Procedure

This document is adapted from the SOP-PCL-013 In vitro test systems

From ARTIALIS Quality Management System (QMS)

1. Purpose

This document is dedicated to the *in vitro* culture of human primary synoviocytes in an inflamed environment used to perform mode of action or screening studies of novel therapeutic solutions intended for joint diseases.

2. Abbreviations

- IL-1β: Interleukin-1β
- PS: Penicillin - Streptomycin

3. Procedure

3.1. Human synoviocytes obtention

Human primary synoviocytes can either be obtained from commercial suppliers (HFLS) or can be isolated from fresh synovial membrane collected during knee replacement surgeries (this sampling of biological material should be submitted to an ethical committee).

3.1.1. Isolation of primary synoviocytes from synovial membrane

An enzymatic digestion of the synovial membrane is performed to isolate primary synoviocytes from the synovial membrane. The synovial membrane is sampled, cut, and placed in DMEM high glucose supplemented with PS (1%) and HEPES (1%) in a hermetic tube. Dissection and digestion of the synovial membrane should be performed on the same day than dissection. The synovial membrane is digested overnight with collagenase (0.5 mg of enzyme per mL of medium) at 37°C under agitation. Following the overnight digestion with collagenase, the solution is filtered using a 70-µm filter and is centrifuged for 10 min at 1200 rpm. This step is repeated three times. After the last centrifugation, the cell pellet is resuspended in DMEM high glucose supplemented with FBS (10%), PS (1%) and HEPES (1%) and the cell suspension is transferred into culture flasks for amplification at 37°C in a humidified atmosphere with 5% CO_2.

3.1.2. HFLS vial thawing

Alternatively, HFLS can be purchased from a commercial supplier and thawed according to the supplier's instructions in DMEM high glucose supplemented with FBS (10%), PS (1%) and HEPES (1%). Synoviocytes shall then be amplified at 37°C in a humidified atmosphere with 5% CO_2.

3.2. Cell culture

Experiments with primary synoviocytes need to be performed between the 3rd and the 9th passage to avoid dedifferentiation. Cells are seeded in 6-well plates at a density of 2 x 10^5 cells/well for the 24h timepoint and 1.5 x 10^5 cells/well for the 72h timepoint. Cells are maintained in culture for 72h to form a semi-confluent monolayer before the *in vitro* experiment. Once the semi-confluent monolayer is obtained (72h after the seeding), the cells are deprived for 24h with DMEM high glucose supplemented with FBS (1%), PS (1%) and HEPES (1%). Then, the cells are treated with the treatments, in presence of an inflammatory reagent (with IL-1β) for 24h or 72h (depending on the treatment indication). In each experiment, a basal condition (medium or mix of medium and excipient), an inflammatory control condition (IL-1β) and a positive control condition (IL-1β with dexamethasone) are included in addition to the treatment conditions. The final concentration of IL-1β is 10 ng/mL and of 1 µM for dexamethasone.

3.3. Assays

Several assays can be performed on supernatants or cell samples collected after the culture (non-exhaustive list):

1) **DNA assay** (Hoechst assay on cell lysates)
2) **Anti-inflammatory properties** (e.g. pro-inflammatory cytokines such as IL-6, IL-8, TNF-α):
    a. assessed by qPCR on cell lysates
    b. assessed by ELISA/Multiplex in supernatants
3) **Anti-catabolic properties** (e.g. MMP-3, MMP-13, ADAMTS-4, TIMP)
    a. assessed by qPCR on cell lysates
    b. assessed by ELISA/Multiplex in supernatants
4) **Oxidation status**
    a. Nitric oxide quantification (Griess assay on supernatants)
    b. assessed by qPCR on cell lysates (e.g. SOD2, COX2, iNOS)

3.4. Outcomes

Previous internal studies have shown that the stimulation with IL-1β allowed to create an aggravated OA environment in terms of inflammatory and pro-catabolic status in HFLS at 24h and 72h. Indeed, a significant upregulation of the gene expressions of IL-6, IL-8 and TNF-α (pro-inflammatory cytokines), COX-2 (pro-oxidative target), MMP-3 and MMP-13 (matrix metalloproteinases involved in collagenolysis) and ADAMTS-4 (disintegrin and metalloproteinase with thrombospondin motifs involved in aggrecan cleavage) was observed after 24h and 72h compared to the non-treated condition. Only ADAMTS-5 remained unaffected by the IL-1β stimulation.

Compared to the IL-1β condition, dexamethasone at 1 µM, considered in this study as positive control, was able to significantly downregulate the gene expression of IL-6, IL-8, TNF-α, COX-2, MMP-3 and MMP-13 and ADAMTS-4 in inflamed HFLS. These effects were observed at both timepoints except for IL-8, MMP-13 and ADAMTS-4 with a modulation only found at 72h. ADAMTS-5 remained unaffected by the addition of dexamethasone. DEX was therefore valuable as positive control to counter the pro-inflammatory and pro-catabolic status in inflamed synoviocytes found in human osteoarthritic joints.