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Research Article

REPRODUCTIVE SAFETY ASSESSMENT OF A POLYHERBAL ANTIHYPERTENSIVE MIXTURE IN GHANA

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ABSTRACT
Medications designed to treat symptoms and cure chronic diseases can also cause unanticipated problems with the reproductive system; even in therapeutic doses. This study was therefore conducted on a commonly used polyherbal antihypertensive product on the Ghanaian market to ascertain its reproductive toxicity in both females and males. Couple-mediated and male-specific toxicities (endpoints) that measure characteristics for successful sexual performance and procreation were investigated in ICR mice. Data obtained indicated that the product did not have any negative effect on mating behavior and fertility in both female and male mice. It did not affect gestation; neither did it have detrimental effect on litter number and litter weight, live birth and weaning index. The product also did not affect caudal epididymis and testis weight. Spermatozoa concentrations, spermatozoa motility and viability after semen analysis were also not affected after semen analysis. The endpoints that measure characteristics for successful sexual performance and procreation were not adversely affected with the use of the product under study, therefore, as far as reproductive toxicity is concerned, the polyherbal antihypertensive mixture is safe to use within limits of the doses administered in this study.
Keywords: Couple-mediated toxicity, Spermatozoa concentration, Mating Index, Fertility Index, Weaning Index, Live Birth Index, Polyherbal antihypertensive (PHA)

INTRODUCTION

Reproductive toxicity is "any effect of chemicals that would interfere with reproductive ability and capacity" with subsequent effect on lactation and the development of the offspring.[1] Medications designed to treat symptoms and cure chronic diseases can also cause unanticipated problems with the reproductive system; even in therapeutic doses.[2, 3] Such medication could affect the female reproduction by decreasing libido (and hence mating) and having negative effects on ovulation, fertilization, implantation, and gestation. They could also affect the developing implant and cause still birth or could affect the health of the offspring. Similarly, these medications could also affect male reproductive abilities by decreasing libido, causing impotence (and hence reducing mating), and/or decreasing spermatozoa quantity or quality which adversely affect fertilization. Pharmacologically mediated male infertility can be an unintended effect of medical treatment or an unanticipated consequence of drug use.[4]

In Ghana, herbal remedies have become widely accepted in the treatment of hypertension, a chronic disease of adults most of whom are in the reproductive ages and beyond. Most male patronize these product because of knowledge that many of the allopathic antihypertensives are commonly associated with erectile dysfunction[5]and other reproductive system impairments.[6] This prompted the present reproductive toxicity studies on a commonly used polyherbal antihypertensive mixture in Ghana. This polyherbal is a prepackaged aqueous preparation made from bark and leaves of *Persea americana* and *Vernonia amygdalina* respectively.

Persea americana (avocado, alligator pear or butter pear) is known to have hypotensive or antihypertensive effects,[7] wound healing properties,[8] antibacterial activity[9] and hypoglycaemic effects.[10] *Vernonia amygdalina* ("bitter leaf") is a widely used medicinal plant in Africa for its antihypertensive effects.[11] Leaves from this plant serve as culinary herb in soup.[12] In traditional Nigerian homes, extracts of the plant are used as tonic in the treatment of cough, feverish condition, constipation and hypertension.[13, 14] *Vernonia amygdalina* extracts may help suppress, delay, or kill cancerous cell by diverse mechanisms.[15, 16] It may provide anti-oxidant benefits,[17] strengthen the immune system through cytokine regulation,[18] and decrease blood glucose.[19]

Koffuor et al., (2011 a) reported previously that the polyherbal anti-hypertensive preparation has CNS depressant, anxiolytic, and probably muscle relaxant activity which affects neurological behaviors. Within limits of acute and delayed toxicity, however, the product is safe to use.[20] Sub-chronic toxicity evaluation of this polyherbal mixture suggests that it may be safe to use when used in lower doses but could potentially provoke liver and kidney damage in higher doses.[21] Further studies have shown that the product shortens the onset of sleep and prolongs the duration of sleep possibly by inhibiting CYP450 enzymes. We therefore proposed that caution should be taken on the concomitant use of this product and allopathic antihypertensives and/or hypno-sedatives as it could potentiate their activity.[22] Here we report on the reproductive toxicity of this polyherbal antihypertensive product in both male and female ICR mice.

MATERIAL AND METHODS

This study was conducted at the Department of Pharmacology, KNUST in compliance with: OECD Principles of Good Laboratory Practices ENV/MC/CHEM (98)17, EEC Good Laboratory Practices (90/18/EEC) and FDA Good Laboratory Practice Standards (Part 58 of 21 CFR).

