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Optimizing Preincubation Conditions for Precision-cut Rat Kidney and Liver Tissue Slices: Effect of Culture Media and Antioxidants

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Abstract—Tissue slices are commonly 'preincubated' before use, but optimal conditions to ensure their maximal viability have not been systematically investigated. The effects of serum-free Dulbecco's minimum Eagle's medium and Ham's nutrient mixture (DMEM/F12) (1:1) culture media with and without phenol red (±PR), or RPMI-1640 and six different antioxidants on the viability of precision-cut rat kidney and liver slices ($200 \pm 5\ \mu m$) were investigated. Slice viability was assessed every 30 minutes over a 2-hour preincubation period and after 24 hours of incubation in a multiwell plate culture system maintained at 37°C. In all cases, preincubation produced a time-dependent significant reduction of ethidium bromide positive nuclei stained in each medium and in both kidney and liver slices. Based on lactate dehydrogenase (LDH) leakage, there are viability differences between the media. In contrast, alkaline phosphatase (ALP) leakage and MTT reduction were less sensitive and did not differentiate between slice viability in each incubation medium. Preincubation of kidney and liver slices in DMEM/F12 medium containing antioxidants, indicated an enhanced viability which was specific for each tissue. Extension of the culture period to 24 hours after 1 hour of preincubation showed up to a further 4–13% leakage of ALP or LDH in DMEM/F12 (±PR) media for both kidney and liver slices and with a further 5–15% decline in MTT viability assay. RPMI-1640 medium on its own was not a suitable medium for maintaining the viability of either kidney or liver slices. However, kidney or liver slices preincubated with DMEM/F12 medium in the presence of some of the antioxidants were satisfactorily maintained for 24 hours. Exposure of slices to atractyloside (ATR) at concentrations of 0.2–2.0 mM in the different media for 24 hours showed a significant increase in enzyme leakage, decline of MTT reductive capacity and increased oxidative damage, with toxicity more elaborate in RPMI-1640 medium. Preincubation of kidney slices with either reduced glutathione (GSH) or α-tocopherol (TOC) and liver slices with either GSH or deferoxamine (DEF) followed by 24 hours of exposure to ATR showed a similar decline in toxicity profile. The antioxidants provided partial protection of slices from ATR toxicity. The results demonstrate the importance of slice preincubation and indicate that slices could be maintained in culture using an appropriate medium, thus providing slices that could serve as a useful alternative *in vitro* system for assessing novel compounds for toxicity.

Keywords: improved preincubation conditions; culture media; antioxidants; superoxide dismutase; rat tissue slices; atractyloside; cytotoxicity.

Abbreviations: ALP = alkaline phosphatase; ASC = L-ascorbate; ATR = atractyloside; DEF = deferoxamine; DMEM/F12 (±PR) = Dulbecco's minimium Eagle's medium and Ham's nutrient mixture F-12 with or without phenol red; GSH = reduced glutathione; LDH = lactate dehydrogenase; MDA = malondialdehyde; MESNA = 2-mercaptoethanesulfonic acid; PBS = phosphate buffered saline; PR = phenol red; SOD = superoxide dismutase; TBA = thiobarbituric acid; TOC = α-tocopherol; TBARS = thiobarbituric acid reactive substance; TCA = trichloroacetic acid.

INTRODUCTION

Precision-cut tissue slice technology offers a most attractive *in vitro* system that is already widely used for pharmacotoxicology, metabolism and cell

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biology studies (Bach *et al.*, 1996b; Barr *et al.*, 1991; Parrish *et al.*, 1995; Vickers, 1994). The method efficiently converts different tissues from any species and each of the major organs into slices with minimal waste. Tissue slices have gained acceptance, and are used preferentially, rather than cell culture systems by maximizing the use of the available tissue. This is essential for human organs where only a limited amount of tissue is typically available. Tissue slices also maintain the correct architectural relationship of all cells present, and intercellular communications are maintained. This helps to ensure both selective toxicity, where one or more cell types are affected (Azri *et al.*, 1992; Ruegg, 1994) and interactive toxicity, where one cell type contributes to the toxicity observed in another cell type (Azri-Meehan *et al.*, 1994; Nosaka *et al.*, 1992).

During preparation, the slices are subjected to both mechanical and oxidative stress injury (Chen and Tappel, 1994; Obatomi *et al.*, 1997), changes that could ultimately limit their viability and useful lifespan, especially for long-term investigations. At present, a large variation in slicing buffers, incubation media and incubation methods makes comparison of different studies difficult. A wide variety of methods and conditions for the preparation and incubation of tissue slices has been reported (Beamand *et al.*, 1993; Fisher *et al.*, 1995a,b), but there are limited data on optimized preincubation protocols that stabilize slices and extend their viability. Producing optimal viability is most desirable when animal and human *in vitro* systems are being used. Indeed, while most workers have given conditions that could improve the incubation of slices, there are no reports of functional viability changes that occur in fresh tissues and during preincubation. Optimized preincubation conditions which increase the slice viability and use will offer researchers a valuable tool for the *in vitro* study of metabolism and potential toxicity of novel compounds.

In an attempt to improve the versatility of tissue slice preparations for mechanistic investigation, we have determined preincubation conditions that will help improve the viability of liver and kidney slices and ensure that these are more appropriate for *in vitro* pharmacotoxicology studies. To achieve this, we have assessed cellular function using fluorescent probes which can be performed within minutes, and provide information about structural characteristics of slices. This was compared with some of the conventional biochemical assays which provide a better picture of the overall state of the slices and in the presence of three widely used culture media (Fisher *et al.*, 1995b), several antioxidants and superoxide dismutase. The slicing buffer of choice is phosphate buffered saline (PBS) with 0.1% agarose which is similar to other buffers used by different researchers (Fisher *et al.*, 1993, 1995b; Parish *et al.*, 1995). The agarose serves as a lubricant to facilitate easy movement of the blade through the tissue and also makes the slices more buoyant, avoiding mechanical stress as the slice is being harvested.

Our aim was to start defining preincubation conditions that produced slices of the highest quality and allowed the widest use of this technique. We report here a simple incubation system in the multiwell plates (immersion system of slices) which is widely used (Dogterom, 1993) as an alternative to the dynamic organ culture system (Fisher *et al.*, 1995a). With the establishment of the most appropriate conditions for preincubation, the system was tested by exposing slices to a well known nephro- and hepatotoxic compound, atractyloside (ATR) (Carpenedo *et al.*, 1974; Georgiou *et al.*, 1988; Obatomi and Bach, 1997). This will provide some additional information necessary to make an informed decision on the appropriate media and antioxidants needed to improve slice viability during preincubation and beyond.

