(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 November 2010 (18.11.2010)

PCT

(10) International Publication Number
WO 2010/131038 A2

(51) International Patent Classification:
A61K 31/375 (2006.01) *A61K 31/573* (2006.01)
A61K 31/445 (2006.01) *A61P 25/04* (2006.01)

(21) International Application Number:
PCT/GB2010/050776

(22) International Filing Date:
12 May 2010 (12.05.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0908174.6 13 May 2009 (13.05.2009) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) **Title**: STEROID CONTAINING COMPOSITION AND USES THEREOF

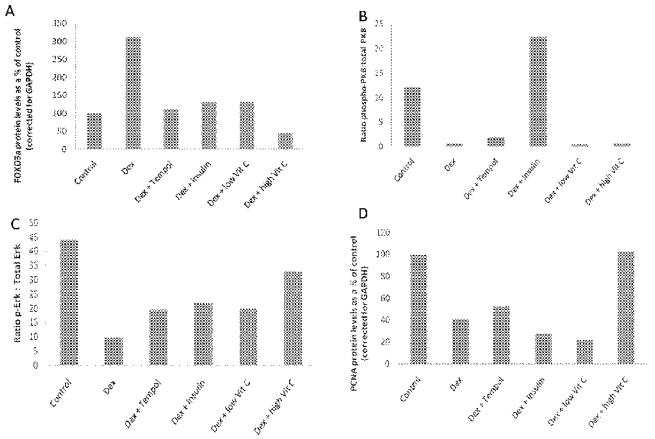

Figure 10

(57) **Abstract**: The present invention provides the use of a steroid in combination with an anti-oxidant or an inhibitor of forkhead signalling, in particular, for the treatment, amelioration or prevention of tissue degeneration and/or pain and/or inflammation and/or for facilitating tissue repair. The invention also provides a composition comprising a steroid and an anti-oxidant or an inhibitor of forkhead signalling.

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STEROID CONTAINING COMPOSITION AND USES THEREOF

This invention relates to the use of a steroid in combination with an anti-oxidant or an inhibitor of forkhead signalling, in particular, for the treatment, amelioration or prevention of tissue degeneration and/or pain and/or inflammation and/or for facilitating tissue repair.

Steroid injections are a common and effective treatment for a variety of conditions in which inflammation causes pain, swelling and other problems. Glucocorticoids, particularly prednisone and cortisone, are commonly used in injections for inflammation and pain. These steroids help reduce inflammation and pain in the body. Cortisone is the most well known injected steroid and it has a dramatic anti-inflammatory effect on tissues, particularly joints and tendons.

Conditions which may be treated with steroid injections include, but are not limited to, sciatica, tennis elbow (lateral epicondylitis), golfer's elbow (medial epicondylitis), joint pain of varying nature (for example osteoarthritis), bursitis of the shoulder, rotator cuff tears, hip or knee inflammation, frozen shoulder, plantar fasciitis, carpal tunnel syndrome, herniated disc and other back pain, synovitis, gouty arthritis, tendinitis, rheumatoid arthritis, neuromas, glaucoma, skin conditions such as psoriasis and eczema, and other painful or inflammatory musculoskeletal, ocular, dermal or articular pathologies. In particular, local glucocorticoid injections are commonly used for the management of the pain associated with tendinitis caused by tendon degeneration and/or rupture. In the UK alone, 500,000 such injections are administered to tendons or joints annually.

The debilitating effects of chronic pain are not only a source of anxiety and distress for the individual, but represent a tremendous cost to society. For example, workers suffering from chronic pain are frequently absent from work for weeks or even longer. Indeed, in the UK over half of all sick days from work are due to musculoskeletal pain, for example, back, shoulder or joint pain.

Whilst glucocorticoids are effective in many cases in pain relief, and are the most potent anti-inflammatory therapy currently available for clinical use, this treatment can however have significant side effects. Glucocorticoids can cause substantial bystander damage in skin, bone and other musculoskeletal tissues and therefore excessive use is a major clinical concern. Collagen producing tissues seem particularly vulnerable to the tissue wasting side-effects of glucocorticoids, yet glucocorticoids are extensively used as anti-inflammatory treatments in these tissues. For example, despite the prevalence of glucocorticoid use for treating tendon pain, it is widely believed by clinicians that administration of steroids to tendon injuries can hinder the healing process, however how this occurs is unknown (Ford & Debender. Southern Medical Journal 1979; 72(7) 827-830; Nichols, Clinical Journal of Sports Medicine 2005; 15(5), 370-375; Watson. J Bone Joint Surg Br. 1985; 67(4), 618-624). Typically, clinicians see an increased incidence of tendon re-rupture in patients to whom steroid treatment has been administered. There is therefore a need to identify the negative effects of glucocorticoids, and indeed other steroids, and to develop protective strategies.

Despite considerable research, no equally effective alternative to steroid treatment is currently available nor has an effective means of protecting against the undesirable effects of steroid treatment been developed.

According to a first aspect, the invention provides i) a steroid and ii) an antioxidant or inhibitor of forkhead signalling, for use in the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair.

According to a further aspect, the invention provides the use of i) a steroid and ii) an antioxidant or inhibitor of forkhead signalling, in the preparation of a medicament for the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair.

According to another aspect, the invention provides a method of treating, ameliorating or preventing pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair, in a subject, comprising administering i) a steroid and ii) an antioxidant or inhibitor of forkhead signalling to the subject. Preferably the method comprises administering a therapeutically effective amount of i) a steroid and ii) an antioxidant or inhibitor of forkhead signalling to the subject.

An inhibitor of forkhead signalling may include an agent which activates a negative forkhead regulator which then inhibits forkhead signalling, alternatively an inhibitor of forkhead signalling may include an agent which inhibits a positive forkhead regulator which then inhibits forkhead signalling. For example, insulin may activate the negative forkhead regulator PKB to inhibit forkhead signalling, whereas vitamin C may inhibit a positive forkhead regulator and thereby inhibit forkhead signalling.

Preferably the steroid and antioxidant or inhibitor of forkhead signalling are for use in the treatment, amelioration or prevention of one or more of

the conditions selected from the group comprising sciatica, tennis elbow (lateral epicondylitis), golfer's elbow (medial epicondylitis), joint pain of varying nature (for example osteoarthritis), bursitis of the shoulder, rotator cuff tears, hip or knee inflammation, frozen shoulder, plantar fasciitis, carpal tunnel syndrome, herniated disc and other back pain, synovitis, gouty arthritis, tendinitis, rheumatoid arthritis neuromas, glaucoma, skin conditions such as psoriasis and eczema, and other painful or inflammatory musculoskeletal, ocular, dermal or articular pathologies. Preferably the steroid and antioxidant are for use in the treatment of a tendonitis. Tendonitis may be caused by a degenerative process or by a physical strain, such as during exercise. Degenerative tendon conditions include rotator cuff tendinitis and Achilles tendinosis

Preferably the steroid and antioxidant or inhibitor of forkhead signalling are provided/administered at the site of disease, injury or in need of treatment. Preferably the steroid and antioxidant or inhibitor of forkhead signalling are provided/administered in a therapeutically effective amount.

The results presented herein demonstrate that when steroids, such as corticosteroids, are administered to tenocytes, the cells found in tendons, cell proliferation decreases, matrix output of both collagen type I and GAG decreases. Pro-apoptotic markers may increase. In addition, there may be an increase in reactive oxygen species (ROS) which may cause a number of downstream effects. More specifically, ROS may cause activation of forkhead transcription factors which may result in cell apoptosis and/or tissue degeneration, or the activation of the forkheads may cause cells to become quiescent, which is also not useful when cell proliferation and tissue repair is needed. This is the first time that an increase in ROS has been associated with steroid injections. It is clear

from the results that steroids, and glucocorticosteroids in particular, induce ROS which play a role in tissue damage and that anti-oxidant strategies are highly effective in reversing the changes.

In use the steroid and antioxidant or inhibitor of forkhead signalling may be administered simultaneously, sequentially or separately.

In use the steroid and antioxidant or inhibitor of forkhead signalling may be in the same or separate compositions.

The steroid and antioxidant or inhibitor of forkhead signalling may be for topical, mucosal, intraarticular, periarticular, intrabursal, intratendonus, peritendonus, intramuscular, intraperitoneal or inhalation administration.

Preferably the steroid and antioxidant or inhibitor of a forkhead regulator are administered by injection.

Preferably the steroid and antioxidant or inhibitor of a forkhead regulator are administered by topical application.

The steroid and antioxidant or inhibitor of forkhead signalling regulator may be administered as a single or multiple dose regimen.

Preferably the steroid and antioxidant or inhibitor of forkhead signalling are for administration to a tendon, and administration is via injection either directly into the tendon or into the area directly surrounding the tendon. Preferably the steroid and antioxidant or inhibitor of forkhead signalling are for the treatment, alleviation or prevention of pain and/or inflammation in the tendon.

Preferably the steroid and antioxidant or inhibitor of forkhead signalling are for administration to a joint, and administration is via injection either directly into the joint or into the area directly surrounding the joint. Preferably the steroid and antioxidant or inhibitor of forkhead signalling are for the treatment, alleviation and/or prevention of pain and/or inflammation in the joint.

Similarly, the steroid and antioxidant or inhibitor of forkhead signalling may be for administration to a joint, and administration may be via injection directly into the joint (intra-articular injection), or into a region near the joint (peri-articular injection).

The steroid and antioxidant or inhibitor of forkhead signalling may be for administration in the eye, for example, for the treatment of glaucoma.

Alternatively, if the steroid and antioxidant or inhibitor of forkhead signalling are intended to act in the lungs or somewhere in the airways then the compositions may be arranged to be administered by inhalation.

Furthermore, if the steroid and antioxidant or inhibitor of forkhead signalling are intended to act on the skin or in a surface wound, then the compositions may be arranged for topical administration. Such compositions may be for use in the treatment of skin conditions such as psoriasis and/or eczema. The steroid and antioxidant or inhibitor of forkhead signalling for topical administration may be formulated as a cream, gel, lotion, emulsion, ointment, paste or spray.

If the steroid and/or the antioxidant or inhibitor of forkhead signalling is for administration by injection, a long lasting (depo) preparation of either or both may be used.

Preferably the steroid is a corticosteroid, more preferably a glucocorticoid. Glucocorticoids are a class of steroid hormone which bind to the glucocorticoid receptor (GR). The invention may use one or more steroids, preferably at least one of the steroids is a glucocorticoid steroid,

Corticosteroids associated with the present invention can be any naturally occurring or a synthetic steroid hormone. Naturally occurring corticosteroids are secreted by the adrenal cortex or generally the human body. Corticosteriods may have glucocorticoid and/or mineralocorticord activity. For the present invention non-limiting examples of corticosteroids may include: dexamethasone, betamethasone, triamcinolone, triamcinolone acetonide, triamcinolone diacetate, triamcinolone hexacetonide, beclomethasone dipropionate, beclomethasone dipropionate monohydrate, flumethasone pivalate, diflorasone diacetate, fluocinolone acetonide, fluorometholone, fluorometholone acetate, clobetasol propionate, desoximethasone, fluoxymesterone, fluprednisolone, hydrocortisone, hydrocortisone acetate, hydrocortisone butyrate, hydrocortisone sodium phosphate, hydrocortisone sodium succinate, hydrocortisone cypionate, hydrocortisone probutate, hydrocortisone valerate, cortisone acetate, paramethasone acetate, methylprednisolone, methylprednisolone acetate, methylprednisolone sodium succinate, prednisolone, prednisolone acetate, prednisolone sodium phosphate, prednisolone tebutate, clocortolone pivalate, fluocinolone, dexamethasone 21 -acetate, betamethasone 17-valerate, isoflupredone, 9-fluorocortisone, 6-hydroxydexamethasone, dichlorisone, meclorisone, flupredidene, doxibetasol, halopredone, halometasone, clobetasone, diflucortolone, isoflupredone acetate, fluorohydroxyandrostenedione, beclomethasone, flumethasone, diflorasone, fluocinolone, clobetasol, cortisone, paramethasone,

clocortolone, prednisolone 21-hemisuccinate free acid, prednisolone metasulphobenzoate, prednisolone terbutate, and triamcinolone acetonide 21-palmitate.

An antioxidant is an agent capable of slowing or preventing or reversing the oxidation of another molecule. In the invention the antioxidant may be vitamin C (ascorbic acid), tempol, glutathione, lactoferrin, edaravone, tiron or any other suitable antioxidant or a salt or derivative thereof. Preferably the antioxidant is vitamin C, or a derivative or salt thereof. The invention may use one or more than one antioxidant.

