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Cytogenetic Effects of Sonication on *Spathosternum prasiniferum* (Grasshopper), *Anabas testudineus* (Fish), and *Mus musculus* (Mammal)

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Because of its superiority and several advantages over other methodologies like X-rays in pinpointing location/site of abnormalities in internal anatomy of human beings without causing any apparent injury to them, the use of ultrasonic sound waves is being increasingly made in modern diagnostics e.g. for detection of various anatomical disorders, blockage or overgrowths (tumours etc.), position of foetus, appendicitis etc. (Simonovsky 2000; Princi et.al. 2000) and therapies (Kremkau 1979; Marmor 1983), thereby inviting attention to test whether its use as a medical tool is otherwise absolutely safe. Contrary to the reports of several workers that ultrasonic irradiation have certain undesirable biological effects, producing enzymatic, physiological, biochemical and genomic (DNA) changes (Hedges and Leeman 1979; Leibeskind et al. 1979; Haupt et al. 1981) a large number of workers failed to confirm such effects particularly by testing effects on DNA of lymphocytes or sperm chromosomes *in vitro* of subjects exposed to ultrasonic irradiation and therefore, advocated its safe use as a tool in medical and therapeutic practices (Miller et al. 1983; Ciaravino et al. 1985, 1986; Becher et al. 1983; Au et al. 1982; Lundberg et al. 1982; Tateno et al. 2000). This controversy prompted us to re-examine the cytogenetic effects *in vivo*, if any, in three different test models viz. grasshopper (invertebrate), fish (lower vertebrate) and mice (higher vertebrate).

MATERIALS AND METHODS

Live adult specimens of grasshopper (*Spathosternum prasiniferum*) of male sex, of fish (*Anabas testudineus*) and mammal (*Mus musculus*) of both sexes served as the test materials for the present study.

Ultrasonic Sound Exposure: For ultrasonic irradiation, the machine used was an ultrasonic cell disruptor (LSL SECFROID SA Microson Model XL 2005, Switzerland) which generated sound waves of 20-23 KHz. The microprobe (tuned to vibrate at 20 KHz) was inserted into water slightly above the experimental specimens submerged in water in a suitable glass jar (containing just enough water to enable fish specimen to submerge its dorsal fin under water and enough water to submerge mice specimens leaving its head free of water). For grasshoppers, the microprobe was directly touched onto the dorsal thoracic part of the specimens. During sonication, ice was placed around the glass jar containing water and the specimen, preventing water inside to become too hot during sonication.

Acute ultrasound exposure series: Half of total number of live specimens were selected randomly and exposed to sonication (treatment group) for 1 minute, given a rest period of 1 minute and then again subjected to 1 minute irradiation at an output percentage of 70. Then they were sacrificed at 2hr, 24hr and 48 hr. A total number of 30 specimens each of grasshoppers and fish and 50 specimens of mice were used for the present investigation.

Methodologies: To study the meiotic chromosomes the routine squash preparation of

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grasshoppers' testes was made at 2, 24 and 48 hrs after sonication and stained with haematoxylin. The conventional citrate-flame-drying-Giemsa stain schedule was adopted for preparation of somatic chromosomes from bone marrow cells of mice and from kidney and gill cells of fish, and germinal chromosomes from testis of mice.

For study of micronucleus induction, routine smear preparations of peripheral blood from fish and bone marrow cells from mice were made at 24 and 48 hr after sonication and were stained with May-Grunwald. Mitotic index was ascertained from bone marrow preparations of mice.

For study of sperm shape anomaly, smeared preparation of sperm of mice sacrificed at 24 and 48 hr after sonication were made from epididymis and stained with Giemsa.

For embryotoxic study, 15 females were exposed to ultrasonic irradiation only once on 2^{nd}, 4^{th}, 6^{th}, 8^{th} or 10^{th} day after conception and they were dissected on the 15^{th} day of gestation to examine any possible embryotoxic effect by examining the absence of any foetus or presence of any abnormal foetus or scar-mark indicating loss of foetus.

