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Prevention of cellular senescence by Metformin in horse tenocytes

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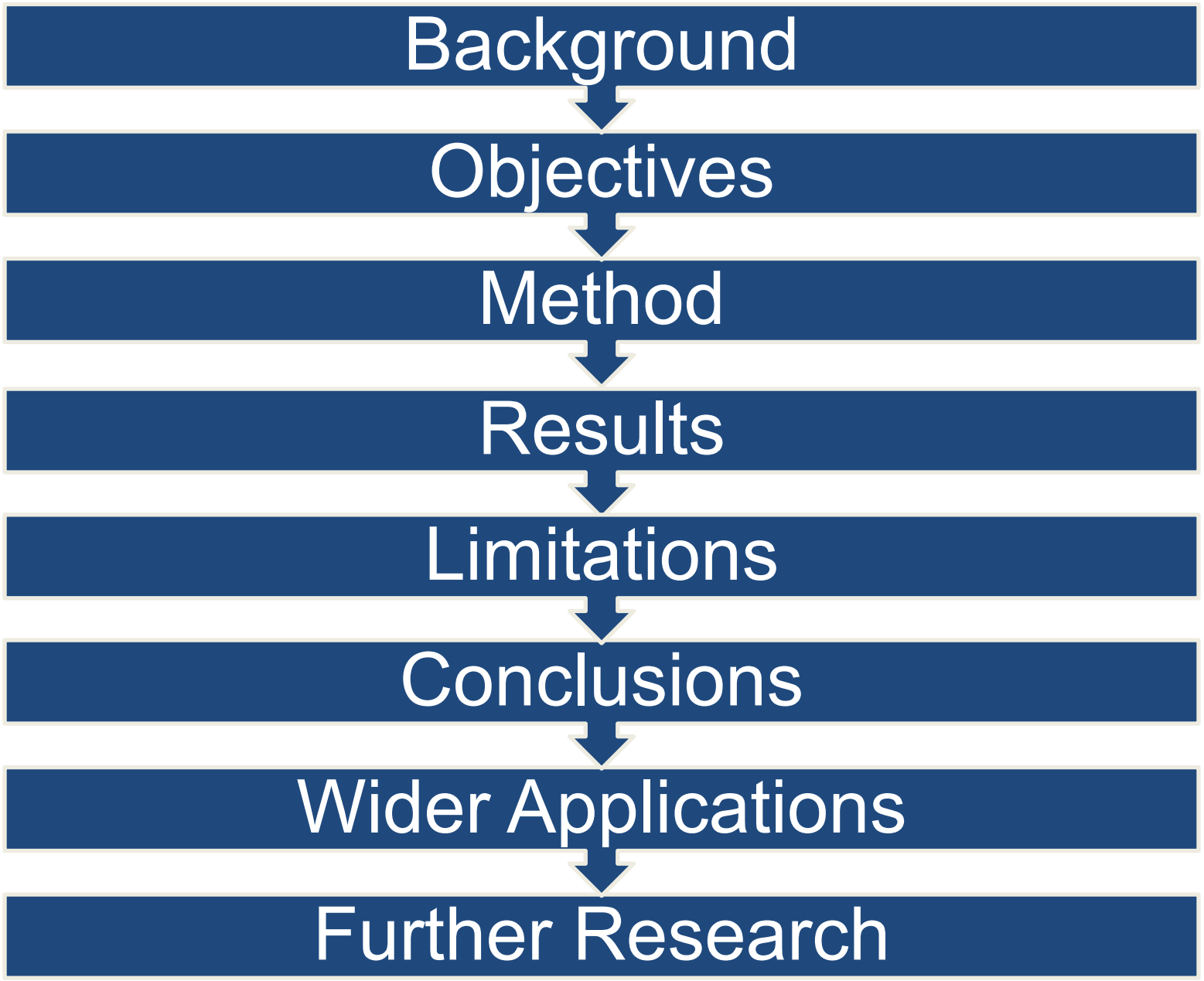
Plan


Background
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Wider Applications
Further Research

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What is the problem?

- Equine tendon injuries – ”one of the most frequent causes of thoroughbred lameness”
- 23-67% reinjury rates

- Risk of SDFT injury rates increases dramatically with age and exercise intensity (Perkins, Reid and Morris, 2005)

- Thus, tendinopathies are a major cause of early retirement and affects both horse’s welfare, owners, trainers and the thoroughbred sport as a whole.

- Used as a model for human tendon disease (Magnusson et al., 2002; Riley, 2005)

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Is prevention a valid solution to reduce injury?

- Prolonged healing and failure to return to normal structure
- “Failure of regeneration suggests that prevention would have a significantly greater clinical impact than therapy”
- “Cumulative subclinical damage”
- “Window of opportunity” for intervention (Patterson-Kane et al., 2012)

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What is senescence and why is it important?

