Osteoarticular cells tolerate short term exposure to Nitisinone – Implications in alkaptonuria.

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Abstract

Objective

Alkaptonuria (AKU) is a rare genetic disease resulting in severe, rapidly progressing, early onset multi-joint osteoarthropathy. A potential therapy, Nitisinone, is being trialled that reduces the causative agent; homogentisic acid (HGA) and in a murine model has shown to prevent ochronosis. Little is currently known about the effect Nitisinone has on osteoarticular cells; these cells suffer most from the presence of HGA and its polymeric derivatives. This led us to investigate Nitisinone's effect on chondrocytes and osteoblast-like cells in an in vitro model.

Design

Human C20/A4 immortalized chondrocytes and osteosarcoma cells MG63 cultured in DMEM, as previously described. Confluent cells were then plated into 24 well plates at 4 x 10^4 cells per well in varying concentrations of Nitisinone. Cells were cultured for 7 days with medium changes every 3rd day. Trypan blue assay was used to determine viability and the effect of Nitisinone concentration on cells. Statistical analysis was performed using analysis of variance and differences between groups was determined by Newman-Keuls post-test.

Results

Analysis of C20/A4 chondrocyte and MG63 osteoblast like cell viability when cultured in different concentrations of Nitisinone demonstrates that there is no statistically significant difference in cell viability compared to control cultures.

Conclusion

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There is currently no literature surrounding the use of Nitisinone in human *in vitro* models, or its effect on chondrocytes or osteoblast like cells. Our results show Nitisinone does not appear detrimental to cell viability of chondrocytes or osteoblast like cells, which adds to the evidence that this therapy could be useful in treating AKU.

Keywords

Alkaptonuria, Ochronosis, Chondrocyte, Nitisinone, Osteoblast

Running title

Osteoarticular cells and Nitisinone

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Introduction

Alkaptonuria is a rare, autosomal recessive disorder of tyrosine metabolism, caused by a single enzyme deficiency. The deficient enzyme is responsible for metabolising homogentisic acid (HGA). The result is an excess of HGA in body tissues which over time causes a triad of clinical features; homogentisic aciduria, ochronosis (darkening) of collagenous tissues and rapidly progressing early onset ochronotic osteoarthropathy[1]. The condition has a long history with an iconic place in medicine, however, until recently little advancement in understanding the progression of the joint disease has been made[2]. Whilst understanding of the factors that cause ochronosis in cartilages, beyond the presence of HGA in tissues is still unknown, the most effective therapy appears to be one which lowers or completely eradicates the causative molecule HGA from the body. This has appeared in the form of Nitisinone which has previously been used to treat Hereditary Tyrosinaemia type I successfully[3]. Nitisinone works by inhibiting the enzyme 4-hydroxyphenylpyruvate dioxygenase. This enzyme is responsible for the formation of HGA from the upstream tyrosine metabolite 4-hydroxyphenylpyruvic acid[1]. Nitisinone is an herbicide; its effective inhibition of the formation of HGA was first noticed in plants[4]. The benefit for AKU patients has been trialled in a randomised trial, looking at hip range of motion as the primary outcome, this was shown to be inconclusive in the Nitisinone group, however, it did show that HGA, the causative molecule, was reduced by 95%[5]. Evidence in a murine model shows that lifetime administration of Nitisinone results in prevention of ochronosis[6]. There is no data in humans about lifetime administration of Nitisinone and its effect on pigmentation, however there is some data detailing the adverse effects as a result of elevated tyrosine levels, resulting in corneal deposition of tyrosine crystals[5]. A recent study, SONIA 1, utilising doses of 1 mg, 2 mg, 4 mg and 8 mg of Nitisinone daily demonstrated that there is a clear dose response in reduction of urinary HGA levels with no serious adverse events. The results of this study will form part of establishing a

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longer term study into understanding the effects of long term administration of Nitisinone, both in terms of the effect on clinical symptoms and long-term safety to patients with AKU[7]. Given that the primary cells affected by the presence of HGA and its polymeric deposits are those in the joint we investigated the effect of Nitisinone on C20/A4 human immortalized chondrocytes and the osteosarcoma cell line MG63.

