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Effects of dietary protein on teratogenicity of polyphenols obtained from the outer coat of the fruit of *Treculia africana*

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The teratogenic effects of a single subcutaneous dose of 2.5 mg kg^{-1} polyphenol given on day 6 of gestation were compared in weanling rats fed 5% protein for 4 weeks followed by normal protein diet (referred to as 5% normal protein diet) for the remainder of the experimental period (I), rats maintained on 10% protein diet (II), and rats on a normal protein diet throughout (III). These different diets are based on total dietary concentration of protein. Twenty-two percent of the rats fed 10% protein diet failed to mate while 39% failure of fertilization was observed. In rats fed 5% normal protein diets, the fertilization rate was 100%. Treatment with polyphenol obtained from the outer coat of the fruit of *T. africana* resulted in significant depression of fetal body weights in rats fed 5% normal protein diet and in rats maintained on normal protein diet throughout. Significant numbers of malformations (gross and skeletal) occurred in all the treated groups. Rats on a 5% normal protein diet and rats on the normal diet yielded a significant number of fetuses with internal soft tissue anomalies. The highest incidence of skeletal malformations occurred in polyphenol-treated rats maintained on 10% protein diet throughout the experiment. Complete rehabilitation took place by replacement of low protein diet with normal protein diet.

INTRODUCTION

Dietary composition has been shown to represent an important determinant of pharmacological and toxicological activities of xenobiotics (Mayura *et al.*, 1983; Maduagwu, 1989; Lawal, 1992).

Drill (1952) and Rouiller (1964) observed increased toxicity of drugs during protein deficiency. This should be expected since the microsomal enzyme system in the liver, which deals with foreign compounds, chemicals and drugs, is known to fall to a low level during feeding of a protein-free diet, or one low in protein content, to rats. Similar findings by Boyd and Krupta (1970) showed that the toxicities of a series of 16 pesticides were increased in rats fed low protein diet.

Protein deficiency decreased tumor induction but increased the toxicity of repeated aflatoxin B doses in rats (Madhvan & Gopalah, 1965, 1968). Increased acute toxicity to aflatoxin during protein deficiency has been observed in chicks and swine (Smith *et al.*, 1971; Sisk & Carlton, 1972).

Nolen (1972) reported that protein deficiency increased the incidence of defective fetuses caused by retinoic acid.

Polyphenols are known to be the most abundant secondary compounds of higher plants, exerting a wide range of biological effects in animals (Lowry & Sumpter, 1980). The high content of polyphenol compounds in the outer coat of *Treculia africana* (Lawal *et al.*, 1987) may explain the general avoidance of the outer coat of fruits by birds and animals. Previous findings (Lawal *et al.*, 1989) gave a value of 3.4 ± 0.2 mg polyphenol per ml of extract of the outer coat of the fruit of *Treculia africana*.

Reverse phase-high pressure liquid chromatography of methanolic tissue extracts (Smith & Stanley, 1989) showed seven major phenolic peaks tentatively identified as 4-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, p-coumaric acid, syringaldehyde, and ferulic acid.

Polyphenols obtained from the outercoat of the fruit of T. *africana*, as well as other unknown factors may alter the absorptive capacity of the intestinal wall, and may reduce the amount of available glucose it contains (Lawal *et al.*, 1989).

Motilva *et al.* (1983) suggested that polyphenols react at the brush-border (where the carriers for sugar transport are located), subtly modifying the membrane proteins and so impairing glucose transport.

O-diphenols in plants readily undergo enzymic or spontaneous oxidation to o-semiquinone radicals or to o-quinones which can combine with various functional groups (amines, thiols, indoles, imidazole) found in proteins (Synge, 1975; Van Sumere *et al.*, 1975), thereby affecting the digestibility and nutritive value of proteins.

Most of the world population is subjected to protein deficiencies. Data from well-nourished laboratory animals cannot be extrapolated to the malnourished human without knowledge of how various nutritional deficiencies might alter the toxic response. Therefore, more information is needed about fundamental biochemical and physiological mechanisms to understand the interaction between nutritional deficiencies and toxic response of chemicals.

The present studies were undertaken to develop information on the teratogenic effect of a single subcutaneous dose (2.5 mg kg^{-1}) of polyphenol obtained from the outercoat of the fruit of *T. africana* when administered to protein-deficient, pregnant rats.

MATERIALS AND METHODS

Approximately 21-day-old weanling female Wistar strain albino rats obtained from the Veterinary Science Faculty, of the University of Ibadan were used. These animals were maintained, after weaning, on diets having different protein concentrations.