MATERIALS AND METHODS

Reagents and materials

ATR (sodium salt), MTT, HEPES, L-ascorbate (ASC), α-tocopherol (TOC), 2-mercaptoethanesulfonic acid (MESNA), DEF, GSH, superoxide dismutase (SOD), DMEM/F12 with or without phenol red (PR), RPMI-1640, low gelling temperature agarose, ethidium bromide and fluorescein diacetate were obtained from Sigma Chemical Co. (Poole, Dorset, UK). All other chemicals were of the highest grade available and purchased from common sources. The Krumdieck tissue slicer and accessories were obtained from Alabama Research and Development Corporation (Munford, AL, USA). The equipment was either autoclaved or sterilized with disinfectants. All media and buffers were filter sterilized immediately before use.

Animals

Adult male Wistar rats (200–230 g) were obtained from Charles River (Kent, UK). The animals were fed standard laboratory chow (Lab. Diet No. 1, Spratts, Barking, Essex, UK) and allowed free access to drinking water. They were housed at $22 \pm 3^{\circ}C$ with a relative humidity of 40–70% under a 12-hr light cycle and acclimatized to these conditions for at least 3 days before use. Both liver and kidneys from each rat were used simultaneously, in order to limit the numbers of animals used for each investigation.

Tissue slice preparation

Rats were sacrificed by cervical dislocation and liver and kidneys excised, through a midventral incision, and immediately kept in ice-cold sterile Krebs–HEPES medium (pH 7.4) gassed with (95% O_2:5% CO_2) until cored. The liver was carefully dis-

sected into lobes and cylindrical cores (8 mm i.d. were made with the tissue supported on a polystyrene foam surface. The kidneys were decapsulated and cored (8 mm i.d.) perpendicular to the cortico-papillary axis. This positional orientation allows identification of kidney slices derived from the cortex region of the organ. Liver and renal cortical slices ($200 \pm 5\ \mu$m) were prepared in the Krumdieck tissue slicer (Krumdieck *et al.*, 1980), filled with ice-cold PBS containing 0.1% (w/v) low melting point agarose. Slices were collected and stored on ice in gassed (95% O_2:5% CO_2) Krebs–HEPES buffer and used within 10 min of slicing. This step served to rinse slices of blood and/or enzymes released from damaged cells during the slicing process. Slice thickness was checked intermittently using the vernier feeler gauge calibrated travelling microscope (Griffin and George, UK) with fine focus.

Culture of slices and incubation procedure

In the first series of experiments on preincubation studies, slices were incubated in a 24-well culture plate containing 1.0 ml per well of either, HEPES-buffered serum-free DMEM/F12 with or without phenol red or RPMI-1640 which were also gassed with 95%:5% O_2/CO_2. For the purpose of this study, DMEM/F12 with phenol red is designated as DMEM/F12 (+PR), DMEM/F12 without phenol red simply as DMEM/F12 and DMEM/F12 with or without phenol red as DMEM/F12 (±PR). The slices were usually incubated in the various media for 30, 60, 90 or 120 min at 37°C on an orbital shaker. Time zero, which is taken as the control value, represents slices incubated for 5 min in the appropriate medium. The preincubation procedure enabled us to identify the medium that maintained maximum viability. In this regard, DMEM/F12 proved to support cell viability best and was subsequently used as the incubation medium.

In the second series of experiments, slices were incubated at 37°C in the DMEM/F12 containing either ASC, TOC, DEF, MESNA or GSH (all at 1 mM concentration) or SOD (10 U/ml) for 30, 60, 90 and 120 min. Time zero, which is taken as the control value, represents slices incubated in DMEM/F12 for 5 min. The antioxidants chosen have different modes of action.

Slices were also incubated in previously gassed (95%O_2:5% CO_2) fresh media (DMEM/F12 ± PR or RPMI-1640) for 24 hr after a 1-hr preincubation in the appropriate media. Some slices were incubated for 24 hr in fresh DMEM/F12 medium after 1 hr of exposure to the various antioxidants.

In the latter part of the experiment, slices were exposed to ATR at concentrations of 0.2–2.0 mM for 24 hr. After 1 hr of preicubation with the different culture media, slices were then exposed to ATR in the same media for 24 hr. Also, kidney slices preincubated in DMEM/F12 medium containing either GSH or TOC and liver slices preincubated in DMEM/F12 medium containing either GSH or DEF were further incubated for 24 hr with ATR in unsupplemented medium.

For each experiment, up to four slices per time point were used and each experiment was repeated three times.

Characterization of assay

The viability parameters used in the present study to assess tissue slice integrity included LDH and ALP (Obatomi and Plummer, 1995) leakage (to measure slice membrane integrity) and MTT conversion to formazan to measure mitochondrial viability (Berridge and Tan, 1993). Lipid peroxidation was measured by the production of thiobarbituric acid reactive substance (TBARS) as a marker of oxidative damage. The number of staining nuclei was assessed using the fluorescein diacetate and ethidium bromide staining method, which allows viable cells (green) to be contrasted against dead cells (red nuclei) microscopically (Edidin, 1970; Obatomi and Bach, 1996). Together these different parameters provide a better picture of the overall state of tissue slice viability.

Biochemical assays

Aliquots of the preincubation and incubation media were analysed for ALP and LDH leakage (Obatomi and Plummer, 1995). Leakage of enzymes into the medium at time zero which is taken as the control value was assessed in fresh slices incubated for approximately 5 min in 1 ml medium. For the preincubation studies, total enzyme activity was determined at each time point and this was taken as the amount of enzyme in media plus that in slices disrupted by addition of 1% (v/v) Triton X-100. For the 24-hr incubation studies, the total enzyme was taken as the amount of enzyme released into the media after 24 hr of incubation plus that in slices lysed with 1% (v/v) Triton X-100. Leakage of the enzymes from the slices into medium was expressed as percentage of total enzyme (100% lysis).

The ability of slices to convert MTT to MTT–formazan was determined by a modification of the procedure described by Mosbann (1983). After each time point of preincubation and incubation, slices were removed, rinsed with PBS and transferred to 24-well culture plates with each well containing 1 ml MTT solution (1.21 mM) and incubated at 37°C for 40 min. After incubation, MTT was aspirated from the wells, rinsed again with PBS and the MTT-formazan product was extracted in 1.0 ml isopropanol after 30 min. Aliquots (100 μl) were transferred into a 96-well plate to determine the absorbance at 570 nm in a scanning spectrophotometer (Anthos Labtech Instruments, Germany). The formazan formed in each slice at a series of time points was expressed as percentage of that of fresh slices which

represents time zero on the plotted graph and also as the control.

The extent of lipid peroxidation in slices during the preincubation period and after 24 hr of incubation was estimated by measuring the concentration of malondialdehyde production in the form of TBARS (Burge and Aust, 1978). The TBA reagent was composed of 0.026 M TBA, 0.92 M TCA and 0.25 M HCl. After incubation, the slices were removed and homogenized in 3 ml of the TBA reagent. The reagent was heated for 15 min in a boiling water-bath. After cooling, the precipitate was removed by centrifugation at 3000 rpm for 10 min and the absorbance of the supernatant determined at 535 nm. The malondialdehyde concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Davenport *et al.*, 1995).

