The Laryngoscope

A Controlled Safety Study of Diindolylmethane in the Immature Rat Model

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Objectives/Hypothesis: Diindolylmethane (DIM), a natural product from cruciferous vegetables, has been shown to be a dietary component that has inhibitory effects on some tumors (e.g., laryngeal papilloma). However, current evidence to support its safety is based on adult humans or mature animals. There is little to show on its safety in children. This study is designed to assess safety in the young rat model.

Study Design: Prospective controlled animal study.

Methods: Forty rats were separated into four treatment groups of 10 rats each, based on the amount of study drug they received in their daily food: 1) immature rats fed a low dose of DIM, (0.6 mg/kg/day); 2) immature rats fed a high dose of DIM (6.0 mg/kg/day); 3) immature rats fed no DIM (control); and 4) adult rats fed a high dose of DIM (6.0 mg/kg/day). At the conclusion of the study we collected blood to compare serum chemistries and vitamin D levels, and harvested organs to observe for any gross or histological changes among the groups. Statistical methods involved one-way analysis of variance and pairwise comparisons with Tukey multiple comparison adjustment.

Results: Although our numbers do not allow for statistical significance, there was no appreciable difference in rat weights among the immature groups, nor was there appreciable difference in serum chemistries, or gross or histological examination of liver, kidney, and bone.

Conclusions: Diindolylmethane seems to have no adverse affects on the rat even when given in doses 3× what we propose to be therapeutic. This adds evidence to the safety of this drug in the pediatric population as a treatment option for recurrent respiratory papilloma.

Key Words: Diindolylmethane, safety, sex immature, rat.

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INTRODUCTION

Cancer chemoprevention therapy is a promising approach for fighting tumors. Unfortunately, currently used agents have significant side effects. It is a well-accepted fact that many human cancers could be prevented or reduced by changing one's lifestyle, including dietary modification. A natural product from cruciferous vegetables, 3,3′-diindolylmethane (DIM) has been shown to be a dietary component that has such inhibitory effects on some cancers and recurrent respiratory papilloma (RRP). However, current evidence to support its safety is mostly based on adult human or mature animal studies. There is minimal evidence in the literature to show its safety in children, in whom this disease process can be the most devastating. In this study, we conducted a safety feasibility experiment in sexually immature rats by collecting blood and tissue samples to screen for possible side effects. Our goal in this study is to determine if oral DIM is a safe drug for young rats, and if DIM poses a greater risk in immature rats when compared to mature ones. By accomplishing this, we will be able to fill a gap in the literature regarding the safety of this drug in this patient population.

MATERIALS AND METHODS

Chemicals

DIM powder was obtained from BioResponse (BioResponse Nutrients, LLC, Boulder, CO). Independent laboratory analysis by Eurofins (Eurofins Scientific Inc, Petaluma, CA) via high-performance liquid chromatography confirmed pure DIM content.

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Animals

Approval for animal use in research was obtained from our animal oversight committee, the Institutional Animal Care and Use Committee (IACUC). Forty Sprague-Dawley rats were purchased from Charles River Inc. (Boston, MA). Thirty rats were sexually immature at 21 days old, and 10 were mature at 60 days old. The animal housing facility was the Center for Advanced Biomedical Research (W-Bldg) at Boston University School of Medicine. To track individual rats within a cage, a rat in each cage had a small piece of its ear clipped.

Design

Rats were separated into four groups based on age and DIM concentration in their food. All rats underwent a 24-hour acclimation period prior to the start of the study. Immature rats were divided into three different treatment groups of 10 each: 1) low-dose DIM (0.6 mg/kg/day); 2) high-dose DIM (6.0 mg/kg/day); and 3) no DIM as a control. The adult group was given a high-dose regimen to serve as a control to the immature group in the event that there were biochemical/histological findings in the young groups. There were five male and five female rats in each of these four groups. The rats were housed two or three per cage (same sex in each cage), and maintained at 12-hour light/dark cycle. Tap water was available ad lib throughout the study, and the food was given after calculating the amount of required food per gram of rat (see Study Food Calculations section).

Study Food Calculations

Prior to initiation of the study, we calculated the feeding requirements of both adult and immature rats. We measured how much food was consumed by each cage per day, over 3 days, then divided that by the total rat weight for the cage. This provided a reasonable estimate of how much was consumed per gram of rat. We calculated that the rats required 15% of their bodyweight/day (including wastage).

