In vitro Inhibition of N-Nitrosomorpholine Formation by Fresh and Processed Tomatoes

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ABSTRACT

We evaluated the ability of fresh tomatoes and processed tomato products (whole, diced, sauce, puree, and juice) to inhibit in vitro the formation of N-Nitrosomorpholine (NMOR), under conditions similar to the human stomach. The amount of NMOR that was formed averaged 23% to 82% that of the controls, on an equal wet weight basis, with paste being the most inhibitory. On an equal soluble solids basis, the amount of NMOR formed in the presence of products averaged 82% to 88% of that formed in the distilled water control. Fresh tomatoes showed greatest inhibition of nitrosation (NMOR formation averaged 80% of control), and processed tomatoes showed similar inhibition (NMOR 82% to 88% of the control). Ascorbic acid content was the strongest predictor of ability to inhibit NMOR formation. Processed tomato products inhibited nitrosation to a similar extent as had been reported for fresh tomatoes.

Key Words: tomatoes, N-Nitrosomorpholine (NMOR), phytochemicals, nitrosation inhibition

INTRODUCTION

EVIDENCE SUGGESTS THAT PHYTOCHEMICALS CONTRIBUTE TO a reduction in risk of cancer among high consumers of such foods. Examples of such phytochemicals are carotenoids, including lycopene in tomatoes. Phytochemicals also act as vitamin precursors (Nguyen and Schwartz, 1999). It has been suggested that a possible mechanism by which phytochemicals may help prevent cancer is by inhibiting the endogenous formation of N-Nitroso compounds (NOC), resulting from in vivo nitric oxide formation (Bartsch and Spiegelhalder, 1996; Hotchkiss et al., 1994).

NOC compounds have been shown to be potent carcinogens in all the animal species in which they have been tested (Hill, 1996). Many NOC (about 300) have been shown to be carcinogenic on at least 40 animal species (Hecht, 1997; Bartsch, 1991). Although humans may be exposed to preformed NOC from various sources, endogenous synthesis of such compounds may present a greater hazard. Studies have suggested a causal relationship between the formation of NOC in the human and the development of various cancers (Hicks et al., 1977; Bartsch, 1991; Ramon et al., 1993).

A variety of fruit and vegetable extracts have been shown to inhibit NOC formation in vitro and in vivo (Kurechi et al., 1980; Sen et al., 1985; Normington et al., 1986; Sato et al., 1986). In a study of the ability of fresh fruit and vegetable extracts to inhibit the formation of N-Nitrosoproline in humans, tomatoes were among the most inhibitory (Helser et al., 1992). The ability of fresh tomatoes to inhibit nitrosation has been attributed to constituents, such as ascorbic acid and phenolics (Helser and Hotchkiss, 1994). Studies have shown fresh tomatoes to be inhibitors of nitrosation, but no work has been published on the effects of processed tomatoes on nitrosation. In the United States, the per capita consumption of processed tomato products is 4 times that of fresh tomatoes (USDA, 1998). The objective of this study was to compare the effects of fresh tomatoes and processed tomato products on inhibition of in vitro formation of N-Nitrosomorpholine (NMOR) and to determine which phytochemical components correlated most strongly with inhibition of nitrosation.

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MATERIALS & METHODS

Reagents

Citric acid, hexane, acetone, petroleum ether, activated magnesia, diatomaceous earth, sodium sulfate, hydrochloric acid, methylene chloride, sodium hydroxide, sodium carbonate, and Folin-Ciocalteau reagent were purchased from Fisher Scientific Co. (Pittsburgh, Pa., U.S.A.). Sodium azide, polyvinylpolypyrrolidone, and morpholine were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Sodium nitrate, methanol, and meta-phosphoric acid were purchased from Mallinkrodt Specialty Chemicals Co. (Chesterfield, Mo., U.S.A.). L-ascorbic acid kit (Test Combination #409677) was purchased from Boehringer-Mannheim (Mannheim, Germany). N-Nitrosomorpholine was synthesized by the method of Lijinsky et al. (1970).

