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DISSERTATION

Effects of Nucleoside Analogues on Protein Expression in Cells of the SerW3 Cell Line

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Content

Abbreviation

AIDS	acquired immunodeficiency syndrome
AJ	adherens junction
APS	ammonium peroxodisulfate
b.i.d.	twice a day
BPA	bisphenol A
BSA	bovine serum albumin
BTB	blood–testis barrier
cAMP	cyclic adenosinemonophosphate
CHO	Chinese hamster ovary
CMV	cytomegalovirus
DDT	dichlorodiphenyltrichloroethane
DED	diethyldithiocarbamate
DEHP	di-2-ethylhexylphthalate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein–Barr virus
EDTA	ethylenediamintetraacetate
EPA	environmental protection agency
ERK	extracellular-signal-regulated kinases
ES	ectoplasmic specialization
FDA	Food and Drug Administration
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
GJ	gap junction
GJIC	gap junctional intercellular communication
GMP	guanosine monophosphate
GTP	guanosine triphosphate

HBV	hepatitis B virus
HIV	human immunodeficiency virus
HN2	nitrogen mustard
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HSV	herpes simplex virus
HSV-TK	herpes simplex virus-thymidine kinase
IF	intermediate filament
i.p.	intraperitoneal
KDa	kilodalton
kg	kilogram
KSHV	Kaposi's sarcoma-associated herpesvirus
LD10	lethal dose 10%
lgG	immunoglobulin G
mA	milliampere
MEHP	mono(2-ethylhexyl)-phthalate
mg/kg	milligram/kilogram
mg/l	milligram/liter
mRNA	messenger ribonucleic acid
pU97	UL97-encoded protein kinase
PBS	phosphate buffered saline
PCP	phenylcyclohexylpiperidine
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidenedifluoride
p,p'-DDE	1,1-bis-(4-chlorophenyl)-2,2-dichloroethene
SCO	Sertoli cell only
SD	standard deviation
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
t.i.d.	three times a day
TEMED	tetramethylethylenediamine
TUNEL	TdT-mediated dUTP-biotin nick end labeling
TJ	tight junction
TP	triphosphate
V	volte
ZO	zonula occludens
ZVZ	varicella zoster virus

Abstract

Nucleoside analogues are important antiviral agents used to treat infections with herpes simplex and other viruses. They have the potential to impair male fertility, however, there is still limited information on this topic. In the experiments presented here, the effects of four nucleoside analogues were studied in vitro utilizing the SerW3 cell line, derived from rat Sertoli cells. Cells were cultured for three days in DMEM supplemented with four concentrations of the drugs: aciclovir and ganciclovir (0.3 mg/l, 1 mg/l, 3 mg/l, 10 mg/l), and famciclovir and penciclovir (3 mg/l, 10 mg/l, 30 mg/l, 100 mg/l), then later compared with the control group by performing Western Blot analysis, in which the expression of two junctional proteins (connexin43 and N-cadherin) and one protein of the cytoskeleton (vimentin) was determined. In addition, at the end of the culture period, immunofluorescence was performed using primary antibodies against connexin43, N-cadherin and vimentin, and a FITC-labeled secondary antibody.

In the Western Blot analysis, aciclovir was found to cause a significant effect at the highest concentration tested (10 mg/l), which is less than the peak concentration measured in patients after intravenous infusion of the drug. The content of connexin43, vimentin and N-cadherin decreased to 49.8 ± 17.2 %, 68.3 ± 7.6 % and 75.3 ± 1.7 % of the control values, respectively (n=3; mean ± SD). Similar effects were observed with ganciclovir (43.2 ± 10.8 %; 76.9 ± 4.5 %; 84.4 ± 10.8 % of the control values). Penciclovir caused less pronounced effects at 10.0 mg/l medium (82.1 ± 20.6 %; 83.0 ± 17.9 %; 76.5 ± 17.7 % of controls). Only a slight effect was observed with the prodrug famciclovir; even at a 10-fold concentration (100 mg/l), only moderate changes were induced. A change in protein content to values of 83.5 ± 1.7 %, 91.2 ± 3.3 %, and 85.3 ± 5.1 %, respectively, was observed for the three proteins when the cells were exposed to famciclovir. Using the immunofluorescence technique, the fluorescence intensity of connexin43, N-cadherin and vimentin was observed to decrease after three days of culture with aciclovir, ganciclovir and penciclovir when compared to the control group, but no aberrant localization was detected.

In these experiments, compared to N-cadherin and vimentin, connexin43 was found to be the best marker for detecting negative effects on these cells. In summary, aciclovir and ganciclovir showed stronger effects on the SerW3 cells compared to famciclovir and penciclovir.

Zusammenfassung

Nukleosid-Analoga sind wichtige antiviral wirksame Arzneimittel, die zur Behandlung von Herpes simplex-Infektionen und anderen Viruserkrankungen eingesetzt werden. Es gibt tierexperimentelle Hinweise, dass sie die männliche Fertilität schädigen können. Die bisher publizierten Daten sind jedoch sehr lückenhaft, insbesondere fehlen direkt vergleichende Untersuchungen. Die hier beschriebenen Experimente sollen einen Beitrag dazu leisten, die Situation zu verbessern. Im Rahmen dieser Arbeit wurden die Wirkungen von vier Nukleosid-Analoga auf SerW3-Zellen untersucht. Die SerW3-Zelllinie wurde aus Sertoli-Zellen von Ratten abgeleitet. Die Zellen wurden für drei Tage in einem DMEM Kulturmedium mit vier verschiedenen Konzentrationen von Aciclovir und Ganciclovir (0,3 mg/l, 1 mg/l, 3 mg/l, 10 mg/l), sowie Famciclovir und Penciclovir (3 mg/l, 10 mg/l, 30 mg/l, 100 mg/l) behandelt. Anschließend wurden zwei Transmembranproteine (Connexin-43 und N-Cadherin) und ein Desmin (Vimentin) per Western Blot analysiert; die Ergebnisse wurden mit denen von Kontrollzellen verglichen. Zusätzlich wurden die Zellen am Ende der Kultur mit Antikörpern gegen Connexin-43, N-Cadherin und Vimentin unter Verwendung eines FITC-markierten sekundären Antikörpers markiert und mit Hilfe der Fluoreszenzmikroskopie untersucht.

In der Western Blot-Analyse zeigte sich, dass Aciclovir in der höchsten untersuchten Konzentration (10 mg/l) einen signifikanten Effekt auf die genannten Proteine hat. Diese Konzentration von 10 mg/l ist geringer, als die mittlere maximale Plasmakonzentration, die bei Patienten nach intravenöser Gabe von Aciclovir gemessen wird. Die Gehalte von Connexin-43, Vimentin and N-Cadherin sinken auf Werte von 49,8 ± 17,2 %, 68,3 ± 7,6 % und 75,3 ± 1,7 % im Vergleich zur Kontrollgruppe (n=3; Mittelwert ± SD). Ein entsprechender Effekt konnte auch mit Ganciclovir beobachtet werden (43,2 ± 10,8 %; 76,9 ± 4,5 %; 84,4 ± 10,8 % im Vergleich zur Kontrollgruppe). Penciclovir zeigte einen geringeren Effekt bei 10,0 mg/l (82,1 ± 20,6 %; 83,0 ± 17,9 %; 76,5 ± 17,7 % im Vergleich zur Kontrollgruppe). Noch weniger ausgeprägt waren die Veränderungen nach Behandlung der Zellen mit dem Prodrug

Famciclovir: nach Exposition mit der 10-fach höheren Konzentration (100 mg/l) konnte für die drei Proteine eine Reduktion des Proteininhaltes auf 83,5 ± 1,7 %, 91,2 ± 3,3 % und 85,3 ± 5,1 % der Kontrollwerte festgestellt werden. In der Fluoreszenz-Mikroskopie wurde bei Benutzung der Antikörper gegen Connexin-43, N-Cadherin und Vimentin in der genannten Reihenfolge eine zunehmende Fluoreszenz beobachtet. Nach Tagen der Kultivierung mit Aciclovir, Ganciclovir und Penciclovir war beim Vergleich mit der Kontrollgruppe tendenziell eine Abnahme der Fluoreszenz aber keine aberrante zelluläre Lokalisation der Proteine zu erkennen.

Im Vergleich der analysierten Proteine stellte heraus, dass Connexin-43 der beste Marker war, um zelluläre Veränderungen durch die untersuchten Arzneistoffe zu beschreiben. Zusammenfassend kann gesagt werden, dass Aciclovir und Ganciclovir einen deutlicheren Effekt als Famciclovir und Penciclovir auf die SerW3 Zellen zeigten.

1 Introduction

1.1 Nucleoside analogues

1.1.1 Chemical structure and clinical use

Some nucleoside analogues are important antiviral agents. They are used to treat viral infections such as herpes simplex virus (HSV), cytomegalovirus (CMV) and varicella zoster virus (VZV) infections. Aciclovir, a guanosine analogue, is one of the most commonly used antiviral drugs.

Aciclovir is a modified nucleoside, in which the sugar ring is replaced with an open-chain structure. It was discovered in 1971 (Schaeffer et al., 1971) and regarded as the start of a new era in antiviral therapy (de Clercq and Field, 2006). Till now, although more new antiviral compounds were developed, aciclovir is still one of the most useful agents against herpes simplex virus infections (HSV-1 and -2) (Gumina et al., 2001) and it is still the first-line therapy for the treatment of varicella zoster virus infections (Freeman and Gardiner, 1996). Because it is widely used for treating herpesvirus infections, aciclovir has been considered to be the "gold standard", against which any new antiviral compounds must be measured (Field, 1996). According to the Full Prescribing Information on Zovirax®, this aciclovir-containing drug is indicated for the acute treatment of herpes zoster, initial episodes and recurrent episodes of genital herpes, as well as varicella (GlaxoSmithKline, 2012; Full Prescribing Information Zovirax®).

Ganciclovir is the 2-hydroxymethyl analogue of aciclovir. It shows higher activity against CMV (Freeman and Gardiner, 1996) and extends the spectrum of activity of antiviral compounds (Field, 1996). CMV occurs commonly in immunocompromised patients, including solid organ transplant recipients (McGavin et al., 2001). Cymevene® (ganiclovir) administered by intravenous infusion is indicated for the treatment of confirmed sight-threatening CMV disease in patients with AIDS and other severely immunocompromised individuals, CMV pneumonitis in bone marrow transplant patients and the prophylaxis of CMV infection. However, ganciclovir is indicated neither for congenital nor neonatal CMV disease, nor in immunocompetent individuals (Roche, 2010; Full Prescribing Information Cymevene®-IV)

possibly because of its negative clinical outcomes. For example, almost two thirds of treated infants had significant neutropenia during therapy in a controlled trial which was performed for the treatment of neonates congenitally infected with CMV (Kimberlin et al., 2003).

When the ether oxygen in ganciclovir was replaced with a methylene substitution, penciclovir was produced (Harnden et al., 1987), which showed an activity profile similar to that of aciclovir but had an activity against HBV (Shaw et al., 1994). By the time of its development, its poor oral bioavailability was already well recognized and thus its prodrug (diacetyl 6-deoxypenciclovir) with the generic name famciclovir was designed (Vere Hodge et al., 1989). Famciclovir is well absorbed after oral administration. Penciclovir reaches higher concentrations in deeper epidermal layers and it is commonly prescribed as topical cream Denavir® (Hasler-Nguyen et al., 2009). When compared with aciclovir, penciclovir has a similar activity against HSV-1 and VZV (Field, 1996), but it is less active against HSV-2 (Simpson and Lyseng-Williamson, 2006). Famvir® is indicated for herpes zoster, recurrent herpes labialis and recurrent episodes of genital herpes including HIV-infected adult patients. It is not licensed for patients under 18 years old (Novartis, 2012; Full Prescribing Information Famvir®). For the treatment of recurrent orolabial herpes in immunocompetent patients, oral famciclovir given as a single 1500 mg dose was approved by the FDA. There were not only clinical trials around higher dose single-day famciclovir therapy (Aoki et al., 2006; Spruance et al., 2006; Bodsworth et al., 2009), but also 2-day therapy with famciclovir (Bodsworth et al., 2008). Single-day famciclovir could be appropriate as a first-line therapy for recurrent orolabial and genital herpes (Modi et al., 2008). Other short-course regimens have also been shown to be as effective as traditional 5-day regimens for recurrent episodes: 3-day therapy with valaciclovir (Leone et al., 2002), 2-day therapy with higher dose aciclovir (Wald et al., 2002). The short-course therapy has several benefits, including convenience, reduced cost and improved compliance (Bodsworth et al., 2009).

1.1.2 Mechanism of antiviral action

Aciclovir is considered to have low toxicity for normal host cells. It inhibits HSV and VZV with high selectivity because only viral thymidine kinase phosphorylates aciclovir to the monophosphate effectively. Aciclovir is first converted into acyclo-guanosine monophosphate (acyclo-GMP), which is further phosphorylated into the active triphosphate form, acyclo-guanosine triphosphate (acyclo-GTP). The substrate aciclovir-triphosphate stops replication of herpes viral DNA resulting in premature chain termination and acyclo-GTP persists in HSV-infected cells after aciclovir is removed from the medium (Elion, 1993).

Like aciclovir, penciclovir rapidly diffuses into both uninfected and HSV-infected cells, but effective phosphorylation occurs only in infected cells carrying HSV-encoded thymidine kinase. Compared to aciclovir, penciclovir demonstrates different intracellular dynamics. Penciclovir is phosphorylated more rapidly than aciclovir within HSV-infected cells, reflecting its higher affinity for HSV-encoded thymidine kinase. Penciclovir-triphosphate also demonstrates a prolonged intracellular half-life in cells infected with HSV (10-20 hours), compared with aciclovir-triphosphate (less than one hour). However, the affinity of penciclovir-triphosphate for viral DNA polymerase is 100-fold lower than that of aciclovir (Field, 1996). The reduced affinity of penciclovir triphosphate for viral DNA polymerase offsets its increased affinity for viral thymidine kinase, which may explain the similar antiviral activity of penciclovir and aciclovir (Gnann, 2007). Hepatitis B DNA polymerase is also sensitive to inhibition by penciclovir triphosphate. In contrast to herpes virus-infected cells, there is no selectivity at the phosphorylation stage, but very high selectivity at the stage of inhibition of DNA polymerase (Field, 1996).

Aciclovir and penciclovir also share a similar mechanism of drug resistance, including deficient thymidine kinase and mutants with altered DNA polymerase. Approximately 95-96% of aciclovir-resistant HSV isolates are thymidine kinase deficient and the remaining isolates are usually thymidine kinase-altered mutants (Pottage and Kessler, 1995). Penciclovir resistance, like that of aciclovir, is associated with mutations principally in the thymidine kinase gene or to a much lesser extent in the DNA polymerase gene, for example a single base change only leads to

a single amino acid substitution (Chiou et al., 1995). In contrast to aciclovir, in which resistance-conferring mutations occur preferentially in genetic hot spots, the mutations that confer penciclovir resistance occur randomly throughout the entire coding region of the viral thymidine kinase (Vinh and Aoki, 2006). The occurrence of resistance is related to the immune state of the patients and the strains of the virus. For example, in one study penciclovir-resistant HSV was isolated from 0.22% of immunocompetent patients and 2.1% of immunocompromised patients (Sarisky et al., 2003). Prevalence of resistance differs between the strains of HSV. Abraham et al. showed a 3.0% prevalence of aciclovir resistance among HSV-1 strains while in HSV-2 strains, it was 7.8% (Abraham et al., 2007). The strains resistant to aciclovir are almost always cross-resistant to other thymidine kinase-dependent drugs such as penciclovir and famciclovir (Hasegawa et al., 1995). When the resistance of a strain depends on altered DNA polymerase, the cross resistance could be incomplete (Morfin and Thouvenot, 2003).

Like aciclovir and penciclovir, ganciclovir is also activated intracellularly and incorporated into the growing chain of viral DNA. Ganciclovir is preferentially phosphorylated by a protein encoded with the UL97 open reading frame on CMV (Littler et al., 1992). The deletion of UL97 resulted in decreased recognition of ganciclovir as a substrate while maintaining the normal protein kinase functions of pUL97 (Lurain and Chou, 2010). The important role of pUL97 in the mechanism of action implies any antiviral agent that depends on pUL97 for phosphorylation or uses pUL97 as an antiviral target will possibly have cross-resistance with ganciclovir. Since the pUL97 also phosphorylates aciclovir, it is not surprising that the exposure of CMV to aciclovir may display reduced ganciclovir susceptibility (Lurain and Chou, 2010). The appearance of a DNA polymerase resistance mutation has also been reported, which more likely results from continuing viral replication after prolonged drug exposure, usually in patients treated for post-transplant primary CMV infection (Hakki and Chou, 2011).

1.1.3 Plasma concentrations of antiviral drugs during therapy

For HSV infection, aciclovir is prescribed at a dose of 5 mg/kg every 8 hours for 5 days as an infusion while for VZV infection 10 mg/kg every 8 hours for 10 days (GlaxoSmithKline, 2006; Full Prescribing Information Zovirax®-IV). The pharmacokinetics of aciclovir has been evaluated in adult patients with normal renal function after one-hour intravenous infusion every eight hours. The peak plasma concentration (C_{max}) is 9.8 ± 2.6 mg/l and 20.7 ± 10.2 mg/l (mean ± SD) following one hour infusion at doses of 5 mg/kg and 10 mg/kg, respectively (Blum et al., 1982; GlaxoSmithKline, 2006; Full Prescribing Information Zovirax®-IV). Neonates receiving a dose of 10 mg/kg had a peak aciclovir level of 13.9±4.2 mg/l (mean ± SD) (Sullender et al., 1987). When the doses were increased to 45 mg/kg/d and 60 mg/kg/d in newborns, the C_{max} (mean ± SD) was 18.82 ± 5.52 mg/l (Kimberlin et al., 2001).

Drug	Administration	Drug in Plasma	Peak plasma concentrations
Aciclovir	Oral multi-dose 800 mg	Aciclovir	1.4 mg/l (Mean)
	Intravenous 5 mg/kg t.i.d.		9.8 mg/l (Mean)
Valaciclovir	Oral single-dose1000 mg	Aciclovir	5.65 ± 2.37 mg/l (Mean ± SD)
Ganciclovir	Oral 1000 mg t.i.d.	Ganciclovir	1.18 ± 0.36 mg/l (Mean ± SD)
	Intravenous single-dose 5 mg/kg		9.0 ± 1.4 mg/l (Mean ± SD)
Valganciclovir	Oral 900 mg daily	Ganciclovir	5.61 ± 1.52 mg/l (Mean ± SD)
Famciclovir	Oral single-dose 1000 mg	Penciclovir	6.6 mg/l (Mean)
Penciclovir	Intravenous single-dose 20 mg/kg	Penciclovir	22.7±4.2 mg/l (Mean±SD)

Table 1 Peak plasma concentrations in adults after administration of nucleoside analogues

When aciclovir is administered orally, only 15-30% of the dose is absorbed (Gnann, 2007). The C_{max} after dosing of a single 200 mg aciclovir capsule or 200 mg solution is 0.3 ± 0.1 mg/l (mean ± SD) (de Miranda and Blum, 1983). When aciclovir was administrated orally 200 mg four hourly, the C_{max} was 0.49 mg/l (range: 0.47 mg/l - 0.54 mg/l). After oral administration of 800 mg six hourly, the mean C_{max} was 1.4 mg/l (range 0.66-1.8 mg/l (GlaxoSmithKline, 2012; Full Prescribing Information Zovirax®). When the prodrug valaciclovir was administered orally, the C_{max} of aciclovir after single-dose administration of 1000 mg was 5.65 ± 2.37 mg/l (mean ± SD) (GlaxoSmithKline, 2013; Full Prescribing Information Valtrex®).

For CMV infection, ganciclovir is recommended at a dose of 5 mg/kg as intravenous infusion every 12 hours for 2 to 3 weeks (Roche, 2010; Full Prescribing Information Cymevene®-IV). At the end of a 1-hour infusion of 5 mg/kg ganciclovir, the C_{max} (mean ± SD) was 9.0 ± 1.4 mg/l (Roche, 2010; Full Prescribing Information Cymevene®-IV). Another study showed that intravenous infusion of 5 mg/kg daily yields a mean C_{max} of ganciclovir in plasma of 11 mg/l (Laskin et al., 1987). The mean C_{max} observed in newborns was 2-fold lower than that observed in adults after intravenous administration of 5 mg/kg over 1 hour (Trang et al., 1993). In pediatric renal transplant recipients, the C_{max} is 11.77 ± 2.82 mg/l (mean ± SD) (Zhang et al., 2003), which is similar to that of adults with normal renal function. In contrast to intravenous administration, concentrations after oral administration are much lower. The bioavailability of oral ganciclovir is approximately 7% and the mean C_{max} is 0.8 mg/l with t_{max} 6 hours after a 1000 mg oral dose (Boeckh et al., 1998). When 1000 mg ganciclovir was administered three times a day, a mean C_{max} of 1.18 mg/l was measured (Roche, 2001; Full Prescribing Information Cymevene®). If the prodrug valganciclovir instead of ganciclovir was administered orally, the C_{max} (mean ± SD) after a single-dose of 900 mg was 5.61 ± 1.52 mg/l (Roche, 2011; Full Prescribing Information Valcyte®).

The dose of famciclovir for the treatment of herpes zoster is 500 mg three times a day for 7 days while for suppressive therapy of genital herpes it is 250 mg two times a day (Novartis, 2012; Full Prescribing Information Famvir®). After single doses of 250 mg and 500 mg in healthy volunteers, the C_{max} of penciclovir was 1.59 mg/l and 3.34 mg/l, respectively (Pue et al.,

1994). Following oral single-dose administration of 500 mg famciclovir, the C_{max} (mean ± SD) was 4.0 ± 0.7 mg/l. The mean C_{max} after a single-dose of 1000 mg was 6.6 mg/l (Novartis, 2012; Full Prescribing Information Famvir®). In infants aged 6 to 12 months after a single 500 mg dose of famciclovir, penciclovir concentrations were similar to those observed in children aged 6 to 12 years and in adults, although the average systemic exposure to penciclovir was slightly lower in infants (<6 month-old) or children (1 to 5 years old) (Blumer et al., 2010).

The pharmacokinetics of penciclovir administered as an intravenous infusion was investigated in 15 healthy male subjects by Fowles et al. in 1992. The highest concentrations were seen at 45 minutes after the start of infusion. The C_{max} (mean ± SD) following doses of 10, 15 and 20 mg/kg, were 12.1 ± 3.1 mg/l, 19.6 ± 7.5 mg/l and 22.7 ± 4.2 mg/l, respectively (Fowles et al., 1992). The pharmacokinetic analysis of penciclovir in 10 volunteers after intravenous infusion of 250 mg penciclovir revealed a C_{max} (mean ± SD) of 3.63 ± 0.72 mg/l (Xu et al., 2007). A comparison of peak plasma concentrations in adults after administration of four nucleoside analogues is shown in Table 1.

1.1.4 Impairment of fertility

Purine nucleoside analogues showed impairment of male fertility in animal experiments with various species (e.g., mice, rats). Effects on cells of male reproductive organs have been observed in in vitro studies as well. The effects were time- and dose-dependent. They were reversible at lower doses but irreversible at higher ones.

When mice were treated orally with 450 mg aciclovir/kg bodyweight in a preclinical study, no adverse effects on reproduction were observed (Moore et al., 1983). In mice treated intraperitoneally with doses of aciclovir ranging from 4 to 48 mg/kg/day for 15 days, testis weight decreased on day 21 and day 28 at 32 and 48 mg/kg/day and day 35 at all dose levels. Sperm motility was inhibited from day 7 to day 35 after the last exposure with a maximum effect from day 28 to day 35, along with decreased sperm counts and increased sperm abnormalities, which could be recovered on day 70, indicating that aciclovir did not affect stem

cell lines of spermatogenesis (Bairy et al., 2009).

Ganciclovir decreased fertility in male mice after daily i.p. injection of 2 mg/kg bodyweight. The impairment at this low dose was reversible but irreversible or incompletely reversible when the dose was 10 mg/kg (Roche, 2010; Full Prescribing Information Cymevene®-IV). When HSV-1-infected mice received 50 mg/kg to 200 mg/kg of ganciclovir by subcutaneous injection over a 6-week period or uninfected mice received 100 mg/kg for 14 days, a marked testicular weight reduction and Sertoli cell only (SCO) syndrome were observed although the bodyweight increased normally (Neyts et al., 1995). For famciclovir, testicular toxicity was observed following chronic administration in mice at doses of 600 mg/kg/day (Novartis, 2012; Full Prescribing Information Famvir®), but no testicular toxicity was observed after chronic administration of 50 mg/kg for 26 weeks.

