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Influence of commonly used pharmaceutical agents on equine bone marrow-derived mesenchymal stem cell viability

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Running title: Effects of anaesthetic, sedatives and corticosteroids on MSCs

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Summary

Reason for performing study: To provide evidence to support recommendations regarding the co-administration of drugs with mesenchymal stem cell (MSC) therapy.

Objectives: To determine the influence of sedatives, local anaesthetic and corticosteroids on MSC viability and proliferation, in comparison to somatic cells derived from tendon (TDCs).

Study design: In vitro cell culture.

Materials and methods: MSCs (n = 3) and TDCs (n = 2) were cultured in media containing a clinically relevant dose range of xylazine, romifidine, detomidine and butorphanol, mepivacaine, methylprednisolone or triamcinolone acetonide. Cell viability in suspension culture was assessed at intervals up to 4 h using the trypan blue dye assay. MSCs in monolayer culture were exposed to the highest concentrations of drug and proliferation was measured using the alamarBlue® fluorescence assay.

Results: Exposure to romifidine or mepivacaine did not significantly affect viability or proliferation rate of MSCs or TDCs, at any of the dosages tested. At the highest concentration of detomidine and butorphanol, MSC viability was significantly reduced compared to controls. Although xylazine exposure caused a significant ($p<0.001$), dose-dependent reduction in MSC viability compared to controls, overall population viability remained good. Conversely, both methylprednisolone and triamcinolone resulted in the rapid death of significant numbers of MSCs ($p<0.001$).

Conclusions: Clinicians can sedate horses and administer nerve blocks to assist in intra-tendinous or intra-thecal injection of MSCs with confidence that these drugs will not impact the viability of implanted cells. However, the concomitant use of corticosteroids is likely to have a severely detrimental effect on cell viability and should not be performed. Similarly, steroid administration into the sheath of a damaged tendon is not recommended.

Introduction

The horse is prone to overstrain injury of the palmar soft tissue structures of the distal limb, particularly the superficial digital flexor tendon (SDFT). Intra-lesional implantation of mesenchymal stem cells (MSCs) has shown significantly improved outcomes and reduced re-injury rates for the treatment of SDFT overstrain injuries [1, 2]. The use of MSCs to treat meniscal tears [3] and osteoarthritis [4-6] has also been trialled in the horse although with variable outcomes. There is currently considerably less well-scrutinised evidence for the clinical use of MSCs to treat other diseases although early stage experimentation suggests promise with regard to their use for a range of tissues [7-9]. However, studies have shown that a large number of cells are lost following injection

into tendons [10-14]. It is therefore important to optimise the survival and retention of implanted cells at the site of injury.

In contrast to humans, chemical restraint of large animals (by standing sedation and/or local anaesthesia) is standard practice [2], and the use of additional drugs may have an adverse effect on the cells. Adverse effects of co-administered drugs can unknowingly hamper a therapeutic effect. Although it is not recommended that corticosteroids and/or antibiotics be administered simultaneously during stem cell implantation, it is possible that this practice persists, particularly if cells are implanted into a synovial joint, or tendon sheath. Therapeutic concentrations of some antibiotics have been shown to have a negative effect on the viability of equine and human MSCs [15-17]. Although the acid pH of the drug-media combination may have contributed to the reported rapidity of cell death, pH buffering served only to delay it [15]. In contrast, non-steroidal anti-inflammatories had positive effects on equine MSC proliferation and migration at low concentrations, whereas higher concentrations had detrimental effects on cells [18]. In addition, the findings of a small study which reported the pronounced and rapid death of equine MSCs following exposure to undiluted mepivacaine (20 mg/mL) [19], are supported by a broader study describing the deleterious effects of several amide-type local anaesthestics on human MSCs [20].

No other drugs have been tested on equine MSCs but, in a study of human MSCs, all commonly used intra-articular corticosteroids reduced cell viability in a dose-dependent manner, although significant differences were apparent between drugs [21]. Cytotoxic effects of corticosteroids have also been reported in human fibroblasts [22] and lymphocytes [23]. However, when exposed to a range of much lower doses (0.001 - 100 mM) of budesonide or dexamethasone cell viability, metabolic activity and morphology were unaffected and the immunomodulatory function of MSCs was actually enhanced, through increased indoleamine-pyrrole 2,3-dioxygenase (IDO) expression [24].

