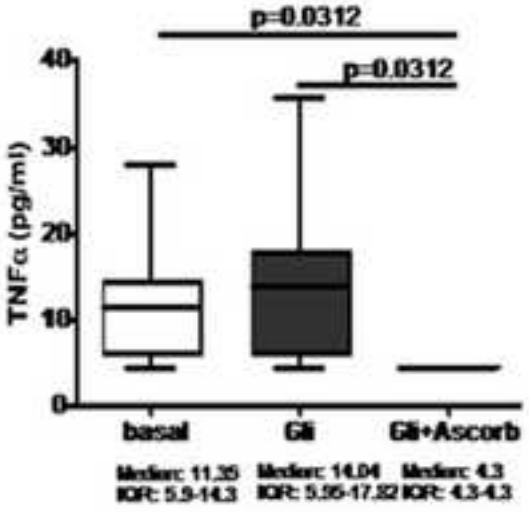

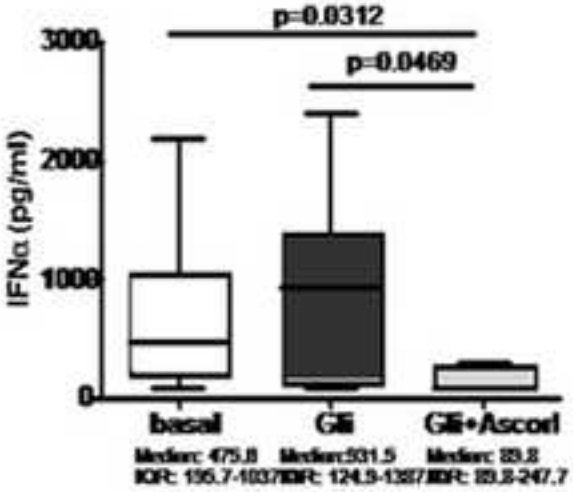

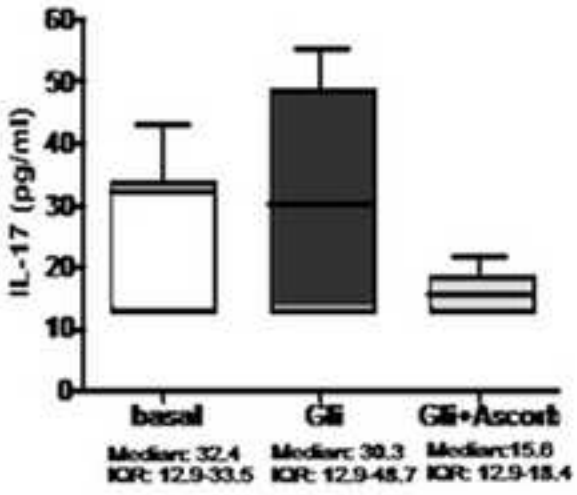

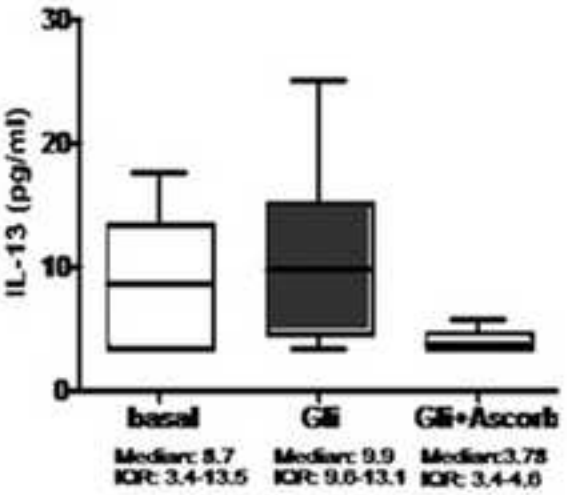

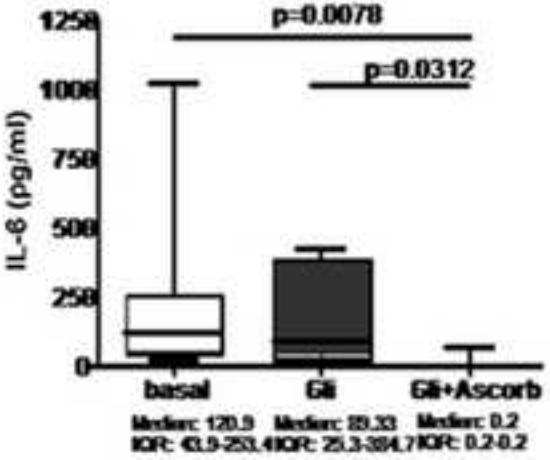

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