Study Food Creation

We decided to put all of the study drug into one fifth of each day's food requirement, giving the other four fifths as normal preformed pellet food. We incorporated DIM with meal mix, 18% protein rodent diet from Harlan Teklad Global diets (Crossett, AR), in ratios that would give us the per gram requirement. The soft meal mix was then formed into pellets by using a standard cake funnel and allowing it to dry via a circulating fan away from direct sunlight. In this manner we were able to ensure that the low- and high-dose groups would consume 0.6 and 6.0 mg/kg/day, respectively.

Study Food Administration

Since drug administration is weight based, at the initiation of the study we weighed all the rats in order to administer the correct amount of study drug. This ensured that all food was consumed, allowing us to achieve a reasonable daily concentration of DIM in the rats. Rats were reweighed every 2 to 3 days, and the new food requirements were recalculated. Enough food was left in the cages to last until the next weigh/feeding day.

Blood Collection and Analysis

Two test rats from group 2, and two from group 3 had blood samples collected via the tail vein on day 7, after fasting overnight. This was done as a dry run to ensure that we were able to collect samples and to ensure the reliability of our laboratory in performing the required analysis. On day 32, all 40 rats were euthanized, after a 24-hour fast, via inhalation of CO_2, and blood samples were collected. From each blood sample 0.5 cm^3 serum was collected, and then sent to the Veterinary Medical Center of Michigan State University (Lansing, MI) for comprehensive chemistry profiles and endocrinology testing.

Bone Density Analysis

After euthanasia, the knee joint was removed and embedded in glycol methacrylate. The samples were sectioned on a rotary microtome withhematoxylin-and-eosin (H&E) staining for microscopic observations.

Histopathology

Gross observation was conducted for liver and kidneys under a microscope in order to identify any variances. The tissue samples from these organs were then processed as per routine procedures and H&E staining in histopathology for microscopic examinations.

Statistical Analysis

The six subgroups (four treatment groups and two different sexes) were analyzed separately, and then compared to each other to determine any significant difference. The data were compared using one-way analysis of variance (ANOVA) and all pairwise comparisons with Tukey multiple comparison adjustment. When outliers or other nonnormality was indicated, simple transformation (e.g., logarithmic) was examined.

RESULTS

General

All 40 rats exhibited normal growth and development throughout the 40-day study period, as evidenced by equivalent growth among the groups (Fig. 1). There were no visible signs of distress or conflict during the entire study period. All rats tolerated the study protocol.

Blood Chemistries

Initially, we conducted chemistry tests with a comprehensive metabolic profile (total of 30 items tested) in a total of eight serum samples collected at day 7 from four rats in groups 2 (high DIM) and 3 (no DIM). There was no statistical difference between the two groups ($n = 8$, $P > .1$).

On day 32, we collected serum samples from all 40 rats, after a 24-hour fast, immediately after they were euthanized. We repeated the same chemistry tests and statistical analysis on these 40 samples (Table I). Differences in means for continuous variables (untransformed and log-transformed to adjust for nonnormality) across the four groups were assessed using ANOVA, and Fisher exact tests were used to compare the distribution of the categorical variables across the four treatment groups. A hierarchical testing approach was implemented. We first looked at overall difference among the four groups, and if the difference was significant at 0.05 level, we then proceeded with analysis of the overall difference within young animals, and the difference between young and adult animals. If the overall difference within young

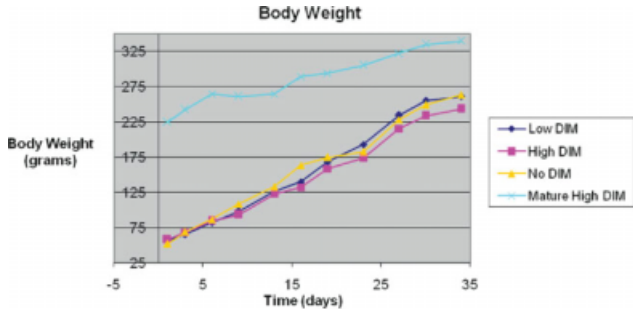

Fig. 1. Rat weights during study. DIM = diindolylmethane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

animals was significant, we tested the difference in high-dose DIM group versus control and low-dose DIM group versus control (Table I).