The purpose of this *in vitro* study was to evaluate the effects on viability and proliferation of equine MSCs and tendon-derived cells of drugs commonly used during the diagnosis or treatment of musculoskeletal disease (sedatives, local anaesthetics and corticosteroids) at concentrations that might be achieved clinically.

Materials and Methods

Isolation of cells: Bone marrow-derived MSCs (n = 3 horses) and somatic cells derived from tendon (TDC, n = 2 horses) were obtained as previously described [25, 26] and stored in Bambanker™ cell freezing medium[a] in liquid nitrogen or at -80°C until use. For experiments, cells were seeded in D10 medium (Dulbecco's Modified Eagle Medium[b], supplemented with fetal bovine serum[b] (10% v/v), 100 U/mL penicillin[b] and 100 U/mL streptomycin[b]) and expanded to required numbers (at 37°C in a humidified atmosphere of 5% CO_2), then detached from the culture flasks by 1% trypsin-EDTA treatment[c] and counted.

Preparation of drugs: Drug suspensions were prepared in D10 as detailed in Table 1. The following assumptions were made in order to calculate drug concentrations: for intravenously administered drugs (sedatives), the usual drug dose was divided by the blood volume of a 500 kg horse (50 L) [27]; for drugs administered intra-synovially (corticosteroids, local anaesthetics), the drug dose was divided by the average volume of a tendon sheath (12 mL, estimated volume of a moderately distended sheath); for drugs administered into the subcutaneous space (local anaesthetics), the concentration will vary with diffusion from the injection site. The effective concentration (0.3 µg/mg tissue, equivalent to 0.3 mg/mL) has been measured in sites adjacent to that of mepivacaine injection in the foot [28] which was used for our choice of minimal concentration, and thereafter increased to reflect cases where the MSC injection site might be more intimately associated with local anaesthetic injection.

Cell viability: Cells were re-suspended at a concentration of 0.5×10^6 cells/mL in 1 mL media containing the test drugs and incubated in 12-well suspension culture dishes. For each of 3 horses, one replicate for each drug concentration (control (D10, no drug), high, medium and low) was tested. At each sampling time point, 20 µL of cell suspension was removed and mixed with 20 µL of 0.4% trypan blue solution[d] for 2 min. Live and dead cells were counted using a haemocytometer micro-chamber under a light microscope [28]. A minimum of 100 cells were counted at each time point to calculate the percentage of live and dead cells. Plates were returned to the incubator until the next sampling time point.

Cell proliferation: The effect of mepivacaine (Intraepicaine)[e], romifidine (Sedivet)[f], detomidine (Dormosedan)[g] and butorphanol (Torbugesic)[g] or xylazine (Rompun)[h] exposure on the proliferation rate of MSCs was assessed using alamarBlue®[i] (AbD Serotec, Kidlington, UK). The proliferation of

cells following steroid exposure was not assessed due to the observed negative effect on viability. 20,000 cells were seeded into each well of a 12 well culture plate[j] and allowed to adhere overnight. The following day a baseline reading of fluorescence was obtained; media was replaced with D10 containing 10% alamarBlue® and incubated in the dark for 4 h. One hundred µL aliquots of media were transferred, in triplicate, to fluoro-microtitre plates[k] and fluorescence was measured at 570 nm (excitation) and 585 nm (emission) (Infinite M200 PRO fluorometer[l]). Cells were washed and media was replaced with 1 mL D10 containing the highest concentration of each drug, or with fresh D10 in the case of the control. After 20 min, 1 h or 4 h, wells were washed twice with PBS and left in 1 mL D10 (no drugs added) overnight. The assay was repeated at 3, 4 and 6 days post drug-exposure.

