Effects of n-3 fatty acids on cartilage metabolism

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Although the clinical benefits of dietary supplementation with n-3 polyunsaturated fatty acids (PUFA) has been recognised for a number of years, the molecular mechanisms by which particular PUFA affect metabolism of cells within the synovial joint tissues are not understood. This study set out to investigate how n-3 PUFA and other classes of fatty acids affect both degradative and inflammatory aspects of metabolism of articular cartilage chondrocytes using an in vitro model of cartilage degradation. Using well-established culture models, cartilage explants from normal bovine and human osteoarthritic cartilage were supplemented with either n-3 or n-6 PUFA, and cultures were subsequently treated with interleukin 1 to initiate catabolic processes that mimic cartilage degradation in arthritis. Results show that supplementation specifically with n-3 PUFA, but not n-6 PUFA, causes a decrease in both degradative and inflammatory aspects of chondrocyte metabolism, whilst having no effect on the normal tissue homeostasis. Collectively, our data provide evidence supporting dietary supplementation of n-3 PUFA, which in turn may have a beneficial effect of slowing and reducing inflammation in the pathogenesis of degenerative joint diseases in man.

n-3 PUFA: Cartilage metabolism: Chondrocytes: Degenerative diseases

Clinical studies on dietary supplementation with n-3 polyunsaturated fatty acids (PUFA), such as those present in fish oils, have demonstrated modulation of inflammatory symptoms involved in the pathogenesis of arthritis (Volker & Garg, 1996; Cleland & James, 2000). However, these studies did not investigate the molecular mechanisms whereby n-3 PUFA supplementation specifically affects the metabolism of cells within the articular joint tissues. However, in a related in vitro study Lee et al. (1985) demonstrated that supplementation of monocytes and neutrophils with n-3 PUFA can elicit anti-inflammatory effects by decreasing leukotriene B4 levels, indicating an inhibition of the 5-lipoxygenase (LOX) pathway, responsible for producing this inflammatory leukotriene from arachidonic acid. Loss of proteoglycan (aggrecan) from cartilage is an early event leading to degradation and joint tissue destruction in degenerative joint diseases, and is attributed to the cleavage of aggrecan at specific peptide bonds within the aggrecan core protein (for references, see Caterson et al. 2000). The principal proteinases responsible for the degradation of aggrecan are termed the aggrecanases, two isoforms of which have been identified as members of the family of proteins known as ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif; ADAMTS-4 and ADAMTS-5; Abbasszade et al. 1999; Tortorella et al. 1999). Aggrecanase activity can be up regulated by exposure of cartilage to pro-inflammatory cytokines, and cartilage explant model systems stimulated with interleukin (IL) 1 or tumour necrosis factor α have been used to mimic the degradative processes involved in aggrecan catabolism during arthritis (Sandy et al. 1991; Ilić et al. 1992; Arner et al. 1998; Little et al. 1999, 2002).

Exposure of cartilage to inflammatory mediators propagates the autocrine production by cartilage of inflammatory cytokines that can contribute to the perpetuation and progression of arthritis (Isomäki & Punnonen, 1997; Curtis et al. 2002). These cytokine-induced degradative processes

Abbreviations: COX, cyclooxygenase; S-GAG, sulphated glycosaminoglycan; IL, interleukin; LOX, lipoxygenase; PUFA, polyunsaturated fatty acids.

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in the synovial joint can be potentiated via the biosynthesis of inflammatory eicosanoids (prostaglandins, leukotrienes and thromboxanes) mediated through the cyclooxygenases (COX; COX-1 and COX-2) and the lipoxygenase isoform 5-LOX (Vane et al. 1998; Brash, 1999).

Recent studies from this laboratory (Curtis et al. 2000) have reported that incorporation of n-3 PUFA (but not other classes of fatty acids) into bovine monolayer cultures causes an abrogation of cytokine-induced inflammatory mediators and degradative enzymes. The purpose of the present study was to expand the earlier findings by utilising a bovine explant in vitro model and pathological late-stage human osteoarthritic articular cartilage supplemented with n-3 PUFA.