Slices were dissolved in 1.0 ml 1.0 M NaOH for at least 24 hr, and protein content was assessed by the Coomassie Brilliant G method (Read and Northcote, 1981) using bovine serum albumin as a standard.

Morphological investigation

Slices were dual stained with the fluorescent probe of ethidium bromide (200 μg/ml) and fluorescein diacetate (5 (g/ml) (Edindin, 1970), transferred to a microscope slide and observed under a fluorescent microscope (Leitz Dialux 20, Leica, Wetzlar, Germany) with excitation of 390–490 nm and emission of 510–515 nm. Time-dependent effects of various media and antioxidants were quantified by measuring the amount of fluorescein retention and the decrease in ethidium bromide exclusion relative to the untreated control. The combination of both fluorochromes results in counter-staining, the nucleus of membrane damaged cells (non-viable cells) fluoresce red while intact cells (viable cells), the cytoplasm fluoresce bright green. The non-viable cells were determined quantitatively as the number of ethidium bromide positive nuclei counted in an area of 0.03 mm^2. At least 10 separate counts were completed for each slice in separate microscopic fields. The average of cells counted is expressed as a percentage of non-viable cells in fresh slices.

Statistical analysis

The data are presented as the mean $\pm$ SE for values compiled from separate animals in which four slices were used in each experiment. Values from these experiments were pooled and compared with concurrent control values by ANOVA followed by multiple comparison. Student's *t*-test was used and $P < 0.05$ was considered to be statistically significant.

RESULTS

Preliminary data showed a linear increase in MTT–formazan production up to 40 min which plateaud thereafter. We determined experimentally that, under these conditions, MTT would maximally penetrate the slices to give maximum MTT–formazan product. Thus, all slices used for mitochondrial function assay were incubated with MTT for 40 min at 37°C in a gently shaking water-bath. All data were expressed per mg protein, (CV < 2%) whereas the error in expressing results in wet weight was high (CV < 12%).

Effect of culture media on slice viability

Incubation of kidney slices in DMEM/F12 or RPMI-1640 media caused a significant ($P < 0.05$) time-dependent increase in LDH leakage with over 6% of total enzyme loss after 120 min (Fig. 1). In contrast, there was a significant ($P < 0.05$) reduction in LDH leakage at 90 and 120 min when kidney slices were incubated in DMEM/F12. The leakage of LDH from liver slices followed a similar pattern in the three media and was accompanied by an initial sharp increase (27–30%) in the first 30 min of incubation which dropped rapidly and stabilized at a significantly reduced level (2–7%) after 60 min of preincubation (Fig. 1). Figure 2 shows a significant increase (up to 9% and above after 60 min and beyond) in ALP leakage from kidney slices incubated in RPMI-1640 medium but not in DMEM/F12 ($\pm$PR) media which showed similar pattern of a significant decrease in ALP leakage to almost half the value obtained in the control tissues and stabilized after 60 min of preincubation. A time-dependent and significant decrease of ALP leakage from liver slices in DMEM/F12 and RPMI-1640 media which also stabilized after 60 min was obtained (Fig. 2). There was also a decrease in ALP leakage from liver slices incubated in DMEM/F12 (+PR) after 30 min but this fall was inconsistent (Fig. 2). Incubation of both kidney and liver slices in RPMI-1640 medium showed a significant decrease in viability by over 30% within 60 min of incubation as assessed by the MTT reduction assay (Fig. 3).

There were significant reductions in the number of ethidium bromide positive nuclei (dead cells) counted on the surface of kidney slices at 60 and 120 min of incubation in DMEM/F12 medium and at 120 min of incubation in RPMI-1640 mediium. There was also a slight but insignificant reduction in the number of these positive nuclei on the surface of the liver slices incubated in DMEM/F12 and RPMI-1640 media but not in DMEM/F12 (+PR) medium (Fig. 4).

There were no changes in TBARS up to 24 hr of incubation, an indication of lack of lipid peroxidation in the slices (Fig. 7) and suggesting no further oxidative damage. All the indicators of via-

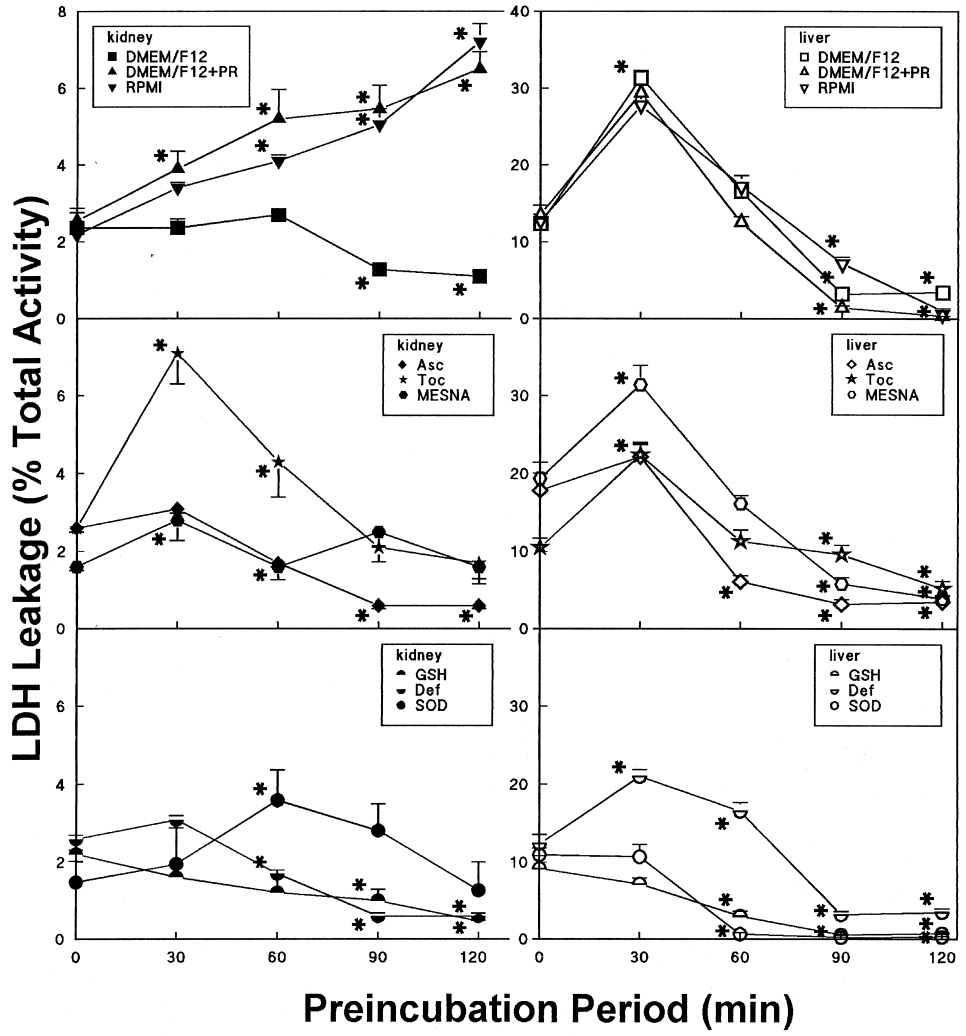

Fig. 1. Effect of different culture media and antioxidants on the leakage of LDH from rat kidney and liver slices incubated for 2 hr. Data are expressed as a percentage of total enzyme in slices. Each value represents the mean ± SE of three experiments in which four slices were used for each condition (*$P < 0.05$, significantly different from control). The control data obtained for each treatment is as described in Materials and Methods.

bility measured indicate DMEM/F12 medium as the most suitable preincubation medium that supports viability of both liver and kidney slices. This medium was therefore the preincubation medium of choice in subsequent experiments.

