J. Pharm. Pharmacol. 2000, 52: 779–784
Received November 17, 1999
Accepted March 20, 2000

Cytotoxicity and Teratogenicity of Chlorhexidine Diacetate Released from Hollow Nylon Fibres

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Abstract

Intra-uterine contraceptive devices are associated with an increased incidence of pelvic infections, possible due to the introduction of vaginal bacteria into the uterus at insertion. One potential means to overcome this problem is the use of a device which releases the antimicrobial agent chlorhexidine although such an approach carries with it the risk of adverse effects on the endometrium and, possibly, teratogenic effects.

Cultured monolayers of endometrial cells were used to assess the cytotoxicity of both chlorhexidine and chlorhexidine-releasing devices. The results indicated that the agent is toxic at concentrations of 1 μg mL^{-1} and that the devices potentiated the toxicity. When the devices were tested in a guinea-pig model, endometrial damage was seen only at the high dose of chlorhexidine, suggesting that there is greater distribution of chlorhexidine in-vivo. Assessment of the teratogenic effects of chlorhexidine in rat embryonic limb bud tissue cells in-vitro showed that the foetal cells were highly susceptible to the toxic effects of chlorhexidine, but that there was no evidence of teratogenicity.

Overall, the findings suggest that chlorhexidine-releasing devices may be a safe means of reducing infections related to intra-uterine devices, but that the chlorhexidine may have a toxic effect on foetal cells.

The use of intra-uterine contraceptive devices (IUDs) is associated with an increased risk of pelvic infection, probably due to the carriage of vaginal bacteria into the uterus at the time of insertion; the relative risk of pelvic inflammatory disease is 1·5–2·6 in IUD users compared with women using no contraception (Grimes 1987). We have previously reported two developments aimed at decreasing
the incidence of device-related uterine infections. The first was the use of hollow, chlorhexidine-releasing hollow nylon fibres (Reynolds et al 1991); the second was a chlorhexidine-releasing hydrogel coating for the IUD marker tails (Bilbruck et al 1994). In comparison with unmodified devices, both prototypes, when inserted transcervically into the uteri of guinea-pigs, significantly reduced the extent of uterine bacterial contamination within 24 h (Reynolds et al 1991; Bilbruck et al 1994). The aim of this study was to explore the potential adverse effects of the chlorhexidine-releasing hollow fibres. Chlorhexidine is a biguanide antiseptic which acts by disruption of the cytoplasmic membrane, especially of Gram-positive organisms. Minimal inhibitory concentrations of chlorhexidine in-vitro, range from 1 μg to 1 mg mL^{-1}, according to the bacterium and the type of culture medium. It was considered suitable for intra-uterine use as it is used widely in similar tissues (0·2-μg-mL^{-1} aqueous solutions are used for bladder irrigation and 5-μg-mL^{-1} solutions in 70% ethanol are used as a pre-operative skin disinfectant) and it is generally considered to be non toxic. The current study used in-vitro and in-vivo techniques to assess possible adverse effects on the endometrium of high concentrations of chlorhexidine, such as those which may occur immediately adjacent to the drug-delivering device. In addition, because of the known failure rate of intra-uterine contraceptive devices: 0·08 pregnancies per 100 women-years for levonorgestrel-releasing devices (Toivonen et al 1991) and the

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use of intra-uterine systems for reasons other than contraception (Luukkainen & Toivonen 1995), the possible teratogenic effects of the chlorhexidine-releasing devices were investigated.

Materials and Methods

Drug release

Hollow nylon fibres (Standard grade Nylon 6, internal diameter: 0·50 mm; external diameter: 0·63 mm; Portex, Basingstoke, UK) of 100 mm length were filled with 100 μg chlorhexidine diacetate in 20 μL ethanol (70% v/v) or with vehicle alone, and the ends were heat-sealed. The fibres were then immersed in 30 mL of 0·9% w/v saline. At various times, samples were removed from the immersion fluid for determination of chlorhexidine concentration by UV absorption at 253 nm, after which they were returned ($n = 10$ in all cases).