5% and 10% protein diets were prepared by adding an appropriate quantity of casein to protein-free test diet (Boyd & Carsky, 1969; Cuthbertson, 1957).

These animals were distributed into three groups. Group I animals were maintained on 5% protein diet for 4 weeks followed by the normal protein diet for the remainder of the experimental period. Group II animals were maintained on 10% protein diet throughout the experimental period, and group III animals were fed on normal protein diet containing 27% casein throughout the experimental period.

The animals were weighed at weekly intervals and examined for their physical condition. When sexual maturity was achieved (13 weeks), the rats were mated with mature males of the same strain in stainless steel breeding cages housed in a temperature-controlled and artificially illuminated room (12 h light/12 h dark) free from known sources of toxic contamination. The day on which a vaginal plug was found was designated day 0 of pregnancy. The male rats were maintained on normal protein diets from weaning.

Pregnant females received single subcutaneous injections of polyphenol obtained from the outer coat of the fruit of *T. africana* (2.5 mg kg^{-1} in 5% sodium bicarbonate ($NaHCO_3$) on gestation day 6.

All injection volumes were 0.1 ml 100 g^{-1} body weight. Some animals from each feeding group were untreated and a few animals of group I were injected with an appropriate volume of solvent. Body weights of pregnant rats were monitored daily to assess their general health.

Pregnant rats were killed with an overdose of ether on day 20 of gestation. The uterine horns were exposed and subjected to visual examination for resorptions. Live fetuses were counted, removed from the uterus, blotted dry, weighed and examined for gross abnormalities. Every third fetus was fixed in 95% ethanol, cleared and stained with alizarin red, S, as described by Schnell and Newberne (1970) prior to examination for skeletal defects.

The remaining fetuses were preserved in Bouin's fluid for subsequent internal soft tissue anomalies by the method of free hand razor sectioning as described by Wilson (1965).

Data for fetal body weights, number of implants, and number of live fetuses were evaluated statistically by Student's t-test or analysis of variance (Gaylor, 1978).

Number of resorptions, gross, skeletal and visceral abnormalities were analyzed using a 2×2 contingency table test (Steel & Torrie, 1960).

The fetus was used as the experimental unit in latter analyses.

In all statistical tests, a probability of $p < 0.05$ was accepted as significant.

Polyphenol extraction from the outercoat of the fruit of *T. africana*

The outer coat of the fruit of *T. africana* was removed using a sharp knife, and 150 g was pounded in a mortar

Table 1. Group distribution and relation of various protein levels to fertilization in the rat. (*Numbers in parentheses indicate percentages)

Protein level %	Total No. of rats	No. of rats failed to mate	No. of rats positive for mating	No. of rats conceived
Group I				
5% protein diet replaced with normal protein diet	31	0	31 (100)	31 (100)
Group II				
10% protein diet	30	7 (23)	23 (77)	9 (30)
Group III				
Normal protein diet	23	0	23 (100)	23 (100)

Table 2. Effects of single subcutaneous dose (2.5 mg kg^{-1}) of polyphenol obtained from the outer coat of the fruit of *T. africana* on implantation, percentages of live and dead fetuses and fetal body weights on the 6th day of gestation in rats[a] maintained on different levels of dietary protein

Treatment	No of litters	No of implants		Resorptions		Live fetuses		Fetal body
		Total/group	Average/litre	Total/group	% of implants	Total/group	% of implants	average (g)
Group I								
(5% protein-normal protein diet) untreated	11	165	15.0 ± 0.6	13	8.7 ± 4.7	152	91.3 ± 5.0	3.9 ± 0.07
Solvent-treated	8	121	15.1 ± 0.8	7	5.45 ± 3.0	114	94.55 ± 3.0	3.8 ± 0.1
2.5 mg kg^{-1} polyphenol treated	12	171	14.3 ± 0.7	24	13.2 ± 3.8	147	87.0 ± 3.8	3.30 ± 0.09[b]
Group II								
10% protein diet untreated	4	40	10.0 ± 1.4	1	2.3 ± 0.3	39	97.70 ± 2.3	2.60 ± 0.31
Polyphenol (2.5 mg kg^{-1})-treated	5	53	10.6 ± 0.5	5	9.0 ± 2.9	48	91.00 ± 3.9	2.30 ± 0.17
Group III								
Normal protein diet untreated	11	157	14.3 ± 0.5	10	6.4 ± 2.3	147	93.6 ± 2.4	3.8 ± 0.04
Polyphenol (2.5 mg kg^{-1})-treated	12	169	14.1 ± 0.6	34	19.8 ± 4.5[b]	135	80.20 ± 4.5[b]	3.10 ± 0.08[b]

[a]% Resorptions and live fetuses are based on the number of implants. Average and percent values are given as mean ± S.E of the mean.
[b]Significantly different from corresponding controls ($p < 0.01$).

into a homogeneous slurry. The slurry was macerated in 300 ml of ethanol/water (50:50 v/v) with continuous shaking. After 24 h, the extract was filtered, and the residue subjected to a second maceration of 30 min and filtered. The filtrates were combined, concentrated by rotary evaporation at 50°C, centrifuged at 15 000 rev. min^{-1} for 20 min and the supernatant made up to 100 ml with distilled water. The extract was then freeze-dried.