Equipment

Gas Chromatograph–Thermal Energy Analyzer. A Hewlett–Packard Model 5890A Gas Chromatograph and a Model 543 Thermal Energy Analyzer (Thermal Electron Corp.; Waltham, Mass., U.S.A.) were used for the detection and quantification of N-nitrosomorpholine. Conditions for analysis by the GC-TEA were: nickel column packed with 10% Carbowax 20 M on 80-100WHP; length, 3 m; O.D., 0.32 cm; I.D., 0.25 cm; support, Chromosorb WHP; helium carrier gas flow rate, 25mL/min; oven, 180 °C; injection port, 200 °C; pyrolyzer, 550 °C; interface, 200 °C; cold trap, 160 °C.

Assays

General procedures. Three cultivars of fresh tomatoes and 16 canned tomato products (3 brands each of whole tomatoes, sauce, puree, and paste; 2 brands each of diced and juice) were purchased from a local supermarket. Assays were performed on 2 samples/product, each analyzed in duplicate and compared using standard curves.

Total Solids. Samples were analyzed by Official Method 985.26 for Total Solids in Tomato Products (AOAC, 1995a) with a CEM microwave Drying Moisture Solids Analyzer Model AVC-80 (CEM Corp., Matthews, N.C., U.S.A.). Results were expressed as % total wet weight.

Soluble Solids. Samples were analyzed by Official Method 970.59 for Soluble Solids in Tomato Products: Refractive Index Method (AOAC, 1995b). Samples were prepared for analysis by filtration followed by centrifugation. Results were expressed as % total wet weight.

Total Phenolics. Total phenolic content was measured by the Folin–Ciocalteau Method for Total Phenolics (Singleton and Rossi, 1965), based on optical density at 750 nm. Results were expressed as mg/100-g total wet weight.

Lycopene. Lycopene was estimated by the procedure of Adsule

and Dan (1979). Pigments were extracted by adding samples (1 g) to 20 mL of acetone and shaking on an electric shaker. Extracted pigments were isolated by filtering the acetone mixture through Whatman 1 filter paper (W&R Balston Ltd., England) and dissolving the filtrate in petroleum ether (20 mL). The optical density of the solution was measured at 503 nm. Results were expressed as mg/100-g total wet weight.

β-carotene. β-carotene was measured by Official Methods 43.014–43.017 for Carotenes in Fresh Plant Materials and Silages, Spectrophotometric Method (AOAC, 1984), based on optical density measurement at 436 nm.

Ascorbic Acid. Ascorbic Acid was measured with an L-Ascorbic Acid Kit (Boehringer-Manheim Test-Combination #409677) and based on optical density measurement at 578 nm. Results were expressed as mg/100-g total wet weight.

In vitro N-Nitrosation Assay. The effect of tomato products on the nitrosation of morpholine was determined by comparison with distilled water in a model system. Test samples were prepared by homogenizing tomato products (undrained) in a Waring Blender (Dynamics Corp. of America; New Hartford, Conn., U.S.A.) and filtering the homogenate through cheesecloth. Filtrates were adjusted to pH 2.5 with 2 N HCl, centrifuged and supernatants collected. Each supernatant was vacuum evaporated to dryness with a rotary flash evaporator (Buchler Instruments; Fort Lee, N.J., U.S.A.), and the residue was redissolved in 1.5 mL distilled water. This solution was used in total for the nitrosation assays. The model system consisted of 1.0 mL citrate buffer (0.6 M, pH 2.0), 1.5 mL test solution (or distilled water control adjusted to pH 2.0 with 2N HCl), and 0.1 mL of morpholine solution (to make the reaction mixture 20 mM in morpholine). The reaction was started by the addition of 0.1 mL sodium nitrite solution (to make the reaction mixture 26mM in sodium nitrite), and the mixture was incubated at 37 °C for 30 min in a screw-capped tube. The reaction was stopped by adding 1 mL of 0.01 M sodium azide (Eisenbrand et al., 1981). The NMOR was extracted with methylene chloride (3 × 1mL). Aliquots (5 to 6 μl) were analyzed by GC-TEA. Concentrations were determined by comparing NMOR peak heights with those of an external NMOR standard (8.9 μg/mL). The NMOR that formed (percent of control) was determined by comparing the amount of NMOR formed from the test samples with that formed from the distilled water controls (Helser and Hotchkiss, 1994). Blank samples with added NMOR were used to determine percent recovery.