An inhibitor of forkhead signalling may act at any point in the pathway of action of a forkhead protein. An inhibitor of forkhead signalling may act by activating or inhibiting forkhead regulators. Forkhead regulators include proteins which can regulate the activity of such proteins as FOXO3a, FOXA1, FOXO4 and FOXO6. Regulators of FOXO3a include Protein Kinase B (PKB), Jnk, hypoxia, progesterone, sirtuins, histone deacetylase, c-AMP binding protein and other growth factors such as those found in human platelet rich concentrate. An example of an activator of a negative forkhead regulator is insulin. As well as being an antioxidant, Vitamin C or a derivative thereof, is an example of an inhibitor of a forkhead activator, wherein ROS is an example of a forkhead activator. The invention may use one or more than one inhibitor of forkhead signalling.

The inhibitor of forkhead signalling may also be an antioxidant.

The steroid and antioxidant or inhibitor of forkhead signalling may be administered following or in combination with a local anaesthetic. Anaesthesia may be administered directly, for example, by local

administration of an anaesthetic such as lidocaine or bupivacaine, or at a distant location, such as by a somatic or neuraxial block.

The steroid and antioxidant or inhibitor of forkhead signalling may be for human use and/or for veterinary applications.

According to a yet further aspect, the invention provides a composition comprising i) a steroid and ii) an antioxidant or an inhibitor of forkhead signalling. Preferably the composition is for use in the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair.

According to another further aspect, the invention provides a composition comprising i) an amount of a steroid and ii) an amount of an antioxidant or an inhibitor of forkhead signalling which is therapeutically effective for the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair,

Preferably a composition according to the invention is in a pharmaceutically acceptable form.

A pharmaceutical composition preferably comprises one or more physiologically effective excipients or auxiliaries. The pharmaceutically acceptable excipients may include carriers, diluents, binders, lubricants, preservatives, stabilizers, dyes, suspending agents, and solubilising agents.

Preferably the active ingredients in a composition according to the invention are greater than 50% pure, usually greater than 80% pure, often greater than 90% pure and more preferably greater than 95%, 98% or

99% pure. With active compounds approaching 100% pure, for example about 99.5% pure or about 99.9% pure, being used most often.

Preferably a composition according to the invention can be used in any method or use of the invention.

The compounds used in the present invention may be made according to procedures well known to those skilled in the art and/or are commercially available.

The compounds used in the present invention may be administered *per se* or in the form of a pharmaceutically acceptable salt. Such salts are well known to those skilled in the art,

Subjects to be treated by the method or using a composition of the invention include both human and animal, and preferably mammal, even more preferably human, horse, dog, cat, sheep, camel, alpaca or cow.

According to a still further aspect, the invention comprises a kit comprising: i) a steroid; ii) an antioxidant or an inhibitor of forkhead signalling; and iii) instructions as to how and in what amount to administer i) and ii). Preferably compositions i) and ii) are in a pharmaceutically acceptable form.

The skilled man will appreciate that any of the preferable/optional features discussed above can be applied to any of the aspects of the invention.

Preferred embodiments of the present invention will now be described, merely by way of example, with reference to the following drawings and examples.

Figure 1 – illustrates the effect of glucocorticoids on cell number and cell proliferation in tenocytes. (A) Treatment of tenocytes with Dex (1nM-100μM) for 7 days resulted in a significant reduction ($p < 0.05$) in viable cells as measured by Alamar Blue fluorescence. (B) Cell proliferation rate apoptosis as measured by the rate of tritiated thymidine incorporation was significantly lower ($p < 0.05$) in tenocytes treated with Dex than in non-Dex treated controls. Graph shown is for tenocytes treated with 1μM Dex. Similar results were obtained for tenocytes treated with 1nM - 100μM (data not shown). (C) The rate of apoptosis as measured by FACS-mediated detection of nick translation was significantly higher ($p < 0.05$) than controls in tenocytes treated with 100μM (but not 10μM or 1μM) Dex for 7 days. Results shown are mean ±SE for three independent experiments. Significant differences between Dex-treated cells and carrier-treated (EtOH) controls are shown (*$p < 0.05$). (D) Protein levels of pro-death and pro-survival proteins as measured by Western blotting. Transient fluctuations in Bim, Nix, BCL-$_{XL}$ and survivin were observed in tenocytes treated with 1μM Dex. Blots shown are representative of three independent experiments.

Figure 2 – illustrates the effects of glucocorticoid treatment of tenocytes on collagen and GAG content in the cell layer. (A) Collagen content in the cell layer (corrected for dsDNA concentration, an indicator of cell number) was significantly lower ($p < 0.05$) in tenocyte cultures treated with Dex (10nM-100μM) for 10 days compared to carrier-treated

controls. (B) However there was no significant difference ($p > 0.05$) in glycosaminoglycan content in the cell layer in Dex-treated tenocytes compared to controls after correction for dsDNA concentration. Results shown are mean ±SE for three independent experiments. Significant differences between Dex-treated cells and carrier-treated (EtOH) controls are shown (*$p < 0.05$).

Figure 3 – illustrates ROS generation in dexamethasone-treated tenocytes as visualised by DCFDA assay. No DCFDA fluorescence was visible in untreated tenocytes but fluorescence was clearly visible in dexamethasone treated cells. Figure 3A shows untreated tencocytes. Figures 3B1 and 3B2 show tenocytes treated with 1μM dexamethasone for 30 minutes, and Figures 3C1 and 3C2 show tenocytes treated with 10μM dexamethasone for 30 minutes. All images are at 200X magnification.

Figure 4 – further demonstrates that glucocorticoid treatment induces ROS generation in tenocytes. ROS production as visualised by dichlorofluorescein fluorescence. No fluorescence was visible in untreated tenocytes (A) however bright fluorescence due to ROS-mediated oxidation of dichlorofluorescein diacetate to dichlorofluorescein was observed in tenocytes treated for 5 minutes with 100µM hydrogen peroxide (positive control) (B). Some fluorescence was also visible in tenocytes treated for 30 minutes with 1µM (C) or 10µM (D) Dex. 200x magnification.

Figure 5 – illustrates that Protein Kinase B (PKB) and JNK have pivotal roles in controlling for the intracellular localisation of forkhead proteins, as depicted schematically in Figure 5A. Figure 5B shows that treatment of tenocytes with 1μM dexamethasone results in an increased level of FOXO3a proteins. Figure 5C shows reduced levels of active PKB, and

Figure 5D shows increased levels of phophorylated JNK, 24 to 48 hours post-treatment with dexamethasone, indicating conditions are appropriate to allow increased forkhead signalling in dexamethasone treated cells.

Figure 6 – further demonstrates that glucocorticoid treatment results in induction of the Forkhead signalling pathway in tenocytes. (A) Protein levels of FOXO3a were significantly increased ($p > 0.05$) in tenocytes following 72h of Dex (1μM) treatment but returned to normal levels by 7 days. (B) FOXO1 protein levels were significantly increased ($p > 0.05$) after 24h of Dex treatment and more or less remained elevated throughout the 7 day treatment period. Results shown are mean ±SE for three independent experiments. Significant differences between Dex-treated cells and carrier-treated (EtOH) controls are shown ($*p > 0.05$). (C) The ratio of P-Akt:Akt was reduced whereas the ratios of P-JNK:JNK and P-ERK:ERK were increased in Dex-treated (1μM) tenocytes. DUSP1/MKP1, a phosphatase which dephosphorylates ERK was detected in Dex-treated tenocytes. Protein levels were measured in cell lysates by Western Blotting. Blots shown are from one experiment and are representative of results obtained in all three experiments. (D) Only low levels of FOXO1 protein were detected by immunocytochemistry in carrier treated tenocytes (left). However in Dex-treated (1μM, 48h) tenocytes, nuclear localisation of FOXO1 protein was evident.(right) 200x magnification.

Figure 7 – demonstrates that treatment of tenocytes with dexamethasone results in reduced protein levels of PCNA, a marker of cell proliferation, 36 to 48 hours post-treatment (Figure 7A), as well as reduced activation of the pro-survival kinase ERK (Figure 7B)

Figure 8 - demonstrates that dexamethasone treatment of tenocytes results in increased levels of the pro-apoptotic proteins Bnip3, NOXA and Bim, but not Bad or Puma, as well as slightly increased levels of Bcl-2 and Bcl-xL, two anti-apoptotic proteins. Bim levels were also increased following dexamethasone treatment however as no Bim was detected in cells not treated with dexamethasone it was not possible to determine the percentage increase in Bim protein levels and hence Bim does not appear in the graph in Figure 8.

Figure 9 - demonstrates that addition of the glucocorticoid receptor (GR) inhibitor RU486 prevents the dexamethasone-induced effect on FOXO3a protein levels (Figure 9A), ERK activation (Figure 9B) and PKB activation (Figure 9C) indicating that these effects are GR-mediated.

Figure 10 - demonstrates that the ROS scavengers vitamin C and tempol, and the PKB activator insulin, protects against the dexamethasone-induced increase in FOXO3a protein levels. Figure 10A illustrates the effect of the compositions 48 hours post dexamethasone treatment. Insulin alone was effective at increasing the level of PKB phosphorylation (Figure 10B), whereas high dose (1mM) vitamin C appeared to be the most effective in rescuing ERK-signalling (Figure 10C) and increasing PCNA levels (Figure 10D) 48-hours post treatment.

Figure 11 - illustrates the effect of the ROS quenchers tempol and vitamin C and the Akt-activator insulin on forkhead protein levels in glucocorticoid-treated tenocytes. Protein levels of (A) FOXO1 and (B) FOXO3a were significantly lower ($p > 0.05$) in tenocytes co-treated with 1μM Dex and 1mM vitamin C and tended to be lower in tenocytes co-treated with 1μM Dex and 0.5mM tempol or 30μg/ml insulin compared to in tenocytes treated with 1μM Dex alone. Protein levels were measured in

cell lysates by Western Blotting. Results shown are mean ±SE for three independent experiments. Blots shown are from one experiment and are representative of results obtained in all three experiments. Significant differences between Dex-treated cells and cells treated with Dex and vitamin C are shown (*$p > 0.05$).

Figure 12 – demonstrates the protection against the glucocorticoid-induced reduction in cell proliferation rate and collagen content of the cell layer by insulin and vitamin C. (A) Cell proliferation rate as measured by 3H thymidine incorporation was significantly higher following 7 days of treatment with Dex (1μM) and either vitamin C (0.5mM or 1.0mM) or insulin (30μg/ml) compared to in cells treated with Dex (1μM) alone. Tempol was cytotoxic to tenocytes following prolonged culture. (B) Collagen content of the cell layer was significantly higher in tenocyte cultures treated with vitamin C and Dex and tended to be higher in cultures treated with insulin and Dex compared to cultures treated with Dex alone. Significant differences between Dex-treated cells and cells treated with Dex and antioxidants are shown (*$p > 0.05$).

MATERIALS AND METHODS

Materials

The following primary antibodies were used: MKP1/DUSP1 (v-15):sc-1199 rabbit polyclonal, Survivin goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 and Bcl-xL rabbit polyclonals (Santa Cruz Biotechnology, Santa Cruz, CA), Noxa mouse monoclonal IMG-349A (Imgenex, San Diego, CA), Bim rabbit polyclonal (Calbiochem, San Diego, CA), FOXO1/FKHR rabbit monoclonal EP927Y and FOXO3a rabbit monoclonal EP1949 (Epitomics, Burlingome, CA), PCNA mouse monoclonal (Zymed Laboratories, San Francisco, CA);

Phospho-PKB and PKB rabbit polyclonals (Cell Signalling Technologies, Beverley, MA), Phospho-JNK and JNK mouse monoclonals JNK-PT48, BNIP3 mouse monoclonal Ana40 and P-ERK and ERK rabbit polyclonals (Sigma, Poole, UK) and Nix/BNIP3L rabbit polyclonal (Alexis Biochemicals, Lausen, Switzerland). DMEM-F12 was purchased from Lonza Group Ltd (Cambrex), Wokingham, UK. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy), ascorbic acid-2-phosphate, dexamethasone, 1,9 dimethylmethylene blue and 2,7 dichlorofluorescein diacetate were purchased from Sigma, Poole, UK. Unless otherwise stated, all other chemicals were purchased from Sigma, Poole, UK and were of the highest purity available.

Tenocyte Isolation

Tenocytes were isolated by explant culture of human hamstring according to published methods (Corps et al. Arthritis & Rheumatism 2002; 46(11) 3034-3040). Briefly, human hamstring excess to requirements for allograft repair of anterior cruciate ligament rupture was cut into 1-3mm^3 pieces. Explants were cultured in DMEM-F12 containing 50% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in 6-well plates until migration of tenocytes out of the tissue sections was observed. Tissue explants were removed and tenocytes were cultured in DMEM-F12 containing 10% FBS and 1% P/S with media changes every 2-3 days. Once 70% confluent, tenocytes were harvested by scraping and cells in each well were transferred to individual 10cm^2 petri dishes. Cells were cultured again to 70% confluence, transferred to 90% FBS/10% DMSO and immediately frozen and stored in liquid nitrogen until later use.