Chronic ultrasonic sound exposure series: In another set of experiment, a total number of 40 specimens of mice were used to examine the chronic or "cumulative" effect, if any, of ultrasonication; mice were subjected to repeated exposure for a total period of 2 min each at an interval of 20 days, in the same way in acute exposure series, so that mice sacrificed at 30, 60, 75 and 90 days after the initial dose actually received 2, 3, 4 and 5 such doses of sonication, respectively. In this series bone marrow chromosome aberration, micronuclei induction and spermshape anomalies were only studied. 20 specimens each were used as treatment and control lots.

Control Series: In case of both mice and fish models, specimens identical in number to that of treatment series were sham exposed to the chambers but not actually irradiated and sacrificed at different fixation intervals served as controls for each group of treatment. Similarly, normal grasshoppers collected from the wild and acclimatised in laboratory condition for different fixation intervals served as controls. The actual numbers of cells examined for different parameters for different species have been mentioned in the respective tables.

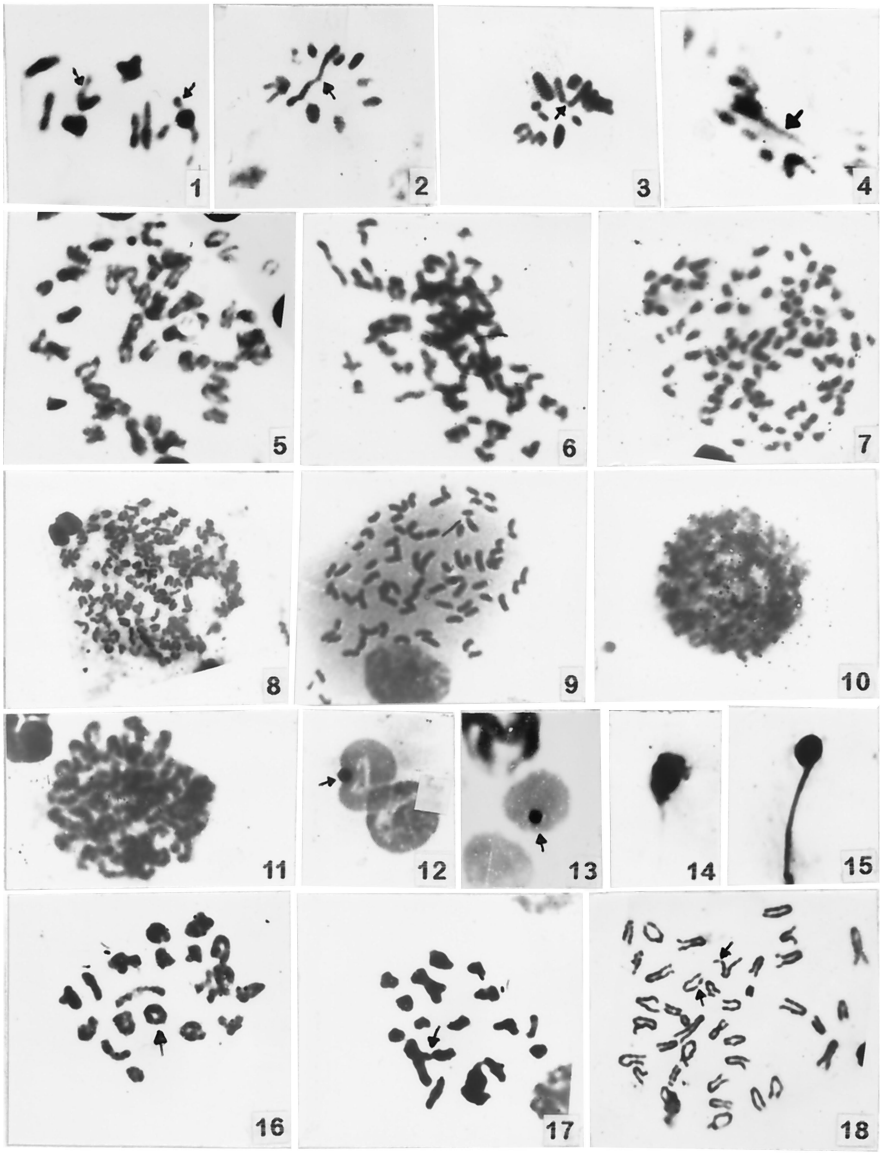
RESULTS AND DISCUSSION

In the ultrasonic sound exposed series, structural chromosomal damages (breaks, fragments, sticky-bridges etc.) as well as damages of lesser significance (stickiness, pycnosis etc.) were observed in different meiotic divisional stages (Figs. 1-4) of grasshoppers and their frequency distribution has been summarized in Table-1. In

Table 1. Frequency distribution of major aberration types observed in grasshopper spermatocytic chromosomes after exposure to ultrasonic sound (T) and in control grasshoppers (C), at different fixation intervals.