- Although senescence is normally associated with aging, it can be triggered in younger animals by certain risk factors such as over-exercise. (Sun et al., 2015)

- **Senescence** can occur via **two mechanisms:**

  - a)**Replicative** senescence due to telomere shortening
  - b)**Stress induced** senescence usually due to an increase in ROS

- In the horse superficial digital flexor tendon (SDFT) cell senescence is upregulated due to DNA damage and cell stress signalling. This initiates the release of proinflammatory cytokines which is the main feature of SASP (Sun et al., 2015; Tsai et al., 2011).

- This suggests a direct relationship between cellular senescence and tendinopathies. *Current research at the RVC sponsored by the HBLB

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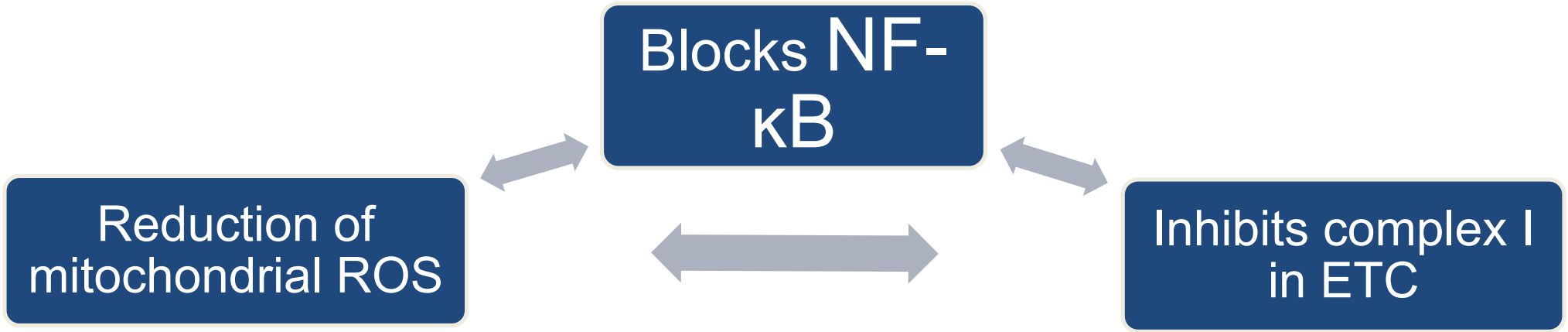
Why Metformin?

- Metformin – established antidiabetic drug; however, unexpected anticancer and antiaging properties were recently discovered in several retrospective studies.

- Metformin inhibits SASP (senescence associated secretory phenotype) and extends lifespan (Anisimov et al., 2008) (Moiseeva et al., 2013) suggesting potential utility in tendon injuries.

- Mechanism not well understood, but main proposed pathways involve:



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Why Dexamethasone as the experimental senescence inducing agent?

- Dexamethasone (corticosteroid) is often used as an anti-inflammatory drug in horses
- However, multiple studies on human fibroblast cells suggest severe adverse effects:
- “dexamethasone reduces both cell number and collagen synthesis in tenocyte cultures in a concentration-dependent manner”
- (Scutt, Rolf and Scutt, 2006)
- As cell senescence is irreversible in vivo, glucocorticoid-induced senescence may result in long- term degenerative changes in tendon tissue. (Poulsen et al., 2013)

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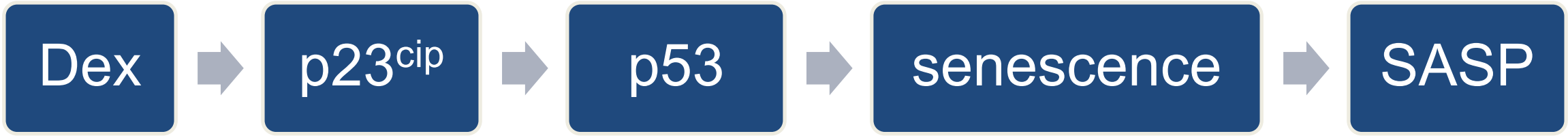
Why Dexamethasone as the experimental senescence inducing agent?

- Overall it is clear that **glucocorticoid** has significant **negative effects** on tendon cells in **vitro**, including **reduced cell viability**, **cell proliferation** and **collagen synthesis**. There is increased **collagen disorganisation** and **necrosis** as shown by **in vivo** studies. (Dean et al., 2014)
- The mechanical properties of tendon are also significantly reduced.