Cell Culture

Tenocytes were defrosted as required and cultured in DMEM-F12 containing 5% FBS. Previous studies by the inventors have shown the cell

phenotype is stable up until passage 5 in tenocytes isolated from tendon explants when passaged sub-confluence (Liang et al. Calcified Tissue International 2008; 83(1) 24; Liang et al. Bone 2008; 42: S57) In the following experiments, cells were passaged at 70% confluence and used up until passage 3. Tenocytes were cultured in DMEM-F12 containing 5% FBS and treated with either dexamethasone (1nM to 100μM) or carrier (10^{-5} – 1% ethanol) for up to 10 days. Media was changed every 2-3 days and fresh dexamethasone or ethanol added as appropriate. Other treatments were as specified in individual experiments. Tenocytes were cultured in 10cm diameter petri dishes for experiments requiring protein quantification by Western Blotting and in 24-well plates for immunocytochemistry and DCFDA assays. Cells were treated with either dexamethasone (0.01μM – 100μM) or carrier (ethanol) for up to 7 days. Media was changed every 2-3 days and fresh dexamethasone or ethanol added as appropriate. Other treatments were as specified in individual experiments.

Determination of Viable Cell Number

Viable cell number was determined using the Alamar Blue assay (BioSource, Camarillo, CA, USA). Cells were cultured in 96-well plates in 5% FBS/DMEM-F12 with treatments as appropriate for individual experiments for 7 days. Culture media was changed every 2-3 days and treatments re-applied. Three hours before the end of the culture period Alamar Blue (1:10 v/v) was added to each well. At the end of the culture period, fluorescence was measured using a Spectramax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as percentage of control.

Cell Proliferation Rate

Cells were plated in collagen-coated 96-well FlashPlates ®(Perkin Elmer, Waltham, Massachusetts, USA) in 5% FBS/DMEM-F12. After overnight serum-starvation, cells were cultured for 7 days in 5% FBS/DMEM-F12 with 1μCi 3H thymidine per well and treatments as appropriate for individual experiments. Incorporation of 3H thymidine into the cell layer was measured by scintillation counting using a MicroBeta Trilux (Perkin Elmer, Waltham, Massachusetts, USA).

Determination of collagen content

The cell layer was collected by scraping and mixed with the cell supernatant to form a homogeneous suspension. Collagen content was determined in the suspension by reference to a standard curve using the Sircol Soluble Collagen Assay following the manufacturer's instructions (BiocolorLtd, Carrickfergus, Northern Ireland). Content of dsDNA, as a surrogate measure for cell number, was determined in the suspension by reference to a standard curve created for bacteriophage lambda DNA using a PicoGreen® dsDNA Quantitation kit as per manufacturer's instructions (Molecular Probes Inc, Eugene, Oregon, USA). Fluorescence was measured using a SpectraMax Gemini XS fluorescence plate-reader (Molecular Devices, Sunnyvale, CA, USA). The amount of collagen per quantity of dsDNA in each sample was determined by calculation.

Determination of glycosaminoglycan content

The amount of sulphated glycosaminoglycans (GAG) in the cell layer was determined using the dimethylmethylene blue (DMB) assay. Briefly, the cell layer was digested with papain for 2 hours at 60°C. DMB colour reagent (40.55mM glycine, 40.55mM NaCl, 9.5mM 0.1M HCl, 0.0016% (w/v) 1,9-Dimethylmethylene blue (DMB) pH 3.0) was added and absorbance read at 520nm using a MRX plate reader (Dynex Technologies

Ltd, Worthing, UK). The amount of GAG in each sample was determined by reference to a standard curve created for chondroitin sulphate. The quantity of dsDNA in each sample was determined using the pico green assay as described above and GAG content was expressed as quantity of GAG in sample per quantity of dsDNA.

Detection of Apoptosis

The rate of apoptosis in dexamethasone-treated tenocytes was determined using the Nick translation assay as previously described. Briefly, following 7 days of treatment with dexamethasone (1μM, 10 μM or 100 μM) or carrier (0.01-1% ethanol), cells were fixed in 4% formaldehyde and permeabilised in ethanol at -20°C. Cells were incubated with 1μM dATP, 1μM dCTP, 1μM dGTP, 1μM biotin-16-dUTP, 1 unit DNA polymerase I in buffer (2.5Mm $MgCl_2$, 50mM Tris, 10μg/ml BSA pH 7.8) containing 10mM β-mercaptoethanol,. Cells were then incubated in the dark with 0.1% triton X, 5% w/v non-fat milk powder, 2.5μg/ml avidin-FITC in 4 X SSC pH 7.0. Incorporation of the avidin-FITC label was measured using a FACS Calibar (BD Biosciences, San Jose, California, USA).

Detection of ROS generation

Cells were treated with 1 μM dexamethasone for 30 minutes. Cells were washed with PBS, incubated with 0.5μM DCFDA at room temperature for 5 minutes, washed thoroughly with PBS then immediately visualised using a fluorescent microscope.

Western Blotting

On ice, cells were washed twice with wash buffer (PBS containing 1% EDTA and 1% PMSF), lysed with lysis buffer (PBS containing 1% EDTA, 1% PMSF and 1% NP-40), harvested by scraping and

sonicated for 15 seconds. Samples were centrifuged for 15 mins and the supernatant collected. The amount of protein in each sample was quantified using the Pierce 660nm Protein Quantification Assay as per the manufacturer's instructions. Protein concentration was equalised across all samples by diluting more concentrated samples with lysis buffer as appropriate. Laemmli buffer containing 0.1M dithiothreitol was added to each sample in a ratio of 2 parts sample, 1 part buffer and samples were boiled in a water bath for 15 minutes. Samples were loaded in an acrylamide gel and proteins were separated by electrophoresis. Proteins were transferred for 90 minutes to a Millipore membrane. Non-specific binding sites were blocked by soaking the membranes in methanol for 30 seconds, air-drying followed by incubation of the membranes in 3% non-fat milk for 1 hour. Membranes were then incubated with the appropriate primary antibody overnight at 4°C. Following thorough washing, membranes were incubated with the appropriate secondary antibody for 45 minutes at room temperature and proteins visualised using either the Pierce ECL detection reagents or Pierce Supersignal detection reagents (Thermoscientific, Rockford, Illinois) using a ChemiDoc-it Imaging System with a Biochemici HR camera (Upland, Ca., US). Band size and signal intensity was quantified using Image J software or Vision Works LS software (UVP, Upland, Ca, USA).

Immunocytochemistry

Tenocytes were grown on 1 cm diameter coverglasses for 48 hours. After fixation in 10% formalin, cells were permeabilised with 0.5% triton X. Non-specific binding sites were blocked by incubating in 1% horse serum. Cells were incubated with FOXO1 and FOXO3a primary antibodies (1:50) followed by goat anti-rabbit secondary antibody conjugated to Alexafluor 488. Cells were visualised using a BX40 Olympus fluorescent microscope

with a DP70 camera (Olympus Life Science Europa GmbH, Hamburg, Germany).

Statistical Analysis

Results were analysed by one-way ANOVA with post-hoc Tukey testing using SPSS 16.0 (SPSS Inc., Chicago, Illinois, ËÍA). A *p*-value of ≤ 0.05 was considered statistically significant. All experiments were repeated at least three times using different tissue donors for each experimental replicate.

RESULTS

Dexamethasone treatment of tenocytes results in reduced cell proliferation

Clinically, 40mg (approximately 100μmol) of synthetic glucocorticoids suspended in 1-10ml of saline are administered in a single injection to treat tendinopathy. The glucocorticoids used are long-acting "depo" preparations (Jacobs. Best Pract Res Clin Rheumatol 209:23(2):193-219). Although the concentration of glucocorticoids administered varies between 10mM-100mM per injection, different tenocytes in the tendon would be exposed to different concentrations of the synthetic steroid depending on their location relative to the injection site. Therefore the effects of a range of doses (1nM-100μM) of the synthetic glucocorticoid dexamethasone (Dex) on healthy human hamstring tenocytes *in vitro* was tested. Treatment of tenocytes with 1nM -100μM Dex resulted in a significant reduction in viable cell number (Figure 1A). The rate of incorporation of 3H thymidine was also significantly lower ($p < 0.001$) than carrier-treated controls indicating a lower rate of cell proliferation following Dex treatment (Figure 1B). Maximal inhibition of cell proliferation rate was observed with 1nM Dex; increasing Dex dose up to 100μM did not lead to a further reduction in the rate of cell proliferation.

The rate of apoptosis as measured by nick translation was only significantly elevated in tenocytes treated with 100μM Dex for 7 days (Figure 1C). Lower doses of Dex did not result in an increased rate of apoptosis at this time-point. However, transient increases in the pro-apoptotic proteins Bim and Nix were observed at 36h post-treatment in tenocytes treated with 1μM Dex. Transient fluctuations in the levels of the pro-survival protein Bcl-XL were also observed whereas levels of survivin were reduced in Dex-treated cells at 5 and 7 days post treatment compared to carrier-treated controls (Figure 1D).

Collagen content in the cell layer is reduced from dexamethasone-treated tenocytes

The amount of both collagen and glycosaminoglycans (GAGs) in the cell layer in Dex-treated cultures was significantly lower than that of ethanol-treated controls ($p=0.002$ and $p=0.01$ respectively; data not shown). After correction for dsDNA (double-stranded DNA) quantity (an indicator of cell number) the difference in the amount of collagen (Figure 2A), but not GAG (Figure 2B), incorporation remained, indicating reduced collagen output on a per cell basis in Dex-treated tenocytes.

Dexamethasone treatment induces ROS generation in tenocytes

Dichlorofluoroscein diacetate (DCFDA) is a non-fluorescent compound which readily diffuses into cells through the cell membrane. In the presence of ROS, DCFDA is rapidly converted to the fluorescent compound dichlorofluorescein (DCF). As cell membranes are relatively impermeable to DCF, it accumulates within cells and hence fluorescence emitted by DCF is indicative of intracellular ROS generation. As can be seen in Figure 3, negligible DCF fluorescence is observed in untreated tenocytes however treatment of tenocytes with either 1μM or 10μM

dexamethasone for 30 minutes results in visible DCF fluorescence indicating ROS generation.

Figure 4 illustrates the results of a similar experiment, again negligible DCF fluorescence was observed in untreated tenocytes (Figure 4A) whilst a strong positive signal was detected with hydrogen peroxide (100mM) treatment (Figure 4B). Treatment of tenocytes with either 1μM (Figure 4C) or 10μM (Figure 4D) Dex for 30 minutes resulted in visible DCF fluorescence indicating ROS generation.

Dexamethasone treatment activates forkhead signalling/forkhead protein levels in tenocytes

The forkhead signalling pathway is a stress-response pathway activated by ROS which has been shown to play an integral role in the regulation of cell proliferation and survival in a range of different cell types (Burgering and Medema, J Leukoc Bio 2003;73(6):689-701; Accili and Arden. Cell 2004;117(4):421-426).

The forkhead proteins FOXO1 and FOXO3a were found to be present in tenocytes. Protein levels of both FOXO3a (Figure 5A and 6a) and FOXO1 (Figure 6b) were significantly increased following treatment with Dex. The Dex-mediated induction of FOXO3a protein levels was time-dependent and peaked at approximately 72h post-treatment. In contrast, FOXO1 protein levels were elevated 24 hours post-Dex treatment and remained elevated up to 7 days post-treatment.

Whilst, the results shown in Figure 5 are results from one patient, the experiments have been repeated with tenocytes isolated from at least three different individuals (both male and female) and similar results were obtained (Figure 6).

Dexamethasone promotes JNK activation but inhibits Akt and ERK activity in tenocytes

The phosphorylation status of forkhead proteins is important for governing their intracellular activity and hence their activity. Akt (Protein kinase B) and ERK (extracellular signal regulated kinase) phosphorylate the forkheads promoting their nuclear export whereas JNK (Jun N-terminal kinase) promotes forkhead nuclear localisation. Phosphorylation of forkheads by phospho-PKB leads to export of the forkheads from the nucleus and hence a loss of forkhead activity. In the de-phosphorylated form forkheads are localised in the nucleus where they regulate transcription by binding to forkhead response elements in target genes. JNK signalling has a pivotal role in activating the phosphatases responsible for dephosphorylating forkheads (Figure 5A).