Materials and methods

Preparation and culture of cartilage explants

Bovine articular cartilage was obtained from a local abattoir and human articular cartilage was obtained as surgical waste from patients undergoing total knee replacement surgery for osteoarthritis (individuals aged 40–87 (mean 75) years), with procedures approved by the Hospital Institutional Review Board. Explants (20–70 mg wet weight) were precultured for 72 h at 37°C in a humidified atmosphere of CO2–air (5:95, v/v) in Dulbecco’s modified Eagle’s medium (Gibco Life Technologies, Paisley, UK) containing: 50 µg gentamicin/ml, 0.5% (v/v) antibiotic–antimycotic solution 100X (Gibco Life Technologies), 10% (v/v) foetal calf serum. Cultured explants were washed (3 × 10 min) in serum-free Dulbecco’s modified Eagle’s medium and maintained for 24 h in individual wells of twenty-four-well tissue culture plates (Costar, High Wycombe, Bucks., UK) containing 1 ml of serum-free Dulbecco’s modified Eagle’s medium with (10–300 µg/ml) or without n-3 PUFA (α-linolenate (α18:3) or eicosapentaenoate (20:5)), or the n-6 PUFA (linoleate (18:2) or arachidonate (20:4)), or monounsaturated and saturated fatty acids (oleate (18:1) and palmitate (16:0) respectively) as previously described (Curtis et al. 2000). All fatty acids were minimum 99% purity from Sigma-Aldrich, Poole, Dorset, UK. Before their addition to culture media, fatty acids were incubated with fatty acid-free albumin, as described by Sicarde & Lagarde (1985). Fatty acid concentrations used in the study were levels which have been established to be achievable in human serum and physiologically relevant (Takita et al. 1996). The culture medium was subsequently removed and explants washed and replaced with fresh serum-free medium (without fatty acid) supplemented with or without 10 ng recombinant human IL-1β/ml; (Totam Biologicals, Peterborough, UK) and maintained in explant culture for 4 d. Incubation periods of 4 d were chosen because they were used in our previous studies utilising bovine tissue (Curtis et al. 2000), and thus provided a direct comparison. At the termination of all cultures the conditioned medium was collected and stored at −20°C until further analysed, and the cartilage explants blotted dry and weighed before fatty acid or RNA extraction. Fatty acids were analysed as previously described (Curtis et al. 2000).

Analyses of cartilage metabolism using lactate production

To evaluate the metabolic state of chondrocyte cultures, the concentration of lactate in the culture medium was measured using a commercially-available lactate assay kit (Sigma-Aldrich Co.). Lactate standards were prepared (0–400 µg/ml) and 5 µl of the standard solutions and appropriately diluted unknown samples were added to a ninety-six-multi-well plate. Lactate reagent (250 µl) was added and incubated for 10 min at room temperature and the absorbance of these samples measured at 540 nm. The effect of culture treatment on lactate concentration was analysed using a two-factor ANOVA and post hoc Bonferroni-Dunn analysis. All data were analysed using the StatView 4.02 package for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA), with P < 0.05 being considered statistically significant.

Measurement of proteoglycan synthesis

Proteoglycan synthesis was measured by radiolabelling cultures for 96 h with H32SO4 (740kBq/ml). Unincorporated label was removed from the culture medium and tissue extracts using a Sephadex G50 desalting column (Amersham Pharmacia, Little Chalfont, Bucks., UK) and total radioactivity (counts/min) in the void volume measured. Total proteoglycan synthesis was measured as total radioactivity in the void volume from the culture medium and the tissue extracts.

Quantification of cartilage proteoglycan degradation

The proteoglycan content of the medium was measured as sulphated glycosaminoglycan (S-GAG) by colorimetric assay using dimethylmethylene blue (Serva, Heidelberg, Germany) with chondroitin sulphate-C from shark cartilage (Sigma, Poole, Dorset, UK) as a standard (Farndale et al. 1986). The effect of culture treatment on S-GAG release (expressed as a percentage of total S-GAG) was analysed statistically using the same procedure as that for lactate production (see p. 000).

