Rev. Int. Contam. Ambient. *14* (2) 79-83, 1998

EFFECTS OF A PYRROLIZIDINE ALKALOID, INTEGERRIMINE, ON MOUSE SPERM MORPHOLOGY

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(Recibido noviembre 1997, aceptado agosto 1998)

Key words: integerrimine, pyrrolizidine alkaloid, sperm morphology, *Senecio*, mice

ABSTRACT

The pyrrolizidine alkaloid (PA), integerrimine, a natural product obtained from *Senecio brasiliensis*, was used at single dose, in two concentrations (18.75 or 37.5 mg/kg) to test its ability to induce morphological abnormalities in spermatozoa of inbred C57BL/6 mice. The anomalies were classified in nine types, six of which presented anomalous sperm heads. The frequency of three types of anomalous sperm heads were increased by integerrimine at the higher dose level, and only one at the lower dose level. The results suggest that integerrimine can modify, at least in part, some step of spermatogenesis.

RESUMEN

El alcaloide pirrolizidínico integerrimina, un producto natural obtenido del *Senecio brasiliensis* fue usado en dosis única, en dos concentraciones (18.75 y 37.5 mg/kg) para verificar su capacidad de inducir anormalidades en el espermatozoide del ratón endocruzado C57BL/6. Las anomalías fueron clasificadas en nueve tipos, seis de los cuales son de la cabeza. La frecuencia de tres tipos de cabezas anormales aumentó por el tratamiento con la dosis mayor de integerrimina y solamente en un tipo se incrementó la frecuencia por la dosis menor. Los resultados sugieren que la integerrimina puede modificar, al menos en parte, algun paso de la esparmatogénesis de los ratones.

INTRODUCTION

The pyrrolizidine alkaloids (PAs) are natural toxins found in many plant genera of wide geographic distribution. The toxicity of these compounds in laboratory animals, veterinary practice and human populations was reviewed by McLean (1970). Study on potential genotoxicity of PAs began with the demonstration of the mutagenic activity of heliotrine in *Drosophila* (Clark 1959).

The species *Senecio brasiliensis* (Sprengel) Less. var. *tripartitus* is very common in the south-central region of Brazil and contains two pyrrolizidine alkaloids, integerrimine and senecionine, the former being more abundant (Motidome and Ferreira 1966). In spite of the fact that several species of *Senecio* are used in folk medicine, to our knowledge no cases of *Senecio* poisoning have been described in humans. Some cases of lethal equine *Senecio* intoxication were described; however only the pioneering experimental studies by Moraes (1952) showed that a *Senecio brasiliensis* predominant pyrrolizidine alkaloid is toxic to rats and mice. Integerrimine has been reported to induce mutation in *Drosophila melanogaster* (Paula-Ramos and Marques 1978), *Tradescantia* (Chies 1983), *Aspergillus nidulans* (Rocha and Azevedo 1986) and *Saccharomyces cerevisiae* (Paula-Ramos *et al.* 1991). It also induces mitotic anomalies in *Allium cepa* (Chies 1983) and presents antimitotic effects with the induction of megalocytosis in rat liver parenchyma cells (Nardi *et al.* 1980, Gimmler and Nardi 1982). In mice it causes abortion, fetal malformations and chromosome aberrations in bone marrow cells (Kvitko and Gimmler 1986, Gimmler-Luz *et al.* 1990). In contrast, integerrimine fails to induce detectable structural or numerical chromosome aberrations in mouse spermatocytes at diakinesis/metaphase I, after exposure of germ cells at pre-leptotene stage (Gimmler-Luz *et al.* 1987).

Among the approaches used for testing damage to germ cells due to drug administration, the examination of the shape of male gametes has been employed (Wyrobek *et al.* 1983, Topham 1983, Kar and Das 1983), but the mechanism of induction of abnormality is not completely understood (Aubele *et al.* 1990). Since male mice treated with integerrimine did not show chromosomal aberrations in spermatocytes (Gimmler-Luz *et al.* 1987), the present study was undertaken to analyze sperm morphology to know if this alkaloid disturbs in some way the spermatogenesis.