Following Dex treatment, the ratios of phosphorylated-Akt:total Akt and phosphorylated ERK:total ERK were reduced (Figure 5C) and the ratio of phosphorylated-JNK:total JNK (Figure 5D and 6C) was increased indicating conditions were appropriate to allow nuclear localisation of the forkheads. DUSP1/MKP-1 (Dual specificity phosphatase 1/Mitogen Activated Protein Kinase Phosphatase -1), a glucocorticoid-responsive phosphatase previously shown to decrease ERK activity in osteoblasts, was also induced in glucocorticoid-treated tenocytes (Figure 6C). Immunocytochemistry confirmed the increase in forkhead proteins and demonstrated increased nuclear localisation of the forkheads in Dex treated tenocytes (Figure 6D).

Dexamethasone treatment may inhibit cell proliferation

As forkhead signalling has been shown to induce cell cycle exit and to inhibit cell proliferation in other cell types, the effect of glucocorticoid

treatment on tenocyte proliferation was studied. Proliferating cell nuclear antigen (PCNA) is a nuclear protein which assists the attachment of DNA polymerase to DNA during DNA replication. Levels of PCNA are increased in proliferating cells and hence PCNA protein levels serve as a marker of cell proliferation. PCNA protein levels were reduced 36-48h following dexamethasone treatment in tenocytes (Figure 7A) suggesting a lower rate of cell proliferation.

The effect of dexamethasone treatment on activation of ERK was also studied, ERK is a member of the MAPK family involved in promoting cell survival and cell replication. Glucocorticoid treatment resulted in a reduced ratio of phosphorylated-ERK:total Erk (Figure 7B) indicating reduced ERK signalling.

Dexamethasone treatment increases the levels of pro-apoptotic proteins in tenocytes

One of the possible downstream effects of forkhead signalling is induction of apoptosis. The two pro-apoptotic proteins Bim and Bnip3 are known to be induced in response to forkhead signalling. Protein levels of various pro-apoptotic proteins were studied and Bim, Bnip3 and NOXA (but not Bad or Puma) proteins levels were found to increase following dexamethasone treatment in tenocytes (Figure 8). Levels of two anti-apoptotic proteins, Bcl-2 and Bcl-xL were also slightly increased in dexamethasone-treated tenocytes (Figure 8). This is not an unusual finding as the balance between pro- and anti-apoptotic signalling governs whether the cell actually undergoes programmed cell death.

Effect of dexamethasone on ERK, PKB and forkhead signalling in tenocytes is glucocorticoid receptor-mediated

RU486 is a glucocorticoid receptor inhibitor. Treatment with RU486 reduced the rise in FOXO3a protein levels seen following 48h of dexamethasone treatment in tenocytes (Figure 9A). RU486 also ameliorated the reduction in both PKB and ERK activation following 48h of dexamethasone treatment in tenocytes (Figure 9B and 9C). These findings indicate that the effect of dexamethasone on forkhead, ERK and PKB signalling in tenocytes is at least partially glucocorticoid receptor-mediated.

Effect of dexamethasone on forkhead signalling is inhibited by vitamin C and insulin

In order to determine if glucocorticoid-induced ROS generation contributed to the increased forkhead signalling protein levels in tenocytes, the effect of two ROS scavengers/antioxidants: Tempol, a superoxide dismutase mimetic, and ascorbic acid-2-phosphate, a stable derivative of vitamin C, on levels of FOXO3a proteins in dexamethasone-treated tenocytes was studied. Both tempol (0.5mM) and vitamin C (1mM) were effective in preventing the dexamethasone-induced increase in FOXO3a protein levels 48h post-treatment (Figure 10A).

To determine whether inhibiting FOXO3a signalling by reducing either FOXO3a protein levels or FOXO3a nuclear localisation would prevent the effects of dexamethasone treatment on indices of cell survival and proliferation a number of studies were performed. For example, insulin is a known PKB activator and hence would be expected to inhibit FOXO3a signalling by promoting nuclear export of FOXO3a. Experiments showed that insulin increased PKB activity in dexamethasone-treated cells (Figure 10B) and was also somewhat

effective at reducing FOXO3a protein levels (Figure 10A), possibly as an indirect effect of nuclear export of FOXO3a as excess cytosolic FOXO3a is degraded by the proteosome pathway. Insulin, tempol and 0.5mM vitamin C all provided some protection against the dexamethasone-induced reduction in ERK phosphorylation, however high dose (1mM) vitamin C was the most effective at preventing both the dexamethasone-induced reduction in ERK activation as well as the dexamethasone-induced reduction in PCNA levels (Figures 10C and 10D).

Figure 11 shows results for a similar experiment in which FOXO1 and FOXO3a protein levels were both considered. In this example, tenocytes were treated for 48h with 1μM Dex and either of the antioxidants Tempol or ascorbic acid-2-phosphate. Tempol had no significant effect on forkhead protein levels at this 48h timepoint however co-treatment of Dex-treated tenocytes with ascorbic acid-2-phosphate resulted in significantly lower levels of both forkhead proteins compared to tenocytes treated with Dex alone. Protein levels of both forkheads tended to be lower in tenocytes treated with Dex and the PKB-activator insulin compared to tenocytes treated with Dex alone however the difference failed to reach statistical significance (Figure 11).

Inhibitory effect of dexamethasone on tenocyte proliferation and collagen synthesis is reduced by vitamin C and insulin

Tempol was cytotoxic to tenocytes after prolonged (7 day) exposure. However, co-treatment of tenocytes with Dex and ascorbic acid-2-phosphate (0.5mM or 1.0mM) or insulin (30μg/ml) ameliorated the Dex induced reduction in tritiated thymidine incorporation (Figure 12A). Collagen content in the cell layer was also higher in tenocyte cultures treated with Dex and ascorbic acid-2-phosphate or insulin compared to Dex alone (Figure 12B). The amount of collagen in the cell matrix in

cultures treated with Dex and ascorbic acid-2-phosphate or insulin was greater than could be explained simply by the higher dsDNA content (an indicator of cell number) in co-treated cultures suggesting ascorbic acid-2-phosphate and insulin may inhibit or over-ride the Dex-induced reduction in collagen-producing activity of tenocytes.

Discussion

Synthetic glucocorticoids are highly effective, clinically essential anti-inflammatory agents utilised for the treatment of a variety of different pathologies in a wide range of different tissues. However glucocorticoid use results in decreased tissue integrity and a reduced capacity for tissue healing leading to a number of undesirable and potentially serious side-effects. In this invention primary human tendon fibroblasts (tenocytes) have been used to characterise the side-effects of glucocorticoids and to develop strategies to protect against the detrimental effects of glucocorticoids.

Tenocytes respond to glucocorticoid treatment in a similar manner to that of cells in other collagen-producing tissues. In the results presented the treatment of tenocytes with nanomolar concentrations of glucocorticoids resulted in a significant and sustained reduction in cell number. At least in the short term, this was primarily due to a decrease in cell proliferation rate rather than an increase in the rate of apoptosis. Collagen content in the tenocyte cell layer was disproportionately lower than would be expected solely based on the lower cell number in glucocorticoid treated cultures in the present study, indicating that glucocorticoid treatment inhibited either the synthesis or secretion of collagen by tenocytes.

In contrast to glucocorticoids, insulin and ascorbic acid-2-phosphate (vitamin C) are known to stimulate cell proliferation and collagen

synthesis (Goldstein et al. Biotechnology 1989;124(2):964-970; Kwack et al. Br J Dermatol 2009;160(6):1157-1162). The present invention demonstrates that antioxidants and inhibitors of forkhead signalling can protect against the deleterious effects of steroid treatment in tenocytes by significantly attenuating the steroid induced reduction in cell proliferation rate and collagen output in tenocytes.

In conclusion, steroids, such as glucocorticoids (including physiologically relevant concentrations of dexamethasone), cause severe and sustained decreases in cell number, cell proliferation and collagen output in tenocytes *in vitro*. These effects are likely to contribute to the compromised tissue repair and increased incidence of tendon re-rupture observed in glucocorticoid-treated patients *in vivo* (Ford and Debender. South Med J 1979;72(7):827-830; Nichols. Clin J Sport Med 2005;15(5):370-375; Watson. J Bone Joint Surg Br 1985;67(4):618-624). Glucocorticoids generate ROS in tenocytes and activate the stress- and ROS-responsive transcription factors FOXO1 and FOXO3A. Strategies which prevent the glucocorticoid-induced increase in forkhead protein levels such as antioxidant (vitamin C) or Akt-activating (insulin) regimens are profoundly effective at restoring normal cell proliferation and collagen synthesis in glucocorticoid-treated tenocytes. This indicates that glucocorticoid-induced ROS generation and activation of forkhead signalling contributes to the manifestation of some of the undesirable side-effects associated with glucocorticoid treatment. The protective effects of insulin and vitamin C, and by analogy other inhibitors of forkhead signalling and antioxidants, on glucocorticoid-treated cells has direct clinical implications for the management of glucocorticoid-treated diseases. Vitamin C in particular may constitute a cost-effective and safe co-treatment strategy for reducing the harmful side effects of steroid treatment.

CLAIMS

1. A steroid and an antioxidant or inhibitor of forkhead signalling, for use in the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair.

2. Use of i) a steroid and ii) an antioxidant or inhibitor of forkhead signalling, in the preparation of a medicament for the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair.

3. A method of treating, ameliorating or preventing pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair, in a subject, comprising administering i) a steroid and ii) an antioxidant or inhibitor of forkhead signalling to the subject.

4. A composition comprising i) a steroid and ii) an antioxidant or an inhibitor of forkhead signalling.

5. A composition according to claim 4 wherein the composition is for use in the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair.

6. A composition comprising i) an amount of a steroid and ii) an amount of an antioxidant or an inhibitor of forkhead signalling which is therapeutically effective for the treatment, amelioration or prevention of pain and/or inflammation and/or tissue degeneration, and/or for facilitating tissue repair,

7. A composition according to any of claims 4 to 6 in a pharmaceutically acceptable form.

8. The use, method or composition of any of claim 1, 2, 3, 5, 6 and 7 wherein the treatment, amelioration or prevention relates to a condition selected from the group comprising sciatica, lateral epicondylitis, medial epicondylitis, joint pain of varying nature, bursitis of the shoulder, rotator cuff tears, hip or knee inflammation, frozen shoulder, plantar fasciitis, carpal tunnel syndrome, herniated disc and other back pain, synovitis, gouty arthritis, tendonitis, rheumatoid arthritis, neuromas, glaucoma, skin conditions such as psoriasis and eczema, and other painful or inflammatory musculoskeletal, ocular, dermal or articular pathologies.

9. The use, method or composition of any of claims 1 to 7 wherein the steroid and antioxidant or inhibitor of forkhead signalling is for the treatment of pain and/or inflammation in a joint or tendon.

10. The use, method or composition of any preceding claim wherein the steroid and antioxidant or inhibitor of forkhead signalling are intended to be provided/administered at the site of disease, injury or in need of treatment.

11. The use, method or composition of any preceding claim wherein the steroid and antioxidant or inhibitor of forkhead signalling are for administration simultaneously, sequentially or separately.

12. The use, method or composition of any preceding claim wherein the steroid and antioxidant or inhibitor of forkhead signalling are in the same or separate compositions.

13. The use, method or composition of any preceding claim wherein the steroid and antioxidant or inhibitor of forkhead signalling are for topical, mucosal, intraarticular, periarticular, intrabursal, intratendonus, peritendonus, intramuscular, intraperitoneal or inhalation administration.

14. The use, method or composition of any preceding claim wherein the steroid and/or the antioxidant or inhibitor of a forkhead regulator are for administration by injection.

15. The use, method or composition of any of claims 1 to 13 wherein the steroid and antioxidant or inhibitor of forkhead signalling is for topical application,

16. The use, method or composition of claim 15 wherein the steroid and antioxidant or inhibitor of forkhead signalling is formulated as a cream.

17. The use, method or composition of claim 16 wherein the cream is for use in the treatment of psoriasis and/or eczema.

18. The use, method or composition of any of claims 1 to 7 and 10 to 14 wherein the steroid and antioxidant or inhibitor of forkhead signalling is for use in eye care.

19. The use, method or composition of claim 18 wherein the steroid and antioxidant or inhibitor of forkhead signalling is for the treatment of glaucoma.

20. The use, method or composition of any preceding claim wherein the steroid is a corticosteroid.

21. The use, method or composition of claim 20 wherein the corticosteroid is a glucocorticoid.

22. The use, method or composition of any preceding claim wherein the antioxidant is selected from the group comprising vitamin C (ascorbic acid), a salt of vitamin C, a derivative of Vitamin C, ascorbic acid-2-phosphate, tempol, glutathione, lactoferrin, edaravone, tiron and any other suitable antioxidant.