Similar experiments were also performed in rats. The administration of aciclovir by intraperitoneal route induced histopathological changes in the testis at doses of 16 and 48 mg/kg, confirming the toxic potential of aciclovir on the reproductive system in male rats (Elham et al., 2013). The effects of short-term treatment of ganciclovir on male reproduction in adult rats were also studied. The animals were treated subcutaneously with either a single-dose of 60 mg/kg daily for 5 days (Gan5day) or with 100 mg/kg administered three times for 1 day (Gan1day). Over 80% of sperm were abnormal in Gan5day group, and only few normal sperm were detected in Gan1day group. Morphological investigation of testes revealed a clear-cut time-dependent effect. Four weeks after treatment distinct alterations were located which were reversible 24 weeks after treatment (Faqi et al., 1997).

Reproductive toxicity of famciclovir was also detected in rats although there was no evidence of adverse effects in the clinical trial (Sacks et al., 1994). According to the studies of Sacks et al., in 34 men who received famciclovir (250 mg b.i.d.) for 18 weeks, proportions of dead, motile or normal sperm or any other semen parameter did not show differences in comparison to 33 placebo recipients. However, in 40 male adult rats, when the doses increased to 135 mg/kg/day for two or four weeks, the mean values of the semen analysis parameters were significantly

reduced when compared with those of the control group. In the recovery group the percentage of abnormal forms was smaller than in the treated groups but greater than in the control group (Helal et al., 2009). For penciclovir, testicular toxicity was also observed in rats treated with a high dose of 160 mg/kg/day intravenously while no adverse testicular effects or impairment of reproductive function were observed at a lower dose of 80 mg/kg/day after 10 to 13 weeks (Novartis, 2004; Full Prescribing Information Denavir®).

In vitro, concentration-dependence is also shown in various assays using primary cells or cell lines. Choi et al. evaluated the cytotoxic effect of ganciclovir on cultured human corneal endothelial cells (HCECs) after 48 h exposure. When exposed to various concentrations (0-20 mg/ml) of ganciclovir, cytotoxic effects were shown in a concentration-dependent manner by a series of cytotoxicity tests. Concentrations of ≥ 5 mg/ml resulted in a significant reduction in cell viability, cell cycle delay, low proliferation rate, and an increased number of apoptotic cells, which indicated activation of the pro-apoptotic pathway. It was suggested that although concentrations of ≤ 0.5 mg/ml did not reduce cell viability, a higher dose did increase the risk of cell damage (Choi et al., 2013). There is another publication on B lymphoblastoid cells, which were exposed to ganciclovir at considerably lower concentrations between 1 to 20 mg/l for 1, 2, 7 or 14 days. During the first two days, high-level exposure to ganciclovir (20 mg/l) was not more toxic than low-level exposure (1 mg/l). When the duration exceeded 2 days, ganciclovir exposure started to decrease the total cell number. It seems that there is a minimal duration of exposure, after which ganciclovir exhibits toxicity (Janoly-Dumenil et al., 2009).

There are some in vitro studies using cell lines, which aimed to compare the cellular toxicity of aciclovir, ganciclovir and penciclovir. In Chinese hamster V97-E cell line and HSV-TK transformed baby hamster kidney cells, ganciclovir was found to have the greatest potential to induce rapid accumulation of cells in S-phase and apoptotic cell death while aciclovir was associated with sustained S-phase arrest and only evoked borderline effects (Thust et al., 1996; Shaw et al., 2001). In primary cell culture, ganciclovir had cytotoxic activity toward KSHV-infected lymphoma cells, while aciclovir had weak or little activity (Fujimuro et al., 2006).

1.2 Spermatogenesis

1.2.1 The process of spermatogenesis

Spermatogenesis is the process of gametogenesis in the seminiferous tubules, during which male gametes are produced. By conception, these gametes, which are specifically called spermatozoa, fertilize the oocyte from the female and turn into a single-celled zygote. Heller and Clermont published data on the duration of spermatogenesis and found that the entire process lasted approximately 64 days and could be divided into several distinct steps: spermatocytogenesis, spermatidogenesis, spermiogenesis, spermiation (Heller and Clermont, 1963). The distinct steps can exist at the same time on the seminiferous epithelium, which consists of many different types of germinal cells. The complexity of this epithelium is simplified by separate stages, which go through organized cycles from Stage I to Stage XII. Stage I is followed by II, followed by III, and so on, till Stage XII, which is then repeated by Stage I (Hess et al., 2008).

Efficiency of spermatogenesis depends on spermatocytogenesis and meiosis and can be measured quantitatively by the number of spermatozoa per day per gram of testicular parenchyma (Johnsona, 2000). It is distinct among different species because of different density and life span of germ cells rather than the differences in testicular size. Cells divide and differentiate during spermatogenesis, the efficiency of which is reflected in the number of spermatogenic stages per cross-section and missing generations within each stage, but not in the arrangement of stages along the tubular length (Johnson, 1995). For impaired spermatogenesis, Hentrich et al. in 2011 distinguished between “pool of founder cells”-related deficiencies (reduced numbers of Sertoli cells, spermatogonia, and spermatogonial stem cells) and “meiotic”-deficiencies (reduced numbers of spermatocytes, meiotic divisions, and spermatids) (Hentrich et al., 2011). Sertoli cell defects could impair testicular microenvironment without affecting the germ cells, and critical impairment of Sertoli cell function apparently induces some types of teratozoospermia, such as oligoasthenoteratozoospermia, as reported by Nakamura and co-authors (Nakamura et al., 2004).

1.2.2 Sertoli cell

Sertoli cells are regarded as a kind of epithelial supporting cells, which are specifically located in the seminiferous tubules. Their name derives from their discoverer Enrico Sertoli, who published a description of them in 1865 and used the terms "tree-like cell" or "stringy cell" referring to these "mother cells". Later other scientists used the name Sertoli to label these cells. The main task of Sertoli cells is to take care of the developing germ cells as “mother” through the stages of spermatogenesis, including offering nutrition, providing structural and metabolic support, as well as consuming the residual cytoplasm during spermatogenesis (O'Donnell et al., 2011). The functions of Sertoli cells have a close relationship to their specific shape. One single Sertoli cell stretches from the basement membrane to the lumen of the seminiferous tubule and stays in close contact with the spermatogenic cells.

Sertoli cells also play a central role in testicular development. In the fetus they act as the organizing center of testis formation until about the time of birth, coordinating the migration and differentiation of all other cell types (Ross and Capel, 2005), such as germ and somatic cell lineages (McLaren, 2000). The process of spermatogenesis can be also regarded as the process of displacement of germ cells through the lateral margins of the surrounding Sertoli cells. During this active cell migration process, intermittent junction disassembly and reassembly occur at the Sertoli–Sertoli cell and Sertoli–germ cell interface (Mruk and Cheng, 2004). Claudin was found to regulate the progression of meiosis during spermatogenesis by promoting germ cell migration across the blood-testis barrier (Chihara et al., 2013). If cross talk between these cells is disrupted, spermatogenic cells fail to migrate and/or orientate properly in the seminiferous epithelium. This thus leads to germ cell apoptosis, premature germ cell depletion from the epithelium, and infertility (Yan et al., 2008). The damaged Sertoli cell environment has also been shown to be a cause of infertility. For example, the development of spermatogonial stem cells was arrested and their differentiation blocked due to the damage to the somatic environment in mice following irradiation (Zhang et al., 2007).

It is also important that the Sertoli cells function normally in some special periods of development of the testis, and the regulation of Sertoli cell proliferation (effective cell number)

and maturation (effective cell function) is vital for normal adult fertility. The total number of Sertoli cells increased in adults compared to newborns but the density of Sertoli cells decreased (Cortes et al., 1987). It is generally assumed that all Sertoli cell proliferation occurs only during fetal and early neonatal life in rodents (until around 15 days) and in the fetal and peripubertal period in higher primates (Marshall and Plant, 1996; Sharpe et al., 2003). The spermatid number and spermatogenic capacity through adulthood depend on the appropriate perinatal development of the Sertoli cell population (Orth et al., 1988). At a species-specific time after birth (puberty), the role of Sertoli cells switches to the support of germ cell differentiation, meiosis, and spermatid transformation (Sharpe et al., 2003). This pubertal maturation of Sertoli cells is associated with a cessation of proliferation, alterations in protein expression and gene transcription, and the formation of a functional blood-testis barrier (BTB) (Sharpe et al., 2003). Thus, the Sertoli cells can broadly be described as having two distinct roles: one of testis formation through differentiation of the primordial gonad and the other in the support of spermatogenesis through functional maturation at puberty.

1.2.3 Sertoli cell line - SerW3

Cultures of the Sertoli cell line can mimic the environment of the BTB and hence may be utilized in toxicological tests. SerW3 is an immature cell line deriving from Wistar rat Sertoli cells (Pognan et al., 1997), which displays morphology and function similar to that of native cells. There is a special time window from 15.5 to 17.5 days in rats, when the development of the fetal genital system is particularly sensitive to reproductive toxicants (Pointis et al., 2011). Because the SerW3 cell line was taken from rats undergoing this sensitive gestational period, such cells are more suitable for studying the effects of reproductive toxicants. Since they act like immortalized but not transformed cells, they are also capable of undergoing apoptosis, a process which allows cellular toxicity to be observed.

1.2.4 Blood-testis barrier

The blood-testis barrier (BTB) is formed between pairs of adjacent Sertoli cells, and provides an isolated environment for the development of germ cells. The BTB is composed of tight

junctions, adherens junctions, desmosomes and gap junctions (Setchell, 2008; Vogl et al., 2008), and divides the seminiferous tubule into two parts: a basal compartment and an adluminal compartment. The cooperation between different junction proteins makes the BTB one of the tightest blood-tissue barriers (Xia et al., 2005). The opening/restructuring of the BTB, which takes place during stages VIII to XI of the epithelial cycle, makes it distinct from other cell barriers. In this process, a "new" BTB is gradually created behind spermatocytes while the "old" BTB is undergoing degeneration, so that the immunological barrier can still be maintained while the spermatocytes are traversing the BTB region (Cheng and Mruk, 2012).

As a physical and immunological barrier, the BTB plays an important role during spermatogenesis. It controls the entry and exit of substances into and from the seminiferous epithelium, including the entry of harmful toxicants and drugs into the apical compartment where germ cell development takes place. Environmental toxicants, such as heavy metals (e.g., cadmium), can cause testicular injury (Parizek and Zahor, 1956; Parizek, 1960; Chiquoine, 1964) and BTB disruption (Setchell and Waites, 1970). When the BTB is disrupted and fails to act as a normal barrier, the apoptosis of germ cells will increase and result in the further impairment of fertility.

1.2.5 In vitro methods to study reproductive toxicity

Around 65% of all animals used in chemical safety testing are used for testing the reproductive and developmental toxicity of xenobiotics (Van der Jagt et al., 2004). In order to reduce the large number of experimental animals consumed by such experiments, alternative approaches for reproductive hazard assessment have been developed (Piersma et al., 2013). In vitro methods can be used for targeted experiments and also for the replacement of some animal experiments in the study of reproductive toxicology, but no in vitro assay can fully address the complexity of reproductive development and function (Mantovani and Maranghi, 2005). Although the cycle of reproduction can be broken down into its individual biological components, in vitro methods nevertheless have limitations because the entire cycle is more than just the sum of its components (Piersma et al., 2013). To understand the complexity of the

reproductive cycle, it has been found to be effective to perform studies in combination with a database or a test battery. A database of repeated dose toxicity data has thus been developed, which has been shown to be a useful tool (Bitsch et al., 2006). A test battery consisting of a large number of different assays is also required by any reproductive toxicity test strategy that includes in vitro assays (Schenk et al., 2010). The EU-Framework 7 Project ReProTect feasibility study examined 10 compounds in 14 different in vitro test systems. In this project, bovine in vitro maturation assay (bIVM) and bovine in vitro fertilization assay (bIVF) were utilized for testing the effects of xenobiotics on fertility (Schenk et al., 2010). Another EU-Framework 7 project, ChemScreen, recently employed a different set of compounds and assays compared to those used in the ReProTect study (Piersma et al., 2013). The US EPA's ToxCast program studied developmental toxicity by analyzing the correspondence between the data from 309 chemicals in 662 alternative in vitro assays, and the data on rats and rabbits from almost 500 different chemicals (Sipes et al., 2011). The primary drawback of the in vitro assays above is that there are no reliable tests for male fertility. For providing more information on male reproductive toxicity, some related cell lines such as SerW3 Sertoli cell lines and MA-10 Leydig cell lines have been established and examined (Ascoli, 1981; Pognan et al., 1997). Such cell lines are commonly employed in tests for male reproductive toxicity. However, the metabolism-related pharmacokinetic parameters of the test compounds are not taken into account by the in vitro test systems to the same extent as by most animal assays (Schenk et al., 2010). This may represent a limitation, since if one compound is not activated in vitro, then the test will show a false negative result.

1.2.6 Junctional protein

During the process of cell migration, the disassembly and reassembly of intermittent junctions has been shown to occur at the Sertoli–Sertoli cell and Sertoli–germ cell interfaces (Mruk and Cheng, 2004). In vitro, Sertoli cells derived from prepubertal rats have been seen to proliferate in a cell density-dependent manner (Griswold et al., 1977) and to cease proliferation upon receiving contact-inhibition signals (Schlatt et al., 1996). The dynamics of normal proliferation rely on the presence of normal junction structures, among which the gap junction connexin43 is

an absolute requirement (Sridharan et al., 2007a, b; Brehm et al., 2007). Environmental toxicants (e.g., $CdCl_2$, BPA, DDT) can disrupt Sertoli–Sertoli cell junctions by either reducing the level or inducing the aberrant localization of occludin, ZO-1, N-cadherin, and connexin 43 (Fiorini et al., 2004; Wong et al., 2004). In response to DDT, junctional proteins such as connexin43, N-cadherin and ZO-1 have been found to be internalized and present in vacuoles (Fiorini et al., 2008).

1.2.6.1 Gap junction

Gap junctions in the region of the blood-testis barrier are located between Sertoli cells, as well as between Sertoli cells and germ cells during spermatogenesis (Russel, 1993). They are assembled by two hydrophilic channels, each of which is formed by six connexin monomers. Besides forming intercellular channels, connexins can also play a critical function in signal transduction (Stout et al., 2004). Connexin43 is the predominant connexin in its family, and has been detected in various animal species, such as rats, mice (Risley et al., 1992; Batias et al., 1999) and humans (Steger et al., 1999; Defamie et al., 2003). Connexin43 has been shown to participate in the formation of homocellular gap junctions between adjacent Sertoli cells, and heterocellular gap junctions between Sertoli cells and spermatogonia or spermatocytes, but not between Sertoli cells and spermatids (Decrouy et al., 2004).

Connexin43 plays an essential role in the control of spermatogenesis, because the gap junction has many important functions. Firstly, the gap junction allows the direct exchange of molecules with a relative molecular mass of up to 1 kDa, such as metabolic precursors, nutrients and second messengers including cAMP and ions (Bruzzone et al., 1996). Through the exchange of molecular signals, also termed gap-junctional intercellular communication (GJIC), the proliferation and differentiation of male germ cells is synchronized (Decrouy et al., 2004; Risley et al., 2002). Connexin43 is also essential for the cessation of proliferation and normal maturation in Sertoli cells (Sridharan et al., 2007a). The connexin43 located between Sertoli cells and spermatogonia is indirectly involved in maintaining the number of germ cells, by controlling their survival rate rather than their proliferation (Gilleron et al. 2009).

Connexin43 has been found to be undetectable in the seminiferous tubules of hypospermic and aspermic patients (Schleiermacher, 1980), whereas it was present in patients with normal spermatogenesis (Kotula-Balak et al., 2007). This result is consistent with the results of studies on mutant mice (Batias et al., 1999). Connexin43-deficient mice have been found to exhibit a 50% reduction in primordial germ cells in fetal testes (Juneja et al., 1999), and testes from Connexin43-null mutant fetuses were found not to display normal proliferation and differentiation of germ cells (Roscoe et al., 2001). Interestingly, the coding sequence of connexin43 could be substituted with the coding sequences of either connexin32 or connexin40 in connexin-knockin mice, indicating that connexin43, connexin40 and connexin32 share at least some vital functions (Plum et al., 2000).

1.2.6.2 Adherens junction

Adherens junctions (AJ) are present in the testes in a specific form, known as ectoplasmic specialization (ES), which includes both Sertoli cell-spermatid interfaces (apical ES) and Sertoli-Sertoli cell interfaces (basal ES). The apical ES is located in the apical compartment, and is crucial not only for cell attachment but also for spermatid movement and orientation in the epithelium. The basal ES is part of the BTB. It is possible to perturb the Sertoli–germ cell adherens junctions without compromising the integrity of the BTB (Xia et al., 2005). This may be because the enhanced production of tight junction proteins, such as occludin and ZO-1, can remedy the transient loss in adherens junction function at the basal ES.

The cadherin superfamily is present in the adherens junction, and the expression of its members has been observed to be period-specific. For example, N-cadherin is expressed from postnatal day 7 through to adulthood, while cadherin-6 is not present at postnatal day 7 and first appears at day 21. The large number and variety of members in the cadherin superfamily have been found to have a critical function for cadherin-mediated cell-cell adhesion in spermatogenesis (Johnson et al., 2000), including cell movement across the seminiferous epithelium (Siu and Cheng, 2004). N-cadherin is a classical cadherin and it interacts with catenins. Lee et al.

showed in 2003 for the first time that the cadherin/catenin complex may indeed be one of the functional units that regulate adherens junction dynamics between Sertoli and germ cells. The cadherin/catenin complex between Sertoli cells as well as between Sertoli and germ cells is structurally linked to actin but not to vimentin (Lee et al., 2003). It was however suggested later that classic cadherins are also associated with vimentin-based intermediate filaments via some adaptors. While N-cadherin has not been found to be associated with vimentin in the seminiferous tubules, it was found to have a connection to vimentin when testis lysates were utilized (Lee et al., 2004).

1.2.7 Intermediate filament

In most cells, the cytoskeleton consists of three major components: microfilaments, microtubules, and intermediate filaments (IFs). Intermediate filaments consist of a family of related proteins, which possess diverse genes but share similar characteristics. They play a role in anchoring germ cells to the seminiferous epithelium (Amlani and Vogl, 1988). Sertoli cells possess a highly organized and quite active cytoskeleton, among which IFs are of the vimentin type (Franke et al., 1979). In situ, the Sertoli cells of 17-day-old rats were found to contain only vimentin intermediate filaments (Guillou et al., 1990). Vimentin occurs in the basal and perinuclear region of the Sertoli cells and radiates towards the apical cytoplasm, where it becomes associated with some specialized membrane structures - ‘‘desmosome-like junctions’’- between Sertoli cells and adjacent germ cells, as well as between the Sertoli cells. Vimentin is expressed regularly in Sertoli cells in tubules with normal spermatogenesis, as well as in tubules with any kind of spermatogenic impairment (Bergmann et al., 1994; Steger et al., 1996).

1.2.8 Stage dependence

The expression of connexin43, N-cadherin and vimentin has been found to change during all the developmental periods and stages of the seminiferous epithelial cycle. Connexin43, for example, has been seen to co-assemble with connexin33 in some Sertoli-Sertoli gap junction plaques, and the assembly of connexin33 and connexin43 has been found to be regulated by the

cycle of the seminiferous epithelium (Risley et al., 1992; Tan et al., 1996; Batias et al., 1999; Batias et al., 2000; Bravo-Moreno et al., 2001). The localization of N-cadherin has also been found to change with the different stages of the epithelial cycle. N-cadherin was initially only immunoreactive in the basal ES between adjacent Sertoli cells, but at stages I-VII it has also been found to appear in Sertoli-elongate spermatid junctions (Johnson and Boekelheide, 2002). Vimentin may play a role in anchoring or positioning Sertoli cells during the early stages of the spermatogenic cycle (Amlani et al., 1988). The distribution and the shape of vimentin have been found to change during the cycle of the seminiferous epithelium and postnatal development (Mali et al., 1987; Steger et al., 1994; Zhu et al., 1997). Colchicine, a microtubule-disrupting agent, has also been found to disrupt vimentin in a stage-dependent manner (Allard et al., 1993).

1.3 Aim of study

First of all, the present study investigates the effects of four different nucleoside analogues on the Sertoli cells. Although some animal experiments on reproductive toxicity have already been conducted, the data available regarding Sertoli cells is still rather limited. The SerW3 Sertoli cell line, which displays some features of native Sertoli cells, is employed to mimic the environment of the blood-testis barrier. If impairments of the Sertoli cells are observed, this will indicate that the nucleoside analogues studied might cause impairment of male fertility. Because the SerW3 Sertoli cell line was derived from immature rat Sertoli cells, the analysis performed will be particularly relevant to the special time window during which the testes are more sensitive to reproductive toxicants than in adulthood.

Secondly, it compares the effects of four nucleoside analogues: aciclovir, ganciclovir, famciclovir and penciclovir. Although the activation of nucleoside analogues in uninfected cells is weaker than in infected cells, and the degree of activation may vary among different cell lines, the results will be useful for providing information on suitability of the SerW3 Sertoli cell line to study the reproductive toxicity of xenobiotics.

Finally, a gap junctional protein, connexin43, an adherens junction protein, N-cadherin, and an intermediated filament protein, vimentin, are tested by Western Blot analysis and immunofluorescence as potential indicators of Sertoli cell damage and impairment of the blood-testis barrier. Western Blot analysis is a quantitative method for revealing alterations in the expression of the target protein, and immunofluorescence can reveal the location and dislocation of that protein. Knowing which marker is more sensitive is useful because using a more sensitive marker would allow the toxicity of environmental toxicants or pharmaceutical agents to be tested even after relatively short-term exposure.