Effects of antioxidants on slice viability

Once it was found that DMEM/F12 medium was most suitable for preincubation we investigated the effects of some antioxidants in the medium on slice viability and injury. ASC, GSH and DEF caused a time-dependent significant reduction of LDH leakage from kidney slices, lower than that obtained in media only (Fig. 1). The leakage of these enzymes stabilized at 60 min. MESNA, TOC (at 30 min) and SOD (at 60 min) caused a significant increase in LDH leakage from kidney slices but the levels steadily decreased thereafter, reaching the same point as the control (Fig. 1). In liver slices, the antioxidants (except GSH) initially (at 30 min) caused a significant increase in LDH leakage which steadily falls with the time of incubation stabilizing after 60 min (Fig. 1). However, it appears that SOD and GSH markedly improve slice viability as LDH leakage was drastically reduced when compared with incubation in each of the media alone.

The addition of GSH and SOD to the medium appeared not to alter ALP leakage from kidney slices compared with the control. Only MESNA appeared to show a significant reduction in ALP leakage from kidney slices after 60 min of incubation, whereas ASC, TOC and DEF did not improve the slice viability in relation to ALP leakage from kidney. All the antioxidants (except ASC

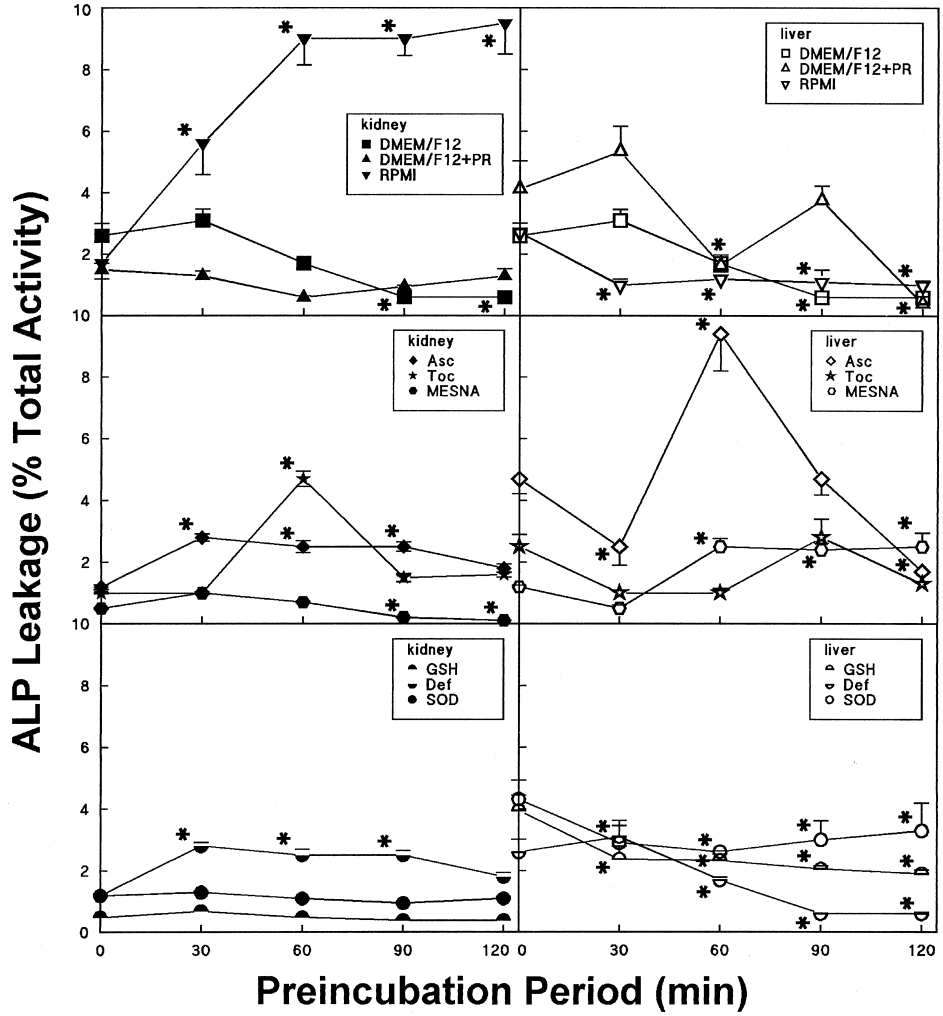

Fig. 2. Effect of different culture media and antioxidants on leakage of ALP from rat kidney and liver slices incubated for 2 hr. Data are expressed as percentage of total enzyme in slices. Each value is the mean $\pm$ SE of three experiments in which four slices were used for each condition (*$P < 0.05$, significantly different from control). The control data obtained for each treatment is as described in Materials and Methods.

and MESNA) caused a significant time-dependent reduction of ALP leakage from liver slices (Fig. 2). SOD clearly caused a significant time-dependent increase in ALP leakage (Fig. 2).

The viability of kidney slices was not affected by the addition of antioxidants and SOD into the medium as assessed by MTT reduction assay. In contrast, viability was significantly reduced (between 10 and 30%) in liver slices by the antioxidants (except ASC) and SOD. The antioxidants therefore produced a negative effect on MTT reduction.

The number of ethidium bromide positive nuclei (dead cells) on the surfaces of kidney slices was reduced with increase in incubation time, but this was only significant in the presence of MESNA and DEF (Fig. 4). In liver slices, ASC,TOC, SOD and DEF reduced the number of ethidium bromide positive nuclei similar to when the incubation was carried out in the media alone. However, GSH and MESNA increased the number of positive nuclei (Fig. 4).

Effects of culture media and antioxidants on 24-hr culture of slices

Table 1 indicates the effect of culture media on 24-hr culture of slices. Compared with 1-hr preincubation values, ALP and LDH leakage from liver and kidney slices were significantly increased in all the three media. The viability of liver slices in the three media as assessed by MTT reduction was significantly reduced but not in kidney slices.

