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DevelopmentandValidationofaGliadinInducedIntestinalEnteropathyRatModelofNon-CeliacGlutenSensitivity

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Abstarct

Ingestion of gluten-containing foods has been linked to a condition known as non-celiac gluten sensitivity (NCGS). Here, we create and verify a rat model of NCGS.

The experimental group received 0.02 M acetic acid solution while the control group received 1.5 mg/g of body weight of gliadin in acetic acid solution. It was administered intragastrically through gavage to rats beginning on postnatal day 2 and continuing for a total of six weeks, three times per week. Changes in body weight, intestinal permeability, histology, proinflammatory cytokines, and IgG antibodies against gliadin (AGA).A lactulose/mannitol solution (500/250 mg/kg respectively) was administered 24 hours before sacrifice, and urine was collected to determine intestinal permeability. Small intestines were obtained, fixed, and hematoxylin and eosin stained for histological analysis. Breast cancer resistance protein (ABCG2) and P-glycoprotein (MDR1a) uptake transporter gene expression in the intestine was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Total anti-gliadin antibodies (AGA), AGA-IgA, AGA-IgM, and pro-inflammatory cytokines were measured in the blood samples taken.

Keywords: Celiacdisease,non-Celiacglutensensitivity,enteropathy,gluten,gliadin,animalmodel

Introduction

Up to six percent of the U.S. population may suffer from non-celiac gluten sensitivity (NCGS), a reaction to eating or drinking anything containing gluten. Joint/muscle discomfort, headaches, weariness, and foggy thinking are only some of the systemic symptoms experienced by people with NCGS [1, 2]. Mass loss, sickness, inflammation, and other illnesses may also coexist [5, 6]. When gluten is removed from the diet, these symptoms lessen, but they return when gluten is reintroduced. Increases in immunoglobulin A (IgA) and/or G (IgG) and positive anti-gliadin (AGA) or anti-deamidatedgliadin peptide (anti-DGP) antibodies have also been found in individuals with NCGS [3, 4].

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Several experimental models of investigation have been presented [7–11] to investigate the pathophysiology of gluten sensitivity. Intragastricgliadin injection in mice has been used to examine gluten intolerance and its effects on the gastrointestinal tract's structure [9]. The jejunal mucosa may not alter or the outcomes may be less than ideal [9, 12]. The clinical alterations seen in gluten-sensitive non-celiac individuals need the development and validation of a model of intestinal enteropathy. Studies in this area are necessary because of the prevalence of illnesses associated with gluten hypersensitivity and the severity of their negative health consequences.

Drug pharmacokinetics may also be affected by the gastrointestinal abnormalities seen in this condition, thus treating these is a priority [13, 14]. Dosage modifications for medications with a restricted therapeutic window may also be made possible by pharmacokinetics research in NCGS and gluten-sensitive individuals. Therefore, it is reasonable to assume that drug disposition is affected by the presence of gluten hypersensitive intestinal enteropathy in a validated rat model of local and systemic inflammation due to changes in intestinal drug metabolizing enzymes, transporters, and tight junctions. By comparing the pharmacokinetic properties of medications administered to rats with gliadin-induced enteropathy with normal rats, this model might be useful. Intestinal drug metabolizing enzyme (DME) substrates, OATP drug transporter substrates, and P-glycoprotein (P-gp) substrates may result in a different pharmacokinetic profile in mutant than in normal rats.

Changes in drug disposal as a result of pathological changes in enterocytes and knowledge of therapeutic dose-response relationships and drug-drug interactions. To evaluate the pharmacokinetics of various medicines, the research aimed to create and verify a rat model that displays pathologic alterations in the intestinal mucosa (villus atrophy and inflammation).

2. MaterialandMethods

2.1 Animal ExperimentalDesign

In this work, we used eight pregnant Wistar female rats (Charles River Labs, Cambridge, MA) and their offspring. When female rats had reached their last stages of pregnancy, they were acclimated to our vivarium. About 10-12 pups were born to each mother. Young rats were kept in the same environment as their mothers and were handled everyday for brief periods of time (1-2 minutes). Cages with vented cage tops were employed so that food dust wouldn't spread from one researcher to the next. The research was not impacted by the use of corn cob bedding since it was wheat gluten-free. The rat mothers and their young were provided with enrichment items such as plastic bones and elbow chambers for crawling and hiding. Every 3 days, the weight of each puppy in both groups was recorded.