2 Material and methods

2.1 Material

2.1.1 Equipment

Equipment	Producer
Analytical balance MC1	Sartorius, Göttigen
Autoclave Classic 400	Varioklav, Oberschleißheim
Centrifuge 5417R	Eppendorf, Hamburg
Centrifuge 5810R	Eppendorf, Hamburg
Centrifuge Biofuge Fresco	Heraeus, Hanau
Control unit HT200W for heating system	Minitube, Tiefenbach
Cryogenic freezing container	Nalgene, USA
Dewar flask(vacuum pump)	WWR, Dresden
Flake ice machine AF80	Scotsman, Frimont
Freezer ULT	Thermo, Rockford
Fusion FX7	Vilber, Eberhardzell
Hemocytometer	Schott, Jena
Hot air oven	Heraeus, Hanau
Incubator	Heraeus, Hanau
Incubator fridge	Heraeus, Hanau
Incubator shaker	Janke & Kunkel, Staufen
Laminar flow hood	Heraeus, Hanau
Magnetic stirrer	Janke & Kunkel, Staufen
Magnetic stirrer with hot plate	Janke & Kunkel, Staufen
Micropipettes 8ch (30-300µl, 20-200µl)	Eppendorf, Hamburg
Micropipettes 10µl, 200µl and 1000µl	Eppendorf, Hamburg
Microscope fluorescence Axiophot	Zeiss, Jena
Microscope Axiovert 40C	Zeiss, Jena
Microscope Olympus CK2	Olympus, Hamburg
Microplate absorbance Reader	Bio-Rad, Munich
Pipetboy IBS	Integra, Switzerland
PH meter PH526	WTW, Leipzig
Powerpac basic power supply	Bio-Rad, Munich
Protein electrophoresis equipment	Bio-Rad, Munich
Roller mixer	Ratek, Boronia
Shaker	Elmi, Riga
Tank blotting system	Bio-Rad, Munich
Thermomixer®	Eppendorf, Hamburg
Vortex mixer	Janke & Kunkel, Staufen
Water bath AL5	Lauda-Brinkmann, New Jersey
Water purification system	Millipore, USA

2.1.2 Chemical reagents

Chemical reagents	Producer	Catalog No.
30% Acrylamid/Bis solution 37.5:1	Bio-Rad, Munich	161-0158
Ammonium persulfate (APS)	Sigma-Aldrich, Munich	A3678
Acetone	Merck, Darmstadt	8.22252
Bovine serum albumin (BSA) powder	Sigma-Aldrich, USA	A3059
Bovine serum albumin (BSA) 2mg/l	Interchim-Montlucon, France	UP36859A
Chemiluminescent substrate	Thermo, Rockford	34080
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Munich	D2650
Dulbecco's modified Eagle's medium	Biochrom, Berlin	TG0415
Ethanol 70%	Herbeta, Berlin	605173
Fetal calf serum (FCS)	Biochrom, Berlin	505S
Gelatin	Fluka, Switzerland	48723
Glycine	Merck, Darmstadt	K10259901
Hydrochloric acid (HCl) 1M	Carl Roth, Karlsruhe	K025.1
Isopropanol	Carl Roth, Karlsruhe	6752.3
Methanol	Merck, Darmstadt	1.06009.2500
Mounting medium	Dako, Denmark	S3023
Sodium chloride (NaCl)	Merck, Darmstadt	1.06009
Sodium hydroxide (NaOH) 1M	Carl Roth, Karlsruhe	K0211
PBS powder w/o Ca^{2+} Mg^{2+}	Biochrom, Berlin	L182-05
PBS with Ca^{2+} Mg^{2+}	Biochrom, Berlin	L1815
PBS without Ca^{2+} Mg^{2+}	Biochrom, Berlin	L1820
Pepstatin	Sigma-Aldrich, Munich	P5318
Phenylmethanesulfonyl Fluoride	Sigma-Aldrich, Munich	78830
Protein molecular weight marker	Bioline, Luckenwalde	BIO-33065
Protein assay kit reagent A	Bio-Rad, USA	500-0113
Protein assay kit reagent B	Bio-Rad, USA	500-0114
Reducing sample buffers	Thermo, USA	39000
Saponin from quillaja bark	Sigma-Aldrich, Deisenhofen	S7900
Sodium lauryl sulfate	Bio-Rad, USA	161-0301
Skim milk powder	Fluka, Switzerland	70166
Tetramethylethylenediamine	Carl Roth, Karlsruhe	2367.3
Tris-HCl	Merck, Darmstadt	1.08219
Triton X-100	Carl Roth, Karlsruhe	T6683
Tris Base	Sigma-Aldrich, Munich	T1503
Trypsin/EDTA	Biochrom, Berlin	L2163
Tween® 20	Carl Roth, Karlsruhe	9127.1

2.1.3 Consumables

Consumables	Producer	Catalog No.
3MM Blotting paper	Whatmann, Dassel	3030-917
Cell culture flask 25 cm^2	NUNC, Roskilde	156367
Cell culture flask 75 cm^2	NUNC, Roskilde	156499
Cell scraper	Santa Cruz, USA	sc-213229
Cover slip 24×50 mm	Menzel, Braunschweig	E309.1
CultureSlides	BD Falcon, Heidelberg	REF354104
EpT.I.P.S. ® (100-5000 µl)	Sigma-Aldrich, Munich	Z640158
NUNC® cryotube®	Sigma-Aldrich, Munich	V7634
Parafilm®	American National Can, USA	WI 54952
Pasteur pipette (plastic)	Sarstedt, Nümbrecht	86.1172.200
Pasteur pipette (glass)	Carl Roth, Karlsruhe	4522
Pipette tip 200 µl	Sarstedt, Nümbrecht	70.760.002
Pipette tip 10 µl	Sarstedt, Nümbrecht	70.113
Pipette tip 1000 µl	Sarstedt, Nümbrecht	70.762.200
PVDF membrane	Roche, Mannheim	0301004001
Safe-Seal® tubes 0.5 ml	Sarstedt, Nümbrecht	REF 72.704
Safe-Seal® tubes 1.5 ml	Sarstedt, Nümbrecht	REF 72.706
Sterile centrifuge tube 15 ml	BD Falcon, Heidelberg	352095
Sterile centrifuge tube 50 ml	BD Falcon, Heidelberg	352070
Sterilized pipette 5 ml	BD Falcon, Heidelberg	357543
Sterilized pipette 10 ml	BD Falcon, Heidelberg	357551
Sterilized pipette 25 ml	BD Falcon, Heidelberg	357525

2.1.4 Antiviral agents

Antiviral agents	Producer	Catalog No.
Aciclovir	Sigma-Aldrich	A4669
Ganciclovir	Sigma-Aldrich	G2536
Famciclovir	Santa Cruz	sc-211498
Penciclovir	Sigma-Aldrich	P0035

2.1.5 Software

Software	Version	Producer
AxioVision 40	4.7.2.0	Zeiss, Jena
Bio-1D	12.12	Vilber, Eberhardzell
Multi-imaging system	15.12	Vilber, Eberhardzell
Microplate Manager	6	Bio-Rad, Munich

2.1.6 Antibody

Method	Primary antibodies	Catalog No.	Producer
Western Blot	mouse anti-connexin43	13-8300	Invitrogen
	mouse anti-N-cadherin	33-3900	Invitrogen
	mouse anti-vimentin	V6389	Sigma-Aldrich
	mouse anti-β-Actin	A5441	Sigma-Aldrich
Immunofluorescence	mouse anti-connexin43	13-8300	Invitrogen
	mouse anti-N-cadherin	ab12221	Abcam
	mouse anti-vimentin	V6630	Sigma-Aldrich
Method	**Secondary antibodies**	**Catalog No.**	**Producer**
Western Blot	goat anti-mouse lgG HRP-conjugated	12-349	Millipore
Immunofluorescence	goat anti-mouse lgG fluorescent-conjugated	12-506	Millipore
	Alexa Fluor® 488 goat anti-rabbit IgG	A-11008	Invitrogen

2.1.7 Buffer

Buffer	Ingredients	Concentration
APS-Solution	Ammonium peroxodisulfate	50.0 g/l
Blocking buffer	Skim milk powder	50.0 g/l
	Tween® 20	1.0 ml/l
Dilution for Antibodies in Immunofluorescence	BSA	5.0 g/l
	Gelatin	2.0 g/l
	Saponin	5.0 g/l
Lysis buffer	Triton X-100	10.0 ml/l
	SDS-Solution (20%)	1ml/l
	NaCl	8.8 g/l
	Tris-HCl (1M, pH7.2)	7.9 g/l
	PMSF	174 mg/l
	Pepstatin	5 mg/l
Running buffer 10×	Tris Base	30.0 g/l
	Glycine	144.2 g/l
	SDS (powder)	10.0 g/l
SDS-solution (20%)	SDS (powder)	200.0 g/l
Separating gel buffer pH8.8	Tris-HCl	157.6 g/l
Stacking gel buffer pH6.8	Tris Base	124.1 g/l
Transfer buffer 2×	Tris Base	5 g/l
	Glycine	24.5 g/l
	Methanol	100 ml/l

2.2 Methods

2.2.1 Aseptic technique and cell culture condition

The cell culture hood was turned on at least 15 minutes prior to commencing work. All solutions and equipment to be used for the culture had to be sterilized, and all appropriate bio-safety procedures had to be followed accordingly. All the bottles used were labeled and stored in the 4°C fridge after use. Each container was sprayed with 75% ethanol each time before it was moved inside the hood, and the solution was protected from exposure to the air outside the hood. The culture media were disposed when it had been more than one month since the bottles were opened. The growth of the cells was monitored daily under a light microscope. In the case of contamination occurring, all the cells inside the incubator would be removed and disposed of into the biohazard bin.

The cells of the SerW3 cell line were maintained in a humidified incubator at 37°C with 5% CO_2. The culture medium was Dulbecco's modified Eagle's medium (DMEM), which contained 3.7 g/l of glutamine. 5% fetal calf serum (FCS) was added to the DMEM for the cell culture while 10% was added for freezing the cells. The culture medium was pre-warmed to 37°C in water before use. 10 ml of the medium or PBS was pipetted into 75 cm^2 flasks while 5 ml was pipetted into 25 cm^2 flasks. To suspend the cells, the medium was pipetted up and down, with efforts to avoid forming bubbles. The cell suspension was then centrifuged at 300xg for 4 minutes. Supernatant was removed with a sterile glass pipette which was connected to a Dewar bottle. When the cells had grown for three or four days and reached confluence, they were split or frozen.

2.2.2 Cell culture and incubation with nucleoside analogues

2.2.2.1 Split the cells

The culture media were aspirated from flasks and the cells were washed once with PBS (without Mg^{2+} and Ca^{2+}). 1.5 ml of trypsin-EDTA was added to the flasks and allowed to distribute evenly across the surface of the cells for a short time, and then immediately aspirated. The flasks were sealed and returned to the incubator for three minutes. After incubation, the cells were checked under a light microscope to determine whether they were fully trypsinized. If the cells were not fully detached from the bottom, the flasks were placed back into the incubator for two more

minutes. When the cells were still attached to the bottom, it was possible that trypsin-EDTA would fail to work effectively, in which case the process of trypsinization would have to be performed again with new trypsin-EDTA. Once the cells were detached from the bottom, 5 ml of culture medium (including 5% FCS) was added into the flask to halt the action of trypsin-EDTA. The detached cells were then washed down into the medium and resuspended evenly. The resuspended cells were collected and centrifuged for 4 minutes at 300xg. During centrifugation, sterilized culture flasks were labeled with the name of the cell line, the number of the new generation, the name of the researcher and the date. After centrifugation, the media above the cell pellet were aspirated carefully and the cells were resuspended in 5 ml of new culture medium. The number of cells was counted with a hemocytometer. After cell counting, 1 ml of medium with cells and 9 ml of medium without cells were mixed together and added into each flask.

2.2.2.2 Freezing cells

Sertoli cells were trypsinized following the same procedure as used for splitting the cells, and the viable cells were counted using trypan blue. When viability was greater than 90%, the cells were healthy enough for storage. The cell suspension was centrifuged to obtain cell pellets. The supernatant was then removed and the cell pellets were resuspended in 5 ml of medium, supplemented with 10% FCS and DMSO. The media containing cells were distributed into cryotubes, with 1.5 ml in each tube. The cryotubes were then immediately transferred to a cryobox and kept at -80°C for at least 24 hours before permanent storage in liquid nitrogen.

2.2.2.3 Thawing cells

Cryotubes were removed from liquid nitrogen and shaken frequently in a 37°C water bath until their insides became transparent. The mixture inside each tube was transferred into a new sterile 15 ml tube, and gently washed with 5 ml of warm medium. Centrifugation was then conducted to remove DMSO residues. The cell pellets were resuspended in 10 ml of new medium and then transferred to a 75 cm^2 flask. After around 12 hours, the cells were checked under a light microscope. Most of them would attach to the bottom of the flask, with only a few cells still floating, and attachment would continue for 24 hours.

2.2.2.4 Incubation with antiviral agents for three days

After the cells reached confluence, they were split into 25 cm^2 flasks or culture slides and cultured with nucleoside analogues for three days, in order to perform Western Blot analysis in the case of the flasks and immunofluorescence in the case of the slides. The cells used for each separate trial had to derive from the same flask. The seeding density was around $5\times10^3/cm^2$, which allowed the cells to reach confluence after three days of culture. All four nucleoside analogues were dissolved in PBS without Ca^{2+} or Mg^{2+}, and the concentration of the stock solution was 0.5 g/l. The final concentrations used for testing the four agents were as follows:

Antiviral agent	Concentration (mg/l medium)
Aciclovir	0(control), 0.3,1,3,10
Ganciclovir	0(control), 0.3,1,3,10
Famciclovir	0(control), 3,10,30,100
Penciclovir	0(control), 3,10,30,100

2.2.3 Western Blot

2.2.3.1 Cell lysis- preparing the sample

The enzyme inhibitors PMSF and pepstatin were thawed from a -20°C freezer and added into a lysis buffer shortly before cell lysis. The cells were washed twice with cold PBS (with Mg^{2+} and Ca^{2+}). After the washing procedure, 400 µl of lysis buffer with inhibitors was added into each 25 cm^2 flask. The cells were incubated on ice for 15 minutes, then scraped from the bottom of the flasks and incubated on ice for another 15 minutes. The cell lysate was then harvested and centrifuged at 9000xg for 30 minutes at 4°C. When the centrifugation was finished, the supernatant in the tube was carefully transferred to the new labelled tubes. If the Western Blot was not performed straight after cell lysis, the samples were stored at -80°C.

2.2.3.2 Protein determination- microplate assay

The total protein concentration was determined using a kit from Bio-Rad, based on the Lowry method, measuring the protein concentration through the use of a Folin phenol reagent (Lowry et al., 1951). The procedures prescribed by that method for measuring protein in a solution were followed. Working reagents included Reagent A and B, and efforts were made to ensure that they

were not mixed with each other before use.

The lysis buffer was used to make a serial dilution of five different concentrations of protein standard BSA ranging from 0.0625 mg/ml to 2 mg/ml. 10 µl of protein standards, standard dilutions or protein samples were pipetted into a clean 96-well plate while 10 ml of lysis buffer was used as the negative control, which was labeled as "#" in the microplate analysis system. A multi-pipette (20-200 µl) was used to add 25 µl of reagent A into each well and 200 µl of reagent B was added into the wells with another multi-pipette (30-300 µl). The mixture in each well had to be pipetted up and down to mix completely, and the production of bubbles had to be avoided. The 96-well plate was then incubated in darkness for 30 minutes at 37°C. The microplate reader was turned on and the positions of the samples (S1-S40) or the six standards (from 0.0625 mg/ml to 2 mg/ml) were marked as below. The absorbances were read at 750 nm.

#	#	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
2 mg/ml	2 mg/ml	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
1 mg/ml	1 mg/ml	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
0.5 mg/ml	0.5 mg/ml	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
0.25 mg/ml	0.25 mg/ml	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
0.125 mg/ml	0.125 mg/ml	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
0.0625 mg/ml	0.0625 mg/ml	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
#	#	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

Table 2: The positions of protein standards, samples and negative controls on a 96-well plate

2.2.3.3 Reduction

When the samples were removed from storage in the -80°C freezer, they had to be warmed up before use on ice for at least half an hour until they had completely thawed. The samples and lysis buffer were mixed in the new tubes to ensure each 20 µl sample contained the same volume of protein. The sample buffer was taken out of the -20°C freezer, warmed at room temperature, and added at a ratio of 1:4 to the samples. Each 20 µl sample needed 5 µl of sample buffer. The samples were then boiled for 10 minutes at 70°C and cooled down to room temperature.

2.2.3.4 SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis)

The SDS-PAGE was prepared following the recipes for polyacrylamide gels below, and stored in

the 4°C fridge for a short period or overnight before use, allowing polymerization to be completed and the gels to become hard. The gels were protected from exposure to light and from drying.

Gel	Ingredients	Volume
Stacking Gel 4%	Acrylamid 30%	0.67 ml
	Tris Puffer pH 6.8	1.25 ml
	SDS (20%)	25 µl
	Distilled water	3 ml
	APS (5%)	50 µl
	TEMED	10 µl
Separating Gel 10%	Acrylamid 30%	3.3 ml
	Tris Puffer pH 8.8	2.5 ml
	SDS (20%)	50 µl
	Distilled water	4.05 ml
	APS (5%)	100 µl
	TEMED	10 µl

First, 20 µl of sample mixture or 10 µl of prestained marker was loaded into the bottom of each well in the gel. The tank for protein electrophoresis was then filled with running buffer, and the electrophoresis apparatus was attached to an electric power supply. At first the gels were running at 80 V for 20 minutes, and then at 120 V for 60 minutes, until the protein reached 5 centimeters in height above the bottom of the gels. The glass plates were then removed from the electrophoresis apparatus, and in order to mark the orientation of the gel, a corner was cut from one side of the gel. The gels were then incubated in transfer buffer for approximately 10 minutes to remove SDS from the running buffer.

2.2.3.5 Blotting transfer

The PVDF membranes and filter paper were cut to a size similar to that of the gels, and one corner of the bottom of each membrane was cut to mark the orientation of the membrane. The members were first soaked in methanol for 10 minutes, then washed in distilled water for 10 minutes and finally soaked with filter paper and fiber pads in transfer buffer for 10 minutes.

The transfer sandwiches were prepared in the following manner: the cassette was opened with the gray side down, one pre-wetted fiber pad was then placed on the gray side of the cassette, a sheet

of filter paper was placed on the fiber pad, the gel placed on the filter paper, the pre-wetted membrane on the gel, filter paper on the membrane, a fiber pad on the filter paper, and finally the cassette was closed with a white latch. During the whole process, it was important to avoid making bubbles or causing different parts to rub against each other. The cassettes were inserted into the tank of the blotting system, along with one ice block, and then the tank was filled with transfer buffer. The blotting system was attached to an electric power supply and ran at 200 mA for one hour. The ice block had to be changed once during the transfer process. After the transfer, the transfer sandwiches were opened and the membranes were immediately soaked in the blocking buffer.

2.2.3.6 Detecting the protein with antibodies

The membrane was blocked with 5% skimmed milk in PBS for 1 hour while being shaken and then washed with PBS. 0.1% BSA/PBS buffer was prepared and used to dilute 10 µl of primary antibody to 1:1000. The membranes with the primary antibody were incubated overnight in a 4°C fridge with shaking. On the next day, the membranes were washed three times in PBS with 0.1% Tween20, each time undergoing 10 minutes of shaking. 0.1% BSA/PBS buffer was prepared and used to dilute 2.5 µl of secondary antibody to 1:4000, followed by the incubation of the membranes with secondary antibody for 1 hour at room temperature. The blotting process was ended with three washes in PBS with 0.1% Tween20, each time lasting 10 minutes.

2.2.3.7 Developing

Before their development, each membrane was incubated in 2 ml of SuperSignal West Pico working solution. This incubation at room temperature lasted for around 5 minutes and involved gentle shaking in darkness. Until the first membrane had finished the development process, the second membrane could not start incubation with the Pico working solution, but could continue the washing process. The relevant Fusion FX7 software was then opened, and the camera was precooled and focused on the platform of the membrane. Exposure commenced after the membrane was placed in the middle of the platform. The time for exposure was normally 6 minutes, which could be prolonged to 12 minutes or shortened to 3 minutes when the bands on the membranes were too weak or too strong, respectively. After the development of all the

membranes was complete, the bands were measured by the bio1D software in order to reveal the differences between the treated group and the control group. When there was a lane for prestained marker on the membranes, it was also necessary to take a picture under the normal light in order to check the protein weight of the main bands.

2.2.4 Immunofluorescence staining

The Sertoli cells were seeded in the labelled chambers with a density of around 8,000-10,000 per well and incubated with four nucleoside analogs for three days. When the culture was finished, the following steps were performed. Before the staining process, the growth of the cells was observed under a light microscope. The cells were then washed twice with cold PBS (without Mg^{2+} and Ca^{2+}), fixed with methanol for 5 minutes at -20°C and washed twice with PBS. The blocking buffer 0.5% BSA/PBS was prepared with BSA in PBS and used to incubate the cells for 15 minutes. After the primary antibody was diluted to 1:10 with antibody dilution, the working solution was added with at least 20 µl per chamber. The chambers were covered by paraffin, stored in the dark at 4°C and incubated overnight. On the next day, the paraffin was removed and the chambers were washed with PBS twice. The 1:40 dilution for the secondary antibody was prepared using antibody dilution. Each chamber needed 20 µl when covered by paraffin but at least 50 µl without paraffin. The incubation lasted for one hour at room temperature in the darkness. From this step on, the cells were kept strictly away from exposure to light. After the incubation of the secondary antibody, the chambers were washed with PBS twice. Following the instructions for the culture slides, the slides were prepared and mounted in mounting solution. At that point, the staining process was finished, and the cells were then observed under the fluorescence microscope. Representative pictures were then taken as soon as possible under the same fixed parameters.

3 Results

3.1 Western Blot Analysis

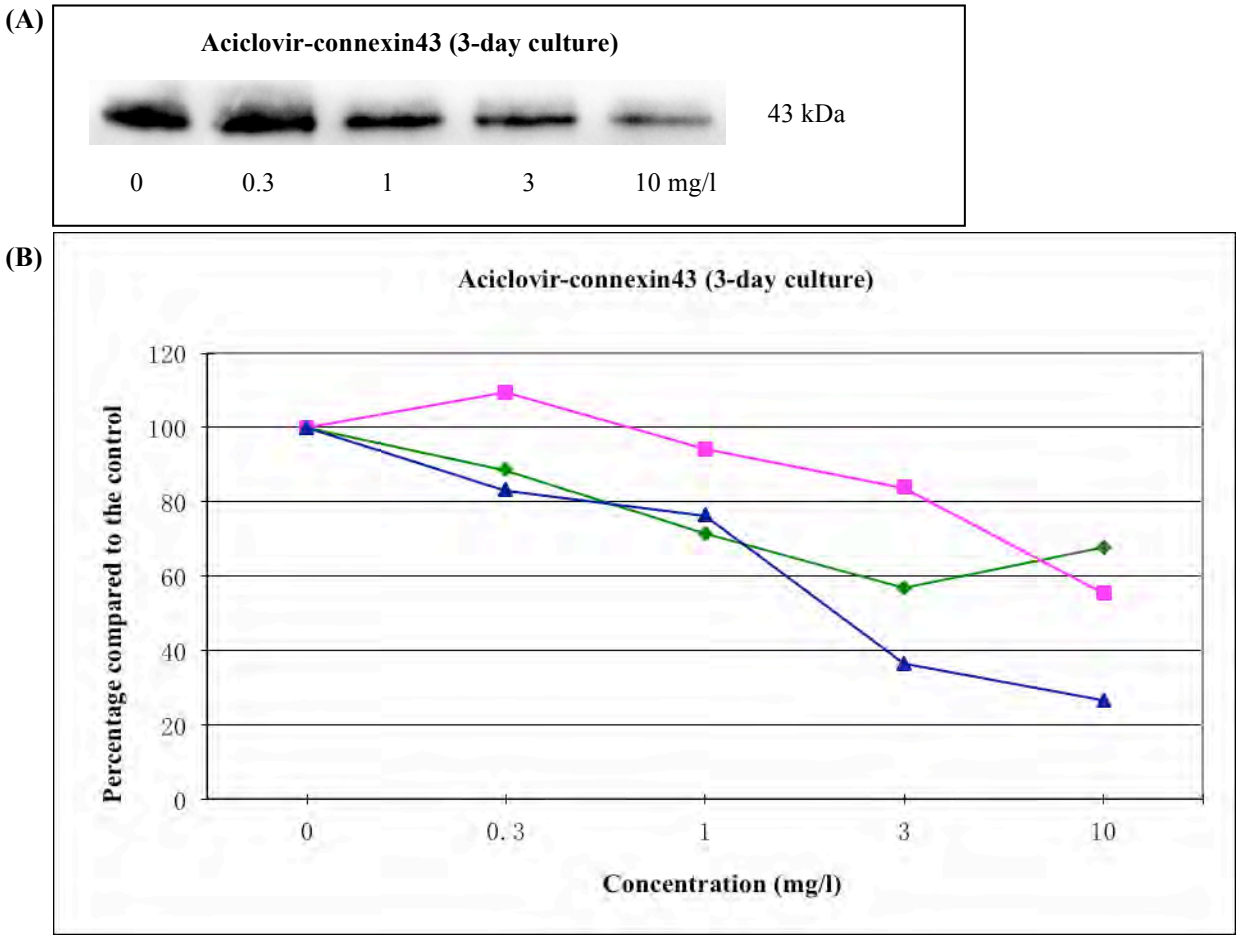


Figure 1:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of connexin43 after incubation for three days with aciclovir at concentrations ranging from 0.3 to 10 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

From the figures above, it can be seen that the amount of connexin43 protein present in the sample, after three days of culture with aciclovir, decreased in a dose-dependent manner, with the highest concentration of aciclovir tested, 10 mg/l, causing down-regulation of connexin43 by more than 50% (Figure 1A). The expression of connexin43 in the sample, compared to that of the control, decreased to 93.7 ± 11.3 % and 80.6 ± 9.8 % (n=3; mean ± SD) when the concentration of aciclovir was 0.3 mg/l and 1 mg/l, respectively. At the two higher concentrations of 3 mg/l and 10 mg/l, the expression of connexin43 was reduced to 59.0 ± 19.4 % and 49.8 ± 17.2 %, respectively (Figure 1B).