- Emerging clinical evidence that shows significant long-term harms to tendon tissue and cells associated with glucocorticoid injections. (Dean et al., 2014)



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Hypothesis

- Metformin will prevent SASP in Dexamethasone induced horse tenocytes

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Aims

- (1) Investigate whether dexamethasone induces senescence in equine tenocytes

- (2) Investigate effect of pre-treatment with metformin on dexamethasone induced senescence in equine tenocytes

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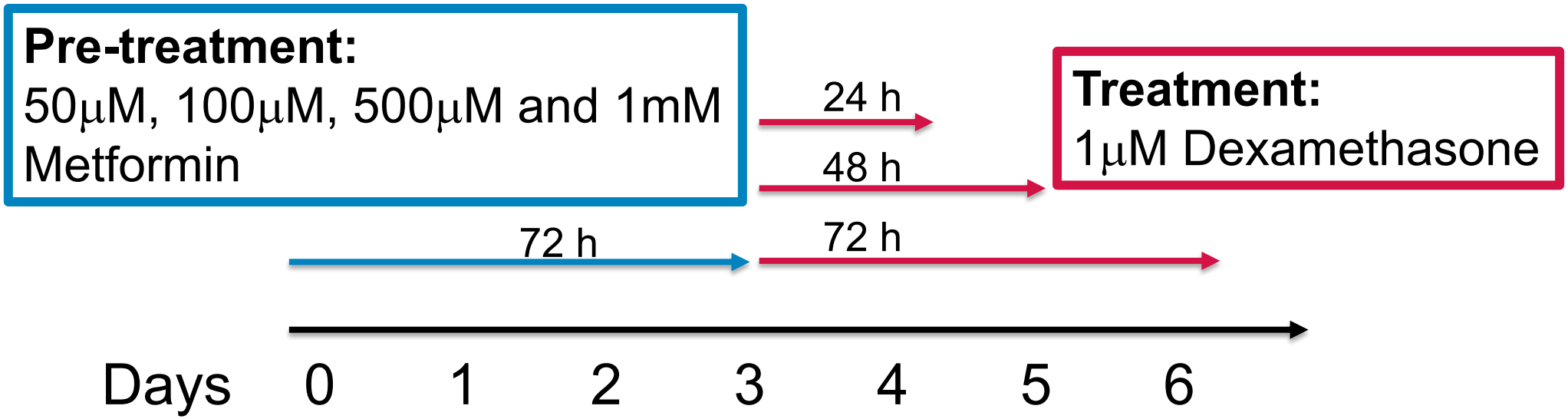
Methods

- Horse SDFT tenocyte were cultures as a monolayer in DMEM supplemented with 10% fetal bovine serum)
- Senescence was measured using Senescence Associated-β-galactosidase (SA-β-gal) commercial kit.