Statistical Analysis. Results were analyzed using regression analysis, analysis of variance (ANOVA), Tukey's pairwise comparisons, and correlations (Minitab Statistical Package, 1996). Regression analysis and ANOVA, with NMOR formed (% of amount formed in the control) as the response variable and ascorbic acid, lycopene, and phenolic contents as predictors, were used to determine which phytochemical constituents were significant predictors of NMOR formation. Correlation coefficients between ascorbic acid, lycopene, and phenolic contents of each tomato product and the degree of inhibition of NMOR formation were used to assess the effects of these components on NMOR formation. ANOVA and Tukey's pairwise comparisons were used to determine significant differences between average values for NMOR formed by different products. Statistical significance was defined at $p < 0.05$.

RESULTS & DISCUSSION

Composition

The total and soluble solids contents ranged (Table 1), respectively, from 5.5% and 3.8% for fresh tomatoes to 28% and 24% for paste. The β-carotene values were below the level of detection (< 0.1 mg/100 g) and were not reported. Ascorbic acid content ranged from 8.0mg/100 g (wet weight) for fresh tomatoes and sauce to 28 mg/100 g for paste. Paste was also higher (water weight basis) in phenolics and lycopene than fresh tomatoes.

Table 1—Compositional analysis of fresh and processed tomato products[a]

Product	Total solids (%)[b]	Soluble solids (%)[b]	Phenolics (mg/100g)[c]	Lycopene (mg/100g)[c]	Ascorbic acid (mg/100g)[c]
			Fresh tomatoes		
Medium	5.5	4.0	12	5.0	6
Beefsteak	5.0	3.5	10	8.0	10
On the vine	6.0	4.0	17	6.5	10
Average	5.5	3.8	13	6.5	8
			Canned products		
Whole tomatoes					
A	6.0	5.5	18	12	9
B	6.0	5.0	11	11	16
C	6.0	5.5	14	10	10
Average	6.0	5.5	14	11	12
Diced tomatoes					
A	7.5	6.5	18	16	12
B	5.0	4.5	12	15	8
Average	6.0	5.5	15	16	10
Tomato sauce					
A	10	9.0	30	7.0	8
B	9.0	8.5	19	13	<3
C	9.5	9.0	26	9.5	9
Average	9.5	9.0	25	10	8
Tomato puree					
A	14	9.5	38	20	35
B	10	9.5	28	26	9
C	10	9.5	38	23	7
Average	11	9.5	35	23	17
Tomato paste					
A	29	24	50	24	21
B	28	24	38	21	32
C	27	23	43	30	29
Average	28	24	44	25	28
Tomato juice					
A	6.0	5.5	34	9.0	22
B	6.0	5.5	25	11	10
Average	6.0	5.5	29	10	16

[a]Averages of two samples, each analyzed in duplicate.
[b] % of Total Wet Weight of Product
[c] mg/100 g of Total Wet Weight of Product

All results for fresh cultivars and most results for processed products fell within reported ranges (Balasubramanian, 1983; Winter and Herrmann, 1986, Cashel et al., 1989; Hertog et al., 1992; Tonucci et al., 1995; FDA, 1997). Lycopene results for paste and sauce were somewhat lower than those published (Tonucci et al., 1995), possibly due to cultivar and/or seasonal differences. Published values for lycopene and ascorbic acid content of diced tomatoes were not available but would be expected to be similar to values for canned whole tomatoes. Total phenolic contents of processed tomato products have not been published. The phenolic, lycopene, and ascorbic acid content of all products increased with increasing total and soluble solids.