MATERIALS AND METHODS

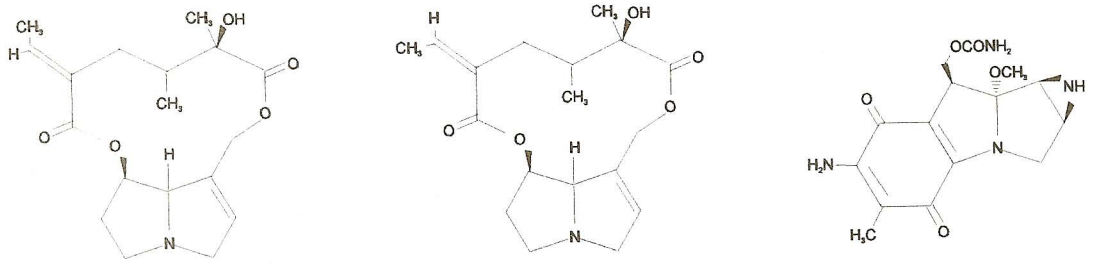
Inbred C57BL/6 mice, 10-12 weeks old, from our own colony, housed under controlled temperature and light conditions in the animal house facility of Instituto de Biociências, Universidade Federal do Rio Grande do Sul were employed. All animals received commercial standard mouse cube diet (Nuvilab, CR1, Moinho Nuvipal Ltda, Curitiba, PR, Brazil) and water *ad libitum*.

Integerrimine (**Fig. 1**) was supplied by Dr. Mario Motidome from Universidade de São Paulo, São Paulo, Brazil. The LD50/24 h for mice was established by Moraes (1952) as 100 mg/kg. Two groups of 6 males each were injected intraperitoneally with integerrimine at a single acute dose of 18.75 or 37.5 mg/kg. At these dose levels integerrimine is known to caused genetic damage as detected by analysis of aberrations in metaphase chromosomes or micronuclei in mouse bone marrow cells (Gimmler-Luz *et al.* 1990, Gimmler-Luz and Erdtmann 1997). The negative control group of 5 males received the vehicle solution (0.1% acetic acid). The positive control group of 4 males was dosed with 100 mg/kg of cyclophosphamide (CP) named Endoxan (Abbot Laboratórios do Brasil Ltda., São Paulo). The application volume was 10 ml/kg of body weight.

RESULTS AND DISCUSSION

Figure 2 shows examples of spermatozoa types observed in the present study, numbered from 0 to 9 and called *Type 0* to *9*. *Type 0* represents the normal form. *Types 1* and *8* possibly represent normal forms (with tail exceptionally folded, perhaps due to technical artifacts) observed with similar frequency in all groups. Although, it is also possible that the spermatozoa *Types 1* and *8* were abnormal forms unresponsive to PA and CP treatments. The other shapes (*Types 2* to *7* and *9*) are considered abnormal. The heads of spermatozoa considered *Type 2* are slightly distorted, *Type 3* have triangular form, *Type 4* are completely distorted, *Type 5* have an elongated head with a narrow or banana-like form, *Types 6* and *7* have abnormal sizes, smaller and larger, respectively. *Type 9* included spermatozoa with abnormal tails. These *Types* closely resemble the abnormal murine sperm observed by Wyrobek and Bruce (1975), Wyrobek (1979) and Komatsu *et al.* (1982).