The mothers were slaughtered by carbon dioxide asphyxiation until they stopped breathing, followed by cardiac exsanguination, on day 21 (when the pups were no longer reliant on their mothers for food). Both litters were separated into a control and gliadin- fed group at the outset. Six weeks was the cutoff for all the groups. Both groups of pups were fed a solution of gliadin (10% in 0.02 M acetic acid, pH=3.22), but the control group also received a blank vehicle (0.02M acetic acid, pH=3.22).

A stainless steel curved feeding needle (24-22 GA/1.5 in; Kent Scientific Corporation, Torrington, CT) was used to intragastrically give doses of the solution (1.5mg/g) every 3 days till end point.

One day before to the assessment, rats in both groups were given a lactulose/mannitol solution. Each rat received an intragastric dosage of 1 mL, which was equal to 500:250 mg lactulose/mannitol/kg of body weight. After fasting for 24 hours, the rats were put in separate metabolic cages so that their urine could be

collected to determine the intestinal permeability to lactulose (7, 15). Each rat's urine lactulose to mannitol ratio was determined.

All rats were checked on regularly (three times a week) to ensure their well-being during the trial. We tracked their weight and any fluctuations in it, their hydration levels, the presence of gastrointestinal symptoms (like diarrhea), the color of their mucus membranes, and their overall body condition by palpating their lumbar spine and hips to determine a body condition score. All of the rats were given individual ratings based on the descriptions and illustrations provided by the Biopharmaceutics Classification System (BCS) (16).

Rats were given 3-4% isoflurane to put them to sleep on day 46, and their intestines were removed. Each rat had a thoracotomy performed to obtain blood samples by puncturing the heart. Rats were killed by cervical dislocation while under heavy anesthesia, after which their blood and tissues were harvested.

2.2 Histological Analysis

Excisedintestinaltissuesectionswerelabeledaccordingtotheregion(duodenum/jejunum)andthen fixed in Bouin's solution(Sigma-AldrichCo.LLC). Tissues were embedded in paraffin cassettesand then thin sections were obtained and stained withhematoxylin–eosin.Tissuesectionslideswereanalyzed under the microscope. Histological analysiswas performed atthe RodentHistopathologyCorefacilityatHarvardMedical School,Boston,MA.

2.3 Lactulose/Mannitol Permeability Assessment

Thedaybeforetheirendpointeachratwasplacedindividuallyinametaboliccagethatseparateditsurinefromfeces.Urinesampleswerecollectedandthevolumewasmeasured.Then,allurinesampleswerestoredat-80°Cuntilthetimeofanalysis.Samples were prepared by adding CarrezClarificationReagent(BioVisionInc.,Milipitas,CA)toprecipitateallproteincontaminantsintheurine.Thesupernatantwascollectedthencentrifugedat12,000×gfor5min.Becausetheconcentrationsoflactuloseandmannitolwereunknown,differentvolumesofurinepersampleforallsampleswereretested.Theratiooftheconcentrationoflactulosetomannitolforeachwasdeterminedandcomparedbetweenbothgroups.

D-MannitolColorimetricAssay

SampleswereanalyzedusingaD-MannitolColorimetric Assay Kit (BioVision). All preparationswereaddedtoa96-wellplateandreadonaspectrophotometer at absorbance of 450 nm, which isthedetectionwavelengthofmannitol.

LactuloseFluorometricAssay

Samples were analyzed using a PicoProbe LactuloseFluorometric Assay Kit (BioVision). All preparationswere added to a 96-well plate and read on a platereader with fluorescence detection capability at thefluorescencemeasureofEx/Em=535/587nm.

2.3 Plasma analyses of AGA, IgA, IgM and pro-inflammatorycytokines

Anti-gliadinantibodies(AGA),ImmunoglobulinA(IgA) and Immunoglobulin M (IgM) by ELISA kits(MyBioSource,SanDiego,CA)wereusedtodetermine the change and presence of these markersbetween both animal groups using plasma samples.Moreover,quantikineELISAkits(R&DSystem)were used to measure the plasma concentrations ofinterferongamma(IFN-γ), Tumornecrosisfactoralpha(TNF-α),interleukin-1beta(IL-1β)andinterleukin-6(IL-6),inbothanimalgroups,inaccordance with manufacturer'srecommendations.