SDS–PAGE and Western blotting

Proteoglycan fragments in the conditioned medium were analysed by Western blot analysis as previously described (Little et al. 1999), using monoclonal antibodies BC-3 (1:1000) recognising the aggrecanase-generated N-terminal neoepitope 372อาร์GSV. … (Hughes et al. 1995) and BC-14 (1:1000) recognising the N-terminal matrix metallo-proteinase-generated neoepitope 342FFGVG … on aggrecan metabolites (Caterson et al. 1995).

RNA extraction and reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from intact cartilage (cultured explants) essentially as described by Reno et al. (1997), and isolated using RNeasy mini-columns and reagents (Qiagen Ltd, Crawley, West Sussex, UK), according to the manufacturer’s protocol, and eluted in sterile water.
Reverse transcription–polymerase chain reaction was performed using an RNA PCR kit (Perkin-Elmer, Warrington, Cheshire, UK) as described previously (Rees et al. 2000), using oligonucleotide primers corresponding to cDNA sequences for the inflammatory mediators shown in Table 1. Following an initial denaturation step of 1 min at 95°C, amplification consisted of forty-five cycles of 1 min at 95°C, 45 s at the primer annealing temperature, 30 s at 72°C, followed by a final extension step of 5 min at 72°C. Our polymerase chain reaction analyses were extended to forty-five cycles in order to maximise our chances of detecting low levels of message expression. The polymerase chain reaction products were visualised on 3% (w/v) agarose gels (containing 0.5 μg ethidium bromide/ml) and their nucleotide sequences verified using an ABI 310 Genetic Analyser (Perkin-Elmer, Warrington, UK).

**Results**

**Fatty acid incorporation into chondrocytes from young v. mature bovine articular cartilage**

In order to determine the starting fatty acid profile of bovine chondrocytes, fatty acids were extracted from control tissue from young (7-d-old) and mature (18-month-old) animals. In young control cartilage (Table 2) the predominant fatty acids were the saturated fatty acids (16:0 and stearate (18:0)) and the monounsaturated fatty acid, oleate. There were no n-3 PUFA detectable in the membrane lipids from control tissue. The percentages of fatty acids for different young animals were similar between animals, as can be seen by the small values for standard deviation shown in Table 2. In contrast, when fatty acids were analysed from mature tissue, the dominance of 16:0 and 18:1 was no longer seen and, notably, there were increased percentages of the n-6 PUFA, γ3:6 and 20:4. In some mature animals there were small traces of the n-3 PUFA α18:3, but no 20:5 or docosahexaenoic acid (22:6) was found. In contrast to the young bovine articular cartilage, there was more variation in the mature bovine samples, as can be seen by the larger standard deviation for fatty acids analysed in these samples. This difference could be due to the variation in diet of the mature bovine animals compared with the younger animals that only received milk. Supplementation of cartilage from both the younger or more mature animals with an n-3 PUFA (α 18:3) resulted in a significant accumulation of this fatty acid in the membranes. In the same way, supplementation of the chondrocytes with an n-6 fatty acid (18:2) resulted in an increased percentage of this fatty acid in the samples from both mature and young animals. The variation in fatty acid composition between animals (less variation in young and more variation in mature) was also observed in samples supplemented with either n-3 or n-6 fatty acids. These data showed the effectiveness of the supplementation system for incorporation of exogenous fatty acids into membrane lipids.

**Time-course of fatty acid incorporation into bovine chondrocytes**

In order to determine the optimum amount of time to allow incorporation of the fatty acids into bovine chondrocytes, explants were incubated with either n-3 PUFA, n-6 PUFA or 16:0 for 4, 8, 12, 24 or 48 h (Fig. 1). At each time point the chondrocytes were harvested and the fatty acid composition analysed to determine whether the supplemented fatty acid had been incorporated into the chondrocyte membrane. At 0h (before any supplementation) there was no α18:3 detectable, whereas both 18:2 and 16:0 were present in the bovine tissue. Up to 8h incubation there was a steady increase in incorporation of all three fatty acids (Fig. 1). Between 8h and up to 48h there was no further increase in incorporation of any fatty acid. Thus, for further studies bovine tissue was incubated with PUFA for between 8 and 24h.