Assessment of toxicity in-vitro

RENT4 rat endometrial cells (Wiehle et al 1990; ECACC No 92092512) were grown in 1·0 mL of Dulbecco's modified minimum essential medium (DMEM) containing 10% heat-inactivated foetal calf serum (FCS), 20 μg mL^{-1} glycine–histidine–lysine tripeptide, 80 int. units L^{-1} insulin, 10 000 U L^{-1} penicillin and 5 μg L^{-1} streptomycin. The cells were maintained at 36·5°C with 5% CO_2 and were seeded into 6-well plates (well diameter: 2·5 cm) at a density of 50 000 cells/well. Toxicity of chlorhexidine was assessed either by placing fibres containing varying amounts of the drug (in 20 μL of 70% ethanol) on to the cultured monolayer of RENT4 rat endometrial cells or by adding chlorhexidine, in not more than 30 μL of 70% ethanol, directly to the culture medium. The cells were maintained in contact with the chlorhexidine or chlorhexidine-releasing fibres for five days after which cell suspensions were produced by incubation with 0·25% trypsin-EDTA solution for 10 min at 36·5°C . Cell viability was assessed directly by trypan-blue exclusion or indirectly by determination of mitochondrial dehydrogenase activity. Each experiment was performed, in triplicate, on 3 separate occasions.

Viable cells exclude trypan blue while dead cells are stained by the dye. Trypan blue (20 μL; 0·5% v/v) was added to cell suspension (20 μL) and the cells not stained by the dye were counted following microscopic examination. Results are expressed as a percentage of a parallel, untreated, control.

The mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of the yellow 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to yield purple formazan crystals which are insoluble in aqueous solutions. MTT (200 μg in 100 μL) was added to 1 mL of cell suspension; the crystals formed were dissolved in acidified propan-2-ol and the UV absorbance of the resulting purple solution was determined at 570 nm against 690 nm for blank solution. The amount of formazan produced is proportional to the number of viable cells.

Assessment of toxicity in-vivo

The deleterious effects on the endometrium of 100-mm hollow fibres containing either 1·0 or 2·8 mg of chlorhexidine diacetate in 20 μL ethanol (70% v/v) or vehicle control were assessed using guinea-pigs as a mammalian model.

Groups of 6 virgin female Dunkin-Hartley guinea-pigs, 300–500 g, housed in wire-bottom cages, were used. On the day of oestrus, as determined by vaginal opening, neuroleptic analgesia was induced by intraperitoneal administration of diazepam (2·5 mg kg^{-1}, Roche Products Limited, Welwyn Garden City, Herts, UK), followed by intramuscular administration of 0·5 mL kg^{-1} Hypnorm (a mixture of fentanyl citrate, 0·315 mg mL^{-1} and fluanisone, 10 mg mL^{-1}; Janssen Pharmaceuticals, Oxford, UK). Following laparotomy a hollow nylon fibre was introduced into one of the uterine horns through a small incision which was subsequently repaired using a silk suture; the abdominal incision was then repaired with surgical clips.

Five days after surgery the guinea-pigs were killed and the uterine horns removed, sectioned, fixed and stained with haematoxylin and eosin. Each section was examined by light microscopy and for each treatment group the numbers of mitotic and dead cells within the endometrial epithelium and stroma and glandular epithelium of the horn containing the fibre (ipsilateral horn) were determined in 10 random fields of 750 μm^2. Values for each horn were compared with those of the contralateral horn. This procedure was licensed under the Scientific Procedures (Animals) Act, 1986 (UK).

Teratogenicity

Female Wistar albino rats (10–12 weeks old) were bred in-house. The rats were mated overnight and the appearance of a vaginal plug by the following morning was considered indicative of pregnancy. The day of the appearance of the plug was designated as day 0 of gestation. On day 13 of gestation

the dams were killed and the uteri were removed aseptically and placed into phosphate-buffered saline prewarmed to 37°C. Limb bud tissues were removed from the embryos post-mortem and were dissociated into individual cells by successive washing in trypsin (1% in phosphate-buffered saline containing 50 mM tris acetate) for 15 min at 37°C. The resultant suspension was then filtered through a sterile nylon filter (10 μm mesh), placed in culture medium consisting of Ham F12 plus 10% FCS and adjusted to $2{\cdot}5 \times 10^7$ cells mL^{-1} (with a viability of greater than 95%). Samples (20 μL) of the cell suspension were delivered to each well of 12-well tissue-culture plates. The cells were allowed to settle and adhere to the dish for 2 h at 37°C after which 1 mL of culture medium was added. To assess the teratogenic effects, chlorhexidine or the chlorhexidine-releasing fibres were placed in the culture wells together with the cells as described for RENT4 cells. The fibre or drug was left in the dish for 5 days and incubated at 37°C, after which the number of differentiated foci were counted using the alcian blue staining method of Flint et al (1984). Each dose of drug was tested, in triplicate, on 3 separate occasions. This procedure was licensed under the Scientific Procedures (Animals) Act, 1986 (UK).