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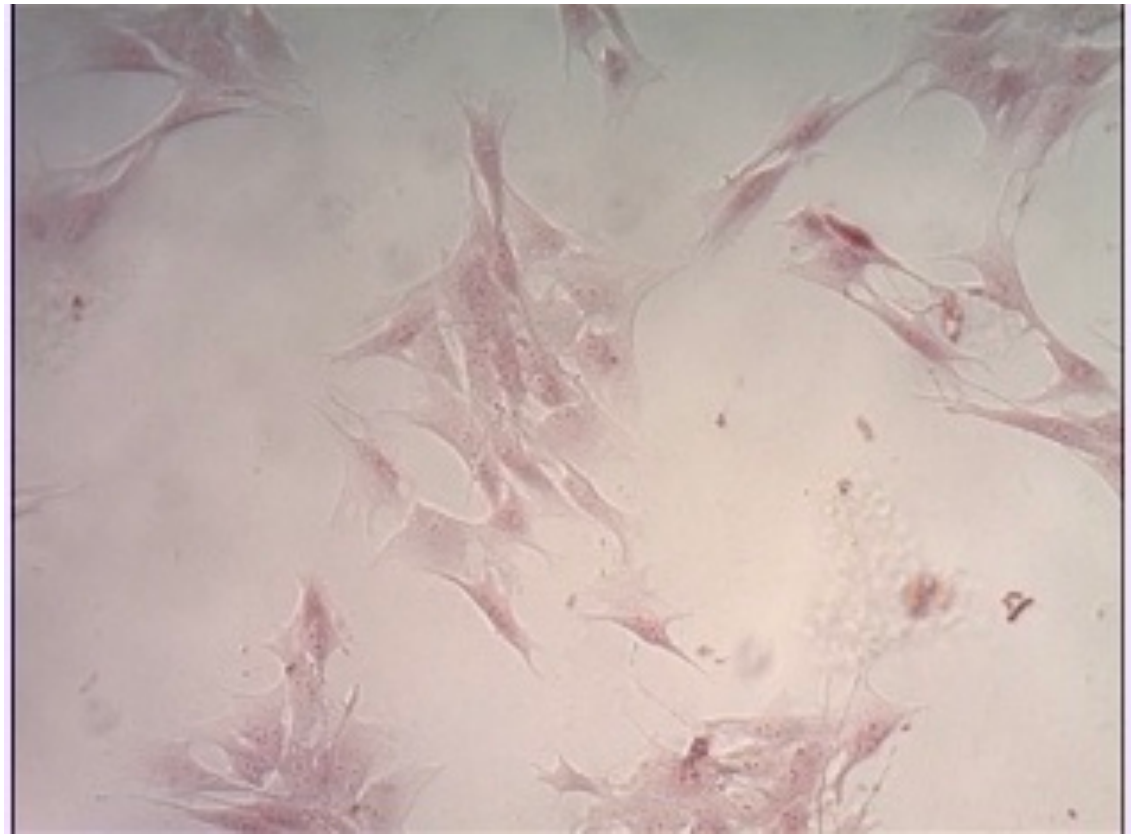
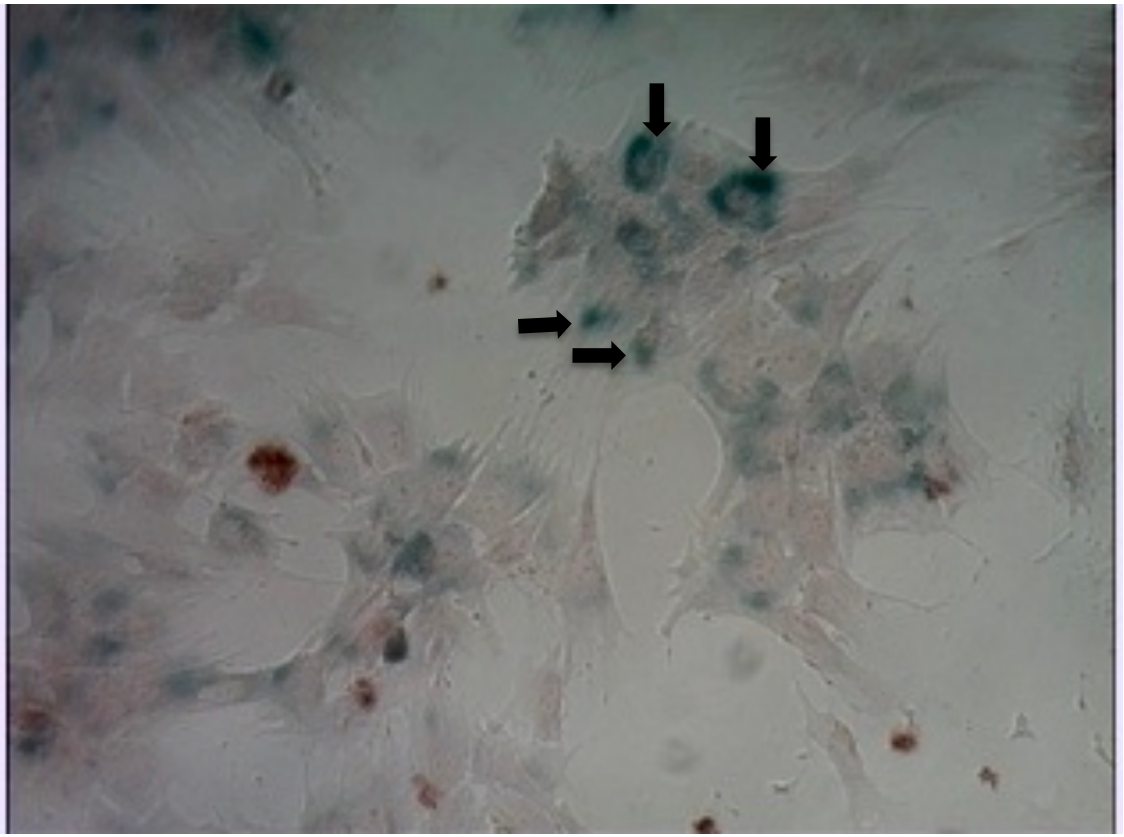
Methods

SA-β-gal **negative** cells



SA-β-gal **positive** cells



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Method – Statistical Analysis

- Two-Way ANOVA (Analysis of Variance) with Replication
- (p=0.05)