Preincubation of kidney slices in the presence of DEF, GSH or SOD caused a significant ($P < 0.01$) decrease in LDH leakage following a 24-hr incubation while there was also a significant decrease in ALP leakage in the presence of all the antioxidants

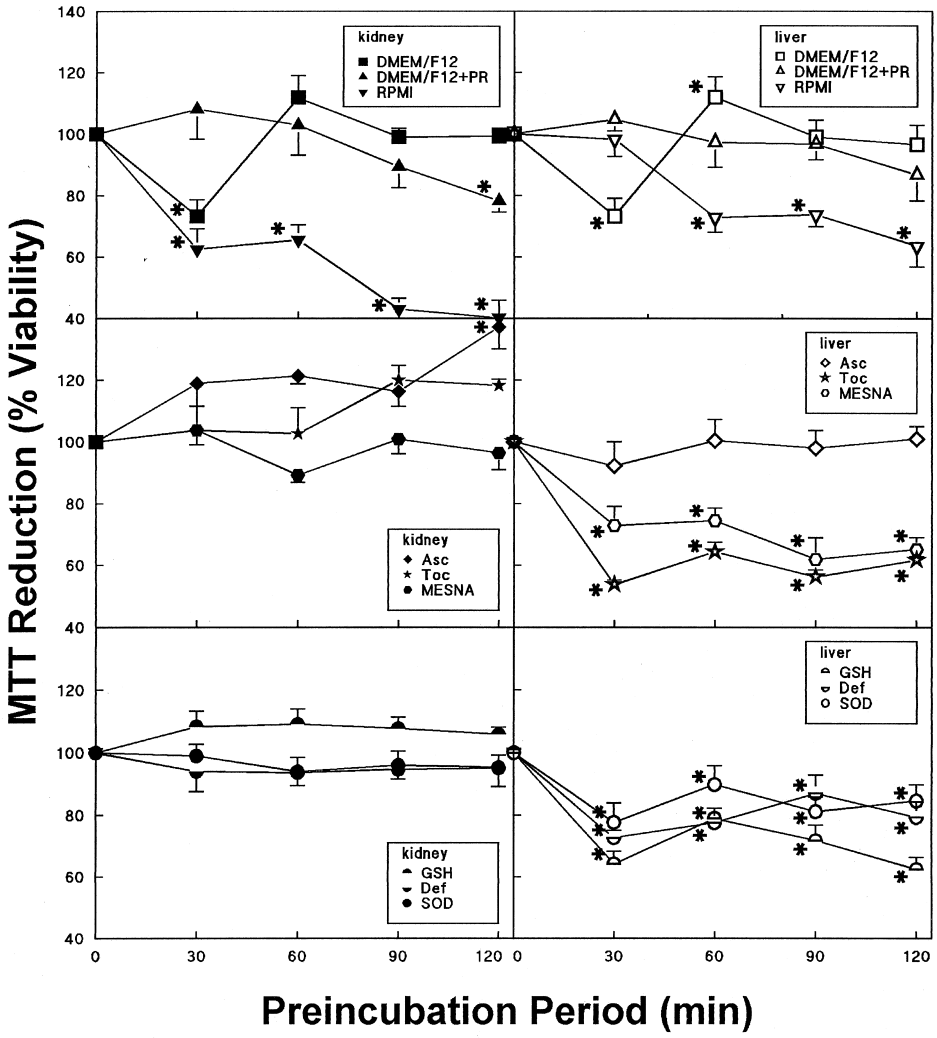

Fig. 3. Effect of different culture media and antioxidants on rat kidney and liver slices using the MTT assay as the viability indicator. Data are expressed as percentage of freshly cut (0 hr) slices. Each value is the mean ± SE of three experiments in which four slices were used for each condition (*$P < 0.05$, significantly different from control). The control data obtained for each treatment is as described in Materials and Methods.

Table 1. Effect of culture of precision-cut kidney and liver slices for 24 hr[a] in either DMEM/F12 with or without phenol red or RPMI-1640 media on viability. Each point represents the mean ± SE of triplicate experiment in which four slices were used

Culture media	Kidney			Liver		
	MTT	ALP	LDH	MTT	ALP	LDH
DMEM/F12 (preinc.)	119.9 ± 7.1	1.7 ± 0.23	2.7 ± 0.15	112.0 ± 6.57	1.7 ± 0.09	16.6 ± 1.11
DMEM/F12 (24 hr)	92.6 ± 4.4	12.2 ± 2.1#	11.7 ± 3.4#	87.4 ± 2.0*	15.3 ± 3.9#	18.5 ± 3.8**
DMEM/ F12 + PR (preinc.)	102.9 ± 9.7	0.60 ± 0.04	5.2 ± 0.77	97.2 ± 7.95	1.65 ± 0.33	12.5 ± 0.8
DMEM/F12 + PR (24 hr)	87.4 ± 5.9	9.68 ± 1.1#	17.0 ± 5.9*	72.8 ± 5.4*	5.6 ± 1.2**	12.7 ± 2.9
RPMI-1640 (preinc.)	65.5 ± 4.91	9.0 ± 0.85	4.1 ± 0.16	72.9 ± 4.91	1.2 ± 0.23	17.3 ± 1.35
RPMI-1640 (24 hr)	71.9 ± 2.4	14.2 ± 2.5*	31.7 ± 4.8#	67.4 ± 1.4*	21.4 ± 3.7#	29.8 ± 3.2**

[a]Slices were preincubated for 1 hr with the three separate media after which the medium was changed and incubated in same medium for 24 hr. MTT was expressed as percentage of fresh values, while ALP and LDH were expressed as percentage of total enzyme.
*$P < 0.05$; **$P < 0.01$; #$P < 0.001$, compared with values obtained after 1 hr of preincubation.

