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Abstract
As developers begin to advance their research of genetically modified (GM) animals from the experimental to the commercial phase, it is expected that regulatory agencies worldwide will soon begin to perform the requisite safety assessment of food products obtained from these animals. In anticipation of these developments, the extensive experience that has accumulated over the last three decades regarding food safety assessments of GM plants and microorganisms is being used to develop new criteria for animal-derived products, taking into account the differences and similarities between plant-based and animal-based food sources. Food safety assessment approaches specifically for GM animals are currently being refined, and efforts at the international level have been consolidated into specific guidelines included in the *Codex Alimentarius*.

Riassunto
Mentre i ricercatori fanno progressi nel processo che va dalla sperimentazione alla fase commerciale degli animali geneticamente modificati, ci si aspetta che le agenzie di regolamentazione a livello mondiale inizino presto a rilasciare le valutazioni sulla sicurezza degli alimenti derivati da questi animali. In attesa di questi sviluppi, viene utilizzata la vasta esperienza accumulata negli ultimi tre decenni sugli alimenti derivanti da piante e microorganismi geneticamente modificati allo scopo di sviluppare nuovi criteri per i prodotti di provenienza animale, tenendo in considerazione le differenze e somiglianze tra cibi di origine vegetale e animale. La valutazione sulla sicurezza dei cibi specificamente derivanti da animali geneticamente modificati è attualmente in fase di perfezionamento e gli

sforzi fatti a livello internazionale sono stati riuniti in specifiche linee guida contenute nel *Codex Alimentarius*.

Keywords: allergenicity, Codex Alimentarius, history of safe use, safety assessment, substantial equivalence, toxicology

1. INTRODUCTION

The safety assessment of food products obtained from genetically modified (GM) organisms is based mainly on: (i) the safety analysis of each new substance that is present in the food, and; (ii) analysis of the whole food product compared to conventional analogues or counterpart foods. The analysis of new substances is similar to the single substance approach of traditional toxicological risk assessments applied to specific chemicals such as food additives and pesticide residues. However, since for most GM animals the new substances are proteins, the analysis mainly focusses on the assessment of toxicological and allergenicity risks (described later in this review).

In contrast, the analysis of whole food products derived from GM animals relies on a comparison with the particular characteristics of the most similar pre-existing foods that have a history of safe use.

The concept of *substantial equivalence* is frequently used to describe the initial stage of the safety assessment process, in which similarities between the new food and its conventional counterpart are confirmed. Any relevant differences in composition or other pertinent characteristics are subject to further investigation to determine whether they have implications for food safety.

More information about *substantial equivalence* and comparative safety assessments are available (OECD, 1993; FAO/WHO, 2003; Kok & Kuiper, 2003).

2. GUIDELINES FOR FOOD SAFETY ASSESSMENT

Extensive experience has accumulated over the last three decades regarding food safety assessments of GM plants. As GM animals developed for commercial purposes are now approaching the market, this early experience is being used for developing criteria for safety assessments that take into account the differences and similarities between plant-based and animal-based food sources.

The current approach for the safety assessment of foods derived from GM animals has been refined over recent years (FAO/WHO, 1991; Kuiper *et al.*, 2001; FAO/WHO, 2003; FAO/WHO, 2007a, 2007b). Although different assessment approaches were initially developed independently by

different organisations worldwide, these efforts have been consolidated into specific guidelines included in the *Codex Alimentarius* (CODEX), which now represents the foremost international guidance on the subject (CODEX, 2003, 2008).

The CODEX is a compilation of standards, methods and guidelines related to food products, developed under the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Food Standards Programme. The objectives of this programme are to protect the health of consumers, ensure fair practices in food trade and harmonise food standards. Although the CODEX is not mandatory *per se*, its observance by governments is enforceable in trade disputes under the World Trade Organization. CODEX standards are produced following the scientific analysis of all relevant information under a set of rules that guarantee a wide scientific and political consensus amongst governments, industry and consumers before guideline can be released (Boutrif, 2003; WTO, 2003).

For these reasons, CODEX guidelines are the most widely accepted reference in the field, and are therefore used as the main reference in the following sections. Nevertheless, it is important to recognise that CODEX standards represent a very wide-ranging technical agreement arising from complex drafting processes. Although in the main its content provides essential guidance, in some areas those working in the field may resort to complementary information sources (Ridley *et al.*, 2004; Lema & Burachik, 2009; Kok *et al.*, 2010).