Data analysis: Within each drug, a linear mixed effects model was used to assess the effect of concentration and time on viability (expressed as a percentage) with a compound symmetry structure used to account for the repeated measures. No comparison was made between drugs. Fixed effects were drug concentration and time. A p value of ≤0.05 was considered significant. Modelling was performed using PASW software (version 18)[m].

Results

MSC viability: Mean cell viability at the start of the experiment was 95%. Exposure to romifidine did not significantly affect viability of MSCs compared to controls. In contrast, xylazine induced a significant, dose-dependent reduction in MSC viability at all tested doses (after 4 h mean viabilities were: high dose 72%, medium dose 73%, low dose 74%; all $p<0.001$ compared to controls). Low dose detomidine and butorphanol resulted in significantly higher viability (93%) than medium (90%) or high dose drug (77%; both $p<0.001$). High dose detomidine and butorphanol significantly reduced cell viability compared to controls ($p = 0.007$). Exposure to mepivacaine did not significantly affect viability of MSCs, at any of the dosages tested (Fig 1).

Both methylprednisolone and triamcinolone significantly reduced MSC viability compared to controls at all concentrations (all $p<0.001$). Cell death occurred almost immediately (within 20 min) following exposure to methylprednisolone[g]. In high dose suspension no live cells were detectable after 1 h and after 4 h in low dose suspension, cell viability was only 35%. Cell death was less rapid with

triamcinolone^{n} (within 2 h) but by 4 h, cell viability was below 50% in all tested concentrations (Fig 2 A, B). Increased concentrations of both corticosteroids resulted in a significant increase in cell death rate (all doses $p<0.001$). Cell viability correlated strongly with increased drug concentration (R^2 value range: 0.74 (mepivacaine) – 0.95 (depomedrone), Fig 2E).

Differentiated cell viability: Mepivacaine did not reduce TDC viability (Fig 1E). However, both methylprednisolone and triamcinolone caused a dose-dependent, significant reduction in TDC viability (all $p<0.001$) with similar kinetics to that shown by MSCs (Fig 2 C, D).

Proliferation: Proliferation rate of MSCs was not significantly affected by exposure to detomidine and butorphanol, mepivacaine, romifidine or xylazine compared to control cells (Fig 3). Duration of drug exposure (from 20 min to 4 h) had no impact (data not shown).

Discussion

Although the precise mechanism by which MSCs implanted into core SDFT lesions result in improved healing is still unknown, it is logical to deduce that survival with or without further proliferation of implanted cells is important for clinical effect. Neither property was affected by exposure to romifidine or to mepivacaine. While the dosages tested were representative of blood concentrations at the point of injection, tissue levels would be expected to be even lower so long as significant haemorrhage did not occur at the time of implantation [29]. Therefore, MSCs implanted into a core SDFT lesion or tendon sheath are likely to encounter concentrations below those tested here. In general, cell viability exposed to even the highest concentrations of drugs tested (concentrations far above those likely to be achieved at the actual site of cell injection) remained high. Nonetheless, viability of MSCs exposed to the highest concentration of xylazine was significantly reduced and although remaining at approximately 75%, this additional loss could be clinically significant. Dead cells at the site of injection could contribute to a local inflammatory reaction. As a result, if prolonged, heavy or repeated sedation of a particularly fractious horse is likely to be necessary, sedation with romifidine, or a combination of detomidine and butorphanol should be considered in preference to xylazine.

The concentration of local anaesthetic to which implanted cells are likely to be exposed, ranges widely depending on proximity to a subcutaneous injection or intra-synovially administered local analgesia. True exposure is likely to be considerably lower than the concentrations used here, since local anaesthetic is injected extra-vascularly in the region of the target nerve and studies using contrast have suggested that diffusion is along the fascial planes rather than into the body of the tendon [30]. A recent study has documented diffusion into adjacent synovial structures after injection of local anaesthetic into the digital flexor tendon sheath although the levels are considerably lower than used here, even after 60 min (maximum of 0.0005 mg/mL), and therefore represent minimal risk to implanted cells [31]. Therefore, combined with the results presented here, the use of a regional nerve block to assist in control of the horse's limb and accurate injection of MSCs into a tendon body or sheath can be employed.