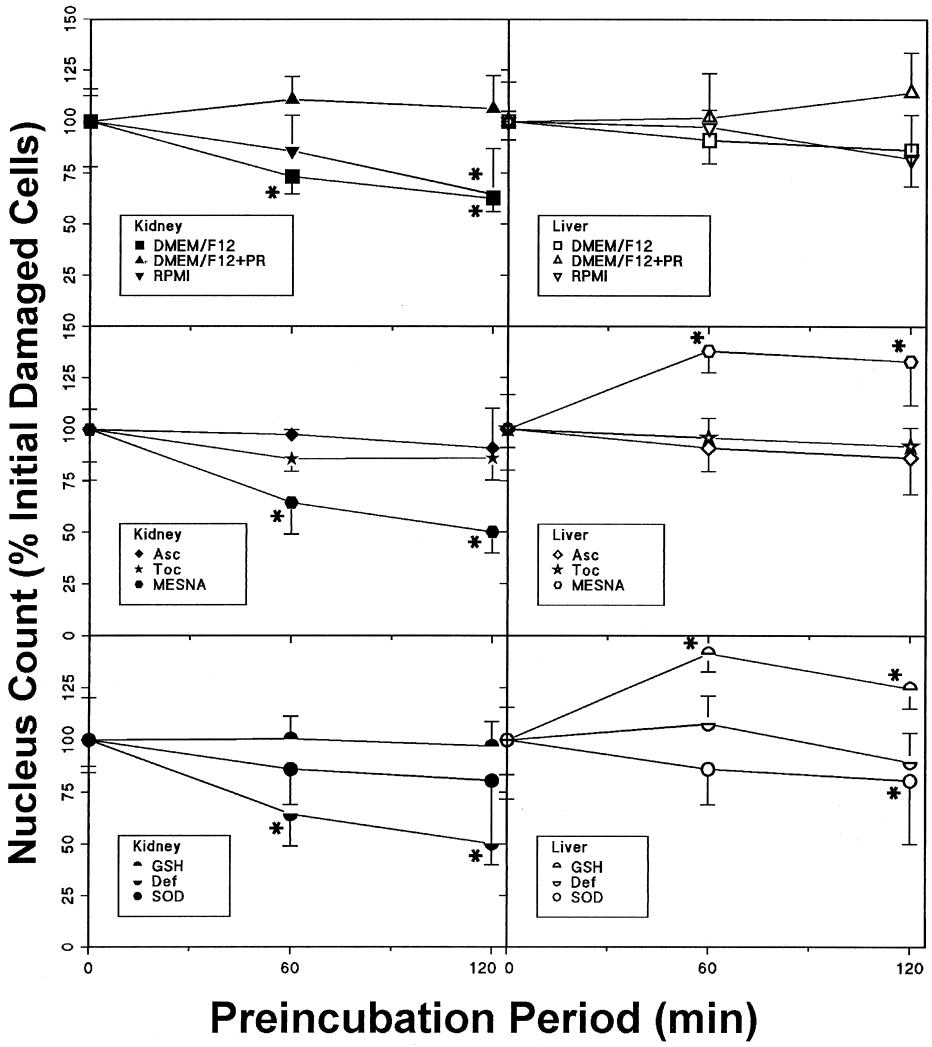

Fig. 4. Effect of different culture media and antioxidants on rat kidney and liver slices as assessed by the number of ethidium bromide positive nuclei counted per field. Data are expressed as mean percentage of control values ± SE (*$P < 0.05$, significantly different from control).

except MESNA (Table 2). However, the presence of MESNA, DEF, GSH and SOD significantly decrease (at least by 35%) the MTT reductive capacities compared with control slices. All the viability parameters showed significant improvements in slice viability when preincubated with ASC and TOC for kidney slices (Table 2).

Preincubation of liver slices in the presence of all antioxidants (except ASC) and SOD caused a significant decrease in LDH leakage following a 24-hr

Table 2. Effect of preincubation of rat kidney and liver slices with antioxidants on a 24-hr[a] incubation period

	Kidney			Liver		
Antioxidants	MTT	ALP	LDH	MTT	ALP	LDH
Control	92.6 ± 4.4	12.2 ± 2.1	11.7 ± 3.4	87.4 ± 2.0	15.3 ± 3.9	18.5 ± 3.8
Ascorbic acid	121.6 ± 5.9**	5.8 ± 1.2**	11.4 ± 3.7	37.6 ± 5.2‡	4.4 ± 1.2**	12.0 ± 4.1
α-Tocopherol	88.2 ± 6.2	5.0 ± 1.5**	8.2 ± 2.7	114.5 ± 18.0*	8.9 ± 1.9	2.7 ± 1.1#
MESNA	53.1 ± 6.1#	18.7 ± 3.8*	27.0 ± 3.8**	97.9 ± 11.8	7.6 ± 0.9*	1.9 ± 0.6#
DEF	39.4 ± 2.3#	0.7 ± 0.04#	1.16 ± 0.1**	92.4“4.4	11.6“4.1	1.26 ± 0.7#
GSH	50.4 ± 3.5#	1.30 ± 0.4#	1.22 ± 0.4**	110.6 ± 7.1**	12.6 ± 5.2	2.58 ± 0.9#
SOD	57.8 ± 2.7#	1.00 ± 0.2#	1.14 ± 0.3**	100.6 ± 4.0**	16.3 ± 3.7	1.31 ± 0.2#

[a]Slices were preincubated with antioxidants for 1 hr in DMEM/F12 without phenol red, after which the medium was changed and incubated in the same medium for 24 hr without any antioxidants. All antioxidants are at concentration of 1 mM except SOD (10 U/ml). MTT was expressed as percentage of fresh values, while ALP, LDH were expressed as percentage of total enzyme. Each point represents the mean ± SE of triplicate experiment in which four slices were used.

*P <0.05; **$P < 0.01$; #$P < 0.001$, compared with controls (24-hr incubation in DMEM/F12-PR medium).

incubation. In sharp contrast, only ASC caused a significant decrease in ALP leakage while other antioxidants and SOD caused no significant changes in the leakage of this enzyme (Table 2). The presence of antioxidants (except ASC) also showed improved liver slices viability by over 10%, as assessed by the MTT reduction assay. All viability parameters clearly indicate improvement in liver slices viability when preincubated in the presence of the antioxidants (except ASC) and SOD and further incubation for 24 hr (Table 2).

Effects of ATR on slices incubated in different culture media

Rat liver and kidney slices were incubated in the different media up to 24 hr in the presence of ATR, and cytotoxicity was monitored by the MTT assay, LDH leakage and lipid peroxidative changes. Whereas LDH leakage and MTT reduction in kidney slices cultured in the three media showed similar toxicity profile, RPMI-1640 appeared to have a more profound effect on the toxicity profile in liver slices (Figs 5 and 6). While there was a dose-related increase of LDH leakage from kidney slices cultured in DMEM/F12 (±PR) media containing ATR, there was relatively insignificant leakage in liver slices except at higher concentrations of ATR.

Oxidative damage measured by MDA production showed a dose-related increase in both liver and kidney slices in the presence of all the media used. However, a significant increase was only obtained in the kidney slices incubated in DMEM/F12 (±PR) media at highest ATR concentration and in RPMI-1640 medium at a concentration of 0.5 M or greater (Fig. 7). However, there was a significant lipid peroxidation in liver slices at all concentrations of ATR and in the presence of the three culture media.

Effects of ATR on slices preincubated with antioxidants

Preincubation of slices in DMEM/F12 containing either GSH or TOC followed by exposure to ATR for 24 hr showed a signficant increase in viability of kidney slices at a concentration of 0.2 mM or greater, but significantly decreased at ATR concentrations of 1.0 mM or greater, as assessed by the MTT assay (Fig. 5). However, there was a significant dose-related increase in LDH leakage from kidney slices preicubated with GSH or TOC before exposure to ATR for 24 hr (Fig. 6). A greater reduction in MDA production was obtained in kidney slices pretreated with GSH or TOC compared with slices that are only exposed to the media (Fig. 7).