Fixation time intervals	Series	% of aberrations in different meiotic stages ± SE					
		Diakinesis	Metaphase I	Anaphase I	Metaphase II	Anaphase II	Total
2 hr	C_1	0.0 ± 0.0	1.9 ± 0.8	2.9 ± 1.8	3.5 ± 2.3	0.0 ± 0.0	1.6 ± 0.7
	T_1	10.7 ± 2.3^c	19.4 ± 1.1^c	29.3 ± 3.7^c	20.7 ± 2.5	37.8 ± 2.4^c	19.6 ± 0.5^c
24 hr	C_1	3.2 ± 2.1	0.7 ± 0.7	1.0 ± 1.0	4.0 ± 2.9	2.0 ± 2.0	2.6 ± 0.9
	T_1	5.3 ± 3.6	16.6 ± 1.9^c	24.1 ± 2.6^c	10.8 ± 1.5	16.6 ± 5.0	13.4 ± 1.4^c
48 hr	C_1	1.0 ± 1.0	1.5 ± 0.9	0.0 ± 0.0	2.0 ± 2.0	0.0 ± 0.0	0.8 ± 0.2
	T_1	2.7 ± 1.1	13.5 ± 1.2^c	14.7 ± 2.3^c	10.5 ± 3.1^a	12.1 ± 3.4^b	9.8 ± 0.6^c

Aberration includes break, constriction, acentric fragment, sticky bridge, dicentric bridge, condensed etc., a= $p<0.05$, b= $p<0.01$, c= $p<0.001$.
No. of Individuals examined in each case =15; cells scored per individual =100.



Figures 1-18. Photomicrographs of spermatocytic complements of grasshoppers showing: break and acentric fragment (Fig.-1), stretching (Fig.-2), constriction (Fig.-3) and a sticky bridge (Fig.-4); somatic metaphase complements of *Anabas* showing a normal plate (Fig.-5) and plates with aberrations like stickiness (Fig.-6), C-mitotic effect (Fig.-7), and somatic metaphase complements of mice showing polyploidy (Fig.-8), c-mitotic effect (Fig.-9) and crumpledness (Fig.-10); stickiness (Fig.-11); poly-chromatic (Fig.-12) and normo-chromatic erythrocytes (Fig.-13) showing MN; sperm showing abnormal head shapes (Figs.-14 & 15); germinal plates of male mice showing, ring (Fig.-16), multivalent association (Fig.-17) and abnormal spermatogonial metaphase with chromatid break (Fig.-18).

general, the frequencies of aberrations were found to be maximum at 2 hr, which declined with the lapse of time. No aberration was noted in the diplotene, either in the sonicated or in normal grasshoppers. In fact, the maximum aberrations were contributed by condensed chromosomes, possibly due to excessive spiralization effect. On the other hand, in case of somatic chromosomes of both fish and mice(Table-2), no break type or any other major type aberrations such as gaps,

Table 2. Frequency distribution of different types of aberrations examined in sonicated fish (T_2) and mice (T_3) and in their respective controls (C_2 and C_3).

Fixation time intervals (hr)	Series		% of aberration ± SE	
	Fish	Mice	Fish	Mice
2	C_2	C_3	0.8 ± 0.2	0.4 ± 0.2
	T_2	T_3	17.4 ± 1.1[a]	29.8 ± 0.4[a]
24	C_2	C_3	0.6 ± 0.3	0.6 ± 0.2
	T_2	T_3	12.0 ± 1.1[a]	19.0 ± 0.9[a]
48	C_2	C_3	1.2 ± 0.2	0.6 ± 0.3
	T_2	T_3	7.6 ± 0.9[a]	18.0 ± 0.7[a]

Aberrations include polyploidy, aneuploidy, stickiness, c-mitotic effect, pycnosis, condensation, crumpled etc.
a= $p < 0.05$
No. of Individuals examined in each series/fixation intervals =5; cells scored per individual =500.