In vitro N-Nitrosation Assay

Recovery of NMOR was 92% (± 5). Test samples from all fresh and processed products tested inhibited in vitro formation of NMOR relative to a distilled water control (Table 2). The amount of NMOR formed by reactions containing tomato product extracts was 10% to 83% of that formed in assays containing distilled water. The greatest degree of inhibition (10% to 35% NMOR formed compared to controls) was demonstrated by the samples of tomato paste while the lowest inhibition (76% to 82% NMOR compared to controls) was shown by the fresh cultivars compared on an equal wet weight basis. As expected, the degree of NMOR inhibition increased as the soluble solids contents of the various products increased. On a fresh weight basis, tomato paste was the most inhibitory and fresh tomatoes the least.

However, when degree of nitrosation inhibition was adjusted to an equal soluble solids basis (based on average soluble solids of fresh tomatoes, 3.8%) the degree of inhibition of NMOR formation by the different products narrowed to 76% to 88% NMOR formed

Table 2—NMOR formed in vitro by soluble solids fractions of tomato products (μg and % of control) and by distilled water controls (μg). Assays were performed on two samples per product, each analyzed in duplicate

	NMOR formed Product (μg)	NMOR formed Control (μg)	% of Control (wet weight)	% of Control[a,b] (equal solids basis)
		Fresh tomatoes		
Medium	41	54	76	77
Beefsteak	41	51	80	78
On the vine	47	57	82	83
Average	43	54	79	80
		Canned products		
Whole tomatoes (in tomato juice)				
A	44	56	79	86
B	41	55	75	81
C	37	53	70	79
Average	41	55	75	82
Diced tomatoes (in tomato juice)				
A	43	57	75	86
B	40	56	72	77
Average	42	56	74	82
Tomato sauce				
A	38	53	72	88
B	37	53	70	87
C	41	61	67	86
Average	39	56	70	87
Tomato puree				
A	30	53	57	83
B	32	54	59	84
C	37	60	62	85
Average	33	56	59	84
Tomato paste				
A	19	54	35	90
B	6	60	10	85
C	14	56	25	88
Average	13	170	23	88
Tomato juice				
A	35	53	66	76
B	45	54	83	88
Average	40	54	74	82

[a]Data standardized to an equal soluble solids content
[b] Significant differences ($p < 0.05$) exist between averages for Fresh and Sauce, Fresh and Puree, Freshand Paste, Whole and Sauce, Diced and Sauce, and Puree and Paste.

Table 3—Total phenolic, lycopene, and ascorbic acid content of tomato products standardized to an equal soluble solids content

Product	Phenolics (mg/100g)[a]	Lycopene (mg/100g)[a]	Ascorbic Acid (mg/100g)[a]
	Fresh tomatoes		
Medium	12	5.0	5.0
Beefsteak	10	8.5	10.0
On the vine	16	6.0	9.0
Average	12	6.5	8.0
	Canned products		
Whole tomatoes (in tomato juice)			
A	12	8.0	6.0
B	8.5	7.7	12.0
C	9.5	7.0	7.0
Average	10	7.7	8.0
Diced tomatoes (in tomato juice)			
A	10	9.5	7.0
B	10	12	7.0
Average	10	11	7.0
Tomato sauce			
A	12	3.0	3.5
B	8	6.0	<1.5
C	11	4.0	3.5
Average	11	4.5	3.5
Tomato puree			
A	15	8.0	14.0
B	12	10	3.5
C	15	9.0	3.0
Average	14	9.0	6.5
Tomato paste			
A	8.0	4.0	2.5
B	6.0	3.5	3.5
C	7.0	5.0	3.0
Average	7.0	4.0	3.0
Tomato juice			
A	23	6.0	16.0
B	18	7.5	7.0
Average	20	7.0	11.0

[a]mg/100 g of the wet weight of the product

compared to controls. On that basis, the processed products were slightly lower than fresh tomatoes in inhibition of nitrosation (Table 2). Inhibition by fresh tomatoes (80% NMOR compared to controls) was highest, while paste exhibited the lowest inhibition (88% NMOR compared to controls). The products with the lowest inhibition of NMOR formation were tomato concentrates (pastes, purees) or prepared from concentrates (sauces), suggesting that the concentration process may degrade components that inhibit nitrosation.