**Effect of fatty acid supplementation on lactate release**

In order to ensure that metabolism of the chondrocytes was not being compromised, lactate levels in the medium were measured. There was no significant difference in lactate

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**Table 1. Oligonucleotide primers used for reverse transcription–polymerase chain reaction (PCR) ( Primer sequences correspond to sequences for human or bovine cDNA deposited to GenBank. Where a mixed base is indicated (i.e. for glyceraldehyde phosphate dehydrogenase (GAPDH)), the sequence also corresponds to the analogous rat cDNA)**

<table>
<thead>
<tr>
<th>Target template</th>
<th>PCR primers</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’TGGYATCGTGGAAGGRCCTCAT</td>
<td>370</td>
<td>53-0</td>
</tr>
<tr>
<td></td>
<td>5’RTGGGWGTGYGCTGTGAAATGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human COX-1</td>
<td>5’AGGAGGTGGGTTCTTCTTCT</td>
<td>413</td>
<td>56-3</td>
</tr>
<tr>
<td></td>
<td>5’AGGGTCTGGGGGCAATGGGTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human COX-2</td>
<td>5’GGCTGTTCCCTTACCTCTCATT</td>
<td>438</td>
<td>48-2</td>
</tr>
<tr>
<td></td>
<td>5’ACATCTTACCTTTGCTCTTAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human 5-LOX</td>
<td>5’GGAACACCGGCAAAAACACTA</td>
<td>274</td>
<td>56-5</td>
</tr>
<tr>
<td></td>
<td>5’CTGGATGGCAAATGGGCCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine COX-1</td>
<td>5’GCCCCAACCATCCACCACATG</td>
<td>287</td>
<td>59-0</td>
</tr>
<tr>
<td></td>
<td>5’CCAGGAAGCGGCCAAACACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine COX-2</td>
<td>5’GCTTCTTCCCTTCTGTCTGAT</td>
<td>229</td>
<td>52-3</td>
</tr>
<tr>
<td></td>
<td>5’CATGGTCTTTTCTTCTGATG</td>
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<tr>
<td>Bovine 5-LOX</td>
<td>5’ATCGATGGGTGGAGTTGGAACC</td>
<td>554</td>
<td>57-5</td>
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<tr>
<td></td>
<td>5’CTGGATGGCAATGGGCCAAT</td>
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COX, cyclooxygenase; LOX, lipoxygenase.
levels in control or IL-1-treated bovine cultures supplemented with n-3 PUFA (Fig. 2(A)) or any other class of fatty acid (results not shown). Analysis of lactate levels in human osteoarthritic cultures also showed no significant difference in lactate with any of the PUFA tested (results not shown).
Nutrient regulation of inflammation

Effect of fatty acid incorporation on proteoglycan synthesis

In order to ensure that incorporation of fatty acids into bovine chondrocytes was not altering proteoglycan metabolism (i.e. changes in proteoglycan metabolism were not due to a decreased proteoglycan synthesis), $^{35}$S labelling of newly-synthesized proteoglycan was examined in explant cultures in the absence and presence of IL-1 (Fig. 2(B)). Addition of IL-1 to bovine tissue resulted in a marked decrease in proteoglycan synthesis (Fig. 2(B)). However, incubation with $n$-3 PUFA in the range 10–100 $\mu$g/ml did not significantly alter the proteoglycan synthesis in either control cultures or those treated with IL-1 (Fig. 2(B)). Similar results were found with $n$-6 PUFA supplementation (results not shown). Collectively these results indicate that cartilage metabolism was not being altered after PUFA supplementation.