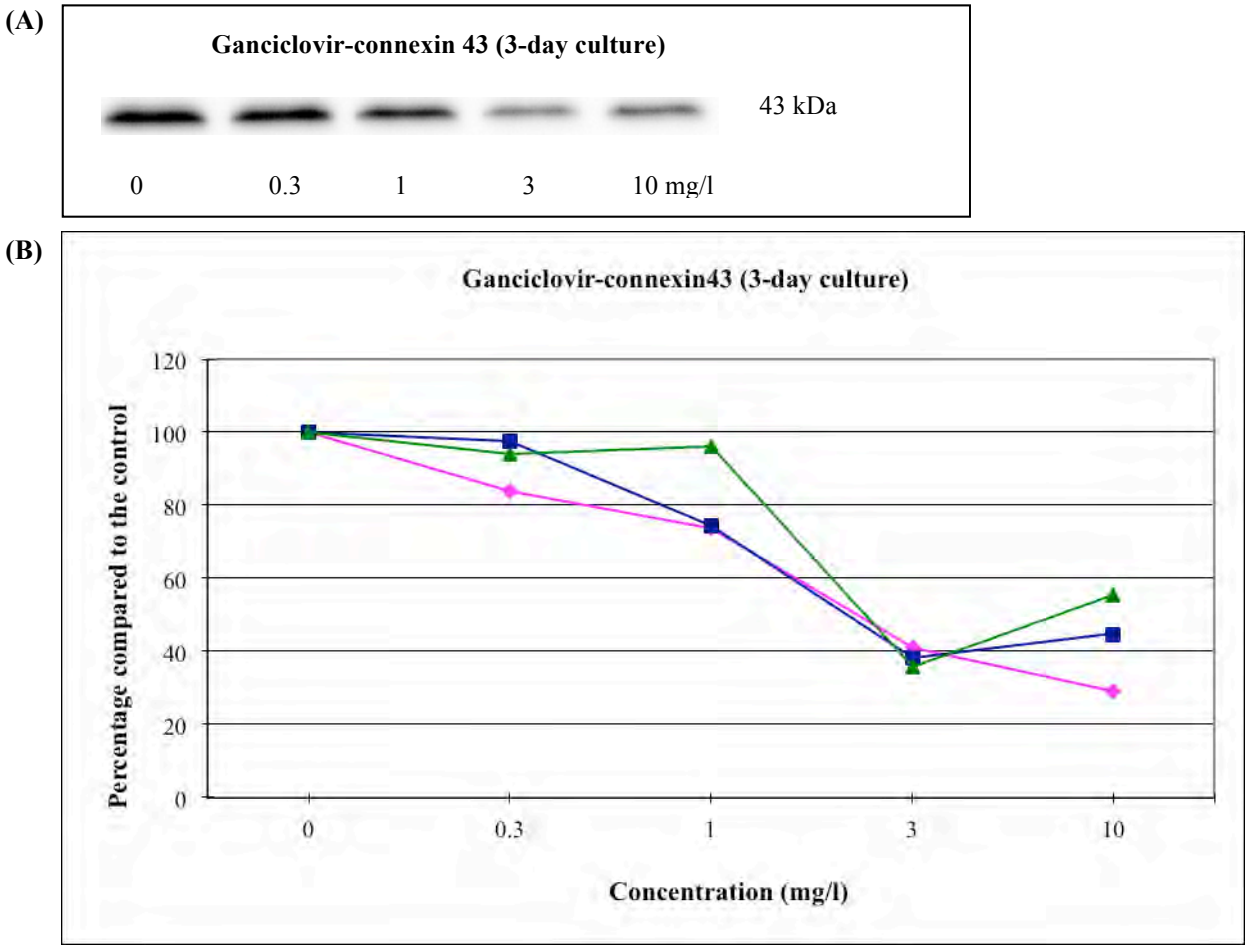


Figure 2:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of connexin43 after incubation for three days with ganciclovir at concentrations ranging from 0.3 to 10 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

From the figures above, it can be seen that, after three days of culture with ganciclovir, the level of connexin43 protein decreased in a dose-dependent manner. At the highest concentration tested (10 mg/l), ganciclovir caused down-regulation of connexin43 by more than 70% (Figure 2A). There were only slight changes in connexin43 levels when the concentration of ganciclovir was below 1 mg/l. The expression of connexin43 within the sample, compared with that of the control, decreased to 91.9 ± 5.8 % and 81.5 ± 10.4 % (n=3; mean ± SD) when the cells were cultured with ganciclovir at doses of 0.3 mg/l and 1 mg/l, respectively. More pronounced effects were apparent at the higher concentrations of 3 mg/l and 10 mg/l, with connexin43 expression reduced to 38.4 ± 2.1 % and 43.2 ± 10.8 %, respectively (Figure 2B).

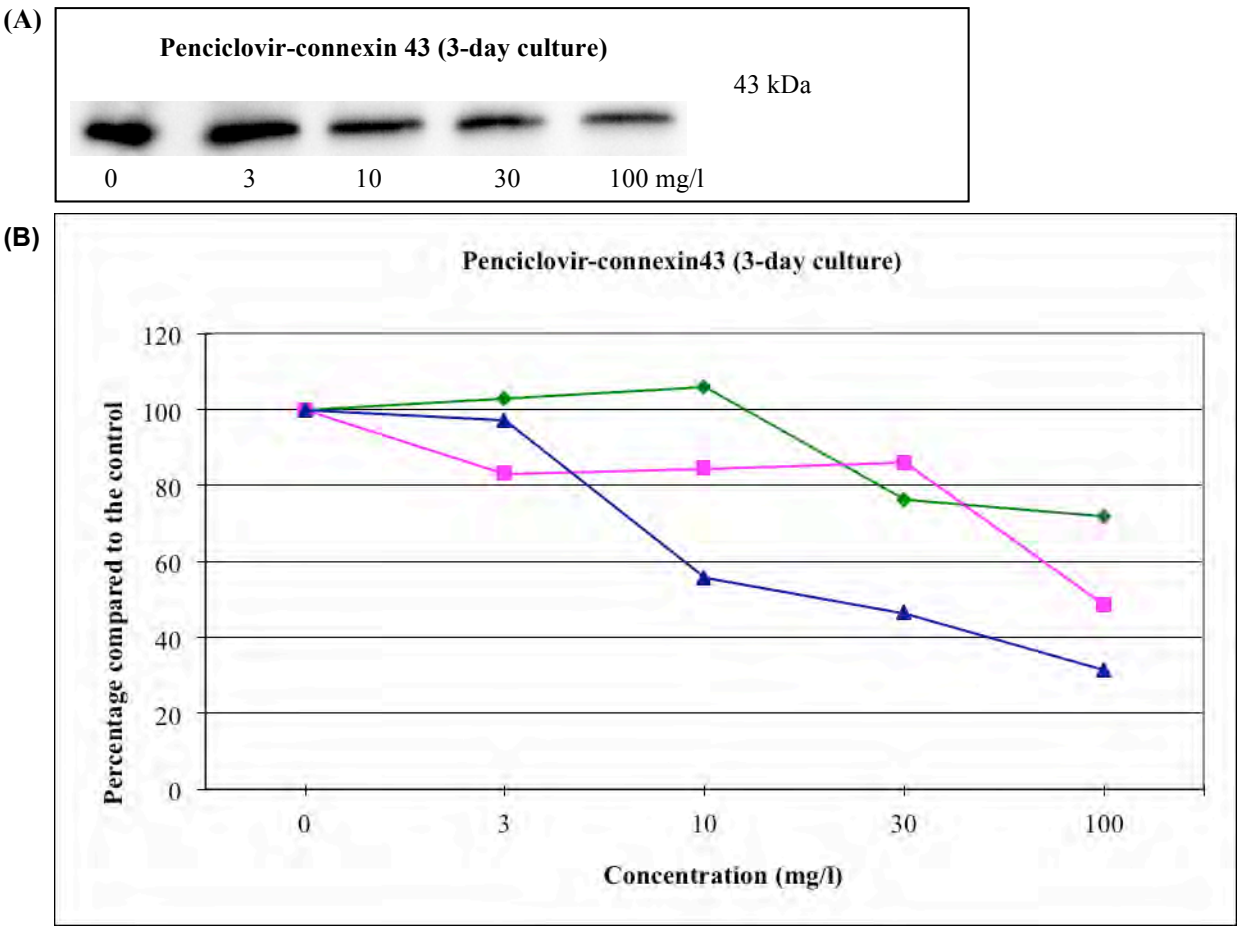


Figure 3:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of connexin43 after incubation for three days with penciclovir at concentrations ranging from 3 to 100 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

Figures above show that, after three days of culture with penciclovir, the expression of connexin43 in the sample had been down-regulated in a dose-dependent manner. The strongest effect was detected at the highest concentration tested, 100 mg/l (Figure 3A). At the two lower concentrations of 3 mg/l and 10 mg/l, the expression of connexin43 decreased to 94.5 ± 8.3 % and 82.1 ± 20.6 %, respectively (n=3; mean ± SD). More pronounced changes were visible at the two higher concentrations of 30 mg/l and 100 mg/l, which demonstrated connexin43 expressions of 69.6 ± 16.8 % and 50.7 ± 16.5 %, respectively (Figure 3B).

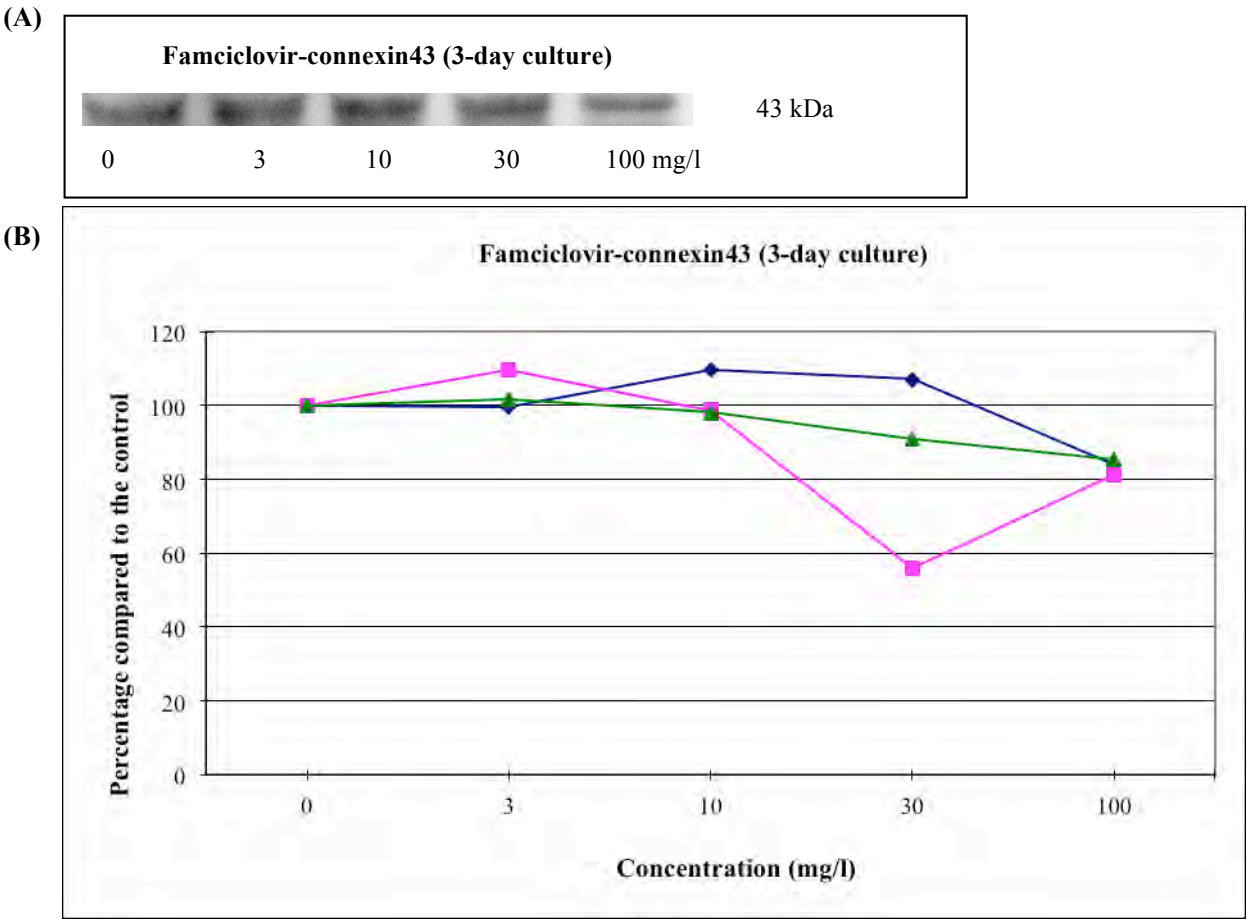


Figure 4:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of connexin43 after incubation for three days with famciclovir at concentrations ranging from 3 to 100 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

From the figures above, little difference can be seen between the treated group and the control group in terms of the different concentrations of famciclovir tested. In the two lower concentrations in particular, connexin43 expression remained at nearly 100% that of the control (Figure 4A, B). At the highest concentration tested (100 mg/l), the expression of connexin43 still remained at around 80%. When the concentration of famciclovir increased from 30 mg/l to 100 mg/l, the values of the expression were reduced to 84.6% ± 21.3% and 83.5% ± 1.7%, respectively (n=3; mean ± SD).

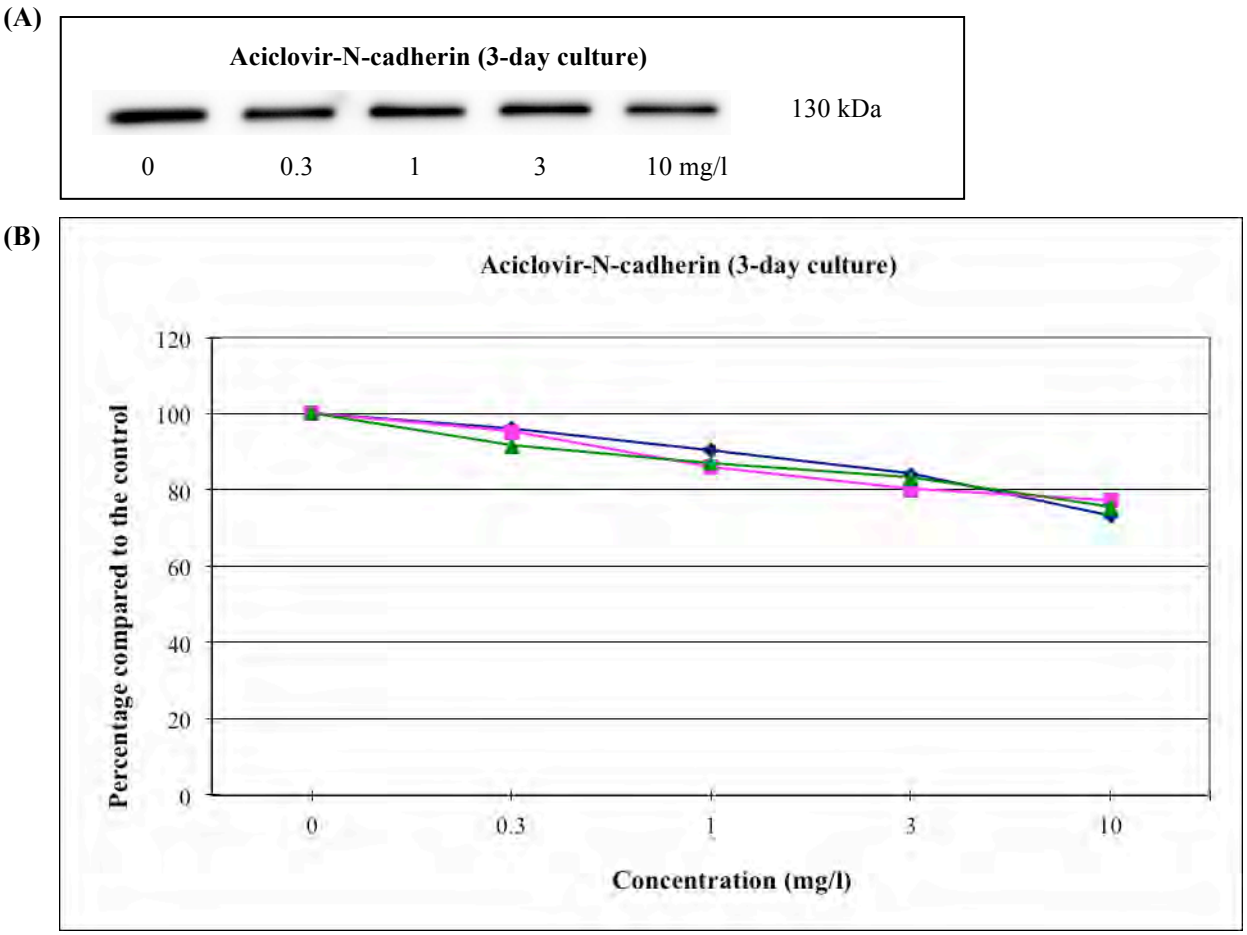


Figure 5:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of N-cadherin after incubation for three days with aciclovir at concentrations ranging from 0.3 to 10 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

Only slight effects were observed after the cells were cultured with aciclovir for three days (Figure 5A) and the level of N-cadherin protein slightly decreased in a concentration-dependent manner (Figure 5B). The expression of N-cadherin were 94.3% ± 2.0% of the control value in the 0.3 mg/l group, 87.8% ± 1.9% in the 1 mg/l group, 82.5% ± 1.7% in the 3 mg/l group and 75.3% ± 1.7% in the 10 mg/l group (n=3; mean ± SD).

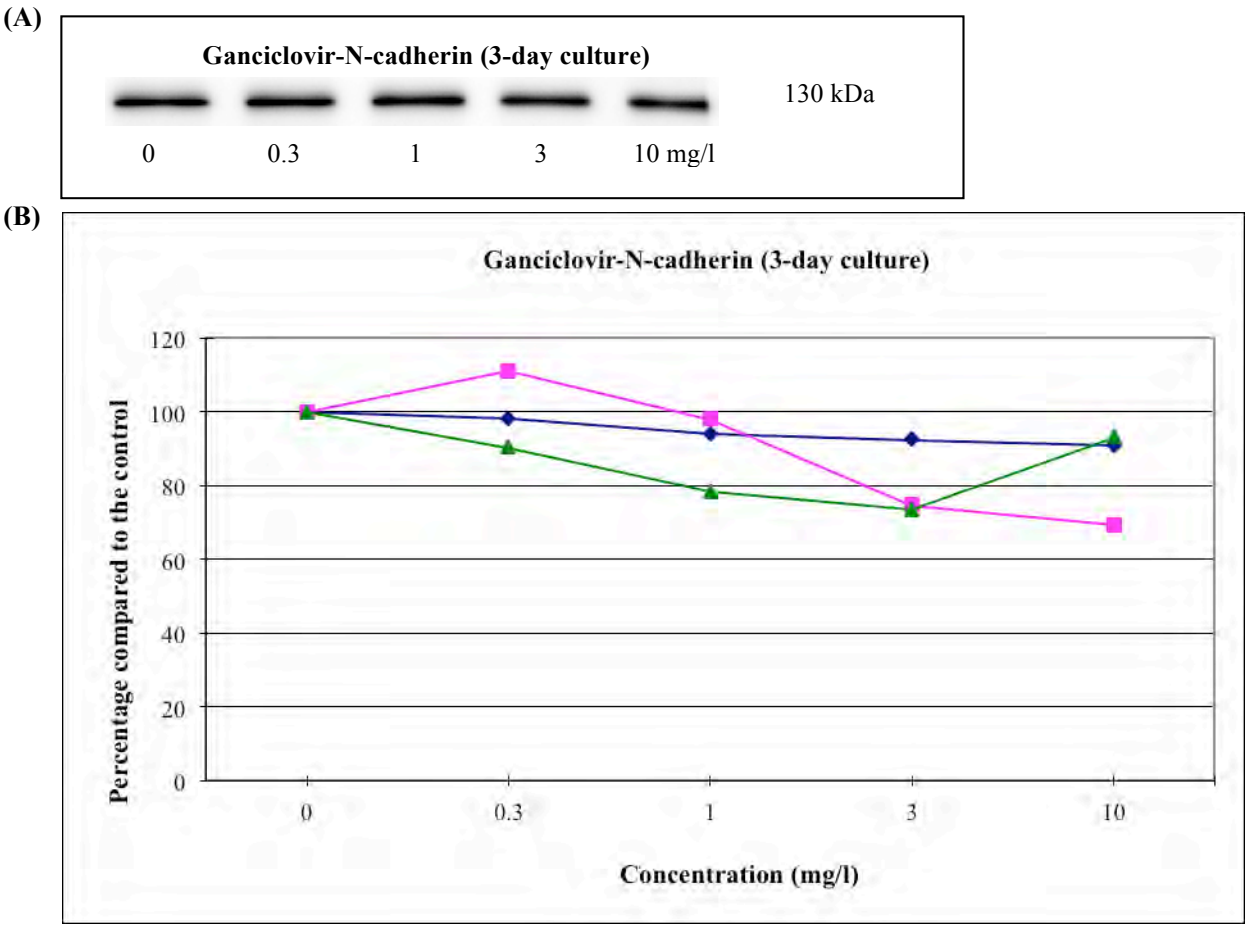


Figure 6:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of N-cadherin after incubation for three days with ganciclovir at concentrations ranging from 0.3 to 10 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

There were no obvious changes in N-cadherin after three days of culture with ganciclovir compared to the control group (Figure 6A). When measured with Bio1D, the mean values at different concentrations were all above 80% but under 100% of the control values (Figure 6B). At concentrations between 0.3 mg/l and 10 mg/l, the expressions of N-cadherin protein were 99.9 ± 8.6 %, 90.2 ± 8.4 %, 80.2 ± 8.7 % and 84.4 ± 10.8 %, respectively (n=3; mean ± SD).

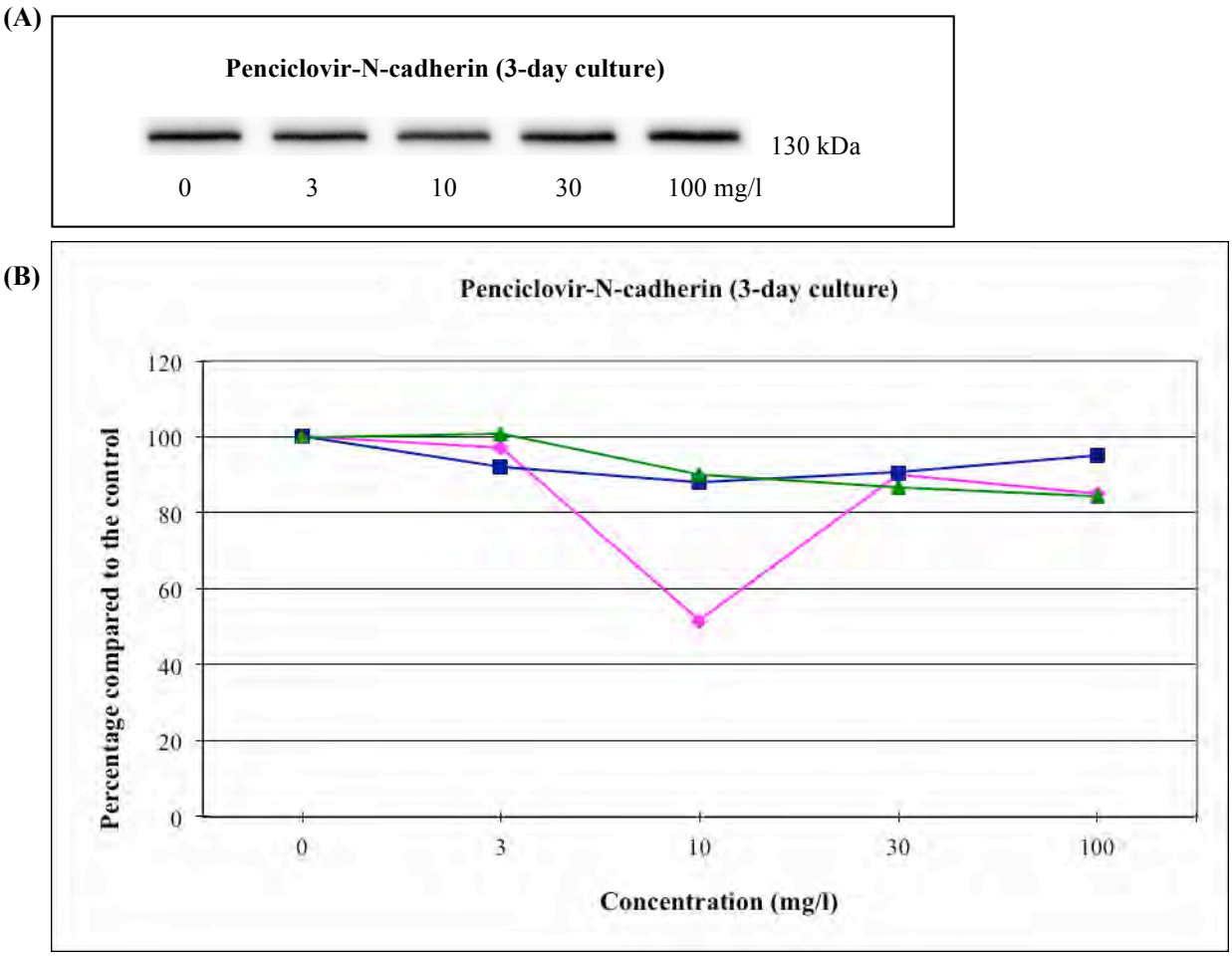


Figure 7:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of N-cadherin after incubation for three days with penciclovir at concentrations ranging from 3 to 100 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

No large differences were observed after three days of culture with penciclovir (Figure 7A). In Figure 7B, all the values are above 80%, but below 100% of the control values, apart from the value of around 50% at a dose of 10 mg/l, which was probably caused by a mistake during the conduction of the Western Blot. At the two higher doses tested (30 mg/l and 100 mg/l), the expression of N-cadherin decreased to 89.0 ± 1.6 % and 88.1 ± 4.9 %, respectively (n=3; mean ± SD).