23. The use, method or composition of any preceding claim wherein the inhibitor of forkhead signalling is selected from the group comprising vitamin C, a salt of vitamin C, a derivative of Vitamin C, ascorbic acid-2-phosphate, and insulin.

24. The use, method or composition of any preceding claim wherein the steroid and antioxidant or inhibitor of forkhead signalling is for administration to a human or an animal.

25. The use, method or composition of claim 24 wherein the animal is selected from horse, dog, cat, sheep, camel, alpaca or cow.

26. A kit comprising: i) a steroid; ii) an antioxidant or an inhibitor of forkhead signalling; and iii) instructions as to how and in what amount to administer i) and ii).

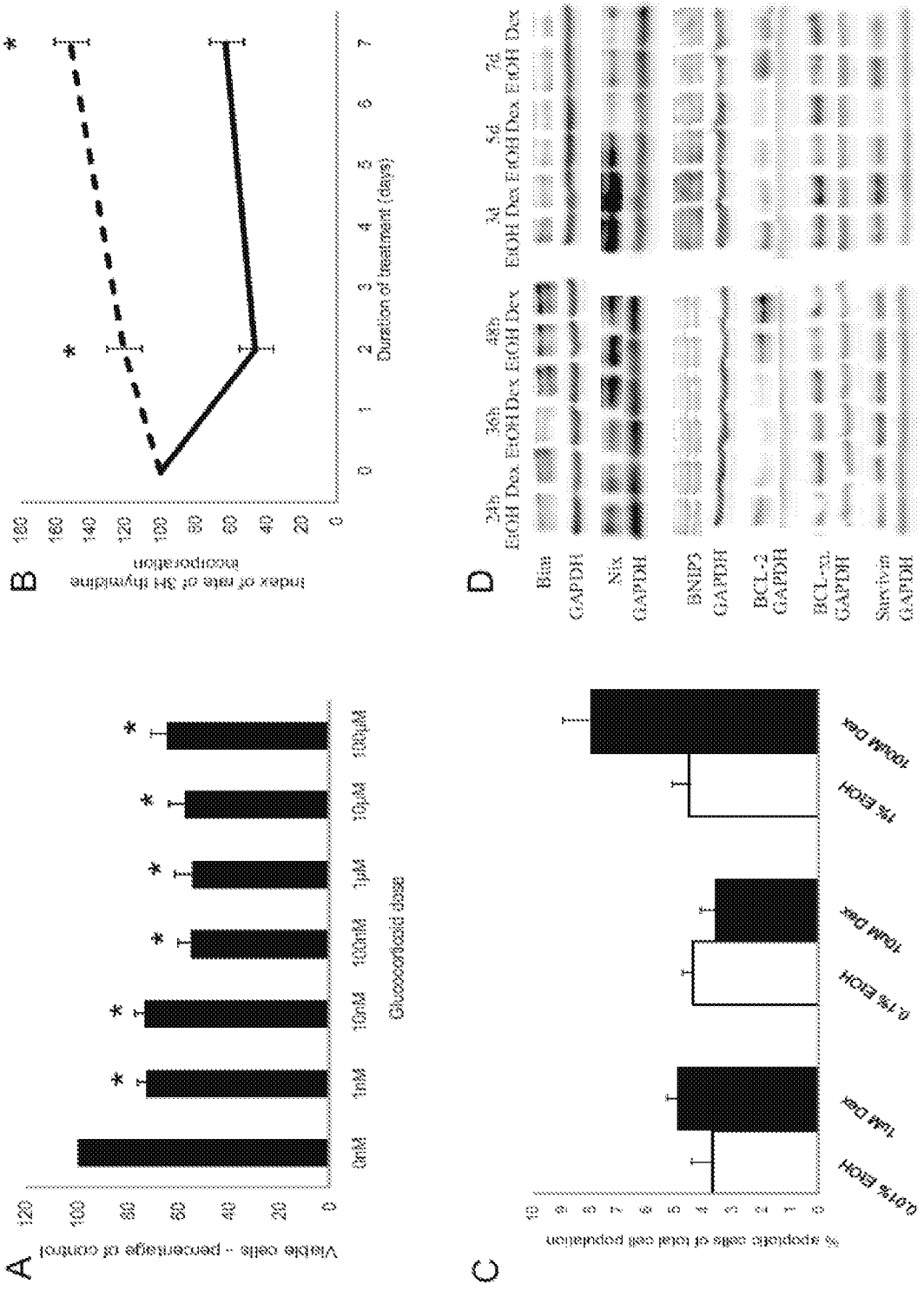
A
Viable cells - percentage of control
Glucocorticoid dose
0nM
1nM
10nM
100nM
1μM
10μM
100μM
B
Index of rate of 3H thymidine incorporation
Duration of treatment (days)
C
% apoptotic cells of total cell population
0.01% EtOH
1uM Dex
0.1% EtOH
10uM Dex
D
24h
36h
48h
3d
5d
7d
EtOH Dex EtOH Dex EtOH Dex EtOH Dex EtOH Dex EtOH Dex
Bim
GAPDH
Nix
GAPDH
BNIP3
GAPDH
BCL-2
GAPDH
BCL-xL
GAPDH
Survivin
GAPDH

Figure 1

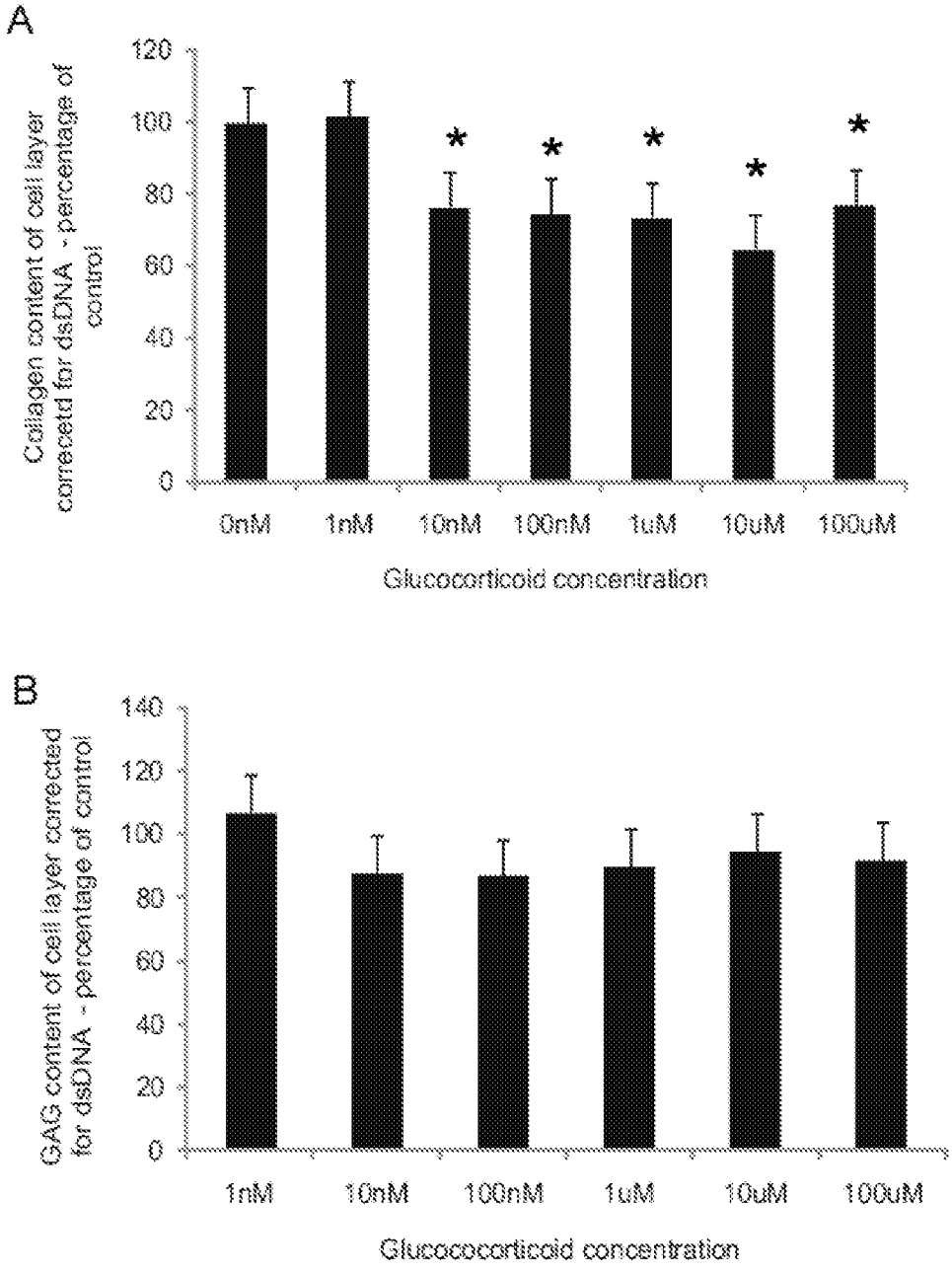
A
Collagen content of cell layer corrected for dsDNA - percentage of control
120
100
80
60
40
20
0
*
*
*
*
*
0nM
1nM
10nM
100nM
1uM
10uM
100uM
Glucocorticoid concentration
B
GAG content of cell layer corrected for dsDNA - percentage of control
140
120
100
80
60
40
20
0
1nM
10nM
100nM
1uM
10uM
100uM
Glucococorticoid concentration