exchanges, fragment etc. could be encountered on somatic metaphase spreads. However, clastogenic effects of lesser significance such as stickiness, crumpledness, condensation, c-mitotic effect, polyploidy etc. could be encountered in quite a good number of metaphase spreads, of both fish (Figs. 5-7) and mice (Figs. 8-11) particularly at the early intervals, which again declined with the lapse of time. There was statistically insignificant increase of micronuclei (Figs. 14-15) in the sonicated series vis-à-vis controls (Table 3). In mice, the mitotic index of bone marrow cells was also found to be slightly enhanced by the ultrasound exposure (Table 3). However, the number of abnormal sperm heads (Figs. 12-13) was more in the sonicated series at both 24 and 48 hr which was statistically significant as compared to controls (Table 3). There were also elevated frequency of aberrations in the

Table 3. Frequency distribution of MI, MN in NCE and PCE in sonicated mice (T_3) against their respective controls (C_3) at different fixation intervals.

Fixation time intervals	Series	MN				MI	SHA
		% of MN in NCE	% of MN in PCE	Total % of MN in NCE and PCE ± SE	P/N ratio	% ± SE	% ± SE
24 hr	C_3	0.06	0.05	0.06 ± 0.02	0.5	0.4 ± 0.06	0.2 ± 0.08
	T_3	0.35	0.63	0.44 ± 0.02[a]	0.8	2.7 ± 0.21[a]	1.8 ± 0.09[a]
48 hr	C_3	0.09	0.08	0.08 ± 0.02	1.0	0.4 ± 0.05	0.2 ± 0.04
	T_3	0.49	0.39	0.44 ± 0.05[a]	0.8	2.1 ± 0.14[a]	1.5 ± 0.10[a]

MN= Micronucleus, NCE= Normochromatic Erythrocytes, PCE= Polychromatic Erythrocytes, MI= Mitotic Index, SHA= Sperm Head Anomaly.
a= $p<0.05$.
No. of Individuals examined in each study/series/fixation intervals =5; cells scored per individual =5000.

Table 4. Frequency distribution of different types of aberrations examined in meiotic chromosomes of sonicated mice (T_3) and in controls (C_3).

Fixation time intervals	Series	% of aberration ± SE
24 hr	C_3	0.3 ± 0.2
	T_3	1.8 ± 0.3[c]
48 hr	C_3	0.4 ± 0.1
	T_3	0.9 ± 0.1[c]

Aberration includes multivalent association, ring, polyploidy, stickiness, pulverisation, chromatid break, terminal association etc.
c $p<0.001$
No. of Individuals examined in each series/fixation intervals =5; cells scored per individual =1000.

germinal chromosomes of mice (Figs. 16-18; Table 4), e.g. breaks, multivalent association, rings, terminal association, pulverisation, stickiness in both M_I and M_{II} stages. In the long-term repeated-dose experiment, in which only mice were used, there seemed to be cumulative effect, the effect being gradually elevated from 30 days through 90 days (see table 5).

Table 5. Frequency distribution of CA, MN in NCE and PCE and SHA in sonicated mice (T_3) against their respective controls (C_3) at different (long term) fixation intervals.

Fixation time intervals	Series	CA	MN				SHA
		% of CA ± SE	% of MN in NCE	% of MN in PCE	% of MN in NCE and PCE ± SE	P/N ratio	% of SHA ± SE
30 d	C_3	0.40 ± 0.25	0.06	0.07	0.06 ± 0.03	0.45	0.18 ± 0.04
	T_3	35.90 ± 0.23[c]	0.25	0.52	0.40 ± 0.09[a]	1.14	5.56 ± 0.11[a]
60 d	C_3	0.38 ± 0.25	0.06	0.06	0.07 ± 0.03	0.45	0.19 ± 0.04
	T_3	23.00 ± 0.77[c]	0.22	0.74	0.42 ± 0.10[a]	0.61	0.88 ± 0.10[c]
75 d	C_3	0.42 ± 0.25	0.06	0.06	0.06 ± 0.03	0.50	0.16 ± 0.04
	T_3	25.60 ± 0.51[c]	0.39	0.65	0.49 ± 0.11[b]	0.60	0.98 ± 0.02[b]
90 d	C_3	0.40 ± 0.25	0.06	0.06	0.05 ± 0.03	0.45	0.18 ± 0.04
	T_3	29.40 ± 0.50[c]	0.55	0.74	0.06 ± 0.09[b]	0.37	1.48 ± 0.11[c]