Animals and Husbandry

Imprint Control Region (ICR) mice at 4 weeks of age were obtained from the Department of Pharmacology, Animal House and acclimated for 2 weeks prior to initiation of dosing. During this period, mice were observed (physical; in-life) daily and weighed. At initiation of treatment, animals were approximately 6-7 weeks old. The mean weight for the

males was 29.2 g and the females 25.6 g. The females were nulliparous and non-pregnant. Individual weights of mice placed on test were within ± 20% of the mean weight for each sex. Animals were housed in stainless steel wire mesh cages during the acclimation and the experimental periods. The mice were kept under ambient light/dark cycle, room temperature and relative humidity. The animal had free access to pelleted mice chow (GAFCO, Tema, Ghana) and water presented to them daily.

The Polyherbal Antihypertensive and Dosing

The polyherbal product under study is an aqueous preparation made from stem bark and leaves of *Persea americana,* and *Vernonia amygdalina* obtained from forests in Ghana. Dosing of the product was done based on the manufacturer's recommendations. ICR mice were grouped (n=5) and received vehicle or 36, 72, or 180 mg/kg of the product by gavage. Dosing was once daily at a volume of 10 ml/kg body weight. Individual dose volumes were calculated based on the animal's most recent recorded body weight. The oral route of administration was used because it is the intended human exposure route.

Reproductive Toxicity in Female ICR Mice

The reproductive toxicity in female mice was carried out by a method described by Ansah et al., (2010).[23] Four groups of female ICR mice (n=10) were used in the study. Treatment group I was the control and received vehicle only. Treatment groups II, III, and IV received 36, 72, and 180 mg/kg of PHA (respectively) daily for two weeks. After the two-week treatment period, the female mice were regrouped by subdividing each treatment group into two (n=5) and labeled as follows: IA, IB, IIC, IID, IIIE, IIIF, IVG, and IVH. Two male mice were introduced into each of the eight female groups. Treatment of female mice in groups IA, IIC, IIIE, and IVG was continued with vehicle, 36, 72, and 180 mg/kg PHA respectively through the gestation period and up to 21 days after parturition. Treatment with vehicle and PHA in groups IB, IID, IIIF, and IVH was discontinued just after males were cohabited with the females. Formation of vaginal plug was taken as evidence of successful mating. The time taken to mate after cohabitation, number of mice mated, impregnation, fertility, gestation period, litter size, live births and offspring survival were observed. Reproductive indices which include Mating Index (MI), Fertility Index (FI), Live Birth Index (LBI) and Weaning Index (WI) were determined.

Reproductive Toxicity in Male ICR Mice

Four groups of male mice (n=5) were used in the study. Treatment group I was the control and animal received vehicle only. Treatment groups II, III, and IV received 36, 72, and 180 mg/kg of PHA (respectively) daily for 21 days. After the treatment period, the male mice were introduced into groups of female mice as described as follows. Female mice were grouped into eight (n=5) as follows: A, B, C, and D. Two male mice from group I (control) were introduced into female group A; two 36 mg/kg PHA-treated males from group II were introduced into female group B; two 72 mg/kg PHA-treated males from group III were introduced into female group C; and two 180 mg/kg PHA-treated males from group IV were introduced into female group D. Formation of vaginal plug was taken as evidence of successful mating. Time taken to mate, number of mice mated, impregnation, fertility, gestation period, litter size, live births and offspring survival were observed. Mating Index (MI), Fertility Index (FI), Live Birth Index (LBI) and Weaning Index (WI) were determined

Evaluation of Fertility in Male ICR Mice

Epididymal Spermatozoa Assay

For epididymal spermatozoa counts, the method described by Meistrich (1989)[24] was used with slight modifications. Four groups of male mice (n=5) were used in the study. Group 1; the control was given received vehicle only. Groups 2, 3, and 4 received 36, 72 and 180 mg/kg respectively of PHA daily for 3 weeks. Animals from each group were then euthanized by cervical dislocation and the wet weight of the left caudal epididymis and testis was taken and recorded to the nearest 0.1 mg. To prevent the loss of secretory fluid, the base of each seminal vesicle was grasped with forceps before removing. By mincing the caudal epididymis in 20 ml physiological saline at 37°C, a spermatozoa suspension was obtained for evaluation of semen parameters using the Ceti magnum-T/trinocular microscope for fluorescence (Medline Scientific limited, UK) under an objective lens magnification 40X as follows:

Spermatozoa Concentration

A drop of the spermatozoa suspension was delivered onto the counting chamber of the Improved Neubauer Haemocytometer (Depth 0.1mm, Area: 1/400 mm^2; Yancheng Cordial Lab Glassware Co. Ltd, Jiangsu, China (Mainland) and allowed to stand for 5 minutes for sedimentation, after which spermatozoa were counted from five large squares (volume: 0.5 mm^3) and spermatozoa concentration expressed as number of spermatozoa per ml.