Our data agrees with previously published results reporting the decreased viability of human MSCs in suspension containing corticosteroids [21]. TDC viability was similarly reduced suggesting a non-specific mode of action on cells in suspension. It was noted that methylprednisolone suspension in culture media was a more basic pH than that of the other drugs studied. Previous *in vitro* studies have also suggested that corticosteroid exposure can inhibit the regenerative properties of human tendon stem cells [32] and may also augment undesirable adipogenesis in murine MSCs [33]. When considering these reasons and the poor viability demonstrated in the current study, the authors would strongly advise against treating with corticosteroids immediately after stem cell treatment.

These experiments were conducted over a relatively short time course and so it is possible that while viability was not altered, delayed apoptosis could have been initiated. Further study is necessary to determine if any of these downstream events occur in MSCs.

In conclusion, equine practitioners should continue to sedate horses and/or can administer a regional nerve block prior to administration of MSCs, as this will not adversely affect cell viability or proliferation. Corticosteroids should not be used concurrently or administered into the sheath of a damaged tendon.

Authors' declaration of interests

No competing interests have been declared.

Ethical Animal Research

Ethical approval for collection of equine tissues was obtained from the Ethics and Welfare Committee at the Royal Veterinary College (URN 2013 1230R 2005). Explicit owner informed consent for participation in this study was not stated but general permission for post mortem examination and use of tissue in research was given.

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Manufacturers' addresses

[a]Anachem, Luton, Bedfordshire, UK.

[b]Invitrogen, Paisley, Renfrewshire, UK.

[c]Sigma-Aldrich, Gillingham, Kent, UK.

[d]Sigma-Aldrich, Poole, Dorset, UK.

[e]Intraepicaine (mepivacaine, 10mg/mL for injection): Dechra Veterinary Products, Stoke-on-Trent, UK.

[f]Sedivet (romifidine, 10mg/mL): Boehringer Ingelheim, Bracknell, UK.

[g]Dormosedan (detomidine, 10mg/mL); Torbugesic (butorphanol, 10mg/mL): Depomedrone (methylprednisolone acetonide, 40mg/mL): Pfizer Inc, Tadworth, UK.

[h]Rompun (xylazine, 2%, 23.32mg/mL): Bayer Healthcare, Leverkusen, Germany.

[i]AbD Serotec, Kidlington, Oxfordshire, UK.

[j]Starlab, Milton Keynes, Buckinghamshire, UK.

[k]SPL Lifesciences, UK.

[l]Tecan, UK.

[m]IBM, UK.

[n]Adcortyl (triamcinolone acetonide, 10mg/mL): E.R. Squibb & Sons Ltd, Uxbridge, UK.

Figure legends:

Fig 1: Cell viability (percentage) measured by trypan blue exclusion assay. Viability of mesenchymal stem cells (MSCs, n = 3 horses) and tendon derived cells (TDCs, n = 2 horses) suspended for up to 4 h in control media (D10 only) or a range of concentrations of A) xylazine (0.02 – 0.005 mg/mL); B) detomidine and butorphanol (0.001 - 0.00025 mg/mL + 0.002 – 0.0005 mg/mL); C) romifidine (0.001 – 0.00025 mg/mL) and D, E) mepivacaine (1.2 - 0.3 mg/mL). Error bars represent 95% confidence intervals.

Fig 2: Cell viability (percentage) measured by trypan blue exclusion assay. Viability of mesenchymal stem cells (MSCs, n = 3) and tendon derived cells (TDCs, n = 2) suspended for up to 4 h in control media (D10 only) or a range of concentrations of A, C) triamcinolone (0.8 – 0.2 mg/mL) and B,D) methylprednisolone (8 – 2 mg/mL). E) Cell viability of MSCs suspended in each concentration of the test drugs. Error bars represent 95% confidence intervals.