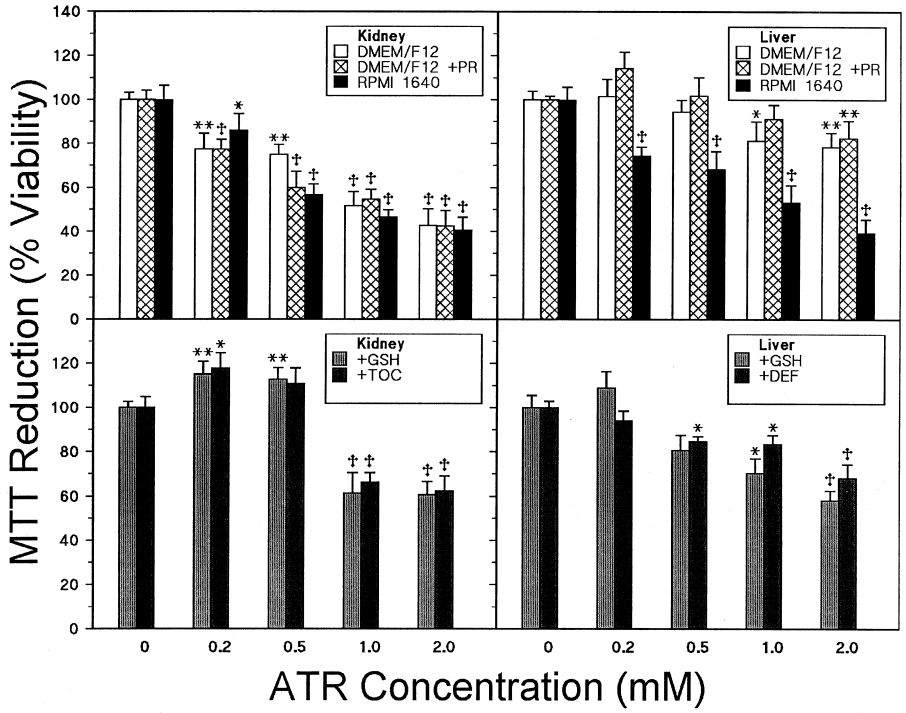

Fig. 5. Comparison of MTT assay in rat kidney and liver slices treated with ATR in the presence of different culture media and preincubated with antioxidants, all incubated for 24 hr. Data are expressed as percentage of the control and the mean ± SE of triplicate determination in which four slices were used. The significance of the differences between control and treated slices are indicated as: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.001$.

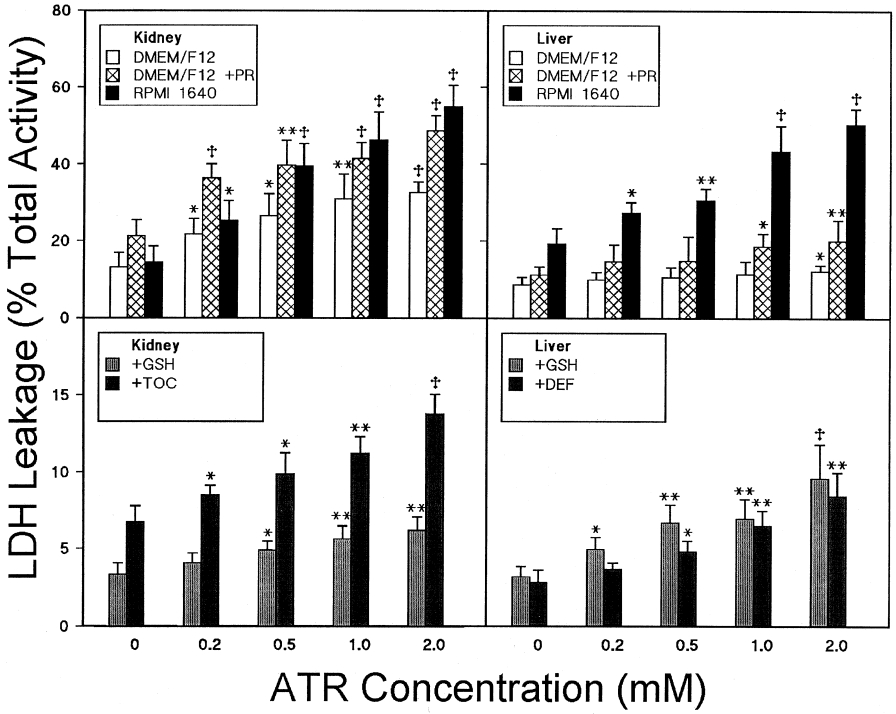

Fig. 6. Comparison of LDH leakage from rat kidney and liver slices treated with ATR in the presence of various different culture media and preincubation with antioxidants, all incubated for 24 hr. Data are expressed as percentage of total enzyme in fresh slices and are given as mean ± SE of triplicate experiments in which four slices were used. The significance of the differences between control and treated slices are indicated as: $*P < 0.05$; $**P < 0.01$; $^{\#}P < 0.001$.

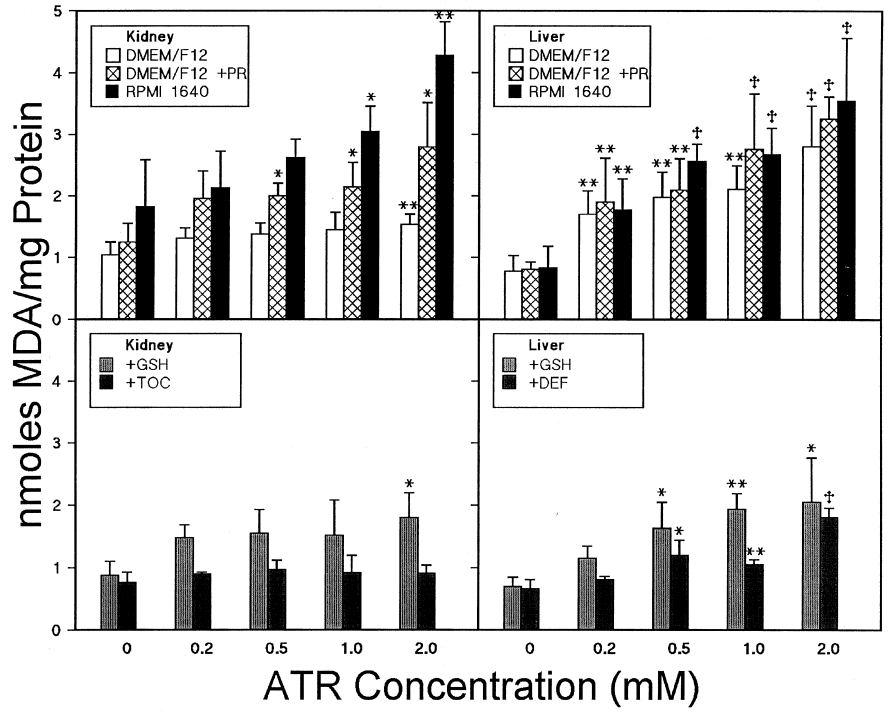

Fig. 7. Comparison of MDA production determined in rat kidney and liver slices treated with ATR in the presence of different culture media and preincubation with antioxidants, all incubated for 24 hr. MDA was measured as an index of lipid peroxidation by the TBA assay. Data are the mean ± SE of triplicate determinations in which four slices were used. The significance of the differences between control and treated slices are indicated as: $*P < 0.05$; $**P < 0.01$; $^{\ddagger}P < 0.001$.