Spermatozoa Motility

After introducing a drop of spermatozoa suspension onto the counting chamber of the Improved Neubauer Haemocytometer, non-motile spermatozoa numbers were first determined from five large squares, followed by a total spermatozoa count (motile and non-motile). The number of motile spermatozoa was calculated. Spermatozoa motility was estimated as: percentage of motile spermatozoa to the total spermatozoa counted.

Spermatozoa Viability

This technique is used to differentiate between live and dead spermatozoa. A drop of the eosin stain was added to the spermatozoa suspension and delivered onto the counting chamber of the on the Improved Neubauer Haemocytometer and allowed to stand for 5 minutes at 37°C. On examination under the microscope (40X), the head of dead spermatozoa were stained red while the live spermatozoa were not stained. Spermatozoa viability was estimated as: percentage of live spermatozoa to the total spermatozoa counted.

Statistical Analysis

The observations are presented as mean ± SD. Significant differences among means of the group were determined by one-way ANOVA using Graph Pad Prism for windows version 5.0 (Graph Pad Software, San Diego, CA, USA) followed by Dunnet's multiple comparison test with level of significance set at $P \leq 0.05$.

RESULTS

Reproductive Toxicity in Female and Male ICR Mice

Data obtained indicated that PHA did not have any detrimental effect on mating behavior in both female and male mice as time taken to mate after cohabitation between PHA-treatments and that of the control were not significantly different, and mating index was always 100 % (Table 1, 2, and 3). It was also observed that there were no serious effects on fertility as the lowest fertility index (recorded in only one treatment group) was 75 % (Table 2). Treatment of both female and male mice with PHA did not affect gestation

period which was approximately 21 days. PHA did not have any effect on litter number and litter weight and all pregnant mice gave birth to live litter (live birth index was nearly 100 %) with weights similar to untreated mice (Table 2 and 3). Almost all litter in all treatment groups was alive at day 21 after birth; recording 100 % weaning index (Table 2 and 3).

Evaluation of Fertility in Male ICR Mice

Weights of the caudal epididymis recorded and that of the testis were not significantly different from the control (Table 4). Spermatozoa concentrations, spermatozoa motility and viability after semen analysis were also not different from that of the control group (Table 4).

DISCUSSION

Reproductive toxicity risk assessment to evaluate the potential toxicity of drugs to the human male and female reproductive systems and to developing offspring focuses on reproductive system function as it relates to sexual behaviour, fertility, pregnancy outcomes, and lactating ability, and the processes that can affect those functions directly. The endpoints that measure characteristics that are necessary for successful sexual performance and procreation are couple-mediated, female-specific, and male-specific.

Couple-mediated endpoints are those in which both sexes can have a contributing role if both partners are exposed. These include mating rate, time to mating (time to pregnancy), pregnancy rate, delivery rate, gestation length, litter size (total and live), number of live and dead offspring (foetal death rate), offspring gender, birth weight, offspring survival, and external malformations and variations. Male-specific endpoints of reproductive toxicity include monitoring organ weights (testes, epididymis, seminal vesicles, prostate, and pituitary) and spermatozoa evaluation (spermatozoa number count) and quality (morphology and motility).

Although PHA did not have any adverse effects on mating behavior in both females and males, a useful indicator of impaired reproductive function may be the length of time required for each pair to mate after the start of cohabitation. An increased interval between initiation of cohabitation and evidence of mating suggests abnormal oestrous cyclicity in the female or impaired sexual behaviour in one or both partners. A fertility index between 80-100 % as observed confirms the "no-effect" on mating behavior. Sexual behaviour reflects complex neural, endocrine, and reproductive organ interactions and is therefore susceptible to disruption by a variety of toxic agents and pathologic conditions. Interference with sexual behaviour in either sex by drugs represents a potentially significant reproductive problem which is an evidence of impaired sexual receptivity and copulatory behaviour.

Treatments did not affect the gestation period, however, significant shortening of gestation, can lead to adverse outcomes of pregnancy such as decreased birth weight and offspring survival. Significantly longer gestation may be caused by failure of the normal mechanism for parturition and may result in death or impairment of offspring if dystocia (difficulty in parturition) occurs.