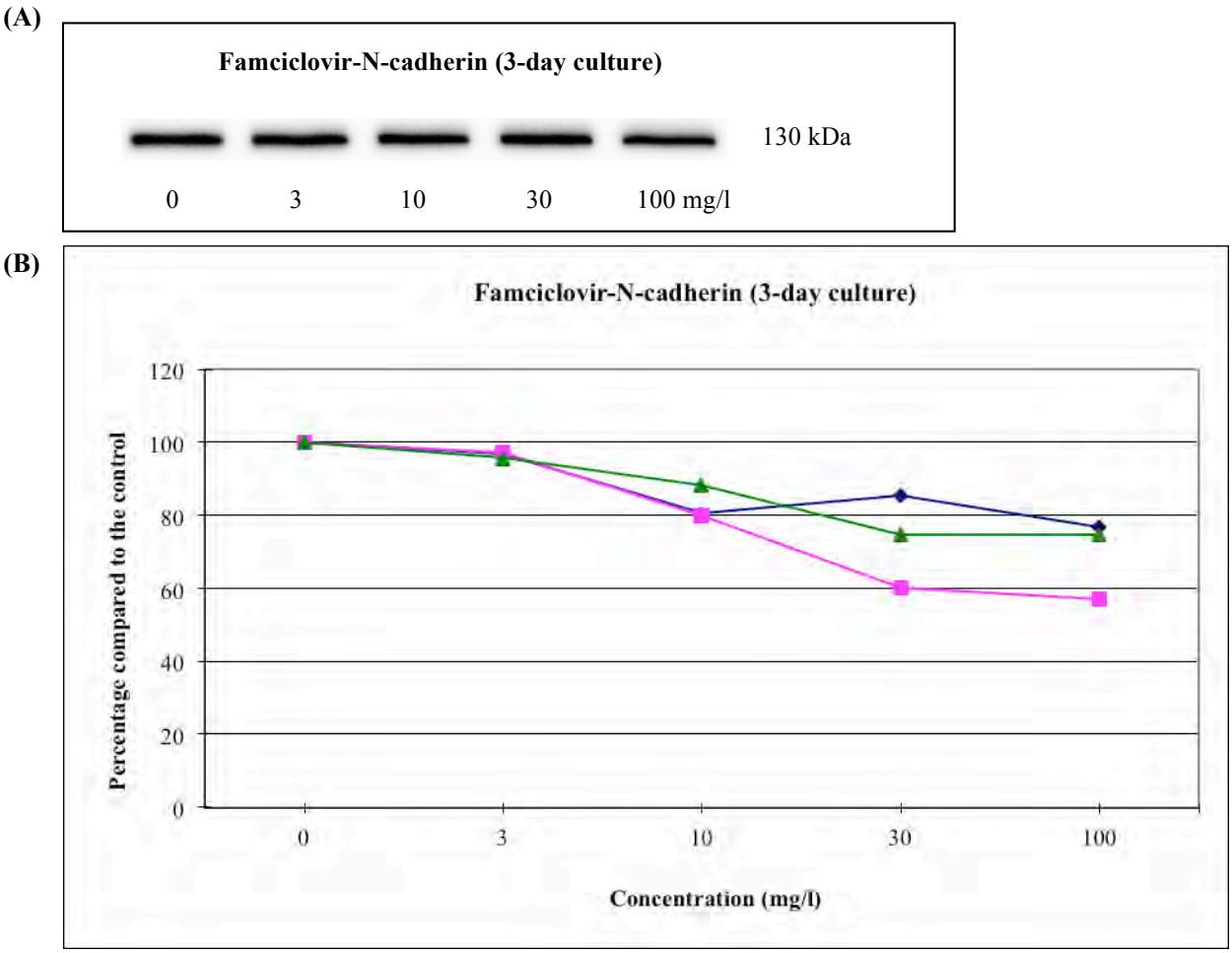
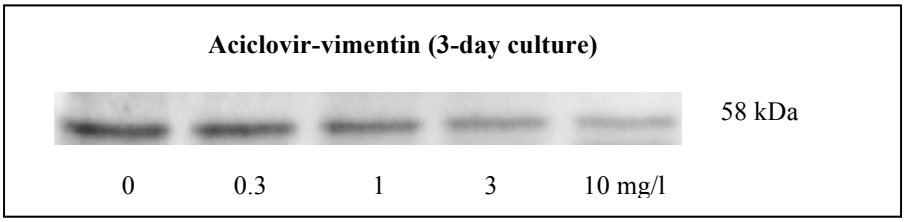


Figure 8:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of N-cadherin after incubation for three days with famciclovir at concentrations ranging from 3 to 100 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

From the figures above, after 3 days of culture no large differences could be seen among the different concentrations of famciclovir tested (Figure 8A). In Figure 8B, the expression of N-cadherin remained between 80% and 100%, even at the highest concentration tested, 100 mg/l. The mean values of N-cadherin expression were 94.4% ± 3.8 %, 88.2% ± 5.0 %, 87.4% ± 9.3 % and 85.3% ± 5.1 % at concentrations from 3 mg/l to 100 mg/l, respectively (n=3; mean ± SD).

(A)



(B)

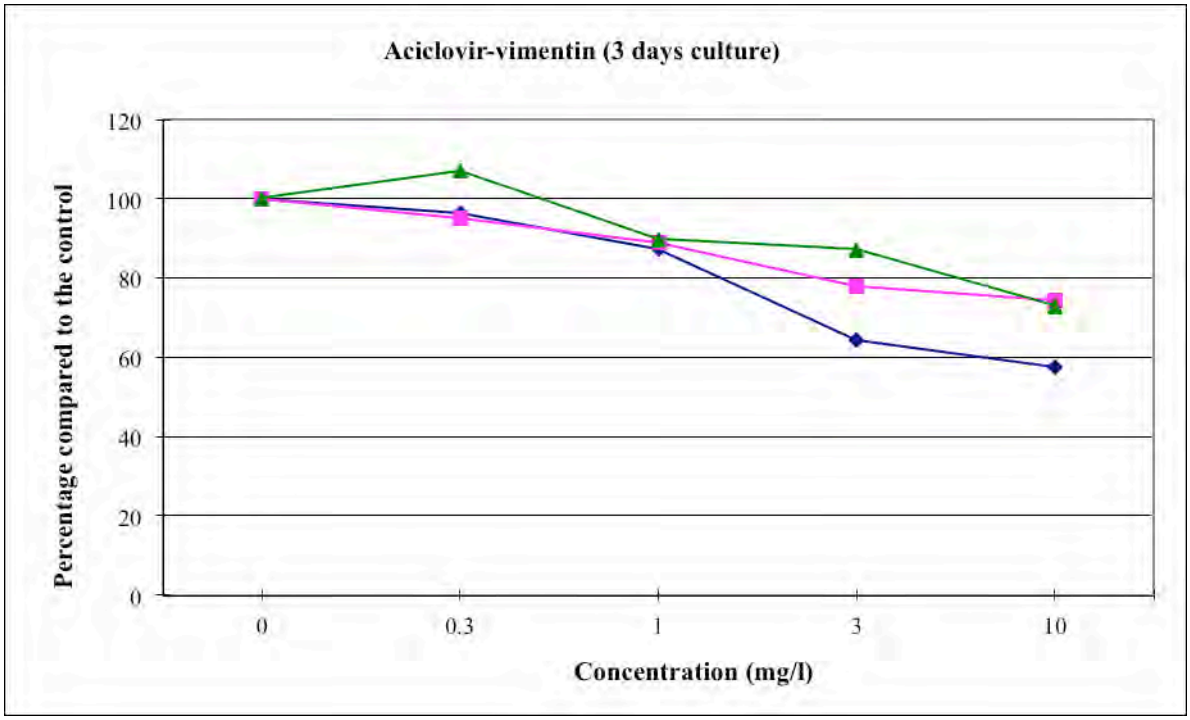


Figure 9:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of vimentin after incubation for three days with aciclovir at concentrations ranging from 0.3 to 10 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

From the figures above, it can be seen that the quantity of vimentin protein decreased in a concentration-dependent manner after three days of culture with aciclovir (Figure 9A). In Figure 9B, it can be seen that the mean value remained above 80% at the two lower concentrations tested; 99.5 ± 5.3 % and 88.7 ± 0.9 % at 0.3 mg/l and 1 mg/l, respectively. More pronounced reductions of the vimentin content were visible at the two higher concentrations tested: to 76.5 ± 9.3 % and 68.3 ± 7.6 % of the control values at 3 mg/l and 10 mg/l, respectively (n=3; mean ± SD).

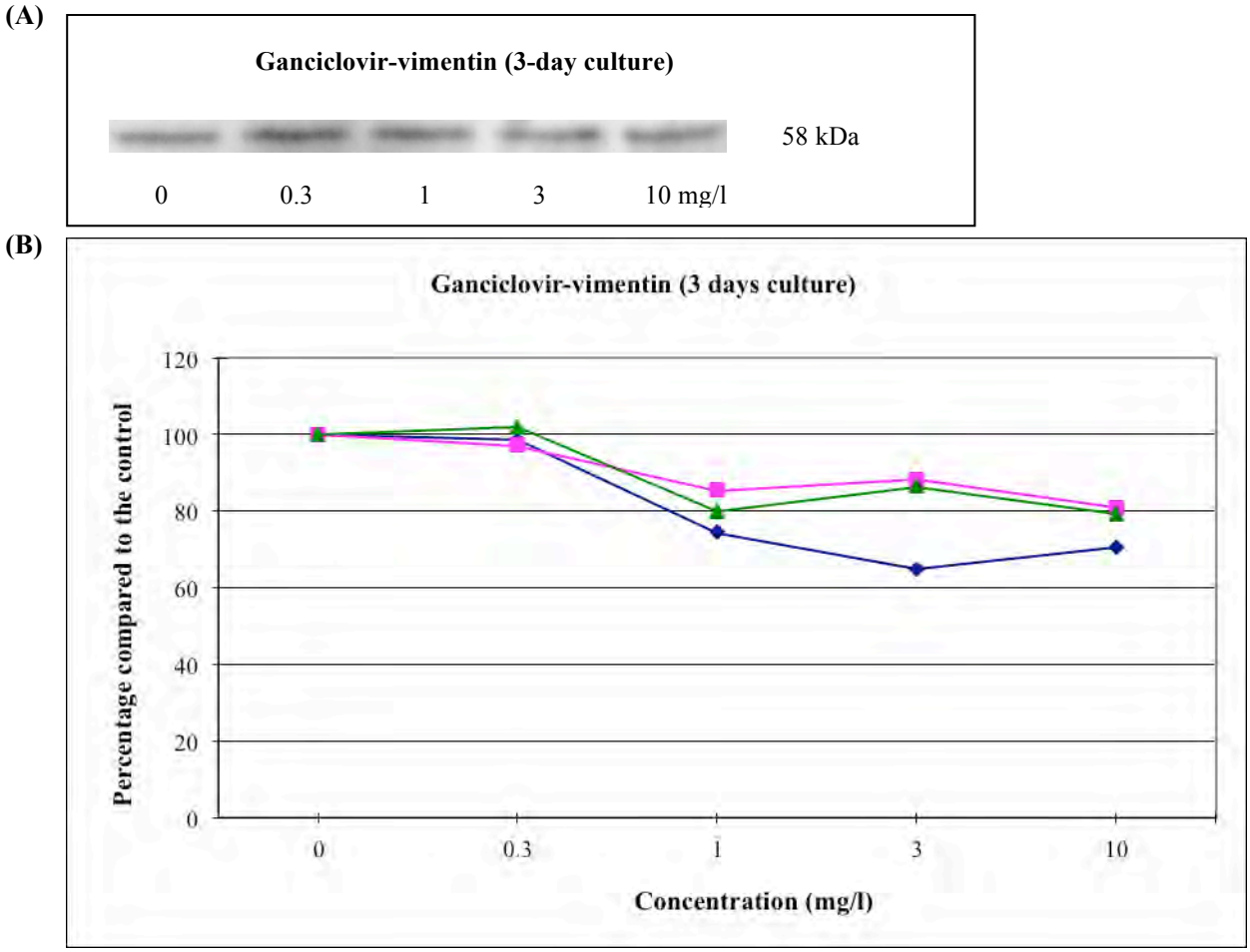


Figure 10:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of vimentin after incubation for three days with ganciclovir at concentrations ranging from 0.3 to 10 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

In Figure 10A, although the level of vimentin present at the concentration of 0.3 mg/l was slightly higher than that of the control group, vimentin otherwise appeared to decrease in a concentration-dependent manner. The expression of vimentin decreased at a lower rate at the two lower concentrations tested (0.3 mg/l and 1 mg/l) than at the two higher concentrations tested (3 mg/l and 10 mg/l). In Figure 10B, the level of vimentin present at the concentration of 0.3 mg/l remained almost the same as that of the control, but it was reduced to 80.0 ± 4.5 %, 79.8 ± 10.6 %, 76.9 ± 4.5 % (n=3; mean ± SD) at the three higher concentrations tested (1 mg/l, 3 mg/l, 10 mg/l), respectively.

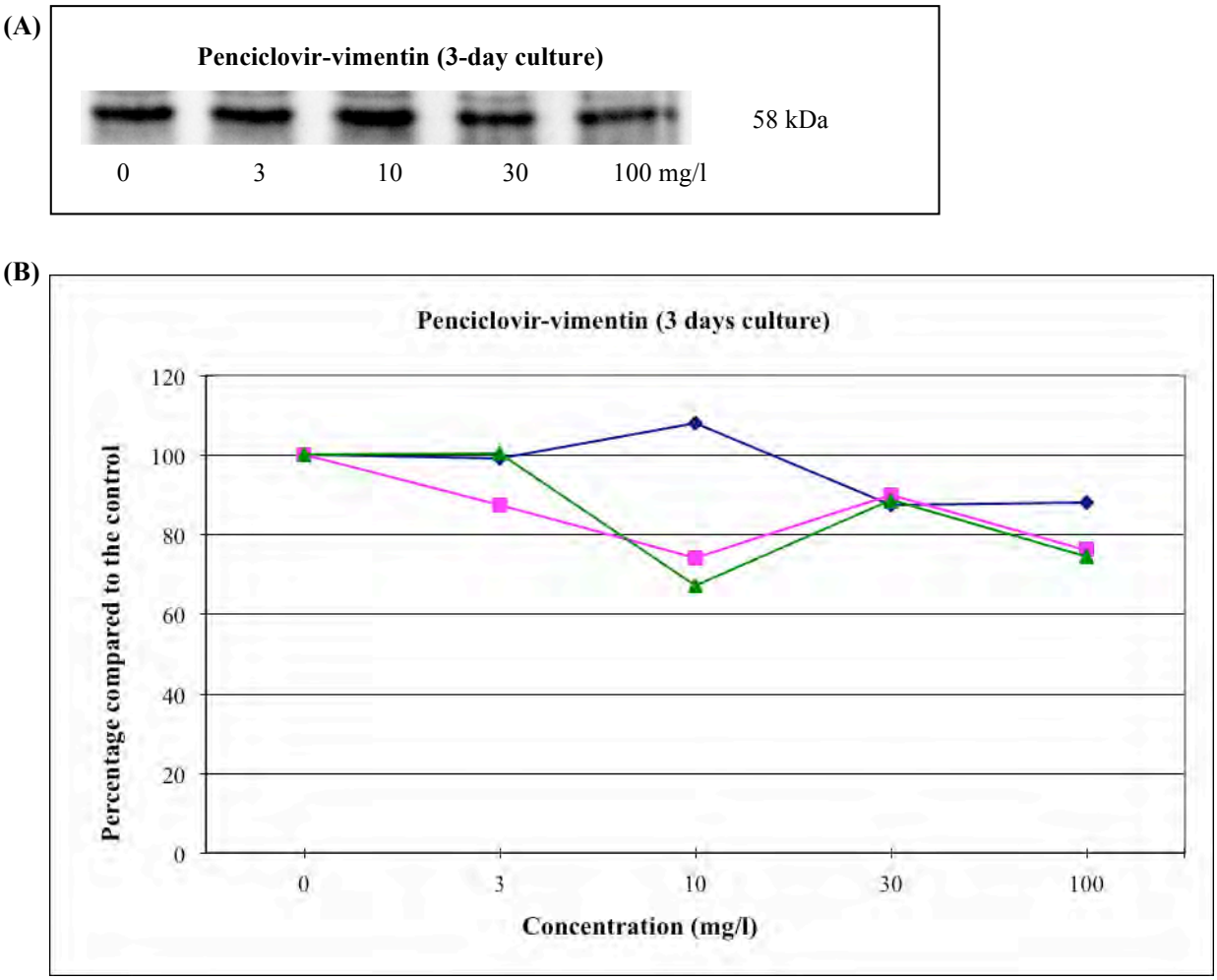


Figure 11:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of vimentin after incubation for three days with penciclovir at concentrations ranging from 3 to 100 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

In Figure 11A, vimentin appeared to slightly decrease in a concentration-dependent manner after three days of culture with penciclovir, except that the level of vimentin present at the concentration of 10 mg/l was slightly higher than that of the control group. Figure 11B shows at the highest concentration tested (100 mg/l), the expression of vimentin was 79.5 ±6.0 % that of the control. At 10 mg/l and 30 mg/l, the expression of vimentin was 83.0 ± 17.9 % and 88.5 ± 1.0 %, respectively (n=3; mean ± SD). The lowest concentration of penciclovir, 3 mg/l, did not have any obvious effects on the level of vimentin present.

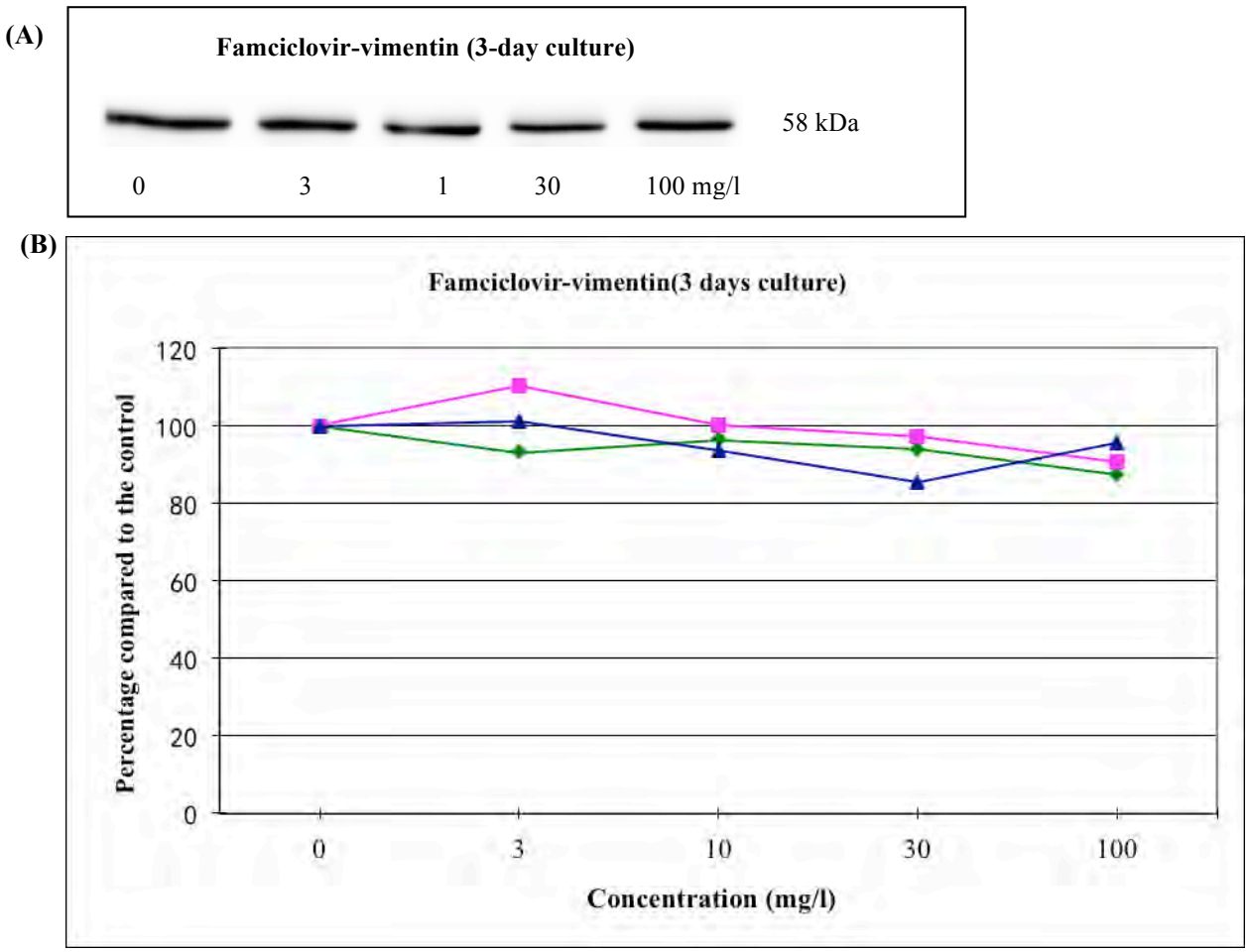


Figure 12:
(A) Result of a Western Blot experiment performed with Sertoli cells, expression of vimentin after incubation for three days with famciclovir at concentrations ranging from 3 to 100 mg/l.
(B) Results from three independently performed experiments. Western Blots were analyzed in three separate experiments. The blue line represents the blot shown in Figure 11A after densitometric analysis.

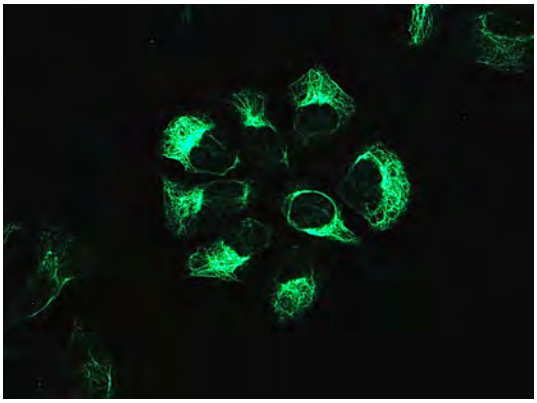
Figures above show that very similar levels of vimentin were present in all samples of cells incubated with various concentrations (Figure 12A, B). There were no obvious differences between the treated group and the control group. After three days of culture with famciclovir at the concentrations tested, the expression of vimentin remained between 80% and 100% that of the control, with the mean value across this period remaining above 90%. When the concentration of famciclovir increased from 10 mg/l to 100 mg/l, the values of the expression were reduced to 96.7% ± 2.7%, 92.3% ± 5.0% and 91.2% ± 3.3%, respectively (n=3; mean ± SD).

3.2 Immunofluorescence

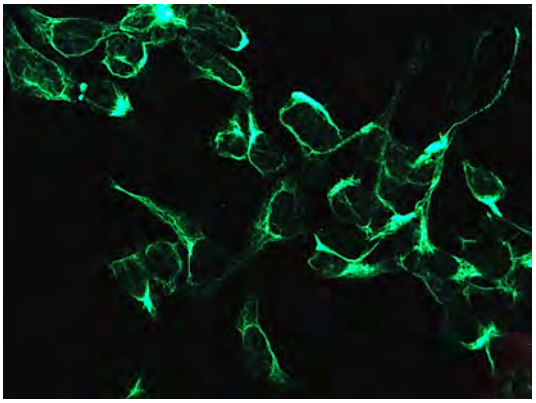
In the figures below, the immunofluorescence staining patterns of connexin43, N-cadherin and vimentin are shown. At a magnification of 400, the bright green fluorescence signal is present in the cellular plasma or on the cell membranes, revealing the shape of the Sertoli cells. Inside the outline of the cells, the nucleus is represented by the dark area.

After three days of culture with different concentrations of nucleoside analogues, the fluorescence intensity of the samples decreased slightly compared to that of the controls, apart from that of the samples cultured with famciclovir, for which no effects were observed. In particular, at the highest concentrations tested, 10 mg/l (aciclovir and ganciclovir) and 100 mg/l (penciclovir), the fluorescence intensity was weaker than that of the control group. Compared to N-cadherin and vimentin, the fluorescence intensity of connexin43 was more significantly reduced. No aberrant localization was detected. The distribution of fluorescence remained the same in the treated and untreated Sertoli cells.

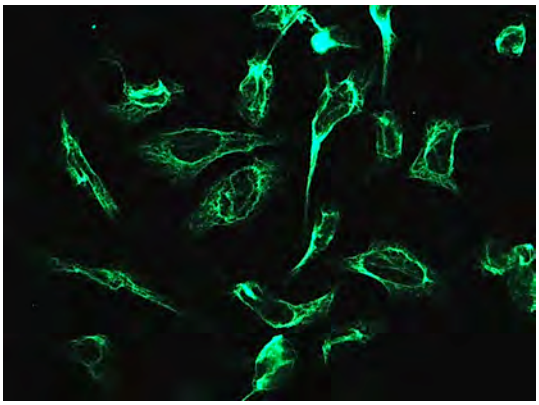
Compared to the cell spin technique, this method showed more advantages when employed with the culture slides, allowing the original locations of the proteins inside the Sertoli cells to be shown, along with the connections between two adjacent cells.