Previous studies have shown fresh tomato extracts inhibited in vitro and in vivo nitrosation (Helser and Hotchkiss, 1994). Our results showed that processed tomatoes also inhibited nitrosation, to almost the same extent as fresh tomatoes (on an equal soluble solids basis). Because conditions for in vitro nitrosation assay were similar to those in the human stomach (pH 2.0, 30 min, 37 °C), it is likely that similar inhibition would take place in vivo after consumption of such foods.

Composition

Results for lycopene, phenolics, and ascorbic acid were adjusted for soluble solids (Table 3), and the highest vitamin C contents (water weight basis) were for juice, fresh tomatoes, and canned whole tomatoes, while the lowest were for paste and sauce, which is generally made by diluting paste. The phenolic and lycopene contents were similar in most products, except for tomato paste, which was somewhat lower compared to the other products.

Multiple linear regression showed that the ascorbic acid content of products was a significant inverse predictor ($p < 0.01$) of the amount of NMOR formed (i.e., NMOR formed decreased as ascorbic acid increased). Ascorbic acid and phenolic content both inversely affected NMOR formation but only ascorbic acid was significant at $p < 0.05$.

Multiple linear regression performed to assess differences in NMOR formed by different brands showed that for juice, phenolics and ascorbic acid content were significant inverse predictors ($p < 0.05$, $p < 0.01$, respectively) of NMOR formed by different brands. The inverse relationship between ascorbic acid and phenolic contents and NMOR formation observed for diced and purees were not significant at $p < 0.05$.

Overall, the strongest inverse relationships were formed between ascorbic acid content of products and NMOR formed. The brand with the greatest inhibition of NMOR formation (Brand A) also had the highest ascorbic acid content. Note that this brand contained ascorbic acid as an added ingredient. Numerous studies have shown ascorbic acid was an effective inhibitor of nitrosation in vivo and in vitro (Mirvish et al., 1972 and Mirvish, 1986; Bartsch, 1991; Knight and Forman, 1987; Kamiyama et al., 1987). Our results confirm those findings for processed tomato products.

In previous studies using a similar in vitro assay method, tomato extract containing 85 mg/L ascorbic acid was reported to inhibit NMOR formation by 60%, whereas the same concentration of ascorbic acid in distilled water only inhibited NMOR formation by 25% (Helser and Hotchkiss, 1994). In our results, tomato extracts

containing 80 mg/L ascorbic acid inhibited NMOR formation by 20%.

Previous studies have shown that different phenolic compounds were effective inhibitors of nitrosation (Pignatelli et al., 1982; Stich et al., 1982; Kurechi et al., 1980). Helser and Hotchkiss (1994) also found phenolic components in fresh tomato extracts (chlorogenic acid and p-coumaric acid) accounted for some of the extract inhibitions of the in vitro formation of NMOR. Generally, products with higher phenolic content inhibited NMOR formation to a greater extent in this work although the correlations were not significant at the $p < 0.05$ level.

The amounts of lycopene and β-carotene in the soluble solids fraction of the tomato products tested were below the levels of detection (< 1.0 mg/100 g). Data from this study did not provide any conclusions about the effects of lycopene and β-carotene on nitrosation. These components may have low aqueous solubility and may have mostly remained with the solids after filtration.

The ascorbic acid content of tomato products was the best predictor of their ability to inhibit NMOR, but other phytochemical components likely also have effects. Our results confirmed the data of Helser and Hotchkiss (1994), showing that, while ascorbic acid accounted for a large portion of the ability of tomatoes to inhibit nitrosation, it was not the only effective component. Inhibition of nitrosation is likely due to a combination, including ascorbic acid and polyphenols. Results also confirmed that concentrating fruits and vegetables may result in the loss of nutritionally effective compounds (Johnson, 1995; Crandall et al., 1983).

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