The analysis yielded significant overall differences in both untransformed and log-transformed values of phosphorus ($P = .008$), total protein ($P = .008$), globulin ($P = .01$), and total cholesterol ($P = .04$); and in log-transformed only alkaline phosphatase (ALP) ($P = .02$), aspartate aminotransferase (AST) ($P = .01$), and creatine kinase (CK) ($P = .006$). All observed differences were due to the difference between young and adult animals. All overall difference tests within young animals were not significant (Table I). The analysis detected no overall significant difference in urea nitrogen, creatinine, sodium, potassium, chloride, Na/K ratio, total CO_2, anion gap, osmolarity calculation, total calcium, magnesium, iron, albumin, glucose, amylase, total bilirubin, direct bilirubin, indirect bilirubin, iditol dehydrogenase (ID), alanine aminotransferase (ALT), hemolysis, chemistry icterus, and chemistry lipemia.

TABLE I.
Chemistry Test Results (n = 40) With 30 Test Items on Day 32.

	Chemistry Tests and Results (*P* Value)				
		Young			
Tests	Overall	Overall	H vs. C	L vs. C	Adult vs. Young
Urea nitrogen					<.05
Creatinine					<.05
Na+					
K+					
Cl−					
NaK ratio					
TCO_2					
Anion gap					
Osmolarity calculation					
Ca+ total					
Phosphorus	<.001				<.001
Magnesium					
Iron					<.05
Protein total	<.001				<.001
Albumin					<.05
Globulin	<.05				<.001
Glucose					
Amylase					
ALP	<.05				<.001
Total bilirubin					<.05
Direct bilirubin					
Indirect bilirubin					
ID					
ALT					
AST	<.05				<.001
CK	<.001				<.001
Total cholesterol					<.05
Hemolysis					
Chemistry icterus					
Chemistry lipemia					

All unmarked items and tests $P > .05$.
H vs. C = high-dose DIM vs. control; L vs. C = low-dose DIM vs. control; TCO_2= total CO_2; ALP = alkaline phosphatase; ID = iditol dehydrogenase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; CK = creatine kinase.

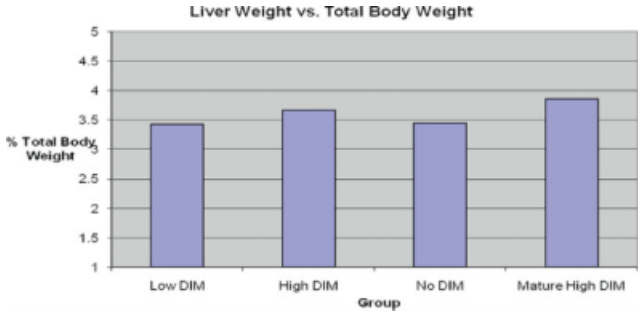

Fig. 2. Liver somatic index (LSI) at day 32. DIM = diindolylmethane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

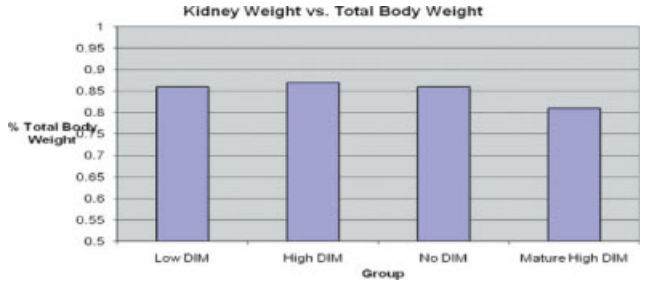

Fig. 3. Kidney somatic index at day 32. DIM = diindolylmethane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Body and Organ Weights

There were no significant differences in diet consumption or weight gain among the three young rat groups over the 32-day period (n = 40, $P > .1$, Fig. 1). There were also no treatement-related effects on the liver and ratio of body and liver weight (liver somatic index, [LSI]), in either sex ($P > 0.5$, Fig. 2). The same was the true on the kidney and the ratio of body and kidney weight (kidney somatic index, [KSI]), (n = 40, $P > .1$, Fig. 3).