Data analysis

The results presented are the means ± s.e.m. of at least 3 separate experiments. The effects of the increasing concentrations of chlorhexidine were assessed using one-way analysis of variance. The results of the assessment of toxicity in-vivo were analysed using the Wilcoxon non-parametric test.

Results

Chlorhexidine release in-vitro

Approximately 13% of the 100-μg chlorhexidine reservoir had been released into the surrounding fluid within the first 24 h of the study. This release continued until approximately 20% had been released by day 7 (Figure 1). The mean rate of release over the first 6 days was $3{\cdot}3\ \mu g\ d^{-1}$.

In-vitro toxicity

Addition of 30 μL of 70% ethanol to the culture medium (1 mL) of the RENT4 cells had no significant effect on viability, as determined by exclusion of trypan blue. The results presented in Figure 2 demonstrate that addition of chlorhexidine

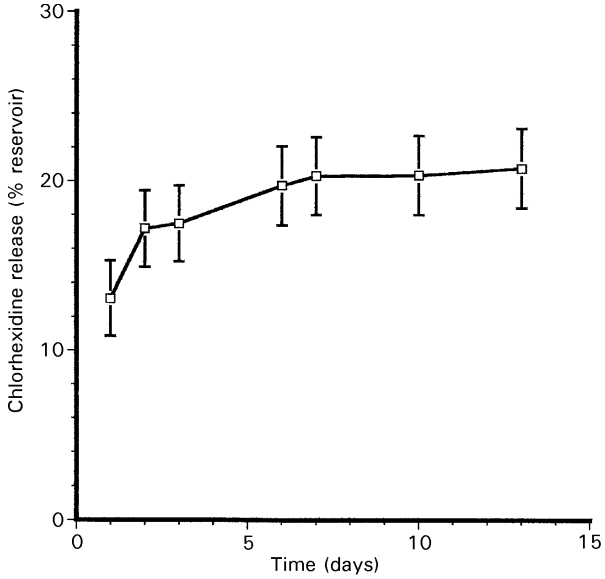

Figure 1. The release of chlorhexidine diacetate in ethanol (70% v/v) from hollow nylon fibres (reservoir: 100 μg in 20 μL) into an aqueous environment. Results represent the mean ± s.e.m. of 10 experiments.

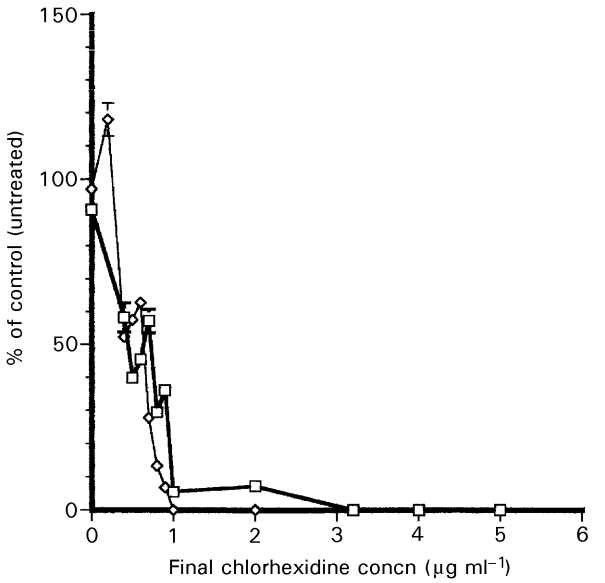

Figure 2. The effects of chlorhexidine diacetate on viability of cultured RENT4 endometrial cells as determined by trypan blue exclusion (□) and mitochondrial dehydrogenase activity (◇). Each point represents the mean ± s.e.m. of 9 determinations.

diacetate directly to the culture medium significantly reduced the endometrial cell viability, as determined by both trypan blue exclusion and mitochondrial dehydrogenase activity ($P < 0{\cdot}005$, one-way analysis of variance) with cell survival being reduced to 50% of control at a concentration of approximately $0{\cdot}5\ \mu g.mL^{-1}$ (1×10^{-6} M). In comparison with direct administration, administra-