3. FRAMEWORK OF THE SAFETY ASSESSMENT

The safety assessment process for foods derived from GM animals is based on a stepwise consideration of relevant information regarding a range of different issues. According to CODEX (2008), this should include an overall description of the GM animal, the genetic modification and the genetic information sources, along with background information on the biology and food use of the conventional counterpart.

3.1. The conventional counterpart

Conventional counterpart is a concept that appears simple in abstract terms, but deserves careful consideration when it is applied. For example, for an animal breed of a species with a known history of safe food use, it may refer to the initial GM animal derived from this breed, as well as the

breeding partners used in developing the GM animal for use as a food source. If applied to foods, conventional counterpart may refer to food derived from conventional counterpart animals, although complementary comparators may be required for certain assessments, e.g. regarding nutritional enhancement (see below).

The following information regarding conventional animals that contribute to the genetic background of GM animals should be taken into account:

• The history of breeding and information on the genotype and phenotype of the animal related to its safety as a source of food, including its known toxicity and allergenicity and the possible presence in foods of toxin-producing organisms or human pathogens.

• Information on the effect of different husbandry conditions (feeding, exercise or growth environment) on food products.

• The history of their safe use as a food source or for food production. This includes information on how the animals are bred and reared; how food products are obtained from them; and the conditions under which such food products are delivered to and used by the consumer (e.g. storage, transport, processing and home preparation). This analysis should also form a basis for predicting the range of derived foods that should be considered for safety assessment.

• The nutritional relevance of the main food products to the general consumer and/or particular subgroups, with particular regard to important nutrients they may contribute to the diet.

3.2. Recombinant DNA sources

For each DNA sequence present in the DNA construct inserted into the genome of an animal, basic information on the source organisms should be considered during the food safety assessment. For microorganism sources, a history of pathogenicity to humans or the recipient animal should be considered, as well as any known phylogenetic relationship to or natural association with human or animal pathogens. Alternatively, for animal sources it is relevant to review breeding history and genotype and phenotype information relevant to food safety, particularly regarding known toxicity, allergenicity and the presence of toxin-producing organisms or human pathogens.

In addition, for animal or viral sources, it may be relevant to assess potential risks derived from unexpected pathogens in source materials such as cell culture media. Finally, for all source organisms it is important to have information on any previous (intended or unintended) presence in the food supply.

3.3. The genetic modification process

A complete food safety assessment requires a detailed understanding of how the genetic modification was made, especially for identifying genetic material that may have been introduced into the recipient animal, whether intentional or not. This requires having access to the following information:

a) The full nucleotide sequence and map of the final vector/construct used, indicating the location and orientation of all genetic components. In addition, every individual genetic component (including open reading frames and regulatory sequences that affect DNA expression and/or function) should be characterised with respect to source organism, size, biological function and its potential for mobilisation or recombination. Finally, the expected function of the transgene in the GM animal should be made clear.

b) The methodology and specific protocol used to introducerecombinant DNA into the recipient animal. Special consideration should be applied if pathogenic organisms have been used as vectors or during the construct assembly, particularly regarding their natural hosts, transmission mode and potential for recombination with other pathogens.

3.4. Genetically modified (GM) animals used for food production

In addition, a final characterisation should be made of the GM animal line that will be introduced for food production, following breeding or back-crossing of the primary GM animal. This is especially necessary if the original transgenic animal was hemizygous and/or mosaic for the transgene. This does not imply that a new assessment will be necessary if a GM animal line already assessed to be safe is later cross-bred with a conventional breed already on the market.

3.4.1. Breeding

It is necessary to provide information on the steps undertaken in any traditional breeding process used, including the use of marker-assisted selection, cloning or other assisted reproduction techniques. This includes, if appropriate, evidence of how the heritability and/or genetic homogeneity

of the transgene were reached.

It is also relevant to consider the background of other animals used in the process (e.g. breeding partners or surrogate mothers), including relevant information on genotype, phenotype, husbandry conditions, and if their history of food use differs from the conventional counterpart.