Chromosome aberrations (CA) include gap, break, centric fusion, translocation, fragment, pulverisation, ring, terminal association, polyploidy, aneuploidy, stickiness, C-mitotic effect, etc.
MN= Micronuclei, NCE= Normochromatic erythrocytes, PCE= Polychromatic erythrocytes, SHA= Sperm head anomaly. a= $p< 0.05$, b= $p<0.01$, c= $p<0.001$.
No. of Individuals examined in each series/fixation intervals =5; cells scored per individual =500.

Embryotoxic effect could not apparently be found in any of the 15 female pregnant mice that received sonication on different days of the conception, because all the mice had intact foetuses as found in their control counterparts. However, further studies are warranted before coming to a definite conclusion.

In the present study, ultrasonic sound waves of moderate intensity, when directly applied on the body surface of grasshoppers, were found to produce more drastic effect on germinal chromosomes of grasshoppers than when they were indirectly given on fish and mice kept under water. However, still some clastogenic effect, of

lesser significance though, was found to occur in the somatic metaphase chromosomes of both fish and mice. Interestingly, the frequencies of aberrations in single dose experiments, declined along with the lapse of time, implying that some of the aberrations had possibly been restituted or repaired. But in the "repeated dose" experiment the effects of CA, MN and SHA, appeared to increase with the lapse of time and with the increase in number of exposure. Thus, it seemed that like that of X-ray, the ultrasonic irradiation effect may also be "cumulative" in nature. Further, the increase in abnormal shapes of spermheads and chromosome aberrations in germinal cells of male mice exposed to ultrasonic sound is an important finding and should be viewed with some concern. However, the apparent lack of embryotoxic effect found in pregnant mice exposed to ultrasonic sound may not exclude the possibility of any genetic disorder to follow in the progeny, an aspect which has not been included in this study but needs more attention and further experimental studies.

The biophysical effects of ultrasound in aqueous solutions can be characterized as thermal effects, cavitation, and direct effects (Hill 1968). The mechanism of ultrasonic action in vivo and in vitro appears to be complex due to the interaction of the three types of modes. In aqueous media, the nonthermal effects of ultrasound have been attributed mainly to cavitation. The collapse of cavitation bubbles produces a variety of free radical species and induces local shock waves, while the oscillation of cavitation bubbles causes hydrodynamic shearing stress (Hill 1968; Baker and Dalrymple 1978). The more drastic effects observed in grasshopper spermatocytic chromosomes may be due to the thermal and direct effects while the fish and mice showing lack of breaktype aberrations may therefore partly be due to the cavitation effect and partly be due to the formation of free radicals within them (Jana et al. 1990a, b 1995). Since ultrasonic sound waves are now being extensively used in many diagnostic procedures, physiotherapy, deep-sea fishing and in hyperthermia treatments for cancer (Kremkau, 1979) for both induction of localized hyperthermia (Marmor 1983) and non-invasive thermometry of internal tumours (Christensen 1983), the possible effects on the genetic system should be carefully studied. More so because degradation of DNA in aqueous solutions and destruction of cells have already been reported to be induced by ultrasound, mostly by the shearing stress of cavitation (Miller and Brayman 1997). Thus it is quite possible that the shearing stress as well as the chemical effects of the free radicals produced during the collapse of ultrasound-induced cavitation bubbles might possibly be responsible for the origin of the clastogenic effects observed in both fish and mice in the present study. Further, the failure to observe induction of SCE at enhanced rate in *in vitro* cultured lymphocytes (Lundberg et al. 1982; Miller et al. 1991) may not reflect the true picture in *in vivo* system, as the possibility of differential response in *in vitro* and *in vivo* systems cannot be totally ruled out (Liebeskind et al. 1979; Ciaravino et al. 1985, 1986). Therefore, although the present study should not discourage the use of ultrasonication totally as a medical tool, certainly its indiscriminate and overuse should be of some risk to the genome and therefore, avoided as far as practicable.