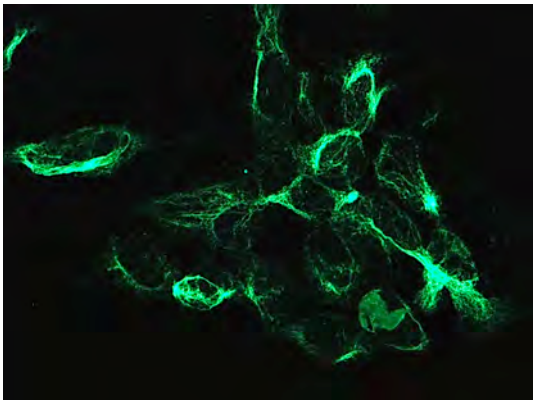
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Connexin43



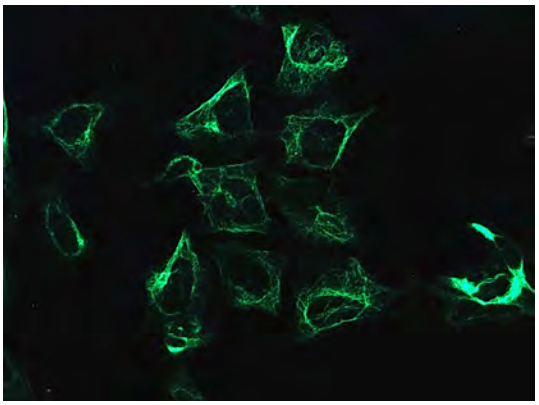
(b) Aciclovir-0.3 mg/l:
Sertoli cells incubated for 3 days with 0.3 mg aciclovir/l culture medium; immunofluorescence using an antibody against Connexin43



(c) Aciclovir-1 mg/l:
Sertoli cells incubated for 3 days with 1 mg aciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

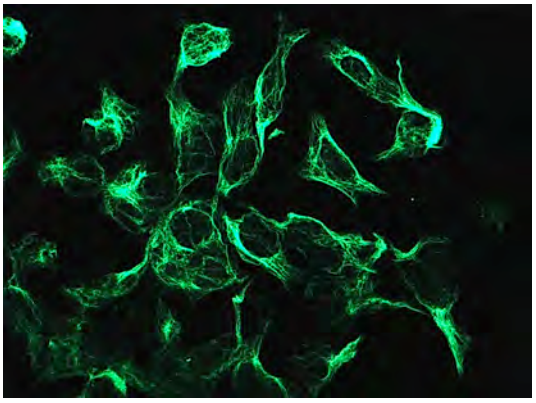


(d) Aciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg aciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

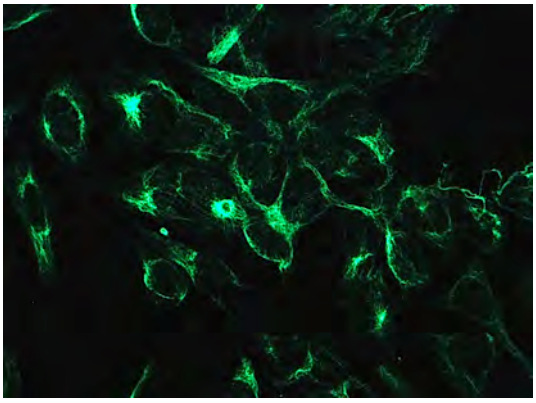


(e) Aciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10mg aciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

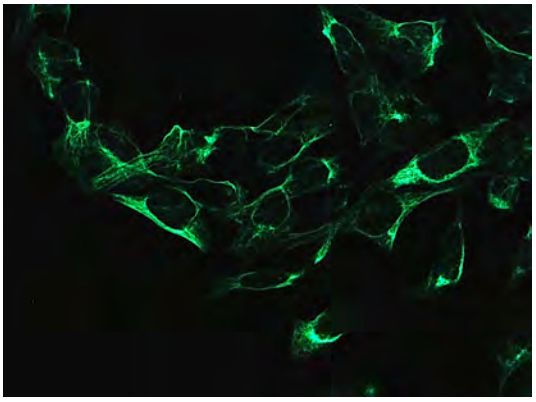
Figure 13: Immunofluorescence of SerW3 Cells using an antibody against Connexin43 after incubation with Aciclovir for three days.
Magnification 400×.



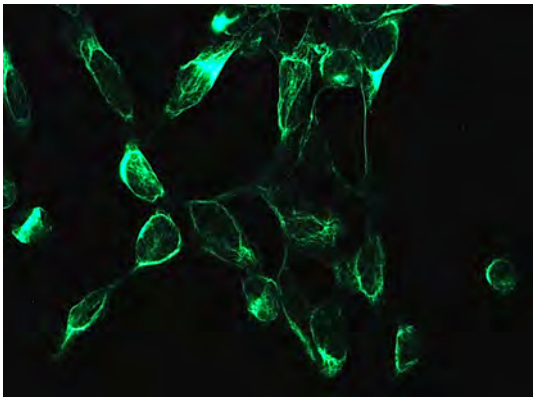
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Connexin43



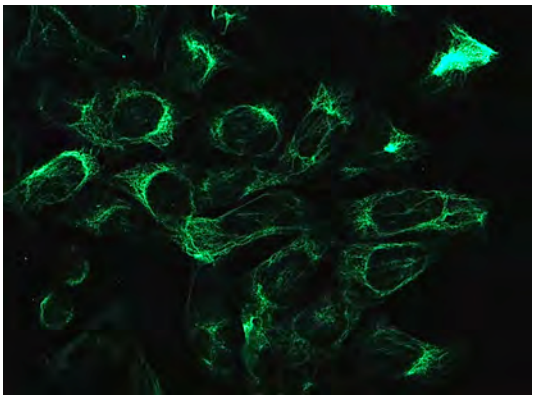
(b) Ganciclovir-0.3 mg/l:
Sertoli cells incubated for 3 days with 0.3 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Connexin43



(c) Ganciclovir-1 mg/l:
Sertoli cells incubated for 3 days with 1 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

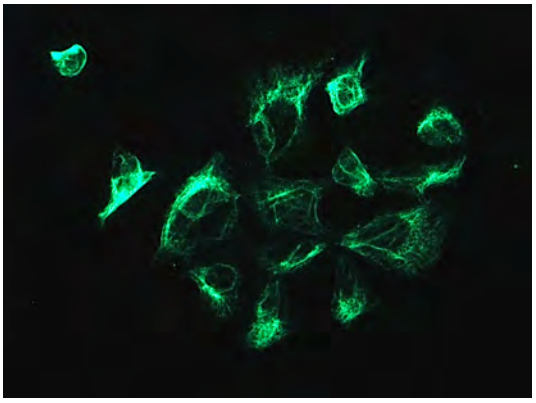


(d) Ganciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

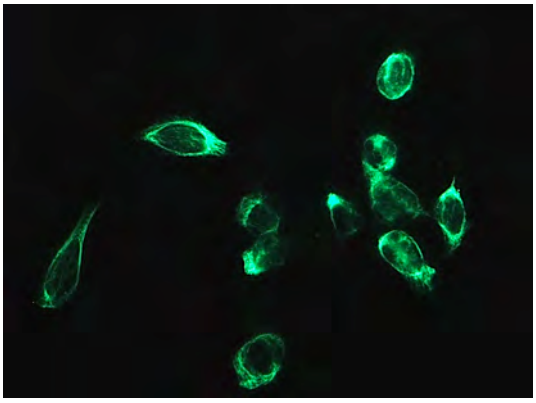


(e) Ganciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

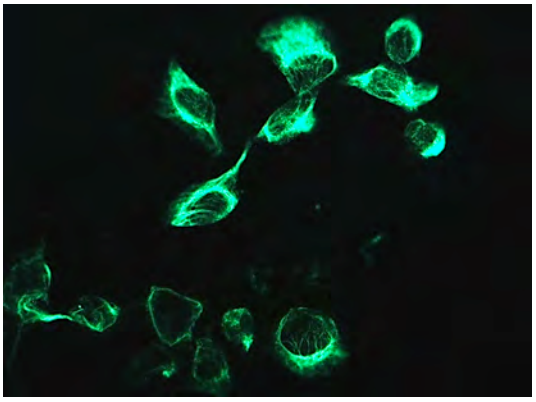
Figure 14: Immunofluorescence of SerW3 Cells using an antibody against Connexin43 after incubation with Ganciclovir for three days.
Magnification 400×.



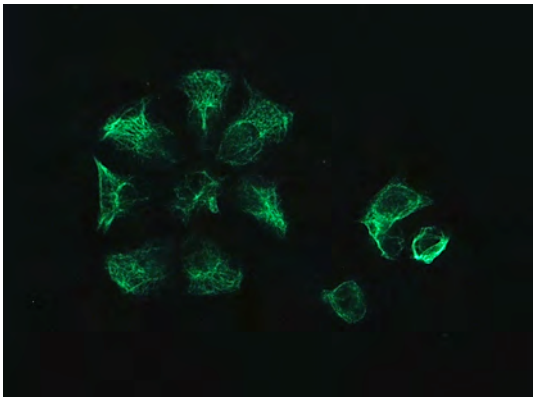
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Connexin43



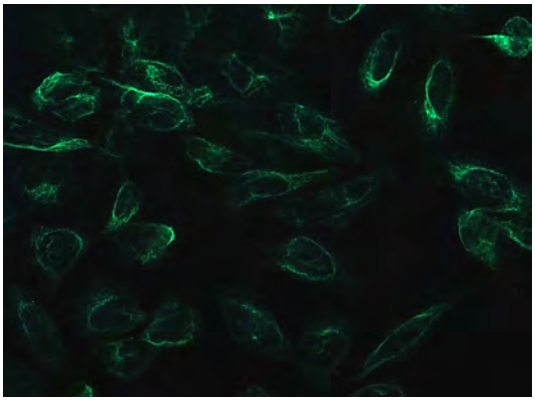
(b) Penciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg penciclovir/l culture medium; immunofluorescence using an antibody against Connexin43



(c) Penciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg penciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

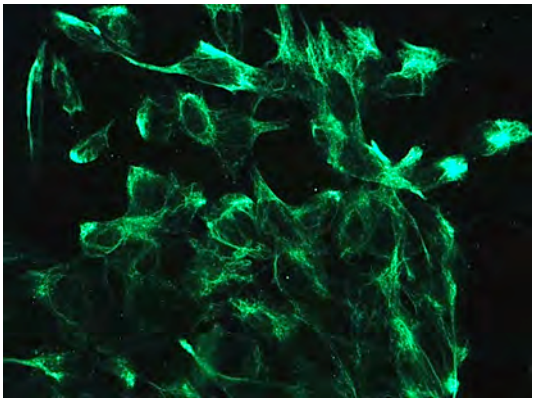


(d) Penciclovir-30 mg/l:
Sertoli cells incubated for 3 days with 30 mg penciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

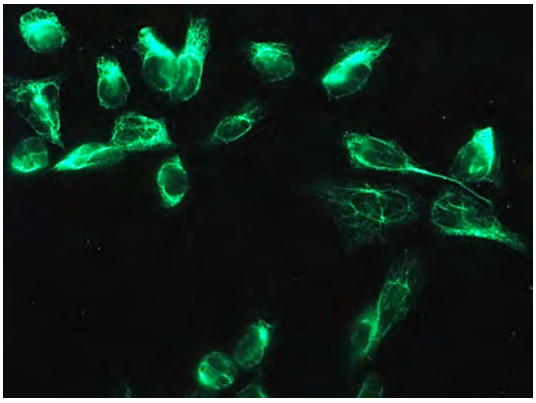


(e) Penciclovir-100 mg/l:
Sertoli cells incubated for 3 days with 100 mg penciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

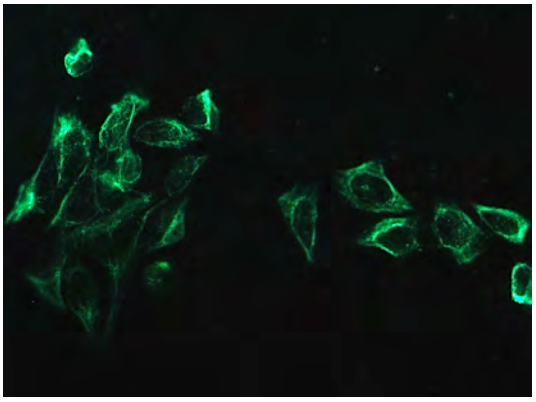
Figure 15: Immunofluorescence of SerW3 Cells using an antibody against Connexin43 after incubation with Penciclovir for three days.
Magnification 400×.



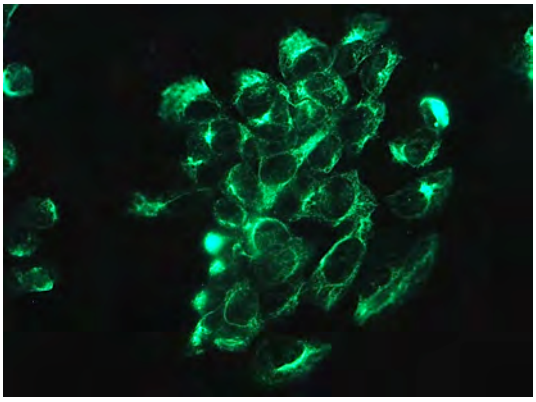
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Connexin43



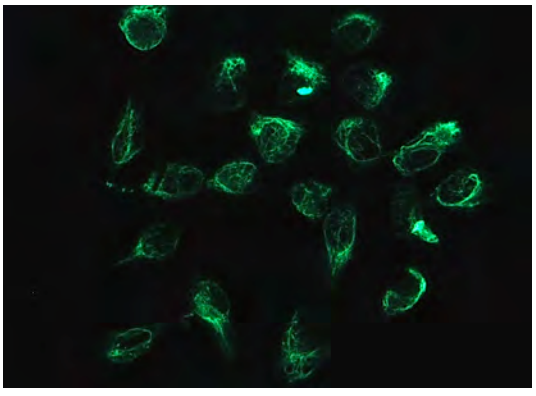
(b) Famciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg famciclovir/l culture medium; immunofluorescence using an antibody against Connexin43



(c) Famciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg famciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

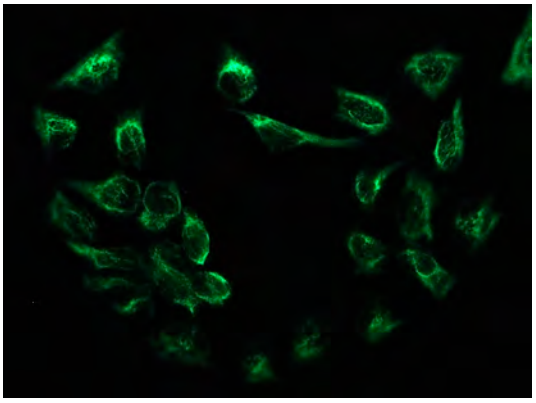


(d) Famciclovir-30 mg/l:
Sertoli cells incubated for 3 days with 30 mg famciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

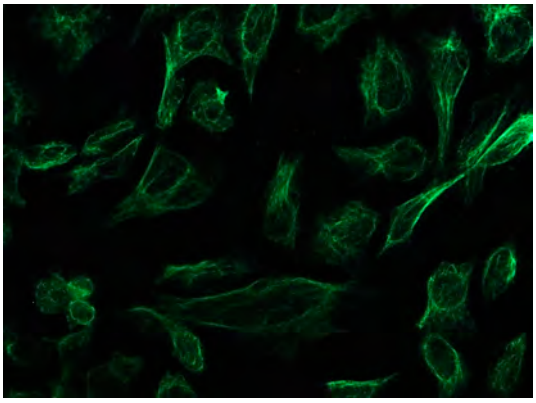


(e) Famciclovir-100 mg/l:
Sertoli cells incubated for 3 days with 100 mg famciclovir/l culture medium; immunofluorescence using an antibody against Connexin43

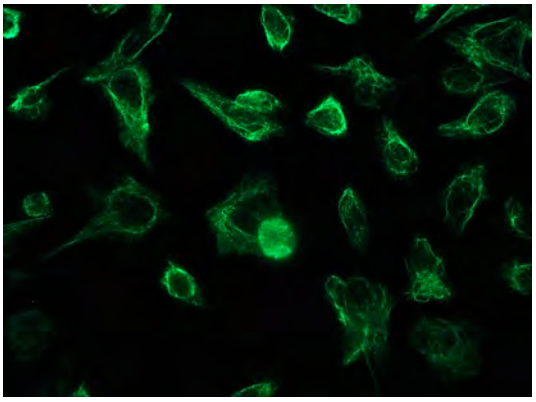
Figure 16: Immunofluorescence of SerW3 Cells using an antibody against Connexin43 after incubation with Famciclovir for three days.
Magnification 400×.



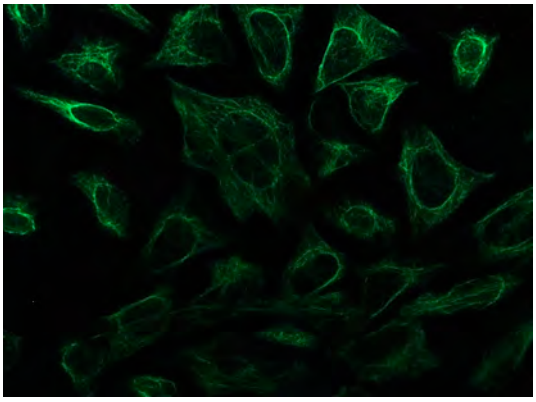
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Vimentin



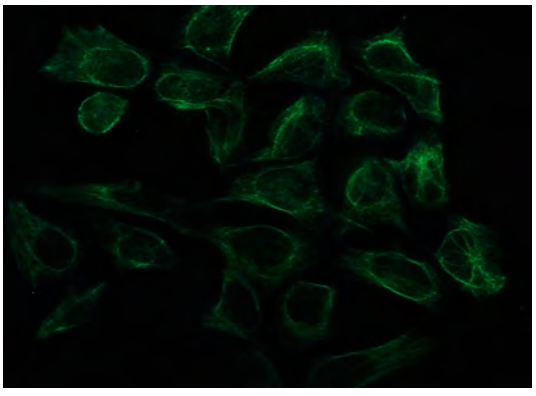
(b) Aciclovir-0.3 mg/l:
Sertoli cells incubated for 3 days with 0.3 mg aciclovir/l culture medium; immunofluorescence using an antibody against Vimentin



(c) Aciclovir-1 mg/l:
Sertoli cells incubated for 3 days with 1 mg aciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

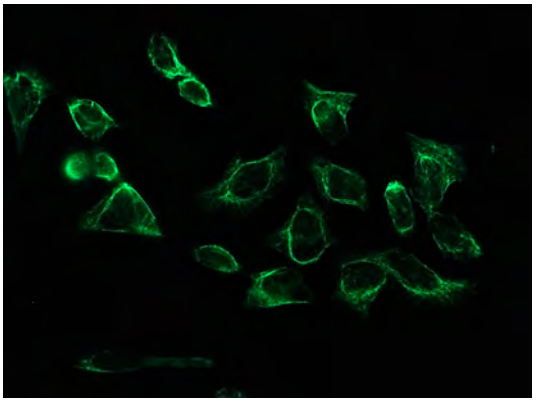


(d) Aciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg aciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

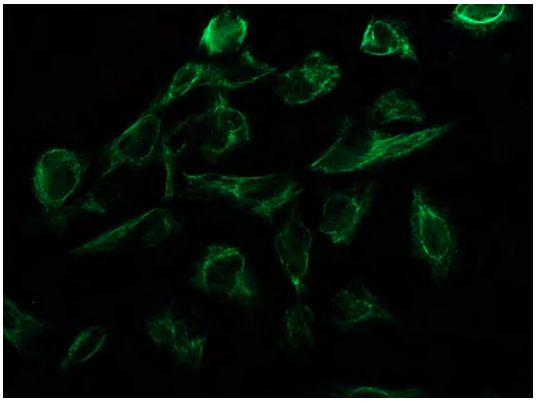


(e) Aciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg aciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

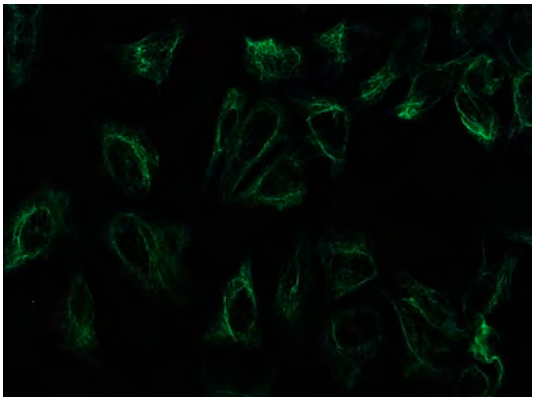
Figure 17: Immunofluorescence of SerW3 Cells using an antibody against vimentin after incubation with Aciclovir for three days.
Magnification 400×.



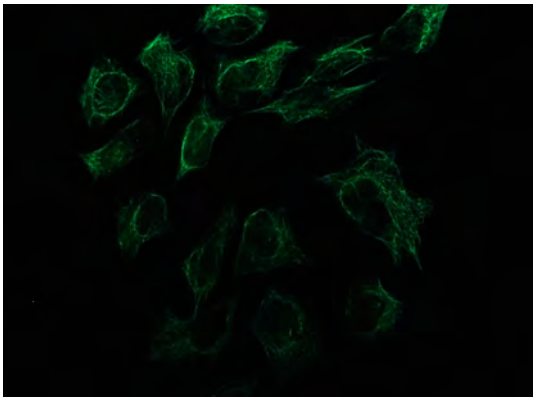
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Vimentin



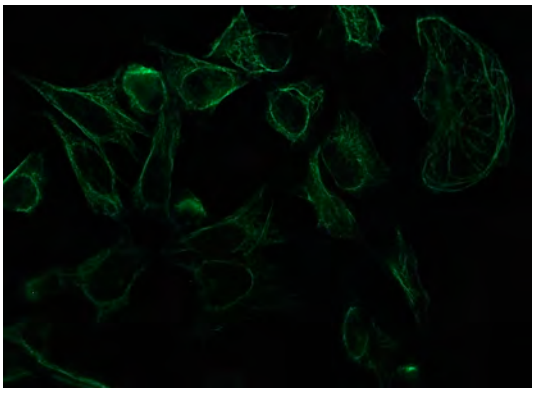
(b) Ganciclovir-0.3 mg/l:
Sertoli cells incubated for 3 days with 0.3 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Vimentin



(c) Ganciclovir-1 mg/l:
Sertoli cells incubated for 3 days with 1 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

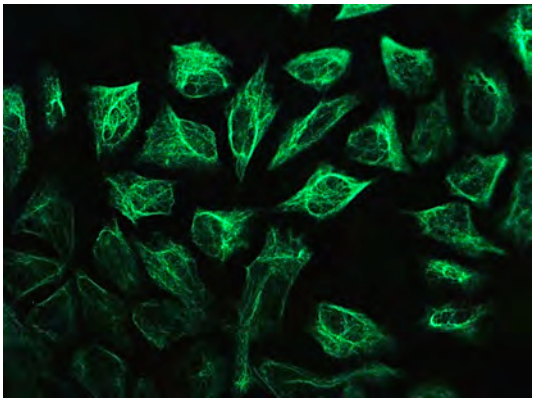


(d) Ganciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

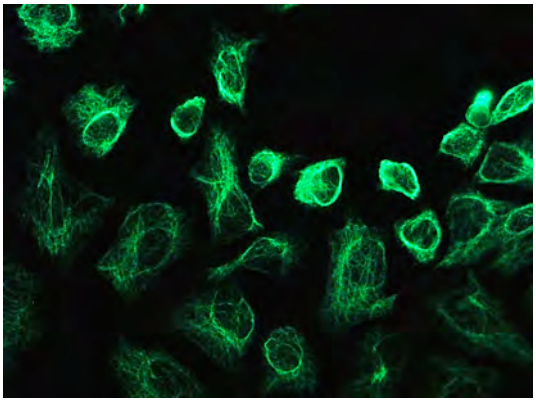


(e) Ganciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg ganciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

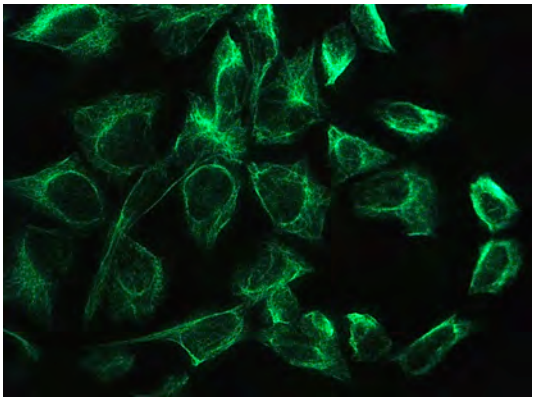
Figure 18: Immunofluorescence of SerW3 Cells using an antibody against vimentin after incubation with Ganciclovir for three days.
Magnification 400×.