3.4.2. Health status

Animal food products have generally been regarded as safe for consumption when derived from animals with an acceptable health status that belong to a species with a history of safe use. Health status has proven to be a robust and broad indicator of safety, and also contributes to a weight-of-evidence approach in GM animals.

Evaluating the health status of a GM animal could provide additional insight into the possible toxicity and bioactivity of newly-expressed substances. Additionally, health parameters can be considered as traits that are influenced by many genes simultaneously (i.e. multigenic), and may therefore constitute an additional assurance on the absence of unintended effects. Information on overall health and performance indicators, physiological measures and other species-specific considerations should be taken into account.

3.4.3. Genetic analysis

Genetic analysis involves characterising the number of insertion sites, as well as the organisation of the inserted genetic material at each insertion site (including information on tandem insertions and the DNA sequence of inserted material and flanking regions).

This information can be used to determine whether the original arrangement of the genetic material used for transformation has been conserved or whether significant rearrangements have occurred during integration. This should form the basis of assessing whether polypeptides expressed from the construct or flanking regions have been modified as a consequence of insertion. Random integration may modify the pre-existing local genetic sequence and have a significant influence on transgene expression. For this reason, safety assessments are made for each transformation event on a case-by-case basis.

3.4.4. Compositional analysis

Key components are substances relevant to nutrition and food safety that are inherently present in foods derived from the conventional counterpart, including nutrients (fats, proteins, minerals and vitamins), anti-nutrients (e.g. digestive enzymes inhibitors), toxicants and allergens. A list of key components should be established. Following this, the levels of each component should be measured in the transgenic animal line and compared with data from conventional counterparts grown under equivalent husbandry conditions. The range of variation within species and breeds should also be considered, if available.

The purpose of the compositional analysis, in conjunction with an exposure assessment, is to establish that pre-existing substances relevant to the nutritional value or safety of the food have not been modified by genetic modification in a way that would adversely affect human health.

3.5. Novel substances

A complete safety assessment requires that any novel substance present in GM animals and/or derived foods as a consequence of genetic modification should be identified. This includes proteins or untranslated RNA expressed from transgenes in the original construct or created by insertion of the recombinant DNA into the genome, as well as any new metabolites produced by the catalytic or other biochemical activity of these substances.

A comprehensive molecular and biochemical characterisation is required for each substance identified. For proteins in particular this may include: biological activities; significant sequence homology to known proteins and the biological activities of those proteins; changes in the pattern of post-translational modifications, and simulated human digestion studies. Proteins should also be subjected to specific toxicity and allergenicity assessments (described below).

Concentrations (and tissue specificity levels, if applicable) of the novel substance should be determined in both the animal and derived foods. In particular, it should be determined whether this information is consistent with both regulatory sequences present in the construct and the expected phenotype.

In addition, if the function of the substance is to alter the accumulation of a specific endogenous substance, the amount of endogenous target

present in the GM animal should be reported. Furthermore, any evidence suggesting that endogenous genes in the GM animal have been affected by the transformation process, intentionally or not, should be used to identify other relevant endogenous substances to use as indicators.

3.6. Toxicity and bioactivity assessment

The goal of this assessment is to verify that genes involved in the expression of toxins or anti-nutrients present in donor organisms, vectors or other biological materials have not been transferred to GM animals.

Conventional toxicology studies may not be necessary when a substance that is novel in the foods derived from the GM animal has previously been consumed safely in other foods, as long as its function in the food and exposure patterns are similar in both cases. In other situations, the use of appropriate conventional toxicology or other appropriate studies on the new substances may be necessary. Furthermore, when the composition of the food is substantially altered, the whole food may be tested for safety using animal feeding studies.

The toxicology and bioactivity safety assessment should take into account: the chemical nature and function of any newly-expressed or up-regulated substances; the concentration range in edible tissues and other food products derived from the GM animal, and; the usual dietary exposure to conventional counterpart foods.

Regarding proteins, the toxicity assessment should initially focus on amino acid sequence similarity between the protein and known protein toxins, stability to heat or processing and degradation in gastric and intestinal model systems.

Appropriate oral toxicity studies should be carried out in cases where the novel protein is not similar to any other protein that has been consumed safely in food. The toxicity of novel proteins is normally tested in laboratory animals, using appropriate dose regimes over a time course of 28-90 days.