Effect of fatty acid supplementation on glycosaminoglycan release

The effect of fatty acid supplementation on the release of proteoglycans (S-GAG) from bovine explant cultures is shown in Fig. 2(C). In the absence of IL-1 exposure there is a low level of S-GAG release in the cultures that is not altered with $n$-3 PUFA supplementation. In contrast, addition of IL-1 to cultures results in a marked increase in S-GAG release into the culture medium. Interestingly, addition of $n$-3 PUFA to IL-1-stimulated cultures causes a dose-dependent decrease in S-GAG release (Fig. 2(C)). Addition of other fatty acids ($n$-6 PUFA, monounsaturated and saturated) has no effect on S-GAG release (data not shown). In comparison, using human osteoarthritic cartilage explants, in control cultures there is a high release of S-GAG (as the tissue is pathological), although an increased S-GAG release is also observed on addition of IL-1 to cultures. Interestingly, $n$-3 PUFA are able to decrease the endogenous S-GAG release both in control and IL-1-stimulated cultures, and the IL-1-induced S-GAG release is dose-dependent (Curtis et al. 2002).

Activity and expression of aggrecanases in fatty acid-supplemented cultures

Western blot analyses with mAb BC-3 (recognising aggrecanase-generated aggrecan catabolites initiating $^{374}$ARGSV …) of proteoglycan metabolites present in culture media from bovine explants that were pre-incubated in the presence or absence of $n$-3 PUFA before continued culture for 4 d in the absence or presence of IL-1 is shown in Fig. 3(A). In control cultures there are no aggrecanase-generated products after 4 d culture. In contrast, addition of IL-1 to cultures results in aggrecanase-generated BC-3 immunoreactive proteoglycan catabolites. However, this IL-1-induced aggrecanase activity was reduced in a dose-dependent manner with the addition of $n$-3 PUFA. Furthermore, supplementation of cultures with $n$-6 PUFA had no effect on (or slightly increased) this IL-1-induced aggrecanase activity. Fig. 3(B) shows Western blot analyses with mAb BC-3 (recognising aggrecanase-generated aggrecan catabolites initiating $^{374}$ARGSV…) of
Fig. 3. Effect of fatty acid supplementation on aggrecanase activity in bovine articular cartilage (A) and in human osteoarthritic cartilage (B) in the absence or presence of interleukin (IL) 1. Explants that had previously been supplemented with or without n-3 polyunsaturated fatty acids (PUFA; 50–300 µg/ml) or n-6 PUFA (50–300 µg/ml) were cultured in the absence (control) or presence of IL-1. Aggrecan metabolites released into the culture media were separated by SDS–PAGE and then immunoblotted with mAb BC-3, recognising the aggrecanase-generated N-terminal neoepitope \(^{374}\text{ARGSV} \ldots\). The migration of pre-stained protein standards are indicated, with their apparent molecular mass at the left.
proteoglycan metabolites present in culture media from human osteoarthritic explants that were pre-incubated in the presence or absence of n-3 PUFA before continued culture for 4 d in the absence or presence of IL-1β. In contrast to the bovine explant cultures, control human cultures demonstrated the presence of aggrecanase-generated BC-3 immunoreactive material catabolites, since these were pathological osteoarthritic cartilage samples. However, this endogenous aggrecanase activity was abolished in control cultures that had been treated with n-3 PUFA. In contrast, cultures pre-treated with n-6 PUFA had no effect on this aggrecanase activity. Furthermore, cultures maintained in the presence of IL-1β exhibited increased aggrecanase-generated aggrecan catabolites compared with control cultures. Importantly, in IL-1β-stimulated cultures that had been exposed to n-3 PUFA, there was a noticeable decrease in the intensity of this IL-1 induced increase in aggrecanase activity. In contrast, pre-incubation with n-6 PUFA before IL-1 stimulation caused a noticeable increase in aggrecanase-generated metabolites, indicative of increases in aggrecanase activity when this PUFA was present.

Replicate Western blot analyses with mAb BC-14, which specifically detects matrix metalloproteinase-generated aggrecan catabolites (Little et al. 1999), demonstrated the absence of any positive immunostaining in bovine and human explants (data not shown), indicating that matrix metalloproteinase degradation within the interglobular domain of aggrecan was not a major contributor to the S-GAG release observed in these explant culture systems.