- Parametric and Multifactorial

- Standard deviation

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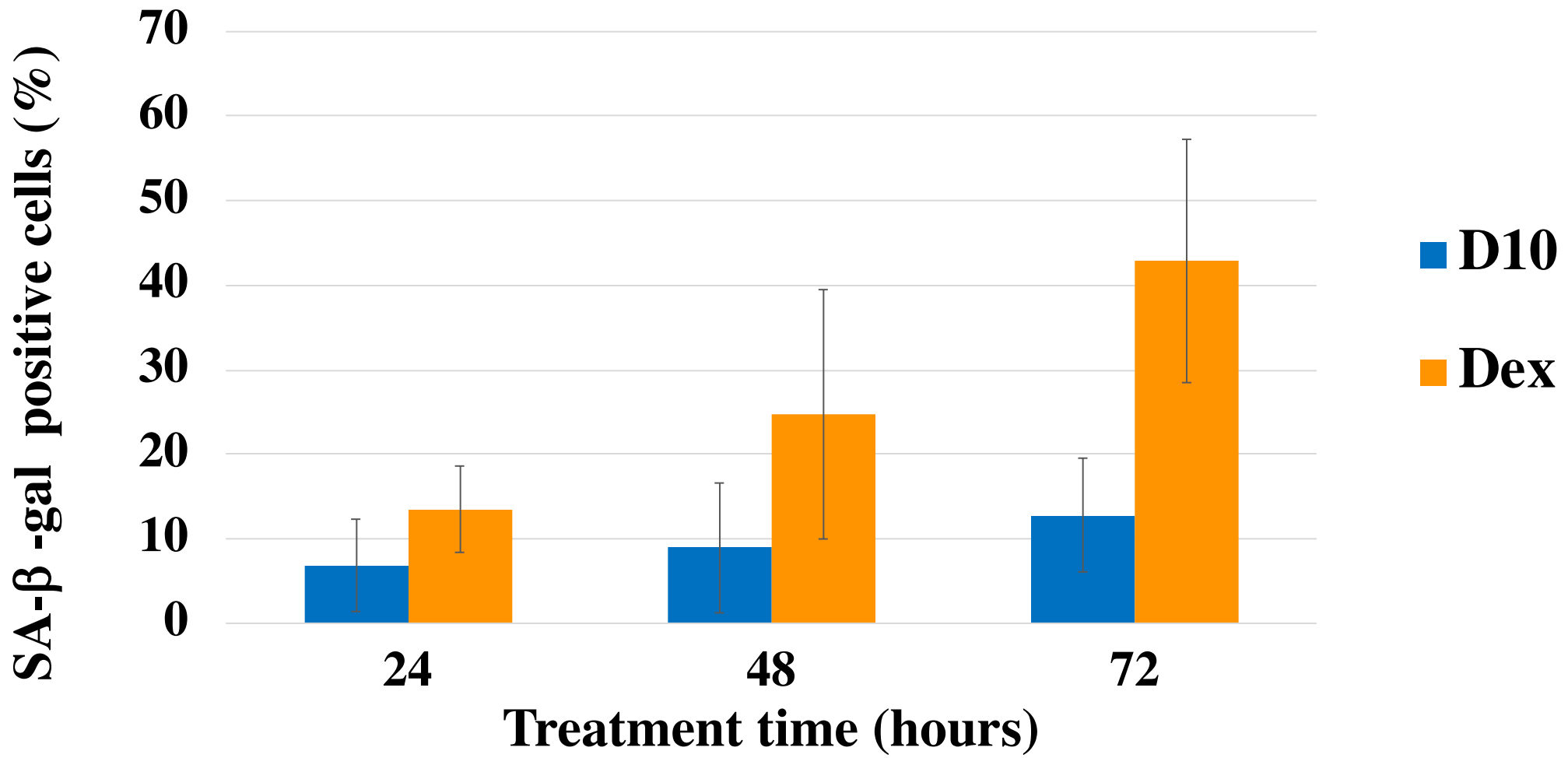
Results