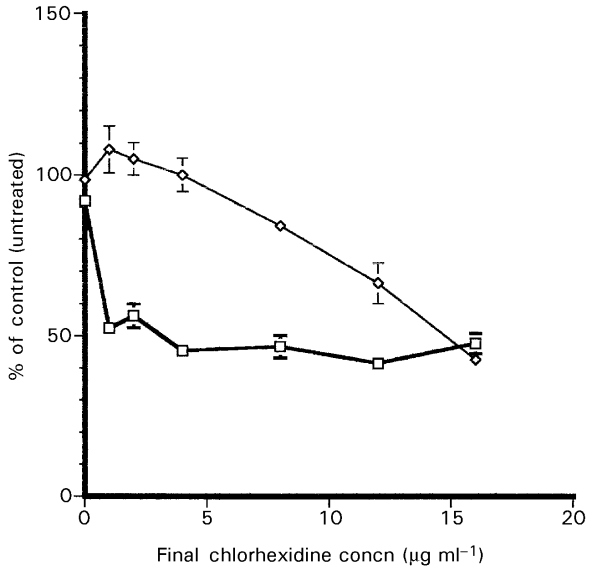

Figure 3. The effects of chlorhexidine diacetate, delivered via a hollow nylon fibre, on viability of cultured RENT4 endometrial cells as determined by trypan blue exclusion (□) and mitochondrial dehydrogenase activity (◇). Each point represents the mean ± s.e.m. of 9 determinations.

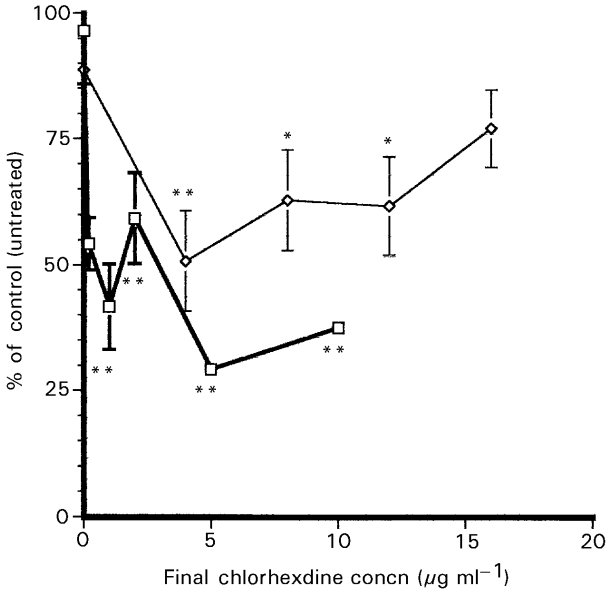

Figure 4. The effects of chlorhexidine diacetate administered either directly (□) or delivered via a hollow nylon fibre (◇), on differentiation/viability of foetal rat limb bud cells in-vitro. Each point represents the mean ± s.e.m. of 9 determinations; $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ compared with vehicle control.

tion of the chlorhexidine via the hollow nylon fibres caused a 50% decrease of viability at a dose of 1 μg when viability was assessed by trypan blue exclusion and 15 μg when assessed by mitochodrial dehydrogenase activity (Figure 3).

In-vivo toxicity

There were no significant differences in the numbers of endometrial mitotic cells or dead cells between the ipsilateral and contralateral uterine horns in those animals implanted with hollow nylon fibres containing 70% ethanol. Similarly, there were no significant differences in animals implanted with hollow nylon fibres containing 1 mg chlorhexidine acetate. In those animals implanted with fibres containing 2·8 mg chlorhexidine there was a significant decrease in the number of mitotic cells and a significant increase in the number of dead cells in the endometrial stroma of the ipsilateral horn ($P < 0{\cdot}05$), but no significant effects in the endometrial or glandular epithelia. It was observed that all fibres, including those containing vehicle alone, appeared to cause an increase in the number of leucocytes within the endometrium, but this effect was not quantified.

Teratogenicity

The results presented in Figure 4 indicate that in comparison with control, chlorhexidine at concentrations as low as 0·2 μg mL^{-1} of cell culture medium produced a significant (approximately 50%) reduction in the staining of differentiated limb bud foci ($P < 0{\cdot}01$, Student's independent *t*-test). When administered via a hollow fibre, the chlorhexidine reduced limb bud differentiation to 50% of the control at a dose of 4 μg within the fibre ($P < 0{\cdot}05$, Student's independent *t*-test); higher doses also significantly reduced differentiation, although paradoxically there was no significant effect at the highest dose of 16 μg.

Discussion

Approximately 20% of a 100-μg reservoir of chlorhexidine was released from the hollow fibres by day 7, highlighting the concentration-dependent (first-order) nature of the chlorhexidine release from these devices. In the subsequent studies of the toxicity of the released chlorhexidine, the fibres were loaded with 1–20 μg chlorhexidine in 70% ethanol. Thus it could be predicted that the rate of release into an aqueous medium would be very slow, somewhat less than 3 μg per day. In the course of these experiments, however, the fibres were incubated for 5 days in tissue-culture medium containing both lipids and plasma proteins. It might be expected, therefore, that the rate of release into the culture medium would be greater than into saline, although the actual concentrations of chlorhexidine in the medium were not determined.

