Feasibility of therapeutic protein production in milk of large animals to increase their affordability

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Global Therapeutic Protein Market

Increase in Number of FDA Approval for Biotherapeutics

- Small Molecules
- Biologics

<table>
<thead>
<tr>
<th>Year</th>
<th>Small Molecules</th>
<th>Biologics</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>2011</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>2012</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td>2013</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>2014</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>2015</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>2016</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>2017</td>
<td>34</td>
<td>12</td>
</tr>
</tbody>
</table>

Global Market Share of Various Biotherapeutics

- Growth factors, Vaccines, mAbs
- Hormones, Insulin, Fusion proteins
- Recombinant Vaccines, mAbs
- Insulin, Recombinant proteins, vaccines, mAbs
- China - 7%
- USA - 40%
- EU - 25%
- All Other - 28%

In spite of Increase in Market share the Production cost till Remain very high

- Equipment: 71%
- Facilities: 18%
- Materials: 8%
- Labor: 4%

Reason is Huge Cost of Infrastructure Establishment and Manpower
## Cost of Presently Available Biotherapeutics

### Approximate cost of Recombinant Protein Therapeutics Available at Market

<table>
<thead>
<tr>
<th>Name of the Molecule</th>
<th>Brand Name (Cost Per Unit)</th>
<th>Requirement (Approximate Expenditure of treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>Novorapid (~650Rs/300IU)</td>
<td>A Individual has to take ~600-900IU minimum in a month (1800-2700 RS)</td>
</tr>
<tr>
<td></td>
<td>Levemir (~900Rs/300IU)</td>
<td></td>
</tr>
<tr>
<td>Blood Coagulation factor 7,8,9</td>
<td>In various brand name (10.5 to 18 Rs/IU)</td>
<td>Each patient with a small cut require minimum 2000IU each time (~20000-40000Rs)</td>
</tr>
<tr>
<td>Interferon</td>
<td>Viraferonpeg (~14000Rs/80microgram)</td>
<td>Depending on treatment (~28000-52000Rs)</td>
</tr>
<tr>
<td>Growth Hormone</td>
<td>Somatrophin (7500Rs/18mg)</td>
<td>Depending on treatment (~7500-15000Rs)</td>
</tr>
<tr>
<td>Bone Morphogenetic Protein 2 (BMP2)</td>
<td>BMP2 2Lakh/1mg</td>
<td>Depending on treatment (?)</td>
</tr>
</tbody>
</table>

### Alternative ----? Production of Biotherapeutic in Animal Bioreactor

**HUGE COST**
Diseases Need Immediate Attention

Guillain-Barre Syndrome

Treatment is to block the antibodies using high-dose immunoglobulin therapy (IVIG).

Osteoarthritis

Treatment require BMP2 and BMP7 Supplementation
Indian Scenario

Indian Population: 1,314,097,616
Per capita income: $5,760
Life expectancy at birth women/men: 69/68 yrs

269 million (21.9% of the total population) reside below poverty line

In India, the current market of approved recombinant therapeutics has been estimated to be about Rs. 5357 million, which is approximately 1.6% of the world market and 3.2% of the total pharmaceutical market of Rs.165 billion in the country.

Carrying 1/6th of the world population, India must be Holding ~15-20% of the world market.

Unaffordability of the present therapeutic by masses is the main reason behind low market share of costly therapeutics.

Requirement of Various Therapeutic Protein is Huge but Need to make the available at Cheaper Cost
Advantages of Animal Bioreactor

**Conventional method of Production of Therapeutics**

- Huge Infrastructure
- Requirement of Electricity
- Land
- Labour shifts
- License
- GMP facility etc.

**Animal Bioreactor Based Production of Therapeutics**

- Very Less Infrastructure
- All-in-One
1. βCasein is the most abundantly expressed protein in buffalo milk. Barłowska et al., 2011.

2. Buffalo Milk contain higher amount of milk protein than cow or goat. Barłowska et al., 2011.

3. Average Total Milk volume is more in Buffalo than other species. Mudgal, 1999.

Indicate High Level of Activity of BUFFALO βCASEIN PROMOTER Can be Utilized to Express Exogenous Protein in the time of Lactation in Higher Amount in Mammary Gland
Expression of Therapeutic Protein in Milk: (isolation of Buffalo βCasein Promoter)

Mammary gland specific promoter

E1 and E2 refer to Exon1 and 2 respectively. I1 refer to Intron1.