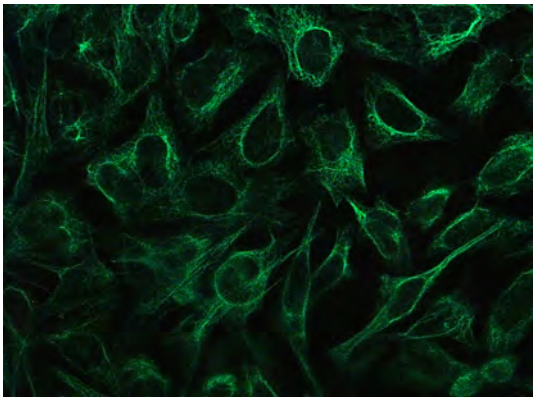
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Vimentin



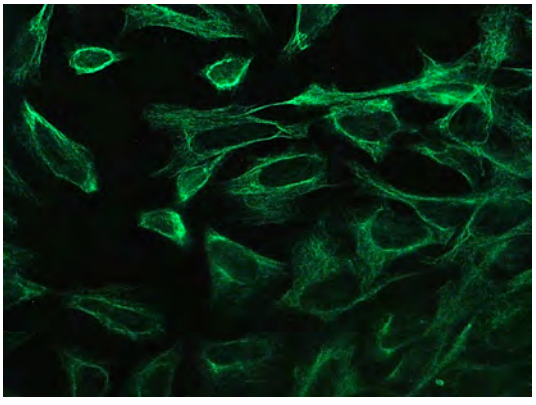
(b) Penciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg penciclovir/l culture medium; immunofluorescence using an antibody against Vimentin



(c) Penciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg penciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

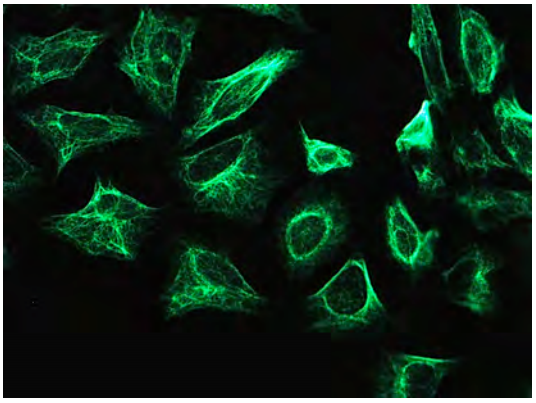


(d) Penciclovir-30 mg/l:
Sertoli cells incubated for 3 days with 30 mg penciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

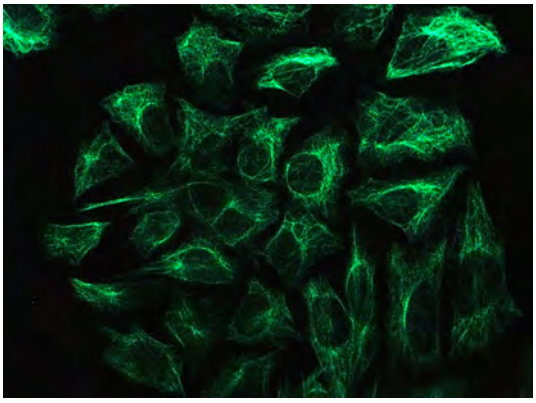


(e) Penciclovir-100 mg/l:
Sertoli cells incubated for 3 days with 100 mg penciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

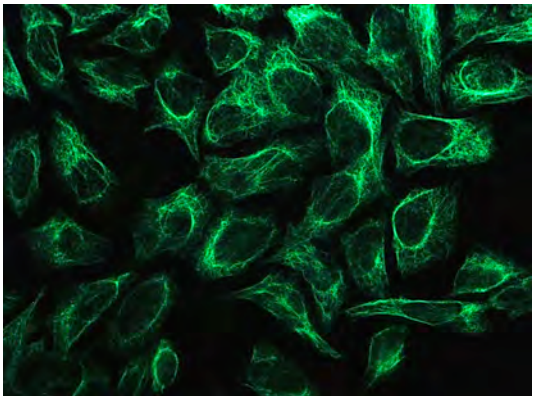
Figure 19: Immunofluorescence of SerW3 Cells using an antibody against Vimentin after incubation with Penciclovir for three days.
Magnification 400×.



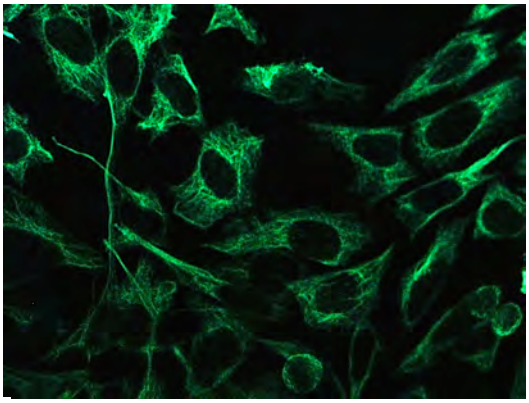
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against Vimentin



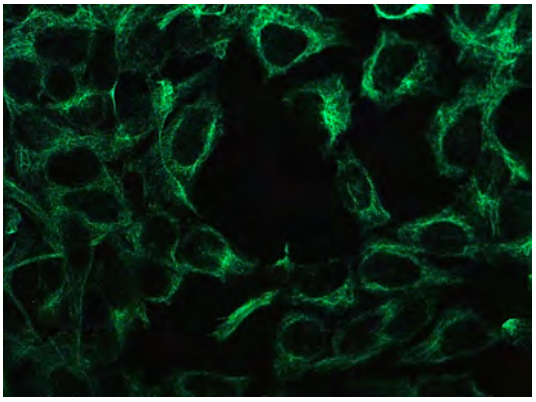
(b) Famciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg famciclovir/l culture medium; immunofluorescence using an antibody against Vimentin



(c) Famciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg famciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

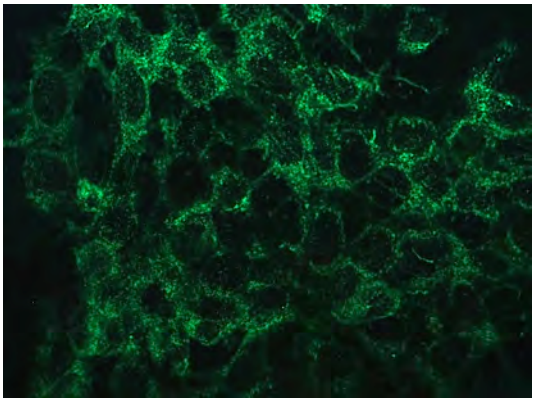


(d) Famciclovir-30 mg/l:
Sertoli cells incubated for 3 days with 30 mg famciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

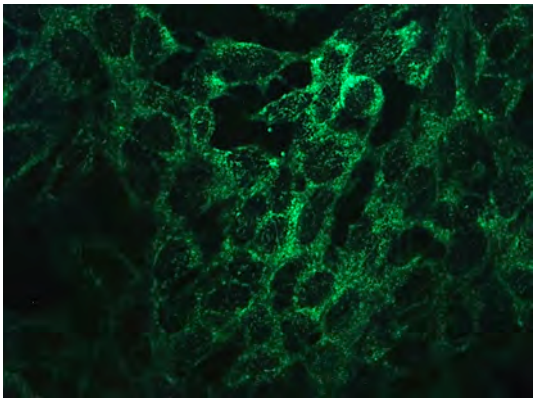


(e) Famciclovir-100 mg/l:
Sertoli cells incubated for 3 days with 100 mg famciclovir/l culture medium; immunofluorescence using an antibody against Vimentin

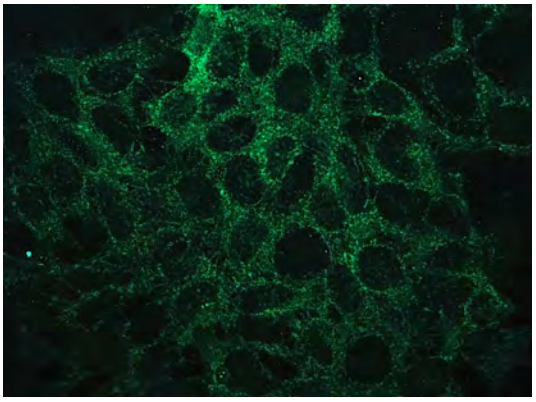
Figure 20: Immunofluorescence of SerW3 Cells using an antibody against Vimentin after incubation with Famciclovir for three days.
Magnification 400×.



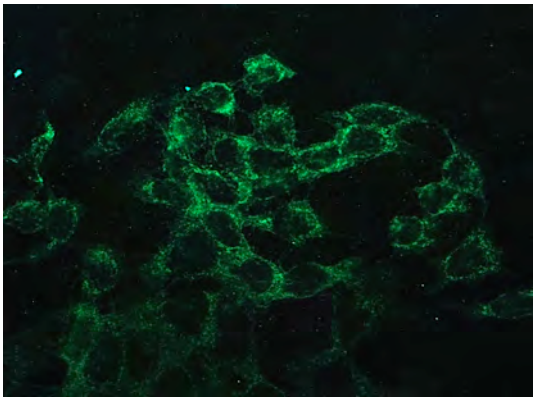
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against N-cadherin



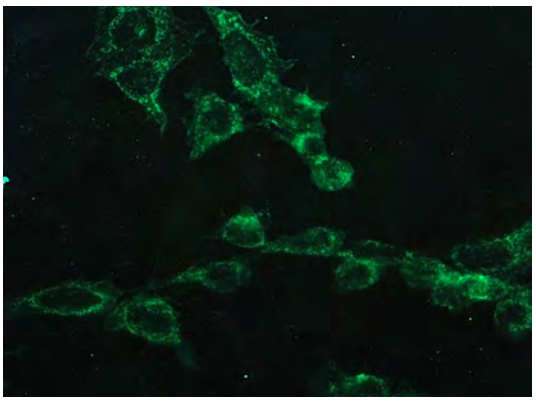
(b) Aciclovir-0.3 mg/l:
Sertoli cells incubated for 3 days with 0.3 mg aciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin



(c) Aciclovir-1 mg/l:
Sertoli cells incubated for 3 days with 1 mg aciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

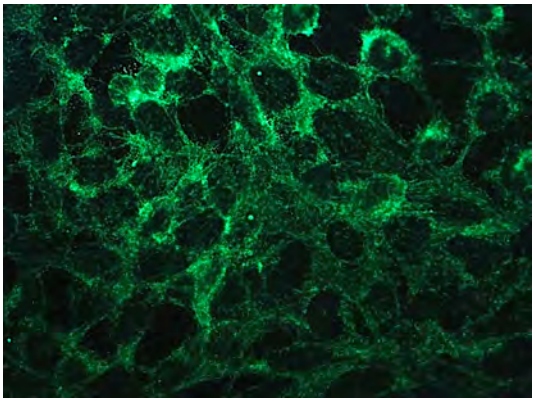


(d) Aciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg aciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

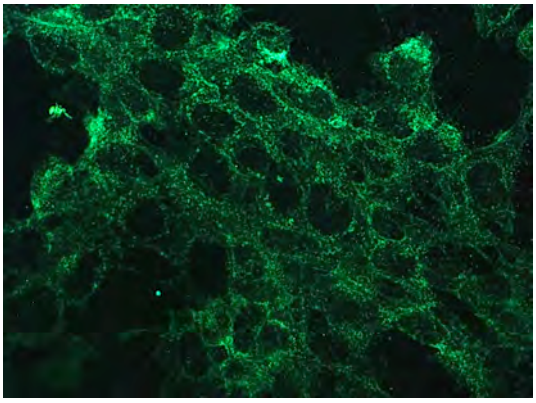


(e) Aciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg aciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

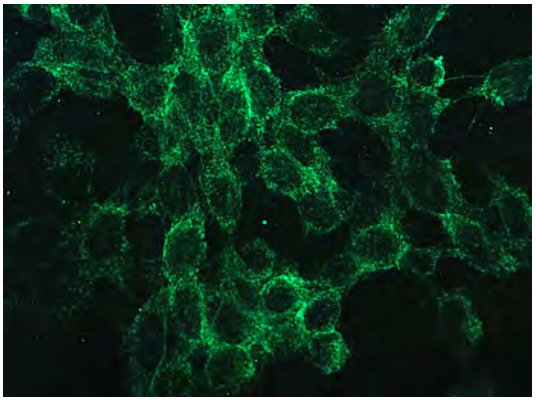
Figure 21: Immunofluorescence of SerW3 Cells using an antibody against N-cadherin after incubation with Aciclovir for three days.
Magnification 400×.



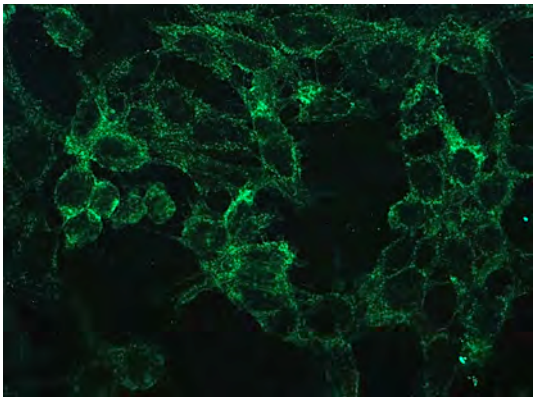
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against N-cadherin



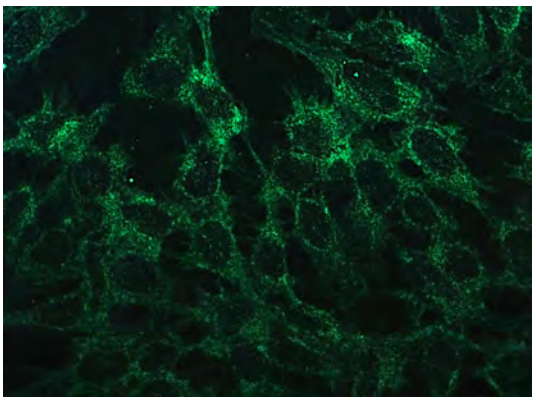
(b) Ganciclovir-0.3 mg/l:
Sertoli cells incubated for 3 days with 0.3 mg ganciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin



(c) Ganciclovir-1 mg/l:
Sertoli cells incubated for 3 days with 1 mg ganciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

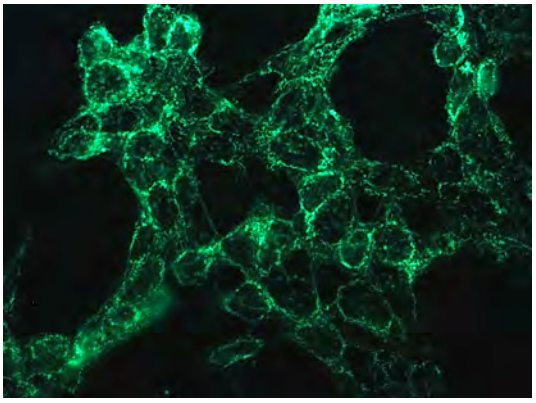


(d) Ganciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg ganciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

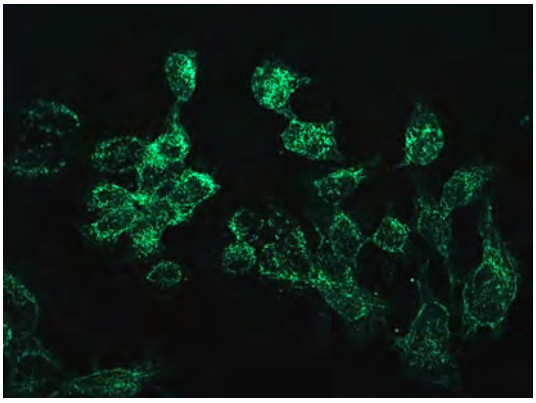


(e) Ganciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg ganciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

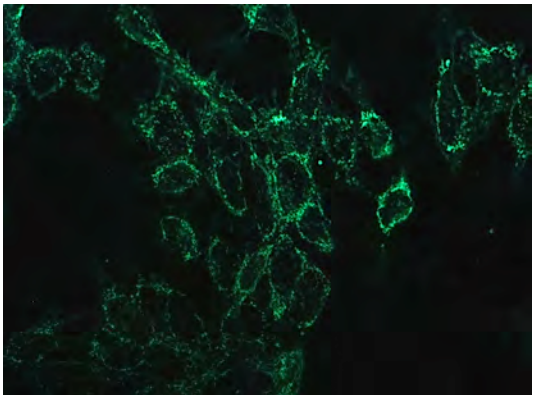
Figure 22: Immunofluorescence of SerW3 Cells using an antibody against N-cadherin after incubation with Ganciclovir for three days.
Magnification 400×.



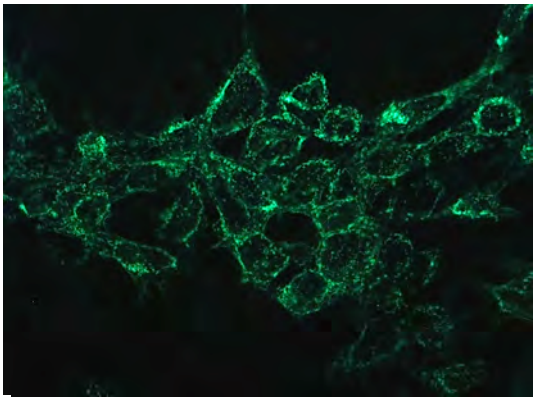
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against N-cadherin



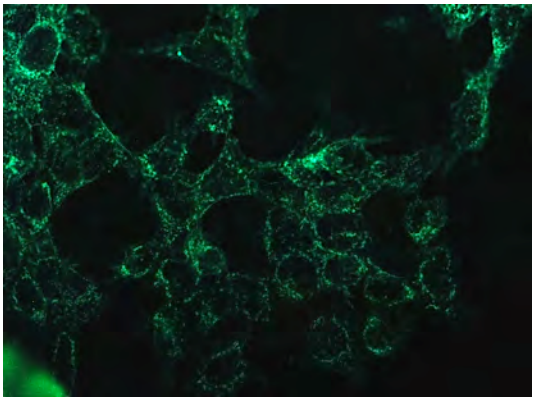
(b) Penciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg penciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin



(c) Penciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg penciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

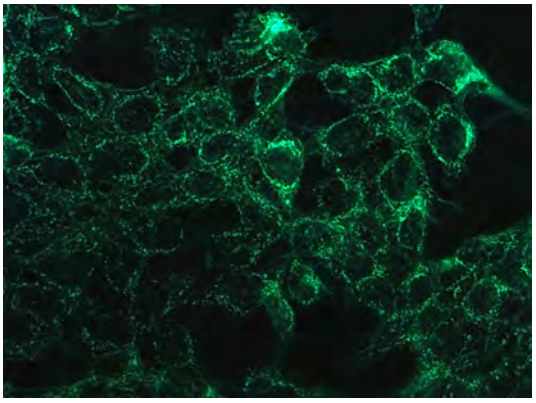


(d) Penciclovir-30 mg/l:
Sertoli cells incubated for 3 days with 30 mg penciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

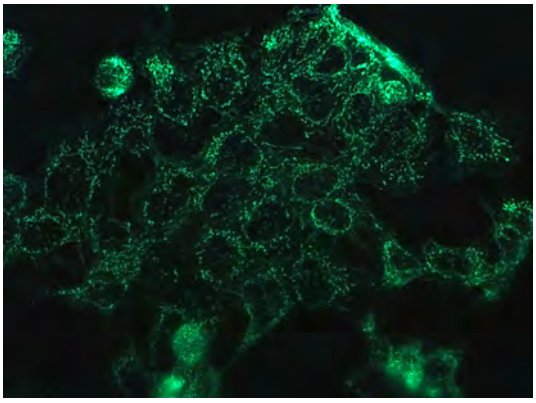


(e) Penciclovir-100 mg/l:
Sertoli cells incubated for 3 days with 100 mg penciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

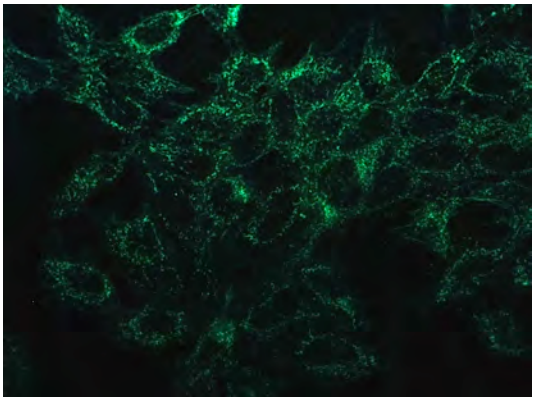
Figure 23: Immunofluorescence of SerW3 Cells using an antibody against N-cadherin after incubation with Penciclovir for three days.
Magnification 400×.



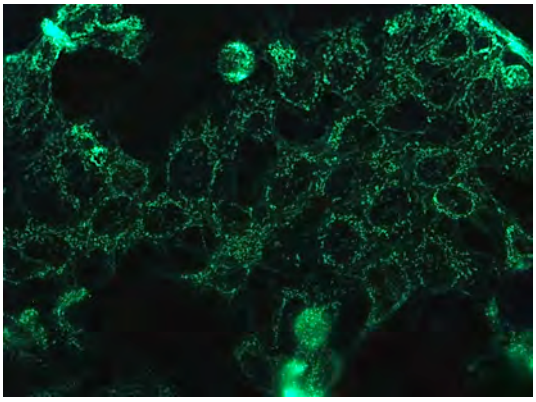
(a) Control: Sertoli cells from SerW3 cell line incubated for 3 days; immunofluorescence using an antibody against N-cadherin



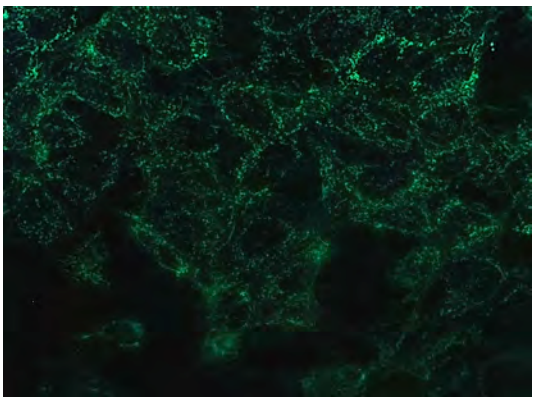
(b) Famciclovir-3 mg/l:
Sertoli cells incubated for 3 days with 3 mg famciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin



(c) Famciclovir-10 mg/l:
Sertoli cells incubated for 3 days with 10 mg famciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin



(d) Famciclovir-30 mg/l:
Sertoli cells incubated for 3 days with 30 mg famciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin



(e) Famciclovir-100 mg/l:
Sertoli cells incubated for 3 days with 100 mg famciclovir/l culture medium; immunofluorescence using an antibody against N-cadherin

Figure 24: Immunofluorescence of SerW3 Cells using an antibody against N-cadherin after incubation with Famciclovir for three days.
Magnification 400×.

4 Discussion

4.1 Reproductive toxicity of nucleoside analogues

Purine nucleoside analogues, a class of antiviral agents, exhibit a potential for testicular toxicity. In animal experiments as well as in vitro, it was shown that nucleoside analogues, including aciclovir, ganciclovir, famciclovir and penciclovir, may have a negative effect on the testis or isolated cells derived from this organ.

In the Western Blot analyses carried out in this dissertation, aciclovir showed a notable negative effect on connexin43 expression at concentrations of 3 mg/l and 10 mg/l, and at 10 mg/l the expressions of vimentin and N-cadherin also decreased compared to that of the untreated Sertoli cells.

In a clinical trial involving 20 male subjects with normal sperm counts, aciclovir was administered orally at doses of up to 1 g per day for up to six months. The results revealed that aciclovir has no clinically significant effect on the sperm count, motility or morphology in humans (GlaxoSmithKline, 2012; Full Prescribing Information Zovirax®). However, animal experiments have revealed different results. In experiments published by Bairy et al., the testis weight of mice decreased after they were treated intraperitoneally with aciclovir at doses ranging from 4 to 48 mg/kg/day continuously for 15 days, and their sperm motility was inhibited, along with decreased sperm count and increased sperm abnormalities being observed. Although the stem cell lines obviously were not affected, because sperm quality had returned to normal by day 70, aciclovir did affect the normal function of spermatogenesis (Bairy et al., 2009). Similar experiments have been performed with rats, which led to similar results. Male rats exposed to aciclovir at doses of 16 and 48 mg/kg showed histopathological changes in the testis and decreased fertility (Elham et al., 2013). This was contrary to previous results from a preclinical toxicity study conducted in 1983, when 15 mature male mice were orally treated with aciclovir at doses of 50, 150 and 450 mg/kg/day for 64 days before mating, and no adverse effects on fertility were observed (Moore et al., 1983). After treatment with the highest dose of 450 mg/kg the concentration of the drug in blood plasma was 11 mg/l.