2.4 Geneexpression

Jejunaltissuesectionsfromallanimalsinbothgroups were stored at -80 °C then, were prepared formRNA isolation as follows: 50-100 mg of tissue washomogenizedwith1mLTRIzolreagent(ThermoFisher Scientific, Waltham, MA), then homogenizedmechanicallyunderdryice.Chloroform(Sigma-Aldrich Co. LLC) was added in volumes of 200 uL toeach sample. The mixture was then centrifuged for 15minat 12,000 × g at 4 °C.Uponseparationtheaqueous phase was removed to a new vial and 500 uLof100%isopropyl(Sigma-AldrichCo.LLC)wasaddedandthencentrifugedundertheprevioustcondition. This process resulted in a pellet. The pelletwaswashedwith1mLof75%ethanol(Sigma-AldrichCo.LLC) and thencentrifugedfor5min7500×gat4°C.Thepelletwasthenallowedtodryandthen50uL of triethanolamine buffersolution(Sigma-Aldrich Co. LLC) was added and mixed well.TheRNAconcentrationsweredeterminedwithaN

anoDropND-2000spectrophotometer(NanoDropTechnologies,Wilmington,DE).Allthesampleswere diluted to a concentration of 133 ng/uL of RNAandthenfurtherused forcDNApreparation.

ThecDNAwassynthesizedusingtheprepareddiluted samples of mRNA along with the transcript ofFirstStrandcDNASynthesiskit(RocheAppliedScience, Indianapolis, IN). Samples were then placedintheSimpliAmpThermalCycler(AppliedBiosystemsInc.,FosterCity, CA)foramplification.

Then,sixprobeswereanalyzedformRNAexpression levels in all samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as ahousekeeping gene to normalize for the expression ofotherprobes.Quantitativereverse-transcriptasepolymerase chain reaction was performed using theAppliedBiosystemsStepOnePlusReal-TimePCRSystemandTaqManFastAdvancedMasterMix (LifeTechnologies).Allprobeswere6-carboxyfluorescein (6-FAM) labeled. The primers ofthe genes measured in this study are listed in Table 1.The relative expression of each gene was analyzedusingacyclethreshold(Ct)by the $2^{-\Delta\Delta Ct}$ method[17].

Gene name	Forward (5' to 3')	Reversed (5' to 3')
GAPDH	5′-CCA TCA CCA TCT TCC AGG AG-3′	5′-CCT GCT TCA CCA CCT TCT TG-3′
CYP3A9	5′-GGA CGA TTC TTG CTT ACA GG-3′	5′-ATG CTG GTG GGC TTG CCT TC-3′
CYP3A18	5′-TCC TGT CTC CAA CCT TCA CC-3′	5′-CAC TCG GTT CTT CTG GTT TG-3′
MDR1/Pg-p	5′-GAT GGA ATT GAT AAT GTG GAC A-3′	5′-AAG GAT CAG GAA CAA TAA A-3′
ABCG2	5′-CCA CTG GAA TGC AAA ATA GAG-3′	5′-CCT CAT AGG TAG TAA GTC AGA CAC A-3′

Table1: Theprimersofthegenesmeasuredinthisstudy.

2.5 Statistical Analysis

Data were presented as the mean ± standard deviation(S.D)foreachvariablestudied.Statisticalsignificance comparing two groups was assessed byStudent's t test. All analyses were performed usingGraphPad Prism 7 software (GraphPad Software, LaJolla,CA);apvalueof<0.05wasconsideredstatisticallysignificant.

2.6 Bodyweightassessment

Animal weight gain was analyzed between gliadin-fed and control groups from day 1 until the endpoint(Figure 1). On day 34 gliadin-fed rats started to showstatisticallysignificantlessweightgainthanthecontrol ($p<0.05$).