The frequencies of different types of abnormal spermatozoa are listed in **Table I**. The integerrimine-treated groups for doses 18.75 and 37.5 mg/kg presented 18.15% and 35.42% abnormal spermatozoa respectively, while the negative control group (dose



Integerrimine Senecionine Mitomycin C

Fig. 1. Chemical structure of the pirrolizidine alkaloid integerrimine and its geometric isomer senecionine (Klásek 1973) and of mitomycin C (Hopkins *et al.* 1991)

The mice were killed 35 days after the treatment, and their epididymes were removed. The sperm examined were at late spermatogonia or early spermatocytes stage at the time of treatment (Oakberg 1957). These cells are described as the most sensitive stages for chemical induced spermatozoa-shape abnormalities (Aubele *et al.* 1990). Sperm suspensions were prepared from each mouse by disrupting the two epididymis in 2 ml of phosphate buffered physiological saline. The suspension was transferred to V-shaped centrifuge tubes to allow the natural sedimentation of the larger tissue pieces. The supernatant was dropped on coded slides, air dried, fixed for 3 min in absolute methanol, and stained with Giemsa. The microscopic analysis was done under 40x and 100x objectives. From each mouse 1000 intact and non-overlapping spermatozoa were examined.

The results obtained for integerrimine-treated and negative control groups were compared following the Kruskal-Wallis test. The Mann-Whitney test was employed to compare the two control groups (negative and positive).

0) presented 16.14% and the positive control group (CP-treated) 27.28%. The results obtained in the negative control group are in agreement with those of Wyrobek (1979). In the group treated with 18.75 mg/kg PA, only the frequency for *Type 3* increased significantly, but in the group dosed with 37.5 mg/kg, significant enhancement of the frequency of sperm head abnormalities was observed for *Types 2, 3* and *5*. These dose levels were shown to increase chromosomal aberrations in C57Bl/6 mice bone marrow cells (Gimmler-Luz *et al.* 1990), while doses between 6.25 and 50 mg/kg failed to induce detectable cytogenetic abnormalities in male meiotic cells (Gimmler-Luz *et al.* 1987). Significantly high frequency of sperm abnormalities observed for 37.5 mg/ kg of integerrimine was mainly due to two animals (numbers 15 and 17). Similar results were obtained by Lähdetie (1988) after vinylacetate exposure. In the CP-treated group the frequencies for *Types 2, 3* and *6* increased significantly, compared to the negative control group.

The mechanisms and the genetic basis of changes in

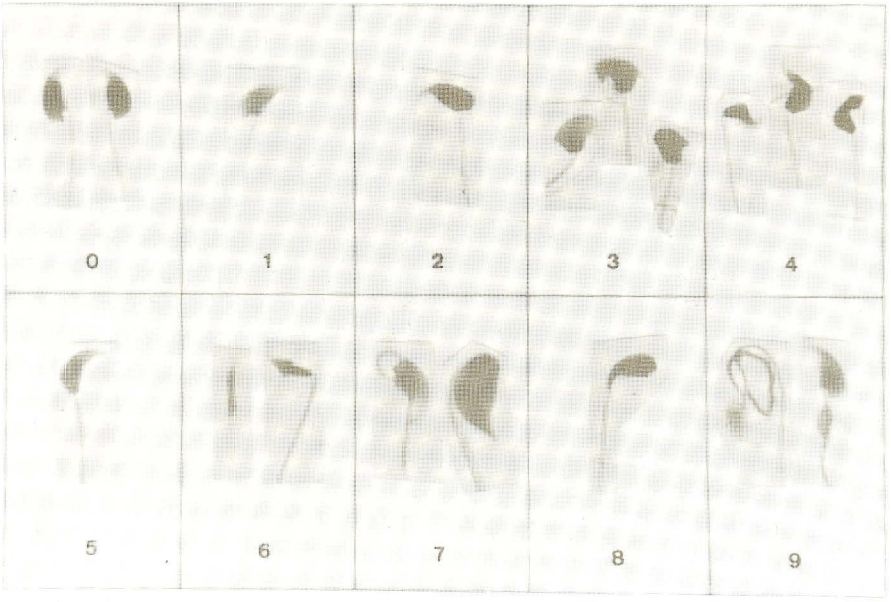


Fig. 2. *Types* of spermatozoa heads observed in C57BL/6 mice

TABLE I. NUMBER OF SPERMATOZOA WITH DIFFERENT *TYPES* OF ABNORMALITIES PER 1000 SPERMS EXAMINED IN INTEGERRIMINE TREATED AND CONTROL GROUPS