Fig 3: Cell proliferation for mesenchymal stem cells. MSCs (n = 3) in monolayers were assayed by alamarBlue® following exposure to high dose detomidine and butorphanol (0.001 mg/mL + 0.002

mg/mL), mepivacaine (1.2 mg/mL), romifidine (0.001 mg/mL) or xylazine (0.02 mg/mL) for 4 h. Control cells were cultured in D10 medium only. Data points are the mean of values obtained from 3 horses and error bars represent the standard error of the mean.

Supplementary Information

Supplementary Item 1: Percentage viability for mesenchymal stem cells (all n = 3 horses) exposed to high, medium and low doses of test drug.

Table 1: Drug concentrations used for viability experiments. Proliferation experiments were performed in media containing the highest indicated concentration.

Drug	Drug concentration (mg/mL)	Clinical dose range	Concentration in blood/ tissues/ synovial cavity mg/mL	Concentration used in culture (mg/mL)			pH of High dose
				High	Medium	Low	
Intravenously injected							
Xylazine[h]	20	0.5-1 mg/kg	0.005-0.01	0.02	0.01	0.005	7.82
Detomidine[g] (in combination with)	10	0.01 mg/kg	0.0001	0.001	0.0005	0.00025	8.2
Butorphanol[g]	10	0.025 mg/kg	0.00025	0.002	0.001	0.0005	
Romifidine[f]	10	0.04-0.12 mg/kg	0.0004-0.0012	0.001	0.0005	0.00025	8.4
Locally injected							
Mepivacaine[e]	20	2 mL at multiple sites	20 (initial concentration)	1.2	0.6	0.3	7.56
Intra-synovially injected							
Methylprednisolone[g]	40	40-80 mg/ sheath/joint	4-8	8	4	2	8.59
Triamcinolone acetonide[n]	10	10 mg/ sheath/joint	0.8	0.8	0.4	0.2	8.51