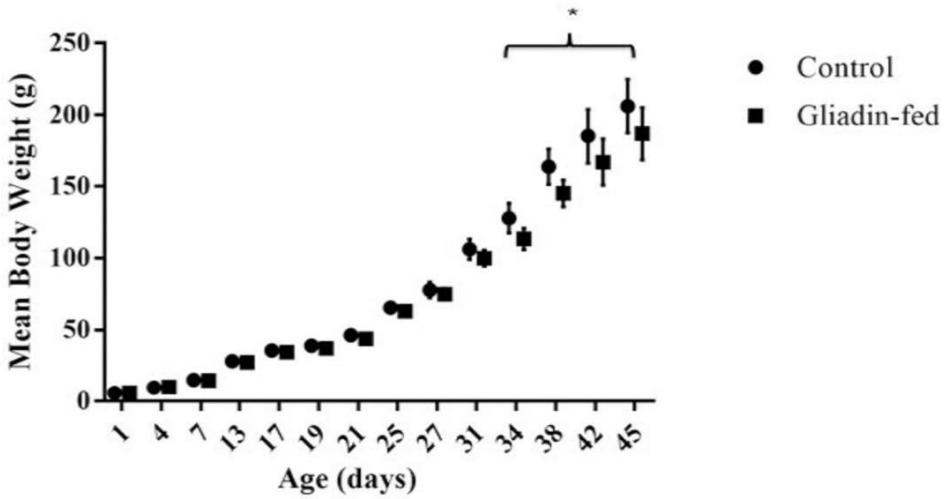

Figure 1: Weight Gain Chart for Control and Gliadin-Fed Groups Days 1-46 (p= 0.0006, p=0.0003, p=0.0184, andp=0.0190,respectively).Datarepresentthemean±standarddeviation(S.D),n=6ratspergroup.

2.7 Histological Analysis

The difference in villus height between gliadin-fed rats and the control in two different intestinal regions, duodenumand jejunum at six weeks is shown in Figure 2A. Intestinal sections from both regions resembled mild inflammationcharacterized by infiltrates of immune cells in the lamina propria, edema at the tips of villi and a few macrophagesandneutrophilsinthelumenofthegutasshowninFigure2B.

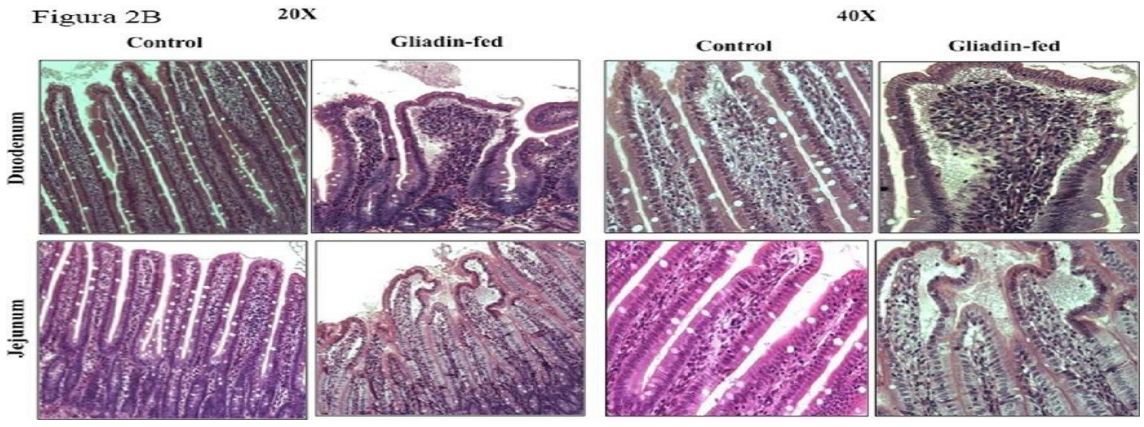

Figure 2: Imageofintestinalsectionsofduodenumandjejunuminhandestaining10X(Figure2A)and20Xand

40X (Figure2B)betweenControlandGliadin-fed Groups atDay46.

3. Discussion

In this study, we used an oral gavage injection of a crude gliadin preparation to create an intestinal environment in newborn rats that mirrored the pathophysiology of celiac disease (CD). Several previously-reported-successful models [7–12] served as inspiration for our own revised model. However, our approach was developed with the stated goal of evaluating pharmacological profiles in terms of their pharmacokinetics. In-depth validation methods were also employed to confirm the accuracy of this model for determining medication elimination rates.

Keeping an eye on the rats' weight was important because of the signs of gluten intolerance. Although the gliadin-fed group had no outward symptoms of diarrhea, their collected feces were much more watery and lighter in color than those of the control group. Gliadin-fed rats' total body weight increase significantly slowed beginning on day 34. The malnutrition that often accompanies these diseases may be to blame. Similar decreases in mouse body mass were seen in another work that attempted to replicate the intestinal abnormalities of gluten-intolerant creatures [9].