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REFERENCES

Au WW, Obergoenner N, Goldenthal KL, Corry PM, Willingham V (1982) Sister-chromatid exchanges in mouse embryos after exposure to ultrasound in utero. Mutat Res 103:315-320

Baker ML, Dalrymple GV (1978) Biological effects of diagnostic ultrasound: A review. Radiology 126:479-483

Becher R, Zimmer G, Schmidt CG, Sandberg AA (1983) Sister chromatid exchange and proliferation pattern after ultrasound exposure *in vivo*. Am J Hum Genet 35:932-937

Christensen DA (1983) Thermometry and thermography. In: Storm FK (ed) Hyperhermia in Cancer Therapy. GK Hall Medical Publishers, Boston, p 223-232
Ciaravino V, Brulfert A, Miller MW, Jacobson-Kram D, Morgan WF (1985) Diagnostic ultrasound and sister chromatid exchanges: failure to reproduce positive findings. Science 227:1349-1351
Ciaravino V, Miller MW, Carstensen EL (1986) Sister-chromatid exchanges in human lyphocytes exposed in *in vitro* to therapeutic ultrasound. Mutat Res 172:185-188.
Haupt M, Martin AO, Simpson JL, Iqbal MA, Elias S, Dyer A, Sabbagha RE (1981) Ultrasonic induction of sister chromatid exchanges in human lymphocytes. Hum Genet 59:221-226
Hedges MJ, Leeman S (1979) 1.5 MHz ultrasound irradiation of human lymphocytes. Int J Radiat Biol Relat Stud Phys Chem Med 35:301-311
Hill CR (1968) The possibility of hazard in medical and industrial applications of ultrasound. Br J Radiol 41:561-569
Jana AK, Agarwal S, Chatterjee SN (1990a) The induction of lipid peroxidation in liposomal membrane by ultrasound and the role of hydroxyl radicals. Radiat Res 124:7-14
Jana AK, Agarwal S, Chatterjee SN (1990b) Membrane lipid peroxidation by ultrasound: Mechanism and implications. J Biosci 15:211-215
Jana AK, Chatterjee SN (1995) Estimation of hydroxyl free radicals produced by ultrasound in fricke solution used as a chemical dosimeter. Ultrasonics Sonochem 2:87-91
Kremkau FW (1979) Cancer therapy with ultrasound: A historical review, J Clin Ultrasound 7:287-300
Leibeskind D, Bases R, Mendez F, Elequin F, Koenigsberg M (1979) Sister chromatid exchanges in human lymphocytes after exposure to diagnostic ultrasound. Science 205:1273-1275
Lundberg M, Jerominski L, Livingston G, Kochenour N, Lee T, Fineman R (1982) Failure to demonstrate an effect of *in vivo* diagnostic ultrasound on sister chromatid exchange frequency in amniotic fluid cells. Am J Med Genet 11:31-35
Marmor JB (1983) Cancer therapy by ultrasound. In: JT Lett, UK Ehman, AB Cox (eds) Advances in Radiation Biology, vol 10. Academic Press, New York, p 105-133
Miller MW, Wolff S, Filly R, Cox C, Carstensen EL (1983) Absence of an effect of diagnostic ultrasound on sister-chromatid exchange induction in human lymphocytes *in vitro.* Mutat Res 120:261-268
Miller MW, Azadniv M, Cox C, Miller WM (1991) Lack of induced increase in sister chromatid exchanges in human lymphocytes exposed to *in vivo* therapeutic ultrasound. Ultrasound Med Biol 17:81-83
Miller MW, Brayman AA (1997) Comparative sensitivity of human erythrocytes and lymphocytes to sonolysis by 1-MHz ultrasound. Ultrasound Med Biol 23:635-638
Princi D, Pantano E, Pantano F, Buccarelli P (2000) Intrauterine fetal death in twin pregnancy. Minerva Ginecol 52:123-126
Simonovsky V (2000) Detection of acute appendicitis using ultrasonography. Rozl Chir 79:215-220
Tateno H, Kimura Y, Yanagimachi R (2000) Sonication per se is not as deleterious to sperm chromosomes as previously inferred. Biol Reprod 63:341-346