Gross and Microscopic Pathology

No significant gross differences among these four groups of either sex at day 32 was noted upon necropsy (Fig. 4) or following histopathology. Most notably, no effects were seen in DIM-metabolized organs such as the kidney and liver. All of the tissues and organs showed a normal structure and appearance. Neither toxicity nor other abnormality was indicated in either low- or high-DIM dose groups, as compared to the controls.

Bone Density

Histomorphometric analysis of decalcified cancellous and cortical bone from the DIM-treated young rats showed normal structures and appearance. Our microscopic results indicated that, in comparison to the control group, the DIM diets were not associated with any additional adverse effects on bone density, structure, or turnover. Additionally, we measured 25-hydroxyvitamin D levels of four rats from the four groups on day 32 to assess for any other clues that new bone creation and resorption was affected. We did not discover any major difference among these four groups ($P > .1$, Table II).

DISCUSSION

RRP is a noncurable resilient disease that requires vigilant surveillance and treatment. The only universally accepted method of managing RRP is surgical resection when airway or voice morbidities necessitate intervention. Cold knife, microdebridement, and the ablative carbon dioxide (CO_2) laser have long been accepted modalities. Unfortunately, there is the unwanted side effect of scar tissue formation due to the multitude of surgeries required in this population. More recently, angiolytic laser therapy via the 532-nm potassium titanyl phosphate[1] and pulsed dye laser[2] have been investigated as methods to manage papilloma by destroying its blood supply, which also confers the benefit of decreasing the resultant scarring that develops.

Medical treatments continue to be explored with hopes of discovering ways to prolong the intervals between surgeries, which are often inevitable. Hormonal

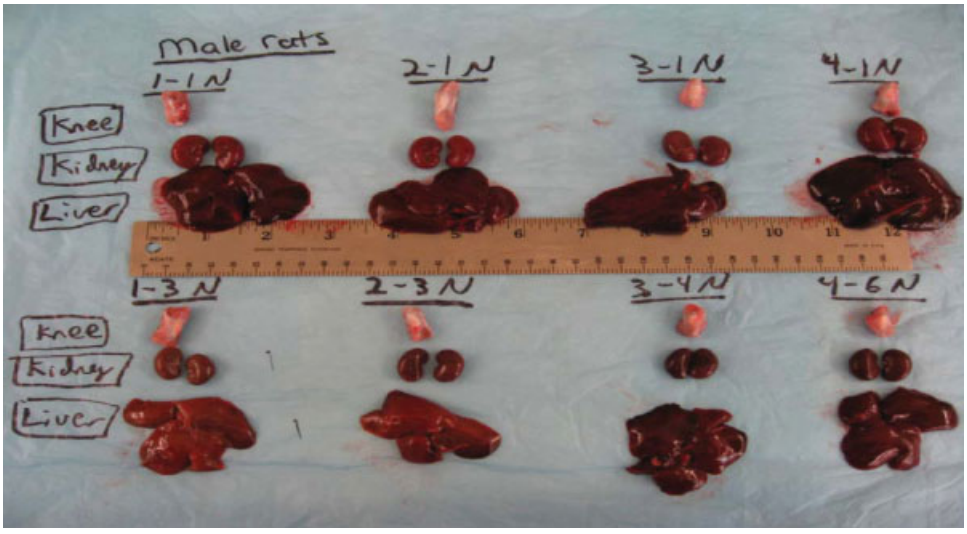

Fig. 4. Gross examination of organs top to bottom: knee, kidney, and liver. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II.
Results of 25-Hydroxyvitamin D (n = 16) on Day 32.

		25-Hydroxyvitamin D, ng/nmol/L			
Rat	Sex	Group 1	Group 2	Group 3	Group 4
2R	Male	29	40	30	49
2N	Male	30	33	31	38
4R	Female	46	41	38	42
4N	Female	35	36	48	32
Mean		35	37.5	36.75	40.25

therapies include DIM and indole-3-carbinol (I3C), which are aimed at altering the effects of estrogens on papillomas, and no serious side affects have been noted to date. Antiviral drugs (e.g., cidofovir) are also at times used to directly target the viral burden of human papillomavirus at sites of papilloma growth, although currently no common serious side affects are noted.[3] Another studied method is by alteration of the patients immune system with interferon alpha; this method has been reserved for severe cases and has also been connected with neurological and developmental abnormalities in children. It is important to understand that preventative measures attack the different causative factors of RRP, and therefore are not mutually exclusive. On the contrary, a multidimensional medical and surgical regimen may provide the best results in these patients.