- **Figure 1.** Effect of dexamethasone on tenocyte senescence



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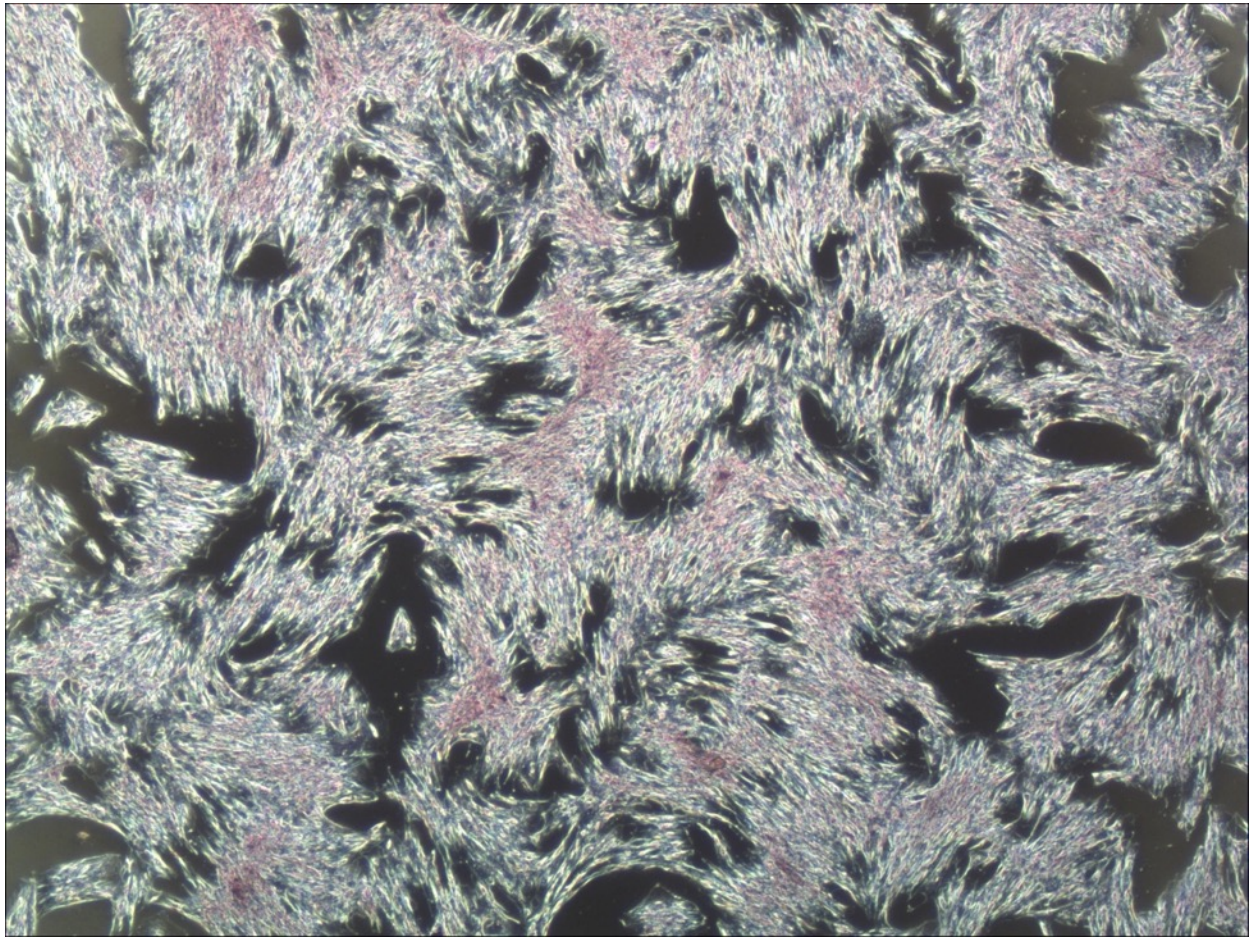
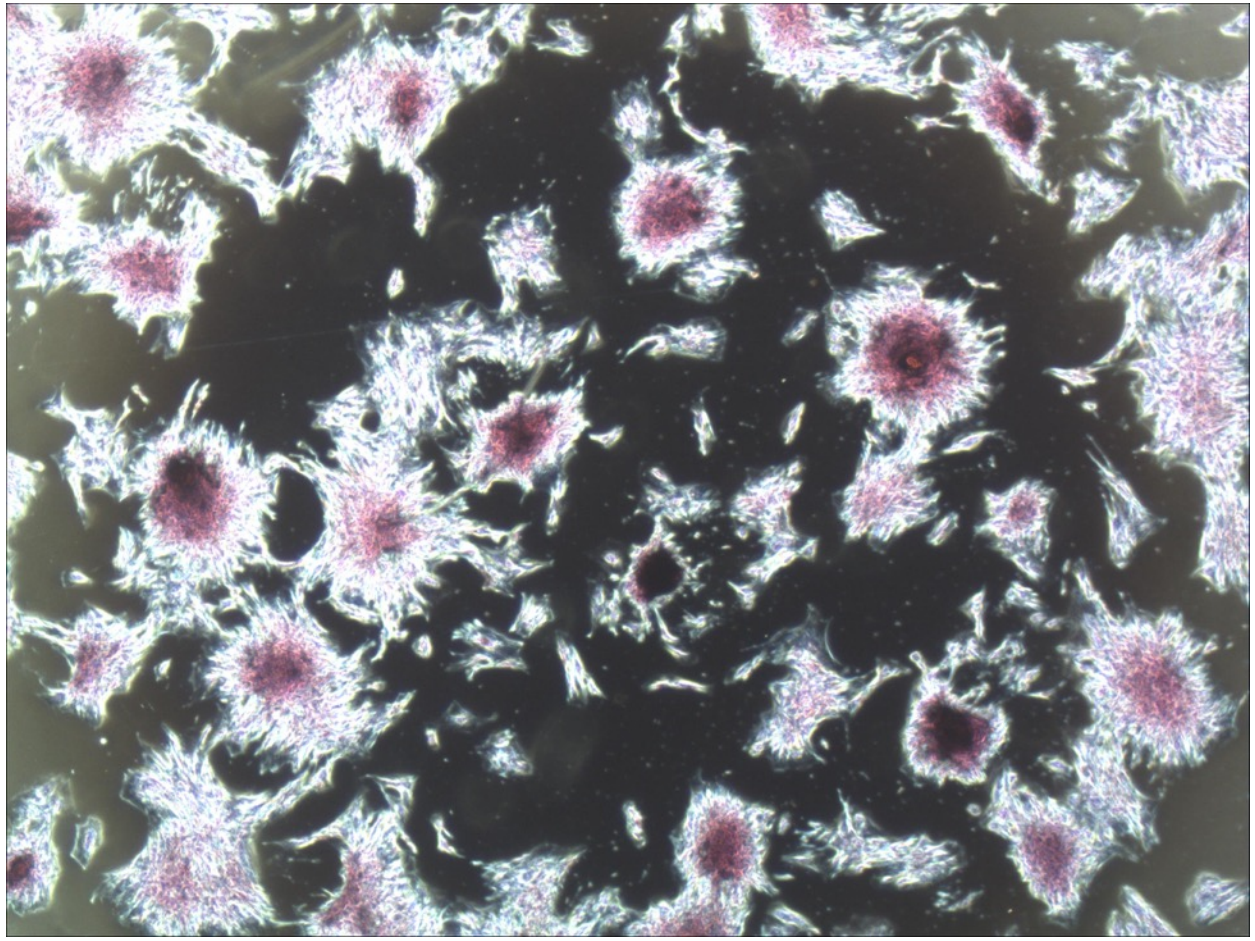
Results – Standard deviation and issue with data collection