Materials and Methods

Human osteosarcoma cells (MG63) were a kind gift from Professor Jim Gallagher (University of Liverpool, UK) and C20/A4 immortalized chondrocytes were a kind gift from Dr Mary B Goldring (Hospital for Special Surgery, NY, USA). Cells were cultured and maintained in 9cm petri dishes containing DMEM (MG63) or DMEM Ham's F12 (C20/A4) supplemented and cultured as described previously[8]. Confluent cells were then plated into 24 well plates at 4 x 10^4 cells per well. All work was carried out following approval from Lancaster University Research Ethics Committee.

Plasticware and Foetal Calf Serum were purchased from Appleton Woods. DMEM was purchased from Gibco. HGA, Nitisinone, Penicillin/Streptomycin, Trypan Blue solution and L-Glutamine were all purchased from Sigma Aldrich, UK. Disposable C-Chip hemocytometers were purchased from Labtech, UK.

A 1mM Nitisinone stock solution was produced and stored at -20°C. Serial dilutions were produced to give DMEM containing Nitisinone at the following concentrations; 1μM, 100nM, 10nM and 1nM. Cells were cultured for 7 days with a medium change every 3rd day. On day 7 cells were washed, trypsinized and counted in disposable C-Chip hemocytometers. Trypan Blue exclusion assay was used to determine viability of cultures.

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Statistical analysis was performed using analysis of variance in GraphPad Prism Version 5 software (La Jolla, CA). Differences between the control and Nitisinone group(s) was determined using Neuman-Keuls post-test.

Results

FIGURE 1 – suggested location

The varying concentrations of Nitisinone showed no significant effect on cell viability in chondrocyte cultures. There was no significant difference between control cultures and concentrations of Nitisinone during 7 days of culture. Interestingly chondrocytes exposed to 100nM and 1nM concentration of Nitisinone showed a greater number of cells than the control cultures. Cultures containing concentrations of Nitisinone above, between and below (1uM, 10nm and 1nM, respectively) those cultures with an increased number of viable chondrocytes,) all showed a reduced, but not significantly, number of viable chondrocytes.

FIGURE 2 – suggested location

The MG63 cells cultured in varying concentrations of Nitisinone showed no significant difference in viable cell number. There is a weak general trend of a decrease in cell number seen across decreasing concentrations of Nitisinone but none of these are significantly reduced. The cells cultured in 100nM show similar viability to the control cultures.

Analysis of C20 chondrocyte and MG63 viability when cultured with Nitisinone at different concentrations, demonstrates that there is no statistically significant difference in the viability of any of the cells cultured with Nitisinone, when compared to the control.

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This implies that Nitisinone at any concentration used in vitro does not impact on chondrocyte viability.

Discussion

This investigation shows promising early results for chondrocytes and osteoblastic osteosarcoma cells when exposed to Nitisinone. Nitisinone is currently being trialled as a potential disease modifying therapy for patients with AKU, it is already approved for use in treatment of hereditary tyrosinaemia type I (HTT-I)[7]. Little is currently known about the extra-hepatic effects of Nitisinone on human cells other than in the eye[9]. There is currently no literature surrounding the use of Nitisinone in human *in vitro* models, or its effect on chondrocytes or osteoblast like cells. Our results highlight that Nitisinone does not appear to be detrimental to cell viability of chondrocytes or osteoblast like cells, particularly at higher concentrations investigated, which adds to the evidence that this therapy could be useful in treating AKU.

Experiments using Nitisinone in murine models of AKU have administered the drug at 4mg/L in drinking water[6] and in single doses at a range of concentrations, with the best results seen at a daily dose of 25μg[10]. In humans, Nitisinone has been administered at concentrations of 0.01 – 0.1mg/kg[11], and single daily 2mg doses[5]. Examination of murine chondrocytes following 67 weeks of administration of 4mg/L Nitisinone appear normal and healthy when viewed microscopically, indicative that Nitisinone exposure does not cause any degenerative changes in the cells. This demonstrates that Nitisinone is effective at preventing pigmentation in AKU, but also does not appear to activate any other pathological processes due to long term exposure[6].