PHA did not have any significant effect on litter size (number) and litter weight and live birth index which could imply that other reproductive endpoints such as ovulation rate, and fertilization rate were normal and implantation number was good. Furthermore, litter size, litter weight and live birth index were not significantly affect probably indicating that there were no pre-implantation or post-implantation losses, as well as internal malformations and variations which could affect the numbers of live and dead offspring.

Birth weight measured on the day of parturition for the PHA treatments and the control were not significantly different from each other. Birth weights, however, are influenced by intrauterine growth rates, litter size, and gestation length (which were found to be normal). Individual pups in large litters tend to be smaller than pups in smaller litters. Thus, reduced birth weights attributed to large litter size was not considered an adverse effect. Weaning index recorded was 100 %. This could be due good postnatal structural and functional development devoid of PHA effect. Offspring survival is dependent on birth weight, sex, and normality of the individual, as well as the litter size, lactational ability of the dam, and suckling ability of the offspring. Although all weight and survival endpoints can be affected by toxicity of an agent, either by direct effects on the offspring or indirectly through effects on the ability of the dam to support the offspring, PHA treatment had no effect.

Weights of the caudal epididymis and that of the testis recorded were not significantly different from the control. Weight is an excellent index of either the biochemical or the anatomical state of reproductive organs. The male reproductive organs for which weights may be useful for reproductive risk assessment include the testes, epididymis, seminal vesicles (with coagulating glands), and prostate. Reproductive organ size (testes and seminal vesicles occupies a place of special importance among morphological measures because of its direct implication in fertility. Reproductive organ sizes are markers of the timing of puberty and testicular weight is connected total spermatozoa count in mice. Since normal testis weight varies only modestly within a given test species. This relatively low inter-animal variability suggests that absolute testis weight should be a precise indicator of gonadal injury.

Spermatozoa concentrations, spermatozoa motility and viability after semen analysis were also not different from that of the control group. Although effects on spermatozoa production can be reflected in other measures such as testicular spermatid count or caudal epididymal weight, no surrogate measures are adequate to reflect effects on spermatozoa morphology or motility. The ability to detect a decrease in testicular spermatozoa production may be enhanced if spermatid counts are available.

It can be concluded that the endpoints that measure characteristics that are necessary for successful sexual performance and procreation were not adversely affected with the use of the product under study, therefore, as far as reproductive toxicity is concerned, the polyherbal antihypertensive mixture is safe to use within limits of the doses administered in this study.

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Table 1: Comparison of time to mate, number of mice mated, and number of females pregnant for female pretreatment with PHA for 14 days only prior to mating, and female mice pretreated with PHA for 14 days prior to mating with continuing treatment.

Treatments	PHA Treatment	Time taken to mating (days)	№ of mice mated	№ of Pregnant females
Pretreatment for 14 days followed by mating and continued treatment during gestation	I A (control)	3.4 ± 0.83	5	4
	II C (36 mg/kg)	3.4 ± 0.89 ^{ns}	5	5
	IIIE (72 mg/kg)	3.2 ± 0.83 ^{ns}	5	4
	IVG (180 mg/kg)	3.2 ± 1.09 ^{ns}	4	3
Pre-treatment for 14 days only followed by mating	IB (control)	3.4 ± 1.14	4	4
	IID (36 mg/kg)	3.4 ± 0.89 ^{ns}	5	4
	IIIF (72 mg/kg)	3.4 ± 0.89 ^{ns}	4	4
	IVH (180 mg/kg)	3.2 ± 1.09 ^{ns}	5	4

Values quoted are Means + SD (n=5). There were no significant different between treated groups and the control as established using one way Analysis of Variance (ANOVA). ns imply P > 0.05.

Table 2: Comparison of reproductive indices for female pretreatment with PHA for 14 days only prior to mating, and female mice pretreated with PHA for 14 days prior to mating with continuing treatment during gestation and up to 21 days after parturition.