Gut alterations in villi morphology are diagnostic of both CD and non-celiac gluten sensitivity [5, 9]. Mild inflammation characterized by infiltrates of immune cells in the lamina propria, edema at the tips of villi, a few macrophages and neutrophils in the lumen of the gut, and the very tips of some villi have granular material in the edema were all observed in the intestine images of the gliadin-fed group in our study. The large dosage of consumed gliadin predicted these findings. Similar levels of villous shortening and moderate inflammation have been observed by those who are celiac and those who are not. The inflammatory properties of the gluten moiety that triggers a cascade of localized events changing the enterocyte were confirmed by our validation research.

In addition, we found that the proportion of lactulose excreted in the gliadin-fed group was significantly higher than in the control group. On the other hand, mannitol is absorbed transcellularly, although this absorption may be impaired by a decline in the number and activity of intestinal epithelial cells available for transcellular transport [15, 19]. Rats given gliadin had lower mannitol excretion rates, but the difference was not statistically significant. It was shown that in the same group, the ratio of lactulose to mannitol was much greater to make up for the reduction in surface area.

Genetic variables linked to CD are a key differentiator in the immunological mechanisms at play in gluten-related illnesses. HLA-DQ2/DQ8 genes are required for the adaptive

T-cells are involved in the stimulation of the immune system and the generation of gliadin-specific IgA antibodies [20, 21]. It's the humoral immune response and the T-cell-independent adaptive immunological response that play a larger role in non-celiac gluten sensitivity than they do in celiac disease. This is primarily because the genetic variables linked to CD are not required for the enzymatic activity of TG2. People with NCGS have an immune response to native gliadin, and those antibodies tend to be IgG and IgM [22]. In our model, there was no difference between the two groups when gliadin IgA antibodies were measured. Due to the absence of essential genetic variables, we predicted this outcome. Gliadin-fed rats, however, revealed a substantial 3-fold rise in non-specific gliadin antibodies. In addition, the latter group had considerably greater levels of gliadinIgM antibodies. These results are consistent with the immune responses seen in NCGS [5, 21].

The plasma levels of IFN-, TNF-, and IL-6 were found to be significantly elevated in gliadin-treated rats, all of which are pro-inflammatory cytokines. Therefore, various issues connected to gliadin treatment for 45 days may be attributable to the model's elevated cytokine production. Studies mimicking autoimmune illness in rodents have been done to examine the effects of cereal grain consumption in this setting. According to one of these investigations, increasing intestinal permeability in diabetic-prone BioBreeding (BBdp) rats caused by giving them a cereal-based diet. In addition, there was a substantial increase in IFN- - in the digestive tract [23, 24]. Lammers et al. also showed that gliadin generated an inflammatory response in both CD patients and healthy controls, therefore their findings are comparable.

The levels by which they are altered in such diseases need to be investigated because of the lack of a sufficient understanding of the role of their levels, the regional abundance and actions of drug metabolizing enzymes, and the interplay of intestinal drug metabolizing enzymes and transporter proteins [26–28]. Researchers looked at the number of enzymes and transporters in the colon that are responsible for drug metabolism since they may have a major effect on drug bioavailability when taken orally. There are many isoforms of CYP3A found in rodents, including CYP3A9/18 and CYP3A62. Significant reductions of 42 and 39 percentage points were seen in the mRNA expression of CYP3A enzymes. The proteins MDR1a and ABCG2 were among those studied because of their roles as drug transporters. Rats given gliadin had significantly lower mRNA expression of these proteins, by 42% and 37%, respectively. This attenuation was predicted on the basis of pathological flaws discovered in the enterocytes of gliadin-fed rats, which were the consequence of gliadin-induced local inflammation that altered the shape of the villi. These results provided critical confirmation of the intended outcome of this rat model study.

4. Conclusions

Finding a treatment for Celiac disease and distinguishing non-celiac gluten sensitivity have lately been hotly debated scientific topics.

This discussion has to include the apparent gap in knowledge around medication disposition characteristics in these disorders. By creating an appropriately equipped rat model,

In order to validate this hypothesis, pharmacokinetic studies may be conducted to learn more about the drug and its effects on the body. This study was a helpful stepping stone on the road to achieving that objective. The effects of gliadin on the rat digestive tract were better understood. The next phase of this experiment, evaluating drug disposition, is close at hand, and it promises to provide positive findings.

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