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Figure 1

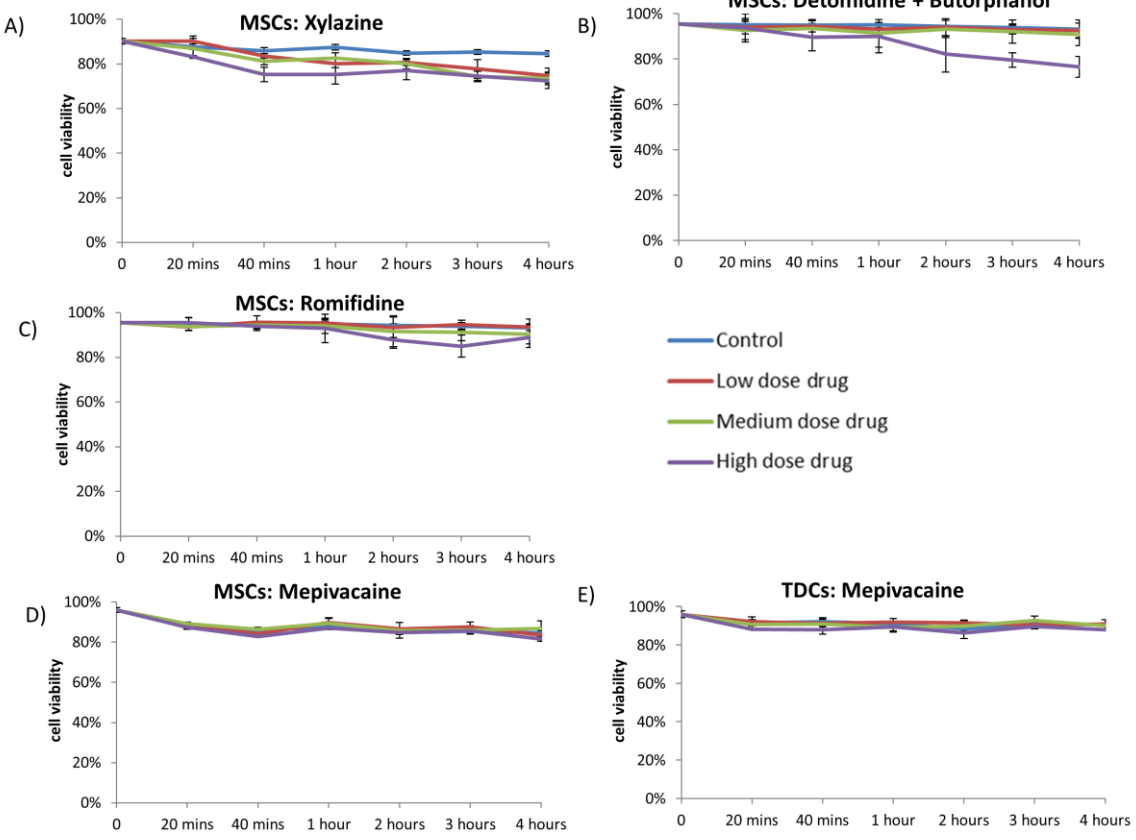

Figure 2

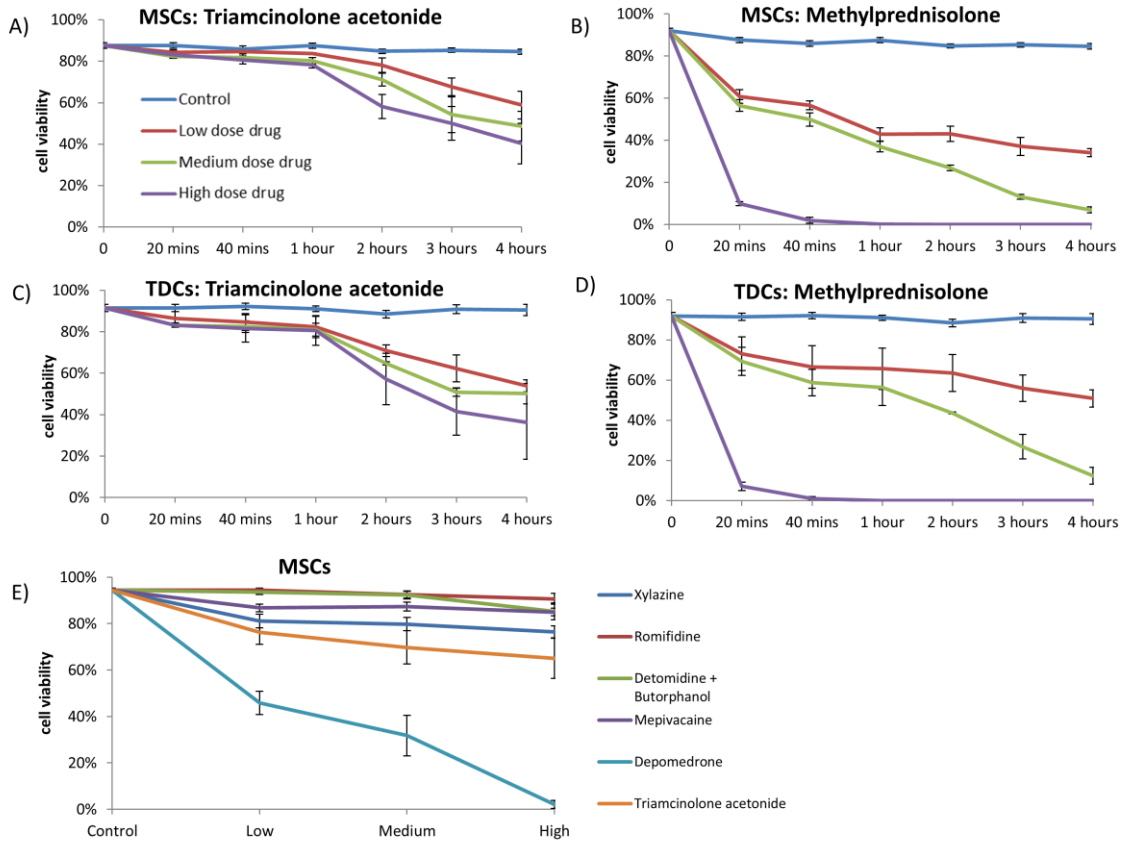

Figure 3