DIM has been widely studied for its anticarcinogenic properties. DIM and its precursor I3C are naturally occurring plant alkaloids formed by the hydrolysis of indole glucosinolate, which is found mainly in cruciferous vegetables such as broccoli and Brussels sprouts.[4] Some past studies have focused on I3C as the active agent, but new evidence leans towards DIM as the true active compound responsible for the antitumor activities. This evidence, coupled with reports of toxicity associated with oral use of I3C, which relates to unfavorable enzyme induction, are pointing toward DIM as the agent of choice.[5]

Although the exact manner in which DIM may produce antitumor effects has not been defined, numerous studies have suggested specific mechanisms as to how DIM may be beneficial for specific tumors, most notably breast and prostate.[6,7] A recent study by Chinnakannu et al.[8] demonstrated that DIM inhibits the progression of synchronized prostate cancer cells from G(1) to S phase by induction of p27(Kip1) and downregulation of the androgen receptor pathway, and that it inhibits proteasome activity in S phase, leading to apoptosis in certain cells. Similar activity has been noted in breast tissue by Howells et al.[9] S-phase retardation and mitotic delay has also been linked to topoisomerase II alpha-catalyzed adenosine 5′-triphosphate hydrolysis by DIM.[10] Another study by Denger et al. suggests that since it is known that DIM is an antagonist of the aryl hydrocarbon receptor (AhR), this modulation may be beneficial because the activation of cyclooxygenase-2 expression by AhR ligands may contribute to inflammation and tumorigenesis.[11] New reports are starting to show that DIM may also be beneficial in certain lung cancers.[12]

The mechanism of action in regard to RRP has been less specific. A leading theory revolves around the estrogen metabolism modulation effects of DIM. Prior studies in humans and rats have noted that cruciferous vegetables and I3C increase metabolism for estrogen creating beneficial estrogens (2-hydroxy estrogens), which increase antioxidant activity, and at the same time reduce 16-hydroxy estrogens, which are not antioxidants and can potentially cause cancer.[13,14]

This study was designed to better understand the implications of using DIM in immature rats and its eventual use in the pediatric population. There is currently minimal data regarding the use of DIM in the treatment of RRP. Prior safety studies have mainly focused on older rats and/or humans. A study by Leibelt et al. compared the safety of DIM to I3C in rats that were 5 weeks old and showed no gross of histological adverse effect or toxicity, but did note that DIM is less efficacious than IC3 in inducing CYP in rats.[15] Human studies have shown a dose of up to 200 mg is well tolerated, and increasing the dose did not add anything to the pharmacokinetics.[16]

Prior reports of the use of this drug in the pediatric population were limited to a case report, where an 8-year-old girl was reported to have experienced the disappearance of RRP over a 27-month period with the use of intralesional and intravenous cidofovir in association with indole-3-carbinol,[17] and in a prospective trial of 18 adults and children with RRP, which showed that in 33% of the subjects a cessation of papilloma growth resulted, and 33% experienced decreased papilloma growth.[18] No previous studies had looked specifically at DIM's safety in the pediatric population.

Clinical chemistry panels failed to uncover any significant differences among the three young rat groups. Statistical differences in some tests (e.g., phosphorus, AST, ALT) were found between the young rat groups and the adult group. This, however, corresponds to a variation that would be expected in the chemistries between young and older animals.

CONCLUSION

The data from this study indicated that DIM is safe, with no significant side effects found, even with DIM at a dose 3× higher than that currently used in practice. It further confirmed results about safety reported from previous studies in adult humans and the adult animal model. There was a concern that in some cases, DIM may influence levels of vitamin D or hormones, which in turn could affect bone density. In this study, no effect on bone density or vitamin D levels was observed following DIM exposure. Additionally, histopathological examination did not demonstrate any changes in the liver or kidneys. This study demonstrated that DIM is a relatively nontoxic compound that seems to be safe for use in the pediatric population. Our results supported its clinical application in the authors' ongoing child papilloma study, and allowed us to begin patient recruitment.

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