	PHA-Treatments	MI (%)	FI (%)	GP (days)	Litter №	LBI (%)	LW (g)	WI (%)
Pretreatment for 14 days followed by mating and continuing treatment during and after gestation	I A (control)	100	80	21.0±0.32	10.4±0.51	100±0.00	1.60±0.083	100±0.00
	II C (36 mg/kg)	100	100	20.8±0.20 ^{ns}	9.2±0.58 ^{ns}	98.2±1.82 ^{ns}	1.68±0.080 ^{ns}	100±0.00 ^{ns}
	IIIE (72 mg/kg)	100	80	21.6±0.24 ^{ns}	10.2±0.58 ^{ns}	100±0.00 ^{ns}	1.63 ±0.082 ^{ns}	94.8±3.37 ^{ns}
	IVG (180 mg/kg)	100	75	21.4±0.24 ^{ns}	9.0±0.45 ^{ns}	100±0.00 ^{ns}	1.62 ±0.65 ^{ns}	100±0.00 ^{ns}
Pre-treatment for 14 days only followed by mating	IB (control)	100	100	21.4±0.40	10.0±0.7	92.6±3.25	1.62 ±0.092	96.4±3.7
	IID (36 mg/kg)	100	80	20.8±0.20 ^{ns}	9.2±0.37 ^{ns}	95.6±2.72 ^{ns}	1.64 ±0.084 ^{ns}	100±0.00 ^{ns}
	IIIF (72 mg/kg)	100	100	21.0±0.24 ^{ns}	10.0±0.78 ^{ns}	100±0.00 ^{ns}	1.67±0.087 ^{ns}	100±0.00 ^{ns}
	IVH (180 mg/kg)	100	80	20.6±0.25 ^{ns}	9.6±0.25 ^{ns}	100±0.00 ^{ns}	1.66 ±0.062 ^{ns}	100±0.00 ^{ns}

Values are Means + SD (N=5). There were no significant different between treated groups and the control as established using one way Analysis of Variance (ANOVA). Mating Index (MI): The number of mated females/Number of females cohabited; Fertility Index (FI): Percentage mated female/Number of pregnant females; Gestation Period (GP); Live Birth Index (LBI): Number of live offspring/Number of offspring delivered; Litter weight (LW); Weaning Index (WI): Number of offspring at day 21/number of offspring delivered.

Table 3: Reproductive indices for female mice mated with male mice treated with 36, 72 and 180 mg/kg PHA for 21 days.

PHA Treatment (mg/kg)	Time taken to mating (days)	№ of mice mated	№ of Pregnant females	MI (%)	FI (%)	GP (days)	Litter №	LBI (%)	LW (g)	WI (%)
A (control)	3.2 ± 0.83	5	5	100	100	21.0±0.32	9.2±0.62	100±0.00	1.60± 0.083	100±0.00
B (36)	3.2 ± 1.09 ^{ns}	5	4	100	80	21.8±0.20 ^{ns}	10.4±0.26 ^{ns}	98.2±1.82 ^{ns}	1.68± 0.080 ^{ns}	100±0.00 ^{ns}
C (72)	3.4 ± 1.14 ^{ns}	5	5	100	100	21.6±0.24 ^{ns}	10.8±0.42 ^{ns}	100±0.00 ^{ns}	1.63 ± 0.082 ^{ns}	94.8±3.37 ^{ns}
D (180)	3.4 ± 0.89 ^{ns}	5	5	100	100	21.4±0.24 ^{ns}	10.3±0.45 ^{ns}	100±0.00 ^{ns}	1.64 ± 0.065 ^{ns}	100±0.00 ^{ns}

Values are Means + SD (N=5). Different between treated groups and the control was established using one way Analysis of Variance (ANOVA); ns imply P > 0.05. Mating Index (MI): The number of mated females/Number of females cohabited; Fertility Index (FI): Percentage mated female/Number of pregnant females; Gestation Period (GP); Live Birth Index (LBI): Number of live offspring/Number of offspring delivered; Litter weight (LW); Weaning Index (WI): Number of offspring at day 21/number of offspring delivered.

Table 4: The Effect of 36, 72 and 180 mg/kg PHA treatment on male-specific toxicities in ICR Mice

Treatments	Paired weight of caudal epididymis (mg)	Paired weight of testis (mg)	Spermatozoa concentration (x 10^6cells/ml)	Spermatozoa motility (%)	Spermatozoa viability (%)
Control (untreated)	0.0237 ± 0.004	0.133 ± 0.048	9.22 ± 1.125	93.45 ± 2.734	96.39 ± 2.78
36 mg/kg PHA	0.0242 ± 0.002 ^{ns}	0.133 ± 0.060 ^{ns}	9.37 ± 0.677 ^{ns}	94.67 ± 2.18 ^{ns}	95.55 ± 2.59 ^{ns}
72 mg/kg PHA	0.0228 ± 0.004 ^{ns}	0.132 ± 0.021 ^{ns}	10.04 ± 0.448 ^{ns}	92.88 ± 1.96 ^{ns}	97.01 ± 3.49 ^{ns}
180 mg/kg PHA	0.0245 ± 0.004 ^{ns}	0.134 ± 0.075 ^{ns}	9.86 ± 0.829 ^{ns}	95.82 ± 3.07 ^{ns}	96.02 ± 2.88 ^{ns}

Values quoted are Means + SD (n=5). Different between treated groups and the control was established using one way Analysis of Variance (ANOVA); ns imply P > 0.05.

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