Figure 2

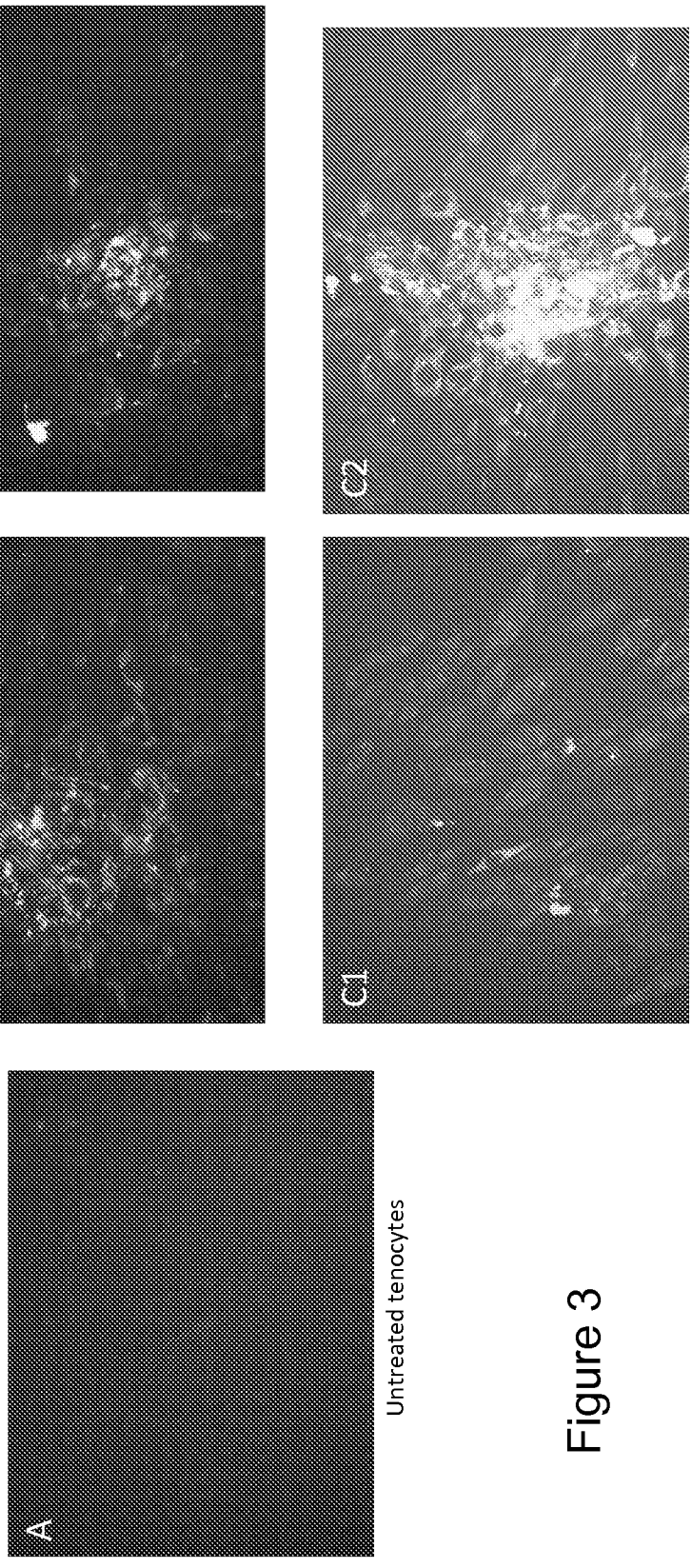

Figure 3

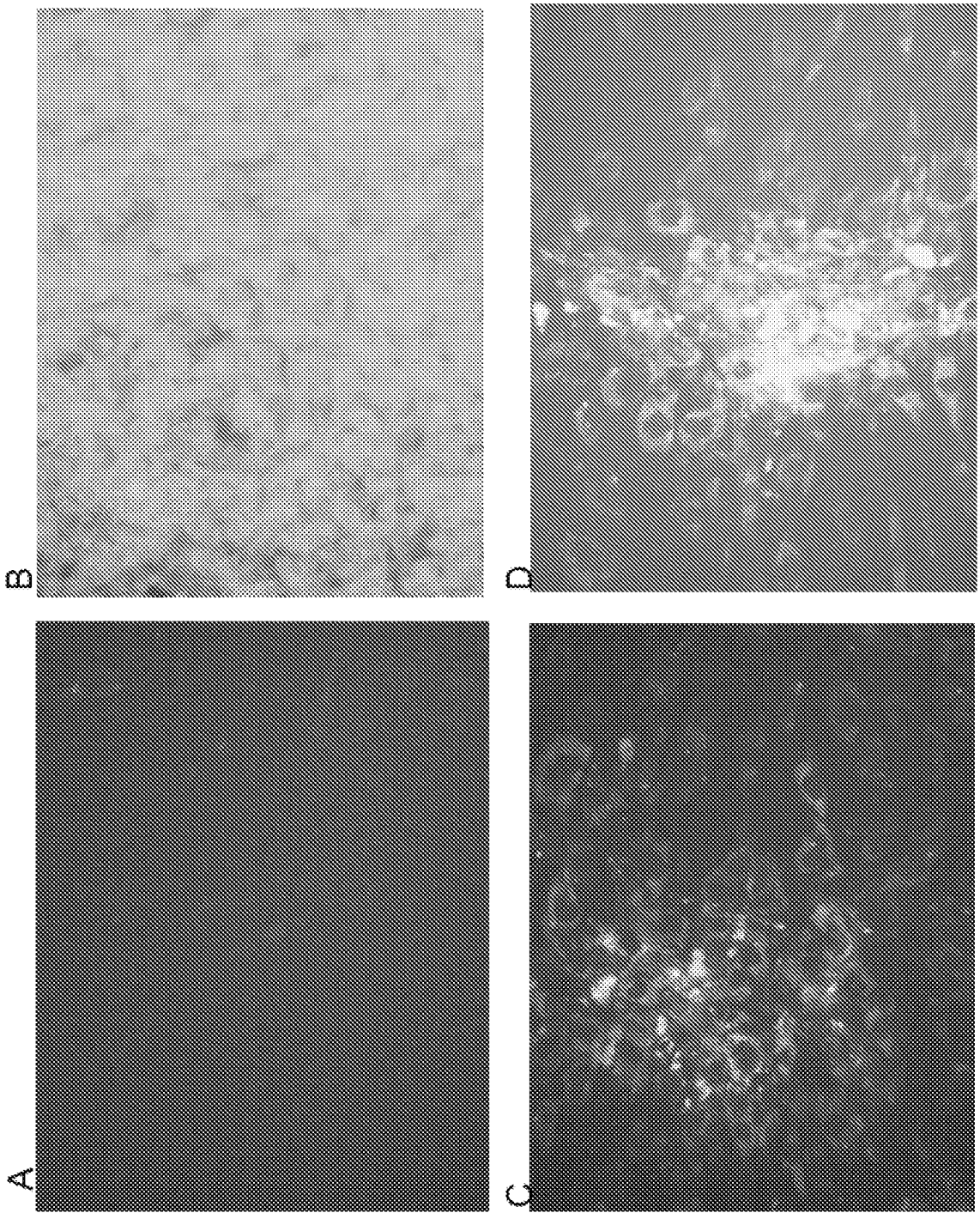

Figure 4

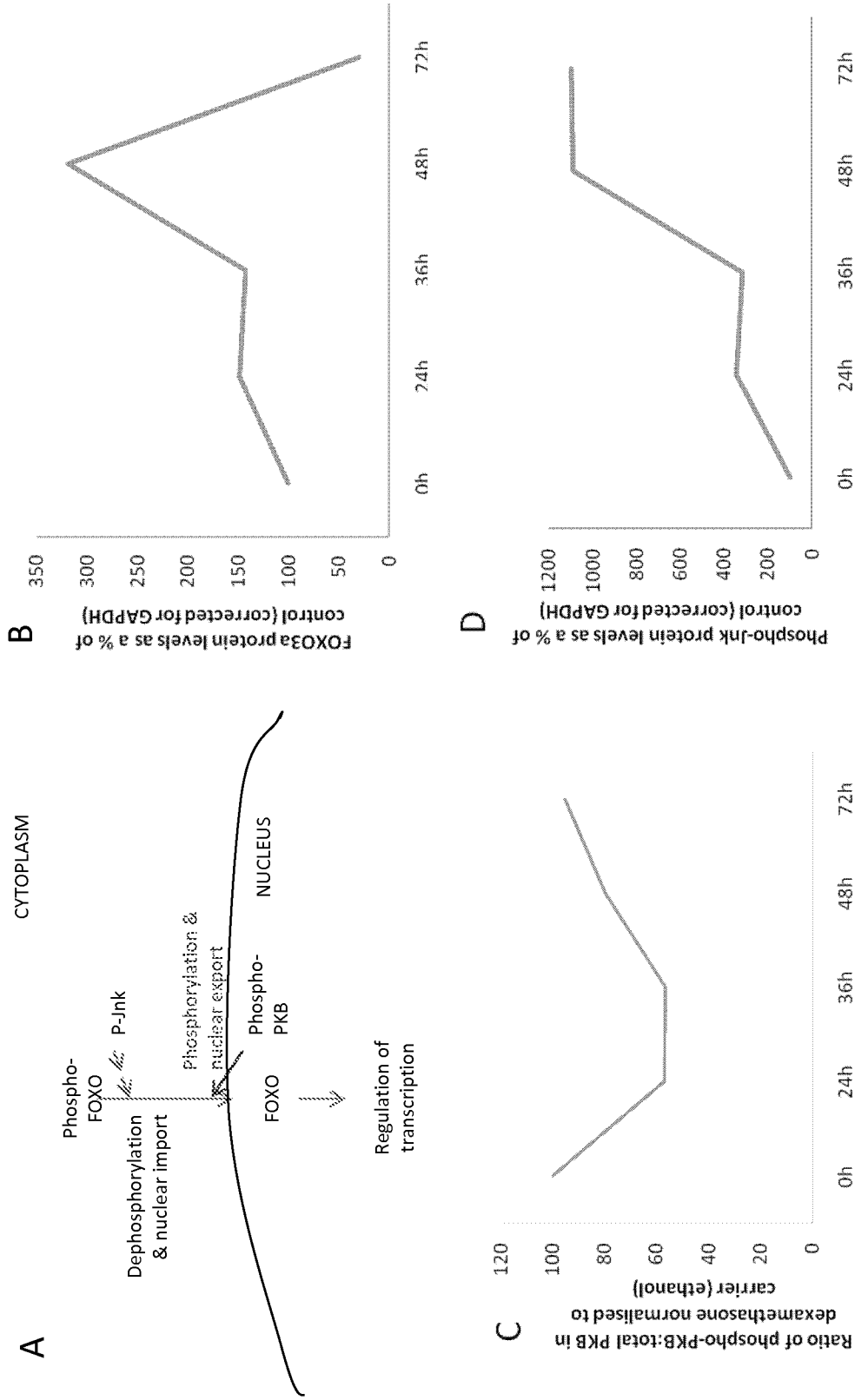
A
CYTOPLASM
Phospho-FOXO
P-Jnk
Dephosphorylation & nuclear import
Phosphorylation & nuclear export
Phospho-PKB
NUCLEUS
FOXO
Regulation of transcription
B
FOXO3a protein levels as a % of control (corrected for GAPDH)
350
300
250
200
150
100
50
0
0h
24h
36h
48h
72h
C
Ratio of phospho-PKB:total PKB in dexamethasone normalised to carrier (ethanol)
120
100
80
60
40
20
0
0h
24h
36h
48h
72h
D
Phospho-Jnk protein levels as a % of control (corrected for GAPDH)
1200
1000
800
600
400
200
0
0h
24h
36h
48h
72h