RESULTS AND DISCUSSION

A total of 90 weanling female rats were used in this study. Table 1 shows the details regarding group distribution and relation of various protein levels to fertilization in the rat.

Of the 30 rats maintained on 10% protein diet, 7 failed to mate and, in 14 rats, failure of fertilization was observed after confirmation of mating.

Table 3. Percent of rat litters with gross visceral and skeletal anomalies from material subcutaneous single dose (2.50 mg kg^{-1}) of polyphenol obtained from the outercoat of the fruit of *T. africana* on the 6th day of gestation in rats maintained on different levels of proteins.

Treatment	No of litters	External examinations		Internal soft tissue examinations		Internal skeletal examination	
		No of fetuses examined	% malformed	No of fetuses examined	% malformed	No of fetuses examined	% malformed
Group I							
5% protein diet (normal protein diet) untreated	11	152	0	95	0	57	0
Solvent treated	8	114	0	70	0	44	2 (4.5)
Polyphenol (2.50 mg kg^{-1})-treated	12	147	16 (10.9)	101	12 (11.9)	45	17 (37.8)
Group II							
10% protein diet. Untreated	4	39	0	27	5 (18.5)	12	3 (25.0)
Polyphenol (2.50 mg kg^{-1})-treated	5	48	11 (22.9)	32	6 (18.7)	16	12 (75.0)
Group III							
Normal protein diet. Untreated	11	147	0	98	2 (2.0)	49	5 (10.2)
Polyphenol (2.50 mg kg^{-1})-treated	12	136	27 (19.9)	93	28 (30.1)	43	18 (41.9)

[a]Numbers in parentheses indicate percentages of live fetuses with the anomaly.
[b]Significantly different from corresponding controls.

Rats maintained on 5% normal protein diets and rats fed normal protein diet from the onset exhibited normal mating tendencies and all animals conceived.

Effects of a single subcutaneous dose of polyphenol obtained from the outer coat of the fruit of *T. africana* (2.5 mg kg^{-1}) injected on day 6 of gestation on total number of implants, percent of resorptions, percent of live fetuses and fetal body weights are shown in Table 2.

There was no effect on the total number of implants regardless of diet. The percentage of implants resorbed was significantly higher, and the percent of live fetuses lower, in treated animals fed normal protein diet. In rats fed 5% normal protein diets and those maintained on 10% protein diet, polyphenol treatment caused no significant increase in the percent of implants resorbed when compared with their corresponding controls. Polyphenol-treated animals maintained on normal protein diet and 5% normal protein diets had statistically significant decreases in fetal body weights compared to the respective controls. There was no significant decrease in fetal body weights in polyphenol-treated rats fed 10% protein diet. The fetal body weights of animals fed 10% protein, but not treated with polyphenol, were much less when compared with control fetuses of other groups. Polyphenol induced various gross, visceral and skeletal malformations in fetuses from rats maintained on different levels of protein (Table 3).

The percent of grossly malformed fetuses was increased significantly in all of the three groups treated with polyphenol obtained from the outer coats of the fruit of *T. africana*. None of the control fetuses from rats maintained on 10% protein diet and 5% normal protein diets showed any gross malformations. The percent of grossly malformed fetuses in treated rats fed 10% protein diet and normal protein diet were 23.0 and 20.0, respectively, whereas, in treated rats maintained on 5% normal protein diet, the percent of grossly malformed fetuses was 11.0.

Major gross malformations after polyphenol treatment were hydrocephaly and anophthalmia (Table 4).

Two fetuses from treated animals maintained on 5% normal protein diets, one fetus from treated rat fed 10% protein diet and 2 fetuses from treated animals maintained on normal protein diet showed omphalocele. One fetus from group I treated with polyphenol showed ectopia cordis.

With regard to visceral anomalies, rat fetuses from treated animals of groups I and III showed significant increases in the percent malformations (12.0 and 30.0, respectively, Table 3). The major visceral anomaly was internal hydrocephaly (Table 4). In rat fetuses from the treated group fed 10% protein diet, no significant increase in the percent of malformations was observed.