The toxicity of ganciclovir was also studied both in vitro and in vivo. Janoly-Dumenil et al., for instance, found that high-level exposure to ganciclovir (20 mg/l) caused a decrease in the total number of B lymphoblastoid cells when the exposure time exceeded two days, and the growth rate of B lymphoblastoid cells showed a reduction of 52% (Janoly-Dumenil et al., 2009; Janoly-Dumenil et al., 2012). Recently, the cytotoxic effects of ganciclovir on cultured human corneal endothelial cells (HCECs) were investigated, and a high concentration was found to increase the risk of cell damage (Choi et al., 2013). In this study, the two higher concentrations of ganciclovir tested (3 mg/l and 10 mg/l) were found to cause a reduction in the expression of connexin43 and N-cadherin, with the change in connexin43 in particular being quite pronounced. The results indicated that ganciclovir has a negative effect on Sertoli cells and possibly on the blood-testis barrier. Impairment of male fertility has been observed in vivo. When HSV-1 infected mice received ganciclovir at doses ranging from 50 to 200 mg per kg over a 6-week period, along with uninfected mice receiving 100 mg per kg for 14 days, a marked reduction in testicular weight was observed, although bodyweight increased normally (Neyts et al., 1995). Ganciclovir has been shown to induce testicular damage and reduce sperm quality in rats, after short-term exposure, with the Gan5 day regimen (a single dose of 60 mg/kg daily for 5 days) found to be more toxic than the Gan1 day regimen (100 mg/kg administered three times at 4 h-intervals in a single day) (Faqi et al., 1997).

No in vitro studies have been conducted on famciclovir or penciclovir. Although famciclovir was found in clinical trials to be negative for reproductive toxicity (Sacks et al., 1994), famciclovir has nonetheless been found to impair spermatogenesis and alter normal sperm parameters in rats (Helal et al., 2009). The difference in results between the clinical trials and animal experiments could be explained by the different doses involved. In the Sacks' study, the subjects received 500 mg of famciclovir each day, while the rats in Helal's experiment received 145 mg/kg (Sacks et al., 1994; Helal et al., 2009). The potential for testicular toxicity of famciclovir can thus not be ruled out. In the results of this dissertation, the Western Blot analyses and the immunofluorescence of the Sertoli cells incubated with famciclovir do not show any clear-cut differences compared to the results of the control. Presumably because Sertoli cells were incapable of metabolizing famciclovir to penciclovir, when penciclovir, the metabolite of

famciclovir, was tested, it altered the expression of the connexin43 and vimentin proteins.

The reproductive toxicity of nucleoside analogues appears to be time- and dose- dependent. The higher the doses tested, the earlier toxicity was observed. When the exposure time was increased, even relatively low doses could lead to impairments of spermatogenesis. In the mice treated with aciclovir at doses ranging from 4 to 48 mg/kg/day, testis weight began to decrease on day 21 and day 28 at doses of 32 and 48 mg/kg/day respectively, while on day 35 it decreased for all dose-levels tested (Bairy et al., 2009). When the corneal endothelial cells were exposed to various concentrations of ganciclovir for two days, cytotoxic effects were also visible and occurred in a concentration-dependent manner. Although low concentrations of ganciclovir did not reduce cell viability, higher concentrations resulted in cell cycle delay, lower proliferation rate and an increased number of apoptotic cells (Choi et al., 2013). During the first two days of exposure, a high concentration of ganciclovir (20 mg/l) did not appear to be more toxic than a low concentration (1 mg/l) on B lymphoblastoid cells. When the exposure duration exceeded two days, however, incubation with ganciclovir reduced the total cell number (Janoly-Dumenil et al., 2009). It was assumed that for each specific concentration of nucleoside analogues tested, there would be a minimal duration required to exhibit toxicity, while for each specific duration of exposure, there would be a minimal concentration. In this dissertation, when the concentration of aciclovir and ganciclovir was ≥ 3 mg/l, the target proteins started to decrease after three days of exposure. For penciclovir, the minimum concentration threshold was approximately 30 mg/l.

The impairments of fertility caused by the nucleoside analogues studied may be reversible. Abnormal semen parameters in mice, for instance, have been shown to fully recover by the 70th day after the treatment (Bairy et al., 2009). Morphological alterations observed in rat testis were also observed to reverse 24 weeks after treatment with ganciclovir (Faqi et al., 1997). There may, however, be a certain high dose beyond which the effects are irreversible. Decreased fertility in mice was shown to be reversible after daily intraperitoneal administration of ganciclovir at a dose of 2 mg/kg, but it was irreversible or incompletely reversible after daily doses of 10 mg/kg (Roche, 2010; Full Prescribing Information Cymevene®-IV). It was assumed that the abnormalities required a relatively long time to be completely reversed, and hence that impaired

fertility would still be detected when examining the mice before they had time to fully recover. After 4 weeks of recovery time from treatment with famciclovir, semen parameters (sperm count, sperm motility and viability) increased in the recovery group, but still less than those of the control group. The percentage of abnormal sperm observed was smaller than that of the treated group but still greater than that of the control group (Helal et al., 2009).

Some studies have aimed to compare the cellular toxicity of aciclovir, ganciclovir and penciclovir in primary cells or HSV-infected cell lines (Thust et al., 2000). Ganciclovir exhibited cytotoxic activity toward lymphoma cells that harbored Kaposi's sarcoma-associated herpesvirus (KSHV), while aciclovir showed weak or no activity (Fujimuro et al., 2006). Ganciclovir was found to have the greatest potential to induce cell death, while aciclovir only evoked relatively minor effects. Treatment with ganciclovir induced rapid accumulation of cells in S-phase and DNA laddering, followed by apoptotic cell death. Penciclovir behaved similarly to a much lesser extent, with only a decrease in the mitotic index and an increase in cell cycle delay being observed (Thust et al., 1996; Shaw et al., 2001). The unrepairable DNA damage, as evidenced particularly by the presence of DNA double-strand breaks (DSBs), may be responsible for the greater toxicity of ganciclovir. The activation of HSV-TK turned ganciclovir from a traditional antiviral drug to a cell killing agent (Ladd et al., 2011). Some cell-cycle genes appeared to mediate the degree of cytotoxicity via cell cycle control pathways, which could explain why some cell lines were more sensitive to ganciclovir than others (Abate-Daga et al., 2010).

Although previous studies have demonstrated that ganciclovir may gain a high degree of toxicity after viral infection, it still remained unclear whether ganciclovir was more toxic than aciclovir towards uninfected cell lines. From the results of the Western Blot analyses conducted in this dissertation, it can be seen that aciclovir had similar effects on Sertoli cells to those of ganciclovir, and penciclovir had relatively slight effects. Ten times the concentration of penciclovir was needed to exhibit effects similar to those of aciclovir or ganciclovir. One possible explanation for this could be the remarkable difference in the activity of the agents in terms of killing HSV-TK-expressing and non-expressing cells. In the CHO cell line, the difference in cellular sensitivity between HSV-TK-positive and HSV-TK-negative cells at LD10

level was 7-fold for aciclovir, 60-fold for ganciclovir and 400-fold for penciclovir (Tomicic et al., 2002), which may also explain why aciclovir could exhibit the same degree of toxicity as ganciclovir in uninfected cell lines. In the Vero cell line, the amount of acyclo-GTP formed in HSV-infected cells was 40 to 100 times greater than in uninfected cells (Elion, 1993), indicating that infected cells may have a different profile compared to uninfected cells (Elion, 1982). It was also reported, however, that in the C127I cell line, the rates of phosphorylation and de-phosphorylation for ganciclovir were similar for infected and uninfected cells (Okleberry et al., 1997). There are also other potential mechanisms for the cytotoxicity of aciclovir. A metabolite of aciclovir may cause the direct renal tubular insult that is associated with aciclovir. Inhibition of this metabolite has been found to offer significant protection from cell death in vitro. This mechanism could partly explain the clinical evidence of nephrotoxicity in the absence of crystalluria (Gunness et al., 2011).

There were also differences seen between primary cell cultures and cultures of cell lines. Studies conducted with primary cell cultures of lymphoma cells showed that neither aciclovir nor ganciclovir displayed cytotoxicity- or apoptosis-inducing characteristics towards uninfected cells (Fujimuro et al., 2006). In primary cell cultures of Sertoli cells, the guanosine analogues could not be activated, possibly because only uridine kinase, but not thymidine kinase activity is localized in such Sertoli cells (Nishikawara and Leung, 1979). In cultures of cell lines, however, the phosphorylation of aciclovir still occurred without the activation of HSV/TK (Furman et al., 1981). Kominsky et al. have pointed out that human glioblastoma cell lines T98G, SNB-19, and U-373 were more than seven times as efficient at phosphorylation of aciclovir into the active phosphate form than normal human astrocytes (Kominsky et al., 2000).

4.2 Alteration of junction proteins under exposure to drugs

Nucleoside analogues have been shown to cause infertility in animal models and toxicity in vitro. Sertoli cells have been the primary cellular targets for a number of pharmaceutical and environmental testicular toxicants (Moffit et al., 2007). Because the SerW3 cell line appeared to be a useful in vitro model for evaluating substances with potential reproductive toxicity, it was

interesting to see whether this model could also be applied for nucleoside analogues.

4.2.1 Alteration of connexin43

Gap junctions play a crucial role in spermatogenesis (Kotula-Balak et al., 2007). Because connexin43 is predominant in the connexin family that forms gap junctions, it is essential for the initiation and maintenance of spermatogenesis (Pointis and Segretain, 2005) and has been considered to be a precocious molecular target for the impairment of spermatogenesis by environmental toxicants (Pointis et al., 2011). The expression of connexin43 has been shown to decrease because of the endocytosis process (Fiorini et al., 2008), which possibly requires ERK activation (Mograbi et al., 2003). To explore the mutation of connexin43 in Sertoli cells under exposure to environmental toxicants, several Sertoli cell lines, such as TM5, TM4 and SerW3 have been utilized (Kang et al., 2002; Fiorini et al., 2004; Fiorini et al., 2008; Zhou et al., 2008). Some pharmaceutical agents have also been tested with Sertoli cell lines. According to previous studies, aminoglycoside antibiotics had a negative effect of the expression of connexin43 (Zabel et al., 2012). In the present study, the connexin43 content, expressed as a percentage of that in the control, decreased to 49.8 ± 17.2 %, 43.2 ± 10.8 % and 50.7 ± 16.5 % (n=3; mean ± SD) when the Sertoli cells were cultured for three days with the maximum concentrations of aciclovir, ganciclovir and penciclovir respectively.

A close relationship between altered testicular connexin43 expression and the severity of impairment in spermatogenesis has also been reported in hypofertile men and in mutant rodents with testicular defects (Steger et al., 1996; Batias et al., 1999； Matsuo et al. 2007). For example, connexin43 mRNA expression was significantly reduced in the testes of infertile patients with secretory azoospermia (Defamie et al., 2003). Another example is Klinefelter's syndrome, which is characterized by numerous degenerated seminiferous tubules and impaired spermatogenesis. A correlation between severe spermatogenic impairment and loss of connexin43 immunoreactivity was observed in the testes of patients and mice with testicular disorders, whereas the connexin43 protein was ubiquitously distributed in the testes of reproductively normal males (Kotula-Balak et al., 2007).

Some experiments have also been conducted using animal knock-out models. Batias et al. demonstrated in jun-d-/-mice that sterile mutants possessed Sertoli-cell-only tubules in which connexin43 content was either reduced or undetectable (Batias et al., 1999). The testes of connexin43-null mutant fetuses did not display normal proliferation and differentiation of germ cells (Roscoe et al., 2001). In 2007, Sridharan et al. generated a Sertoli cell-specific connexin43 knockout (SC-Cx43 KO) mouse in which connexin43 was not present in the Sertoli cells but was expressed normally in organs such as the heart. SC-Cx43 KO mice showed continuous Sertoli cell proliferation and delayed maturation in adulthood, indicating that connexin43 played key roles in Sertoli cell development (Sridharan et al., 2007b). Defamie et al. suggested that connexin43 mRNA and protein expression may be a functional marker of undifferentiated Sertoli cells (Defamie et al. 2003). Sertoli cells may also reveal a positive signal for the gap junctional protein in some SCO syndrome phenotypes as long as the Sertoli cells are fully differentiated (Anniballo et al., 2011). Because the dysregulation of connexin43 has been related to the malfunction of spermatogenesis, it can be predicted that reduction in connexin43 expression could possibly impair male fertility. Because no testicular alteration was reported in men with connexin gene mutations (Lai-Cheong et al., 2007), it has been postulated that altered connexin expression is a consequence of impaired testicular function rather than the cause of such testicular pathology (Pointis et al., 2010).

Connexin43 has been found not only to be a sensitive marker for the impairment of spermatogenesis, but also to be involved in the mechanism of cellular toxicity by controlling the balance between cell proliferation/differentiation/apoptosis, which is a prerequisite for maintaining certain levels of spermatozoa (Chevallier et al., 2013). Cell proliferation and apoptosis may share some common signal pathways. Some kinases may have dual roles as integral components of the cell cycle and as regulators of apoptosis (Kasten and Giordano, 1998), so it is not surprising that connexin43 appeared to play a role in both processes. Kolaja et al. found that both these processes could take place simultaneously. When GJIC was decreased by 30-35%, proliferation increased nearly 3-fold while apoptosis increased 2-fold (Kolaja et al., 2000). This may have been because the gap junction is a common element in the early stages of apoptosis and mitosis, and as each process progressed, a common process of change in GJIC

occurred (Wilson et al., 2000). Cell-killing signals initially generated by a single cell could spontaneously initiate apoptosis. Connexin43 may support coupling of the apoptotic cells with their healthy neighbors, hence resulting in clusters of dying cells (Krutovskikh et al., 2002). However, when connexin43 is defective, it may be possible to avoid toxicity. Yao et al. investigated the role of gap junctions in aminoglycoside-induced injury to renal tubular cells using two tubular epithelial cell lines, NRK-E52 and LLC-PK1. The former expressed abundant connexin43 while the latter was gap junction-deficient and resistant to aminoglycoside-induced cytotoxicity. Gentamycin-elicited cytotoxicity in NRK-E52 cells was also significantly attenuated by gap junction inhibitor α-GA (Yao et al., 2010).

4.2.2 Alteration of vimentin

Some environmental toxicants have been shown to induce a collapse in the amount of vimentin in Sertoli cells. The expression of vimentin in Sertoli cells can be used as an indicator for the loss of mechanical support for spermatogenic cells and the subsequent apoptosis of spermatogenic cells (Tay et al., 2007).

In 1996, Richburg and Boekelheide studied the dislocation of vimentin filament in the Sertoli cell and the incidence of testicular germ cell apoptosis after the treatment of 28-day-old Fischer rats with mono-(2-ethylhexyl)-phthalate (MEHP). An early collapse of vimentin filaments was observed, followed by a progressive increase in the perinuclear condensation of the vimentin filaments and germ cell apoptosis (Richburg and Boekelheide, 1996).

It was later found by Tay et al. (2007) that MEHP studied in 21-day-old C57Bl/6N mice and their Sertoli cell cultures could also result in the reduction of vimentin and the apoptosis of Sertoli cells. In vivo, a correlation was evident between the increase in apoptotic cells and vimentin disruption in treated mice. In vitro, both the number and size of vacuoles in Sertoli cell cytoplasm increased, and the immune signal of vimentin gradually disappeared as time and dose increased (Tay et al., 2007).

A study has also been conducted on the cytoskeleton vimentin disruption resulting from nitrogen mustard (HN2) exposure. The results suggested that one of the mechanisms of reproductive toxicity may be the disruption of the vimentin structure and down-regulation of vimentin expression in Sertoli cells (He et al., 2007). In the results of the Western Blot analyses carried out in this dissertation, the expression of vimentin as a percentage of the control decreased to 68.3 ± 7.6 %, 76.9 ± 4.5 % and 79.5 ± 6.0 % after Sertoli cells were cultured for three days with the maximum concentrations tested of aciclovir, ganciclovir and penciclovir respectively (n=3; mean ± SD).

Vimentin has been considered to be involved in the mechanism of cellular toxicity, and it was recently found that vimentin possibly plays a role in nuclear alteration, with the overproduction of vimentin found to be indicative of a senescence phase in cultured cell populations (Litwiniec et al., 2013). Vimentin can be rapidly proteolyzed into fragments by caspases, with the cleavage of vimentin disrupting the cytoplasmic network of intermediate filaments, amplifying cell death and promoting apoptosis (Byun et al., 2001). Majumder et al. also showed that the cleavage of vimentin was sufficient to reduce HEL cell viability (Majumder et al., 2011). This was consistent with the results of a later study by Dinsdale et al., in which apoptotic cells showed the activation of caspases together with the cleavage of vimentin. Vimentin has been speculated to be responsible for controlling the intracellular distribution of caspases during apoptosis (Dinsdale et al., 2004).

From the results of a study conducted on bovine testis, vimentin has been found to be present in the cytoplasm of prespermatogonia I, but disappeared when the cell turned into prespermatogonia II. In prepubertal supporting cells the vimentin content increased, and in the postpubertal adult the positive filament bundles created a flame-like pattern around the unstained nucleus (Steger et al., 1994). The vimentin filaments of Sertoli cells in rats may also be regulated cyclically in a stage-dependent manner. During stages XII-V of the epithelial cycle, the Sertoli cells have been found to show a reaction in the perinuclear area, with vimentin-positive projections extending towards the developing spermatid bundles. During stages VI-XI these extensions were small and narrow (Mali et al., 1987). According to the results of observations

made via immunofluorescence in the present study, vimentin was distributed evenly in the cell plasma, and it decreased slightly after three days of culture with aciclovir, ganciclovir and penciclovir.

It was important to use specific inhibitors to avoid vimentin degradation during cell lysis. Due to the occurrence of Ca^{2+}, the specific band of vimentin would completely disappear after 2 to 3 minutes without proper inhibitors (Nelson and Traub, 1983). In the present study, the specific band of vimentin was undetectable when the PMSF used was not effective. The regulation of the degradation of vimentin possibly followed patterns specific to the particular cell lineage used. In human monocytic leukemia THP-1 cells, protease activity against vimentin is not inhibited by pepstatin or PMSF (Honke and Wada, 1997), unlike the activity of Ca^{2+}-activated vimentin-specific protease (Nelson and Traub, 1981; Nelson and Traub, 1983).

4.2.3 Alteration of N-cadherin

The expression of N-cadherin has been regarded as one of the early targets for different classes of reproductive toxicants in SerW3 Sertoli cells (Fiorini et al., 2004). The alteration of the expression of N-cadherin may be secondary to the endocytosis process of N-cadherin. N-cadherin was for instance internalized and present in vacuoles in response to DDT (Fiorini et al., 2008). Mislocalization of N-cadherin was recently explained to be related to the overexpression of SHP2 and the up-regulated BTB disruptor ERK1/2 (Puri and Walker, 2013). Interestingly, different toxicants cannot only up-regulate but also down-regulate the expression of N-cadherin. For example, in the same publication by Fiorini in 2004, DED reduced N-cadherin expression while the other environmental chemicals tested (PCP and DDT) did not show any effect on N-cadherin (Fiorini et al., 2004). Two studies based on animal experiments in vivo reported up-regulation of N-cadherin after treatment with DEHP and BPA in 2006 and 2009, respectively (Sobarzo et al., 2006; Salian et al., 2009). More recently, in vivo and in vitro studies have revealed that p,p'-DDE can increase the expression of N-cadherin mRNA, while at the same time the level of N-cadherin protein is reduced (Yan et al., 2013).

Carette et al. investigated in 2013 the potential influence of toxic metals on the Sertoli cell

barrier, employing the seminiferous tubule culture model in order to mimic the in vivo situation for toxicological studies on spermatogenesis (Geoffroy-Siraudin et al., 2010; Carette et al., 2013). N-cadherin was found to remain unaltered when the gap junction protein connexin 43 was strongly delocalized from the membrane to the cytoplasm of Sertoli cells. In the results of the Western Blot and immunofluorescence studies conducted in this dissertation, the expression of N-cadherin did not change significantly after three days of culture with the four nucleoside analogues tested.

N-cadherin has been reported to exert an anti-apoptotic effect on some kinds of cells. N-cadherin has been found to support the survival of hepatic stellate cells (HSC), and the apoptosis of HSC may be promoted when the N-cadherin is cleaved into smaller fragments (Murphy et al., 2004). N-cadherin-mediated cell-cell contacts have been shown to initiate anti-apoptotic signaling for the survival of vascular smooth muscle cells (VSMC), with the cell-cell contacts providing the cells with protection from apoptosis (Koutsouki et al., 2005). N-cadherin has also been found to contribute to the progression of human hepatocellular carcinomas (HCCs) by exerting anti-apoptotic effects (Gwak et al., 2006). N-cadherin has however also been reported to have the opposite effect in some cases, with the overexpression of N-cadherin being found to decrease osteoblast survival in vitro and in vivo due to its negative control of proliferation (Haÿ et al., 2009).

4.3 Future perspective

The work in this dissertation revealed alteration in the expression of two junctional proteins, connexin43 and N-cadherin, and one intermediate filament protein, vimentin, after three days of exposure to four nucleoside analogues in the SerW3 Sertoli cell line, via employing Western Blot analysis and immunofluorescence.

Although immunofluorescence could provide an image of the distribution of the proteins studied, Western Blot analysis was the only way to quantitatively analyze the level of expression of the proteins. Because the alterations of the proteins and their mRNA could differ from each other (Yan et al., 2013), it would be interesting to study the alteration of mRNA through the use of a real-time reverse transcription polymerase chain reaction process (RT-qPCR), which has been utilized by other labs for studying the effects of environmental toxicants. Connexin43 for instance has been investigated through RT-PCR along with immunofluorescence and Western Blot analyses to study the effects of gossypol (Zhou et al., 2008). Besides RT-qPCR, some tests on apoptosis (e.g., V-FITC, TUNEL) could also be used to show the correlation between apoptosis and the alteration of the target protein. Vimentin has already been shown to be associated with an increase of TUNEL-positive cells (Tay et al., 2007).

Besides connexin43, N-cadherin and vimentin, there may be other potential indicators for the dysfunction of Sertoli cells. For example, environmental toxicants (e.g., $CdCl_2$, bisphenol A, DDT) could also disrupt Sertoli–Sertoli cell junctions by either reducing the levels or inducing the dislocation of occludin and ZO-1 (Fiorini et al., 2004; Wong et al., 2004), and hence these toxicants could be selected as candidates for further study.

The effects of nucleoside analogues on the male reproductive system were found to be reversible in studies conducted on various animal species. It might hence make sense to conduct further studies on the SerW3 Sertoli cell line in order to explore whether the alterations of the target protein are also reversible in vitro.

4.4 Conclusion

Although in some clinical trials aciclovir and famciclovir were shown to have no negative effects, other studies have found that high doses of nucleoside analogues do in fact result in impairments of spermatogenesis, even after short-term exposure. A series of in vitro studies, which were performed on different cell lines, also indicated that nucleoside analogues can induce cellular toxicity. In this dissertation, the Sertoli cell line SerW3 was employed, which was considered as an excellent model for studying male reproductive toxicity.

In the Western Blot analysis, aciclovir was found to have a clear-cut effect after a three-day culture period, especially at the highest concentration tested, 10 mg/l, which produced a similar effect to that of ganciclovir. At 10 mg/l, penciclovir caused less pronounced effects compared to aciclovir and ganciclovir. The effects observed with famciclovir at its highest concentration of 100 mg/l were not pronounced, presumably because the Sertoli cells were incapable of metabolizing famciclovir to penciclovir. For each specific time of exposure, there was a minimal concentration at which the agents evoked negative effects. When the concentrations of aciclovir and ganciclovir were ≥ 3 mg/l, the target protein started to alter after three days of culture, and for penciclovir this concentration threshold was 30 mg/l. Compared to N-cadherin and vimentin, connexin43 appeared to be more reliable for detecting cytotoxicity in SerW3 Sertoli cells. In comparison to connexin43, the expression of N-cadherin only changed slightly after three days of culture with nucleoside analogues.

The effects of the four nucleoside analogues tested could hardly be quantified via immunofluorescence. Immunofluorescence did however provide the possibility to observe the distribution of connexin43, N-cadherin and vimentin in the Sertoli cells. The fluorescence intensity of the cells decreased slightly after their exposure to aciclovir, ganciclovir and penciclovir, but aberrant localization was not detected. Because the location of vimentin changed during the different stages of the seminiferous epithelium, immunofluorescence was useful for discovering the pattern of distribution of vimentin in SerW3 Sertoli cells.

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6 Affidavit / Eidesstaatliche Versicherung

„Ich, Runan Qiu, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Effects of Nucleoside Analogues on Protein Expression in Cells of the SerW3 Cell Line selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

7 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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