Regarding non-protein substances that have not previously been safely consumed in food, the safety assessment should follow a case-by-case rationale, considering the chemical nature and biological effects of the chemical and the expected dietary exposure. The range of analyses that should be performed may include toxicokinetic, acute/sub-chronic/ chronic toxicity and carcinogenicity, and immunological, reproductive and

developmental toxicity evaluations (Barlow *et al.*, 2002; OECD, 2011).

For newly-expressed bioactive substances, GM animals should be evaluated for the potential effects of those substances as part of the overall animal health evaluation. Moreover, consideration should be given to dietary exposure where the substance is likely to remain bioactive following consumption.

3.7. Allergenicity assessment

Typically, food allergies are exacerbated immune responses to food proteins and are mediated by immunoglobulin class E (IgE) antibodies. At least 160 conventional foods are associated with allergic reactions (Hefle *et al.*, 1996; Miescher & Vogel, 2002; Beyera & Teuber, 2004) that occur randomly in a small proportion of the population.

GM organisms usually express proteins that are novel components of the derived foods and these should therefore be assessed for their potential to cause allergic reactions. The assessment should include the potential for cross reactivity with IgE raised against the same or similar proteins by sensitised individuals. In addition, it should be determined whether a protein that is completely new or is presented in a different way to the usual food supply is likely to induce an allergic response in some individuals, thus leading to an adverse reaction after subsequent dietary exposure to the same or a similar protein.

A stepwise approach is recommended in order to ascertain the likelihood that the novel protein is a food allergen (Metcalfe *et al.*, 1996; FAO/WHO 2000; FAO/WHO 2001; Metcalfe 2005; CODEX, 2008. It should be noted that currently available criteria is intended only for assessing the allergenicity of newly-expressed proteins and not for assessing putative impacts of the genetic modification of foods that were *a priori* allergenic (e.g. shrimp), for instance any changes to the expression and presentation of pre-existing allergens (e.g. allergen down-regulation to result in a hypoallergenic foodstuff).

3.7.1. Source of the protein

Every report of an allergy (or unclassified hypersensitivity reaction) associated with the donor organism should be considered, as well as studies aimed at identifying the allergen responsible, and the availability of sera for testing GM animal-derived foods.

3.7.2. Amino acid sequence homology

A sequence comparison should be performed between the newlyexpressed protein and the best available database of known allergens in order to search for similarities that would suggest that the new protein is likely to cross-react with known allergens. Examples of possible criteria for a positive result (using standard alignment tools such as FASTA or BLASTP) are >35% identity over an 80 amino acid-long sequence or complete identity across a stretch of 8 contiguous amino acids (Fiers *et al.*, 2004; Goodman *et al.*, 2007; CODEX, 2008).

3.7.3. Immunoassays

In vitro assays for specific binding to IgE class antibodies should be performed for proteins known to be allergenic or that display significant sequence homology to known allergens, as long as sera from individuals with clinically-validated allergies to that source or allergen are available. A negative result should be confirmed by additional tests, as reported previously (Bruijnzeel-Koomen *et al.*, 1995; Bindslev-Jensen & Poulsen, 2007).

3.7.4. Biomolecular characteristics

Biomolecular characteristics repeatedly found in food allergens may help to explain their allergenic potential. Nevertheless, since such characteristics may also be found in non-allergenic proteins (or are absent from other allergens), they can be taken only as warning signs indicating the need for complementary studies. An example of these is digestive stability, measured as protein resistance to degradation in the presence of pepsin under simulated gastric conditions (Astwood *et al.*, 1996; Thomas *et al.*, 2004).

In addition, stability to food processing, in particular to storage time and temperature, is considered relevant for assessing potential allergenicity, as labile proteins are less likely to have an opportunity to interact with the immune system and thus becoming food allergens.

Another important characteristic is the presence of post-translational modifications, particularly glycosylation. Glycan residues can act as antibody epitopes and also affect protein susceptibility to degradation. Furthermore, as glycosylation patterns depend both on protein sequence and host glycosylation pathways, transgenesis may alter glycosylation patterns and thus induce allergenicity in glycosylated proteins.