Similarly, preincubation of liver slices in DMEM/F12 containing either GSH or DEF also showed a significant reduction in viability only at ATR concentrations of 1.0 mM or greater (Fig. 5), LDH leakage (Fig. 6) and lipid peroxidation (Fig. 7) at 0.5 mM or greater, respectively.

DISCUSSION

Precision-cut liver and kidney slices are widely used tissues for pharmacotoxicology and drug metabolism studies (Bach *et al.*, 1996b). Understanding the mechanism of renal and hepatic injury using slices is highly dependent on optimal tissue viability being maintained in the presence of appropriate culture media throughout the period of experiment. This is important, since control parameters change significantly over time, which will provide an unstable system for toxicological studies. For tissue slices to be useful for long-term studies, appropriate preincubation conditions and factors that affect this must be established in untreated control slices.

The assessment of slice viability depends on the use of multiple cytotoxicity criteria. Thus, in our hands, enzyme leakage appeared to be very reliable and comparable to morphological assessment, whereas MTT assay was inconsistent and not very dependable (Obatomi *et al.*, 1998). It has been suggested (Smith *et al.*, 1986) that media containing a continuous supply of oxygen be used for preincubation and subsequent incubation. However, in the present study, increasing the oxygen supply from 95% to 100% did not enhance slice viability either in a preincubation studies or at 24 hr.

In the present study we have used the multiwell plate incubation system (where tissue slices are submerged in medium, gassed and placed on a gyratory shaker) which is relatively simple and highly economical system (Dogterom, 1993; Smith *et al.*, 1986) that is an alternative to the roller culture system (Fisher *et al.*, 1995a). The culture media selected for our study are based on their previous use both for primary renal and hepatic cell cultures (Gray *et al.*, 1983; Gsraunthaler *et al.*, 1990; Paine *et al.*, 1990; Smith *et al.*, 1986) and for kidney (Keith *et al.*, 1995; Ruegg, 1994) and liver (Fisher *et al.*, 1995b; Lake *et al.*, 1993; Vickers *et al.*, 1993) slices. DMEM/F12 (±PR) kept liver slices more viable for the period of preincubation, and up to 24 hr, whereas only DMEM/F12 medium kept kidney slices more viable during the same period. However, RPMI-1640 medium was less suitable for maintaining the viability of both kidney and liver slices, during preincubation and up to 24 hr. The suitability of DMEM/F12 medium is especially relevant for the kidney slices since phenol red, which is a pH indicator, is known to be actively transported in the proximal tubule of the kidney (Bach *et al.*, 1996a; Morin *et al.*, 1996) and could also interfere with drug metabolism (Fisher *et al.*, 1995a). The results of the fluorescent probe clearly indicate that damaged cells are removed during the preincubation period presumably as dead cells slough off the cut surface. The FDA/EB assay can detect and quantify reproducibly the membrane permeability of tissues in a few minutes and can be used routinely for screening conditions or chemicals that may affect the cell membrane.

We have assessed the effects of preincubating slices with antioxidants on maintaining viability during a short- or long-term incubation period. The antioxidants selected are either lipid soluble (TOC) or water soluble and are known to act by scavenging the free radicals that were generated in aqueous phase and/or in phospholipid membrane (Takenaka *et al.*, 1991) probably during slicing, preincubation or the 24-hr incubation periods. These antioxidants are not known to have effects on metabolism and probably do not interfere with the test media. It is of interest to note that no significant lipid peroxidative changes were observed in either kidney or liver slices during preincubation and up to 24 hr incubation studies either with the media alone or even when antioxidants are added. However, most of the antioxidants tested were found to increase viability in both kidney and liver slices during the preincubation period and up to 24 hr of incubation as assessed by all the viability parameters. This is not surprising, since there have been previous reports of the effective support of viability of kidney and liver tissues in the presence of various antioxidants (Beamand *et al.*, 1993; Haraldsson *et al.*, 1995; Paller and Eaton, 1995).

The objective of this study was to identify the optimal preincubation conditions which could prolong slice viability for subsequent utilization for 24 hr in multiwell plate incubation. To our knowledge, this is the first time that such systematic data are presented that highlight and support the significance of preincubation and factors which could affect this. We have been able to demonstrate that precision-cut kidney and liver slices can be well maintained in DMEM/F12 without phenol red, and that this was the most suitable media that totally supports the viability of slices. We have also demonstrated that both kidney and liver slices can be conveniently maintained in culture for a period up to 24 hr and that preincubation with some antioxidants can further improve tissue viability.

ATR is a diterpenoid glycoside known to cause fatal renal and hepatic necrosis in animals and man (Carpenedo *et al.*, 1974; Georgiou *et al.*, 1988; Obatomi and Bach, 1997). We have used the system developed by us to assess the *in vitro* cytotoxicity in kidney and liver slices under the above conditions. Our results using both enzyme leakage and MTT assays showed this compound to be highly toxic to the kidney at all concentrations used. The extent of toxicity was also compounded by the medium used.

While both DMEM/F12 (±PR) media showed a similar toxicity profile, RPMI-1640 showed a greater toxicity. It is interesting to note that few peroxidative changes were obtained in the kidney slices. The liver slices responded differently to ATR toxicity. There was little change in the LDH leakage and MTT assay at the various concentrations of ATR and using all media. However, there was an extensive lipid peroxidation in the slices at all concentrations of ATR and the effect was aggravated in the presence of RPMI-1640. It is interesting to observe that preincubation of both kidney and liver slices in medium containing some antioxidants before exposure to ATR reduced the susceptibility of these tissues. Although the mechanism of ATR toxicity is not fully understood, previous study (Obatomi *et al.*, 1998) showed that a short-term (3 hr) exposure of rat and pig kidney and liver slices to ATR caused a significant depletion of GSH in both tissues and also an increased lipid peroxidation only in the liver. This led to the conclusion that the mechanism of ATR might be by oxidative process. The protective effect of antioxidants as obtained in this study may then suggest that the antioxidants were acting against generation of free radicals and thereby reducing the toxicity of ATR in these tissues.

These data represent a systematic assessment of three media and six antioxidant systems in two tissues and how these conditions can affect the viability that may alter the tissue response to toxins. We have established the most appropriate medium and antioxidants that could support the viability of tissues and provide a suitable environment to study tissues exposed to various toxins. In all the tests of viability performed in this study, it becomes clear that both LDH and fluorescent probe have similar sensitivity for both kidney and liver slices while ALP was only sensitive with kidney slices and the MTT assay is least sensitive of all. The reductive capacity of MTT as a marker for cell viability is currently in question. From our result, it is obvious that the assay does not always correlate with other markers of cell death and the suspected mitochondrial involvement of this reaction is reported to be only about 40% while extramitochondrial NADH and NADPH-dependent reaction account for the remaining activity (Berridge and Tan, 1993).

Our results have shown that preincubation of tissues is highly desirable to provide the most viable tissues for pharmacotoxicology studies. The leakage of enzymes, especially LDH, and the use of fluorescent probes are shown to be most useful markers for judging the viability of both kidney and liver slices.

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