Figure 5

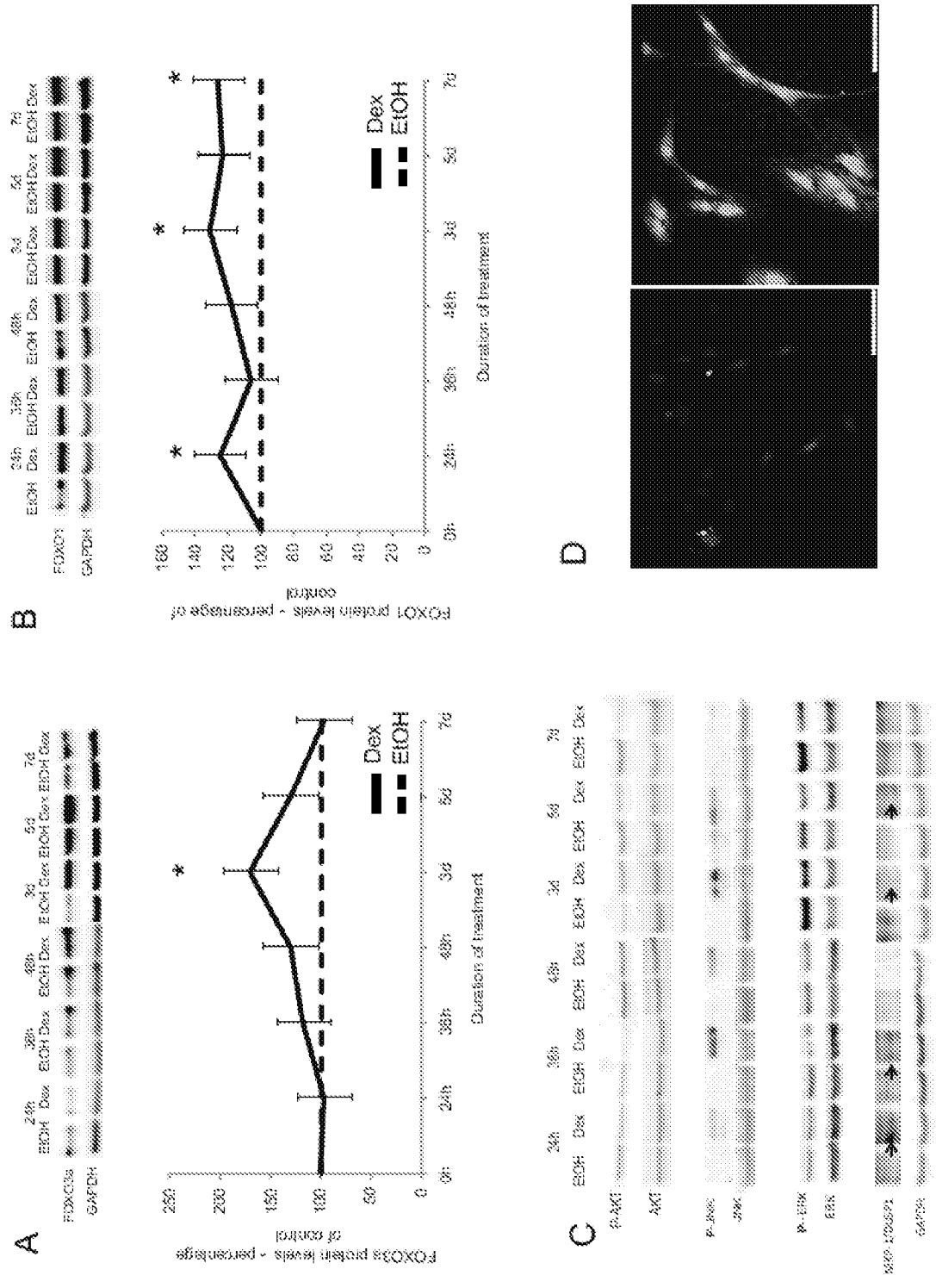

Figure 6

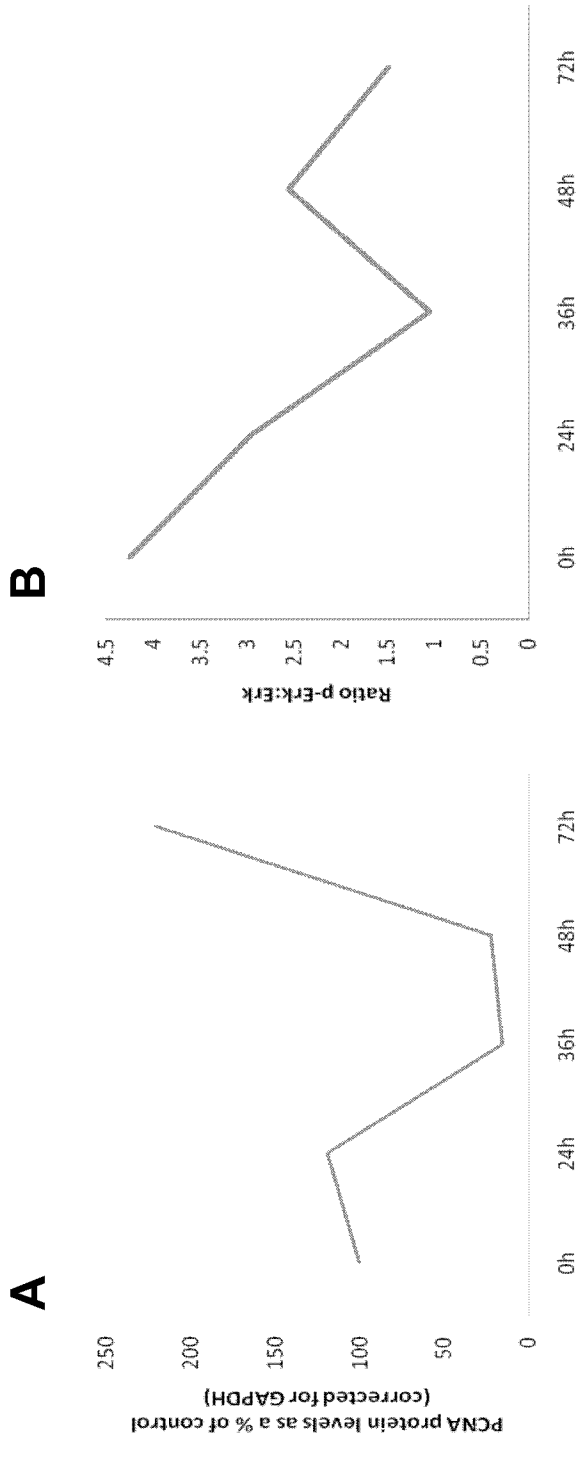
A
PCNA protein levels as a % of control
(corrected for GAPDH)
250
200
150
100
50
0
0h
24h
36h
48h
72h
B
Ratio p-Erk:Erk
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0h
24h
36h
48h
72h

Figure 7

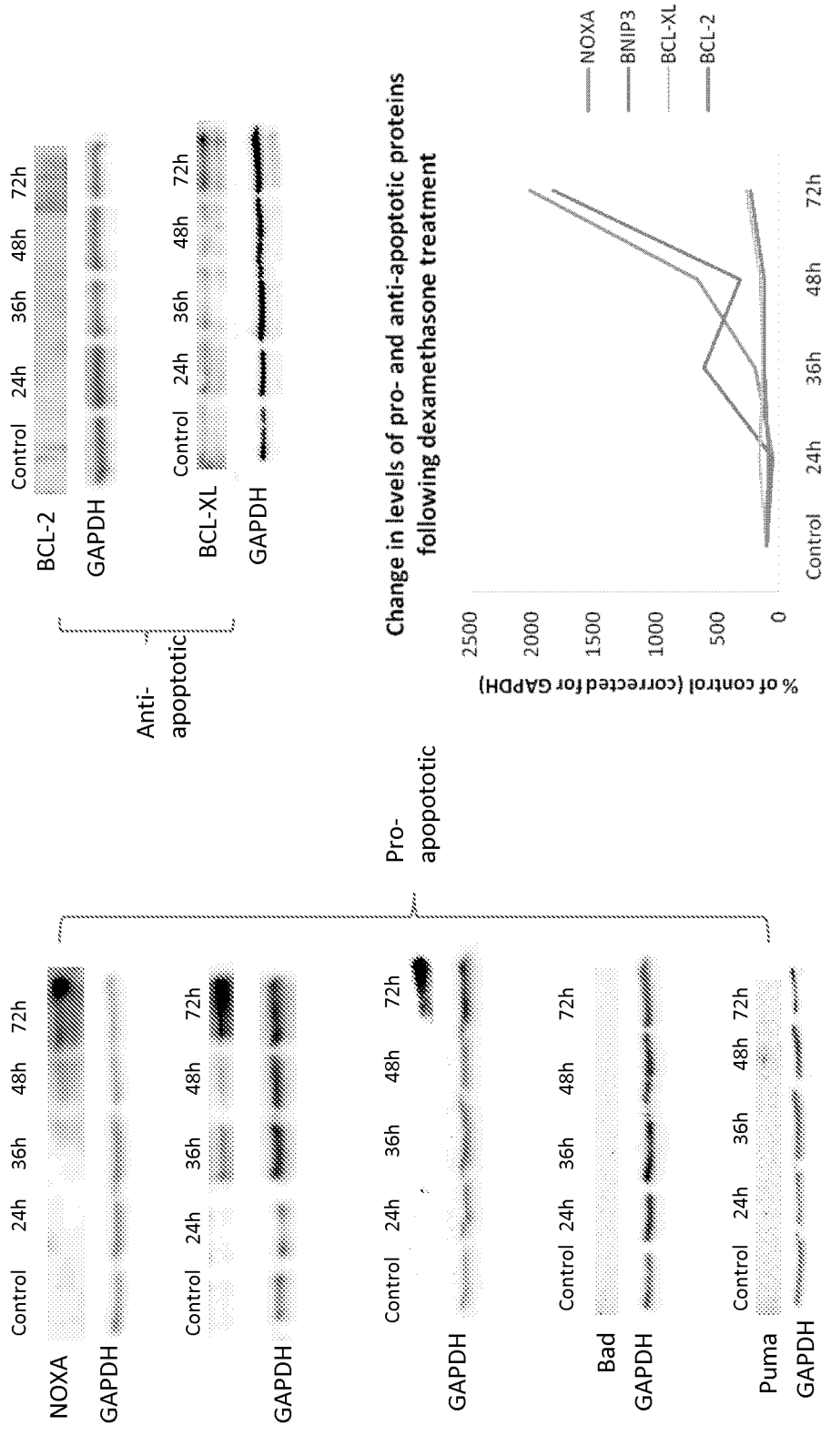
Control 24h 36h 48h 72h
BCL-2
GAPDH
BCL-XL
GAPDH
Anti-apoptotic
Change in levels of pro- and anti-apoptotic proteins following dexamethasone treatment
% of control (corrected for GAPDH)
2500
2000
1500
1000
500
0
NOXA
BNIP3
BCL-XL
BCL-2
Control 24h 36h 48h 72h
Pro-apopototic
NOXA
GAPDH
Bad
GAPDH
Puma
GAPDH

Figure 8

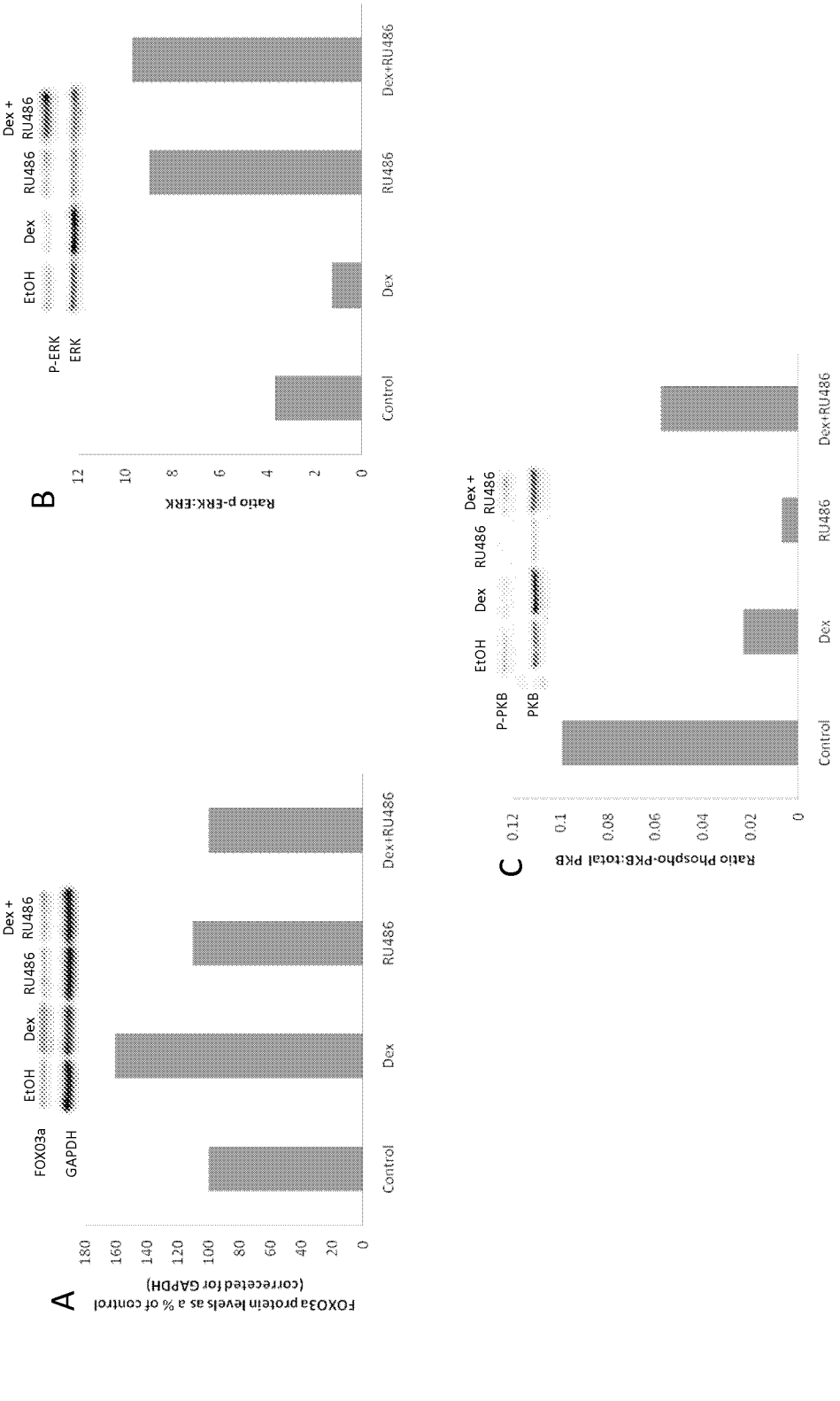
A
FOXO3a protein levels as a % of control (corrected for GAPDH)
180
160
140
120
100
80
60
40
20
0
FOX03a
GAPDH
EtOH
Dex
RU486
Dex + RU486
Control
Dex
RU486
Dex+RU486
B
Ratio p-ERK:ERK
12
10
8
6
4
2
0
P-ERK
ERK
EtOH
Dex
RU486
Dex + RU486
Control
Dex
RU486
Dex+RU486
C
Ratio Phospho-PKB:total PKB
0.12
0.1
0.08
0.06
0.04
0.02
0
P-PKB
PKB
EtOH
Dex
RU486
Dex + RU486
Control
Dex
RU486
Dex+RU486