Finally, other characteristics that may relate to the allergenicity of particular proteins (Breiteneder & Mills, 2005) include molecular size, the presence of repetitive substructures, the ability to form aggregates, rheomorphism and binding to ligands or to lipid membranes. Their relevance to allergenicity of transgenic proteins should be considered in a case-by-case basis.

3.7.5. History of safe use

If the source of the novel protein expressed in a transgenic organism has a history of safe use as a food (in particular, if it is not known to be allergenic), this could provide safety reassurance as long as the protein expressed in the GM animal is equivalent in terms of sequence, structure and post-translational modifications, and if the expected consumption levels and food processing are similar.

3.8. Other considerations

3.8.1. Accumulation of xenobiotics or microorganisms

It should be considered whether the traits acquired by the GM animal could lead to an increased risk of zoonoses or the accumulation of xenobiotics (e.g. through veterinary drug residues).

3.8.2. Transfer of antibiotic resistance genes

In assessing the safety of animal foods harbouring antibiotic resistance genes (which are used for the selection of transformed cells in several, but not all, transformation methods), it is important to establish if the antibiotic(s) involved have clinical and veterinary relevance. The goal is to determine whether antibiotic resistance genes could be accidentally transferred to pathogenic bacteria, thus potentially compromising therapies for the treatment of infectious diseases (Jonas *et al.*, 2001; Van den Eede *et al.*, 2004), although this possibility is thought to be unlikely (Einspanier *et al.*, 2001; Chambers *et al.*, 2002). Alternative selection technologies that do not use antibiotic resistance genes should be considered (Lema & Burachik, 2009).

3.8.3. Food Storage and Processing

Implications of the expected food storage and processing conditions should be analysed particularly when: (i) the genetic modification changes food processing or shelf life; (ii) a substance from a source with a history of safe food use is intended to be made available under different processing conditions (e.g. to be eaten raw instead of cooked), or; (iii) the newly-expressed substances may alter the stability of key food components.

3.8.4. Intended nutritional modification

Some animals have been genetically engineered to improve the nutritional value of the foods derived from them or for their own health benefits. This can be done, for instance, by increasing the content of a nutrient, incorporating a new one or reducing the content of a pre-existing toxicant.

With regard to food safety assessment, in these cases it may be necessary to assess different consumption scenarios while considering the particular patterns of food consumption in the target population. The dietary exposure assessment should consider the bioavailability of the relevant substances, as well as the usual consumption level of the conventional counterpart and/or other foods that are likely to be displaced.

Guidance for the safe fortification of foods is available (CODEX, 1991). However, in contrast with conventional food fortification, foods derived from GM organisms may require further characterisation of variability in concentration of novel compounds, the range of chemical forms present in the food and their combined bioavailability. For nutritional impact studies, it may be relevant to consider the use of special comparators of an analogous composition, such as fortified foods or conventional foods of a different origin.

4. CONCLUSION

Given that the first GM animals are now approaching the marketplace, there is a lot of interest in developing the technical capacity for assessing the safety of foods derived from these animals. However, an analysis of current literature suggests that such assessments will be performed in a very similar manner to the current standardised practice for foods derived from GM plants.

Therefore, after considering a few differences specific to animals, it is not anticipated that the necessary alterations to established procedures would require the extensive lag phase originally anticipated for adapting the guidance and assessment methods for foods derived from GM plants and microorganisms.

Overall, it is important to note that current guidelines only permit foods derived from GM animals that belong to a species with a history of safe use to be used as food sources. For this reason, assessments can only arrive at a conclusion regarding whether the food product of a GM animal is as

safe as the conventional counterpart food, according to the best available scientific knowledge.

The reliability of this discipline has already been established by the numerous assessments of products from GM plants, which have proven to be safe. However, this does not preclude the future development of methods and tools to complement the current assessment strategy. It is likely that some updates may take place in allergenicity assessment, since this is based on the rapidly evolving fields of immunology and protein function analysis. For instance, it may be advisable to carefully monitor developments in targeted serum screening, the use of animal models, protein structure analysis and T-cell epitope prediction (Knippels *et al.*, 1998; Dearman *et al.*, 2000; Weber *et al.*, 2003; Matsuda *et al.*, 2006; Prescott & Hogan 2006; Prescott *et al.*, 2006; Lema & Burachik, 2009)

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