Furthermore it is also highly unlikely that the drug distribution within the culture medium was homogeneous, with the concentrations probably being greater in the immediate vicinity of the fibre.

Both assays of cell viability indicated that chlorhexidine is highly toxic to endometrial cells in vitro, the IC50 (concentration required to reduce cell viability by 50%) being approximately $0{\cdot}5\ \mu g\ mL^{-1}$ (1×10^{-6} M). When the same doses of chlorhexidine were administered within hollow fibres, marked toxicity (50% reduction in viability) was detected by the method of trypan blue exclusion at doses as low as $1\ \mu g$, although the MTT method did not detect toxicity until doses of approximately $15\ \mu g$. This difference probably reflects the mechanism of action of chlorhexidine and the duration of the study. There are two distinct phases of chlorhexidine action: low concentrations induce leakage of intracellular components due to an effect on the cell membrane (Rye & Wiseman 1964), but as time or concentration increases the chlorhexidine enters the cell and precipitates cytoplasmic and membrane proteins (Hugo & Longworth 1966). The former phase is detected by the trypan blue method, whilst the MTT method would detect only the second phase.

Importantly, the results indicate a greater degree of toxicity of the chlorhexidine-releasing devices than would be predicted from the estimated rate of chlorhexidine release. Such a finding suggests that there is non-homogeneous distribution of the chlorhexidine within the medium surrounding the fibre although viable RENT4 cells were seen adjacent to the fibres.

We have previously reported that nylon hollow fibres containing 1·65 mg chlorhexidine are able to exert an antimicrobial effect in-vitro and that fibres with a coating containing chlorhexidine were able to reduce the extent of uterine microbial contamination when inserted transcervically in guinea-pigs (Reynolds et al 1991; Bilbruck et al 1994). In the present study, the effects on the endometrium of fibres containing 1·0 and 2·8 mg chlorhexidine were assessed. All fibres, including those containing vehicle alone, caused an increase in the number of leucocytes within the endometrium. This is indicative of an inflammatory response which may have been induced by the physical presence of the fibre within the uterus, or perhaps by the introduction of microorganisms at the time of device insertion, although this is unlikely as all devices were sterilized with 70% ethanol before insertion and the contained chlorhexidine is known to be antimicrobial. There were no other signs of any deleterious effects of the control fibres or of the fibres containing 1 mg chlorhexidine. Those fibres containing 2·8 mg chlorhexidine were seen to induce endometrial damage as evidenced by an increase in the number of dead cells and a decrease in the number of mitotic cells in the endometrial stroma. The dose required to produce endometrial toxicity in-vivo ($>1{\cdot}0$ mg) is therefore much greater than the dose producing toxicity in-vitro ($1-15\ \mu g$). These results would suggest that within the physiological environment, where drugs administered to the uterus might be rapidly distributed by the capillaries or lymph system, the high concentrations of chlorhexidine that might be expected to accumulate in the immediate vicinity of the drug-releasing fibre are without adverse effect, although toxicity was seen at the higher dose.

One possible adverse property of a chlorhexidine-releasing fibre is its effect on foetal development. The ability of a drug to inhibit limb bud differentiation by more than 50% has been shown to be significantly correlated with teratogenic effects in-vivo (Flint et al 1984). The results of the limb bud assays show a reduction in limb bud differentiation, indicating a possible teratogenic effect. These results were, however, confounded by the cytotoxic actions of chlorhexidine. As described earlier, chlorhexidine concentrations as low as $1\ \mu g\ mL^{-1}$ were seen to be toxic to endometrial cells. The foetal cells appeared to be more susceptible to the effects of the drug, thus a 50% reduction in viability occurred at concentrations as low as $0{\cdot}2\ \mu g\ mL^{-1}$. The results of the limb bud assay therefore reflect a cytotoxic effect of chlorhexidine on foetal cells, rather than an inhibition of differentiation. These findings suggest that the chlorehexidine-releasing device may have a toxic effect on foetal cells, but there was no evidence of teratogenicity.

In conclusion, this study has shown that chlorhexidine-releasing hollow nylon fibres are cytotoxic when tested in-vitro, but that there is evidence of endometrial toxicity in-vivo only when the fibres contain high doses. In contrast, foetal cells appear to be more susceptible to the toxic effects of chlorhexidine although there is no evidence of teratogenicity.

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