**Fig. 4.** Analysis of mRNA expression of inflammatory mediators in extracts of normal bovine articular cartilage (A) and human osteoarthritic cartilage (B) supplemented with fatty acids before 4 d of explant culture in the presence or absence of interleukin (IL)-1β. mRNA expression was analysed in cartilage explants that were pre-incubated in the presence or absence of n-3 or n-6 polyunsaturated fatty acids (PUFA; 100 or 300 µg/ml) before continued culture in the presence or absence of IL-1β (10 ng/ml). Polymerase chain reaction (PCR) product sizes are indicated. The sequences of the oligonucleotide primers used are shown in Table 1. COX, cyclooxygenase; LOX, lipoxygenase; GAPDH, glyceraldehyde phosphate dehydrogenase.
Analysis of mRNA expression of inflammatory mediators in fatty acid-supplemented cultures

Analysis of mRNA expression for inflammatory mediators in bovine articular cartilage explants is shown in Fig. 4(A). Expression of COX-1 in these cultures is constitutive, with no effect from supplementation with any class of fatty acid or IL-1 treatment. In contrast, expression for COX-2 is absent in control cultures, and is induced with the addition of IL-1. However, supplementation of IL-1-treated cultures with n-3 PUFA results in a loss of expression of COX-2 induced by IL-1 exposure, whilst addition of n-6 PUFA has no effect. Similarly, expression of 5-LOX is absent in control cultures and is induced by addition of IL-1. This IL-1-induced expression is also lost on supplementation of cultures with n-3 PUFA, whilst n-6 PUFA supplementation has no effect. Similar analysis of mRNA expression in extracts of the human osteoarthritic cartilage explants is shown in Fig. 4(B). In keeping with the fact that this explant material was pathological tissue, mRNA expression for COX-2 and 5-LOX was present in control cultures, in addition to the expression of the constitutive COX-1 enzyme. Of particular note, however, was the finding that the expression of mRNA for COX-2 and 5-LOX was abolished in cultures supplemented with n-3 PUFA. In contrast, addition of n-6 PUFA caused no change in the expression of these pro-inflammatory enzymes. In IL-1-treated human osteoarthritic explants the expression of COX-1, COX-2 and 5-LOX was unchanged. However, supplementation of IL-1-treated cultures with n-3 PUFA resulted in the loss of expression of COX-2 and 5-LOX, whilst there was no effect on the constitutive COX-1 enzyme. Addition of n-6 PUFA had no effect on the expression of any genes examined.

Conclusions

The current study has utilized in vitro culture models, with both normal bovine articular cartilage and pathological human osteoarthritic cartilage, to demonstrate that supplementation with n-3 PUFA can result in a decrease in degradative and inflammatory factors indicative of cartilage pathology in degenerative joint diseases. Importantly, when comparing human osteoarthritic cartilage with the normal bovine tissue, the human tissue has activity and expression of mediators of joint destruction (aggrecanases) and inflammation (COX-2 and 5-LOX) in unstimulated tissue because at harvest it was pathological, whereas bovine tissue required IL-1 exposure to induce these degradative and inflammatory factors. Moreover, all the pathological markers present in the diseased human articular cartilage and those induced by IL-1 in the bovine tissue could be abrogated or reduced by previous culture for 24 h with n-3 PUFA. Thus, n-3 PUFA may play a potential role in halting or slowing degradative and inflammatory factors contributing to the progression of disease. Our data have also demonstrated that n-3 and n-6 PUFA are readily incorporated into chondrocyte membranes after only 8–24 h of culture before further incubation for 4 d. We also demonstrated that this incorporation of PUFA into chondrocytes had no detrimental effects on cartilage metabolism in terms of lactate production and proteoglycan synthesis in either the absence or presence of subsequent IL-1 exposure.

Further studies are needed to investigate how n-3 PUFA control mRNA levels and activities of aggrecanases and inflammatory mediators in the bovine and human in vitro models of arthritis, and whether a common mechanism exists for the alteration of gene transcription by n-3 PUFA. These findings add further weight to the epidemiological and clinical studies that have reported the benefits of dietary supplementation of n-3 PUFA in reducing pain and inflammation in human arthritic diseases (Volker & Garg, 1996; Cleland & James, 2000; Calder, 2001; Calder & Zurier, 2001), and suggest molecular mechanisms as to how these benefits occur.

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