Untreated tenocytes

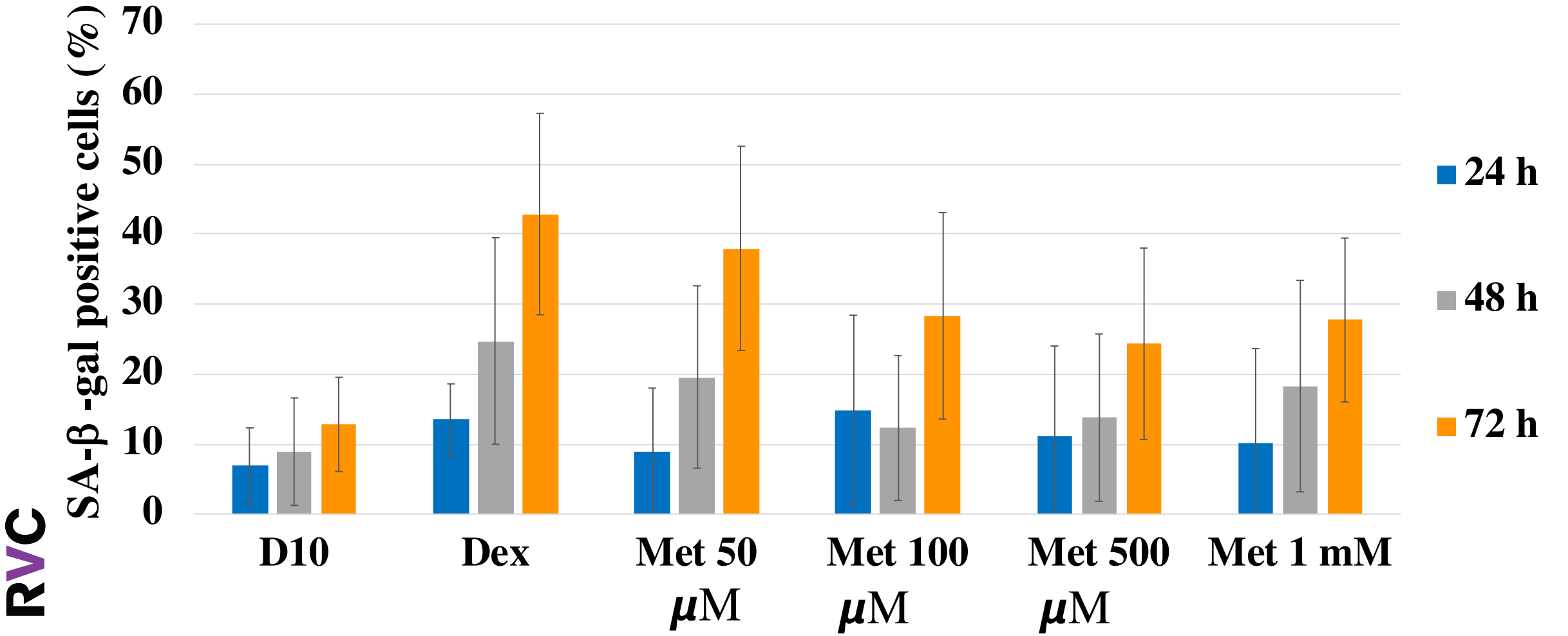


Tenocytes treated with 1μM dex for 48 h



Results

- **Figure 2.** Effect of Metformin on dexamethasone induced senescence



Results - Statistical Analysis

ANOVA Outcomes: (P – values)	Dex vs. D10	Metformin
Treatment	$4.2 \cdot 10^{-9}$	$2.2 \cdot 10^{-19}$
Time	$9.1 \cdot 10^{-13}$	0.001
Interaction	$7.2 \cdot 10^{-5}$	0.064

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Limitations and Solutions

- Fields of view - samples from the same well could have high variation in the number of SA-β-positive cells, depending on where the image was taken. This led to high standard deviation.
- Percentages from some of the groups weren't normally distributed (skewness up to 1.97)

- Nevertheless, Two-Way ANOVA with replication was the best test to use.
- 5 fields of view were taken per well.