Table 4. Fetal malformations (gross, internal soft tissue and skeletal) associated with prenatal exposure to different polyphenols (2.50 mg kg^{-1}) on day 6 in rats[a] maintained on different levels of protein. Protein levels (%)

	Group I: 5% protein diet			Group II 10% protein diet		Group III Normal protein diet	
	Untreated	Solvent-treated	Polyphenol (2.50 mg kg^{-1})	Untreated	Polyphenol (2.50 mg kg^{-1})	Untreated	Polyphenol (2.50 mg kg^{-1})
Extenal No.							
Examined	152	114	147	39	48	147	136
Hydrocephaly	—	—	12 (8.16)	—	7 (14.6)	—	24 (17.6)
Anophthalmia	—	—	7 (4.76)	—	6 (12.5)	—	7 (5.4)
Omphalocele	—	—	2 (1.36)	—	1 (2.08)	—	2 (1.47)
Ectopia cordis	—	—	1 (0.68)	—	—	—	—
Microphthalmia	—	—	2 (1.36)	—	—	—	—
Internal soft tissues							
No. examined	95	70	101	27	32	90	93
Internal hydrocephaly	—	—	12 (11.9)	4 (14.8)	4 (12.5)	—	19 (20.0)
Hydronephrossis esophagus	—	—	—	1 (3.7)	1 (3.1)	2 (2.0)	1 (1.1)
Shift in position	—	—	—	—	2 (6.3)	—	10 (10.8)
Skeletal No. examined	57	44	45	12	16	49	43
Bipartite vertebral centra	—	6 (13.3)	6 (13.3)	—	5 (31.3)	—	7 (16.3)
Bipartitis sternebrae	—	—	6 (13.3)	—	3 (18.8)	—	6 (13.3)
Sternebrae genesis	—	—	2 (4.4)	2 (16.6)	6 (37.5)	—	3 (6.9)
Rudimentary rib	—	2 (4.5)	8 (17.8)	2 (16.6)	4 (25.0)	5 (10.2)	9 (20.9)
Fused ribs	—	—	10 (22.2)	—	3 (18.8)	—	4 (9.3)
Extra ribs	—	—	1 (2.2)	—	4 (25.0)	—	—
Missing ribs	—	—	1 (2.2)	—	–9	—	1 (2.3)
Broken ribs	—	—	—	—	—	—	1 (2.3)
Incomplete ossification of sternebrae	—	—	—	—	1 (6.25)	—	—

[a]Number in parentheses indicate percentage of live fetuses with the respective anomaly.

Mild internal hydrocephaly occurred spontaneously in 44.8% of the fetuses from a single rat (untreated) fed a 10% protein diet.

With regard to skeletal defects, the percent of skeletal malformation was increased significantly by polyphenol treatment in all the three groups (Table 4). However, the highest incidences occurred in fetuses from treated rats fed the 10% protein diet. In this group 75% of the fetuses were affected, whereas the percentages of skeletal malformations were only 36 and 42 in fetuses from treated rats fed 5% normal protein diet, and normal protein diets, respectively. The major skeletal defects involved vertebrae, sternebrae, and ribs (Table 4).

Most of the world population is subject to protein deficiencies. Therefore the fundamental biochemical and physiological mechanisms as related to toxic response of chemical and nutritional deficiencies must be thoroughly investigated.

The findings in this investigation suggest that dietary protein deficiency may increase the susceptibility of the animal to the teratogenic effects of polyphenol obtained from the outer coat of the fruit of *Treculia africana* with the major increased susceptibility related to skeletal development. Mayura *et al.* (1982, 1983) reported that a single dose of 1.75 mg kg^{-1} subcutaneous injection of Ochratoxin A, was teratogenic in rats.

Protein deficiency has effects on reproduction in rats. With 10% protein diet, 23% of the rats failed to mate and 30% of rats failed to fertilize after showing a positive test for mating.

Rats fed a normal protein diet treated with polyphenol, obtained from the outer coat of the fruit of *T. africana*, showed a significant increase in the percentage of fetal resorptions, a corresponding decrease in percent of live fetuses and depression in fetal body weights when compared with corresponding controls.

Polyphenol induced various gross, visceral and skeletal malformations in all the three groups fed with different levels of protein.

CONCLUSIONS

1. Dietary protein deficiency may increase the susceptibility of animals to the teratogenic effects of polyphenol obtained from the outer coat of the fruit of *Treculia africana*.
2. Various gross, visceral and skeletal malformations might be induced by polyphenol administration in rats fed different levels of proteins.

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