Dose (mg/kg)	Animal	*Types* of anomalous spermatozoa										Total of spermatozoa		
		1	2	3	4	5	6	7	8	9	other	with anomalous form	with anomalous size	with anomalies (%)
N control	1	29	80	15	3	10	2	9	8	16	3	137	11	175 (17.5)
(0)	2	17	88	18	6	8	2	7	8	7	0	137	9	161 (16.1)
	3	20	69	14	8	4	3	10	10	4	1	115	13	143 (14.3)
	4	16	86	16	3	3	2	1	26	5	0	124	3	158 (15.8)
	5	25	108	14	5	4	4	1	8	1	0	156	5	170 (17.0)
$\bar{X}$		21.4	86.2	15.4	5	5.8	2.6	5.6	12	6.4	0.8	133.8	8.2	161.4 (16.14)
SD		5.5	14.3	1.7	2.1	3.0	0.9	4.3	7.9	5.8	1.3	15.5	4.2	12.34
PA	6	21	95	29	7	1	3	6	9	5	0	153	9	176 (17.6)
(18.75)	7	20	114	37	2	7	5	1	2	8	0	180	6	196 (19.6)
	8	20	119	19	6	12	4	1	16	9	0	176	5	206 (20.6)
	9	17	77	21	2	11	1	4	12	3	0	128	5	148 (14.8)
	10	16	102	19	8	13	6	6	23	13	0	158	12	206 (20.6)
	11	13	74	25	1	19	1	6	15	1	2	132	7	157 (15.7)
$\bar{X}$		17.8	96.8	25*	4.3	10.5	3.3	4.0	12.8	6.5	0.3	154.5	7.3	181.5(18.15)
SD		3.1	18.6	7.0	3.0	6.1	2.1	2.4	7.1	4.4	0.8	21.6	2.7	21.1
PA	12	17	107	21	8	15	3	7	15	16	1	168	10	210 (21.0)
(37.50)	13	20	157	36	3	14	13	6	17	2	1	230	19	269 (26.9)
	14	7	120	22	6	14	3	17	8	6	1	169	20	204 (20.4)
	15	9	434	120	33	26	24	3	18	7	2	622	27	676 (67.6)
	16	15	113	19	4	9	1	8	19	14	1	160	9	203 (20.3)
	17	4	322	112	35	33	27	4	11	15	0	506	31	563 (56.3)
$\bar{X}$		12	208.8*	55*	14.8	18.5*	11.8	7.5	14.7	10	1	309.2*	19.3*	354.2* (35.42)
SD		6.3	136.8	47.7	14.9	9.0	11.4	5.0	4.3	5.8	0.6	202.3	8.8	210.1
P Control	18	40	155	45	7	13	12	5	27	4	1	260	17	309 (30.9)
CP	19	18	125	45	6	3	10	11	31	5	0	197	21	254 (25.4)
(100)	20	16	110	50	0	6	22	4	12	8	0	182	26	228 (22.8)
	21	21	150	49	9	9	14	6	25	15	2	238	20	300 (30.0)
$\bar{X}$		23.8	135*	47.3*	5.5	7.8	14.5*	6.5	23.8	8	0.8	219.3*	21.0*	272.8* (27.3)
SD		11.0	21.2	2.3	3.9	4.3	5.3	3.1	8.2	5.0	1.0	36.0	3.7	38.3