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Conclusions

- Treatment with 1μM dexamethasone alone progressively induced cellular senescence in equine tenocytes with time.
- Pre-treatment with Metformin gave inconsistent results, however a negative trend between the number of SA-β-gal positive cells and the concentration of Metformin was found.
- Dexamethasone has a detrimental effect on equine tenocytes even at normal circulating concentration, and thus must be used cautiously.

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Conclusions

- Statistical analysis shows that both the effect of time, treatment and interaction of the two are all significant in regards to the comparison of Dexamethasone treated vs. control samples.

- The effects of Metformin were less clear but appeared to suppress dexamethasone induced cellular senescence. However further investigation of metformin as an anti-senescent preventative measure is warranted.

- There was a trend for a dose-related decrease in SA-β-gal positive cells with Metformin

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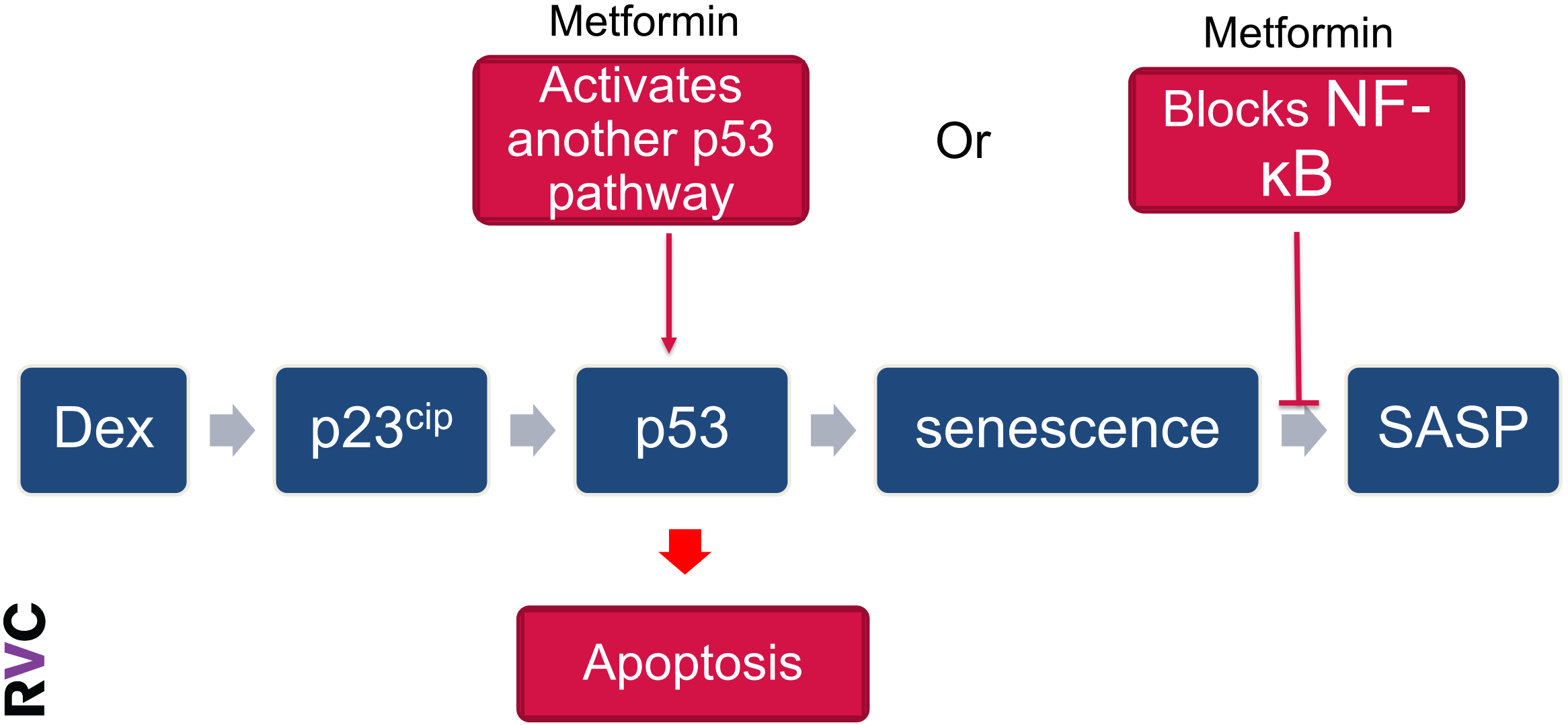
Conclusions – Potential Mechanism



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Wider Applications

- If the results bear out with more detailed research, Metformin might be used as a preventative treatment to reduce the cumulative subclinical damage, if given to athletes under high intensity training.

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Further Research

- (a) More precise method of measure of senescent phenotype is needed – e.g. measure SASP-regulated cytokines, more quantitative and reliable then SA-β-gal colour assay

- (b) Method of inducing senescence with a mechanism more similar to the naturally occurring process could be a better model of stress induced senescence
  - Hydrogen Peroxide (ROS dependent)

- (c) The work needs to be repeated with more horse samples

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