Our results demonstrate that human articular cells tolerate the presence of Nitisinone in the short term and these results provide additional evidence for the safety of Nitisinone in human

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cells used as in vitro cultures. Longer term studies of patients with AKU on Nitisinone are required to detail the full extent of the effect the drug may have on articular tissues. To the authors knowledge there is no data in the published literature examining joint tissues in patients with HTT-1 who have been administered Nitisinone, providing further indication that the drug does not affect osteoarticular cells in the longer term, particularly given that doses of Nitisinone used in HTT-1 are higher than those used in AKU[7, 12, 13]. The effect of Nitisinone on osteoarticular cells must be considered of importance given that osteoarticular cells have the capacity to produce ochronotic pigment in loco, contributing to the effect and burden of ochronotic osteoarthropathy in patients[14]. The presence of Nitisinone and its effect on osteoarticular cells likely removes any contribution by these cells to the production of HGA or any of its polymeric derivatives, identical to the effect in the liver.

Early investigations into the administration of Nitisinone demonstrated that corneal injury can first be detected as soon as 1 week after dosing, but usually takes longer to reach its peak at around 4 weeks[15]. More recent studies show that in some investigations no patients demonstrated any corneal symptoms whilst being on Nitisinone for a number of years[16].

Our results from the chondrocyte cultures show that at least 2 concentrations show greater viability than the control cultures, suggesting a potential beneficial effect of the Nitisinone on the cells. As well as removing HGA from the body, Nitisinone has been shown to reduce pain in patients who take the drug for more than 1 week[11], the mechanism for this is unclear but supports the theory that there may be other beneficial effects of Nitisinone.

There is still an unanswered question regarding timing of Nitisinone administration to AKU patients. The effectiveness of Nitisinone at reducing HGA, the molecule responsible for causing the devastating pigmentation that leads to arthropathy, suggests that Nitisinone would be beneficial to patients as early as possible since the initial onset of pigmentation within

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collagenous tissues is currently not known. The initiation and early pigmentation may occur years before any clinical signs or symptoms manifest. Whilst this data is not known, what has been shown is that once the pigmentation is present in tissues it cannot be broken down[2], supporting an early intervention with Nitisinone until understanding of the initiating factors or timing is known. Nitisinone is administered to infants with tyrosinaemia type I who show good tolerance and developmental progress[17], but their condition is much more life threatening than AKU and it may require separate studies in infants with AKU to determine whether and when Nitisinone should be administered to them.

Our results demonstrate promising short term tolerance of Nitisinone by osteoarticular cells, a longer term examination of the effects may be beneficial in providing a complete understanding of effects over a longer period, utilising the variety of models of AKU that exist as well as studies of surgical waste samples from patients with AKU who have been administered Nitisinone. The lack of a correlation between the serum tyrosine levels and the development of corneal opacities suggests that there may potentially be other factors involved, potentially patient specific, in producing adverse effects seen in individuals administered Nitisinone[18].

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Author Contributions

AT and MB conceived the study and supervised JM and DJ.

AT, JM & DJ undertook experimental procedures.

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All authors contributed to interpreting data, drafting, reading and approving the final version of the manuscript.

Role of the funding source

Funders had no input into study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1: Viability of C20 cells supplemented with Nitisinone for 7 days. Number of viable cells expressed as the number of cells/ml. Results are the mean of 4 cultures with 5

counts/culture. Error bars = SEM. There are no statistically significant differences denoted between control and Nitisinone treated groups.

Figure 2: Viability of MG63 cells supplemented with Nitisinone for 7 days. Number of viable cells expressed as the number of cells/ml. Results are the mean of 4 cultures with 5 counts/culture. Error bars = SEM. There are no statistically significant differences denoted between control and Nitisinone treated groups.

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