E2 contain milk secretory signal peptide sequence

Restriction Digestion Analysis
1. PstI & NotI
2. NotI
M = 1kb Ladder. (Neb)
Generation of Transgenic Animal for Production of Therapeutic Protein in Milk

In mice Successfully standardized the Testicular Transgenesis by Electroporation

Extrapolating the same system to large animal

Biotherapeutics at Very low Cost
TRANSGENESIS = **GERMLINE** GENE Integration

Consequences

BT – Brinjal, Mustard !!!

**NON** - **Germ** Line Gene integration
INTRODUCTION: Difficulties in Available Alternative Approaches

Invivo Transfection of Mammary Gland for Transgene Delivery

- Adenovirus Mediated Invivo Transfection
- Gene Gun
- Retro Virus Mediated Transfection
- Various Chemical Induced Transfection

1. Disturbed Tissue Architecture.
2. Failed to Deliver transgene in maximum number of cells in the gland.
3. Expression stable for some few days only.
4. Very low level of expression.


ALTERNATIVE?

Virosome Mediated Delivery of Transgene in The Mammary Gland
INTRODUCTION: Sendai Virus

SENDAI VIRUS

Negative Stranded RNA Virus of the family *Paramyxoviridae*.
**INTRODUCTION:** Sendai Virosome (HNF-V)

Sendai Viral Membrane + F – Glycoprotein and Hemagglutinin-Neuraminidase = HNF – Virosome
PREPARATION OF SENDAI VIROSONE

Culture and Harvesting of Sendai Virus

Seeding of Sendai Virus in Egg Cell and Cultured in 37°C

Incubation for 2/3 days

Break Open Egg Shell and harvest the culture

Centrifugation and washing

Processed and stored for future use.
Preparation of HNF-Virosome

Detergent Solubilisation

P DNA

HNF-Virosome (DNA Entrapped)

SENDAI VIRUS

SDS PAGE Analysis Of Protein from HNF – Virosome
Normal postnatal development of the murine mammary gland.

Defatted and carmine-red stained inguinal sub-dermal fat pads of wild type female mice, visualizing the epithelial tree of the mammary gland.
DEVELOPMENT OF MAMMARY GLAND IN MICE

- Estrogen
- Progesterone
- Prolactin
- ERα
- PR
- PrlR
- Ductal elongation/bifurcation
- Side branching
- Alveologenesis/lactogenic differentiation
- Rudimentary ductal system
- Puberty
- Estrous cycles
- Pregnancy
- CLD = Cytoplasmic lipid droplet.
- MFG = Milk Fat Globule.
- CLD = Cytoplasmic Lipid Droplet.
- TJ = Tight Junction.
- GJ = Gap Junction.
- D = Desmosomes.
- BM = Basal Membrane.
- ME = Myoepithelial Cell
- LE = Luminal Epithelial Cell.

- Lumen
- Myoepithelial cell
- Luminal cell
- Basement membrane
- Fibroblast
- Adipocyte
- Suprabasal cell
- Golgi
- RER
- N
- Capillary
- BM
- LE
INTRADUCTAL PERFUSION DELIVERY OF HNF-V IN MICE MAMMARY GLAND
INTRADUCTAL PERFUSION DELIVERY OF HNF-V IN MICE MAMMARY GLAND
INVIVO DELIVERY OF TRANSGENE BY HNF-V IN MICE MAMMARY LUMINAL EPITHELIAL CELLS
Observation on Day 5 of Lactation

IFNγ, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. IFNγ is an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression.

To treat osteoporosis, Chronic granulomatous disease (CGD) etc.
Direct Transfection of Mammary Gland by HNF-Virosome:

**Observation of In Vivo EGFP fluorescence in Mice Mammary Gland**

- HNF-V Treated (Lactating)
- HNF-V Treated (Non-Lactating)

**Isolation of Milk from Mammary Gland**

- HNF-V Treated (Lactating)
- HNF-V Treated (Non-Lactating)

**Western Blot of hIFNγ from Mammary Tissue Lysate**

- hIFNγ (~17kd)
- β-Actin (~42kd)

**ELISA for hIFNγ – from human kit available in market**

**Expression of hIFNγ in Rabbit Milk (μg/ml)**

- 5.6μg in 1ml milk
- ND: Not Detected

**Direct Transfection of Mammary Gland of New Zealand White Rabbit**
DNA is injected directly into the mammary gland.

Initiated work in goats.

Rabbit is not a bad bioreactor.

Betacasein Promoter

Gene of Therapeutic Protein