CP = cyclophosphamide; N = negative; P = positive; PA = integerimine; SD = standard deviation; $\bar{X}$ = mean; * = different from negative control group ($p < 0.05$)

spermatozoa form are not clear. Goldberg *et al.* (1977) suggest multiple roles of proteins in spermatozoa differentiation. The ability to induce spermatozoa-shape alteration has been proposed to be related to the germ-cell mutagenic capacity of a chemical (Wyrobek *et al.* 1983). Topham (1983) reports that multiple genes that control spermatozoa shaping seem to be expressed at the diploid level before meiotic segregation, thus the haploid genotype in the mature sperm has no influence on its own morphology. The author suggests that the abnormal chromosome complement does not imply an abnormal sperm morphology. On the other hand, synthesis of mRNA encoding rSMP-B protein, probably involved in spermatogenesis, can be demonstrated during the postmeiotic haploid phase of rat and rabbit spermatogenesis (Wang *et al.* 1990). It was also speculated that the spatial changes due to structural rearrangements of the karyotype could allocate DNA sequences to a functional domain regulated differently from that selected during evolution (Redi and Garagna 1992). The increased abnormalities due to dietary restriction suggest that the sperm anomalies were not always induced by exogenous mutagens (Komatsu *et al.* 1982).

It has been demonstrated that many PAs, including senecionine (**Fig. 1**), an integerrimine isomer, induce DNA cross-links which consist primarily of proteinase-sensitive cross-links (Hincks *et al.* 1991). Kim *et al.* (1995) observed that about half of the DNA cross-links induced by pyrrolic PAs involved proteins. Eastman *et al.* (1982) have found that senecionine and seneciphylline bind covalently to hepatic DNA, RNA and proteins. Although protein binding is approximately the same, senecionine binds more to RNA whereas seneciphylline is the strongest alkylator of DNA. The senecionine transalquenal metabolite (t-4HH) was shown to bind to protein but not to the nucleic acid of rat liver *in vivo* (Grasse *et al.* 1985). The authors suggest that these metabolites have their action on DNA probably mediated by interaction with sulphydryl containing proteins. Mitomycin C (MMC) and the PAs, cyclic diesters of retronecine (**Fig. 1**), possess a spatial relationship of the two potentially electrophilic centers, that suggests a similar scheme in adduct formation on DNA (Hopkins *et al.* 1991). A similar pattern of DNA cross-linked proteins was seen in isolated nuclei treated with pyrrolic PAs and those treated with MMC (Kim *et al.* 1995). The sperm head abnormalities induced by MMC are mainly banana-shaped heads in C57BL/6 strain (Wyrobek and Bruce 1975) and folded forms in Swiss outbred mice. Those forms are similar to *Type 5*, and *3* respectively, increased after integerrimine treatment (**Fig. 1** and **Table 1**).

The absence of observable chromosome aberrations in male germ cells after integerrimine treatment (Gimmler-Luz *et al.* 1987) is in contrast with the present results, but is in agreement with the absence of induced dominant lethal mutation after monocrotaline, jacobine or seneciphylline treatment of mice (Walton and Cumming, 1978). Those latter results indicate a lack of chromosomal aberrations in male germinal cells, the main factor involved in dominant lethality. Alternatively we could demonstrate that integerrimine caused, in low but significant frequency, structural chromosome alterations in mouse bone-marrow cells (Gimmler-Luz *et al.* 1990), which was again confirmed by micronucleus examination in polychromatic erythrocytes (Gimmler-Luz and Erdtmann 1997). When integerrimine was tested in *Drosophila melanogaster* using the wing somatic mutation and recombination test, the results indicated that the principal cause of anomalies is mitotic recombination instead of somatic mutation that included small deletions, point mutations, chromosome aberrations, etc. (Campesato *et al.* 1997).

To summarize, the observed alterations of sperm shape allow us to suggest that the PA integerrimine can modify some steps of spermatogenesis in mice. Although these steps can not be determined, our previous negative results indicate that detectable chromosome aberrations in spermatocytes are not directly involved in the process.

ACKNOWLEDGEMENTS

We thank Dr. Mario Motidome (Universidade de São Paulo, SP, Brazil) for supplying integerrimine and Dr. Nance B. Nardi for critical suggestions regarding this manuscript. This work was supported in part by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), Financiadora de Estudos e Projetos (FINEP) and Laboratório de Genotoxicidade, Universidade Federal do Rio Grande do Sul (GENOTOX).

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