Figure 9

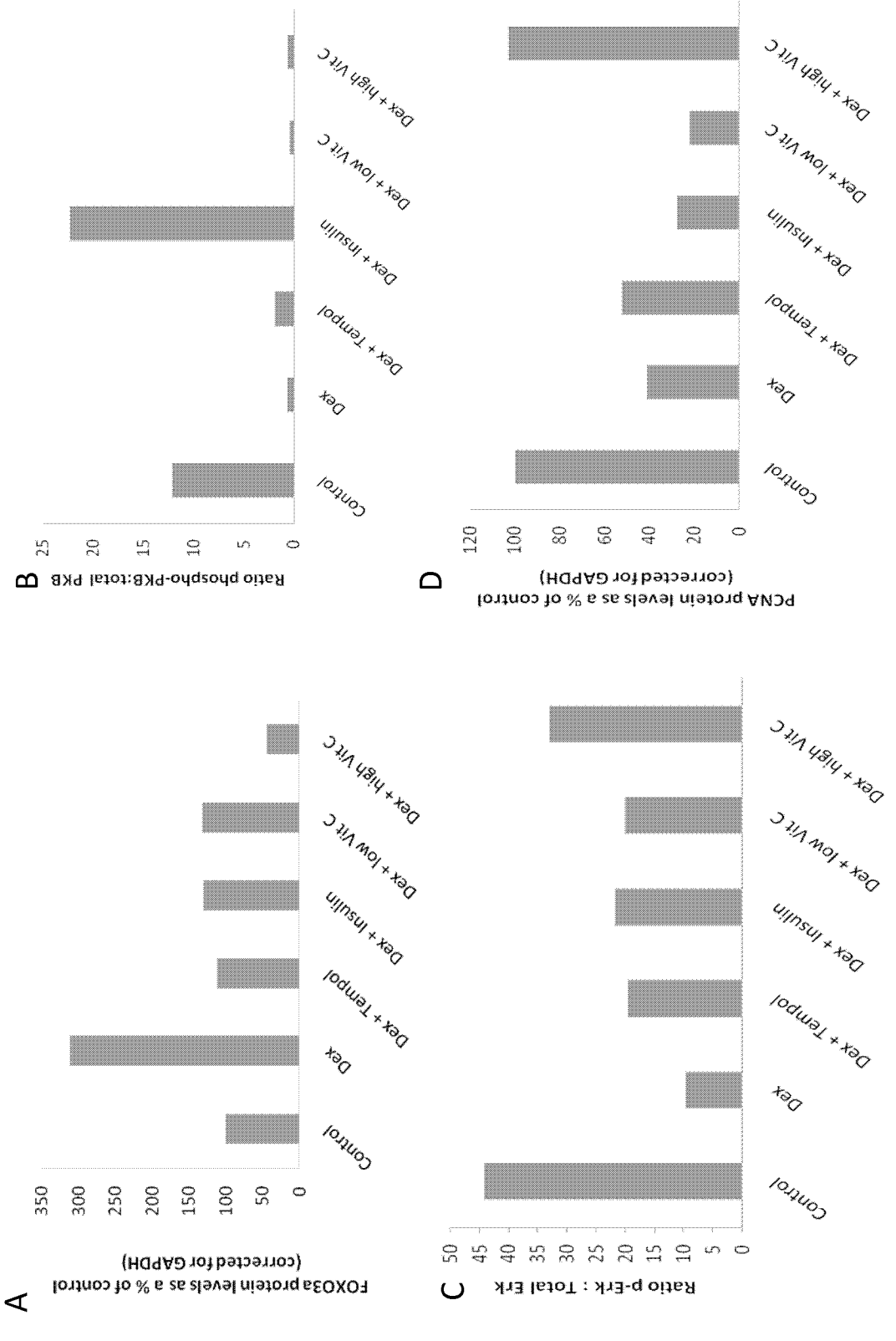
A
FOXO3a protein levels as a % of control (corrected for GAPDH)
0
50
100
150
200
250
300
350
Control
Dex
Dex + Tempol
Dex + Insulin
Dex + low Vit C
Dex + high Vit C
B
Ratio phospho-PKB:total PKB
0
5
10
15
20
25
Control
Dex
Dex + Tempol
Dex + Insulin
Dex + low Vit C
Dex + high Vit C
C
Ratio p-Erk : Total Erk
0
5
10
15
20
25
30
35
40
45
50
Control
Dex
Dex + Tempol
Dex + Insulin
Dex + low Vit C
Dex + high Vit C
D
PCNA protein levels as a % of control (corrected for GAPDH)
0
20
40
60
80
100
120
Control
Dex
Dex + Tempol
Dex + Insulin
Dex + low Vit C
Dex + high Vit C

Figure 10

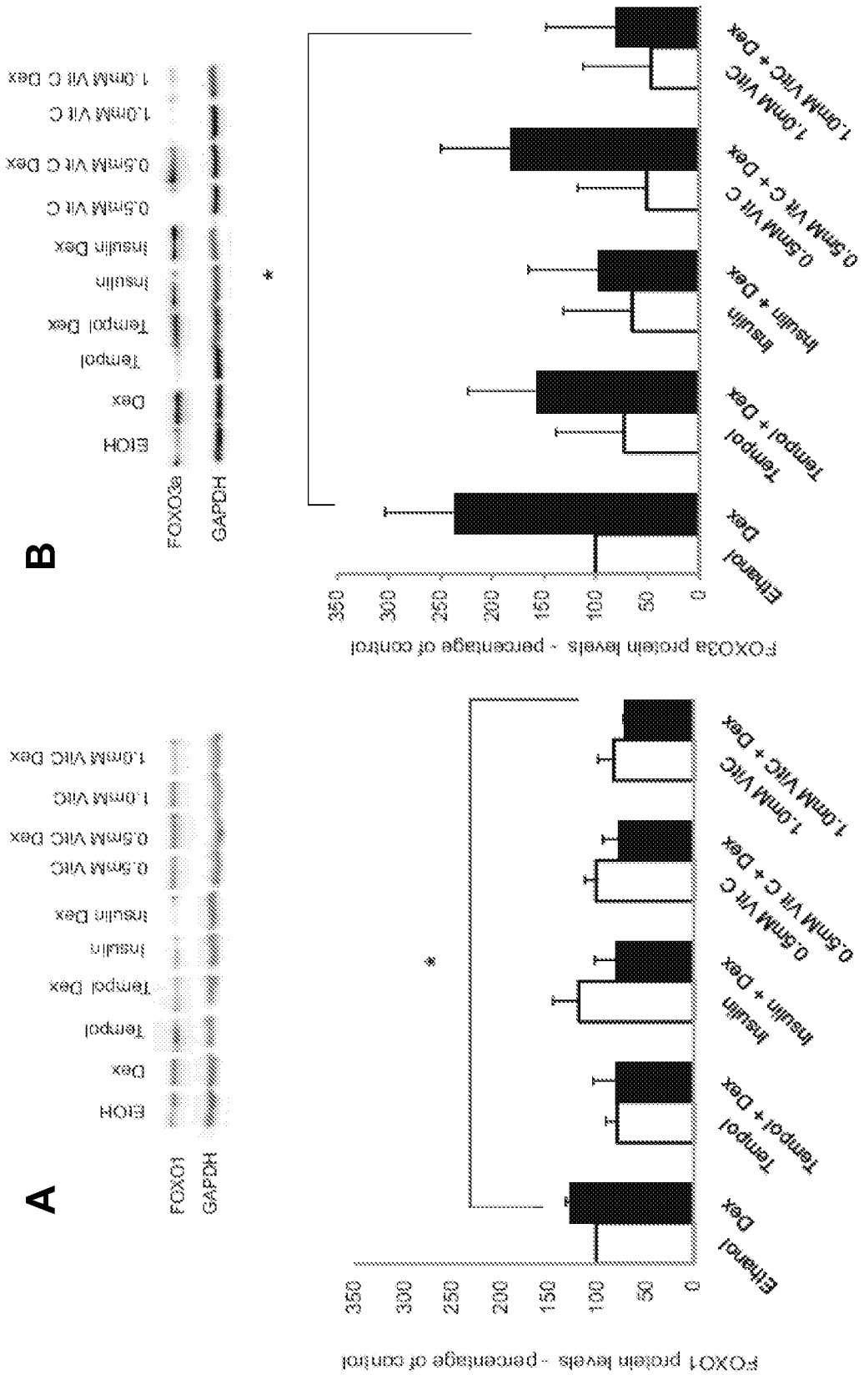
A
FOXO1
GAPDH
EtOH
Dex
Tempol
Tempol Dex
Insulin
Insulin Dex
0.5mM VitC
0.5mM VitC Dex
1.0mM VitC
1.0mM VitC Dex
FOXO1 protein levels - percentage of control
Ethanol Dex
Tempol
Tempol + Dex
Insulin
Insulin + Dex
0.5mM Vit C
0.5mM Vit C + Dex
1.0mM VitC
1.0mM VitC + Dex
B
FOXO3a
GAPDH
EtOH
Dex
Tempol
Tempol Dex
Insulin
Insulin Dex
0.5mM Vit C
0.5mM Vit C Dex
1.0mM Vit C
1.0mM Vit C Dex
FOXO3a protein levels - percentage of control
Ethanol Dex
Tempol
Tempol + Dex
Insulin
Insulin + Dex
0.5mM Vit C
0.5mM Vit C + Dex
1.0mM VitC
1.0mM VitC + Dex

Figure 11

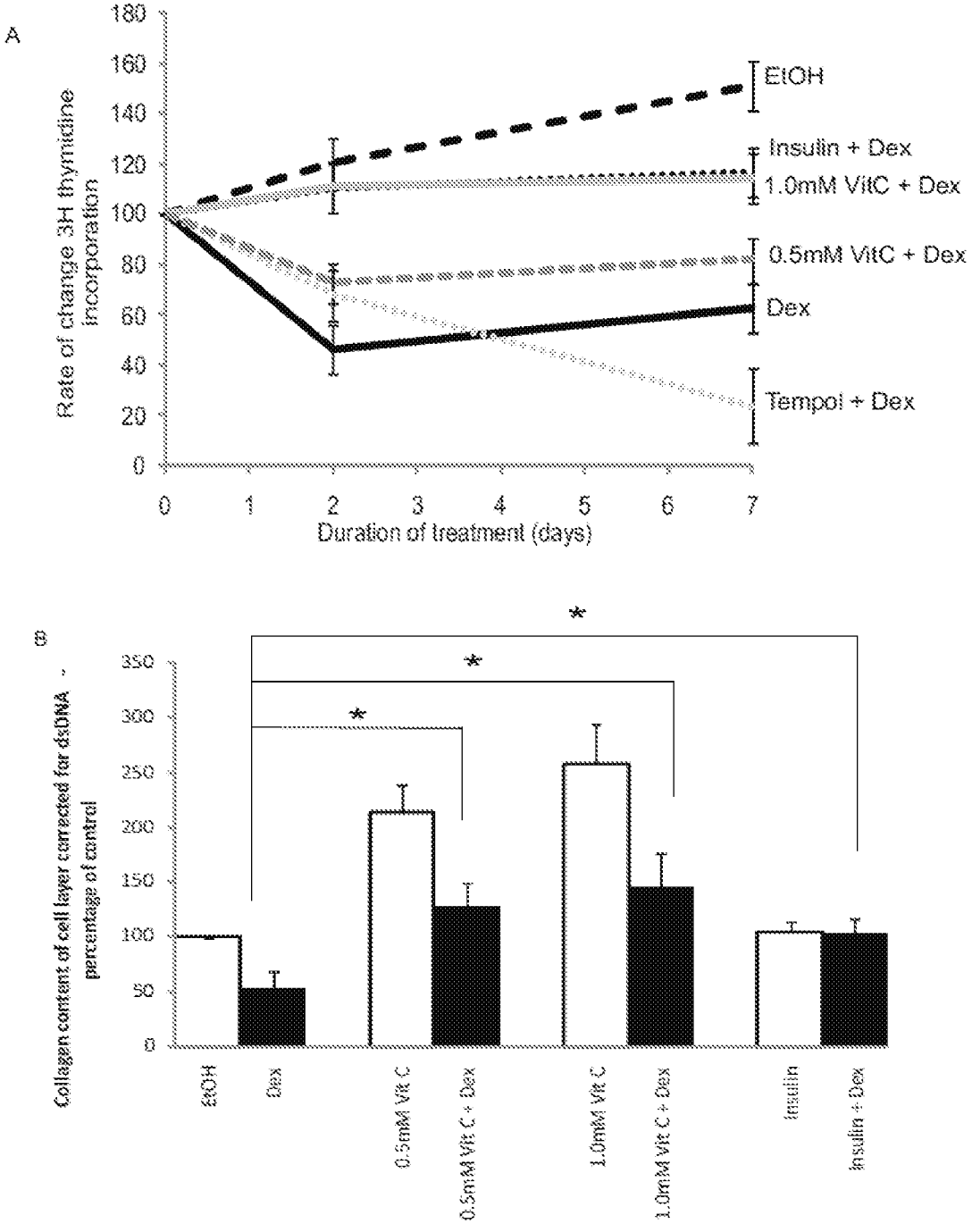
A
Rate of change 3H thymidine incorporation
180
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Duration of treatment (days)
EtOH
Insulin + Dex
1.0mM VitC + Dex
0.5mM VitC + Dex
Dex
Tempol + Dex
B
Collagen content of cell layer corrected for dsDNA - percentage of control
350
300
250
200
150
100
50
0
*
*
*
EtOH
Dex
0.5mM Vit C
0.5mM Vit C + Dex
1.0mM Vit C
1.0mM Vit C + Dex
Insulin
Insulin + Dex

Figure 12